RECENT PROGRESS IN HORMONE RESEARCH

Proceedings of the Laurentian Hormone Conference

VOLUME I

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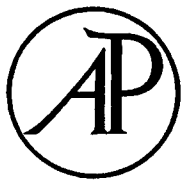

1947
ACADEMIC PRESS INC., PUBLISHERS
NEW YORK, N. Y.

Second Printing, 1954
Library of Congress Catalog Card Number: Med 47-38

PRINTED IN THE UNITED STATES OF AMERICA
FOR ACADEMIC PRESS INC.
125 EAST 23rd STREET, NEW YORK 10, N. Y.

PREFACE

After its 1943 meeting at Gibson Island the Hormone Conference of the A. A. A. S. was invited by the Montreal Physiological Society to meet in Canada in 1944. After a day in Montreal the membership met for its regular sessions in the Laurentians at Mont Tremblant. The location and circumstances of the meeting were such that the members voted unanimously for a return to Canada, and at the 1945 meeting voted to call the assembly the Laurentian Hormone Conference with the wish that it might be continued regularly. Publication of the papers and discussion was requested.

The discussions of each paper were recorded by a stenotypist and included in this volume. The editor is grateful to the authors and discussants for their generous assistance in the editing of these discussions.

The holding of the conference and the preparation of the book manuscript were made possible by contributions from Ayerst, McKenna and Harrison Ltd.; Sharp and Dohme, Inc.; E. R. Squibb and Sons; Roche-Organon; The Glidden Company; Mallinckrodt Chemical Works; The Upjohn Company; Hoffman-LaRoche, Inc.; Armour and Company; Ben Venue Laboratories, Inc.; Parke, Davis and Company; Ciba Pharmaceutical Products, Inc.; Difco Laboratories, Inc.; American Home Products Corporation; The Schering Corporation; White Laboratories, Inc.; Winthrop Chemical Company, Inc.; Des Bergers-Bismol Laboratories; Charles E. Frosst and Co.; and Horners Ltd.

The committee is much indebted to the authors for the inclusion in their papers of much unpublished material. It is hoped that the publication of critical evaluations and work-in-progress by leading investigators will be valuable not only as records of knowledge and accomplishment but as incitements to research. The spirit of inquiry dies without criticism and discussion, and it is largely the purpose of these conferences to nourish that spirit. The hormones are often regarded as regulators of the rates of numerous vital processes. We hope that these papers will act as hormones to the creative processes of students and scholars in this far-flung field.

Shrewsbury, Mass. GREGORY PINCUS

On the Role of Acetylcholine in the Mechanism of Nerve Activity

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I. Introduction

For more than a century nerve activity was conceived in electric terms only. The analysis of the nerve action potential, the electric spike, constituted the only means of studying nervous action. And yet, 150 years after Galvani's discoveries, H. S. Gasser (7) compared the electric spikes to the ticks of the clock. Both are only signs of activity in the underlying mechanism: "It follows then that if spikes are but manifestations of activity in the inherent mechanism of nerve fibers, the story of nerve is by no means told when the spikes are described. We need to know something about the mechanism which produces them—how it is maintained, its capacity for work, and when and how the work is paid for." Study of the physical aspect alone may give us valuable information. But, for a thorough understanding of the mechanism of nerve activity, it is necessary to know the chemical reactions involved.

The special function of the nervous system is that of carrying messages from one distant point of the body to another. This process may be subdivided into three successive phases: First, a stimulus reaching a neuron has to initiate an impulse; that is the problem of the "primary disturbance," as Keith Lucas called it, by which a propagated impulse is produced. Second, the impulse once initiated has to be propagated along the axon; that is the problem of conduction. Finally, the impulse arriving at the nerve ending has to be transmitted either to a second neuron or to an effector cell. Early in this century the idea was evolved that a chemical compound may be connected with the third phase, namely, the transmission of the nervous impulse from the nerve ending to the effector cell: T. R. Elliot suggested, in 1905, that adrenaline may be the transmitter of the impulse from the sympathetic nerve ending to the effector cell. He based this idea on the similarity between the adrenaline and the effect of stimulation of sympathetic nerves on the effector organ. In 1921, Otto Loewi found that following vagus stimulation of the frog's heart a compound appeared in the perfusion fluid which, if transmitted to a second heart, produced an effect similar to that of vagus stimulation. Accepting

*Most of the work presented here was aided by grants of the Josiah Macy, Jr., Foundation and the Dazian Foundation for Medical Research.

the basic idea of Elliott, he concluded that this compound, later identified with acetylcholine, is released and acts on the heart cell directly. Loewi's concept of "neurohomoral" transmission was widely accepted among physiologists.

In 1933, Dale tried to extend this idea of a "chemical mediator" of the nerve impulse to the neuromuscular junction and to the ganglionic synapse. His theory was based essentially on the same type of evidence as previously applied by Otto Loewi in the case of autonomic nerves. In this case, however, the theory encountered strong opposition. Besides many contradictions and difficulties discussed by Eccles (5), there were two main objections. The first was the time factor. This factor was of lesser importance in the case of the slowly reacting cells innervated by the autonomic nervous system. But the transmission of nerve impulses across neuromuscular junctions and ganglionic synapses occurs within milliseconds. No evidence was available that the chemical process can occur at the high speed required, and Dale and his associates admitted this difficulty. The second objection was still more fundamental. According to leading neurophysiologists like Sherrington, Fulton, Gasser, and Erlanger, the excitable properties of axon and cell body are basically the same. The electric signs of nervous action therefore do not support the assumption that the transmission of the nerve impulse along the axon differs fundamentally from that across the synapse (6, 10, 11).

The idea of a chemical mediator released at the nerve ending and acting directly on the second neuron, thus appeared to be unsatisfactory in many respects.

II. Approach to the Study of the Mechanism of Nervous Action

Two features of nervous action are essential to an understanding of the problems and difficulties involved: the high speed of the propagation of the impulse and the infinitely small energy required. In medullated mammalian nerve the impulse travels at a rate of 100 meters per second and the energy required per impulse per gram nerve is less than 1/10 of a millionth of a small calorie. The recording of such an event offered many difficulties even with the use of specialized physical methods. A really adequate electrical recording instrument became available only with the introduction of the cathode ray oscillograph by Gasser and Erlanger. Still more difficult was the detection of the energy involved. It is not surprising that Helmholtz who first demonstrated heat production in muscle failed to demonstrate it in nerves. Even A. V. Hill was unable to detect any heat production in nerves for a long time, and only when he and his associates developed thermo-

electric methods of an amazingly high degree of perfection, did it become possible to measure amounts of heat of such a small order of magnitude as produced by nerve activity.

If even physical methods encountered so many obstacles, it is obvious that the study of the chemical reactions connected with an event of this kind must offer serious difficulties. No adequate methods are available for determining directly chemical compounds appearing in such infinitely small amounts and for such short periods of time. However, the development of biochemistry, especially during the last twenty years, has shown that in such cases much information may be obtained by the study of biocatalysts. Nearly all chemical reactions in the living cell are catalyzed by enzymes. Since Buchner's demonstration, in 1897, that fermentation may occur in cell-free extracts, a great number of enzymes have been isolated. The study of these enzymes *in vitro* has elucidated many chemical reactions, known to occur in living cells, which could not be followed by direct chemical determination of the compounds established. Especially for an event occurring with such a high speed as the propagation of the nerve impulse, analysis of the enzyme systems involved appeared to be the most promising approach.

Enzyme studies alone are, however, not sufficient for the elucidation of a biological mechanism, since there are so many simultaneous reactions in the complex system of the living cell. It is necessary to correlate enzyme activities with events in the intact cell. The most conspicuous example of such an approach is the development of muscle physiology. By the pioneer work of A. V. Hill and O. Meyerhof, many physical and chemical changes have been correlated and our concept of the mechanism of muscular contraction went through a real "revolution" according to an expression of A. V. Hill.

The investigations which will be presented in this paper are based partly on the study of the enzyme systems involved in the formation and hydrolysis of acetylcholine (ACh). Besides the study of the enzymes *in vitro*, their activities could be correlated at several instances with events in the living cell recorded by physical methods. The facts established show that the original theories of the role of ACh have to be abandoned. They have provided evidence for a new concept of the role which the ester may have in the mechanism of nerve activity. According to this concept, the release and removal of ACh is an intracellular process occurring at points along the neuronal surface and directly connected with the nerve action potential.

The facts on which the new concept is based have been recently reviewed and discussed (15). Only the most essential features will be presented today.

1. The Time Factor

ACh is inactivated by the enzyme choline esterase which hydrolyzes the ester into choline and acetic acid. The first essential result of the studies of this enzyme has been the evidence of its high concentration in nerve tissue: Significant amounts of ACh may be split in milliseconds; that is the period of time required for the passage of a nerve impulse. Consequently, the potential rate of ACh metabolism is sufficiently high to justify the assumption that it parallels the rate of the electric changes and may therefore be directly connected with the nerve action potential.

The special case in which this problem of the time factor has been studied and received a satisfactory answer is the frog's sartorius muscle. A small fraction of this muscle is free of nerve endings. By determining the concentration of choline esterase in this part of the muscle, in the part containing nerve endings and in the nerve fibers, it is possible to calculate the concentration of choline esterase at the motor end-plates. Since the number of end-plates in a frog's sartorius is known, the amount of ACh which may be split during one millisecond at a single motor end plate can be calculated. This turns out to be 1.6×10^9 molecules of the ester. About one-third of the enzyme at the motor end-plate is localized inside the nerve ending. On the assumption that one molecule of ACh covers about 20 - 50 square A°, the amount which may be hydrolyzed during one millisecond at one end-plate would cover a surface of 100-250 square microns.

A high concentration of choline esterase, of an order of magnitude similar to that at motor end-plates, exists at all synapes whether central or peripheral, whether mammalian or fish, whether vertebrate or invertebrate. In mammalian brain, for instance, 10^{14} to 10^{15} molecules of ACh may be inactivated per gram tissue during one millisecond. This corresponds to about 10-100 millions of square microns of neuronal surface.

These experiments removed one of the chief difficulties encountered by the theory that ACh is involved in the transmission of nerve impulses. They established that the ester may be metabolized at the high speed required for a chemical reaction directly connected with such a rapid event.

The difference between synaptic region and fiber is, however, only quantitative. The concentration of choline esterase is high everywhere in nerves although it rises at the region of synapses.

2. Localization of Choline Esterase at the Neuronal Surface

The second essential feature is the localization of choline esterase in the neuronal surface. Direct evidence for this localization has been offered with experiments on the giant axon of squid (*Loligo pealii*) (1). This axon

was made known to biologists by the work of J. Z. Young, F. O. Schmitt, and their associates. It has a diameter ranging from 0.5 to 1.0 mm. The axoplasm may be extruded and thus separated from the sheath. The axoplasm was found to be practically free of choline esterase. Most of the sheath is connective tissue to which are attached two thin membranes each only a few micra in thickness. The whole enzyme activity is in the sheath.

This exclusive localization of choline in the neuronal surface has been found only in the case of this enzyme. Respiratory enzymes are localized nearly completely in the axoplasm. Bioelectric phenomena occur at the surface. The high concentration of the enzyme at the surface suggests that ACh may be connected with conduction along the axon as well as with transmission along the synapse. This view is consistent with the conclusion of Erlanger that the mechanism of these two events is fundamentally the same.

The high rate of ACh metabolism and the localization of the enzyme at the neuronal surface made possible the assumption that the ester is connected with the electrical manifestations of nerve activity. But for the interpretation of the actual role of ACh the activity of the enzyme had to be connected with an event in the living cell. Such a correlation has been established in experiments carried out on the electric fish. It was found that the activity of the enzyme in these organs parallels the voltage of the action potential.

3. *Parallelism Between the Enzyme Activity and the Voltage of the Nerve Potential*

The powerful electric discharge in these organs is identical in nature with the nerve action potential of ordinary nerves. The only distinction is the arrangement of the nervous elements, the electric plates, in series. The potential difference developed by a single element is about 0.1 volt, which is the same order of magnitude as that found in ordinary nerves. In the species with the most powerful electric organ as yet known, *Electrophorus electricus,* the so-called electric eel, several thousand elements are arranged in series from the head to the caudal end of the organ. Thus the voltage of a discharge amounts to 400 - 600 volts on the average, and in some specimens, more than 800 volts have been observed. In *Torpedo* another species with a powerful electric organ, the elements are arranged in dorso-ventral direction. Since it is a flat fish, the number of plates usually does not surpass 400 to 500, and consequently, the discharge is only 30 to 60 volts on the average. In the large *Gymnotorpedo occidentalis* found on the North American east coast, especially in the water surrounding Cape Cod, the number of plates in series and, consequently, the voltage may be more than twice as high.

In 1937, the electric tissue was introduced by the writer as material for the study of the role of ACh in the transmission of the nervous impulse. An extraordinary high concentration of choline esterase was found in the strong electric organs of *Torpedo* and *Electrophorus electricus*. These organs hydrolyze in one hour amounts of acetylcholine equivalent to one to five times their own weight. In the larger specimens the organs have a weight of several kilograms, so that the amount of acetylcholine which may be split in these organs may amount to several kilograms per hour or several milligrams in one-thousandth of a second. These are significant amounts. They make possible the assumption that ACh is directly connected with the action potential and may even generate it. For in this case the compound must appear and disappear in milliseconds. If speculation were to be excluded, the only means of removing this compound so rapidly is enzymatic action. The high concentration of a specific enzyme appeared particularly significant in view of the chemical constitution of these organs: They contain 92 percent of water and only 2 percent of protein.

In a weak electric organ of the common Ray, the concentration is relatively low. If in the three species mentioned, voltage and number of plates per centimeter are compared with the concentration of choline esterase, a close relationship becomes obvious (13, 26).

A more detailed analysis has been carried out on the electric organ of *Electrophorus electricus*. This species is particularly favorable for such studies, since the number of plates per centimeter and consequently the voltage per centimeter, decrease from the head to the caudal end of the organ. The choline esterase activity decreases in the same proportion (See Fig. 1). If the electric changes are recorded and compared with the enzyme activity at the same section, a close parallelism is obtained between voltage and enzyme concentration. This is found not only in regard to the variations which occur in the same specimen, but even for the variations between the individuals which are quite considerable (17, 19).

In a great number of experiments carried out on fish of various sizes and at different points, covering a range of the action potential from 0.5 to 22.0 volts per centimeter, the quotient Ch.E/V was found to be $k = 20.7$ with a standard deviation of only ± 0.7 or 3.7 per cent (18). This is a good agreement for biological material and emphasizes the significance of the constant. If the choline esterase concentration is plotted against the voltage per cm., the line which correlates the two variables passes apparently through the 0 point. This supports the assumption of a direct proportionality between physical and chemical events measured. Combined with other observations, a direct connection of acetylcholine with the action potential becomes highly probable.

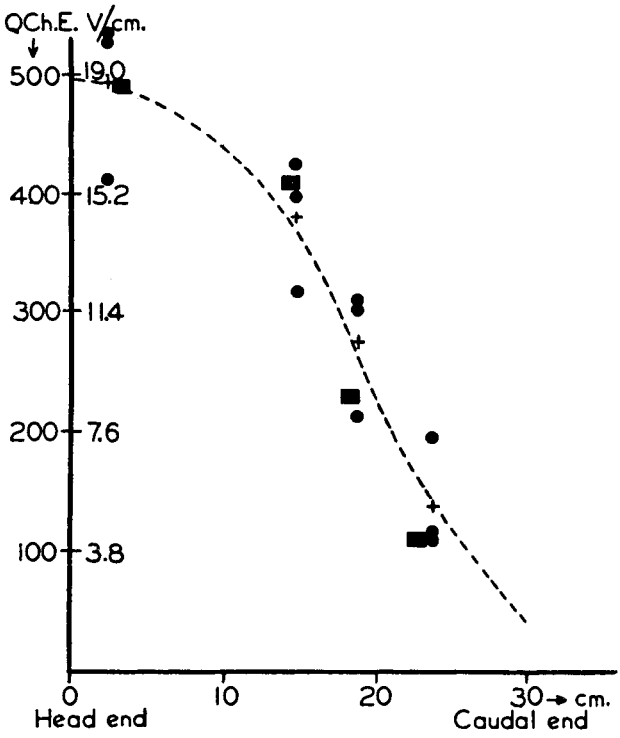

FIG. 1

Action Potential and Choline Esterase Activity in the Electric Organ of *Electrophorus electricus*.

Abscissae: Distance from the anterior end of the organ in cm. Ordinates: QCh. E. (mg. of ACh split by 100 mg. of fresh tissue in 60 min.) and V/cm.

● average QCh. E. from a single piece of tissue.

+ average QCh. E. values from pieces of the same section.

■ V/cm.

The voltage developed in the discharge depends upon the electromotive force, the current, and the resistance. Two assumptions therefore appear possible concerning the manner in which ACh may act: it may produce electromotive force directly by action on the surface, or it may decrease the resistance by increasing the permeability of the boundary. Resistance and electromotive force are closely related properties. So far, the evidence from experiments on nerves is in favor of a change in resistance and increased permeability. On the basis of alternative current impedance measurements, carried out on the giant axon of squid, Cole and Curtis calculated that the resistance drops during the passage of the impulse from 1000 ohms to about 25 ohms per square centimeter (3). In experiments on the

electric tissue a comparable drop in resistance was found by Cox, Coates, and Brown (4). There is no conclusive evidence that electromotive force is actually produced during the passage of the impulse. The material available so far is therefore consistent with the assumption that the parallelism found between voltage and ACh metabolism may be due essentially to the effect of the ester on the resistance of the boundary or, what is equivalent, on its permeability.

4. On the Role of Acetylcholine in the Mechanism of Nervous Action

Thus we arrive at the following picture of the role which ACh may have in the mechanism of nerve activity: According to the membrane theory which is most widely accepted among physiologists, the nerve is surrounded by a polarized membrane. The polarized state of the membrane is due to a selective permeability to potassium ions which are many times more concentrated inside the axon than outside. During the passage of the impulse, the permeability of the membrane to negative ions is increased and a depolarization occurs. The rapid appearance and removal of ACh may play an essential role in this change in permeability. The depolarized point becomes negative to the adjacent region, and flow of current results. This flow of current stimulates the next following point. There again acetylcholine is released and the whole process repeated. The impulse is thus propagated along the axon. At the nerve ending the process is fundamentally the same; the flow of current transmits the impulse across the gap. Other factors, like increased surface and consequently, decreased resistance, may enhance the efficiency of the process. Whereas in earlier theories ACh was considered as a "neurohumoral" or "synaptic" transmitter, *i.e.*, a substance released from the nerve ending and acting directly on a second neuron, in the new concept the transmitting agent is always the electric current, the action potential, but the current is generated by changes in the membrane, in which the release of ACh is an essential event.

The picture is consistent with the idea of the propagation of the nerve impulse as developed by Keith Lucas and Adrian. It becomes unnecessary to assume that the transmission along the axon differs basically from that across the synapse. The assumption of a special mechanism at the synapse different from that in the axon as emphasized before was one of the chief difficulties which had to be overcome for reconciling the original theory with the conclusions of the electrophysiologists. This appeared necessary for any satisfactory answer to the problem. If it is true that physical methods alone are unable to explain the mechanism in a living cell, it is equally true that conclusions based on chemical methods should not be in contradiction to those obtained with physical methods in view of the much higher sensitivity of the latter.

5. *Specificity of Choline Esterase*

In all the experiments on the activity of the enzyme, it was assumed that choline esterase is specific for ACh. In such a case, not only is the conclusion justified that the substrate metabolized is ACh; but also the activity of a specific enzyme determined *in vitro* may well be used as an indicator for the potential rate of metabolism of the substrate occurring *in vivo*. As pointed out by Schoenheimer and Rittenberg (33), one of the most important general results of the work with isotopes is the conclusion that "enzymes do not lie dormant during life but are continuously active." This, of course, does not imply that all enzymes are working at an optimal rate at every moment. In cells like nerve and muscle a considerable difference has to be expected between the resting condition and a state of activity. Lactic acid formation, for instance, occurs in anaerobic condition even in the resting muscle; but during tetanic stimulation, the rate increases several thousand times. Similarly, it cannot be expected that an enzyme directly connected with the events during the passage of the impulse is equally active in resting condition. It appears possible, however, and even probable, that enzymes are, to some extent at least, present in excess above the optimum usually required. But all experience in enzyme chemistry appears to indicate that a correlation exists between the concentration of a specific enzyme in a cell and the rate at which its substrate is metabolized.

It appeared imperative therefore to demonstrate the specificity of the enzyme for ACh in all those tissues which were used in the investigation leading to the new concept. The ester linkage in ACh shows no peculiar properties. It has, therefore, to be expected that the ester can be hydrolysed by other esterases and, on the other hand, that choline esterase can hydrolyse other esters. Specificity in this case would be expected, on the basis of analogy, to be only relative, not absolute: Choline esterase might be expected to split ACh at a higher rate than other esters, whereas other esterases might be expected to behave differently. By testing a number of substrates, a pattern may be obtained which makes it possible to distinguish specific choline esterase from other esterases (27, 28).

In the variety of nerve tissues which have been used as basis for establishing the new concept, the enzyme was found to be an esterase specific for ACh, *viz.*, mammalian brain, lobster nerve, squid fiber containing the giant axon and the electric tissue. All show a similar pattern, typical for choline esterase. In contrast, the hydrolysis patterns of the esterase of other organs—liver, kidneys, and pancreas—differ greatly from that of choline esterase. The esterase in these tissues shows several variations, but this could be expected, since the physiological substrate is unknown and proba-

bly varies in the different organs. They should be referred to as unspecified and not unspecific esterases, because they may well be specific for substrates not yet specified. Only in muscle (free of nerve endings) was a pattern similar to that of choline esterase obtained. It is possible that propagation of an impulse in the muscle fiber has the same mechanism as in the nerve fiber. But the presence of choline esterase alone is not sufficient to permit any conclusion.

Of special interest is the pattern obtained with purified choline esterase. The enzyme extracted from the electric organ of *Electrophorus electricus* has been purified to a degree where 1 milligram of protein splits 78,000 milligrams of ACh per hour. The rates of hydrolysis of different substrates is exactly the same as those obtained with freshly homogenized electric tissue. Thus, the enzyme tested in fresh electric tissue is the same as that which is highly purified and the parallelism established between voltage and enzyme activity becomes particularly significant.

6. *Affinity of Drugs to Choline Esterase*

The specific character of choline esterase has found further support in studies in which the affinity of certain drugs to the specific enzyme has been compared with that of other esterases not specified for ACh (29). In these studies two enzyme preparations were used representing specific choline esterases: One from electric tissue, the other from the nucleus caudatus of ox brain. The esterases from horse serum and from guinea pig pancreas were used as unspecified esterases.

Significant differences in affinity were found with a number of compounds. As in the case of the specificity tests with choline and with non-choline esters, the differences are mostly quantitative. Quinine, quinidine, and cocaine have a low affinity to choline esterase whereas the affinity to other esterases is high. The weak effect of quinine on choline esterase in contrast to the strong inhibition of other esterases may be of interest in connection with the powerful anti-esterasic effect of atebrine (34). This may be relevant for the mode of action of antimalarials.

A specific affinity was found with caffeine which acts exclusively on choline esterase. The affinity is high enough to be compatible with the assumption that the well-known action of caffeine as a general stimulant of the central nervous system may be referred to its effect on the choline esterase.

Other drugs tested were found to be equally weak inhibitors of both types of esterases, *e.g.*, nicotine, or relatively strong inhibitors, as for instance strychnine and veratrine. The affinity of some of these compounds to choline esterase may be relevant to their mode of action.

7. *Choline Esterase During Growth*

Another correlation between ACh metabolism and function has been established in experiments on embryonic tissues. It could be demonstrated in a great variety of cases that during growth choline esterase reaches its high concentration in nerve tissue and at motor end plates at a time when function develops.

One example may be given. The enzyme concentration in growing tissues does usually increase continuously until it has reached a maximum. In most cases the increase is slow in the beginning, then, for a certain period, the concentration rises sharply and then again the increase becomes slow and finally reaches a high level at which it persists.

In contrast to this usual pattern a very different one was found in the muscle of chick embryos. The choline esterase concentration reaches high values around the twelfth to the fourteenth day, that is, at a time when the muscular movements begin. The concentration then increases still slightly, but after hatching the values begin slowly to fall and three weeks later are less than 10% of those shortly before and at hatching (See Fig. 2). This unusual curve may be explained in the following way: At hatching the muscle fibers are small and therefore the end plates in a given weight of muscle are relatively numerous. Later the fiber grows and the number of end plates per unit of weight decreases. The fact that the concentration of choline esterase is so high at the early stages of development when the number of end plates per unit of tissue weight is high is consistent with the assumption that the concentration of choline esterase at the end plates is high at that period (14).

There are a number of other observations available which show the coincidence between the appearance of the high concentration of choline esterase and the beginning of function. They will be discussed elsewhere (16); the observation quoted may suffice as illustration.

8. *The Energy Source of the Nerve Action Potential*

Another support for the connection of ACh with the nerve action potential was found on the basis of the energy transformations involved. The electric organ offers a suitable material for investigating the chemical reactions which supply the energy for the nerve action potential. Both electrical and chemical energy released are in the range of possible measurement, whereas in ordinary nerves, the methods available are not adequate for quantitative analysis. The organ of *Electrophorus electricus,* for reasons discussed elsewhere, is again particularly favorable for such a study.

Measurements carried out on these fish have revealed some facts about the chemical source of energy for the action potential. The electric energy

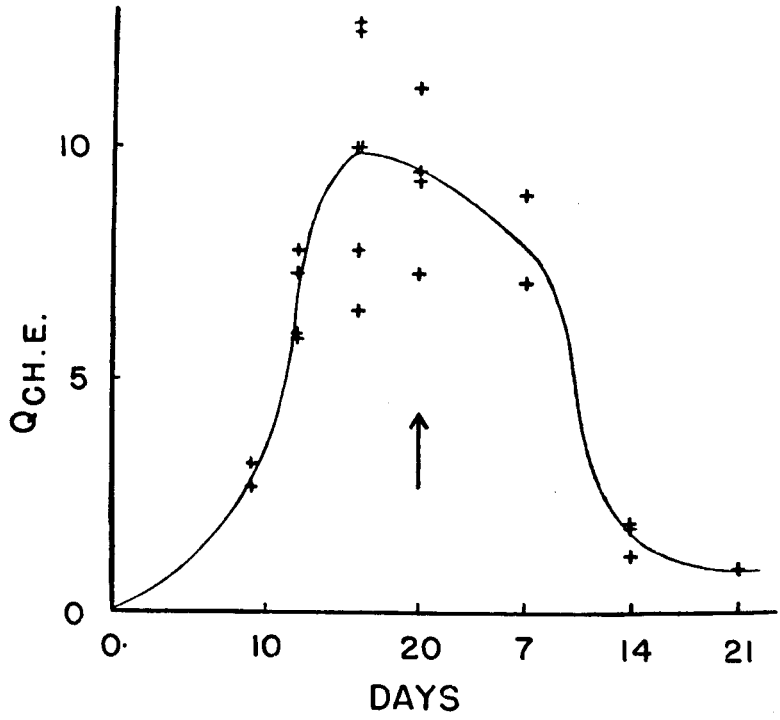

FIG. 2

Course of QCh. E. During Growth in Breast Muscle of Chicken.

Abscissae: Days of incubation and after hatching. The arrow marks the day of hatching. Ordinates: QCh. E. (mg. of ACh split by 100 mg. of fresh tissue in 60 min.)

released externally per gram and impulse was found to be 8 x 10^{-6} gcals. This is the maximum external energy which may be obtained under the condition that the external resistance is approximately equal to the internal. The total electric energy is in this case about six times as high as the external, or about 48 x 10^{-6} gcals. per impulse per gram electric tissue. Under the same conditions and tested simultaneously, the energy released by the breakdown of phosphocreatine was found to be about 32 x 10^{-6} gcals. per gram and impulse (average of 15 experiments). Lactic acid formation released about 17 x 10^{-6} gcals. per gram and impulse, averaging 7 experiments (20). The energy of the lactic acid is probably used to phosphorylate creatine just as in muscle where the phosphopyruvic acid transfers its phosphate via adenosine triphosphate to creatine ("Parnas reaction"). The figures are consistent with the conclusion that energy-rich phosphate bonds are adequate to account for the energy of the action potential. Hence, if the primary alterations of the surface membrane during the passage of

the impulse are due to the release of ACh, the figures suggest that phosphate bonds may yield the energy for the synthesis of ACh.

The amounts of ACh actually released during a discharge are not known. But the amounts of ACh which may be split by one gram of electric tissue during one discharge may be used as indication. This amount is about 5×10^{-6} millimole. The amount actually released may be smaller, since the enzyme may be present in excess, but the figures indicate the order of magnitude. The amount of prosphocreatine actually split per gram and impulse is about 3×10^{-6} millimole. Thus, the amounts of ACh and phosphocreatine metabolized seem to be of the same order of magnitude.

One of the essential facts supporting the new concept is, as repeatedly emphasized, the extremely high concentration of choline esterase at the neuronal surface, making possible a rate of ACh metabolism sufficiently high to parallel the electrical changes. In electric tissue, the rate may be at least 100,000 times, but probably close to 1,000,000 times as high as that of respiration. We have to distinguish, however, between the possible rate and the absolute amounts metabolized. ACh is released and hydrolyzed within a very short period. The actual duration of 1,000 discharges is about three seconds. The recovery may require one to two hours during which the rate of respiration may be increased. If the absolute amounts of ACh possibly metabolized are compared with those of the phosphorylated compounds actually metabolized and with the rate of respiration, a satisfactory picture is obtained.

9. *The Formation of Acetylcholine by Choline Acetylase*

It appeared essential to test whether or not energy-rich phosphate bonds are really the energy source of acetylcholine formation as suggested by these investigations. Evidence for the correctness of this conclusion would show that the energy of the primary recovery process is really used for the resynthesis of the compound, the release of which is supposedly essential in initiating the nerve impulse. It would, therefore, at the same time, constitute a new support for the assumption that the release of the ester may be essential for the alterations of the membrane necessary for the propagation of the impulse.

In accordance with this assumption, a new enzyme, choline acetylase, could be extracted from brain which in cell free solution, under strictly anaerobic conditions in the presence of adenosine triphosphate forms ACh (21, 22, 24, 25).

The enzyme may be extracted from homogenized brain. From one gram of fresh rat or guinea pig brain, an enzyme solution may be prepared which forms 120 to 140 μg of ACh per hour (24). Up to 200 μg/g/hr were ob-

tained. In larger brains (cat, calf) the values are considerably lower.

Presence of eserine and fluoride is necessary to inhibit the action of choline esterase and adenosine triphosphate respectively. Inhibition of the latter enzyme is necessary since otherwise the breakdown of adenosine triphosphate occurs too rapidly. Fluoride inhibits this breakdown but it does not interfere with the transfer of energy-rich phosphate bonds as has been shown by Ochoa (30).

The enzyme may also be extracted from powder of acetone dried brain. Extracts prepared from one gram of powder form on the average 0.6 to 1.0 mg. of ACh per hour, but in some experiments a formation up to 1.5 mg. of ACh has been obtained. In these extracts the enzyme is about twice as pure as in those obtained from fresh tissue: One gram of protein may form three to four milligrams of ACh in 60 minutes (22).

Since acetone inactivates choline esterase, this enzyme is largely or sometimes completely inactivated in the extracts prepared from powder of acetone-dried brain, so that addition of eserine may have either a small effect or practically none on the formation of ACh. Adenosine triphosphatase is also removed in extracts from acetone-dried brain. No addition of fluoride

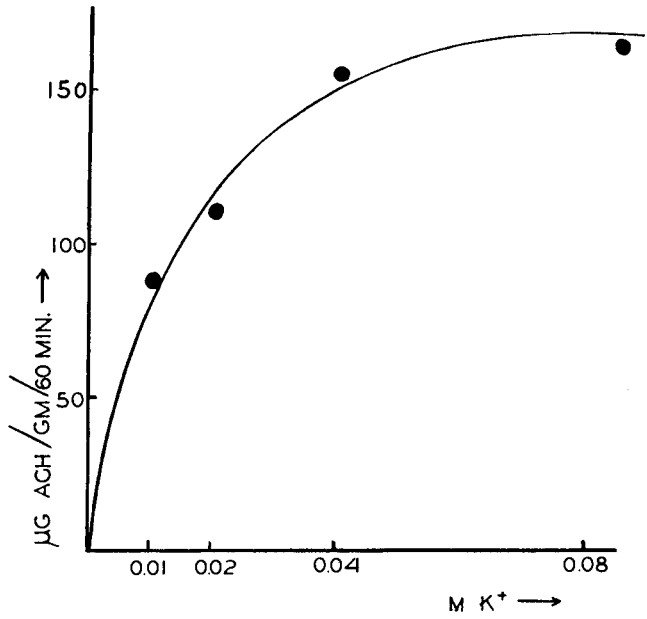

FIG. 3

Rate of Acetylcholine Formation in Brain Extract as Function of the Concentration of Potassium.

is therefore required. For instance: in one experiment 820 μg of ACh were formed per gram an hour, with no eserine 780 μg, without fluorine 810 μg. It has thus been demonstrated that the enzyme mechanism responsible for the formation of the ester is not identical with the hydrolyzing enzyme.

The enzyme requires the presence of potassium in high concentration, close to that found in brain (Fig. 3) (22). It contains active sulfhydryl groups which are readily inactivated by monoiodoacetic acid or copper in low concentration. The —SH groups are easily oxidized by air. The rate of ACh formation is considerably lower in air than in strictly anaerobic conditions. Addition of cysteine increases the rate of formation markedly (24). For instance, in one experiment carried out in air, 74.5 μg of ACh were formed per gram per hour; with cysteine 115 μg of ACh. In nitrogen without cysteine 143 μg were found.

On dialysis, the enzyme rapidly loses its activity. This suggests that the enzyme requires a coenzyme. Addition of potassium (in 0.04 M. concentration), or glutamic acid (in 0.02 M. concentration), reactivates it partly if extracts of fresh tissue are used. Only the naturally occurring 1 (+)- form is effective (21, 22, 24). With potassium and glutamic acid, 50 to 80 per cent of the original activity may sometimes be restored (see Fig. 4). Further addi-

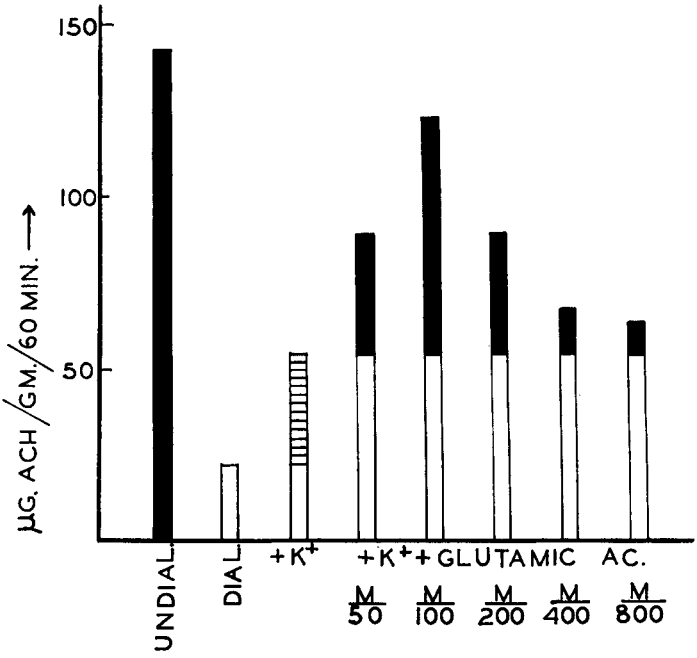

FIG. 4

Effect of 1(+)- Glutamic Acid in Varying Concentrations on Dialyzed Choline Acetylase without and with 0.04 M. K^+.

tion of cyanide or replacement of glutamic acid by cysteine may reactivate the enzyme nearly completely. 1 (+) –alanine has also some effect; other amino acids have either a weak effect or none. Citric acid has an effect nearly as strong as glutamic acid, whereas dicarboxylic acids have practically no effect (21, 24).

The effect of both glutamic and citric acid, although weaker than that of cysteine, appears of interest. Cysteine, like glutathione, may enhance the activity of many enzymes containing –SH groups which have been oxidized during preparation, whereas the effect of glutamic acid cannot be explained by action on the –SH groups of the enzyme.

Price, Waelsch, and Putnam (31) have observed a favorable effect of glutamic acid on patients suffering from petit mal attacks. The interest in the effect of glutamic acid on choline acetylase is, by this clinical observation, further increased, since a relation between the clinical effect and the enzyme reactivation is easily conceivable.

The oxidation products of amino acids, *i.e.*, α–keto acids, (pyruvic, phenylpyruvic, oxyphenyl pyruvic acid and α–keto glutaric acid have been tested) have a strong inhibitory effect on the formation of ACh when present in 10^{-3} to 10^{-4} M concentrations (22). These concentrations are close to those which occur in living cells. Pyruvic acid is known to be a "physiological anticonvulsant" (32). The strong inhibitory effect of α–keto acids on the formation of ACh is therefore obviously of physiological as well as clinical interest.

10. Choline Acetylase in the Axon

Feldberg (8) claimed recently to have evidence for a synthesis of ACh in the nerve fiber *in vitro* and that this synthesis becomes impossible two days after section of the fiber, *i.e.*, at a time when conductivity is still preserved. He considers his results as a difficulty for the assumption of a role of ACh in conduction.

The conditions and methods used by Feldberg are, however, inadequate for testing the synthesis of ACh. He worked under conditions where he had no supply of energy necessary for the synthesis: He chopped nerve fibres of cats and sheep and kept them for two hours in oxygenated Locke solution, assuming to have the same condition as Quastel working with brain slices. There is however, a decisive difference: Brain slices have a high rate of respiration since the cells remain intact and there is therefore sufficient energy to build up adenosine triphosphate, which, as shown by Nachmansohn and his associates, is the immediate energy source for ACh synthesis. In chopped fibers, even of cold-blooded animals, but still faster in those of warm-blooded animals, the respiration disappears very rapidly.

In Feldberg's experiment, there is therefore no energy source available. He found after two hours of incubation, a difference of one or two μg ACh per gram nerve between control and experiment. We consider this difference due to different methods of extraction for control and experiment after incubation. His own figures show that if the two methods of extractions are compared without incubation, the difference is the same as between control and incubated nerve. So for instance (page 44), he finds in the control 0.9 μg per gram, after two hours of incubation 2.0 μg per gram, or an increase of 1.1 μg per gram. But if he compares the two extraction methods, the two values given in the same series are 7.8 and 10.9 respectively, or an absolute increase of 3.1 μg per gram. For the first experiment, Foldberg calculates a difference of 122%, in the second case of 40%, and concludes that the higher increase in per cent is a real synthesis. There is obviously no evidence that under these conditions, formation of ACh occurs.

It has been tested whether the formation of ACh by choline acetylase occurs in the peripheral fiber as well as in the brain. This should be the case if the new concept and the conclusions of the previous investigations on the role of the ester in the axon are correct. It has been found that choline acetylase may be extracted from peripheral nerve fibers as well as from brain; the rate of formation of ACh in extracts prepared from the sciatic nerve of the rabbit was found to be 70 to 90 μg per gram an hour (23). This is remarkably high, especially in view of the large amount of connective tissue and myelin present in those fibers.

Two days following section of the sciatic nerve when conduction is still possible, the enzyme concentration has decreased by only 25%. On the third day when conduction has stopped, there is a decrease of about 65%. The high rate of formation of ACh in the peripheral fiber and the observations on the relation between function and ACh synthesis during degeneration are consistent with the concept that ACh is directly associated with conduction.

11. Nerve Action Potential and Inhibition of Choline

One of the essential supports of the older theories of "neuro-humoral" or "chemical" transmission was the observation that when applied to synaptic regions, ACh may have a stimulating action. No action can be obtained with the ester when applied to the axon. Lorente de Nó kept bullfrogs' sciatic nerves in 2% solution of ACh for many hours and did not find any effect on conductivity. He considers his failure to obtain an effect on the axon by ACh as proof against the new concept of the role of ACh in the mechanism of nerve activity. It has been pointed out (27).

that ACh is a quaternary ammonium salt and that such compounds generally cannot penetrate the lipoid membrane. Therefore, these compounds can be expected to have no effect on the axon since axons are always surrounded by a lipoid membrane even though it may be rather thin.

The problem has been approached in a different way (2). If ACh is the depolarizing agent and if the function of choline esterase is to remove the active ester so that polarization again becomes possible after the passage of the impulse, then, inhibition of the enzyme should alter and, in sufficiently high concentration, abolish the nerve action potential.

Eserine is known to be a strong inhibitor of choline esterase. This compound is a tertiary amine and may therefore, if undissociated, penetrate the lipoid membrane. Experiments carried out on the giant axon and on the fin nerve of squid have shown that eserine alters and finally abolishes the nerve action potential. Within a few minutes in eserine, amplitude, length, and duration of the action potential recorded with the cathode ray oscillograph are markedly changed, and in 15 to 20 minutes the conductivity has been abolished. When the nerves are put back into sea water they quickly recover and conductivity reappears. The reversibility of the effect is consistent with the fact that inhibition of choline esterase is easily reversible *in vitro*.

Strychnine, another inhibitor of choline esterase, was also found to alter and in higher concentrations to abolish the nerve action potential reversibly.

Thus a new relationship has been established between enzyme activity and nerve action potential, in this case using the peripheral axon.

Prostigmine has, *in vitro,* the same effect as eserine, but it has no effect on the nerve action potential. Prostigmine is like ACh a quaternary ammonium salt and it cannot penetrate the lipoid membrane. This has been demonstrated by the following experiment. The axoplasm of the nerves kept in eserine was extruded and the presence of the compound was tested by the inhibitory effect on purified choline esterase. Even in thousandfold dilution the axoplasm from a portion of a single axon showed by the inhibition of esterase easily detectable quantities of eserine. The axoplasm of nerves kept in prostigmine had no inhibitory effect on choline esterase even when diluted.

These observations explain why ACh and prostigmine applied externally act only on nerve endings which do not have a myelin sheath but are inactive when applied to the axon. Only on electric tissue the power of ACh to produce an action potential may be demonstrated (9): Injection of ACh leads to changes in potential of the same direction as those observed during the discharge. But electric tissue is an accumulation of nerve endings which

in contrast to the axons are not protected by the lipoid membrane and therefore they do react. This may also be the explanation of the famous observation of Claude Bernard on the effect of curare, since, according to recent observations, the active principle of curare is a quaternary ammonium salt (12, 25). The peculiarity of the synapse to react to injected ACh can no longer be referred to a difference in the fundamental physico-chemical process underlying the propagation of the nerve impulse, but to the difference in histological structure.

III. Discussion

It may be of interest to discuss the neuro-humoral theory in the light of recent developments and to analyze the two basic experiments which form the main support for the hypothesis that the ester is actually liberated at the nerve ending and, having crossed the synapse or motor end plate, acts directly on the second neuron or on the muscle fiber. The two observations are: (1) The stimulating action of ACh when applied to synaptic regions and, (2) the appearance of the ester in the perfusion fluid following nerve stimulation. It has just been explained why the effect of ACh applied externally is limited to the nerve ending. In any case, a stimulating effect is not necessarily a physiological effect but may well be a pharmacalogical one. The same action may indeed be produced by other compounds.

Evidence for the appearance of ACh in the perfusion fluid following nerve stimulation was an important observation since it suggests that ACh may be connected with nerve activity. The appearance of a compound in the perfusion fluid, however, is not sufficient evidence for concluding that the compound acts outside the cell. Many compounds of intermediate cell metabolism may appear outside the cell. This is due to the fact that all enzymatic reactions follow a logarithmic curve. Therefore, if even the greatest part of a compound is rapidly metabolized by the intracellular enzymes, a small fraction may persist long enough to escape enzymatic action and leak out from the cell. This apparently may happen also to ACh in spite of the high concentration of choline esterase inside the cell. But, as we know from experiments on degenerated nerves, only part of the enzyme is localized inside the cell. Part of it is present at the outside. The enzyme outside may serve to protect the effector cell from the disturbing effect of ACh leaking.

In order to verify the assumption that the amount of ACh actually released from the nerve ending is sufficiently high to produce a stimulating effect on the second unit, Dale and his associates attempted two sets of experiments. They determined the minimum amount required to produce a

stimulus and compared it to the amounts released. However, in both cases tested a puzzling discrepancy was found: In the case of the superior cervicle ganglion only 1/40,000 of the amount of ACh necessary to produce a single response appeared in the perfusion fluid per impulse. In the case of the muscle, only 1/100,000 of the amount of ACh necessary to produce a single twitch was collected. This difference is so considerable that in itself it forms a serious difficulty for accepting the idea that ACh is the direct transmitter of the impulse. The situation is complicated even more by the fact that these infinitely small amounts of ACh can only be found in presence of eserine which should inhibit their destruction. The high enzyme concentration at the neuronal surface forms a formidable barrier for the crossing of the ester. Even without regarding the existing discrepancy, it is very difficult to believe that under physiological conditions, that is, in the absence of eserine the small amounts of ACh released can cross the barrier and still arrive in sufficient concentrations for producing a response. The small amounts found under these conditions are easily explained if we assume that ACh is released inside the cell and that the amounts which appear in the perfusion fluid are those which escaped hydrolysis and have been preserved due to the presence of eserine.

The investigations presented are an attempt to conceive the mechanism of nerve activity in terms of physics and chemistry which is the ultimate goal of all our research in biology and medicine. One or two facts, however well established and suggestive, would not be sufficient for any theory, but if a great number of facts point in the same direction, then they support each other and potentiate the value of each of them. Even so, many factors remain unknown, and since biological mechanisms are very complex, dogmatic statements should be avoided. New facts may change the picture. For the time being, the concept presented appears to be the best integration of the great quantity of physical and chemical data available on the mechanism of nervous function.[1]

DISCUSSION

R. Beutner: The fundamental difficulty in electrophysiology is lack of knowledge concerning the electrical mechanism capable of producing such electrical variations as are observed on nerve and muscle. The whole field is approximately in the same condition as was our knowledge on a disease like beri-beri before the vitamins were discovered; at that time, beri-beri was investigated by bacteriological methods and a causative bacillus found. In electrophysiology an hypothesis has been substituted for the lacking knowledge on the electrical mechanism. This is the widely adopted theory of selective ionic permeability introduced by Ostwald in 1895 as a working hypothesis,

[1]This lecture is based on an article written for "Currents in Biochemistry," edited by David E. Green (Interscience Publishers, in press).

later tentatively introduced into electrophysiology by Bernstein. Although widely adopted, the theory has no basis in facts. It operates on assumptions which are foreign to electrochemistry, postulating the existence of hypothetical membranes in living tissues permeable to only one kind of ion. Michaelis and his collaborators believed that dried collodion membranes had such properties. But thorough investigation of the dried collodion membranes shows that they operate through an entirely different mechanism.

T. C. Barnes and the speaker have tried to find a new foundation for basic electrophysiological questions by setting up artificial cell systems, so-called "oil cells," in which certain materials as occurring in living tissues are placed in mutual contact with each other. For instance, cholesterol or other lipoids are in contact with saline and the existing potential difference is measured. Subsequently, diminutive amounts of acetylcholine are added to this saline in dilutions of 1 to a million or less, and this diminutive addition produces sizable changes in the electrical potential difference. I believe that we can claim that such *in vitro* experiments are better than the remote models with which the speaker experimented in the earlier development of this line of work. At that time oils were used consisting of substances such as nitrobenzol and guaiacol, never present in living tissues. Our experiments with lipoids suggest at once a relationship between the negative variation of nerve and the acetylcholine generated in it since obviously the acetylcholine when generated in nerve comes in contact with lipoids and thus produces a phase boundary potential difference as it does in our *in vitro* experiments.

In our *in vitro* experiments lipoid layers are used which are several centimeters thick. In contrast, the lipoidal membranes contained in nerve are only a few millimicrons or at best microns thick. We assume that the acetylcholine is generated inside of such a thin membrane on one side, thus producing the up-stroke of the spike potential or negative variation. As the acetylcholine diffuses to the other side of the thin membrane—a distance of only a few millimicrons—it reaches the phase boundary on the opposite side creating a potential difference of equal and opposite magnitude; this would be the cause of the down-stroke of the spike potential.

The essential difference of the explanation to which we have been led through these *in vitro* experiments is the existence of phase boundary potential differences on either side of the lipoid membrane, whereas the old ionic permeability theory assumes the existence of only one single potential difference located inside a thin membrane and entirely neglecting the existence of the two phase boundary potentials, the existence of which is brought to light by the study of thick lipoid layers made of biological material.

An important point is the increased electrical conductivity of the nerve after stimulation, an effect which the old permeability theory attempts to explain through greater permeability of the membrane. However, the thick lipoid layers of our models are rendered better conducting by addition of acetylcholine to them which leads to the conclusion that the increased conductivity of the nerve after stimulation is also the result of better conductivity of the lipoid material of its membranes. This better conductivity of the lipoid need not be associated with greater permeability.

W. T. Salter: I simply should like to ask Doctor Nachmansohn to elaborate upon his very important work by a statement about adrenergic substances. I was fortunate enough, a few years ago, to hear first-hand some of his important work on acetylcholine. Of course, we were all interested in the very important advances he was making in describing how the transfer of energy occurred in connection with nerve conduction. What intrigued me most, however, was the pharmacologic aspects of the

problem. Dr. Nachmansohn was working with a substance which pharmacologists had studied for years as an important element in the field of the autonomic nervous system. In that field, a good deal of the earlier description of physiologic phenomena was couched in terms of an antagonism between cholinergic and adrenergic substances. One cannot help wondering whether the same thing may not be true in connection with the central nervous system. For example, Walter Cannon long ago postulated that epinephrine would increase the action of the nervous system and would revive it under circumstances calculated to produce fatigue. In more recent years, Burn at Oxford has also shown that epinephrine has an important influence upon nervous action within the spinal cord. Certainly epinephrine has an important effect on synaptic transmission.

In view of these actions of adrenergic substances, one cannot help wondering whether some sort of interplay between cholinergic and adrenergic substances does not occur also in the field which Doctor Nachmansohn is studying. Therefore, I should like him to discuss briefly the possibility that adrenergic hormones may play an important part in the physiology of the brain and the central nervous system. Is there any evidence in his work that adrenergic and cholinergic substances exhibit some sort of physiologic interplay?

R. G. Hoskins: The previous speaker mentioned "the adrenalin reaction." That phrase should not be used unless more adequately particularized. The response to adrenalin may vary diametrically, depending upon conditions. For instance, it may stimulate or inhibit intestinal activity. It can increase blood pressure or decrease it. Generally speaking, if applied abruptly, adrenalin brings out the traditional sympathetic-stimulating effect. But if the drug is applied gradually, as by slow intravenous infusion, it proves to be a sympathetic depressant.

D. Nachmansohn: It appears necessary to make a clear cut distinction between facts and interpretations. All of the experimental evidence presented indicates that the release of acetylcholine is an intracellular process closely associated with the nerve action potential. Without these facts any theory connecting acetylcholine with the action potential would be baseless. How the finer mechanism of this association may be pictured is admittedly still open to discussion.

The interpretation given is based on the membrane theory. This theory may not be the final word, but it is widely accepted among physiologists, as Dr. Beutner himself admits, and it appears at present to be the best assumption available. It is not appropriate to attempt to discuss fully the pros and cons of the membrane theory, at this point, but I would like to mention recent observations of Webb and Young which seem to give a strong experimental support for the theory. Those authors find that the diffusion potential which would arise if the membrane were permeable to potassium ions during the passage of the impulse, is very close to the action potential observed. This interesting observation as well as the well known fact that following the passage of the impulse potassium ions leave the nerve fiber indicate that the membrane theory is more than a pure speculation, although it is probable that sooner or later it will be modified.

The correlation found between the rate of acetylcholine metabolism and voltage may be explained in two ways as outlined in the lecture: The ester may either produce electromotive force or it may decrease the resistance. Both are possible. Dr. Beutner pleads for the first possibility on the basis of his model experiments. He asks why should it not be possible that acetylcholine produces electromotive force in the nerve membrane as it does in his model experiment? It certainly is possible, but a model

experiment can only show what is possible. It never has the strength of an argument derived from experiments which were carried out on the living cell itself. From experiments on nerves, there is, thus far, no conclusive evidence available that electromotive force is actually produced, whereas the experiments on the giant axon and on the electric organs mentioned indicate that a sharp drop of resistance does occur.

Therefore, it seems to me that at present a possible interpretation of the relation found is the assumption that acetylcholine acts on the surface by decreasing the resistance rendering the membrane permeable to anions; but I am not committed to that and would gladly accept another interpretation if any new facts were presented demonstrating convincingly that electromotive force is actually produced during the passage of the impulse. Nothing is more dangerous in the interpretation of biological mechanisms than dogmatic statements. As Hopkins remarked at the International Congress in Stockholm in 1926: "All dogmatic statements about any aspect of the phenomena of life is apt to be checked by the ultimate discovery that the living cell is before all things a heretic."

Dr. Salter's question points indeed to a most intriguing aspect of the problem, one which promises interesting developments. The material presented concerns exclusively the role of acetylcholine in the physico-chemical mechanism of nerve activity. The data indicate that the ester has essentially the same role in the conduction of the nerve impulse in all nerves, whether central or peripheral, afferent or efferent, vertebrate or invertebrate.

No comparable data is available to permit any hypothesis by which physico-chemical adrenaline acts. Previous theories that adrenaline is the antagonist of acetylcholine and acts as an inhibitor where acetylcholine acts as an accelerator or vice versa cannot be maintained in the light of recent observations. Dr. Hoskins' statements form an excellent illustration. In his extremely stimulating paper in the last *Physiological Reviews,* Burn arrives at interesting conclusions as to the effects of adrenaline. He too abandons the older schematic and rather simplifying ideas of an antagonism to acetylcholine. I agree with Dr. Salter that there is reason to believe in a role of adrenergic substances in the brain and central nervous system as well as in the periphery. Sooner or later, we will come to a better understanding of the precise function of these compounds and their interplay with acetylcholine, but at present, there is no clue as to the intrinsic mechanism of the action of adrenaline.

G. Pincus: I should like to ask Dr. Nachmansohn if the apparent inability to recover acetylcholine after stimulation, as tried by Dale, can in any way be accounted for quantitatively by amounts of esterase inside the nerve?

D. Nachmansohn: Yes, it can. Without eserine, no traces of acetylcholine are recovered in the perfusion fluid according to Dale and his associates. Consequently, there is no reason to believe that acetylcholine appears outside the cell under physiological conditions. This may be different if the cells are damaged by long perfusion. The latter may be the explanation for the fact that Kibjakow did find acetylcholine in the perfusion fluid without eserine. He was, in fact, the first to demonstrate the release of acetylcholine from preganglionic fibers and to suggest a "chemical mediation" by acetylcholine from neuron to neuron. His evidence was not accepted because Dale and his associates did not find any acetylcholine in the absence of eserine and his observations were therefore discredited. But, I believe that this is not justified. It is, in fact, the same situation as with the observations on the heart; Ascher claimed that Loewi's observations can only be reproduced in hypodynamic hearts. It seems highly probable that some kind of damage is necessary in order to find acetylcholine outside the nerve cell, but the amounts found are extremely small even in the

presence of eserine. In the superior cervical ganglion of cats, it is less than 1% of that which can be metabolized *inside* the cell within a millisecond. This is an order of magnitude which we would expect if a compound is an intermediate metabolite but leaks out because the mechanism of its removal has been impaired. But if even under these conditions, the amount which appears outside the cell is only 1/100,000 of that necessary to produce a response, this finding cannot be considered as evidence for "chemical transmission." It appears difficult to believe that without eserine, *i.e.*, with the physiological barrier intact, the ester released would be able to cross the synapse and arrive at the membrane of the effector cell in sufficiently high concentration for producing a stimulus. It would be necessary to find amounts of acetylcholine of a very different order of magnitude outside the cell in order to make the assumption of a direct chemical transmission more acceptable, even without considering all other obstacles. The small amounts found are easily conceivable in the light of the new concept.

M. L. Tainter: I wonder if I could ask Dr. Nachmansohn to comment on a related problem? He has presented data establishing that the acetylcholine is pretty well locked up in the cell. I would appreciate his telling us something about the distribution of the choline esterase. I have had occasion to see massive intravenous injections of choline esterase which however produced only insignificant increases in the blood choline esterase level. I wonder if he has an explanation for this?

D. Nachmansohn: What preparation of esterase did you inject?

M. L. Tainter: Both true and pseudocholine esterase.

D. Nachmansohn: I do not accept either these terms which seem to me not a very lucky choice nor the criteria on which they are based. There exists an enzyme in the body which has the specific function to remove acetylcholine during nerve activity. This enzyme we call choline esterase. Besides, there are a great variety of other not yet specified esterases. They too, may be specific but for another substrate. The choline esterase too has not an absolute but only a relative specificity. This is not surprising since the ester linkage of acetylcholine has no peculiar features. But by testing a number of compounds, we have established a pattern which makes it possible to distinguish choline esterase from other esterases. In this way, it has been demonstrated that the specific choline esterase is found in all nerve tissue as outlined in the lecture.

In human serum, the esterase is not choline esterase but an unspecified esterase. The observation that intravenous injection of a number of esterase preparations do not increase the level of the esterase in the blood is not surprising. Even in the test tube, some esterases, especially if purified, do not maintain the activity for a long time except under special precautions. Many enzymes introduced into a foreign organism lose their activity. It is, however, not impossible that some preparation may be found which keeps its activity for quite a period of time and which increases the level of the esterase in the blood.

I doubt, however, that any effect can be expected from the activity of an esterase injected into the blood. The effects observed may not be due to the esterase activity, but may be some side effects which are not yet explained. As outlined in my lecture, there is an overwhelming evidence for the assumption that the release and removal of acetylcholine is an intracellular process. The traces found outside the cell in perfusion fluids are artefacts due either to severe damage to the nerve cell or to the poisoning of the mechanism which physiologically prevents this appearance outside the cell, namely, the inactivation of choline esterase by eserine. It is difficult to believe that an esterase injected from outside should have any effect on the acetylcholine metabolized inside the nerve. The enzyme molecule is large and would probably never reach the site of action.

An influence on an intracellular reaction may however be obtained by the use of small molecular compounds which penetrate into the cell and which may interact with the active enzymes, either inhibiting or accelerating their activity.

(The first session of the 1945 Conference is adjourned).

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Hormones and Mating Behavior in Vertebrates[1]

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I. Introduction

In electing to discuss the effects of hormones upon mating behavior in vertebrates I had two purposes in mind: first, to sketch in broad outline the evidence suggesting that certain hormones do in some fashion affect behavioral responses, and second, to advance tentative interpretations regarding ways in which endocrine products exert their control over an animal's response to its environment.

In approaching the first of these objectives we will have time to consider relatively few of the studies reported in the literature; but I have done my best to make a representative selection. Although most of the existing data have been derived from experiments with lower mammals and birds, I have when possible included the results of investigations upon fish, amphibia, reptiles, and primates with a view to implementing a series of phylogenetic comparisons.

An appreciation of the similarities and differences between various species, orders, and especially classes of animals is fundamental to the realization of an evolutionary interpretation of the problems at hand; and it is becoming increasingly evident that the method of phylogenetic comparison constitutes a promising approach to the practical understanding of the effects of hormones upon human behavior. That such an understanding can ever be reached by the study of man alone is doubtful, but it must be sought in an exposition of general principles, a comprehension of the sequence of evolutionary changes of which the hormone-behavior relationships of man represent merely one end-point.

II. Survey of the Evidence

It is established beyond reasonable doubt that the mating activities of many species are heavily dependent upon particular endocrine secretions. To illustrate the sort of relationship which exists we may consider first the ovarian control of courtship and mating in females of several vertebrate species.

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[1]All of the author's personal researches cited herein were supported by grants from the Committee for Research in Problems of Sex, National Research Council.

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1. OVARIAN HORMONES AND MATING BEHAVIOR IN FEMALES

a. Lower Mammals and Submammalian Vertebrates

The several reactions which combine to form the well-integrated pattern of estrous behavior in the female cat include approach to the male, loud purring, head rubbing, rolling on the ground before the male, and executing various subsequent adjustments to the male's reactions. The male's initial response usually consists of exploring the female's genital region, and this investigation results in an intensification of the female's courtship behavior. The first step in the copulatory act occurs when the male grips with his teeth the loose skin of the female's neck. In response to this stimulus the female assumes the mating crouch, resting the anterior part of her body upon the flexed forelimbs and elevating the hindquarters. As the male mounts, the female moves her tail to one side and displays the "treading" reaction which consists of alternate stepping movements of the hind limbs. After several preliminary copulatory thrusts the male achieves full intromission which is maintained for a very short time.

Almost at the moment that penis insertion occurs the female emits a loud cry, and within a few seconds she turns and dislodges the male if he has not dismounted voluntarily. As soon as the male withdraws, the female passes into a frenzy of "after reactions" which consist of vigorous rolling, twisting and turning, and intermittent licking of the vaginal orifice. The after reactions may continue without abatement for several minutes; and during this interval the female actively repells the male if he approaches. Termination of the after reactions marks the revival of receptivity, at which time the female will permit and even seek additional copulatory contacts.

This complex sequence of reactions can be shown to depend upon estrogen. Spayed females never exhibit estrous behavior no matter how frequently and vigorously they may be solicited by the male; but if the ovariectomized female is injected with estrogen she comes into heat within one or two days and proceeds to display the entire mating pattern when placed with a sexually active male (6). Furthermore, although normal females usually remain in heat for only a few days, the spayed individual may be maintained in a constantly receptive condition for months by the continued administration of small doses of estrogen (author's unpublished observations).

As far as is known this behavioral change can be elicited exclusively by estrogenic substances. It apparently does not involve directly the activity of endocrine organs other than the ovary, for the normal sexual pattern is obtained in spayed and hypophysectomized females under the influence of exogenous estrogen (53).

In some fashion concerning which we know very little the follicular hormone brings about a pronounced change in the behavior of the female cat; and a similar situation appears to obtain in the case of females of many other species.

The female dog's heat reactions, like those of the cat, are elicited by estrogen, for the spayed bitch displays all of the elements of the normal mating response when treated with estrogen (51, 49).

The sexually receptive reactions of female guinea pigs and rats are heavily dependent upon ovarian hormones, but in these species estrogen does not act alone. Studies of the induction of estrous responses in spayed individuals reveal that normal mating activity is produced only if the female is primed with an injection of estrogen and then subjected to the administration of progesterone approximately 48 hours later. When this regime is followed, the spayed animal comes into heat some 24 hours after progesterone treatment (12, 19, 20).

We know less about the hormonal control of courtship and mating in submammalian than in mammalian species, but there is good reason to believe that ovarian hormones are intimately involved in the breeding activities of at least some birds, reptiles, and fishes.

Bilaterally ovariectomized poulards do not squat and allow the courting rooster to tread, but the injection of estrogen restores this form of behavior (34. 35). Gonadectomized female laughing gulls, *Larus atricilla,* do not respond to the male's courtship displays, but estrogen treatment evokes full sexual receptivity including the assumption of the typical stooping posture and the execution of "food-begging" responses which constitute an important preliminary to coition (64).

When she is in mating condition, the female lizard, *Anolis carolinensis,* displays various postural reactions which facilitate the male's copulatory performance. These responses cannot be elicited in spayed or out-of-season females, but administration of estrogen causes the appearance of typical "estrous" behavior in gonadectomized specimens (45).

The sexually active female jewel fish, *Hemichromis bimaculatus,* indulges in characteristic swimming movements in response to the male's advances. During courtship the male and female cooperate in cleaning the surface of a smooth stone (in the laboratory an inverted flower pot) in preparation for egg laying. At the height of courtship the pair swims in slow circles over the surface upon which the eggs are to be laid, the female depositing the eggs and the male fertilizing them as soon as they are laid. The spayed female jewel fish does not respond to the male's courtship, and will not indulge in cleaning behavior or go through oviposition movements; but these reactions reappear in the operated individual following the administration of estrogen (63).

The small, brilliantly colored Siamese fighting fish, *Betta splendens,* exhibits a very interesting reproductive pattern. The male first builds a bubble nest by spitting out one bubble at a time at the surface of the water. When this structure is completed the female joins the male directly beneath the nest; whether she is lured into position by the male or pays the visit on her own initiative is not known. At any rate soon after she arrives the male enfolds her in a nuptial embrace, placing his body immediately above and at right angles to the female's, and bending sideways in a complete half circle so that his head hangs down on one side and his tail on the opposite side of the female. While the pair is in this position the female extrudes a few eggs at a time, and they are fertilized by the male whose genital pore is in close approximation to hers. As the eggs drift slowly downward through the water the male releases the female and darts after them, takes them in his mouth, and swims upward to the bubble nest where each egg is carefully deposited. In some instances the female cooperates in this transferal of eggs. Although the remainder of the reproductive pattern is equally interesting it is of no importance for present purposes.

The significant point is that the female's role in this complex series of responses appears to depend heavily upon ovarian hormones. It has been shown that such behavior cannot be elicited in spayed female fighting fish unless they are injected with estrogen, and that when such hormone administration is carried out the entire pattern reappears in normal form (63).

Many other experiments have been conducted with various species of lower vertebrates (11), and although some exceptions may exist, it is clear that in the majority of cases the mating activities of the female occur only in the presence of critical concentrations of ovarian hormones.

b. Primates

The universality of the foregoing generalization is sharply challenged as soon as we turn our attention to the primates. It is true that female monkeys usually do not execute the sexual presentation or permit copulation by the male unless the ovaries contain mature follicles. Furthermore the spayed female rhesus ordinarily is sexually inactive, and can be brought into full breeding condition by the administration of estrogen (5). Nevertheless, observers who have studied the behavior of these monkeys in the wild state report that copulatory presentation occasionally takes place when the female is not in estrous, and it has been noted that in this species the female sometimes employs her sexual charms for what are called "various social purposes" (28).

If in the female monkey we may detect the beginnings of emancipation from the control of ovarian hormones, it is apparent that the chimpanzee exhibits an even higher degree of freedom from endocrine control over her mating activities.

The female chimpanzee's ovarian cycle does of course have a definite effect both upon her sexual responsiveness and upon her ability to arouse the male. During the female's follicular phase a male in an adjoining cage shows more sexual excitement (erection) prior to the time that the two animals are allowed to enter the same enclosure; and during this same stage the female's interest in the male is heightened as reflected by several alterations in her behavior. She grooms the male more often, moves toward him or his cage more frequently, exhibits sexual presentation repeatedly, and permits copulation readily. During the luteal phase of the cycle the male-female relationships are altered, and the frequency of play noticeably increased (106).

In this anthropoid species, however, the actual occurence of copulation is strongly influenced by extraphysiological factors, and the female often shows sexual presentation and permits intercourse at any point in her ovarian cycle (104). In fact, despite the undeniable importance of ovarian hormones upon chimpanzee behavior, observers have suggested that mating behavior depends more upon individual differences and upon the consort than upon the sexual status of the particular female. Considering the evidence pertaining to the rhesus monkey and to the human, it appears that the position of the chimpanzee is intermediate between these two with respect to the extent to which such behavior is affected by cyclic fluctuation in the female's sexual status (106).

We have seen that the sexual behavior of females of lower mammalian species is rather rigidly controlled by ovarian hormones, that that of the monkey is somewhat more variable, and that in the chimpanzee even more latitude obtains between hormonal conditions and mating behavior. What then is the case with respect to the human female?

Here the evidence is ambiguous and opinion very much divided; but there is little doubt that sexual desire and the capacity for full sexual response are much less directly dependent upon ovarian conditions in woman than in the female of any other species. Some authorities would have it that gonadal hormones are almost without effect (39), while others conclude that, "the role of the hormone [in controlling the sex urge in women] is such that libido may be spoken of as a test tube chemical equation" (46).

The experimentalist is inclined to view somewhat skeptically the majority of endocrinological studies dealing with human sexual behavior. In the

first place we are still more or less in the dark as regards the meaning of "normal" in this field. Animal studies define normal sexual behavior in lower species in terms of observed responses shown by large numbers of individuals, usually derived from inbred strains, and tested repeatedly under reasonably constant conditions. Most investigators dealing with human material have up to the present been limited to verbal reports from relatively small, heterogeneous groups. Too frequently convention prevents even a frank verbal exploration of the situation; and even when this is not the case the subject's report of his or her own reactions is notoriously unreliable and subject to all sorts of conscious or unconscious distortion.

However, in spite of these inherent sources of error there is good reason for believing that sexual behavior in the human female is at most only partially dependent upon gonadal secretions. And, viewed in the light of more objective findings with regard to lower primate and sub-primate species, this presumptive fact points to a speculative but thought-provoking hypothesis. Phylogenetic comparisons suggest that in their sexual responses females of the more highly evolved species exhibit less rigid dependence upon ovarian hormones than do the lower forms. Furthermore the degree of independence seems to increase progressively from the lower to the higher primates. It may therefore be suggested that in the course of evolution, particularly during that phase in which the primate stock appeared and differentiated, the manifold changes which occurred included certain ones by virtue of which the female's mating activities became progressively less dependent upon gonadal hormones, and more subject to other forms of control. What these more recent, alternative forms of control may have been we shall reserve for subsequent consideration.

2. TESTICULAR HORMONES AND MATING BEHAVIOR IN MALES

Thus far we have considered only the sexual activities of female animals; but the role of gonadal hormones in the courtship and mating of the male is of equal interest. Our survey of this aspect of the main problem may begin with a consideration of that much-studied species, the Norway rat.

The sexual activity of rats, like that of most rodents, is extremely simple, —so much so in fact that the term "courtship" seems hardly appropriate. Nevertheless the pattern normally includes various precopulatory responses which appear to stimulate both the male and the female and to result in a rising tide of sexual excitement which culminates in coition.

When placed with an estrous female the male rat approaches and examines his cage mate, paying particular attention to the genital region and to the head. The receptive female responds to the male's preliminary investigations by darting away from him with a characteristic, hopping gait, and

then coming to rest in a semi-crouched position. Simultaneously she shakes her head very rapidly in the lateral plane, thus producing a distinct vibratory motion of the ears. The male follows, intensifying his explorations, and once more the female moves away. This pattern of brief retreat with a display of estrous reactions on the part of the female, accompanied by persistent pursuit on the part of the male, may continue for several minutes if the male is not extremely active sexually; but the majority of males soon dispense with further investigatory responses and proceed to mount the female when she comes to a stop at the end of one of her short, darting runs. The male's forepaws, which clasp the female's sides just behind her front limbs, are moved downward and backward in a series of fluttering, palpating movements. The male's intromittive attempts which follow consist of short, rapid, piston-like thrusts of the pelvic region.

Erection of the penis apparently takes place during the execution of the preliminary copulatory thrusts, after several of which the male executes a much deeper and more forceful thrust that inserts the penis in the vagina. Intromission is quite brief, lasting scarcely more than a second, and the male breaks away from his partner, releasing his clasp and throwing himself backward with a vigorous lunge which carries him several inches away from the female. Assuming a sitting position, the rat proceeds to manipulate and clean the penis.

There follows another copulation identical with the first, and then another and another. Intromission occurs once during each period of contact but it is not always accompanied by ejaculation. The emission of the ejaculate and formation of the vaginal plug takes place only after several completed copulations have been achieved.

When ejaculation occurs the observable mating pattern is plainly modified. Its first parts proceed as described, but after insertion is achieved the male does not abruptly terminate contact with the female. Instead, intromission is prolonged by firm pressure of the male's hindquarters against those of the female; and simultaneously the male releases his fore-paw clasp and slowly elevates both front legs. After several seconds he slips slowly off the female and displays the genital cleaning responses which follow ordinary copulation.

Prior to the occurrence of ejaculation the copulatory acts follow one another in quick succession, as many as five or six occurring each minute; but when ejaculation takes place the male gives every sign of temporary sexual fatigue. For several minutes thereafter he pays very little attention to the female, and her most active display of heat reactions cannot stimulate him to renewed sexual aggressions. Within five minutes or so his potency is restored and the mating activities are resumed.

a. Effects of Prepuberal Castration

What is the importance of testis hormone in the behavior described above? The answer is found in studies of the effects of castration and hormone therapy; but it is of interest to note that the effects of gonadectomy vary markedly according to the age of the male at the time of operation. If the testes are removed well before puberty, some rats fail to exhibit any copulatory responses when adulthood is attained (92). However, individual differences are marked, and many prepuberally castrated males respond to the receptive female with the occasional execution of incomplete sexual attempts. The male mounts the female, employing the normal sexual clasp but dismounting with no further display of coital behavior. Other individuals mount, palpate the female with the forepaws, and display pelvic thrusts, but then slip weakly off without showing the final, vigorous thrust essential to complete copulation. Upon rare occasions a very few prepuberal castrates show the complete copulatory pattern including the final thrust and backward lunge which terminates contact; but even these males do not display the ejaculatory response (15).

The effects of prepuberal castration upon the males of other mammalian species have been studied less extensively than in the case of the rat, but various observations indicate that a somewhat similar situation may hold.

Several experimenters have reported the occurrence of mating responses in prepuberally castrated male guinea pigs, although all agree that the sexual behavior is less vigorous and complete than that of the intact male (57, 87, 84).

I removed the testes from a pet cat before it became sexually mature, and several months thereafter noted that the animal frequently displayed recognizable elements of the normal mating pattern when offered a small pillow or folded cloth. These reactions included gripping the material with the teeth, crouching over it in a straddling position, and carrying out a few, weak pelvic thrusts. Semi-erection of the penis took place during some of these performances.

Observations of the sexual activity of a prepuberally castrated male chimpanzee have revealed a surprising amount of mating behavior. Confronted with a female in the stage of maximal genital swelling, this animal covered her repeatedly and showed as many as ten copulations within an hour's time, although ejaculatory responses never occurred (29).

It seems clear that the incompleteness and infrequency of the prepuberally castrated male's sexual performance is due directly to the absence of testis hormone, for in every species which has been so investigated the administration of androgen has resulted in the appearance of normal mating activity. Prepuberally castrated male rats (85), rabbits (40), and guinea

pigs (87) respond to androgen treatment in adulthood with an increase in the frequency of complete copulation and with the appearance of the ejaculatory reaction. The prepuberally castrated male chimpanzee which copulated but failed to ejaculate, reacted to androgen therapy with the execution of the ejaculatory pattern (29).

Findings such as those cited above suggest an important difference in the sexual activities of male and female mammals. When females of most mammalian species below the primate level are deprived of ovaries, whether the operation be performed before or after puberty, the manifestation of neat responses is abolished promptly and permanently. In contrast, even the prepuberally gonadectomized male often displays at least a small amount of incomplete and abortive mating behavior. There is, in addition, some slight indication that the frequency and completeness of such behavior on the part of the male may be noticeably greater in the case of the primates than in the lower forms.

b. Effects of Castration in Adulthood

If we turn to a consideration of the effects of castration in adulthood the data are more numerous and the difference between the sexes becomes more striking.

(1) *Fish.* The elaborate courtship and mating performance of the jewel fish and the Siamese fighting fish were described earlier, and it will be recalled that spayed females of these species fail to participate in this reproductive pattern. In contrast, if the male is castrated and the female intact the behavior may continue to appear for several months. Gonadectomized males exhibit normal nest building, courting, and fertilizing movements although they are of course sterile (63). Whether these reactions would eventually disappear in castrated fish we do not know, for the tests were not continued long enough to provide an answer.

(2) *Amphibia.* In the case of amphibia the evidence is more complete. Castration of the male frog during or just before breeding season is reported by some workers to be without any immediate effect upon the animal's tendency to approach and embrace other frogs; and normal mating activity may continue through the entire season (42, 89, 97). Nevertheless sexual behavior is eventually eliminated or greatly reduced, and several months after castration the male frog does not utter the sex call, fails to clasp or does so very weakly (37, 65, 80), and when placed upon a female the male shows none of the fertilizing movements typical of intact specimens (60).

That the postoperative loss of mating behavior is due to absence of gonadal secretions is indicated by the fact that castrated male frogs which

are given grafts of testis tissue (65), or injected with extracts of the same gland (90, 37), soon resume their display of all elements in the reproductive performance.

Several experiments have established that castration of the male skink, *Eumeces fasciatus* (69), and American chameleon, *Anolis carolinensis* (62), is followed by loss of sexual behavior, although data do not reveal how promptly the behavioral change follows the operation. It has been reported, however, that castrated males respond to andogren administration with the revival of the normal mating pattern.

(3) *Birds.* The behavioral effects of castration in male birds of domestic species are well known, and various studies indicate that the sequelae to this operation are approximately the same in wild forms. Male bronze turkeys, *Meleagris gallopavo* (66), ruffs, *Philomachus pugnax* (81), herring gulls, *Larus argentatus* (21), and laughing gulls, *Larus atricilla* (64), exhibit partial or complete loss of their characteristic courtship and copulatory patterns as a result of testicular ablation. Complete sexual quiescence is not always attained, for capons, although they do not ordinarily tread the squatting hen, do execute some elements of the masculine sexual pattern such as the waltzing response (34), and may occasionally display the entire male mating reaction (43). Furthermore one observer (75) has reported the occurrence of some mating activity in male pigeons with congenital absence of gonadal tissue.

Sporadic and casual observation is sufficient to reveal in a general way the sexually-depressing effects of castration in males of many avian species, but only repeated and well controlled tests are capable of determining the exact extent and speed of the behavioral change. Investigations of this type dealing with castrated male pigeons have shown that courtship and copulatory responses are gradually reduced over a period of several postoperative months; and that some residual sexual responses survive indefinitely in more than half of the gonadectomized males of this species (26, 27).

Although the data are too few to justify any final conclusions, there is some indication of a difference in the effects of castration upon sexual behavior in the male and the female. For instance, ovariectomized poulards give no indication of sexual receptivity, while capons continue indefinitely to display at least some elements of the normal masculine mating pattern.

The sexual performance of castrated male birds returns to normal if androgen is administered. Findings of this nature have been described for the capon (34), the laughing gull (64), and the herring gull (21). Although castrated pigeons have not been treated with androgen, Halpern and I have noted that males of this species which have been rendered

sexually inactive as a result of brain injury frequently display a revival of courtship and mating behavior under the influence of injected testosterone propionate (unpublished observations).

(4) *Mammals.* The well known effects of postpuberal castration in male mammals seem to warrant three generalizations. First, loss of the testes is almost inevitably followed by reduction in the susceptibility to sexual arousal and the capacity for mating performance. Second, this behavioral change is characteristically gradual and progressive, rather than abrupt and complete as in the case of the female. Third, the loss is rarely complete, and some weak manifestations of sexuality are usually retained indefinitely.

Qualitative observations have established the applicability of these principles in the case of many farm animals such as the bull (70) and horse (92), and to various species investigated in the laboratory including the rat, rabbit (95, 96), and the monkey (98). In some instances detailed, quantitative examinations have been conducted, and it is from such sources that we obtain the most useful descriptions of the exact nature of postoperational change.

The effects of castration upon mating performance in the rat are characterized by marked individual differences. Some animals cease to copulate within one week after gonadectomy while others continue to show such behavior for several months. Specifically, one study (95) has revealed that the copulatory response is lost in 33 per cent of male rats before the end of the first postoperative month, in 45 per cent by the end of the second month, 57 per cent by the end of the third month, 74 per cent by the end of the fourth, 79 per cent by the end of the fifth, and 91 per cent by the end of the sixth month after castration. Turning attention to changes in the performance of individual animals it is found that the first reaction to be eliminated by testicular removal is the ejaculatory response. Next to disappear is the complete copulation; and finally the abortive copulatory attempts become fewer, although they may never be completely abolished.

When it is recalled that the mating behavior of the female rat is promptly and completely eradicated by ovariectomy, the difference between the sexes in this species is rather striking.

Adult castration in the male rabbit is followed by much the same sort of changes in sexual behavior as have been described for the rat (96).

We have no carefully plotted "curves of decay" to show the progressive changes consequent to castration in other mammalian species, but one observer has noted that pronounced impotence is not established in the male monkey until half a year after gonadectomy (98), and we have seen that in the case of the chimpanzee a considerable amount of mating activity persists for years after prepuberal testis removal (29).

In attempting to estimate the effects of adult castration upon sexual ability in man one immediately encounters numerous sources of ambiguity, most of which are identical to those mentioned in connection with consideration of the human female. Individual differences are even more pronounced in man than in the lower forms. Qualitative and quantitative criteria for sexual "normality" differ from one study to the next, and there is no objective, generally reliable base line against which to measure any sort of behavioral change.

Despite the many factors which contribute to the general confusion a few facts emerge with reasonable clarity. In the majority of cases castration appears to result eventually in at least partial reduction in ability to copulate, and there is frequently a lowering of sexual excitability. Although in many instances these losses are quite marked, some capacity for arousal usually is retained, and such partial elements of response as penile erection are rarely completely eliminated.

There are, on the other hand, numerous reports to indicate that some men may retain full sexual power for many years after complete castration. What proportion of the total cases falls in this class it is impossible to determine; but it should be noted that such prolonged survival of completely normal ability is not found in males of any other species; and this gives us some reason to suspect that in the human male, as in the female, gonadal hormones are less essential to the manifestation of sexual performance than they are in lower mammals. There is, in addition, some slight indication that sub-human primates, particularly the chimpanzee, may stand somewhere between man and the lower mammals with respect to the importance of testicular hormone to mating ability.

It is generally acknowledged that male mammals which have suffered loss of sexual ability as a result of castration may be restored to normal by the administration of androgen. Results of this nature have been described for the rabbit (40), guinea pig (84), rat (58), chimpanzee (29), and human (33, 55a, 97a). Experiments with rats suggest that the revival of mating responses under the influence of exogenous androgen follows a course which is precisely the reverse of that described for the postoperative loss of such reactions. Small initial doses of the hormone result in an increase in the frequency of incomplete copulatory attempts; continuation of treatment is accompanied by the reappearance of complete copulation; and lastly the ejaculation response is restored (15, 93).

In the case of many human males the power of androgen to increase sexual excitability and to restore the capacity for coital responses testifies to the importance of this hormone in normal males despite the fact that in its absence some individuals may experience few symptoms of deprivation (24, 68).

3. BISEXUAL MATING BEHAVIOR

One special aspect of the relationship between hormones and mating activities lies in the question of reversal of sexual behavior or "homosexuality." The facts in the case are reasonably clear cut and obvious as far as the lower animals are concerned, but the entire area of thought is badly confused by a widespread tendency to apply fuzzy concepts of human sexual psychology to the interpretation of mating behavior in other species.

So far as human homosexuality is concerned there is little that can be said in a constructive sense. Although some endocrinologically-minded clinicians have essayed to demonstrate an hormonal basis for such deviations, the most charitable critic must agree that the outcome of these attempts have been singularly unconvincing (44, 102). This is not to say that endocrine imbalance can be ruled out automatically, but it should be self-evident that any understanding of potential endocrine factors in homosexuality will not be forthcoming until we achieve a reasonably satisfactory exposition of the importance of hormones to so-called "normal" sexual activity. It is, for example, quite pointless to state that the psychological peculiarities of the male homosexual are due even in part to excessive amounts of estrogen when we cannot even estimate the importance of estrogen to feminine behavior in women.

There have been many reports of "homosexual" behavior in animals below man, but the application of this vague term serves to confuse rather than clarify the important issues involved. A realistic and objective point of view must be based upon full description of the type of sexual relationships which are known to occur.

a. *Male animals.* In several vertebrate species sexually active males give no evidence of discriminating between other males and females, but regularly attempt to mate with any other individual regardless of sex. In such cases the responses of the partner govern the subsequent phases of the relationship. When the male leopard frog, *Rana pipiens,* clasps another male the clasped animal struggles violently, sounds the "warning croak" and usually is released promptly. The clasped female, in contrast, remains quiet and silent; the male's grip is therefore maintained and oviposition follows (60).

In various species of birds and mammals the male ordinarily attempts to copulate only with the receptive female, but under conditions of intense sexual excitement indiscriminate mating activity may occur. When one male is mounted by another he normally extricates himself as quickly as possible, whereas the female reacts with the execution of receptive behavior.

The explanation for instances such as these seems to lie in absence or temporary impairment of discriminatory ability on the part of the male;

and any analogy between this behavior and homosexuality in humans seems far-fetched and misleading.

A somewhat different interpretation is indicated for cases in which male animals exhibit sexual responses characteristic of the receptive female. This occurs infrequently, but both Stone (94) and I (14) have seen male rats respond in feminine fashion when mounted by other males. This behavior is especially noteworthy because (a) it has appeared exclusively in males which were extremely vigorous copulators when placed with females, (b) it occurs primarily when the males are highly aroused by previous copulations with estrous females, and (c) it can be abolished by castration and revived by the administration of either androgen or estrogen (9).

I take this evidence to indicate that the males in question were equipped with a neuromuscular constitution capable of mediating either the masculine or the feminine mating pattern. Their responses are therefore accurately described as *bisexual*, and the tendency to show either pattern appears to be heavily dependent upon gonadal hormones. (As a matter of fact, synthetic androgen revived both masculine and feminine responses in the castrated male, whereas exogenous ovarian hormones evoked the entire feminine pattern and only part of the masculine response.)

It seems quite likely that many if not all male animals are similarly constituted, although in most cases the mechanisms for feminine behavior are refractory to external stimulation. Various studies have revealed that under the influence of exogenous ovarian hormones female mating responses can be evoked in males of several avian and mammalian species (11). Males which spontaneously exhibit bisexual behavior may be presumed to differ from the majority in an atypical degree of responsiveness on the part of the neuromuscular mechanisms governing the feminine reactions, and in the extreme susceptibility to high degree of sexual excitement. Such males are unique only in the sense that certain nervous circuits are hyperexcitable; and no hormonal abnormality need be postulated to account for their bisexual mating responses. In these individuals a condition of intense arousal tends to result in activation of the mechanisms for feminine behavior when the appropriate stimuli are applied.

b. *Female animals.* The occurrence of masculine sexual responses in female animals is more common than is the appearance of feminine behavior in males. Execution of portions of the male's copulatory pattern with mounting and coital movements is regarded as a normal accompaniment of estrous in such mammalian species as the sow (2), cow (70), lion (31), and guinea pig (105); and in many birds including the great crested grebe and water hen (82), pigeon (100, 27), penguin (77), and canary (85a), the female may mount the male during courtship ceremonies.

A. Zitrin and I (unpublished observations) have observed female cats which mounted other females, gripped the neck in typical masculine fashion, and executed pelvic movements. In some strains of Norway rats a high percentage of virgin or sexually-experienced females readily and repeatedly mount other females, displaying palpating movements of the forepaws and typically masculine pelvic thrusts (16).

It is important to note that behavior such as that described above is shown by perfectly normal females which have not been subjected to any sort of experimental treatment. When they are in heat such animals receive the male, are readily impregnated, and bear normal, viable young. There is no reason to suppose that their display of masculine sexual behavior is caused by hormonal abnormalities. On the contrary, the most reasonable interpretation would seem to rest upon the assumption that female animals inherit neuromuscular mechanisms capable of mediating masculine behavior as well as those controlling the feminine mating performance; and that when the external stimulus situation is such as to call forth the male pattern this sequence of responses is apt to appear. At a later point we shall consider the probable role of gonadal hormones in bisexual mating activity of both males and females.

III. Theoretical Interpretations

1. Sources of variation

It is impossible to discuss in abstract or generalized terms the effects of hormones upon behavior, for various types of behavior are influenced by endocrine secretions in different ways, and the variation includes differences in the degree as well as in the nature of the hormonal effect.

A great many behavior patterns may be modified as a result of drastic upset in the metabolic level of the organism, and inasmuch as several glandular products contribute to homeostasis it follows that such function represents an indirect but potent type of hormonal control over behavior. The effect of hormones upon some forms of behavior is restricted to this type of general control.

There are other behavior patterns which are likewise subject to indirect control by hormones that contribute to the maintenance of normal physiological processes, but at the same time depend upon more restricted functions of still other endocrine products. For example, the occurrence of mating behavior in male rodents and cattle is affected by pronounced changes in metabolic rate, and thyroidectomy eliminates such behavior in these species (74, 67). In addition, however, the male's sexual activity depends upon the presence of testis hormone, and in the absence of androgen such responses fail to appear despite the fact that metabolic efficiency is not seriously impaired.

Reactions which are affected specifically by particular hormones may be subdivided according to the degree of dependence upon such effects. Thus the female mouse's mating behavior is so heavily dependent upon ovarian hormones that in the absence of these substances the behavior is lacking (101). In contrast, the female's tendency toward maternal responses is specifically affected by prolactin, but in this case the contribution of the hormone is less essential, and the behavior can be elicited in its absence (52).

It is well to note in passing that some behaviors appear to be directly dependent upon single hormones; while others are subject to direct control by combinations of two or possibly more secretions. For example, the male rat's sexual performance is controlled by androgen, while that of the female depends upon the synergistic action of estrogen and progesterone.

2. PRINCIPAL AVENUES OF CONTROL OVER BEHAVIOR

To avoid loose thinking in our analysis of potential mechanisms of hormonal control over behavior we should recognize that diffuse chemical substances circulating with the blood cannot "organize" or "direct" overt responses. In other words we can assign no executive function to the hormones. On the contrary, it may be accepted as self-evident that the only way in which endocrine secretions can alter an animal's reaction to its environment is by modification of the structure or function of various bodily tissues which mediate overt behavior.

Three obvious loci of potential hormonal action are the sensory receptors, the effector structures, and the integrative mechanisms of the central nervous system. In practice we can deal separately with these constituents of the total response, but it is to be noted that the division is one of expedience, and reflects a degree of independence which does not actually exist; for in the behaving organism stimulation, integration, and reaction are so inextricably interwoven and so highly interdependent that to consider one of the processes as a separable and independent function is to lose sight of the basic nature of the processes involved.

a. Receptor Mechanisms

It is conceivable that hormones may change behavior by inducing alterations in the sensitivity of peripheral receptors. One might suppose, for example, that one effect of testis hormone in seasonally breeding male mammals is a qualitative or quantitative alteration of olfactory sensitivity with the result that the odor of the estrous female is more easily perceived and serves as the instigator of sexual responses. Along similar lines we might hypothesize that after castration the male rat undergoes gradual changes in olfactory capacity resulting in loss of ability to smell the characteristic

odor of the receptive female (assuming, of course, that such an olfactory differential actually exists between estrous and diestrous females).

Some of Dr. Curt Richter's work (72, 73) dealing with diet selection in rats suggests the alteration of gustatory sensitivity consequent to glandular change. Adrenalectomized animals are said to display marked increase in the ability to detect the presence of salt in their drinking water. Severance of the taste nerves destroys this ability, but we cannot be certain that the changes which were induced by adrenal loss were restricted to peripheral receptors, since more centrally located nervous analyzers may have been involved.

There are qualitative observations to the effect that administration of androgen to senescent dogs results in a "sharpening of the senses" (91), and more objective experiments reveal that old men receiving testosterone propionate show improved visual efficiency in terms of increased fusion frequency of flicker (86). It has also been stated that many women experience hyperacuity of olfaction from 24 to 48 hours before the onset of menstruation until several days after cessation of flow (38).

None of these scattered reports provide any critical evidence to support the theory that hormonally induced behavioral change depends in part upon altered peripheral sensitivity. The fact of the matter is that the student of behavior deserves and can expect no help from endocrinological experiments until he learns a great deal more about the actual nature of the sensory stimuli involved in complex behavior patterns. To date nearly all of our opinions are based upon *a priori* reasoning rather than experimental evidence. It is frequently asserted that the male dog is attracted to the bitch in heat by virtue of the odor which accompanies her estrous condition; but the only basis for this assumption is the evidence of casual and uncontrolled observation; and in the case of other mammalian species which have been carefully investigated similar assumptions have proven incorrect (8, 23). It follows that until we determine what sensory receptors are involved in an animal's mating activity it is fruitless to speculate as to the possible effects of hormones upon those unknowns.

One exception may be allowed to the preceding generalization. Practical experience and experimental data combine to indicate that genital sensations contribute to the intensity and completeness of sexual performance. In this case, however, there is very little evidence to indicate the effects of hormones upon such peripheral sensitivity. To be sure there are descriptions of heightened sensitivity of the penis which occurs in eunuchs treated with androgen (55), and a few clinicians state that female patients experience increase in sensations from the external genitals after certain types of hormone therapy (78). But we must realize that in human subjects the

importance of suggestion is great, and in the absence of carefully controlled tests evidence such as that cited above is to be accepted with reservations. (There is, of course, no serious obstacle to the methodical study of this question with animal material. One could, for instance, measure quantitatively the amount of direct stimulation necessary to induce full erection in a male mammal, and subsequent tests of the effects of castration and androgen administration might well prove revealing.)

In considering the possible effects of hormones upon genital sensitivity, it is desirable that we distinguish clearly between a simple alteration of the peripheral threshold and the presumed sensory change which some writers have postulated as the origin of sexual impulses. It has often been assumed that the impulse to mate is evoked by sensations from the sex accessories (32, 59); but experimental evidence contradicts this point of view. For example, surgical removal of the uterus and vagina, or partial destruction of the penis fail to eliminate mating behavior in rats (4, 15). However, although we cannot regard sensations from the genitals as "prime movers" in the initiation of sexual behavior, this does not imply that the stimulation of these regions is without effect. On the contrary, both male and female rats exhibit reduced sexual performance in the absence of such stimulation.

b. Effector Mechanisms

In proposing the possibility that hormones influence behavior by stimulating or inhibiting the development of effector mechanisms we are thinking not so much of the more general types of effect such as altered muscular strength or increased body size, but of the involvement of specialized morphological structures employed specifically in particular behavioral responses. Some of the most clear-cut examples of such effector units are the various accessory and secondary sexual characters.

During breeding season the male two-lined salamander, *Eurycea bislineata,* develops elongate monocuspid teeth which are used to stimulate the female in courtship and mating; and the growth of the "courtship teeth" is governed by testis secretion (61). Male birds of certain orders (*Anseres, Cracidae, Crypturi, Ratitae*) possess copulatory organs somewhat similar to the mammalian penis, and the growth of these structures is affected by gonadal hormone (36). Annual growth of the antlers in the Virginia deer, *Odocoileus virginianus borealis,* is inaugurated under the influence of anterior pituitary hormones; and in the fall the velvet is shed and the horns harden in response to testicular androgen (103).

In these few illustrations we see examples of hormonal control over special morphological characters normally employed in particular behavior

patterns; but it must be added that there is little conclusive evidence to show that the form as well as the result of the response pattern is affected by the possession of these structures.

This brings out a significant point, namely that the importance of any structure to the execution of a pattern of behavior cannot be determined deductively, but must be investigated experimentally. In our consideration of the effects of hormones upon behavior we are interested in the overt character of behavioral responses, and not in their biological outcome. Thus we have described as "normal" the sexual behavior of castrated male mammals, although such individuals are of course sterile. Similarly the male rodent deprived of testes, seminal vesicles, and coagulating gland will, under appropriate hormone therapy, display the behavioral reactions normally accompanying ejaculation, despite the fact that emission of seminal fluid and formation of a plug are impossible (92, 85). For present purposes it is important therefore to recognize that our concern is only with the observable characteristics of the behavior pattern.

With these reservations in mind we may return to the examination of hormonal control which is exerted through the alteration of effector structures. Although, as noted above, the mating performance of male rats does not depend upon the vesicles or coagulating gland, it is markedly affected by alterations in the penis; and normal penis growth fails to occur if the testes are removed soon after birth.

Holz and I (15) recently found that male rats castrated at birth show abnormal mating behavior following injections of androgen in adulthood. The sexual activity of such animals is characterized by a very high frequency of incomplete mating responses, scarcity of complete copulations, and absence of the ejaculatory reaction. Analysis of the data has convinced us that the explanation rests upon the fact that the rats castrated in infancy possessed copulatory organs too small to permit intromission, and in the absence of penile sensations which are normally derived from contact with the vagina, the overt mating pattern was modified. We checked these conclusions by removing a portion of the penile bone in noncastrated adult rats, and found that the postoperative copulatory behavior of such animals was exactly the same as that of males castrated at birth. The results of this study suggest that testis secretions control mating behavior in part by virtue of their stimulating effect upon penis growth during early life.

A comparable situation probably exists in the case of female mammals. Ovarian hormones are essential to normal growth and development of the vagina (1), and there is reason to suppose that direct stimulation of the vagina during mating gives rise to sensations which serve to intensify the

female's receptive responses. It is known that estrous female rats which are temporarily resistant to the male often become much more receptive after a few copulations (7); and although females surgically deprived of the vagina may show normal heat responses during initial contacts with the male, such animals display a gradual reduction in responsiveness which is probably attributable to absence of vaginal stimulation (4).

c. Integrative Mechanisms

We have seen that certain hormones affect behavior through their control over the development of certain morphological structures which are essential to the execution of various reaction patterns; and we have considered the possibility that endocrine products may contribute in another manner by altering the functional capacity of peripheral receptor mechanisms. There remains for consideration the possible effects of hormones upon integrative mechanisms of the central nervous system. Although the sensory receptors and the effector mechanisms upon which a behavior pattern depends are fully developed and normally responsive to stimulation, the response will not appear unless the central nervous structures interposed between these terminal elements are functionally organized.

As an introductory example it will be helpful to study the effect of one hormone upon a relatively simple behavioral pattern. In the process of micturition the adult male dog lifts and crooks one hind leg, whereas the female assumes a squatting position. Puppies of both sexes squat during urination, but the male begins to display leg elevation at about 20 weeks of age. Males which are castrated in infancy fail to shift from the squatting posture, but continue throughout life with the feminine micturition pattern. However, if castrated males are injected with androgen the typical male posture soon appears (17). Here we have an example of a relatively simple type of motor integration which is heavily dependent upon one hormone; and comparable illustrations may be drawn from the behavior repertoire of females of several mammalian species.

(1) *Sexual behavior of the female.* If the diestrous or spayed female guinea pig is stroked lightly on the rump she "humps" her back and moves away from the stimulus; but the estrous female or the castrate which has been injected with estrogen and progesterone reacts to the same stimulus by standing still, arching the back concavely and raising the perineal region. The motor response to a simple tactual stimulus is thus profoundly altered in the presence of ovarian hormones.

When one grips a female cat in heat by the loose skin at the nape of the neck and simultaneously taps the perineum with the finger, the animal responds by lowering the forequarters, raising the pelvic region and treading

with the hind feet. The same stimuli applied to the diestrous female do not elicit these reactions. The ovariectomized female is equally unresponsive; but if estrogen is administered, the spayed animal's reactions are identical with those of the intact female during the period of heat. As in the case of the female guinea pig, this situation seems to indicate hormonal control of the motor reactions induced by simple tactual stimulation.

Physiologists have attempted to identify the nervous structures responsible for the motor patterns described above, and it appears that certain areas in the hypothalamus are essential to the estrous responses of the female guinea pig (22) and cat (6). Of course it does not follow automatically that these neural mechanisms are affected by the ovarian hormones, for it is possible that the integrative functions of the hypothalamus are unaltered by hormones, and that the endocrines actually control only the short arc reflexes in the spinal cord (54). However, the weight of evidence suggests that in the cases described the hormones do induce a functional change in the hypothalamus although they may also increase the activity of spinal centers.

Modification of the relatively simple motor reactions which constitute the basis of estrous responses are comparatively easily observed, but the condition of sexual receptivity consists of more than these postural adjustments to tactual stimulation. It includes a tendency to seek the company of the male, and to stimulate his advances by the spontaneous display of courtship responses. For example, the female rat which is in heat, or the spayed individual treated with ovarian hormone will repeatedly cross an electrified grid to get to the male; but the diestrous female or the untreated castrate displays this behavior much less frequently than the female in heat (99). It will be recalled that the female chimpanzee during her period of maximal genital swelling shows an increased tendency to approach the male, to groom his coat, and to invite copulation by the display of sexual presentation. These more complex portions of the estrous pattern undoubtedly are hormonally conditioned but we cannot as yet identify the nervous mechanisms involved.

(2) *Sexual behavior of the male.* The integrative functions involved in the mediation of the male's sexual behavior appear to be considerably more complex than those responsible for the female's reproductive pattern. Any satisfactory analysis of the masculine mating pattern must take into consideration the possibility of at least three levels of integration.

Proceeding from the simple to the complex, we may first consider those types of integration which occur at a low spinal level; and a foremost example is seen in the reflexes of the copulatory organ. In the male cat penile erection is produced by stimulation of the second sacral roots,

while stimulation of the internal pudendal nerves results in ejaculation, and subsidence of erection follows stimulation of the sympathetic trunks and hypogastric nerves (83). There is good evidence to indicate that androgen exerts a direct influence upon the spinal mechanisms mediating erection; and this localized effect seems to be at least partially independent of any possible hormonal influences over higher centers, for it can be induced in the absence of more widespread manifestation of sexual response.

Brain-operated male rats injected with large amounts of androgen exhibit priapism although they show no tendency whatsoever to mate with the receptive female; and the response of the copulatory organ occurs under conditions of isolation as frequently as when an estrous female is present (13). Along somewhat similar lines it has been reported that androgen administration to the human male may induce frequent erection which is not accompanied by any increase in desire for sexual contact (33).

