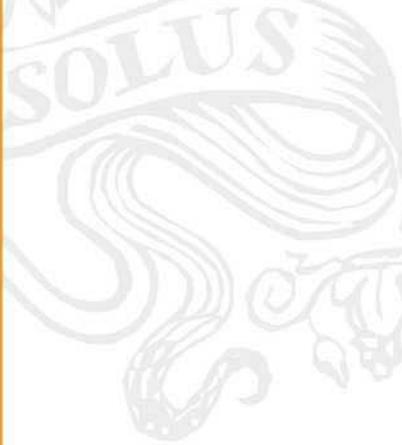




VITAMINS AND HORMONES

Volume IV



Robert S. Harris &
Kenneth V. Thimann

VITAMINS AND HORMONES
VOLUME IV

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VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

Edited by

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VOLUME IV



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Editors' Preface

With the end of the war and the gradual untangling of the biological and medical problems of the postwar world, we shall doubtless see a large upsurge in the volume of research. The return of workers to peace-time research from their temporary diversion to war problems, and the reinstatement of universities and research institutions abroad will both contribute to this. The need for critical reviews in this and other fields will, therefore, doubtless prove greater than ever.

The reestablishment of normal international scientific relations will also make it possible for Vitamins and Hormones to reflect more fully world scientific opinion. Despite the influence of war conditions on the first four volumes, contributions have been published from England (several), Switzerland, Palestine, Argentina and Australia, (besides the United States and Canada), and it is our hope that a larger number of colleagues abroad will be able to participate in the future.

An interesting feature of the present trend is the increasing interrelationship between vitamin and hormone research. This is exemplified in three of the articles in the present volume, and it justifies the Editors' initial feeling that the bringing of reviews of these two fields under one cover would prove realistic and helpful.

The present volume has been compiled in the unsettling "aftermath" atmosphere, and delays and difficulties have been inevitable. The Editors wish to express their thanks to the contributors, whose patience and concentration under such conditions has led to the production of very valuable reviews.

KENNETH V. THIMANN

ROBERT S. HARRIS

August, 1946

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The Newer Hematopoietic Factors of the Vitamin B-Complex

By J. J. PFIFFNER AND ALBERT G. HOGAN

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I. INTRODUCTION

Many years ago Castle (17, 95), in his work on pernicious anemia, demonstrated a parallel distribution in nature of the "extrinsic factor" and the vitamin B complex. Since that time an extensive literature, largely clinical, has sprung up on the relationship of various known and unknown members of the B complex to hematopoiesis. In recent years numerous studies in the fields of animal and bacterial nutrition, carried on in many different laboratories, have yielded results which direct attention to a group of new compounds which are intimately concerned with growth and the formation of both red and white blood cells. This article represents an attempt to correlate the findings of these recent studies in the light of present day knowledge and to review the field with particular emphasis on the problem of identification and chemical and nutritional interrelation of these newer hemopoietic factors. No effort has been made to survey the literature on the relationship of the better known members of the B complex to hemopoiesis nor on the general problems of the nutrition of the chick, rat, monkey or lactic acid bacteria.

In retrospect it would appear that the earliest observations on the hemato-

poietic activity of this group of then unknown substances were made by Lucy Wills (111) in 1931. She observed the striking effect of liver and yeast extracts on the macrocytic anemia of pregnancy which occurs commonly in India. The effect was not obtained with purified antipernicious anemia principle. She and Bilimoria (113) reproduced the nutritional deficiency in monkeys. These latter observations were extended by Day and his co-workers (24) and others (118, 104). Because of difficulties of assay in man and monkeys little progress was made in concentrating the active factors. Observations on the development of nutritional anemia in chicks (32, 54, 70) speeded up isolation work which was accelerated by the application of microbiological (70, 87, 62, 92, 37) and enzymatic-microbiological methods (5, 71).

In the literature these newer hematopoietic and related factors have been referred to as vitamin M, norit eluate factor, vitamin Bc, folic acid, *Streptococcus lactis* R factor, *Lactobacillus casei* factor, new *Lactobacillus casei* factor, vitamins B₁₀ and B₁₁, vitamin Bc conjugate, *Streptococcus lactis* R stimulating factor, potential *Streptococcus lactis* R stimulating factor, potential folic acid, and folic acid complex. These terms were adopted for convenience by various groups of workers to indicate a substance or substances which could be defined by some measurable biological effect. Certain of these factors have been isolated as crystalline compounds, some have been obtained as concentrates, while still others are known only in crude natural vitamin carriers. Analysis of the literature is rendered more difficult, particularly for those not actively working in the field, by the fact that some workers have adopted the terms of others and altered the connotation. When different avenues of research, each with its own terminology, become confluent it is to be expected that there will be a certain temporary confusion in nomenclature. Although certain of the above factors are known as chemical entities and identity in some instances suspected, in no case has the identity of any two been unequivocally established by accepted chemical methods. In reviewing the facts, therefore, the authors will try as much as possible to employ the terminology adopted by those whose results are under discussion. In this way it is hoped to avoid further confusion which might arise as a result of premature assumption concerning chemical identity. The development of a system of nomenclature acceptable both to chemists and physiologists will no doubt follow in the wake of further chemical progress.

II. NORIT ELUATE FACTOR

In 1939 Snell and Peterson (86) reported in abstract form that liver or yeast extract was necessary for the growth of *L. casei* in a hydrolyzed casein medium. Earlier in their studies on the nutritional requirements of this and related organisms they had demonstrated the indispensability

of riboflavin, pantothenic acid and nicotinic acid for growth. They found that liver or yeast extract could be separated into two indispensable fractions by treatment with norit in acid solution and elution of the adsorbate with pyridine-alcohol mixtures. A number of properties of the factor in the eluate were given at that time. The following year (87) they pointed out in their detailed paper that the fraction not adsorbed by norit could be largely replaced in the medium by pyridoxine but that the filtrate also contained some other unknown growth factor. This second filtrate factor they later showed to be biotin (35). They were unable to find a known compound which would give a growth response comparable to that obtained with the norit eluate, and they referred to the unknown substance(s) as the norit eluate factor. The best sources were liver, yeast, malt sprouts and cereal grains. A study of the properties of the factor in yeast concentrates led them to suggest that the substance was a rather strongly basic compound, having some acidic properties and possibly being of a purine nature. Their purest preparation produced half maximum fermentation in a concentration of 0.055 γ per cc. of medium. In a subsequent paper in 1941 Hutchings, Bohonos and Peterson (35) described a simplified method of concentrating the norit eluate factor in liver extract about 100 to 200 times. They showed that the active principle could be inactivated with ethanolic HCl and that the activity could be regenerated in 50% yield with sodium carbonate. Along with this evidence, pointing to the presence of a carboxyl group in the norit eluate factor, they also presented evidence indicating the presence of an amino group since their concentrate lost activity on treatment with nitrous acid, acetic anhydride and benzoyl chloride. A number of other properties of the factor were given but no preparation was described which had greater activity than the products described a year previously. Hutchings *et al.* (34) demonstrated that concentrates of the norit eluate factor contained a chick growth factor and that the concentration of both factors ran parallel, that is, they were both adsorbed on norit and superfiltrol and could be eluted with ammonia in aqueous alcohol. Inactivation experiments demonstrated that the norit eluate factor and the chick growth factor were sensitive to the same reagents.

Peterson and his students (35) used *L. casei* as the test organism in their fractionation work. They recognized, however, the necessity of the norit eluate factor for the growth of *Lactobacillus delbrückii*, *Propionibacterium pentosaceum* and *Streptococcus lactis* R¹.

¹ Krueger and Peterson (45) have recently called attention to the work of Niven and co-workers (66) who have demonstrated that *Streptococcus lactis* R is an enterococcus, specifically *Streptococcus fecalis*. During the past few years the term *Streptococcus lactis* R and the initials SLR have been incorporated into the designation for several unidentified nutritional factors. For the sake of clarity in discussing these factors the term *S. lactis* R is used throughout this article.

III. FOLIC ACID

In 1941 Mitchell, Snell and Williams (62) reported the preparation of a concentrate from spinach which was very active in stimulating the growth of *S. lactis* R. The basis of their test medium was a hydrolyzed casein digest similar to that employed by Snell and Peterson (87). The medium was supplemented with a number of purines and pyrimidines including adenine, guanine, xanthine and uracil (85, 61).

In concentrating the growth factor Mitchell *et al.* (62) used methods involving successive adsorptions and elutions from norit, fractionation of lead and silver precipitates, followed by chromatographic fractionation on fullers' earth. Their most active preparations produced half maximum growth in a concentration of 0.00012 γ per cc. These workers felt that they had a growth factor in nearly pure form and suggested the name *folic acid* for the factor since their source material was green foliage. Folic acid was defined as "the active principle required for the growth of *S. lactis* R under specified conditions" (85). Their concentrates also stimulated the growth of *L. delbrückii* and *L. casei*. When fed to rats their spinach concentrates appeared to have a slight effect on the rate of growth but the limited number of test animals rendered the observations of questionable significance.

In a series of papers which appeared in 1944 Mitchell, Snell and Williams (63, 27, 64, 59) presented the results of their fractionation work in detail. Starting with large quantities of fresh spinach, they succeeded in concentrating the *S. lactis* growth activity to a point where the product was 137,000 times as active as their microbiological standard (Wilson's Liver Extract B).² Products of such high potency however were not characterized. The best concentration procedure involved repeated adsorption on charcoal and elution with aqueous ammonia or aniline. This was followed by precipitation of the activity with lead and regeneration of the precipitate with ammonium sulfate; precipitation of the silver salt and regeneration with ammonium chloride; adsorption on fullers' earth at pH 1 and elution with ammonia water; adsorption on alumina and fractional elution with dilute methanol and dilute methanol containing 2% of ammonia. Further purification was effected by chilling an acidified aqueous solution of the concentrate. The insoluble fraction was again chromatographed on alumina. The more potent eluates were combined and sub-

² According to Williams' method (109) of expressing potency of folic acid concentrates, crystalline vitamin Be has a potency in the neighborhood of 200,000. To convert assay results in the literature expressed in terms of "folic acid of potency 40,000" into terms of crystalline vitamin Be it is necessary to divide by 5. If the microbiological growth activity in the spinach concentrates is due to a single compound and if that compound (folic acid) is identical with crystalline vitamin Be from liver then material of potency 137,000 would represent a product of about 65-70% purity.

jected to acid precipitation from cold water yielding amorphous products of high activity in low yield (63). Highly active products were free of halogens, phosphorus and sulfur. The analytical values recorded for one of their better preparations (potency 80,000) were as follows, C 45%; H 3.6%; N 19.2%. These investigators expressed the view that the impurities consisted in large part of substances of a very similar nature to folic acid since comparable analytical values were obtained on products with a wide range of potency. By diffusion experiments on some of their purified concentrates they fixed the molecular weight of folic acid at 400 ± 50 and proposed an approximate empirical formula of $C_{15}H_{15}O_8N_5$. The products were fluorescent and the intensity of fluorescence ran parallel with micro-biological potency in diffusion experiments (27).

The folic acid concentrates were inactivated by much the same type of reagents which inactivated the norit eluate factor of Snell and Peterson (87, 35) such as acetic anhydride, benzoyl chloride, strong mineral acids and oxidizing agents. The activity was lost under esterification conditions (methanol-H₂SO₄) and was partially regenerated on treatment with alkali (1 N alcoholic KOH). Complete inactivation occurred on treatment of folic acid concentrates with methyl iodide, nitrous acid, hypobromite and hydroxylamine. No glycol group could be detected on periodate oxidation of the concentrate and quantitative acetylation experiments failed to demonstrate the presence of polyhydroxy groups (27).

The purified concentrates stimulated the growth of four different strains of yeast, and were essential for the growth of *L. casei*, *L. delbrückii* and *Clostridium tetani* (27, 83). On the whole, there are no striking qualitative differences in the chemical or physiological properties of the folic acid concentrates and those of the norit eluate factor. Judging from the quantities required for half maximum growth of the test organisms (*S. lactis* and *L. casei*) the folic acid concentrates were several hundred times as concentrated as the norit eluate factor concentrates of Snell and Peterson (87). Hutchings, Bohonos and Peterson (35) discussed the identity of folic acid and the norit eluate factor.

IV. LACTOBACILLUS CASEI FACTORS

In 1941 Stokstad (91) reported the isolation of a nucleotide essential for growth of *L. casei*. He used the assay technique of Snell and Peterson (87) as a guide in his fractionation work. The concentrate was prepared from liver extract by adsorption of the activity on norit and elution with ammoniacal aqueous methanol followed by fractional precipitation of manganese salts with methanol. The most active preparation produced half maximum growth in a concentration of about .02 γ per cc. The material contained phosphorus, gave a positive color test for pentose and liberated

phosphate and ribose on acid hydrolysis. Stokstad expressed the view that this growth factor was a dinucleotide made up of both a purine and pyrimidine component. The growth effect could be partially replaced by guanine and thymine.

In a later communication (92) Stokstad reported the preparation of a microbiological growth factor from liver and from yeast both of which were free of phosphorus. The preparation from liver yielded a methyl ester which was obtained in yellow gelatinous form by repeated precipitation from ethanol. The methyl ester had the following percentage composition: C, 52.7; H 4.8; N 20.1. Small acicular or bladed crystals, singly or in aggregate, formed on slow evaporation of a methanol solution on a microscope slide. The product from yeast yielded a crystalline methyl ester. The hydrolyzed esters from the liver and yeast compounds had equal potency when tested on *L. casei* but the yeast compound was only one-half as active as that from liver when *S. lactis* R was used as the test organism. The acids from liver and yeast had the same ultra-violet absorption spectrum when measured in 0.1 N NaOH and at pH 7.0. Because of the wide difference in growth activity on *L. casei* and *S. lactis* R, Stokstad expressed the view that the yeast compound differed from the liver product. The compound from liver caused half-maximum growth in a concentration of 0.000055 γ per cc. which compares with 0.00005 γ per cc. reported for crystalline vitamin Bc (70). In view of the analytical values cited above for the methyl ester of the liver compound and the potency of the free liver acid on *L. casei* Stokstad felt that his compound from liver was probably identical with crystalline vitamin Bc from liver (70).

Crystalline vitamin Bc has since been isolated (5) from a yeast concentrate following enzymatic digestion. It is difficult, therefore, to accept Stokstad's compound from yeast as being different from his liver compound on the basis of only comparative microbiological potency on *L. casei* and *S. lactis*. His compounds from both sources had the same ultraviolet absorption spectrum. The $E_{1\text{cm}}^{1\%}$ values are 70% of those later recorded for crystalline vitamin Bc by Bloom *et al.* (10). The ultraviolet absorption curves at neutral reaction of *L. casei* factor (92), folic acid (59) and vitamin Bc (10) are compared in Fig. 1.

The isolation of a third compound referred to as a new *L. casei* factor was announced in 1944 by Hutchings, Stokstad, Bohonos and Slobodkin (36). This compound was obtained as the crystalline free acid (small needles or threads), barium salt (needles) and methyl ester (small needles or threads). No elementary analytical data were recorded. The absorption spectrum was found to be very similar in 0.1 N NaOH to that recorded by Stokstad (92) for the *L. casei* factor from liver but the $E_{1\text{cm}}^{1\%}$ values were only 70 to 80% as high. Whereas the new *L. casei* factor was

85 to 90% as active on *L. casei* as the factor from liver, it was only 6% as active on *S. lactis* R. Hutchings *et al.* (36) reported that this factor was active in the nutrition of the chick. The source of the new *L. casei* factor was a fermentation residue (1). This compound may be a conjugated form of the *L. casei* factor from liver. Day *et al.* (25), for example, pointed out that the growth activity for *S. lactis* R was markedly increased by digestion with an enzyme preparation from chicken pancreas.

Recently, in an important preliminary note, Angier *et al.* (1) announced the synthesis of the *L. casei* factor from liver. The synthetic and natural

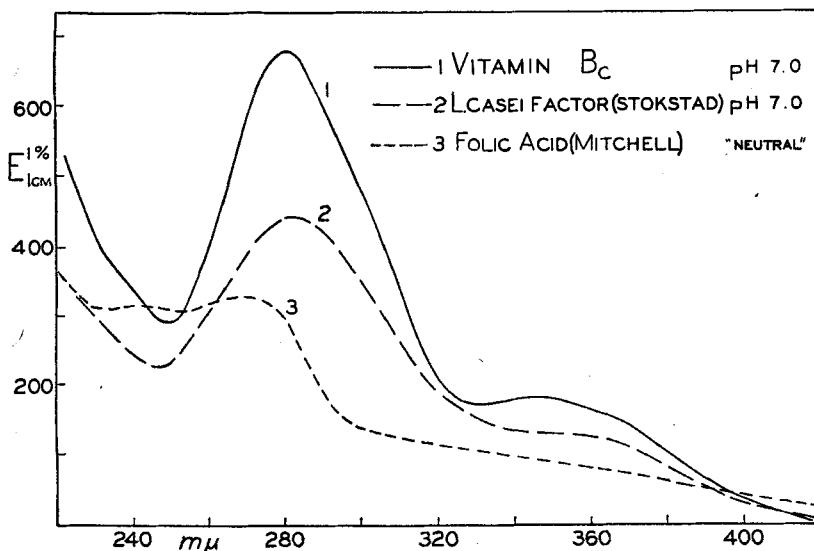


Fig. 1

A Comparison of the Ultraviolet Absorption Curves of Vitamin B_c from Liver at pH 7.0 (10), *L. casei* Factor from Liver at pH 7.0 (92) and Folic Acid from Spinach at "Neutral" Reaction (59)

compounds were identical crystallographically, in ultraviolet and infra-red absorption properties, and in microbiological growth activity as measured on *L. casei* and *S. lactis* R. The ultraviolet absorption maxima of the compound are about 29% higher than the values recorded earlier by Stokstad (92) and Hutchings *et al.* (36).

V. *STREPTOCOCCUS LACTIS* R FACTOR (SLR FACTOR)

In 1943 Keresztesy, Rickes and Stokes (37) reported the isolation of a substance which was very active in stimulating the growth of *S. lactis* R but only slightly active for *L. casei*. They used the assay method of Mitchell,

Snell and Williams (62). The source of the substance was not disclosed nor was the substance characterized except by the ratio of growth stimulating activity on *S. lactis* R and *L. casei* which was 2500:1. The SLR factor was found to be inactive in rat leucopenia by Sebrell (83). Although this substance fitted the definition of Mitchell, Snell and Williams (62) for folic acid the ratio of growth activity cited above indicates that the folic acid concentrates of spinach probably contained little of this substance. Stokes, Keresztesy and Foster (90) found, however, that *S. lactis* R and several related organisms, when grown in the presence of the SLR factor, produce folic acid in the medium. They used the term folic acid here to indicate any substance or mixture of substances which would stimulate the growth of *L. casei* under defined conditions. The conversion was independent of growth since they observed that washed cells, on incubation in an aqueous solution of the SLR factor, produced folic acid as measured by the growth of *L. casei*. Whether the *L. casei* active substance(s) formed by *S. lactis* R in the presence of the SLR factor is the same as the substance(s) in green foliage (folic acid) remains an open question. The possibility exists that the *L. casei* activity produced from the SLR factor by *S. lactis* R is due to the formation of the new *L. casei* factor of Hutchings *et al.* (36). Stokes (89) found that thymine stimulated the growth of *S. lactis* R in the absence of folic acid. However, such growth was not accompanied by the formation of material stimulatory to the growth of *L. casei*. Folic acid may have been produced but conjugated in a manner which destroyed its *L. casei* growth stimulating power.

VI. VITAMIN Bc

In the course of their studies on synthetic diets for chicks the Missouri investigators noted sporadic cases of anemia, and Hogan and Parrott (32) devised a ration that produced anemia with some degree of consistency. The difficulty was in obtaining vitamin carriers deficient in the antianemia agent but reasonably adequate in other essential vitamins. The fraction of pork liver which was soluble in 95% ethyl alcohol was fairly satisfactory. When this material was included in the basal diet the survival period was lengthened but the chicks were markedly subnormal in weight, the number of erythrocytes and the red-cell volume often became reduced to one third the normal levels, and the percentage of hemoglobin was frequently reduced to less than one half the normal amount. The anemia was of the macrocytic, hyperchromic type and the erythrocytes were less fragile in hypotonic salt solution than normal. It was concluded that the anemia was due to the absence of an unrecognized vitamin which, for convenience, was designated as vitamin Bc.