Another type of integration which probably occurs in the cord is that responsible for gross bodily activity involved in copulation. Following complete lumbar section, manipulation of the genitals elicits in the male dog a series of reactions strongly suggestive of copulatory behavior. These include bilateral extension at the knees, ankles, and hip joints, depression of the tail, and a downward curving of the hind quarters (79). In this particular instance we have no evidence to indicate the possible effects of hormones upon the integrative structures involved; but there are data from other species which point to the conclusion that motor responses of this order may be directly affected by endocrine secretions. Male lizards, *Anolis carolinensis*, which have been implanted with pellets of androgen display exaggeration and prolongation of the postural adjustments involved in normal mating (62). Often these reflexes are so strongly stimulated by the hormone that the copulatory position may be maintained by a male which is forcibly separated from the female and held in the observer's hand.

These indications of hormonal effects upon spinal mechanisms are important; but from the psychological point of view the most interesting hormonally-induced change in the male's behavior is reflected in the altered susceptibility to sexual arousal.

Many factors contribute to the arousal and maintenance of sexual excitement in the male, but limitations of time and space prevent us from considering the evidence in detail. Suffice it to say that available data indicate that in male mammals there is no single, critical sensory stimulus upon which the male's arousal depends. On the contrary, this phenomenon involves activity in several different types of sensory receptors. In the male rabbit (23) and rat (8), vision, olfaction, touch, and probably other types of sensory data contribute to the male's sexual response. Impulses arriving via

afferents from various peripheral receptors exert mutually facilitative effects upon centrally located nervous circuits. Psychologically speaking, the adequate stimulus seems to be a pattern based upon the activation of several sensory modalities. Any single type of stimulus may be dispensed with providing others are present. Under normal conditions each makes its own contribution to the final result, and in the absence of any one avenue of stimulation the arousal of sexual excitement is partially impaired, although not eliminated.

The concept of an organized multi-sensory stimulus necessitates the postulation of still another type of integration essential to the mating performance of the male, namely the correlation of nervous impulses from different sensory mechanisms. It seems unlikely that such correlative function could be mediated at a spinal level or by the midbrain circuits presumably responsible for the final integration of the motor side of the pattern. The probability is that response to a multi-sensory pattern of stimulation depends upon the activities of higher nervous centers which exert a facilitative control over the activities of motor mechanisms. The location of such hypothetical integrative units is uncertain, but they are probably to be found within the forebrain. It is likely furthermore that progressive encephalization of function which has characterized the evolution of the vertebrate forebrain has involved some change in the nature of the nervous structures contributing to integrative functions of this order.

So far as actual evidence is concerned it can be stated definitely that in such lower mammals as the rat and cat the cerebral cortex contributes in some important fashion to the arousal of sexual excitement. Lowered responsiveness to sexual stimulation is to be understood as distinct from impaired copulatory ability; for on the one hand we are dealing with the results of integration of peripheral stimuli, and on the other with the organization of motor reactions. Injury to the neopallium in some animal species results in lowered susceptibility to arousal but is without effect upon the physical ability to copulate.

This difference is clearly apparent in results obtained with the rat. Male rats in which approximately 50 per cent of the cerebral cortex has been removed frequently show no tendency to mate with the estrous female (7a). However, when such individuals are injected with androgen they once more become capable of sexual arousal and proceed to display normal mating behavior (13). Completely decorticate male rats and cats pay very little attention to the female in heat; and although subsequent androgen treatment induces some signs of sexual responsiveness in both species, the full mating pattern cannot be revived.[3]

[3]Experiments with male cats were conducted by the author and Dr. A. Zitrin. Results are being prepared for publication.

Results such as these suggest that under normal conditions androgen and nervous activity within the cerebral cortex both contribute to sexual arousal. If the contribution of the cortex is decreased by virtue of brain injury, administration of exogenous hormone may exert a compensatory effect sufficient to make up for the reduced cerebral component. But if the entire cortex is destroyed no amount of hormone therapy can restore normal function.

From the evidence at hand it is impossible to determine whether androgen exerts a facilitative effect upon the sexual functions of the cortex itself or whether the hormonal effects are exerted exclusivly upon lower centers which are subject to stimulation by fibers from the cortex. In the first case the compensatory effects of postoperative androgen therapy could be accounted for by assuming the induction of an increased efficiency in the residual cortical tissue. In the second instance the behavioral phenomena could be referred to heightened sensitivity of lower centers resulting in an increased responsiveness to the reduced cortical stimulation.

(3) *Differences between the sexes.* At this point it should prove fruitful to survey briefly certain differences in the neurohormonal control of sexual behavior in male and female mammals. Two sets of observations appear complementary. (a) In subprimate females ovarian hormones seem to be a *sine qua non* for mating performance; whereas in males of the same species some sexual behavior is possible in the absence of testis hormone, although such activity is infrequent and incomplete. (b) In males of lower mammalian species the cerebral cortex is somehow involved in sexual arousal, and complete decortication eliminates mating, although studies of cats (6) and rats (10) show that ablation of the cortex does not interfere significantly with the female's copulatory performance.

It is difficult to escape the conclusion that these differences are causally related, that the absence of an essential cerebral component in the sexual performance of the female may account for the exclusive nature of the hormonal control over mating activities. This suspicion is strengthened by evidence suggesting that in the case of the male the cerebral cortex and gonadal hormone appear to exert complementary, coordinated effects upon sexual behavior.

From a theoretical point of view these speculations have an important bearing upon certain phyletic comparisons which have been suggested earlier in this survey. It has been noted that the rigid control which ovarian secretions exert over sexual activity in females of lower mammalian species is relaxed slightly in the case of lower primates, becomes less apparent in apes, and is difficult to detect in the human female. At the same time it is well known that the cerebral cortex increases progressively in size and

complexity as we pass from lower mammals to monkeys, anthropoids, and finally to the hominidae.

It is within the bounds of possibility that the change in degree of hormonal control and the progressive encephalization of complex physiological and psychological functions are interrelated phenomena, that the developing cerebral cortex has gradually assumed a larger and larger responsibility for the mediation of sexual activity in the female, and finally that increasing importance of neocortical factors has been responsible for proportionate decrease in the dependence of such behavior upon hormonal influences.

In the case of male animals it has been shown that both the cerebral cortex and the testis hormone are essential to normal mating behavior in lower forms. Furthermore it appears that the male chimpanzee and human are capable of considerably more sexual activity in the absence of testis secretions than are males of other mammalian species. Once again, our knowledge of evolutionary changes in forebrain structure and function lead us to wonder whether in the male as in the female there may not have been a partial transferal of control over sexual behavior from the gonadal hormones to the cerebral cortex.

d. Hormonal Effects upon Nervous Tissue

Having explored in summary fashion various nervous mechanisms which appear to be affected by hormones, our final task is to analyze the various ways in which endocrine secretions might bring about alterations in the function of central nervous tissue; and here three major possibilities present themselves.

(1) *Morphological change.* One potential effect of hormones upon nervous tissue is the induction of periodic growth and regression of nerve fibers. Any theory of this nature is of course out of step with orthodox concepts indicating the absence of growth or regeneration in the central nervous system of adult vertebrates, particularly those of homeothermic species (30). Despite this rather formidable source of opposition it is nevertheless possible that minute degrees of extension and retraction of fine fibers may occur (25), and some experimenters have suggested that chemical influences are capable of inducing such changes (88). At present, however, this appears more or less an outside possibility, and in general one is inclined to agree with Lashley (50) in his conclusion that hormonal control of behavior is probably not mediated through the induction of morphological changes in the nervous system.

(2) *Stimulation or Inhibition.* A second manner in which hormones might conceivably influence nervous function involves the direct stimulation or inhibition of activity within critical nervous circuits. During the period of estrus

the majority of female rats show a great increase in the amount of running activity, and it has been suggested that ovarian hormones directly stimulate the nervous centers responsible for this behavior (71). Critical proof of this theory is lacking, although it may be pointed out that a similar explanation could be applied to the effects of androgen upon penis reflexes in the male. The concept of direct chemical stimulation or inhibition of nervous mechanisms responsble for relatively simple reflexes is acceptable from the neurophysiological viewpoint, but it is somewhat more difficult to imagine a similar effect upon extremely complex behavior which is obviously mediated by a hierarchy of integrative mechanisms.

(3) *Alteration of sensitivity to external stimulation.* The most widely accepted theory purporting to explain the effects of hormones upon the nervous system postulates neither morphological change nor direct stimulation. It assumes, on the contrary, that glandular products act upon particular nervous mechanisms in such a way as to alter their sensitivity to external stimulation (6, 76, 84, 107, 3, 7). According to this view we are to regard hormones not as organizing agents, nor as primary stimuli, but as sensitizing factors capable of increasing or decreasing the responsiveness of nervous mechanisms to impulses from afferent fibers.

If we accept this line of thought it follows that the behavioral effects of a given endocrine product will be determined by the functional characteristics of the sensitized nervous mechanisms as well as the chemical composition of the hormone; and it has been pointed out repeatedly (56) that the occurrence of a given response rests as heavily upon the capacity of a tissue or organ to respond as upon the hormone administered. For example, androgen facilitates *croaking* in the male frog, *crowing* in the rooster, *cooing* in the pigeon, and *calling* in the male cat. Similarly, in more complex types of reproductive behavior the actual form of the overt response must be governed by the functional organization of the executive mediators.

This concept may be illustrated by an analogy. It has been determined that acetylcholine stimulates central nervous mechanisms responsible for respiration; and one explanation (41) involves the assumption that the chemical "potentiates" the effects of all nervous impulses impinging upon every synapse involved in the nervous control of breathing. Observation reveals, however, that despite the presumed equal stimulation of all synapses in the respiratory center, the rhythm of inspiration and expiration is maintained. The preservation of a smoothly integrated response is referred to the structural organization of the central nervous mechanisms involved. In other words acetylcholine provides a steady state of sensitization thus potentiating all incoming impulses; and the final form of the reaction pattern is governed entirely by functional relationships within the executive centers.

Transferring this analogy to overt mating responses, we might assume that the actual organization of the motor reactions is a product of the structural and functional characteristics of the nervous mechanisms involved, and that the effect of the hormone is to potentiate effects of incoming nervous impulses.

Accepting this hypothetical explanation as a useful working tool we are still confronted with the necessity of explaining why a specific hormone should act upon one set of nervous mechanisms and not upon others; why androgen, for example, should increase the stimulability of mechanisms for masculine sexual behavior, and leave relatively unaltered the excitability of other mechanisms which in themselves are known to be responsive to other hormones. No satisfactory answer is yet available, but it may be worth while to suggest that the solution may eventually be found to lie in an affinity of various nervous circuits for the specific chemical composition of particular hormones.

Thus the integrative mechanisms for masculine sexual behavior may by virtue of their characteristic biochemical constitution react specifically to androgen, while those for feminine behavior are sensitive to ovarian hormones. This would account not only for the hormonal restoration of normal mating responses in spayed animals, but for the elicitation of feminine behavior in males, and masculine behavior in females under the influence of heterologous gonadal hormones. Both sexes possess nervous structures capable of mediating the reproductive pattern of the opposite sex; but if the reversed behavior is to appear two factors are necessary. The appropriate external stimuli must be applied to the animal, and the corresponding integrative mechanisms must translate the peripheral stimulation into a pattern of motor response. Administration of the gonadal hormones of the opposite sex sensitizes the heterologous behavior mechanisms and the mating pattern changes accordingly.

Although this line of interpretation is purely speculative, there is ample evidence to suggest differential effects of various chemicals upon different types of peripheral nerve endings. The selective effects of several local anesthetics reveal that different types of nervous tissue respond in different but constant fashion to the same chemical substance (18). Combined with present knowledge of variation in chemical constitution of different parts of the central nervous system (47, 48) such data suggest the possibility of complex neurochemical relationships which may eventually be revealed as a primary cause for the selective behavioral effects of certain hormones.

IV. Summary

This review of the existing evidence together with the accompanying survey of theoretical interpretations leaves us with a plethora of unanswered questions, a few established facts, and a wealth of stimulating ideas for future research.

Summarizing the situation as it appears now we may make the following points regarding the effects of hormones upon mating behavior.

1. Certain hormones affect behavior by virtue of their control over metabolic processes throughout the organism. Such effects are indirect and widespread and involve many unrelated types of response.

2. Some types of behavior appear to be independent of any additional form of hormonal control, but others exhibit more specific and intimate affiliations with the secretions of particular glands.

3. Various hormones exert specific effects on particular behavior patterns in different ways. It is possible but not yet established that in some cases the functions of critical sensory receptors are altered in the presence of the essential hormones. In other instances the hormone is essential to the growth and development of a given effector structure which is employed in the execution of the behavioral response. And finally, some hormones appear to exert direct effects upon integrating mechanisms within the central nervous system.

4. Although we do not know the exact manner in which hormones bring about a change in nervous function it seems likely that their task is to increase or decrease the sensitivity of critical nervous circuits to external stimulation; and it has been suggested that the specificity of hormonal effects rests upon biochemical affinities between particular hormones and given nervous structures.

DISCUSSION

G. Pincus: One of the possibilities that may have been investigated is the behavior of the castrated female receiving testis hormone. I think it would be interesting to know whether the response is, shall we say, undiluted in the castrated female? Also, if the decorticate female receives testis hormone, is the male pattern possible at all in view of the presumed role of the cortex in male behavior?

That was a very interesting observation on the effects of castration on the extremely young male rat. The implication is that testis hormone is inadequate to restore complete maleness in the sense of morphology of the genitalia. Has there been any indication as to what is necessary to effect such reestablishment? Is another hormone necessary or is it purely a matter of developmental sequence?

F. A. Beach: Intact or castrated female animals do not respond as completely nor as intensely to androgen as do males. The ejaculatory pattern, for example, is rarely observed in the androgen-treated female. Similarly, the feminine reactions induced in castrated males by administration of ovarian hormones are quantitatively and quali-

tatively inferior to those elicited in the spayed female under the same treatment. The behavioral effects of gonadal hormones are a function of the genetic constitution of the individual as well as the chemical composition of the hormone.

I have not investigated the effects of androgen upon decorticated female rats, but it seems safe to predict that such animals would not exhibit masculine sexual responses no matter how much hormone was administered, because it is impossible to revive copulatory performance in the decorticated male by androgen treatment.

It is doubtful whether any sort of hormone therapy in adulthood could bring about the development of a normal penis in a male rat which had been castrated at birth. It seems that the copulatory organ passes through a critical developmental stage sometime during the first three post-natal weeks; if testis hormone is absent in this phase the subsequent administration of androgen will not induce normal growth. If the testes are removed after this stage has passed, growth is retarded but will be renewed in adulthood under the effects of exogenous androgen.

H. B. Friedgood: As a footnote to the interesting data which we have just heard about mating behavior, one might recall certain experiments which were carried on in Dr. Cannon's laboratory some years ago. We experimented with anestrous cats during the months of October, November, and December and injected them with FSH and LH according to the technique of Hisaw. Injections of FSH were given daily for five days and on the last day we also administered an intravenous injection of a small amount of LH.

Previous to therapy, these anestrous cats had no interest at all in matters of sex. As a result of treatment with FSH and LH, the animals went through typical cycles of mating behavior including coitus.

In this connection it is interesting also to recall some of Dempsey's experiments in which he showed that the physiological effect of estrogenic treatment, insofar as estrous behavior in guinea pigs is concerned, depends on an intact hypothalamus. It is clear from this and other studies that the nervous system is one of the essential components of the mechanism responsible for characteristic sex behavior.

Lastly, one might call your attention to certain clinical observations on one of the effects of androgens in the human female. As far as mating behavior is concerned, there is evidence to believe that the administration of androgens to frigid females results in a definite improvement of their libido. Judging from the cases which I have studied, it would appear that the change in reaction is due to a local increase in sensitivity of the external genitalia principally in the clitoris, which enlarges perceptibly with such therapy.

F. A. Beach: The observation that androgen may improve sexual responsiveness in "frigid" women is particularly interesting in connection with reports of the revival of a very low level of estrous behavior in spayed female rats treated with testosterone propionate.

R. McCullagh: Dr. Beach's very interesting discourse introduces the question of the role played by adrenal steroids in the behavior patterns of animals. Steroids are, generally speaking, quite nonspecific and it is common that steroids from one organ may exhibit pharmacological properties usually associated with another organ. For example, although the castrated rabbit is depleted of androgens as indicated by atrophy of the secondary sex glands, the blood of such an animal contains a normal amount of comb-growth producing material which is under ordinary circumstances considered testicular in origin. In this case it is possibly derived from the adrenal glands.

It might be of interest to study behavior patterns in castrated adrenalectomized ani-

mals and to investigate the pharmacological effect of numerous steroids from this point of view.

F. A. Beach: The point is well taken, and we have under way an experiment to determine whether the masculine sexual reactions of spayed female rats can be eliminated by subsequent adrenalectomy.

K. E. Paschkiss: Have there been any comparative studies of the effects of natural estrogens as compared with synthetic compounds (stilbestrol, etc.) with regard to behavior patterns?

I have another question: You mentioned castrated rats which have maintained a partial male behavior. Is there any evidence that such rats develop neurosis? The question, of course, is prompted by the psychological reaction of the human eunuchoid.

F. W. Lorenz: Stilbestrol will elicit the complete sexual pattern in hens, although it is apparently ineffective in quail. Females of the latter species become receptive when treated with androgen.

F. A. Beach: There have been very few comparative studies with mammalian material to measure quantitatively the relative effectiveness of natural and synthetic estrogenic compounds, but such evidence as is available suggests that the behavioral effects are at least qualitatively similar.

The problem of so-called "neurotic" behavior in lower animals is in a state of confusion due largely to the uncritical adoption of psychiatric terminology by comparative psychologists. I would hesitate to label any behavior shown by rodents as "neurotic;" but it is worth noting that highly aroused male or female rats which are prevented from normal coital reactions may exhibit "substitute" reactions such as frantic digging in the sawdust on the cage floor, or may become very aggressive, attacking any other rat in the vicinity.

F. C. Koch: I thought that the following experiment might be of some interest in this connection. Early in our studies on the physiological effects of the male hormone we were limited to responses to low doses, such as the comb-growth reaction in the capon. Other physiological responses such as behavior could be studied only after the organic chemists supplied us with pure testosterone propionate in adequate amounts.

The experiment I wish to refer to involves the treading response in the capon. Most of us probably know that it is very rare indeed that a capon treads a pullet. The particular capon we used in this study was caponized when about two weeks old and was kept in a colony without roosters and hens. It was used routinely for the assay of androgenic material and was found to be a reliable capon in that the comb regressed regularly after each assay period.

Having established this fact we decided to attempt to produce sex behavior. The capon then received gradually increased doses of testosterone over a period of three months. In this interval we measured the comb two or three times a week and produced an enormous comb. After the dosage had been stepped up tremendously the capon now behaved like a normal cockerel in crowing, courting, and treading. We then ceased the injections and two days later the regression of the comb was already measurable, but the crowing and treading phenomena were retained for at least two months. In other words, the comb-growth response, although a more sensitive physiological response to male hormone, requires continuous treatment for maintenance, whereas the behavior response requires large doses of hormone and long treatment but when once established is retained for several months without treatment.

D. Ingle: One of the ultimate objectives of such studies must be to determine whether

or not individual differences in sex drives have an endocrine basis. I would like to ask Dr. Beach whether the differences in the intensity of mating drives among intact animals can be ironed out by treatment with sex hormones?

F. A. Beach: There are no data bearing directly upon either aspect of the question. I do not believe that differences in sexual responsiveness among normal animals are simply reflections of differences in hormone level. On the contrary, the histological normality of the accessories in non-copulating male rats suggests that testicular hormone is secreted in normal amounts.

At the present state of knowledge it seems more likely that the root of the behavioral differences lies in the central nervous system. In some individuals the critical neural mechanisms may be somewhat refractory to the sensitizing effects of the gonadal hormones. Experiments with guinea pigs have shown that in most colonies there are a few females which, although they run normal ovarian cycles, never show heat behavior. When such individuals are spayed and injected with estrogen and progesterone it is found that normal heat responses can be induced, but the amounts of ovarian hormones necessary to evoke such behavior are far in excess of those adequate to produce receptivity in normal castrated females.

In reply to the second part of the question I can only say that there appear to exist all degrees of refractoriness to hormonal facilitation. Some non-copulating male rats will mate normally when the natural supply of testis hormone is augmented by administration of exogenous androgen; but a few such cases never display coital reactions even though very large doses of testosterone propionate are injected.

R. G. Hoskins: There is one aspect that has not been touched upon, the matter of psychogenic amenorrhea. This can be illustrated by a striking example that came to my attention some years ago. A woman reporter had occasion to visit a city morgue one day, and as she stepped through the door the morgue keeper picked up a severed head from a corpse and bowled it down the floor to her feet. She was menstruating at the time but promptly ceased and did not resume the function for three years. Her doctor told me he had two other equally striking cases of psychogenic amenorrhea. What can be said as to the mechanism of such effects?

F. A. Beach: Current issues of English medical journals are filled with accounts of amenorrhea in women whose home towns were heavily bombed during the war. The most logical interpretation of such phenomena would seem to rest upon the assumpiton that psychological trauma may result in pituitary dysfunction due to the intimate functional relations between the hypothalamus and the hypophysis, and that pituitary failure is subsequently reflected in interference with normal ovarian cycles.

H. B. Friedgood: Dr. McCullagh mentioned in his remarks that there is a lack of specificity of hormones on the psychological reaction of individuals afflicted with homosexuality. Homosexual people testify to this themselves. I have studied several male homosexuals who asked for therapy with the intent of trying to alter their behavior. In a purely empirical fashion they were given testosterone propionate in large doses. It is interesting to note that instead of altering their behavior, their homosexual tendencies became strongly exaggerated as a result of this therapy. The psychological aspects of homosexuality play an important part in its pathogenesis. Presumably, when the intensity of the psychological component is markedly accentuated even small doses of the male sex hormone simply intensify the drive along the direction in which the individual is already headed.

F. A. Beach: As far as human beings are concerned it seems fairly safe to say that although the *strength* of the sexual drive may be altered by an increase or decrease

in the level of gonadal hormone, the *direction* or manner of expression of sexual excitement is governed primarily by experiential factors.

E. P. McCullagh: A few clinical experiences might be worth mentioning in this respect. Five of my patients who have severe prepuberal hypogonadism are married. Two of these were married before they had any treatment. Both are approximately 30 years of age and both claimed to have reasonably satisfactory sex relations. The three others who have been married since they have had treatment have normal sex relations, apparently; and all five, whenever treatment is discontinued or if pellets are absorbed, return and want the treatment, not only for the sense of vigor and well-being which they experience, but also for an increase in sex drive and potence. In short, in these individuals severe testicular failure is consistent with moderate degrees of sexual potency. Sex drive and potency, however, are distinctly increased by testosterone therapy in cases in which testosterone deficiency exists.

In adult castrates we have observed two individuals who were married some years after castration and had had no treatment. One of these men thought it unnecessary for him to have treatment and did not take any treatment. We have not seen, as far as I can recollect, any individuals castrated in adult life who had had sex experience before who did not notice a distinct diminution in sex drive and potence, although they were not necessarily lost by any means.

Boys who come to us, of the type usually classified as adiposogenital dystrophy, are, in our experience, brought commonly by the mother because she knows that they do not show the normal aggression of boys their age and feels that they are not maturing normally. We have come to doubt very seriously, however, that many of these boys have pituitary failure, in the sense that we originally believed they had. In fact, most of those studied in the past year show what appears to be adequate gonadotrophic substances in the urine. In spite of this, some of these boys respond to chorionic gonadotrophin injections with increased aggressiveness and decided genital growth.

We followed for a period of about two years, in cooperation with the psychologists at Western Reserve Univeristy, eighteen homosexuals, seventeen men and one woman. We saw some who appeared to us to show clinical evidences of weak maleness as far as hair growth is concerned, but most of them showed no such evidence whatever. Their abnormality was evident clinically in their mannerisms and demeanor only. Six of these men were treated, and two of them appeared to us to have a distinct increase in sex drive which did not deviate from the previous pattern, so far as we could tell.

In children who present clinical evidence of pubertas praecox associated with adrenal tumor and sometimes with high 17-ketosteroid excretion there is usually no evident trend toward adult sexual behavior.

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Steroids Derived From the Bile Acids: 3, 9-Epoxy-Δ^{11}-Cholenic Acid, an Intermediate in the Partial Synthesis of Dehydrocorticosterone

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I. Introduction

The membership of this group includes very few who are primarily interested in organic chemistry and for this reason I shall limit my remarks to a general discussion of the pathway which has been followed in the partial synthesis of one of the hormones of the adrenal cortex which is oxygenated at C_{11}. Through the use of 3(*a*)-hyrdoxy-Δ^{11}-cholenic acid as an intermediate, Reichstein and his associates have succeeded in the preparation of dehydrocorticosterone; however, the yield was so small that the method could not be used for large scale preparation of this hormone.[1] For the past several years a chemical study of the hormones of the adrenal cortex has been carried on in the biochemistry laboratory of the Mayo Foundation, and recently a new method of introducing oxygen at C_{11} was discovered. The details of the work and the proof of structure of the various intermediate compounds are given elsewhere (2, 9, 10, 18). I shall act as reporter for the group of workers who have carried out this investigation. They are: Lewis L. Engel, Vernon R. Mattox, Warren F. McGuckin, Bernard F. McKenzie and Richard B. Turner. I shall describe the work through the use of a series of figures (Fig. 1).

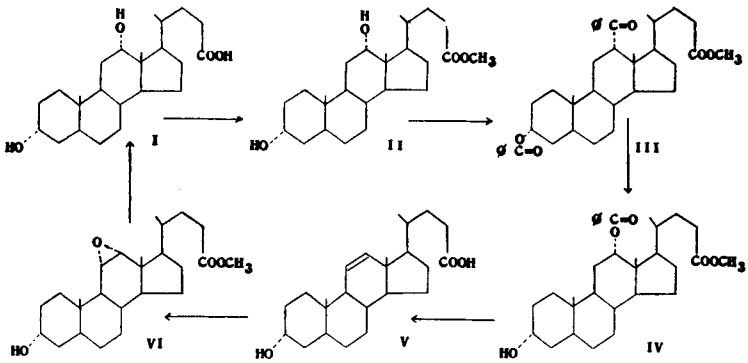

FIG. 1

II. Experimental

Desoxycholic acid was converted to 3(α)-hydroxy-Δ^{11}-cholenic acid (V) through pyrolysis of the methyl ester of the C_{12}-benzoate (IV). The oxide (VI) was formed with perbenzoic acid and this in turn was reduced in acetic acid with a trace of hydrochloric acid to give the starting material, desoxycholic acid. The configuration of the groups at C_3, C_{10} and C_{12} will be discussed later.

The double bond C_{11}:C_{12} as shown in Fig. 2 will add 1 mole of bromine.

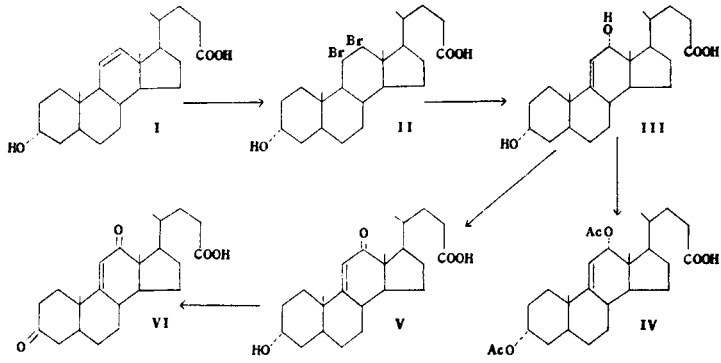

Fig. 2

Only one crystalline dibromo derivative has been separated. Under the influence of sodium hydroxide both atoms of bromine are removed and a compound is formed which has been shown to be 3(α), 12-dihydroxy-$\Delta^{9,11}$-cholenic acid (III) (2, 15). This can be converted to the diacetate (IV), the 3(α)-hydroxy-12-keto-$\Delta^{9,11}$-cholenic acid (V) and finally to the 3,12-diketo compound (VI).

The 3-keto-12-hydroxy-$\Delta^{9,11}$-cholenic acid (III, Fig. 3) can be made from II with alkali by a process analogous to the preparation of III from II of Fig. 2, or through protection of the hydroxyl group at C_{12} as an acetate and oxidation of the 3(α)-hydroxyl group to a ketone as shown in Fig. 3: I→ IV→ V→ VI→ III.

The hydroxyl group at C_{12} of 3(α), 12-dihydroxy-$\Delta^{9,11}$-cholenic acid (I, Fig. 4) is a part of an allylic system and is readily replaceable with acetoxyl or methoxyl groups and halogen (II and IV, Fig. 4, and II, Fig. 5). In addition it is possible to prepare the C_3-keto compounds with methoxyl at C_{12} (III, Fig. 4) and with chlorine at C_{12} (VI, Fig. 5). The last mentioned compound is prepared from I, Fig. 5, through replacement of the C_{12}-hydroxyl with acetate, oxidation of the C_3-hydroxyl to a ketone, and replacement of the C_{12}-acetate with chlorine. The methoxyl group of IV

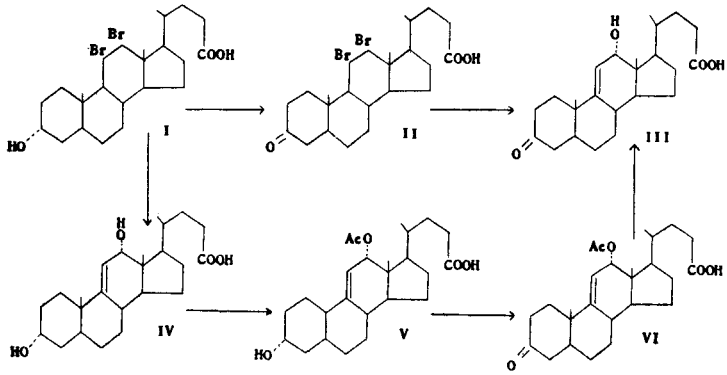

FIG. 3

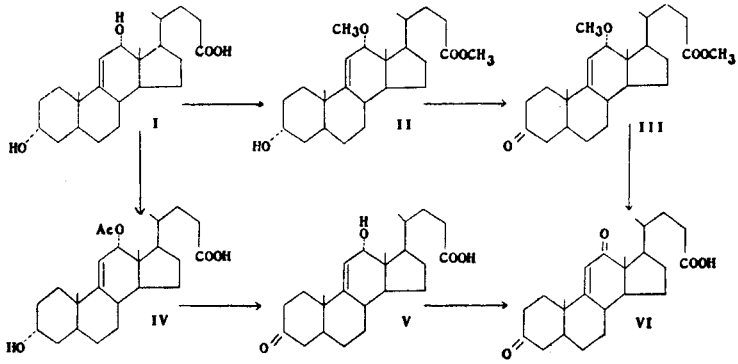

FIG. 4

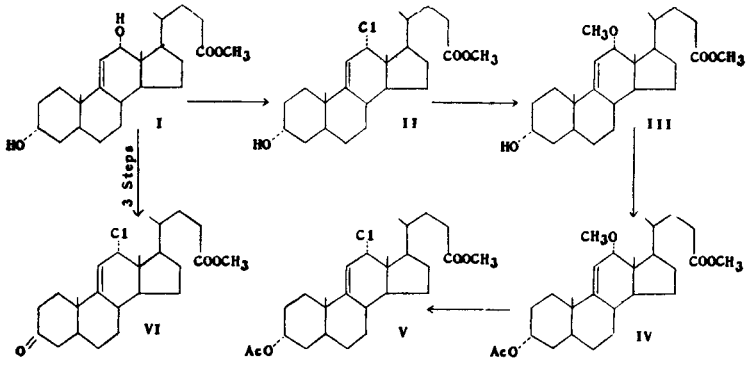

FIG. 5

may be replaced through the use of hydrogen chloride to give methyl 3(α)-acetoxy-12-chloro-$\Delta^{9,11}$-cholenate (V, Fig. 5).

The three compounds which have a double bond C_9:C_{11}, an atom of chlorine at C_{12} and a hydroxyl, ketone and acetoxyl, respectively, at C_3, form an interesting series of derivatives. The last two mentioned compounds do not have a free hydroxyl group at C_3, and Fig. 6 shows that the halogen

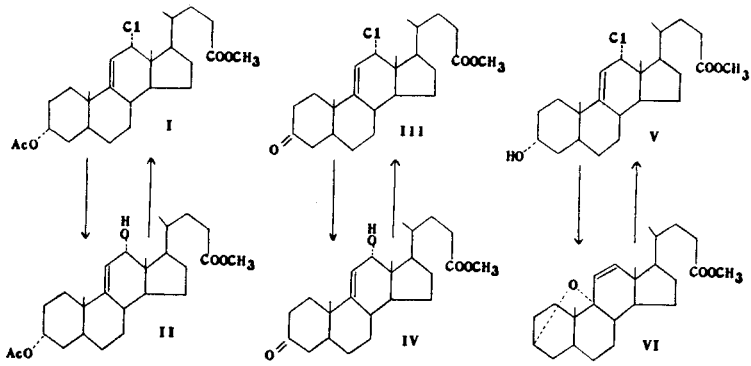

FIG. 6

at C_{12} is replaceable with the hydroxyl group when these compounds are treated with silver salts in aqueous acetone. However, when methyl 3(α)-hydroxy-12-chloro-$\Delta^{9,11}$-cholenate (V, Fig. 6) is treated with pyridine or in a two-phase mixture of water and chloroform the chlorine at C_{12} is removed as hydrochloric acid and through an allylic rearrangement the double bond is shifted to C_{11}:C_{12}. Loss of a proton from the hydroxyl group at C_3 results in formation of a 3, 9-cyclic ether (VI, Fig. 6). A probable mechanism for this reaction is illustrated in Fig. 7. Solvation of the atom of halo-

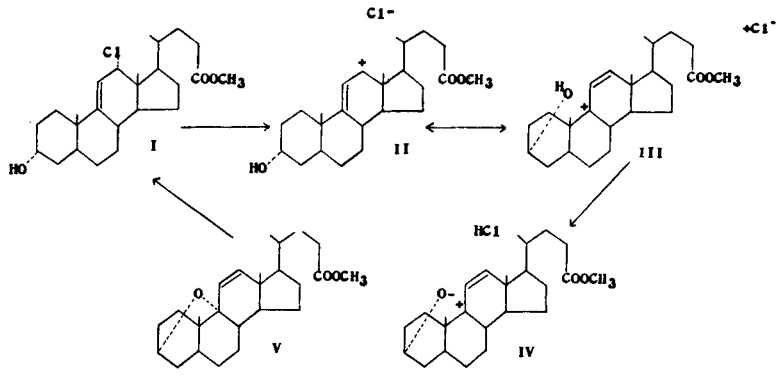

FIG. 7

gen yields a carbonium ion (II and III) which is stabilized by addition of the oxygen at C_3 to C_9 with simultaneous loss of a proton.

The highly labile nature of methyl 3, 9-epoxy-Δ^{11}-cholenate (I, Fig. 8) is illustrated by the reactions shown in Fig. 8. With hydrogen chloride the

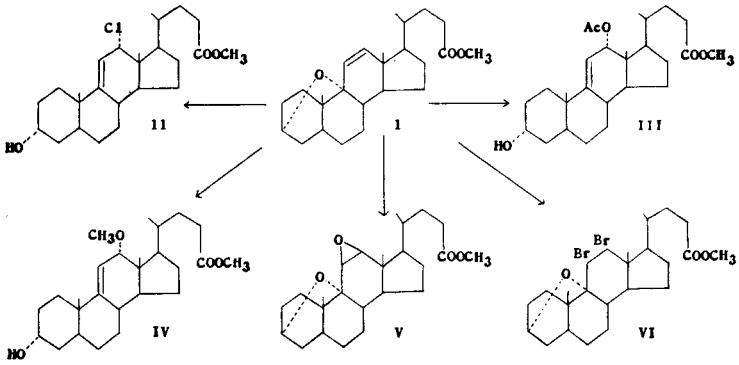

FIG. 8

3(a)-hydroxy-12-chloro compound (II) is regenerated. With methanol and a trace of mineral acid the C_{12}-methoxy compound (IV) is formed and acetic acid and a trace of mineral acid yield the C_{12}-acetoxy derivative (III). Perbenzoic acid forms an 11, 12-oxide (V), and 1 mole of bromine is rapidly absorbed to give the 11, 12-dibromo compound (VI).

Catalytic hydrogenation of methyl 3, 9-epoxy-Δ^{11}-cholenate does not reduce the C_{11}:C_{12} double bond. Neither the double bond nor the cyclic ether is affected in neutral ethanol, but in the presence of a trace of hydrogen ion the epoxide group is weakened and absorption of hydrogen with a shift of the allylic system after hydrolysis yields 3(a)-hydroxy-$\Delta^{9, 11}$-cholenic acid as the first product of hydrogenation.* Further uptake of 1 mole of hydrogen yields lithocholic acid. A suggested mechanism is shown in Fig. 9.

The steps which furnish a method for introduction of oxygen at C_{11} are given in Fig. 10. 3(a), 12-Dihydroxy-$\Delta^{9, 11}$-cholenic acid (I) is converted to methyl 3(a)-hydroxy-12-chloro-$\Delta^{9, 11}$-cholenate (III) through the methoxy intermediate (II). The chloro compound (III) is treated with pyridine or a base in a two-phase water-chloroform mixture to give practically a

*Formula IV is not shown as an intermediate compound but to indicate a transition from II to V. Whether ethoxyl, acetoxyl, or other group actually adds to the carbonium ion and is removed by hydrogenolysis has not been shown except in regard to rates of reaction for the hydrogenolysis of the acetoxyl and methoxyl groups at C_{12}. These are slow compared with the rate for conversion of II to V which is very rapid.

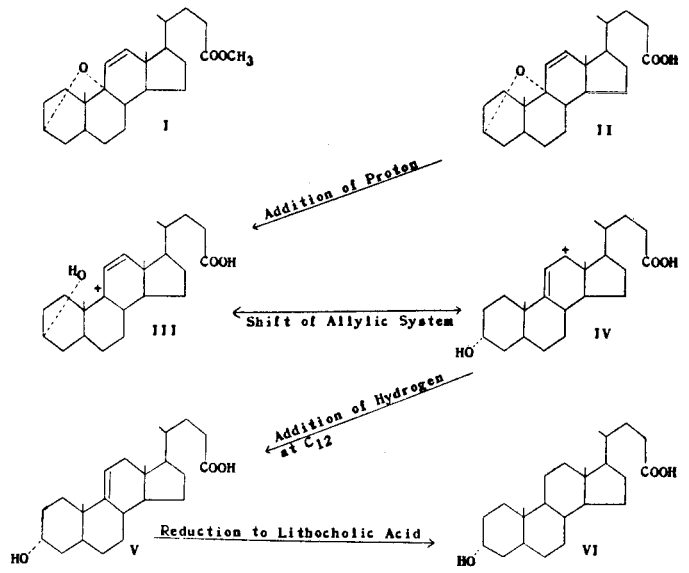

FIG. 9

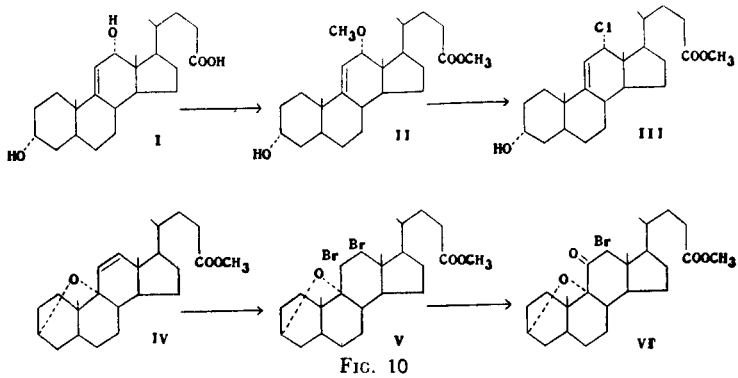

FIG. 10

quantitative yield of the 3, 9-epoxide (IV). Absorption of 1 mole of bromine at −50° provides the intermediate dibromo derivative V and also an isomeric dibromide of similar structure but with different configuration of the atoms of bromine at C_{11} and C_{12}. Investigation of these two dibromo compounds suggests that they are 11(α), 12(β) m.p. 123° and 11(β), 12(α), m.p. 143°. The higher melting compound can be used to introduce the acetoxyl or hydroxyl group at C_{11}. Subsequently it was found that the action of silver chromate gave a 95% yield of the C_{11}-keto-C_{12}-bromo derivative (VI). The proof of structure is furnished by cleavage of the cyclic ether and restoration of the 3(α)-hydroxy group. This step is illustrated in Fig. 17.

Degradation of the side chain of cholic acid and related compounds by the method of Barbier and Wieland (1, 20) has given yields of diphenylethylenes which approximated 50 to 60% of the theoretical yield, and oxidation of the ethylenes has in turn given the next lower acid in yields of 50 to 60%. Through the use of methyl 3, 9-epoxy-11-keto-12-bromocholanate and at a temperature of 0° it has been possible to form the diphenyl ethylene derivative (II, Fig. 11) in a yield of more than 90% and to obtain the

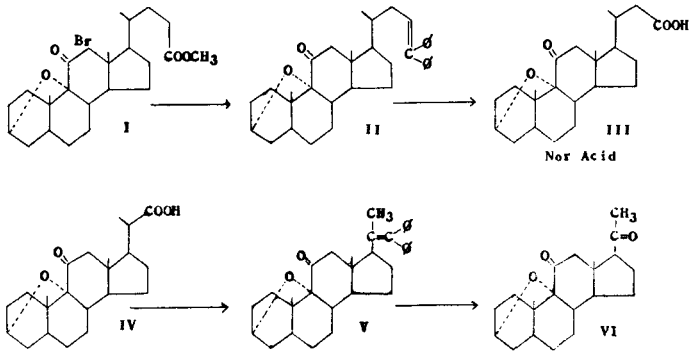

FIG. 11

nor acid (III) through oxidation of the ethylene II in a yield of 90% based on the weight of the ethylene. The conversion of the methyl cholanate (I, Fig. 11) to the methyl ester of the nor cholanic acid III was accomplished with an over-all yield of more than 80%, and since five steps were involved the average yield for each step was 96%. It should be pointed out that the atom of bromine at C_{12} is removed during the formation of the diphenyl carbinol and therefore does not require a separate procedure. The last part of the degradation of the side chain to the C_{20}-ketone is indicated in formulas IV, V, and VI, Fig. 11. The ethylene prepared from the nor acid (III) closely resembled the ethylene from the methyl cholanate (I) and the oxidation of the ethylene from III yielded the bisnor acid (IV).

Preparation of the diphenyl ethylene (V) from the methyl ester of IV did not proceed satisfactorily for two reasons. At low temperatures the Grignard reagent did not affect the methyl ester of the bisnor acid (IV), and the starting material was recovered unchanged. Even at room temperature the addition of the phenylmagnesiumbromide to the ester caused precipitation, and formation of the diphenyl carbinol did not proceed. Addition of benzene and other possible solubilizing agents was ineffective, but it was then found that ethyl morpholine provided a satisfactory medium. In the presence of this organic base the yield of the diphenyl ethylene (V, Fig. 11) was approximately 80%. The oxidation of this ethylene to

give the C_{20}-ketone (VI) was not satisfactory when chromic acid was used but treatment of V, Fig. 11, in methanol-ethyl acetate with ozone gave an excellent yield of the desired product (VI).

The methyl group at C_{21} was removed by the method devised by Hoehn and Mason (5), and the 3, 9-epoxy-11-ketoetiocholanic acid (IV, Fig. 12),

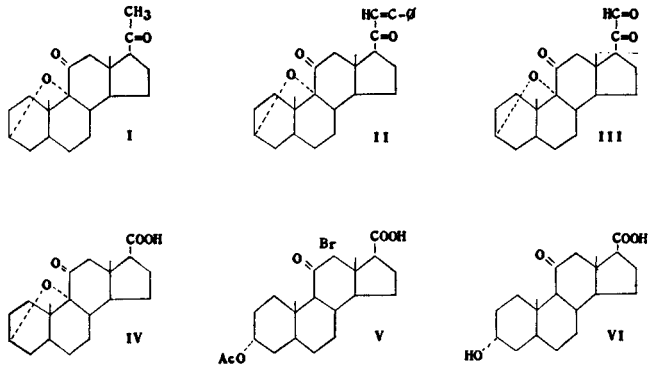

FIG. 12

was separated in a yield of about 75 per cent. Cleavage of the 3, 9-epoxy group with hydrogen bromide gave compound V, Fig. 12, and debromination gave the 3(α)-hydroxy-11-ketoetiocholanic acid (VI). The rupture of the cyclic ether will be discussed later (Fig. 17).

The compounds I, II, III, and IV shown in Fig. 13 are the essential intermediates which have been described for the conversion of desoxycholic acid (I) to the 3, 9-epoxy-Δ^{11}-cholenic acid (VIII). Although compounds II and III furnish a pathway, there remains much to be desired both from the standpoint of labor involved and from that of the yield. These two unsatisfactory steps could be avoided if 3(α)-hydroxy-12-ketocholanic acid (V, Fig. 13) could be used for introduction of the double bond C_9:C_{11} and the hydroxyl group at C_{12}. Schwenk (16) had observed that selenium dioxide could be used to introduce the double bond, and an extended investigation in the biochemistry laboratory of the Mayo Foundation has increased the yield of VI, Fig. 13, to more than 90% of the theoretical amount. Catalytic hydrogenation of VI converted the ketone at C_{12} into a hydroxyl group almost quantitatively to give VII and this compound in turn gave VIII with an over-all yield from I of about 60%. This compares very favorably with a yield of less than 10% when VIII was prepared through compounds II, III, and IV, Fig. 13.

The steps I, V, VII, and VIII, Fig. 13, were carried out as shown in Fig. 14. Methyl desoxycholate (I) was acetylated at C_3 in glacial acetic

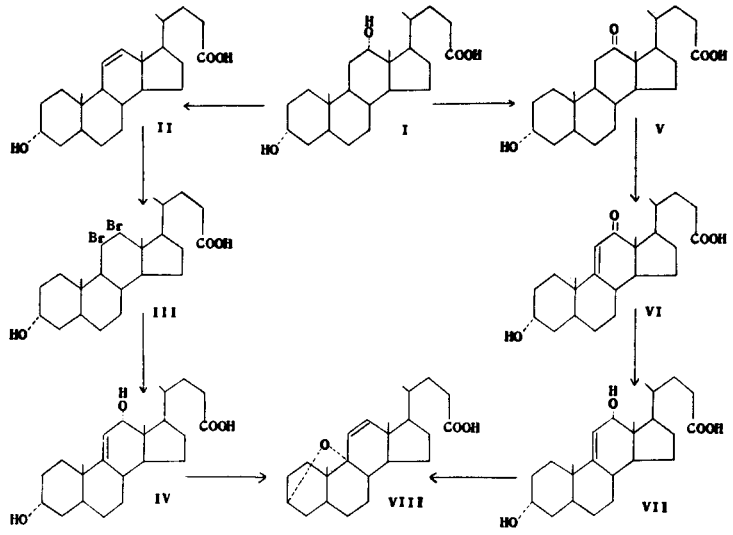

FIG. 13

acid with 0.20 N hydrochloric acid. Under these conditions the hydroxyl group at C_3 is completely acetylated, but less than 3% of the hydroxyl group at C_{12} is esterified. Chromic acid is added to oxidize the C_{12}-hydroxyl group to a ketone to give III, Fig. 14, and the double bond C_9:C_{11}

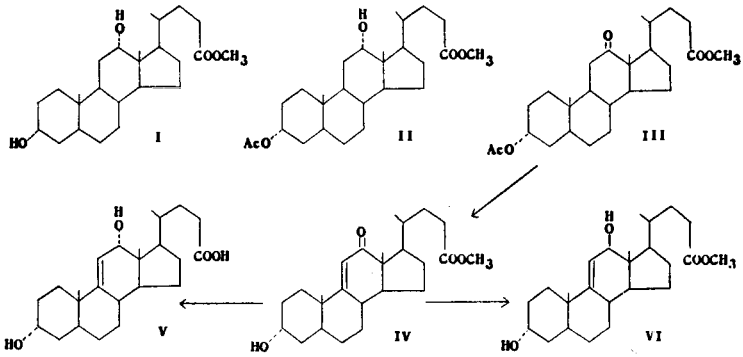

FIG. 14

is formed by oxidation with selenious acid in a mixture of chlorobenzene and acetic acid 4:1. A small amount of hydrogen chloride catalyzes the oxidation to give IV. Neither the double bond C_9:C_{11} nor the ketone can be hydrogenated in neutral methanol or ethanol but in a mixture of ethanol and acetic acid 1:1 the ketone is almost completely reduced to give V and VI which are epimeric at C_{12}. About 80% of the $C_{12}(\beta)$-epimer (VI, Fig. 14) is formed.

Figure 15 shows the relationship between the α and β configurations of the substituent at C_{12}. After esterification of the carboxyl group with diazomethane both of the epimeric forms I and III can be acetylated in acetic anhydride and pyridine to give the methyl esters of the diacetyl derivatives (IV and VI) and these can be hydrolyzed in alkali to give the start-

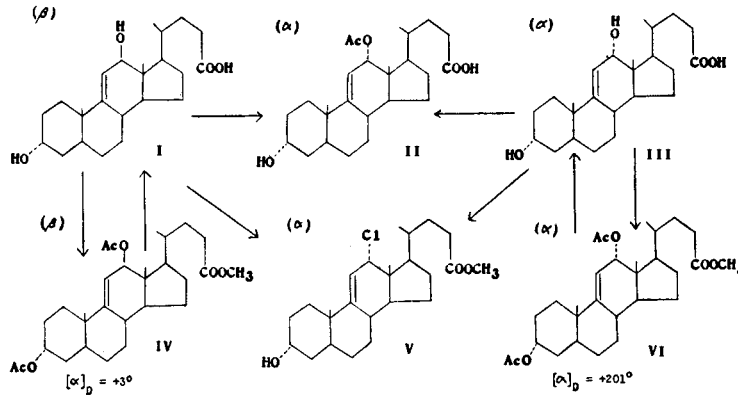

FIG. 15

ing materials (I and III), but in acetic acid and a trace of sulfuric acid the hydroxyl groups of both I and III are replaced to give the same C_{12}-acetoxyl derivative (II), and with hydrogen chloride the methyl esters of both I and III give the same C_{12}-chloro compound (V). These reactions indicate that in acid mediums the C_{12}-hydroxyl groups of I and III are replaced with the acetoxyl group or with chlorine through the formation of a carbonium ion which is common to both epimers and suggest that the group which enters is on that side of the plane of the molecule which is least hindered. It is for this reason that the α configuration is assigned to II, III and V since the $C_{12\alpha}$ position is *trans* to the methyl group at C_{13} and is therefore less hindered than is the β position. There is a wide difference between the specific rotations of the α and β configurations at C_{12}. For the 3, 12-diacetates the values are $+201°$ and $+3°$ respectively. This relationship will be discussed further in connection with Tables 1, 2, and 3.

Both α and β-hydroxyl groups at C_{12} (I and II of Fig. 16), the α-acetoxyl group (III) and the C_{12}-methoxyl group (IV) are removed by hydrogenolysis to give 3(α)-hydroxy-$\Delta^{9,11}$-cholenic acid (V) as the primary reduction product. It has already been mentioned that V is also formed by reduction of 3, 9-epoxy-Δ^{11}-cholenic acid (VI). It is interesting to note that although the compound methyl 3(α), 12(α)-diacetoxyl-$\Delta^{9,11}$-cholenate (VI, Fig. 15) can be reduced to the methylene group at C_{12} the epimer, methyl 3(α), 12(β)-diacetoxyl-$\Delta^{9,11}$-cholenate (IV, Fig. 15), cannot be

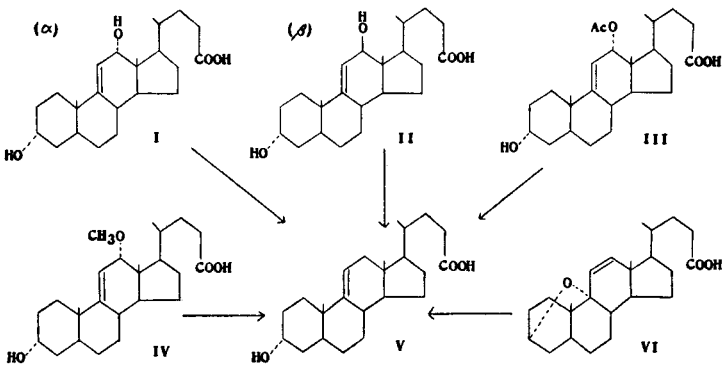

FIG. 16

reduced under the same conditions. Presumably this is because of steric hindrance of the β form at C_{12}.

In Fig. 12 it was indicated that the 3, 9-epoxide could be opened to form the 3(α)-hydroxy-11-keto-12-bromo derivative of etiocholanic acid but it was found that the 3, 9-epoxide derivative of cholanic acid could be opened more easily with hydrogen bromide. At present it is not possible to explain the influence of the length of the side chain on the stability of the 3, 9-cyclic ether, but the differences observed are striking and for the best results we have usually opened the 3, 9-epoxide of the nor acid. In keeping with the relation between the length of the side chain and the ease of rupture of the 3, 9-epoxide, the diphenyl ethylene (II, Fig. 11) prepared from methyl 3, 9-epoxy-11-keto-12-bromocholanate (I, Fig. 11) opened to give an excellent yield of 3(α)-hydroxy-11-keto-12-bromobisnorcholanyldiphenylethylene.

In connection with the rupture of the 3, 9-epoxide and the influence of the length of the side chain at C_{17}, another observation has been recognized as of importance. I refer to the position of the atom of bromine which is found after cleavage of the cyclic ether. The bromine could be at C_3, C_9 or C_{12}. It can be stated that approximately 90% of the bromine is present at C_{12}. Proof for this statement was obtained by rupture of the cyclic ether in methyl 3, 9-epoxy-11-ketocholanate with hydrogen bromide to give a compound which was identical with an authentic sample of methyl 3(α)-acetoxy-11-keto-12-bromocholanate which was prepared by the method devised by Reichstein and his associates (14).* A probable reaction mechanism for the formation of the last mentioned compound is shown in Fig. 17. The 3, 9-cyclic ether appears to be weakened by the formation of the double bond

*We are indebted to Dr. L. H. Sarett of Merck and Co., Inc., Rahway, New Jersey, for the preparation of this compound.

C_{11}:C_{12} through enolization of the ketone at C_{11}. The enol ether which results could then exist as a hybrid carbonium ion. Addition of a proton to the oxygen at C_3 and shift of the double bond as shown in the equilibrium between III and IV, Fig. 17, afford the conditions under which bromine

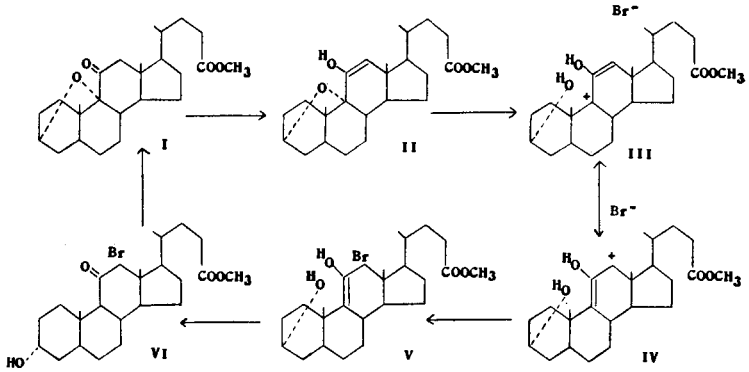

FIG. 17

adds to the carbon at C_{12} to give V and the C_{11}-keto form (VI). The arrow between compounds VI and I, Fig. 17, indicates that by loss of halogen acid from C_{12} and the hydroxyl group at C_3, the 3, 9-epoxide can be re-established. This step has been carried out in alkaline solution. The probable course of the changes which occur, illustrated in Fig. 18, is essentially a reversal of the addition of hydrogen bromide to the 3, 9-epoxide as shown in Fig. 17.

Formation of the 3, 9-epoxy structure in the bile acids furnishes the first direct chemical evidence for the configuration of certain parts of desoxycholic acid. Construction of Stuart models shows that the cyclic ether can

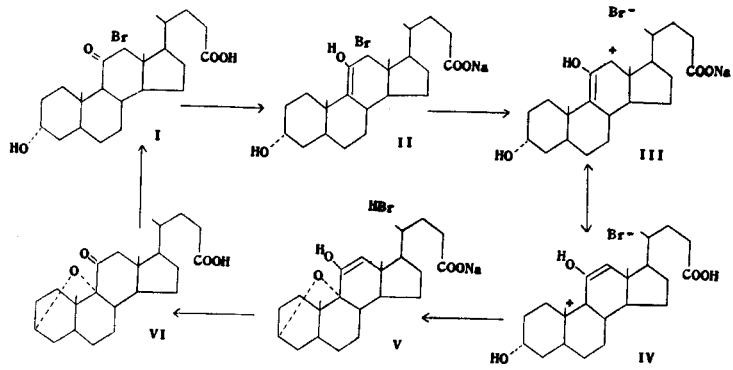

FIG. 18

be formed only when the hydroxyl group at C_3 is in the α position, *trans* to the methyl group at C_{10}, and also when the arrangement between rings A and B is *cis*, as in *cis*-decalin. It therefore is important to secure unequivocal evidence for the structure under consideration. As shown in Fig. 10, methyl 3, 9-epoxy-Δ^{11}-cholenate will add 1 mole of bromine and give the dibromide m.p. 143° (I, Fig. 19). Silver acetate in acetic acid yields

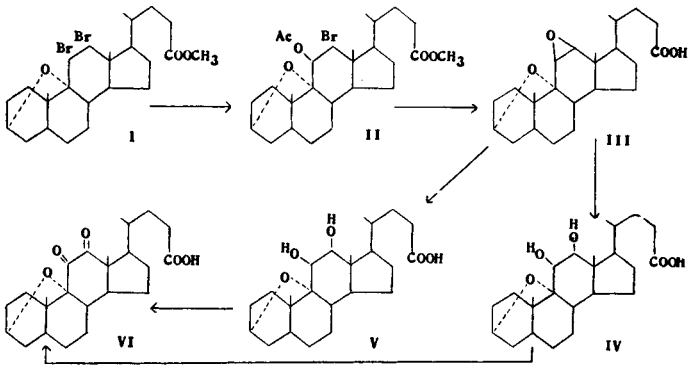

FIG. 19

a monoacetoxymonobromo derivative (II), and sodium hydroxide removes the bromine and acetyl group to form the oxide (III). The oxide in turn is opened in acetic acid with a trace of mineral acid, and after hydrolysis the two 11, 12-glycols (IV) and (V) are obtained. Oxidation of each of these glycols results in the formation of the diketone (VI). This series of steps establishes the positions of the double bond and of the epoxy group in the compound 3, 9-epoxy-Δ^{11}-cholenic acid.

Figure 15 shows the relationships between the derivatives of $\Delta^{9,11}$-cholenic acid with epimeric configurations at C_{12}. Those compounds which were stable in acid mediums were considered to have the α configuration at C_{12} with the substituent *trans* to the methyl group at C_{13} since the experimental evidence indicates that they were formed through a carbonium ion as an intermediate and the entering group would be added to the side least hindered. When the specific rotations of these epimeric pairs of compounds were tabulated (Tables I and II) it became evident that all of the

TABLE I

Specific Rotation of C-12 Epimeric Pairs $\Delta^{9,11}$—Cholenic Acid Series

High	Compound	Low	Solvent
+104°	3(α)—OH, 12—OH	+28°	Methanol
+206°	3(α)—OH, 12—OAc	−16°	Methanol
+201°	3(α)—OAc, 12—OAc	+3°	Methanol

TABLE II

Specific Rotation of Derivatives of Methyl $\Delta^{9,11}$—Cholenate with Substituents at 3 and 12 in the α Position

Compound	Rotation	Solvent
3(α)—OH, 12(α)—Cl	+149°	Chloroform
3(α)—OH, 12(α)—Br	+205°	Chloroform
3(α)—OH, 12(α)—OCH_3	+130°	Methanol
3(α)—OAc, 12(α)—OCH_3	+140°	Chloroform
3(α)—OAc, 12(α)—OH	+111°	Methanol
3(α)—OAc, 12(α)—Cl	+154°	Chloroform
3—Keto, 12(α)—OH	+71°	Chloroform
3—Keto, 12(α)—OCH_3	+110°	Chloroform
3—Keto, 12(α)—Br	+182°	Chloroform

compounds to which the α form had been assigned had specific rotations which were much higher than those of the epimeric pairs.* For example, the values are +201° and +3° for the two epimeric pairs of the 3(α), 12-diacetate. Previous work on epimeric forms at C_7 has shown that introduction of the double bond C_5:C_6 raises the value but does not change the relationship between the high and low rotating forms (21, 22). It therefore seems safe to conclude that in the derivatives of cholanic acid all the members with higher rotations have the same configuration at C_{12}; it follows that the lower rotating compounds have the opposite spatial arrangement at C_{12}. This is susceptible of experimental proof. It was found that only desoxycholic acid and those compounds derived from it had the higher rotations (Table III).

TABLE III**

Specific Rotation of C-12 Epimeric Pairs Cholanic Acid Series

High	Compound	Low	Solvent
+48°	3(α)—OH, 12—OH	+38°	Dioxane
+70°	3(α)—OAc, 12—OH		Methanol
+94°	3(α)—OAc, 12—OAc	+57°	Acetone
+83°	3—Keto, 12—OAc	+44°	Acetone

*The values given in Table III are taken from reference 11 except for the compound 3(α)—acetoxy—12—hydroxycholanic acid.

**Table II gives the specific rotations of a number of derivatives of 3(α), 12(α)—$\Delta^{9,11}$—cholenic acid.

From analogy with the derivatives of $\Delta^{9,11}$-cholenic acid, it may be assumed that the hydroxyl in desoxycholic acid at C_{12} is trans to the methyl group at C_{13} and is in the α position. The configuration of the hydroxyl group in desoxycholic acid is a matter of more than academic importance since the literature already contains structural formulas with hydroxyl groups at C_{11} and C_{12} definitely assigned to positions (6, 17, 8, 11, 12; 13, 19). It now seems certain that many of these structures are not correct and I feel that the relationships on optical activity which are discussed in this paper contribute good evidence which conforms with much other chemical work (4, 3). The work of Reichstein and his associates indicates that the side chain at C_{17} in desoxycholic acid is on the side of the plane of the molecule which is opposite to the hydroxyl group at C_{12}. The side chain at C_{17} of desoxycholic acid, of progesterone, and of the cortical hormones therefore is in the β position. As a consequence the hydroxyl group at C_{17} in 17-hydroxy-11-dehydrocorticosterone is in the α position. For the sake of the reader it is hoped that a uniform structure which accurately depicts the chemical relationships may soon be adopted by workers in the steroid field.

DISCUSSION

Dr. Gallagher: I would like to congratulate Dr. Kendall, as I have many times privately, on the completion of a masterly piece of research.

The epoxy compound appears to offer many advantages particularly in the initial Grignard reaction. He gave the impression that this reaction would occur readily at a low temperature with the other bile acids. I think this property is a peculiarity of the epoxy structure. The yields are very poor at low temperature with the 3, 11-dihydroxycholanic acid. One point, of interest to this group particularly, is the course of the reduction of the $\Delta^{9,11}$ compounds. These appear to yield exclusively cholane derivatives and no evidence has thus far been obtained for the formation of the urane compounds of Marker. I think that is quite significant.

Possibly the most interesting results of this work and that of others, are on configuration of substituents at C_{12}. The importance to the biologist lies in the bearing which this has on the configuration at carbon 11. The configuration of groups at C_{11} and C_{12} are intimately related and all of the evidence, including Reichstein's chemical evidence, points to the fact that the hydroxyl at 12 of desoxycholic acid is, as Dr. Kendall has indicated, in the alpha configuration. The consequence is that the hydroxyl group at C_{11} of corticosterone is in the beta configuration, as I have indicated in our report.

One other question I would like to ask is whether the epoxy structure in the 3, 9-epoxy-11, 12-diketo compound is labile to acid.

Dr. Kendall: In regard to the question of temperature of the Grignard reactions, it does apply to several compounds with a hydroxyl group at C_3 as well as to the 3, 9-epoxy structure. At icebox temperatures the most important factor is whether the intermediate products of the reaction are soluble or not. With 11-hydroxy or some other compounds which have a low solubility, the yield may be poor.

With regard to formation of uranes, the only compound that we have completely reduced is lithocholic acid. This reduction has not given anything but lithocholic acid that we have identified.

The 3, 9-epoxy-11,12-diketone is unstable to acid. Not only is it highly labile but decomposition occurs and only oily products have been obtained.

Dr. Heard: I wonder would Dr. Kendall care to discuss two general points which arise out of the dissertation he has given. First, in connection with the over-all yield from desoxycholic acid to dehydrocorticosterone, he quoted Reichstein's realization of 0.04 per cent. What is the yield obtained via the epoxy route just described?

Also, does Dr. Kendall consider it advisable to introduce the 11 oxygen atom before or after the degradation of the side chain?

Dr. Kendall: In regard to the question of yield, we have been more concerned with the development of the best yield at each step than we have been with the preparation of dehydrocorticosterone in quantity. I think the 3(α)-hydroxy-11, 20-diketone can be prepared in a yield between 10 and 20 per cent. The 3(α)-hydroxy-11-ketoetio acid, I believe, can be obtained in yields of between 5 per cent and 10 per cent.

One of the greatest troubles with the preparation of dehydrocorticosterone is the very last step. The yields of the intermediates are 70 per cent, 80 per cent and in some cases 90 per cent, but introduction of the double bond in ring A is not satisfactory. If that one step could be modified it would change the whole picture. I think it is an advantage to introduce the oxygen at C_{11} at the cholanic stage, because the degradation of C_{11} derivatives is easier than that of C_{12}. Introduction of oxygen could be done at any stage but it is very easy to introduce oxygen at C_{11} in the cholanic stage and then proceed with the degradation of the side chain.

Dr. Gregory Pincus: I may have misunderstood, but what is the implication that if there were a beta hydroxy group at C_{12} one might have quite different biological activity than if one has an alpha hydroxy group at C_{12} of desoxycholic acid or possible derivatives therefrom?

Dr. Kendall: The biologic activity of 3(α), 12(α)-dihydroxycholanic acid would probably be different from that of 3(α), 12(β)-dihydroxycholanic acid. Caglioti and Giacomello, on the basis of studies of x-ray defraction patterns, concluded that the hydroxyl group at C_{12} in desoxycholic acid is in the plane of ring C, and that this position is responsible for certain physical properties of the acid. If the position of the hydroxyl group were changed from α to β I believe there would be as significant changes in the biologic activity as there are in the melting point, specific rotation and other physical properties of the acid.

Dr. Jensen: What method do you employ to reduce the ketone at C_{12} without reducing the double bond C_9:C_{11}?

Dr. Kendall: The ketone can be reduced either with aluminum isopropoxide or better with hydrogen catalytically with platinum. The reduction takes place very satisfactorily with hydrogen. Just one mole of hydrogen is rapidly absorbed.

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Some Advances in the Partial Synthesis of Adrenal Cortical Steroids†

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I. Introduction

As a result of the brilliant chemical investigations of Reichstein, Kendall, Wintersteiner, and their collaborators (30) many steroids have been isolated from the adrenal gland, their chemical constitution established and in the majority of instances their biological activity determined. Certain of these compounds are unique among the steroids in that they have an oxygen atom present as a ketonic or hydroxyl group at carbon 11, a structural feature which appears to be responsible for or intimately associated with the biological activity of these compounds. Since a natural product oxygenated at carbon 11 is not as yet available for the partial synthesis of the adrenal cortical steroids, many attempts have been made to introduce either a ketone or hydroxyl group in the steroid nucleus at this position without at the same time introducing another substituent in the C ring (1, 2, 3, 4, 6, 24, 26, 37, 38). These attempts were uniformly unsuccessful until 1943 when Reich and Reichstein (28) prepared 11 ketocholanic acid and established for the first time a procedure by means of which the partial synthesis of an adrenal cortical hormone was made possible. In this report I shall review briefly the experiments of Reichstein and his colleagues and discuss some of our own results which have led to a realization of a similar result. The work of Kendall and his associates who likewise reached the same goal is reported elsewhere in this volume (16).

II. Work of Reichstein and Collaborators

As starting product for his synthetic attempts Reichstein chose the naturally occurring bile acid desoxycholic acid. After the successful outcome of model experiments on 12-hydroxycholanic acid (1) the removal of the hydroxyl group from carbon 12 was accomplished by pyrolysis of both 3-keto (I and IV) (5) and 3-acetoxy (V) (17) derivatives of desoxycholic acid with the formation of $\Delta^{11, 12}$-lithocholenic acids (III and VI) (Fig. 1). Similar experiments were carried out upon bisnordesoxycholic acid (18) and etiodesoxycholic acid (19).

†A portion of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development, and the University of Chicago.

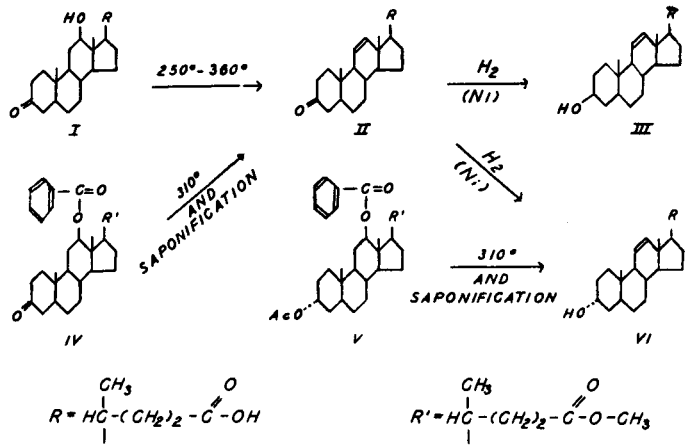

FIG. 1

The acids unsaturated in ring C were treated with hypobromous acid (Fig. 2) (bromacetamide in aqueous solution), and it was found that the hydroxyl group attached to carbon 11 with the halogen atom at C 12 (VIII) (20, 27, 28). Two side products of the reaction were identified. One (VII) represented normal addition of a molecule of bromine to the double bond; the other, a dibromomonohydroxy ester (X), was identified by oxidation and dehalogenation to methyl $\Delta^{9,11}$-12-ketocholenate (XI). Oxidation of the 11-hydroxy-12-bromo ester with CrO_3 followed by reduction of the bromoketo ester yielded the 11 keto steroid. In practice it was unnecessary to separate the primary products of the addition; the mixture was directly oxidized, then debrominated, and the desired product separated

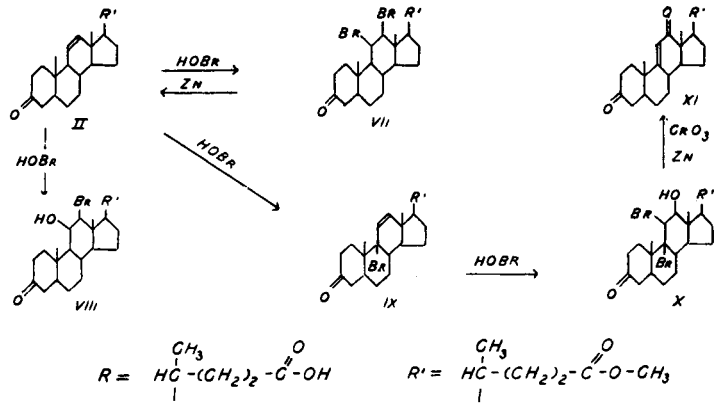

FIG. 2

from the reaction mixture. The reactions applied to methyl 3-keto-$\Delta^{11, 12}$ etiolithocholenate (XII) and the corresponding 3(α)-acetoxy ester (XVIII) are summarized in Figure 3. Partial reduction of methyl 3, 11-diketoetiolithocholenate yielded predominantly the β configuration (XV) at carbon 3 (21).

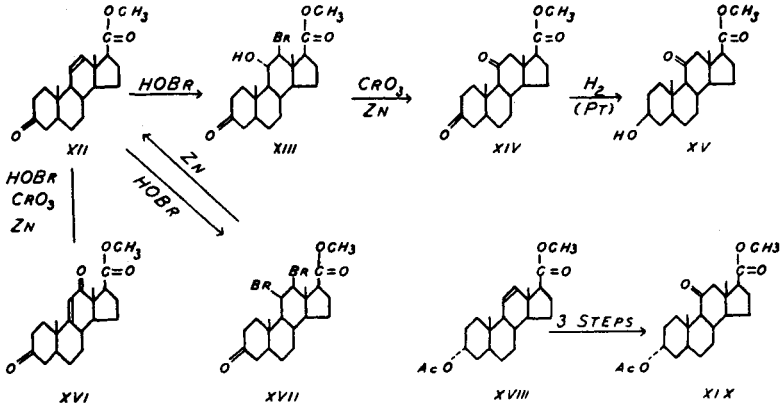

FIG. 3

It was possible now to prepare dehydrocorticosterone by essentially similar procedures to those previously developed by Steiger and Reichstein (35) for the synthesis of desoxycorticosterone. The reactions (22) are summarized in Figure 4. It is noteworthy that monobromination of 3, 11, 20-triketo-21-acetoxypregnane (XXV) proceeded smoothly and in good yield; the dehydrobromination of the product, however, was accompanied by con-

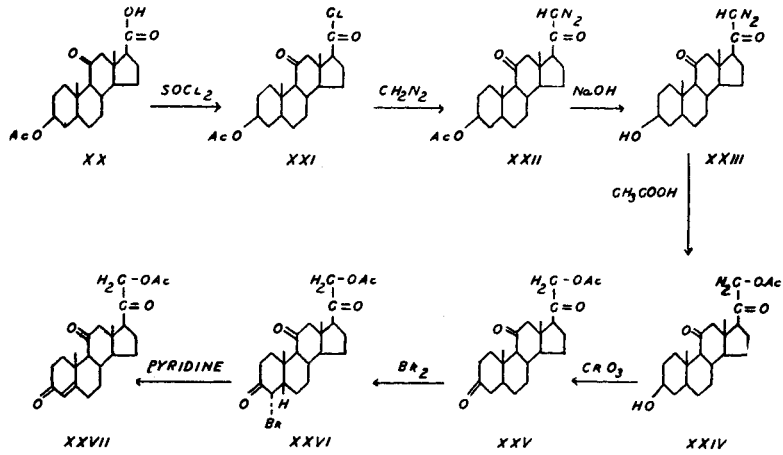

FIG. 4

siderable loss. The final product (XXVII) was dehydrocorticosterone acetate and upon saponification yielded the free ketol identical in all respects with the compound obtained from the adrenal gland.

The direct application of these results to the synthesis of corticosterone was not immediately possible. The hydroxyl at carbon 11 in the latter compound is very difficult to esterify and therefore is not readily protected against reaction with thionyl chloride when the etio acid is converted to the ketol side chain of the cortical hormones. It became necessary, therefore, to devise a new procedure for the synthesis of the side chain which would be applicable in the presence of an easily oxidized, unprotected hydroxyl group. This was accomplished by taking advantage of the reaction of lead tetraacetate with a reactive methyl or methylene group (7, 8, 29, 33). The necessary starting product was obtained from methyl 3(β)-acetoxy-11-ketobisnorcholanate (XXVIII) (Fig. 5) by catalytic re-

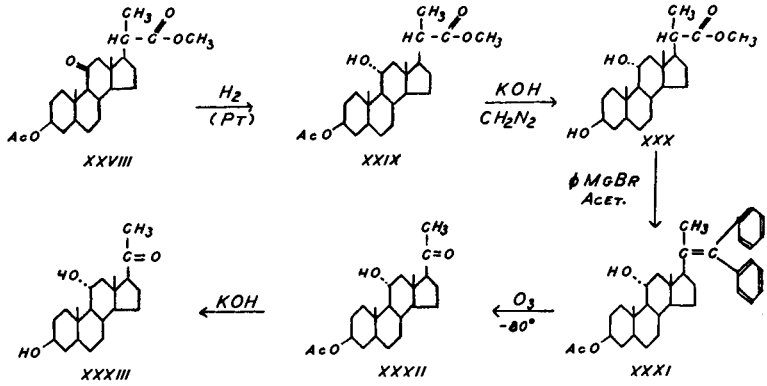

FIG. 5

duction (which apparently yielded exclusively the 11(α) isomer*). The bisnor ester was converted to the corresponding diphenylethylene (XXXI) which upon ozonolysis yielded the pregnane ketone (XXXII) (9). When treated with lead tetraacetate under strictly controlled experimental conditions this substance yielded 3, 11-dihydroxy-21-acetoxy-20 ketopregnane (XXXIV) (Fig. 6). Oxidation of the acetoxy ketol with aluminum phenate and acetone afforded the 3 keto compound (XXXV) which, by the reactions used in the preparation of dehydrocorticosterone, was converted to an α-β unsaturated ketone (XXXVII) (10). The product of hydrolysis was identical with corticosterone isolated from adrenal glands. These experimental results afforded a partial synthesis of adrenal cortical hormones and pro-

*The configuration designation is that of von Euw, Lardon and Reichstein (29). There is considerable reason to believe that this is incorrect and that the configuration at carbon 11 of the product is actually β.

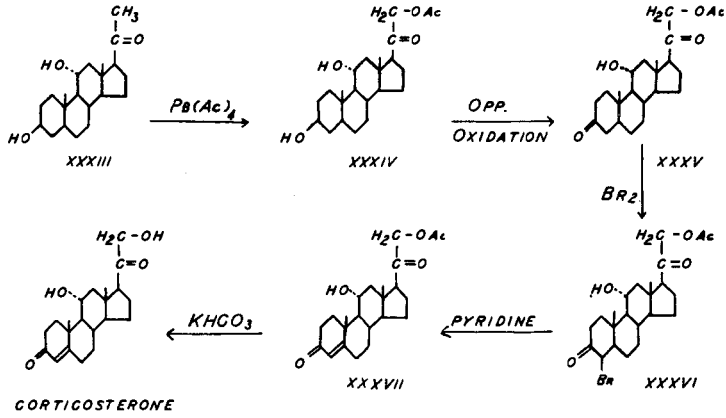

FIG. 6

vided a fitting climax to the long arduous work by which Reichstein and his associates had isolated and characterized a large number of steroids from the adrenal gland.

III. EXPERIMENTAL

Our early work as well as that of others on partial synthesis of adrenal steroids in this country was greatly facilitated by the preparation of Δ^{11-12} lithocholenic acid by Professor Kendall and his associates (25) who succeeded in developing a process for making this compound. This work had been done independently before Reichstein's publications were received in this country. In attempting to utilize Δ^{-11}-lithocholenic acid for the preparation of C11 oxygenated compounds, we investigated some of the properties of the 11(a)-12(a) epoxide (XXXIX) of this substance which can be pre-

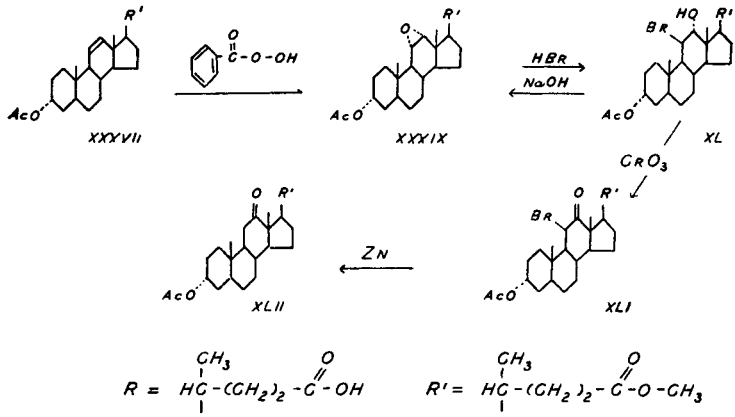

FIG. 7

pared by treatment with perbenzoic acid (Fig. 7). The epoxide reacted instantly with hydrobromic acid to form a beautifully crystalline bromohydrin (XL) which could be readily oxidized to the corresponding ketone (XLI) (13). Removal of halogen from this product yielded the 12-keto derivative of cholanic acid (XLII) proving that the halogen had occupied C11. The yields in these steps were essentially quantitative.

We then investigated the acetolysis of the epoxide. If this reaction proceeded as expected from the action of HBr on the epoxide, we should anticipate the formation of an 11-acetoxy-12-hydroxy derivative of cholanic acid (XLIII). This was found to be the case (Fig. 8), although side

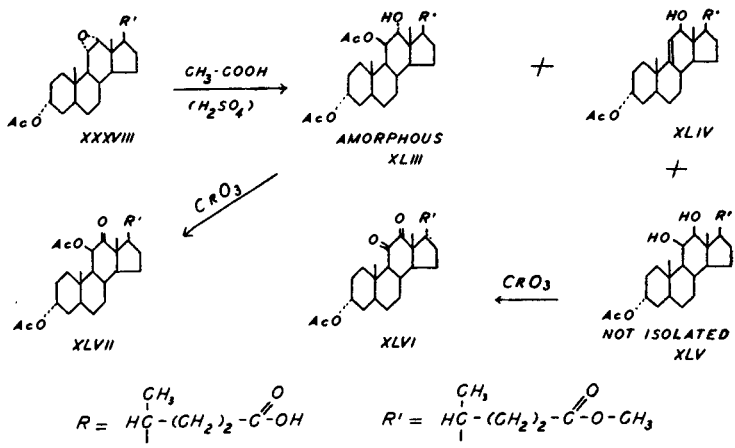

FIG. 8

products XLIV and XLV were also formed. The principal reaction product, methyl 3, 11-diacetoxy-12-hydroxycholanate (XLIII) was isolated (23), and the acetoxy group at C11 was found to be quite resistant to alkaline hydrolysis. The trihydroxy acid obtained by more vigorous saponification could not be reacetylated to a triacetate. These were expected properties of the compound since the C11 hydroxyl group of the cortical steroids has been shown to be markedly hindered in its reactions. An unexpected finding, however, was that methyl 3, 11-diacetoxy-12-ketocholanate (XLVII) obtained from CrO_3 oxidation of the acetolysis product readily formed ketonic derivatives. Since Marker and Lawson (26) had previously described a compound to which this structure had been assigned and since Longwell and Wintersteiner (24) had shown that it did not form ketonic derivatives the ready formation of both an oxime and a hydrazone by the compound we obtained demanded explanation. The reasons will be dealt with later. Since, however, we were in possession of a 12-hydrazone of 3, 11-dihydroxy-

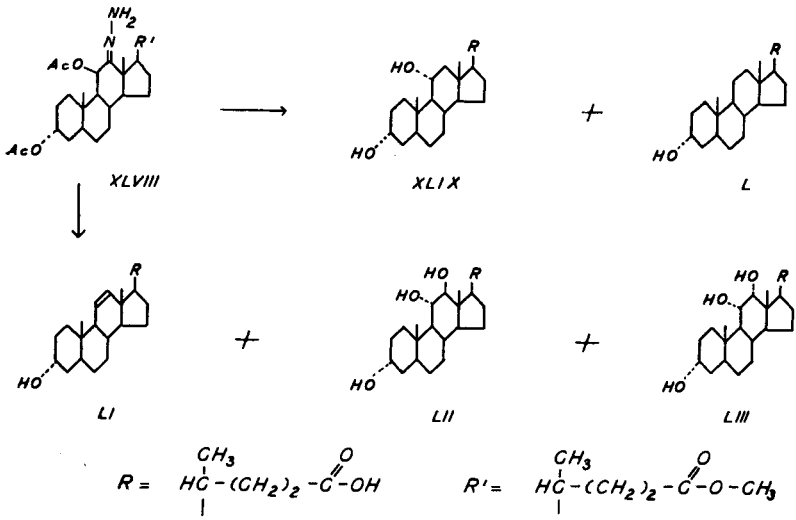

FIG. 9

cholanic acid (XLVIII), a Wolff-Kishner reduction was possible (Fig. 9). The reaction was unexpectedly complicated, but a compound (XLIX) was isolated which from the elementary analysis was unquestionably a dihydroxy cholanic acid. It was not identical with either desoxycholic acid or 12-epi-desoxycholic acid and the provisional conclusion was made that the compound was a 3, 11-dihydroxycholanic acid. Its properties are recorded below.

PROPERTIES OF 3(α), 11(α)-DIHYDROXY CHOLANIC ACID

1. Stable toward dehydration with concentrated HCl in acetic acid.
2. Oxidized to 3, 11-diketo cholanic acid identical with compound prepared by independent methods.
3. The diketo acid forms only a monosemicarbazone.
4. The 11 monoketo ester does not form an oxime.
5. Both hydroxyl groups acetylate very readily so that partial acetylation at 3 has not been possible.
6. Partial saponification of the diacetate proceeds smoothly so that the 11 acetate is obtained in high yield.

It appeared, therefore, that we were dealing with a compound having a hydroxyl group at C11 in the opposite configuration to that of the C11 hydroxyl of the cortical steroids.

We had thus succeeded in preparing a bile acid with a hydroxyl group at C11 having no other substituent in ring C. The procedure was, however, limited by the poor yields of $\Delta^{11,12}$-lithocholenic acid obtained by the pyrolytic method, and the yield in the acetolysis of the epoxide was also un-

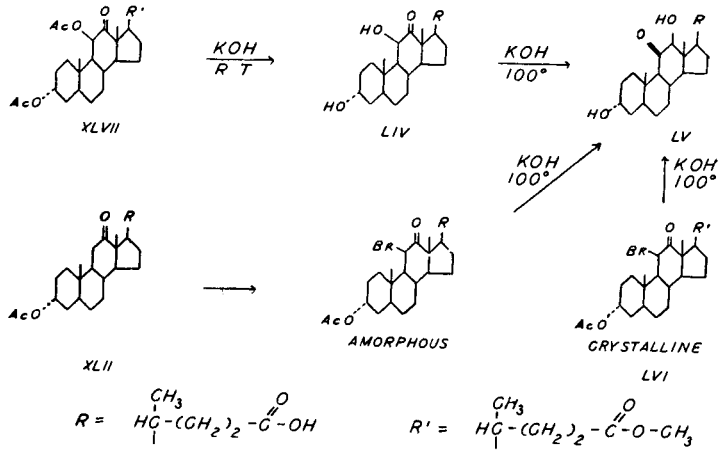

FIG. 10

satisfactory. A clue to a more desirable route was provided by the behavior of methyl 3, 11-diacetoxy-12-ketocholanate (XLVII) upon saponification Fig. 10). Vigorous alkaline hydrolysis of the product yielded the acid (LV) isolated by Marker and Lawson and by Longwell and Wintersteiner. If the hydrolysis was effected at room temperature or lower, a different compound (LIV) was obtained which was characterized by the formation of a beautifully crystalline insoluble sodium or potassium salt. Like the acetate from which it was prepared the product formed ketonic derivatives. We concluded, then, that if we were able to hydrolyze a substituent at C11 under sufficiently mild conditions we could obtain a ketol which would form a hydrazone and which could be converted to an 11 hydroxy bile acid.

We accordingly brominated methyl 3(α)-acetoxy-12-ketocholanate (XLII) (Fig. 11) at room temperature and were able to separate two crystalline bromoketo esters (XLI and LVII) (14). One of these (XLI), present in the mixture in very small amount, was identical with the product obtained by oxidation of the bromohydrin from the epoxide; the other (LVII) was a different compound; both had in the meantime been isolated by Seebeck and Reichstein (34). The two bromoketones differed markedly in their behavior when hydrolyzed by aqueous base at room temperature. The one obtained in small amount (XLI) was readily hydrolyzed, the other (LVII) was hydrolyzed much more slowly. The first yielded a 3, 11-dihydroxy-12-ketocholanic acid similar to but not identical with the Marker and Lawson acid. The second bromoketone was hydrolyzed to an acid identical with the 3, 11-dihydroxy-12-ketocholanic acid which we had previously obtained from acetolysis of the epoxide of lithocholenic acid. It

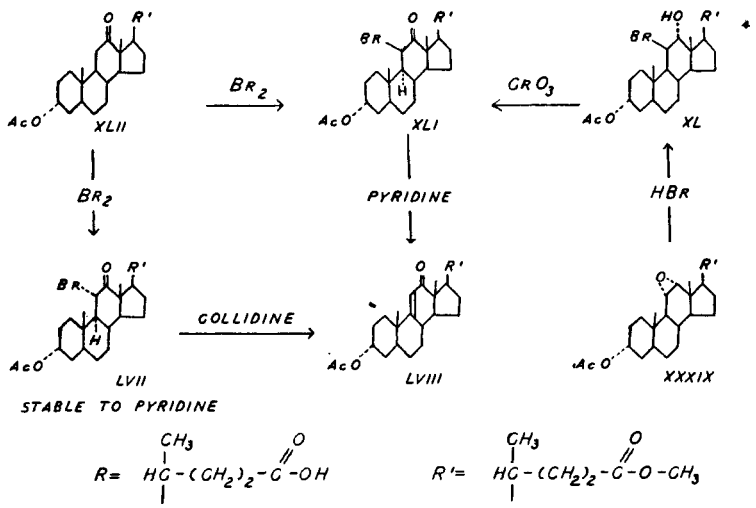

FIG. 11

is important to note that these two acids which differ only in the configuration of the hydroxyl group at C11 exhibit properties which are characteristic of the two epimers. One of them readily forms a diacetate proving the 11 hydroxyl is comparatively unhindered (12) while the other forms only a monoacetate. Both compounds form ketonic derivatives showing that the ketone group is at C12. Both yield 3, 11 dihydroxycholanic acid upon Wolff-Kishner reduction (14, 12). These experiments provided a more satisfactory method for the preparation of a bile acid with a hydroxyl group at C11.

IV. Discussion

In order to offer a logical interpretation of these reactions it is necessary to discuss briefly the configuration of substituents in ring C of the steroid nucleus. Assuming that the two angle methyl groups at carbon 10 and carbon 13 are perpendicular to the plane of the nuclear rings in a projection formula, reference to a molecular model immediately indicates that a substituent at carbon 11 *cis* to these methyl groups is subject to considerable steric hindrance. Similarly a substituent *trans* to these methyl groups is not sterically hindered and should readily form derivatives. A ketone group at carbon 11, from the model, appears to be subject to considerable steric hindrance from the methyl groups. On the other hand a substituent at carbon 12 in either configuration does not appear especially masked by the adjacent groups.

It seems clear, from molecular models at least, that a substituent at

carbon 11 *cis* to the angle methyl groups or in β configuration according to the generally accepted convention, should be sluggish in its reactions toward any reagent which must approach this group from the side of the molecule toward which the substituent and the angle methyl groups project. Thus a β-hydroxyl group at carbon 11 should not readily esterify. This has been shown to be the case with one of the diastereoisomeric 11-hydroxy-12-keto derivatives of cholanic acid where the failure to react is especially striking since the hydroxyl is activated by the neighboring carbonyl group. This substance has therefore been designated β at carbon 11. More important, however, is the demonstration that the other member of the pair, *i.e.*, α configuration at C11, readily forms esters and behaves, in fact, as one would be led to anticipate from inspection of molecular models. These results are substantiated by the similar reactions of the 3, 11, 12-trihydroxycholanic acids, of which the four possible epimers in ring C have been examined both in our laboratory and that of Dr. Oskar Wintersteiner of the Squibb Institute for Medical Research (11, 39). Two of these readily form triacetates, whereas the other two form only diacetates. From these considerations it would be plausible to assign the α configuration at carbon 11 to the 3, 11-dihydroxycholanic acid which we isolated and the β configuration to the 3, 11-dihydroxycholanic acid synthesized by Lardon and Reichstein (20).

These conclusions can be supported by independent reasoning from a different series of ring C derivatives. The configurations assigned to the two diastereoisomeric methyl 3(α)-hydroxy-11-bromo-12-ketocholanates both by Seebeck and Reichstein (34) and by us (14) are in agreement. One of these, the 11(β)-bromo epimer is identical with the product formed from the 11(α), 12(α)-epoxide** of lithocholenic acid after treatment with hydrobromic acid and oxidation. Upon hydrolysis under mild conditions this substance forms an 11-hydroxy-12-keto acid in which the hydroxyl group *a priori* should have the α configuration since a Walden inversion almost invariably accompanies a replacement reaction of this type. In point of fact the product behaves precisely as anticipated since it readily forms an acetate at carbon 11. Conversely the 11(α) bromo ester which, under the

**The formation of this substance from $\Delta^{11, 12}$-lithocholenic acid is an argument in favor of the configuration in Ring C. It is almost quantitatively formed, if, indeed, it is not the exclusive product, when the unsaturated acid or its derivatives is treated with perbenzoic acid. It is reasonable that the formation of an epoxy group at C-11 would be hindered in one configuration and not in the opposite. Since the reagent can, in effect, approach the double bond from only one side of the molecule the production of a single isomer is not surprising. Inversion at the carbon (C-11) to which the anionic substituent becomes attached would thus lead to β configuration. This is in agreement with the experimental results.

assumption that a Walden inversion takes place, should yield a β hydroxyl at carbon 11 when subjected to hydrolysis, forms a product which is markedly hindered toward acetylation.

The behavior of both 11-bromo and 11-hydroxy derivatives toward reagents which tend to eliminate the substituent at 11 together with the hydrogen at carbon 9 with the formation of a 9, 11 ethylenic bond is likewise in agreement with the configurations we have assigned. Thus the 11 hydroxyl group in the β configuration is readily lost as a molecule of water under the influence of dehydrating agents (13). This is the case both in the presence and absence of a 12 keto group. The 11(β)-bromo-12-keto esters are easily dehydrobrominated to the α-β unsaturated keto ester with pyridine while the 11(α)-bromo derivatives are quite stable toward similar treatment (34) (Fig. 11). Since elimination of trans substituents is the favored reaction in epimeric pairs (26) these results are in harmony with the configuration both at C-11 and that which has been suggested at C-9 (31, 32).

It would therefore seem to be inescapable that the substances synthesized by Reichstein and to which he has assigned 11-α configuration are in fact 11(β) compounds. Accordingly corticosterone should be designated 11(β), 21-dihydroxy-3, 20-diketo-Δ^4 pregnene. Likewise the other 11-hydroxy cortical steroids, which have thus far been isolated, have the β configuration of the 11 hydroxyl group since they are esterified at C11 with difficulty or not at all (30).

An 11(α)-hydroxy bile acid, since it can be easily acetylated, offers certain advantages in the degradation of the side chain as a preliminary to the introduction of the carbohydrate-like side chain of the cortical steroids.

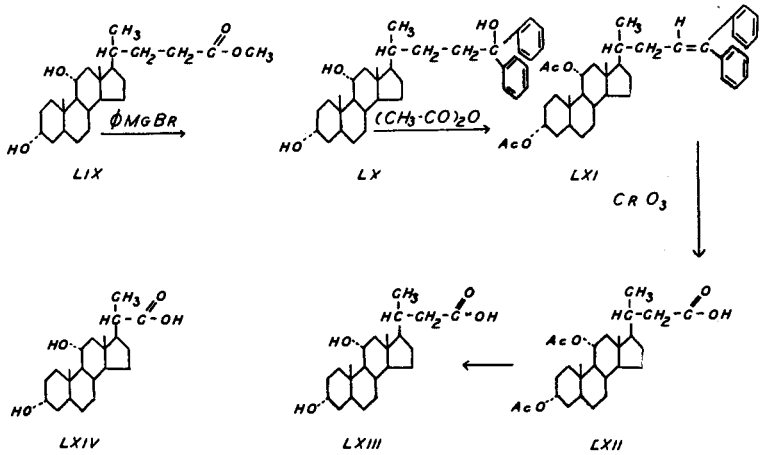

FIG. 12

The final product, an isomer so far not isolated from adrenal glands, should moreover provide interesting biological information. We, therefore, converted 3(*a*), 11(*a*)-dihydroxycholanic acid to the corresponding etio acid using the stepwise degradation of the diphenylethylenes to the pregnane ketone LXVII and the procedure of Hoehn and Mason (15) for the final steps (Figs. 12 and 13). 3(*a*), 11(*a*)-dihydroxyetiocholanic acid was also prepared directly from etiodesoxycholic acid by slight modifications of the same procedure which had been used with desoxycholic acid.

The 3(*a*), 11(*a*)-dihydroxyetiocholanic acid (LXX) (Fig. 14) after ace-

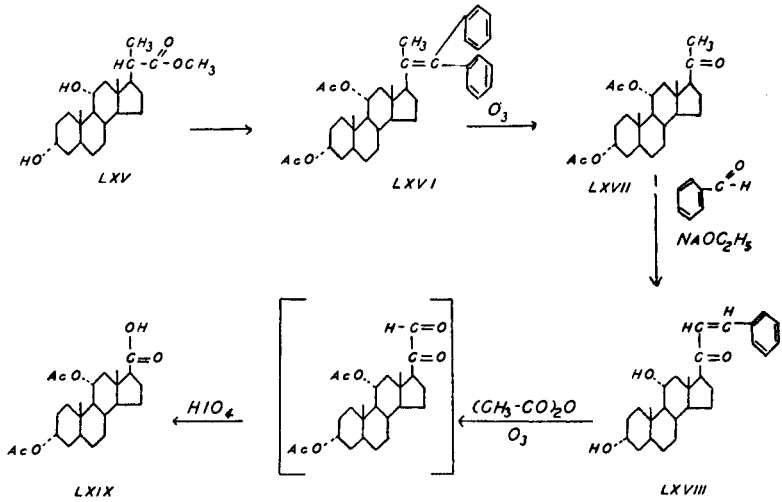

FIG. 13

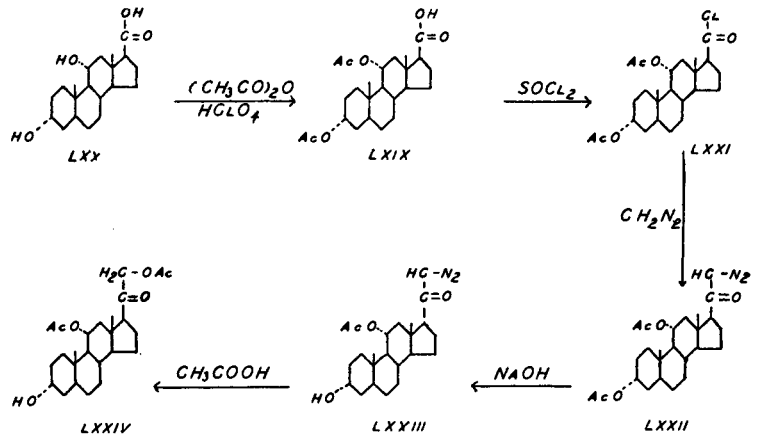

FIG. 14

tylation readily yielded an acid chloride (LXXI) which upon treatment with an excess of ethereal diazomethane formed a diacetoxy diazo ketone (LXXII). Partial hydrolysis removed the acetoxy group at C3 and the diazoketone was readily converted to the acetoxy ketol (LXXIV) upon warming with glacial acetic acid. These reactions are similar to those of Steiger and Reichstein (35) for the partial synthesis of desoxycorticosterone. Oxidation of the unprotected hydroxyl group at C3 (Fig. 15) yielded the di-

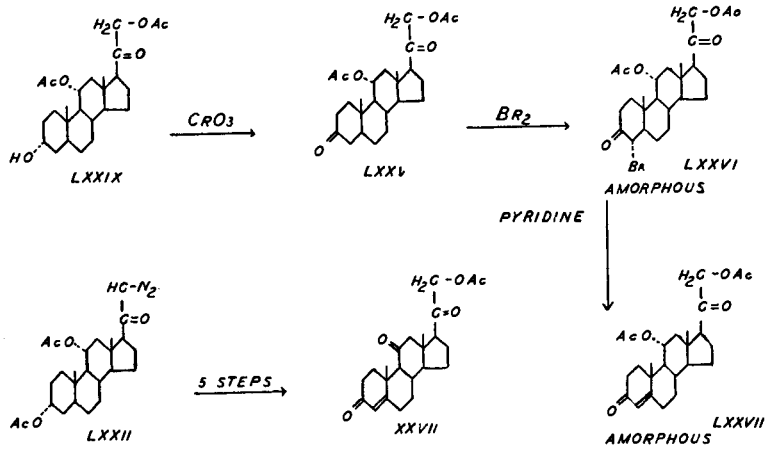

FIG. 15

ketone (LXXV). Bromination of this substance followed by the removal of the elements of HBr with pyridine yielded a substance which has not as yet been obtained in a crystalline state. It is the diacetate of 11 iso-corticosterone. Preliminary biological tests by Dr. D. Ingle of the Upjohn Laboratories and by Dr. K. Dobriner of Memorial Hospital indicate that the biological activity measured by glycogen deposition in the livers of adrenalectomized mice and weight maintenance of adrenalectomized rats is of a low order. It should be emphasized that these are preliminary results obtained with an amorphous product. The possibility that this compound or a closely related substance may be a constituent of the "amorphous fraction" from adrenal glands merits further work.

Since the aim of the investigation was the production of an adrenal steroid it was of some interest to prepare dehydrocorticosterone acetate from 3(*a*), 11(*a*)-dihydroxyetiocholanic acid. This was accomplished (Fig. 15) by a series of reactions similar to those outlined in Figures 13 and 14. The diazo ketone LXXII was subjected to a more prolonged alkaline hydrolysis so that both acetoxy groups were removed. Oxidation then yielded a triketone which upon warming with acetic acid yielded XXV (Fig. 4). The latter

upon bromination and dehydrobromination was converted to dehydrocorticosterone acetate identical with the known compound.

SUMMARY

1) The experiments of Reichstein and his collaborators leading to the preparation of dehydrocorticosterone and corticosterone have been reviewed.
2) An alternative procedure leading to the preparation of dehydrocorticosterone acetate and the diacetate of 11 iso corticosterone have been presented.
3) The evidence bearing upon the configuration of the C11 hydroxyl group of corticosterone and related compounds has been reviewed.

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The Relation of Cholesterol and Ascorbic Acid to the Secretion of the Adrenal Cortex*

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I. INTRODUCTION

Sooner or later in the study of the function of an endocrine gland it becomes of importance to determine the circumstances under which an increased supply of its particular hormone is made available to the organism. Such a question demands for its solution first an understanding of the circumstances causing an increased secretion and, secondly, a knowledge of the manner by which the increased supply of hormone is made available by the secretory cells of the gland. The mechanism of the biological synthesis and release of any hormone is imperfect at the present

*These studies were assisted by grants from the Fluid Research Fund, of the School of Medicine, Yale University.

time. This is particularly true in the case of the adrenal cortex, whose characteristic hormone has only recently been synthesized in small amounts by a laborious technic (8).

II. Methods for Study of Adrenal Cortex Activity

The methods that have been developed for the determination of an increased secretory activity of the adrenal cortex are as follows:

1. *Urinary Excretion*

The determination of the urinary excretion of cortical hormone, using as a method of assay either the ability of this hormone to increase the liver glycogen of rats and mice (5, 17), or its capacity to protect adrenalectomized animals against the effects of exposure to cold (15). The use of these technics has clearly demonstrated the increased urinary excretion of cortin in man after trauma burns and infections. The large amounts of urine required limit its use to man or the larger animals.

2. *17-Keto-Steroids*

The excretion of 17-keto-steroids has been suggested by some investigators as a measure, at least in part, of adrenal cortical function in man. However more recent studies, particularly those in which urinary "cortin" was determined on the same urine samples, tends to throw some doubt on the value of such measurements, for it has been reported from several sources that 17-keto-steroid excretion may be decreased or remain at normal levels at the time when "cortin" excretion is rising. The 17-keto-steroid excretion, at least in the male, is also complicated by the formation of these substances from the testicular hormone, and until methods are devised for more specific determination of the keto-steroids with C11 substitution, the question of their value, as a measure of normal adrenal cortical function, must remain uncertain. Both the preceding methods have, however, the advantage that they can be carried out on men or large animals without the use of operative procedures.

3. *Adrenals*

Vogt (20) has recently shown that the blood plasma drawn from the adrenal veins of large dogs contain sufficient quantities of cortical hormone to be determined by the Selye-Schenker method of assay. Surprising values were obtained for the 24-hour output of hormone from the adrenal glands of dogs. In some experiments quantities equivalent to 250 cc. of present-day commercial extract were estimated to be secreted, that is, if the rates as determined during the experiment were continued throughout the day. This

high figure may be fallacious, since the operative procedure in itself must have raised the output of hormone almost to maximal levels, although, as Vogt (19) has also shown, further increases in secretion could be evoked by splanchnic nerve stimulation or by epinephrine. So far as animal experimentation is concerned, this method is extremely promising.

4. *Hormone Content of Gland*

One of the most widely used methods for the study of variations in endocrine activity is the determination of the actual quantities of hormone present in the gland under different conditions. One general difficulty of this method is to decide whether a decreased content of hormone in the gland indicates a greater secretion or a decreased formation, a matter that can only be settled by actual measurement of hormone released into the blood. A special difficulty in the case of endocrine glands secreting steroid hormone is the small quantity present in them at any one time. This, in the case of the adrenal cortex, makes it impossible to directly measure alterations in the hormone content of the gland even by biological technics.

5. *Associated Metabolic Changes*

There is, however, one other approach to the problem. It may be assumed that an increased output of hormone, particularly in the gland that possesses only a small reserve, is accompanied by characteristic metabolic changes associated with the synthesis and release of the hormone. This is particularly so when a precursor or an essential constituent of the hormone is present in the gland in quantities much greater than those present in the other tissues. Two instances of this are now known. The first, which is not within the scope of this paper, is the relation of iodine metabolism to the thyroid hormone; the second is the presence in the adrenal cortex of two chemical substances, both of which appear to be specifically related to the formation of the hormone, and both of which appear to reflect the changes in its secretion. These substances are cholesterol and ascorbic acid.

a. *The Cholestrol and ascorbic acid content of the adrenal.* The outstanding chemical characteristics of the adrenal cortex are its high content of cholesterol and ascorbic acid. No other organ of the body except the corpus luteum approaches the organ in its high concentrations of *both* these substances.

Table I gives the values obtained for adrenal cholesterol and ascorbic acid in rats after various periods of "Nembutal" anesthesia* whose body temperature was maintained within normal limits throughout.

*It should be emphasized that when the anesthetic is injected intraperitoneally it

TABLE I
Adrenal Cholesterol and Ascorbic Acid Levels in Normal Rats Nembutal Anesthesia

Period of Anesthesia	No.	Adrenal Cholesterol g./100	Adrenal Ascorbic Acid mg./100 g.
Intraperitoneal—10-15 mins.	23	4.05 ± 0.14	370 ± 14* (D)**
" —2 hours	6	4.19 ± 0.15	431 ± 17 (R&A)***
Subcutaneous—1 hour	6	3.95 ± 0.27	420 ± 31 (")
" —1½ hours	6	4.09 ± 0.21	422 ± 26 (")
" —2 hours	6	3.77 ± 0.26	406 ± 20 (")
" —2½ hours	6	4.17 ± 0.30	430 ± 31 (")
24-day old rats	45	3.03 ± 0.08	314 ± 6 (D)**

*Ascorbic Acid value on 6 rats only.
**Dye titration method.
***Roe and Keuther method.

Another characteristic of the adrenal cholesterol is that approximately 90% of it is present in the gland in ester form. This is to be compared with the 50% of ester found in the liver and the 10% found in the brain. It is generally understood that a high proportion of esterified cholesterol is indicative of a high rate of turnover of both the steroid and its associated fatty acids.

Although in the past there has been considerable speculation concerning the possible relationship between the adrenals and the general cholesterol metabolism of the body, no definite association has been shown. The fact that the adrenal cholesterol was capable of wide fluctuation under different circumstances was, however, known. Recently we have reviewed the literature on this subject (10) and have pointed out that a lowering of adrenal cholesterol is associated with exposure of the organism to a variety of stresses, both internal and external.

The high concentration of ascorbic acid in the adrenal furnished the first source for its isolation in pure form. Here again considerable fluctuations in its content have been observed under various circumstances and the relation of the vitamin to the natural resistance of the organism has also been suggested (6). Furthermore, Zwemer, Lowenstein and Pines (22) have suggested that the adrenal contains water-soluble steroids in which the steroid is associated with ascorbic acid. In a private communication Lowenstein has informed me that it is possible to isolate compounds of this type which possess the biological activity of the cortical hormones.

should be given in normal saline at body temperature. The intraperitoneal injection of cold water solutions will in itself cause a lowering of adrenal ascorbic acid and cholesterol. Volatile anesthetics, unless carefully administered, to small animals easily cause respiratory anoxia, and are contraindicated for studies of this kind. Respiratory anoxia, however produced, causes a rapid depletion of adrenal ascorbic acid.

b. *Effect of adrenotrophic hormone on adrenal cholesterol and ascorbic acid.* The demonstration that cholesterol and numerous other substances of biological importance had a common chemical structure has led to the hypothesis that cholesterol might undergo transformation in the body into such related substances as the steroid hormones and bile acids. Recently it has been shown by Block, Berg and Rittenberg (2) by use of cholesterol tagged with deuterium that this substance is actually converted into cholic acid, while Bloch (1) has found that the feeding of cholesterol labelled in this manner to women is followed by the excretion of pregnanediol containing the isotope in the urine. This last experiment is of particular interest in relation to similar transformations in the adrenal cortex, since pregnanediol is the urinary excretory product of progesterone, a substance of very similar composition to the adrenal cortical steroids.

Having in mind the possibility that the adrenal cholesterol might indeed be the precursor of the adrenal hormones and that alterations in its concentration in the gland would thus be indicative of a formation or liberation of the hormone, we first studied the effect upon the adrenal cholesterol of highly purified adrenotrophic hormone (A.C.T.). When it became apparent that this trophic hormone actually did specifically deplete the adrenal cholesterol, we then also determined its effects upon the adrenal ascorbic acid.

In the *rat* a single injection of 4 mg. per 100 g. of body weight of highly purified A.C.T. (4 mg./100 g.) is followed by a rapid fall in adrenal ascorbic acid and a slower fall in adrenal cholesterol* (10, 11, 13, 14).

Some 20 minutes after the injection the *adrenal ascorbic acid* has been reduced by 30% and in 1 hour by 60%. It then begins to rise again and by the ninth hour after injection has returned to normal.

The fall in adrenal *cholesterol* does not reach its maximum until the third hour after these amounts of A.C.T., and the return to normal is prolonged, the initial level not being regained until almost 24 hours after the injection.

These effects of A.C.T. upon the composition of the adrenal are accompanied by definite indications of an increased output of the cortical hormones themselves. Thus, it has been shown that the liver glycogen rises as the cholesterol and ascorbic acid content of the adrenal fall, the liver glycogen reaching a maximum between the 6th and 9th hour after injection (10). Furthermore, as White and Dougherty (3) have shown, the maximum lymphopenia after A.C.T. injection into rats is also reached be-

*This is a large dose. A detectable effect is produced by quantities of 0.25 mg. per 100 g. while 1 mg. per 100 g. eicits about the same response as four times this amount.

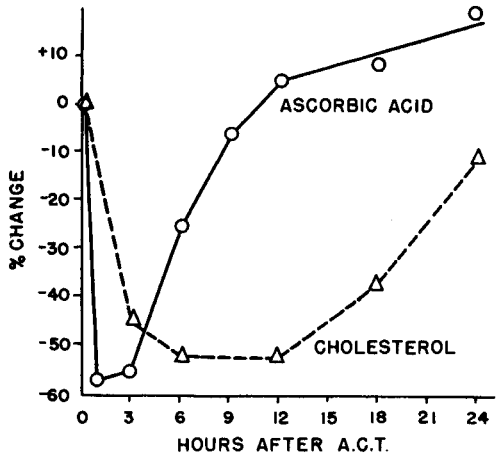

FIG. 1

The Effect of a Single Injection of Adrenotrophic Hormone (A.C.T.) 4 mg./100 g. of Body Weight on the Adrenal Cholesterol and Ascorbic Acid of the Rat (17).

tween the 6th and 9th hour. Both these effects, the increase in liver glycogen of fasted animals and the lymphopenia, are specific effects of the adrenal cortical hormone, and their association with these alterations in the composition of the adrenal gland itself are, we believe, highly suggestive of a relationship between them. Similar changes are observed in hypophysectomized rats, provided not more than three days has elapsed since the operation. After a longer period the response becomes sluggish and then disappears. Evidently, the atrophic cells of the gland require a preliminary period of reconstruction and reconstitution before they can again exhibit the prompt response seen in intact animals. The gradual fall in adrenal ascorbic acid in the glands of hypophysectomized rats with time has been described by Tyslowitz (16).

The guinea pig, like man, is an animal that is unable to synthesize ascorbic acid, and it was of obvious interest to determine the effect of A.C.T. in this species. It was found (12) that this trophic hormone produces the same rapid fall in adrenal ascorbic acid followed by a slower fall in cholesterol as was found in the rat. However, while the rate of reformation of adrenal cholesterol is at about the same rate as in the rat, that of ascorbic acid is definitely retarded. This substance reaches its maximum decline in the gland about the 3rd to the 6th hour after A.C.T. injection. It remains at this low level until at least the 12th hour, by which time the level in the rat has returned to normal, and then begins to rise slowly. 24 hours after the injection it is still some 10% less than normal. This slow rise in

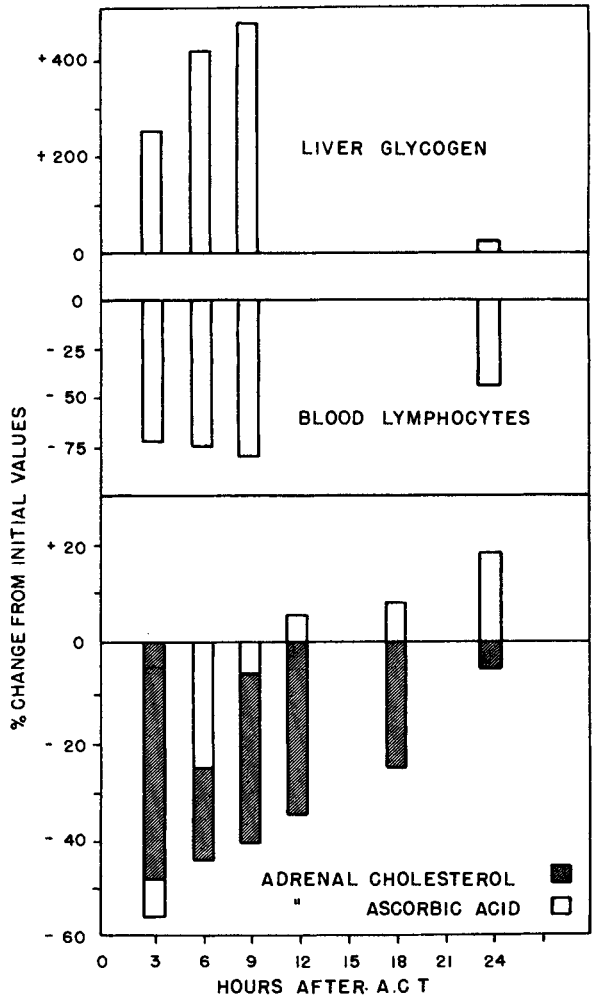

FIG. 2

The changes in (a) Liver Glycogen, (b) Circulating Blood Lymphocytes, and (c) Adrenal Cholesterol and Ascorbic Acid of the Rat Following a Single Injection of Adrenotrophic Hormone (7, 15, 17).

animals not receiving any ascorbic acid during this period is presumably due to the withdrawal and storage of the vitamin from the blood by the cells of the gland.

This slow restoration of adrenal ascorbic acid after stimulation of the guinea pig gland by A.C.T. raises some interesting possibilities if indeed there is an actual association between the cortical secretion and the vitamin.

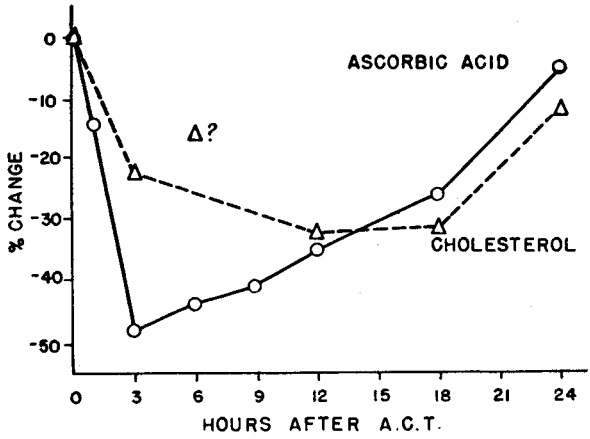

FIG. 3

The Changes in Adrenal Cholesterol and Ascorbic Acid of the Guinea Pig Following the Injection of a Single Dose (4 mg./100 g.) of Adrenotrophic Hormone (17).

For as will be seen shortly, various noxious stimuli rapidly reduce the adrenal ascorbic acid, and since in the guinea pig and man ascorbic acid synthesis is impossible, then the adrenal is dependent on the amounts initially present in the gland or those that can be withdrawn from the blood stream. A strong and continuous stimulation of the gland might, therefore, reduce the ascorbic acid content to a point where the secretion of the hormone-vitamin complex might be greatly retarded. This occurring at a time when the demand for hormone is high might lead to a state of relative cortical insufficiency and would, if it occurred, furnish a sound reason for the administration of extra cortical hormone during periods of stress. At the moment the possibility of such an event is based solely on hypothesis, but it is an hypothesis perhaps worthy of further study.

To return to more factual material, it should be emphasized that comparable changes in the cholesterol and ascorbic acid levels of other tissues do not occur following the injection of A.C.T. We have examined brain, liver, kidney, spleen, heart, skeletal muscle, and lymphoid tissue, and only inconsequential alterations are found.

Since so far as is known at the present time, A.C.T. has no other physiological effect except the stimulation of adrenal cortical secretion with a consequent display of the characteristic activities of these hormones, we may conclude that the changes in adrenal cholesterol and ascorbic acid that follow the injection of A.C.T. are indicative of an increased rate of formation and release of the hormone from the gland.

c. *Adrenal cholesterol and ascorbic acid as precursors of the cortical Hormones.* The evidence for the ultimate conversion of cholesterol into the cortical hormones (or hormone) by the cells of the gland is admittedly at the present time indirect. The circumstantial evidence, however, appears to be strong, and this view is strengthened even more by the demonstration that cholesterol is converted into progesterone in man. It would be extremely interesting to devise experiments in which the cholesterol and ascorbic acid content of the corpus luteum was followed under conditions in which progesterone secretion was known to be occurring.

The association of vitamin C with the formation and secretion of cortical hormone was first suggested by Zwemer and Lowenstein. Lowenstein, in still unpublished work which he has kindly permitted me to quote, has now isolated from the adrenal a water-soluble steroid in which he believes C is attached to ring D of the steroid nucleus apparently by carbon-to-carbon union. According to Lowenstein this compound which has a high degree of instability in water solution breaks down in an acid medium to form a steroid without cortical activity and vitamin C. The latter has been isolated and its anti-scorbutic properties confirmed. This important claim has yet to be substantiated but if correct greatly clarifies the alterations in adrenal ascorbic acid we have observed following the injection of A.C.T. It might also account for the rapid and early depletion of ascorbic acid in response to stimulation of the gland since this may represent the discharge of preformed hormone which is followed by a slower change in cholesterol as new quantities of hormone are formed.

6. *The Effect of Various Stresses on Adrenal Cholesterol and Ascorbic Acid*

It now appears to be established that one of the effects of stress, whether arising within or without the organism, is an increased demand for cortical hormone. The reasons for this increased requirement are not understood, but presumably they are first met by an increased rate of secretion of A.C.T. Indeed, in all discussions of this problem it must be clearly realized that the response of the anterior pituitary is not only the first but also the essential one in the events leading to adrenal cortical activation. The nature of the stimuli, whether nervous or humoral, that evoke this response are quite indefinite at the moment. Once the pituitary response has occurred, the adrenal cortex of a normal animal promptly responds by greatly increasing the output of hormone.

It is evident then that if the changes in adrenal cholesterol and ascorbic acid following the exogenous administration of A.C.T. are a humoral effect of this trophic hormone, then similar alterations in the composition of the

gland should occur when animals are placed under circumstances in which it is known that an increased secretion of cortical hormone is necessary for survival.

Such experiments have been carried out either by ourselves or others under the following conditions, as (a) Hemorrhage (b) Burns (c) Cold (d) Muscle trauma and (e) Painful stimulation of peripheral nerves. The results of experiments by other investigators not specifically designed along the same lines, yet dealing in essence with the same problem, have been reviewed elsewhere (10).

(a) *Hemorrhage.* The experiments on hemorrhage formed part of a study carried out on shock (11). Although we failed to find any alleviation of severe or irreversible shock by the use of adrenal cortical hormone, we did observe that even hemorrhages that did not have a fatal outcome were accompanied by marked changes in the composition of the adrenal cortex.

Two degrees of hemorrhage were studied. In the first, blood equivalent to about 2% of the body weight was withdrawn from the tail veins of rats over a period of one hour. From such a hemorrhage practically all healthy, normal or hypophysectomized rats recover spontaneously. Nevertheless, as the figure shows (Fig. 4), the adrenal ascorbic acid and cholesterol (Fig. 5) continue to fall for some hours and even 24 hours later has not returned to normal levels. The similarity of these curves to those given by a single injection of purified A.C.T. is very striking and illustrates the coincidence of results obtained by an exogenous or endogenous stimulation of the gland by A.C.T. In sharp contrast to these effects is the absence of any response of these glandular constituents to similar hemorrhage in the hypophysectomized animal (Fig. 6). The cholesterol and ascorbic acid levels of liver and brain in both types of animals are unaffected. There appears to be a slight lowering of plasma cholesterol and a rise in plasma ascorbic acid in normal rats.

In the second type of hemorrhage studied, blood equivalent to 3% or more of the body weight, was withdrawn from the tail veins over a period of 1 hour. After such a degree of hemorrhage most animals will collapse and die within the next hour. Such deaths are to attributed in large part to the acute oligemia and not to shock. However, in order to prolong the period of survival of these animals and thus allow time for severe and irreversible shock to develop, they were transferred shortly before this initial circulatory collapse with a volume of blood inadequate to effect complete recovery. The quantity of this transfusion was so adjusted that a second collapse occurred in less than 3 hours. At this time a volume of blood sufficient to completely replace that initially removed was given. In spite of this total replacement of the blood volume, all animals so treated remained in shock and

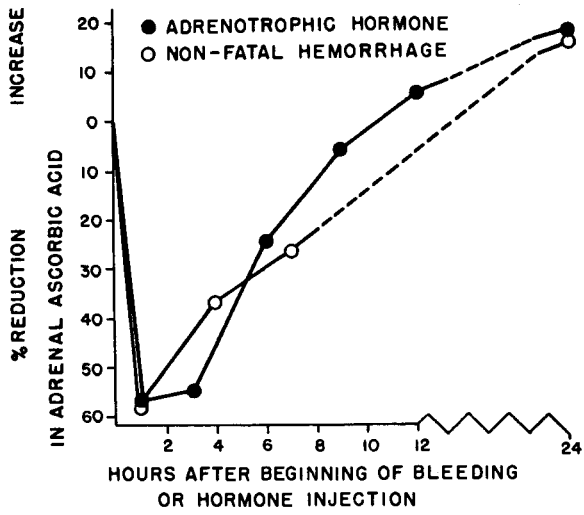

FIG. 4

The Effects (a) of Hemorrhage (2% of body weight) and (b) Adrenotrophic Hormone (4 mg./100 g.) on the Adrenal Ascorbic Acid of Rats (14).

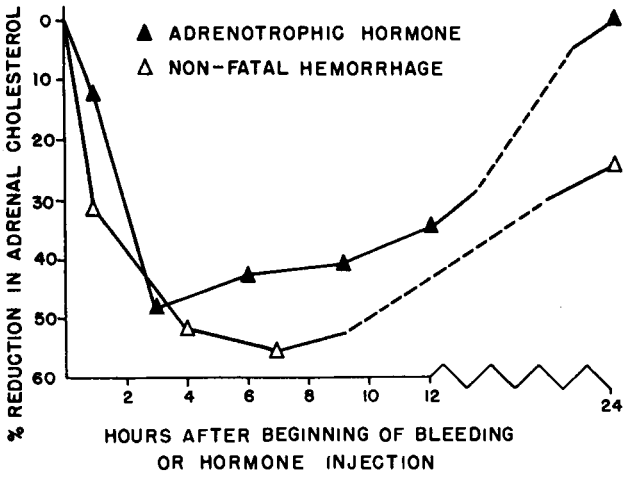

FIG. 5

The Effects (a) of Hemorrhage (2% of body weight) and (b) a Single Injection of Adrenotrophic Hormone on the Adrenal Cholesterol of Rats (14).

died within a few hours. The state to which they were reduced by this procedure is, we believe, truly "irreversible shock," and it may be repeated that such animals cannot be saved by injecting cortical hormone alone or in combination with ascorbic acid, at least in the amounts we have used.

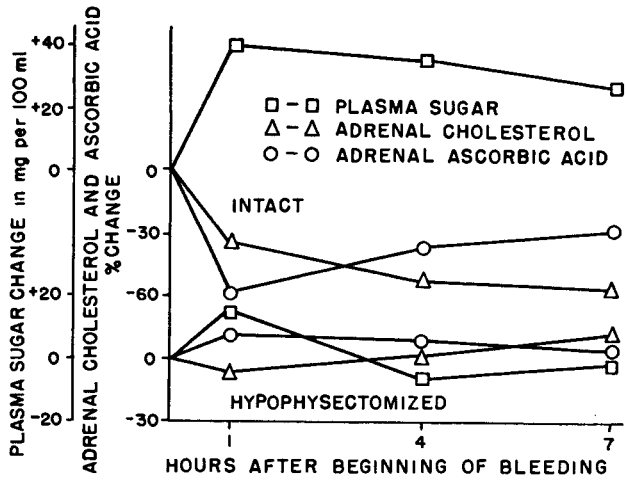

FIG. 6

The Effect of Hemorrhage (2% of body weight) on the Adrenal Cholesterol, Ascorbic Acid, and Blood Sugar of Normal and Hypophysectomized Rats (14).

Although the adrenal ascorbic acid and cholesterol were not determined at the end of the initial bleeding in this group of animals, it is to be assumed that they were reduced to at least the same degree as in the former group. The further behavior of the adrenal constituents is, however, quite different, since in this group they showed no inclination to return to normal and, indeed, were actually lower at the time of death than in the intermediate stage (Tables II and III). This may in part be due to the inadequate oxygenation of the gland as a consequence of the reduced blood flow, or it may indicate a continuing and perhaps unsatisfied demand by the tissues for cortical hormone. It will be noted that in severe shock the liver loses ascorbic acid and the plasma level is increased some six-fold.

Since hypophysectomized animals will not tolerate this degree of hemorrhage and other procedures, it was not possible to carry out comparable studies on their adrenals.

TABLE II

Adrenal, Liver, Brain and Plasma Ascorbic Acid Following a Fatal Hemorrhage

Values expressed as mg./100 g. of fresh tissue and mg./100 ml. of plasma. Each value represents the average and standard error of 5 to 7 animals.

	Controls (not bled)	Bled	
		Intermediate Stage	Terminal
Adrenal	403 ± 15.2	231 ± 11.6	205 ± 7.9
Liver	24.0 ± 1.10	20.5 ± 2.18	13.2 ± 0.99
Brain	39.6 ± 0.72	41.3 ± 0.69	38.9 ± 1.37
Plasma	0.51 ± 0.058	2.57 ± 0.128	3.0 ± 0.476

TABLE III

Adrenal, Liver, Brain and Plasma Cholesterol Following a Fatal Hemorrhage

Values expressed as mg./100 g. of fresh tissue and mg./100 ml. of plasma. Each value represents the average and standard error of 5 to 7 animals.

	Controls (not bled)	Bled	
		Intermediate Stage	Terminal
Adrenal	5.84 ± 0.314	2.58 ± 0.414	1.53 ± 0.140
Liver	0.391 ± 0.0239	0.325 ± 0.0093	0.330 ± 0.0101
Brain	1.89 ± 0.031	1.85 ± 0.040	1.93 ± 0.041
Plasma	55 ± 2.6	43 ± 1.8	42 ± 3.5

It should again be emphasized that we do not imply that the response of the adrenal cortex is the sole factor in resistance to hemorrhage nor that adrenal cortical insufficiency is the cause of irreversible shock. Indeed, it is our opinion that while the adrenal cortical response is a physiological one and necessary for adjustment of the organism to unfavorable circumstances, a point is reached at which both it and other compensatory mechanisms fail. When this point is reached, so called "irreversible" shock appears, and marked and serious derangements of cellular metabolism dominate the picture. These are not relieved by cortical hormone nor, for that matter, by restoration of the normal oxygen transport capacity of the blood.

(b) *Burns and scalds.* It has long been recognized that the adrenal cortex may be seriously damaged following severe burns or scalds. Hemorrhages and other changes in the substance of the gland are a common occurrence. Harkins and I (4) have found that normal rats scalded by dipping the body up to the neck in water at 70° C. for 5 seconds show, some 6 hours later, a 70% reduction in adrenal cholesterol. Adrenal ascorbic acid was not determined in these experiments. However, similar treatment of rats hypophysectomized 3 days previously had no effect on the adrenal cholesterol.

TABLE IV

*The Effect of Hypophysectomy in Preventing the Usual Changes in the Adrenal Total Cholesterol Following Experimental Scalds in the Rat**

Preparation	Experiment	No. of Animals	Rise in Hemato-crit %	Av. Body Wt. g.	Adrenal Wt. as Mg.	% Body Wt.	Adrenal Total Cholesterol Mg./100 mg. Adrenal Wt.
Control Rats	Control	5	0**	190	17		3.7 ± 0.6
Control Rats	Burn 70° 5″	8	11	231	15		1.0 ± 0.1
Mock Hypophysectomy	Burn 70° 5″	3	16	171	22		1.5 ± 0.2
Hypophysectomy	Burn 70° 5″	8	13	167	13		5.7 ± 0.6

*All animals killed at the end of 6 hours.

**Actually the control hematocrits fell an average of 6 per cent. For clarity of tabulation, this fall is used as a baseline of reference for the other figures given below in the same column.

(c) *Exposure to cold.* Exposure to cold is another potent stimulant of the adrenal cortical secretion. Hypophysectomized or adrenalectomized rats will succumb to exposures to cold that are easily tolerated by normal animals and may be protected against these deleterious effects by the injection of cortical extracts.

We have exposed fasted normal rats to temperatures of 0-4° C. for periods of 1 to 24 hours. Hypophysectomized rats were similarly exposed for periods up to 4 hours. Any longer exposure than this is attended by a high mortality and even the exposure of hypophysectomized rats for 4 hours causes a fall in body temperature to around 94° F. The body temperature of normal rats remains about normal after even longer periods of exposure.

In normal rats the adrenal ascorbic acid falls after one hour of exposure, but as this is continued it rises again to normal levels by the sixth hour. The cholesterol again falls later than the ascorbic acid and remains low throughout exposures up to 24 hours. Hypophysectomized animals show no changes in adrenal ascorbic acid and cholesterol even though they are rendered moribund by this treatment.

TABLE V

Effect of Exposure to Cold on the Adrenal Cholesterol and Ascorbic Acid of Normal and Hypophysectomized Rats

	No.	Adrenal Cholesterol g./100 g.	Adrenal Ascorbic Acid** mg./100 g.
Normals			
Normals—room temp.	23	4.05 ± 0.14	370 ± 14
Cold room*—1 hour	4	3.69 ± 0.74	230 ± 26
" " —2-2½ hours	11	2.70 ± 0.19	290 ± 17
" " —4-4½ hours	17	2.40 ± 0.17	———
" " —6-6½ hours	18	2.07 ± 0.20	420 ± 35
" " —24 hours	9	2.19 ± 0.42	———
Hypophysectomized			
Controls—room temp.	13	5.60 ± 0.43	418 ± 26
Cold room—1 hour	4	5.90 ± 0.32	379 ± 12
" " —4 hours	8	5.72 ± 0.42	379 ± 18

*Cold room maintained at temperature of 0-4° C.
**All determinations by dye titration method.

In *guinea pigs,* as may be expected from the effects of injected A.C.T., exposure to cold produces a rapid and sustained fall in adrenal ascorbic acid which lasts as long as the exposure is continued. The adrenal cholesterol is also markedly and persistently lowered.

(d) *Trauma.* Although we have not determined the effect of trauma involving extensive muscle injury on the composition of the adrenal, an interteresting and satisfactory study of this has recently been published by

TABLE VI

Effect of Exposure to Cold on Adrenal Cholesterol and Ascorbic Acid of Guinea Pigs

	No.	Adrenal Cholesterol g./100 g.	Adrenal Ascorbic Acid** mg./100 g.
Room temp.—no fast	6	4.27 ± 0.93	137 ± 6
" " —fasted 18 hrs.***	8	4.49 ± 0.24	114 ± 12
" " —fasted 42 hrs.	3	3.20 ± 0.10	105 ± 3
Fasted 18 hrs. + cold room* 1 hr.	7	3.59 ± 0.80	99 ± 7
" " " + " " 4 hrs.	6	3.21 ± 0.40	104 ± 9
" " " + " " 12 hrs.	4	2.91 ± 0.33	90 ± 13
" " " + " " 24 hrs.	4	1.25 ± 0.36	51 ± 9

*Cold room maintained at temperature of 0-4° C.
**All determinations by dye titration method.
***Last injection of ascorbic acid at the beginning of the fast.

Popjak (7). This investigator showed that rats shocked by application of tourniquets to one hand limbs had, 24 hours after release of the ligatures, lost some 64% of their adrenal cholesterol without, however, any change in the neutral fat, phospholipid or protein content of the glands. The water content had increased slightly. In other words in these experiments the major change in the adrenal resulting from the trauma and shock was the fall in cholesterol ester. This accounted for the loss of stainable lipids.

Popjak also observed in the traumatized group of rats a marked widening and greater staining intensity of the zone of the adrenal stained after treatment with phenyl-hydrazine, a reaction which is attributed by some to the presence in this zone of the keto-steroid hormones.

(e) *Painful stimuli.* Many stresses that evoke an increased adrenal cortical secretion are accompanied by pain of a greater or lesser degree. In order to determine the effect of painful stimuli on the adrenal cholesterol and ascorbic acid we have under both general and local anesthesia exposed the sciatic and brachial nerves of rats. The nerves were then divided and the central ends stimulated electrically by a "Thyratron" stimulator at intervals of a minute for 15 seconds until 15 such bursts of stimulation had been given.

The results, Table VII, are interesting inasmuch as even these brief periods of painful stimulation cause a fall of some 40% in adrenal ascorbic acid. Cholesterol, however, is unchanged at the end of the period of stimulation, but during the next two hours falls to some extent. Evidently even brief periods of pain cause an activation of the adrenal cortex. These experiments also illustrate the rapidity with which the gland responds. Again no such response is evoked in hypophysectomized rats, illustrating once more the

TABLE VII

Effect of Stimulation of Central End of Sciatic or Brachial Nerves on Adrenal Cholesterol and Ascorbic Acid of Normal and Hypophysectomized Rats Nembutal (Subcutaneous) Anesthesia

	No.	Adrenal Cholesterol g/100 g.	Adrenal Ascorbic Acid*** mg./100 g
Normal rats			
Sciatic nerve dissected; no stimulation	4	3.89 ± 1.0	441 ± 16
Stimulation of sciatic nerve for 15 minutes*	6	3.96 ± 0.38	270 ± 3
Stimulation of sciatic nerve one-half hour after stimulation	6	4.16 ± 0.21	268 ± 4
Stimulation of sciatic nerve two hours after stimulation	6	3.08 ± 0.33	237 ± 18
Brachial nerves dissected; no stimulation	8	4.52 ± 0.38	416 ± 13
Stimulation of brachial nerves for 15 minutes*	4	3.88 ± 0.28	264 ± 19
*Hypophysectomized rats***			
Controls	13	5.60 ± 0.36	418 ± 26
Stimulation of sciatic nerve for 15 minutes	2	5.95	412

*In all cases the central end of the nerve was stimulated for 15 seconds at 1 minute intervals for 15 minutes by means of a "Thyratron" stimulator.

**Rats used three days after operation.

***All determinations by Roe and Keuther method.

dependence of the adrenal response on a preliminary activation of the anterior pituitary.

The association of this response with the liberation of epinephrine from the adrenal medulla is suggestive, and the effects of the latter hormone on the adrenal cholesterol and ascorbic acid will now be described.

7. *Effect of Epinephrine*

The fact that such diverse forms of stress as cold, burns, hemorrhage, and painful nerve stimulation all cause a depletion of adrenal cholesterol and ascorbic acid, raises the question as to what common denominator is possessed by such stimuli. For, as we have seen, these alterations in adrenal composition depend on a preliminary activation of the anterior pituitary, and it is difficult to believe that the response of this organ is entirely non-specific.

Three mechanisms may control the secretory activity of the anterior pituitary in so far as the liberation of adrenotrophic hormone is concerned. These are (a) excitation of a nervous secretory mechanism (b) changes in the composition of the blood traversing the gland which in turn excites the

secretion of adrenotrophic hormone (c) alterations in the level of cortical hormone in the blood. This last view has been advanced by Sayers and Sayers (9) who found that the previous administration of cortical hormone to rats exposed to cold (4° C) for one hour prevented the usual fall in adrenal ascorbic acid.

The question of a direct nervous control of anterior lobe secretion is a debatable one at the moment, but in so far as the types of stress outlined above are concerned there is very definitely one type of nervous activity associated with them all. This is an excitation of the sympathetic nervous system and the release of its specific hormone, epinephrine.

It was, therefore, of interest to determine the effects of epinephrine given in doses within the physiological output of the gland, on the adrenal cholesterol and ascorbic acid.

Epinephrine given subcutaneously or intravenously causes an unmistakable fall in adrenal ascorbic acid and cholesterol in intact rats but has no such effect on hypophysectomized animals.*

TABLE VIII

Effect of Epinephrine on Cholesterol and Ascorbic Acid of Normal and Hypophysectomized Rats

	No. Animals	Duration of Expt. (hours)	Adrenal Cholesterol g.%	Adrenal Ascorbic Acid mg. %
		NORMAL RATS		
Controls	14	——	4.14 ± 0.17	423 ± 13
*Saline** 0.5cc./100g./hour intravenous	4	1.0	3.53 ± 0.15	439 ± 18
*Epinephrine*** 1 x 0.02mgs./100g. subcutaneous	6	2.0	2.88 ± 0.28	233 ± 24
4 x 0.02 mgs./100g. subcutaneous	10	4.0	2.26 ± 0.22	216 ± 9
0.0003 mgs./100g./min.	6	2.0	2.31 ± 0.18	104 ± 17
		HYPOPHYSECTOMIZED RATS		
Controls	4	——	5.74 ± 0.43	398 ± 38
Epinephrine 4 x 0.02 mgs./100 g. subcutaneous	9	4.0	5.34 ± 0.37	411 ± 13
0.0003 mgs./100g./min. intravenous	2	2.0	6.04	357

*Saline (0.09%) contained 20 mg.% glutathione.
**Epinephrine in saline (0.9%) containing 20 mg.% glutathione.

*It should be noted that epinephrine has its normal glycogenolytic effect in hypophysectomized animals.

These experiments would indicate that the effect of epinephrine on these adrenal constituents is also mediated by the anterior pituitary.

They do not, however, tell us whether epinephrine is acting in its role of a specific stimulant to structures innervated by the sympathetic nervous system or whether its well-known effects on metabolism have altered in some way the composition of the blood and thus excited the secretion of adrenotrophic hormone.

However, in the case of epinephrine we are dealing with a substance whose effects are far more specific than those produced by such stresses as trauma, burns, etc., and yet which brings about comparable changes in the adrenal cortex.

If the activation of the adrenal cortex depends on release of epinephrine and consequent stimulation of the adrenotrophic secretion, then animals with complete adrenal denervation or demedullation should have some difficulty in meeting stresses. This they may have, as the recent work of Wyman and his colleagues (21) indicates, although such animals do not exhibit the great sensitivity to stress shown by hypophysectomized or adrenalectomized animals. We are attempting to determine whether adrenal cortical transplants show a diminution of cholesterol or ascorbic acid when their hosts are subjected to stresses of the kind used in the experiments on normal animals. There are technical difficulties in such experiments that up to the moment have prevented us from obtaining a decisive answer.

Even if it should be known that epinephrine evokes a response from the anterior pituitary by its direct effect on the secretory cells of this organ, it does not imply that these are actually innervated by the sympathetic nervous system. The situation may be similar to that existing in skeletal muscle where this hormone has a striking metabolic effect on cells which apparently do not possess any sympathetic nerve supply.

On the other hand if we accept the liberation of epinephrine as producing its effects by imposing a "stress" on the organism we are still at a loss to account for such an effect by a substance whose sympathomimetic action is so well established. Vogt's experiments (18) indicate that continued exposure to epinephrine for 1-3 weeks produces adrenal lipid changes in rats only in the presence of the anterior pituitary. Our acute experiments indicate the same thing so far as adrenal cholesterol and ascorbic acid is concerned.

III. Conclusions

The whole question of the factors concerned with an increased liberation of cortical hormone as a consequence of the exposure of the organism

to adverse circumstances evidently requires further investigation. We are continuing our studies at the present time, but until more evidence is available the hypothesis that epinephrine is an essential agent in the process should be regarded as a tentative one only.

However, we believe the subject to be one of importance particularly in relation to the large amount of interesting work that has been conducted in the last few years on the metabolic changes accompanying and following the exposure of men and animals to a variety of stresses. It is not too much to say that a new phase of metabolism has been and is being uncovered by these investigations. The endocrine responses to such stresses must play a considerable role in the metabolic adjustments that are evoked by them, although as in the case of the action of all hormones they do not initiate but only regulate the rate of such changes.

Discussion

F. Beach: I would like to ask one question which has to do with the manner in which the pituitary itself is stimulated. Has anyone checked the effect of section of the pituitary stalk, leaving the gland intact on adrenal cortical function?

C. N. H. Long: Uotila found that stalk section in the rat did not prevent the usual adrenal hypertrophy when the animals were exposed to cold. It is possible that the alterations in the adrenal cholesterol and ascorbic acid may be used to determine whether the adrenal cortex has actually been stimulated by various types of operative procedures or stimulations of the hypothalamic area.

D. J. Ingle: Several years ago we did the experiment of destroying the infundibulum and found that adrenal cortical hypertrophy would still occur in response to stress and that atrophy would occur in response to an excess of adrenal cortical extract. This was not a crucial experiment since destruction of the stalk does not completely denervate the anterior pituitary. Moreover we have not studied the functional activity of the adrenal cortices by these new methods which Dr. Long and his co-workers have introduced.

C. N. H. Long: I am very interested to hear that Dr. Ingle has carried out experiments of this kind. Perhaps I can talk with him afterwards about transplantation of adrenals and how to obtain a uniform size of transplant?

G. Pincus: I would like to call Dr. Long's attention to this presumable role of ascorbic acid in the corpus luteum. A number of years ago Dr. Berkman and I published a paper on the content in the corpus luteum of the rabbit. We found that the concentration of ascorbic acid rose markedly during the period when the corpus luteum was establishing itself. When it reached its maximum size the content of ascorbic acid was much higher than other portions of the ovary and remained constant as long as the corpus luteum was functional.

We also followed ascorbic acid in the pituitary glands in the rabbit and there one found indications of a release of ascorbic acid following copulation, which was rather marked, and paralleled other data on the release of the gonadotrophic hormone. There was a return after ovulation and it continued during pregnancy at a high level. In these two centers ascorbic acid has been followed and we also followed the glutathione content of these glands. We found that in the corpus luteum particularly the

glutathione paralleled the ascorbic acid quite faithfully and from a number of studies found the intimate relationship between glutathione and ascorbic acid demonstrated, generally in cells under certain conditions. I would like to ask Dr. Long if any glutathione determinations have been made?

C. N. H. Long: We have made a few determinations of glutathione. There is no change of any consequence. We have not made any determinations of ascorbic acid in the corpus luteum.

R. G. Hoskins: In our studies on the schizophrenic psychosis in which adaptation is notably faulty we have found that subjects of this disorder practically always show a low or very low blood-ascorbic acid titre. As Dr. Pincus is reporting at this meeting, the patients also exhibit inefficient adrenal cortex activity. The blood cholesterol on the other hand is commonly about normal. Giving enough ascorbic acid to the patients to bring the blood titre up to normal has not seemed to have any beneficial effect on the psychosis.

K. E. Paschkis: The findings of Dr. Long, that adrenaline stimulates the anterior pituitary to secrete or liberate adrenocorticotrophic hormone, may explain fluctuations of adrenal cortical function.

Several years ago our group studied a large number of patients suffering from weakness and exhaustion, in many instances associated with low blood pressure, cases of a type described as "mild hypoadrenia." We wanted to find out whether this syndrome, not infrequently found in certain neurotics, was really due to an adrenocortical hypofunction. In some of them we found high chloride excretion with the test procedure of Cutler, Power and Wilder. When the test was repeated at short intervals in such subjects we found a wide variation from low to high chloride excretion. We tentatively interpreted this as due to fluctuations in adrenal cortical function in individuals who showed numerous signs of imbalance, both in the physical and psychic sphere. None of the patients had Addison's disease, and we believe that the fluctuations of adrenal cortical function were the effect rather than the cause of their general instability. The trouble was that there is no evidence for a nerve supply of the adrenal cortex. Now, however, it is easily understandable that an autonomous imbalance may lead to rapid and wide fluctuation of adrenaline secretion via the sympathetic, and that the changes of output of adrenaline in turn would induce fluctuation of the secretion of the adrenal cortex via the pituitary adrenocorticotrophin.

R. L. Noble: I should like to ask if you have studied the chemical changes in the adrenals of an animal receiving a high protein intake?

Does a guinea pig which has been made scorbutic show symptoms or biochemical changes which would indicate a decreased hormone production from the adrenals?

C. N. H. Long: We have not studied the effect of high protein diets on the adrenal ascorbic acid or cholesterol. We are engaged in a study of the adrenal cortical function in scurvy. We have found the adrenal hypertrophy that occurs in scorbutic pigs is not a consequence of inanition as is the case in certain other vitamin deficiencies, but is a consequence of the lack of vitamin C. The size and ascorbic acid content of the guinea pig adrenal is very variable, and constant values cannot be obtained unless the vitamin is given daily by injection. We do this by putting the pigs on a scorbutic diet and injecting 20 mg. a day of ascorbic acid. Withdrawal of the vitamin for 24 hours is followed by a lowering of adrenal ascorbic acid. We are endeavoring to test in several ways whether the adrenal of the scorbutic guinea is responding in a normal manner to the adrenotrophic hormone. No conclusions can be drawn at the present time.

N. Talbot: It appears that the adrenal cortex can, on the one hand, be stimulated

to produce and accumulate its hormones within the gland, and on the other hand to secrete these hormones into the circulation. Are these changes induced by a single adrenocorticotrophic hormone or are they due to two hormones with strikingly different characteristics?

C. N. H. Long: It cannot yet be answered as to whether a single trophic hormone enhances synthesis and release of the cortical hormones. The adrenotrophic preparation we have used appears to be a single entity, as judged by the available physicochemical and biological tests. This substance produces both the changes in adrenal chemistry and an increased secretion of cortical hormones.

W. T. Salter: Perhaps I could contribute to that question by asking Dr. Long whether there are any blocking agents which prevent the release of the cortical hormone as yet discovered?

C. N. H. Long: I have no information as to whether it is possible to block the secretion of cortical hormones by chemical means.

G. Pincus: I would like to take a minute to reinforce Dr. Long's discussion on the importance of ascorbic acid and the adrenal function in the guinea pig. We have studied 17-ketosteroid excretion in the guinea pig and, following Dr. Long almost exactly, we found that under ordinary dietary conditions there are great variations in the 17-ketosteroid output. We obtained values for 48 hours, varying from 1/10 mg. of ketosteroids to 3/10 mg. per pig. When these pigs had greens in their diets and were given ascorbic acid injections daily, the amount of 17-ketosteroids became much more constant at above the maximum level that we observed in the pigs raised at random.

R. D. Rawson: Dr. Talbot just asked the question as to whether or not there were two hormones which have an action on the adrenal, one to cause the release of the hormone, and two, to cause the collection of the hormone. I cannot give an answer to this question, but I think it is interesting that in our studies on the effect of the thyrotrophic hormone on the loss and collection of radioactive iodine by the thyroid, we have observed that the iodine loss and iodine recovery can be plotted on a curve quite similar to that presented today by Dr. Long. We have observed that thyroids labelled with radioactive iodine and then treated with one injection of thyrotrophic hormone lose about 70 to 80% in 24 hours. We have also observed that an increase in the collection of radioactive iodine is not demonstrable until between 48 and 72 hours. I don't think that we have evidence in support of the theory that small doses of thyrotrophic hormone would maintain the high level of iodine. My own interpretation has been that one of the primary actions of thyrotrophic hormone is to cause release of the stored thyroid hormone and that the subsequent collection of iodine follows as a result of the gland's being made avid for iodine by its previous loss of hormone.

Dr. Long, you asked a question, "Does this adrenalin effect apply to other pituitary hormones?" At your suggestion a year ago we started studying the effect of adrenalin on the thyroids of intact animals. We have observed a similar change to what you observed, but the change observed in the thyroid was much slower than that observed in the adrenal cortex. We have observed that by giving adrenalin to the immature female rat an increase in the thyroid cell height could be demonstrated. Indeed, the mean cell height of the treated animals was practically twice that observed in the control animals at the end of 96 hours. There was a small change demonstrable at the end of 72 hours. It is possible that the reaction in the thyroid is slower than that in the adrenal cortex, and it is also possible that with our histometric method we are not able to pick up changes as quickly as you can with your chemical method.

H. B. Friedgood: Dr. Rawson's remarks about the effect of epinephrine on the secre-

tion of hypophseal hormones recalls an experiment which Dr. Cannon and I did some years ago. We were concerned with reproduction of the syndrome of exophthalmic goiter by means of phrenico-sympathetic anastomosis. Dr. Cannon's earlier work was confirmed inasmuch as exophthalmos and an elevated metabolic rate appeared in several cats. It was decided at that time that the mechanism involved might be different from that which Dr. Cannon suggested originally. It occurred to us that the thyroid might have been activated via the cervical sympathetic innervation of the adenohypophysis, particularly after Dr. Pincus and I observed that faradic stimulation of the cervical sympathetics resulted in maturation of ovarian follicles in the rabbit, presumably by activation of the gonadotrophic hormones of the adenohypophysis. The fact that epinephrine affects the secretion of certain of the adenohypophysial hormones is in accord with these data.

Dr. Long's remarks about the effect of epinephrine on the functional activity of the hypophysis also fit in with the conception that a neurohumoral mechanism plays an important role in the regulation of the secretion of the thyrotrophic and gonadotrophic hormones of the adenohypophysis. Whether or not epinephrine and the sympathetic nervous system affect the functional activity of the adenohypophysis directly or indirectly through the hypothalamus is a problem which is in need of further elucidation.

C. N. H. Long: That is extremely interesting. We have not worked with the thyroid, and it may be in the case of the thyroid that it is a gland with a more sluggish response. There are probably different relationships and responses in the different endocrine glands, and different times are required for the release of hormone, and also. as Dr. Rawson has indicated, there is a difference in the techniques used.

J. S. L. Browne: In connection with the use of transplanted adrenals to settle the question of the relation of the sympathetic nervous system to the discharge of hormone from the adrenals, is it not difficult to prevent regrowth of certain sympathetic fibres along blood vessels even in transplanted organs?

Further with regard to the question of the relation of the sympathetic nervous system to the secretion of corticoids by the adrenal, you will recall that I showed a case last year who had hypertension and an increased amount (177 glycogen units) of urinary corticoids. After a bilateral splanchnic neurectomy there was in the first few days the usual post-operative rise in urinary corticoids and then at a single observation made two months after operation the level was 60 glycogen units, a normal value and a normal blood pressure. It is of course impossible to conclude that these two changes were causally related, nor that the fall in urinary corticoids was directly related to the splanchnic neurectomy.

With regard to the relation of the hypophysis to adrenal cortical secretion, we observed a case of panhypopituitarism who ordinarily had no detectable amounts of urinary corticoids in the urine. She developed acute pyelitis with a temperature of 101° F. During the acute infection there were 26 units of urinary corticoids per 24 hours. (The normal level is 30-60 units and in a normal individual with infection the values may rise to 100-200 units.) After the acute stage had passed the level again fell to less than was detectable by Dr. Venning's method. Administration of pituitary corticotrophin prepared by Dr. Neufeld in Dr. Collip's laboratory, raised the level to 32 glycogen units. This shows that in this individual the adrenal was capable of responding to pituitary corticotrophin and suggests that there was a small amount of pituitary tissue left which responded to the infection leading to the rise of urinary corticoids above the usual level for this individual, the response to stress being, however, much less than in the normal individual.

With regard to the relation of ascorbic acid to the production of the adrenal cortical hormone, Dr. Andreae in our laboratory and Johnson, Lund, Levinson, Taylor and their collaborators at the Boston City Hospital have found that there is a rapid change in ascorbic acid metabolism after trauma such as burns, fractures, etc. Crandon and Lund showed that it took approximately two weeks on a vitamin C-free diet for the level of blood ascorbic acid to fall to the level found in scurvy and several weeks more after this for actual symptoms of failure of formation of cement substance and fibroplasia to occur. Dr. Andreae found that immediately after burns there is a marked retention of ascorbic acid. For example there is practically no ascorbic acid in the urine of one individual for 21 days after a burn, the intake after the 8th day was 500-600 mg. per day. This is not due to destruction of the ascorbic acid by something in the urine, nor to failure of absorption of ascorbic acid from the gastro intestinal tract, nor to the appearance of dehydroascorbic acid in large quantities. In three burn patients the blood ascorbic acid was normal one hour after the burn, but fell at the end of 24 hours to very low levels in spite of an intake of 500-1000 mg. in the 24 hours. In these cases it requires about 5-6 g. total intake to cause a rise in ascorbic acid excretion. There is thus evidence for a rapid destruction or utilization of ascorbic acid after burns, fractures, etc. Dr. Venning has examined the urinary corticoid excretion in these cases. We do not know the quantitative relations between the ascorbic acid and production of corticoids by the adrenal if indeed they are related. It seems unlikely that these large amounts of ascorbic acid would be used in the increased formation of corticoids after trauma.

Further individuals not given extra ascorbic acid showed the usual rise in urinary corticoids after burns and trauma. I am not sure whether we have as yet done the urinary corticoids in the burned patients who were given the large amounts of ascorbic acid?

E. H. Venning: Yes, we have done them.

J. S. L. Browne: Was there any difference between the amount of corticoid excreted in these cases and those not given extra ascorbic acid?

E. H. Venning: No, the levels were about the same.

C. N. H. Long: Dr. Browne has put forward some interesting points. The responses of transplanted adrenals to various stresses has been examined in the light of the chemical changes presented in this paper. As I said, the hypothesis that sympathetic stimulation (and release of epinephrine) is an essential link in the pituitary-adrenal cortex activation is at present not completely established. The hypothesis is, however, capable of further experimental test and this we hope to do in the near future.

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Studies of the Role of the Adrenal Cortex in the Stress of Human Subjects

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I. Introduction

It is clear from numerous studies of adrenal cortex activity that stressful performance in experimental animals results in adrenal cortex hypersecretion followed, in many instances, by hyposecretion. This has been called the "alarm reaction" and the total sequence of physiological changes involved the "adaptation syndrome" (18, 19). Direct histological and biochemical examination of adrenal tissue has been undertaken with stressed animals but this is not possible with human subjects. Three measures of adrenal cortex secretion have been used: the urinary concentration of cortin, the excretion of 17-ketosteroids, and the alterations in the number of circulating lymphocytes. Although evidence has been presented for an increased output of cortin in the urine following traumatic damage and surgical procedures (2, 3), methods for the quantitative extraction and assay of corticoid substances are still in the stage of development. Accurate microchemical procedures for urinary 17-ketosteroid analysis are available, and blood lymphocyte counts involving a minimal experimental error can readily be made.

The urinary 17-ketosteroids, as ordinarily measured, are not exclusively indices of adrenal secretion (11). In men, for example, a certain moiety of these substances (estimated at 10% to 20% of the total) represent metabolites of testis hormone. Furthermore, chromogenic materials which are not 17-ketosteroids contribute to a variable extent to the usual colorimetric reaction with alkaline alcoholic *m*-dinitrobenzene. The effect of such chromogens can largely be obviated by the use of the more specific $SbCl_3$, reaction (12). It is also possible that there are 17-ketosteroids which derive from neither adrenal cortex nor testis steroid precursors (4). Nonetheless there is abundant evidence that most of the urinary 17-ketosteroid derives from adrenal cortex steroid (1, 4, 10, 11). This paper will present data indicating that a variety of stressful activities lead to increases in urinary 17-ketosteroid output.

In animals the stimulation of adrenal cortex secretion by pituitary corticotrophin results in a lymphocytopenia (5) as do various stresses (6). The lymphocytopenia is accountable to 11-oxygenated corticosteroid (5, 20). Data presented in this paper will indicate the utility of this measure of adrenocortical function in man.

The data on urinary steroid output presented in this paper were obtained by the application of methods of extraction and microdetermination recently described (14). It should be emphasized that the 17-ketosteroid determinations were made on the separated ketonic neutral fraction for reasons set forth fully elsewhere (11, 17). The absolute output values will therefore tend to be less than those obtained by the usual methods with the total neutral fraction.

II. The Rate of 17-Ketosteroid Secretion in Men

In order to assess the effects of stressful procedures of limited duration, it is necessary to determine the rate of 17-ketosteroid output for similar intervals under normal conditions. An investigation was made of the 17-ketosteroid output of a group of healthy young men throughout the day and night in a succession of 24-hour periods (13). The data clearly demonstrated a minimal excretion during the period of sleep. Moreover, there was an average tendency for the 17-ketosteroid output to the maximal during the hours shortly following waking, with a fairly regular decline thereafter (16). This is illustrated in Fig. 1, wherein the mean values for the

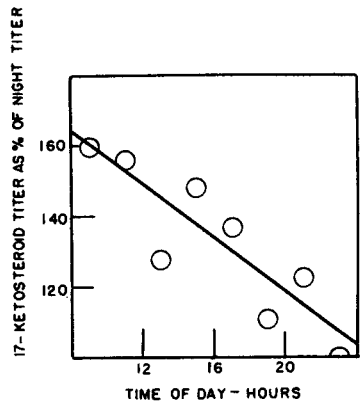

Fig. 1

The Output of 17-Ketosteroids as a Function of Time of Day.
Data on 97 collections from seven young men. Note maximal output in the early morning hours with subsequent decline. Time is given on a 24 hour clock in which 24 is midnight. (From the *Journal of Aviation Medicine.*)

median times indicated are plotted as a percent of the night output. It may be seen that the early morning output is approximately 60% above night level.

To determine if the increased 17-ketosteroid output during the day might

be a result of the relative increase in urine volume ordinarily observed during the day, forced diuresis experiments were undertaken. The data of a typical experiment are presented in Fig. 2 (15). It may be seen that the large

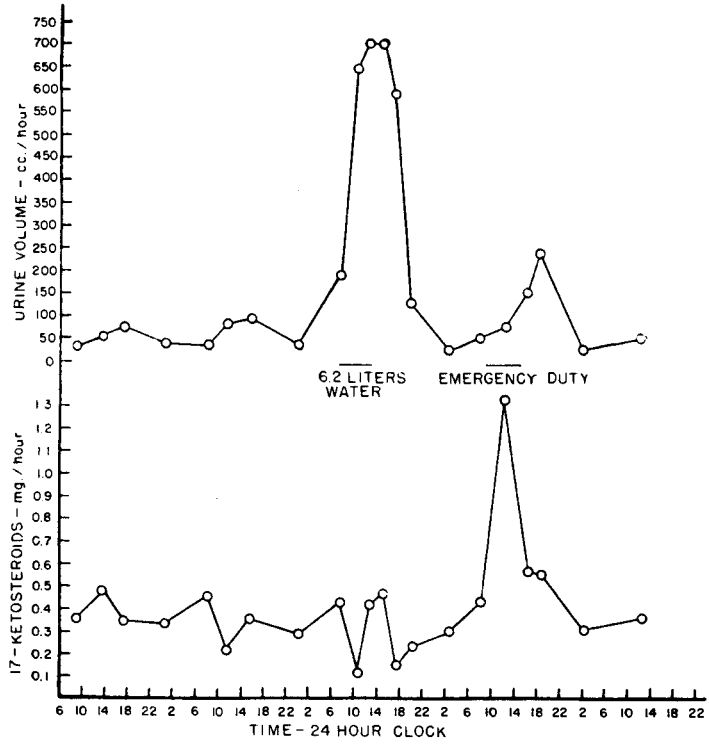

FIG. 2

A Forced Diuresis Experiment.

After two controls days the subject ingested 6.2 liters of water over the time indicated by the horizontal line in the figure. Note the large diuresis with no marked alteration in 17-ketosteroid output. A call to emergency hospital duty on the succeeding day is marked by a large rise in 17-ketosteroid output followed by a definite diuresis.

induced diuresis was not accompanied by any significant increase in 17-ketosteroid excretion; it should be noted in contrast that a call to stressful emergency duty led to a significant 17-ketosteroid increase followed by a clear diuresis.

The data of Fig. 1 are assembled from hourly outputs that differ widely, but when the outputs during the day are taken as a percent of the night output a rather good agreement is found. In normal healthy men the night excretion range is 0.25 to 0.80 mg./hr. (*m*-dinitrobenzene reaction values), and the early morning range 0.35 to 1.30 mg./hr. Each individual

tends to vary about a certain characteristic level so that the man who has a mean night output of 0.40 mg./hr. does not reach the excretion level of 0.80 mg./hr. The basis for the wide difference observed between individuals is a matter of interest and merits investigation.

III. Pursuit-Meter Operation and 17-Ketosteroid Excretion

Early in 1941 our laboratories initiated an investigation of adrenal cortex activity under the stress of flying. Before undertaking studies in the field, determinations were made of the 17-ketosteroid outputs of a group of university students while operating the Stevens' serial coordination meter (16). A photograph of the apparatus is presented in Fig. 3. The seated operator,

Fig. 3

The Stevens Pursuit-Meter.

The operator, using airplane stick and pedal controls, operates a pointing light the beam of which can be seen on the spiral pathway. The light, falling on photocells at the center and ends of the pathways, extinguishes a guiding light and another lights elsewhere more or less at random. When the light beam falls off the spiral pathway an error is scored automatically from the photosensitive screen backing.

by means of an airplane stick and pedal rudder controls, manipulates a pointing light directed at the photosensitive screen overlaid with two spiral pathways. At the ends of the pathways is a photoelectric cell next to a hooded pilot light. As the pointing light beam falls on a photocell the adjacent pilot light goes out. As one light is extinguished another goes on, and the operator must move the pointing light beam along the spirals to extinguish it. If the beam falls off the spirals onto the photosensitive screen an error is recorded. The student volunteers undertook a series of three to six hour

experiments involving the continuous operation of the pursuit-meter. Control urines were collected preceding each run as well as specimens covering the period of each run. From the 17-ketosteroid output of the control sample, the "expected" value for the period of the run was calculated. In each instance the observed "flight" value was above the expected value and the difference was the excess 17-ketosteroid due to the psychomotor stress involved.

In Fig. 4 this stress output is plotted against the mean error score of each

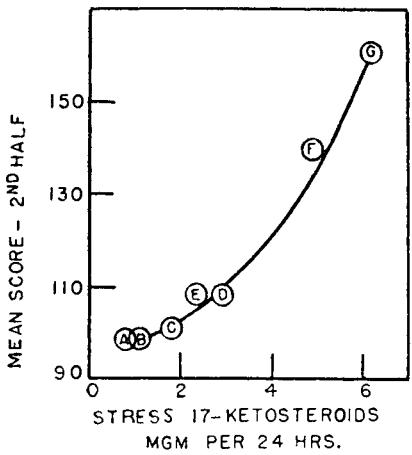

FIG. 4

Excess 17-Ketosteroid Excretion in Males.

The mean error score (ordinate) during the second half of a series of runs plotted against the excess of 17-ketosteroid excreted (abscissa) by each of 7 male subjects. Note that poor performance is correlated with excessive 17-ketosteroid output. (From the *Journal of Aviation Medicine.*)

of the subjects. It may be seen that the poorer the score the greater the stress output. If the error is a measure of the degree of stress involved, then the 17-ketosteroid increased represents a quantitative stress response.

Fig. 5 is a photograph of the Hoagland-Werthessen pursuit meter (16). It employs the same stick and rudder controls of the pointing light as the Stevens' meter, but the operator is required to bracket with the light beam the two photocells located at the end of the randomly moving rod. By means of suitable recording devices the percentage of time the light covers the photocells is measured as well as the duration of each bracketing. The operation of this pursuit-meter requires more concentrated psychomotor coordination than the Stevens' meter, and experienced airforce pilots liken an hour of its operation to an hour of close formation flying. With con-

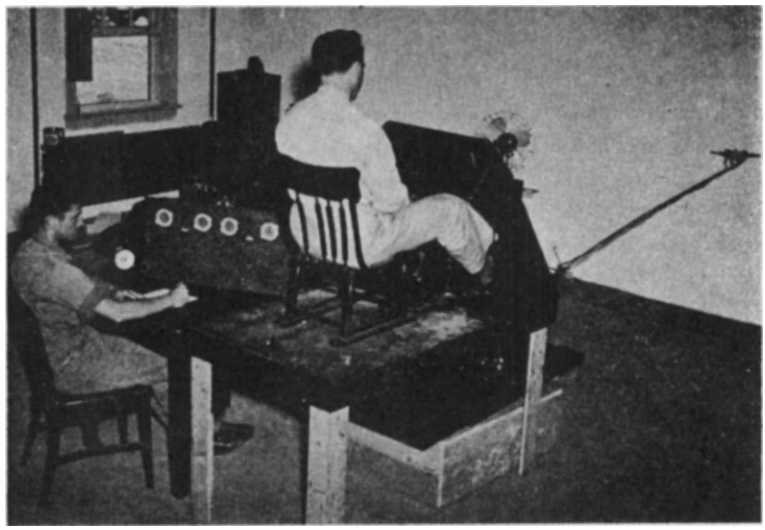

FIG. 5

The Hoagland-Werthessen Pursuit-Meter.

The operator, seated on the platform, operates a pointing light that must bracket the two photocells at the end of the rod (right). The rod moves irregularly at random. The time that the light covers both cells and the duration of each coverage is recorded by the clocks of the recorder on the table at the left.

tinuous operation there is a gradual decline in the percentage of time the light on the target (Fig. 6) and in the duration of each bracketing (seconds per hit). This decline is accentuated when the oxygen of the atmosphere is reduced (Fig. 7). The rate of decline in efficiency occurs whether the target

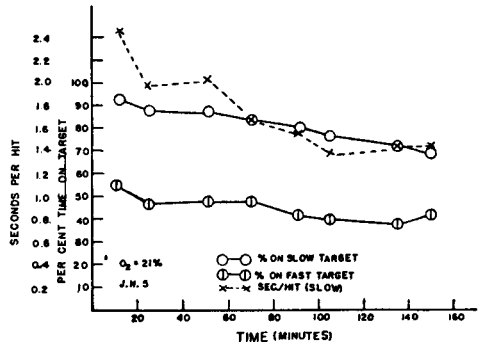

FIG. 6

The Scores of a Male Subject Operating the Hoagland-Werthessen Pursuit-Meter at 21% Oxygen (sea level).

Note the decline of score values with time by each measure of scoring ability. (From the *Journal of Aviation Medicine.*)

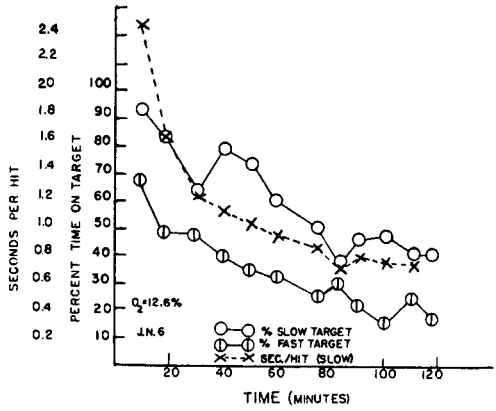

FIG. 7

The Scores of the Subject of Fig. 6 at 12.6% Oxygen.

Note the accelerated decline of scoring ability with time over that observed at 21% oxygen. (From the *Journal of Aviation Medicine.*)

moves slowly or rapidly (Figs. 6 and 7). A measure of this decline may be had by dividing the mean scores for the second half of each run by the mean scores of the first half. This we have called the fatigue ratio (R).

When the fatigue ratio is plotted against altitude, a regular decline with increasing altitude is observed particularly at oxygen tensions corresponding to 5000 feet or above. The stress 17-ketosteroid output increases with increasing altitude (Fig. 8). Again the implication is clear that the more difficult the job of coordination the greater the urinary output of adrenal cortex secretion metabolites.

IV. The Stress of Flying

That airplane flight itself causes an increased 17-ketosteroid excretion is demonstrated by data on two sets of pilots: (1) flight officers on routine instructing duties at low altitudes and (2) test-pilots operating at varying altitudes.

Urine specimens were collected from 16 instructor-pilots. Again two samples were collected each day, a control sample and a sample covering the period of flight duty. For each period of flight duty a record of actual flying time was made. Data on 152 flights are presented in Fig. 9, in which the percent increase of 17-ketosteroid excretion over the expected values is plotted against the percent time the fliers were air-borne. Here it is clear that the 17-ketosteroid output increases directly as the percent time in air increases.

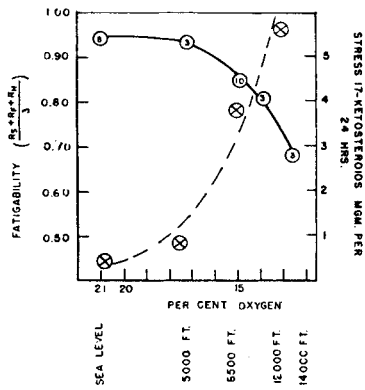

FIG. 8

Fatigability from Hoagland-Werthessen Pursuit-Meter.

An index of fatigability derived from the Hoagland-Werthessen pursuit-meter scores of three male subjects is plotted on the ordinate (left) against the per cent oxygen (altitude) on the abscissa. The numbers in the circles indicate the number of pursuit-meter scores averaged for each point. The crossed circles are data from the same subjects on the excess output of 17-ketosteroid (broken curve). Note that increasing altitude is accompanied by poorer performance and increasing 17-ketosteroid excretion.

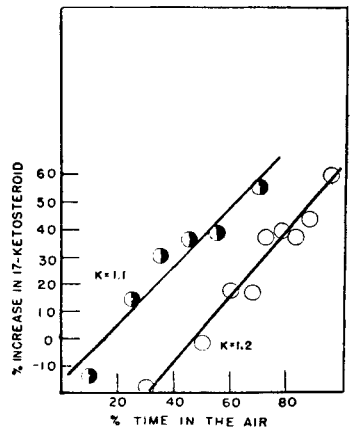

FIG. 9

The Percentage Increase in 17-Ketosteroid Output Plotted Against the Median Percent Time Spent in the Air for 152 Flights of 16 Army Instructor-Pilots Open Circles) and for 56 Flights of 7 Pratt and Whitney Test Pilots (Half-Circles).

Note that the curves for these two groups have essentially the same slopes (measured by K) but that at any given % time the test pilots exhibit a greater ketosteroiduria.

Similar data on 57 flights of 7 test pilots are presented in Fig. 9. It is notable that whereas the curves for the two sets of pilots have essentially the same slope constants, a greater absolute increase occurs in the test pilot data. The test pilots would seem to be more stressed by their particular jobs.

One feature of the data of Fig. 9 may well be commented on here. In the instructor-pilot data on periods involving brief proportions of actual flight time, there is recorded a decrease of 17-ketosteroid output over expectation. This would imply that any increased excretion due to flying is followed by a hypo-excretion, *i.e.*, a compensatory phase. We shall return to this consideration later.

V. The Stress of Daily Life

The foregoing data indicate that stressful psychomotor activity increases 17-ketosteroid excretion. In the light of this finding let us consider again the significance of a diurnal rhythm of 17-ketosteroid excretion. It would appear that ordinary daily activity is most stressful in the morning and declinigly so through the day. Alternatively there is a rhythm of 17-ketosteroid excretion that is endogenous, probably reflective of rates of adrenal secretion.

In Fig. 10 are presented data on one test of these alternatives. Urine

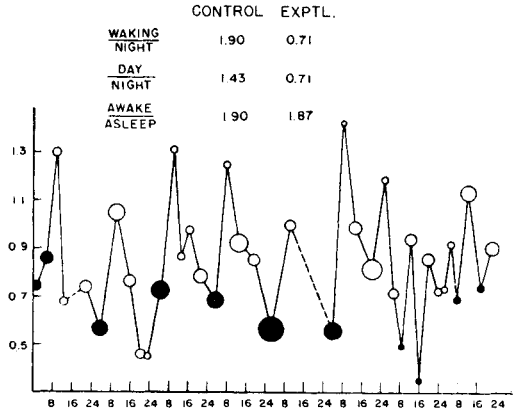

FIG. 10

17-Ketosteroid Output During Normal and Abnormal Sleep Schedules.

The 17-ketosteroid output in a male subject during four days of normal activities with sleep at night and on three succeeding days in which the subject slept at intervals during the day and remained awake at night. The closed circles represent periods of sleep. Note that minimal output values occur during the periods of sleep regardless of time of day. Abscissa: time of day on a 24 hour clock in which 0 hour is midnight. Ordinate: 17-ketosteroid output in mg. per hour.

collections covering the period of sleep (closed circles) and various periods while awake (open circles) were made for several successive days of normal sleep-waking activity. Then the night's sleep was omitted followed by brief sleeps in the morning and again in the late afternoon for two successive days. Activities during waking periods were walking, reading, writing, eating as in an ordinary day. The results appear to be unequivocal. Each period of sleep during the day is marked by a low 17-ketosteroid excretion rate and each waking by a definite output rise. The initial period of insomnia is marked by a rise indicating a possible stress in staying awake.

A further test of the effect of daily activity was afforded by a study of 17-ketosteroid excretion in day workers and night-shift workers at the American Optical Plant in Southbridge, Massachusetts. For four days three specimens per day were collected from the two groups: (1) a specimen covering the period of sleep, (2) a specimen covering one to two hours following waking (3) a specimen covering the first three or four hours of work. In the case of the 10 day-shift workers who went to work at 7 a.m. to 8 a.m. there were three successive periods. In the case of the nine night-shift workers who went to work at 4 p.m. to 5 p.m. there was a period of several hours between the waking specimens and the work specimens. The mean data on 17-ketosteroid excretion in the two groups are presented in Fig. 11. In both groups the sleep values are the lowest and a rise occurs after waking, but among the night shift workers the work specimens show a further rise whereas this is not the case in the day workers. Either going to work is more of a stress for the night shift men or the waking hours are less stressful. In any event the data on the night shift men indicate that activities and not time of day are prime factors in governing the rhythm of 17-ketosteroid excretion.

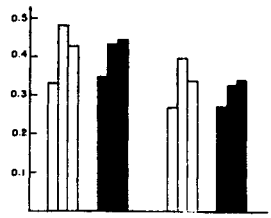

FIG. 11

17-Ketosteroid Output of Day-Shift and Night-Shift Workers.

Mean 17-ketosteroid outputs (in mg./hr.) of day-shift workers (open rectangles) and night-shift workers (solid rectangles) as measured by the *m*-dinitrobenzene reaction (rectangles at left) and the $SbCl_3$ reaction (right). The first rectangle in each group is the mean output during sleep, the second during one to two hours following waking and the third during the first few hours of work. See text for discussion.

The implications of these findings are interesting: waking involves a notable stress in terms of adrenocortical response, the evocation of the response is rather prompt, a hormonal mechanism for adapting to the activities of daily life appears.

VI. Stress Responses of Normal and Psychopathic Subjects

The Worcester Foundation for Experimental Biology, the Memorial Foundation for Neuro-Endocrine Research, and the Research Service of the Worcester State Hospital have been engaged in a joint research on adrenocortical function in mental patients. A preliminary report of certain stress studies has been made (8). Initially we undertook a study of the 17-ketosteroid output of a group of Army patients assigned to the Worcester State Hospital. Seventeen of these men and twelve normal young men of the same age grouping were used as subjects in the following experiment:

(a) For four days in a control week urines were collected covering the periods 7 to 9 p.m. and 9 p.m. to waking (5:30 a.m. to 7 a.m.).
(b) During four days of the following week the subjects clad in shorts entered a cold room at 50° F. from 7 to 9 p.m. Urine collections covering the period of cold exposure and the subsequent 9 p.m. to waking period were made.

In Figs. 12 to 14 the data of these experiments are presented graphically. The mean values presented for each group are 7 to 9 p.m. 17-ketosteroid outputs taken as a percent of the subsequent night values. These data demonstrate:

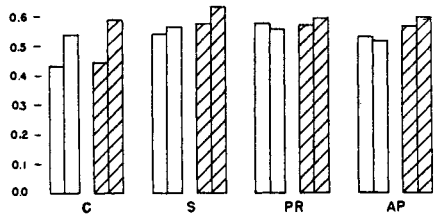

Fig. 12

The Mean 17-Ketosteroid Outputs (in mg./hr. of 12 Non-Psychotic Men (C), 7 Schizophrenic Men (S), 5 Psychotic Men Diagnosed as Having Remission (PR) and of All 17 Mental Patients Studied (Including C, S, and 5 Men with Miscellaneous Diagnoses).

The open rectangles are values taken during a control week, the cross-hatched values during an experimental week. In each pair of rectangles the first represents the mean values for the period of sleep, the second for the 7 to 9 p.m. period preceding sleep. During the 7 to 9 p.m. period of the experimental week the subjects were in a cold room at 50° F. Data obtained by the *m*-dinitrobenzene reaction.

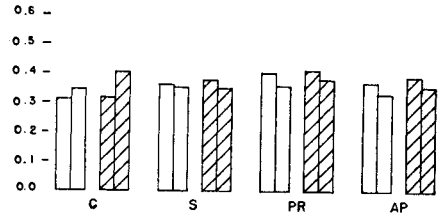

FIG. 13

Mean 17-Ketosteroid Outputs Plotted as in Fig. 12 but Measurements Were Made by the $SbCl_3$ Reaction.

(a) That by each measure of steroid output employed [the *m*-dinitrobenzene reaction (Fig. 12), the $SbCl_3$ reaction (Fig. 13), the non-ketonic steroids (Fig. 14)], the normal subjects exhibit a larger output during the 7 to 9 p.m. period than during the 9 p.m. to waking period and that the cold exposure increases the 7 to 9 p.m. output;

(b) That by all measures employed the patients taken as a whole excrete less neutral steroid during 7 to 9 p.m. than at night and that this is true of all groups;

(c) That the cold stress is reflected by a relative increase in 17-ketosteroid output in almost all groups when the *m*-dinitrobenzene reaction is used for measurement, but that the typical normal increase measured by the $SbCl_3$, reaction fails in the patients taken as a whole and is clearly unobservable in the schizophrenic men and the psychotic men diagnosed as recovered; similarly the relative increase in non-ketonic steroid excretion observed in normal subjects does not occur in the patient groups.

A more intensive study, still in progress, of schizophrenic men was then initiated. In Figs. 15, 16 and 17 are presented data on five schizophrenic

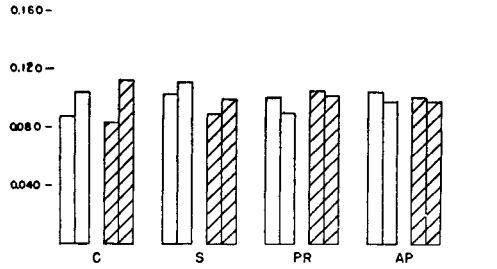

FIG. 14

Mean Non-Ketonic Steroid Outputs Plotted as in Fig. 12.

Measurements made by applying the $SbCl_3$ reaction to the neutral non-ketonic urine fraction. Data expressed as mg. color equivalents to an androsterone standard.

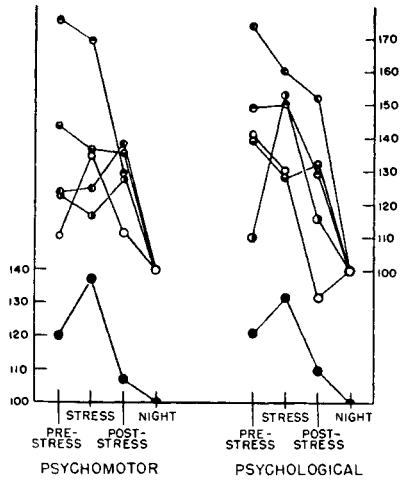

FIG. 15

The Mean Changes in 17-Ketosteroid Outputs of Subjects Operating a Pursuit-Meter.

The lower curves represent the mean data for three non-psychotic men. The upper curves are the individual means of five psychotic men. The 17-ketosteroid output value (mg./hr.) for the nights preceding the pursuit-meter operation is taken as 100; the pre-stress, stress, and post-stress values are taken as a % of the night output. The *m*-dinitrobenzene reaction was employed for obtaining these data. Psychomotor (pursuit-meter) stress was used in the experiments the data of which form the curves at the left; psychological tests were employed in the case of the data at the right.

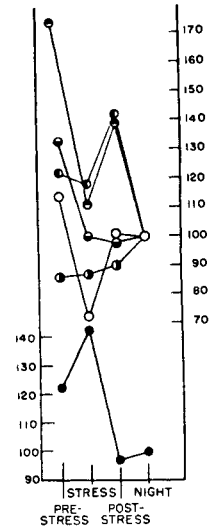

FIG. 16

Data on the Urinary α-Ketosteroids of the Subjects Represented in Fig. 15, Using the $SbCl_3$ Reaction.

The data are calculated as in Fig. 15.

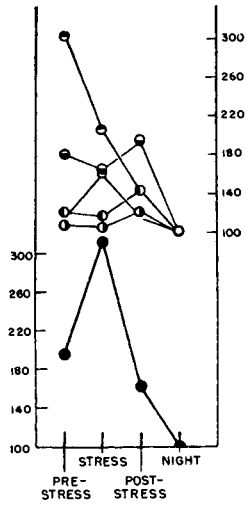

FIG. 17

Data on the Urinary β-Ketosteroids of the Subjects Represented in Fig. 15, Using the *m*-dinitrobenzene Reaction on the β-Fraction.

The data are calculated as in Fig. 15.

subjects and three normal controls subjected to stress of an hour's pursuit meter operation daily for six to seven days. Urines were collected covering (a) the period of sleep preceding the experimental day, (b) a prestress period of two to three hours, (c) the period of stress and (d) a post-stress period of two to three hours. The normal subjects exhibit a typical ketosteroid response by each measure employed, namely, a "stress" rise in output followed by an abrupt decrease. The percentage increase is greatest in the β-ketosteroid measurements (Fig. 17). Although one of the patients (#1, Fig. 15) exhibits a normal curve when the *m*-dinitrobenezne reaction is employed for measurement, it should be noted that none exhibit normal curves by other methods of measurement.

Consideration of the foregoing data suggests that the typical adrenocortical response to stress exhibited by normal men is deficient in psychotic subjects.

VII. Lymphocytopenia as a Stress Response

We have sought and found in circulating lymphocyte changes a more specific index of adrenocortical secretion in stress. This is interesting in view of Dougherty and White's (5) demonstrations that lymphopenia is a response to the sugar-active 11-oxygenated corticosteroids and none other. Here is the evidence:

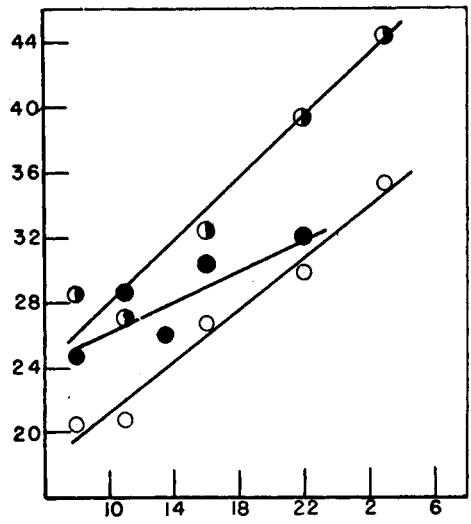

FIG. 18

Diurnal Variation in Blood Lymphocytes.

Variations of blood lymphocyte number according to time of day in 6 normal men (open circles), 6 normal women (half circles) and 6 psychotic men (closed circles). Abscissa: time on a 24 hour clock, in which midnight is 0 hour. Ordinate: lymphocytes in hundreds per cu. mm. (From the *Journal of Clinical Endocrinology*.)

First, both men and women exhibit a diurnal change in number of circulating lymphocytes which accords remarkably with the 17-ketosteroid excretion rhythm (7). In Fig. 18 are presented data on six normal men and six normal women which indicate a minimal count in the morning followed by a rise in count continuing through the day into sleep. This is what we would expect if adrenocortical secretion is maximal in the morning, declining thereafter. Six psychotic men exhibit irregular diurnal variations, and the mean rate of increase from waking to night is very much less than in normal subjects (Fig. 18).

Second, the stress of pursuit-meter operation under anoxic conditions is marked, in normal subjects, by a lymphocytopenia which lasts for some time after the end of the operation. This is illustrated in Fig. 19 in the representation of data on six normal subjects operating the Hoagland-Werthessen pursuit meter (9) for one hour. Note the relative lymphocytopenia accompanying the stress with the incomplete recovery by 90 minutes after the end of the stress. The three psychotic men given the same stress exhibit a lymphocytosis under stress rather than a lymphocytopenia (Fig. 19).

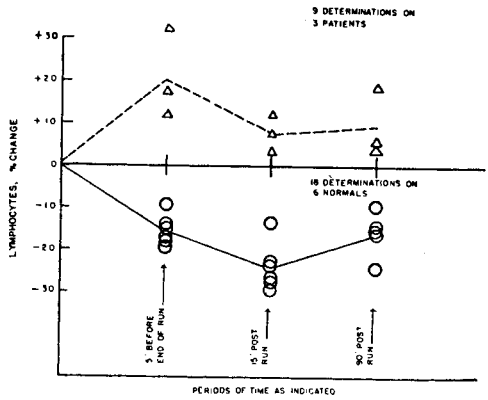

FIG. 19

Lymphocytes in Normal and Psychotic Subject at Hoagland-Werthessen Pursuit-Meter.

The mean change of lymphocyte number in 6 normal subjects (open circles) and three psychotic subjects (open triangles) in the course of operating the Hoagland-Werthessen pursuit-meter under anoxic conditions. Note the lymphopenia developed in the normal subjects, the lymphocytosis of the psychotic subjects. (From the *Journal of Clinical Endocrinology*.)

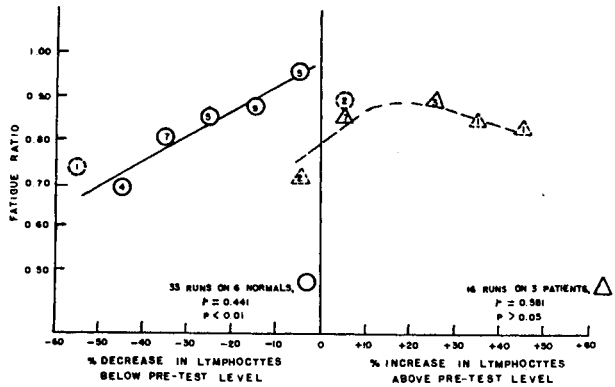

FIG. 20

The Percentage Change in Circulating Lymphocytes Plotted Against the Fatigue Ratio for Normal (circles) and Psychotic (triangles) Subjects Operating the Hoagland-Werthessen Pursuit-Meter under Anoxic Conditions.

Note the correlation between the degree of lymphopenia and the fatigue ratio in the normal subjects. The numbers in the symbols indicate the number of observations averaged for each point. (From the *Journal of Clinical Endocrinology*.)

Hoagland, Elmadjian and Pincus (9) have demonstrated that the lymphocytopenia is quantitatively correlated with the fatigue ratio (Fig. 20) and that it is accompanied by a ketosteroiduria (Fig. 21) in normal subjects, whereas such correlations did not occur in the data for the psychotic subjects (Figs. 20 and 21).

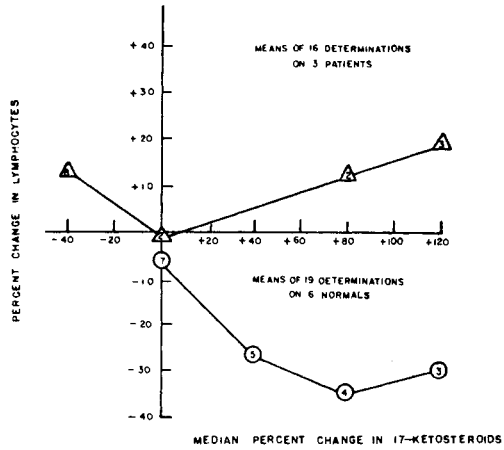

FIG. 21

The Median Percent Change in 17-Ketosteroid Output Plotted Against the Percent Change in Circulating Lymphocytes.

Subjects as in Figs. 19 and 20. Note that in the normal subjects lymphopenia and ketosteroiduria are correlated. (From the *Journal of Clinical Endocrinology*.)

Third, the stress of exposure to heat (105° to 110° F.) and high humidity (90% to 100% R.H.) for one hour causes in normal men a lymphocytopenia (Fig. 22). Psychotic men subjected to the same stress exhibit on the average a lymphocytosis (Fig. 22). In the psychotic subjects (with one exception) this lymphocytosis is followed by a post-stress fall in lymphocyte number, whereas in the normal subjects a post-stress rise in lymphocyte number occurs (Fig. 23) (15).

These data appear to demonstrate that there occurs in non-psychotic men an adrenocortical secretory response to a variety of stresses. They go further: they indicate a diurnal rhythm of adrenocortical activity that appears to reflect the stresses of daily living. Psychotic men, on the whole, fail to exhibit this typical stress response. It is either markedly diminished, or fails to appear promptly. The psychotic men whom we studied are men who broke down under the particular stresses of their lives. Is the poor functioning of the physiological stress-response mechanism etiologic to the mental breakdown? This we propose to investigate.

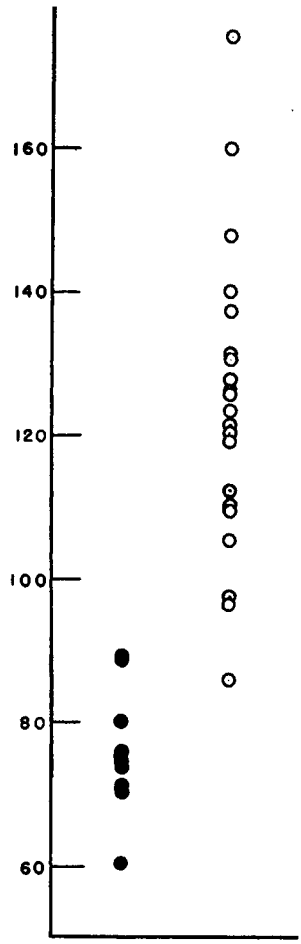

FIG. 22

Lymphocytes in Normals and Psychotics after High-Temp. Exposure.

The percentage change in circulating lymphocyte number after one hour's exposure to 105° F. at 85% to 95% relative humidity in a group of normal men (closed circles) and a group of psychotic men (open circles). The pre-exposure blood lymphocyte number is taken as 100. Note the relative decline in blood lymphocyte number in the normal men and the tendency for lymphocytosis in most of the psychotic men. (From the *Journal of Clinical Endocrinology*.)

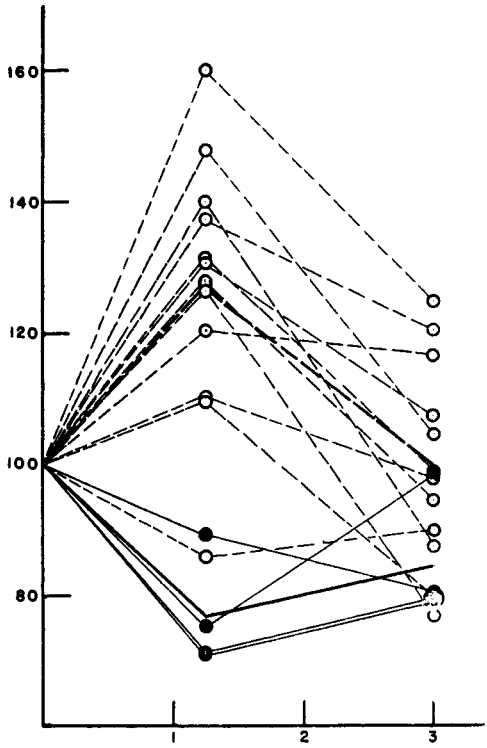

FIG. 23

The Percentage Change in Lymphocyte Number Immediately After the Hot Room Exposure and at 3 Hours After Entering the Hot Room in Normal Men (Closed Circles) and Psychotic Men (Open Circles).

The pre-exposure blood lymphocyte number is taken as 100. Note the drop with a subsequent return in most of the normal men, and the rise with subsequent fall in most of the psychotic men. The mean values for each group are given by the heavy solid (normals) and dotted (psychotics) lines. (From the *Journal of Clinical Endocrinology*.)

DISCUSSION

R. G. Hoskins: Dr. Pincus' presentation covered quite a lot of psychiatry. In effect, schizophrenia represents in various ways an inability to adapt. We have been interested for years in trying to particularize the failures in adaptation. I get a little different slant on the meaning of these data than Dr. Pincus suggests. I doubt if the difficulty is that waking-up is not as annoying to a schizophrenic as to other people but that the schizophrenic is metabolically clumsy and inefficient. The reason why this interpretation appeals to me more than the one suggested by Dr. Pincus is that much the same sort of picture came out in neurotics as in schizophrenics, and the neurotics are definitely over-responsive.

The use of the lymphocyte count as the index of corticosteroid output is a method

that promises to spare much laborious analysis. The fact that the lymphocyte count goes up in schizophrenic subjects under conditions that cause a fall in normals suggests that two factors that determine the lymphocyte count are separately implemented and must be dealt with individually. These are the rate of discharge of the lymphocytes from the tissue depots and the rate of destruction of the discharged cells. The alternative suggestion that the rise in the patients is due to over-facile discharge is incongruous with the fact that their metabolic processes are generally relatively sluggish.

T. Dougherty: There are some indications of a diurnal change in circulating lymphocytes in certain strains of mice. However, the number of peripheral lymphocytes may be markedly altered as the result of numerous factors. Lymphopenia may be induced, for example, by certain dosages of x-rays, by heat, by cold, by altitude, or even by merely boxing mice. The lymphocytes disappearing as a result of such stimuli are replaced, but the basis of the balance between utilization and delivery of lymphocytes is still unresolved. One piece of evidence that is worth mentioning is the apparent role of the adrenal cortex in antibody formation. We have previously shown that corticosteroid may induce a marked outpouring of antibody into the blood stream of rabbits immunized a long time previously. Presumably the antibody stored in the lymphatic tissue is released as a result of the corticosteroid effect. On the other hand it is almost impossible to immunize an adrenalectomized animal. This might imply that antibody formation by lymphatic tissue as well as antibody release is affected by the adrenal cortex.

G. Pincus: The interpretation of the facts about psychotic subjects that we have presented must, I think, go slowly. The defective alarm reaction responses may be found in other pathologies, for example. The few data that we do have on psychoneurotic subjects indicate abnormal diurnal rhythms different from those psychotic subjects. In a number of psychoneurotics exaggerated morning rises in 17-ketosteroid output occur. It may well be that a classification of mental subjects on the basis of their adaptive responses might afford a physiologically usable grouping but only the accumulation of critical data will resolve this probability.

Concerning the balance between lymphocyte production and destruction, I agree with Dr. Dougherty that the problem is open. However, recent studies with Mr. Elmadjian in our laboratories indicate a possible role of the thyroid in increasing the number of circulating lymphocytes. We have found, for example, that thyreotropic pituitary extract induces a significant lymphocytosis in normal or adrenalectomized rats, but fails to do so in thyroidectomized animals. Lymphocytosis with hyperthyroidism has frequently been reported. It is therefore possible that the stress lymphocytosis observed in our psychotic subjects is due to a thyroid response. This response may also occur in normal subjects but be overridden by the large adrenal response.

H. Sobotka: According to studies carried out on the Neurological Service and in the Electro-Encephalographic Laboratory at Mount Sinai Hospital by Drs. I. S. Wechsler and H. Strauss (Clinical and Electroencephalographic Studies of Changes of Cerebral Function Associated with Variations in the Blood Sugar, *American Journal of Psychiatry,* **102**: 34-39, July 1945), there seems to be a correlation between "morning malaise," hypoglycemia, and delta activity in the EEG. The considerable number of subjects interrogated and observed could be subdivided into two groups. More than half suffered from one or several symptoms of morning nervousness; a minority do not. It would be interesting to learn of the effect of insulin shock on ketosteroid output.

G. Pincus: We have no data on subjects examined according to the criteria men-

tioned by Dr. Sobotka. However, we have taken lymphocyte counts on subjects in sugar tolerance tests and during insulin shock. In normal men the blood lymphocytes vary inversely as the blood sugar during the course of the tolerance test. In psychotic men this relationship either fails completely or is only partially manifested. When insulin is given to psychotic men in convulsive doses the lymphocyte count rises in varying amount in different individuals. When sugar is given to men in the convulsive state there is usually a pronounced fall in blood lymphocytes. Since we do not have data on normal subjects receiving insulin, interpretation is difficult.

Electroshock treatment which fails to lead to remission fails to alter either the diurnal rhythm of 17-ketosteroid output or the diurnal changes in lymphocytes of the psychotic subjects thus far examined. Electroshock-induced remissions of involutional women was generally accompanied by a normalization of the 17-ketosteroid diurnal rhythm (Hoagland, Malamud and Kaufman, *Psychomatic Medicine,* in press).

R. D. H. Heard: Dr. Pincus has given an admirable account of the behavior of the adrenal under stress. He has pointed out the limitations on the usefulness of 17-ketosteroid output and the difficulties of the direct estimation of urinary adrenocorticosteroids. On the assumption that all biologically active corticosteroids are reducing substances in that they possess a ketol side-chain we attempted to work out a chemical method of their estimation. This involved the extraction of the lipoid-soluble corticoids and the determination of the reducing activity of the neutral lipid fraction obtained. A standard method of measurement was evolved and data were collected on a number of urine extracts assayed for glycogenetic activity by Dr. Venning. The data tended to parallel each other in most cases. In certain hypoadrenal cases in which there is no urinary glycogenetic activity a definite but low reducing activity is found. In Cushing's syndrome both biological and reducing activity are markedly increased. The details will be published shortly (*Journal of Biological Chemistry,* in press).

N. Talbot: We can confirm and extend the observations just reported by Dr. Heard. By means of a similar procedure (Talbot, Nathan B., Saltzman, A. H., Wixom, R. L., and Wolfe, J. K., *Journal of Biological Chemistry,* **160**: 535, 1945) assays of the urinary corticosteroid-like reducing agents have been made on a number of normal and abnormal subjects. The results obtained appear to be at least in rough agreement with those obtained by bioassay on similar subjects. High values were found in patients with active Cushing's Syndrome and in patients recently burned; low values occurred in patients with severe hypopituitarism, hypothyroidism, and hypoadrenocorticism. Patients with hyperadrenocorticism and virilism, in contrast to patients with Cushing's Syndrome, had approximately normal values. The urinary corticosteroid measurements apparently are distinct from measurements of urinary 17-ketosteroids and thus far seem to give information concerning the rate of production of the adrenal cortical "S" hormone of Albright.

A. White: I would like to ask Dr. Pincus several questions. These relate, perhaps, to elucidating the degree of function of the adrenal cortex in schizophrenics. The first question would be: What is the level of circulating lymphocytes in the so-called "normal" schizophrenic? Is information available regarding the degree of resistance of the schizophrenic to stress or the capacity of the schizophrenic to develop circulating antibodies in response to immunization? Another question is of some importance because of a possible relation to the lesion in schizophrenia. I wonder if Dr. Pincus has injected adrenotrophic hormone into the schizophrenic to determine whether the adrenal cortical response is normal. This study may have a bearing upon pituitary function and perhaps its neurological implications.

G. Pincus: While the average numbers of circulating lymphocytes in the psychotic men we have examined tends to be higher in the day than those of the normal men, it is probably lower at night due to the difference in diurnal trend (see Fig. 18). However, individual variations are large and generalization is best avoided.

• Concerning the response to adrenocorticotrophin, that is obviously requisite and will be studied. Our program for the study of the alarm reaction defect involves tracing the defect to its origins. We now know that the response of psychotic men to corticosteroid administration is a lymphopenia as in normal men. Therefore, their responsivity to adrenal cortex hormone is not defective. If they react to corticotrophin administration with a lymphopenia, it seems clear that a responsive adrenal secretory mechanism must be present and that the defect lies either in the pituitary itself or in the neural (?) mechanism activating the pituitary. We hope to have this whole reaction sequence characterized in mental patients.

Dr. Hoskins may be able to reply to your inquiry about immunization in schizophrenics.

R. G. Hoskins: I do not recall any specific studies on immunity in schizophrenia that bear on the problem of antibody formation. The schizophrenics are vulnerable to tuberculosis to which the lymphocytes have some special relationship but they generally die of the same thing as the rest of us. They are, however, less prone to hypertension and other psychosomatic disorders that result from inner tension. That seems to be one of the few ways in which their psychosis does them any good.

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The Relation of the Anterior Pituitary Hormones to Nutrition

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I. Introduction

If the broad definition of the term is taken, the title covers such a wide field that it would not only embrace portions of other papers to be presented here, but space would not permit its full consideration. Sherman (59) defines nutrition as "The assemblage of processes concerned in the growth, maintenance or repair of the living body as a whole or of its constituent parts or organs." We shall, therefore, make two limitations in our present paper.

First, we shall use the term nutrition to apply to those general processes which define the over-all needs for foodstuffs. Thus we shall consider the questions of absorption of food, the utilization of fat and carbohydrate, of nitrogen balance, and the nutritional value of proteins. Second, since the purpose of this conference is to consider the work with which the speakers themselves are familiar, the discussion will be directed toward a correlation of the work of others with our own studies using the forced-feeding technique for the absolute control of diet. We shall not discuss the relation of the pituitary hormones to the vitamins.

In the following paragraphs the terms "endogenous" and "exogenous" are used. The work of Schoenheimer (58) and his coworkers has brought the old connotations of these terms into disrepute. The author believes, however, that there is a significant meaning to these terms. When food is taken into the body a certain portion of it is directly utilized for energy and functioning structures. The balance is incorporated into masses which are again utilized as the need in the more active tissues arises. While there is constant interchange in these "storage" tissues, the balance between entrance and release is under controls which differ from those affecting the utilization of the circulating foodstuffs. The term "endogenous" is therefore used to indicate those processes affecting the mobilization of foodstuffs from these storage tissues.