The procedure for producing anemia was improved somewhat by O'Dell

and Hogan (69). These workers reported that the vitamin is adsorbed on fullers' earth and on Amberlite IR4 and readily eluted with aqueous ammonia. It was also adsorbed on charcoal but, on elution, recovery was unsatisfactory. The vitamin is more stable in alkaline than in acid solution and is precipitated more or less completely by lead, mercury, zinc, silver, and by phosphotungstic acid. It is quite soluble in glacial acetic

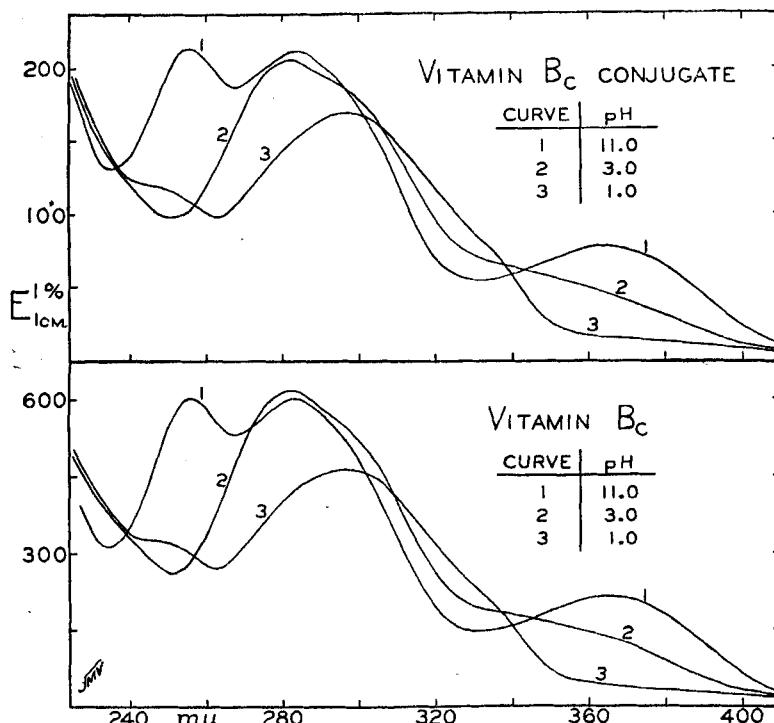


Fig. 2

A Comparison of the Ultraviolet Absorption Properties of Vitamin Bc Conjugate and Vitamin Bc. Reproduced from *Science* 102, 228 (1945)

acid, phenol and hot methanol but insoluble in the other common organic solvents.

Mills, Briggs, Elvehjem and Hart (54) observed that a concentrate of the norit eluate factor from liver which promoted growth of chicks on a purified ration (34) also prevented the development of anemia. They suggested that the norit eluate factor and vitamin Bc of Hogan and Parrott (32) may be identical. About a year later the isolation from liver of a crystalline compound which had both antianemic activity in the chick and

microbiological growth activity was reported by Pfiffner, Binkley, Bloom, Brown, Bird, Emmett, Hogan and O'Dell (70). The compound was named tentatively vitamin Bc.

Crystalline vitamin Bc was described as a yellow compound separating from water in orange-colored spherulites exhibiting crossed extinction. On repeated recrystallization it separated in the form of thin yellow spearhead shaped platelets. The compound had no melting point. Analyses reported on an ash-free specimen were: C 50.50, 50.63%; H 4.78, 4.78%; N 19.91% (70). In a later paper (5) it was pointed out that these analytical data were obtained on an incompletely dried sample and the following set of analytical figures were recorded for an ash-free crystalline liver product: C 52.44, 52.46%; H 4.28, 4.49%; N 19.8, 19.6%. The crystalline compound from liver yielded a crystalline methyl ester which had less than 5% of the microbiological activity of the original acid. The original acid could be regenerated from the crystalline methyl ester (70). The ultraviolet absorption properties of crystalline vitamin Bc were described (10) and the curves at several pH levels are reproduced in Fig. 2. The compound is only moderately sensitive to ultraviolet illumination. Although impure products are strongly fluorescent the pure compound was reported to exhibit a barely detectable trace of blue-green fluorescence in ultraviolet light. This trace of fluorescence may have been caused by decomposition of the compound during the examination since, on continued exposure, fluorescence is markedly increased.

Crystalline vitamin Bc was reported to produce approximately half-maximum growth of *L. casei* in a concentration of 0.00005 γ per cc. of culture medium (70). It was found to have the same potency on *L. casei* and *S. lactis* R (5, 45).

In a later report by Campbell, Brown and Emmett (14) it was stated that a deficiency of vitamin Bc in the diet of the chick interfered with feathering, markedly retarded gains in weight, and decreased the red blood cell volume, the red blood cell count and the percentage of hemoglobin in the blood. In addition there was a reduction in the number of leucocytes and thrombocytes. It was estimated that the ration must contain 100 γ of vitamin Bc to support a normal rate of growth, and 40 γ to maintain a normal thrombocyte and erythrocyte count, and normal hematocrit and hemoglobin percentages. The authors stated that the optimum requirement for leucocyte production was about 400 γ of vitamin Bc, but inspection of the data indicates that 100 γ would maintain the leucocyte counts in the normal range.

One might suppose that the activity of vitamin Bc when administered orally is due to the synthesis of other factors by intestinal bacteria. To test that possibility Campbell, Brown and Emmett (15) administered the crys-

talline vitamin both orally and subcutaneously, as supplements to the basal ration. The data indicate that 20 γ daily per chick of the crystalline material was sufficient, and gave the same results by either method of administration.

Angier *et al.* (1) have reported on the chick growth and antianemia activity of synthetic *L. casei* factor from liver. When 50 γ of the compound per 100 g. of ration was included in the diet the chicks made an excellent response. They grew as rapidly and maintained as high a level of hemoglobin as did chicks on a diet of natural feedstuffs.

Briggs *et al.* (13) found that a concentrate of folic acid (potency 60,000) prepared at the University of Texas, presumably from spinach, accelerated the growth rate of chicks and increased the hemoglobin in the blood when fed at a level of 50 γ per 100 g. of ration.

VII. VITAMIN Bc CONJUGATE

Yeast is a rich source of chick antianemia activity but it contains very little microbiological growth activity for *L. casei* or *S. lactis* R. The amount of microbiological growth activity is not significantly increased by autolysis. However, Bird *et al.* (6, 7) demonstrated that the microbiological growth activity of yeast extract on both *L. casei* and *S. lactis* is increased on digestion with kidney extract. The increase in microbiological growth activity, expressed in terms of crystalline vitamin Bc, accounts for the chick antianemia activity of the undigested yeast concentrates (7). Presumptive evidence that the chick antianemia activity in yeast is due to vitamin Bc held in conjugated form was supplied by Binkley *et al.* (5) who isolated crystalline vitamin Bc from yeast concentrates following enzymatic digestion. These investigators pointed out that the chick antianemia activity in yeast is non-protein in nature. It is dialyzable through cellophane and is not precipitated by heat in acid solution, by saturated ammonium sulfate at pH levels between 3 and 7 or by trichloroacetic acid. A conjugated form of vitamin Bc having similar properties is also present in liver extracts.

More recently Pfiffner *et al.* (71) reported the isolation of a crystalline compound from yeast which is antianemic in the chick but which is almost devoid of microbiological growth activity for either *L. casei* or *S. lactis* R. They refer to this compound as vitamin Bc conjugate. It was crystallized from 5% sodium chloride solution in the form of yellow birefringent spherulites. On repeated recrystallization it separated as rosettes of microscopic needles. Its elementary composition on an ash-free basis was reported as C 49.61%, H 5.36%, N 14.79%. Tests for phosphorus and sulfur were negative. The compound has no melting point but decomposes above 200°C. It yields a crystalline methyl ester which melts with decomposi-

tion at 212–215°C. (corr.). The following analytical values were reported for an ash-free sample of the crystalline methyl ester: C 50.94, 50.91%; H 6.04, 6.10%; N 14.20%.

Evidence was obtained along three different lines that this crystalline compound is a conjugated form of vitamin Bc. (a) The shapes of the ultraviolet absorption curves are almost identical with those of vitamin Bc at several pH levels, the curves for the two compounds differing only in the $E_{1\text{cm}}^{1\%}$ values, (Fig. 2). If there is one molecule of vitamin Bc per molecule of conjugate then a molecule of the latter is 2.8 times as large as a molecule of vitamin Bc. (b) One γ of the crystalline compound is equivalent to only .003 – .006 γ of vitamin Bc using *L. casei* as a test organism and only .002 γ using *S. lactis*. However when the compound is degraded with a crude enzyme extract of hog kidney the digestion mixture contains an amount of vitamin Bc (as measured microbiologically on both organisms) equivalent to that predicted from the ultraviolet absorption constants. (c) When the crystalline compound was assayed for antianemia activity in the chick by the prophylactic assay technique (14, 15) the results obtained likewise coincided with ultraviolet absorption measurements. The foregoing observations constitute strong circumstantial evidence that vitamin Bc exists as such in this new crystalline compound in a chemically bound form. Direct proof, namely, the chemical degradation of the crystalline compound to vitamin Bc, has not been reported as yet. From a consideration of ultraviolet absorption properties in conjunction with elementary analyses on vitamin Bc and vitamin Bc conjugate it is clear that the non-vitamin Bc portion of the conjugate molecule is nitrogenous in character.

From a metabolic point of view the question of the identity or non-identity of vitamin Bc conjugate in liver extract with crystalline vitamin Bc conjugate from yeast is of fundamental importance. Physiological evidence indicates that vitamin Bc performs its chemical function in the cell when in conjugated form. Does the conjugate function as such or must it, in turn, be linked to a protein moiety? Repeated attempts to separate a water-soluble protein complex from yeast have yielded only negative results. However, the water-insoluble fraction from plasmolyzed or autolyzed yeast always contains a significant amount of chick antianemia activity. It would appear that about 50% of the vitamin Bc activity in the yeast cell is bound to water-insoluble cellular constituents.

The occurrence in yeast and liver of a conjugated form of vitamin Bc having a relatively small molecular weight and essentially no microbiological growth activity appears to offer an explanation for the results obtained at the University of Arkansas on Vitamin M, those at the University of Wisconsin on vitamins B₁₀ and B₁₁, and those at the University of

Cornell on Factors R and S. These nutritional antianemia principles are discussed below.

In her early work Wills (111, 112, 115) found that crude liver extract, but not highly refined anti-pernicious anemia principle was active in relieving macrocytic anemia of pregnancy which occurs commonly in India. The striking effect of crystalline vitamin Bc from liver on hemopoiesis in the chick was cause for speculation as to its possible identity with the active factor in the crude liver extracts used by Wills. But Wills had equally good success with yeast extracts and yet yeast extract contains only a trace of vitamin Bc. The isolation of vitamin Bc conjugate from yeast extract resolves this paradox and offers ground for suggesting that vitamin Bc may be the limiting nutritional factor in tropical macrocytic anemia.

VIII. VITAMIN Bc CONJUGASE

The enzyme(s) which is concerned with splitting vitamin Bc from its conjugate has been referred to as vitamin Bc conjugase (6). It has also been referred to as the enzyme which splits *S. lactis* R stimulating factor from inactive precursor substances in yeast (57). In this discussion we use the term vitamin Bc conjugase for convenience because it has been demonstrated that the enzyme(s) in question will act upon the chemically pure substrate, vitamin Bc conjugate. There is the possibility that more than one enzyme may be involved in the reaction and that enzymes from different sources may not be identical. When working with crude yeast extract more than one substrate may be concerned. Conjugase activity is found in most tissues of the body (rat, rabbit, chicken, hog, ox) including the bone marrow but appears to be particularly rich in hog liver, hog kidney, hog intestine and chicken pancreas (57, 6, 47). It is also present in potatoes and in almonds. Present conclusions regarding the quantitative distribution of the enzyme must be considered of a tentative nature, however, since most observations to date have been made with crude yeast extract as substrate. Such extracts contain strong inhibitors. Crude tissue extracts (liver particularly) which have been used as enzyme sources likewise contain inhibitors. In no instance has the nature of the inhibitors been established (8). Autolysates of hog kidney or chicken pancreas are very rich sources of the enzyme (6, 55). The fact that autolysis appears to increase the concentration of enzyme may be due to destruction of tissue inhibitors. Both tissue and yeast inhibitors are thermostable. The pH optimum of hog kidney conjugase was reported as 4.5 (6), that of chicken pancreas as 7 (47). The enzyme in crude almond extract exhibits an optimum pH of 7 (6) but partially purified enzyme, 4.5 (8). Vitamin Bc conjugase has not been identified with any of the common proteolytic en-

zymes or with kidney nucleosidase, acid phosphatase of almond or potato, alkaline phosphatase of small intestine or β -glucosidase of almond (6). The possible activity of phosphatase was investigated before vitamin Be conjugate was isolated in pure form and found to be free of phosphorus. The enzyme in desiccated hog kidney and in almond meal is very stable but partially purified products lose activity over a period of a few weeks.

Methods of preparing active protein fractions from hog kidney autolyses (6) and from chicken pancreas (47) have been described. Laskowski *et al.* (47) suggested as a provisional unit of enzyme activity "the amount of enzyme which will produce 1 γ of the factor (as folic acid of potency 40,000)² per hour when incubated at 37°C. with 200 mg. of the yeast extract (Difco); total volume 11 cc., incubation 4 hours" (47). In later work on the purification of chicken pancreas conjugase Mims and Laskowski (56) propose a new unit of enzyme activity. They suggest a unit which is "ten times the amount of enzyme which produces 0.1 γ of vitamin Be when incubated with 1 γ of vitamin Be as conjugate during 1 hour at 32°C. in presence of 0.01 *M* CaCl₂ in 0.2 *M* borate buffer pH 7.8, total volume 1 cc." These units are adequate for comparative purposes but, as yeast extracts and concentrates vary in inhibitor content, the results obtained have no absolute significance.

Highly active enzyme preparations were obtained by Mims and Laskowski (56) from chicken pancreas in the following manner. The finely hashed tissue was allowed to autolyze for 24 hours at pH 8 at 32°C. The fraction which precipitated between 40 and 80% saturation with ammonium sulfate at pH 7 was precipitated with cold alcohol and fractionated a second time with ammonium sulfate. Calcium ion in 0.01 *M* concentration was found to activate this purified conjugase. Products were obtained which assayed 700,000 of their new units per mg. of protein.

The isolation of crystalline vitamin Be conjugate from yeast made available a pure substrate and Bird *et al.* (8) adopted a unit of enzyme activity which is that amount of enzyme which produces 1 γ of vitamin Be from crystalline vitamin Be conjugate in one hour under specified conditions. For measuring the enzyme activity of hog kidney preparations, the reaction is carried out at 37°C. in the presence of 0.05 *M* acetate buffer at pH 4.5 for one hour, the reaction mixture containing an amount of vitamin Be conjugate equivalent to 1 γ of vitamin Be per cc. and a concentration of enzyme such that 0.2 to 0.8 γ of vitamin Be per cc. per hour is released. Water extracts of fresh hog kidney assay about 600 such units per g. of kidney tissue.

In a study of the kinetics of hog kidney conjugase, using crystalline vitamin Be conjugate as substrate, it was found that the optimum pH for the reaction is approximately 4.5. At a concentration of 0.5 unit of enzyme per cc. and in the presence of a large excess of vitamin Be conjugate, the

release of vitamin Bc is linear over a period of at least 4 hours when incubated at 37°C. When the substrate contains 2.8 γ of vitamin Bc conjugate per cc. (equivalent to 1 γ of vitamin Bc per cc.) the amount of vitamin released in 1 hour at 37°C. is directly proportional to the amount of enzyme present up to a concentration of enzyme sufficient to convert 80% of the conjugate to the free vitamin. When hog kidney conjugase at a concentration of 0.7 unit per cc. is incubated with increasing amounts of vitamin Bc conjugate, half maximum velocity of the enzyme reaction is reached at a concentration of 4.5 γ vitamin Bc conjugate per cc. (8).

The rôle of this enzyme in the intermediary metabolism of vitamin Bc and its functional relationship to hematopoiesis are yet to be explored.

IX. OTHER NUTRITIONAL ANTIANEMIA FACTORS

a. *Vitamins B₁₀ and B₁₁*. Hutchings *et al.* (34) and Mills *et al.* (54) reported that a concentrate of the norit eluate factor prepared from liver contains factors that are essential for the chick. If they are not provided, chicks grow slowly, are anemic and fail to develop normal feathering. The chicks grew more rapidly when they received solubilized liver, from which the norit eluate was prepared, than they did on the norit eluate itself, which indicated the existence of an unidentified factor in solubilized liver. Briggs, Luckey, Elvehjem and Hart (11) prepared a number of fractions from the norit eluate and concluded that it could be partially separated into at least two fractions by fractional precipitation with ethanol. One of the fractions was essential for feather development and was named vitamin B₁₀. The other was required for growth and was named vitamin B₁₁. The various fractions were assayed for folic acid with *S. lactis* R and there was no parallelism between the amount of folic acid in the fractions and their biological activity. The authors concluded that neither vitamin B₁₀ nor B₁₁ could be identified with folic acid. They suggested that folic acid may also be required by the chick but their data did not permit a final decision on that point. These observers (12) continued their attempts to separate vitamins B₁₀ and B₁₁ but did not achieve complete separation. If a fraction produced maximum feathering it at least improved the growth rate; and if a fraction supported the maximum rate of growth it at least produced good feathers. The authors were convinced, however, that though the functions of the two vitamins may overlap, their principal effects are entirely different. It was also observed that there was no correlation between the quantities of folic acid as determined by assay with *S. lactis* R and *L. casei*; and there was no correlation between vitamin B₁₀ and B₁₁ on the one hand, and the amount of folic acid as determined by either method. The authors concluded that the norit eluate contained two folic acids or, in all, at least four biologically active compounds. The chicks on the basal

diet were not severely anemic, but some of the fractions with little folic acid activity by either method of assay, were more effective in preventing anemia than were some of the fractions with higher folic acid activity. The properties of the two vitamins were quite similar. They were relatively insoluble in most organic solvents and they were more stable in alkaline than in strongly acid solution. They were adsorbed most completely in acid solutions and they were precipitated more or less completely by lead, zinc, silver and barium. The Wisconsin group (13) also reported that anemia was not completely prevented by either vitamin B_{10} or B_{11} , and that fractions which were low in vitamin B_c increased the percentage of hemoglobin in the blood. They suggested that an additional factor is required for hemoglobin formation.

The observation of Binkley *et al.* (120) that vitamin B_c may exist as a conjugate with slight microbiological activity, casts grave doubts on the reliability of the folic acid assays as indicating the true amount present. It is not impossible that the activity of both vitamin B_{10} and B_{11} was due to folic acid, or to vitamin B_c , according to the terminology followed. Since estimates of the quantities of vitamin B_c supplied were based on microbiological assay there is a wide degree of uncertainty as to the exact amounts the chicks received. It is entirely possible that practically all of the activity observed in the various fractions was due to the presence of vitamin B_c . However, since the rate of growth by chicks as reported by Campbell *et al.* (14, 15) is inferior to the rate observed when crude vitamin carriers are included in the ration, one would suppose that there is at least one additional unrecognized vitamin.

Luckey, Briggs, Elvehjem and Hart (48) investigated pyridoxal, pyridoxamine and α -pyracin as substitutes for vitamins B_{10} and B_{11} and concluded that they were completely ineffective for both growth and feather development.

b. Factors R and S. It had been shown by Kline, Keenan, Elvehjem and Hart (38) that the antidermatosis vitamin is destroyed by long-continued exposure to dry heat, and Bauernfeind and Norris (4) attempted to determine whether this vitamin is required by the mature fowl as well as by the young growing chick. When chicks consumed the heated diet they grew slowly and developed dermatosis as would be expected. When the heated diet was supplemented with a rice bran filtrate dermatosis was prevented and the weights were improved, but were still subnormal. The authors concluded that an unrecognized vitamin in addition to the antidermatosis vitamin, had been destroyed by the heat treatment. The unrecognized vitamin was present in yeast and liver, was soluble in water, adsorbed on fullers' earth and was not destroyed by heating in an autoclave.

When hens were supplied with the heated diet the hatchability of their

eggs declined almost to zero. When this diet was supplemented with an alcohol precipitate of a water extract of yeast there was a slight increase in hatchability. Schumacher and Heuser (79) had reported that the alcohol precipitate is an excellent source of the new factor. If the heated diet was supplemented with a rice bran filtrate there was again a slight increase in hatchability. If, however, the alcohol precipitate and the rice bran filtrate were both added to the heated diet there was a marked increase in hatchability.

When Schumacher, Heuser and Norris (80) attempted to concentrate the alcohol precipitate factor they decided that it contained at least two factors required by the chick. An acid extract of yeast was treated with 10 volumes of alcohol and the precipitate separated from the filtrate. The factor which remained in solution was designated as Factor R. The factor carried down by the precipitate was designated as Factor S. Factor R could be precipitated, in somewhat higher concentration, by neutralizing the acid filtrate. When the basal diet was supplemented with either Factor R or Factor S there was some increase in weight. When both were included the chicks grew at the normal rate.

Record and Bethke (75) reported that the ration of Schumacher, Heuser and Norris (80) is deficient in choline. When Record and Bethke added either choline or Factor R to their experimental diet the rate of growth of the chicks was increased. If both were added at the same time there was still another increase in the rate of growth. In no case did Factor S give any response in growth.

In subsequent studies Hill, Norris and Heuser (31) improved the original basal diet by the addition of choline. They confirmed the earlier report from the same laboratory, that the rate of growth is not accelerated by Factor S alone. However, practically a maximum rate of growth was obtained by Factor R alone, presumably because it was contaminated with Factor S. Assays for folic acid with *S. lactis* R revealed that there was no correlation between the folic acid content of any of the yeast fractions observed and their growth-promoting activity. It was concluded, therefore, that neither Factor R nor Factor S is identical with folic acid. The chicks on the basal diet of natural feedstuffs were not anemic. These authors also used a basal diet of the synthetic type to impose more rigorous conditions. The chicks on the basal diet were anemic and few survived until the end of the experimental period. When Fraction R was included in the diet the concentration of hemoglobin in the blood was normal but the weights were slightly subnormal. When Fraction S was included the weights were still lower and the chicks were anemic. When both were included at once the chicks were normal in all respects. Although it was concluded that neither Factor R nor Factor S was folic acid, this conclusion

may have been in error since direct microbiological assay is without significance when applied to conjugates. Factor R, and an adsorbate of Factor R, were more effective in supporting growth than they were in preventing anemia. The adsorbate was as effective as Factor R itself in supporting growth, but it was less effective in stimulating hemoglobin formation. The authors concluded, therefore, that Factor R itself is not the antianemia agent. To correlate the observations of Hill *et al.* (31) with those of other workers it is necessary to assume that Factor R is, or contains, vitamin Bc conjugate.

c. *Factor U.* Stokstad and Manning (93) attempted to rear chicks on a semisynthetic diet in which the water-soluble vitamins were supplied by crystalline thiamine, a whey adsorbate and whey filtrate. Chicks grew slowly on this diet but when yeast was included the weights of the chicks were markedly increased, presumably because it supplied a new growth factor in adequate amount. As sources of the new factor yeast could be rated excellent; wheat bran, wheat middlings and alfalfa leaf meal, good; rice bran as fair; corn and molasses as poor. The factor was insoluble in most organic solvents, although it was somewhat soluble in methanol. It was adsorbed on fullers' earth at pH 1, and on charcoal at pH 1 or 8. It was not destroyed by boiling at either a pH of 1.7 or 11.0. The new factor was designated Factor U.

Not long afterwards, it was shown by Stokstad, Manning and Rogers (94) that Factor U was a mixture. Their original basal diets were deficient in pyridoxine as well as in the essential unrecognized vitamins. It may be that the basal diets were also deficient in other recognized vitamins, but it seems probable that the activity of Factor U was chiefly due to vitamin Bc conjugate in the crude yeast fractions.

d. *α - and β -Pyracins.* During the course of attempts to improve the microbiological method of estimating folic acid, Scott *et al.* (82) observed that when pyridoxine was treated with hydrogen peroxide a preparation was obtained which promoted the growth of *L. casei*. A consideration of the probable reactions indicated that the active product was the known lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine (29). When tested, this compound accelerated the growth of *L. casei* in the same way as did pyridoxine after treatment with hydrogen peroxide. The properties of the compound suggested some relation to Factor R and its physiological activity was tested on chicks by adding it to the synthetic diet of Hill and co-workers (31) along with Factor S. The compound had anti-anemia activity and accelerated the growth rate during the first week. This acceleration did not extend beyond the first week, as the growth rate was then retarded because of other deficiencies.

In a later report by Scott, Norris, Heuser and Bruce (81) the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine was named

α -pyracin, and the isomeric 4-carboxy lactone was named β -pyracin. The object of the investigation was to determine the effectiveness of a combination of crystalline *L. casei* factor from a fermentation residue (36) and the pyracins in supporting growth and in preventing anemia. Factor S was included to determine whether it also is required to prevent anemia. When supplied alone, new *L. casei* factor and both of the pyracins were ineffective in hemoglobin formation and in accelerating the growth rate although the new *L. casei* factor did reduce the mortality rate. The combination of new *L. casei* factor with either of the pyracins completely prevented anemia and also accelerated the growth rate. β -Pyracin was more effective than was α -pyracin in improving the growth rate although the rate was still subnormal. It was suggested by the authors that the failure to grow normally was due to lack of Factor R. Briggs, Luckey, Elvehjem and Hart (13, 48) reported that chicks on their rations gave no response to the 5-carboxy lactone in growth, or in hemoglobin or feather formation. Campbell, Brown and Emmett (16) were unable to attribute any complementary action to β -pyracin in preventing anemia or poor growth when fed in conjunction with crystalline vitamin Bc to growing chicks.

e. *Extrinsic Factor.* Castle and his associates (18) recently tested all known members of the vitamin B complex for extrinsic factor activity in patients with pernicious anemia. Only negative results were obtained but they are of particular interest since among the factors tested was a folic acid concentrate and crystalline folic acid, both prepared from a fermentation residue (36). Folic acid here refers to the new *L. casei* factor which is probably not a constituent of spinach and which, therefore, would not be present in the folic acid concentrates of Mitchell, Snell and Williams (63). Castle *et al.* (18) direct attention to negative results obtained by other investigators for extrinsic factor activity in pernicious anemia with folic acid concentrates, but the natural source of the concentrates is not stated. As different compounds may be involved the negative observations of one group may not necessarily be confirmatory of those of another.

X. VITAMIN M AND THE POTENTIAL *STREPTOCOCCUS LACTIS* R STIMULATING FACTOR

Wills and Stewart (117) produced a macrocytic hyperchromic anemia in monkeys by feeding a diet, similar to one in common use among the poorer classes in Bombay, made up of polished rice 40, margarine 15, white bread 45, salt, cod liver oil, 3 cc. per animal, and tomato or carrot, 25 g. per animal. Later on each monkey was given 0.5 g. daily of iron and ammonium citrate. As the anemia developed the animals "lost weight, became inactive, tended to walk and sit with feet tightly clenched, and lost the hair from the tail." In later stages there was definite edema apparently related to a vitamin B deficiency. The number of red blood cells decreased to approximately one-

third the normal number, the hemoglobin decreased to about one-half the normal level, and the color index increased by about 20%. The number of white blood cells decreased to about one-third of the normal number. The condition was cured by marmite.

In a continuation of the investigation, Wills, Clutterbuck and Evans reported that none of the animals became anemic under 3 months, and a few required over a year. The anemia was cured by liver extract, administered orally or parenterally, but it was not cured by the liver fraction used to treat pernicious anemia. The distribution of the anemia-preventive factor in yeast, marmite, wheat germ, liver, and the fact that it was water-soluble, suggest that it is related to the vitamin B-complex.

Somewhat similar studies on men and women with a typical macrocytic anemia were described by Wills and Evans (116). Various extracts were administered with about the same response as had been previously reported for monkeys. The liver extracts prepared for the treatment of pernicious anemia were ineffective but crude liver or autolyzed yeast extracts were effective. It was concluded that the active agent was probably identical with the one which prevents nutritional macrocytic anemia of monkeys. It was not identical with any of the vitamins that had been recognized at that time.

A syndrome that was probably identical with the one described by Wills and collaborators was produced by Day, Langston and Shukers (24) who gave monkeys a diet similar to one used to produce cataract in rats. The animals did not develop cataract but they died of a nutritional disease characterized by anemia, leucopenia, gingivitis and diarrhea. Normal monkeys have from 4.5 to 6.0 million erythrocytes and 10 to 30 thousand white cells per cu. mm. Some of the experimental animals had only about 20% of the normal number of red cells and 10% of the normal number of white cells. The gingivitis was the only consistent physical finding and the average survival period on the deficient diet was 56 days. Since the deficiency was prevented by dried brewer's yeast, it was concluded that the syndrome was due to the lack of a member of the vitamin B complex. According to Langston, Darby, Shukers and Day (46) the syndrome is also prevented by Cohn's liver extract Fraction G. The same deficiency developed when monkeys consumed the Goldberger diet, and it likewise was prevented by a liver extract (23). Day, Langston and Darby (22) demonstrated that nicotinic acid did not prevent the blood dyscrasia and that it did not prevent the gum ulceration. The factor that prevents the nutritional cytopenia was designated vitamin M. Topping and Fraser (97) and Tomlinson (96) gave monkeys a diet very similar to the modified Goldberger diet used by Day and collaborators and observed about the same type of symptoms. Wilson, Doan, Saslaw and Schwab (118) gave monkeys

a modified Goldberger diet and also a synthetic diet. They developed leucopenia on both. Some of the monkeys were moderately anemic but the degree of anemia was inconsistent and not parallel with the degree of leucopenia. A normal white cell equilibrium was restored by intramuscular injection of a folic acid concentrate. This concentrate was prepared from yeast autolysate by the method of Hutchings *et al.* (35) and probably contained a significant amount of vitamin Bc conjugate. Waisman and Elvehjem (104) confirmed Wilson *et al.* (118) as to the effectiveness of a folic acid concentrate (norit eluate of liver prepared by the method of Hutchings *et al.* (35)). Monkeys which received their synthetic diet lost weight and there was a sharp decline in the number of leucocytes. When the folic acid concentrate was added to the diet the weights and the white cell counts became normal.

Totter *et al.* (101) observed that there was no correlation between vitamin M activity in the monkey and folic acid activity in a number of vitamin M-rich materials, particularly dried brewer's yeast. Five g. of dried yeast when fed daily prevented nutritional cytopenia and contained 21 γ of folic acid by assay with *S. lactis* R. A monkey which received 3 g. of dried liver powder daily was not protected, although the powder contained over 7 times as much folic acid as did the 5 g. of yeast. This comparison indicates that the amount of folic acid measured by *S. lactis* R accounts for only a small part of the activity of vitamin M. In view of later work, it is certain that the assay for folic acid gave a result that was much too low. Totter, Mims and Day (100) found that, on incubation of yeast with fresh rat liver, they were able to markedly increase the folic acid content as measured by growth stimulation of *S. lactis* R. They interpreted the data in terms of a synthesis of folic acid from precursors in yeast. Later these workers (57) prepared a crude enzyme fraction from rat liver which formed folic acid from yeast or yeast extracts. Since there was no evidence that the microbiological growth stimulant was chemically the same as folic acid, these workers referred to the growth factor which is formed as the *S. lactis* R stimulating factor and the compound(s) from which it is formed as potential *S. lactis* R stimulating factor. They succeeded in concentrating the latter factor about 50 times by adsorption on norit or superfiltrol at pH 3 to 4 and eluting with 2% ammonia water or pyridine-alcohol-water mixtures. Further purification was effected by fractionating the concentrated eluates with ethyl alcohol. Such concentrates were active in preventing the leucopenia and granulocytopenia in rats fed a purified diet containing 1% succinylsulfathiazole. The biological activity in the rat paralleled the potential *S. lactis* R stimulating factor content. These results, taken in conjunction with the findings of other observers, prompted Mallory *et al.* (50) to suggest the identity or close relationship of vitamin M,

potential *S. lactis* R stimulating factor and vitamin Bc conjugate. The isolation of vitamin Bc conjugate in crystalline form from yeast, its low micro-biological growth activity, its ready conversion enzymatically to vitamin Bc, and its activity in correcting the anemia and leucopenia in deficient monkeys (26), support this view.

Day and others (25) tested the effectiveness of a highly purified preparation of the new *L. casei* factor from a fermentation residue (36) in cytopenic monkeys. The white blood cell and granulocyte counts returned to normal levels within a few days after intramuscular injections of 4 or 4.5 mg.

XI. RELATION OF SULFA DRUGS TO NUTRITIONAL RÔLE OF THE NEWER HEMATOPOIETIC FACTORS

The discussion of this topic will be somewhat abridged as Sebrell (83) has recently published an excellent review. Marshall and his associates (51) announced that sulfaguanidine reduced the concentration of coliform bacteria in the feces of mice. The more recent literature in this field was reviewed by Miller (53). Black, McKibbin and Elvehjem (9) explored the possibility that the synthesis of vitamins by intestinal bacteria could be inhibited by the feeding of sulfaguanidine. They gave rats a synthetic diet which contained sulfaguanidine at levels of 0.5 to 2.0%. A considerable proportion of the animals which received the higher drug dosage died. When 0.5% of the drug was added to the basal diet the rats gained one-third as rapidly in weight as did the controls. If 0.5% of the drug and 0.3% of a liver extract were both added to the basal diet the rats grew at a normal rate. It was suggested by the authors that the synthesis of unrecognized essential vitamins by intestinal bacteria was inhibited by sulfaguanidine, and that these vitamins were present in the liver extract. Later investigations have lent some support to this point of view though it does not explain all the facts which have been reported. Mackenzie, Mackenzie and McCollum (49) independently carried out a similar study. They gave rats a synthetic diet which contained 1 or 2% of sulfaguanidine. Bleeding developed from the anterior corner of the eye, which later involved the whole eye. The symptom was prevented by *p*-aminobenzoic acid in rats which received 1% sulfaguanidine, but not in those receiving 2%. It was always prevented by yeast. The thyroid glands were hyperemic and 3 or 4 times larger than the glands of animals which did not receive sulfaguanidine. Another important observation was contributed by Spicer, Daft, Sebrell and Ashburn (88). Rats which received sulfaguanidine or succinylsulfathiazole, grew at almost a normal rate the first week and then the rate declined until there was practically no growth after the third week. Leucopenia and agranulocytosis developed consistently and anemia was observed in some cases. The abnormalities were prevented, or cured, by whole

liver and by liver extracts. Daft, Ashburn and Sebrell (20) observed that some of the rats were anemic. Nielsen and Elvehjem (65) and Martin (52) reported that folic acid (norit eluate factor from liver) counteracts growth inhibition of succinylsulfathiazole. These observations are in accord with the finding of Mitchell and Isbell (60) on the synthesis of folic acid by rat intestinal flora.

It was observed by Kornberg, Daft and Sebrell (39) in a comparison of sulfathiazole, sulfadiazine and sulfamilamide, that the incidence of blood dyscrasias was lower in the sulfamilamide group than in either of the others. The incidence of severe granulocytopenia was higher, and the incidence of severe anemia was lower, in the rats that received sulfathiazole than in those that received sulfadiazine. Oral treatment with liver extracts corrected the granulocytopenia in four days and the anemia in ten days although the diet still contained the sulfa drugs. According to Daft and Sebrell (21) the granulocytopenia and leucopenia previously described can be cured by either crystalline vitamin Bc or crystalline *L. casei* factor.

It was reported by Kornberg, Daft and Sebrell (40) that a small percentage of rats which received synthetic diets developed granulocytopenia. The condition was corrected promptly by four oral administrations of 25 γ of crystalline *L. casei* factor for each of four days. Axelrod and co-workers (3) had previously observed anemia in a large proportion of rats which received the basal diet with no added sulfa drugs. It may be that at least some of the symptoms described by Gyorgy, Goldblatt, Miller and Fulton (28) had a similar etiology, though the syndrome may have been complicated by a deficiency of vitamins not available when their work was done. The animals developed low platelet, white blood cell and red blood cell counts, granulocytopenia and a low percentage of hemoglobin.

Mallory, Mims, Totter and Day (50) gave rats a synthetic diet to which 1% of succinylsulfathiazole had been added. The rats grew slowly and the number of white blood cells and of granulocytes was reduced. A supplement of yeast extract, which contains little preformed *S. lactis* R stimulating factor, was more effective in stimulating the growth rate and in maintaining normal leucocyte and granulocyte counts than was a liver extract which contained 15 times as much of the preformed factor. In contrast with this, when the dosage was properly chosen there was satisfactory correlation between the quantity supplied of potential *S. lactis* R stimulating factor, on the one hand, and the growth rate and white cell and granulocyte counts on the other. The type of yeast extract employed in these studies would be expected to contain significant quantities of vitamin Bc conjugate.

Axelrod, Bosse, Gross and Gregg (2) included 1% and 0.5% sulfaguanidine, and 0.5% of sulfasuxidine in their experimental diet. As has been described previously, growth was at first retarded, then inhibited, and the

animals developed leucopenia. The combination of biotin and *p*-amino-benzoic acid did not stimulate the rate of growth in rats that received 1% of sulfaguanidine but it did elicit variable growth responses in animals that received 0.5% of sulfasuxidine. There was also a variable and delayed leucocyte response in rats that received either 1% of sulfaguanidine or 0.5% of sulfasuxidine. There was an immediate and sustained growth response in rats that received 0.5% sulfaguanidine when treated with the norit eluate factor from liver plus biotin, but this treatment gave only a temporary response in rats that received 1% of the drug. When the rats received 1% of sulfaguanidine the lymphocyte response was gradual but was immediate in those that received 0.5% of sulfaguanidine or sulfasuxidine. The granulocyte response was prompt at all levels of the sulfa drugs. Methylacetamide had consistent granulopoietic activity when supplied in daily doses of 40 and 80 mg., but it did not stimulate the formation of lymphocytes and it did not elicit a response in growth.

It was shown by Higgins (30) that rats on synthetic diets developed hypochromic anemia if they were given promin or promizole. Vitamin Bc concentrate was administered in daily doses of 80 γ of vitamin Bc by both the curative and prophylactic procedures and displayed a marked anti-anemic effect.

An unexpected observation was made by Michaud, Maass, Ruegamer and Elvehjem (122) that dogs are not adversely affected by the addition of succinylsulfathiazole to their diets. The amount of added succinylsulfathiazole varied from 0.5 to 2.0%, and the amount of hemoglobin in the blood was reduced to 6% by bleeding in order to obtain a more definite measure of the rate of regeneration. Surprisingly enough the normal hemoglobin level was restored as rapidly when succinylsulfathiazole was included in the diet as when it was omitted. Kornberg, Tabor and Sebrell (41) applied a bleeding technique to rats fed succinylsulfathiazole and succeeded in producing a severe anemia. The new *L. casei* factor from a fermentation residue (36) was found to have both a preventive and corrective action on the hemorrhagic anemia.

In 1942 Williams, Cheldelin and Mitchell (110) reported that milk had a very low content of folic acid. This observation prompted Welch and Wright (105) to design a folic acid-low diet for the rat using whole milk powder as a base. They and their co-workers (120) reported that rats which consumed 20% of succinylsulfathiazole in a diet of dried milk had lowered leucocyte counts and a decreased percentage of circulating neutrophiles. If the succinylsulfathiazole made up 10% of the ration the animals had a borderline deficiency of folic acid. If the amount of the drug was less than 10% there was no evidence of blood dyscrasia in any of the animals, and the folic acid content of the organs of these animals was high compared

to controls on a purified diet in which casein, sucrose and corn oil were used. On the basis of these data Welch and Wright (105) suggested the occurrence in milk of a factor or factors which could be utilized by the rat in lieu of folic acid. They later demonstrated (120) a 20- to 50-fold increase in the *S. lactis* R growth activity of milk powder following digestion with the rat liver enzyme preparation of Mims, Totter and Day (57) which forms *S. lactis* R stimulating factor from yeast concentrates.

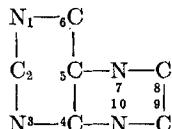
The results of Welch and Wright are explainable on the known properties of crystalline vitamin Bc conjugate. Whether vitamin Bc conjugate itself or some closely related compound exists in milk is an unsolved problem. Its isolation from milk would be the only direct answer. As in the case of liver, the isolation of the conjugated microbiological growth factor from milk and a demonstration of its chemical relation to crystalline vitamin Bc conjugate from yeast would have considerable metabolic significance.

XII. XANTHOPTERINE

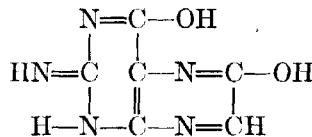
During the past ten years xanthopterine has held a controversial position in relation to its possible rôle in hematopoiesis. More recently nutritional experiments have been reported which have been interpreted to indicate a close biological and chemical relationship to folic acid, vitamin M and vitamin Bc. The fact that crystalline vitamin Bc was shown to be a yellow compound may have tended to stimulate more recent speculation.