The following points will be emphasized:

1. There are adaptive mechanisms to dietary changes which are not under the control of the endocrine system.

2. Both fat and carbohydrates can be readily used in the hypophysectomized animal, and fat will spare carbohydrate.

3. Exogenous protein is readily catabolized but cannot be used to any significant extent for anabolic processes in the absence of the growth hormone.

4. The apparent nutritive value of a protein is, therefore, dependent on pituitary function.

5. Since the pituitary gland is very sensitive to nutritive deficiency, it may well be that the variations in the nutritive value of foodstuffs are in part dependent on their specific influence on pituitary metabolism.

It will be observed in this discussion that much more is known about the effect of hormones on processes involved in nutrition than is known about the degree to which nutritive substances act by way of the hormones. The student of nutrition has rarely taken cognizance of the fact that the animal on which he experiments is a carefully balanced dynamic system controlled in many ways by internal secretions. The fact that the influence of the endocrine glands is often offset by changes in appetite has also tended to obscure their role when the animals have been fed *ad libitum*.

Conversely, it is seldom that the endocrinologist has emphasized the nutritive significance of his observations although when one stops to look upon them, one realizes their importance. The first observations on the effects of the pituitary gland indicated its influence on nutrition. Pierre Marie called attention to the connection of tumors of the pituitary gland with gigantism, and excessive visceral growth in acromegaly. Aschner (4) in his experiments on dogs noted the cessation of growth when the pituitary gland was removed and further observed the effect on the caloric balance of damage to the hypothalamus. He carried out experiments which clearly showed that obesity resulted in a dog when the hypothalamus was damaged but not when the hypophysis alone was removed.

The first quantitative experiments which throw light on the changes in the sources of energy produced by variation in pituitary function are the classic experiments of Lee and his co-workers, using rats treated with pituitary extracts and rats from which the pituitary gland had been removed. Table I is taken from the pages of Lee and Shaffer (31). It gives the changes in the composition of the carcass minus the digestive tract which resulted when groups of animals were carried on balanced feeding experiments from three to ten weeks, one group being injected with a crude pituitary extract while the other served as a control. The daily amount of food eaten by the controls was fed to the injected animals. In spite of the similar food intake it will be observed that there was a great difference in the composition of the carcasses particularly of the weight gain. The injected animals had a much greater increase in water and fat-free dry tissue and a smaller increase in the ether extract than the controls. As

TABLE I

Summary of Total Gains and Composition of Gains in Control and Treated Rats. M. O. Lee and N. K. Shaffer, *J. Nutrition*, **7**: 337, 1934

	Body Length cm.	Live body weight g.	Empty carcass weight g.	Composition of empty carcass weight gain, g. and %					
				Water	Ether ext.	Fat-free dry tissue	Total N	Ash	Energy Cal.
12 controls Total gain	18.2	764	716	324	281	111	15.4	22.7	3161
% initial datum	7.4	32.1	31.3	22.8	96.7	19.2	20.7	23.1	59.3
% gain in E. C. W.				45.2	39.3	15.5	2.15	3.16	[1]14.41
12 treated Total gain	33.1	1295	1217	771	162	284	37.9	45.1	2820
% initial datum	13.5	54.9	53.7	54.9	56.3	49.6	51.5	46.4	53.5
% gain in E. C. W.				63.3	13.3	23.4	3.12	3.71	[1]2.32
Excess gain of treated	14.9	531	501	447	—119	173	22.5	22.4	—341
% of initial datum	6.1	22.5	22.1	31.8	—41.5	30.2	30.6	23.0	—65
% excess E. C. W. gain				89.2	—23.8	34.5	4.49	4.47	[1]—0.68

[1]Calories/g. of empty carcass weight.

a consequence while the injected animals increased 55% in body weight against an increase of 32% for the controls, the actual caloric gain in the bodies of the injected animals was only 2820 calories while the smaller controls had increased their caloric stores by 3161 calories. Obviously weight gain was not correlated with energy stores. Since these differences were on the carcass after the digestive tract was eliminated they did not involve the error of unabsorbed material.

Fig. 1 is taken from the paper of Lee and Ayres (30). It is a chart of the weight loss when the control animals were fed the amount of food which the hypophysectomized animals voluntarily ate. Both groups lost weight because of the decrease in appetite in the hypophysectomized rat. The controls however lost significantly less weight than those in which the pituitary gland had been removed. This is explained by the composition of the weight loss. While the control animals had largely lost ether extractable substances and had actually lost no significant amount of nitrogen, the hypophysectomized animals had lost a considerable amount of nitrogen and a much smaller amount of fat. They had also lost more water than the controls.

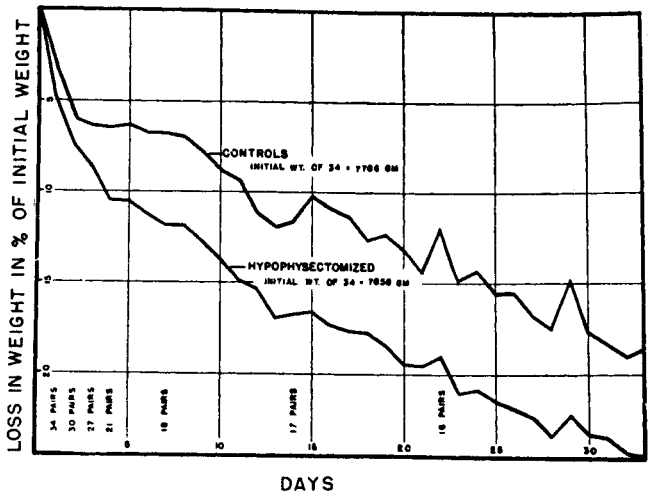

FIG. 1

Relative Rate of Weight Loss by Hypophysectomized and Control Rats on Paired Feeding. M. O. Lee and G. B. Ayres, *Endocrinology* **20**: 484, 1936.

As an introduction to our subject, this work is highly instructive. Also, there are a number of pertinent conclusions to be drawn. Obviously nitrogen balance in the two groups must have been different. The function of the pituitary gland, therefore, is going to affect the study of nitrogen balance. Second, if we were using this type of experiment to determine the nutritional value of the protein, the result we should get would be greatly influenced by the presence or absence of the hypophysis.

Since inanition is a complicating factor in the hypophysectomized animal, we have developed a series of diets in our laboratory which enables one to produce gains in weight in rats fed two or three times a day by stomach tube. This has enabled us to control amount, composition, and periodicity of feeding. The work of C. N. H. Long (33) in connection with his studies on the hypothalamus has demonstrated the importance of this last factor in the total metabolism. In the discussion to follow our own work has always been carried out on animals fed in this way.

II. Absorption of Foodstuffs

The absorption of foodstuffs is affected by the pituitary gland largely through its thyrotrophic action. In 1934 Phillips and Robb (45) reported that the rate of intestinal absorption of glucose was reduced by 30% in the young hypophysectomized rat. This was confirmed by Bennett (11). In 1940 Phillips and Gilder (46) found that the absorption of glucose by the young hypophysectomized rat fell within the range of absorption found in

the adult animal. They observed that the rate of absorption of glucose decreased with increasing age in the normal animal until a plateau was reached at the adult stage. They also carried out forced feeding experiments in this study. In this procedure the rats were given the amount of diet which would maintain approximately a 2-gram increase in weight per day. Under these circumstances the hypophysectomized animal had a rate of intestinal absorption of glucose which was as great as that of similar normal animals. It would seem then that part of the reduction in absorption observed by others was due to the decreased food intake.

These studies involved the administration of only one foodstuff, glucose. Under ordinary circumstances, however, a mixture of foodstuffs enters the intestinal tract and one substance may exert an influence on the rate of movement, digestion and absorption of others. In 1942, Samuels, Reinecke, and Ball (54) studied the rate of absorption of carbohydrate, fat, and protein, when carbohydrate-protein or fat-protein diets were fed by stomach tube. These studies were on adult animals given a food intake which just maintained the original body weight in the controls. In the hypophysectomized rats there was apparently a reduced movement of food as well as a reduced rate of absorption of all three of the major foodstuffs (Table II).

TABLE II

Distribution of Foodstuffs Two Hours After Meal by Stomach Tube.

Groups	A Carbohydrate-fed, Controls	B Carbohydrate-fed, Hypophysectomized	C Fat-fed, Controls	D Fat-fed, Hypophysectomized
Number of Rats	8	5	9	5
Fats, percentage				
in stomach			22.4	28.4
in small intestine			7.2	10.5
absorbed			70.4	61.1
Carbohydrate, percentage:				
in stomach	25.0	33.6		
in small intestine	4.8	5.8		
absorbed	70.2	60.6		
Nitrogen, percentage:[1]				
in stomach	37.5	53.0	32.2	46.6
in small intestine	21.3	26.8	22.4	24.9
absorbed	41.2	20.2	45.4	28.5

[1]This nitrogen includes the nitrogen of the digestive juices.

The rate of absorption of carbohydrate and fat was reduced to about the same extent, approximately to 83% of the rate of the normal animal. The absorption of protein appeared to be more markedly reduced although figures were complicated by the fact that the nitrogen in the digestive juices was included.

The reduction in absorption appeared to be eliminated when thyroxin was given to the hypophysectomized animal. This would indicate that the reason for the reduced rate of movement and rate of absorption was the reduced function of the thyroid gland. It has been demonstrated by a number of workers that the rate of gastric motility and the rate of absorption in hypothyroidism is less than normal (3, 42), while the opposite is true in hyperthyroidism. The effects seem to be general on all of the foodstuffs.

While the rate of intestinal absorption appeared to be reduced because of the reduced thyroid function, an over-all study of the fecal excretion of the foodstuffs in the four groups of animals during a 17-day balance period indicated that the difference seen in hypophysectomized and control animals was not significant (Table III). In those animals fed fat and pro-

TABLE III

Absorption of Foodstuffs During Balance Experiments

Group	Number of rats	Percentage absorbed over balance period		
		Fats	Carbohydrate	Nitrogen
A. Carbohydrate-fed, controls	9		98.6	81.9
B. Carbohydrate-fed, hypophysectomized	6		98.3	76.4
C. Fat-fed, controls	12	43.2		86.6
D. Fat-fed, hypophysectomized	7	89.1		86.5

tein, the hypophysectomized rats absorbed 89% of the fat and 86% of the nitrogen. In the carbohydrate-fed animals the carbohydrate absorption was 98% in both groups. The absorption of nitrogen was 82% in the controls and 76% in the hypophysectomized rats. There is always a slightly greater loss of foodstuffs in the feces of the hypophysectomized animals, but the amount is less than 10% of the total. Any significant differences in internal metabolism and the utilization of foodstuffs cannot therefore be attributed to differences in over-all absorption.

Judovits and Verzar (27) reported that intestinal absorption of glucose was reduced in adrenalectomized animals and attributed this reduction to a failure in the phosphorylation mechanism. Deuel, Hallman, Murray, and Samuels (14) found no significant reduction in absorption of glucose or amino acids when adrenalectomized animals were maintained on salt solution. Althausen and Anderson (2) made a careful study of intestinal absorption of glucose in the adrenalectomized rat. They found that while adrenalectomized animals given tap water had a significant reduction in the absorption of glucose, rats which were maintained on salt solution had rates of absorption which were well within normal limits. Apparently the reduction in absorption is not due to any specific effects of the adrenal cortex but to disturbances in electrolyte balance. Since the adrenotrophic hormone

largely influences the formation of compounds involved in carbohydrate metabolism, it probably plays a minor role in absorption.

Verzar and Laszt (63) also reported that fat absorption was reduced in adrenalectomized rats, presumably because of reduced phosphorylation. Barnes and coworkers (5, 6, 7) were unable to confirm this when the rats were maintained in good condition with salt administration. They did, however, observe that the lacteals were not filled with emulsified fat as in the normal controls. Bavetta and Deuel (9, 10), using younger animals, did find a 25% reduction in the absorption of hydrogenated cottonseed oil but normal absorption of tributyrin. In the former case, they reported an accumulation of fatty acids in the gut. Frazer (19) has reported that there are different pathways for the absorption of neutral fat and for fatty acids. It may be that one of these pathways is more dependent on the adrenal cortex. Again it may simply be that Bavetta's young animals were not in as good salt and water balance as those of Barnes. At any rate, here again the more important factor appears to be that associated with electrolyte metabolism and would, therefore, not be so dependent on the adrenotrophic hormone.

III. Utilization of Carbohydrate and Fat

1. *Non-endocrine Factors*

Before turning to the effect of hormones on the utilization of foodstuffs, it will be well to note certain factors which they do not control. When we began our work on hypophysectomized rats we were struck with the apparently well maintained carbohydrate levels when fat-protein diets were fed. We, therefore, carried out a study on the effects of high carbohydrate and high fat diets on carbohydrate and fat stores during subsequent fasting (55). As shown in Table IV, we found that animals previously on a high fat diet used carbohydrate more slowly during subsequent fasting both in the presence or absence of the pituitary gland.

The effect of hypophysectomy was seen in the low glycogen and fat levels in the livers, but the decrease in disappearance of glycogen on the fat diet was similar whether or not the pituitary gland was present. The same adjustment is seen in the tolerance to insulin (49) (Fig. 2).

Similar observations have been made by Guest (22) and by Mackay, Carne, Wick and Visscher (36) in animals fed high protein diets. Both groups found that, while the glycogen content of the livers at the end of feeding was lower in the protein-fed rats than in those on a high-carbohydrate diet, the reverse was true after twenty-four hours of fasting. The blood sugar levels were also higher in the protein-fed rats after fasting and the fasting ketosis was less.

TABLE IV

Liver Lipids and Glycogen in Hypophysectomized and Control Rats Fed Equicaloric Amounts of High Carbohydrate and High Fat Diets.

	High fat diet		High carbohydrate diet	
	Hypophysectomized	Controls	Hypophysectomized	Controls
Not Fasted:				
No. rats	6	9	6	9
Fatty acids mg./100 g. body wt.	245	585	98	241
Non-saponifiable mg./100 g. body wt.	16.8	10.9	10.0	8.3
Glycogen mg./100 g. body wt.	51.3	95.6	108	140
Fasted 30-33 hours:				
No. rats	14	15	9	9
Fatty acids mg./100 g. body wt.	127	529	87	224
Non-saponifiable mg./100 g. body wt.	19.3	12.0	9.3	7.7
Glycogen mg./100 g. body wt.	9.1	19.7	0.16	2.5
Glycogen lost during fasting mg./100 g. body wt.	42.2	75.9	107.8	137.5
Mean mol. wt. fatty acids	290 ± 1.8	286 ± 1.9	294 ± 2.0	279 ± 1.9
% fat in carcass	10.80	9.53	13.23	8.14

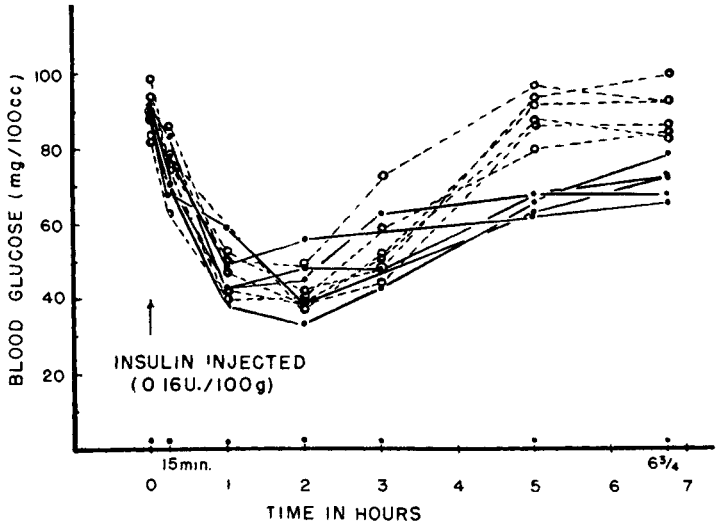

FIG. 2

Diet and Insulin Tolerance of Normal Rats

Blood sugar curves of normal adult male rats injected with 0.16 U. of insulin/100 g. after 36 hours' fast. These animals had previously been fed by stomach tube for 6 weeks equicaloric amounts of the special diets:

High Fat Diet .....

High Carbohydrate Diet ·_____·

These are apparently adjustments in the metabolism of the peripheral tissues. The rate of fall of the blood sugar in eviscerated rats was much less if they had previously been maintained on a high fat diet than after a high carbohydrate diet, while the metabolic rate was the same (50).

After a high protein diet, the fall of blood sugar in eviscerated rats was intermediate between the high fat and high carbohydrate groups. The same was true if the rats had been starved for 48 hours or longer after a mixed or high carbohydrate diet (48).

The adjustment requires time. When rats were maintained for five weeks on the fat diet they survived longer after evisceration without glucose injection (17-24 hours) than similar animals kept for only three weeks (average 15½ hours). Eviscerated rats previously on a high carbohydrate diet survived 6½-10 hours. This time factor has led to the supposition that an adjustment in intracellular enzyme systems must occur.

The kidney seems to play an important part in these adjustments. Reinecke (47) has shown that in the eviscerated rat which has been previously fasted the kidney is adding glucose to the blood stream in significant quantities.

The adjustments in utilization of the foodstuffs which are brought about by the endocrine system must be looked upon, therefore, as superimposed upon these slower, more basic adaptations, enabling the organism to meet more rapid and more extensive changes in nutritional environment.

2. *Endocrine Factors*

The utilization of dietary carbohydrate and fat is affected by the pituitary gland both through the trophic action of its hormones on other endocrine glands and through its direct metabolic action.

The rate of disappearance of glucose from the blood of an hypophysectomized rabbit is very high. Greeley (21) found that it was necessary to inject glucose at the rate of 700 mg./kg./hr. to maintain the blood sugar of fasting hypophysectomized rabbits. This could not be accounted for by an increase of glycogen or of liver fat. Its oxidation would require nearly all of the oxygen consumed by the animal. Russell (51) likewise found in hypophysectomized rats that the amount of glucose required to maintain the blood sugar by continuous injection after evisceration was twice that required in preparations with the hypophysis intact. Yet we have found that rats from which the pituitary gland had been completely removed could maintain themselves for weeks on high fat diets, and even for some time on diets which contained practically no other sources of energy except fat (57). It is obvious then that such animals can obtain their energy from fat if it is available. The rapid disappearance of glucose in the fasting hypophysec-

tomized animal must be related to an inability to mobilize endogenous materials which can be converted into glucose or which will spare glucose. That the rate of mobilization of endogenous fat stores is affected by the pituitary gland is indicated by the studies of Barrett, Best and Ridout (8). By using deuterium as a tracer in the mouse, they found that the extra fat appearing in the liver after injections of anterior pituitary extract was derived from previously stored fat and not from carbohydrate or protein.

Much of the effect on the utilization of carbohydrate and fat in the hypophysectomized animal may be due to the atrophy of the adrenal cortex. Britton and his coworkers (12) demonstrated the marked disturbance which occurred in the carbohydrate metabolism of the adrenalectomized animal. The work of Long and Lukens (34) showed that this was largely due to a decrease in the rate of gluconeogenesis from body protein; and that in the hypophysectomized cat, at least, the major abnormalities in carbohydrate metabolism during fasting were due to lack of the adrenotrophic hormone.

Not only does removal of the adrenal gland or atrophy of the adrenal cortex affect gluconeogenesis; it also affects mobilization of stored fat. Samuels and Conant (53) found that the rate of disappearance of fat from muscle and abdominal stores was delayed by adrenalectomy; the rate of disappearance from the liver was unaffected. This may be an indirect effect of the influence on protein catabolism.

The administration of adrenocorticotrophic hormone to hypophysectomized rats will not only relieve the insulin sensitivity but will increase liver glycogen storage and raise the blood sugar level. Lucke and coworkers (35) in 1933 were the first to call attention to the role of the adrenals in the pituitary action on insulin resistance.

There is evidence for a direct and an indirect effect of the growth hormone. Marx, Herring, and Evans (38) using a purified growth hormone preparation in fasting hypophysectomized rats showed that in such rats not only was nitrogen stored but the hypoglycemia was accentuated and typical hypoglycemic convulsions were produced. On the other hand, the same group of workers (37) using the purified preparation, have confirmed the observations of Mirsky (40) and of Gaebler and Robinson (20) that growth extract produces a definite increase in urine glucose when administered to depancreatized rats. Gaebler and Mirsky also observed an increase in nitrogen excretion rather than the decrease as observed in animals with the pancreas present. There would seem to be, therefore, an indirect effect via the pancreas which is dominant under ordinary circumstances. This decreases gluconeogenesis while there is a direct effect that increases it. This

last may be of significance in connection with the problem of partial pancreatic diabetes.

The thyrotrophic hormone will, through its indirect influence on the total metabolism of the body, affect the utilization of dietary carbohydrate, since more will be oxidized and less converted into fat.

The gonadotrophic hormones also influence carbohydrate utilization indirectly. Deuel and his coworkers (13, 24) demonstrated that female animals have an increased tendency toward ketosis on any given diet or during fasting, and that there was a greater accumulation of fat in the livers. Ingle (26) has been able to produce evidence of diabetes when estrogens were administered to hypophysectomized-adrenalectomized rats which were maintained with constant doses of pituitary and adrenal extracts. These compounds appear to increase fat metabolism and decrease carbohydrate utilization directly.

While it is obvious, then, that the hormones of the hypophysis will affect the utilization of carbohydrate and fat directly or indirectly, the peripheral adjustments to diet which take place may, in part, offset this.

The experiments of Samuels, Reinecke, and Ball (54), illustrated in Table V, show that the hypophysectomized animal can use either carbohydrate or fat, and when maintained at caloric equilibrium with diets containing adequate protein, the only definite change in body composition is a loss of water. The sources of energy are shown in Table VI. Carbohydrate and fat were used equally well, and there was no loss of protein in the hypophysectomized rats. There was evidence of the protein-sparing action of carbohydrate in both types of animals.

III. Maintenance of Nitrogen Balance

The phases of nutrition which are most markedly affected by the hormones of the anterior hypophysis are those associated with protein metabolism. The hypophysis affects protein metabolism in several ways. It affects it both directly and indirectly through the growth hormone. The adrenotrophic hormone through its action on the adrenals will also influence nitrogen balance. The gonadotrophic hormones have an indirect action through their influence on the production of the androgens and the thyrotrophic hormone will have an influence because of its stimulating action on the thyroid gland.

The growth hormone has been prepared in relatively pure form by several workers and has been shown to have a positive influence on formation of protein in adrenalectomized-hypophysectomized rats (61). Smith early demonstrated that pituitary extracts could produce growth in thyroidectomized animals (62). Therefore, it is not acting indirectly through these

TABLE V

Change in Body Composition on Diet Containing 15% of Calories as Protein
(Gram/100 g. of original weight)

Number and Type of Animal	Original composition[1]				Final composition				Change in composition				
	Carbohydrate	Fat	Protein	H_2O and salts	Carbohydrate	Fat	Protein	H_2O and salts	Weight	Carbohydrate	Fat	Protein	H_2O and salts
6 Hypophysectomized rats, high carbohydrate diet	0.37	9.55	17.77	72.3	0.42	13.32	18.53	66.5	—1.2	+0.05	+3.77	+0.76	—5.8
9 Control rats, high carbohydrate diet	0.36	8.89	18.01	72.7	0.45	8.43	19.03	75.1	+2.9	+0.09	—0.46	+1.02	+2.4
6 Hypophysectomized rats, high fat diet	0.37	9.55	17.77	72.3	0.26	11.08	18.93	66.1	—3.6	—0.11	+1.53	+1.16	—6.2
9 Control rats, high fat diet	0.36	8.89	18.01	72.7	0.36	10.11	19.76	72.3	+2.5	0	+1.22	+1.75	—0.4

[1]Based on analysis of rats from same groups killed at beginning of experiment.

TABLE VI

Energy Consumption of Rats on Diets Containing 15% Protein (Calories per 100 sq. cm. of original body surface per day)

Number and Type of Animal	Carbohydrate	Fat	Protein	Total
6 Hypophysectomized rats, high carbohydrate diet	10.29 ± .06[1]	—1.92 ± .66	1.22 ± .05	9.58 ± .70
9 Control rats, high carbohydrate diet	10.35 ± .09	0.35 ± .37	1.25 ± .40	11.95 ± .40
6 Hypophysectomized rats, high fat diet	—0.022 ± .006	10.12 ± .33	1.44 ± .08	11.56 ± .31
9 Control rats, high fat diet	—.045 ± .004	10.56 ± .46	1.31 ± .07	11.81 ± .52

[1]Standard Error.

organs. Its action on protein anabolism does seem to have some connection with pancreatic function, however. Mirsky and Swadesh (40) first pointed out this relation. They found that the non-protein nitrogen of the blood rose less rapidly in nephrectomized dogs if they were injected with anterior pituitary growth extract than in the normal nephrectomized animals. They were not able to find this difference, however, in eviscerated or depancreatized-nephrectomized dogs. In such dogs the rate of accumulation of non-protein nitrogen was greater than in the normal animals. They concluded that there were two actions of the pituitary extract, a direct stimulation of protein catabolism and an indirect stimulation of anabolism by the pancreas. These findings were confirmed by Gaebler and Robinson (20).

Since the cortical hormones with oxygen on carbon 11 increase the rate of gluconeogenesis from protein, they therefore tend to create a negative nitrogen balance. Evans, Simpson and Li (17) have shown that the adrenotrophic hormone decreases the rate of growth of male rats, either intact or gonadectomized, but has no influence on adrenalectomized rats maintained on salt solution. It must, therefore, act through the adrenals.

The hypophysis also influences nitrogen balance through the gonadotrophic hormones, particularly the interstitial cell-stimulating hormones. Kochakian and Murlin (29) first demonstrated the influence of androgens on nitrogen balance in dogs and Kenyon and his co-workers as well as others have extensively studied this in humans (28). They have shown that a positive balance can be created by the administration of androgenic steroids. This anabolic effect is particularly observable in gonadectomized animals but occurs in normal individuals in a variety of states of nutrition. The recent studies of Simpson, Marx, Becks and Evans (60) indicate, however, that in rats testosterone acts only in the presence of the pituitary growth hormone. When the steroid was injected into hypophysectomized

animals, there was no influence on body weight. If growth hormone were given, however, testosterone considerably increased the rate of growth. It would seem then that there must be some pituitary growth hormone present to have any general anabolic effect, but that it can be increased by the androgenic steroid.

The thyrotrophic hormone of the pituitary gland will also affect nitrogen balance through its influence on the production of thyroxin. Thyroxin is apparently important for growth to a different degree at different ages. Salmon (52) has shown that if rats are thyroidectomized at birth the growth extract of the pituitary gland will have no effect. On the other hand in weanling rats Smith (62) early demonstrated that growth would be resumed in hypophysectomized-thyroidectomized animals if growth hormone alone were given. However, the effect of the growth hormone was greater if thyroid were given at the same time. This has been confirmed by Evans, Simpson and Pencharz (18). They also showed that thyroxin in the thyroidectomized-hypophysectomized rats at these older ages had no affect unless growth hormone was given at the same time. Administration of thyroxin to thyroidectomized rats at low levels increases the positive balance toward that of normal rats of the same age. On the other hand if enough thyroxin is administered to a thyroidectomized rat to increase the metabolic rate beyond normal, the nitrogen balance is reduced. It, therefore, appears that there is an optimal level of thyroxin for the greatest positive nitrogen balance.

The ability of the body to deaminate protein does not seem to be interfered with by any of the endocrine glands, particularly the pituitary gland. Houssay and coworkers (25) maintained hypophysectomized dogs on an all protein diet and found that it would maintain the blood sugar and liver glycogen of these animals. Drury and Greeley (16) studying the increased glucose utilization rate of hypophysectomized rabbits found that if amino acids were injected instead of glucose, the glucose need was lowered. The studies of Samuels, Reinecke, and Ball (54), previously referred to, had shown that the adult animal without a pituitary gland would maintain nitrogen equilibrium if given an adequate diet.

The question of what the tissues of a young hypophysectomized rat would do with protein in excess of that required for maintenance of the original weight was not answered by these experiments. Experiments were carried out by Samuels and coworkers (56) on young male rats weighing 60-100 g. One of each set of littermates was hypophysectomized, another was killed immediately and analyzed to determine original composition, and a third was used as the normal control. The hypophysectomized and control rats were then placed on a stomach tube diet in amounts sufficient to maintain

an increase in body weight in the control of approximately 2 g./day. Table VII gives the caloric and nitrogen balances on three such sets. Six sets were carried through the experiment, but loss of some sample along the way made it impossible to calculate complete balances on the other three.

TABLE VII

Caloric Balance on Young Force-Fed Rats

Rat #	4	5	7	8	10	11
Type of preparation	Hypophysectomized	Control	Hypophysectomized	Control	Hypophysectomized	Control
Days on Diet	22	22	32	32	54	54
Total Intake, Calories	725.4	745.1	1053.9	1053.9	1822.5	1822.5
Unabsorbed Food, Calories						
Carbohydrates	27.5	19.3	37.8	27.3	74.0	47.2
Protein	21.5	14.6	24.3	17.5	41.1	25.9
Fat	72.1	50.9	65.2	47.4	138.9	86.3
Total unabsorbed, Calories	121.1	84.8	127.3	92.2	254.0	159.4
Increase in N stored, Calories	6.9	34.2	16.2	33.4	17.9	61.4
Increase in Fat Stored, Calories	96.7	80.6	245.2	34.1	398.9	81.6
Total Caloric Increase	103.6	114.8	261.4	67.5	416.8	143.0
Calories Used	500.7	545.5	665.2	802.0	1155.7	1520.1
Metabolic Rate Cal/day	22.8	24.8	20.8	25.1	21.4	38.2
Metabolic Rate Cal/sq.m./day	10.3	10.7	10.0	11.6	9.3	12.1

Absorption of foodstuffs was somewhat less in the hypophysectomized rats but this was a relatively unimportant factor in the total result. The four most striking differences seen in this table are the much smaller storage of nitrogen, the great storage of fat, the smaller gain in weight, and the lower metabolic rate in the rats without a pituitary gland.

The nitrogen balances in these rats are shown in Fig. 3. The protein was utilized almost equally well by both types of animals there seems to be no abnormally low limit to the ability of the hypophysectomized rat to metabolize protein. There was a marked difference in the way in which it was utilized, however. As in the case of Lee's animals, which were on a subnormal intake, the balance between catabolism and protein synthesis was shifted in the catabolic direction by elimination of the hypophysis. More nitrogen appeared as urea in the urine and, since the metabolic rate was lower, the carbon chain of the amino acids was in part converted to fat.

Similar results have been obtained both by us and by Levin (32) in adult animals receiving amounts of food which produced gains in weight, although the protein increment in the adult controls was not so great. It

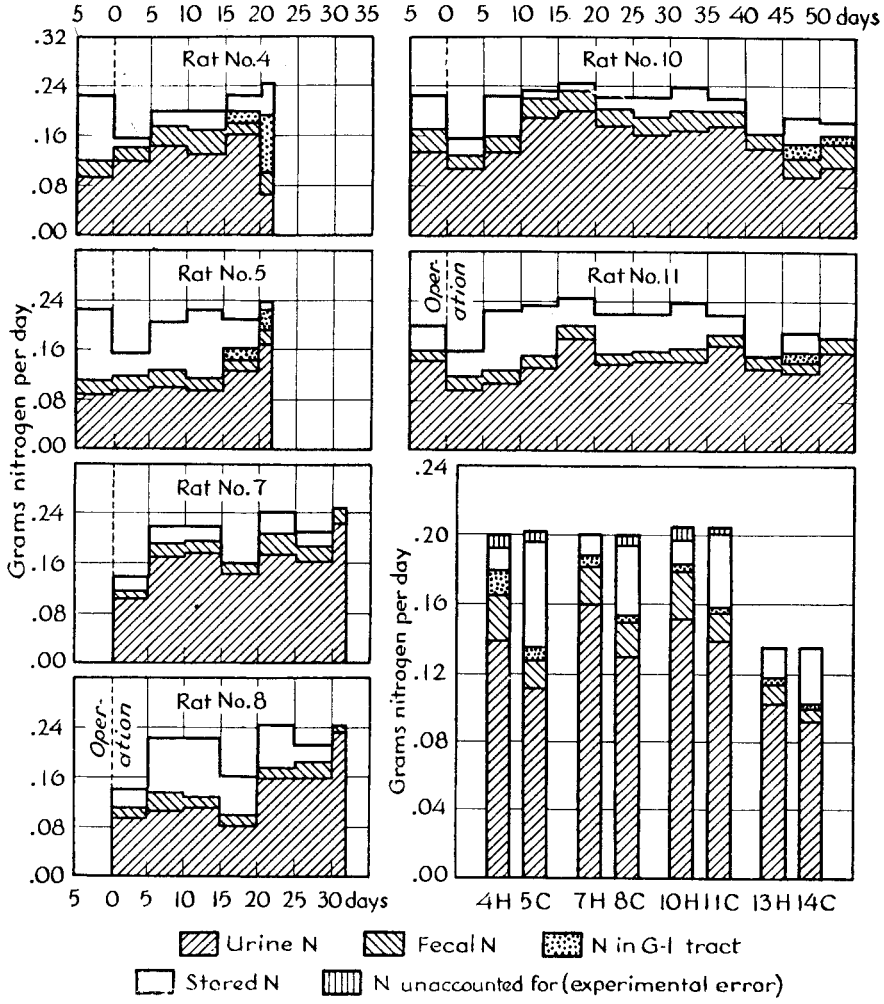

FIG. 3

Nitrogen Balances of Hypophysectomized and Control Rats During Forced Feeding
The numbers of the rats correspond to those given in Table VII. Rats #4 and #5, #7 and #8, #10 and #11, #13 and #14 were paired littermates. The graphs with days as abcissae represent the balances by five-day periods. The bar graphs represent the distribution of the total nitrogen divided by the number of days the animal was on the experiment.

seems, therefore, that the catabolic processes in connection with the metabolism of dietary protein are not depressed by removal of the pituitary gland but that the synthetic processes are considerably limited.

The ability to synthesize protein for general growth of the organism appears to depend primarily on the so-called growth hormone, although the

metabolic rate of the cells must be stimulated to a certain level by the thyroid hormone before synthesis can take place at a normal rate. We carried out some experiments similar to those of Lee except that adult hypophysectomized rats were used which were maintained on a diet sufficient to cause a slow increase in body weight in normal adult animals. A purified growth preparation obtained from the Wilson Laboratories was injected into one group, together with thyroxin and Upjohn's adrenal extract. The effects on total weight and on composition are shown in Table VIII. The

TABLE VIII

Effect of Pituitary Growth-Promoting Extract (Wilson) on Size and Composition of Hypophysectomized Rats

No.	Start Cont. 5	Uninjected fed 6	Injected fed 7
Wt. start, g.	239 ± 4.9*	244 ± 4.0	238 ± 1.7
Wt. end, g.		277 ± 3.2	326 ± 4.9
Wt. livers, g.	6.36 ± .17	8.96 ± .17	15.86 ± .90
Wt. kidneys, g.		1.484 ± .062	2.60 ± 0.17
Wt. spleen, g.	0.478 ± .033	0.479 ± .015	2.630 ± .26
Wt. abdominal fat depots, g.	8.98 ± .80	14.01 ± .42	10.80 ± .70
Wt. Carcass less viscera, g.	156 ± 2.4	165 ± 2.5	206 ± 3.7
fat in livers, %	4.63 ± .07	5.76 ± .07	6.46 ± .11
nitrogen in livers, %	2.70 ± .06	2.33 ± .04	2.28 ± .14
water in livers, %	76.9 ± .65	76.0 ± .44	77.5 ± .66
nitrogen in carcasses, %	2.84 ± .035	2.75 ± .021	2.82 ± .047

*Standard error of the mean.

nitrogen and water content of both the livers and whole carcasses increased when compared either with the group killed at the start of the experiment or with the uninjected rats fed for the same length of time with the same amount of diet. The total fat of the injected rats was also less than in either of the other groups, but the absolute fat content of the livers was higher than at the start of the experiment. The concentration in this organ was not significantly different, the gain being accounted for by the increased amount of liver tissue. As in the case of the young hypophysectomized rats when compared with their paired controls, the uninjected rats had used less energy during the experimental period even though they weighed less at the end; the high fat content accounted for the excess energy.

Preliminary experiments with purified adrenotrophic extracts indicate that the opposite effect on protein metabolism is obtained. Whether the diet is high in carbohydrate, fat, or protein, the carcasses apparently contain less protein than uninjected hypophysectomized rats fed equal amounts of the same diets.

It seems then that in the normal animal there is an interplay between these two hormones on the protein metabolism of the body.

V. Nutritional Value of Proteins

If the ability to synthesize protein is dependent on pituitary function, the concept of nutritional value of a protein is involved. Nutritional value is taken to mean the tendency of a protein to be used anabolically, either to spare endogenous protein or to increase storage of protein. To determine the influence of hypophysectomy on this value we studied the effect on adult rats of diets containing progressively less protein (57). Because we wished to determine the ability of the hypophysectomized animal to maintain itself on fat, we first adapted the rats to a high fat diet in which 15% of the calories were furnished by protein, and then transferred them to diets of lower protein content. As long as the intake of protein was sufficient to maintain nitrogen balance and the total caloric intake supplied more than the daily requirement, the hypophysectomized rats, as in the case of the young rats studied, excreted more nitrogen than the controls. At a level of 5% protein calories, the two groups excreted about the same amount. With an all fat diet the hypophysectomized rats excreted slightly less nitrogen (Table IX). This is simply an example of the inability to mobilize endogenous protein and the equal inability to store exogenous protein which follows pituitary removal.

Table IX

Nitrogen Excretion on High Fat Diets Containing Different Amounts of Protein

	Nitrogen excreted mg. N/100 g. body weight per day	
	Controls	Hypophysectomized
15% Protein Calories	.1192	.1239
5% Protein Calories	.0489	.0517
0% Protein Calories	.0360	.0332

The fall in nitrogen output compared with that from fasting is shown in Fig. 4.

Allison and Anderson (1) in a recent article have given expression in equation form to the biological value of protein. When this is applied to our data in the region of negative nitrogen balance the results illustrated in Table X are obtained. Pituitary function has a great influence on the so-called biological value of a protein.

This is brought out also when the data on the young rats are used (Table XI). The results on three pairs of rats are presented to illustrate the uniformity of the difference.

It would seem that catabolic processes are not limited by deficiency of the pituitary hormones. The ability of the rat to synthesize protein from exogenous sources, however, is dependent on pituitary function. If this is

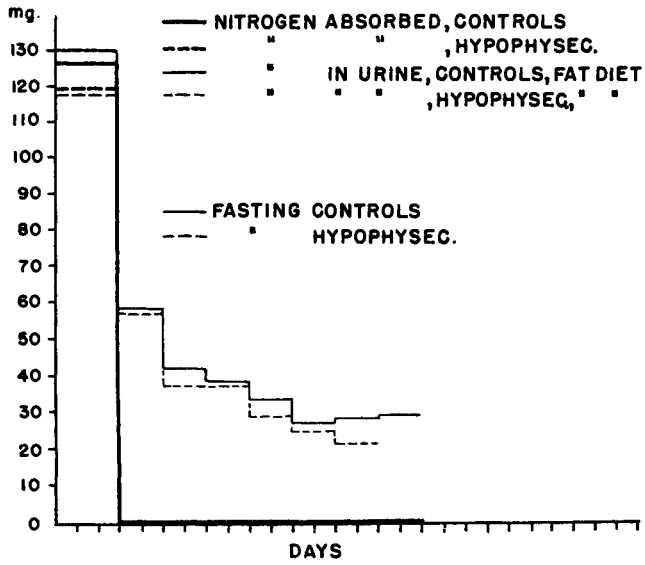

FIG. 4

Urine Nitrogen on Low Protein Intake mg. per 100 g. Body Wt. per Day

Nitrogen Output in Intact and Hypophysectomized Adult Male Rats when Changed from a Fat-Protein Diet Containing 15% of the Calories as Protein to an Equicaloric All-Fat Diet.

TABLE X

Biological Value of Ovalbumin
(Modification of Method of Allison and Anderson)
(*J. Nutrition* 29: 413, 1945)
Based on mg. N/100 sq. cm. body surface/day

	Hypophysectomized	Normal
Fecal N, no protein (FN′)	7.38	5.98
Fecal N, 5% protein (F N)	14.9	7.99
N Intake, 5% protein (N I)	29.5	29.5
Urine N, no protein (U N′)	10.80	16.14
Urine N, 5% protein (U N)	19.13	19.19
Absorbed N (A N) = N I — (FN—FN′)	22.0	27.5
Biological Value (B V) = $\frac{\text{AN} - (\text{UN}-\text{UN}')}{\text{AN}}$	0.63	0.89

true the anabolic efficiency of a protein will depend upon two general factors: its specific influence on the synthesis of the growth hormone and its value for synthesis of protein by cells in general. So far as the author is aware, no one has made a systematic study of the anabolic value of different proteins in hypophysectomized animals maintained on constant levels of growth hormone.

TABLE XI
Biological Value of Proteins in Young Rats
mg. N/100 g. Original Body Weight/day

	Rat #4 Hypoph.	Rat #5 Control	Rat #7 Hypoph.	Rat #8 Control	Rat #10 Hypoph.	Rat #11 Control
Fecal N, no protein (FN′)	10.2	8.40	10.2	8.40	10.2	8.40
Fecal N, 15% protein (FN)	36.8	25.4	30.6	23.9	29.1	19.1
N intake (N I)	193.3	202.5	207.2	216.0	202.0	210.0
Urine N, no protein (U N′)	30.5	34.2	30.5	34.2	30.5	34.2
Urine N, 15% protein (U N)	132.8	110.2	160.0	124.8	153.0	130.7
Absorbed N (A N)	166.4	185.5	186.8	200.5	183.1	199.3
Biological Value (BV)	0.38	0.59	0.31	0.55	0.33	0.52

VI. INFLUENCE OF NUTRITION ON PITUITARY HORMONES

The fact that the apparent nutritive value of a protein may depend on its influence on the synthesis of the growth hormone of the hypophysis illustrates the importance of the problem of the effect of nutrition on the pituitary gland. Some information has been obtained but much more is needed.

We know most about the influence of nutrition on the gonadotrophic hormones. Mason and Wolfe (39) first showed by transplantation experiments that the gonadotrophic activity of the hypophyses of female rats is lowered by inanition. Moore and Samuels (41) found that the decreased activity of the cells of the secondary sex glands of the male rat observed in thiamine deficiency could be produced in the presence of high thiamine intake if the food of such animals were limited to that of the thiamine-deficient group. In either case the development of the glands could be restored if gonadotrophic extracts were given, even though the rat might die of the deficiency. They concluded, therefore, that the primary hormonal deficiency was a decrease in pituitary gonadotrophic activity.

When food intake was limited, either by feeding a diet deficient in thiamine or restricting the amount of an adequate diet, Drill and Burrill (15) found that the estrous cycles of rats became irregular and finally ceased. Restoration of food intake led to a restoration of estrous cycles. If the animals were maintained on a limited food intake but injected with pituitary gonadotrophic extracts, the rats immediately went into prolonged estrus. The dose was no larger than required for a normal rat during the diestrous period. Guilbert and Goss (23) fed diets containing various levels of protein and found that if the protein content of the diet was below 7% the estrous cycles became prolonged and, in most instances, ceased. Here again the injection of gonadotrophic extracts led to immediate estrus. It seems, therefore, that the formation of gonadotrophic hormones in the pituitary gland is very sensitive to either a caloric deficiency or lack of protein.

The adrenotrophic hormone seems also to be affected by undernutrition although probably not so easily as the gonadotrophs. Mulinos and Pomerantz (43, 44), in describing "pseudohypophysectomy" produced by chronic submaximal undernutrition, mention that if they withheld all water and food the adrenal cortex underwent hypertrophy in a short time but if they fed suboptimal quantities of food, after a period of time the adrenals underwent atrophy. The acute reaction they attributed to an outpouring of adrenotrophic hormone, the "alarm reaction" of Selye. The chronic effect of undernutrition, however, was to decrease the formation of the adrenotrophic hormone in the pituitary cells.

These workers also called attention to the effect of chronic undernutrition on growth of the skeleton and showed that injection of pituitary extracts would cause resumption of skeletal growth and decrease in the rate of weight loss on the same diet.

These observations all point to the sensitiveness of the pituitary gland to nutritional deficiency and indicate that many effects of undernutrition are mediated through it.

VII. Conclusion

In conclusion I should like to summarize the evidence I have presented.

1. There are adjustments of the cells to dietary changes which are apparently not under the control of the endocrine system.

2. Besides the obvious influence on nutrition exerted by pancreatic and thyroid function, the anterior lobe of the pituitary gland affects the utilization of exogenous material through its hormones.

3. Utilization of foodstuffs for catabolic processes is not seriously affected. Excess sources of energy are readily stored as fat.

4. The balance between synthesis of protein from, and deamination of, amino acids of foods is shifted in the direction of the former by the growth hormone.

5. The control of the mobilization of specific tissues by pituitary hormones as in the case of the adrenotrophic hormone, has an indirect effect on the general nutrition of the animal.

6. The pituitary gland is in turn readily affected by nutritive deficiency.

7. It may well be that the variation between different proteins or amino acid mixtures in their ability to be used anabolically is in part due to their influence on the metabolism of the pituitary gland rather than on their influence on the body cells in general.

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DISCUSSION

D. J. Ingle: It has been my opinion for some time that those of us in the endocrine field are likely to over-emphasize the role of the endocrines in the control of metabolic processes. My thinking about this has been shaped by the work of Dr. Samuels and his associates more than by anyone else. I remember that when I went to work in the laboratory of Dr. Frank Mann 11 years ago, he discussed this problem with me and said, in effect, the following:

"There is no single metabolic process which is controlled exclusively by one body organ. One possible exception is the production of glucose by the liver, and," he added, "perhaps some day it will be found that other tissues can form glucose from non-carbohydrate sources." Now Dr. Samuels and his associates have shown us that the kidney also can form glucose. I have listened to the discussion of metabolic problems by learned men in the field who gave an extensive consideration of the subject without even making a bow in the direction of the endocrine system.

These studies by Dr. Samuels and his associates show that certain metabolic adjustments to diet can occur independently of the adrenal and pituitary glands. Being influenced by those studies, we have carried out experiments along the same lines and I would like to mention one of them briefly. It has been debated whether protein can be metabolized normally in the absence of the adrenal cortical hormones.

In the following experiment we force-fed normal rats on high sodium chloride intake. After a control period of about 2 weeks, the adrenals were removed from half of the animals and the controls were sham-operated. Within the first two days following operation the adrenalectomized rats excreted less nitrogen. By the third day there was a post-operative rise in each group—which was greater in the adrenalectomized animals than in the controls. At no time did the urinary non-protein nitrogen fall below the pre-operative level. At the end of 14 days we fasted the animals for a 10-day period and quite to our surprise the adrenalectomized animals continued to excrete just as much non-protein nitrogen as did the control animals.

I am not quite prepared to explain why our results are different from those of earlier studies, except that most of them have been for a short time. Most investigators have not studied animals previously adapted to the diet, and if the animals were fasted it was immediately following adrenalectomy.

These results are not unlike those shown by Dr. Evelyn Anderson and her associates a few years ago. In her studies on fasting adrenalectomized rats the administration of sodium chloride kept them in good condition, and the animals were not markedly deficient in the excretion of non-protein nitrogen.

I would like to emphasize the importance of the force-feeding method in studies of this kind. There are all kinds of secondary dietary changes coming to complicate the picture in adrenal and pituitary insufficiency. In addition, it is well-known to students of comparative psychology and some physiologists that there are important differences in the voluntary activity of intact animals and those that have had either the adrenal or pituitary glands removed. Such animals have become almost entirely inactive, whereas intact animals show a marked increase in voluntary activity during fasting so that their energy output must be much greater.

L. T. Samuels: I think that Dr. Ingle's experiments are extremely interesting and his point regarding the matter of activity is a very well taken one. That enters into the matter of differences in carbohydrate and fat diets. I think one reason there is less difference in the metabolism between fat-fed hypophysectomized and normal animals is that the normal animals are less active when they are on a high fat diet, and therefore the total caloric output is reduced.

There is another piece of work which Dr. Ingle has recently published which is important in work on eviscerated animals. He has shown that the life of such a preparation is prolonged by continuous injection of isotonic saline solution. The blood glucose also appears to be maintained. Dr. Ingle's work illustrating the importance of keeping the kidney functioning, or at least introducing salt solution continuously to maintain glucose levels, is something which puts all our work on eviscerated rats back where we must check it again.

There is one thing I might mention in this matter of adjustment to diet. Yesterday in the discussion of Dr. Long's paper, regarding the matter of adaptation, Dr. Selye mentioned the fact that adrenalectomized and hypophysectomized rats do become adapted if stresses are repeated at a low level. In other words, the adaptation is not dependent on either the pituitary or the adrenal. In those conditions of physical change or metabolic stress where energy is required from endogenous sources, might it not be that the reason the immediate effects are so notable is that the immediate supply of energy is from the breakdown of protein to supply carbohydrate as affected by the adrenal hormones? If this effect is not too severe at the start, eventually the cellular metabolism changes and these animals now can offset stresses by utilizing whatever fat or protein is readily available. Thus they are no longer dependent on the sources of carbohydrates controlled by the adrenal.

R. H. Barnes: I would like to make a few remarks concerning the interpretation of the biological value of proteins. According to the original definition as presented by Karl Thomas and later modified by H. H. Mitchell, the biological value of a protein is the percentage of the absorbed nitrogen that is retained by the body. Retained nitrogen is the nitrogen utilized in the maintenance of the nitrogenous integrity of tissues and that which is used in the building up of new tissues.

Mitchell recognized that the apparent biological value of a protein could change under such conditions as changing the amount of protein ingested. More recently it has been shown that the apparent biological value of a protein might be increased considerably if the measurement is made in an animal that is severely depleted in protein. In fact under such conditions Allison and his associates have recorded apparent biological values of egg protein that are far in excess of 100. Since it is ridiculous to speak of biological values greater than 100, the figure that is obtained should not be given this designation. Allison and his associates have preferred to call this a nitrogen balance index. The probable reason for an apparent increase in nitrogen retention under the above conditions is a change in "endogenous" urinary nitrogen that may not be determined by the usual technic of measuring endogenous excretion. This is cited as an example of an apparent change in the biological value of a protein that should be more accurately referred to as a change in endogenous urinary nitrogen under conditions of protein feeding.

It is possible that the changes in the biological value of protein that Dr. Samuels has shown are actually changes in the excretion of endogenous urinary nitrogen under conditions of protein feeding. Such changes in endogenous nitrogen might not be apparent during periods of feeding a protein-free diet. I do not believe that this view changes in any way the importance of the interpretation that Dr. Samuels has given to the influence of pituitary hormones on the utilization of protein. However, it is possible that endocrine influences upon protein metabolism such as those just described are not directly related to the biological value of proteins.

L. T. Samuels: I think as regards the matter of endogenous excretion, we have insofar as is possible, used methods which would give similar results, the equivalent

of those Allison used on the dog. We have fed caloric intake which was more than adequate for the animal without protein and then simply have substituted a certain number of calories as protein without changing the total caloric intake. We have done so for 5% and 10% protein and had a similar degree of slope, but on higher protein intakes the slope changed because we were then getting well above the level of minimum nitrogen balance. In all of these balance experiments rats were placed on the diets immediately after hypophysectomy so that no abnormalities could develop beforehand.

It is true that we cannot be sure that there are not changes in endogenous metabolism but I think that, particularly when one considers the findings in the young animals, the effect must be on the utilization of exogenous protein. The original conclusion is that the hormone output is a factor in evaluation of the relative ability of proteins to form new tissue.

E. C. Kendall: Dr. Ingle spoke of the influence of the adrenal on endogenous protein. I would like to ask one question: whether you continued the sodium chloride all through the fast?

D. J. Ingle: Yes.

E. C. Kendall: If the sodium chloride had been stopped and water given, then the animals would have died promptly?

D. J. Ingle: Such animals die in a few days.

E. C. Kendall: Dr. Samuels has referred to the influence of the hormones of the adrenal cortex on metabolic processes. I would like to report some work which shows that we must refine our viewpoint and consider these hormones as individual substances qualitatively different one from the other. Dr. Heilman and I have carried out an investigation on the effect of implantation of pellets of corticosterone, dehydrocorticosterone and 17-hydroxydehydrocorticosterone (Compounds B, A, and E respectively) on four strains of normal mice. In age the mice varied from young (21 days old) to adult (212 days). Both sexes were used. The diet was Purina Fox Chow mixed with oats. Approximately 1.0 mg. of hormone was absorbed each day from the pellets during a period of five weeks. The results indicated that Compounds B and E caused a loss of weight. A slight gain in weight in young mice and a significant gain in old mice followed implantation of Compound A. With both A and B the most striking result was a marked gain in body fat and loss of protein both in percentage of body weight and in the weight of protein compared with that of controls which were litter mates or closely matched in weight. Compound E produced loss of weight but the percentages of protein and fat were not altered. It thus becomes evident that each of these hormones has a specific effect. In addition compound E produced lesions in the muscles including cardiac muscle. On the original diet compound A produced lesions in the muscles in only one of twenty-two mice, but when Purina Fox Chow alone was given as a diet, compound A produced lesions in a high percentage of mice and many died apparently of circulatory failure associated with cardiac lesions.

L. T. Samuels: I think those observations are very interesting. Apparently with these compounds the catabolism of protein can be increased to a point where it can exceed synthesis even in animals whose caloric intake leads to a storage of fat. I think it would be a good thing to carry this out on rats controlled by stomach tube feeding, so that you would know where a given amount of food would go. Of course, animals which lost weight as with compound E do not take in enough food to meet their energy requirements. An appetite factor entered in here. Either they did not eat so much or they must have had a higher metabolic rate. The food must be present either as a metabaloid or it must be burned up.

A. White: I think I am correct in saying that Dr. Samuels made the statement that the corticotrophic hormone has an indirect effect on catabolism of protein. Then we have Dr. Ingle's statement that adrenalectomized animals, after a period of force-feeding and a period of adjustment or adaptation to a particular kind of diet, appeared to excrete as much or more nitrogen than unoperated control rats. Also, Dr. Kendall stated that animals implanted with pellets of desoxycorticosterone can greatly augment the loss of protein from the body. Therefore, we have reports that excess doses of desoxycorticosterone markedly accelerate protein catabolism, a suggestion that the loss of adrenal does not decrease nitrogen loss, and the suggestion of Dr. Samuels that the effect of corticotrophin is an indirect one. These data may seem in conflict with one another. Perhaps the variable which enters is the nature of the experimental conditions.

There is no argument or disagreement with the fact that there may be metabolic processes which can continue in the absence of the endocrine glands. I think we are all aware of the fact that the endocrine glands do not initiate metabolic processes but control their rates, *e.g.*, the normal animal versus the thyroidectomized animal. Furthermore, it has been common experience that different strains of rats and mice vary in their behavior toward various types of endocrine experimentation. We may have genetic, environmental, and dietary influences on mammalian cells. The bacteria and other lower forms can adapt themselves to alterations in environment. Experiments in Professor Ivy's laboratory have indicated that the composition of the digestive secretions may be varied by dietary alterations. The nature of the dietary constituents to be handled apparently influences the type of secretory activity of the cells whose secretions are concerned with the digestion of foodstuffs. Therefore, the previous dietary treatment of the animal may affect the results obtained. Moreover, experiments designed to examine the effects of extirpation of an endocrine gland will be influenced by the length of the post-operative period preceding metabolic study. It is likely that the composition of an animal one day post-operative is different from the composition of an animal 2 or 3 weeks post-operatively; consequently, an experiment started immediately after hypophysectomy may yield different results from an experiment which is started two or three weeks following hypophysectomy.

Finally, I would like to mention some experiments Dr. Dougherty and I have published in abstract form in the *Anatomical Record* which, in a sense represent the opposite experimental approach from that employed by Dr. Kendall, who administered excess adrenal steroids. We have removed the animal's source of adrenal steroids by adrenalectomy. As you know, Dr. Dougherty and I have come to look upon the lymphocytes as target cells of those adrenal cortical steroids oxygenated in position 11. We have previously discussed the contribution of the lymphocytes to normal and immune globulin production. Dr. Paul Cannon has demonstrated that one of the proteins of lymphocytes, gamma globulin, has a high nutritive value.

We have postulated that lymphoid tissue may contain a part of the reserve or deposit protein which investigators have sought for many years. Our experiments support a role of lymphoid tissue as a reserve store of protein. Normal mice fasted for 48 hours lose approximately 3 grams of body weight, and show 50% decrease in the total lymphoid tissue. The lymphoid tissue nitrogen also decreases approximately 50% under these conditions. If one fasts an adrenalectomized animal for a similar time, there is no decrease in total lymphoid tissue weight or nitrogen content. Indeed, the lymphoid tissue is slightly increased in size. Thus, a fasted, adrenalectomized animal with an extraordinarily severe need for protein is unable to get at this reserve protein in lymphoid tissue as is the animal with adrenals intact.

Therefore, we suggest that the adrenocorticotrophic hormone is concerned with the mobilization of a portion of the protein reserve, and that one of the functions of the adrenal cortical steroids is concerned with the release of this reserve protein from lymphocytes.

L. T. Samuels: I did not want to indicate, directly or indirectly, that I was not thinking of this matter of the release of lymphoid tissue.

With regard to the matter of change in the composition of the animal after operation, that, of course, is an important thing, but both Dr. Levin and ourselves have found that in hypophysectomized animals, if we maintain the animals both before and after operation on an adequate but not excessive diet, the only change which is significant is the decrease in the water content of the animal. There is a change in the liver, however, but the total composition of the body shows no significant variation.

A. White: Would you grant that there may be a change in the distribution of the constituents among the various organs and that, therefore, there might be a difference in response?

L. T. Samuels: Yes, I will grant you that.

Dr. Selye: Dr. Samuels mentioned the possibility that the reason why hypophysectomized or adrenalectomized animals may become more resistant to certain damaging agents after a certain time following operation is that the metabolic processes necessary, for instance, for the endogenous mobilization of sugar, might readjust themselves in such a manner that later on, after, let us say, several days or a week after the operation, the animal acquires a greater resistance.

L. T. Samuels: I think that after a period of time and after repeated treatment, changes occur in the metabolites the cell uses to offset the strain.

Dr. Selye: Under the influence of stress or a damaging agent? I think that is perfectly correct. I feel that adaptation cannot occur quite as well in the absence of the pituitary. For the proper results in acquiring adaptation these glands are most useful.

In connection with the changes occurring during adaptation to various diets, it may be worth mentioning that in the "general adaptation syndrome," which develops under the influence of chronic exposure to any type of strain, there is what Dr. Collip and I called a "shift in pituitary hormone production." This is characterized by an increased production of corticotrophic hormone (manifested by hypertrophy of the adrenal cortex) but a decreased secretion of gonadotrophic, growth, and lactogenic hormone. We interpreted this shift as due to the fact that during exposure to severe damaging agents, it is more important for the organism to produce an excessive amount of corticotrophic hormone, which helps to combat stress, than to maintain normal sexual functions or growth.

In view of this shift in pituitary hormone production, during the adaptation syndrome, we always find some involution of the gonadal tissue which, in females, tends to be accompanied by irregularities of the cycle, such as, anovular estrus or anestrus ovulation. As a little known curiosity, it may be mentioned that rats fed lima beans show a particularly pronounced and instantaneous involution of the ovaries, which is so severe and accompanied by so little evidence of general damage, that it must be considered due to more specific factors than those which cause the gonadal atrophy during the adaptation syndrome.

In connection with this paper, I would also like to call attention to the fact that crude corticotrophic hormone-containing anterior lobe preparations cause nephrosclerosis in the rat, especially if the animals are fed a high sodium diet. We believe that this effect may be due to the liberation, by the adrenals, of mineral-active corticoid

hormones which (like desoxycorticosterone) are known to cause nephrosclerosis. This view is supported by the observation that both the nephrosclerosis caused by desoxycorticosterone and that caused by the pituitary extracts is very effectively counteracted by the administration of high carbohydrate and low protein diets. It is much more difficult to understand why even the adrenotrophic effect of the pituitary extracts is diminished on such high carbohydrate rations.

Be this as it may, I think that the various papers and discussion remarks made at this meeting give some support to the view, which we tried to defend for some time, that there are several adrenotrophic hormones which differ in their physiologic actions. To summarize this rather important question, I think we may say the following:

There are certain corticoids which selectively influence gluconeogenesis, fat deposition in tissues (as shown by Dr. Kendall, during this conference), or mineral metabolism. In addition to these corticoids, we know that the adrenal cortex can also produce testoid hormones, similar in their physiologic effects to those produced by the testis itself. I would like to suggest that, in order to simplify the very complex terminology necessary to describe these pharmacologic actions of the cortical hormones, we designate them, respectively, as gluco-corticoids, lipo-corticoids, mineral-corticoids and testoids produced by the adrenal cortex. This terminology clearly indicates that the first three-mentioned effects imitate certain normal physiologic actions characteristic of the adrenal cortex, while the last-mentioned effect is especially typical of the testis. If this terminology of the adrenal cortical hormones be accepted, it would also give us convenient names for the corresponding trophic hormones of the pituitary, which are responsible for the fact that the adrenal cortex can selectively elaborate one or the other type of adrenal hormone. These latter pituitary principles could then simply be designated as: gluco-corticotrophic, lipo-corticotrophic, mineralo-corticotrophic, and testo-corticotrophic principles of the anterior lobe.

Finally, I would like to ask Dr. Kendall whether the lesions he observed in animals overdosed with corticoids resembled nephrosclerosis and periarteritis, that is to say, changes which we noted as a result of over-dosage with desoxycorticosterone? It would also be instructive to know whether the diets of the animals used by Dr. Kendall were high in sodium and protein, since in our experience, as we have said above, such rations favor the development of corticoid overdosage phenomena.

E. C. Kendall: Diet was not analyzed in either case for sodium; I don't think there was much difference between the two diets. Adding salt to the diet, not in this experiment but in some others, did not make very much difference as far as these hormones were concerned.

In answer to Dr. Selye: The kidneys were not examined, either, for changes in weight or structure. We did make a study of the lesions in the muscles; they appeared to be similar to those described by you and others which were produced by the administration of some of the hormones of the adrenal cortex. In our investigation death was probably due to heart failure rather than renal failure.

The Role of Hydrolytic Enzymes in Some of the Metabolic Activities of Steroid Hormones[1, 2]

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I. Protein Anabolic Properties of Androgens

In 1935 (20, 33) it was reported from our laboratory that a "male hormone" extract prepared from normal male urine produced a decrease in urinary nitrogen excretion and an increase in body weight of castrated male dogs. The fecal nitrogen excretion was not affected. The decrease in urinary nitrogen was accounted for by a parallel decrease in urea, which suggested that protein metabolism had been altered. The only other urinary nitrogen constituent to show any significant changes was creatine. This substance decreased during the period of androgen treatment and increased slightly or "rebounded" on cessation of treatment. The appearance of creatine in the urine of these dogs was demonstrated to be of dietary (beef heart) origin and not a metabolic disturbance as a result of castration. All of the above effects could be obtained after a single injection of the androgen preparation.

The energy metabolism of the dogs was not affected by single injections even though the protein metabolism was affected. If the injections were continued, there appeared, in time, a slight increase, 10%, in the energy metabolism of the "fat" dog but no change in that of a "thin" dog. Analysis of the calorie balance of the animals indicated that there was an increase in fat metabolism to compensate for the sparing of the protein.

It was thought at the time of the investigation that the effects obtained were due to the androgenic substances present in the urine extracts. The most potent substance known at that time was androsterone. While these experiments were in progress, a highly potent substance, testosterone, was

[1]The investigations herein reported were aided by grants from the Committee on Scientific Research of the American Medical Association, Josiah Macy Jr. Foundation, and Ciba Pharmaceutical Products Inc.

[2]The author is indebted to Dr. E. C. Kendall for the 11-dehydrocorticosterone; Dr. J. J. Pfiffner for the 17-hydroxy 11-dehydrocorticosterone; Drs. K. Dobriner and S. Liebermann for the etiocholanol-3(α), one-17; pregnanol-3(α), one-20 and allopregnanol-3(α), one-20; Dr. M. Ehrenstein, for the androstanediol-3(β), 5, dione-6; 17; and to Drs. E. Oppenheimer, C. R. Scholz and A. St. Andre of Ciba Pharmaceutical Products Inc. for the other steroids many of which were prepared specially for these studies by Dr. St. Andre.

synthesized and also isolated in a small amount from bull's testes. These discoveries were preceded by the synthesis of a substance, Δ^4-androstenedione-3, 17, which had androgenic and chemical properties intermediate between those of androsterone and testosterone. Therefore, this compound was prepared in our laboratory and studied for its protein anabolic properties (34). Later testosterone and its acetate became available and they were studied (26). The results obtained with these compounds were similar to those obtained with the urine extracts. In the experiments with Δ^4-androstenedione-3, 17 blood N.P.N. and urea levels were determined in order to eliminate the possibility that the decrease in urinary urea was a kidney threshold effect. In none of the experiments was there any increase in these blood constituents. Indeed there was a slight decrease. Also, the blood lipids were not changed (32).

An analysis of the data indicated that the maximum nitrogen retention was related to the size or protoplasmic mass of the dog. Thus the factor, maximum nitrogen retained per kilogram body weight per day, was devised.

The above observations were confirmed and extended to man by Kenyon and his associates (*cf.* 13) and have been the subject of numerous investigations by many laboratories (1, 27, 42).

II. Site of Deposition of New Tissue

Although there was no doubt that the accessory sex organs were increased in size during androgen administration, nevertheless this newly formed tissue could account for only a small fraction of the retained nitrogen. Therefore, other tissues must have been increased in size. The increase in body weight suggested greater muscle mass. Objective evidence of this supposition was provided by the observation (40) that castration decreased the size of the skeletal muscles of the male guinea pig to those of the female. Injection of testosterone propionate resulted in a gradual but general hypertrophy of the skeletal muscles. These experiments have been successfully repeated (15) and extended to other steroids.

Another tissue that is influenced by androgens is the kidney (*cf.* 14, 25, 27, 45), an effect that is most pronounced and uniform (25) in the mouse. Since the kidney is an important metabolic organ, changes in its size probably are a reflection of the metabolic demands placed upon the body as a result of treatment. Therefore, a study of the available steroids for both their renotrophic and androgenic properties might provide a valuable "screening process" for the selection of steroids with greater renotrophic (metabolic?) than androgenic properties (14). That such a compound or compounds might exist was suggested by the fact that the urinary androgenic

extracts contain marked protein anabolic properties yet they do not contain any compounds similar to testosterone. Furthermore, urine extract equivalent to only 0.9 mg. of testosterone was needed to produce maximum nitrogen retention in the castrated dogs. Thus, the material(s) present in urine is at least ten times as potent as testosterone in stimulating protein anabolism.

Since the most uniform response (25) is obtained in the kidneys of mice, these animals were selected for the experiments. Furthermore, instead of administering the steroids[2] by injection of oil solutions (25), they were implanted as pellets of uniform weight and size (14 ± 1 mg.). The amount of steroid absorbed was increased by implanting two or more pellets and decreased by mixing the compound with various proportions of cholesterol (46, 10, 11). The method of pellet implantation is not only more efficient but also more nearly simulates normal physiological conditions.

There is a very wide range in the rate of solubility among the pure compounds (Table I). The lowest rate of absorption is 0.1 to 0.2 mg./30 days

TABLE I

The Effect of Various Steroid Pellets on the Kidney, Seminal Vesicles and Prostate and Thymus of the Mouse

Steroid	No. of mice	Steroid absorbed	Change from Castrated Controls[1] Kidneys %[2]	Seminal vesicles + prostates %[2]	Thymus %[2]
		30-day Experiments			
Testosterone	9	8.3	108	2860	—91
Testosterone propionate[3]	11	4.4[11]	99	2700	—94
17-Methylandrostanediol-3(α), 17(α)	7	2.6[17]	98	1380	—76
17-Methyltestosterone[4]	6	8.5[12]	96	2700	—97
Androstanol-17(α), one-3	6	2.6	79	2390	
Androstanediol-3 (α), 17(α)	11	1.7	77	1245	—82
Normal Mice	16	——	61	1962	—42
17-Vinyltestosterone	8	8.1[13]	53	1100	—36
Testosterone acetate 3, prop-17	2	1.3[14]	42	1800	—79
Desoxycorticosterone acetate[5]	3	9.3	40	—9	—18
Δ[4]-Androstenedione-3, 17	6	10.6	32	1570	—88

[3]Recently we have noted a striking increase (68%) in the size of the kidneys of rats treated for 126 days with 2.5 mg. testosterone propionate (Perandren) per day. These rats were maintained on an adequate prepared diet.

TABLE I (continued)

Steroid	No. of mice	Steroid absorbed	Change from Castrated Controls[1] Kidneys %[2]	Seminal vesicles + prostates %[2]	thymus %[2]
17-Ethyltestosterone	9	5.1[15]	32	700	—24
17-Methylandrostanediol-3(β), 17(α)	5	0.4	29	—9	—12
17-Hydroxy, 11-dehydrocorticosterone[6]	2	15.1[16]	27	—9	—100
Androstanedione-3, 17	7	9.2	19	327	—58
Androsterone	6	3.7	18	54	—46
Androstanediol-3(α), 17(α), acetate-3	3	0.7[16]	17	—18	—24
17-Methyl Δ[5]-androstenediol-3(β), 17(α)	6	0.9	17	82	—9
α-Estradiol[7]	6	2.6	15	54	—70
17-Ethynylandrostanediol-3(β), 17(α)	5	0.5	13	—18	+9
Dehydroisoandrosterone	5	10.9	13	73	—24
17-Ethynyl Δ[5]-androstenediol-3(β), 17(α)	5	1.0	5	0	+3
17-Ethynyltestosterone[8]	6	0.5	5	0	+12
Androstanediol-3(β), 17(α)	6	0.2	5	18	—15
3, 17-Dimethyl Δ[3, 5] androstadienol-17(α)	6	2.6	4	—9	+9
Progesterone[9]	5	5.6	3	9	—15
Testosterone benzoate	6	0.2	2	27	—21
11-Dehydrocorticosterone[10]	4	17.6[16]	2	—45	—27
Androstanediol-3(β), 5, dione-6, 17	1	6.8	2	—45	+12
Isoandrosterone	8	9.3	1	18	—33
cis-Testosterone	4	3.6	1	18	—9
Δ[5]-Pregnenol-3(β), one-20	5	0.8	0	—9	+3
3-Methyl Δ-[3, 5] androstadienol-17(α)	6	5.7	—1	9	0
Androstenediol-3(β), 17(α)	7	0.7	—2	18	—24
Androstenediol-3(β), 17(β)	3	2.1	—3	—18	0
Allopregnanol-3(α), one-20	2	0.1	—5	0	—12
Androstanediol-3(α), 17(α), diacetate	5	0.2	—6	—18	—21
Etiocholanol-3(α), one-17	1	7.9	—8	+18	—6
Pregnanol-3(α), one-20	1	2.9	—16	—36	—18

TABLE I (continued)

Steroid	No. of mice	Steroid absorbed	Change from Castrated Controls[1] Kidneys %[2]	Seminal vesicles + prostates %[2]	thymus %[2]
		20-day Experiments			
Testosterone + Desoxycorticosterone	4	7.2 / 13.9	137	2410	—85
Testosterone	2	6.0	99	2260	—79
Desoxycorticosterone	4	12.0	24	—9	—18
Testosterone + Desoxycorticosterone	4	(3.4 (8.8	86	1270	—85
		10-day Experiments			
Testosterone	9	3.3	60	1130	—61
Testosterone + α-Estradiol	5	3.2 / 0.7	58	736	—61
17-Methyltestosterone	3	3.1	56	1090	—76
Testosterone propionate	5	1.7	55	1110	—79
Androstanediol-3(α), 17(α)•	4	0.8	46	527	—58
Androstanol-17(α), one-3	4	0.7	40	1060	—58
Desoxycorticosterone	4	10.2	22	91	—55
α-Estradiol	5	0.6	20	191	—49
11-Dehydrocorticosterone	5	12.5	40	—18	—97

(From Kochakian, C. D. *Am. J. Physiol.* **142**: 315 (1944)).

[1]The average values of 28 castrated mice: Kidneys 263 (231-292), seminal vesicles and prostates 11 (7-13), thymus 33 (23-43).

[2]Per cent of averages.

[3]Perandren.

[4]Metandren.

[5]Percorten.

[6]Pfiffner's compound F. (Kendall's compound E).

[7]Ovocylin.

[8]Lutocylol, anhydrohydroxyprogesterone, pregnenynolone.

[9]Lutocylin.

[10]Kendall's compound A.

[11]Equivalent to 3.7 mg. of testosterone.

[12]Equivalent to 8.1 mg. of testosterone.

[13]Equivalent to 7.5 mg. of testosterone.

[14]Equivalent to 0.94 mg. of testosterone.

[15]Equivalent to 4.7 mg. of testosterone.

[16]Only traces of the steroid remaining at autopsy (*cf.* Fig. 1).

[17]Equivalent to 2.5 mg. androstanediol-3(α), 17(α).

and the highest rate of absorption is demonstrated by the adrenal cortical compounds which are about 90% absorbed in only 10 days (Fig. 1).

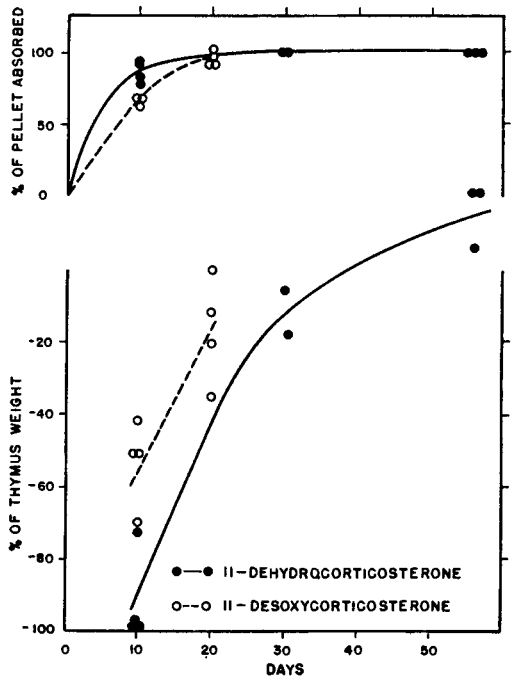


FIG. 1

Thymus Weight and Steroid Pellet Absorption.
From Kochakian, C. D. *Am. J. Physiology* **142**: 315 (1944)

Esterification markedly reduces the rate of absorption of the various steroids.

More than half of the compounds show both renotrophic and androgenic activity (Table I), and many of the apparently inactive steroids exhibit activity when a greater amount of the material is made available to the animal (Table II).

The increase in renotrophic activity is not related to androgenic activity (Table II, III; Fig. 2-9) but is related to chemical structure. The 17(α)-hydroxyl group is necessary for maximum activity of the molecule. The replacement of this group by a ketone (Δ^4-androstenedione-3, 17; androstanedione-3, 17) greatly reduces and the introduction of a 17(β)-hydroxyl (cis-testosterone) completely obliterates all activity.

The 3(β)-hydroxyl group also tends to decrease (isoandrosterone) or completely remove activity (androstanediol-3(β), 17(α)) but its effect is offset somewhat by the introduction of unsaturation (dehydroisoandrosterone) or a methyl group in the 17 position (17-methylandrostanediol-3(β), 17(α) and 17-methyl Δ^5-androstenediol-3(β), 17(α).

TABLE II

The Effect of Castration and Certain Steroids on the Kidney and Seminal Vesicles and Prostates of the Mouse

Treatment	No. of mice	No. of pellets implanted	Steroid absorbed mg./30 days	Increased wt.[2] Kidneys %	Kidneys per mM x 288[3]	Sem. Ves. & Pros. %	Sem. Ves. & Pros. per mM x 288[3]	Incr'd. Kid wt. Incr'd. S. V. & Pr. wt. mg./mg.
Normal mice	12	—	—	57	—	1920	—	0.74
Cholesterol	8	1 or 2	0.0[4]	(11)	—	(265)	—	—
Δ^4-Androstenedione-3, 17	2	2	19.7	60	8	1950	11	0.73
17-Methylandrostanediol-3(β), 17(α)	7	3	1.8	60	92	290	19	4.90
17-Methyl Δ^5-Androstenediol-3(β), 17(α)	4	4	2.4	32	37	400	15	2.47
Testosterone Benzoate	2	5	0.9	28	97	673	99	0.98
Androstanediol-3(β), 17(α)	4	4	2.0	27	30	0	0	0
17-Ethynyltestosterone[1]	2	3	1.8	—2	0	50	3	—

[1]Lutocylol, anhydrohydroxyprogesterone, pregnenynolone.
[2]Change from values of castrated control (cholesterol treated) mice.
[3]The millimoles of each steroid have been multiplied by the molecular weight, 288, of testosterone.
[4]Range of values ± 0.15 mg.

TABLE III
The Renotrophic-Androgenic Ratio of Various Steroids

Steroid	Increased kidney weight/increased seminal vesicle and prostate weight		
	Duration of Experiment		
	30 days	20 days	10 days
17-Methylandrostanediol-3(α), 17(α)	1.70	—	—
Androstanediol-3(α), 17(α)	1.48	—	2.09
Testosterone + α-Estradiol	—	—	1.89
Testosterone + Desoxycorticosterone	—	1.36	1.61
17-Vinyltestosterone	1.14	—	—
17-Ethyltestosterone	1.09	—	—
Testosterone	0.90	1.05	1.27
Testosterone propionate	0.88	—	1.20
17-Methyltestosterone	0.85	—	1.23
Androstanol-17(α), one-3	0.79	—	0.90
NORMALS	0.74		
Testosterone acetate-3, propionate-17	0.55	—	—
Δ^4-Androstenedione-3, 17	0.49		

From Kochakian, C. D. *Am. J. Physiol.* **142**: 315 (1944).

Of special significance is the decrease in androgenic activity without a comparable decrease in renotrophic activity. This effect is attained by the saturated 3(α), 17(α) diols (Tables I, II, III and Figs. 2-9) (*cf.* 3) and also by the implantation of a pellet of either α-estradiol or 11-desoxycorticosterone (Table I, II) simultaneously with testosterone.

The methyl group has a variable effect on the activity of the various steroids. It neither enhances nor decreases the activity of testosterone, but it definitely increases the activity of androstanediol-3(β), 17(α) and androstenediol-3(β), 17(α) when it is present on the 17 carbon atom.

In contrast to the 17-methyl group, the 17-ethyl group greatly decreases the potency of testosterone. The 17-vinyl group also decreases the activity but to a lesser degree than the ethyl group and the 17-methyl group completely removes all activity. The decrease in activity may be due in part to the lower rate of absorption as a result of addition of these groups but some of the change must be due to the chemical nature of the alkyl groups. There is absorbed almost as much 17-vinyl testosterone as testosterone or 17-methyl testosterone and about twice as much as testosterone propionate. Also the amount of 17-ethyltestosterone absorbed, though less than that of testosterone or 17-methyltestosterone, is greater than that of testosterone propionate.

Testosterone, testosterone propionate, and 17-methyltestosterone exhibit the greatest effect on both kidney and seminal vesicles and prostate weight. Esterification of testosterone results in a greater efficacy of the material, *e.g.*, testosterone propionate, testosterone acetate-3, propionate-17; but when the procedure results in too great a decrease in the rate of absorption

of the hormone, *e.g.*, testosterone benzoate, then the physiological activity also is no longer apparent.

The hydroxy ketosteroids known to be present in urine show no or slight activity in both renotrophic and androgenic activities. Experiments with a crystalline fraction containing a mixture of all of the α-hydroxy ketosteroids present in normal male urine also did not exhibit any activity. The failure of this mixture of steroids to show an appreciable renotrophic or androgenic effect suggests that the highly potent protein anabolic property of "androgenic" urine extracts is not due to ketosteroids but probably to the α-hydroxy non-ketonic fraction which composes a large part, 42%, of the urine preparations (30). Furthermore, not only are the known hydroxy ketones of urine feeble in their androgenic and renotrophic properties but also at least two of them, androsterone and dehydroisoandrosterone, possess no or very little protein anabolic properties (*cf.* 42).

All of the adrenal cortical steroids show variable but significant renotrophic activity. The failure of 11-dehydrocorticosterone to show any renotrophic activity in the 30-day tests probably is due to the rapid rate of absorption of this material. It is practically all absorbed in 10-20 days. A similar effect is noted with 11-desoxycorticosterone; this compound produces a small but definite increase of the sex organs and a decrease in the size of the thymus for the 10-day but not for the 20-day experiment (Table I).

Neither progesterone nor its related compounds including the synthetic product, anhydrohydroxyprogesterone (17-ethynyltestosterone) influence any of the organs studied.

The one estrogen, α-estradiol, investigated showed significant effects on both the kidneys and the seminal vesicles and prostates; but when combined with testosterone, it decreased the androgenic activity but did not affect the renotrophic activity of this compound.

It is pertinent that the increase in kidney size varies with the nature of the metabolic demands imposed upon the organism by the steroids. The greatest increases in kidney size are obtained with those compounds that have protein anabolic properties (42). On the other hand, only small and variable increases in the size of the kidney result after the administration of the steroids that stimulate protein catabolism. The effect of 11-desoxycorticosterone on kidney size has been correlated with mineral metabolism (6).

Many of the steroids were studied at various dose levels (Figs. 2-9). Testosterone, testosterone propionate, 17-methyltestosterone and androstanol-17(α), one-3 (Figs. 2-5) produce as rapid and as great an effect on the seminal vesicles and prostates as on the kidneys, but the diols (Figs. 6-9, Table II) produce a much slower and smaller effect on the accessory

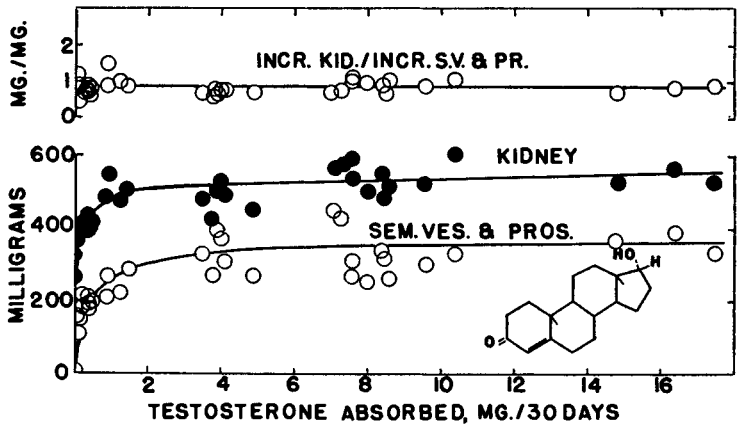


FIG. 2

A Comparison of the Renotrophic and Androgenic Activities in Castrated Mice of Testosterone at Different Doses.

The androgenic and renotrophic values for castrated mice, zero dose, represent the averages of 8 mice. (*cf.* Table II). From Kochakian, C. D. *Am. J. Physiol.* **145**: 549 (1946).

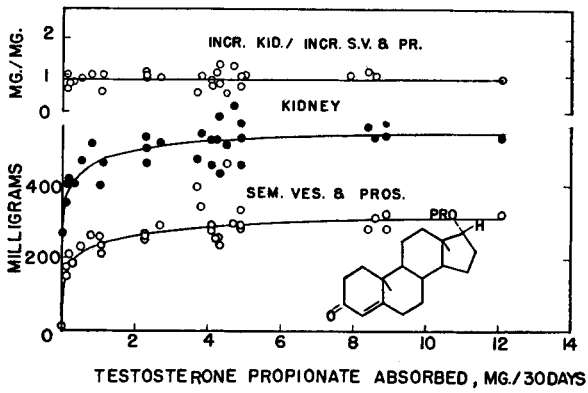


FIG. 3

A Comparison of the Renotrophic and Androgenic Activities in Castrated Mice of Testosterone Propionate at Different Doses.

sex organs than the kidneys, especially in the lower more efficacious doses (Figs. 8, 9). Once the maximum rate of increase of the organs has been attained further increase in dose produces only a slight increase in size of the organs. The height of the maximum response, however, is not dependent entirely on the dose but also on the duration of treatment. The 10-day experiments show a lower maximum, and continued treatment gives a higher maximum (18).

When the increase in kidney weight is divided by the increase in seminal vesicles and prostate weight (the renotrophic-androgenic ratio), testo-

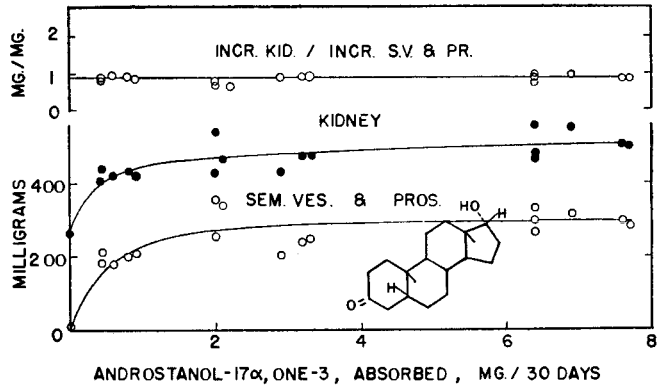


FIG. 4

A Comparison of the Renotrophic and Androgenic Activities in Castrated Mice of Androstanol-17(α), one-3 at Different Doses.

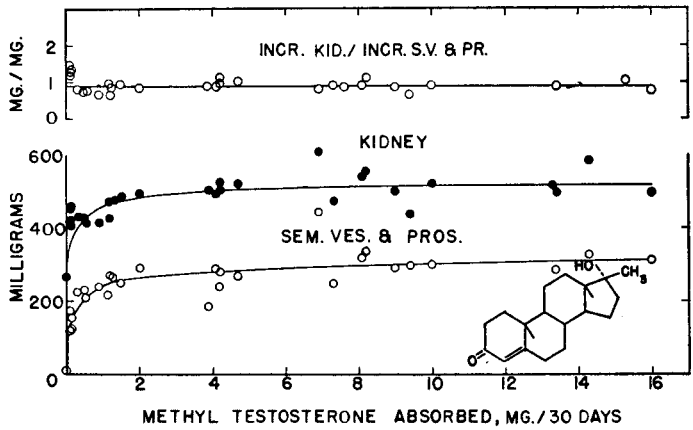


FIG. 5

A Comparison of the Renotrophic and Androgenic Activities in Castrated Mice of Methyltestosterone at Different Doses.

sterone and its derivatives (Figs. 2-4) and androstanol-17(α), one-3 (Fig. 5), produce a ratio slightly less than one which is the same over the dose range used. The diols (Figs. 6-9, Table II), on the other hand, produce a ratio much greater than one which gradually decreases with increase in dose.

The renotrophic efficacies of testosterone; testosterone propionate; 17-methyl testosterone; androstanol-17(α), one-3; 17 methyl androstanediol-3 (α), 17(α), and androstanediol-3(α), 17(α) not only are the same when compared on a mole basis but also show the same rapid decrease in efficacy with increase in dosage. The other compounds with the possible exception

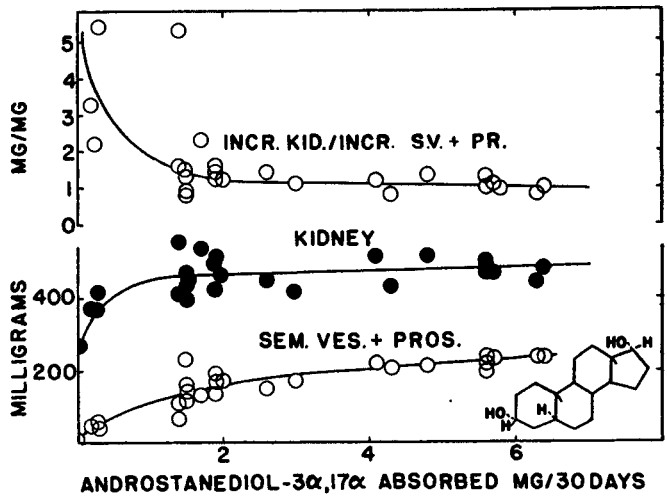


FIG. 6

A Comparison of the Renotrophic and Androgenic Activities in Castrated Mice of Androstanediol-3(α), 17(α) at Different Doses.
From Kochakian, C. D. *Am. J. Physiol.* **145**: 549 (1946).

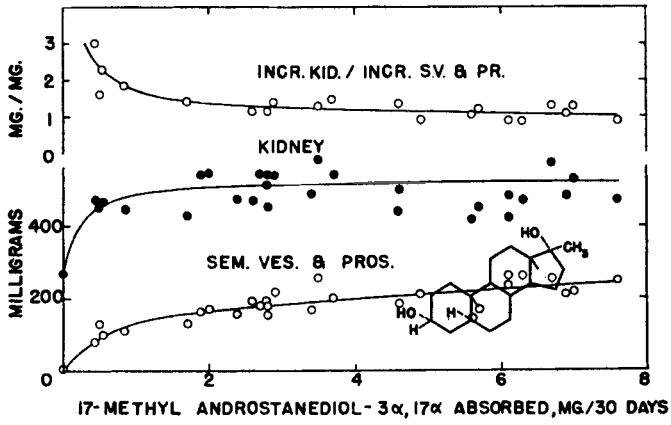


FIG. 7

A Comparison of the Renotrophic and Androgenic Activities in Castrated Mice of 17-Methylandrostanediol-3(α), 17(α) at Different Doses.

of 17-methylandrostanediol-3(β), 17(α) which is only slightly less active than the above steroids, show varying degrees of lower efficacy (Table II, Fig. 10).

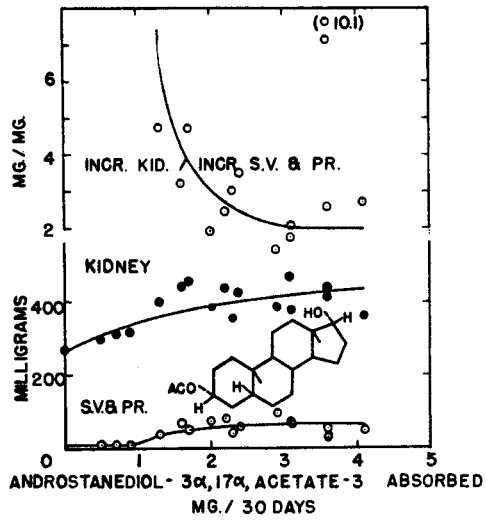


FIG. 8

A Comparison of the Renotrophic and Androgenic Activities in Castrated Mice of Androstanediol-3(α), 17(α), Acetate-3 at Different Doses.

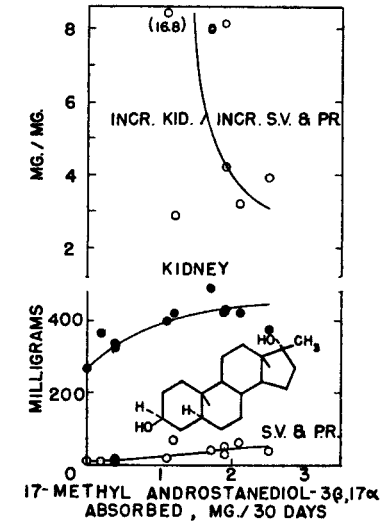


FIG. 9

A Comparison of the Renotrophic and Androgenic Activities in Castrated Mice of 17-Methylandrostanediol-3(α), 17(α) at Different Doses.

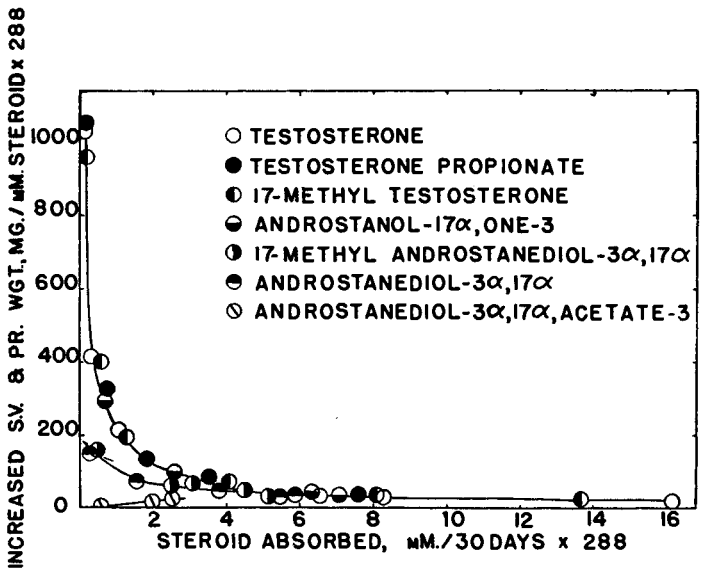


FIG. 10

The Renotrophic Efficacy in Castrated Mice of Seven Steroids at Different Doses. From Kochakian, C. D. *Am. J. Physiol.* **145**: 549 (1946).

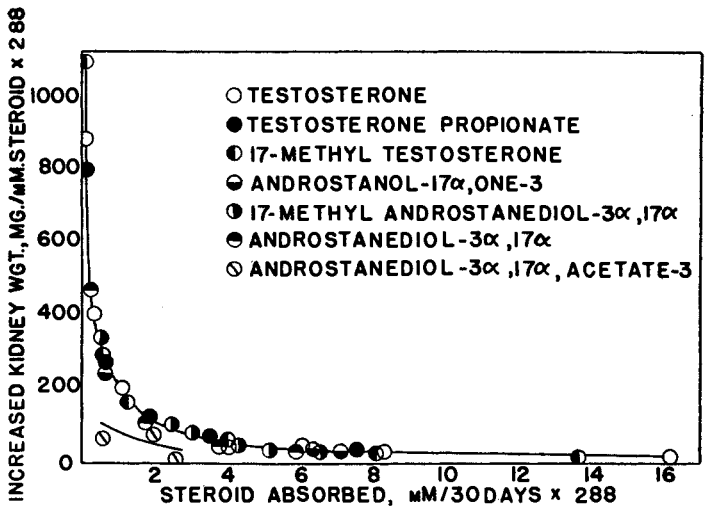


FIG. 11

The Androgenic Efficacy in Castrated Mice of Seven Steroids at Different Doses. From Kochakian, C. D. *Am. J. Physiol.* **145**: 549 (1946).

Testosterone; testosterone propionate; 17-methyltestosterone and androstanol-17(α), one-3 possess the same androgenic activity when compared on a weight equivalent basis (Fig. 11). The diols on the other hand are much less efficacious at the lower levels but have the same efficacy when excessive amounts of steroid are made available. A further decrease and even an inhibition of androgenic activity is obtained by acetylation (Table II, Fig. 8) or the substitution of a 3-(β) hydroxyl group for the 3-(α) hydroxyl group (Table II).

At one time in the course of this investigation the mice did not receive adequate care due to technical difficulties, consequently they were in a state of undernutrition as indicated by the body weights. As a result there was a marked decrease in the weight of the kidneys and seminal vesicles and prostates of the normal mice but only a relatively small decrease in these organs of the castrated mice (Table IV). The decrease in size of the

TABLE IV
Effect of the Nutritive State on the Body, Kidney and Seminal Vesicles and Prostate Weghts

Nutritive State	Mice	No. of mice	Fasting body wt.	Sem. ves. + pros. mg.	Kidney mg.
N[1]	Normal	12	22.5	222	414
U[2]	Normal	3	13.9	69	222
N	Castrated	8	20.7	11	265
U	Castrated	6	16.1	8	235

From Kochakian, C. D. *Am. J. Physiol.* **145**: 549, (1945).
[1]N = Normal nutrition.
[2]U = Undernutrition.

organs of the normal mice is very likely due to the loss of their endocrine stimulus through "inanition hypophysectomy." These same organs in the castrated mice lose relatively small amounts of tissue mass because they have already regressed to a basal level as a result of surgical removal of their endocrine stimulus. When, however, the stimulus for growth of both of these organs is restored by the administration of pellets of the various steroids (Table V), there is a marked difference in the response of the two organs in the underfed castrated mice. The seminal vesicles and prostates increase in size to as great an extent as in the well-fed animals. The kidneys, on the other hand, show a very greatly decreased response. Thus the increase in size of these two organs under steroid stimulation is for entirely different purposes. The accessory sex organs increase in size for their own special functions and at the expense of other tissues of the body which are probably broken down to provide the necessary materials (*cf.* 41).

TABLE V

Effect of the Nutritive State on the Response of the Kidney and the Seminal Vesicles and Prostates to Steroid Stimulation

Nutritive State	Treatment	No. of mice	Fasting body weight g.	Steroid absorbed mg./30 days	Increased wt.[5] S. V. + Pr. mg.	Kidney mg.
N[1]	Testosterone	4	20.8	0.37	189	154
U[2]	"	1	16.4	0.50	188	87
N	"	4	22.4	1.15	243	242
U	"	1	17.2	1.20	272	128
N	"	7	20.6	4.34	300	203
U	"	5	17.1	4.40	214	122
N	"	3	22.4	16.20	354	278
U	"	1	18.7	17.80	313	196
N	Testosterone[3] propionate	5	21.6	2.33	257	227
U	"	4	16.3	2.67	207	97
N	17-Methyltesto-sterone[4]	4	23.3	0.14	128	152
U	"	1	18.0	0.12	122	64
N	"	6	22.8	4.26	263	244
U	"	3	17.3	3.54	232	144
N	17-Methylandro-stanediol-3(α), 17(α)	5	22.5	3.20	197	254
U	"	2	16.0	3.80	168	67
N	Androstanediol-3(α), 17(α)	3	22.1	0.25	36	120
U	"	1	18.9	0.30	36	17
N	Androstanediol-3(α), 17(α), Acetate-3	12	21.9	2.50	48	151
U	"	4	14.7	3.20	45	60

From Kochakian, C. D. *Am. J. Physiol.* **145**: 549, (1945).

[1]N = "Normal" nutrition

[2]U = Undernutrition.

[3]Perandren.

[4]Metandren.

[5]Change from castrated control (cholesterol treated) mice, *cf.* Table II.

The kidney, on the other hand, increases in size to store reserve protein which it synthesizes under the stimulus of the particular steroid hormones. In contrast to the accessory sex organs, however, the kidney during inanition gives up the newly fabricated material. Thus the kidney may be considered as an intermediary in some phase of protein fabrication.

None of the steroids affected the weight of the liver or intestine of the mice. The more potent steroids, however, had a marked effect on body weight (Table VI, Fig. 12).

III. Enzymes Influenced by Steroid Hormones

Since many of these steroids influence growth processes, it seemed that a study of various enzymes would provide information in the mechanism of their action.

TABLE VI

The Effect of Steroids on Growth of the Castrated Mouse Treated for 30 Days[1]

Steroid	Mice	Change in body weight g.	Change in body weight %[2]
Desoxycorticosterone acetate	3	5.6	230
Androstanol-17(α), one-3	6	4.8	182
17-Methyltestosterone	6	4.8	182
Testosterone acetate-3, propionate-17	2	4.8	182
Androstanediol-3(α), 17(α)	11	4.5	165
Testosterone	9	4.4	159
Dehydroisoandrosterone	5	4.4	159
17-Vinyltestosterone	8	4.4	159
Testosterone propionate	11	4.3	153
17-Methylandrostanediol-3(α), 17(α)	7	4.1	141
Testosterone + isoandrosterone	4	4.0	135
Androstanedione-3, 17	7	3.9	129
Δ^4-Androstenedione-3, 17	6	3.9	129
Androstanediol-3(α), 17(α), acetate-3	3	3.5	106
Testosterone Propionate (2 pellets)	5	3.4	100
17-Ethyltestosterone	9	3.4	100
Androstanediol-3(β), 17(α)	6	3.4	100
17-Methylandrostanediol-3(β), 17(α)	5	3.0	76
17-Ethynylandrostanediol-3(β), 17(α)	5	2.9	70
Isoandrosterone	8	2.8	65
Androstanediol-3(β), 5, dione-6, 17	1	2.6	53
17-Ethynyl Δ^5-androstenediol-3(β), 17(α)	5	2.5	47
Δ^5-Androstenediol-3(β), 17(α)	7	2.5	47
17-Methyl Δ^5-androstenediol-3(β), 17(α)	6	2.4	41
3, 17-Dimethyl $\Delta^{3,5}$-androstadienol-17(α)	6	2.4	41
Androsterone	6	2.3	35
Δ^5-Androstenediol-3(β), 17(α)	3	2.3	35
Testosterone Benzoate	6	2.2	29
Progesterone	5	2.1	24
17-Ethynyltestosterone	6	2.0	18
3-Methyl $\Delta^{3,5}$-androstadienol-17(α)	6	2.0	18
Cistestosterone	4	1.9	12
Allopregnanol-3(α), one-20	2	1.9	12
Etiocholanol-3(α), one-17	1	1.8	6
Pregnanol-5, trione-3, 6, 20	1	1.7	0.0
Castrate	22	1.7[3]	—
Δ^5-Pregnenol-3(β), one-20	5	1.2	—29
Pregnanol-3(α), one-20	1	1.2	—29
α-Estradiol	6	0.1	—94
17-Hydroxy, 11-dehydrocorticosterone	2	—3.2	—288

(Kochakian, C. D. Unpublished).

[1]See Table I.

[2]Difference from castrated controls.