In 1889 Hopkins (33) called attention to the occurrence of a yellow pigment in the wing scales of butterflies of the class *Pieridae*. He obtained the water-soluble pigment as a yellow powder. A study of its properties prompted him to remark on its possible chemical relationship to uric acid. Wieland and Schöpf (107) in 1925 prepared the yellow pigment in crystalline form and named the compound xanthopterine. Pterine³ was suggested by them as a class name for the pigments of butterfly wings. In a long series of painstaking researches extending over a period of 15 years

³ The spelling xanthopterin and pterin have been used widely in English speaking countries. In keeping with suggested rules of nomenclature of the American Chemical Society (*J. Am. Chem. Soc.* 50, 3074 (1928)) the ending -ine is preferred, namely, xanthopterine, pterine, pteridine, the last term being a trivial name for the ring system



Wieland and his students (106, 108, 73) proved by degradation and synthesis that xanthopterine has the following structure.



Improved methods of synthesis have since been reported (44, 93). An excellent review by Schöpf (78) on the chemistry of the pterines has appeared recently. In 1936 Koschara (42) isolated a yellow pigment from normal human urine. It occurred in a concentration of about 1:1,000,000. He found that the pigment had many properties in common with xanthopterine from butterfly wings but, in view of certain analytical differences, the identity was not firmly established. He named the compound from urine uropterine. Seven years later Koschara (44) proved that uropterine is in fact xanthopterine.

Tschesche and Wolf (102) reported in 1936 that xanthopterine from human urine caused a striking increase in the red blood cell count of rats which had been rendered anemic on a goat's milk diet. It required only a few γ per rat per day. The minute doses of xanthopterine employed seemed to rule out the possibility of copper as a contaminant. The following year these same workers (103) reported that not only xanthopterine was active in the anemic rat but that positive results were also obtained with leucopterine, erythropterine, guanopterine (which has since been shown to be isoguanine (72)) and tyrosine. Tschesche and Wolf used the animal test devised by Rominger and Bomskov (76). Rominger (77) tried to check the results of Tschesche and Wolf with the same sample of xanthopterine the latter workers used. He obtained only negative results.

In 1941 Simmons and E. R. Norris (84) reported that xanthopterine, either natural (prepared from liver) or synthetic, caused a striking increase in the erythrocyte count of young anemic salmon. They used relatively large doses (10 to 40 mg. per kilo of body weight) (68). Xanthopterine was found by Totter and Day (99) to stimulate growth and to correct, at least partially, the leucopenia in rats on a succinylsulfathiazole diet, but they were unable to duplicate these findings (100) and only negative results were reported from several other laboratories (3, 121, 21, 74). O'Dell and Hogan (69) found it entirely inactive in correcting the anemia of vitamin Bc deficient chicks. However, positive results have been obtained with the monkey. Totter *et al.* (101) reported that xanthopterine caused a reticulocyte response in vitamin M deficient monkeys, that it gave rise to increases in red and white blood cell counts, and that it delayed the onset of nutritional cytopenia. Castle and co-workers (18) tested xanthopterine for its

possible extrinsic factor activity in pernicious anemia with negative results.

In 1943 Wright and Welch (121) presented data on the production of folic acid (growth stimulation of *S. lactis* R) by digestion of rat liver with human urine. Urine itself contained only a trace of folic acid activity. They succeeded in concentrating the urine principle(s) 2000 times. The behavior of the urine principle in concentration methods suggested to these workers its possible identity with, or close relationship to, xanthopterine. Synthetic xanthopterine when digested with rat liver also formed folic acid. Totter, Mims and Day (100) and Stokes, Keresztesy and Foster (90) confirmed the production of folic acid on digesting xanthopterine with rat liver suspensions. Subsequently Wright *et al.* (119) discussed the complexity of the reactions involved in the production of folic acid from rat liver without added xanthopterine. Although they reaffirmed their view that rat liver produces folic acid from xanthopterine *in vitro* the results do not force the conclusion. It is still an open question as to how much of the production of folic acid was due to the autolytic release of vitamin Bc from vitamin Bc conjugate. In this connection it is of interest that Mims, Totter and Day (57) were unable to produce *S. lactis* R stimulating factor from xanthopterine with a partially purified but still crude rat liver enzyme preparation. Positive results were obtained only with liver "brei" which contains variable amounts of vitamin Bc conjugate.

E. R. Norris (67) found that folic acid concentrates from spinach were active when tested for antianemia activity on trout but these results are difficult to interpret since Mitchell (58) reported that the concentrates may have contained several *per cent* of xanthopterine.⁴ On the other hand Mitchell (59) accepted a comparison of the ultraviolet absorption properties of folic acid concentrates and impure xanthopterine (50–60% pure) as evidence for the presence of a structural unit very similar to xanthopterine in the folic acid molecule. A set of comparative curves as recorded by Mitchell is reproduced in Fig. 3. Bloom *et al.* (10) compared the ultraviolet absorption properties of crystalline vitamin Bc with those of pure xanthopterine. These workers concluded from their data that a pyrimidopyrazine ring structure may be present in vitamin Bc. They pointed out that flavins, alloxazines and pterines have similarities in their ultraviolet absorption properties and that the striking similarity in the ultraviolet absorption properties of folic acid concentrates and impure xanthopterine may be fortuitous. Some striking differences in the ultraviolet absorption properties of xanthopterine and vitamin Bc are apparent from a study of Figs. 4 and 5.

⁴ Koschra (43) however was unable to detect the presence of xanthopterine in green grass.

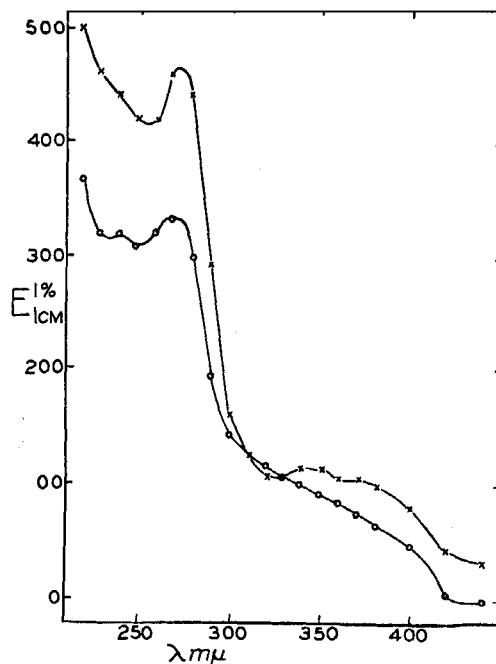


Fig. 3

A Comparison of the Ultraviolet Absorption Curves of Xanthopterine and Folic Acid from Spinach. (Reproduced from H. K. Mitchell, *J. Am. Chem. Soc.* **66**, 276 (1944), Fig. 4. — — — — —, folic acid, potency 63,000; x — x — x, xanthopterine). The pH at which the determinations were made was not indicated.

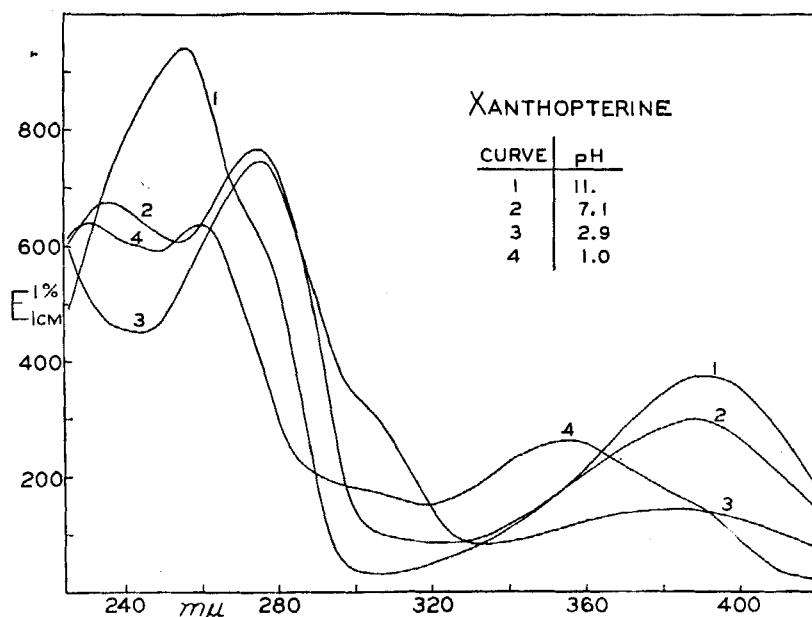


Fig. 4

Ultraviolet Absorption Curves of Xanthopterine at Several pH Levels. Reproduced from E. S. Bloom, J. M. Vandenbelt, S. B. Binkley, B. L. O'Dell and J. J. Pfiffner, *Science* **100**, 295 (1944)

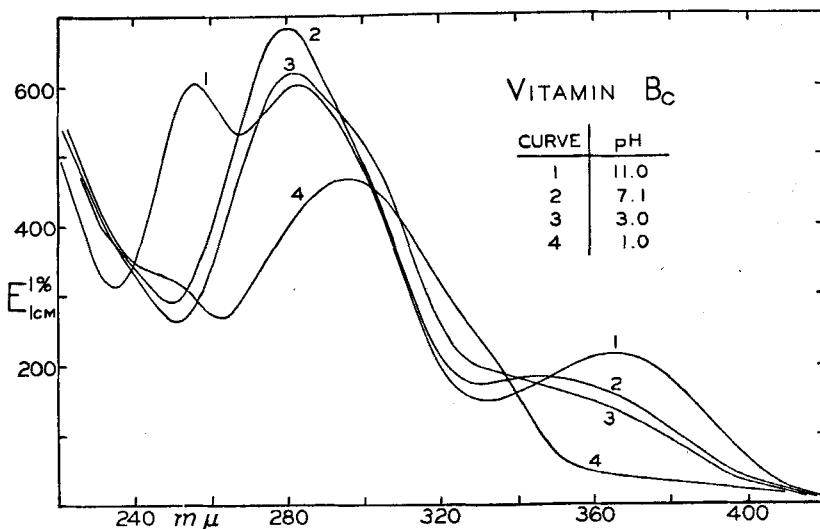


Fig. 5

Ultraviolet Absorption Curves of Vitamin Bc at Several PH Levels. Reproduced from E. S. Bloom, J. M. Vandenbelt, S. B. Binkley, B. L. O'Dell and J. J. Pfiffner, *Science* 100, 295 (1944).

XIII. SUMMARY

The subject reviewed in the foregoing pages is at a stage of investigation where few final conclusions can be drawn concerning the chemical identity of the numerous nutritional factors having hematopoietic activity in the chick, rat and monkey. Five crystalline products have been isolated. They are (1) vitamin Bc, (2) *L. casei* factor from liver, (3) *L. casei* factor from yeast, (4) new *L. casei* factor from a fermentation residue, and (5) vitamin Bc conjugate. Crystalline vitamin Bc and the three *L. casei* factors are powerful growth factors for *L. casei*. Crystalline vitamin Bc conjugate which is almost devoid of growth effect on *L. casei* releases vitamin Bc on digestion with a specific enzyme.

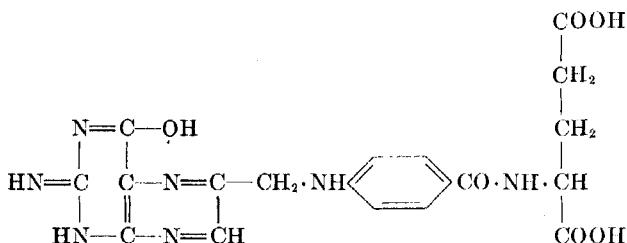
It appears extremely probable that Stokstad's *L. casei* factors from liver and yeast are identical with vitamin Bc, although Stokstad, himself, concluded that the yeast compound differed from the liver compound on the basis of microbiological assay. The major physiologically active component of folic acid is probably identical with or chemically very closely related to vitamin Bc. The crystalline new *L. casei* factor is not identical with any other recognized hematopoietic factor. On the other hand, crystalline vitamin Bc conjugate may be identical with several nutritional factors which have been recognized as having hematopoietic activity but little microbiological growth activity. They are vitamin M, vitamins B₁₀

and B_{11} and Factors R and S. There may be a specific feather factor for the chick as postulated by Briggs, Luckey, Elvenjem and Hart (11), but the antianemic activity of concentrates of vitamin B_{10} (the feather factor) is probably due to vitamin Bc conjugate.

A fourth compound, the *S. lactis* R factor (SLR factor), although not active in prevention of rat leucopenia and stimulating only slightly the growth of *L. casei*, is of direct concern in the general problem because of its ready biological conversion to a factor or factors stimulatory to *L. casei* growth. It is not known, however, whether the substance(s) formed has antianemic activity.

There is not sufficient chemical evidence available to justify a broad generalization, but it would seem reasonable to assume that the hematopoietically active compounds have a common structural unit. The successful degradation and synthesis of *L. casei* factor from liver has been announced but no chemical details are available.

Addendum. July 26, 1946. Angier *et al* (Science, **103**, 667 (1946)) have recently disclosed the results of their degradative and synthetic studies on the liver *L. casei* factor. The compound has the structure



N-[4-[(2-amino-4-hydroxy-6-pteridyl)-methyl] amino] benzoyl]-glutamic acid

They suggested the name pteroylglutamic acid. A comparison of synthetic *L. casei* factor supplied by Angier *et al* with vitamin Bc isolated from liver and yeast showed them to be identical (Pfiffner *et al*, J. Am. Chem. Soc. **68**, 1392 (1946)). In contrast to the liver *L. casei* factor which yields one mole of glutamic acid Angier *et al* (Science, **103**, 667 (1946)) found that the fermentation *L. casei* factor yields 3 moles while vitamin Bc conjugate was reported by Pfiffner *et al* (J. Am. Chem. Soc. **68**, 1392 (1946)) to yield 7 moles of glutamic acid. Assuming normal peptide linkages in the fermentation *L. casei* factor and vitamin Bc conjugate these two compounds may be designated respectively pteroyldiglutamylglutamic acid and pteroylhexaglutamylglutamic acid. The conjugase enzymes do not hydrolyze the methyl ester of pteroylhexaglutamylglutamic acid and hence can be classified as carboxypeptidases. The identification of the conjugase enzymes as carboxypeptidases is of particular interest since most of the pteroylglutamic acid in natural foodstuffs occurs in conjugated form. Numerous clinical studies have demonstrated the beneficial effect of pteroylglutamic acid in Addisonian pernicious anemia, nutritional macrocytic anemia, macrocytic anemia of sprue, of pellagra and of pregnancy (for literature citations

see L. J. Berry and T. D. Spies, *Blood* **1**, 271 (1946)). Bethell *et al* (Univ. Mich. Hosp. Bull. **12**, 42 (1946)) and Welch and Heinle (private communication) however observed an impaired ability to utilize the yeast conjugate in pernicious anemia patients in relapse. In normal subjects a markedly increased urinary excretion of free vitamin followed administration of the yeast conjugate while in pernicious anemia patients only a slight increase was observed. On the contrary, Sharp *et al* (Proc. Central Soc. for Clin. Res., Nov. (1943) in *J. Am. Med. Assoc.*, **124**, 734 (1944)) found an increased urinary excretion of free vitamin after oral administration of yeast conjugate in pernicious anemia and more recently (personal communication) has observed as high concentrations as 4.960 mg. of free vitamin per 24 hour urine following the daily ingestion of 28 mg. of yeast conjugate in a case of severely relapsed pernicious anemia. These clinical observations focus attention on the relationship of the folic acid group of compounds to the extrinsic food factor of Castle and the anti-pernicious anemia active substances in stomach and liver tissue.

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Nutrition and Resistance to Infection

The Strategic Situation

By HOWARD A. SCHNEIDER

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I. INTRODUCTION AND DEFINITIONS

"General impressions are never to be trusted. Unfortunately when they are of long standing they become fixed rules of life, and assume a prescriptive right not to be questioned. Consequently those who are not accustomed to original inquiry entertain a hatred and horror of statistics. They cannot endure the idea of submitting their sacred impressions to cold-blooded verification. But it is the triumph of scientific men to rise superior to such superstitions, to desire tests by which the value of beliefs may be ascertained, and to feel sufficiently masters of themselves to discard contemptuously whatever may be found to be untrue."

—FRANCIS GALTON.

To declare that it is one's purpose to deal with the relationship between nutrition and resistance to infection is to draw attention once again to a relationship made conspicuous more by reiteration than by demonstration. Like a cynical political doctrine, the phrase "nutrition and resistance to infection" means many things to many men, and its fascination is similarly perennial. The notion, so readily entertained, that there exists in fact a relationship between nutrition and resistance to infection, is probable because the whole thing sounds so much like "common sense." That there is also a mite of wishful thinking included may be inferred from the use of the word "resistance," with its happy connotations for man, and the

complete neglect of the equally logical word "susceptibility." A cloak of respectability is also provided for these notions by a history extending back to the origins of medicine, including the Hippocratic writings. But the search in medical history for prophetic intimations of the link between diet and infection, although a fascinating study, need not detain us here.¹

If the wishes of the reader are to be anticipated correctly, what we will need to examine here is our ability to document, in terms of modern information, first, the *fact* of the influence of diet on resistance to infection, and second, the nature of the nutritional factors which are effective. That we begin so pessimistically is due to no more than what the critical reader will have expected if he has dipped at all into the voluminous and controversial literature in this field. Where there is a babble of voices there may be only nonsense—or there may be an obscured truth.

A word or two might be offered in explanation of the addition of "The Strategic Situation" to the title of this chapter. This was done to indicate the nature of the analysis which it is intended to pursue. When a field in science has been thoroughly defined and its aims and methods have been made clear to all, then a periodic review of the progress in this field will be in the nature of a résumé of "tactical" successes and failures. The goal is clearly defined, the field of action is commonly understood and agreed upon and the reviewer notes the progress of the attack. Most of the "reviews" of the present day fall into this category. But from time to time new relationships between recognized fields of science appear and in these new areas, initially, there must be an examination of the "strategic" situation. Understanding must be reached as to the nature of the phenomena which are the proper subject of study in the new field and, in general, as to what disciplines might rationally be admitted to effective use. It appears that in the early days of such a new field a "strategic" review might also serve a good purpose. Since the science of modern nutrition is still young, and since fundamental concepts of infection are still undergoing revision, it seems doubly necessary, as a beginning, to survey the "strategic situation" in the field of nutrition and resistance to infection.

Although it may be poor policy to begin with a threat of tedium, there seems no escape from the necessity of starting with definitions,² for, as the King of Hearts recommended in "Alice in Wonderland," it is well to "Begin at the beginning and go on till you come to the end: then stop."

¹ Aycock and Lutman (1), in a recent review of vitamin deficiency as an epidemiological principle, put forward two interesting views on the origin of the idea that dietary deficiency is a factor in resistance to infection. One is the long association between famine and pestilence; the other, the association between vitamin A deficiency and xerophthalmia (not an infectious process *per se*, but oft-times quickly followed by secondary "infection").

² The reader will be more charitable, perhaps, if he will recall that among his fellows there may well be specialists in other fields who will find some of these definitions, while not profound, at least reassuring.

The word "nutrition" has taken on an added meaning ever since the days of Lunin, Funk and Hopkins and it will certainly not be pursuing a novel course if, when the word is used here, it is meant to include primarily the biochemical aspects, qualitative and quantitative, of an "adequate" diet.³

By "infection" we mean the process of interaction of two living entities, the infectious agent (bacterium, virus, protozoon, helminth, fungus, etc.) and the host (*Vide Zinsser, Enders and Fothergill (3)*).

The observable effects of this interaction between host and infectious agent are termed "infectious disease" and several terms are commonly employed to describe the *degree* of this interaction. Thus, if the interaction does not occur, although the infectious agent has been presented to the host, the latter is described as "resistant," or better, "insusceptible." Used in this way, the terms have an absolute meaning. In the same circumstances, the infectious agent would be designated as "avirulent." When interaction does occur and is so extensive that the host dies, no one will quarrel with us if we then designate the host as "susceptible" and the infectious agent as "virulent." When employed in this connection, the words "susceptible" and "virulent" also have an absolute meaning. But very often, indeed most of the time, these terms will have a relative meaning, *i.e.*, hosts will be "more resistant" or "less resistant," and infectious agents will be "more virulent" or "less virulent." The interaction of host and infectious agent is capable of producing a whole spectrum of phenomena which can be represented schematically as in Fig. 1.⁴

It is at once obvious that in attempting to apply the terms "resistant" or "susceptible" to a host, represented at "D" in Fig. 1, a point of reference is necessary. Thus a host described at "D" would be more resistant than

³ This amounts to a statement of the main "strategic" aim of modern nutrition. The goal is described in terms of normal physiology; the "tactics" are the methods of chemistry and biochemistry. McCollum, Orent-Keiles and Day (2), in examining the present concept of nutrition, state: ". . . in recent decades the principal emphasis has been on the discovery of indispensable nutrients, or the primary components of an adequate diet. We now regard an adequate diet as composed qualitatively of many chemically discrete components provided in such states of combination that they are utilized efficiently. Moreover, the adequate diet must contain these essentials in approximately the proportions required by the body in order to promote optimum efficiency and the prolongation of physiological well-being. Also, the adequate diet must contain a minimum of injurious factors, *e.g.*, selenium and fluorine. Since the necessary experimental procedures are well-defined, it appears probable that the next one or two decades will witness an essentially complete solution of the present major nutritional problem. This is the discovery of all the indispensable nutrients for man and some of the domestic animals."

⁴ Since we are here mainly concerned with phenomena observable in the host, Fig. 1 has been constructed in terms of the host reaction. Another similar spectrum can be constructed, of course, from the standpoint of attributes of the infectious agent, *i.e.*, a "virulent-avirulent" spectrum.

hosts described at "E," "F" or "G," but would just as correctly be termed as more susceptible than hosts described at "C," "B" or "A." When we speak of "nutrition and resistance to infection," therefore, we really mean "nutrition and resistance-susceptibility to infection."

Now, although Fig. 1 may be of use in presenting the aspect of interaction of host and infectious agent in theory, this spectrum is not of practical utility at present. There are two reasons for this. (1) There is at this time no truly objective basis which experimenters agree is of use in measuring *degree* of illness.⁵ (2) In many types of infection, particularly if the infectious agent is a "natural" one, it is the experimenter's frequent experience to find that laboratory animals vary a great deal in degree of re-

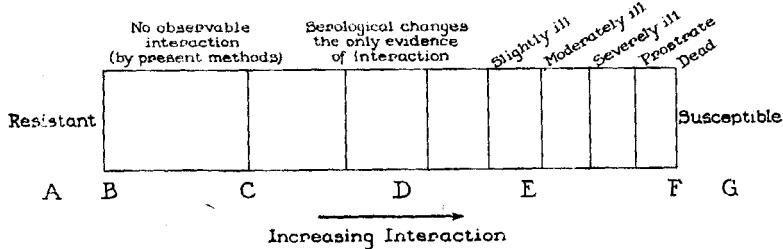


Fig. 1

sponse to infection. But there is one result of infection upon which all investigators can more easily agree, namely, the death of the host. Consequently most of the intelligible, and reproducible, data in the field of infection are stated in terms of mortality and not morbidity. Differences in host response to infection are usually reported in one or the other of two forms. Host populations are compared, for equal doses of the infectious agent, in terms of *per cent* mortality (or survival);⁶ or some percentage of

⁶ Horsfall (4, p. 212) has attempted the quantification of response of mice to influenza virus by grading the amount of pulmonary consolidation observed at necropsy. His method appears to be equally as valid as traditional methods based on mortality. Zinsser, Castaneda and Seastone (5), working with murine typhus in rats and guinea pigs, considered the increase in numbers of Rickettsiae recoverable from the peritoneum and tunica as an index of increased susceptibility. Special features of some infections provide still other indices. Thus the percentage of parasitized erythrocytes is commonly used as a criterion in studies of malaria.

⁶ English experimental epidemiologists (6) have made use of the life-table method in evaluating the effectiveness of experimental procedures, including diet, upon herds of mice under risk of infection. For practical reasons, these workers limited the expectation of life, \bar{e}_x , to a "cage life" of 60 days, using the notation ${}_{{}_{60}}E_x$. The method has several advantages, but it has not been generally used. The reader is referred to the monograph of these authors (6).

mortality is chosen, usually either 50% or 100%, and the host population described in terms of the dose of infectious agent necessary to achieve the chosen percentage of mortality, *i.e.*, the population is "titrated" to a given "end-point." These are the pragmatic bases of measuring resistance-susceptibility.

One other pertinent aspect of resistance to infection remains to be examined before we can proceed to consider the influence of nutrition. This aspect deals with the biological bases underlying the resistance phenomenon. Thus, resistance may be either (1) innate, or (2) acquired. Innate resistance is a specific biological attribute of a host and is a consequence of the specific genetic constitution of the host that exhibits it. This kind of resistance is independent of any previous experience the host may have had with an infectious agent and is revealed the first time the host is exposed to a given infection. Innate (genetic) resistance is responsible for the wide differences in resistance to various infectious agents displayed by different animal species and is probably the dominant biological factor of practical importance controlling the dissemination of infectious disease between different animal species. In addition to the divergence between species, differences in innate resistance also obtain within a species. These intra-specific differences in innate resistance occur in laboratory animals such as the rat (7) and the mouse (8), and they may be of such magnitude that an experimenter cannot afford to ignore them. Moreover, these intraspecific differences in innate resistance are not of interest solely in the laboratory for there is a growing appreciation that they exist in man as well, and have been found whenever a systematic analysis has been performed. Thus, Puffer (9), in her monograph on susceptibility to tuberculosis, is able to present in summary form the argument of the importance of innate (genetic) factors for infectious disease of such diverse etiology as leprosy, rheumatic fever, poliomyelitis and tuberculosis.

This innate (genetic) resistance, in which immunologic reactions apparently play no part, is sometimes termed "natural resistance" in both the intra- and interspecific forms.

The second biological basis of resistance to infection is that of acquired resistance, or acquired immunity. Here resistance follows as a result of a primary experience with an infectious agent or some active fraction thereof. As a consequence of this experience, the host responds by the production of antibodies whose presence confers upon it the property of resisting the original inciting infectious agent. This kind of resistance is termed "immunity." Immunity may be acquired in either of two ways, actively or passively. It can be acquired actively by exposing the host to sublethal doses of the infectious agent, as often happens in the natural spread of disease, or by injecting into the host various non-infectious forms of the infectious agent or its fractions or products. Immunity may be acquired

passively by transfer of immune bodies from a mother to her young across the placental membranes, or by ingestion of the protective substances by the young during the first days of life in the colostrum; or immunity may be passively acquired by an animal through injection into it of the serum of

TABLE I

*Several Kinds of Resistance to Infection**

- I. Innate resistance ("natural resistance").
Genetic basis; between or within species.
- II. Acquired resistance (immunity).
 - A. Active. (The host participates in the formation of immune bodies.)
 1. Naturally acquired. (By sublethal experience with the infectious agent.)
 2. Artificially induced. (By injection of an immunologically active form of the infectious, or specifically related, agent.)
 - B. Passive. (The host does not participate in the formation of immune bodies.)
 1. Naturally acquired (congenital).
 2. Artificially induced. (By injection of immune bodies produced in another host.)

* Immunologists have recently added still another kind of acquired active resistance which has its biological basis in the "interference phenomenon." Resistance here follows prior infection with an immunologically unrelated infectious agent having a cellular predilection similar to that of the test infecting agent. The phenomenon is of short duration and any relationship of nutrition to it remains completely unexplored.

another animal which had been rendered immune. These several kinds of resistance to infection are summarized briefly in Table I (*Vide* Topley and Wilson (10), p. 763).

There are, then, several kinds of resistance to infection in animals, each with its distinct biological basis. If one would deal with the relation of nutrition to resistance to infection, then he ought to keep in mind the particular kind of resistance he is considering. When this is done, as will be attempted in the following pages, one may be astonished to discover great gaps in the knowledge in this field that are still completely unexplored. The large bulk of publications dealing with nutrition and resistance is almost exclusively devoted to nutrition and *natural* resistance. As has been seen, natural resistance is a genetic phenomenon, yet the mere mention of the word "genetics" is a rare thing in present-day papers on nutrition. Let us be content to agree on the point that, in order to be precise in a consideration of nutrition and resistance to infection, it will be necessary to recognize that there are several kinds of resistance, and to speak of the relation of nutrition to this or that kind of resistance.

Although there are several kinds of resistance to an infectious agent, there is only one kind of susceptibility. To realize this is to acknowledge at once the more fundamental nature of the phenomenon of susceptibility. If there were no susceptibility with which to begin, there would be no necessity of a concept of resistance. The fact that a given animal is capable of interaction with an infectious agent and, thus, by definition, is susceptible, is a biological attribute of that animal. Like any other biological attribute in the animal kingdom, it will be found, as wings or webbed feet, to be part of the biological make-up of some species, absent in others, and, in general, subject to the same genetic principles that underlie the differences between the species. Susceptibility, or lack of it, is part of the biological description of a species; and to say, for example, that the mouse, *Mus musculus*, is susceptible to infection by *Salmonella enteritidis* is to include such susceptibility in the list of biological attributes appended to the creature all admit to be a "mouse." Susceptibility here is a part of "mouseness" and it may be anticipated that those procedures which will diminish "mouseness" will diminish, to greater or lesser degree, the susceptibility of the mouse. Recent literature indicates that nutrition is such an influence and that it operates in such a way.

II. NUTRITION AND SUSCEPTIBILITY TO INFECTION

The basic technique of experimental nutrition is the production of aberrant physiology by withholding from the experimental animal some essential dietary item. Two results can be distinguished, one direct and greater, the other indirect and smaller. Thus, when vitamin D is withheld from an animal requiring it, the processes of bone calcification are slowed and even interrupted. That is a direct effect. The affected animal is less representative of its species just to that extent that it lacks "normal" calcification. An indirect effect can be observed in the unkempt condition of the hair-coat of the affected animal. The normal attribute of a well-groomed coat of hair is not achieved in full, although the association of vitamin D deficiency with this event may not be easily traced, and the non-specificity of vitamin D in this relation is readily apparent. Other nutritional factors can influence the state of the hair-coat, but the relation between vitamin D and bone calcification is direct and intimate.

So with susceptibility to infection, certain dietary deficiencies can be shown to diminish susceptibility (and thereby increase "resistance"), but our knowledge is still too meager to enable us to discern whether the effect is, as described above, direct or indirect.

It is difficult to trace at this time the origins of the appreciation of the relation between nutrition and susceptibility. On the basis of present experience, the guess may be hazarded that the conception first introduced itself to a significant extent when investigators of infectious diseases

turned to a study of the filterable viruses in the first decade of this century. The intimate and parasitic association of virus and host cell seems to enhance the demonstration that an ill-fed animal is less susceptible than a well-fed one. The filterable virus seems to need a well-nourished cell for its continued existence and multiplication.

One of the earliest demonstrations of this phenomenon was that of Rous (11) who, in his experiments on fowl sarcoma virus, emphasized that healthy, well-nourished fowls were more susceptible to the virus than the thin and ill. Olitsky, Traum and Schoening (12) found that guinea pigs suffering from malnutrition were more resistant to infection when inoculated with the virus of foot-and-mouth disease than were normal, healthy animals. Similar relations are evident for another host, the rabbit, and another infectious agent, vaccinia virus (Rivers, 13). The effect of underfeeding on susceptibility to vaccinia virus has been studied in more detail by Sprunt (14) who found that fasting rabbits were indeed more resistant to intracutaneously admitted virus than rabbits fed a normal diet. The difference, in terms of virus titer, was about 1 logarithmic unit, or tenfold. Sprunt, however, is not content to ascribe these results solely to nutritional causes. He has suggested, on the basis of India ink injection experiments, that fasting may cause an apparent increase in resistance, at least in the rabbit, by bringing about tissue edema with a consequent restriction of the dissemination of the virus particles. The suggestion has some point and is supported by Sprunt's observations on the effects of supplying or withholding water during the fast. The maximum reduction in susceptibility was observed when water was supplied during the fast and tissue edema rendered maximum.

New evidence that a nutritional deficiency may decrease susceptibility has come from an unexpected quarter, malaria research. Seeler and Ott (15) have reported that riboflavin deficiency markedly decreased the severity of *Plasmodium lophurae* infection in chicks. These authors used a low riboflavin diet containing 20 γ per 100 g. of diet, and a high riboflavin diet, containing 2,000 γ per 100 g. After 11 days, the birds on each diet were divided into two comparable groups, one intended for infection and the other to serve as non-infected controls. The progress of *P. lophurae* infection was followed by counts of parasitized erythrocytes in the circulation. By this criterion the course of infection was less severe in the riboflavin-deficient birds since in them the peak of parasitized red cells (approximately 3%) was $\frac{1}{2}$ the level reached in the riboflavin-high group (approximately 17%). As the peak of infection was passed this distinction disappeared, the most dramatic difference having been observed on the 7th postinfection day.

Seeler and Ott recognized the possible rôle of inanition in these experiments, for the low-riboflavin birds consumed only $\frac{1}{2}$ as much food as the high-riboflavin group. Riboflavin restriction might thus have been con-

comitantly restricting the intake of other important items. However, restriction of the intake of the high riboflavin diet to amounts consumed by birds on the low riboflavin diet had no depressing effect on the parasitization peaks. There is thus good reason to believe that the decreased parasitization peaks observed by Seeler and Ott are specifically referable to riboflavin deficiency. But the meaning of this is complicated by other observations made by the same authors in the course of their experiments. Although with low riboflavin diets parasitization peaks were low, in the same groups mortality was higher (61%) than it was among the infected birds on a high riboflavin diet (29%). Thus, it would be small comfort to a bird on a low riboflavin diet to learn that his parasitization peak would have been low, if he had only lived to experience it! Apparently there is no escape from this dilemma in interpretation. The mortality among the uninfected riboflavin-deficient animals is not high enough to account for the increased mortality in the infected riboflavin-deficient birds. Inanition, as Seeler and Ott suggest, may play a rôle in these mortality differences, but one is still left with a choice of a suitable criterion for measuring the infection process. In the face of the data at hand at present, two opposite conclusions are possible: (1) Riboflavin deficiency in chicks *decreases* susceptibility of *P. lophurae*, as measured by the *per cent* of erythrocytes parasitized. (2) Riboflavin deficiency *increases* susceptibility, as measured by mortality rates. Further clarification is needed.

Recent reports dealing with nutrition and poliomyelitis have contributed further data on the way in which nutritional deficiency can decrease susceptibility. The main thesis of these reports is that a deficiency of thiamine or a restriction of caloric intake, will decrease the susceptibility of mice to intracerebral inoculation of the Lansing strain of poliomyelitis virus. Since two independent groups of workers are in essential agreement on this point, the relation of dietary deficiency to decreased susceptibility in the case of poliomyelitis infection is probably the best explored instance of what we have here essayed to stamp as a general phenomenon. The Philadelphia group (Foster, Jones, Henle and Dorfman) has recently presented an excellently controlled analysis with some improvements in technique (16; earlier papers: 17, 18, 19). This report makes a direct comparison of the relative effectiveness of thiamine deficiency and food restriction (paired feeding) to the extent that these two procedures can lower the susceptibility of mice to poliomyelitis. Decreased susceptibility (or increased resistance) was estimated on three bases: 1) a prolongation of the incubation period of the virus following inoculation, 2) a decreased incidence of paralysis and 3) a decrease in mortality rate. Although, at various times following infection of the animals with the virus, significant differences were found in terms of decreased paralysis and mortality which could be ascribed to thiamine deficiency and underfeeding, yet these differ-

ences tended to disappear as the postinfection period of observation was extended. A difference of 57% on the 15th-postinfection day had dropped to an insignificant proportion by the 28th day. Hence the one outstanding effect of thiamine deficiency and of underfeeding on the course of experimental poliomyelitic infection is a prolongation of the incubation period. Indeed, the Wisconsin group of investigators were able to show in similar experiments that thiamine-deficient mice, inoculated with the Lansing strain of poliomyelitis virus but not exhibiting any signs of paralysis, did subsequently become paralyzed when given sufficient thiamine (Rasmussen, Waisman, Elvehjem and Clark, 20). A delay in incubation time as a sole effect does not add much encouragement to the hope that such dietary procedures will lead to an effective means of dealing with the infection known as poliomyelitis, at least in an epidemiological sense, but from a biological point of view, these experiments are meaningful in that they shed some light on the nutritional nature of the parasitism existing between virus and susceptible cell.

The question remains whether in the experiments of the Philadelphia and Wisconsin groups the effects of thiamine deficiency are to be considered as specific, or whether the results of the vitamin deficiency are explicable on the basis of inanition and are non-specific. Foster *et al.* and Rasmussen *et al.* both believe that there is more to the thiamine effect than can be attributed to inanition. However, the margin of this difference is small, as indicated in the published experiments of both groups, and at the moment the case for the specificity of thiamine in lowering susceptibility to poliomyelitis infection can hardly be considered as established.

Rasmussen and his associates (20) extended their experiments to include infection of mice with the virus of mouse poliomyelitis (Theiler's virus) but the effect of thiamine deficiency in this instance was by no means as marked as in the case of the Lansing strain of virus and may, in fact, be non-existent. That another kind of nutritional deficiency, that of pantothenic acid, may also result in lowered susceptibility in mice has been shown by Lichstein, Waisman, Elvehjem and Clark (21). In this instance pantothenic acid deficiency decreased susceptibility to Theiler's virus of mouse poliomyelitis (GDVII strain) but did not do so when the mouse-adapted strain of human poliomyelitis virus, the Lansing strain, was used as the infecting agent. Thus, accumulating data have begun to emphasize the intricate nature of the relationships between hosts and pathogens. Certain manipulations of host nutrition may have significance for one strain of pathogen and have little or no significance for other strains, although these very strains of a pathogen may elicit what appears to be similar phenomena of a disease. If laboratory experimentation is to have any meaning for the world of nature, which, it may be assumed, is the desired object of study, then investigators may well have to pay more attention in the future

to the suitability of the infection model they employ. This point was recently explored by Olitsky (22) in the instance of various "strains" of mouse poliomyelitis virus. Various clinical, pathological and epidemiological features of mouse poliomyelitis (Theiler's disease) recommend the murine virus and malady as a suitable model for study of problems of the human disease. Yet examination of several of the characteristics of the GDVII strain of Theiler's virus, which is quite generally employed, has convinced Olitsky that this strain is unsuitable as a "model" for the study of human poliomyelitis. The original strain that Theiler isolated (TO strain) is capable of producing a syndrome which more closely duplicates the features of classical poliomyelitis. There appear to be no reports in the literature on the effect of nutrition on infections produced with the latter agent.

In referring to unpublished experiments, Pinkerton (23) has stated that he has observed a prolongation of the incubation time of Lansing strain poliomyelitis virus in riboflavin-deficient mice.

In the field of bacterial infections there are a few reports that dietary deficiencies are capable of decreasing susceptibility. In our own laboratory we have observed that mice on a fat-free diet are less susceptible to infection with *S. enteritidis* by mouth than mice receiving the same purified diet containing 5% cottonseed oil (24). On the basis of a single experiment, involving only two litters of rats, West, Bent, Rivera and Tisdale (25) claimed that pantothenate-deficient rats were less susceptible to infection by nasal insufflation with broth culture of Type I *pneumococcus* admixed with 8% mucin. On the contrary, Robinson and Siegel (26), after more extensive study, came to the conclusion that pantothenic acid-deficiency had no effect on the susceptibility of rats to intratracheal insufflation with broth cultures of Type I *pneumococcus* in mucin suspension. Although there will be occasion further on to refer again to this paper of Robinson and Siegel, one comment might be made here. The data these workers report suggest that there exist in natural foodstuffs certain dietary factors as yet unrecognized which enhance the *susceptibility* of the rat to infection with the *pneumococcus*. Thus, on a "synthetic" diet, rats infected with the *pneumococcus* suffered a mortality rate of 47%, while rats receiving a stock diet of natural foodstuffs had a significantly greater mortality rate, 80%. The point appears worthy of further investigation.

Bloomfield and Lew (27) have reported a decrease in susceptibility of rats to chronic ulcerative cecitis due to B complex deficiency. Controls fed 10% yeast had a 50% higher incidence of the lesion over a period of several months. Unfortunately the etiology of rat cecitis remains unknown and a lack of this information prevents a design of more accurately controlled experiments.

There is also a suggestion that a deficiency of the vitamin B complex is capable of lowering the susceptibility of rats to still another type of infection, namely, trypanosomiasis. Reiner and Paton (28) were among the first to draw attention to the phenomenon. They found that rats on a B complex-deficient diet, when infected with *Trypanosoma equiperdum*, exhibited a prolonged incubation time over that of the controls. This prolongation was only, on an average, one day over the usual period of 4.5 days. However, sufficiently large numbers of rats were used to satisfy statistical criteria. It is of interest to note that trypanosomes made their appearance in the B complex-deficient animals at the same time as in the B complex-supplied controls and reached similar levels of concentration in the blood stream. These facts tend to strengthen the interpretation that the effect of the deficiency was on the host, *i.e.*, that it decreased the host's normal attribute of susceptibility.