[3] $\sqrt{\frac{\Sigma d^2}{N}} = \pm 0.53$

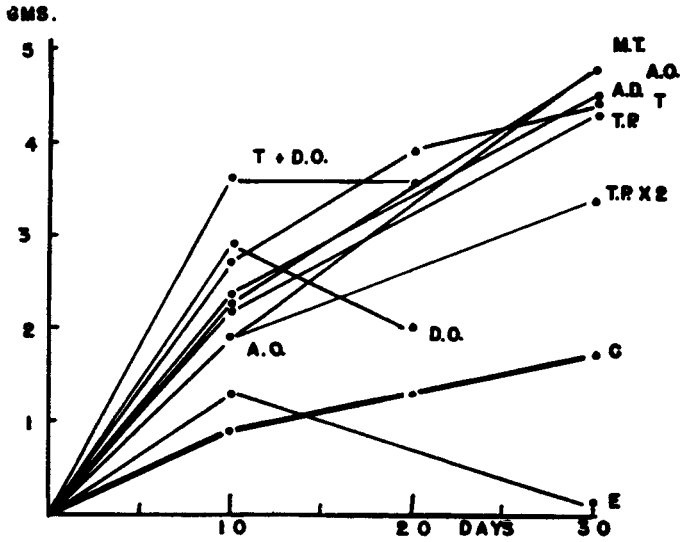


FIG. 12

Effect of Duration of Treatment on the Response of the Body Weight of the Mouse to Various Steroids.

Testosterone plus Desoxycorticosterone (T. + D.O.) as separate pellets; 17-methyltestosterone (M.T.); Androstanol-17(α), one-3 (A.O.), Androstanediol-3(α), 17 (α) (A.D.); Testosterone (T.); Testosterone propionate (T.P.); Two separate pellets of Testosterone propionate (T.P. x 2); Desoxycorticosterone (D.O.); Castrated (control) mice (C.); and α-Estradiol (E.).

1. *Phosphatases*

Testosterone propionate decreases the "alkaline" (pH 9.8) and increases the "acid" (pH 5.4)[4] phosphatase of the kidneys of normal and castrated

TABLE VII

The Effect of Various Steroids on the "Alkaline" and "Acid" Phosphatases of the Kidney of the Mouse

Treatment	No. of mice	Steroid absorbed mg.	Kidney mg.	"Alkaline" Phosphatase Total % change[1]	"Alkaline" Phosphatase Per gram % change[1]	"Acid" Phosphatase Total % change[1]	"Acid" Phosphatase Per gram % change[1]
Normal	12	—	414	—4	—42	+85	+13
Testosterone	4	0.14	389	—10	—41	+62	+9
"	4	0.37	417	—38	—58	+69	+6
"	4	1.15	505	—53	—75	+100	+6
"	7	4.06	469	—49	—50	+96	+8
"	1	8.5	477	—58	—77	+92	+7
"	3	16.2	541	—59	—81	+148	+13

[4]The pH 4.9 recommended by Robinson and Gutman (44) as the optimum for blood was used in our initial study but we found that the optimum for tissue is pH 5.4.

TABLE VII (continued)

The Effect of Various Steroids on the "Alkaline" and "Acid" Phosphatases of the Kidney of the Mouse

Treatment	No. of mice	Steroid absorbed mg.	Kidney mg.	"Alkaline" Phosphatase Total % change[1]	"Alkaline" Phosphatase Per gram % change[1]	"Acid" Phosphatase[a] Total % change[1]	"Acid" Phosphatase[a] Per gram % change[1]
Testosterone propionate[2]	4	0.20	392	—28	—52	+38	—9
"	4	0.88	458	—48	—71	+77	+1
"	5	2.33	490	—35	—70	+96	+2
"	1	4.10	446	—75	—85	+85	+8
17-Methyl-testosterone[3]	4	0.14	415	—6	—44	+77	+5
"	4	0.57	424	—37	—62	+77	+9
"	4	1.30	466	—58	—76	+92	+6
"	6	4.26	507	—55	—77	+92	—2
"	1	8.10	540	—62	—82	+123	+7
"	4	14.40	525	—65	—83	+119	+8
17-Ethynyl-testosterone[4]	2	1.75	257	—13	—14	+4	+1
Androstanol-17(α), one-3	5	0.65	424	—36	—61	+42	—14
"	2	6.4	518	—54	—77	+88	—1
"	4	7.1	505	—62	—80	+88	—2
Δ^4-Androstene-dione-3, 17	2	19.7	420	—46	—66	+62	+1
Androstane-diol-3(α), 17(α)	3	0.25	383	—14	—37	+15	—18
"	1	1.5	470	—30	—60	+96	+9
"	4	3.8	472	—52	—73	+73	—6
"	4	5.5	464	—55	—75	+89	+4
"	3	5.9	466	—64	—80	+127	+25
Androstanediol-3(α), 17(α), Acetate-3	6	2.3	421	—13	—47	+65	+1
"	8	3.0	399	—41	—62	+46	—6
Androstane-diol-3(β), 17(α)	4	2.0	333	—14	—30	—4	—23
17-Methylandro-stanediol-3(α),17(α)	4	0.6	459	—53	—73	+65	—7
"	5	3.2	517	—53	—77	+81	—8
"	6	5.5	448	—62	—78	+58	—8
"	5	6.9	507	—65	—82	+135	+21
17-Methylandro-stanediol-3(β),17(α)	7	1.8	420	—27	—55	+65	+3
17-Methyl Δ^5-Andro-stenediol-3(β), 17(α)	4	2.4	347	+13	—18	+39	+3
Δ^5-Pregnenol-3(β), one-20	1	4.6	305	+18	0	+19	+3

From Kochakian, C. D. *Am. J. Physiol.* **145**: 118 (1945).

[1]Change from average values of 8 castrated mice.

"Alkaline" phosphatase: total units = 118, units/gram = 456.

"Acid" phosphatase: total units 2.6, units/gram = 10.0.

[2]Perandren.

[3]Metandren.

[4]Lutocylol, anhydrohydroxyprogesterone, pregnenynolone.

mice (16). These enzymes of the liver and intestine are not affected. Therefore, the latter two tissues were not studied in the following experiments (Table VII, Fig. 13) (31).

Castration does not affect the total amount of "alkaline" phosphatase but increases the amount per gram of kidney. On the other hand there is a decrease in the "acid" phosphatase which is approximately proportional to the decrease in kidney size.

All of the steroids that increase the size of the kidney decrease the "alkaline" phosphatase but increase the "acid" phosphatase. In both instances the changes are related to the change in kidney size as shown for testosterone in Fig. 13.

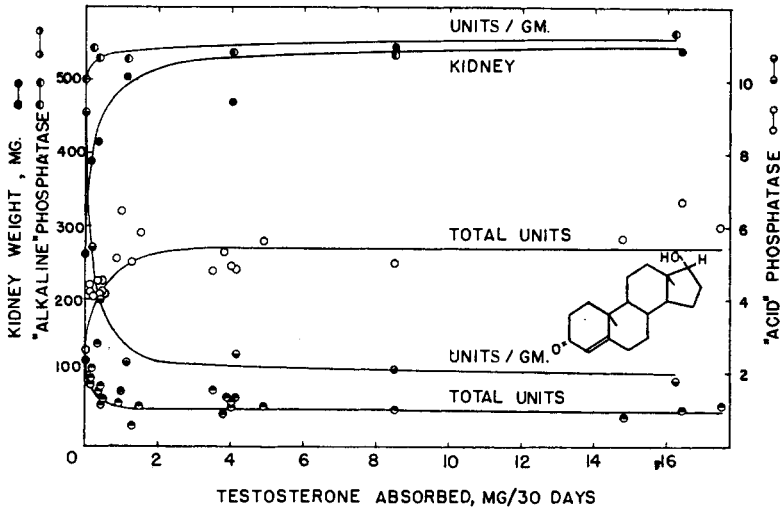


FIG. 13

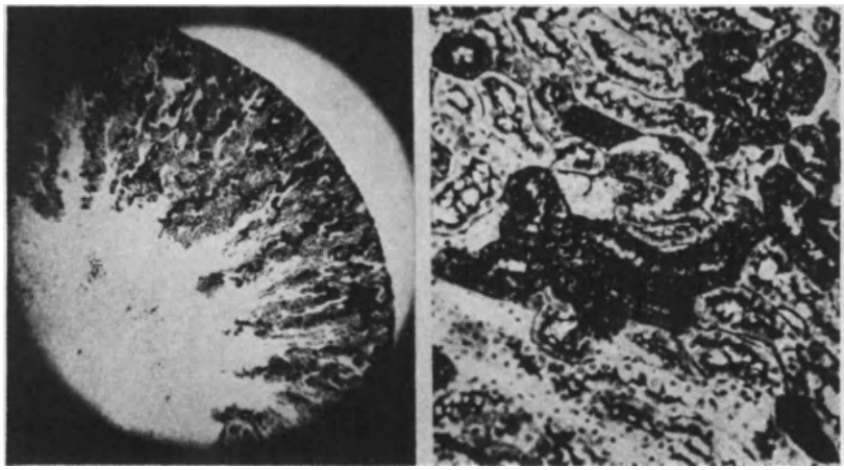
The Effect of Testosterone on the "Alkaline" and "Acid" Phosphatases of the Kidney of the Mouse.

The values for the total units of both enzymes are plotted separately but the values for the castrated mice, the units/g. of kidney and the kidney weights are plotted as their respective averages.

From Kochakian, C. D. *Am. J. Physiol.* **145**: 118 (1945).

The nature of these changes in the cells of the kidney have been studied[5] for "alkaline" phosphatase by the histochemical technic of Gomori (12) as modified in our laboratory (29). The normal mouse kidney (Fig. 14A, B) in agreement with previous reports (12) shows "alkaline" phosphatase in the cells of the proximal convoluted but not the distal tubules. It is present at

[5]Dr. V. Emmel of the Anatomy Department assisted in the "reading" of the prepared histological sections.

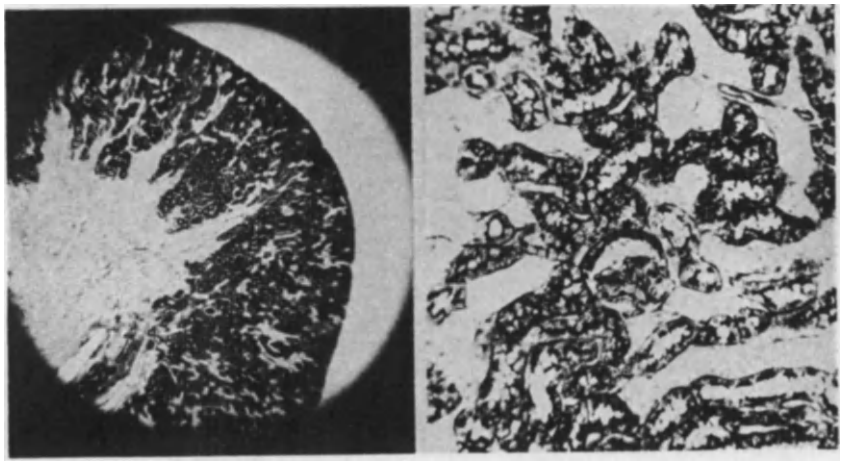


A (25 x) B (200 x)

FIG. 14

The "Alkaline" Phosphatase of the Kidney of a Normal Mouse. Kidney Weight: 419 mg.; "Alkaline" Phosphatase. 195 units/g.

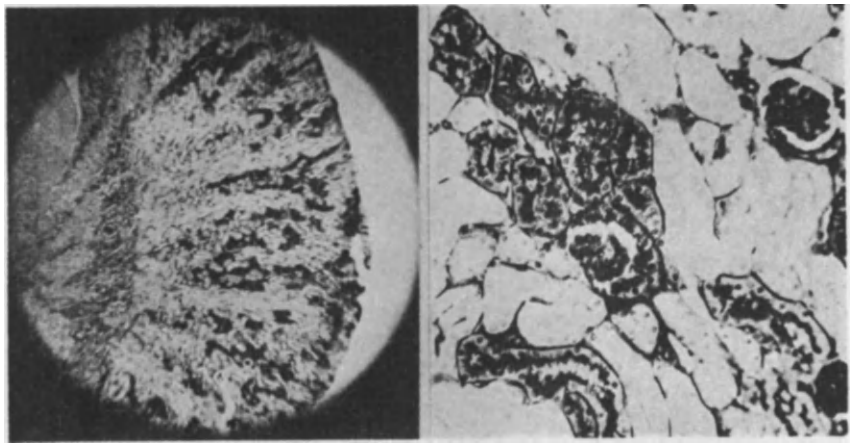
the turn of Henle's loop and in the capsule about the glomerulus. There is no enzyme in the medullary rays. In the castrated mouse the cells of the



A (25 x) B (200 x)

FIG. 15

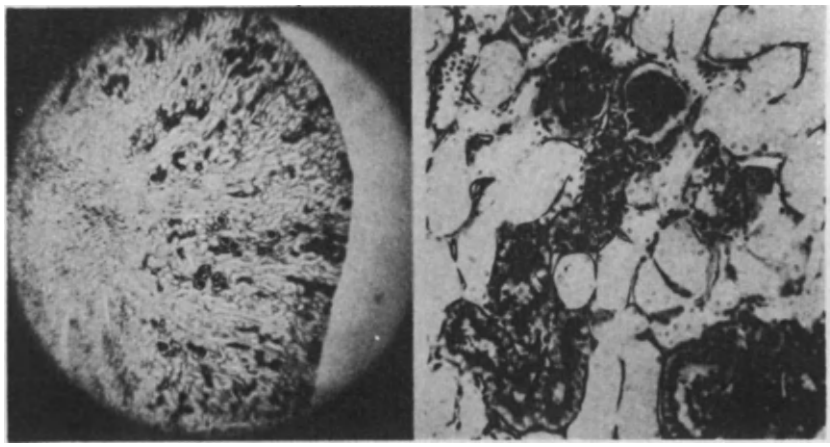
The "Alkaline" Phosphatase of the Kidney of a Castrated Mouse. Kidney Weight: 280 mg.; "Alkaline" Phosphatase: 414 units/g.



A (25 x) B (200 x)

FIG. 16

The "Alkaline" Phosphatase of the Kidney of a Testosterone-Treated Mouse.
Kidney Weight: 477 mg.; "Alkaline" Phosphatase: 57 units/g.



A (25 x) B (200 x)

FIG. 17

The "Alkaline" Phosphatase of the Kidney of a 17-Methyl Androstanediol-3(α), 17(α)-Treated Mouse.
Kidney Weight: 482 mg.; "Alkaline" Phosphatase: 63 units/g.

kidney decrease in size (*cf.* 45) with a resulting greater concentration of the enzyme in the cells (Fig. 15A, B). When the kidney is stimulated to increase in size, as for example after testosterone (Fig. 16A, B) or 17-methylandrostanediol-3(α), 17(α) (Fig. 17A, B) treatment, there is an

apparent "washing out" of the "alkaline" phosphatase in the distal end of the proximal convoluted tubules with a somewhat greater concentration in the portion nearest the glomerulus. In addition, the cells have greatly increased in size.

The similarity of the action of all the steroids studied indicates that these hormones have a similar effect on the metabolic processes influenced by these two phosphatases.

The very great decrease of the "alkaline" phosphatase both total and per gram of tissue amounts is due not only to the formation of greater amounts of non-"alkaline" phosphatase tissue but also by a decrease in the amount present in the distal portion of the proximal convoluted tubules which is only partially compensated for by a greater amount of the enzyme in the cells nearest the glomeruli. This shift in the concentration of the enzyme suggests that the kidney is striving to recover substances (phosphates ?) as quickly as possible from the glomerular filtrate.

The "acid" phosphatase, on the other hand, increases as the kidney increases in size (31).

2. *Arginase*

Since arginase is assumed to be concerned with urea formation (37, 36 but *cf.* 2), a study (17, 19) of this enzyme should provide some information concerning the mechanism of the protein metabolic activities of steroid hormones.

The liver of the mouse contains the greatest amount of arginase. The other tissues contain relatively little or no enzyme[5] (Table VIII).

The arginase activity of the liver and intestines are not affected by

TABLE VIII
Arginase Content of Various Tissues of the Mouse

Tissue	Arginase		
	Units/g.	Standard[5] deviation	Ratio
Liver[1]	11,200	±2500	100.00
Intestine[1]	730	±85	6.52
Kidney[1]	33	±6.8	0.30
Seminal vesicles and prostate[1]	5	±0.9	0.04
Testes (Immature)[2, 4]	0	—	0.00
Testes (Mature)[3, 4]	0	—	0.00

From Kochakian, C. D. *J. Biol. Chem.* **155**: 579 (1944).
[1]Average values from 11 mice of 110 ± days old.
[2]Pooled testes of 6 mice, 30-45 days old.
[3]Determination made on 4 mice, 110-120 days old.
[4] $\sqrt{\Sigma d^2/N-1}$
[5]The lack of this enzyme in the testes of both immature and mature mice is of interest because spermatozoa are rich in arginine.

either castration or steroid stimulation. The enzyme content of the kidney, on the other hand, undergoes marked changes especially after stimulation by certain steroid hormones (Table IX). Castration produces an increase in enzyme per gram of tissue but not in total amount. Thus the increase after castration is due to the decrease in kidney mass.

Most of the steroids which increase the size of the kidney also increase the amount of arginase. There are several instances, however, of definite and considerable increases in kidney size accompanied by a decrease in the arginase of the tissue. It seems, therefore, that certain steroids bring about a decrease while others increase the arginase of the kidney without any correlation in size of the organ. This, however, is not entirely true because dose, rate of increase in weight of the kidney, as well as chemical structure of the steroids, influence the changes in arginase activity (19). Testosterone; testosterone propionate; androstanol-17(α), one-3, and androstanediol-3(α), 17(α) (Figs. 18-21) decrease the arginase activity at the lower dose levels.

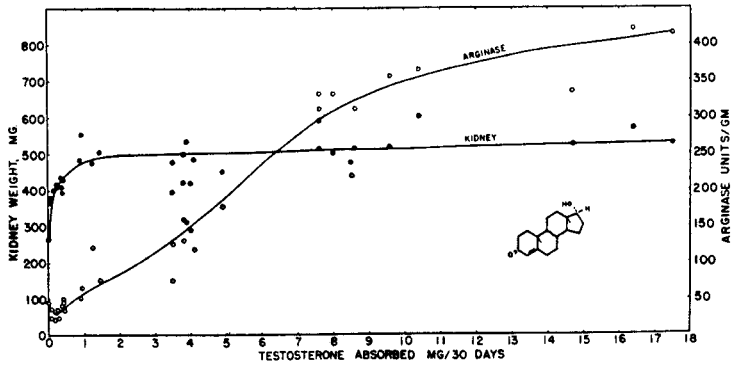


FIG. 18

The Effect of the Dose of Testosterone on the Arginase Content of the Kidney of the Castrated Mouse.

The kidney of the normal mouse weighs 414 mg. and has 27 arginase units/g. From Kochakian, C. D. *J. Biol. Chem.* **161**: 115 (1945).

When the dose is increased so that the kidney increases to greater than normal size, there is an increase in arginase activity which continues to increase even though there is no further significant increase in kidney size. Furthermore, if the duration of treatment is limited to 10 days (Fig. 22), there is an increase in arginase activity even before maximal increase in kidney size is attained. Thus it would seem that the change in arginase activity is indicative of the rate at which the cells are being stimulated and the size of the organ is not a complete reflection of its cellular metabolic processes.

The presence of a 17-methyl group in the very active steroids, *e.g.*, 17-

TABLE IX

Changes in the Arginase Activity of the Kidneys of Castrated Mice Treated for Thirty Days with Pellets of Various Steroids

Steroid	Mice	Steroid abs'd. mg.	Kidney mg.	Arginase Total Units	Total % Diff.[1]	Per g. Units	Per g. % Diff.[1]
17-Methyltestosterone	5	8.8	498	179	1278	359	632
Testosterone	7	8.3	539	182	1300	335	584
Testosterone propionate	6	4.4	501	99	666	200	308
17-Methylandrostanediol-3(α), 17(α)	7	2.6	521	94	624	181	269
Androstanol-17(α), one-3	4	2.9	454	57	338	115	135
α-Estradiol	5	2.7	316	28	116	92	88
Androstanediol-3(α), 17(α)	5	1.6	451	39	200	84	71
17-Vinyltestosterone	8	8.1	401	31	138	76	55
Testosterone acetate-3, propionate-17	2	1.3	374	25	92	66	35
Dehydroisoandrosterone	4	10.8	295	17	31	58	18
Δ^5-Androstenediol-3(β), 17(α)	4	0.6	249	14	8	53	8
Androsterone	3	3.8	301	16	23	52	6
Cistestosterone	2	3.6	257	14	8	52	6
Androstanedione-3, 17	4	8.6	303	14	8	49	0
Castrate	19	—	257	13[2]	—	49[3]	—
Pregnenol-3(β), one-20	5	0.7	263	13	0	48	—2
3, 17-Dimethyl $\Delta^{3,5}$-androstadienol-17(α)	5	2.8	274	13	0	48	—2
3-Methyl $\Delta^{3,5}$-androstadienol-17(α)	5	6.0	264	13	0	47	—4
Androstanediol-3(β), 17(α)	3	0.2	287	14	8	46	—6
Δ^4-Androstenedione-3, 17	4	10.3	344	15	12	43	—12
17-Ethynyltestosterone	6	0.5	275	11	—15	42	—14
Progesterone	5	5.6	273	12	—8	42	—14
17-Ethynyl Δ^5-androstenediol-3(β), 17(α)	5	1.0	292	13	0	42	—14
Testosterone benzoate	4	0.3	268	11	—15	41	—16
Allopregnanol-3(α), one-20	2	0.1	249	10	—23	41	—16
Etiocholanol-3(α), one-17	1	7.9	242	10	—23	38	—22
Pregnanol-3(α), one-20	2	2.6	260	10	—23	37	—24
17-Ethyltestosterone	9	5.1	346	13	0	36	—26
17-Ethynylandrostanediol-3(β), 17(α)	5	0.5	298	11	—15	35	—29
Isoandrosterone	6	9.4	264	9	—31	33	—33
Normal	11	—	386	13	0	33	—33
17-Methyl Δ^5-androstenediol-3(β), 17(α)	6	0.9	306	10	—23	32	—35
17-Methylandrostanediol-3(β), 17(α)	4	0.3	337	10	—23	30	—39

From Kochakian, C. D. *J. Biol. Chem.* **155**: 579 (1944).

[1]Change from values of castrated control mice.

[2] $\sqrt{\Sigma d^2/N-1} = \pm 2.1$

[3] $\sqrt{\Sigma d^2/(N-1)} = \pm 7.7$

TABLE X

Effect of Nutritive State on the Increase in Kidney Arginase after Steroid Stimulation

Nutritive State	Treatment	No. of mice	Fasting body wt. g.	Steroids absorbed mg.	Kidney mg.	Arginas Total units	Arginas Per g. units
N[1]	Normal	12	22.5	—	414	11	27
U[2]	"	3	13.9	—	222	5	21
N	Castrated	8	20.7	—	265	10	38
U	"	6	16.1	—	235	12	48
N	Testosterone	4	20.8	0.37	419	17	41
U	"	1	16.4	0.50	352	7	21
N	"	4	22.4	1.15	507	37	79
U	"	1	17.2	1.20	393	29	77
N	"	7	20.6	4.34	468	64	137
U	"	5	17.1	4.40	387	42	110
N	"	3	22.4	16.2	543	212	390
U	"	1	18.7	17.8	401	155	336
N	Testosterone propionate	5	21.6	2.33	492	36	72
U	"	4	16.3	2.67	362	26	72
N	17-Methyl-testosterone	4	23.3	0.14	417	25	54
U	"	1	18.0	0.12	329	11	33
N	"	6	22.8	4.26	509	122	241
U	"	3	17.3	3.54	409	62	159
N	17-Methylandro-stanediol-3(α),17(α)	5	22.5	3.2	519	98	187
U	"	2	16.0	3.8	332	65	198
N	Androstanediol-3(α), 17(α)	3	22.1	0.25	385	8	23
U	"	1	18.9	0.30	282	8	28
N	Androstanediol-3(α), 17(α), acetate-3	12	21.9	2.5	416	8	20
U	"	4	14.7	3.2	325	11	33

From Kochakian, C. D. *J. Biol. Chem.* **161**: 115 (1945).
[1]N = "Normal" nutrition.
[2]U = Undernutrition.

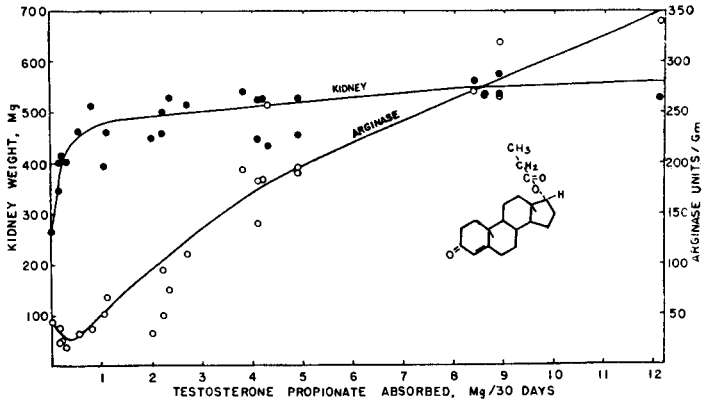


FIG. 19

The Effect of the Dose of Testosterone Propionate on the Arginase Content of the Kidney of the Castrated Mouse.
From Kochakian, C. D. *J. Biol. Chem.* **161**: 115 (1945).

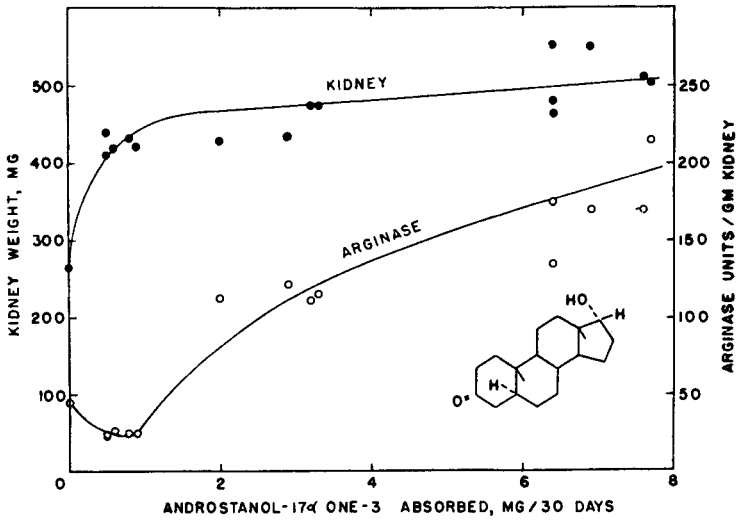


FIG. 20

The Effect of the Dose of Androstanol-17(α), one-3 on the Arginase Content of the Kidney of the Castrated Mouse.

From Kochakian, C. D. *J. Biol. Chem.* **161**: 115 (1945).

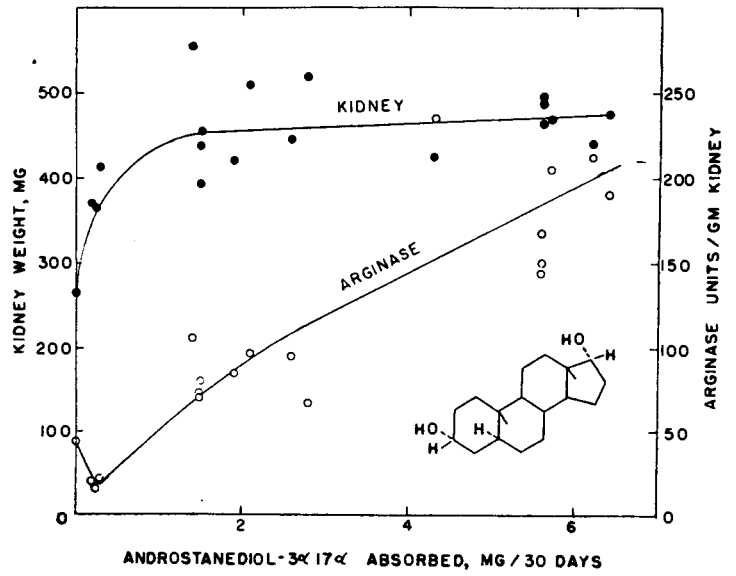


FIG. 21

The Effect of Dose of Androstanediol-3(α), 17(α) on the Arginase Content of the Kidney of the Castrated Mouse.

From Kochakian, C. D. *J. Biol. Chem.* **161**: 115 (1945).

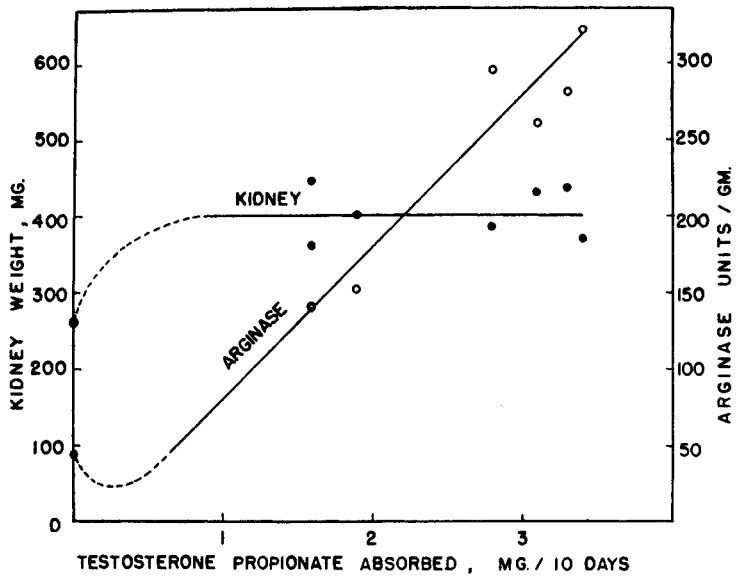


FIG. 22

The Effect of the Dose of Testosterone Propionate on the Kidney Arginase of the Castrated Mouse.

These mice were treated for only 10 days. Note that the same amount of steroid absorbed in 10 days as in 30 days (Graph 15) produces a greater increase in arginase but not in kidney weight.

From Kochakian, C. D. *J. Biol. Chem.* **161**: 115 (1945).

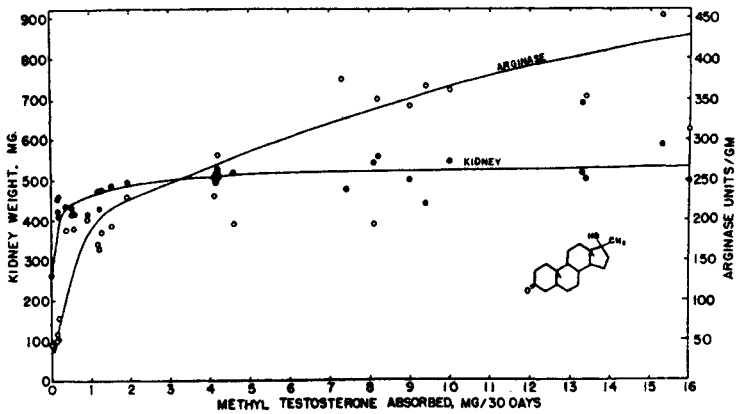


FIG. 23

The Effect of the Dose of 17-Methyltestosterone on the Kidney Arginase of the Castrated Mouse.

From Kochakian, C. D. *J. Biol. Chem.* **161**: 115 (1945).

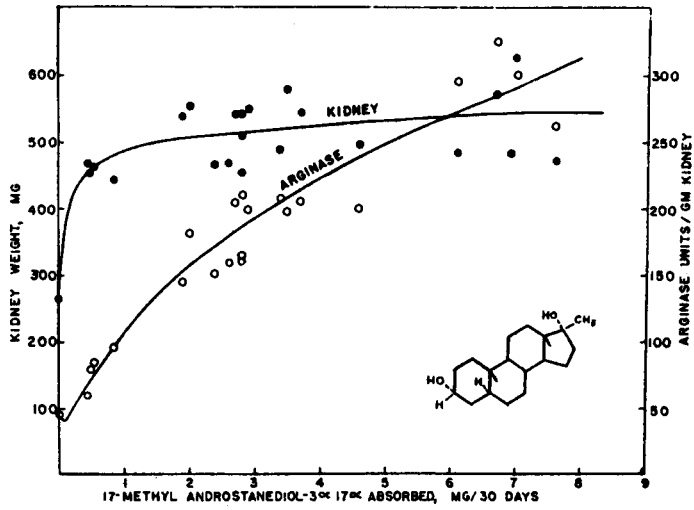


FIG. 24

The Effect of the Dose of 17-Methylandrostanediol-3(α), 17(α) on the Kidney Arginase of the Castrated Mouse.
From Kochakian, C. D. *J. Biol. Chem.* **161**: 115 (1945).

methyltestosterone (Fig. 23) and 17-methylandrostanediol-3(α), 17(α) (Fig. 24) causes an immediate increase in the arginase activity. On the other hand, the less active steroids (Fig. 25) bring about a prolonged decrease in arginase activity.

The changes in arginase activity are apparent even in animals that are undernourished (Table X, *cf.* Table V). Indeed the amount of enzyme per gram of tissue is with a few minor exceptions as great as that found in the well-fed mice. The increase in total amount of arginase, however, is less in the kidneys of the undernourished mice due to the smaller size of the organ. The kidneys of the castrated animals and those which received ineffective amounts of steroids show no change in the total arginase but a slight and probably significant increase in arginase per gram due to the decreased size of the tissue mass.

Since it was known that many factors enhance the activity of arginase, several experiments were run to determine the general nature of the induced enhancement of enzyme action. The addition of 0.5 mg. testosterone to samples of incubation mixture containing homogenisate of kidney did not produce any significant change (—4.5%). The mixing in equal amounts of homogenized kidney tissue from non-treated castrated mice with that of treated animals (Table XI) resulted in a simple summation of activities.

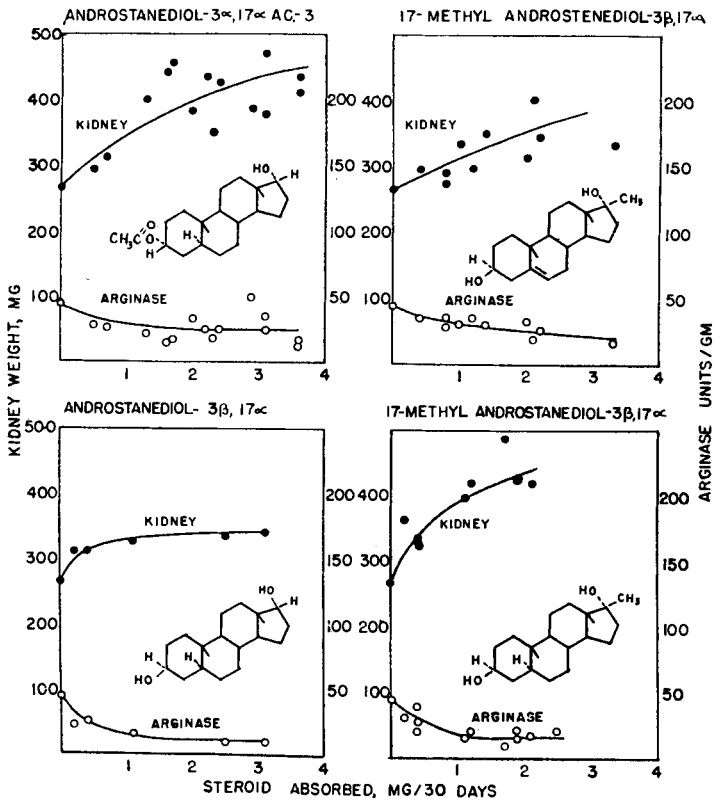


FIG. 25

The Effect of Less Active Steroids on the Kidney Arginase of the Castrated Mouse. The increase in kidney size is not accompanied by an increase in arginase because the rate of absorption of the steroids is not rapid enough to provide the necessary stimulus to the cells.

A similar result was obtained by mixing kidney samples of mice treated with testosterone and α-estradiol. As a further test a mixture of a non-treated and a testosterone-treated kidney sample was run without the "activator," cobaltous chloride. As was to be expected there was a decrease in the activity of the individual samples but the mixture decreased proportionately. It seems, therefore, that the increases in arginase activity of the kidney noted after treatment with the various steroids is not due to the production of an activator but to the production of more of the enzyme.

It is of interest that the steroid hormones concerned with protein anabolism affect only the enzymes (including d-amino acid oxidase which has been studied only for testosterone propionate (5) of the kidney and not

TABLE XI
Concerning the Nature of the Increased Kidney Arginase After Treatment With Steroids

Treatment	Arginase			
	Units	Ave. units	Mixture[1] units	Diff. %
Methyltestosterone Castrate	5.03 0.59	5.62	5.49	—2.3
Testosterone Castrate	4.21 0.63	4.84	4.40	—9.1
Testosterone Castrate	3.92 0.68	4.60	4.20	—8.7
Testosterone Castrate	2.81[2] 0.45[2]	3.26	3.00[2]	—8.0
Testosterone α-Estradiol	5.25 1.84	7.09	7.13	+0.6

From Kochakian, C. D. *J. Biol. Chem.* **155**: 579 (1944).
[1]The two samples of homogenized kidney tissue were mixed in equal quantities.
[2]The same sample as for the preceding test but water substituted for the coboltous chloride solution in the substrate.

those of the liver or intestine. This is of even greater interest in the case of arginase because the presence of this enzyme in the kidney has been more or less ignored because of its relatively insignificant amount as compared to that in the liver (*cf.* Table VIII). It seems that, contrary to expectation, much of the metabolic effects of the protein anabolic steroids are mediated through the kidney. Since arginase occurs in the proximal convoluted tubules (47) it might be even assumed that the cells in this tissue are stimulated to recover for protein fabrication more of those materials that ordinarily would be excreted. Edlbacher (7) as a result of his extensive studies has postulated that one of the functions of arginase may be to provide suitable nitrogen forms for protein synthesis.

One synthetic process, the formation of glycocyamine, by the transfer of the amidine group of arginine to glycine, occurs in the kidney (4) (Fig. 26). This compound then is methylated in the liver to form creatine. Can it be then that the increase in arginase activity of the kidney produced by the protein anabolic steroids is at least in part for the formation of glycocyamine? These steroids do affect creatine formation and excretion (4, 13, 33, 48). In order to explain such a reaction one only need assume that arginase promotes "glycinolysis" of arginine in the kidney instead of hydrolysis as in the liver. This is entirely compatible with our knowledge of reactions in organic chemistry.

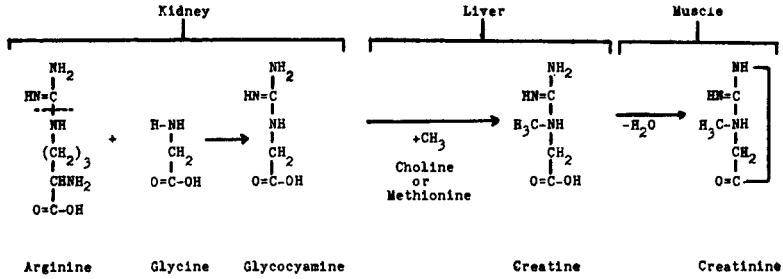


FIG. 26

IV. CAN ANDROGENS PREVENT THE PROTEIN CATABOLIC ACTIVITIES OF ADRENAL CORTICAL STEROIDS?

In order to obtain further insight into the mechanism of the protein-anabolic steroids, it was decided to determine whether these compounds could counteract protein-catabolic processes in the body. One of the properties of the C11 adrenal cortical steroids is to stimulate the conversion of protein to carbohydrate (39). It has been suggested that this process is mediated through the arginase of the liver (8, 9). Therefore, not only these phenomena but also related processes were studied in acute experiments of the Reinecke-Kendall type (43) with and without testosterone propionate treatment (Table XII).

The well established effects in the fasting adrenalectomized rat of a decreased formation of liver glycogen and a decreased excretion of urine nitrogen with increases after administration of the C11 adrenal cortical steroids were observed. The administration of testosterone propionate on the days following adrenalectomy and at the start of the injection of the adrenal cortical extract[6] did not alter the effects of the latter preparation (Table XII). The ineffectiveness of the androgens, however, may be due to the overwhelming effect of the repeated injections of the adrenal cortical extract.

The inability of the adrenal cortical preparation to restore the liver arginase content of adrenalectomized rats to normal is in agreement with observations in mice. Crystalline C11 adrenal cortical steroids do not increase the liver arginase of castrated mice even though protein catabolism is increased as indicated by a decrease in body weight and lesions in the skeletal and cardiac muscles (22, 23, 24). These compounds, also, do not cause appreciable in-

[6]The adrenal cortical extract was obtained in several batches, one of which was supplied by Dr. M. H. Kuizenga of the Upjohn Laboratories and the others were purchased from local pharmacists.

TABLE XII

Rats	Treatment	No. of rats	Urine Nitrogen	Liver			Kidney	
				Arginase	Glycogen	"Alkaline" Phosphatase	Arginase	"Alkaline" Phosphatase
			Per cent change from adrenalectomized rats given 0.9% NaCl as drinking water					
			%	%	%	%	%	%
Normal		8	+53	+113	+363	—10	+29	—12
Adrenalectomized		8	+18	—22	+8	—23	—4	—13
"	Desoxy. Ac. 1 mg./day	6	+12	+6	+9	—31	+14	+15
Adrenalectomized + 0.9% NaCl	A.C.E.[1] 9 x 0.5 ml./hr.	13	+68	+11	+560	+198	+17	+3
"	A.C.E.[1] 9 x 1.0 ml./hr.	3	+70	—	+1740	+272	—	—12
"	A.C.E.[1] 9 x 20 ml./hr.	6	+104	+6	+1340	+236	+5	—18
"	T.P.[2] 2 x 2.5 mg./day + A.C.E. 9 x 0.5 ml./hr.	11	+62	+37	+372	+187	+96	+37
"	T.P.[2] 2.5 mg./day + A.C.E. 9 x 2.0 ml./hr.	6	+130	+11	+1197	+246	+117	+37

(Kochakian, C. D., and Vail, V. N. Unpublished).
[1]A.C.E. = adrenal cortex extract (aqueous Upjohn).
[2]T.P. = testosterone propionate (Perandren, Ciba).

creases in the liver arginase of normal rats (8, 9). Apparently, the increase in arginase obtained by Fraenkel-Conrat *et al.* was not due to increased protein catabolism induced by the C11 adrenal cortical compounds but was a secondary phenomenon. Maintenance of life and growth has been observed (38) in young adrenalectomized rats injected with amounts of adrenal cortical steroids used by Fraenkel-Conrat *et al.* All of the evidence, therefore, indicates that the increase in arginase, which was really a restoration towards normal, observed by Fraenkel-Conrat *et al.* is more likely related to anabolic rather than catabolic processes. Furthermore, arginase is not concerned with the urea formation induced by the adrenal cortical steroids. It is of interest that Bach (2) has postulated that urea may be formed in the liver by a system which excludes arginase.

The injection of adrenal cortical extract produces a striking increase in the "alkaline" phosphatase (35) of the liver which parallels the increase in glycogen (Table XII). The "acid" phosphatase is not affected. The site of the increase in the "alkaline" phosphatase was determined by the Gomori histochemical technic (12) as modified in our laboratory (29).[7] The liver of the untreated animal has a small amount of the enzyme (black areas) in the periphery of the lobules (Fig. 27A) and very little in the cytoplasm of the cell (Fig. 27B). The administration of the adrenal cortical extract stimulates the production of the enzyme in all of the cells (Fig. 28 A, B). Thus, the increase in this enzyme is for metabolic processes (glycogen formation ?) stimulated in the cell. These processes, however, do not seem from preliminary experiments to be associated with a conversion of glucose to glycogen but more likely for the mobilization of small carbon residues obtained from amino acids. The formation of glycogen after the administration of carbohydrate is not accompanied by an increase in "alkaline" phosphatase but is after feeding a high protein diet.

The arginase and "alkaline" and "acid" phosphatases of the kidney are not affected by adrenalectomy or the administration of the adrenal cortical extracts. Testosterone propionate, however, increases both the arginase and "alkaline" but not "acid" phosphatase of the kidney. Similar effects have been observed in castrated rats (28). The increase in arginase is in agreement with that obtained in the mouse but that of "alkaline" phosphatase is not and remains to be explained.

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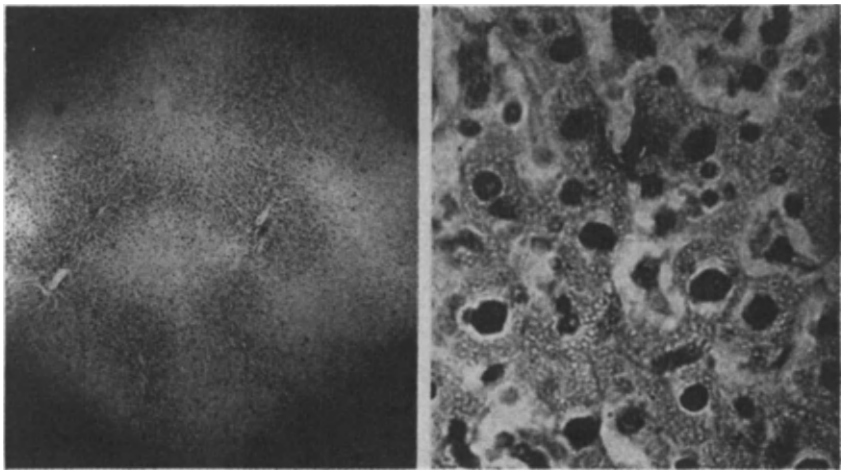
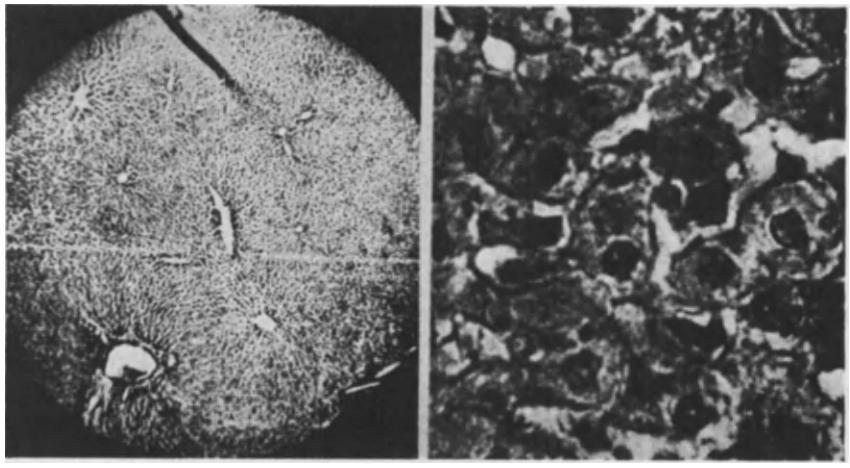
[7] I am indebted to Charles Luttrell for assistance in preparing and "reading" these tissues.



A (25 x) B (200 x)

FIG. 27

The "Alkaline" Phosphatase of the Liver of an Adrenalectomized Rat Receiving 0.9% Sodium Chloride as Drinking Water.
"Alkaline" Prosphatase: 4.4 units/g.
From Kochakian, C. D., and Luttrel, C. Unpublished.



A (25 x) B (200 x)

FIG. 28

The "Alkaline" Phosphatase of the Liver of an Adrenalectomized Rat Receiving 0.9% Sodium Chloride as Drinking Water, Daily Injections of Testosterone Propionate, and on the Last Day Adrenal Cortical Extract (Upjohn).
"Alkaline" Phosphatase: 21.6 units/g.
From Kochakian, C. D., and Luttrel, C. Unpublished.

V. Summary

Certain androgens stimulate the growth of not only the accessory sex organs but also the skeletal muscles and the kidneys. These effects do not parallel each other. Saturated diols preferentially stimulate the kidney. The renotrophic effect is a property of the protein-anabolic but not the protein catabolic steroids. In undernutrition the accessory sex organs respond maximally to steroid stimulation but the kidneys do not.

The metabolic effects of the protein anabolic steroids apparently are mediated at least in part through the kidney. The enzyme activities of this tissue are markedly altered by the various steroids while those of the liver and intestine are not.

The lack of parallelism among the various physiological effects and even at different doses indicates that no one criterion is adequate for a comparison of the physiological properties of the steroid hormones.

The metabolic effects of the protein catabolic steroids in contrast to the protein anabolic steroids are mediated primarily through the liver. The urea formed by excess protein catabolism under intensive adrenal cortical extract treatment apparently is formed by some system which does not include arginase. The daily administration of testosterone propionate is not sufficient to counteract the subsequent protein catabolism induced by intensive adrenal cortical extract injections.

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DISCUSSION

F. D. W. Lukens: Have any such studies on enzymes been done in connection with growth hormone? Has any distinction between the nitrogen retaining effect of the steroids (*e.g.,* testosterone) and the nitrogen retaining effect of the growth hormone been developed by these methods?

Another question: If the phosphatases are changed in the kidney, is it possible that phlorizin would affect the kidney phosphatase?

C. D. Kochakian: Fraenkel-Conrat, Simpson and Evans have reported a decrease in the arginase content of the liver of rats after the administration of anterior pituitary growth hormone. On the other hand we have found that the steroid hormones with

protein-anabolic properties have no effect on the arginase of the liver of rats, mice or guinea pigs. The California group also found that testosterone propionate has no effect on the liver arginase of rats.

Kritzler and Gutman (*Am. J. Physiol.* **134**: 94 (1941)) were unable to find any significant change in the "alkaline" phosphatase of the kidneys in chronically or acutely phlorizinized rats and dogs.

K. E. Paschkis: I believe it is clear that the sex hormones are growth stimulators if one uses this term in the broadest sense. They stimulate growth of certain target organs, the androgens that of the male accessories, the estrogens that of the uterus, vagina, and breast. This does not necessarily mean that the sex steroids are essential for over-all growth. As a matter of fact such is certainly not the case as far as male sex hormone is concerned as evidenced by the excessive growth in length of male castrates or eunuchoids.

And yet testosterone exerts growth effect in certain dwarfs as was first reported by J. S. L. Browne. This observation has since been confirmed by a number of clinicians. The nitrogen retention following administration of testosterone (the metabolic facet of "growth") has been described by Kochakian and by Kenyon and his collaborators.

In our laboratory we are now studying some aspects of this N-retention induced by testosterone. The rat's response is the same before and after adrenalectomy if the animal is well maintained by sodium chloride and well adapted to forced feeding. Also adrenalectomy itself does not change the level of N excretion. The adrenals therefore neither influence nor mediate the testosterone effect.

Further experiments are under way on hypophysectomized rats in order to determine whether the N retention induced by testosterone is mediated by the pituitary gland (stimulation of growth hormone secretion?).

C. D. Kochakian: Growth is a very general term. There is not one but many types of growth. So it is not surprising that castrated animals increase in size. The important thing is whether the nature of the increase is the same or different from that in the normal animal.

In the experiments on adrenalectomized rats reported here, we also determined nitrogen excretion. We found no indication that testosterone propionate caused nitrogen retention in these animals. This, however, does not mean that the adrenals are necessary for the protein anabolic effect of the androgen. There are other factors which must be considered.

With respect to the pituitary, naturally we would like to know whether the protein anabolic effect of the steroids is mediated through this organ. We have observed nitrogen retention in a hypophysectomized-castrated dog injected with 25 mg./day of testosterone propionate.

Dr. Selye: I would like to take this opportunity to correct an error which occurred in one of our earlier publications on renotrophic action. We have examined the renotrophic action of a large number of androstane and pregnane derivatives and found that the histological changes were always the same irrespective of the chemical nature of the compound. They consisted mainly of a hypertrophy and hyperplasia of the convoluted tubules without any obvious alterations in the renal glomeruli. These histologic changes were accompanied by an enlargement of the kidney and an increase in its weight. We therefore assumed that one may take the increase in renal weight as an indicator of renotrophic action. Since that time we found this assumption to be incorrect. Desoxycorticosterone acetate (DCA) also causes marked renal enlargement but not as a result of a true renotrophic action but merely because the compound pro-

duces nephrosclerosis with the formation of hyaline casts which occlude the tubular lumina. This occlusion leads to a retention of urine in the proximal parts of the nephron and consequently to an increase in renal weight. It is incorrect, therefore, to say—as we did in earlier publications—that DCA possesses a true renotrophic action.

In view of this qualitative difference between the effect of true renotrophic steroids and DCA I think it would be very interesting to compare the biochemical changes (*e.g.*, enzyme content) produced by renotrophic steroids on the one hand, and nephrosclerotic compounds such as DCA on the other hand.

As regards Dr. Kochakian's investigations on renotrophic steroids I have little to add as he always manages to keep two or three steps ahead of us in this field. It may be worth mentioning, however, that we succeeded in augmenting the renotrophic action of steroids as well as that of renotrophic pituitary extracts by simultaneous treatment with threshold doses of thyroxin. Curiously, the same hormone (thyroxin) also augments the nephrosclerotic effect of DCA.

C. D. Kochakian: I would like to further emphasize Dr. Selye's remarks about the renotrophic properties of the various steroids. As I indicated in my lecture the greatest renotrophic effects were obtained with those steroids which have protein-anabolic properties. However, the protein-catabolic steroids of the adrenal cortex, desoxycorticosterone and α-estradiol also increase, although only slightly, the size of the kidney. Dr. Durlacher and his associates have suggested that the effect of desoxycorticosterone on kidney size is associated with electrolyte excretion.

I think the observations of Dr. Selye on the pituitary factor are extremely interesting. I hope he will be able to isolate the active factor.

N. T. Werthessen: Dr. Kochakian brought up the question of various dosages of testosterone. Apparently the efficiency in inducing nitrogen retention increased with the dosage in his experience. I am particularly interested in whether α-estradiol has been studied over a wide dosage range. From Dr. Kochakian's graph I got the impression that the effect rose and then decreased with an increment in α-estradiol administration. I wonder if he would enlarge on the relationship and the range of the dosage? Has he employed progesterone?

C. D. Kochakian: The efficacy of the renotrophic and androgenic effects of the active steroids decreases with increase in dose. The arginase content of the kidneys, however, increases with increase in dose.

α-Estradiol was studied at only one dose level but for two periods of treatment—10 and 30 days. The amount of material absorbed from the pellet evidently was too great because there was a definite decrease in the body weight in the mice treated for the longer period of time.

Progesterone never has shown any activity in all of the studies—renotrophic, androgenic, and enzymatic—we have carried out.

Studies on Steroid Hormones in Experimental Carcinogenesis*

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Introduction

At least three different reasons have been cited for the pursuance of studies on the role of steroid hormones in experimental carcinogenesis. In the first place the chemical similarity between the steroid hormones and the carcinogenic hydrocarbons was interesting although subsequently the elucidation of the detailed chemical structure of the steroid hormones and carcinogenic hydrocarbons revealed that their different biological activities are associated with such specific molecular characteristics that, in most instances, there is little possibility of overlap. Some carcinogenic hydrocarbons may possess estrogenic activity, but large amounts of the carcinogen must be used to induce estrous reactions. A reciprocal biological overlapping, that is, carcinogenic activity of estrogens, exists under certain circumstances that will be presented later. In the second place the sex-limited appearance of tumors of certain tissues and organs led some investigators to believe that the gonadal hormones are of significance in tumorigenesis. Furthermore the influence of reproductive activity and the relationship of abnormal or unusual gonadal activity to tumors of certain types afforded instances of apparent gonad-tumor relationship. Finally, after it had been demonstrated that the steroid hormones, particularly those from the ovary, were predominantly stimulators of growth, it was assumed that they might likewise be stimulators of abnormal growth. Periodic stimulation and regression of the accessory reproductive organs occur throughout life in the normal mammalian female. It is possible that, if the stimulatory phase were continued indefinitely, unusual growth would occur; tissues might become independent of influences normally restricting their proliferation and malignancy might ensue. Others have suggested that a tissue stimulated excessively and continuously may become senile, and, because cancer arises more commonly in old organisms, in this manner might predispose to cancer.

Studies of the writer were undertaken because of the interest in the growth-stimulating capacities of the steroid hormones. It was observed, however, that many reactive tissues did not remain permanently responsive to continuous stimulation by exogenous steroids under the experimental con-

*The original investigations reported herein have been supported by grants from the Anna Fuller Fund and the Jane Coffin Childs Memorial Fund.

ditions employed. Frequently the tissues that have ceased to respond in the usual manner during continuous stimulation seem most liable to tumor formation.

A general survey of the relation of steroid hormone to carcinogenesis can probably best be taken up by considering separately the various end organs in which neoplasia has appeared in animals under experimental conditions. Because, for the greater part, the different estrogenic chemicals have shown no qualitative differences in their relation to neoplasia, reference will be made to specific estrogenic chemicals only when it is considered desirable. Furthermore, because so many of the neoplasms arising in estrogen-treated animals are species or strain specific, emphasis is placed, where possible, upon the reasons for the differences in responses of different homogeneous strains that, within the same species, might account for the observed differences in response. It is believed that awareness of and eventual explanation of strain and species differences in response are important because, without too much stretch of the imagination, it is possible to compare observations made in the heterogeneous human population, for example, with populations of mice from several different pure strains. If such an interpretation can be even partially accepted, a stimulus that might result in the onset of neoplasia in one strain or in one individual of a heterogeneous group, such as man, might be quite inadequate in another population, or in another individual. Aside from a possible application to the explanation of experimental tumorigenesis a discovery of the factors responsible for the differences in response of animals within the same species, but of different homogeneous populations, would contribute significantly to the understanding of some fundamental biological processes.

Several papers have been published recently that have covered the extensive literature on the relation of steroid hormones to carcinogenesis, so the writer will consider here chiefly those observations with which he is personally familiar rather than attempt a general review (9, 14, 21, 33, 42, 48, 60).

II. Uterine and Cervical Tumors

1. *Uterine and Uterine Cervical Tumors.* Carcinomas have appeared in the uteri of rabbits treated for long periods of time with estrogenic hormones or gonadotrophic preparations of the pituitary. In the latter case the animals presumably receive estrogen from their own ovaries (58). Hybrid rabbits, obtained by crossing animals of two partially inbred strains, show a high incidence of a syndrome indicating a high level of intrinsic estrogens and also a high incidence of adenocarcinoma in the uterus (32).

A uterine carcinoma has been described in one mouse that received estro-

gen (45). Epithelial malignances of the uterine horns or uterine fundi of estrogen-treated rats, guinea pigs, monkeys or dogs have not been described although animals of these species have been subjected to frequent experimentation. The uteri of these animals, however, do show unusual responses to prolonged treatment with estrogens. Excessive hyperplasia of the endometrial glands occur in some species; the glands sometimes perforate the myometrium and occupy a subserosal position (adenomyosis). Both the luminal and glandular epithelia sometimes undergo squamous metaplasia (22, 50, 68). Acute or chronic pyometra occurs in some species. If large amounts of estrogen are given to mice for long periods of time the endometrial stroma becomes hyalinized (Fig. 10) and the myometrial glands decrease in number (22). The uteri apparently become refractory under excessive stimulation. Neoplasia of the uterus proper, nevertheless, does not occur in such animals.

2. *Uterine Cervix.* Malignant epithelial tumors appear quite frequently in the uterine cervices and upper vaginas of mice that have been treated with estrogens for long periods of time (1, 22, 56, 64). These tumors may become very large, infiltrate the adjacent pelvic tissues and/or metastasize to the regional lymph nodes (Stage IV, Figs. 3, 5) (24). In addition to the invasive and metastatic lesions many cervical tumors have been observed that have greatly enlarged but have not extended beyond the organ of origin (Stage III). It is believed that these locally invasive but smaller and less extensive lesions represent earlier stages in the origin of cervical malignancy. Many small invasive lesions that extend only into the mucosa have been observed near the external os and along the external surface of the cervix and in the vaginal fornices (Stages I and II, Figs. 1, 2). It has been possible to follow the neoplastic process in the mouse's cervix from the stage of earliest infiltration of the mucosa by moderately anaplastic cells, to more extensive invasion involving the greater portion of the mucosa, to localized overgrowths greatly enlarging the organ, and finally to definite malignancy with extension to the regional lymph nodes, and adjacent tissue.

A large number of mice from several different inbred strains have been studied and no strain differences in the appearance of the uterine cervices have been observed; mice of all strains, provided they tolerate treatment for a sufficiently long period of time, show these lesions. The incidence has been from 40 to 60% in a few small groups if mice surviving for less than one year are excluded (1). Uterine cervical tumors have occurred in mice that have received several different estrogenic chemicals (Table I). The duration of treatment is a very important factor; very few cervical tumors appear in animals treated for less than one year, and the incidence is highest among animals treated for 450 to 600 days. Untreated control mice

TABLE I

*The Incidence of Uterine Cervical Lesions Among Estrogen-treated Mice of Several Inbred Strains and Two Hybrid Groups**

Strain**	No. of Female mice observed for uterine neoplasia	No. of uterine or cervial lesions	Average survival of all mice	Average survival of mice with cervical lesions	Stage of lesion*** I	II	III	IV
C_3H	76	10	264	339	4	2	2	2
CBA	26	10	319	442	3	4	3	
$C_{12}I$	19	5	192	191	3	1	1	
A	23	2	200	197		1	1	
C_{57}	15	0	181	—				
N	14	0	161	—				
JK	6	0	204	—				
F	4	0	74	—				
CC_1	25	15	538	552	5	2	3	5
CC_2	26	10	450	542	4	2	1	3

*Data from papers 22 and 1 cited in reference.
**CC_1-F_1 hybrids C_{57}♀ x CBA♂.
CC_2-F_1 hybrids C_{57}♂ x CBA♀.
***See text.

from the same groups that have been studied most extensively have shown no malignant, epithelial, cervical growths although the average age at death exceeds that of the estrogen-treated mice. The mice given estrogen acquire, in this instance, tumors of a type not observed in untreated animals.

The site of origin of the larger tumors cannot be determined but the smaller infiltrative lesions may be specifically located. They appear most frequently in the cervical canal, which, in the mouse, is lined by low stratified squamous epithelium, the external os, and in the fornices of the vagina but rarely at the squamomucal junction at the internal os. A few lesions arise in the vaginal wall of the fornices and in the dorsal vaginal wall.

3. *Early Responses of the Cervix to Estrogen.* Estrogens incite excessive growth and cornification of the thick vaginal and thinner cervical, stratified, squamous epithelium (22). In some mice the vaginas become filled and distended with desquamated cornified epithelial cells. After a few months the vaginal epithelium usually becomes moderately refractory to estrogen; the epithelial layers are thinner, may contain leucocytes, and in some areas, show few, if any, cornified cells (Figs. 4, 9). Usually, but not always, it is in such an atrophic or moderately atrophic epithelium that the earliest invasive lesions are noticed.

The stroma of the uterine cervix exhibits unusual responses to estrogen; it hypertrophies tremendously, increasing the size of the organ several times, and frequently assumes the appearance of a pseudomucinous type of connective tissue. Grossly the enlarged cervices are firm and white; the

blood supply appears quite deficient except just beneath the basement membrane. Unlike the stroma of the endometrium, the mucosal stroma of the cervix shows no evidence of hyalinization.

4. *Influence of Androgens on the Cervix.* Simultaneous administration of testosterone propionate and estrogen tends to decrease but not completely prevent tumors of the uterine cervix (22). The smaller, locally invasive epithelial lesions arising in mice so treated show less evidence of cornification than they do in estrogen-treated mice (Figs. 7, 8). The larger lesions that have infiltrated more extensively are almost indistinguishable from those of estrogen-treated animals.

The vaginal epithelia of the mice which received androgen and estrogen simultaneously showed no cornification. The epithelium was excessively folded, giving the impression that the epithelial surface was unusually large (Fig. 7). Even the epithelium of the cervical canal was folded. The stromal hypertrophy of the cervix was prevented, the cervix being the size of that of a normal mouse. The connective tissue fibers were unusually prominent.

Among the small series of animals that received estrogen and progesterone simultaneously, infiltrative epithelial lesions and cancer also occurred. (Fig. 6). Further investigations must be undertaken, however, before it will be possible to say whether or not progesterone will increase or decrease the incidence of such lesions.

Uterine cervical tumors have not appeared among mice that received testosterone propionate alone for long periods of time.

5. *Other Factors Considered in Relation to Cervical Tumors.* The uterine cervix, in its relatively exposed position, is subject to trauma. Foreign bodies are found frequently in the vagina; it is continually exposed to such substances as hair, fragments of bedding material and other debris. Attempts have been made to rule out the influence of chronic septic inflammatory reactions by transplanting cervices of young mice, prior to the opening of the vagina, into subcutaneous sites of other animals (23). When the hosts received estrogens, however, the transplants became enlarged and cystic. Evidences of epithelial invasion did not occur in any of the transplants. These experiments cannot be considered to afford conclusive proof that trauma and inflammation played significant roles in cervical carcinogenesis because the distended grafts in their foreign locations are quite abnormal. Furthermore the uteri of estrogen-treated mice are septic (65). The uteri of almost all mice observed on the fourth day after a single injection of estrogen contained bacteria. Among animals treated for

long periods the sepsis favors uterine epithelial metaplasia, but as mentioned above tumors did not appear.

6. *Spontaneous Cervical or Vaginal Tumors in Mice Showing Genital Anomalies and Possible Ovarian Dysfunction.* Spontaneous uterine cervical carcinomas have occurred in 5 mice of one inbred strain (Figs. 11, 12). A total of 33 females that have lived beyond 400 days have been examined up to this time. Mice of this strain have shown such a high incidence of imperforate vaginas and of sterility that the strain has been difficult to maintain. Attempts are being made to maintain this strain by outcrossing to animals of other strains. The tumors in these animals were all quite extensive at the time they were observed, large portions of the vagina being involved. The tumors were anaplastic; malignant epithelial cells were found in the adjacent lymphatics (Fig. 12) and had invaded around the nerve trunks. In one mouse the lumbar nodes and liver were also invaded. It is possible that the imperforate vaginas are due to maternal humoral disturbances during late fetal development because somewhat similar anomalies can be induced by injecting testosterone during pregnancy. Rats or mice born of mothers treated in this manner also show modification of ovarian activity which may also be prevalent in the mice of this line.

III. Testicular Tumors

Tumors of the interstitial cells of the testes also arise in estrogen-treated mice (35, 7, 62). Unlike the uterine tumors, however, they occur only in animals of certain inbred strains and not in others; a specific genetic background is necessary for their appearance. It is possible that the incidence of such tumors is higher among animals receiving stilbestrol than other estrogens, but the difference would be only quantitative (Table II).

The tumors may become very large, not only replacing the normal testicular tissues, but also increasing the size of the testes several times; are yellow to a yellow-brown in color, and may contain, when large, hemorrhagic and necrotic areas. They frequently metastasize to the perirenal nodes and less frequently to the lumbar and mediastinal nodes.

Subsequent to the injection of estrogen the seminiferous tubules atrophy; the interstitial cells first hypertrophy, then gradually disappear; at about the time of their disappearance large brown cells (macrophages) appear (36). Among estrogen-treated animals of some strains small islands of hyperplastic interstitial cells appear in the otherwise atrophic interstitial tissue (Figs. 13, 14). These islands increase in size, displace the surrounding testicular tissue, and if growth continues long enough, infiltrate the tunica albuginea and increase the size of the testes. Most of the tumors

TABLE II

*The Incidence of Testicular Tumors Among Mice of Several Strains That Have Received Estrogen**

Strain	Investigator	Estrogens used	No. of mice	No. with tumors
A	Hooker & Pfeiffer (36)	estradiol benzoate	24**	10
A	Hooker & Pfeiffer (36)	stilbestrol	32**	29
JK	Gardner (19)	triphenylethylene	13	7
A	Gardner (19)	triphenylethylene	17	7
C_3H	Garnder (19)	triphenylethylene	14	1
CBA	Gardner (19)	triphenylethylene	5	0
N	Gardner (19)	triphenylethylene	3	0
C	Shimkin, Grady, Andervont (62)	stilbestrol	62	13
C(C)#	Shimkin & Andervont (61)		74	23
C(C_3H)	Shimkin & Andervont (61)		67	20
White label	Bonser (5)	triphenylethylene	39	3***
IFS	Bonser (5)	triphenylethylene	20	4
RIII(RIII)#	Bonser (5)	triphenylethylene	24	0
RIII(CBA)	Bonser (5)	triphenylethylene	47	14
RIII(A)	Bonser (5)	triphenylethylene	16	7
CBA	Bonser (5)	triphenylethylene	36	0
CBA(RIII)	Bonser (5)	triphenylethylene	26	0
A	Bonser (5)	triphenylethylene	31	21
A(RIII)	Bonser (5)	triphenylethylene	9	8

*A summary of data presented in the more recent publications from several laboratories.
**Survived 8 months or more.
***Includes adenomas and carcinomas.
#Designations in parenthesis indicate the strain used to foster nurse young.

are composed of large cells with vesicular nuclei and abundant, moderately granular cytoplasm (Fig. 15). Mitotic figures do not occur frequently in these cells. Areas of small, anaplastic cells (third generation) may occur scattered throughout the tumor, or even make up the greater part of the tumors in some animals. Mitotic figures occur more frequently among such cells. Hooker and Pfeiffer (36) have described in detail a sequence of cellular changes in the development of interstitial cell tumors.

Among animals of strains in which testicular tumors do not occur subsequent to estrogen-treatment the testes show similar early changes, that is, the interstitial cells at first hypertrophy and are later replaced by large macrophages containing debris and brownish pigment. The localized, hyperplastic areas of interstitial cells do not appear. Why mice of some strains develop such tumors subsequent to estrogen therapy and others do not is not known; at this time they can only be ascribed to vague genetic differences.

The tumors grow subsequent to transplantation into genetically related estrogen-treated animals, but do not, or rarely, grow if estrogen is not injected (5, 17). In untreated animals the transplants disappear within a

few weeks or months and can only be located by examining the sites of transplantation under ultraviolet light; small areas of the subcutaneous tissues where the tumors are implanted fluoresce a bright orange color. Histological examination of such areas reveals macrophages containing a brownish pigment and the usual connective tissue cells. No tumor cells can be identified. If estrogen-treatment is begun up to 7 months subsequent to transplantation these "rests"—remnants of the graft—begin to grow (17). They grow as rapidly as new transplants made at the time of institution of estrogen-treatment. The transplants remain dormant in the subcutaneous sites until the proper environment is attained for their growth.

When a transplanted interstitial cell tumor has begun to grow, estrogen treatment may be stopped, and the tumors continue to grow or persist (17), indicating that the treatment has induced some irreversible change in the environment of the host. The hypophysis is not necessary for the persistence or growth of transplanted testicular tumors for at least 37 days subsequent to the operation.

Spontaneous interstitial cell tumors occur rarely in mice. None have been described among mice of those inbred strains that, most frequently, acquire such tumors subsequent to estrogen-treatment but 3 have appeared in susceptible hybrid mice. Those that have been described resemble the tumors arising in estrogen-treated animals but, for the greater part, contain more of the smaller and anaplastic cells (18).

It is possible that mice of strains that acquire testicular interstitial cell tumors subsequent to estrogen-treatment possess some genetic tendency for the development of such tumors spontaneously, and that estrogen merely induces their appearance at an earlier age. However, this statement has not been demonstrated to be a fact at this time. It must also be considered possible that mice of the susceptible strains metabolize estrogens in a different fashion, and that although the end organ is the same as in mice of other strains, the substance acting upon the end organ directly or indirectly might be different. The answers to these problems must await future experimentation. The tendency to acquire testicular tumors is transmitted by both males and females of the susceptible strains to their hybrid young (unpublished). There is no evidence of a special maternal transmission of this tendency (61). Testicular tumors may occur in animals with pituitaries of normal size, or in animals with pituitary tumors, in the latter instances when the proper parents are used to transmit the tendency for pituitary tumors.

The interstitial cell tumors produce androgen (8, 35). When they become moderately large the amount of androgen produced is adequate to restore the atrophic seminal vesicles and prostates of the estrogen-treated hosts. Evidences of the presence of androgenic hormone in animals with testicular

tumors have also been observed as revealed by the structure of the salivary glands and scrotum.

It has been assumed that estrogen may not act directly upon the testes in producing such tumors but that it incites an increased production of the luteinizing hormone (LH) by the pituitary, which in turn stimulates the interstitial cells. To test this hypophysis Pfeiffer and Hooker (57) treated animals of the susceptible strains with large amounts of gonadotrophic hormone for long periods of time. In animals treated with these hormones the early stages of interstitial cell changes up to the stage of nodule formation occurred but no definite tumors developed in such animals. Antigonadotrophic effects, however, limit the interpretation from such experiments. Pregnant mare serum was the only gonadotrophic hormone that could be employed satisfactorily.

IV. Pituitary Tumors

Extensive chromophobic hyperplasias and adenomas of the pituitary also occur among estrogen-treated mice, but at high incidences only among mice of certain inbred strains. (28, 59). The tumors may become very large, some exceeding 250 milligrams, in fact some of them weigh almost as much as the mouse's brain. In mice with such tumors the cranial sutures are pushed apart and the head is misshapen. Upon gross examination the largest pituitary tumors vary in appearance; many of the tumors are fleshy and moderately firm, and others are hemorrhagic to variable extents (Figs. 16-18).

For purposes of presentation here any enlargement of the pituitary exceeding 12 milligrams, or approximately 6 times the normal weight of the female mouse's pituitary, is considered tumorous. Some of these growths are undoubtedly merely chromophobic hyperplasias such as have been described frequently among estrogen-treated rats and mice. The larger tumors, and some of the smaller tumors, however, appear to be true adenomas. They consist of large chromophobic cells which contain large vesicular nuclei, and the non-granular, slightly basophilic cytoplasm contains a large Golgi apparatus (Fig. 19). Vascular sinusoids separate the tumorous cells giving sections of the tissue a cord-like or alveolar appearance. Mitotic figures may be very frequent.

Among the mice in the laboratory at New Haven pituitary tumors have occurred at a high incidence only in animals of the C_{57} strain or in their hybrids (Table III). Almost all mice of the C_{57} strain that have received estrogen for over 400 days have pituitary tumors. Also, hybrid mice obtained by mating mice of the C_{57} strain with mice of the CBA (12), A, or C_3H (unpublished) strains acquire pituitary tumors after prolonged estro-

TABLE III

The Incidence of Pituitary Tumors (Pituitaries Exceeding 12 mg. in weight) Among Mice of the C_{57} strain and First Generation Hybrids of the C_{57} and CBA Strains That Had Received Different Doses of Several Estrogens

Strain or Hybrid Group	Sex	Number	No. with tumors	Per cent with tumors	Average age at death of tumorous mice
C_{57} (28)*	M	42	12	28	381
C_{57}	F	26	3	11	399
CBA♀ x C_{57}♂ (12)	F	28	9	32	562
C_{57}♀ x CBA♂	F	30	16	53	599
CBA♀ x C_{57}♂	M	24	18	75	480
C_{57}♀ x CBA♂	M	23	90	83	513

*The mice of the C_{57} strain that did not acquire pituitary tumors died when less than 400 days of age.

gen treatment. Both male and female mice of the C_{57} strain transmit the tendency to their first generation hybrid young.

Susceptible, estrogen-treated, male mice tend to acquire pituitary tumors at an earlier age, and the tumors attain a larger size than in female mice. Either the pituitaries of the females are different, or the females metabolize estrogens that are administered in a different manner than the males. The simultaneous administration of testosterone propionate inhibits but does not entirely prevent the appearance of pituitary tumors.

Approximately one half of the pituitary tumors that have been transplanted subcutaneously in estrogen-treated, related hosts have grown in one or more of the recipients. Growth of the tumors has been slow, they rarely become apparent less than one year subsequent to transplantation and rarely attain large size until about the time the host shows extensive hypophyseal hypertrophy. The tumors have uniformly failed to grow in animals that have not received estrogen. Up to this time it has been impossible to follow the tumors for more than three serial transfer generations, even if the hosts have received estrogen.

Some evidence is available, indicating that the tumors, once formed, persist or even continue to grow after the discontinuance of estrogen. Treatment was discontinued in one series of hybrid mice at an age at which most of the animals had pituitary tumors. The mice were followed for periods up to 138 days subsequent to the discontinuance of treatment. At the time of autopsy they had pituitary tumors of about the same size as those in animals that had been maintained on continuous estrogen treatment. These observations differ somewhat from those reported by Nelson (52) who described complete recovery of the hypophyses in rats within 6 months of the discontinuance of estrogen-treatment.

The tumorous pituitaries apparently secrete some gonadotrophic hormone.

Subsequent to the discontinuance of estogren the seminal vesicles and prostates recover and resemble those of normal males. Transplantation of bits of hypophyseal tumors into the subcutaneous tissues of hypophysectomized mice also revealed the presence of gonadotrophic hormone as well as some growth hormone. The hormonal content of the tumors was much less than that of normal pituitary tissue, as revealed by this technic (unpublished).

V. Lymphoid Tumors

The incidence of lymphoid tumors—lymphosarcoma and lymphatic leukemia—is also increased among the estrogen-treated inbred mice of some strains. Studies on mice of seven different inbred strains showed that subsequent to estrogen-treatment the incidence of lymphoid tumors was strikingly increased in three, slightly increased in two more, and not changed in two (26) (Table IV). The tumors usually arise in the thymus and, in

TABLE IV

Lymphoid Tumors Among Control and Estrogen-treated Mice of 7 Different Strains (26)

	Controls			Estrogen-treated		
Strain	Number of animals	Number of tumors	Percentage of tumors	Number of animals	Number of tumors	Percentage of tumors
C_3H	481	5	1	747	109	14.4
CBA	62	2	3.2	445	67	15.1
PM	58	0	0	143	22	15.4
A	82	0	0	94	3	2.1
C_{57}	59	3	5.0	170	3	1.8
JK	37	1	2.7	64	3	4.7
$C_{12}I$	43	0	0	136	8	5.9

many animals, are limited to this organ. In some animals, in addition to the thymus, the adenoid tissues throughout the body are involved, and many leukemic lymphocytes may be found in the blood and most of the body's tissues (Figs. 20, 21). In a few animals leukemia may develop without any appreciable involvement of the thymic region.

The lymphoid tumors are malignant. They infiltrate the surrounding tissues if they grow as sarcomatous masses, and invariably grow when transplanted into related hosts. After transplantation for a number of generations some lymphoid tumors grow in animals of several different strains, but none have grown in mice of all strains.

The incidence of lymphoid tumors among the untreated control mice of seven strains studied ranged from 0 to 5%. Among the estrogen-treated mice, including animals that received several different dose levels of hormone, it ranged from 1.8 to 15.4%. The larger the dose of the hormone, providing it was compatible with the continued existence of the animal, the higher the incidence of leukemia. Mice that received estrogen in large doses

for a period of 10 weeks and then were observed until death showed a higher incidence of leukemia than did those animals treated continually throughout their life span, indicating that, whatever the effect was that estrogen had in relation to the development of this neoplasia, it occurred early in the course of treatment.

Among the estrogen-treated mice more tumors occurred in female than in male animals of the strains studied. All the estrogenic chemicals given in large amounts increased the incidence of tumors among the susceptible animals. There is no evidence that one chemical was more effective than another.

Hybridization of mice of two strains susceptible to estrogen-induced leukemia showed an augmented incidence among the first generation young. Approximately 45% of the hybrid young obtained by crossing two susceptible strains (C_3H and PM) died with lymphoid tumors (25) (Table V).

TABLE V

The Incidence of Lymphoid Tumors Among Control and Estrogen-treated Hybrid Mice (25)

Hybrid Group*	Number of mice	Number of tumors	Percentage of tumors	Estrogen-treated Number of animal	Estrogen-treated Number of tumors	Estrogen-treated Percentage of tumors
C_3H x A	97	2	2	221	23	9
CBA x A	92	0	0	166	10	6
C_{57} x C_3H	103	1	1	220	28	12
PM x C_3H	95	0	0	167	69	43
JK x C_3H	95	2	2	154	9	5
C_{57} x CBA	62	4	6	105	12	11
C_{57} x A	102	16	16	206	16	8
JK x A	91	8	7	164	0	0

*Reciprocal male and female matings are represented in each group, for example, in the first group hybrid young of both of the following matings are represented (C_3H♀ x A♂) and (C_3H♂ x A♀). The symbols underlined designate the strains which show a high incidence of lymphoid tumors subsequent to estrogen-treatment.

The young obtained by crossing one strain susceptible to estrogen-induced lymphoid tumors and one strain resistant to estrogen-induced leukemia gave young showing intermediate incidences of the lymphoma. The hybrid young obtained by crossing two strains resistant to estrogen-induced lymphoid tumors gave equivocal results. There was no evidence of a maternal transmission of the tendency to have leukemia subsequent to estrogen-treatment.

Mice that received testosterone propionate and estrogen simultaneously acquired lymphoid tumors no more frequently than did the controls. Testosterone completely prevented the lymphoid-tumor inciting action of estrogen. Testosterone by itself did not alter the incidence of leukemia.

Mice treated with estrogenic hormone show damaged lymphoid tissue. Estrogen, even in the absence of the adrenal gland, induces thymic dissolution. It is also known that the adrenal steroids are extremely active in this respect and that in the presence of the adrenals estrogens are more active in the destruction of lymphoid tissue. It is possible that estrogens may be acting, in part, indirectly in inciting the assumption of malignancy. The antagonistic action of testosterone would permit either an assumption of direct or indirect action of estrogens. The higher incidence of lymphomas among female mice than males of some strains susceptible to the spontaneous occurrence of the neoplasm indicates a possible humoral factor in susceptibility.

Other agents such as roentgen rays and carcinogenic hydrocarbons also increase the incidence of lymphomas of mice of some strains (39). It is of interest that these different agents are not necessarily effective in mice of the same strains, that is, a strain in which the incidence of lymphoma is increased subsequent to estrogen-treatment may show no lymphomas when treated with carcinogenic hydrocarbon and vice versa.

VI. Adrenal Tumors

Although large adrenal tumors have not been found among estrogen-treated animals, they have appeared subsequent to gonadectomy in guinea pigs and mice. Mice of certain strains gonadectomized at an early age acquire adrenal cortical tumors 200 or more days later (13, 66). The tumors frequently attain large size, and among mice of some strains they metastasize (67). The tumors formed in susceptible mice give evidence of the production of female sex hormone, the mammary glands of their hosts were developed, submaxillary glands showed evidence of feminization, and among females the uteri and vaginas were well developed, some of them showing hyperplastic and cystic endometria.

Histologically these tumors were composed of adrenal cortical cells at different stages of differentiation (Figs. 22, 23). They were well vascularized, showed little evidence of degenerative change, and contained a delicate connective supporting tissue.

In addition to the development of the mammary glands, uteri and salivary glands of the animals with adrenal cortical tumors, the tumor-bearing animals excrete in their urine and feces aproximately three to four times as much estrogenic material as do normal animals of the same age with intact ovaries (11). Enough estrogenic hormone was produced by those tumors in some strains to facilitate the appearance of mammary cancer. Woolley, *et al.* has observed that adrenal tumors among castrated mice of one strain produce androgenic responses in their hosts (67).

Among animals receiving estrogenic hormone for long periods of time irregularities of the adrenal cortex are extremely common. Localized areas of proliferation may penetrate the capsule of the adrenal gland and grow into the adjacent connective tissue (Fig. 25). These changes in the adrenal cortices are particularly prevalent among those strains and hybrid groups which acquire pituitary tumors when treated with estrogen. So far, however, none of the adrenal tumors developed to the stage attained by those appearing in castrated animals (unpublished).

Degenerative changes appear frequently in the perimedullar zone of the adrenal glands of mice (10). A number of years ago this zone of degeneration was designated "brown degeneration" and was associated with the tendency of mice of certain strains to have mammary cancer (Fig. 24). However, when an incerased number of strains of mice were added no correlation could be found between the incidence of mammary cancer, although definite strain differences in the extent of perimedullary degeneration existed (4). Also amyloid degeneration frequently occurs in the perimedullary region of the adrenals in old mice, but no relation existed between the tendency for amyloid degeneration to appear and the tendency to develop cancer of the mammary glands.

VII. Mammary Tumorigenesis

Studies undertaken many years ago by Loeb (47) demonstrated the relationship between the ovaries and the tendency for mice to have mammary cancer. Mammary cancer in mice is limited to females and occurs rarely in females of some stocks if they are castrated early in life. Later it was found that male mice bearing ovarian transplants also acquire mammary cancer at about the same incidence as virgin females. Subsequent to the original investigations of Lacassagne (41) a number of investigators have observed mammary cancer among male mice given pure estrogenic chemicals (9, 14, 21, 33, 42, 48). It was found, however, that mammary cancer did not develop in male or female mice of all strains following the injection of estrogen. Those strains in which mammary cancer does not appear spontaneously do not acquire a high incidence of tumors, even when treated with estrogen. In other words, the tendency for mammary cancer to develop is limited in part by genetic or transmitted factors or influences.

Investigators at the Roscoe B. Jackson Memorial Laboratory (37) first reported that females tend to transmit the tendency to have mammary cancer whereas males are relatively unable to transmit this tendency. Bittner (3) then discovered that the tendency to have mammary cancer is transferred to the young while suckling; the milk carries a mammary tumor in-

citer or influence. The inciter is active in genetically susceptible animals, but of very limited activity in animals that are not genetically susceptible to mammary cancer (2). Thus, in mice, three etiological factors in mammary carcinogenisis must be considered, namely, (1) the hormonal influence, (2) the genetic constitution of the animal, and (3) the maternal mammary tumor inciter transmitted through the milk. The hormonal influence will be considered here, the genetic and the mammary tumor influence will be mentioned where it is necessary.

Detailed studies of the mammary glands of mice susceptible and resistant to mammary cancer have been made, and differences of mammary structure have been associated with the tendency to have mammary tumors. The mammary glands of tumor-resistant animals are uniform throughout; they may present variable stages of ductal and aveolar development but within the same animal one gland or portion of a gland does not differ significantly from another gland or another part of the same gland (Table VI) (29).

TABLE VI

Number of Nodules in the Mammary Glands (A) at Different Stages of the Estrous Cycle; (B) in Different Strains; (C) in Different Age Groups (29)

	Number of nodules					Total mice
	None	1 to 3	4 to 9	10 to 27	Over 27	
(A) Stage of cycle						
Diestrus	3	5	2	4	5	19
Proestrus	3	—	2	1	1	7
Estrus	3	2	—	1	1	7
Postestrus	2	1	1	1	1	6
(B) Strain						
CBA	1	4	—	3	1	9
N	8	1	—	—	—	9
A	1	1	3	2	—	7
D	1	—	1	2	4	8
C_3H	—	3	—	1	4	8
(C) Age of animal						
100-150 days	3	3	—	—	—	6
200-250 days	5	4	3	3	2	17
300-350 days	3	1	2	5	7	18

The mammary structures of old tumor-susceptible mice differ from region to region; localized nodules of alveoli or ducts and alveoli are found from place to place along otherwise atrophic duct systems. Some of these nodules contain alveoli lined by a secretory type of epithelium, some nodules are composed of small hyperplastic alveoli and ducts, and others are composed of more or less adenomatous glandular epithelium. In some of these nodules small areas appear that are histologically identical with the larger adenocarcinomas. It is believed that these localized hyperplastic nodules are early stages in the process of abnormal growth that lead to malignancy, in

other words sequentially related, but it is not impossible that they are diverse manifestations of the action of similar etiological agents. Other nodules show squamous metaplasia of the parenchyma, and, usually associated with it, round cell infiltration of the stroma. It is thought that this represents a degenerative state and that nodules at all stages of development may regress, not all of them progressing to malignant growths.

A somewhat similar clinical history of mammary carcinogenesis occurs in rabbits in that a stepwise series of abnormal types of mammary growth precede the assumption of adenomatous and eventually malignant or autonomous disease (31). The onset of malignancy among both mice and rabbits is thus commonly antedated by abnormal but non-malignant growths. Some mammary tumors, however, may arise without obvious preliminary abnormal growth.

Subsequent to the injection of estrogenic hormones, mammary glands of mice from the tumor-resistant strains show variable amounts of development; but they are structurally uniform throughout as are the glands of the normal females of such strains (12) (Fig. 26). Many nodules appear in the mammary glands of estrogen-treated mice from strains that are genetically susceptible to mammary tumors and that have the milk-borne mammary-tumor inciter (Figs. 27, 28). In addition to the discrete nodules mentioned previously as occurring in the untreated animals, large, diffuse adenomatous growths appear that arise from one point along the branching ducts (12, 27) (Fig. 32). As in the untreated mice, it is from these localized hyperplastic nodules that the smallest histologically identifiable adenocarcinomas appear (Fig. 30). Engorgement and distention of the mammary ducts with a concretion-like secretory material occurs in both the estrogen-treated, mammary tumor resistant and susceptible animals (Fig. 28, 29). The cystic condition of the ducts, which may extend into some of the smaller alveolar processes, cannot be associated with the malignant change (12). It is possible, by injecting males with estrogenic hormone, to increase the incidence of mammary tumors so that it is comparable to that occurring in normally breeding females of similar stocks. Whether estrogen does any more than to develop the mammary glands to provide a morphological structure from which cancer can develop or whether it has some direct influence in the instigation of the carcinogenic process otherwise, is not known. Certainly it cannot dominate in the absence of a proper genetic environment and in the absence of the mammary tumor inciter.

By reciprocal mating of male and female mice of a tumor-resistant and tumor-susceptible strain it is possible to obtain genetically identical hybrid offspring (in the absence of sex-linked factors), and to have one group of animals with the maternal mammary tumor inciter and one group without it (60,

37, 60). The young that receive the maternally transmitted mammary-tumor inciter, both males and females, acquire mammary cancer when estrogens are injected, and the breeding females acquire mammary cancer spontaneously (Ref. 60 tabular summary). Interestingly, more tumors usually appear among the breeding females than appear among the estrogen-treated animals, especially among those animals that do not possess the mammary tumor influence (12) (Table VII).

TABLE VII

The Incidence of Mammary Tumors Among Estrogen-Treated Male and Female Mice and Among "Force-Bred" Controls of Several Reciprocal Hybrid Groups

Hybrid group*	No. of estrogen-treated mice	No. with mammary tumors	Average survival	No. of control females	No. with mammary tumors	Average survival
HC_1 (C_{57} x $\underline{C_3H}$)	105	0	392	37	13	661
HC_2 ($\underline{C_3H}$ x C_{57})	115	55	302	42	38	300
PC_1 ($\underline{C_3H}$ x PM)	86	27	312	43	41	337
PC_2 (PM x $\underline{C_3H}$)	81	3	316	39	0	591
A_{71} (A x C_{57})	107	31	288	36	35	300
A_{72} (C_{57} x A)	98	1	358	31	7	635
JC_1 ($\underline{C_3H}$ x JK)	60	23	385	39	38	391
JC_2 (JK x $\underline{C_3H}$)	96	1	352	40	10	372
AB_1 (A x CBA)	93	61	311	42	41	261
AB_2 (CBA x A)	72	23	301	37	30	393
AC_1 (A x $\underline{C_3H}$)	108	78	254	33	31	292
AC_2 ($\underline{C_3H}$ x A)	114	67	288	35	35	238

*Strain designation underlined contributes mammary tumor inciter. The first strain mentioned denotes the maternal parent.

Many tumors that develop in mice without the mammary tumor inciter are histologically different, for the greater part, than those that appear in mice with the inciter. Many of them, although they attain the diameter of a centimeter or more, grow slowly, show extensive necrotic areas, and extensive epithelial metaplasia (12). They usually appear in very old animals. It is possible that there are two types of tumors of the mammary glands in mice; one type which is induced by the mammary tumor inciter in the proper environment and one type which is independent of the known mammary tumor inciter. Experiments reported recently from several laboratories indicate that carcinogenic hydrocarbons induce tumors of the mammary glands of animals that have no genetic or maternal influence for the acquisition of mammary tumors (6, 40, 55, 63). Some of these tumors differ histologically from those occurring among the tumor-susceptible mice.

Mice have been treated with several different dosages of estrogens; amounts that are adequate to promote the growth of mammary glands may

still be inadequate to induce a very high incidence of mammary tumors in mice otherwise susceptible. Mice receiving very large doses of estrogen (50 μg. of estradiol benzoate weekly, for example) acquire few mammary tumors, and usually have smaller mammary glands than do animals that receive smaller amounts (16.6 μg. weekly) (15). Large amounts of estrogen inhibit the usual development of the mammary glands (Figs. 31, 32). It is possible that they also inhibit the malignant transformation of the mammary glands or fewer tumors occur (1), because the mammary glands are smaller and afford less substance for malignant change, or (2) because the animals are in relatively poor condition due to the "toxic" effects of the treatment.

Mice of the tumor-susceptible strains that have received estrogen and testosterone propionate simultaneously show fewer mammary tumors than animals of similar origin that are given estrogen alone (20, 43, 44, 46); the glands are smaller than those of their estrogen-treated controls and show few localized hyperplastic nodules (20) (Figs. 33, 34). Although animals so treated survive for a long period of time, the incidence of mammary tumors is low (Tables VIII, IX). The mammary inhibiting influence of testosterone when given with estrogen is of particular interest because both of the substances when given alone induce growth of the mammary glands. The amount of testosterone necessary to reduce the incidence of mammary cancer, and the length of time over which it must be given, would preclude its use in the prevention of mammary cancer.

Several investigators have observed a lower incidence of mammary tumors in female mice of tumor-susceptible strains when testosterone propionate is injected, especially when the treatment is started early in life and before the age at which spontaneous tumors appear (34, 38, 49, 51) (Table IX). It is probable that the reduction of the tumor incidence in such animals is due to the ovarian inhibition coincident with the treatment as well as to the effect of the androgen.

1. *Influence of the Pituitary on Mammary Growth, Nodules, and Tumors.*

TABLE VIII

Influence of Simultaneous Administration of Estrogen and Testosterone upon the Incidence of Mammary Tumors in Mice of Different Strains

		Estrogen and testosterone			Estrogen		
Investigator*	Strain	No. of mice	No. with tumors	Age when tumor appeared	No. of mice	No. with tumors	Age when tumor appeared
Lacassagne (44, 43, 46)	RIII	37	20	—	—	—	—
Gardner (20)	C_3H	180	17	424	118	51	325

*Several different levels of estrogen and androgen were used and treatment was started at different ages.

TABLE IX

Incidence of Mammary Tumors Among Control and Androgen Treated Female Mice of Several Different Strains

		Testosterone treated			Control		
Investigator*	Strain	No. of mice	No. with tumors	Age when tumor appeared	No. of mice	No. with tumors	Age when tumor appeared
Nathanson and Andervont (51)	C_3H	20	6	11 months	20	20	11 months
Jones (38)	C_3H	12	3	15-24 months	38 V	18	—
		16 B	16	9 months	16 B	16	9 months
Loeser (49)	A	10	—	—	12	8	10-14 months
Heiman (34)	C_3H	108	21	—	486	52%	—

*Several doses of androgen were used in the different experiments grouped together here. Treatment was started at different ages and continued for irregular periods of time. B-mice had been pregnant. V-mice were virgin.

Mammary tissue stops growing and involutes in the absence of the hypophysis. It is of interest to determine whether or not the hypophysis is necessary for the proliferation of the localized hyperplastic nodules. Because they grow in an environment that is inadequate to maintain growth of the adjacent mammary tissue, it is assumed that factors might be essential for their maintenance other than those that are required for the maintenance of normal mammary tissue. Mice with mammary glands containing numerous hyperplastic nodules were hypophysectomized, and removed at variable periods subsequent to hypophysectomy (16). Localized hyperplastic nodules were found subsequent to hypophysectomy, although the number was probably reduced. Some nodules had at least attained a state at which they were independent of hypophyseal control. Increased numbers of degenerating nodules appeared in the glands of such animals, indicating that at least a portion of the nodules required the presence of the pituitary for their progressive growth. Mammary tumors in hypophysectomized animals continued to grow, and in several instances new tumors were first detected subsequent to hypophysectomy. Histologically the tumors were not particularly different from those of intact controls.

2. *Mammary Tumors in Estrogen-treated Animals of Other Species.* A number of investigators have observed mammary tumors in rats subsequent to estrogen injection. Such tumors do not or rarely develop in untreated animals of the stocks used. Prolonged periods of treatment are required and large doses of estrogen are much more effective than smaller doses (30, 52, 54). In some experiments the mammary tumors regressed subsequent to the discontinuance of estrogen-treatment and were transplantable only when the hosts were treated (54). Even when estrogen was

administered the simultaneous injection of progesterone resulted in regression of the tumors (53). A number of other studies have been made on the action of steroid hormones on the growth characteristics of transplanted fibroadenomas in rats (14, 33, 42, 48, 60).

Monkeys and guinea pigs given large amounts of estrogen for long periods of time have failed to acquire mammary tumors (21).

VIII. Summary

Estrogenic and genetic factors (mammary tumor inciter also in mammary carcinogenesis) are of interdependent etiological significance in at least four types of experimentally induced tumors in mice of different strains (Table X). These tumors are of lymphoid, mammary, testicular and hypophyseal origin. The evidence for significant differences in the genetic influence on the action of estrogens on tumors of the uterine cervix in mice is not definite at this time.

The tendency for mice to have mammary cancer is transmitted by the female through the milk (mammary tumor inciter, Bittner) to genetically susceptible male and female mice although estrogenic stimulation, intrinsic or extrinsic in origin (also some unknown hypophyseal hormone, possible lactogenic hormone) is required for their development. A series of abnormal types of growth characterized by localized proliferation of mammary tissue at scattered sites in the glands precedes and accompanies the assumption of malignancy. Recent experiments indicate that mammary tumors are induced also by carcinogenic hydrocarbons in the presence of well developed mammary glands, and without a known mammary tumor inciter.

TABLE X

*A Summary of the Types of Tumors Appearing Among Mice of Different Strains when Subjected to Estrogenic Hormones.**

Strain	Mammary tumors**	Testicular tumors	Lymphoid tumors	Pituitary tumors	Uterine cervical tumors
A	+++	+++	—	—	++
C_3H	++++	—	+++	—	+++
CBA	+++	—	+++	—	+++
C_{57}	—	—	—	+++	+
JK	—	++	±	—	?
PM	—	—	++	±	++
$C_{12}I$	++	—	±	—	++

*Mammary tumors are the only tumors that occur spontaneously with frequency in mice of these strains and then only among female mice, whereas they appear in estrogen-treated males. Testicular or pituitary tumors have not been found in untreated controls. Lymphoid tumors appear in a low percentage of mice of all strains and uterine cervical tumors only among the PM strain of mice.

**++++ indicates a relatively high susceptibility and — no significant susceptibility to the tumors mentioned. +++, etc. indicates intermediate degrees of susceptibility.

The tendency of estrogen-treated mice to have lymphoid tumors is transmitted by both male and female mice of the susceptible strains to their hybrid offspring. Hybrid young obtained from reciprocal crosses of two strains susceptible to "estrogen-induced" lymphomas showed an augmented incidence of lymphomas. The rôle of estrogens in lymphomagenesis is completed during the first ten weeks of treatment. Large doses are more effective than small ones.

Pituitary tumors (chromophobic adenomas) appear frequently among estrogen-treated mice of the C_{57} strain (rarely among mice of other strains). Both male and female mice of the C_{57} strain transmit the tendency for their estrogen-treated hybrids to acquire such tumors.

An endocrine imbalance instituted by castration of mice of some strains is associated with the appearance of adrenal tumors that produce hormones acting on the male or female accessory reproductive organs.

The incidence of tumors appearing in estrogen-treated mice with the possible exception of the uterine cervical tumors, is reduced or the age at the time of appearance increased by the simultaneous administration of the androgen, testosterone propionate. The incidence of mammary, pituitary and lymphoid tumors that appear among mice given adequate doses of testosterone and estrogen have been studied most extensively.

Untreated mice of a strain (PM) in which the several females exhibited imperforated vaginas and a high incidence of sterility have shown several cervical or vaginal carcinomas.

By proper hybridization, mammary, lymphoid, testicular and hypophyseal tumors arise in mice of the same group; actually three different tumors have been found in the same animal. An antagonism does not exist between the tendency to acquire pituitary and testicular tumors, for example.

Discussion

F. Beach: Certain strains of mice tend to develop mammary cancer and hyperplasia or tumor of the adrenal cortex following removal of the ovaries; and Smith has reported many females subjected to prepuberal ovariectomy show in adulthood signs of cyclic estrogenic stimulation. The vagina opens postoperatively and the vaginal epithelium exhibits rhythms of cornification. Have you noticed any indication of a vaginal cycle in the mice which you describe?

W. U. Gardner: I think we must assume that some adrenal tumors and hyperplasias produce estrogen or a hormone having estrogenic activity. It is interesting that, as Dr. Beach said, the adrenal may produce estrogen or estrogenic material subsequent to castration in mice, and that in mice some cycles will occur. I would assume that there might be a relationship between the adrenal and the pituitary in much the same way that there is between the ovary and the pituitary. It is interesting that some adrenal tumors produce androgenic hormone. Dr. Woolley has stated that those arising in one strain produce predominantly androgen, and those arising in another

strain produce estrogenic hormone. The adrenal tumors that we have observed produced estrogenic hormone.

With regard to behavior of the animals, after administration of testosterone propionate the animals became pugnacious, and not only were they willing to bite if molested, but they were willing to go out of their way occasionally to do so. In a few testosterone-treated female mice we have seen scrotal-like development, particularly in mice of the A strain.