From the foregoing observations it is obvious that there is a growing fraction of the literature on nutrition and infection which supports the general thesis that nutritional deficiency is capable of increasing resistance to infection by virtue of lessening the biological attribute of the host, susceptibility. This has now been shown for several hosts and for such diverse infections as those caused by protozoan, viral and bacterial species. The question arises, Can any generalization be made as to which dietary factors are effective in decreasing susceptibility through their deficiency in the diet? The data are still too meager for a real reply to this question, but if the reader will allow, an opinion may be hazarded. If one assumes the relationship of diet to susceptibility to poliomyelitis to be typical, it is already apparent that, for a given strain of virus, one kind of dietary deficiency may be effective while the effect of another deficiency may be quite trivial. For other infections the situation may be reversed. The safest generalization at the present time may be the following: All dietary factors are not equally concerned in the achievement by an animal of a particular biological attribute. Susceptibility is a biological attribute of a host. One might therefore expect that all dietary factors would not be equal in their effect on susceptibility. And since susceptibility is specific, *i.e.*, susceptibility to one disease does not insure susceptibility to all diseases, one may not be far wrong in anticipating that a dietary factor intimately involved in susceptibility to one disease will be of petty influence in the susceptibility of the same host to another disease.

Another question might be asked. What hope can be attached to the imposition of dietary deficiency in hosts as a means of reducing the effects of infection? This is thorny ground. At the present time, even in the case of severe deficiencies, the most that can be said is that the desired end, decreased susceptibility, is hardly worth the cost. The impaired physiological well-being of the deficient animals is sound reason for pause. More-

over, the choice is a poor one: Impaired physiological well-being on one hand, and susceptibility on the other, or "small choice in green apples." The suggestion that a degree of dietary deficiency might be achieved which would be effective for reduction of susceptibility but of slight deleterious effect for the host remains but a pious wish and hope.

III. NUTRITION AND NATURAL RESISTANCE TO INFECTION

In the preceding pages the phenomenon of dietary factors in their contribution to *susceptibility* has been treated briefly. This is a somewhat recent point of view which may reflect a certain broadening of the outlook of investigators in the field of nutrition and infection. It now seems pertinent to turn to a consideration of the components capable of enhancing *resistance* when they are consumed in the diet. This turn-about, it should be emphasized, is merely a matter of convenience in an examination of the subject and is not meant to imply a fundamental inconsistency. In later pages, a reconciliation of the two viewpoints in a general theory will be essayed.

The bulk of the published literature dealing with nutrition and resistance to infection consists of reports on nutrition and *natural* resistance to infection. There are probably three reasons for this singular preoccupation of investigators in the field and their avoidance of nutrition and *acquired* resistance. 1) There has been a valid interest in the factors influencing the outcome of the *primary* contact of a host with a pathogen. Should the host survive this initial contact, then the immune response which follows is, from one view, but an added luxury which now makes doubly certain that a host which has demonstrated its ability to survive an initial infectious contact will now certainly survive a second contact, and probably even a greater dose of the infecting agent. 2) Most investigators have encountered technical pitfalls enough in the focusing on biochemistry, genetics, nutrition, physiology, bacteriology, parasitology, etc. (in any one or several of which an amateur's standing was scrupulously maintained), without taking on, in addition, the complexities of immunology. 3) There has been what is almost a silent recognition of the necessity of dealing with the problem directly: Let dietary effects be established first. Then will come the time to investigate whether immunologic phenomena are involved.

It will not aid our purpose here if we set forth in detail once again the considerable literature surrounding the relationship between nutrition and *natural* resistance. Happily for our ends this has been done recently and competently. In 1934 the reviews of Clausen (29) and Robertson (30) appeared. In 1941 Perla and Marmorston (31) treated the topic at some length in their volume "Natural Resistance and Clinical Medicine." More recently the critical review of Aycock and Lutman (1) has appeared (1944). The debt owed these reviewers is considerable and it is only be-

cause they have performed their bibliographic service so well that we are emboldened, the better to pursue our strategic purpose, to select but a few of the investigations reported in these reviews and, in company with additional more recent work, to form some opinion as to where we stand.

Historically, the idea that the consumption of proper food enhances the ability of an animal to resist infection antedates by a few thousand years the quite opposite notion which has been examined in the preceding pages.⁷ The association between famine and pestilence has inspired comment until it has become almost trite to mention it.⁸ Attempts to document, by means of experiment, the assumed causal connection between failing nutrition and failing resistance to infection, however, date from the growth, in modern times, of the present concepts of nutrition. The advent of the concept of the vitamins, with their almost magical association with the maintenance of life fired the imagination of investigators and set loose a flood of efforts to connect diet and resistance to infection. Although it was stated in many different ways, and sometimes left unstated, the general trend of reasoning was as follows: One of the necessities of continued life is the ingestion of the vitamins. One of the attributes of living is the ability to resist infection. *Ergo*, the ingestion of vitamins is necessary for resistance to infection. But let us not pause for dialectics. For good or ill, it was on some such basis that the hope was born, and reborn countless times, that diet and resistance to infection would be connected. What have been the results?

1. *Inanition*

Famine and starvation can be of two kinds, quantitative and qualitative. The total intake of an otherwise adequate diet can be restricted in amount, or one or several essential dietary components can be restricted to inadequate proportions, while the total intake of other items may or may not remain adequate. The first is inanition; the second, malnutrition. There are surprisingly few reports on the effect of inanition on natural resistance to infection. What information is available comes from the more carefully controlled experiments of recent date in which investigators, recognizing the anorexia which accompanies vitamin deficiencies, especially thiamine, have included inanition controls either by paired feeding or by arbitrary underfeeding. In the instance of a virus infection (poliomyelitis) in mice, Foster *et al.* (18) found that restriction of the intake of a stock diet to 40% of the usual amount consumed prolonged the incubation period of the infection. On the other hand, underfeeding chicks to the extent of

⁷ *Vide* Aycock and Lutman (1).

⁸ Sigerist (32) has recently presented an equally logical account of the association between famine and disease, especially rodent-carried disease. The failing food supply drives the rodent population into closer association with man and the opportunity for contact is thereby increased.

50% of normal consumption was found by Seeler and Ott (15) to decrease survival to *P. lophurae* infection by 50%. In the case of a bacterial infection—Type I *pneumococcus*—Robinson and Siegel (26) found that restricting the intake of a “synthetic” diet by rats to the inanition levels of rats in thiamine and pantothenic acid deficiency did not effect any change in survival rate. In our laboratory we (33) have found the same to be true for mice subjected to infection with *S. enteritidis*.

It is obvious that the data are still too meager to warrant a generalization as to the relationship between inanition and natural resistance to infection.

2. Malnutrition

If the relation of *inanition* to natural resistance to infection has been left pretty much on the shelf, then the relation of *malnutrition* to natural resistance to infection is a subject that is quite shop-worn—many have examined it but few have bought. There may be a fundamental reason for this constant interest yet continuing indecision—and it is, we believe, not far to seek.

As has been remarked earlier in these comments, the only experimental criterion of relative resistance or susceptibility to infection which can be said to be above reproach is that of rate of mortality, or survival. Decreased survival following infection means decreased resistance to infection. But many essential dietary deficiencies, by themselves, ultimately cause death. The course of infection takes time. Thus, an infected animal which is suffering from an essential dietary deficiency proceeds, in time, under a double jeopardy. To find, therefore, that a deficient animal dies in greater numbers than an animal which is not deficient proves nothing, for the increased rate of mortality in the deficient animals may be due to the deficiency directly and independently of the infection. It is apparent that *uninfected* animals maintained under the same conditions and for the same length of time are a type of control which is necessary. Yet this simple control is all too frequently absent from many of the experiments in the literature and so an interpretation of results is rendered exceedingly difficult, if not impossible. The point involved here is a fundamental one in the methodology of experiments in dietary deficiency and infection, and might be stated in general terms as follows: An experiment can be admitted as evidence of the causal relation between nutritional deficiency and decreased natural resistance only if it can demonstrate 1) that the mortality rate of infected, deficient animals is greater than that of infected, adequately fed animals, and 2) that this increased mortality is greater than that of *non-infected* animals subjected to the same deficiency for the same length of time.⁹

⁹ It would be well to maintain the uninfected animals on the deficient diet for an even greater length of time in order to estimate the imminence of death due to dietary

Indeed, it may be more than coincidence that the most frequent claims for the effect of a vitamin on natural resistance in experimental animals have been raised for those vitamins, such as A and C and thiamine, deficiencies of which bring about death soon after the deficiency has made itself evident, while vitamins such as D and K, deficiencies of which do not result in death so quickly, find few supporters to press their claims.

a) *Vitamin A.* If the reader has ever heard of vitamin A at all, it is assured that he has likewise heard of vitamin A as the "anti-infective" vitamin. This is not only nonsense; it is dangerous nonsense.¹⁰ How did this situation come about?

It is often considered tiresome and picayunish to quarrel about the names of things, but the history of science is replete with instances in which a name applied to a phenomenon has influenced to no slight degree the attitudes, and even contents, of subsequent investigations in the field. Witness the influence of the word "vitamin," originally coined by Funk as "vitamine" from his view (erroneous) that the accessory food factors so important for the maintenance of life were, chemically, amines. If one would deny the propriety of associating the term "anti-infective" with vitamin A, then it may be instructive to examine first the circumstances under which vitamin A thus came to be dubbed.

In 1928 Green and Mellanby (34) published a paper entitled "Vitamin A as an Anti-infective Agent." They began this article as follows:

"The assigning of names descriptive of some particular function to distinctive vitamins has been a useful, *although probably only temporary*, (italics author's) step in the development of knowledge of these elusive entities, because their identification has so often depended upon the appearance of definite syndromes in animals whose diets have been deficient in certain respects. The use of the words 'antiscorbutic,' 'antineuritic,' and 'antirachitic' in describing specific vitamins is an example of this, and from a clinical standpoint the nomenclature has been of great value. Vitamin A has always presented special difficulties to a clinically descriptive term because it has depended to such a large extent on a purely physiological criterion—namely, growth in young animals—for its detection. It is in consequence often referred to as the 'growth-promoting' vitamin. Since

deficiency, for it is entirely possible that the infection might in itself increase the demand for the deficient factor and thus precipitate a "deficiency death" earlier than would be the case in the absence of the infection. One would then be dealing with the effect of an infection *on* nutritional requirement, which is another matter and one which should not be confused with the effect of diet on the course of an infection. The point is more than academic and failure to make this distinction clear has led to confusion.

¹⁰ I heard a nutritionist,—save the mark!—with a gleam of triumph, publicly "deduce" that a young patient under discussion must have received a diet deficient in vitamin A in early life because he had a bout of pneumonia when he was 2 years old!

the recognition of vitamin D (the antirachitic vitamin) as an entity distinct from vitamin A, those with experience of nutritional work have felt that to call vitamin A the 'growth-promoting' vitamin is a misnomer, for good growth often takes place in its absence if the diet is otherwise complete. In fact, when growth ceases owing to the single absence of vitamin A from the diet, it often means that the animal is definitely ill—in the sense, as, will be seen below, of its having developed some, and often a widespread infective condition. Indeed, the present paper supplies evidence in favour of the term 'anti-infective' being applied to vitamin A."

It is obvious that Green and Mellanby, in search of a useful appellation for vitamin A from a clinical standpoint, rejected "growth-promoting" because of, what seemed to them, the contradictory nature of the finding that animals could grow on a vitamin A-free diet. At the time their paper was written, there was a general lack of appreciation of the ability of animals to store vitamin A, especially in the liver, although 5 years earlier Steenbock, Sell and Nelson (35) had clearly shown that this was so, and that growth gains of animals, when placed on vitamin A-free diets, were directly influenced by the previous vitamin A history of the animal. Overlooking this finding, Green and Mellanby turned to the well-known effects of illness in arresting growth. In their minds, animals stopped growing on vitamin A-deficient diets because they became ill, as they suggested, from infectious causes. Here, by a gratuitous assumption, is the root of the "anti-infective" myth.

These roots are intertwined with those of another association between vitamin A and "infection," the relation between vitamin A deficiency and xerophthalmia. It is small wonder that early investigators thought of "infection" when they were confronted with the sticky discharges, edematous eyelids, and general evidences of inflammatory reaction in the eyes of rats on vitamin A-deficient diets. And it is only by hindsight, fortified by the information obtained from systematic, pathological investigations, such as those of Mori (36), Wolbach and Howe (37), and Goldblatt and Benischek (38), that it has become possible to distinguish what is primary and what is secondary. Primarily, vitamin A deficiency results in metaplasia of columnar, cuboidal and transitional epithelia to a squamous, keratinizing type. In favorable localities, such as the alveoli of glands, folds of the conjunctiva and mucous membrane surfaces, the accumulation of such keratinized detritus has sometimes been regarded, mistakenly, as pus. At the same time these accumulations have furnished suitable soil for the multiplication of bacteria, in themselves harmless but, with their products, accumulating to serve as sources of irritation sufficient to provoke local inflammatory reactions. These latter effects are, however, all secondary to vitamin A deficiency and follow the primary effect of metaplasia of the epithelia. Moreover, these local accumulations of bacteria

in masses of keratinized cells can hardly be called "infection" in the sense that it was defined earlier in this chapter. At best, they are "local infections," are certainly not systemic, and have no epidemiologic significance. The metaplasia of epithelia in vitamin A deficiency, of course, results in certain morphological changes, such as a diminution of mucus secretion and a loss of ciliary motion. These changes have evoked from certain authors *mechanistic explanations* of a loss of resistance, but such plausibilities will merit attention only after the *fact* of lowered natural resistance has been established, and this has not yet been accomplished.

Because, then, the special facts of the pathology of vitamin A deficiency speciously present the appearance of spontaneous "infections," much of the literature on vitamin A and natural resistance is suspect. The "pus" and "abscesses" often reported have more prosaic explanations and meaning. Even the oft-quoted epidemic of bronchopneumonia in Mellanby's (39) puppies on a diet deficient in vitamin A does not withstand too close scrutiny.¹¹

There are, however, a few reports on experimentally induced infections in vitamin A-deficient animals. Boynton and Bradford (40) reported an interesting study in which vitamin A-deficient rats showed a higher mortality rate than did control animals when injected intraperitoneally with a bacillus of the *mucosus-capsulatus* group. This bacillus had been isolated from the middle ears of rats deficient in vitamin A. Although the number of animals used was small, Boynton and Bradford claim to have shown that in this instance resistance, in the presence of a deficient diet, had been lowered 2 to 4 weeks before the deficiency had made itself evident by the cessation of growth. Rats deficient in vitamin D showed no diminution in resistance. Since wide variation exists in the time necessary to deplete rats of their stores of vitamin A, the fact that Boynton and Bradford did not include vitamin A-deficient, but uninfected, controls limits the value of their results.

It is interesting to note that one of the pioneer investigators in the field of nutrition and resistance to infection was more conservative in interpreting the results of his experiments than were many of those who followed him. As early as 1923, Werkman (41) had demonstrated that by producing vitamin A deficiency in the rat, he was thus able to bring about the death of

¹¹ The following is taken verbatim from Mellanby's paper (39). It is not often quoted.

"In some cases, at least, the bronchopneumonia developed in animals which not only had the diet defect described above, but also had been taken out of their indoor kennels into the open air, where it was usually cold and windy and often wet, in order that their running powers should be determined. The opportunity, in fact, was presented to them of catching a 'chill.' The dogs on the diets containing vitamin A were also placed under the same conditions, but their resistance was apparently sufficient to make the low temperature of the external conditions of no account."

such animals by intraperitoneal injection of the anthrax bacillus. Normal rats are not susceptible to anthrax. Werkman went on to show that similar results could be obtained in the instance of vitamin B (complex) deficiency. The argument for specificity of vitamin A in relation to lowered resistance was thereby nullified. By further investigation into the physiology of both vitamin A- and vitamin B complex-deficient animals, Werkman concluded that such animals became altered in a nonspecific way, presenting effects similar to those observed in total starvation. Rectal temperatures were depressed 3 to 4°F. The same conditions prevailed in rabbits and pigeons. From our vantage point, in time, the further opinion may be added that infecting debilitated animals verging on the moribund with organisms of no epidemiological significance for the species and by routes far from the natural routes of contact, may lead to results which are more of a tribute to the ability to produce special phenomena in the laboratory than they are indicative, as models, of processes which are meaningful in the world of natural events.

There does not appear to be need to continue to labor the point. Inasmuch as information available at the present time does not warrant a description of vitamin A as the "anti-infective vitamin," the term might well be dropped.

b) *The Vitamin B Complex and Natural Resistance.* If the reader has been persuaded that the various principles upon which have been based criticisms of claims that vitamin A exerts an influence on natural resistance to infection are valid grounds for rejection of such claims, then he will, no doubt, be interested in further discussion of the influence of other nutritional factors contained in the reviews already cited (1, 29, 30, 31). Lists of the publications on which the opinions offered herein are based are presented in these reviews. Reports which have become available since the appearance of these reviews will need separate study, especially when they present strategic gains which will be of assistance in clarifying the current position. It is true that, in undertaking an exposition in this manner, one invites the criticism that such evaluation will be biased. It is a charge that is freely admitted, for who is ashamed of being "biased" in favor of the truth as he sees it?

It is to be expected that more recent investigations, equipped with the advances of nutritional experimentation and better able to utilize diets of chemical definition, would offer a clearer understanding of the relations of the B vitamins to natural resistance to infection. Thus, Wooley and Sebrell (42) have reported an interesting series of experiments dealing with the influence of riboflavin or thiamine deficiency on fatal experimental pneumococcal infection in mice. Purified diets were used and the B vitamins were added as the chemical compounds. Choline, nicotinic acid, inositol, pyridoxine, thiamine, riboflavin and calcium pantothenate were

supplied in adequate amounts in the control diet, and the respective deficiencies were produced by lowering either thiamine or riboflavin to low levels (0.5 γ per g. of diet), so that the mice ingesting these diets grew very slightly or not at all. Hence, the mortality risk of the experimental diets was small over a period of time (3 to 4 weeks) which was long enough to allow deficiencies to make themselves evident and to allow time as well for the infection course. Wooley and Sebrell gave additional recognition to the necessity for the uninfected control by subjecting control groups to a mock infection procedure, using, in place of a culture of *pneumococci*, sterile broth and defibrinated rabbit's blood which were inhaled by the lightly anesthetized mice. The mortality rate was negligible. *Pneumococcus* Type I was employed for the infected groups. In one such experiment thiamine- and riboflavin-deficient groups (50 mice at risk in each group) suffered an approximately 35% greater mortality rate than controls. Because of the variation in virulence encountered in cultures of *pneumococcus* it was not easy to reproduce this experiment. In another experiment, in which a lower attack rate was met with, the differences were not as great, being about 12%. In some experiments the difference was even less.

By paired feeding, in the instance of riboflavin deficiency, Wooley and Sebrell claim to have demonstrated that the lowered resistance encountered is not due to the accompanying inanition. The numbers of mice used, however, were smaller than in their previous experiments and the differences observed, though suggestive, are not statistically convincing. The rôle of inanition in thiamine deficiency was not investigated. These workers made another interesting observation. In both riboflavin and thiamine deficiency, the daily administration of these vitamins in amounts 5 to 10 times that supplied the controls, at the time of infection and thence-forward, did not reduce the rate of fatalities. If anything, such treatment increased the number of deaths. An interpretation of this finding is best left until further confirmation of the phenomenon is at hand.

The experiments of Wooley and Sebrell demonstrate clearly the difficulties of experimentation with the *pneumococcus* as an epidemiological model. Mice and *pneumococci* have had a long history together in experimental medicine, but for proper strategic ends which are not applicable in the present instance. In the majority of cases mice have been employed to detect the outcome of operations performed on the *pneumococcus* with anti-serum or drug, sometimes outside the animal and sometimes in the peritoneal cavity. The attempt to change the biological characteristics of the mouse by diet and thereby affect the fate of a pathogenic invader is, however, another matter. In the latter case it is of extreme, yes, decisive importance whether host and pathogen bear any relationship to each other in an epidemiological sense. What epidemiological significance, for example, does the *pneumococcus* have for mice in nature? The answer is, None. One

might expect, therefore, that in the laboratory the bringing together of mouse and *pneumococcus* as an epidemiological model would provide phenomena interesting enough to excite study, and perhaps bizarre enough to win acclaim. But let us not delude ourselves that we are thereby studying the events of the natural world.