G. Pincus: I have been especially struck with the very thorough review given by Dr. Gardner and would like to express my admiration for his marshalling the data. One point particularly seems of special significance, and perhaps Dr. Gardner would develop it further, that is, the inhibitory effects of large amounts of estrogen particularly on mammary tumors. Is the induction of leukemia more likely in animals receiving such amounts? Again, can the effect of androgen, particularly testosterone, on mammary tumors be attributable to the estrogen derived from the metabolic turnover of the androgenic steroid? It has been shown by a number of investigators (originally by Dr. Koch) that there appears in the urine of individuals receiving testosterone fairly good amounts of estrogen. Is it at all possible that the inhibiting effect of testosterone might not be due to such derived estrogen, added to estrogen already given for mammary tumor induction? I notice that you get very small glands in some of the testosterone-treated animals, and apparently you have rather poorly developed or less well-developed glands in the animals receiving large doses of estrogen. I should like to ask whether any studies of estrogen excretion have been made in animals receiving these various doses of estrogen? Is there any qualitative difference in the types of estrogen excreted by these rats, in these contrasting types of experiments?

W. U. Gardner: At first we thought that one of the reasons for the decreased incidence of mammary tumors among animals receiving large amounts of estrogen was the increased incidence of leukemia. On following that idea further, however, the data available does not indicate that to be the case. The larger amount of estrogen an animal received, providing it is compatible with the continuance of life, the higher the incidence of leukemia. Of course this statement applies only to those strains in which estrogens may induce lymphoid and mammary tumors.

The mammary glands of animals receiving both estrogen and testosterone propionate consist essentially of ducts which extend over a small area and show no alveoli, whereas the glands of the estrogen-treated mice are larger, and after prolonged treatment have variable amounts of alveolar development. Mice given very large doses have small but completely developed glands and a good many of them show evidence of lactation. They are completely developed mammary glands. If I might theorize, I might assume that with large amounts of estrogen, the mammary cells become secretory and their growth potentialities have been impaired. I rather doubt that the decreased incidence of tumors of the mammary glands of estrogen-androgen-treated animals can be explained by assuming that it has an additive effect on the estrogens, because testosterone propionate will, by itself, grow mammary glands, but it requires almost as much per day to induce mammary growth in the mouse as was given per week in the experiments to which reference was made. I doubt that even if some conversion did occur the additional amount of estrogen formed from testosterone would be sufficiently large, unless, in the presence of estrogen, the metabolism of androgen is modified. Qualitatively, also, the type of mammary gland developed in the animals given large doses of estrogen is different from that of the estrogen-androgen-treated mice. Unfortunately, no experiments have been done on the excretion of hormone by these animals.

F. C. Koch: Coming from Chicago, I naturally am interested in the difference in reactions by the different strains. I have in mind the hereditary studies of Dr. Maud Slye. I am wondering whether you can give us any information as to whether these different strains which show such different susceptibilities to the development of tumors also show differences in other reactions to hormones. Do these rats when spayed show differences in reactions to estrogens and when normal do these different strains show consistent differences in reactions to estrogens and gonadotrophins? Are they more susceptible to hormones as well as to susceptibility to developing cancer? Are the general physiological reactions different?

W. U. Gardner: Concerning the gonadotrophins I can say nothing. The several strains show differences in the minimum amount of estrogen required to induce vaginal cornification. For instance, an ovariectomized mouse of one strain will respond to one unit of injected hormone while this amount will be inadequate or excessive in castrated animals of other strains. These experiments have been reported by other investigators. The differences in sensitivity to minimal doses of estrogen cannot be associated with the tendency to develop mammary tumors. Furthermore, a number of years ago it was noted that in mice of one tumor-susceptible strain there was a relatively high ratio of days showing estrus in the cycle, compared to days of diestrus. Additional studies have shown no consistent correlation between the duration of estrus and the tendency of mice to have cancer. So far there is no indication of any unusual level of estrogenic hormone production that can be associated with cancer, although there are strain differences.

I. T. Nathanson: We have a few data which might be pertinent to this discussion. The first is the effect of testosterone on the incidence and growth of mammary tumors developing in mice of a high tumor strain. Female mice of the C_3H strain, born at approximately the same time and kept under similar experimental conditions, were used as test animals. All were bred at maturity and the litters were sacrificed after delivery which occurred between four and four and a half months of age. They were then divided equally so that there were sister litter mates in each group. One group received testosterone propionate, 0.6 mg. three times weekly for four months, and the control series received only the vehicle during the same period of time. Of the treated group, only 30% developed spontaneous mammary carcinomas. All arose within four months and were single tumors. All of the control animals (100%) developed one or more tumors by the eleventh month of life. The mechanism by which the tumor incidence was decreased may be explained as follows: Testosterone inhibits certain secretions of the anterior pituitary which in turn leads to ovarian atrophy. As a result the breasts undergo atrophic changes since they are dependent upon ovarian and pituitary secretion. In other words tumors cease to develop when there is no active breast tissue. Testosterone *per se* has no effect on the growth of spontaneous mammary carcinoma in C_3H mice. It is assumed, therefore, that those tumors which did develop in the treated group were microscopic when treatment was commenced and were not influenced in their growth rate.

Another study was concerned with the excretion of estrogens and 17-ketosteroid-like material in the urine of high (C_3H) and low (C_{57}) mammary tumor strain mice. As far as could be determined no significant difference could be detected between the strains in the excretion of these substances. The estrogenic output was of the same order of magnitude as that reported by Dorfman and Gardner. Therefore, judged from urinary excretion rates there is as yet no evidence that high mammary tumor strain mice form or metabolize estrogens or 17-ketosteroid-like material in any significantly

different fashion from a low tumor strain. Estrogens are to be regarded as an essential but not a specifically carcinogenic factor in the development of spontaneous mammary tumors in mice.

In recent years strong evidence has been presented which indicates that nutrition of the host is an important factor in the development of certain tumors in mice. Balanced diets but lower in calories than those taken by the controls results in a considerable decrease in the incidence of these tumors. The simple addition of fat to these diets will significantly increase the incidence of these tumors. In our experience, the administration of estrogen as compared to testosterone impairs the nutrition of animals treated over a long period of time. Therefore, I would like to stress the necessity of giving attention to the nutrition changes in the animal, in evaluating the effect of any agent or in the induction, incidence or growth of animal tumors.

W. U. Gardner: I think at this time we might add a fourth factor as essential for mammary cancer in mice. We then have genetic, mammary tumor agent, humoral and nutritive factors, influencing mammary carcinogenesis. Recently we have undertaken some investigations on the growth of the animals given large amounts of estrogen to inhibit mammary growth. In some experiments undertaken with Dr. White, saline extracts of pituitary tissue containing growth hormone were administered with estrogen; amounts of the pituitary hormone adequate to maintain growth. The estrogen-treated animals were on the same diet as the untreated controls and the animals receiving estrogen plus a saline solution of the pituitary grew as rapidly as the untreated mice and both grew more rapidly than the estrogen-treated mice. The mammary glands of mice receiving estrogen alone showed as much growth as the estrogen-plus-pituitary-treated mice. If it is assumed that growth represented good alimentation on the part of the animals receiving the pituitary hormone, the mammary growth was no greater in the mice that received the pituitary extract. It seems as if growth of the mammary glands is specifically inhibited by large doses of estrogen and inhibited independently of its effect on general body growth. One thing that I think one has to be very careful about in the growth study or nutrition experiments is that reproductive behavior is considered. Castration will reduce the incidence of tumor. If the animals are starved or on a reduced caloric intake, one of the earliest reactions is that reproduction stops. I should not be surprised, under such circumstances, that few mammary tumors occur.

In Tannenbaum's study on reproductive activity in animals on restricted caloric intake the animals were starved so that they became cannibalistic, eating their young, but pregnancy still occurred. Nevertheless the number of tumors was reduced.

H. B. Friedgood: In his scholarly interpretation of the experimental work done on steroid hormones in carcinogenesis, Dr. Gardner has given us some very important data on the problem of therapy. There are several other points which one might consider insofar as that aspect of the problem is concerned. The first is concerned with an experiment which Hisaw carried out some ten years ago with castrate female monkeys. As I recall it he produced metaplasia of the cervical mucosa with hormone therapy and was able subsequently to make it disappear with progesterone. Whether that work has been carried further, I do not know. It would be interesting to have Dr. Gardner give us his opinion on this matter.

A second point which may be of interest in this connection is the occurrence of fibromyomata of the uterus after long continued estrogenic therapy in guinea pigs. Is this observation applicable to the pathogenesis of this condition in humans?

Finally, I would call your attention to some experiments which we did some time

ago. We were working with a group of hens which became virilistic spontaneously. All of these hens, which, as you know, have only a left ovary, showed complete destruction or atrophy of this gland. The destruction was due either to an extensive hemorrhage or tuberculosis. The atrophic ovary on the right side was found to be the site of a tumorous growth. These "tumors" were not malignant according to definition. Histological study of the latter revealed that some of the "tumors" were composed of epithelial elements which were organized into tubular structures similar to those of the normal testicle. Others were composed of more primitive testicular structures reminiscent of the arthenoblastomas described by Meyer and others. So far as we could tell, the "tumors" resulted from the following sequence of events. Destruction of the left ovary probably brought about hyperactivity of the adenohypophysis, which then stimulated the atrophied medulla of the right ovary. Since the latter is related embryologically to androgenic tissue, it seems feasible to attribute the virilism to a secretion from the medullary tissue of the right ovary. Subsequent experiments confirmed this impression in that the injection of hypophyseal gonadotrophic hormones reproduced the same virilistic syndrome in otherwise normal hens.

W. U. Gardner: Concerning progesterone therapy, I recall again a great amount of work to which reference was not made, work done by people in this room. I think Dr. Noble could answer your questions better than I. It is extremely interesting and significant that in rats Dr. Noble and his associates found that progesterone prevented the formation of tumors in estrogen-treated animals or caused the regression of tumors already growing. We have tried similar experiments in mice and it has not worked. The mouse and the rat differ in that respect. Why these strange variations should occur, I think should add significance or at least interest to the problem; it should certainly multiply the problems for further analysis. I agree that the work of Dr. Lipschütz is very interesting indeed. I think these tumors should be called fibromas rather than myofibromas. I have seen a number of them that Dr. Nelson observed in this country, some that Dr. Dessau had in Holland and some of those that Dr. Lipschütz had. It is interesting that these tumors occur in guinea pigs and that the only other species in which they occur frequently is man. These proliferative tissues look histologically malignant; they are completely prevented by adequate treatment with progesterone and testosterone. Progesterone, I believe, is found to be the most active inhibitory substance. As I have said, they are invariably reversible upon discontinuance of treatment. They arise, as nearly as I can tell, from the mesothelium or from the immediate sub-adjacent tissues.

R. L. Noble: I might add a word on the question of progesterone antagonism which is one of clinical importance. Experimentally in rats it is possible to give progesterone and observe some regression of mammary tumors. In our experiments we used a local strain of hooded rats. In these rats after implantation of oestrone pellets, a very high percentage developed multiple mammary tumors. Histologically it was difficult to tell whether the tumors were simply extremely hyperplastic adenomas or malignant growths. Out of some 60 animals there were four or five tumors which appeared to be definitely infiltrative and from that criteria seemed to be malignant. Metastases occurred in the lungs in one case. We were unable to transplant the tumors into normal rats or rats bearing oestrone pellets. Transplants into the back of the same animal having the mammary tumors, however, would grow. These tumors regressed rapidly on removal of the pellets of oestrogen and apparently you cured this condition by removing the stimulus. Similarly it was found that, if you inject progesterone in large doses and leave the pellets intact, tumor regression occurred, but in this case it was

not so marked or so rapid as when the pellets were removed. To satisfactorily settle the question as to whether these tumors should be considered as truly malignant would require further work. Until that time I feel one cannot state with certainty that either removal of the pellet or injection of progesterone causes regression of an experimental malignant mammary tumor.

E. P. McCullagh: Dr. Gardner's very interesting paper brings to mind the question of fertility in the human male which is sometimes of considerable clinical interest. The protein-like hormones which stimulate the testes are not always effective in producing spermatogenesis. All mammals tend to become refractory to certain of the gonadotropins. Such refractoriness is not usually exhibited in the case of the steroids. We have been able to demonstrate that several androgenic steroids are capable of maintaining spermatogenesis in the hypophysectomized rat. It is possible that this observation could be of clinical interest. In regard to refractoriness to protein-like hormones and thinking of Prof. Collip's activity in the field of antihormones, I might mention that the testes of the mink are normally intra-abdominal except during the breeding season. Upon the injection of the serum of pregnant mares containing gonadotropins, the glands descend into the scrotum within a few days. However, continued injection will not maintain the glands in this position, which is a further indication of the development of resistance to this type of glandular treatment.

W. U. Gardner: We have certainly had abundant opportunity to confirm Dr. McCullagh. Testosterone propionate will maintain spermatogenesis in the absence of the pituitary. It will prevent in mice and pigeons, two species that we have studied, spermatogenic-inhibiting action of large amounts of estrogens; I am quite sure of that. Testosterone propionate when given with estrogen tends to decrease the incidence of testicular interstitial cell tumors but does not prevent them. Those that do occur also appear at a later age and after more prolonged treatment.

Concerning prostatic cancer in experimental animals little can be said. These tumors have been studied extensively in man and will probably be commented upon by Dr. Nathanson. In mice we have never observed a prostatic cancer. Urine retention is extremely frequent in mice and it may be sufficient to contribute to cause of death. The urethra is occluded in the cavernous region or at the membrano-cavernous junction rather than in the prostatic region. I have not been able to associate this with metaplastic responses of the coagulating glands or seminal vesicles in any way. Marked reactions of the seminal vesicle complex does not occur, however, in mice of all strains. I have never seen it in mice of the C_3H strain. The prostate of the estrogen-treated mice changes to a much slighter extent than that of the seminal vesicle. There is a decrease in the height of the epithelium and some curtailment of secretory activity.

We have treated dogs with estrogen for periods of over two and one half years, and the male dogs have invariably died with urinary retention but all with greatly enlarged prostates. The prostates showed extreme squamous metaplasia and cystic enlargement. The urethra was obliterated in the prostate region but as in the mice was occluded at the beginning of the cavernous urethra.

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FIG. 1

Section of a Small Infiltrative Lesion that Arose in the Lower Cervical Canal of a Mouse of the CBA Strain that had Received 16.6 μg. of Estradiol Benzoate Weekly Beginning at 2 Days of Age and Continuing for 70 Weeks. Stage I Lesion. × 100.

FIG. 2

Section Through the Lowermost Part of the Cervical Canal and the Vaginal Fornices of a Hybrid Mouse (C_{57}♀ x CBA♂) that had Received 16.6 μg. of Estradiol Benzoate Weekly for 70 Weeks, Beginning at 33 Days of Age.

The thin stratified epithelium of the cervix has given rise to a Stage II infiltrative lesion. × 25 approx.

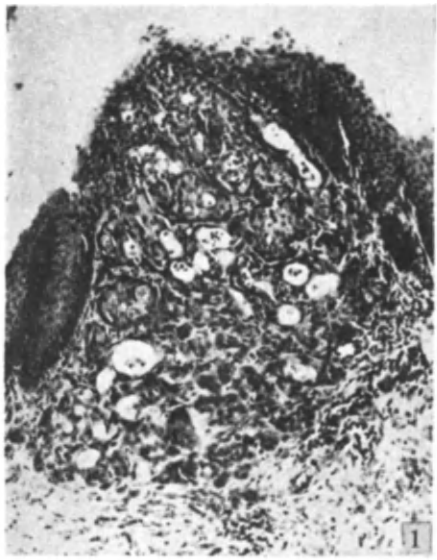
FIG. 3

Tumor of the Uterine Cervix of a Mouse of the C_3H Strain that had Received 22,000 I.U. of Estradiol Benzoate in 44 Weeks Beginning at the Age of 29 Days. Stage IV Lesion. × 240.

FIG. 4

Section Through the Lower Cervix and Vaginal Fornices of a Hybrid Mouse (C_{57}♀ x CBA♂) that had Received 16.6 μg. of Estradiol Benzoate Weekly in 77 Weeks Beginning at 38 Days of Age.

The fibrous stroma is lined by a low stratified squamous epithelium showing no evidence of infiltration. Compare with Fig. 3. × 25 approx.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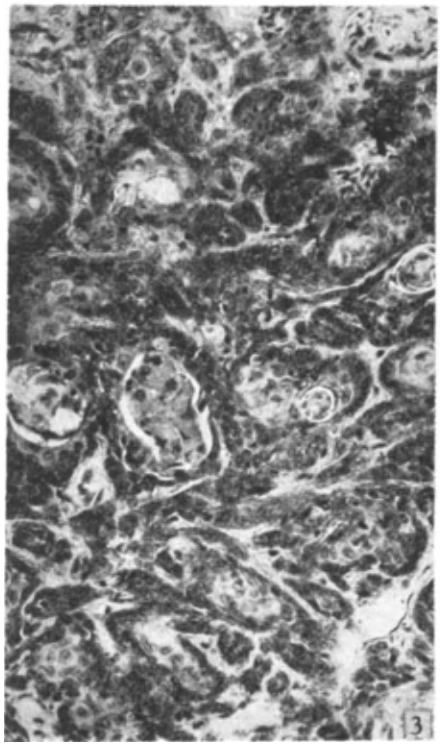
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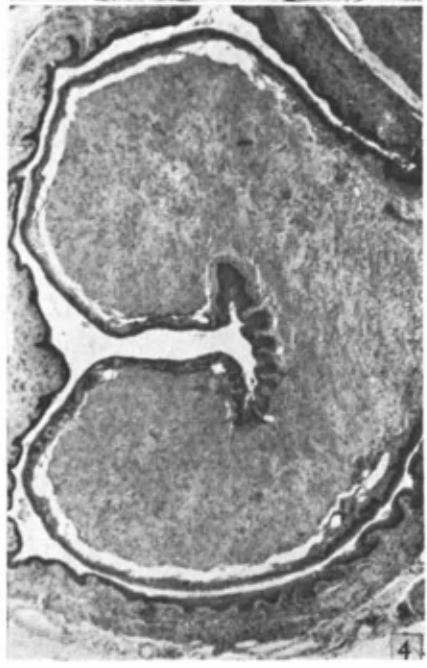
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FIG. 5

Section Through the Lower Vagina of a Hybrid Mouse (C_{57}♀ x CBA♂) Showing a Carcinoma of the Cervix or Upper Vagina that Involved the Entire Lower Genital Region (Stage IV).

The mouse had received 1.116 mg. of estradiol benzoate in 67 weeks—16.6 μg. weekly. × 8 approx.

FIG. 6

Stage IV Lesion, Possible Carcinoma, of the Uterine Cervix of a Mouse of the C_3H Strain that had Received 0.2 mg. Progesterone and 16.6 μg. of Estradiol Benzoate Weekly for 39 Weeks, Total 7.8 mg. and 0.63 mg. Respectively. × 90.

FIG 7

Epithelium of the Vaginal Fornix (lower right), Cervix, and a Small Infiltrative Growth (left) of a Mouse of the C_3H Strain that had Received 2.5 mg. Testosterone Propionate and 16.6 μg. of Estradiol Benzoate for 47 Weeks—Total 117.5 and 0.783 mg. Respectively. × 90.

FIG. 8

A Carcinoma of the Cervix of a C_3H Strain Mouse that had Received 107.25 mg. of Testosterone Propionate and 0.716 μg. of Estradiol Benzoate in 291 Days; 2.5 mg. and 16.6 μg. Weekly, Respectively. × 90.


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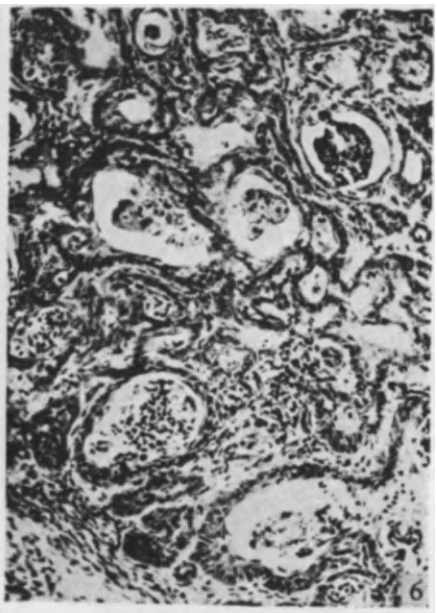
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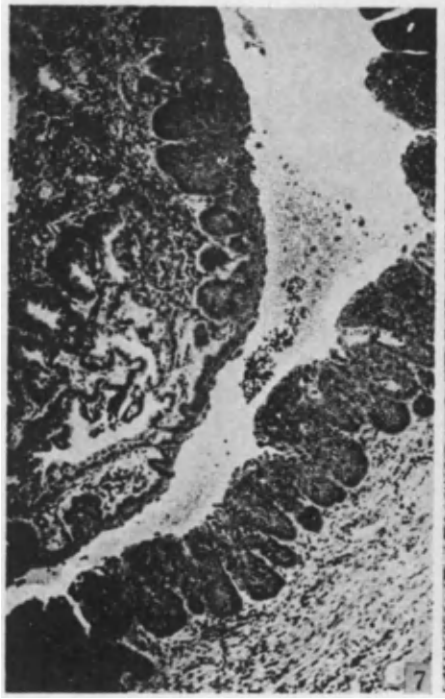
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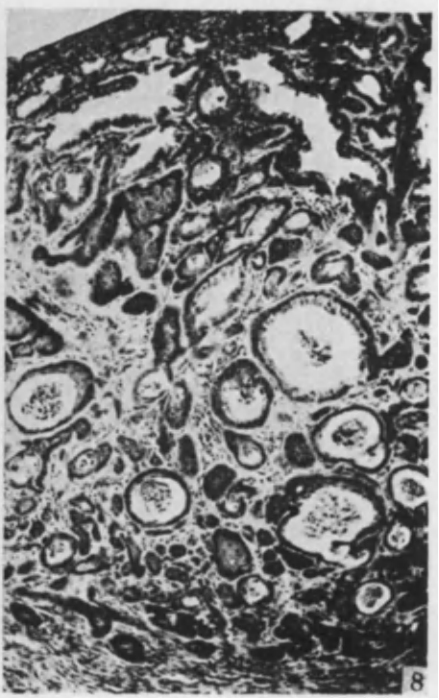
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FIG. 9

Cervical Mucosa of a C_3H Mouse that had Received 50 μg. of Estradiol Dipropionate Weekly for 25 Weeks—Total of 2.5 mg.

The stratified squamous epithelium shows a thin cornified layer. The fibrous stroma is poorly vascularized. × 90.

FIG. 10

Uterine Mucosa and Myometrium of the Above Mouse.

The mucosa shows a marked hyaline transformation of the stroma and the tall columnar epithelium, in places pseudostratified, shows local degeneration. × 180.

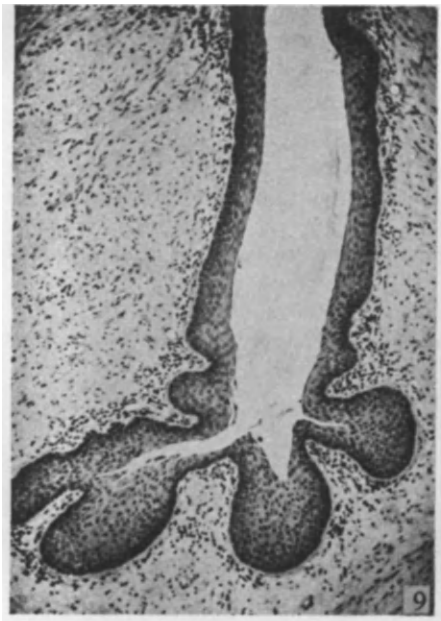
FIG. 11

A Section Through the Upper Vagina of an Untreated PM-stock Mouse 420 Days of Age Showing a Part of an Invasive Carcinoma of the Cervix or Upper Vagina. × 18 approx.

FIG. 12

Lymphatic Invasion of a Carcinoma of the Cervix or Upper Vagina of an Untreated PM-stock Mouse 625 Days of Age.

A portion of the anaplastic tumor appears at the lower border. × 180.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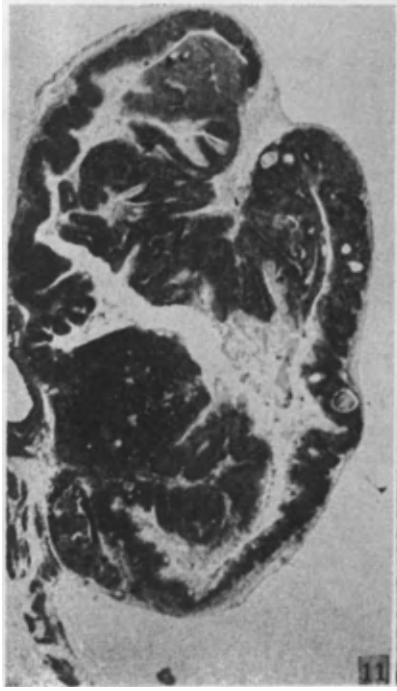
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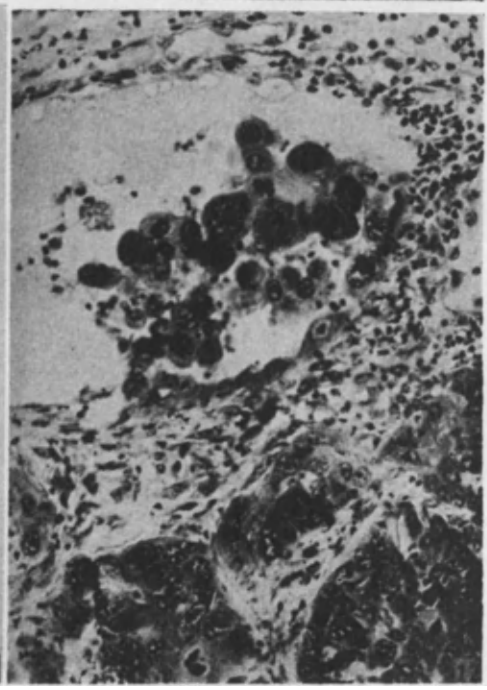


FIG. 13

The Testis of a Mouse of the JK Strain that had Received 280 mg. of Triphenylethylene in 398 Days.

The testis was nearly normal size, pale yellow, and contained a small nodule of Leydig cells. × 6.

FIG. 14

An Interstitial Cell Tumor Occupying the Greater Part of the Left Testis of an Estrogen-Treated A-Strain Mouse.

This mouse had been given weekly injections of 5 mg. of triphenylethylene for 340 days—total dose of 220 mg., 4 weekly injections were omitted.

FIG. 15

A Section of an Interstitial Cell Tumor in a Hybrid Mouse ($C_3H♀$ x $A♂$) Showing Large Closely Packed Interstitial Cells and Extension to Replace the Few Atrophic Seminiferous Tubules Persisting near the Border of the Testis.

Treatment: 50 μg. of estradiol dipropionate weekly for 45 weeks. × 90.

FIGS. 16, 17, 18

Opened Skulls of 3 Mice to Show the Pituitary Adenomas and a Hypertrophied Pituitary of Estrogen-Treated Mice. × 3 approx.

16. Hypertrophied pituitary of a CBA-strain mouse—weight 4.5 mg. The mouse received 16.6 μg. of estradiol benzoate weekly for 55 weeks.

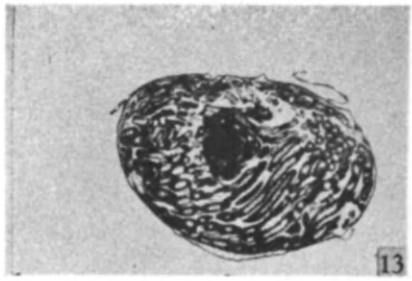
17. A hypophyseal tumor, weighing 87 mg. after several fragments had been removed for transplantation, that arose in a C_{57}-strain mouse. This mouse had received 0.813 mg. estradiol benzoate in 49 weeks—16.6 μg. weekly.

18. A hypophyseal adenoma weighing 53.5 mg. in a male mouse of the C_{57} strain. This mouse had received 0.96 mg. of estradiol benzoate in 55 weeks—16.6 μg. weekly.

FIG. 19

A Section of the Chromophobic Tumor of the Pituitary of the Mouse Described in Fig. 17 Above.

It consisted of hypertrophied and hyperplastic chromophobic cells arranged in dense cords.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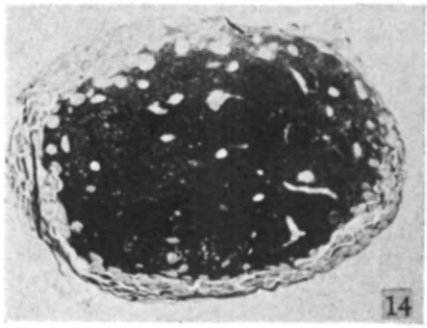
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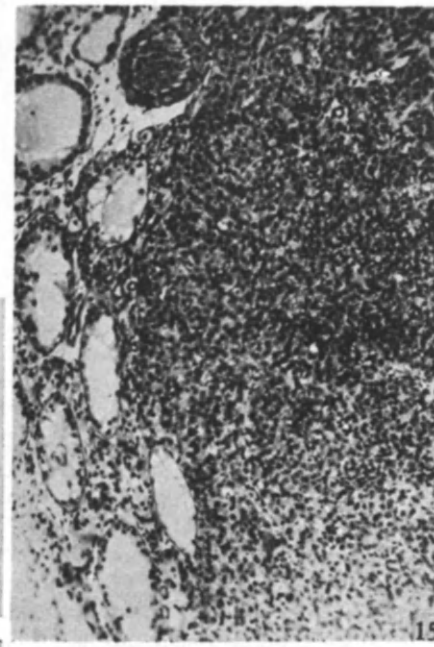
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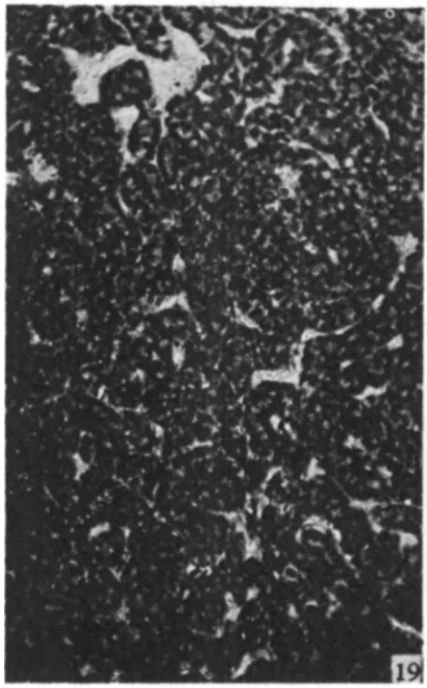
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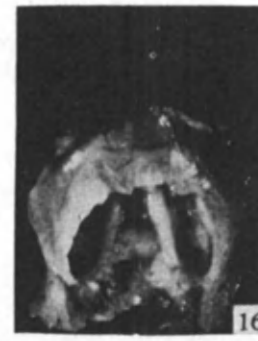
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FIG. 20

A Partially Dissected Mouse to Show a Mediastinal Tumor, Large Liver and the Greatly Enlarged Spleen and Lumbar Lymph Nodes of an Animal with Generalized Lymphomatosis.

The mouse had received 0.75 mg. of estradiol benzoate in 30 weeks, 25 μg. weekly.

FIG. 21

A Cross-Section Through the Mediastinum of a C_3H-Strain Mouse with a Large Lymphoid Tumor that had Extended into the Pericardium, Large Blood Vessels, Lungs and Intercostal Muscles.

This mouse had received 0.75 mg. of estradiol benzoate in 30 weeks, 25 μg. weekly.

FIG. 22

An Adrenal Cortical Tumor that had Arisen in a NH-Strain Mouse that had been Castrated at 43 Days of Age and Was Killed When 619 Days Old.

The greater part of the tumor consists of cords of small cells. At the periphery and scattered throughout the tumor some of the cells contain a vacuolated cytoplasm. × 25 approx.

FIG. 23

A Section of an Adrenal Cortical Tumor in a NH-Strain Mouse that had been Ovariectomized When 65 Days Old and Killed 607 Days Later.

The mouse had a mammary adenocarcinoma and showed other evidence of estrogenic stimulation. × 180.

FIG. 24

A Section of an Adrenal Gland of an Estrogen-Treated Mouse Showing Marked Capsular Hyperplasia and Perimedullary Brown Degeneration. × 90.


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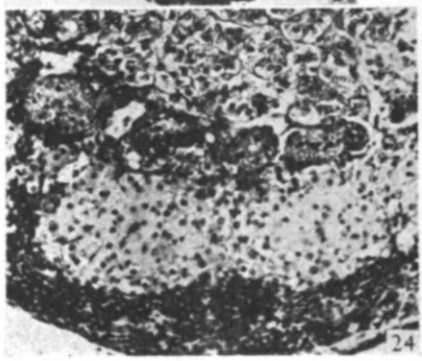
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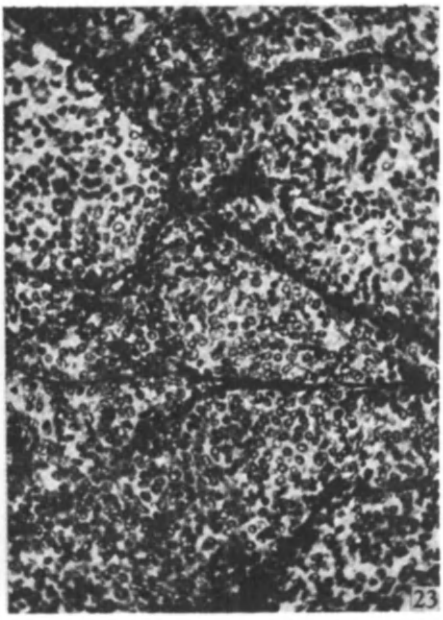
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FIG. 25

Photomicrograph Through the Center of an Enlarged Adrenal Gland.

This hybrid mouse (CBA x C_{57}) had received 1.05 mg. of estradiol benzoate (16.6 μg. weekly) and was killed when 528 days old. Normal cortical tissue is seen at the upper left and the adjacent tissue is medullary. An extensive overgrowth of cortical cells has greatly enlarged the gland. Such cortical overgrowths are especially common in mice with large pituitary tumors.

FIG. 26

A Mammary Gland of the Second Pair of a Female Hybrid Mouse (C_{57} x CBA).

There is no mammary tumor inciter, and the mouse had received 16.6 μg. of estradiol benzoate weekly during 492 days. No localized overgrowths of nodules form in the glands of such animals. Alveoli or lobules may or may not be present but are uniformly distributed when present.

FIG. 27

A Mammary Gland of the Second Pair from a Female Hybrid Mouse (CBA x C_{57}).

Mouse has mammary tumor inciter and had received 16.6 μg. of estradiol benzoate weekly during 417 days. Three localized overgrowths of mammary tissue occurred in this one gland.

FIG. 28

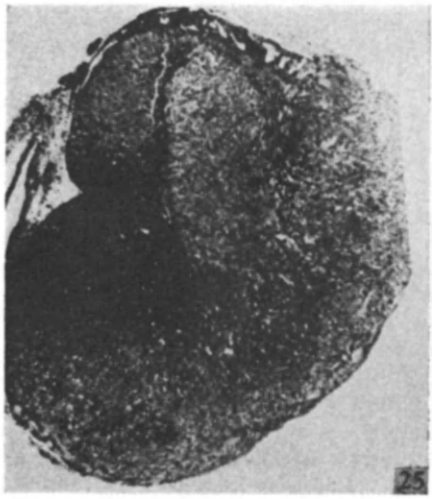
An Alveolar Nodule and Mammary Ducts of the Above Mouse (Fig. 27) at Higher Magnification.

Note the beaded appearance of the ducts. These intraductal nodules upon section sometimes show a concentric layering.

FIG. 29

An Area of the Mammary Ducts of a Mouse.

There is no mammary tumor inciter (C_{57} x CBA), and the mouse had received 1.13 mg. of estradiol benzoate in 481 days (16.6 μg. weekly). The ducts are distended with concretion-like accumulations in the same manner as those of mice with the tumor-inciter, but no mammary adenocarcinomas or hyperplastic nodules develop.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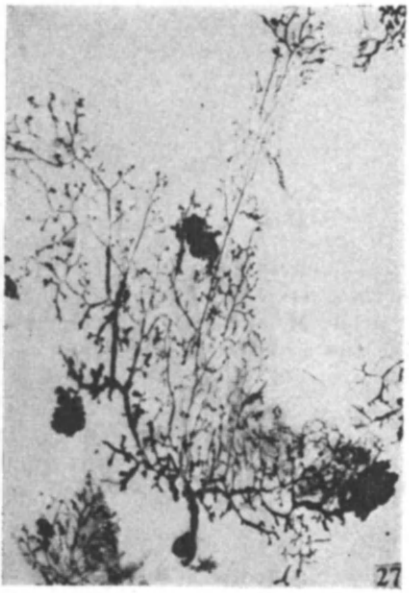
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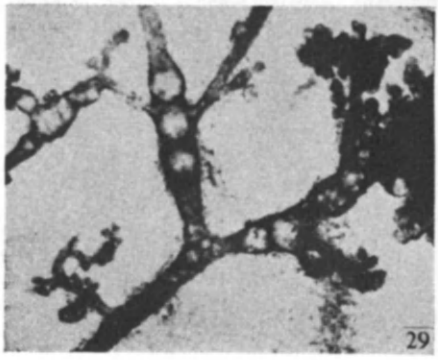
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FIG. 30

A Photomicrograph of One of the Mammary Nodules and Adjacent Mammary Gland. Hybrid Mouse has mammary tumor inciter and had received 1.38 mg. of estradiol benzoate in 583 days. This nodule is similar histologically to a mammary adenocarcinoma. × 90.

FIG. 31

Three Mammary Glands of a Male Mouse of the C_3H Strain.

The mouse had received 50 μg. estradiol benzoate weekly for 211 days (total of 1.5 mg.). The mammary glands are very small. Nodules are present.

FIG. 32

Mammary Gland of a Male Mouse of the C_3H Strain That had Received 25 μg. of Estradiol Benzoate Weekly for 255 Days (Total of 0.9 mg.).

The gland is larger than those of mice receiving larger doses (Fig. 31 above). This gland contains one diffuse adenomatous nodule.

FIG. 33

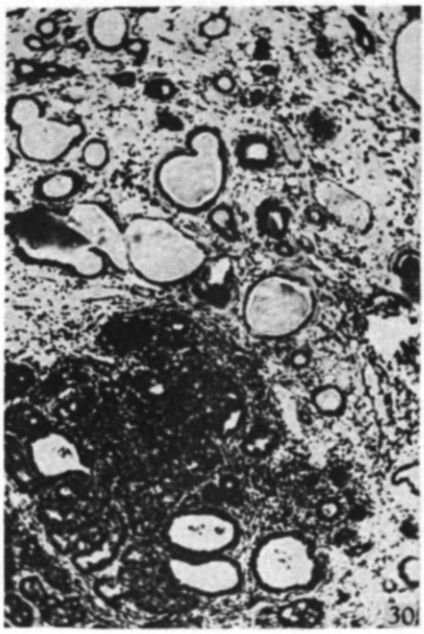
Mammary Gland of a Female Mouse of the Tumor-Susceptible C_3H-Strain.

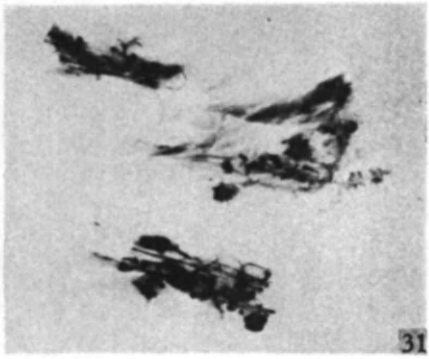
Mouse had received 1 mg. of testosterone propionate and 16.6 μg. of estradiol benzoate weekly for 320 days. The number of nodules and tumors is greatly reduced by the administration of the androgen, and the ducts are relatively atrophic.

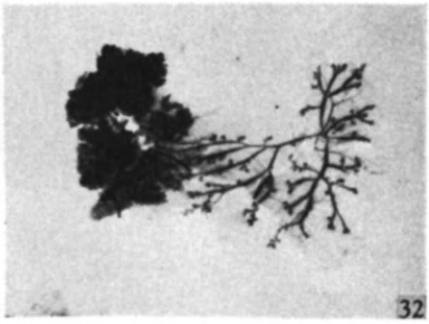
FIG. 34

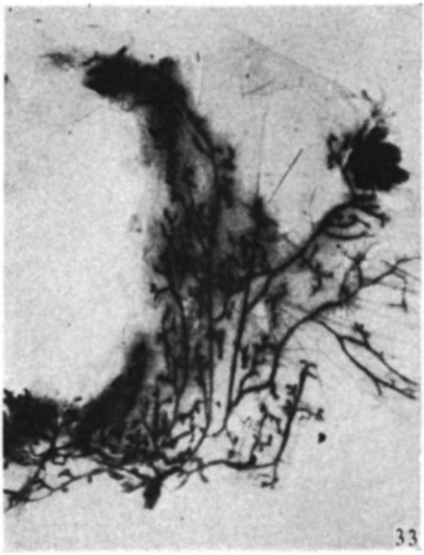
Four Mammary Glands of a Male Mouse of the Tumor-Susceptible C_3H Strain.

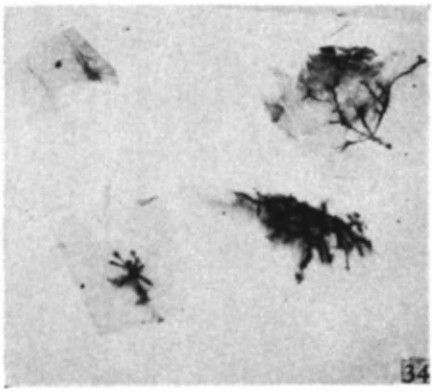
The mouse had received 53.75 mg. of testosterone propionate and 1.76 mg. of estradiol benzoate in 374 days. The glands are small and contained no nodules. Few tumors develop in such animals.











Endocrine Aspects of Human Cancer*

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I. Introduction

Early investigations concerning the relation of hormones to cancer relied chiefly on indirect evidence, for it was not until the isolation and identification of the action of certain of the sex hormones that the problem could be pursued on a more direct basis. It is now an established fact that organs such as the breast, uterus, and the prostate gland, which are common sites of cancer, are under endocrine control. Therefore, it appeared plausible that a causal relationship might exist between the hormones and the genesis of tumors of these organs. Moreover, a number of carcinogenic hydrocarbons that have been isolated or synthesized were found to have a basic chemical structure similar to that of cholesterol and the known steroids of the gonads and adrenal glands. Thus, experimental and clinical studies were further stimulated by the idea that atypical metabolism of these hormones might lead to formation of at least several types of cancer.

The present report is an attempt to summarize and evaluate some of the more significant aspects of the relation of the hormones to carcinogenesis in the human being. It is beyond the scope of this paper to cite animal experiments, particularly since these are thoroughly reviewed in another publication in this series.

II. Tumors of the Breast

1. Carcinoma of the Breast

The peak of age incidence in 2165 cases of cancer of the breast at the onset was about forty-seven years (76). The median age was fifty-two years, that is, as many cases occurred before that age as after it. Approximately a third of the patients were under forty-five years of age, another third from forty-five to fifty-five, and the remainder older than fifty-five. Taylor (107) found that about one-third of the cases occurred during the period of mature ovarian function, another third five years before and after the menopause, and the remainder in women whose ovarian function had ceased five years or more. Thus, the two studies are in

*This is publication number 621 of the Cancer Commission of Harvard University and number 117 of the Pondville Hospital.

agreement, but it must be pointed out that susceptibility to cancer of the breast steadily increases with age (76). Further data on the relation of breast cancer to the menopause were supplied by Olch (80). According to his studies 72 per cent of normal women pass through the menopause from forty to fifty years of age, whereas 55 per cent of those with cancer are still menstruating at the age of fifty. It was concluded that almost five times as many women with breast cancer had a delayed menopause as compared with normal women. The average age of the menopause is cited by most observers as forty-eight years. Since the median age in cancer of the breast is approximately fifty-two years (76), we may speculate further on a possible endocrinologic relationship. Ovarian dysfunction is common at the climacteric. As a result, the breast may be so altered as to provide a suitable substrate for the development of cancer. Data on the length of the menopause are not readily available. This may be of importance for, even though amenorrhea is one of the primary signs of the climacteric, the ovary may continue to be active and secrete estrogens for a relatively long period thereafter (75). Since corpus-luteum function is usually diminished or absent during the menopausal years, the normal cyclic change disappears and estrogen stimulation may continue unopposed. Cancer of the breast may appear many years after castration, even when the latter has been performed at an early age. Since estrogens presumably of adrenal origin are still recovered from the urine of patients who have been castrated, and from the urine of postmenopausal women (75) this may well be a factor in the development of breast disease. Herrell (39) reviewed the records of a large number of patients in the same age group with and without cancer of the breast. The findings disclosed that in the cancer group the incidence of complete ovariectomy before the tumor appeared was 1.5 per cent. The incidence in the noncancer group was 15.4 per cent, or ten times as great. These observations are in essential agreement with data obtained from animal experiments, but it cannot be conclusively assumed that the same mechanisms are necessarily operating in the different species.

It is not known whether women who develop breast cancer have a higher percentage of menstrual disturbances than those who are free of the disease (72). In one study, however, it was found that the menstrual pattern changed shortly before the discovery of the disease (107). So far as can be determined there is no gross effect of the menstrual cycle on the primary tumor. The rapidity of growth of the primary tumor during pregnancy (99) and the appearance of a tumor in the second breast during another pregnancy (111), which may ensue subsequent to treatment of the first lesion, is well recognized. Changes during lactation are usually

more striking (99). Whether these effects are caused by hormones elaborated in pregnancy or lactation or to the generalized metabolic changes has not been established (104).

Breast disease in general is frequently associated with disease or abnormalities of the uterus and ovaries, and in this respect cancer of the breast is no exception (72, 107). For example, Taylor (105) found that cancer of the breast and endometrium may arise in the same patient. This information suggests that the entire reproductive system may be subject to the same abnormal stimulus. Nulliparous women have a higher incidence of cancer of the breast than those who have borne children (54). This appears to be the reverse of the situation in the mouse. Some believe that early weaning of the child or faulty lactation conduces to the development of breast cancer, in that they result in stasis and breakdown products which may cause irritation (1, 54, 60). A study of 350 children of women with breast cancer revealed that 72 per cent were nursed for at least six months, and of the remainder a small number were deliberately weaned or not nursed because of inadequate milk supply (107). Further studies of lactation are indicated, since there is a possibility of an abnormal physiologic state in some patients that in itself may predispose to cancer.

It is the opinion of some that the syndromes described under the term chronic cystic mastitis are of endocrine origin. Moreover, it has been suggested that certain types of chronic cystic mastitis are precancerous. Observations in this regard have been based on the coexistence of the two diseases, the development of cancer in previously proved cystic disease, and the study of changes leading to cancer in the experimental animal and the human being. The important literature concerning this problem has recently been reviewed by Lewis and Geschickter (55), Warren (113), Logie (59), and Foote and Stewart (24). Their conclusions were based on an analysis of their own material as well. Of the recent writers, Lewis and Geschickter are not convinced that most types of chronic cystic mastitis are precancerous. Warren and the further statistical confirmation of Logie strongly support the contention of those who postulate a definite causal relationship between the two conditions. Foote and Stewart conclude that "statistical and morphologic studies indicate that chronic cystic mastitis does play a role in the development of human breast cancer. How large this is we cannot state. Our studies suggest that its part consists largely in those papillary hyperplasias which in some individuals, for reasons unknown, become cytologically atypical. In other words, some people cannot handle the hyperplasias which to others seem controllable and innocuous." The discrepancies may be partly explained by the criteria set up for the diagnosis of chronic cystic mastitis and the type of material used in the

studies. Moreover, in some of the investigations histologic confirmation of the clinical diagnosis was not carried out in all cases. The methods of analyses are also dissimilar. Warren's studies based on histologic examination demonstrate that when age specific rates are used, the breast cancer attack-rate for women with chronic cystic mastitis in the premenopausal age group (from 30 to 49 years) is 11.7 times as great as that of the general female population in the same period. Past the age of 50 it is 2.5 times as great and for the entire group 4.5 times as great. Corrections were made for chronic cystic mastitis in the normal population. These findings are significant, since the group with the highest incidence was that in which the ovaries were still active. Therefore, if chronic cystic mastitis is of endocrine origin, then it must be postulated that hormones are responsible at least in an indirect fashion for the development of cancer of the breast.

Castration was independently suggested by Schinzinger (95) and by Beatson (7) as an adjunct to the treatment of breast cancer. Many have since resorted to this procedure and in later years have substituted X-ray for surgical extirpation of the ovaries (3). Benefits obtained are more obvious in pre-menopausal patients. There is little evidence that castration is of much value in the post-menopausal group. A favorable response to castration is manifested by relief of pain, increase in appetite and weight, regression of bone metastases and occasionally pulmonary metastases. Effects on the primary tumor or lymph node metastases are unusual. However, any beneficial response is only transient. It has been concluded by those with an extensive experience that castration may be expected to result in temporary improvement in from 15 to 30 per cent of patients with advanced, recurrent or metastatic disease (2, 103). It cannot be demonstrated as advantageous when employed as a prophylactic measure in patients in whom the chance for eradication of the disease by accepted methods is favorable. In some cases, laboratory evidence reveals discrepancies regarding the efficacy of X-ray castration as compared to ovariectomy (75). X-ray was adequate in some cases as judged by the urinary estrogen excretion, but in others fluctuations in the output appeared, even though the patients were amenorrheic. Obviously in this latter group castration was incomplete so that the results observed in such cases are inconclusive.

Farrow and Adair (21) have recently reported a favorable response following orchiectomy manifested by a partial regression of the primary tumor and osseous metastases in a male with cancer of the breast. Since this first communication, additional information regarding the beneficial effect of orchiectomy in cancer of the breast has become available (2, 20, 74). Treves *et al.* (110) believe that orchiectomy for male breast cancer may parallel the striking results of the same procedure observed in advanced

cases of prostatic cancer. In contrast to the effect of castration in cancer of the female breast, no alteration was noted in a young patient, whereas the remainder of six patients, all of whom responded favorably, were 63 years of age or older. There can be no doubt that the changes resulted from removal of gonadal secretions. Whether this is the sole factor or whether the changes occur secondarily to an effect on other glands of internal secretion or to metabolic alterations must be considered.

A number of reports have appeared on the possible development of cancer in the breast after prolonged administration of estrogens (4, 5, 71, 81). There is no proof that this was the direct cause of the tumor, but there is certainly a possibility that it was contributory. With the large numbers of females who are now receiving estrogens over long periods for various endocrine disturbances, particularly the menopause, one would expect the development of more cases if the hormone were directly responsible. However, when the life span, the time, and the dosage required to produce the lesion in a susceptible mouse are translated into human equivalents, it appears that a long period of observation is necessary for a possible solution of the problem. In the case of Auchincloss and Haagensen (5) the effect of estrogens seemed to resemble that seen in the mouse, and was of an unusual histologic type. They have cautioned against indiscriminate use of the hormone over a long period of time or in large doses, when there is a family history of cancer, without initial and repeated examination of both breasts; and in patients with chronic mastitis, cancer, or any form of neoplasm either before or after surgical or radiation treatment. Such advice is valuable and should be considered by all those who use the hormones.

Attempts have been made to influence the course of cancer of the breast by the administration of hormones. It was found that carcinogenic hydrocarbons possessed the property of retarding the growth of normal and malignant tissues in experimental animals (34, 35). Since some of these compounds are similar in chemical structure to the estrogens, the latter are now the subject of investigation relating to cancer of the breast. Several of these substances have been used. Observations thus far indicate that they may cause a definite although apparently temporary partial regression of the primary tumor in some patients (8, 36, 70, 87). Ulcerations decrease in size or become epithelialized, masses become smaller, the skin changes in appearance and cicatrization has been observed. Histological evidence of degenerative changes in the primary tumor (36) and in the lymph node metastases (70) were also noted. Interestingly enough, these beneficial effects are confined for the most part to the post-menopausal group. For example, in a report of the Section of Radiology of the Royal

Society of Medicine: of 69 patients under 58 years of age, 43 were unimproved, and none showed spectacular improvement. Of 52 patients over 58 years of age, at least 17 had improved and 6 or 7 were said to have shown spectacular improvement (87). The writer's experience is similar to these observations (70).

In view of a possible relationship between estrogens and cancer of the breast, androgens have also been suggested as a treatment of the disease. Reports are few concerning this method of therapy (1A, 23, 58, 58A, 112A). At any rate, favorable responses have been noted in several patients. In general, the beneficial results have been obtained in younger patients. Prudente (84) has used androgens as a prophylactic agent to prevent recurrence in patients who have had a radical mastectomy. He presented data which indicates that there was a significant reduction in recurrent disease and an increase in life expectancy in patients who had axillary metastases. Farrow and Woodard (22) have noted that in the pre-menopausal group skeletal metastases are apt to occur early, whereas in the post-menopausal group development of skeletal metastases is relatively late. They have also reported a marked acceleration of osseous metastases after intensive androgen or estrogen therapy and have suggested that there may be a direct effect on the secondary deposits themselves. An alternative theory is that these changes are due to an indirect effect which by removal of certain mechanical barriers, possibly as a result of altered metabolism, permits the metastases to grow unhampered. They, too, have considered this possibility. This is suggested by their observations of a marked decalcification of bone and a rise in concentration of calcium in the serum and excretion in the urine.

These reports are exceedingly difficult to evaluate at present. Until these methods of treatment receive further trial under carefully controlled conditions in large clinics, the subject must be left entirely open. Nevertheless, several facts are of interest. Castration may be of benefit in pre-menopausal women, whereas, estrogenic hormones appear to be most effective in post-menopausal women. By contrast, orchiectomy seems to exert a more beneficial effect on cancer of the breast in men in the older age group. The effects of testosterone in relation to age of the patient cannot be evaluated at present, because of insufficient data.

a. Excretion Studies

To date there have been relatively few studies on the excretion rates of the various sex hormones in patients with cancer of the breast. The results obtained thus far may be listed as follows:

(1). *Gonadotrophic hormone.* There is no consistent alteration in the ex-

cretion of pituitary gonadotrophic hormone in patients with cancer of the breast. Zondek (120) has reported an increased excretion of the gonadotrophic hormone of placental origin in a pregnant woman with mammary cancer. He was of the opinion that it was due to the cancer as the values fell precipitously after radical operation in the eighth month of pregnancy. This finding in a single case is difficult to interpret.

2). *Estrogens.* Ross and Dorfman (90) made several determinations on each of four patients in the pre-menopause with breast cancer and could detect no significant variation from normal. These findings have been corroborated by the study of a larger series by Taylor and Twombly (108) and by Nathanson (73). The latter reported slightly lower values than normally found in some cases, whereas others had normal or slightly elevated daily levels. In general, there was no great deviation from normal, and although the average for the entire group was slightly below that of normal women of the same age, it was not considered significant. The excretion cycle of some of the patients appeared atypical in that the peak of estrogen excretion usually observed at the midmenstruum was either absent or delayed. The significance of such a configuration is equivocal. In cancer of the male breast, Yolton and Rea (117) have reported values comparable to that of normal males of the same age.

(3). *Androgens and 17-Ketosteroids.* Normal androgen values were found by Ross and Dorfman (90) in their series. The excretion of 17-ketosteroids in the patients studied by Taylor and Twombly (108) and by Nathanson (73) were usually within normal range, but in general averages for the groups were slightly lower than those usually found for similar normal individuals. Pearlman (82) has reported a lowered 17-ketosteroid excretion in most patients with any type of cancer; these findings are in accord with those of others (18). In the cancer of the male breast the androgen excretion has also been reported as normal (117).

Taylor and Twombly (108) studied the effect of various steroid hormones on the excretion rates of the sex hormones in individuals with and without cancer of the breast. They concluded that there was no striking difference although variations did appear in the cancer patient. Studies on patients in the menopause with cancer of the breast show essentially the same excretion rates as women in the same period without the disease.

Several conclusions may be drawn from these excretion studies. (1) Although variations do occur, there is no significant deviation from normal in the excretion rates of the sex hormones in women with breast cancer. (2) There is no proof that abnormal secretions of the hormones do not accompany or precede the development of the cancer, since excretion levels are not absolute indicators of secretory activity. (3) Carcinoma of the

breast may be independent of hormonal influence once it develops. (4) The findings do not prove that the sex hormones are not involved in the disease, but they do indicate that normal excretion values may be found in the presence of the cancer during the period of active ovarian function.

From the foregoing data, it can be concluded that there is no proof that the hormones are the direct cause of carcinoma of the breast. It is possible and likely in some cases that they are indirectly responsible, inasmuch as they may produce precancerous changes or may provide a suitable substrate so that another agent may act.

2. Fibroadenoma of the Breast

These tumors seldom arise before puberty or after the menopause. Generally, they are detected in the second and third decades. The lesions are usually slowly growing, and although they may pass unnoticed for a long time, it is possible that they arise in the early years after the menarche, when atypical menstrual activity is not infrequent. The disease is said to occur oftener in persons of a definite constitutional type, namely nulliparous women with a relative underdevelopment of the pelvic organs and breasts (107). The menstrual cycles are seldom abnormal when the patients are first seen, although some give a history of a period of abnormal cycles following the menarche (72). Definite changes have been observed, however, in the size and the histologic appearance of the tumors during the menstrual cycle (51) and during pregnancy and lactation (28, 53). Estrogens have been given over a long period to patients in the presence of and following the removal of fibroadenomas (72). No new tumors have been observed that could be attributed to the hormone, since additional lesions frequently arise spontaneously. The significance of excretion studies of the sex hormones are as yet equivocal (72). Observations suggest that the origin of fibroadenomas may be explained partially on the basis of endocrine dysfunction. It is also possible that they arise as a result of atypical stimulation, especially since they usually occur when the metabolism and secretion of the sex hormones are at their height and may be abnormal.

III. Tumors of the Uterus

1. Cancer of the Cervix

Cancers of the cervix arise more frequently in multiparous women than those who have not borne children. Hofbauer (41) advanced the theory that the increased incidence of cancer of the cervix in this group resulted from intensive hormone stimulation during pregnancy rather than from birth injuries. The median age of cancer of the cervix is about forty-nine years (115). Thus, it is not infrequent to find the disease in women passing

through the menopause, when the symptoms may be attributed to the climacteric syndrome. It is of interest that Meigs (63) has noted the absence of hot flashes in patients with cervical cancer who have received radiation therapy. Cancer of the cervix is occasionally associated with cancer of the breast and myomas of the uterus but less so than is carcinoma of the endometrium (106). In our clinics, 3 women have developed metaplasia of the cervical epithelium after estrogen administration. The lesions regressed after treatment was discontinued in two patients, but in the third case an early cancer was diagnosed, which was subsequently confirmed by hysterectomy. Other observers have reported the development of cervical cancer in 3 of 43 patients treated by estrogens for senile vaginitis (27). Yet other investigators failed to find such lesions after treating over 200 women with estrogens over periods varying from a half to five and a half years (26). In a considerable number of patients cervical polyps have developed after estrogen administration to post-menopausal women and in the presence of granulosal-cell tumors. Squamous metaplasia is seen fairly often in cervical polyps, but factors such as infection and trauma seem to play a role. The development of carcinoma in a cervical polyp is rare. Hormone excretion studies to date are few, and no conclusions can be drawn from them (106). Hence, at the present time, the relation of these cancers to the estrogenic hormone is indeterminate.

2. Hyperplasia of the Endometrium

This lesion can be produced with comparative ease, especially in the absence of the ovaries in animals (119, 121), and in women (118), particularly after the menopause. It arises spontaneously at the menopause in patients in whom ovulation is apparently deficient and in the presence of granulosal-cell or thecal-cell tumors of the ovary. These tumors frequently give rise to elevated titers of estrogenic hormone in the urine. Investigators are of the opinion that the hyperplasia is produced by continuous unopposed stimulation by estrogens, probably as a result of ovarian dysfunction in which the balancing effect of the corpus luteum is absent, *i.e.*, the normal endometrial cycle with its rest periods disappears. These assumptions are based on studies in animals (56) and in patients in whom persistent large unruptured follicles have been found in the absence of a recent corpus luteum (96). Thus an excess of estrogens appears to be unessential, especially since it has been shown that hyperplasia is not produced as easily in animals with estrogens if progesterone is administered simultaneously. The histologic changes vary from a proliferation of the endometrium to those simulating early adenocarcinoma (106). The fact that these effects may be reversed by treatment with androgens and progesterone (98) or following castra-

tion by either radiation or surgery further incriminates estrogens as the initiating factor. According to Taylor (106), the origin of cystic hyperplasia of the human endometrium as a result of unopposed stimulation with estrogens on the basis of ovarian dysfunction may be regarded as established. He further points out that the finding of endometrial hyperplasia in conjunction with a tumor of the reproductive tract is presumptive evidence that estrogens are of some significance in the origin of the neoplasm as well. Regarding endometrial hyperplasia arising in the years after the menopause, the explanation is more difficult. As already pointed out, the estrogenic hormone may be found in varying amounts in the urine long after the menopause. Excretion studies of urinary estrogenic hormone suggest an increased urinary output, but these need confirmation (106).

3. Cancer of the Endometrium

This type of cancer is less frequent and usually occurs later in life than cancer of the cervix, suggesting that it may arise on a relatively senile endometrium. The median age in 331 cases was sixty-one (115). According to statistical studies the disease is somewhat more frequent in nulliparous women and in those with a late menopause. Crossen and Hobbs (13) reported that 60 per cent of women with endometrial cancer continued to menstruate after the age of 50, whereas only 15 per cent of women without the disease were still menstruating beyond this age period. Randall's (86) findings are in accord with the previous authors. He states that approximately 75% of the patients with endometrial cancer develop the disease within 15 years after a spontaneous menopause. In order to carefully evaluate his data, therefore, he chose for controls only those patients without the disease who were examined 16 or more years after spontaneous cessation of the menses. It was found that only 8 per cent of the controls menstruated beyond the fiftieth year as compared to 36 per cent of those with endometrial cancer. On the other hand others (61, 109) have not found a significant increase of the disease in women who have a late menopause. Randall (86) has presented data which indicate that when postmenopausal bleeding occurs, women having menorrhagia during the climacteric have three and a half times greater chance of developing cancer than those in whom there is no menorrhagia. It is possible that in some patients the menorrhagia was due to fibroids. He also calls attention to the infrequency of hot flushes and atrophy in the vulvar and vaginal tissues of women who have post-menopausal bleeding as a result of carcinoma of the endometrium, *i.e.*, endometrial carcinoma is rare in women whose signs and symptoms suggest deprivation of the estrogenic hormone. In this regard Herrell (40) reported that he had been unable to find carcinoma of the

endometrium in a castrated woman, although Smith (101) found three cases of this type of cancer occurring 15 years or more after oophorectomy. Several cancers of the endometrium have apparently arisen after long continued administration of estrogens (62). As in cancer of the breast this is difficult to evaluate and may be coincidence. It would appear that more cases would have come to attention, considering the sensitivity of the endometrium at any age to the hormone and the large number of women who are receiving estrogenic therapy for various reasons. Time may be an important factor and perhaps at a later date the probability of such an occurrence will be better defined. Nevertheless, there are now a number of cases reported in which endometrial cancer has been found in association with granulosal-cell tumors of the ovary (63, 106). The incidence of cancers of the endometrium found in patients with this type of ovarian tumor is said to vary from 10 to 20 per cent. Smith (101) made the observation that a high percentage of post-menopausal ovaries of patients with endometrial cancer reveal varying degrees of thecal-cell hyperplasia, the incidence of which was higher than that in the ovaries of elderly women with other conditions. Jones and Brewer (52) in most of their post-menopausal patients with endometrial cancer found the ovaries small and an absence of follicles in the great majority. Taylor (106) points out that in view of the rarity of the tumor* it is hardly conceivable that the associated endometrial lesion was merely coincidence. He states, therefore, that in these particular cases it is difficult to deny the sequence of increased estrogen stimulation, hyperplasia of the senile endometrium and, finally, cancer. Since endometrial hyperplasia is part of the picture, it is of interest to investigate the significance of this lesion as a precancerous change. Endometrial hyperplasia is fairly frequent, but endometrial carcinoma is relatively rare, and although some believe that hyperplastic changes are a predisposing factor, long-term studies of these cases have shown that few patients eventually develop cancer (106). Furthermore, even though cancer is sometimes found arising in association with endometrial hyperplasia, some are of the opinion that such an occurrence is purely accidental. It is of interest that Smith (101) was unable to find a commensurate proliferation in the sections of endometrium available in patients with associated thecal-cell hyperplasia and cancer of the uterus. Herrell (40) noted that in 96 per cent of cases there was a persistent proliferative type of endometrium, which was thought to be due to the unopposed action of the estrogenic hormone. Jones and Brewer (52) concluded that hyperplasia and anovulatory cycles did not often precede endometrial cancer developing in women before the menopause. In post-climacteric

*Granulosal-cell tumor.

women with cancer of the fundus, the endometrium was atrophic in all but a few instances. It must be remembered, however, that as in the case of the relation of chronic cystic mastitis to cancer of the breast, agreement concerning the histologic criteria is of importance. Fibromyomas of the uterus are frequently associated with cancer of the endometrium (9, 61, 63, 79, 106). It has been suggested that fibroids may contribute to the etiology of endometrial cancer (114). However, the consensus of most investigators indicates that endometrial cancer develops as frequently in non-fibroid as in fibroid uteri. Randall's recent studies (86) confirm this point of view and he states that the presence of fibroids does not predispose the individual to the development of endometrial cancer. Fibromyoma of the uterus is a common lesion. Nevertheless, the relatively high incidence of fibroids in patients with endometrial cancer which usually develops at a much later age, suggests that both processes may in part be related to a common dysfunction, possibly of endocrine origin. Finally, it should be pointed out that cancer of the endometrium is more frequently associated with cancer of the breast and fibromyomas of the uterus than is cancer of the cervix. Other observations of interest which may be factors in the development of endometrial cancer are those of Corscaden (12), who noted that the disease appeared to be more common in women with wide hips and short fingers and those of Scheffey *et. al.* (93), who found a high incidence of diabetes.

Hormone excretion studies of significance in this disease have not been reported.

It appears from the accumulated data that carcinoma of the endometrium may be related to endocrine dysfunction. The significant findings for the most part have been observed in post-menopausal women, since possible etiologic factors in the younger women are generally obscure. Factors other than endocrine are probably involved. Nevertheless, the facts at hand demand further investigation regarding the role of hormonal influences in this type of tumor.

4. Fibromyomas

Many believe that fibromyomas of the uterus result from ovarian dysfunction. Numerous attempts to induce fibromyomas in such species as mice, rats, and rabbits have been unsuccessful. The guinea pig, however, is singularly susceptible in that these tumors may be induced simply by the injection of estrogenic hormones (57, 68, 78), thus demonstrating an excellent example of species susceptibility to tumor formation. Evidence for an endocrine dysfunction as an etiologic factor in the production of fibroids in the human female is controversial. Witherspoon (116) was impressed by the association of follicle cysts of the ovary and endometrial hyperplasia with

fibroids, and suggested excessive estrogenic stimulation as a cause of the latter two lesions. He was of the opinion that hyperplasia was an early manifestation and was followed by the development of fibroids if the stimulus was continued for a longer period of time. Brewer and Jones (10) failed to find an unusual incidence of cystic changes in the ovary and stated that ovulation and corpus luteum formation occurred in the same order and frequency as in normal women. Meyer (64) stressed that patients with fibroids menstruate normally, undergo typical cyclic changes and become pregnant, all of which is against ovarian function as a cause of the disease. A certain percentage of fibroids regress or shrink after the menopause or castration, suggesting at least a partial dependence upon ovarian function. This may be also related to the blood supply, inasmuch as fibroids with vascular adhesions to extrapelvic organs may continue to grow after the menopause even with atrophy of the ovaries and uterus (106). There are reports on the regression of fibroid tumors after the administration of testosterone and progesterone, but these are difficult to assess. A study of the excretion rates of the sex hormones in this disease has failed to reveal any gross abnormalities (25). Thus far, the only convincing evidence for a hormonal etiology is the regression of the tumors following cessation of ovarian activity and the information obtained from studies on a single species of experimental animal.

IV. Tumors of the Prostate Gland

1. Hypertrophy of the Prostate Gland

Benign prostatic hypertrophy, that is, nodular hyperplasia, occurs in men over forty years of age. The disease increases with advancing age, so that between the ages of eighty and ninety, 75 per cent of men have some degree of nodular hyperplasia (65). Obstructive symptoms, however, reach a maximum at about sixty-three years. As suggested by Moore (65), this may mean that the etiologic agent becomes active after forty years of age, reaches its maximum soon after sixty and then decreases in intensity, but still remains capable of producing additional or new nodules. Some statistics indicate that benign hypertrophy is less frequent in single men (31). Nodular hyperplasia of the prostate does not occur in the posterior lobe. Moszkowicz (69) showed that the structure of the prostate in the pseudohermaphrodite is dependent on the type of gonad present: the prostate is composed only of the middle and lateral lobes in the presence of ovaries, whereas in the presence of testes the entire prostate is found. These observations indicate that the posterior lobe is primarily a masculine organ, whereas the other lobes are ambisexual, that is, they can be stimulated by

both estrogens and androgens (65). There is no direct relation between the histologic appearance of the testes and benign prostatic hypertrophy. A survey of the literature by Moore (65) revealed, however, that, in a series of 28 eunuchs, eunuchoids and those with pituitary infantilism in whom the secondary sex characteristics were lost before the age of forty, there was not a single case of the disease, even though they all lived to be over forty-five years of age. The incidence would be quite high in a similar age group in the normal population. Hence, it appears that the disease does not develop in the absence of the testes.

The changes in the prostates of patients with testicular tumors are not consistent. Cases occur in children in whom signs of adrenal insufficiency are accompanied by those associated with adrenal hyperfunction, particularly the masculinizing syndrome. In these children there is a hyperplasia of the prostate gland similar to that occurring in adults with functioning adrenocortical tumors (16). In primary Addison's disease, the prostate does not differ from that in normal subjects (65). Estrogens and androgens have been used in the treatment of benign prostatic hypertrophy, and although beneficial effects are said to occur, the consensus at present is that they are infrequently of any value (37). Castration has also been reported as beneficial, but this too is controversial (49). Excretion studies of the sex hormones suggest a decrease in androgens and estrogens in patients with hypertrophy of the prostate gland as contrasted with the levels in normal men of the same age (17, 67). Thus these observations, although not conclusive, suggest an endocrine dysfunction as a possible etiologic factor in the disease.

2. Cancer of the Prostate Gland

The median age at the onset in 235 cases with cancer of the prostate was sixty-five (115). It is rare below the age of thirty and increases steadily with advancing age. In fact in the eighth decade of life it is one of the most frequent of all carcinomas (19). Prostatic cancer is rare in the Chinese (42) and has not been recorded in eunuchs or eunuchoids (65). Routine autopsy studies of the prostate in man reveal an incidence of 10 to 15% of latent carcinoma (66, 88). In one series of unselected consecutive autopsies on 50 men over fifty years of age, however, the prostate was examined by serial section and the incidence of occult carcinoma was 46 per cent, although prostatic carcinoma was not the cause of death in any case (6).

Although castration was previously suggested and occasionally used as a treatment for cancer of the prostate, it was not until the excellent work of Huggins and his group (45, 47) on the effect of the hormones on the pros-

tate and that of the Gutmans (33) on acid phosphatase that investigation of the hormonal relation to the disease received a real stimulus. The prostate in childhood contains small amounts of acid phosphatase, but the level rises considerably after puberty (32). This enzyme, which is present apparently in large amounts only in the prostate, seems to be directly related to the secretory activity of the gland (30). Serum acid phosphatase levels are frequently elevated in patients with cancer of the prostate when the tumor has grown beyond the capsule of the gland and particularly in those with bone metastases (33, 38, 89, 102). Serum alkaline phosphatase levels are also above normal in many cases in which osseous metastases are part of the clinical picture (33). Excessive acid phosphatase can be demonstrated by histochemical examination of neoplastic prostatic tissue (50).

Castration and injection of estrogens produce little change in the acid phosphatase level if it is normal before such therapy (14). Castration and injection of estrogens, however, into patients with prostatic cancer having marked elevation of the acid phosphatase result in a sharp reduction of the levels of the enzyme, but seldom to normal (46). Administration of androgens produces a sharp rise in the serum acid phosphatase (46). Alkaline phosphatase following castration or injection of estrogens into patients with metastatic disease from cancer of the prostate gland, may rise, show no change, or decrease, either at once or after a latent period (14). A rise in alkaline phosphatase levels following castration indicates increased osteoblastic activity and is frequently evidence of a favorable response. These observations have been of inestimable value in the diagnosis of the extent and recurrence of the disease.

Castration or the administration of estrogens, or both, produce apparent regressions of the prostatic lesions and their metastases in many patients (11, 14, 50, 85). Serial biopsies of the tumor reveal marked degenerative changes in the cells when stilbestrol is given over a fairly long period of time (94). Administration of androgens seems to increase the activity of the process (43, 46).

Excretion studies of the sex hormones in the untreated cases before and after castration or hormone administration are of interest. In general, the 17-ketosteroid excretion is low, but it is consistent with that of normal men of the same age group. Following castration a decreased estrogen excretion has been reported, whereas the 17-ketosteroids after a slight initial fall may rise above the pre-treatment level (14, 92, 97). Pituitary gonadotropin increases in the urine following castration, as is the case in menopausal or castrated women or in men who have undergone the climacteric. Administration of estrogens alone or in conjunction with castration may result in no

change or a decrease in the 17-ketosteroid excretion; a decrease in the urinary gonadotrophic levels; a considerable increase in the estrogenic output (14). Thus it appears that the means by which these two methods of therapy produce their similar beneficial effect may be different. On the basis of excretion studies of the sex hormones, one may speculate regarding the mechanism involved. Estrogens in sufficient dosage appear to exert an inhibitory effect on certain functions of the pituitary gland, which in turn results in a decreased stimulation of organs such as the gonads and adrenals, which may produce androgens. Corroborating this point of view are the findings of Nelson (77) who reported that repeated biopsies during stilbestrol treatment indicated a decline in the apparent activity of the interstitial cells of the testes. Orchiectomy, although removing one source of androgens, may permit increased pituitary activity, thus stimulating organs such as the adrenal gland to increased androgen production. This supposition is supported by the rise in 17-ketosteroids noted subsequent to castration. Since recrudescence of prostatic cancer usually occurs following an initial improvement, these findings point to an extra-gonadal source, possibly the adrenal, as an important factor in the control of the disease. Therefore, if the adrenal is actively producing androgens, removal of the testes is not sufficient to eliminate androgenic stimulation. The lack of rise of 17-ketosteroids following estrogenic therapy indicates that estrogens may be one method of combating adrenal overactivity. Against this, however, are the facts that patients treated by estrogens as a primary procedure have reactivation of the disease and that orchiectomy following ineffective estrogen therapy may be followed occasionally by improvement in the individual.

Concerning the therapeutic value and marked clinical improvement following castration or estrogen administration, or both, there can be no doubt. It is still unsettled which form of therapy is the best. Castration or estrogens alone or in combination either simultaneously or successively all have their adherents. The greatest hopes for the treatment, however, have not materialized. Certain cases do not respond to treatment at all, whereas others show striking clinical improvement and relief from pain. It was originally thought that the difference in response might be related to the histologic type of tumor, *i.e.*, those tumors resembling the adult gland appeared to be more responsive (43). However, in the final analysis no significant difference was observed (44). Most patients who benefit from castration or estrogenic therapy have recurrence of the signs and symptoms within a few years and many are not relieved for more than a few months (14, 43, 100). Some, however, live without symptoms or obvious signs of activity of the disease for long periods of time, but as yet there is no definite evidence of true curability. Thus, on the basis of present evidence, castra-

tion or hormone therapy should be reserved for palliative purposes only in advanced or recurrent cases and should not be resorted to as the primary treatment in the operable prostatic cancer without metastases. It must be remembered also, that cancer of the prostate varies in its course and degree of malignancy so that the untreated patient or those receiving palliative surgical measures may live for a long time without benefit of any other form of therapy (115).

It is agreed by most observers that the cases of greatest interest are those which fail to respond to treatment or are those which do not show a sustained improvement after an initial favorable response. As was stated above the adrenal gland has been implicated as a possible source of an agent responsible for reactivation of the disease. Of considerable interest in this regard is the recent paper of Huggins and Scott (48). They reported on the results in four patients who had bilateral adrenalectomy following failure to control the prostatic cancer by orchiectomy. Three patients died within a short period of time, but the fourth survived for 116 days, as a result of increased knowledge in the maintenance of these patients. The 17-ketosteroids fell to almost zero, and urinary androgens were absent. There was a continued excretion of small amounts of estrogen. There was a significant sustained reduction in alkaline phosphatase levels, with little change in the acid phosphatase. There was a transient improvement but the disease continued to progress, although at an apparently retarded rate until death.

The foregoing information indicates that the sex hormones may be intimately concerned with the production and control of prostatic cancer. Regression of the disease following castration or anti-androgenic measures implies that androgens are necessary food substances for certain prostatic cancer cells, and that without such nourishment they may deteriorate. The phenomenon is one of the first concrete demonstrations that an alteration of the status of the host has brought about a regression of a malignant neoplastic process. It indicates that certain types of tumors may be dependent on hormonal, chemical or metabolic influences. Thus, at least as far as some cases of cancer of the prostate are concerned, the concept of autonomy of the cancer cell must be revised.

V. Tumors of the Testes

Tumors of the testicle are essentially diseases of the young. Approximately 75 per cent of the lesions arise in patients under the age of forty, and the average age incidence is about thirty-five (112, 115). In other words, these tumors appear at a time when the male is most active sexually. This age incidence is distinctly different from that of most types of malignant neoplasm and suggests other etiologic factors. It is also well recog-

nized that tumors of the testicle occur more frequently in men with undescended testes. Gilbert and Hamilton (29) found that in 345 of 835 reported cases of teratoma of the testicle the lesions were intra-abdominal. Eleven per cent of the 345 occurred in pseudohermaphrodites. This does not mean that when unilateral cryptorchidism is present the tumors always appear in the undescended testis. In one study it was found that the tumor occurred in the normal gonad in 23 of 744 patients with unilateral cryptorchidism (29) and in another series the incidence was 6 in 27 cases (112). These findings, however, suggest a possible hormonal factor.

Attempts to influence the course of testicular tumors by hormones have been made by Twombly (112). Administration of antigonadotrophic principles resulted in the increase of the chorionic gonadotrophic hormone titers. Estrogens were also tried on 2 patients without any striking change. Saleeby (91) suggested bilateral gonadectomy and cites a case in which the patient seemed to improve after the procedure was carried out. Evaluation is complicated, however, by the fact that the patient had x-ray therapy as well.

VI. Other Tumors

A discussion of other tumors which may possibly be produced or influenced by hormones, as judged by animal data, is omitted inasmuch as little information is available in relation to the human being. It is beyond the scope of this review to consider tumors of the endocrine organs themselves, other than that of the testes since there are certain aspects of these tumors which are pertinent to this discussion. The functioning type of endocrine tumors are of the greatest interest and considerable data is being accumulated regarding their behavior and hormonal activity. It is possible that such studies will yield valuable leads in the clarification of the origin and alteration of tumors arising in tissues normally stimulated by the hormones.

VII. Discussion

The available clinical and experimental information emphasizes the necessity for the endocrine system in the development and growth of organs such as the breast, uterus and prostate gland. The gonads and the hypophysis are especially important, for without one or the other development will not occur or will cease entirely. Furthermore, the removal of these organs leads to regressive changes in fully developed tissues normally stimulated by these endocrine organs. Thus, it has been contended that some tumors may be definitely related to or caused by alterations of function or secretion of certain of the glands of internal secretion. Factual data for such a thesis have been reviewed under each entity. However, a general discussion of

the possibilities involved seems necessary since there may be common denominators.

1. Status of the Individual

Factors other than endocrine must be considered. Chief among these are heredity, race, color, and constitutional type. They are frequently interrelated but each may be of special significance. While the evidence to support these possibilities is far from conclusive, one may justifiably speculate on them. The physiologic status of the individual, endocrine metabolism and secretion, and susceptibility to the disease may depend to a considerable degree on these fundamental backgrounds. These basic states in some measure may thus determine the type and degree of response of the organism to normal or superimposed stimuli. This would help to explain the variety of changes in organs such as the breast, which have been attributed to the same agent, *e.g.*, estrogens.

The stage of life and state of the particular organ when abnormalities appear may, also, determine the type and degree of response to a given stimulus. It has been shown that diseases of the same organ which arise during various age periods vary considerably in their histologic structure and clinical manifestations. Moreover, the degree of development and structure of the organ may differ regardless of age as a result of variations in the physiologic status of the individual. For example, Dieckmann (15) has shown that the extent of development of the breast particularly at puberty is subject to wide variations among different individuals. Previous pregnancies and normal and abnormal lactation account for breast changes not encountered in the virgin female. Other processes, such as inflammatory disease may likewise alter the state of certain organs. Thus, some organs may exhibit wide varieties of histologic structure under different conditions. Hence, these dissimilar substrates may vary considerably in their response to a single stimulus and may determine to some degree the abnormal changes which may appear subsequently.

2. Character of the Stimuli

Foremost among the probable stimuli which may be responsible for disease of the breast, prostate and uterus, are those of the endocrine system. The hormones which have been the subject of the most intensive study are the estrogens and androgens. Nevertheless, the possibilities suggested by investigation may apply to other hormones as well. How may these hormones produce these atypical states? Several possible modes of action seem tenable. (1) Physiologic amounts of the hormone may act upon

tissue which is more sensitive than normal. This susceptibility may be related to the metabolic status of the host, to special characteristics of the cells, or to a change in the tissue as a result of the activity of other agents. (2) It has been demonstrated that estrogens are destroyed by the liver. Normal quantities of the hormone may be secreted, but because of defective function of the liver, abnormal quantities may be released to the tissues. (3) Excessive quantities of the hormone may be responsible, but, except in cancer-susceptible animals, the effects are limited. (4) Atypical hormones may be produced, which may have carcinogenic activity. This is possible since the steroid hormones are closely allied to several of the known synthetic carcinogens. It is conceivable that the formation of carcinogenic agents from hormones may occur as a result of a faulty metabolism due to a change in the physiologic state of the host. (5) Lack of a hormone, which normally stimulates an organ, may lead to degenerative or regressive changes, which might then allow other agents to act.

The nervous system may be involved. The action may be direct, but it is more likely that such stimuli may exert their effect by causing excesses or alterations of hormonal secretion.

3. Endocrine Dysfunction

The close interrelation of various endocrine organs suggests that a disturbance of one gland could affect the function of the others. Because of the possible mechanisms involved there is a lack of conclusive evidence to justify the acceptance of any one factor. It can be stated at present that there is little evidence to support the thesis that the hormones in themselves are directly responsible for the production of neoplastic disease. It is possible, however, that they may be the exciting factors in the production of a suitable substrate or a precancerous lesion and in this fashion may contribute to the development of a malignant process.

4. Hormonal Influence on Tumors

There is accumulating evidence that the alteration of the endocrine or metabolic status of the host by the administration of hormones or by castration may exert an influence on certain tumors, particularly those of the breast and prostate gland. This is a concrete demonstration that certain tumors are to some degree dependent upon hormonal influence. If one will accept these facts, the concept of autonomy of the cancer cell, at least in some organs, must be revised. Although there is no conclusive evidence that cancer of the breast or prostate gland may be "cured" by the methods described, the fact that alterations do occur should lead to intensive inves-

tigation regarding the mechanism of regression and progression of these tumors.

5. The Significance of Hormone Excretion

Urinary excretion levels of the sex hormones represent only the end product of metabolism. It is not definitely known whether they give true indices of the blood levels, the rate of secretion or destruction, and the utilization of the hormones by the tissues. Furthermore, they do not reveal the exact nature or relation of the various components of the hormones excreted. Thus far, the study of hormone excretion is of the greatest value in patients who have functioning tumors of the adrenal glands and the gonads, such as cortical hyperplasias, adenomas and carcinomas of the adrenal glands, granulosal-cell and thecal-cell tumors and arrhenoblastomas of the ovaries and teratoid tumors and interstitial-cell tumors of the testes. The excretion rates in these cases are of inestimable help in making the diagnosis and in following the course of the disease. Recent studies on the isolation of the sex hormones suggest that patients with cancer may excrete estrogens and 17-ketosteroids in a different fashion from persons without cancer. New steroids have also been found in the urine of cancer patients, which have not been identified in the urine of normal individuals to date. The usual relation of the individual components of both the estrogens (83) and 17-ketosteroids (18, 82) may be atypical as well as the ratio between the total estrogenic and 17-ketosteroid complexes. It should be recognized that alterations in excretion levels or in the specific type of hormone excreted may not necessarily be characteristic for patients with cancer. They may merely represent deviations from normal that occur only as a result of disturbed metabolism in the sick person. They indicate, however, a distinct abnormality and as such are significant in the study of the disease.

VIII. Conclusions

A considerable number of data bearing on the relation of the endocrine organs to tumors have accumulated. The administration of sex hormones to experimental animals has resulted in the production, the augmentation and the inhibition of benign and malignant tumors. These changes are limited to definite types of tumors in different species, as well as to certain strains in any one species of animal. Thus, there are other factors that determine the reaction of a tissue to a hormonal stimulus, and the susceptibility of an animal to the induction of neoplasm. It is difficult to interpret these facts in terms of human cancer. Nevertheless they are of extreme importance in the study of the origin and course of cancer in general.

Strong evidence exists to indicate that endocrine factors are associated with some human tumors. There is as yet no conclusive proof that these influences are directly concerned with cancer, although an increasing number of cases are coming to light in which cancer developed after intensive estrogen therapy in organs such as the uterus and breast, which are normally stimulated by these hormones. It is possible that this is coincidence, but the association cannot be ignored. Present evidence suggests that the sex hormones are not in themselves carcinogenic. It is likelier that, as a result of excessive stimulation or atypical metabolism, the tissues of susceptible persons are conditioned to the action of a carcinogenic agent.

The administration of hormones or the alteration of the hormonal or metabolic status of the host by castration may exert a profound influence on cancers of the breast and prostate gland. The fact that alterations occur should lead to intensive investigation regarding the mechanism of regression and progression in these tumors. The concept of autonomy of the cancer cell in general must be revised in light of these recent observations.

There is no question that castration or hormonal administration have been of great benefit, as an adjunct to the treatment of a number of advanced cases of cancer of prostatic or mammary origin. Such treatment should be reserved for palliative purposes only, since there is at present no substitute for established methods in the operable patient. There is little to support the therapeutic value of castration or hormones in any other type of cancer.

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Discussion

W. U. Gardner: Dr. Strong, in our laboratory, has been inbreeding animals that he has selected, toward resistance to tumors at the site of injection of carcinogenic hydrocarbons. He has been quite successful in this and has been able to obtain a strain of mice that acquire a low incidence of subcutaneous tumors but a very high incidence of cancer of the stomach subsequent to subcutaneous injections of carcinogen. In another line he has developed, and is at present investigating, the incidence of subcutaneous sarcomas is low but the incidence of mammary carcinoma is high following similar treatment. These animals have no mammary tumor agent as far as we know, also no genetic background of such tumors. We have to be careful and specific when we define a carcinogen. Each of us should define the term as we conceive it so that there will be as little misinterpretation as possible. For example, using the appearance of local tumors as an index of carcinogenecity, mice of those lines acquiring gastric or mammary tumors would be relatively resistant or conversely the carcinogen might be considered quite inactive. The host upon which the agent acts should not be overlooked. It is known that carcinogenic hydrocarbons increase cancers of the lung, even when they are administered subcutaneously. These points must also be borne in mind when the carcinogenecity of estrogens is considered too; for unknown reasons it might be etiologically associated with breast cancer in some patients but not in all.

The utilization of experimental animals for studies on the effect of therapy on cancer has been disappointing. In mice the mammary tumors have not shown a decrease in growth following large doses of estrogen or testosterone. After tumors appear and large amounts of several steroid hormones are given the tumors continue to grow.

It is possible that some mammary tumors arising in older mice as those in older patients, may be inhibited by estrogens. It is very possible that we have in mice, for the reason mentioned, more than one type of mammary cancer as appears to be the case in man.

J. S. L. Browne: I have a question which I should like to ask Dr. Nathanson, with regard to the incidence of tumors of the testes, which, as he said, is higher in undescended testes. Is there any information as to whether the incidence decreases if these testes are made to descend by operation? Is it possible that the nature of development of these undescended testes determines tumor formation rather than their position? I think that is very important from a therapeutic point of view.

In connection with Dr. Huggins' case after bilateral adrenalectomy, who died on the 116th day after operation, it was my impression that the carcinoma continued to progress.

The third point I would bring out: he has mentioned the importance of the status of the individual as regards incidence of cancer and as regards the rate of progress of the disease, the importance of the general health of the individual. I would also like to bring out that this is true in connection with therapy, particularly with androgens, that one has to distinguish, in therapy, between the effect on one organ and the effect on the body as a whole. If androgens produce a general effect on the body of depositing body protein one might improve the general nutritional condition of the patient, but the tumor may continue to grow. In this connection I might quote again a case I mentioned last year. A man who had an interstitial cell tumor of the testis, whom Dr. Pierre Masson of the University of Montreal invited us to study. He was a heavy labourer and came for examination because he found he could not bend over when he was using his pick and shovel. He did not feel weak; in fact he felt fairly well. However, there was a reason for his failure to bend over, his liver was so large that it became compressed between the pelvis and the ribs. He had 1,000 mg. 17-ketosteroids a day. He died suddenly after a hemorrhage. Up to the day of his death he protested that he should not be in hospital. He had not lost weight. Under ordinary circumstances such an individual would have been cachectic with the extensive metastases which he had and I wonder whether the quantity of androgens secreted by the tumor did not have something to do with this. Dr. Masson informs me that it is characteristic of these patients that they work until the day they die.

I. T. Nathanson: I do not believe there is sufficient information to draw any conclusions regarding the subsequent incidence of tumors in originally undescended testes, which have been delivered into the normal position by operative procedures. As a rule, this operation is performed in youth and as far as can be determined the testis begins to grow and function in a normal fashion, once it is in the scrotum. There is evidence that there is either diminished or a complete lack of development and function of a testicle which is out of its normal environment. As Dr. Browne suggested, it appears that the condition of the testis rather than the position *per se* may be a determining factor in the formation of tumors. As was pointed out, however, even when unilateral cryptorchidism is present, tumors may arise only in the apparently normally descended gonad. One cannot be sure that the involved testis was normal in development and function, even though it was in its natural position. Thus, by the same reasoning, tumors could appear in a previously undescended testicle, which was delivered into the scrotum in youth.

The carcinoma in the patient of Dr. Huggins continued to progress, but apparently at a slower rate after bilateral adrenalectomy.

The mechanism of relief from pain and the sense of well being experienced in some patients with cancer, treated by testosterone, is difficult to determine. It may be related to an induced metabolic change rather than to a direct effect of the hormone. Moreover, we have found considerable variation in the response of the individual cancer patient to testosterone therapy as regards pain and general improvement. Similar observations have been made by others. This may indicate that the physiologic or metabolic state of the host may be a determining factor in the degree of response.

D. R. McCullagh: Dr. Nathanson's interesting observations bring up the question of certain glandular interrelationships. Martins and Rocha, as well as others, demonstrated some years ago that the pituitary gland could be inhibited by the administration of testicular substance. There is considerable indication that the decrease in hypophyseal activity is not the result of the administration of androgens.

It is known that definite relationships exist between the prostate and the pituitary glands. Legendary tales, as well as scientific investigations, indicated that uncooked bull moose or beef testes inhibit the pituitary gland and alleviate urinary symptoms associated with prostatic hypertrophy. It is known that various steroids act as pituitary depressants. A pharmacological problem of considerable clinical importance would be development of steroids or other drugs which would control the activity of the pituitary gland.

G. Pincus: Do hormone excretion studies cast light on mammary cancer etiology?

I. T. Nathanson: We have carried out a very large number of assays of total estrogens and 17-ketosteroids in patients with cancer, primarily as a screening to determine if the excretion rates in any particular type is significantly different from that in patients who do not have cancer. Judged from the total output there is a suggestion that some abnormality may exist in certain individuals with cancer. However, they are sick people and the variations do not seem to be much different than that found in persons with other illnesses. Thus, it should be emphasized again that the general state of the host should be considered in the evaluation of any deviation from normal excretion.

H. Selye: In connection with these two extensive symposia on hormones and tumor formation, I would like to bring up for your consideration an instance of hormonally controlled neoplastic growth which is rarely envisaged as such, namely, endometriosis.

My questions are:

(1) Is endometriosis a true tumor and (2) is it under endocrine control? Personally, I am tempted to answer both these questions in the affirmative. Endometriosis exhibits all the criteria which we usually regard as characteristic of neoplastic growths. It grows independently of the requirements of the host and invades the host organism. It can metastasize, not only by direct spread, but even to distant organs through the blood stream. Thus metastatic endometriosis has been noted in the lung and in extremities, for instance, the arm. Contrary to common opinion, an extensive survey of the relevant literature, which I recently completed, in connection with the two volumes on ovarian tumors, in my *Encyclopedia of Endocrinology* (now in press), I found a surprisingly large number of instances in which endometriosis underwent malignant transformation and, in certain areas, assumed the characteristics of typical carcinoma tissue.

It is more generally admitted that endometriosis is controlled by hormones. This view is supported by the fact that it never occurs in males, it never appears before puberty, and it regresses during the menopause. Furthermore, during gestation, when progesterone formation is probably proceeding at a very high level, endometriomas undergo pro-

gestational transformation, thus indicating sensitivity to hormonal stimuli. The incidence of endometriomas is particularly high in women with follicular cysts or folliculomas; that is, under conditions of excessive endogenous formation of folliculoid hormones. Surgical or x-ray castration has cured endometriosis in all cases so far published. Preliminary observations concerning the treatment of endometriosis with testoid compounds, are very encouraging.

In view of our ignorance of the fundamental factors determining neoplastic growths, it is hardly justifiable at this time to enter into any extensive academic discussion concerning the neoplastic nature of endometriosis, but I feel that the facts which I have just mentioned do not warrant the separation of endometriosis from the neoplastic diseases. Since endometriosis is obviously under hormonal control, it may perhaps deserve more attention than it has hitherto received from investigators interested in hormonally conditioned neoplasms.

I. T. Nathanson: We are interested, indeed, in the remarks of Dr. Selye regarding a hormonal control of endometriosis. Many observers are of the same opinion and there is ample evidence to justify the conclusions. Endometriosis is a common disease and is said by some to occur in 40 per cent of women, particularly those without children. I do not wish to enter into a controversy as to what constitutes a true tumor. One may perhaps call endometriosis a tumor by virtue of the fact that it is a mass, but I should hesitate in calling it a malignant tumor. A malignant tumor is one that kills and as far as I am aware, endometriosis does not cause death except in an unusual case where the disease has invaded a vital organ. In this sense, *i.e.*, direct invasion, endometriosis may perhaps be called a tumor, but inflammatory diseases may also invade vital organs. It rarely goes to lymph nodes. There is no doubt that it goes to distant organs probably via the blood stream, but there are other lesions such as benign adenomas of the thyroid, mixed tumors of the parotid gland, and hemangiomas which may do likewise. These latter lesions have never been considered malignant. It is conceivable that the spread through the blood stream may occur by direct venous invasion and from this, small portions of tissue may break off much like a thrombus. Some may consider this to be a criterion of malignancy. As for transformation of endometriosis into malignant tissue, I believe it would be well to be cautious in the interpretation of such change without subjecting the available material to a thorough review.

The theories of origin, *i.e.*, implantation or serosal (embryological), are still a matter of dispute. Most individuals lean toward the latter idea.

I thoroughly agree that the lesion should be given much more attention from the standpoint of hormonal control.

The Effect of Hormones on Osteogenesis in Man[1]

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In this survey of the effects of various hormones on osteogenesis, I will leave out many of the "ifs," "buts," and "ands" in order to cover the entire field. Furthermore, I will refer largely to work from my own group. This is not that I regard it so highly but that I am more familiar with it. This work has been done with the help of a large number of collaborators. Much of it has already been published; the more recent work which has not been published has been carried out in conjunction with Dr. Edward C. Reifenstein, Jr., Dr. Anne Pappenheimer Forbes, and Dr. Lawrence Kinsell.

I. Certain Aspects of Osteology in General

1. *Types of Osteogenesis*

There are three types of bone formation: (a) endochondral, (b) membranous, and (c) endosteal.

The steps in the formation of bone from cartilage are: (a) proliferation of cartilage cells with increase of intercellular cartilaginous substance, (b) arrangement of more mature cells into rows, (c) calcification of intercellular cartilaginous substance between rows, (d) the breaking of blood vessels into the lacunae containing the cartilaginous cells, (e) the laying down of bony matrix (osteoid) by osteoblasts on to the surfaces of the calcified cartilaginous trabeculae left after the blood vessels have broken into the lacunae, and (f) the deposition of a calcium-phosphate-carbonate salt into the osteoid.

The term "membranous bone" is usually reserved to designate that bone which is formed directly from specialized mesenchymal tissue in the embryo without there having been any preceding cartilaginous phase. I will use this terminology, perhaps somewhat loosely, to include periosteal bone formation.

By "endosteal bone formation" I refer to that appositional bone laid down in the cortex and trabeculae of bone as a part of the constant remodel-

[1]The expense of these studies was partly defrayed by grants from the Josiah Macy, Jr. Foundation, the National Research Council (Committee for Research in the Problems of Sex), and the Mary Gove Pittman Fund. A bed supported by the Mallinckrodt Chemical Company on the Metabolic Ward was used for part of these studies.

A part of the work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Massachusetts General Hospital.

ing of bone which takes place during growth and after growth has ceased.

In the final analysis it will be noted that all three of these types of bone formation are essentially the same, namely the laying down by osteoblasts of an extracellular substance called osteoid, and the deposition into this osteoid of a calcium-phosphate-carbonate salt. However, as will be seen later, certain hormones have a selective action on one of the three types of bone formation, certain others on another, etc. And so it perhaps will be useful to continue to speak of them as three different processes.

2. *Definition of Bone*

It should be noted that bone is first a tissue and secondly a calcified or phosphorized tissue. In order to emphasize this point I will use as my definition of bone "any tissue containing bone matrix". This definition allows then for two subclassifications, uncalcified bone and calcified bone. According to the above definition, rickets, as will be seen below, is a disease characterized by too much bone.

3. *Remodeling of Bone*

In adult bone, and even more so in growing bone, there is a constant remodeling going on. There are places where bone is being resorbed and others where bone is being laid down. Both of these processes go on at one and the same time. This can best be demonstrated in a condition where both processes are speeded up, namely, osteitis fibrosa generalisata (see Fig. 1). One is fortunate in the study of bone since both of these processes can be seen under the microscope, osteoblasts where bone is being formed, and osteoclasts where bone is being destroyed. It is probable that anabolism and catabolism are more or less a constant feature of other tissues, but with most tissues (*e.g.*, muscle) one can only say that the tissue is increasing or decreasing without being able to conclude whether the process, which leads to the imbalance, is a disturbance of anabolism or catabolism.

4. *Why Bone Can Be Formed in One Locality at the Same Time It Is Being Destroyed in Another*

It has probably occurred to many of you to ask yourselves how the calcium-phosphate-carbonate salt can be deposited in one region while being resorbed in another. Obviously, there is some local factor where it is being deposited which favors its deposition or some local factor where it is being resorbed which favors absorption. We need not disturb ourselves too much about what that factor is at this time. However, since alkaline phosphatase is found wherever bone or teeth are being made, a possible explanation, widely accepted, holds that this substance increases the concentration

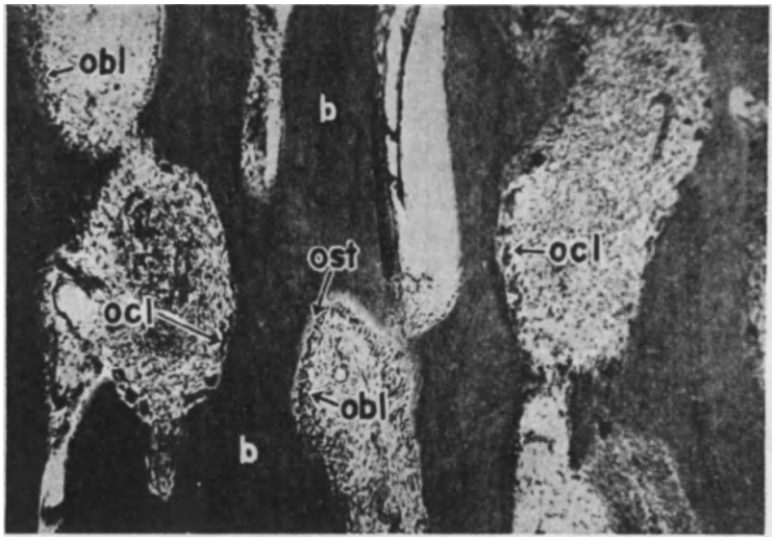


FIG. 1

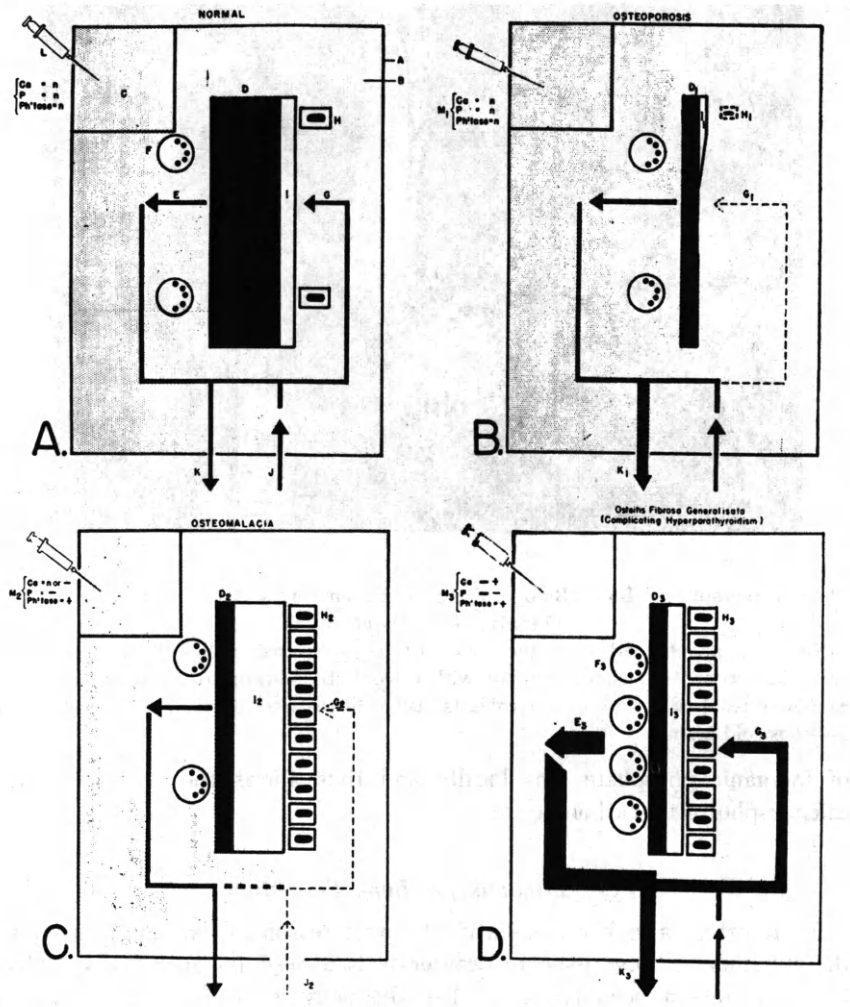
Photomicrograph of Bone Biopsy From a Patient with Hyperparathyroidism and Osteitis Fibrosa Generalisata.

Note that about one-half of the bone surfaces is covered with osteoclasts indicating bone destruction, and about one-half with osteoblasts indicating bone formation. Note narrow osteoid seams. obl = osteoblasts, ocl = osteoclasts, b = bone trabeculae, and ost = osteoid seam.

of inorganic phosphate ions locally and thus allows the deposition of a calcium-phosphate-carbonate salt.

5. *Stimulus for Bone Formation*

To resort to a rather unscientific form of reasoning, one might say that the stimulus for any tissue to proliferate is a need for such tissue. Thus, there becomes a need for skin when the body is not completely covered with skin. Presumably, there is a need for bone when the skeleton is not sufficiently rigid for the uses to which it is put. Thus, the need for bone will depend on the stress and strain to which the skeleton is subjected. A person who sits at a desk will need much less skeleton than one who is carrying out physical work. I picture the skeleton as a rigid tissue with a huge surface area. Over most of this area I think of calcium phosphate as being slowly resorbed from the bone in response to physical chemical laws; however, where the bone becomes too weak as the result of this absorption to withstand the stresses and strains, the osteoblasts lay down osteoid and new bone formation takes place.



6. *Osteoporosis, Osteomalacia, and Osteitis Fibrosa Generalisata*

It is important to make clear the differences between three types of metabolic disease which are characterized by too little calcified bone. According to the definitions here being used, it will be noted that only the first of these, osteoporosis, is characterized by too little bone.

In osteoporosis (see Fig. 2B) the decrease of bony tissue is due to the fact that the osteoblasts lay down too little bony matrix; that matrix which is laid down is normally calcified. Thus, since osteoporosis is a disorder of tissue metabolism, not of calcium or phosphorus metabolism, one is not

FIG. 2

Schematic Diagrams to Show the Speaker's Conception of Differences Between Normal Bone (*A*), Osteoporosis (*B*), Osteomalacia (*C*), and Hyperparathyroidism with Osteitis Fibrosa Generalisata (*D*)

A—body limits; B—body fluids; C—body serum, a compartment of fluid easy to tap for analysis; D—mass of bone with two surfaces, one at which bone is being resorbed and one at which it is being laid down; E—arrow indicating by its size the rate of resorption of calcium and phosphorus; F—osteoclast; G—rate of deposition of calcium and phosphorus; H—osteoblast laying down osteoid (I); J—calcium and phosphorus entering the body from the gastrointestinal tract; K—calcium and phosphorus leaving the body by the kidneys or by other exits; L—syringe obtaining serum for analysis; and M—blood values (n = normal, + = high and — = low).

A. Normal. Note that the calcium and phosphorus going into bone equals that coming out of bone and that part of that which comes out goes back in.

B. Osteoporosis. Note decrease in the mass of bone (D1); primary hypoplasia of osteoblasts (H1); decreased deposition of osteoid (I1); decreased deposition of calcium and phosphorus (G1); increased excretion of calcium and phosphorus (K1), and normal blood values (M1).

C. Osteomalacia. Note decreased mass of calcified bone (D2); hyperplasia of osteoblasts because of increased susceptibility to stresses and strains (H2); increased deposition of osteoid which is inadequately calcified because of abnormal serum calcium and phosphorus values; decreased deposition of calcium and phosphorus (G2); primary difficulty in absorbing calcium and phosphorus from the gastrointestinal tract (J2), and abnormal blood values (calcium normal or low, phosphorus low and phosphatase high).

D. Osteitis Fibrosa Generalisata Complicating Hyperparathyroidism. Note increased excretion of calcium and phosphorus in the urine (K3); increased resorption of calcium and phosphorus (E3); increase of osteoclasts (F3); decreased mass of bone (D3); increased formation of bone by osteoblasts (I3) because of increased susceptibility to stresses and strains; increased deposition of calcium and phosphorus (G3) because serum is not undersaturated in respect to calcium phosphate (*i.e.*, the serum calcium level is sufficiently high almost to offset the low serum phosphorus content); and the high phosphatase level (M3). Figure reproduced from Albright *et al.* (4).

surprised to find normal serum calcium and phosphorus levels. The serum alkaline phosphatase level, the index to osteoblastic activity, is likewise normal, not low as one might at first though expect. A normal phosphatase level really means a relatively low level if one considers the fact that the skeletal mass, being decreased, is more subject to stresses and strains, the usual stimulus to osteoblasts.

In osteomalacia (see Fig. 2C) there is too little calcified bone due to the fact that there is a disorder of calcium or phosphate metabolism of such a nature that the calcium-phosphate-carbonate salt is not deposited in the newly formed osteoid. The bony tissue, therefore, is less resistant to stresses and strains, and this results in an over-production of osteoid by the osteoblasts. This, in turn, results in a high serum alkaline phosphatase level.

In osteitis fibrosa generalisata (see Fig. 2D) there is a decrease in bony tissue as a whole because of increased bone resorption. This leads to decreased bone strength, and this in turn to an increased activity on the part of the osteoblasts and hence to a high serum alkaline phosphatase level. The commonest cause of osteitis fibrosa generalisata is hyperparathyroidism which, if present, is associated with low serum phosphorus and high serum calcium levels.

II. Hormones and Osteogenesis

1. *Effect of Hormones on Epiphyseal Closure*

Obviously, if a hormone causes epiphyseal closure, it stops endochondral bone formation. The total growth of any one epiphysis depends on the rate of growth and the time during which growth continues. A hormone such as testosterone may increase the rate of growth, but, by causing early closure of the epiphyses, decrease the time during which growth continues; the net result may be a decreased growth (see Fig. 12). However, in the discussion to follow, I will entirely lose sight of the effect of hormones on epiphyseal closure, and leave that for Dr. Nathan Talbot to discuss (pages 359-369).

2. *Effects of Parathyroid Hormone on Osteogenesis*

It is not my intention to discuss in detail the action of parathyroid hormone on bone. I will, however, briefly sketch my own conception of how this hormone acts.

In endocrinology one is familiar with the conception that the administration of a hormone causes widespread disturbances. The question frequently arises whether a particular disturbance at one locus is the cause of the disturbance at a second locus, or whether the disturbance at the second locus is the cause of the disturbance at the first locus. In para-

thyroidology there has resulted a controversy over the question of whether the hormone acts primarily on bone with a resulting disturbance in the calcium and phosphorus metabolism, or whether it acts primarily on calcium and phosphorus metabolism with a resulting disturbance in bone. I have been a strong proponent of the second possibility; the Montreal School, headed by Dr. J. B. Collip, has favored the first. It is rather amusing that, just as the Montreal School (37) swung around somewhat to my point of view, Ingalls, Donaldson, and Albright (32) have had to admit that there may be a direct action on bony tissue. I still believe, however, that the main action of the hormone is on the phosphorus and calcium metabolism.

The administration of parathyroid hormone is followed by four cardinal metabolic changes: an increase of calcium in the serum, a decrease of phosphorus in the serum, an increase of calcium in the urine, and an increase of phosphorus in the urine. If one assumes that the four cardinal metabolic changes are interrelated phenomena, which almost certainly must be the case, the most plausible sequence of events which one would arrive at, even without experimentation, is that the initial effect is an increased excretion of phosphorus in the urine, that this leads to a low serum phosphorus level, that this leads to increased pulling of calcium and phosphate ions into the serum from the bones or gut, that this results in an elevation of the serum calcium level, and finally that this leads to an increase in the amount of calcium in the urine (see Fig. 3). There is considerable experimental data

Parathyroid	+
↓	
Ur. P.	+
↓	
Ser. P.	—
↓	
Bone Resorp.	+
↓	
Ser. Ca.	+
↓	
Ur. Ca.	+

FIG. 3
Sequence of Adjustments to Parathyroid Hormone.

to support the above sequence of events. Albright and Ellsworth (5) found that the administration of parathyroid extract was immediately (within one hour) followed by a phosphate diuresis and that this preceded the other sequelae. Harrison and Harrison (30) showed that parathyroid hormone decreases the re-absorption in the kidney tubules of the phosphorus of the glomerular filtrate.

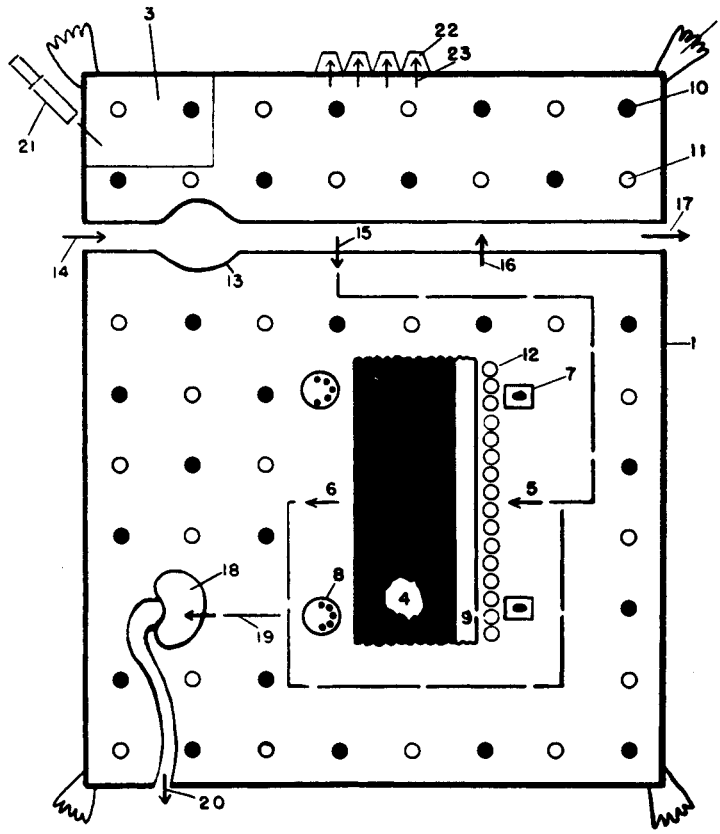


FIG. 4

Diagramatic Representation of Calcium Metabolism In Isoparathyroid State To Be Compared With Similar Diagrams for Hyperparathyroidism-With-Bone-Disease (Fig. 5), Hyperparathyroidism-Without-Bone-Disease (Fig. 6), and Hypoparathyroidism (Fig. 7)

(1) confines of body; (2) rudimentary appendages to make body more realistic; (3) special compartment of body fluids to represent serum; (4) rectangular mass representing calcified bone and having two surfaces, one to the left where bone is being resorbed and one to the right where bone is being formed; (5) arrow indicating rate of calcium deposition into bone forming surface; (6) arrow indicating rate of bone resorption from bone-resorbing surfaces; (7) osteoblasts laying down uncalcified matrix (9) on bone forming surfaces; (8) osteoclasts on bone resorbing surface; (9) uncalcified osteoid tissue laid down by osteoblasts; (10)black dots representing calcium ions in body fluids; (11) white dots representing phosphate ions in body fluids; (12) localized increase of phosphate ions along bone depositing surfaces resulting from action of phosphatase; (13) gastrointestinal tract; (14) calcium entering the body in the food; (15) calcium being absorbed from the gastrointestinal tract; (16) calcium being re-excreted into the gastrointestinal tract; (17) calcium being lost in the feces; (18) kidneys; (19) calcium passing through the kidneys prior to excretion; (20) calcium being lost in the urine; (21) syringe obtaining serum for analysis; (22) tooth; (23) calcium being deposited into tooth during tooth's formation.

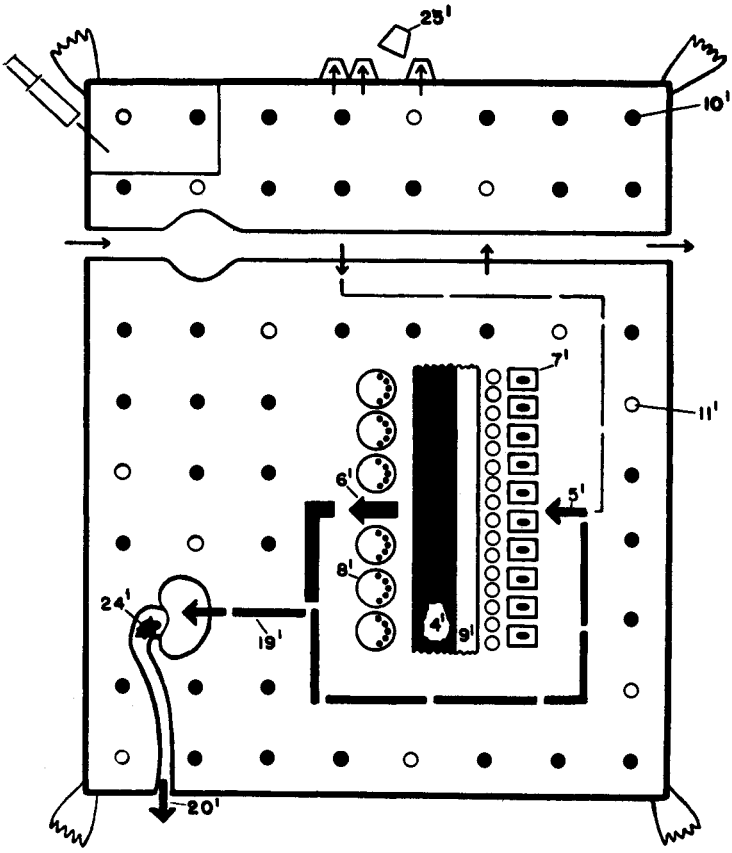


FIG. 5

Diagramatic Representation of Calcium Metabolism in Hyperparathyroidism-With-Bone-Disease to be Compared with Calcium Metabolism in the Isoparathyroid State (Fig. 4)

Note decrease in bone mass (4_1), increase in number of calcium ions in body fluids (10_1), decrease in number of phosphate ions in body fluids (11_1), increased calcium resorption from bone resorbing surfaces (6_1), increased number of osteoclasts (8_1), increase in bone formation with large number of osteoblasts (7_1), ability of calcium to be deposited in the newly formed osteoid tissue as shown by arrow 5_1 and normal width to osteoid seams (9_1), tendency to kidney-stone formation (24_1), and absence of decalcification in teeth although an individual tooth may fall out because of faulty bone (25_1).

In Figs. 4, 5, 6, and 7 are depicted in schematic form my conception of the calcium and phosphorus metabolism in the normal state, in hyperparathyroidism-with-bone-disease, in hyperparathyroidism-without-bone-disease, and in hypoparathyroidism, respectively.

3. *Hyperparathyroidism-With-Bone-Disease*

It will be noted in Fig. 5 that, in the bone disease of hyperparathyroidism, endosteal bone formation is increased as a compensatory mechanism to the increased bone resorption. Furthermore, in spite of the low serum phosphorus level, the serum calcium level is sufficiently high so that the bony

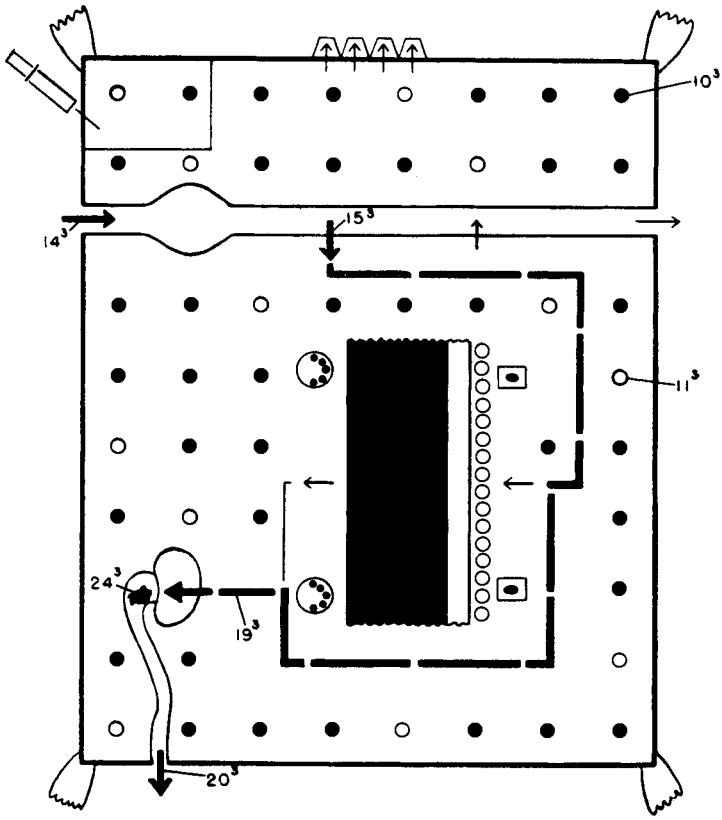


FIG. 6

Diagramatic Representation of Calcium Metabolism in Hyperparathyroidism-Without-Bone-Disease to be Compared with Calcium Metabolism in Normal State (Fig. 4) and That in Hyperparathyroidism-With-Bone-Disease (Fig. 5)

Note that condition coincides with hyperparathyroidism-with-bone-disease in having an increased number of calcium ions in body fluids (10_3), a decrease in phosphate ions (11_3), an increase of calcium excretion in the urine (20_3), and a kidney stone (24_3); note, however, that there is no diminution in bone mass, no increase in bone destruction or in bone formation and that increased calcium excretion in the urine is entirely supplied by increased calcium intake and absorption (14_3 and 15_3).

matrix which is laid down is normaly calcified. In keeping with this is the fact that, when the disease occurs in growing children, there is no lack of calcification of the zone of provisional calcification of the cartilage, *i.e.*, there is no rickets.

4. *Hyperparathyroidism-Without-Bone-Disease*

The fact that one can have hyperparathyroidism without bone disease is quite strong evidence in favor of the thesis that the hormone does not act

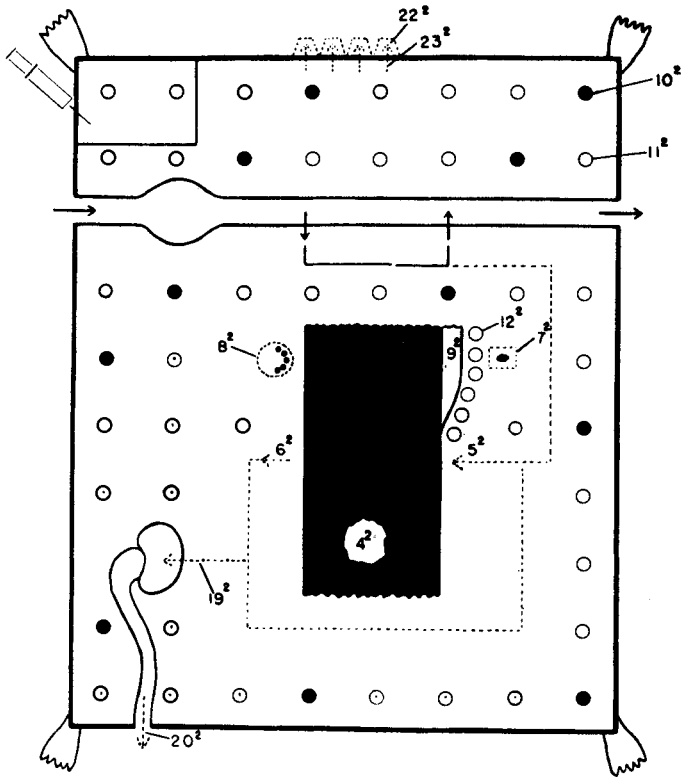


FIG. 7

Diagramatic Representation of Calcium Metabolism in Hypoparathyroidism to be Contrasted to That in a Normal Individual (Fig. 4) and That in Hyperparathyroidism (Figs. 5 and 6)

Note decrease in the number of calcium ions in body fluids (10_2), increase in number of phosphate ions in body fluids (11_2), absence of calcium excretion in the urine (20_2), decrease in bone resorption (6_2) with a decrease in osteoclastic activity (8_2), resulting increase in bone mass (4_2), and compensatory decrease in bone formation with decreased osteoblastic activity (7_2). Note also decreased deposition of calcium into teeth (23_2) with resulting a-calcification of teeth (22_2).

primarily on bony tissue. Out of the first sixty-four cases of hyperparathyroidism proved by operation at the Massachusetts General Hospital, twenty-nine had no evidence of bone disease. Furthermore, in a patient with a severe degree of hyperparathyroidism (serum calcium *circa* 14 mg./100 cc.), it will be noted in Fig. 8 that the calcium excretion was relatively little affected as the calcium intake varied from 100 mg./day to 2,500 mg. Thus, depending on whether this patient was on a high calcium intake or not, he was in positive or negative calcium balance. This is evidence against the concept that the parathyroid hormone stimulates osteo-

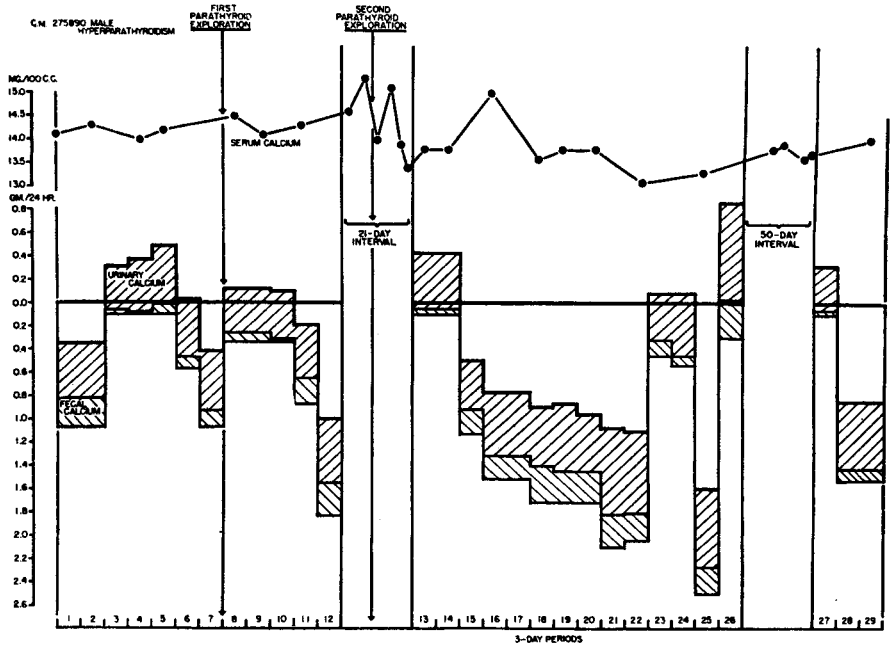


FIG. 8

Metabolic Data on C. M. (M.G.H. #275890) to Show Effect of Calcium Intake on Calcium Balances in Hyperparathyroidism (13)

For construction of figure see paragraph in italics under Fig. 14. Note that serum calcium is very high, circa 14 mg., during entire experiment and is not influenced by negative parathyroid explorations. Note that the calcium excretion in the urine is very high and by and large does not fluctuate materially with changes in calcium intake. When calcium intake is high, patient is in a strongly positive calcium balance; when calcium intake is low, patient is in a negative calcium balance. Note in the sixth metabolic period that the calcium intake was between 0.5 and 0.66 g./day and the calcium output was approximately the same; therefore 0.6 to 1.0 g. of calcium per day in the intake (*i.e.*, about one quart of milk) would have kept this patient in positive calcium balance. Data recharted from Bauer, Albright, and Aub (13).

clasts to destroy bony tissue. But, it might be argued that the high calcium intake allows increased bone formation to compensate for an increased bone destruction. If such were the case, a patient with "hyperparathyroidism-without-bone-disease" would have a high serum phosphatase level, and his bones would show histologically many osteoblasts and osteoclasts. Such is not the case; the phosphatase level is normal and the bone histology shows no evidence of increased activity. [Biopsy material from such a case was briefly discussed at this point. It will not be reproduced here but can be found in a previous paper (9).]

In summary, therefore, it will be seen that the parathyroid hormone effects osteogenesis only secondarily; thus, under certain conditions an excess leads to increased bone resorption which indirectly causes compensatory increased endosteal bone formation. Similarly, in hypoparathyroidism there is decreased bone resorption and secondarily decreased bone formation (see Fig. 7). The parathyroid hormone has no obvious effect on endochondral or membranous bone formation.

5. *The Effect of the Thyroid Hormone on Osteogenesis*

In the absence of the thyroid hormone, as in cretinism or juvenile myxedema, there is marked retardation in growth at the epiphyseal cartilages. The hypothyroidism present in panhypopituitarism, though not usually complete, is of sufficient degree to prevent optimal growth; thus, when such an individual is made to grow with testosterone therapy (*vide infra*) the rate of growth is considerably lessened if thyroid hormone as well is not given. This is well shown in Fig. 12 (*q.v.*). Whether epiphyseal growth is more rapid than normal in hyperthyroidism is a controversial point; it will be discussed more fully by Dr. Nathan Talbot. Further evidence that the thyroid hormone effects endochondral bone formation is the delayed bone age of patients with cretinism or juvenile myxedema. Furthermore, such children often develop a condition of the upper femoral epiphyses which by x-ray is almost indistinguishable from Legg-Perthes' Disease ("osteochondritis deformans juvenilis"), but which in reality is due to islands of cartilage in the epiphyseal heads which have not been converted into bone (1). For a further discussion of the effect of thyroid on endochondral bone formation (*i.e.* growth) see Johnston and Maroney (34).

Aub, Bauer, Heath, and Ropes (12) found the urinary and fecal calcium excretions increased in hyperthyroidism and decreased in hypothyroidism; they also presented some evidence to suggest that the bones are demineralized in hyperthyroidism. Aub, Albright, Bauer, and Rossmeisl (11) found that administration of thyroid hormone to patients with hypoparathyroidism results in a rise in the serum calcium level and an increase in the urinary

calcium and phosphorus excretions. Albright, Bauer, and Aub (3) showed that the increased calcium excretion resulting from the thyroid hormone is not due to an accompanying acidosis. I think it is most likely that the bone demineralization in hyperthyroidism is to be attributed to an osteoporosis; the relatively normal values for serum calcium, phosphorus, and alkaline phosphatase in hyperthyroidism support this thesis. I think, furthermore, that the cause of the osteoporosis is a deficiency in nitrogenous elements with which to build the matrix as a result of the negative nitrogen balance usually accompanying hyperthyroidism. The cases studied by Aub *et al.* (12) were in negative nitrogen balance. The fact that Kinsell, Hertz, and Reifenstein (35) were able to cut down the hypercalcuria in a case of hyperthyroidism by decreasing the negative nitrogen balance by the administration of testosterone propionate is in harmony with this thesis. Such an explanation does not explain the high fecal calcium excretion. This may be the result of lack of absorption secondary to the increased intestinal rate as suggested by Pugsley and Anderson (42).

In summary, therefore, it would seem that the thyroid hormone stimulates, or at least is necessary for, normal endochondral bone formation and that it may indirectly inhibit endosteal bone formation through its calorigenic property by leading to a negative nitrogen balance. I know of no observations which suggest that it has any action on membranous bone formation.

6. *Effect of "The" Pituitary Growth Hormone on Osteogenesis*

I will accept as established by the work of Dr. Herbert M. Evans the existence of "the" pituitary growth hormone in contradistinction to the various trophic hormones which stimulate satellite glands to produce hormones which effect growth, *e.g.*, thyrotrophic hormone. The purified pituitary growth hormone causes, as evidence of stimulation, a widening of the proliferating zone of the epiphyseal cartilage of an hypophysectomized rat (Marx, Simpson, and Evans (36)); the term "chondrogenesis" has been applied to this property.

Unfortunately, almost all if not all of the clinical syndromes involving over- or under-production of the growth hormone have, at the same time, disturbances in the production of other hormone. This makes the interpretation of the effect of "the" growth hormones more uncertain. Let us examine some of these syndromes.

There are three syndromes produced by an excess of the pituitary growth hormone such as is seen with eosinophilic adenomas,—(a) acromegaly, (b) moderate gigantism with acromegaly, and (c) gigantism without acromegaly. Acromegaly results if the production of excess growth hormone does

not start until after the skeleton has matured. Further growth at the epiphyses of the long bones is impossible, and the clinical abnormalities of the skeleton are those due to excessive periosteal ("membranous") bone formation coupled with a re-awakening of endochondral bone formation at certain restricted sites (*vide infra*). Moderate-gigantism-with-acromegaly results when the excess production of growth hormone starts before the skeleton has matured but where the tumor of the pituitary does not interfere with the production of gonadotropic hormone by the basophile cells; hence the individual grows too rapidly until the skeleton matures, but it does eventually mature which precludes further growth in height without preventing the changes characteristic of acromegaly. Gigantism results when the excess production of growth hormone sets in before the skeleton has matured and where the pituitary tumor interferes with the normal function of the basophile cells so that sexual maturity and hence skeletal maturity never take place; when this results, the individual continues to grow indefinitely but endochondral and membranous bone formation proceed in a normal ratio to each other and acromegaly does not assert itself.

After closure of the epiphyses, there is still a possibility for restricted endochondral bone formation at certain sites. Erdheim (24) showed in a classical post-mortem study, and we can confirm his findings by x-ray studies (see Fig. 9), that the cartilaginous end-plates of the vertebrae in acromegaly again start producing endochondral bone which together with increased periosteal bone formation accounts for the increased width of the vertebrae in the antero-posterior diameters (see Figs. 9 and 10). For a discussion of the vertebral changes in acromegaly in the English language see Waine, Bennett, and Bauer (53).

There is a type of dwarfism which I like to call panhypopituitarism, in which the evidence is quite clear that all elements of the anterior pituitary are deficient. Thus, there is no secondary sexual development and the excretion of follicle-stimulating hormone in the urine is nil; the individual is hypo-glycemia unresponsive, which is evidence for an under-production of the adrenal-cortical "sugar hormone" and hence of under-production of "the" corticotrophic hormone*; the 17-ketosteroid excretion is practically absent which is evidence for an underproduction both of the hormone secreted by the cells of Leydig and of the adrenal-cortical "nitrogen hormone" and hence of that trophic hormone (in the speaker's opinion the luteinizing hormone) which controls the production of both of these hormones; and the basal metabolic rate is low as evidence of a deficient thyrotrophic hormone.

*As will be discussed below I believe that there are two pituitary trophic hormones that stimulate the adrenal cortex, "the" corticotrophic hormone and the luteinizing hormone.

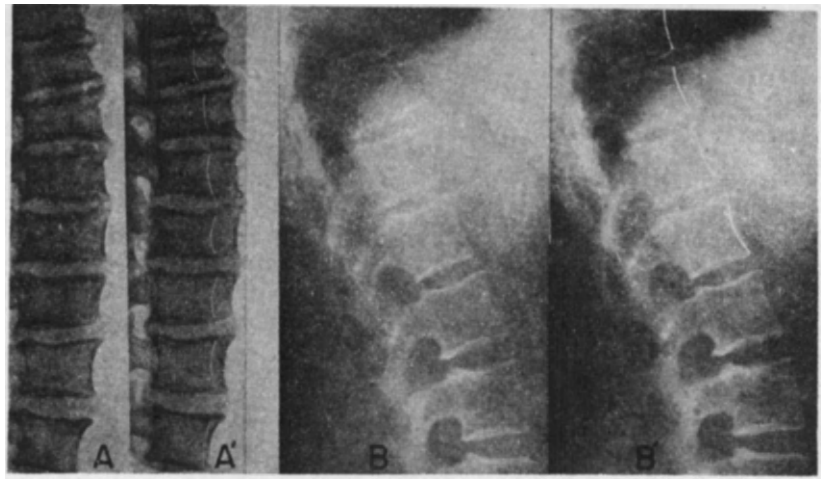


FIG. 9

Vertebral Changes in Acromegaly

(A) X-ray of spine taken at post-mortem from Erdheim (24); (B) X-ray of spine taken *in vivo* (A.P. #135659); (A_1) and (B_1) same as (A) and (B) except that white lines have been drawn in to separate the new growths from the original vertebrae. Note that in both Erdheim's case and our own the maximum changes occur at about the level of the ninth thoracic vertebra. Note, as Erdheim pointed out, that the vertebrae, in contrast to the usual rule, decrease in size from above down; note in (A_1) and (B_1) that this decrease is due to a decrease in the new bone formation.

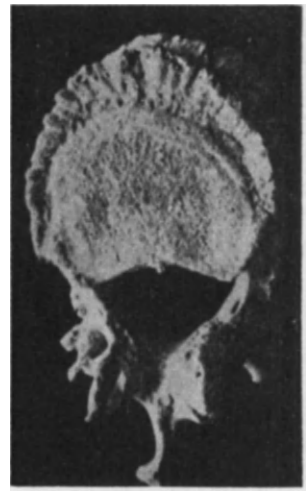


FIG. 10

Vertebra From a Patient with Acromegaly to Show New Bone Formation

Note that new bone formation is greatest on the anterior surface and least on the lateral surfaces. This explains why an acromegalic patient can often bend freely sideways but not frontward. [Reproduced from Erdheim (24)].

Such individuals grow very slowly, presumably due to lack of growth hormone; since they do not mature, they continue to grow indefinitely at a very slow rate. I had one such patient who worked with the circus and who lost his job at the age of forty because he had become too tall. The effect of thyroid and testosterone propionate therapy on two such individuals is shown below in Figs. 11 and 12.

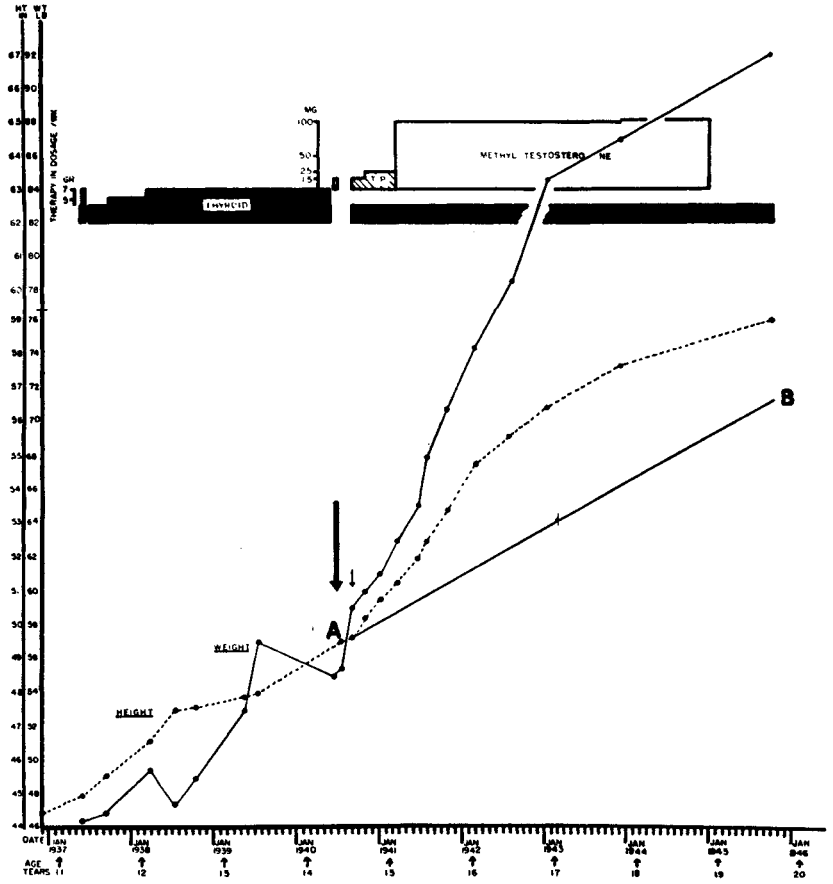


FIG. 11

Height and Weight Chart Before and After Initiation of Testosterone Therapy on Patient M.L. #64011, Suffering from Panhypopituitarism

Note that thyroid therapy may have had an unsustained effect on height; note, however, marked effect on both height and weight of testosterone propionate (methyl testosterone later) therapy which was inaugurated at the age of 14½. The line AB indicates what would have been his growth curve had he continued to grow at the same rate after testosterone therapy as before. Note that therapy was kept at a fairly low dosage in the hope of avoiding too much sexual development and closure of the epiphyses. Details of case history are briefly extracted elsewhere as Patient 52 (25).

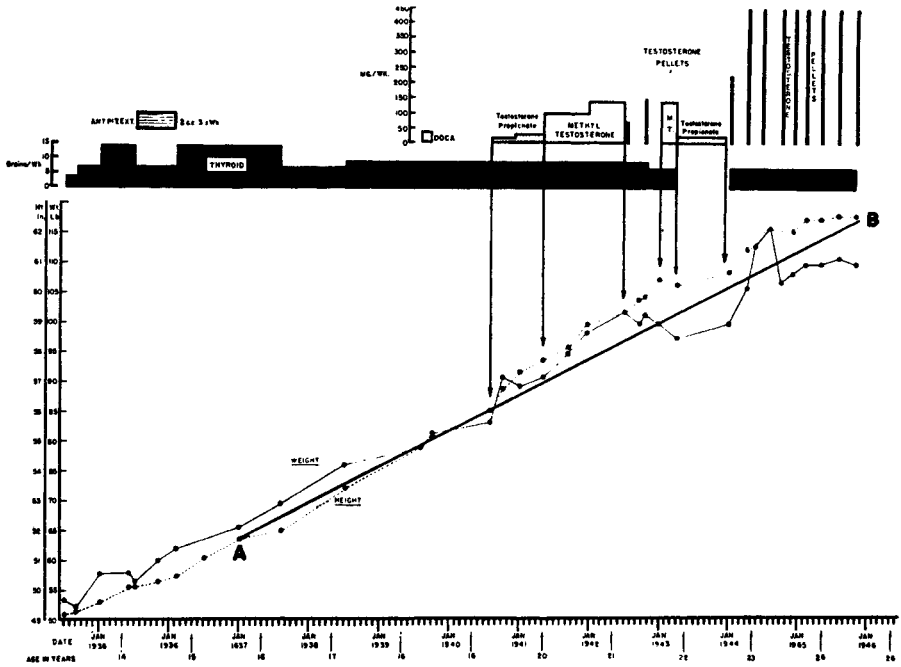


FIG. 12

Growth and Weight Curves of Patient D. A. (M.G.H. #199423) with Panhypopituitarism (Pituitary Dwarf)

Note approximately 6 years of control on thyroid therapy alone; note definite increase in growth rate at institution of testosterone propionate therapy (later methyl testosterone). The line AB indicates the rate of growth before testosterone propionate therapy was introduced. Note decline in growth rate when thyroid medication was omitted while testosterone propionate therapy continued. Note that after almost six years of testosterone therapy his growth rate because of maturation of the skeleton has decreased so that his actual height is probably no greater than it would have been had he received no therapy; this statement presupposes that without therapy his growth rate would have remained constant, which is probably the case. Case history is abstracted as Patient No. 51 elsewhere (25).

When it comes to the effect of "the" growth hormone on endosteal bone formation it is hard to know what to say. Presumably this hormone stimulates growth of all tissues and it would be surprising if endosteal bone were an exception. However, many cases of acromegaly show demineralization of their skeletons. I would like to discuss three possible explanations which come to mind.

There are those who attribute the demineralization to an accompanying hyperparathyroidism. For a discussion of this aspect, see Perlman (39). The argument rests mostly on the fact that hyperplasia of the parathyroids

and even parathyroid adenomas commonly accompany eosinophilic tumors of the pituitary. However, almost all organs are hypertrophied in acromegaly, so hyperplasia in itself argues more for a compensatory increased production as opposed to an over-production. Against the demineralization being due to a hyperparathyroidism are the following three characteristics of acromegaly: the absence of a high serum phosphatase level, the elevation rather than a lowering of the serum inorganic phosphorus level (unpublished data), and the absence of a hypercalcemia. This does not mean that hyperparathyroidism due to a parathyroid adenoma may not be a relatively frequent complication of acromegaly because of the tendency for adenomas to develop in hyperplastic tissue; indeed, in our clinic we have a patient with kidney stones and acromegaly who has a high serum calcium, a normal rather than a high serum phosphorus, and marked hypercalcuria.

The second possibility as an explanation of the demineralization in acromegaly is that eosinophilic tumors of the pituitary may produce an excess of "the" corticotrophic hormone and thus lead to an overproduction of the "sugar hormone" and that this in turn may lead to an osteoporosis (see discussion below under Cushing's Syndrome). The hyperplasia of the adrenal cortex which accompanies acromegaly might support this hypothesis especially since the 17-ketosteroid excretion is not elevated in acromegaly (25) so that the hyperplasia is not due to an over-production of the 17-ketosteroid precursors of the adrenal cortex (*vide infra*). The fact that the "sugar hormone" inhibits "the" growth hormone (*vide infra*) does not preclude its over-production in acromegaly since in that condition there may be too much growth hormone to be entirely inhibited. This question can easily be settled by assay of the urine of acromegalics for its cortin and 11-oxysteroid contents (*vide infra*); this is underway.

A third possibility, to which the speaker rather leans, is that the increased protoplasmic mass in acromegaly (see enlargement of all organs) requires a higher nitrogen intake to keep in nitrogen balance. Hence the likelihood of an inadequate nitrogen intake is increased; this favors the possibility of there being too little material for the osteoblasts with which to build bony matrix which in turn results in osteoporosis. In other words, there is a demand for amino acids by all tissues and the bony matrix loses out because of a low priority rating. The normal serum phosphatase level favors osteoporosis. This explanation is in agreement with the findings of Bauer and Aub (14) who studied the calcium metabolism on a neutral, low calcium, rather low nitrogen (*circa* 10 g./day) diet on five acromegalic patients. Four of the five patients excreted much more calcium in the urine than the normal controls. The fecal calcium excretions were normal. Furthermore, the four patients who had hypercalcuria were in negative nitrogen balance while the patient who did not have hypercalcuria was in positive nitrogen

balance; what is more, the patient who had the most hypercalcuria was in the greatest negative nitrogen balance, but we have unpublished data on 2 cases of acromegaly both of whom received a high nitrogen intake. Both these patients had markedly negative calcium balances in spite of nitrogen equilibrium. One of these patients received estrogen which had an effect exactly similar to that seen with post-menopausal osteoporosis, namely a lowering of both fecal and urinary calcium excretions and the restitution of a positive calcium balance.*

In summary, therefore, "the" growth hormone has a marked stimulating effect on endochondral and membranous bone formation. As for endosteal bone formation there are no satisfactory data; the demineralization so common in acromegaly is probably not a direct manifestation of the growth hormone, but is to be attributed to some indirect effect, possibly an osteoporosis due to lack of some important nitrogenous element.

7. *Effect of Gonadal Hormones on Osteogenesis*

A short digression on the somatotrophic action of testosterone is in order at this point. For further discussion and references see Albright (2).

That the action of the hormones manufactured in the male gonads is not confined to the growth of the sexual organs and the development of the secondary sexual characteristics was well known long before testosterone was heard of. One has only to compare the physique of a bull with that of a steer. With the availability of testosterone propionate as a therapeutic agent it soon became apparent that this substance has a marked effect on muscular development. With its administration, a eunuchoid patient can put on forty pounds of muscle in a short period of time even without exercise. But the somatotropic action of testosterone does not stop with its action on muscles. It is capable of accelerating growth in patients suffering from panhypopituitary dwarfism (see Figs. 11 and 12); in such subjects all tissues, including epiphyseal cartilages, grow faster under the influence of testosterone therapy. Whereas Simpson, Marx, Becks, and Evans (49) were unable to maintain skeletal growth in hypophysectomized rats with testosterone propionate, they were able to show that this hormone prevented bone growth from completely stopping and that it accelerated the growth induced by "the" pituitary growth hormone in hypophysectomized rats. Metabolic studies indicate that following therapy with testosterone propionate there is retention not only of nitrogen but likewise of potassium, phosphorus, and sulphur in such proportions that the ratios of retained nitrogen to retained potassium, phosphorus, and sulphur, respectively, are close to those found in protoplasm (see Fig. 13).

*A fourth possibility is that the osteoporosis in acromegaly is secondary to lack of gonadal hormones which, in turn, is secondary to destruction of the gonadotrophic hormones by the pituitary tumors.

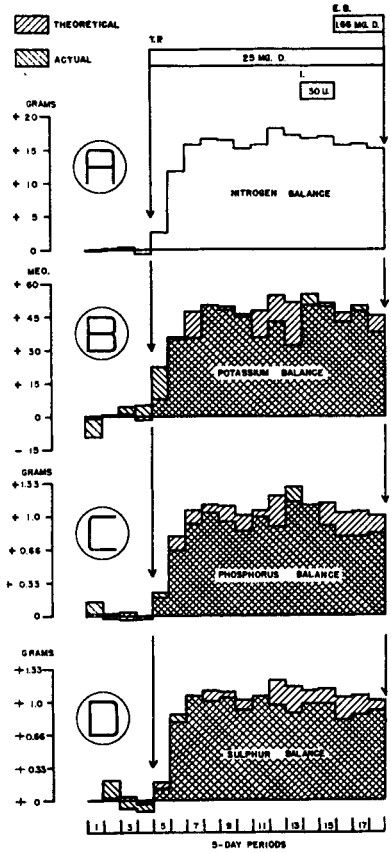


FIG. 13

A Comparison ofr Deviations in the Nitrogen, Potassium, Phosphorus, and Sulphur Balances as a Result of Testosterone Propionate Therapy in a Patient with Cushing's Syndrome (B.V. #74372)

The balances are charted as deviations from the averages of the four control periods. T.P = testosterone propionate; I. = insulin; E.B. = estradiol benzoate; D. = dosage per day; U. = units per day. The data for potassium are based on analyses for urinary excretion alone.

The chart has four divisions: A = the measured nitrogen balance; B = the measured nitrogen balance superimposed on the theoretical nitrogen balance explainable by the potassium balance; C = measured nitrogen balance superimposed on the theoretical nitrogen balance explainable by the phosphorus balance (after the phosphorus theoretically retained with calcium had been subtracted); D = measured nitrogen balance superimposed on the theoretical nitrogen balance explainable by the measured sulphur balance. For further discussion see Reifenstein, Albright, and Wells (45); for original data see Albright, Parson, and Bloomberg (6). (This figure is reproduced by permission of the *Journal of Clinical Endocrinology*.)

We can, therefore, conclude that testosterone propionate and other closely related steroids have an anabolic effect on many if not all tissues; among the tissues so affected is the growing epiphyseal cartilage and, as I will discuss later, the endosteal bony tissue.

8. *Effect of the Gonadal Hormones on Endosteal Bone Formation*

For a number of years in our laboratory we have been interested in the effect of estrogens (actually estradiol benzoate and estradiol diproprionate) and androgens (testosterone proprionate) on endosteal bone formation in various kinds of osteoporosis (4, 8, 6, 2). Since osteoporosis by definition is a lack of endosteal bone formation, it is a good condition in which to observe the effect of hormones on this function. Our work was stimulated by that of Dr. William U. Gardner and Dr. Carroll A. Pfeiffer on the effect of estrogens and androgens on the skeletons of pigeons and mice. For an excellent summary of this work and that of other authors working with animals, I would refer you to a recent review article (27).

In clinical medicine one can list the following conditions where one encounters osteoporosis: (1) disuse atrophy where the normal stimulus to osteoblastic activity is absent, (2) extreme old age where the bony tissue like other tissues (*cf.* hair, skin, muscles) atrophies, (3) malnutrition where the protein requirements of the body are not fulfilled and the bony matrix, like other tissue, is depleted, (4) Cushing's Syndrome, the nature of the osteoporosis of which will be discussed below, (5) an idiopathic osteoporosis where the cause of the condition remains obscure, and (6) the postmenopausal state where, we believe, the difficulty is a deficiency in estrogen to stimulate the osteoblasts.

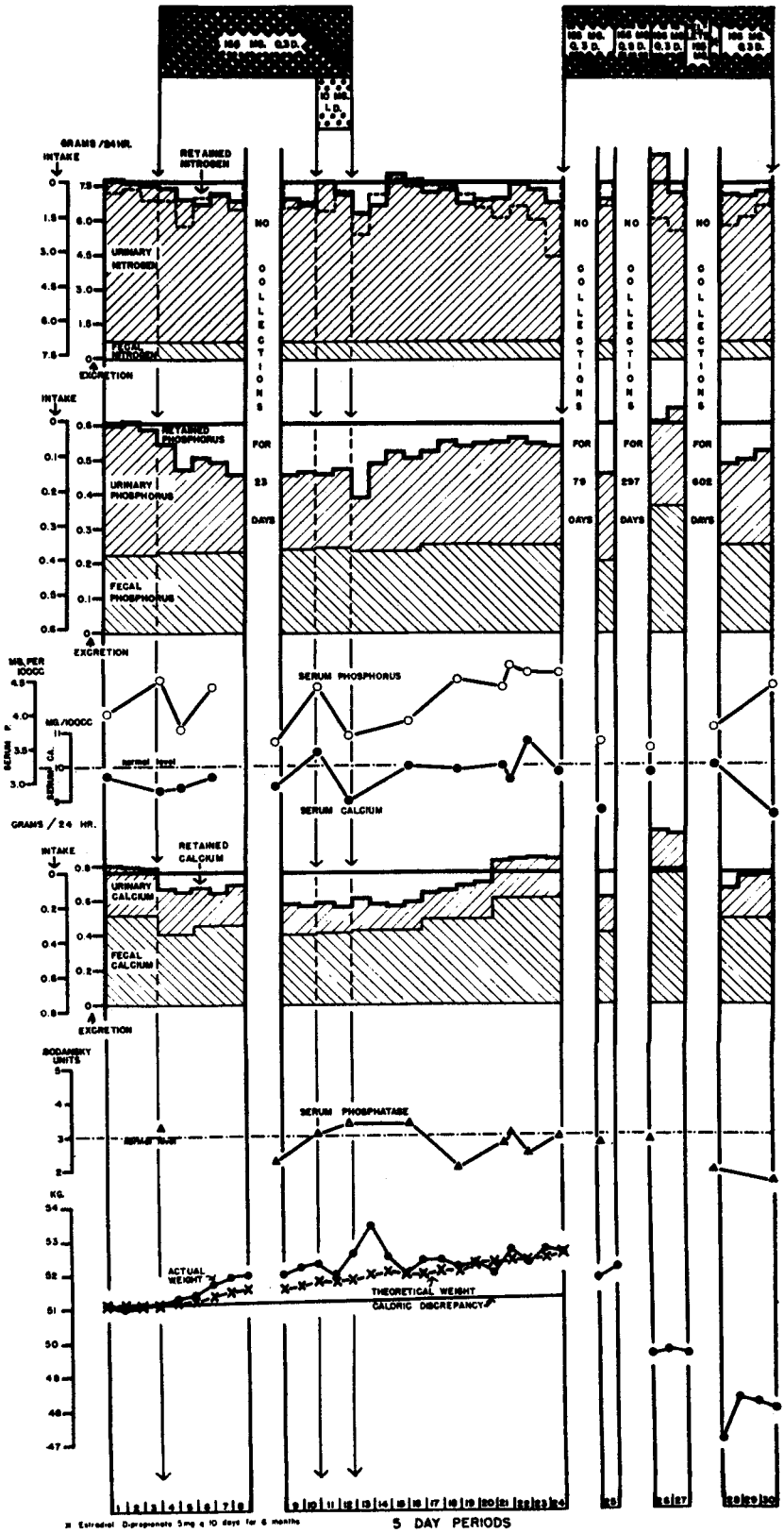
Osteoporosis in man, when due to a systemic cause, has a marked predilection for the vertebrae and pelvis and, to a lesser extent, the skull, and seldom involves the extremities. The lamina dura around the teeth, as seen by x-ray, is characteristically intact, an important differential point in the differential diagnosis between this condition and osteitis fibrosa generalisata. This distribution of the malady, while uneven, has a rhyme and a reason to it, and does not rule out an hormonal disturbance, which characteristically should have a generalized distribution, as the etiological factor. For an analogous example, Gardner found that estrogens cause dissolution of the pelvic bones of mice while stimulating bony proliferation in the femora.

A word about the clinical aspects of post-menopausal osteoporosis appears indicated. A moderate degree of osteoporosis of the spine is almost physiological after the menopause; its degree increases as the time during which the post-menopausal state has existed increases. The condition may progress to collapse and deformity of multiple vertebrae. It is surprising how

much deformity there can be without symptoms. The tendency is to overexaggerate the seriousness of collapsed vertebrae and to immobilize a patient in a plaster cast for a long period of time with this condition. Such therapy, by stopping stresses and strains, only serves to increase the osteoporosis. Furthermore, I feel that an artificial menopause, as opposed to a physiological one, leads to a more complete absence of gonadal hormones and hence to a more serious degree of osteoporosis.

In Figs. 14 and 15 will be seen in chart form the effect of estradiol therapy on the calcium, phosphorus, and nitrogen metabolisms of two patients with post-menopausal osteoporosis; in Fig. 16 will be seen similar data on the effect of estradiol dipropionate alone and in combination with testosterone propionate on a male patient with senile osteoporosis. It is concluded from these experiments and other similar ones that estradiol therapy puts a patient with post-menopausal or senile osteoporosis into a positive calcium balance, presumably by stimulating the osteoblasts; secondly, that testosterone has a similar action to estradiol; and thirdly, that testosterone plus estradiol have a greater effect than either agent alone. This synergistic action of testosterone and estradiol is in agreement with the findings of Bloom, McLean, and Bloom (17) and of Pfeiffer and Gardner (41) on pigeons but contrary to the findings of Gardner and Pfeiffer on mice (26).

Although several investigators, including Dunn (23) and Rakoff, Cantarow and Paschkis (43), have reported a beneficial action from a purely clinical point of view of estrogens in the treatment of Cushing's Syndrome, and although the osteoporosis in this condition is very similar to that seen in the post-menopausal state, Albright, *et al.* (6) concluded from metabolic studies (calcium, phosphorus, and nitrogen) on two patients that estrogen was without effect. Perloff, Rose, and Sundaman (40) also reported an inconclusive effect of estrogen on the calcium metabolism in Cushing's Syndrome. A further analysis of the data of Albright *et al.* (6), however, shows that their conclusion is not justified. It is true that estrogen had no beneficial effect on the nitrogen balance which was in marked contrast to the effect of testosterone proprionate. On the other hand, it is quite clear that estrogen administration did benefit the calcium balance. Thus, in their patient No. 1 (see Fig. 24), estrogen therapy, while apparently adversely affecting the nitrogen balance, increased the calcium balance; later in the same patient the addition of estrogen administration to that of testosterone propionate further improved the calcium balance. In their patient No. 2, estrogen therapy was started before the metabolism study was initiated, so its effect is hard to evaluate; none the less, further metabolic studies undertaken forty days after omitting estrogen show that the calcium balance had changed from a positive to a negative one (see Fig. 25).



* Estradiol Dipropionate 5mg q 10 days for 6 months

FIG. 14

Metabolic Data Showing Effect of Estradiol Benzoate on Nitrogen, Phosphorus, and Calcium Metabolisms of Patient (S.F. #156453) With Post-menopausal Osteoporosis

Metabolic data in this and other figures are arranged according to the following scheme. There is a horizontal base line; intake is charted on a reverse scale downward from this base line; the fecal and urinary excretions are then measured from the intake line upward toward the base line. If the output (feces and urine) exceeds the intake, the final level will be above the base line; if it does not, the final level will be below the base line. Thus a positive balance will be indicated by a clear area below the base line; a negative balance by a shaded area above the base line. Since about 97% of the body phosphorus is contained either in bone (Ca/P ratio equals 2.23) or as an integral part of protoplasm (N/P ratio of muscle equals approximately 14.7), the scales for the nitrogen, phosphorus and calcium metabolism are so chosen (1 g. of P equivalent to 2 g. Ca and 15 g. of N) that the surface representing the balance of phosphorus should approximately equal the sum of the surfaces representing the balances of calcium and nitrogen. Dotted line in the nitrogen metabolic data represents the theoretical nitrogen balance based on the phosphorus and calcium balances.

Note especially the initiation of a positive calcium balance on the administration of estradiol benzoate, the return to a negative calcium balance 9 periods (45 days) after cessation of therapy, that both urinary and fecal calcium excretions were affected, that there was a slight tendency to a positive nitrogen balance with estradiol benzoate therapy, that the phosphorus balance followed the nitrogen and calcium balances, that the increase in the phosphorus balance with estradiol benzoate therapy was the result of a decreased urinary phosphorus excretion, that the serum phosphorus values which tended to be high were on the whole lower during estradiol benzoate therapy, and finally, that periods 26, 27 were out of line with respect to the calcium and phosphorus balances probably due to erroneously high fecal values resulting from too short a metabolic study. The added medication in periods 11 and 12 was progesterone, 10 mg. daily. For further clinical data from this patient see Albright *et al.* (8).

(These data are recharted from data of Albright *et al.* (4)).

In unpublished data from our laboratory we have found both estradiol dipropionate and testosterone propionate ineffective in combatting the osteoporosis in cases of idiopathic osteoporosis.

Cuthbertson (19) has shown that negative nitrogen, phosphorus and

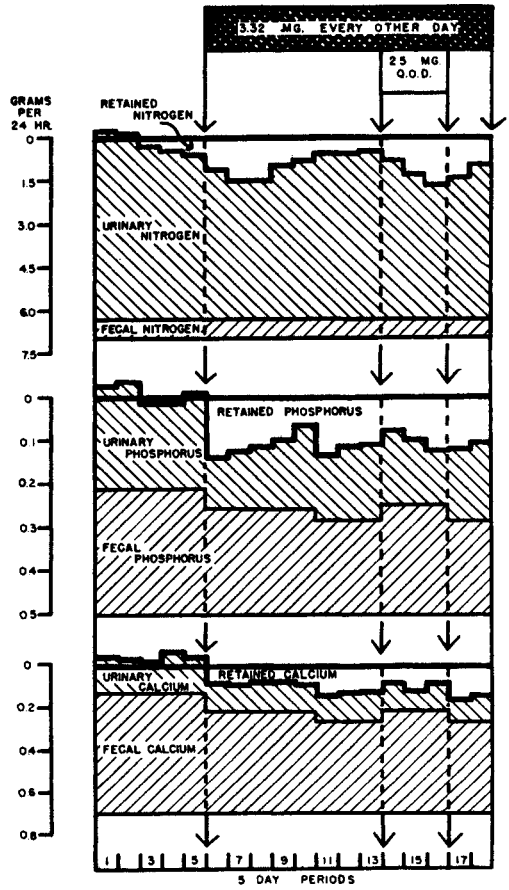


FIG. 15

Metabolic Data Showing Effect of Estradiol Benzoate and Testosterone Propionate Therapy on Nitrogen, Phosphorus, and Calcium Metabolisms in Patient E.P., #203540, With Post-menopausal Osteoporosis

For construction of chart see paragraph in italics under Figure 14.

Note, as in Figure 14, that decreased calcium excretion with estradiol benzoate therapy results from decrease in both urinary and fecal components; note marked effect of testosterone therapy during periods 14, 15, and 16 on nitrogen balance. For discussion of clinical data see Albright *et al.* (8). The data on this figure was recharted from Albright *et al.* (4).

calcium balances follow fractures; the findings have been confirmed by Howard, Parson and Bigham (31) and by ourselves (unpublished data) How much of this negative calcium balance following trauma is to be attributed to the "adaptation syndrome" of Selye (*vide infra*), and how much

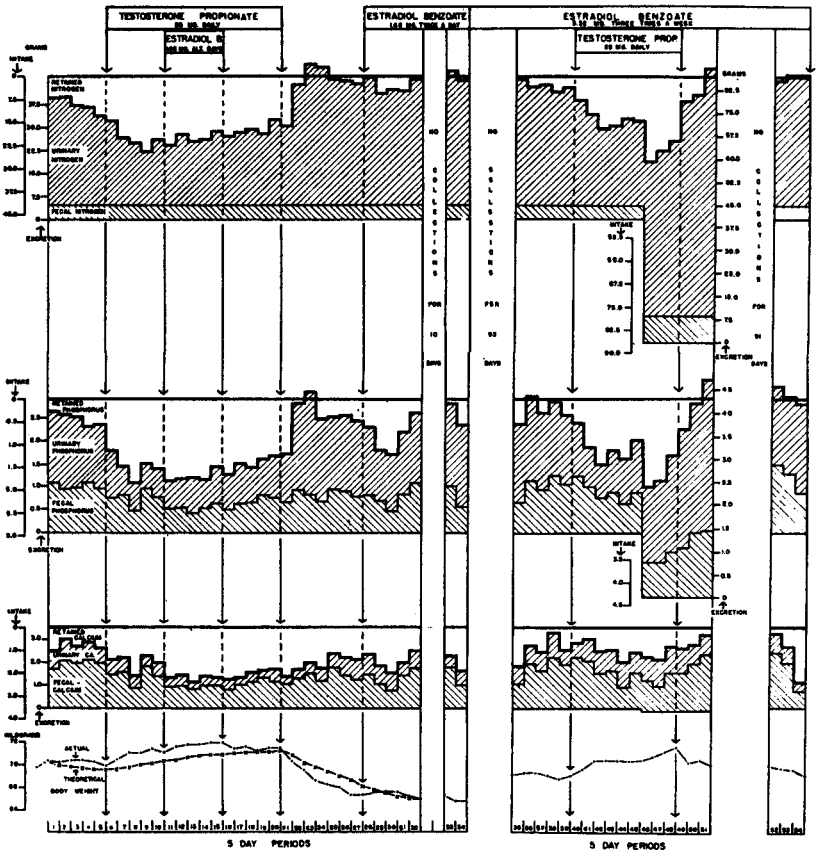


FIG. 16

Metabolic Data Showing Effect of Testosterone Propionate Alone and in Combination with Estradiol Benzoate on Nitrogen, Phosphorus, and Calcium Metabolisms in a Male Patient (M.H., #278511) with Senile Osteoporosis

For construction of chart see paragraph in italics under Figure 14.

Note that testosterone propionate had an effect on calcium metabolism similar to that of estradiol benzoate with regard to both fecal and urinary excretions, that estradiol benzoate therapy added to that of testosterone propionate further improved the calcium balance, that testosterone propionate had a greater effect on nitrogen and phosphorus metabolisms than did estradiol benzoate. These data have not hitherto been published and will be gone into in more detail in a later paper from this clinic.

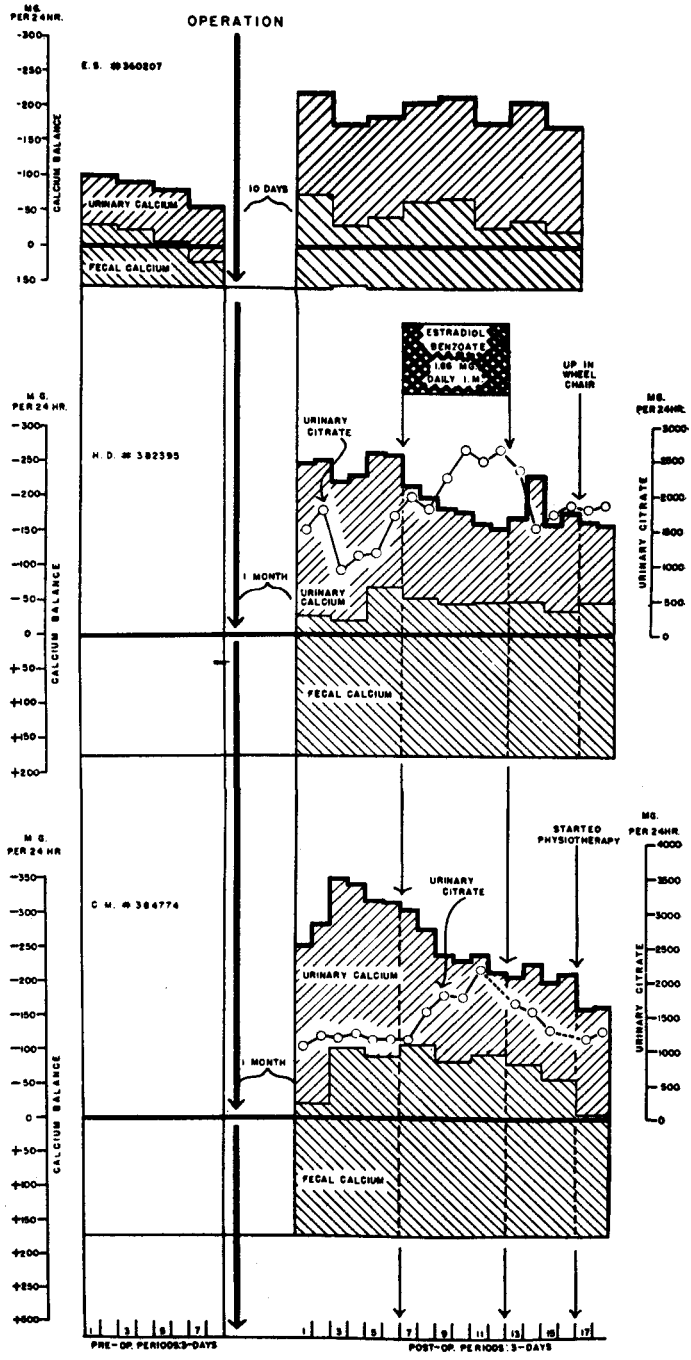

OPERATION
E. S. # 360207
10 DAYS
URINARY CALCIUM
FECAL CALCIUM
CALCIUM BALANCE
MG. PER 24 HR.
ESTRADIOL BENZOATE 1.66 MG. DAILY I M.
UP IN WHEEL CHAIR
H. D. # 382395
URINARY CITRATE
1 MONTH
URINARY CALCIUM
FECAL CALCIUM
STARTED PHYSIOTHERAPY
C. M. # 384774
PRE-OP. PERIODS 3-DAYS
POST-OP. PERIODS: 3-DAYS

FIG. 17

Effect of Estradiol Benzoate on Negative Calcium Balance Following Orthopedic Operation

For construction of figure see paragraph in italics under Figure 14. Data pertain to 3 patients: E.S., #360207, H.D., #382395, and C. M., #384774. The first patient was studied before and after an orthopedic operation; the second two only after operation. Patients 2 and 3 received estradiol benzoate during convalescence. Note in E.S. that the calcium excretion in both urine and feces was increased after operation and that there was no definite tendency to a return to pre-operative levels by the end of the investigation, 64 days after the operation. Note in patients H.D. and C.M. that both were in a markedly negative calcium balance following operation and that in both the administration of estradiol benzoate 1.66 mg. daily intramuscularly from periods 7 through 12 caused a marked decrease in the urinary calcium excretion. The urinary citrate excretions recorded were carried out by Dr. Ephraim Shorr and do not pertain to the present discussion. These studies will be published in greater detail in a later paper.

to atrophy of disuse I do not know. We have data which strongly suggest that the negative calcium balance following orthopedic operations can be ameliorated or overcome by estradiol therapy (see Fig. 17).

There are very little data on the effect of estrogen on endochondral bone formation in man. It seems quite clear that large doses of estrogen inhibit bone growth in animals (Gardner and Pfeiffer (27)). Zondek (58) ascribes this inhibition to a decreased formation of pituitary growth hormone since the simultaneous administration of growth hormone with estrogen prevented the decreased growth rate. Johnston (33) found decreased calcium balances in young girls who received moderate doses of estrone for six to eighteen days. On the other hand, Dr. Nathan Talbot has called the author's attention to the accelerated growth rate exhibited by children with granulosa cell tumors of the ovary. This suggests a stimulating effect of estrogen on bone growth. Furthermore, the speaker has been prejudiced in favor of a stimulating action of estrogens on growth by the short stature in patients with ovarian agenesis. This will be discussed in more detail later. Suffice it to say here that the speaker is inclined to attribute the short stature in ovarian agenesis to an accompanying decreased production of androgens by the adrenal cortex (17-ketosteroid excretion in such patients is definitely reduced); furthermore, he has been inclined to attribute the decreased androgen production by the adrenal cortex to lack of estrogen to stimulate the luteinizing hormone to stimulate androgen production. In favor of this point of view is the fact that these patients do seem to respond to small doses of estrogen by an acceleration of growth, although this is a hard experiment to control; large doses of estrin seem to have the opposite effect. Against the above point of view is the failure of small or large doses of estrin to consistently raise the 17-ketosteroid excretion in these patients. I think we should leave the question of the effect of estrogens on growth wide open while we await further experimental data.

The author is aware of no good data on the effect of progesterone on osteogenesis in man. This subject will therefore be passed over.

To summarize, testosterone stimulates both endochondral and endosteal bone formation; estrogen has a questionable effect on endochondral bone formation but stimulates endosteal bone formation; progesterone has not been sufficently studied to allow any statement. The effect of gonadal hormones on membranous bone formation likewise remains obscure.

9. *Effect of the Adrenal Cortical Hormones on Osteogenesis*

One would gain the impression from the papers we have heard during the past two days that there is only the one adrenal cortical hormone with its threefold function: (a) to antagonize the action of insulin, (b) to facili-

tate the conversion of protein into sugar (gluco-neogenesis), and (c) to cause involution of the thymus and the lymphatic tissue. This hormone has been called the "sugar" or "S" hormone. Dr. Gregory Pincus, when he called attention in his interesting paper to the parallelism between the 17-ketosteroid excretion in the urine and the decrease in the lymphocytic count following stresses, implied that the precursor of the urinary 17-ketosteroids is an "S" hormone. I feel that the urinary 17-ketosteroids are an index, not of the "S" hormone, but of a second hormone which is similar but not identical with testosterone both as to its androgenic and somatotrophic properties, which governs the growth of axillary and pubic hair in females, and the production of which is physiological and begins at puberty. I will speak of this hormone as the "nitrogen" or "N" hormone.

I had not intended to stop and review the evidence for the existence of the "N" hormone but, since I have found considerable skepticism concerning it, I will enumerate some of the clinical evidence. The evidence obtained from laboratory animals has been recently reviewed by Parkes (38). Both forms of evidence, except for the isolation of adreno-sterone by Reichstein (44) from the adrenal cortex, are admittedly circumstantial.

Before coming to the clinical evidence, I will at least mention the following observations from animal experiments. Hypertrophy of the adrenal cortex has been produced by preparations of luteinizing hormone (LH) containing insignificant amounts of adreno-cortico-trophic hormone and by preparations of adreno-cortico-trophic hormone containing insignificant amouts of LH; in hypophysectomized animals no restoration of the lipoid content, and no disappearance of the sudanophobe zone of the adrenal cortex occur after administration of LH, while these changes do occur after administration of the adreno-cortico-trophic hormone (Golla and Reiss (29), (28)). These observations suggest that, besides "the" adreno-cortico-trophic hormone which stimulates "S" hormone production in the adrenal cortex, there is another trophic hormone, LH, which stimulates the production of another hormone in the adrenal cortex, presumably the "N" hormone (see Fig. 18). Further evidence that the luteinizing hormone effects the adrenal cortex is summarized elsewhere (46).

The clinical evidence for the existence of an "N" hormone includes evidence that the adrenal cortex in the female gives rise to the precursor of the urinary 17-ketosteroids, evidence that the growth of axillary and pubic hair in the female is controlled by the precursor of the urinary 17-ketosteroids, evidence that the "S" hormone of the adrenal cortex is not excreted as a urinary 17-ketosteroid and does not influence the growth of axillary and pubic hair, and, finally, evidence that the excretion of 17-ketosteroids in the urine does not parallel the excretion of "S" hormone. Let us examine this evidence point by point:

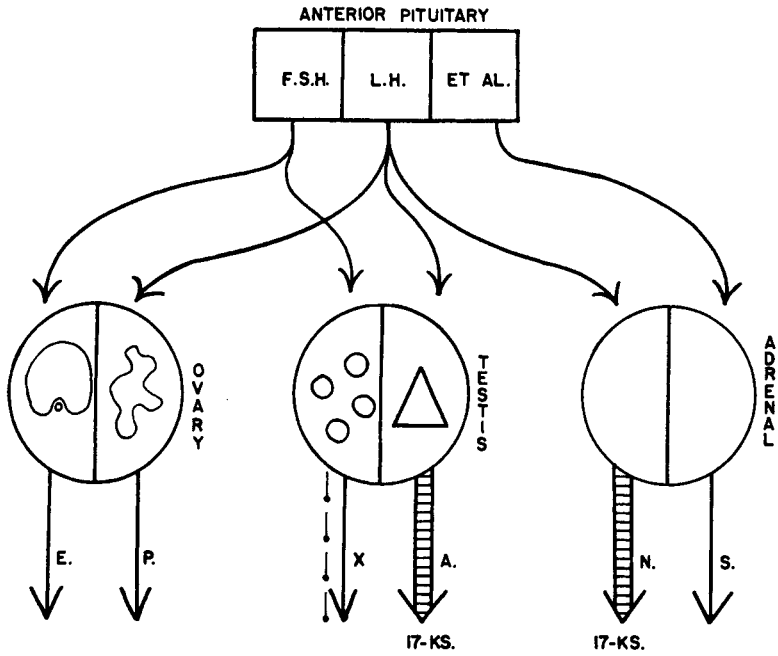


FIG. 18

Schematic Diagram to Show Normal Interrelationships Between Pituitary, Gonads, and Adrenal Cortex

The pituitary is divided into three compartments: FSH for follicle-stimulating hormone, LH for luteinizing hormone, and "et al" for remaining hormones. Striped arrows are used to represent 17-ketosteroid precursors. E = estrogen, P = progesterone, X = hormone thought to be produced by tubules of testis, A = androgen, N = "N" hormone, and S = "S" hormone. (Reproduced from Reifenstein *et al.* (46) by permission of the *Journal of Clinical Investigation.*)

(1) The adreno-genital syndrome (adrenal virilism, pseudohermaphroditism) in females, characterized by virilism, somatic precocity, and a high excretion of 17-ketosteroids, may result from either a hyperplasia or tumor of the adrenal cortex. This suggests that the adrenal cortex produces a testosterone-like steroid. To be sure, the strength of this evidence is greatly vitiated by the possibility that such tumors or such hyperplasias might arise from fetal rests.

(2) Gonadectomy in either sex has little if any effect upon the 17-ketosteroid excretion in the urine; Callow, Callow, and Emmens (18) found substantial amounts of androgens by biological assay in the urine of eunuchs and ovariectomized women. This is strong evidence that the adrenal cortex is an important source of the precursor of 17-ketosteroids and of urinary androgens.

(3) Gonadectomy in either sex after puberty does not prevent the growth of axillary and pubic hair being maintained at the level and configuration found in normal adult females. This is evidence that the gonadal function is not necessary for this degree of growth of axillary and pubic hair.

(4) Addison's Disease in the female, even when accompanied by normal ovarian function, is characterized by an absent or very low 17-ketosteroid excretion, and by an absent or very low production of axillary and pubic hair. This suggests that the ovaries are a negligible source of the precursor of the urinary 17-ketosteroids and do not directly control production of axillary and pubic hair.

(5) Addison's Disease in the male is associated with an only moderate decrease in urinary 17-ketosteroids and essentially normal production of axillary and pubic hair. This suggests that the absence of 17-ketosteroids in the female patient with Addison's disease is not due to an effect of the Addison's Disease on the gonadal production of 17-ketosteroid precursor and that the "S" hormone is not necessary for the production of axillary and pubic hair.

(6) The 17-ketosteroid excretion in either sex does not appear until puberty. In the female, this observation is consistent with the thesis that the adrenal cortex starts producing a second hormone at puberty.

(7) Pubic and axillary hair in the female appear at the same time as the 17-ketosteroid excretion in the urine becomes positive. This observation, tógether with observation #6, is consistent with the thesis that the precursor of the urinary 17-ketosteroids in the female controls the development of axillary and pubic hair.

(8) There are three conditions, other than Addison's Disease in the female, which lead to absence of growth of axillary and pubic hair: panhypopituitarism, myxedema, and old age; all three are characterized by very low or absent 17-ketosteroid excretions in the urine. This suggests that in the female the growth of axillary and pubic hair is controlled by the precursor of the urinary 17-ketosteroids.

(9) In a patient with panhypopituitarism, the rubbing of an ointment containing testosterone into the skin of one axilla will cause axillary hair to grow in that axilla and not the other. This suggests that axillary hair growth is dependent on a testosterone-like hormone.

(10) Axillary and public hair are absent before puberty when there is every reason to believe that "S" hormone is being produced; indeed, Dr. Nathan Talbot has informed me that the evidence to date suggests that the "11-oxysteroid" excretion (*vide infra*) before puberty (infancy not included) is of the order of magnitude of that found after puberty. This suggests that the "S" hormone is not a precursor of the urinary 17-ketosteroids and is not connected with axillary and pubic hair growth.

(11) Certain cases of Cushing's Syndrome, a condition almost certainly due to over-production of "S" hormone (*vide infra*), do not have high urinary 17-ketosteroids. This also suggests that the "S" hormone is not a precursor of the urinary 17-ketosteroids.

(12) The amounts of "11-oxysteroid" (50) and of "S" hormone determined biologically (52) do not parallel the amounts of 17-ketosteroids in the urine. This in itself, is strong evidence that they are excretory products of different hormones.

(13) Another argument for the presence of an "N" hormone in the adrenal cortex is to be found in a syndrome recently brought to the fore (7, 51, 57). This syndrome is characterized by (a) primary ovarian agenesis with lack of development of breasts and uteri, and a high excretion of follicle-stimulating-hormone, (b) short stature (*circa* 4'7") with low 17-ketosteroid excretion (*circa* 3.8 mg./24 hours), slightly delayed bone age, and decreased but not absent axillary and pubic hair, and (c) a normal responsiveness to an insulin induced hypoglycemia. I believe the findings under "a" are to be attributed to the ovarian agenesis with resulting lack of estrin production and compensatory overproduction of follicle-stimulating-hormone, that the findings under "b" are due to decreased but not absent production of "N" hormone, and that the findings under "c" indicate a normal production of "S" hormone. There are two schools of thought as to the cause of the low "N" hormone production; one school holds that the syndrome represents a widespread endocrine disorder which effects not only the ovary but also the adrenal cortex; the other school, to which I rather adhere, believes that the fundamental endocrine disorder is an ovarian agenesis, that this results in a decreased production of LH because of a lack of stimulation of LH production by estrin, and that this in turn leads to lack of "N" hormone production by the adrenal cortex since LH in the female is thought (*vide supra*) to stimulate not only the gonads to produce progestin but also the adrenal cortex to produce "N" hormone. Regardless of which of the above explanations is correct, the fact remains that the presence of a low 17-ketosteroid excretion with a decreased amount of axillary and pubic hair suggests that the growth of said hair is dependent on the precursor of the 17-ketosteroids; furthermore, the very presence of axillary and pubic hair, albeit in reduced amounts, in the presence of ovarian agenesis is evidence that its growth is not controlled by an ovarian hormone.

(14) There are several points of difference between the syndrome just discussed and panhypopituitary dwarfism with which it is often confused. The following difference is pertinent to the present discussion: whereas estrin therapy to either syndrome leads to breast and uterine growth, in the patient with ovarian agenesis it results in production of axillary and

pubic hair while in the patient with panhypopituitarism it has no effect on the growth of this hair. My interpretation of this observation can best be set forth in diagramatic form (see Figs. 19 and 20).

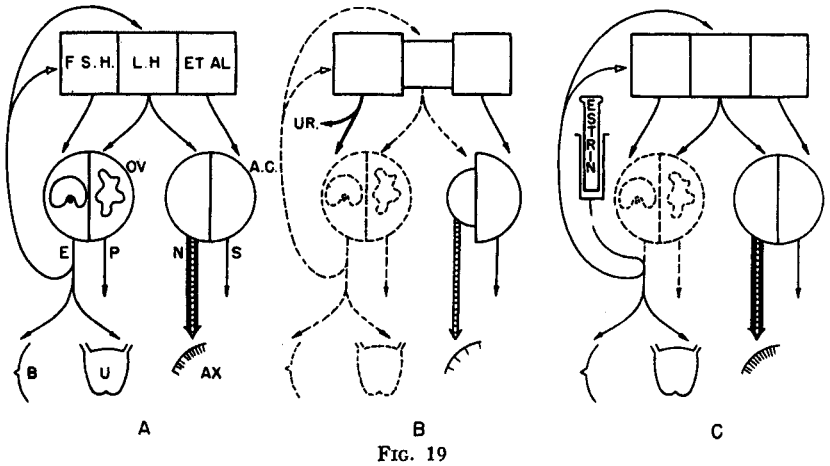


FIG. 19

Schematic Representation of Speaker's Conception of Pituitary—Ovarian—Adrenal Cortical Interrelationships in (A) Normal, (B) Ovarian Agenesis and (C) Ovarian Agenesis Under Estrogen Therapy

F.S.H. = follicle-stimulating hormone
L.H. = luteinizing hormone
et al = remaining pituitary hormones
E = estrogen
P = progesterone
N = "N" hormone
S = "S" hormone
arrows with open heads = inhibiting arrows
For discussion see text.

Fig. 19A indicates my interpretation of certain hormonal interrelationships in the normal, Fig. 19B the disordered hormonal balance in the patient with ovarian agenesis, and Fig. 19C the effect of estrin therapy on said imbalance. Figs. 20A, 20B, and 20C are three corresponding diagrams for the patient with panhypopituitary dwarfism. Note in Fig. 19C that estrin therapy in the patient with ovarian agenesis causes stimulation of breast and uterine tissue directly, whereas it causes growth of axillary hair indirectly via the pituitary; note in Fig. 20C that estrin therapy in the panhypopituitary dwarf causes the same stimulation of breast and uterine tissue directly, but that it has no effect on axillary hair growth since the chain leading to the stimulation of this tissue is broken at the pituitary link. These observations suggest that estrin *per se* is not a direct stimulator or growth of axillary hair.

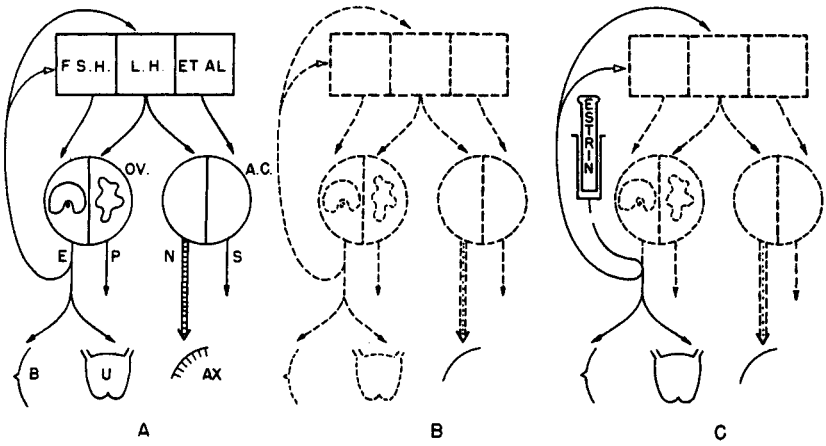


FIG. 20

Schematic Representation of Speaker's Conception of Pituitary-Ovarian-Adrenal Cortical Interrelationships in (A) Normal, (B) Panhypopituitarism, and (C) Panhypopituitarism Under Estrogen Therapy (*cf.* Figure 19)

For discussion, see text; for further discussion, see Albright *et al.* (7).

With these fourteen points and the animal experiments summarized by Parkes (38), I will rest my argument in defense of the existence of an "N" hormone in the adrenal cortex.

10. *Cushing's Syndrome; A Hyper-Adreno-Corticism With Respect to the "S" Hormone*

In two previous communications (2, 6), I and my colleagues have assembled evidence that most of the manifestations of Cushing's Syndrome, with the exception of those resulting from a disturbance of electrolyte metabolism, are the result of a hyper-adreno-corticism with respect to the "S" hormone. Three new pieces of evidence can now be added to support this contention: (a) Talbot *et al.* (50) find high titers of 11-oxysteroids* in the urine of patients with Cushing's Syndrome. For example, in our most recent case, they found a value of 5+ mg./24 hours whereas the upper limit of normal is about 0.3 to 0.4 mg./24 hours. (b) The earlier observations of Anderson, Haymaker, and Joseph (10), and of Weil and Browne (54, 55), who found by biological methods increased amounts of "S" hormone in the urine of patients with Cushing's Syndrome, can now be confirmed by improved techniques. Thus, using a modification of the Dobriner adaptation to mice (21) of the Reinecke and Kendall (47) test for "S" hormones on

*The term "11-oxysteroid" introduced by the speaker is somewhat inaccurate; the term used by Talbot was "corticosteroid-like substances."

rats, Miss Grace Griswold in our laboratory was able to demonstrate in this same patient with Cushing's Syndrome 72 mouse units/24 hours whereas the upper limit of normal is about 6 units/24 hours. (c) It is now quite clear from the work of Dougherty and White (22) and others, that the "S" hormone causes degeneration of the thymus and lymphoid tissue with a resulting decrease in the lymphocytic count; de la Balze, Reifenstein, and Albright (20) were able to demonstrate a marked lymphocytopenia in ten cases of Cushing's Syndrome as compared with a lymphocytosis in twenty cases of Addison's Disease. When this new evidence is added to the old, I think it is safe to conclude that whatever bone changes are found in Cushing's Syndrome are the result of an excess production of "S" hormone.

11. *The Effect of "S" Hormones of the Adrenal Cortex on Osteogenesis*

A better understanding of the effect of "S" hormone on osteogenesis will be gained if we discuss the effect of the "S" hormone on protoplasm in general. Most investigators have been satisfied with the concept that the "S" hormone facilitates the conversion of proteins into sugar (gluconeogenesis) which, if carried to excess as in Cushing's Syndrome, results in an overproduction of sugar and a lack of protoplasm. It has been our concept (2) that protoplasm in general, like the protoplasmic matrix of bone (*vide supra*), is constantly being anabolized and catabolized at one and the same time; a factor which increases catabolism would lead to very much the same net result as a factor which inhibits anabolism, but there would be some differences; it is my belief that the "S" hormone is anti-anabolic rather than catabolic. I will cite six pieces of evidence: (a) An inability to attain a positive nitrogen balance rather than a propensity to go into negative nitrogen balance would be characteristic of an individual suffering from an excess of an anti-anabolic principle as opposed to an excess of a catabolic principle; studies on patients with Cushing's syndrome have seldom revealed markedly negative nitrogen balances. (b) The minimal nitrogen excretion (excretion on a high-caloric, low-protein diet) is not high in Cushing's Syndrome (2); this evidence is against there being an excess of a catabolic principle in Cushing's Syndrome. (c) The negative nitrogen balance which follows operations, injuries, and burns is little influenced by a moderate excess of nitrogen in the intake; it, too, is thought to be due to an excess of the "S" hormone (*vide infra*); if the "S" hormone were catabolic one should be able to compensate for it by supplying an excess of the materials needed for anabolism just as in osteitis fibrosa generalisata where bone destruction is primarily too great one can attain calcium equilibrium by supplying an excess of the materials necessary to build bone. (d) There are certain anabolic processes which are irreversible and thus

cannot be wiped out by catabolism, for example, growth at an epiphyseal cartilage; if the "S" hormone were a catabolic principle, it should not inhibit epiphyseal growth, a purely anabolic function; Wells and Kendall (56) obtained cessation of growth in rats with Compound E and corticosterone, both "S" hormones; Becks, Simpson, Li, and Evans (15) likewise obtained cessation of growth (achondrogenesis) with andreno-corticotrophic hormone; finally a patient of ten years and eleven months with Cushing's Syndrome studied in our clinic showed almost complete cessation of growth during the active stage of the disease (see Fig. 21). (e) Becks, Simpson, Marx, Li, and Evans (16) working with hypophysectomized rats found that growth hormone activated the epiphyseal cartilage and thus promoted endochondral bone formation, that adreno-corticotrophic hormone by itself had practically no effect on the epiphyseal cartilage, and finally that adreno-corticotrophic hormone given together with the growth hormone prevented the effect of the growth hormone. It is quite clear from these experiments that the "S" hormone, produced as a result of stimulation of the adrenal cortex with the adrenocorticotrophic hormone, inhibited the anabolic action of the growth hormone and was therefore anti-anabolic. (f) A study of the one tissue where it is possible to distinguish under the microscope between anti-anabolism and catabolism histologically, namely bone, shows very clearly that the disturbance in the bone in Cushing's Syndrome is an anti-anabolic one; thus one finds osteoporosis, where the primary difficulty is lack of bone formation by the osteoblasts, not osteitis fibrosa generalisata, where the initial step is the resorption of bone.

The anti-anabolism in respect to protoplasm of the "S" hormone in Cushing's Syndrome is diagramatically represented in Fig. 22 where it is contrasted with the increased anabolism due to an excess of the "N" hormone in the adreno-genital syndrome. This anti-anabolism of protoplasm in Cushing's Syndrome accounts for not only the osteoporosis, but the muscular weakness, the thin skin, probably the easy bruisability, and possibly the atrophy of the lymphoid tissues and thymus.

An excess production of the "S" hormone, as discussed by Dr. J. S. L. Browne at the meeting here last year, is also met after injuries, operations, and burns, and probably constitutes a fundamental adjustment in the adaptation syndrome described by Selye (48). This, together with the atrophy of disuse, may account for the increased calcium excretion after fractures (19) confirmed by Howard *et al.* (31) and orthopedic operations (see Fig. 17).

In summary, therefore, the "S" hormone inhibits endochondral, endosteal, and probably membranous bone formation.

12. *The Effects of the "N" Hormone of the Adrenal Cortex on Osteogenesis*

The adreno-genital syndrome offers an excellent opportunity to study the effect of an excess of "N" hormone on osteogenesis. Children with this affliction grow much more rapidly in every way than do normal children (see Fig. 21), but, due to early closures of the epiphyses, end up by being normal in height or slightly short. The bony structure itself of the skeleton

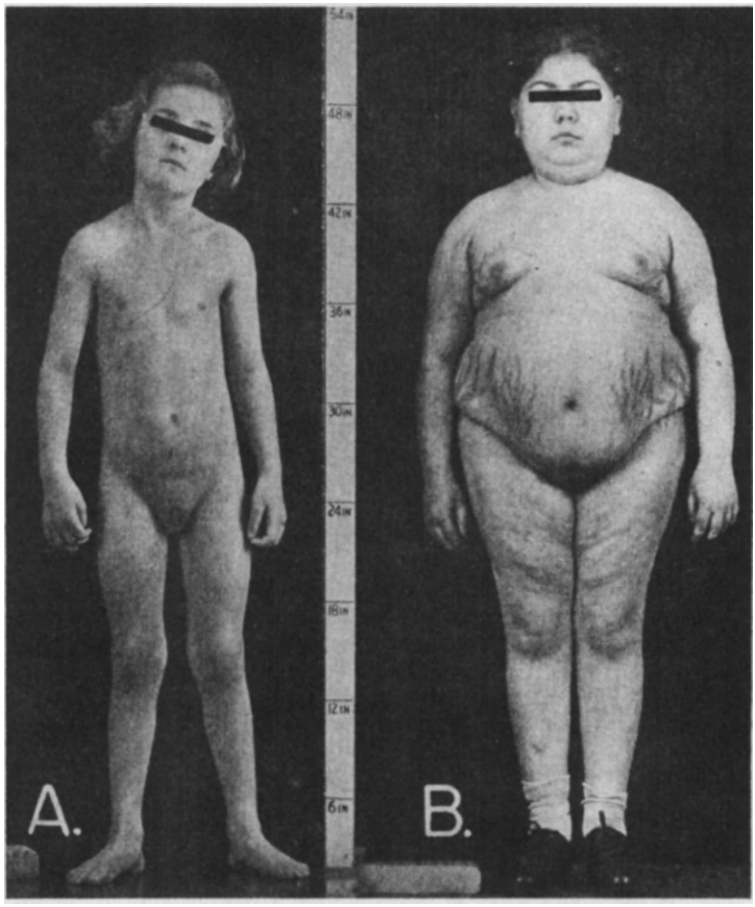


FIG. 21

Photographs to Contrast Adreno-Genital Syndrome with Cushing's Syndrome.

A = Patient C.O., #169536 at 5 years and 6 months with adreno-genital syndrome; B = Patient I.G., #350260 at 11 years and 10 months with Cushing's Syndrome. Scale applies to both photographs. Figure reproduced from Albright (2).

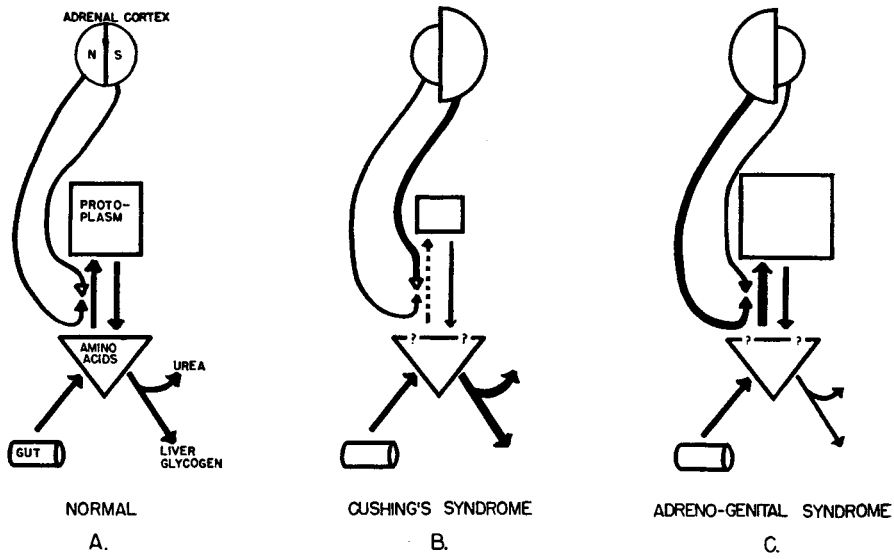


FIG. 22

Schematic Diagrams to Show Speaker's Interpretation of Pathological Physiology in Cushing's Syndrome (B) and Adreno-genital Syndrome (C) as Compared with the Normal (A)

Note in Cushing's Syndrome that there is an increased production of "S" hormone from the adrenal cortex, that this inhibits the production of protoplasm from amino acids (an arrow with an open end at the point is an inhibiting arrow), that this results in a deficiency of protoplasm, and that there is accordingly an increased deaminization of amino acids with an increase in glycogen production and an increase of nitrogen excretion in the urine. Note in the adreno-genital syndrome that there is an increased production of "N" hormone, that this stimulates anabolism of amino acids into protoplasm, that this results in an increase in the protoplasmic mass and a decrease in the deaminization of amino acids into glycogen with a resulting decrease in nitrogen excretion in the urine. (Question marks in triangles representing amino acids are introduced to show the speaker's lack of information as to whether the amino acids are increased or decreased.)

of such children is dense but probably not pathologically so. Adults who develop this condition do not develop the characteristics of acromegaly; this suggests that periosteal bone is not stimulated by the "N" hormone.

It appears, therefore, that "N" hormone is opposite in its action to "S" hormone as regards endochondral and probably endosteal bone formation. In both of these respects "N" hormone is like testosterone. Furthermore, "N" hormone is similar to growth hormone as regards epiphyseal growth, but not as regards membranous bone formation.

13. *Effect of Addison's Disease on Osteogenesis*

Since Addison's Disease is a pan-hypoadrenocorticism, there is in this condition a deficiency of both "N" and "S" hormone. Since these hormones have the opposite actions on the skeleton one is not too surprised that the skeleton is virtually unaffected in Addison's Disease. Growth may not be impaired if the electrolyte balance is controlled and if there are no secondary complications.

14. *Effect of Therapy with Testosterone on Bone Lesions of Cushing's Syndrome*

It follows from the above discussion that an excess of "N" hormone should neutralize an excess of "S" hormone. In the absence of a preparation of "N" hormone, it became of interest to study the effects of testosterone compounds on cases with hyper-adreno-corticism with respect to "S" hormone. Figure 23 shows in diagrammatic form the changes in the disordered homeostasis to be anticipated by such therapy. In Figures 24 and 25 appear in chart form some nitrogen, phosphorus, and calcium metabolic data obtained by Albright, *et al.* (6) on two patients with Cushing's Syndrome to demon-

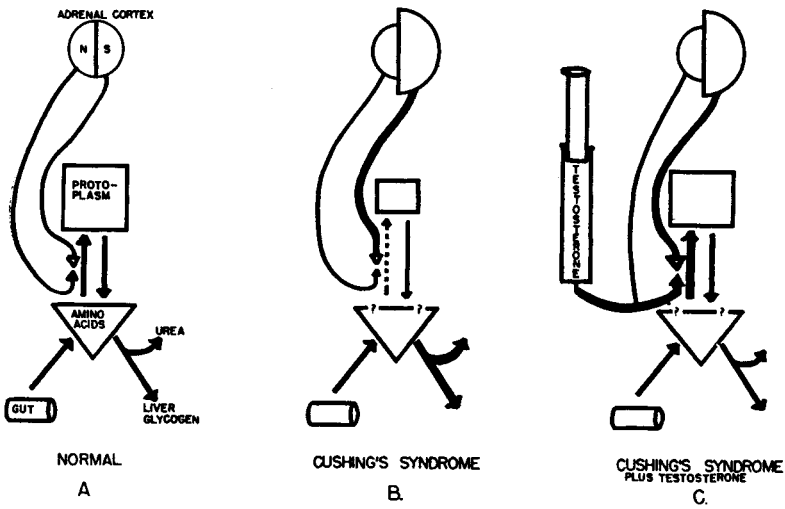


FIG. 23

Schematic Diagrams to Show Speaker's Interpretation of Pathological Physiology in Cushing's Syndrome (B) as Compared with Normal (A) and the Effect of Testosterone Therapy on the Pathological Physiology (C)

Note in "C" that testosterone adds to the action of "N" hormone in stimulating anabolism of protoplasm and overcomes the inhibiting action of the excess of "S" hormone.

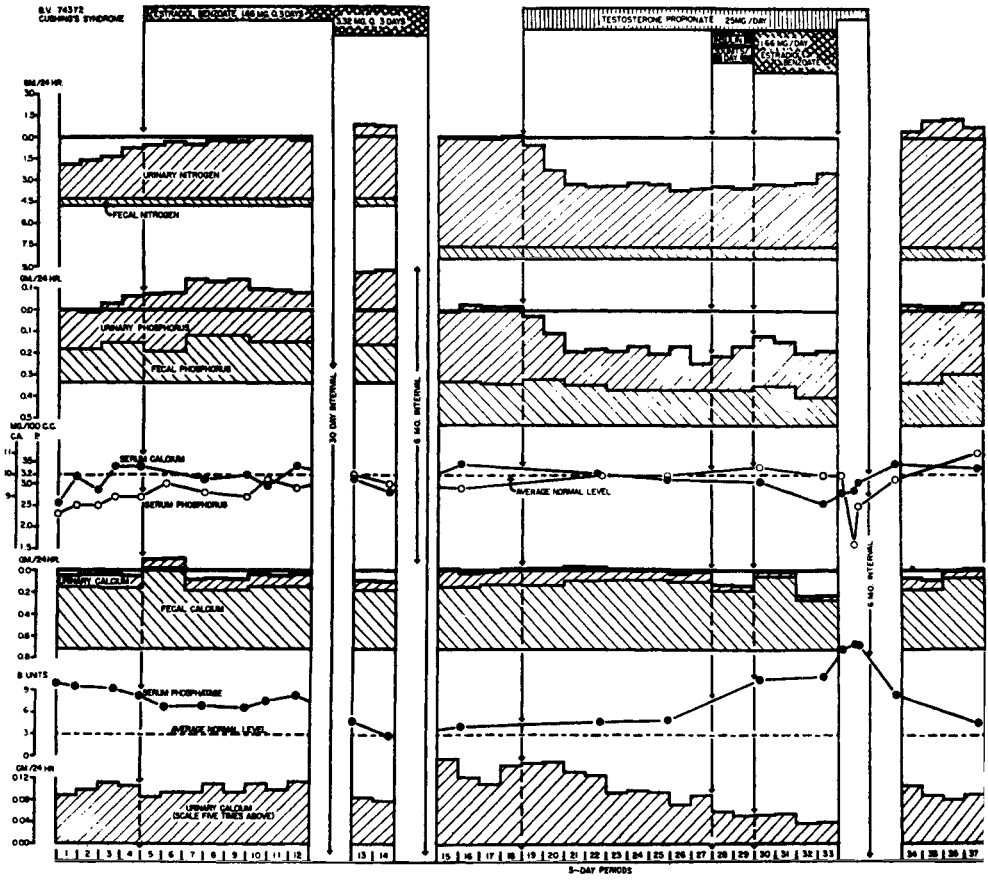


FIG. 24

Metabolic Studies to Show the Effect of Estradiol Benzoate, Testosterone Propionate and Insulin on the Nitrogen, Phosphorus, and Calcium Metabolisms of a Patient with Cushing's Syndrome (B.V., #74372)

For construction of chart see paragraph in italics under Figure 14.