Robinson and Siegel (26) have recently published their findings in a study of the influence of the B vitamins on the natural resistance of rats to induced pneumococcal lobar pneumonia. They found that rats deficient in riboflavin or pantothenic acid were as resistant as rats receiving the same basal diet supplemented with adequate quantities of these vitamins. Thiamine and pyridoxine deficiency seemed to lower resistance somewhat, but the mortality risk of these deficiencies in uninfected animals was not measured. That some risk, at least in the thiamine-deficient animals, was present, is indicated by the comment that small doses of thiamine had to be fed as daily supplements "in order to maintain the lives of rats fed the thiamine-deficient diet."

A deficiency of biotin has appealed to some investigators as influencing natural resistance to protozoan infection. Trager (43) found that biotin-deficient ducks and chicks had significantly higher red cell parasitization peaks following inoculation with *Plasmodium lophurae* and, in the case of ducks, with *P. cathemerium*. Pantothenic acid deficiency produced no such effect. Seeler, Ott and Gundel (44) have confirmed the findings of Trager relating to this effect of biotin deficiency in chicks and have demonstrated its transitory nature. Differences between biotin-deficient birds and controls (receiving 0.0001% crystalline biotin) were statistically significant on the 5th day following inoculation, but were not so on the 3rd day or after the 7th day. Unfortunately, little is known of hemodynamics in biotin deficiency. What possible significance these observations can have for the epidemiology of malaria remains obscure.

Caldwell and György (45) found that, in biotin-deficient rats, the duration of *Trypanosoma lewisi* in the blood stream was extended in time over controls fed a diet of natural foodstuffs. The assumption that biotin was directly responsible for this effect rests on observations on four rats, all from the same litter.

The report of Ross and Robertson (46) on the lowering of natural resistance of rats to *Salmonella* infection by B complex deficiency is often quoted. However, the small number of animals employed in their study, the variation in dosage, the questionable permissibility of summing together experiments in which the attack rate varied widely and the lack of recognition that rats vary innately in their resistance to *Salmonella* infection, all would seem to militate against a ready acceptance of these experiments of Ross and Robertson as substantial proof.

In the field of natural resistance to nematode infection, the rôle of the B

vitamins is not much clearer. In an early paper, Ackert and Nolf (47) reported that in B complex-deficient chickens the administration of 500 embryonated eggs of *Ascaridia lineata* was followed by the finding of a slightly greater number of worms in the intestines 3 weeks later than were found in controls fed baker's yeast. The decreased peristalsis in B complex deficiency in chicks could well account for the more congenial habitat for these worms, as Ackert and Nolf suggested and, what is probably more important, feeding the B complex was just as favorable for the worms as it was for the chicks. Worms in chickens receiving yeast were reduced in numbers but the worms themselves were bigger. More recent studies, such as those of Watt (48), who used the nematode *Nipostrongylus muris* with the rat as host, illustrate the empirical nature of the methods employed in evaluating resistance and the wide variation of behavior in the nematode populations, even in control groups, from experiment to experiment. Watt's experiments indicated that higher nematode populations resulted following infection in thiamine- and riboflavin-deficient rats. The duration of these effects was not studied.

The appearance of diarrhea in monkeys fed the "vitamin M"-deficient diet of Langston, Darby, Shukers and Day (49) and the isolation of *Shigella paradyssenteriae* (Flexner) from their stools have suggested to some workers that vitamin M deficiency results in a lowered resistance to bacillary dysentery. Interpretation of data in this regard is rendered difficult since many of the captured monkeys used experimentally harbor *Shigella* and the appearance of diarrhea in itself is not diagnostic. Verder and Petran (50) observed diarrhea in three vitamin A-deficient *rhesus* monkeys and found *B. dysenteriae* (Flexner) postmortem in the colon of one and the cecum of another. Agglutinins for *B. dysenteriae* were found in both the vitamin A-deficient monkeys and two control monkeys receiving vitamin A. Janota and Dack (51) fed the vitamin M-deficient diet of Langston *et al.* (49), which is a modified Goldberger diet, to captive *rhesus* monkeys. Dysentery appeared and isolation from the stools of *B. dysenteriae* (Flexner) X type was possible in some cases. Agglutinin titers rose somewhat from the levels which had been observed before the animals were fed the deficient diet, and the evidence that the animals harbored the infection before the experiment began was strengthened by the finding of the allegedly causative organism in the stools. The rise in agglutinins is noteworthy and may be invoked as evidence of an "infection," although the rise was slight.

Saslaw, Schwab, Woolpert and Wilson (52) have contributed to an understanding of these observations by employing an experimental diet of greater chemical definition. To a synthetic, B complex-free diet they have successfully added the known B vitamins including riboflavin, thiamine, nicotinamide, calcium pantothenate, pyridoxine, para-aminobenzoic acid, inositol, choline, pimelic acid and glutamine, all without preventing appearance of so-called vitamin M deficiency with its characteristic signs of cyto-

penia. These workers also observed the occurrence of dysentery on this diet and in some cases isolated *Shigella*. Dysentery did not appear in monkeys kept on a "normal" diet. In addition, experimentally induced infection with *S. hemolyticus*, Group C, and influenza virus A, intranasally, resulted in the death of ten of thirteen monkeys on the synthetic diet, while only two deaths occurred in a group of thirty-two monkeys maintained on a "normal" diet and infected similarly. These data are certainly suggestive and focus attention on the dietary difference between the synthetic diet and one of natural foodstuffs. Whether this difference may be described in terms of vitamin M, which was originally defined in its rôle in the prevention of cytopenia, or whether other unknown substances are involved, cannot be stated at the present time. Further work will be awaited with interest.

c) *Vitamins C and D and Natural Resistance to Infection.* In 1937 Watson (53) wrote: "Reference to the voluminous literature, and especially to such reviews as those of Clausen (1934) and Robertson (1934), will reveal a mass of confusing and contradictory statements based on experiments carried out on an inadequate scale, and without regard to simple statistical requirements. The available evidence, taken as a whole, would seem to suggest that vitamins B and D have little, if any, influence on resistance, while in the case of vitamin C the observations recorded are particularly confusing and difficult to interpret."

Pijoan and Lozner (54) in 1944 concluded that ascorbic acid has but two known uses: 1) the prevention and 2) the treatment of scurvy. To which I add, in 1945, "Amen!"

d) *Other Dietary Items and Natural Resistance to Infection.* So far as the published record will permit of judgment, there is no persuasive evidence that such items as fat, various carbohydrates, the essential mineral elements and the quantity or quality of protein intake have any profound effect on natural resistance to infection, although it should be remembered that little work has been done in this respect. Convincing evidence has, however, been brought forward to show that in the realm of natural foodstuffs there exist items as yet unrecognized and only partially defined, which are capable of exerting an influence on natural resistance. It seems almost as though the dramatic effects of the known items of essential nutrition had induced many investigators to attempt to make a case for these items in relation to resistance to infection—an attempt on which many have foundered. It is often overlooked that, in so doing, nutritional experimenters were abandoning the methodology of their science which had already yielded such rich results. For the items that are now included in the list of recognized dietary essentials have each won their right to be included in such a list by having provided an answer to certain specific experimental questions. Thus, in the case of many of the vitamins, the experimental question was: "What are the chemical items of a diet which

will support the growth of young animals?" Any chemical compound which has been shown to be part of the answer to that question has been admitted to the list of dietary requirements, and has remained there. Sometimes the question asked dealt with some other biological attribute of the "normal,"¹² as: "What dietary factors are necessary for normal blood coagulation rates?" Vitamin K was the answer that time.

But that any of these items should necessarily be directly concerned in answering another question, such as: "What dietary items influence natural resistance to infection?" involves a considerable gap in reasoning and, indeed, flies in the face of biological experience. The more logical way to proceed, it may be argued, is to ask the above question and then repair to the world of natural foodstuffs for an answer, just as did the early workers in classical nutrition. On this basis, one could compare the effects of various diets composed of natural foodstuffs derived from different sources, as did Hart, McCollum, Steenbock and Humphrey (55) in 1911; or one could compare the effects of a "synthetic" diet, containing all of the known items, with a control diet of natural foodstuffs as has been done ever since McCollum led the way into the modern era of nutritional research and persuaded investigators of the utility of experimentation with a small laboratory animal, the rat.

It is noteworthy that both of these approaches have been tried, and with encouraging results, not by nutritionists but by the proponents of the two schools of experimental epidemiology which arose as a direct result of the baffling epidemiological problem presented by the great influenza pandemic of 1918-19. The English school, led by the late Prof. W. W. C. Topley, initiated the attack by giving attention to the epidemiological model which was to be employed. A convenient host, the mouse, was used together with a pathogenic agent of epidemiological significance for the mouse, *S. aertrycke* (*Bac. typhi-murium*), one of the "mouse typhoid" agents. The effect of diet on natural resistance in this model was investigated by M. Watson (53), a member of the group at the London School of Hygiene and Tropical Medicine. She found that natural resistance was increased to *per os* infection by dried skim milk, but the active material was not further identified.

The American School of experimental epidemiology was led by the late Dr. L. T. Webster at the Rockefeller Institute for Medical Research in New York. As early as 1924 Webster and Pritchett (56) reported that mice fed on a McCollum complete diet, consisting of whole wheat, casein, milk powder, butterfat, NaCl and CaCO₃ were more resistant to mouse typhoid infection than similar mice fed on bread and pasteurized milk supplemented by an oatmeal and buckwheat mixture and dog biscuit. Both of these diets were capable of supporting the mice in fully normal life

¹² The statistical concept of "modal" is really more precise.

cycles and, in fact, the more susceptible diet was the routine stock diet used for the maintenance of huge colonies of mice in the Institute. The analysis of the items responsible for these differences in natural resistance proved more difficult (57) and the criticisms leveled by Watson (53) were justified.

A fresh start was made at New York in 1940 by Schneider and Webster (58). In the interim the American school had been led by its studies into an investigation of the innate, genetic aspects of host constitution in natural resistance to infection, which culminated in Webster's publications published in the period between 1932 to 1939 (8, 59, 60, 61). In these experiments Webster showed that from a single stock of mice, *free of the test infectious agent*, by inbreeding and selection, it was possible to derive two separate lines of mice which were uniformly and predictably widely divergent in their susceptibility to *S. enteritidis*. These facts held, moreover, whether the mice were tested individually, or whether they were exposed to the infectious agent under conditions of epidemicity such as obtained when the mice were assembled into herds. Another important point was established: susceptibility to one disease, mouse typhoid, could exist in the same animal that was resistant to another infectious agent, the virus of St. Louis encephalitis. Indeed, resistance and susceptibility to each of these two diseases could be established by breeding techniques in such an independent fashion that it was actually possible to derive four lines of mice in which the four permutations of resistance and susceptibility to two diseases were represented. That resistance to one disease and susceptibility to another can thus be so clearly shown to co-exist in the same animal should underline the concept of specificity of host reaction to infectious agent, and makes extremely unlikely the advisability of entertaining a notion of panresistance. The same thesis may hold true for the relation of diet to infection, and we may well prepare ourselves for the concept that when dietary factors are found which are of value, they will be specific for certain infections, although a given factor may have significance for a group of infectious agents which, in common, are similar in their interaction with certain aspects of host physiology.

But speculation aside, there is one conclusion from which there is no escape. If, as Webster has so clearly demonstrated, natural resistance to infection is controlled by innate, genetic factors which obey the laws of modern Mendelian genetics, then, when one seeks to influence natural resistance to infection by diet, he is, in effect, seeking to influence the way in which genetic factors, genes, reach, or fail to reach, their biological goal, the expression of a biological character. This is physiological genetics—and every experimenter who seeks to alter natural resistance to infection is experimenting in physiological genetics.¹³

¹³ The interested reader will find Goldschmidt's book "Physiological Genetics" (62) a modern account of the content of this field.

Strategically, then, investigations on the relation of diet to natural resistance to infection, are investigations in physiological genetics.¹⁴

But the nutritionist who seeks to apply his science to other biological problems may find, as I believe he finds here, that he can no longer neglect genetics. Some biological characters, reflecting perhaps the consequences of natural selection and evolution, are totally or partially under the control of the genes. How, in such circumstances, to affect the character by diet will, for precision, need more information of "nutritional physiological genetics" than is now possessed. The report of Schneider and Webster (58) is no more than a documentation of this viewpoint. These investigators found that nutritional factors were significant for the natural resistance of mice to *S. enteritidis* infection *per os* when the host had a certain genotypic composition. For nutrition to be measurably effective the mice had to be genetically *heterogeneous* (outbred). When the genetic composition of the mice was *homogeneous* (inbred, selected), the same nutritional factors were without effect. This introduction of the concept of genetic heterogeneity as a prerequisite for the influence of nutrition on natural resistance may be another indication of the necessity of including in epidemiological models of the laboratory certain genetic aspects of the natural world if it is to be hoped that laboratory studies will yield information *about* the natural world. Epidemics are a natural phenomenon; genetic heterogeneity is a characteristic of the freely interbreeding hosts which are part of the epidemic phenomenon.

Geneticists have been persuasive in convincing students of biological research of the necessity of having genetically *homogeneous* material for their experiments. To come forward and argue that for a nutrition experiment one needs genetically *heterogeneous* material is, at first sight, rash. But the apparent contradiction is not a real one. The crux of the argument lies in "biological material for *what kind* of experiment?" If the investigator wishes to study the precise *details* of a biological process, he will require experimental material which will present him with this process uniformly. Let us say he wishes to study the cellular events leading to cancer. Then

¹⁴ The idea may be advanced that *all* nutritional research is, strategically, physiological genetics. It may be that thus far the nutritionist has had to pay scant heed to Mendelian genetics because he has taken his problems where he found them and, in retrospect, he found them where genetic influence is not sharply particulate and hence not prominent. Growth, for example, is a biological character which is controlled by so many genes that it is extremely unlikely that breeding methods, even carelessly pursued, result, in the animals, in any embarrassing excursions up and down the scale of rate of growth. If such did occur, one could visualize the effect on growth experiments. The mere fact that this possibility has been excluded by the biological bases of growth has made it possible to neglect genetics to a large extent. At my home university, the University of Wisconsin, nutritional investigations are pursued in the Biochemistry Building. Right next door is the Genetics Building. The two buildings might well be in different worlds. (Up until this moment I have had friends in both buildings.)

he will do well to listen to the argument of the geneticist and use genetically uniform material. The cellular events will then be presented to him for study in a uniform and reproducible manner. When the investigator has learned *all* of the details of the process, he *might* be in a position to devise an effective means of dealing with the process. He would, in other words, be able to intervene intelligently. Thus, ever since the days of Schleiden and Schwann, most medical investigators have stood at the door of the cell, the ultimate indivisible biological unit of function and process, and have sought to pry open that door to win the secrets it has denied. All such investigators had best heed the geneticist if, in returning again and again to the attack on that door, they would be guided to the same door.

The basic philosophical approach of modern nutrition does not, however, seem to be of this kind. The nutritionist is an environmentalist—and he has some successes under his belt to show for it. In the days before the recognition of vitamin D, for example, there were competent studies which provided evidence for the opinion that rickets was a disease under the influence of heredity (see Hess, "Rickets, Osteomalacia, and Tetany," 63). The newer knowledge has not invalidated those studies one whit, but the knowledge that such a factor as vitamin D exists has made it possible to devise a new environment in which hereditary factors are of less and less importance as far as the incidence of rickets is concerned. What under one environment was unfortunate heredity has been circumvented by providing a new environment containing vitamin D. This was no small victory, and it was accomplished in the face of complete ignorance, which exists to this day, of the cellular biochemistry in which vitamin D participates in normal calcification. The pragmatic condition for the experiments which led to this happy end was that the animals used were genetically constituted so that they were plastic enough to respond to the environment. (Diet, after all, is part of the environment, a part with which every animal certainly comes into intimate contact.)

If the *desideratum* of nutritional experiment is plasticity to the nutritional environment, then it is important that there be an understanding of the genetic conditions under which such plasticity is achieved. I would be bold enough to suggest that genetic heterogeneity is necessary for such plasticity. It should be remembered, however, that there are limits to genetic heterogeneity. There is no escape from the consequences of the evolutionary history of a species. Many biological characters seem to be settled irrevocably and no range of variation, either in genetic or environmental circumstances, seems to indicate that there is any hope of affecting such characters. Under such circumstances the strategy of the cellular interventionist is the only strategy left. To return to nutrition and resistance to infection, the range of variation in host response to disease indicates that there is still time left on the evolutionary clock to make use of this other strategy, the strategy of environmental circumvention, before

the slow grinding of the irresistible processes of natural selection removes the decision from the environmental, and man-controllable, world and entrusts it to the world of heredity.

With reference again to the nutritional aspects of the paper of Schneider and Webster, the experiments showed that mice outbred on a diet of wheat, whole dried milk and salt were more resistant (about 25%) than mice fed a "synthetic" diet containing the known vitamins essential for good growth and maintenance. The superior resistance supported by the natural foodstuff diet was traced to the whole wheat, when this component was added to the synthetic diet. The addition of whole dried milk to the synthetic diet was without effect.

IV. NUTRITION AND ACTIVELY ACQUIRED RESISTANCE TO INFECTION

It is a curious thing that, although the literature on nutrition and natural resistance to infection is so abundant, studies on the relationship of nutrition to actively acquired resistance, either naturally acquired or artificially induced, are sparse indeed. Practically the only papers devoted to the subject are those in the field of superinfection with nematodes, such as the report of Spindler (64) who found that vitamin A-deficient rats, following a second infection with *Nippostrongylus muris* larvae, continued to expel more eggs for a longer period of time than did controls on a "normal" diet and subjected to a similar second infection. It is difficult to judge from this report whether vitamin A was specifically responsible for this phenomenon.

Watt (48), using *Nippostrongylus muris* as an infecting nematode, found that, when rats on a "normal" diet were hyperimmunized by means of serial infection with 2,000 larvae and then placed either on a low thiamine diet or the same diet with adequate thiamine, after 68 days the low-thiamine rats were less immune than the adequate-thiamine controls. The immunity was challenged in this instance by a single dose of 2,000 *N. muris* larvae and the worms in the intestines were counted 12 days later. The low-thiamine rats had ten times as many worms as the controls. The size of the worms was not measured.

V. NUTRITION AND PASSIVELY ACQUIRED RESISTANCE TO INFECTION

Here again the literature is scanty for both naturally acquired (congenital) and artificially induced passive resistance to infection. Watt (48) found that hyperimmune serum obtained from thiamine-deficient rats was less potent than hyperimmune serum from rats on adequate thiamine in conferring resistance to normal rats when these sera were administered intraperitoneally concurrently with a test infecting dose of *N. muris* larvae. Similar findings obtained in the instance of riboflavin deficiency.

VI. NUTRITION AND PROCESSES REGARDED AS CONTRIBUTING TO RESISTANCE TO INFECTION

Thus far attention has been paid to experiments in which infective processes themselves have actually been invoked in the experimental procedures, and the infectious process has been part of the phenomena actually observed. Immunology, however, has set forward certain biological processes, the operations of which are represented as part of the ability of the host to resist infection. Among these processes are those of antibody formation of various kinds and the special activity of certain cells such as the phagocytes and the reticulo-endothelial system. In dealing with the relation of nutrition to these processes, it must be remembered that these relations will be valid for resistance to infection only as completely as these processes can be shown to be functionally important for the specific host and infection to which attention may be directed.

1. Nutrition and Antibody Formation

Werkman (65) found that rats and rabbits deficient in vitamins A or B (complex) were equally as capable of producing agglutinins, precipitins, hemolysins or bacteriolysins as control animals receiving these vitamins. Similar findings were encountered for the pigeon deficient in vitamin B (complex).

Greene (66) found that vitamin D did not influence antibody formation in the rabbit, although there was some slight indication of lowered titers in A deficiency.

Zilva (67), in pioneer work published in 1919, used rats and subjected them to the following dietary deficiencies: Low Fe, Ca, K, Na, Cl, and P; low or incomplete protein (8, 12% casein, 18% gliadin); vitamins A, B (complex) and C. He found no changes in the capacity to form agglutinins or amoebocyte, except in the low P diet. In guinea pigs he found no change in complement, agglutinins or amoebocyte in underfeeding or in scurvy.