Note that estradiol benzoate did have a slightly beneficial effect on the calcium balance (compare periods 13 and 14 with periods 1 through 4) in spite of an adverse effect on the nitrogen and phosphorus balances. Note markedly beneficial effect of testosterone propionate on nitrogen, phosphorus, and calcium balances; note steady decrease in urinary calcium excretion, shown in magnified form at the bottom of the chart, throughout testosterone therapy with accompanying rise in serum phosphatase level. Note that the addition of estradiol benzoate to the testosterone propionate therapy (periods 30 through 33) apparently improved the calcium balance. Periods 15 through 33 are those analyzed in Figure 13. (Recharted from Albright *et al.* (24) except that intakes were changed from period 15 on to correspond with values obtained by analysis of diet rather than by calculation from tables.)

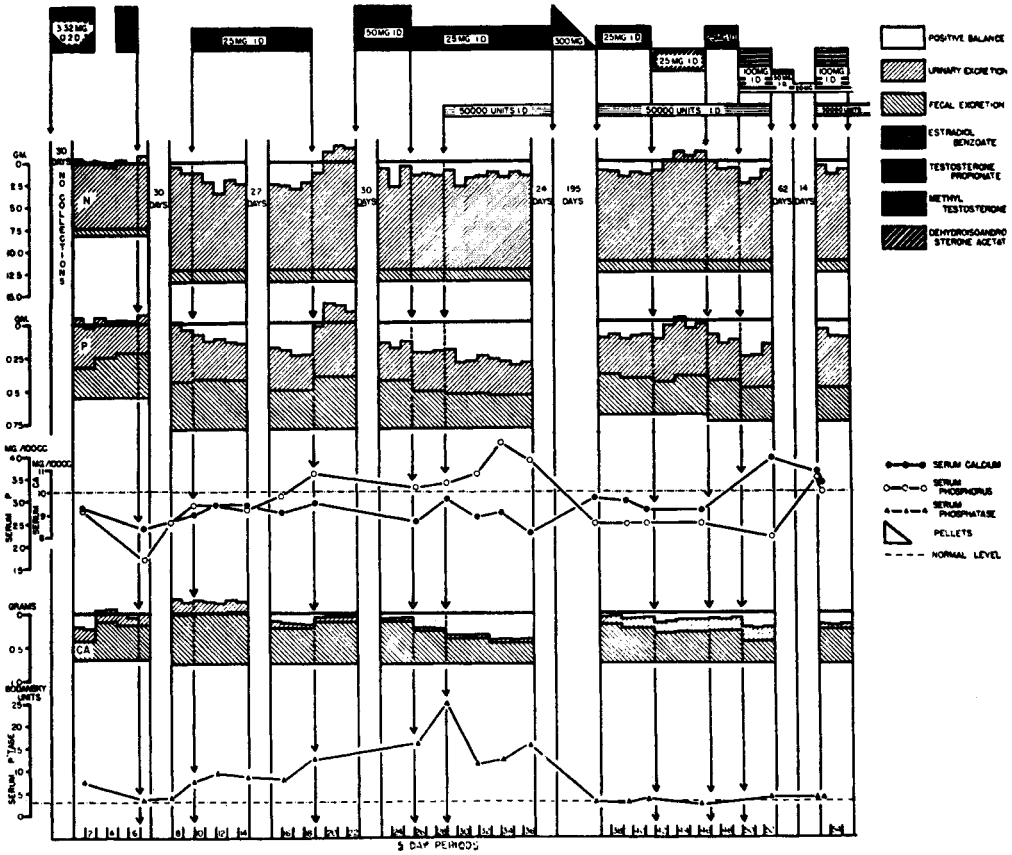


FIG. 25

Metabolic Data on R.B. (#3397) with Cushing's Syndrome to Show Effect of Therapy with Estradiol Benzoate, Testosterone Propionate, Dehydro-iso-androsterone Acetate, Methyl Testosterone, and Vitamin D

For construction of figure see paragraph in italics under Fig. 14. Whereas data are not very satisfactory to judge effect of estradiol benzoate, note in period 8 that calcium balance was negative forty days after cessation of estradiol benzoate therapy. Note that testosterone propionate and methyl testosterone have an almost immediate beneficial effect on nitrogen balance, whereas their effect on the calcium balance was cumulative (compare periods 10 through 14 with periods 15 through 18). Note that serum phosphatase level continued to rise as the calcium balance improved which suggests that the osteoblasts were stimulated to increased activity. Note that dehydro-iso-androsterone acetate was ineffective in inhibiting the rebound in the nitrogen and phosphorus balances on omission of testosterone propionate therapy (period 43). For further clinical data and for the actual metabolic data in the first 36 periods, see Albright *et al.* (6).

strate the effect of estradiol benzoate, testosterone propionate, dehydro-iso-androsterone acetate, and methyl testosterone. The parts of these studies which pertain to the effect of estradiol benzoate have already been discussed (*vide supra*).

Testosterone propionate had a markedly beneficial effect on the nitrogen, phosphorus, and calcium balances in both patients; the effect on the calcium balance seemed to increase with duration of treatment (compare in Fig. 25 periods 10 to 14 with periods 15 to 18). Furthermore, with the improvement in calcium balance with testosterone propionate therapy, there was a rise in the alkaline phosphatase level. Methyl testosterone had a similar effect to that of testosterone propionate; dehydro-iso-androsterone acetate apparently had no effect. The first patient (see Fig. 24) received testosterone propionate, 25 mg. daily, for fourteen 5-day metabolic periods. It was calculated that she retained nitrogen at the rate of 8% per month of the nitrogen content of the entire body of a normal individual her size, or about 19% for the seventy days during which she received the medication. With this positive nitrogen balance there was likewise a marked improvement in her calcium balance with an elevation in the serum phosphatase level, the index to osteoblastic activity. It will be seen, as discussed above in Fig. 13, that her phosphorus retention when corrected for the amount retained with calcium in the bones ($\frac{Ca}{P}$ = *circa* 2.0) was about proportional to the nitrogen retention if one uses the ratio of nitrogen to phosphorus in muscle tissue (N/P equals *circa* 15/1); it will be remembered, furthermore, that potassium and sulphur were also retained proportionately to their ratios with nitrogen in muscle. It is suggested from these observations that testosterone propionate therapy in Cushing's Syndrome stimulates the anabolism of protoplasm which, in bone tissue, means stimulation of osteoblasts to lay down osteoid tissue.

15. *Predilection of "S" Hormone of Adrenal Cortex for Spine and Pelvis*

The "S" hormone, as judged by its effect on patients with Cushing's Syndrome (*vide supra*) has a marked predilection for the spine and pelvis. Conversely, if one were to recover from Cushing's Syndrome, one would expect to see changes in the spine and pelvis and no changes in the extremities.

In Fig. 26 are shown the x-ray findings of the lumbar and lower thoracic spine of a female patient (I. G. 360260) aged 15 years at time of second x-ray (7-10-45) before and approximately fifteen months after cessation of the activity of her Cushing's Syndrome. A more detailed case history will appear elsewhere; suffice it to say that the agent responsible for overcoming

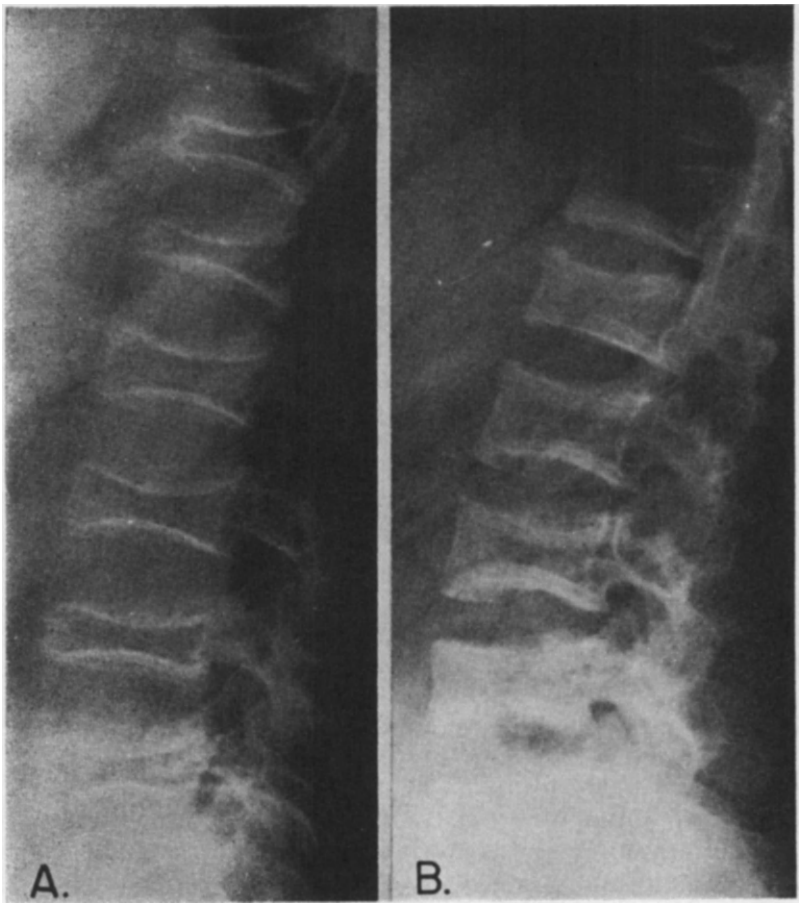


FIG. 26

X-rays of Lumbar and Lower Thoracic Vertebrae of Patient With Cushing's Syndrome (I.G., #350260) Before Cure (A) and Approximately Fifteen Months After Cure (B)

Note in "B" that intervertebral discs have been squeezed together, and that centers of vertebrae remain radio-translucent in marked contrast to dense bone which has been newly laid down. For further analyses of changes see Figs. 27 and 28.

the activity is not definitely known. The improvement may have been spontaneous; it may have been the result of testoterone therapy; it may have been the result of x-ray irradiation of the pituitary. The time sequence suggests the last. It will be noted in Fig. 26B that the intervertebral discs, which had balooned out into the non-resisting vertebrae, were squeezed together again as a result of the growth pressure. In Fig. 27 are depicted in

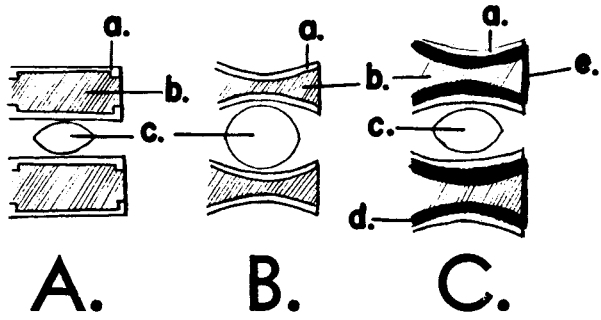


FIG. 27

Schematic Representations of Speaker's Interpretation of Vertebral Changes in Fig. 26 Before Cure (B), Approximately Fifteen Months After Cure (C) as Compared With Normal Findings for the Same Age (A)

a. = cartilaginous end-plate of vertebra
b. = bony part of vertebra
c. = nucleus pulposus
d. = bone laid down after cure by cartilaginous end-plate
e. = bone laid down after cure by periosteum

Note in "B" that nucleus pulposus is expanded at expense of vertebrae because of decreased resistance of vertebrae. Note in "C" that nucleus pulposus has been partially squeezed together again by growth pressure.

diagrammatic form the speaker's interpretation of: (A) the normal vertebral findings in an individual her age, (B) the findings in the patient before recovery, and (C) the findings in the patient after recovery.

Fig. 28 represents a tracing of the vertebrae shown in Fig. 26 together with an analysis of the comparative widths of the vertebrae and intervertebral discs before and after recovery.

Note in Figs. 26 and 27 that the marked change in the vertebrae following recovery is almost entirely due to the laying down of dense new bone; that bone which existed before the cure remains very osteoporotic. It was only because this patient was still growing that these remarkable changes could be demonstrated. It takes a long time in the adult to demonstrate increased density of the vertebrae after alleviation of the activity in Cushing's Syndrome. In Fig. 29 is seen the x-ray of the wrist taken approximately fifteen months after recovery. One is impressed by the lack of a zone of increased density adjacent to the radial epiphyseal cartilage; this is strong evidence that the "S" hormone was not affecting bone being laid down at the wrist.

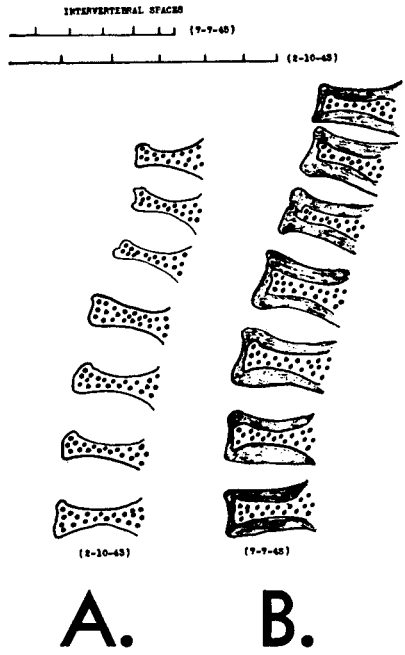


FIG. 28

Tracing of Vertebrae Shown in Fig. 26: (A) Vertebrae on 2-10-43 Before Cure; (B) Vertebrae on 7-7-45 Approximately Fifteen Months After Cure

Note that osteoporotic centers of vertebrae in (B) have same configuration as whole vertebrae on 2-10-43 (A). Note that the sum of the widths of the six intervertebral discs in (B) is shorter than the sum in (A) in spite of the fact that the total length of the vertebrae in (B) is greater than the total length in (A).

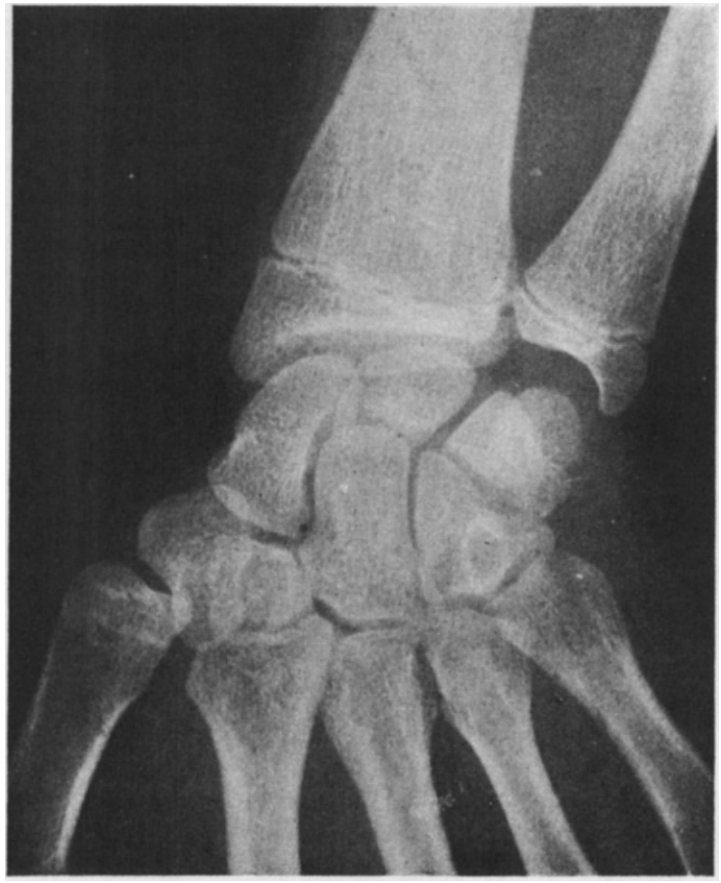


FIG. 29

X-ray of Wrist of Patient I.G., #590260, on 7-3-45 Approximately Fifteen Months After Cure of Cushing's Syndrome

Note absence of any change in density of that bone most recently laid down in juxtaposition to the epiphyseal cartilages.

TABLE I

Summary Table of Effect of Various Hormones on Three Types of Osteogenesis

Hormone	Endochondral Bone	Endosteal Bone	Periosteal ("Membranous") Bone	Remarks
Parathyroid	no effect	no direct effect (see remarks)	no effect	excess may lead to increased bone destruction and secondarily to compensatory increased endosteal bone formation
Thyroid	stimulates	no direct effect (see remarks)	no effect	excess may lead to negative nitrogen balance and secondarily to starvation osteoporosis
"The" Growth Hormone	stimulates	no good data (see remarks)	stimulates	demineralization in acromegaly probably a secondary phenomenon
Testosterone Propionate and Methyl Testosterone	stimulates	stimulates	no effect	
Estradiol Benzoate and Dipropionate	large amounts inhibit; small amounts may stimulate	stimulates	no effect	
Progesterone	insufficient data	insufficient data	insufficient data	
Adrenal cortical "S" hormone	inhibits	inhibits	insufficient data	
Adrenal cortical "N" hormone	stimulates	stimulates	no effect	

SUMMARY

I will summarize my remarks by the following table (Table I):

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DISCUSSION

Dr. K. W. Thompson: I might say that we have to grant that the patients in these conditions that Dr. Albright has portrayed are right. The disturbance is really there and there is a solution to the mystery somewhere. The charm of Dr. Albright's presentation is that he not only presents some of the facts that they have discovered, but he also tries to tell us how these may be interpreted. The refreshing thing about this talk and about Dr. Albright's work is that he is perfectly willing to present a theory, and just as soon as some information is posed that might change it, he willingly changes it and takes up a new theory. Sometimes in our meetings a person will stick to a theory and worry it until we are all so worried that we do not know what to do.

Let me call now for some discussion on this most important subject.

D. J. Ingle: Dr. Kepler used to delight in telling me that much of our supposedly new information on endocrine function from the physiological laboratory had long been known to the clinicians and that the alert clinical observer of experiments carried out on patients by nature could ascertain and describe many of the fundamental metabolic relationships. I was reminded of this when I read Dr. Albright's Harvey Lecture (1943) and was reminded of it again tonight.

In our experiments on over-dosing animals with adrenal cortical steroids and with adrenocorticotrophic hormone we have found that most of the changes which Dr. Albright has included in Cushing's syndrome can be reproduced experimentally. We have induced diabetic states in normal animals which are remarkably resistant to insulin. Other investigators have induced hypertension and an increased deposition of fat with these compounds. The nitrogen balance becomes negative and the tissues of the body tend to lose their substance. Can osteoporosis be produced in normal animals by over-dosage with the cortical steroids? No one has looked for it and such experiments must be carried out.

There are two other points: First, the relationship of the S hormones to growth. They are not entirely incompatible with growth. In the immature adrenalectomized rat each of the C-11-oxygenated cortical steroids is capable of sustaining life and a subnormal rate of growth when given in small amounts. With a little higher intake of these steroids the nitrogen balance becomes negative, but during the continued administration of the steroid adaptation occurs so that the nitrogen balance again becomes slightly positive and the animal gains slowly. Dosage can be increased to a point where the nitrogen balance remains negative and the animal eventually dies. This factor of adaptation to the effect of the hormone as seen in experimental animals, I think, fits in with Dr. Albright's concept of the sequence of changes in Cushing's syndrome. Although these patients are usually in a slightly positive nitrogen balance at the time of study, there is evidence that early in the disease there must have been a negative nitrogen balance with loss of substance from bone, skin, and muscle.

My second point is in regard to Dr. Albright's concept that the S hormone is antianabolic rather than catabolic in action. I believe that the studies of Doctors White and Dougherty show clearly that as far as lymphoid tissue changes are concerned the loss of tissue is so very rapid that there must be catabolic effect.

F. Albright: I wish to thank Dr. Ingle for his very interesting discussion. I am not so sure that the fact that lymphoid tissue disappears so rapidly on the administration of "S" hormone indicates that its breakdown must be increased as opposed to its build-up being decreased. We do not know much about the normal rate of turnover of bodily tissues. From the work of Schönheimer, tissue exchange must be very rapid.

W. W. Gardner: Upon hearing Dr. Albright's report it seems a point not brought out this morning might be considered here. The femora of mice that received large amounts of estrogen over long periods of time have the marrow largely replaced by bone. The femora of mice given comparable doses of estrogen, but also androgen (testosterone) simultaneously show no new bone formed; in fact the bones were thinner than normal. The androgen completely inhibits the osteogenic action of estrogen. Is it possible that in Cushing's syndrome there is a relatively large amount of androgen, or some substance acting like androgen, which might explain the osteoporosis? I realize that it is dangerous to hazard a guess but, as I recall from the paper that Dr. Albright presented, that seems to be true. I would like to hear Dr. Albright's comment on that.

The amount of ash in femora of estrogen-treated mice was one-third greater than that of the controls when the treatment was continued for 5 weeks.

F. Albright: Unfortunately, all experimental animals do not react the same. In the dove testosterone magnifies the stimulating action of estrogen on endosteal bone formation. In the rat, on the other hand, testosterone inhibits and estrogen stimulates. The human is like the dove and not like the rat. In Cushing's Syndrome I think the interfering hormone is not an androgen but the "S" hormone.

J. S. L. Browne: Dr. Albright's remarks are always very stimulating and illuminating, refreshing, and dynamic.

I wanted to bring out certain points about the relation of the S hormone to protein anabolism or catabolism. As Dr. Albright has pointed out, in Cushing's Syndrome there is an increased amount, in most cases, of a substance which he has called the S hormone. It is true that in one case of Cushing's Syndrome, although this hormone was elevated to a value of 250 glycogenic units, in the order of five times the average amount, the patient who had the disease for 10 or 12 years was in a slightly positive nitrogen balance. That may be either a wearing off or an adaptation effect, as suggested by Dr. Ingle, or there may be some other reason. The ketosteroids were not very much raised. Furthermore, Dr. Venning has shown that during pregnancy the level of the S hormone may reach values of the order of two or three hundred glycogen units, and while we have not done nitrogen balances, it seems reasonable to suppose that pregnant women are in positive nitrogen balance. This may possibly be interpreted as follows: In pregnancy the effect of the S hormone is offset by some other anabolic influence. One might speculate that the S hormone makes protein more easily mobilizable within the body. If there is no anabolic focus within the body, the mobilized protein is catabolized and appears as urinary nitrogen. If there is an anabolic focus, such as a growing foetus, then the protein from the maternal organism is transferred to the growing uterus and foetus and the over-all nitrogen balance may be positive. It is obvious that the nitrogen balance as ordinarily determined is not an index of the internal translocations of protein from one part of the body to another.

In connection with the testosterone in Cushing's Syndrome, we can confirm Dr. Albright's results as to its therapeutic effect. Further, Dr. Venning has shown that in the same case I mentioned, within a matter of four days or so after the commencement of the daily administration of 25 mg. of testosterone propionate, the high value 206—250 glycogenic units of the S hormone had fallen to normal (60 G.U.). That would sug-

gest, possibly, that the influence of testosterone in this particular type of case is not only its own metabolic action in whatever way it may be mediated of causing positive nitrogen balance and protein anabolism, but that the testosterone might also act by repressing a high production of S hormone of the adrenal in these cases. This repression of secretion of S hormone by testosterone is unusual in that a hormone is repressing the production of another hormone or hormones which are antagonistic to it in their metabolic effect. Usually it is the production of substances which act similarly in metabolism which is repressed.

F. Albright: We have had one case of Cushing's syndrome, very marked, who started with a very high 11-oxysteroid level (method of Dr. Nathan Talbot) which came down to almost normal under testosterone therapy. This confirms Dr. Browne's findings.

R. G. Hoskins: Two thoughts struck me as I listened to this brilliant exposition. In the case of Cushing's syndrome the author said in effect, regarding the hypertension, "We will just omit that and go on to the other things." I wonder why we do that? That hypertension does not just happen, as some inconsequential accident. The finding affords good evidence that in addition to the other factors that have been discussed this week the adrenal cortex affords still another, a "pressor" factor. It is probably not a ketosteroid. It can readily be demonstrated in glycerine extracts of adrenal cortex substance. It is orally effective. We have published a number of papers on the influence of such extract. It was perhaps unfortunate that we emphasized the fact that the schizophrenic subject reacts unmistakably. We got the same reaction but in lesser degree in normal people. We made many attempts to derive a potent pressor extract in a medium other than glycerine but succeeded only once. Dr. Baird Hastings has told me that he has had a similar experience and is, like ourselves, unable to account for the illusiveness of the active material.

Another point in Dr. Albright's presentation that interests me particularly has to do with the therapeutic effects of androgen. It is obvious that the normal male vertebrate organism is so equipped and adjusted as to operate under the simultaneous influence of gonadotrophin and androgen. As used therapeutically, however, androgen is largely a self-antidoting agent. Unless the dosage is kept well under the usual limits, the androgen has an effect of paralyzing the anterior pituitary in its gonadotrophin-producing function so that the end result in the medium dosage ranges is approximately nil. As much endogenous androgen is lost as exogenous is gained. It is not until a sufficiently large dosage is given, more than enough to compensate for the pituitary-paralyzing effect, that positive results are seen. It is my impression from our own work that if one will curb his impatience and keep to the lower dosage level—comparable to that utilized in implants of the crystaline androgen—equally good results can be obtained with much less expenditure of material than if he goes in for pituitary paralysis.

F. Albright: In answer to Dr. Hoskins' first question, I did not discuss the effect of adrenal cortical hormones on hypertension since my subject is the effect of hormones on osteogenesis. To be strictly honest, however, I do not know the cause of the hypertension in Cushing's Syndrome. Perhaps Dr. Kepler will say a word about this.

As to the decrease in the endogenous production of a hormone when one administers an excess of said hormone, it is true in one of our Cushing's cases that the anabolic effect of testosterone therapy wore out with time. Our first thought was that we might have stopped the endogenous production of androgens; that this was not the cause of the wearing-off effect, however, was shown by the fact that doubling the dose of testosterone administered did not overcome the wearing-off effect.

R. G. Hoskins: What was the cause of the change?

F. Albright: Perhaps lack of potassium. In Cushing's Syndrome there is probably a depletion of potassium as the result of an excess excretion in the urine; since potassium is also needed for the intracellular fluid of protoplasm, it may very well be that availability of potassium is a limiting factor in the amount of protoplasm that can be built up with testosterone therapy. This is under investigation.

Dr. Selye: During the last ten years or so it has become a habit of Dr. Albright and myself at all scientific meetings to disagree violently on the mechanism of parathyroid hormone action.

Today during Dr. Albright's presentation I have again made an elaborate list of all points in favor of my theory and against the concept according to which the hormone acts primarily upon the renal excretion of phosphorus. Now, however, I find that I just do not have the courage to go through the usual routine again and I shall reduce my remarks to two polite questions, which are not even concerned with the parathyroids.

(1) Why limit all our discussions concerning adrenotrophic hormone and adrenal over-activity to excessive production of N hormone and S hormone respectively? There is good reason to believe that under certain conditions the adrenal cortex produces an excess not only of nitrogen retaining testoid (N hormone), or sugar active corticoid (S hormone) principles, but also of salt-active and perhaps even fat-metabolism-active corticoids. Actually an overactivity of the adrenal cortex can produce manifestations of excessive virilization and nitrogen retention (testoid), diabetes (sugar activity corticoid), hypertension, and nephrosclerosis (salt-active corticoid), or abnormal fat deposition, as in Cushing's disease or adreno-genital syndrome (probably due to excessive formation of the fat-active corticoids discussed by Dr. Kendall). I should like to re-emphasize at this occasion that since the degree of overactivity in these various respects does not necessarily run parallel, it appears highly probable that separate corticotrophic hormones of the pituitary are responsible for the production of each of these corticoid hormone types. I wonder if Dr. Albright would agree with this interpretation.

(2) Dr. Albright presented as a more or less definitely established fact that the luteinizing hormone of the pituitary stimulates the adrenal cortex. I wonder whether this is based entirely on clinical evidence; I know of no experimental observations which would support such a contention.

F. Albright: Firstly, about the hypertension! I left it out because it had nothing to do with bone.

Secondly, as to the fat deposits in Cushing's Syndrome, I think we should keep the following two concepts in mind. (1) The disorder in Cushing's Syndrome is not one of increased or decreased caloric consumption but one of a change in the metabolic mixture; if one burns more protein and the same amount of carbohydrate, one will consume less fat. (2) Fatty tissue is after all first a tissue and secondly a tissue containing fat. There may be an atrophy of fatty tissue in certain parts of the body in Cushing's Syndrome analogous to atrophy of other tissues (*cf.* bone, skin, muscle) and, as a result, an increased deposition of fat where there is no atrophy.

Thirdly, as to the evidence that LH stimulates the production of the "N" hormone of the adrenal cortex in addition to its effect on the gonads, there is considerable. I cannot quote chapter and verse. We have reviewed this evidence in a recent paper (Reifenstein *et al.*, *J. Clin. Endocrinol.* **24**: 416, 1945). Much of the evidence is admittedly circumstantial. I first entertained this concept when we made a study of the causes of eunuchoidism (*Trans. Assoc. Amer. Phys.* **56**: 43, 1941). It seemed clear that there are two etiological sub-groups: (1) a condition similar to castration where the follicle-stimulating hormone is in excess and where the 17-ketosteroid excretion is of

the order of magnitude of that in a normal female, and (2) a condition where the follicle-stimulating hormone is not increased (which at once suggests that the deficiency is not primarily gonadal), and where the 17-ketosteroid excretion is much lower than that of a normal female. It was quite clear that in the latter condition there is interference with the production of 17-ketosteroid precursors not only in the testes but also in the adrenal cortices; this suggested that the trophic hormone of the pituitary stimulating each of these tissues is one and the same and in this form of eunuchoidism is primarily lacking. I admit there are other possibilities but this is one piece of evidence. I would also mention again as more direct evidence that Golla and Reiss (38) (39) produced hypertrophy of the adrenal cortex with a preparation of luteinizing hormone containing insignificant amounts of adrenocorticotrophic hormone.

G. Pincus: As far as 17-ketosteroids representing N or S hormone, I think the answer should be made when we discover the true precursors of 17-ketosteroids and know what they are. One known precursor of 17-ketosteroids has been isolated from adrenal tissue, androstenedione; another probable one is adrenosterone, both are androgenic, and a third reported androgen is 17(β) hydroxyprogesterone, but the quantitative production of these by adrenal tissue is not known. It is not impossible that certain sugar-active steroids, or even hormonally inactive corticosteroids may also be 17-ketosteroid precursors. For example, oxidative scission of the side chain of a 17-hydroxylated compound like 17-OH corticosterone, or 17-OH allopregnantetrol might yield 17-ketone.

The other point is this: It seems to me that if in Cushing's disease one has an overactive S hormone production and if, as indicated by the work of Dr. White and Dr. Dougherty, 11-oxygenated steroids are produced as a result of the corticotrophic stimulation, then one ought to be able to find some evidence of corticotrophin production in Cushing's syndrome. But there is evidence of hyperpituitary activity. In animals, an activitation of the adrenal cortex clearly depends on the pituitary. There are several ways of determining whether there is hyperactivity, and I should say that one of them would be to devise a good method of testing the excretion of corticotrophin into the urine. Another method might be a study of the lymphocyte production and circulation, to study the lymphatic tissue in Cushing's syndrome. I would like to know whether that is any evidence for a high production of corticotrophin?

I would like to refer to what Dr. Selye said about LH, that it may stimulate the adrenal cortex and produce an N hormone. I know of no determinations of such production, *per se,* but certainly someone should be able to demonstrate, perhaps without great difficulty, that the administration of LH actually leads to the production of 17-ketosteroids.

F. Albright: We studied the white counts in ten cases of Cushing's Syndrome (*vide supra*) and they work out very nicely. The polymorphonuclear counts are high and the lymphocyte counts low.

There are two pieces of evidence pointing to increased corticotrophic hormone production in Cushing's Syndrome: (1) the evidence for increased "S" hormone production, and (2) the response which certain cases make to x-radiation of the pituitary.

As regards the effect of LH on 17-ketosteroid production, we were able to show in our studies on eunuchoidism just mentioned that chorionic gonadotrophin, which is very similar to LH, increases the 17-ketosteroid production in those cases where the eunuchoidism is due to LH lack.

E. J. Kepler: Dr. Albright and I both subscribe to the doctrine that a poor but provocative theory is better than no theory at all, provided it does not grow into an

obsession. His capacity, when additional factual material becomes available, to modify his point of view should convince anyone that his theories are always theories and never obsessions.

For a long time I had the hope that, if the biochemists finally succeeded in isolating all the urinary adrenal cortical steroids which are excreted in cases of Cushing's syndrome, the pathologic physiology of this condition would be obvious and theories no longer would be necessary. It appears now that this hope was premature and perhaps not too well founded. Almost without exception none of the steroid compounds which have been obtained from cortical tissue can be extracted from urine, and conversely none of the compounds that have been extracted from urine can be obtained as such from adrenal cortical tissue. Consequently, when a crystalline steroid of adrenal origin is finally isolated from the urine in a case of Cushing's syndrome, we are still in the dark regarding its glandular ancestors. So, until methods have been developed which will circumvent this difficulty and enable us to say with assurance that a given urinary adrenal steroid must have been derived from this, that, or the other glandular adrenal steroid, we probably will have to be content with theories.

My concepiton of the pathologic physiology of Cushing's syndrome differs here and there from that expressed tonight by Dr. Albright. I assume that, in Cushing's syndrome, there is an excessive production of biologically active cortical steroids. This assumption does not exclude the possibility of production of abnormal or unusual cortical steroids in cases of adrenal cortical tumor, and it includes the possibility of a disproportionate overproduction of one or more cortical steroids relative to others in cases of adrenal cortical hyperplasia or tumor. That such a disproportion may occur is documented by reports of unusual cases of pseudohermaphrodism associated with adrenal cortical hyperplasia, in which there occurred craving for table salt and ultimately signs and symptoms of acute adrenal cortical insufficiency. In these cases clinical and histologic observations indicated an overproduction of androgenic steroids by hyperplastic androgenic cells at the expense of the cortical cells and steroids necessary to maintain life.

Classified functionally, adrenal cortical steroids fall roughly into three, and possibly four, main groups. I use the word "roughly" because these compounds have complex biologic properties which overlap so that it is impossible to segregate them into iron-clad categories.

Group I includes the "salt and water hormones," steroids having biologic properties similar to those of the synthetic product desoxycorticosterone acetate. An overproduction of such substances might be expected to raise the blood pressure, lower the concentration of serum potassium, cause the retention of sodium, and thereby by some unknown mechanism produce hypochloremic alkalosis. All of these phenomena occur in Cushing's syndrome. The disturbance in the metabolism of potassium might well be a contributing, if not a crucial, factor in the myocardial and voluntary muscle weakness which so frequently characterizes this disorder.

Group II is composed of those steroids, such as 11 dehydro-17 hydroxycorticosterone (compound E of Kendall), which affect the metabolism of protein and carbohydrate. The "S" hormone of Dr. Albright belongs in this category. An overproduction of such substances, as he so ably has pointed out, might account for the diabetes, osteoporosis, muscle weakness, and atrophy of the skin associated with Cushing's syndrome. If the walls of the smaller blood vessels were weakened, hemorrhagic tendencies and easy bruising might be expected. The striae might also be explained after a fashion but one would still be perplexed by the predilection of these lesions to occur in some regions of the body to the exclusion of others.

Group III is made up of those steroids which have androgenic, estrogenic, and progestational properties; the group includes Dr. Albright's "N" hormone. Of these, the androgenic steroids appear to be the most important clinically. In their action some of them resemble testosterone fairly closely; others do not. That the latter statement is correct is indicated by the fact that a male unuch is still a eunuch even though, as sometimes occurs, he excretes urinary 17-ketosteroids in normal or even excessive amounts. (In this connection it is well to remember that all androgens are not 17-ketosteroids, that all 17-ketosteroids are not androgens and that the various adrenal androgens vary in their degree of "androgenicity.") An overproduction of such testosterone-like adrenal "androgens" might account for the hirsutism, amenorrhea, and possibly the acne that occur in cases of Cushing's syndrome. By stretching the imagination, one might also explain the erythrocytosis which occasionally occurs in this disorder, since it has been shown by McCullogh and others that administration of testosterone is sometimes followed by increased formation of red cells. Other compounds might prove to be even more potent in this respect. The florid color which is so characteristic of Cushing's syndrome and which is not necessarily associated with erythrocytosis also suggests an overproduction of some compound having testosterone-like properties. In this connection one may recall the red face shown in the photograph of the formerly pallid elderly man whom Dr. Albright treated with testosterone.

In brief then, rather than assume an underproduction of "N" hormone, I prefer for the time being to contemplate a more kinetic process characterized by overproduction of one or more testosterone-like adrenal "androgens" which are limited or lacking in their capacity to deposit protein. Such androgens need not be 17-ketosteroids nor, after having been metabolized and finally excreted in the urine, they need not show androgenic properties when assayed biologically. If this were the case, a woman with Cushing's syndrome might readily excrete urine which was low, normal, or increased in its content of 17-ketosteroids, weak in androgenic properties when assayed biologically, and yet have masculine distribution of hair and other attributes of masculinity.

Group IV being largely hypothetical can be dismissed quickly. At the moment it contains only one member; namely, dehydrocorticosterone (compound A of Kendall). Its capacity to increase the total amount and the percentage of fat has been mentioned at this meeting. When produced in excessive amounts, it and others like it might cause deposition of fat at the expense of muscle without violating the law of conservation of energy. The superficial resemblance of the patient with Cushing's syndrome to Kendall's A-treated rats needs no further comment.

K. W. Thompson: When I looked up all the cases in the literature a while ago with Dr. Cushing and later on with Dr. Eisenhardt, we found that there were patterns that ranged from practically pure virilism to full-fledged Cushing's syndrome, and in between the two full-blown types there were borderline cases, very difficult to deal with in our statistics. For instance, there would be patients with pure virilism and adiposity, and some with a little diabetes; and then there were those with osteoporosis or with hypertension added, with a partial appearance of Cushing's syndrome.

I am willing to believe that there are these mixed syndromes which Dr. Kepler suggested just now, and the proportions in which the various hypersecretions occur are what determine the final syndrome. Beyond that I would not want to go. I would not want to make a statement as to which hypersecretion comes first.

We also felt that the patient's condition would become a great deal worse and the diabetes would be relentless when the nitrogen requirements increased. When they had a craving for meat, the diabetes was increasing. Those two things seem to be an indication of a relentless progress.

K. E. Paschkis: In this discussion the question of excessive secretion from the adrenal cortex of desoxycorticosterone-like compounds in Cushing's syndrome has been raised. While such compounds have probably nothing to do with skeletal changes they would account for the hypertension so frequently present in Cushing's syndrome. I should like to draw attention to a simple test for salt retention which was described by Dr. A. Cantarow from our institution several years ago. The test is essentially a Cutler Power-Wilder test in reverse. Patients are given an excess of NaCl for 2½ days and the Cl concentration in the urine passed during a four-hour period on the last day of the test is determined. In cases of Cushing's syndrome, the Cl concentration is very much lower than in normal individuals, and in the absence of kidney damage, the Cl retention can probably be attributed to excessive secretion of desoxycorticosterone-like compounds by the adrenal cortex.

My other point is apropos of Dr. Pincus' suggestion that in cases of Cushing's syndrome with pituitary adenoma there might be an excessive secretion of adrenocorticotrophic pituitary hormone. The latter would stimulate the adrenal cortex and thus produce the symptoms of Cushing's syndrome. This mechanism has been frequently suggested by clinicians, first, I believe, by J. Bauer. Attempts to demonstrate the presence of excessive amount of adrenocorticotrophic hormone have been made. Jores tested by injecting serum into intact mice. Sera from Cushing's cases produced a higher increase of adrenal weight than did control sera. We have used this method in two cases of Cushing's syndrome and found the same response. Because results obtained on the intact mouse are, of course, open to criticism, we then employed the baby chick for assay. Riddle and Bates have shown that the chick adrenal is much less responsive to unspecific stimuli ("alarm reaction") than is the mammalian adrenal. We prepared human urine extracts and found increased adrenocorticotrophic activity in cases of Cushing's syndrome.

E. Oppenheimer: I am afraid I am not going to make a contribution. I just want to ask a question. It concerns a rare disease, a congenital condition, namely, osteogenesis imperfecta.

I would like to know how this disease fits into the diagrams which you have shown. We have been in touch with three of these cases. One was treated by Dr. Vest of the University of Virginia. In view of the fact that this condition is alleged to disappear at puberty, it was thought that the production of premature puberty with androgen would favorably influence the condition. The patient was about 6 or 7 years old and had the typical history of this disease. He was treated for six months and after a short interruption the injections were started again. The therapy produced no X-ray changes in the bones, but marked signs of puberty with loss of fat around the hips and the muscles. While not able to walk before treatment, he now moves on crutches.

The second case was under the observation of another physician, but died of meningitis after some clinical improvement under testosterone propionate therapy.

The third case concerns a girl who is under the treatment of Dr. Berry in Summit. In this case estradiol dipropionate was used at the age of 5. She is now 7½ years old and was recently scheduled for surgical repair of the badly healed fractures.

In the literature, Chess, Chess, and Keeton reported, some years ago, a case of osteogenesis imperfecta in the Proceedings of the Central Society for Clinical Research.

From what we have heard today one can visualize and imagine what androgenic or estrogenic treatment may perform, but as far as I know the blood calcium and the blood phosphorus are normal, and I cannot remember to have seen data concerning the

phosphatase. I certainly would like to hear from Dr. Albright whether he believes that androgens or estrogens might influence the condition.

F. Albright: At last a question which suggests that I have been talking about bone! Osteogenesis imperfecta, as I am told, is similar to osteoporosis as defined this evening except that instead of primary lack of osteoblasts there is a deficiency of the osteoid produced by the osteoblast. The serum calcium and phosphorus levels are normal; in contrast to osteoporosis the serum phosphatase level is often increased.

My colleague, Dr. Marion Ropes, is studying the effect of estrogens and androgens on this condition. She has a pair of identical twins, to one of whom she is giving estrogen. Whereas there seems little question that estrogen and androgen both have a favorable effect on the calcium balances in this condition, I don't think she is yet prepared to advocate their general use.

Certain Factors Which Influence the Rate of Growth and the Duration of Growth of Children*

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I. Introduction

The ultimate stature of children is determined by (a) the rate of growth and (b) the duration of growth. The term, growth, is used here to signify an increase in stature which results from the construction or anabolism of new protoplasmic (skeletal) tissue.

Broadly speaking, the factors which influence the rate of growth may be considered as follows:

1. The growing end-organ (tissue protoplasm including skeleton). This may vary its capacity to grow or its sensitivity to growth stimuli. These characteristics are probably determined by heredity, maturity, and certain hormones.

2. The "internal environment," which stands for the cardiovascular, pulmonary, gastro-intestinal, renal, etc. systems. These assimilate and transport building materials to the growing end-organs and excrete the products of catabolism. In this discussion, unless exceptions are taken, it will be assumed that these systems are functioning normally.

3. Certain glands of internal secretion which through their hormones stimulate or inhibit end-organ growth. The central nervous system, through its influence on the anterior pituitary, partly controls the activity of these glands.

4. Nutrition, which provides fuel and other essential nutrients for the survival and building materials for the growth of the organism.

The maximum duration of growth is determined largely by the time of epiphyseal union which is the terminal step in skeletal maturation. The rate of maturation is determined largely by the factors mentioned above.

Nature fortuitously has provided us with patients suffering from an excess or from a deficiency of certain of these factors. Such excesses or deficiencies may either (a) accelerate, (b) have no effect upon, or (c) result in a cessation of growth or skeletal maturation (see Table I).

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*These studies were supported by a grant from the Commonwealth Fund of New York.

This is an abstract of observations to be published in full and with references in "Advances in Pediatrics," Vol. 2. Interscience Publishers Inc., New York, N. Y.

TABLE I

Effect of an Excess or a Deficiency of Certain Factors upon the Rate of Growth and of Skeletal Maturation of Children

Quantity of factor	Factor	Resultant effect on: Rate of growth or anabolism	Resultant effect on: Rate of skeletal maturation
Excess	Anterior Pituitary Gr.	+++*	0
	Thyroid	— to +	0 to +
	Testicular androgen	++	++
	Ovarian estrogen	0	++
	Adrenal Cortical 17-KS	+	++
	Adrenal Cortical CHO	≡	?0
Deficient	Anterior Pituitary Gr.	≡	?—
	Thyriod	≡	≡
	Testicular androgn	0 to —	=**
	Ovarian estrogen	0	=**
	Adrenal Cortical 17-KS	0	0
	Adrenal Cortical CHO	?0	?0

Symbols

+ = A tendency to accelerate anabolism or skeletal maturation.
0 = There is no effect.
— = A tendency to diminished anabolism or retarded skeletal maturation.
* = The number of plusses may decrease with advancing epiphyseal maturation.
** = During adolescence only.

II. Hormones

1. Pituitary Growth Hormone

Prepuberal patients with an eosinophilic adenoma of the anterior pituitary have the following interesting characteristics: (1) they grow rapidly; (2) they show no acceleration in skeletal maturation; (3) they usually do not have symptoms or signs of hyperthyroidism or of hyperadrenocorticism or hypergonadism. Accordingly it seems that the pituitary growth hormone selectively promotes growth without accelerating skeletal or sexual maturation.

Patients with a destructive lesion of the pituitary grow abnormally slowly and show retarded skeletal maturation. These patients probably lack not only the pituitary growth hormone, but also pituitary thyrotropin, gonadotropin, and adrenocorticotropin. Hence, they tend to be deficient not only in growth hormone but also in thyroid, gonad, and adrenal cortex hormones. Because castrate or eunuchoid children and children with Addison's disease tend to grow essentially normally (see below), the slow growth of pituitary dwarfs does not appear to be due to deficiencies of gonadal or adrenal cortical hormones. Inasmuch as thyroid therapy fails to restore to normal

or even at times to accelerate the growth rate of pituitary dwarfs, the slow growth of such patients is not explained satisfactorily by thyroid deficiency. Accordingly it seems probable that the slow growth of the pituitary dwarf is referable to a deficiency of the pituitary growth hormone. It is not known which factor is responsible for their retarded skeletal maturation.

It is not clear when the pituitary growth hormone becomes of major importance in determining the rate of growth of children. The fact that dwarfism in infancy can almost always be explained by a disturbance in some factor other than the pituitary (such as malnutrition, hypothyroidism, chondrodystrophy, renal disease, or the like) suggests that the pituitary growth hormone may not be important during the first year or two of life. Thus the growth of very young children may not be dependent upon the growth hormone, but may occur as the result of an intrinsic tissue tendency to grow. On the other hand, there are a sufficiently large number of children over 4 years of age with dwarfism apparently secondary to an authentic pituitary lesion to indicate the importance of the pituitary growth hormone in such older children. Finally there is a possibility that the production of pituitary growth hormone normally diminishes during the latter part of adolescence. This might explain the diminution in growth rate which normally occurs shortly before the menarche in girls, the failure of certain young eunuchoid adults with open epiphyseal lines to continue to grow rapidly, and the failure of normal adults to become acromegalic. As an alternative it may be postulated that as the growing end organ matures during the latter part of adolescence it loses some of its capacity to grow in response to the pituitary growth hormone. This idea will be considered again below.

2. *Thyroid*

Thyroid is secreted throughout the life span.

Hypothyroid children grow abnormally slowly. There is probably more than one explanation for this fact since hypothyroidism leads to extensive disturbances in body economy. Certain of these, such as impaired cardiovascular function and diminished food intake (due to poor appetite) are easily defined; others, such as a tendency to hypopituitarism and possibly a decreased sensitivity of the growing end-organs to growth promoting stimuli are suggested by records of animal experimentation.

When thyroid is administered to athyreotic children, they tend to grow much more rapidly. However, the rate of growth is not proportional to the dose of thyroid. That is, thyroid apparently facilitates growth without

per se stimulating growth. In this connection it will be remembered that thyroid does not promote growth in pituitary dwarfs.

A lack of thyroid results in retarded skeletal maturation.

Children with thyrotoxicosis are found to be taller than average at the onset of their disease. Approximately 1/3 of these children become tall prior to evident hyperthyroidism. Though tall at the onset of the thyrotoxicosis their ultimate stature is usually only average normal. Thus they anticipate normal growth, but rarely become giants. Metabolic studies indicate that hyperthyroidism *per se* does not result in accelerated growth. On the contrary, if the energy metabolism is abnormally elevated so that the patient is in negative caloric balance, catabolism (which is the opposite of growth) exceeds anabolism. Here it is interesting to note that skeletal growth is one form of anabolism which cannot easily be reversed. That is, while there may be catabolism of skeletal constituents, skeletal dimensions are ordinarily maintained unless fractures develop. Increases in growth rate following moderate increases in thyroid concentration may be due to an increased intake of food.

Since there is a possibility that thyrotoxicosis is due to an increased production of anterior pituitary thyrotrophic hormone, there is also a possibility that the increased growth observed at or preceding the onset of thyrotoxicosis may be the result of a simultaneous increase in the secretion of anterior pituitary growth hormone. This type of thesis might also explain the increased stature commonly observed in juvenile diabetics at the onset of that disease.

Hyperthyroidism does not ordinarily result in accelerated skeletal maturation.

It is concluded that physiologic concentration thyroid facilitates or permits growth to occur, but it is not *per se* a growth (anabolic) hormone. Likewise, it permits but does not stimulate skeletal maturation.

3. Testicular Androgens

The size of the penis serves as an index of testicular androgen production. According to this index, the testes are essentially quiescent until approximately 12 years of age in normal boys. Therefore growth and maturation of younger boys is probably not dependent upon testicular hormones.

Testicular hyperfunction (as in testicular interstitial cell tumor) prior to 12 years leads to accelerated statural growth, accelerated skeletal maturation, increased muscular development as well as precocious development of the genitalia.

There appears to be a close relation between masculine muscular development and testicular androgen production.

Testicular hypofunction in boys over 12 years of age does not result in obviously slow statural growth but does lead to retardation in skeletal and sexual maturation. Because the delay in skeletal maturation permits long bone growth to proceed for an unusually long period, testicular hypofunction may result in unusually tall stature.

The role of the testes in normal growth will be discussed below.

4. Ovarian Estrogens

Uterine size is used as an index of ovarian estrogen production. This index suggests that the ovaries commence to secrete estrogens actively at about the 8th year of life in girls. As for the testes the ovarian hormones probably do not exert an important influence upon the growth and maturation of girls prior to this age.

The scanty available observations on young children with ovarian granulosal cell tumors suggest that excess estrogens accelerate skeletal maturation as well as secondary sexual development. Though some of these children tend to grow rapidly, others do not. Estrogen therapy (medicational hyperestrinism) in normal preadolescent girls has only a minor effect upon the anabolism of calcium and nitrogen as determined by metabolic balance studies.

A lack of ovarian hormones is without effect upon the growth and maturation of the preadolescent girl. On the other hand, ovarian deficiency leads to retarded skeletal and sexual maturation in girls of adolescent age. However, their growth curves do not deviate markedly from the normal.[1]

5. Comments on the Relation of the Testes and Ovaries to the Ultimate Stature of Boys and Girls

The gonadal hormones may under pathological circumstances induce precocious epiphyseal maturation and hence markedly shorten the duration of growth. This phenomenon ultimately results in dwarfism. On the other hand, eunuchoid individuals with retarded skeletal maturation and hence "open" epiphyses may, but often do not, attain an unusually tall stature. That is, some factor other than epiphyseal union or gonadal activity has a bearing upon the duration of growth during adolescence.

[1]There is a group of congenitally anovarian girls who are dwarfed. These children frequently manifest other congenital malformations such as webbed neck, coarctation of the aorta, etc. Some investigators maintain that the dwarfism is due to the lack of ovaries. However, because there are other girls who lack ovaries and yet grow normally it is here contended that ovarian deficiency, when it occurs in association with dwarfism, is not necesssarily the cause of dwarfism. It seems more probable to us that the dwarfism is a result of associated, congenital pituitary growth hormone deficiency or end-organ defect.

In the normal as in the abnormal child, epiphyseal maturation is probably catalysed by the sex hormones during adolescence. There is also a possibility that the epiyhyses gradually lose their capacity to grow as they approach the state of complete maturity. This is suggested by the fact that the maximum growth rates attained by early maturing children exceed those of children who mature later. If this thesis is correct, it follows that the ovarian estrogens and testicular androgens by catalysing epiphyseal maturation may be indirectly responsible for the dimunition in growth rate which sets in shortly before the menarche in girls and sexual maturity in boys.

In these respects it is of interest that the ovaries of girls become active about 2 years earlier than do the testes of boys. Likewise epiphyseal closure occurs on the average approximately 2 years sooner in girls than is the case with boys. Cessation of growth in girls follows the menarche by about 5 years regardless of the menarcheal age. Cessation of growth in boys follows the estimated time of sexual maturity by a similar period of time. This sex difference in the age of onset of gonadal function permits boys to grow for a longer period than girls. This in part explains why men are taller than women. The other reason for this sex difference in ultimate stature is that adolescent boys grow somewhat more rapidly than adolescent girls. It is presumed that boys grow faster than girls during adolescence because the testicular androgens have an appreciable growth promoting effect whereas the ovarian estrogens apparently do not.

6. Adrenal Cortices

The adrenals secrete several types of hormones including those which regulate: (a) the water and electrolyte metabolism and (b) the protein and carbohydrate metabolism. Insofar as these types of hormone are necessary to the maintenance of life and an efficient internal environment, they are essential to growth. In addition, the adrenal cortex secretes substances which are excreted as urinary 17-ketosteroids. These substances are not produced in detectable quantities until the 9th or 10th year of life.

Adrenal cortical deficiency does not result in retardation of statural growth or of skeletal or sexual maturation provided the patient can be maintained in reasonably good health by sodium chloride and, if necessary, desoxycorticosterone acetate therapy.

Hyperadrenocorticism with Cushing's Synodrome in children may not be characterized by an increased excretion of 17-ketosteroids in the urine but is accompanied by an increase in the urinary output of 11-oxycorticosteroid-like substances. Apparently because these hormones are either anti-anabolic or catabolic the growth of these children is extremely slow. Recent infor-

mation suggests that trauma, infections, etc. result in an increased production of these 11-oxycorticosteroids by the adrenals (the alarm or adaption reaction). It is postulated, therefore, that the temporary cessation of growth noted in certain children during periods of illness may be due in part to an increased production of such anti-growth hormones.

Hyperadrenocorticism with virilism (as in certain types of adrenal cortical cancer) in young children is usually characterized by an abnormally elevated urinary 17-ketosteroid output, an increase in the rate of growth and especially in the rate of skeletal maturation. It may be noteworthy that they do not develop acromegaly following closure of the epiphyses.

It is concluded that the adrenal cortical 17-ketosteroid precursors are of minor importance in determining the rate and duration of growth of normal children. In this respect it is of interest that during the latter half of adolescence while the urinary 17-ketosteroid output steadily increases, the rate of growth steadily decreases. Their influence is felt chiefly when they are present in relative excess over the testicular androgens or ovarian estrogens (as in castrates) or in absolute excess (as in true hyperadrenocorticism with virilism). Under such conditions they resemble testicular androgens in their action.

III. Central Nervous System

While the anterior pituitary gland directs growth and maturation processes through its growth, thyrotrophic, gonadotrophic and adrenocorticotrophic hormones, it further appears that the anterior pituitary is itself subject to regulatory influences. Among these the central nervous system may be of great importance. This is indicated by observations on young children with somatic and sexual precocity induced by a variety of neurologic lesions especially of the floor of the third ventricle. In such children the nervous system stimulates or activates the pituitary to "normal" adolescent activity at an abnormally early age. Such phenomena as the foregoing suggest strongly that the central nervous system exerts a general, overall regulatory influence upon growth and maturation processes by its effects upon the anterior pituitary body.

IV. Nutrition

Normal children cannot grow if their dietary intake of building materials (protein, minerals, vitamins, etc.) is inadequate. However, an adequate dietary intake of building materials does not guarantee normal growth, for children receiving sufficient protein but an insufficient total caloric intake (negative caloric balance) also may fail to grow. It appears that the factor which ultimately determines whether or not such calorically

malnourished individuals grow (at the expense of body fat stores) is the pituitary growth hormone. In the absence of this hormone the organism tends to utilize the dietary protein to meet the energy requirements; in its presence, protein is diverted from such catabolic channels for use in tissue anabolism. The additional caloric deficit thus created is made up by increased fat catabolism. Accordingly it is proposed that a state of negative caloric balance may tend to result in a decrease in pituitary growth and incidentally gonadotrophic hormone production. On the other hand, children who have become obese as a consequence of excess eating of carbohydrates tend to show accelerated growth and maturation. Thus a strongly positive caloric balance may favor mild hyperpituitarism.

It appears therefore that nutrition is of importance in two chief respects. First, it may limit growth as in protein malnutrition which results in a lack of essential building materials. Second, it apparently influences the activity of the anterior pituitary gland and hence the rate of growth and of maturation.

V. Summary with Comments on Factors Determining the Growth of Normal Children (Table II)

Assuming a healthy organism and an adequate intake of building materials (protein, minerals, etc.), after the second year or so of life the chief growth-promoting agent is the pituitary growth hormone. Thyroid permits or facilitates this action. In adolescent boys, the testicular androgens act as secondary growth-promoting hormones. In addition, they stimulate masculine muscular development. In adolescent girls, the ovaries probably do not stimulate growth, but may favor mineralization of bones. The adrenal cortical 17-ketosteroid precursors are of unknown and probably minor importance in the normal growing child. The central nervous system exerts a supervisory influence upon anterior pituitary and hence other endocrine functions. A deficit of building materials can limit growth. Furthermore, a simple

TABLE II

Relation of Factors to Normal Growth and Maturation

Factor	Statural growth	Skeletal maturation
Anterior Pituitary Gr. H.	+++	0
Thyroid	Facilitates or Permits	
Testicular androgen	+*	+++*
Ovarian estrogen	0	+++*
Adrenal Cortical 17-KS	± 0	± 0
Central Nervous System	Overall Regulatory Influence	
Nutrition	Overall Regulatory Influence	

*During adolescence.

deficit or excess of dietary calories may modify pituitary function and hence the rate of growth and maturation.

Epiphyseal fusion precludes further normal long bone growth. The capacity of these end organs to grow may diminish prior to complete maturation (or fusion). The pituitary growth hormone does not appear to markedly influence skeletal maturation. Provided the child has a reasonably normal quota of thyroid, this hormone also is relatively unimportant in this respect. During adolescence the testicular androgens and the ovarian estrogens accelerate skeletal maturation processes. The adrenal cortical 17-ketosteroid precursors are of unknown and probably minor importance.

Heredity presumably sets the stage for all of the foregoing.

Discussion

J. S. L. Browne: I would like to congratulate Dr. Talbot on the remarkably clear analysis. With regard to the question of the action of androgens on growth, I am inclined to attribute to them, perhaps, a somewhat greater effect than Dr. Talbot has done. I think that unequivocal evidence that they do this, directly, apart from a possible action through the anterior pituitary growth hormone, is not available. It is, however, true that one can produce very definite growth effects with androgens in individuals who have at least considerable anterior pituitary deficiency. I am inclined to attribute the puberty growth spurt in the male to endogenous androgens. One can, in males who are underweight and fail to grow at the normal rate or to mature sexually seen in the age period 13 to 17, definitely induce a marked acceleration in the growth rate by the administration of androgens. One can, further using these individuals as internal controls through a period of years, start and stop the increased growth rate by giving and withdrawing the testosterone.

After a period of time in some of these cases, about the age of 17, the phenomenon of spontaneous puberty occurs, so that when we stop the androgen for the fourth or fifth time, for a period of three or four months, these patients continue to grow, their testes increase in size and they mature spontaneously.

Now with regard to the question of the effect of testosterone or other androgens on skeletal maturation, I think we have to be careful about the time relations of this. I would point out that normally the androgens are present in normal amounts from the age of 13 or 14 on. There is a very considerable growth which takes place during that period, so that while it is true that androgens do mature the epiphysis, the concept that they do so at such a rate as necessarily to limit what ordinarily would be the normal growth, is perhaps not correct.

As regards the influence of androgens upon skeletal maturation in the period of childhood before 8 years of age, Dr. Talbot is, I am sure, correct in the statement that normally they do not play that role. However, even if injudiciously, I did treat a boy of 8 who was grossly underweight, underheight, with only one carpal centre present, with testosterone, which, incidentally produced marked muscular development, growth in height, and development of the penis. Within a matter of three months, four more carpal centres appeared. Treatment lasted for six months, but skeletal maturation continued after this and reached approximately age 10-11. In cases in which the skeletal age in older individuals was say thirteen at the time of beginning testosterone therapy there was not this very rapid skeletal maturation. It is possible that when

the skeleton is exposed to testosterone at an age period and degree of development at which it is not normally subjected to its influence rapid maturation takes place. At an age period say 13 or above when the skeleton is normally exposed to androgens the effect is no more rapid than that which occurs at normal puberty.

As regards the influence of estrogens on growth, it is my opinion that dwarfed girls, some of them. do show that estrogen does have a stimulatory effect, not very marked and not prolonged. It is also my impression that there is a definite difference between the effect of estrogens and androgens upon the epiphyses. An individual, a girl, age 14, had delay in epiphyseal development; she had a very small epiphysis for the head of the radius, a thin line. She was given estrogen and grew a matter of 2″ in about six months, and on cessation of the estrogen she ceased to grow. During that period that thin epiphysis did not grow appreciably, but united to the shaft as it was, without further growth.

Under testosterone propionate, in both males and females, the epiphysis of the head of the radius thickens and begins to grow down over the shaft and ultimately unites. From that, I think certain further evidence is needed, whether the skeletal maturation effect of androgens is not different from that of estrogens and that the latter may tend to cause growth and more rapid closure.

Silverberg and Silverberg have demonstrated a difference in the effect of the two types of hormones on epiphyseal cartilage.

N. Talbot: We have made somewhat similar observations on a fourteen year old dwarfed boy who was given androgen therapy intermittently over a period of time. Methyl testosterone in doses of about 30 mg. per day by mouth resulted in an increase both in growth rate and in the 17-ketosteroid output, which rose from approximately 0 to essentially normal values during a period of three months. On the first two occasions when the methyl testosterone therapy was discontinued, both the 17-ketosteriod output and the rate of growth returned practically to pretreatment levels. On the third occasion, following the administration and discontinuation of methyl testosterone treatment, the patient continued to grow at a normal rate and the 17-ketosteroid excretion was maintained within normal limits. During the subsequent years off therapy the boy's growth and sexual and skeletal maturation have progressed normally. This type of observation has made us wonder whether methyl testosterone may under certain circumstances stimulate the anterior pituitary to produce adrenocorticotrophic and possibly other hormones.

I. T. Nathanson: Some years ago Dr. Aub and I studied the urinary excretion rates of the sex hormones in normal and abnormal children. It was found that there was a positive correlation of 17-ketosteroids with the growth rate rather than the chronological age. In addition, as was to be expected, there was a correlation between the 17-ketosteroid levels and creatinine excretion. It could not be demonstrated that estrogens bore the same relationship to growth, although there was a steady rise in excretion as the children matured. In the female there was a rapid rise of estrogens several years before the menarche which was associated with clinical evidence of sexual metamorphosis. We also studied children with various types of functioning tumors in the endocrine organs. Dr. Talbot discussed several of these children from the standpoint of growth. The excretion levels of the sex hormones are also of interest. In the child with pubertas praecox occurring in association with a hamartoma in the midbrain there was an elevation of the urinary titres of gonadotrophins, estrogens, and 17-ketosteroids, which were consistent with those usually found in adult young females. The urinary excretion of 17-ketosteroids and estrogens in children with adrenal cortical

hyperplasias or tumors are moderately to markedly elevated, whereas the gonadotrophic titre is not. Children with granulosa cell tumors excrete considerable quantities of estrogens, but the 17-ketosteroid and gonadotrophic levels are not elevated. Thus it appears that assay of the urinary sex hormones may be of value in arriving at a diagnosis when it is obscure clinically as well as indicating which hormones may be involved in any growth abnormality.

Some effects of sex hormones on the anterior pituitary are also of interest in relation to this paper and that of Dr. Long. Studies by us indicate that the injection of a single dose of testosterone propionate in immature female rats will result in stimulation of the ovaries, thyroid, parathyroid and adrenal glands, which were the principal organs examined. A single dose of estrogen will also stimulate the ovary: the other organs were not examined. Using the colchicine technic for doing mitotic counts, Dr. Brues and I found that the greatest activity in the thyroid and parathyroid cells occurred about 96 hours after the injection of testosterone. Measurements of the height of the cells of the thyroid acini by Dr. Rawson correlated well with the mitotic activity. Follicle stimulation in the ovary was at is height in 96 hours as well. Interestingly enough the peak of mitotic activity in the adrenal glands appeared at 48 hours. In the immature male rat the testes show greatest mitotic activity at 48 hours also. These changes do not occur in the absence of the pituitary, which therefore suggests that the sex hormones under certain conditions stimulate secretion of hormones of the pituitary which in turn activate the target organs.

The effect of testosterone on growth was strikingly demonstrated to us by the administration of the hormone to normal boys with adolescent mammoplasia (mastitis). The lesion is very common and it is our opinion that it is an integral part of puberty. Spontaneous regression is the rule and this usually occurs coincidentally with signs of maturity such as voice change, hirsutes, etc. We were interested to see if it was possible to hasten regression with the use of testosterone. This usually proved to be the case, but one boy in particular was of interest. He received 30 mg. of testosterone propionate a week for almost six months. During that time he grew five inches in height, his voice changed, hirsutes appeared and there was evidence of skeletal and somatic maturation. In other words the hormone appeared to hasten the pubertal metamorphosis.

E. J. Kepler: There might be less confusion and disagreement regarding the influence of the endocrine organs on growth if more measurements were made on the comparative rate of growth of different parts of the body. Impressions obtained from measurements restricted to height and weight alone may be very misleading. There are a number of cases recorded in the literature which drive this point home. In 1934 Le Marquand and Dorothy Russell studied a case of sexual and somatic precocity associated with an intracranial tumor which sprung from one of the mamillary bodies. The patient was a boy aged 14 months. Careful anthropologic measurements were made during the child's illness. If only height and weight were considered it would have been possible to conclude that eventually the child would become a giant. However it was shown that growth at first was confined to the long bones. Later the long bones ceased to grow but the spine continued to elongate. The authors showed that had the child lived he would have had the habitus of an achondroplastic dwarf. A similar case was reported by Cappell and Dott. The validity of the conclusions drawn from these two cases is shown by a third case which I had the opportunity of studying. The patient was a young woman aged nineteen years, as I recall. Some time in her latter childhood just prior to the onset of puberty she showed signs of somatic

and heterologous sexual precocity. About 10 years later when I first saw her she had a large malignant tumor mass in the upper right abdomen which presumably originated from the right adrenal cortex. Her height was 54 inches (135 cm.) and she had a habitus typical of achondroplastic dwarfism.

Finally, there is a case reported by Sacchi in which sexual and somatic precocity was induced by an interstitial cell tumor of the testis. Although detailed measurements were not given, the habitus of the patient, judged by his photograph, makes one suspect that he is shorter than normal, has unduly short extremities and a relatively long spine—in brief, the antithesis of eunuchoidism.

A similar trend is evident in the photographs of another patient (unpublished case of Dr. Reforzo Membrives) who also had an interstitial tumor of the testis.

These four cases suggest that both adrenal and testicular androgens first stimulate growth of long bones, subsequently inhibit their growth by causing epiphyseal union to occur and finally bring about lengthening of the vertebral column.

I should like to ask Dr. Talbot if he has made any observations that throw any light on the problem of diabetic dwarfism. As you know, since the isolation of the "diabetogenic" principle there has been a tendency to consider diabetes mellitus as being the result of overproduction of this substance by the anterior pituitary body. At the same time and often by the same authors, there has been a tendency to ascribe the sexual and somatic infantilism that occurs among diabetic children to an under production of growth hormone by the same organ. Personally, I am inclined not to believe that the anterior pituitary body is the primary malefactor in either instance.

N. Talbot: In response to the query concerning diabetic dwarfism the following may be said. Of the diabetics in our pediatric clinic a great majority are of normal height and weight. The few who are underweight appear to be children whose nutritional status is poor either because of difficulties in regulating the degree of glycosuria or because of faulty dietary intake. In these connections the observations of W. N. Bartlett are of interest. He has shown in a study of diabetic children that even without insulin therapy they would grow satisfactorily, as evidenced by a positive nitrogen balance, if their caloric intake was adequate, but that they failed to be in positive nitrogen balance when the caloric intake fell below certain appropriate values. In the group which he studied a major part of the calories was provided in the form of fat. In other words, it would be my guess that the most common cause of dwarfism in diabetics is a caloric balance which is not sufficiently positive to permit protoplasmic anabolism rather than a primary disturbance of the pituitary, adrenal, etc. function. There is, of course, the possibility that caloric malnutrition may lead to various degrees of "physiologic" hypopituitarism.

R. G. Hoskins: During the earlier stage of the development of endocrinology there appeared a fashion of publishing many intricate diagrams of glandular interrelationships. We seem to now be going through a period of rediagraming the subject but in a more adequate way, recognizing that the essential advantage of a diagram is the setting forth of a few salient relationships free from confusing complications. However, there is one fairly complex aspect of the inter-relations that must perhaps be kept in mind. The anterior-pituitary gland does not operate in a vacuum but is itself a target organ for the various hormones—presumably all those which influence growth processes and nutrition processes of the body generally.

Dr. Talbot might be asked if he does not think that in his chart of thyroid relationships he should not include the anterior pituitary as an important target structure. Thus might be understood the well-known fact that under the influence of thyroid—

itself a catabolic agent—anabolism and hence growth can be markedly stimulated. An assumption that the thyroid hormone stimulates the secretion of pituitary growth hormone seems to be genuinely helpful.

N. Talbot: I agree with Dr. Hoskins that a number of hormones, including thyroid, gonadal and adrenocortical hormones, and the central nervous system influence the secretory activity of the anterior pituitary. That is, the pituitary is a target organ.

F. W. D. Lukens: Measurements of skeletal maturation are so useful, particularly after androgenic therapy, that we place a great deal of emphasis upon them. I wonder if Dr. Talbot now has any evidence concerning factors which influence the maturation or termination of development of visceral organs. Do the viscera also mature and finish their growth as a result of any of these agents?

N. Talbot: Relatively little information on this subject is available at present. It should provide an interesting field for further investigation.

F. C. Koch: In discussing Dr. Browne's remarks, Dr. Talbot said treatment of a dwarfed boy was effected by giving methyl testosterone by mouth. He also said there followed an increase in the excretion of urinary 17-ketosteroids. That is a great surprise to me. In all of the studies we have conducted and are familiar with (which I will emphasize were done on normal and eunuchoid adults) no increase in 17-ketosteroids was noted after the oral administration of methyl testosterone. Is it possible that the metabolism of methyl testosterone is different in the young than in the adult?

E. C. Reifenstein, Jr: It is my interpretation that thyroid hormone *per se* does not have a specific action on catabolism of protoplasm. Thyroid hormone does have the specific action of increasing the energy requirements. If these requirements are met with adequate calories, no catabolism of protoplasm occurs; if these requirements are not met with adequate calories, catabolism of protoplasm occurs to supply energy. The evidence for this interpretation is supplied by the investigations of Lauter and Jenke (2), who showed that hyperthyroid patients on high caloric, high carbohydrate, high fat, low protein diets had normal "minimum nitrogen excretions." This interpretation reconciles the loss of weight of hyperthyroid patients with the anabolic properties of the thyroid hormone.

To supplement the discussion of Dr. Koch, we can confirm that the administration of methyl testosterone does not increase the urinary 17-ketosteroid excretion; furthermore, administration of this compound not only does not increase but actually decreases the excretion over a period of time (3). Since methyl testosterone is not itself excreted as a 17-ketosteroid, it is possible to determine the effect of this testosterone compound on the endogenous production of urinary 17-ketosteroids. The fall in the 17-ketosteroid level during methyl testosterone therapy has been demonstrated in a normal male, in two males in Addison's disease (the data on one of whom were kindly supplied by Dr. Edwin J. Kepler), in two normal females, in two females with hyperadrenocorticism of the Cushing's-syndrome-type, in two females with hyperadrenocorticism of the adreno-genital-syndrome-type, and in a castrate male. We believe that the decrease in the level of the urinary 17-ketosteroids of both testicular and adrenal cortical origin is due to inhibition by methyl testosterone of a pituitary trophic hormone which normally stimulates both the testis and the adrenal cortex to produce the 17-ketosteroids or their precursors. We suggest that this trophic hormone is the gonadotrophic hormone which in the female is called the luteinizing hormone.

My third point has to do with the very interesting girl whom Dr. Talbot is following—the girl who was castrated at birth. This child has grown and developed quite normally to her present age of 11. It may be more than coincidence that her present

height is almost exactly that of the "ovarian" dwarfs (1). If she continues to grow from now on she will produce evidence that more than ovarian failure is responsible for the short stature of the ovarian dwarfs; if she stops growing at this point she will be, in fact, an ovarian dwarf, and will produce evidence that the short stature is due primarily to the ovarian failure.

R. D. Rawson: I am interested in Dr. Talbot's suggestion that thyroid hormone has a nutritional effect on the cell and that rather than being a growth hormone *per se,* it simply facilitates the action of the pituitary growth hormone. I wonder if the same action of the gonadal and the adrenal cortical hormones might not also be suggested. Another thing which interests me is what was suggested by Dr. Talbot that the nutrition of the body has some influence on its response to the hormone.

A few years ago Dr. Kendall and one of his associates reported that patients treated with thyroxin in amounts great enough to raise their basal metabolic rates to a level of about +30, when put on a vitamin B-1 deficient diet, had a fall in the metabolic rate to a level of about —10 or even lower, though the treatment with thyroid hormone was continued. When the vitamin B-1 was replaced in the diet the basal metabolic rates again rose to the previous level of +30.

N. T. Werthessen: One phase of the pharmacology of the steroids which I do not think has been discussed adequately is the matter of dosage. Another is the age of the patient relevant to the dosage employed. Dr. Lawrence and I were able to show in some unpublished studies that small doses of the oral estrogens given to young women (0.1 mg. or less per day) were able to raise estrogen excretion rates from castrate levels to normal. The normal rates persisted for months after cessation of therapy. The same estrogens in later life in the higher dosages given for menopausal syndrome are presumed to inhibit the pituitary. The simplest explanation of our findings was that the low dosage had stimulated the pituitary to normal secretory activity. The experimental basis for this explanation can be found in the works of Fevold, Greep and Hisaw. I should like to suggest, therefore, that (1) mode of administration (2) length of period of administration of particular dosage (3) patient's age (4) rate of secretion by the patient's own glands all be considered before ascribing to one of these agents the lack or possession of a particular therapeutic property. On the basis of our experience it appears more than probable that the apparent pharmacological effects of a particular substance may be completely different when studied at the extremes of a wide range of dosage.

N. Talbot: With reference to the comments of Doctors Koch and Reifenstein, to the best of our knowledge methyl testosterene is not metabolized to urinary 17-ketosteroids. Therefore we have tended to conclude that the increases in 17-ketosteroid output noted in the boy I mentioned in answering Dr. Browne's remarks signify that in this subject the drug activated the anterior pituitary to secrete more adrenocorticotrophic or possibly gonadotrophic hormone, which in turn stimulated the adrenals or testes to produce more 17-ketosteroids. It is very likely that a more prolonged use of the drug would have resulted in a reversal of this effect with an inhibition of the pituitary adrenocorticotrophic and/or gonadotrophic hormone production. Such a relationship would be in keeping with the thesis expressed by Dr. Werthessen on the relation between the response of the host, the susceptibility of the host, and the dose or concentration of hormone. In other words, while the metabolism of methyl testosterone is probably the same in the child as in the adult, its physiological influence on hypophyseal activity may differ.

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Experimental Renal Hypertension with Special Reference to Its Endocrine Aspects

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I. Introduction

The view that the kidney is the source of an internal secretion essential for the normal organism is one that was stressed many years ago, particularly by the French physiologists (25), but which has long ago been discarded as based on neither convincing nor accurate data (22). However, the intensive research of recent years on the problem of hypertension has again indicated that the kidney, by some humoral if not by an incretory mechanism, may be concerned in the pathogenesis of hypertensive cardiovascular disease as observed so commonly in man and as produced with ease in the experimental animal. It is now almost generally admitted that the condition as induced in the experimental animal is identical with that observed in the human patient and hence that a study of the former may lead to an elucidation of the pathogenesis and suggest a rational therapeutic approach to what constitutes perhaps the most important practical condition encountered clinically.

II. Experiments

Experimental chronic hypertension has been produced in all the commonly used laboratory animals. A variety of procedures may be used, all of which have in common some interference with the normal kidney. One may reduce the blood flow to the kidney by applying a clamp to the renal artery as in the Goldblatt procedure (7); constrict the renal parenchyma with an enveloping material (29); deform the kidney with a simple ligature (10); remove part of the renal tissue (2); administer known nephrotoxic agents such as lead salts (4, 8), etc. We have found the application of a "figure-of-eight" constricting band around the organ as most convenient for general laboratory investigation (10).

The fact that hypertension follows manipulations on the kidney, as well as the observed high incidence of hypertension in the human with kidney disease can leave little doubt that experimental as well as many cases of clinical hypertension are secondary to dysfunction of the kidney. The

mechanism by which alteration in renal function induces hypertension might however be explained in one of several different ways. The fact that the resulting elevation in the blood pressure is not abolished by severing the nerve supply to the organ excludes a reflex nervous mechanism as the responsible agency. The view which has gained almost universal acceptance but which, as shall be shown later, is entirely incompatible with the facts at hand, is that the kidney produces a humoral pressor substance which maintains the observed rise in blood pressure. The basis for this view was the fact first observed by Tigerstedt and Bergmann in 1898 (41) that extracts of renal tissue, when injected, exert a pressor action due to the presence of a constituent which they designated as renin. It is now believed that this pressor property results from the interaction of renin with an activator present in the blood which forms hypertensin or angiotonin (27, 30).

The following demonstrated facts speak, however, against the "renin" or "pressor" hypothesis: some of them disprove it; others make it very improbable.

1. *The Absence of a Pressor Substance in the Blood in Chronic Experimental or Clinical Hypertension*

The most careful study has failed to reveal the presence of a pressor agent in the blood of patients suffering from chronic hypertension. That these methods are capable of detecting small amounts of such pressor substances has been shown by injection of angiotonin and the demonstration of its presence in the blood (7). The only instances in which pressor substances may be found in the blood are in acute glomerulonephritis, in eclampsia, and in the experimental animal, immediately after inducing ischaemia of the kidney by applying a clamp to the renal artery (27). In these conditions it is probable that a pressor agent is circulating in the body which causes a rise in blood pressure. The absence of such pressor substances in chronic hypertension speaks against the view that such a humoral agent is responsible for the observed elevation of blood pressure.

2. *The Effect of Pithing and Injecting Renin on the Blood Pressure*

As Dock (5) has shown, pithing a hypertensive rabbit results in a drop in blood pressure. However, the pithed rabbit still reacts to the injection of small amounts of renin (angiotonin) by an elevation in blood pressure. It must be concluded, therefore, that the mechanism responsible for the maintenance of the elevation of blood pressure is not dependent upon circulating renin, angiotonin).

3. *The Effect of Removing an Injured Kidney in Unilateral Hypertension*

In a certain percentage of animals, hypertension may be induced by an operation on one kidney (11, 18). Following such an operation, the removal of the injured kidney does not result in a return of the blood pressure to normal (11, 18, 31). Were the injured kidney responsible for the liberation of a pressor substance, its removal should obviously result in a return of the blood pressure to its normal levels. The failure of earlier workers to observe this phenomenon was due to the fact that they induced acute hypertension which was relieved by nephrectomy. This, however, does not occur in the chronic hypertensive. The data based on the human subject in which removal of a kidney was claimed to result in a lowering of the blood pressure is also open to criticism (39).

4. *The Effect of Unilateral Nephrectomy on the Blood Pressure*

Although unilateral nephrectomy, in general, does not cause a rise in blood pressure, this operation will, in a small percentage of animals, induce such an elevation and in fact, the incidence with which an elevation in blood pressure occurs is the same as that which follows an operation on one kidney (11, 18).

5. *The Effect of Bilateral Nephrectomy in the Hypertensive Animal*

Bilateral nephrectomy does not generally result in an elevation of blood pressure because sufficient time does not elapse between the time of the operation and the time required for a rise in blood pressure to manifest itself. The development of hypertension following an operation on the kidney does not usually manifest itself for several weeks. The immediate rise sometimes seen after constriction of the renal artery is probably unrelated to the permanent rise which manifests itself later. In general, at least several weeks must elapse before chronic hypertension ensues. For this reason, one would hardly anticipate that bilateral nephrectomy should result in a rise in blood pressure. However, if the kidneys are removed from an hypertensive animal the blood pressure does not return to normal promptly as one would expect if the kidneys were responsible for the production of a circulating pressor substance. It is only terminally with the imminence of uremia that the blood pressure begins to fall.

6. *Effect of Bilateral Nephrectomy in Parabiotic Animals*

One can study the effects of complete removal of the kidneys on the blood pressure in parabiotic twins (24). When this is done, it is found that the removal of both kidneys from a pair of such parabions results in a rise

in pressure of the nephrectomized twin, with normal or only slightly elevated effects in the unoperated partner (20). This experiment precludes any pressor substance being formed by injured renal tissue as a source of hypertension.

7. *The Effect of Removing a Normal Kidney in an Animal with a Unilaterally Affected Kidney*

If one operates on one kidney of an animal, the removal of the other normal kidney results in a prompt elevation of blood pressure (6, 11, 18). It is thus, apparently, the removal of normal tissues which results in the elevation of blood pressure, rather than the mere presence of injured renal tissue.

It will be noted that all of the experiments just cited point to an absence of normal renal tissue rather than the presence of abnormal tissue which leads to hypertension. This led early to the hypothesis that there was an antagonism between some antipressor substance produced by the kidney and the pressor agent. However, if we discard the pressor hypothesis we may simply postulate that the normal kidney produces an incretory substance necessary for the maintenance of normal blood pressure levels. In the absence of this essential agent a diseased state is induced which is characterized by an increased general peripheral resistance and a rise in blood pressure (9).

A third possible explanation of the mechanism of renal hypertension is to assume that the excretory function of the kidney is interfered with and that the presence of some toxic catabolite gives rise to the observed disease. This theory savors of the general detoxifying function which has been attributed to every endocrine organ prior to the discovery of its function and has so little to support it as to merit no further comment.

8. *The Use of Renal and Other Extracts in Hypertension*

The experiments already cited which pointed to a deficiency of some normal constituent formed by the kidney led to the search in renal extracts for a constituent which might replace the need by the organism for this deficient principle (21, 22). In considering these renal extracts one must differentiate between those which are effective only when administered parenterally and which are derived from relatively small amounts of renal tissue, and the extracts which are effective when administered orally. The former are not dialyzable and are now believed to exert their effects through some nonspecific reaction (34). On the other hand, the orally administered extracts which are obtainable only by concentrating large amounts of renal tissue are readily dialyzable (23). Whether or not the latter represent a

true physiological principle elaborated by the normal kidney and a deficiency of which in the hypertensive is responsible for the elevation in blood pressure is still a matter of conjecture. From a practical standpoint the use of renal extracts has never proved feasible because of the low concentration in which the active substance occurs in the kidney. However, this in no way invalidates the possible theoretical significance of the observations cited, inasmuch as it is generally true that in the case of many endocrine organs (9) only small amounts are obtainable from natural sources (for example, from the ovary or testis).

More recently, it has been possible to lower the blood pressure in experimental animals and a few patients by the administration of a substance obtained by oxidation of certain oils (12). Whether or not this principle bears any relationship to the one present in renal tissue, and its significance also remains unestablished. However, the oils do provide a more practical source than kidneys for a substance which is at least effective in modifying the elevated blood pressure observed in hypertension.

The lowering of the blood pressure which follows the administration of these oils is not due to the presence of vitamin A since this effect is still elicited after destruction of this vitamin (12). The observed effect is thus unrelated to the increased renal blood flow and increased tubular activity ascribed to vitamin A concentrates (40).

9. *The Effect of Sodium Restriction on the Level of the Blood Pressure*

The fact that restriction of salt is capable of lowering the blood pressure in clinical hypertension was observed many years ago. However, this procedure, being based on clinical observation without adequate experimental confirmation, has not been generally accepted. We have recently shown that drastic sodium restriction in hypertensive rats results in a rather dramatic drop in blood pressure (14). The same results were obtained in some patients (15) but not in all. Experiments demonstrated that the decline in blood pressure was due to a restriction of sodium and that chloride or other anions were not involved in this effect (14).

The question arises as to how the sodium ion is related to the level of the blood pressure. Feeding excessive amounts of sodium chloride raises the blood pressure only slightly in normal or hypertensive animals (19). As is well known, sodium chloride elevates the blood pressure in patients suffering from adrenal cortical insufficiency (9) and also, according to Ambard, raises the abnormally low blood pressure of patients suffering from tuberculosis and other chronic and acute infections. It would seem at present unjustified to conclude that the observed reduction in blood pressure which

follows drastic sodium restriction is anything more than a symptomatic response, particularly since it does not prove effective in all cases. Until more is learned about the mechanism involved and the changes in such factors as blood volume, and cellular and extracellular fluid changes occurring during sodium restriction, one can only speculate concerning the manner in which the observed reduction in blood pressure occurs.

10. *The Relation of the Pituitary and Adrenals to Hypertension*

Some years ago we reviewed (16) the relation of the endocrine system to hypertension. Except for the rare chromaphil cell tumors which induce paroxysmal hypertension, the conclusion was reached that no abnormality of the known endocrine glands was fundamentally involved in the production of essential hypertension. The demonstration of the effects of drastic sodium restriction on the blood pressure immediately raises the question as to the possible role certain endocrine organs, particularly the adrenal cortex, might play in the pathogenesis of hypertension. Indeed, if one were to follow the example of oversimplification and schematization which endocrinologists are addicted to, one might be tempted to attribute hypertension to an abnormal retention of sodium induced by over-activity of the adrenal cortex and attribute the latter in turn to over-stimulation of the anterior pituitary, the ultimate source, as previous speakers on this symposium have so often insisted, of all endocrine activity. Such fanciful conjecture, however, cannot be supported by the known facts.

There is no good evidence to indicate that the true hormone of the adrenal cortex induces an abnormal retention of sodium in the organism. In such disorders as the Cushing syndrome in which abnormal steroids are elaborated by the disordered adrenal it may be assumed that these, like desoxycorticosterone, induce the observed retention of sodium which, in turn, may be responsible for the hypertension observed in this disorder (17, 36).

Several authors have, from time to time, demonstrated an hypertrophy of the adrenals in patients dying of hypertensive cardiovascular disease but this does not occur consistently. The excretion of 17-ketosteroids which is accepted as a measure of adrenal cortical activity is also not elevated in hypertensives (1). There is thus no valid evidence to attribute to the adrenal cortex a primary role in the causation of hypertension.

The effect of various steroid substances on the kidney has been well established (3, 26, 32, 35, 37, 38) and described in detail by Kochakian elsewhere in this Symposium. The relation between the observed morphological changes in the kidney to the blood pressure, which is often elevated to hypertensive levels, and to the salt and water metabolism of the organism require further investigation before their significance in relation to the problem of hypertension is established.

III. Summary

The available evidence points to the kidney as the site of origin of a humoral agent involved in the pathogenesis of hypertension. The basis for this view has been reviewed and the possible nature of this relationship discussed. The capacity of certain steroids to induce changes in the kidney and elevate the blood pressure as well as the changes in blood pressure induced by alterations in the sodium and water metabolism of the organism present problems of endocrine interest.

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Discussion

A. C. Corcoran: Last night there was a great enthusiasm for discussion on the subject of hypertension. Are there any comments?

F. W. D. Lukens: Is there any evidence that any of these extracts are steroid in nature? What about your renal extracts? Have you ever tried to get them by using fat solvents?

K. W. Thompson: One of the most interesting points, I think, Dr. Grollman, was the fact that pithing a hypertensive animal causes a drop in blood pressure. How does that fit in with the idea that there is a disturbance in the salt and water metabolism or in some hormonal factor in hypertension? I should think Dock's experiment would indicate that it is a central phenomenon. I think your studies on parabiotic rats were a marvelous experiment and ought to be made a great deal of, somehow.

Dr. Selye: The simplified diagram which Dr. Grollman has shown us cannot be discarded as entirely imginative. In fact, it was just about along the lines of a paper recently sent to the press by Dr. Massey and myself. Dr. Grollman has sketched on the blackboard a diagram similar to the one we have in press.

I think some emphasis ought to be placed upon the difference between the action of desoxycorticosterone and the other steroids. To my mind there is absolutely no correlation between the effects of desoxycorticosterone and the effects of other steroids. Indeed, we could not confirm the effects of any steroid except desoxycorticosterone, as far as blood pressure is concerned. As far as the effect of salt is concerned, its addition in the diet with practically every steroid resulted in a rise in blood pressure. Only desoxycorticosterone causes nephrosclerosis; and only desoxycorticosterone, among those steroids assayed by us, has any salt-retaining capacity. There may be a different experimental arrangement but I may add that testosterone not only failed to cause nephrosclerosis and hypertension but actually prevented the nephrosclerosis developing. The effect on the kidney has been discussed in detail by Dr. Kochakian at this conference, but testosterone also has a beneficial effect on the kidney and by that effect prevents damage from desoxycorticosterone.

Dr. Grollman made no reference to the ability of the desoxycorticosterone to produce nephrosclerosis, although his views concerning the mechanism of renal hypertension may make it relevant. One should emphasize the striking coincidence both in human patients and experimental animals that nephrosclerosis and hypertension develop in parallel.

I would also like to know what evidence there is that the steroids other than desoxycorticosterone exert a sodium retaining effect. I am not aware of any convincing evidence along that line, of a sodium-retaining effect outside of the one compound, desoxycorticosterone.

In connection with the observation on salt metabolism, I have some experimental

observations to report which fully confirm Dr. Grollman's point of view, that when animals are given desoxycorticosterone on a low sodium intake, it is extremely difficult to induce hypertension, and thus when one gives 1% sodium chloride instead of ordinary tap water for drinking, to animals receiving desoxycorticosterone, the incidence of nephrosclerosis is greatly increased compared to the effects of the same dose of desoxycorticosterone alone. That led us to attempt a curative procedure by administering ammonium chloride which would presumably influence the sodium excretion. In the course of our work on the production of nephrosclerosis by desoxycorticosterone, we found that if ammonium chloride was given instead of sodium chloride, then nephrosclerosis was prevented and hypertension failed to occur. In fact, we can even add 1% sodium chloride to the diet and treat with desoxycorticosterone and still not get nephrosclerosis if you add 1% ammonium chloride. The question came up, is it the ammonium or the chloride that brings about this effect and what is the mechanism of this inhibition? Other ammonium salts also proved effective. Thus ammonium nitrate and ammonium sulfate had an inhibitory effect. We tried other chlorides and they were ineffective and we finally concluded that only sodium increased the nephrosclerosis due to desoxycorticosterone, and no other cation. On the other hand, any acidic salt which would cause an excess of acid in the body in the course of metabolism would antagonize the action of desoxycorticosterone, presumably because it would increase the loss of sodium. Thus, a number of electrolytes could be found as having a preventive effect upon the development of hypertension and I think that could be explained on the basis of Dr. Grollman's findings that sodium is the ion conducive to hypertension.

H. Sobotka: We are interested both in hypertension and antipressor substances, and at the same time we are interested in marine oils. I wonder if Dr. Grollman would care to elaborate on what mechanism he attributes the effect of marine oils to, and his ideas about the role of pressor substances as such. Does he believe that the effect of these marine oils, or perhaps the peroxide which may be generated in these highly unsaturated fish oils, act on the pressor substance or on the pressor mechanism?

A. Grollman: I cannot answer Dr. Luken's question as to whether the principle present in renal and marine oil extracts which is effective in lowering the blood pressure is a steroid. It has not, as yet, been isolated. If it be a steroid, it must be present in a combination which renders it water soluble. In fact, with purification the active material becomes progressively more water-soluble as it becomes less lipoid-soluble.

In answer to Dr. Thompson's question regarding the significance of the drop in blood pressure which occurs on pithing a hypertensive animal, this observation indicates that the intact cord is necessary for maintenance of the elevated blood pressure. The functional integrity of the heart is also essential for maintaining the elevation in blood pressure, for cardiac failure both in man and in the experimental animal results in a drop in blood pressure. However, it is unjustifiable to conclude from these observations that overactivity of either the nervous system or the heart is the primary cause of the elevation in blood pressure. Nor do the observations detract from the significance of the observations which point to a relation between the sodium content of the body and the blood pressure. Even were the latter the primary cause of the hypertension—a view to which I do not subscribe—a fall in blood pressure might still follow removal of the normal nervous regulators of the circulatory system.

I am unable to account for the failure of Dr. Selye and other observers to detect a rise in blood pressure following the chronic administration of large doses of steroids other than desoxycorticosterone. The latter substance is by far the most effective in

inducing hypertension, in animals as well as in man, but occasionally one also observes hypertension in the human following overdosage with testosterone propionate, for example. The capacity of steroids other than desoxycorticosterone, to induce salt and water retention, was demonstrated some years ago by Harrop and Thorne. (*cf. Symposia of the Cold Spring Harbor Biological Association,* VOL. **5**, 1937).

In connection with Dr. Selye's comments, regarding nephrosclerosis, I feel that one must differentiate between the occcurrence of this pathological change in the kidney, which is a consequence of chronic hypertension, and other mechanisms which can ininduce hypertension, primarily. Any mechanism which will induce nephrosclerosis may secondarily induce hypertension but the latter may be present before there is any anatomic evidence of nephrosclerosis. For this reason, I have preferred to emphasize the observed elevations in blood pressure as indicative of hypertension rather than nephrosclerosis which is a consequence of this disorder.

In answer to Dr. Sobotka's question, I have no idea as to the exact mechanism whereby oxidized marine oils lower the blood pressure in hypertension. If one accepts the view that chronic hypertension is due to a deficiency rather than to the production of a pressor substance, then it would be logical to assume that in marine oils we are supplying the hypertensive organism with a substance which, in part at least, replaces this deficiency. It is quite possible, of course, that the active agent present in marine oils is related to the quinones with which Friedman, and others associated with Dr. Sobotka, have been concerned.

Dr. Selye: We do, contrary to many, feel that in certain groups of hypertensive patients, we can consistently lower the blood pressure by the administration of ammonium chloride. It must be given in an enteric coated tablet, and all such enteric coatings vary considerably in the rate at which ammonium chloride can be absorbed. Sometimes when we have sent our hypertensive patients for X-ray treatment, the roentgenologists have been excited by the finding of large masses of undissolved tablets in the gastrointestinal tract. It is a problem to know how the treatment can be used. We have already had four or five very successfullly treated cases.

A. C. Corcoran: Dr. Grollman's view of renal hypertension is quite different from that which we had once considered as the likely one. It is, of course, true that renin has not been demonstrated in the blood of chronic hypertensive animals or human beings. We have never, to my knowledge, found angiotonin in the blood in chronic hypertension. However, Dr. Page has made some interesting experiments, extremely open to debate, but which I am quite sure are valid, in which he injected samples of normotensive and hypertensive plasma into a rabbit's ear and quite consistently found a vasoconstrictor substance in the blood of the hypertensive. Its nature is not known. It is not ultra-filterable. It is reasonably stable and where it exists, its concentration increases in a day or two when the plasma is kept in the icebox. I may say that some vasoconstrictor appears in the blood following the increase of renin and persists for some time after the pressor effects of the renin have subsided.

As regards the participation of the nervous system in the development of hypertension, it is generally believed that hypertension may be induced by nervous influences to which there are added primary humoral influences. Relevant to this discussion is the fact that high spinal anesthesia in so-called neurogenic hypertension results in a drop in blood pressure. Early in the case of humoral hypertensives, a similar drop occurs, but this is not true at the later stage of hypertension. An interesting point of view has been advanced, although the evidence is far from complete, by Sapperstein, Ogden and Page of California. They presuppose two stages of renal hypertension, one

stage in which the renin pours out and the angiotonin thus produced elevates the blood pressure. Nephrectomy in that stage reduces the blood pressure which subsequently stays down. After the animal (the rat in this case) has been hypertensive for some six or more weeks, the sympathetic nervous system takes over and maintains the hypertension. In this stage the blood pressure tends to remain higher after nephrectomy and some of the antisympathetic agents, which are not effective in reducing the blood pressure during the first stage now act. So, as a working hypothesis we can assume that humoral agents are only effective early in the development of hypertension and then as time goes on, there is a stage in which the vasomotor center takes over, at which time indeterminable concentrations of pressor substances are formed but blood pressure remains elevated. That point of view we are prepared neither to support nor to deny. It requires additional experimentation and data.

There has lately appeared a paper from Argentina which is very interesting. It shows that in adrenalectomy there is a marked decrease in the concentration of renin in the blood, which fell from a level of .4 to the vanishing level. With that decrease goes, in the adrenalectomized hypertensive animal, a decrease in the level of the blood pressure. This article went on to show that the administration of desoxycorticosterone restores the blood pressure and, curiously enough, restores the concentration of this renin substance in the blood. These experiments are, I think, of considerable interest.

As to the fish oils, we have had only some clinical experience with them. We have given vitamin A concentrate in large quantities and could not show any fall in blood pressure. There was an increase in renal blood flow, in urea and insulin clearance, and in glomerular filtration rate, but no drop in blood pressure.

As to the low sodium, from the clinical point of view it is interesting that several approaches are being made to that, not the least interesting of which is Dr. Kempner's rice diet. He has claimed remarkable effects, which seem to have occurred in more of a nephritic pattern than hypertension. That is essentially a low sodium diet and a distasteful one. We gave it to four patients over a period of six weeks and made three of them dislike us a great deal. There were no material effects on the blood pressure levels and one of them became much worse during the time. I think that this demands further experimentation.

As to renal extracts, we have been using various sorts of these for several years, and using them by injection rather than as Dr. Grollman has used them, orally. Whatever the effect may be clinically, the patient improves to some extent. It is, therefore, I suppose, as justifiable a treatment as sympathectomy, which one of my friends who works in hypertension was reported to have called "legal mayhem." The injection of renal extracts, however, is a procedure which is accompanied by frequent foreign protein reactions. One might regard a reduction in blood pressure as one of the consequences of adrenal changes, which follow the injection of foreign protein. However, whatever it is I don't think that we would be justified in contenting ourselves with the term "non-specific" for a reaction which has kept people alive, which has made hemorrhages vanish from the eyes and induces other beneficent effects in malignant hypertension. For non-specificity must sometimes be made more specific by detailed analysis of the reaction.

A. Grollman: In answer to Mrs. Selye's comments, one would expect large doses of ammonium chloride to aid in depleting the organism of sodium. In fact we have used enteric coated ammonium chloride for this purpose in conjunction with sodium restriction. However, we have not observed the marked drops in blood pressure from ammonium chloride which Mrs. Selye reports, even when acidosis was induced.

It is very true, as Dr. Corcoran has pointed out, that the reaction of the hypertensive animal or human patient differs to some extent with the level and duration of the hypertension. However, the observed differences are better explained, I feel, on the basis of the secondary organic changes which occur in the blood vessels as a result of long standing hypertension rather than by the theory of Ogden and his collaborators quoted by Dr. Corcoran. Thus the effect of nephrectomy in causing an elevation in blood pressure is not explicable on the assumption of the existence of a pressor humoral agent .

Vitamin A, as Dr. Corcoran said, is not responsible for the observed drops in blood pressure induced by various marine and vegetable oils. In fact, as we have demonstrated, pure vitamin A has no such effect. It is some compound associated probably with the unsaturated acids present in certain oils which is the active agent rather than a compound associated with any of the vitamins.

The rice treatment for hypertension owes its effectiveness, we believe, to its incidental low sodium content rather than to any metabolic virtue of rice as claimed by Kempner. The same is probably also true for the "fruit," "strawberry" and other monotonous diets which have been advocated from time to time, particularly in Europe.

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* Numbers in parentheses are reference numbers. They are included to assist in locating references in which the authors' names are not mentioned in the text.

Example: Abbott, W. E., 123 (4). *144,* indicates that this author's article is reference 4 on page 123. Numbers in italic refer to the page on which the reference is listed at the end of each article.

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