Cannon, Chase and Wissler (68) have presented data recently to show that rabbits, on a diet of carrots and protein-free mixture of sucrose, starch, lard, salts and a purified "roughage" ("Ruffex") and subjected to plasma-apheresis, exhibit a decreased capacity to form agglutinins to *Eberth. typhosus* vaccine and *Salmonella paratyphi* vaccine. Although these workers would attribute the results to protein insufficiency, the fact that the experimental diet was deficient in many other nutrients makes such an interpretation doubtful. Further work along these lines appears to be indicated. There is no denying that Cannon *et al.* speak eloquently for the *plausibility* of their views, but plausibility is not *proof*.

2. Nutrition and Phagocytic Activity

Findlay and Mackenzie (69) concluded that normal phagocytic activity

(*in vitro* test) in vitamin-deficient rats was not depressed. Vitamins A, B (complex) and C were tested. Werkman (70) investigated phagocytosis in rats and rabbits with vitamin A and vitamin B (complex) deficiencies. The *in vitro* determination of phagocytic indices in normal or immune animals did not reveal any effect of the vitamins. When determined *in vivo*, slight differences could be found both in non-immune and in immune animals subjected to vitamin A or B deficiency. "As the differences were small, their significance from the standpoint of disease resistance is not to be overemphasized." Werkman finally concluded "that the depression of the phagocytic activity does not result through a failure of the animal to elaborate opsonins, but as the result of a depressive agent acting on the phagocytic mechanism. Temperature may be of significance in this respect, as the body temperature is greatly lowered during vitamin starvation."

Recently Cottingham and Mills (71) have reinvestigated the question of whether a vitamin deficiency severe enough to retard growth produces a reduction in phagocytic activity. Thiamine, riboflavin, pyridoxine, pantothenic acid, choline, ascorbic acid and a combined deficiency of vitamins A and D are all implicated. Inositol and p-aminobenzoic acid are without effect. Protein deficiency also depresses phagocytic activity according to Mills and Cottingham (72). The statistical basis for the observations made by these investigators is not quite clear. There were no inanition controls included.

3. Nutrition in Relation to Serum-Complement

Ecker *et al.* (73, 74, 75, 76) proposed that a correlation exists between ascorbic acid and serum-complement in guinea pigs and in human beings. However, these investigators may have been misled in accepting "average" values in the determination of complement, for Kodicek and Traub (77) were able to show that when the experiments were repeated and the data subjected to statistical analysis, no such correlation was observed. The careful investigations of Spink, Agnew and Mickelsen (78, 79) likewise failed to bring to light any evidence that ascorbic acid and serum-complement were in any way related. Spink, Agnew, Mickelsen and Dahl (80) have reported experiments to demonstrate that vitamin C has no connection with the bactericidal action of human serum.

VII. STRATEGY AND PROSPECTS

A survey of the minutiae involved in a consideration of the possible relationship of nutrition to resistance-susceptibility to infection, even as cursory as this survey has been, cannot but have a chastening effect on the most sanguine individual. The permutations and combinations of different hosts and different pathogens present a staggering array and the various biological bases of resistance-susceptibility add elements of complexity.

It is all too evident that attempts to come to grips with the problem are harassed by a lack of agreement on and, more fundamentally, understanding of the biological aspects of the infection process. In all truth, most of the experiments in this field are contributions to a kind of phenomenology—a kind which is part of the painful fumblings which seemingly must be undergone until at last a theory, even a tentative and possibly incorrect one, has been drawn which has a base broad enough to justify its utility in lending meaning and direction to attempts at further understanding. No such theory exists, and it is too early to expect one to be drawn yet; but in order to avoid scientific nihilism and to provide a target for criticism where none now exists, a sketchy hypothesis may be essayed while a recapitulation is made of the more general aspects of the survey which has just been concluded.

When an infectious agent is presented to a potential host, the first important consideration which will dictate whether or not infectious disease will result, is whether the host possesses the biological attribute of "susceptibility." For a given infectious agent, certain species will have such an attribute while other species will lack it completely. This is the cornerstone upon which all that follows is built. Now, for a given infectious agent, whether a particular host will or will not have the property of susceptibility is determined by the same consequences of evolution that determine whether a species has or does not have any other biological character upon which one may want to turn his interest. "Susceptibility" is, therefore, a biological character, like having scales instead of feathers, or gills instead of lungs, with this difference, however: the degree of variation of susceptibility within a species, to a given agent, is great enough to suggest that the evolution of this character is in mid-flight and that the final decision has not been reached. The pressure of natural selection has worked more slowly, or the introduction of the infectious agent has been, in evolutionary time, more recent than, say, has been true in the case of snakes to whom evolutionary forces have presented the fiat, "Keep your scales." It is almost as if evolution had decided in favor of scales for snakes but had not yet made up its mind as to whether all mice should be susceptible to *S. enteritidis*.

There is, then, evidence for treating susceptibility to infection as a biological character involved in evolutionary processes. It is of prime importance, therefore, to consider the genetic basis of susceptibility for the same reasons that genetics is at the bottom of an understanding of evolution. At first sight this appears to be a surrender of susceptibility into the hands of the geneticist as far as manipulation of the character is concerned, but this is not so. The infant science of physiological genetics has accumulated evidence enough to show that a gene does not operate in a vacuum, and whether or not it achieves a full expression of the biological character it sets out to determine is dependent upon 1) the myriad other genes with

which it finds itself in association and 2) the environment. The importance of these contingent conditions is not always the same and may range from being decisive to ineffectual. In fact, when a particular gene finds itself in a population of other genes (due to the processes of evolution) which uniformly allow the first gene to express an observable character, there is then an example of the classical gene about which the geneticist has succeeded in educating everyone. This is the instance of the gene for "brown eyes" and its dominance over the gene for "blue eyes," for the "color gene" dominant over the "albino gene." Although the original preoccupation of geneticists with these sharp, clearly observable characters has been well publicized, the field of biology in general has remained conspicuously unaware that the geneticists themselves no longer regard so fundamental a property as "dominance" as an innate and unalterable property of a gene (see Fisher, "The Evolution of Dominance," (81)). In one population of genes, a given gene may operate as a dominant; in another the same gene may operate as a recessive. For a heterozygous individual this is a crucial matter. If the given gene determines, say, susceptibility, then this individual would be susceptible, or insusceptible, as the circumstances set forth above would dictate.

All this is still the province of geneticists; and all is manipulable by the method of operation of the geneticist, *i.e.*, breeding. However, what is of interest here is the manipulation of the environment, specifically, diet. It must now be evident that diet will be of influence in the determination of a character in those circumstances where the gene population in which a particular gene is operating is of such composition as to allow a chemical factor in the diet to be decisive in its influence upon the gene. In one case it has been seen how the genes themselves in their myriad interactions and synthetic activities provide the micro-environment of the cell favorable for the expression of a given gene. In these circumstances it would be a matter of complete indifference to the success of this particular gene whether the animal involved did or did not consume a diet containing the important chemical factor. However, when the gene population fails to provide this decisive micro-environment, then it becomes of first importance whether the macro-environment (diet) supplies the decisive chemical factor. Under these conditions success attends the gene when one diet is fed; failure when it is lacking.

Without defining the specific make-up of the gene population we have tried here to picture briefly the circumstances under which the effect of diet on a character is important, and those under which it is trivial. Two questions now present themselves: 1) How can one manipulate genetic constitution experimentally so as to provide material suitable for studying the effect of diet on a given character? 2) What importance will the dietary factor have in the natural world? The questions will be considered separately.

1) In detailing the genetic manipulation necessary to provide material amenable to nutritional influence for a given character, one is on thin ice

indeed. As has been mentioned previously, nutritionists have thus far been busy enough with nutritional problems as they have found them, but if one insists on dealing with nutrition and resistance-susceptibility, then he will have to deal with the genetic circumstances under which this character is found and, if possible, in order to facilitate further studies, it may be desirable to increase the amenability of the material to nutritional influence. In our own experiments we have observed that inbred, selected, genetically homogeneous stocks of mice are independent of diet as it affects natural resistance, while the same test diets were effective in altering the resistance of an outbred, genetically heterogeneous stock of mice. Naturally, we would like to enhance this effect still more and the obvious limitation in striving for genetic heterogeneity is the variation which was existent in the population of laboratory mice at the start. When one recalls the past history of laboratory stocks of mice, one is made uncomfortably aware that right now we are in possession of only a small fraction of the genetic heterogeneity originally part of the species in the wild. Nutritional experiments with wild genotypes might thus yield information on this question and at the same time illuminate the second question which can now be considered.

2) What importance does a dietary factor assume in the natural world after its recognition by laboratory experiment? The only true answer will be obtained by trial, but it is not unreasonable to assume that those nutritional factors will be of certain importance which are recognized by experiments which in themselves contain the pertinent elements operative in the natural world. We must therefore learn to expect some disappointments in repairing to the natural world for trial of laboratory-won information. That information may bear the stamp of the consequences of the man-made manipulations to which the original experimental material has been subjected. For the subject at hand it is possible to be more specific: It is not enough to bring mouse (or any other laboratory animal) and infectious agent together in order to study the nutritional aspects of infection; it is important to consider *what kind* of mouse as it reflects the genetic consequences of evolution and man's domestication. We may, I think, be encouraged that nutrition does have a significance for infection in the natural world by considering that, in order to make the rôle of nutrition obvious in experiment, it is necessary to introduce genetic heterogeneity. This fact leads away from the specious "control" of the laboratory back to an appreciation of the delicate balance of forces in the world of nature.

If there is any merit in this view of the relation of nutrition to biological characters, what then will be expected of nutrition and susceptibility to infection? Even in a single species and for a single infectious agent, an array of circumstances as diverse as the following may be expected:¹⁵

¹⁵ For simplification, susceptibility will be treated as though controlled by a single gene.

- 1) In some animals genetic constitution will be such as to allow the complete expression of susceptibility independently of diet. (The homozygote, or the heterozygote in the appropriate influential gene population.)
- 2) In some animals genetic constitution will be such as to leave the issue of susceptibility capable of influence by diet. (The heterozygote in the appropriate indifferent gene population.) On "normal" diets susceptibility will be achieved. Deficient diets will decrease the achievement of susceptibility (*i.e.*, induce "resistance") to the degree that the specific dietary factor is involved in the expression of the gene. The delayed incubation time in thiamine deficiency in poliomyelitis is probably an effect of this order. Dietary factors more directly concerned with susceptibility have yet to be found, although they have not been sought in the most likely place, the world of natural foodstuffs.
- 3) In some animals the genetic constitution will be such as to fail to provide susceptibility under any circumstances. (The homozygous state of the allele of "susceptibility," or, in another species, the total lack of any such allelic system; the basis, probably, of species insusceptibility.)

After one has dealt with susceptibility, it is permissible to treat of "resistance." "Resistance" here is to be distinguished from "insusceptibility," the former following upon an initial interaction with the infectious agent, either dynamically and simultaneously in the early stages of infection as in "natural resistance" or by the procedures of "acquired resistance." In any event, the ability to respond to the effects of infection in a defensive way is as much a biological character as the initial ability to respond to the infectious agent itself. Hence we may expect to find relations for nutrition to "resistance" to be influenced by the same genetic considerations as those outlined above for susceptibility. The experimental evidence indicates that such dietary factors capable of influencing at least natural resistance do exist. These factors are found in the world of natural foodstuffs, are at present chemically uncharacterized, and are apparently different from the recognized vitamins.

In summary then, subject to the important limitations of genetic constitution of the host, it may be said that nutritional factors may contribute either to susceptibility or to resistance to infection. What any one diet will do may well be but a resultant of these opposed forces. The nutritional factors themselves may be divided into two categories: 1) primary factors and 2) secondary factors. The primary factors, profound and direct in their influence, are yet to be isolated from natural products. The secondary factors, ranging from those weak in their influence to completely trivial effects, comprise most of the nutritional essentials recognized at the present time.

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Manifestations of Nutritional Deficiency in Infants

By F. W. CLEMENTS

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I. INTRODUCTION

Satisfactory nutrition, at any age, but especially in infancy, is a complex process depending upon an adequate supply of all nutrients. The vast expansion of knowledge of vitamin deficiencies during the last two decades has tended to focus attention on these, and to obscure the fact that those nutrients which provide the sources of energy and the elements for growth, together with water, are also of primary importance for the infant.

In this review, therefore, the effect on the growth and health of the infant, of undernutrition, and of deficiencies of water, protein and certain minerals, as well as of the vitamins, will be considered.

Furthermore, the growth and development of an infant is a continuous process which commences with the fertilization of the ovum, progresses through the intrauterine existence, is subjected to violent changes at birth and is sustained in postnatal life. For it to be satisfactory, an adequate supply of all nutrients is necessary during both prenatal and postnatal life.

The prenatal phase has been the subject of considerable study during the last 15 years. Foremost among the investigators is Needham (1) who, although he has worked mostly with avian material, has nevertheless contributed much toward a fuller knowledge of human fetal nutrition. Both Huggett (2) and Windle (3) have covered various aspects of the problem in reviews.

The effect of inadequate supplies of one, or several, nutrients on the health, birth weight, and 'birth vigor' (4) of the newborn infant, has been studied by a number of workers.

It has been shown by many workers that the state of nutrition (5, 6, 7, 8, 9, 10, 11) and even the level of dietary intake of the mother during preg-

nancy (12, 13) do not affect the birth weight or length, although Gerschenson's (14) findings do not agree with this.

Investigations by Lady Juliet Williams in Wales (15, 16, 17, 18, 19, 20), by a group of workers in Toronto (21-23) and by the People's League of Health in London (24) have all drawn attention to the possibility of a close relationship between inadequate diet during pregnancy and the incidence of still-births and miscarriages, the complications of labor and the postnatal health of the infant.

Further, Mrs. Burke in Boston (25-27) and Cameron and Graham in Glasgow (28)—using the questionnaire in retrospect method—found a relationship between the maternal diet during pregnancy and the health of the infant at term.

The whole question of the influence of prenatal maternal diet on infant health has been recently reviewed by Warkany (29). For the present review it is important to recognize that the level of maternal diet may exercise a profound effect on the health of the fetus, and this fact alone warrants an inquiry into the possible variations in the level of the fetal stores of each of the nutrients in the newborn infant.

II. UNDERNUTRITION

Undernutrition results from insufficient calorie intake and may occur at any time during infancy and in both breastfed and artificially fed infants. In the latter class of subject, the undernutrition may arise from the use of too weak a milk mixture, but, in the opinion of Moncrieff (30), it is more frequently the result of not enough food in both breast and artificially fed infants.

While breast feeding is to be preferred to artificial feeding, it is not in the best interests of the infant to persist with it without offering a supplement of a cow's milk mixture if the secretion of the mammary glands is insufficient to meet the requirements of the infant. This, unfortunately, is too often the cause of undernutrition in breast fed infants.

Moncrieff has drawn attention to the fact that artificially fed infants who suffer from undernutrition do so because the calorie value of the mixture on which they have been fed has been calculated according to the child's actual weight which may be several pounds below the average for its age. Thus a vicious circle of chronic starvation is initiated. Not infrequently chronic infection leads to anorexia and this to undernutrition.

There is no special pathology or pathological biochemistry in these cases.

Clinical Manifestations of Deficiency

The onset is insidious and is without characteristic signs. If the weight has been recorded regularly, a steady failure to gain at the normal rate is

the usual finding. If the infant is brought for medical examination and there are no previous weight records it is usually found to be several pounds under weight for its age and in relation to its birth weight.

The infant looks pinched and miserable, and often has a bluish tinge in the mucous membranes of the mouth and eyes. The natural rosy tint of the skin is missing and there is a marked pallor. The skin is dry and lies loosely over the subcutaneous tissue; the muscular tone is poor and there is undue coldness of the extremities. The fontanelle is frequently depressed and there is shrinkage of the tissue about the eyes, leaving them large and staring. The tongue is usually dry and coated and the abdomen frequently distended.

A question to the mother will reveal that the infant has for the previous few weeks passed less than average amounts of urine and that constipation is severe and persistent. Without treatment this condition would progress to the marasmic state described so vividly in text books.

III. PROTEIN

Adequate supplies of protein are necessary for satisfactory health and in infancy those proteins rich in the essential amino acids are indispensable for growth.

1. Physiology

In the intestinal tract, proteins are converted into amino acids and these easily pass through the gut wall to the blood stream. Little or nothing is known of the behavior of amino acids once they enter the tissue cells. The end result is that they form tissue proteins, but whether all cells can synthesize all their protein requirements has not yet been demonstrated. However, it is well recognized that the liver is the principal site for the manufacture of plasma proteins (31).

Albumin and globulin are the principal proteins in human plasma, the main function of the albumin fraction being to maintain a balance between water and electrolytes in the blood and in the body tissue.

Ray and Phatak (32) give a value of 7.0 g. per 100 cc. for the total plasma protein of the newborn infant. Hickmans *et al.* (33) found a range of 4 to 7 g. per 100 cc. during the first two weeks of full term infants, with the majority of the readings occurring below 6.0 g. In full term infants over two weeks of age these authors recorded values ranging from 4.7 to 7.4 g. per 100 cc. The value for premature infants during the first four weeks ranged from 3.7 to 5.4 g. per 100 cc. Rennie (34) found a range of 6.04 to 8.0 g. per 100 cc. for infants 3 to 23 months of age, with an average of 7.08 g. per 100 cc.

In the infant, protein is required for building additional cells, the essen-

tial process in growth. If the diet contains adequate supplies of protein, some will be available for storage in the muscles and, as every gram of protein carries with it approximately 4 grams of water (35), the deposition of protein in a muscle builds up the volume of that muscle and, hence, its tone.

2. Sources of Protein in Infancy

The developing fetus obtains its nitrogen requirements from the maternal blood in the form of amino acids which, being highly diffusible, pass easily through the placenta. During the last month of pregnancy, the daily deposition of protein in the fetus is nearly twice as great as it is during the last three months and four times greater than the deposition throughout fetal life (2). Thus, the mature infant at term has a reasonable reserve of protein, mainly in its muscles, which contain approximately 300 g. of protein per liter of water (36). The concentration in liver is higher, being 450 g. of protein per liter of water. Stearns (36) has shown that the full term fetus at birth contains about 60 g. of nitrogen; Shohl (37a) gives 55 g.

Human milk contains from 1.1 to 1.7% protein and, thus, breast fed infants may obtain from 5.5 to 17 g. of protein per day. The intake of artificially fed infants will depend upon the formulae used.

Zahorsky (38) maintains that infants tolerate a high percentage of protein and there is little danger of giving too much milk protein.

3. Biochemical Pathology of Deficiency States

When the diet contains inadequate supplies of protein, the reserves are drawn upon to supply the needs of the essential tissues, particularly the blood (39). The body endeavors to keep the concentration of plasma proteins within the normal range because of its important effect upon osmotic pressure of the tissue fluids. There is, however, a lower limit below which the reserves cannot be withdrawn and, when this is reached, the protein level in the plasma falls, reducing the colloid osmotic pressure of the blood and thus permitting an escape of fluid from the blood into the extracellular spaces. Dodd and Minot (40), who investigated the albumin, globulin and total protein in cases of edema in infants, found low values for albumin in almost all cases. The total protein was also low in the majority, although in a few it was normal, owing to a high globulin value, values as low as 1.8 g. per 100 cc. being obtained for the serum albumin.

4. Clinical Manifestations of Deficiency

Fully breast fed infants do not as a rule suffer from protein deficiency, for, if the quality of the milk is low, so also, as a rule, is the quantity, and the infant presents signs of undernutrition before those of protein deficiency.

Infants who have been fed on insufficient quantities of a badly balanced artificial diet are, however, likely to suffer from protein deficiency. The point is frequently made that nutritional edema in children and adults occurs with a diet low in calories and with insufficient protein (41d, 42). The small quantity of protein present in the diet, which might suffice if calories were adequate, is used for energy production. Although this general principle holds for infants, it is possible for clinical manifestations of inadequate protein intake to develop when the diet is grossly deficient in protein, particularly of animal origin, though adequate in calories.

The first sign of protein deficiency is loss of weight or, as is more likely, failure to gain in weight at the normal rate. The other outstanding feature is poor muscle tone. The muscles, particularly those of the legs, are thin and fail to fill out the tissues beneath the skin. It is our experience, that, in an otherwise normal baby, poor muscle tone is commonly associated with inadequate protein in the diet (43).

Marriott and Jeans (44) are of the opinion that when the protein content of the diet is insufficient "growth is slow, resistance to infection is decreased, the musculature becomes flabby."

Infants, even with advanced protein deficiency, do not develop the edema typically seen in adults, but become pale and doughy-looking owing to waterlogging of all tissues. When a balanced diet is reestablished, they lose weight and become noticeably thinner (45). The advanced form may occur at any age but, in our experience, it is most likely to happen after 12 months of age. Investigators in Europe during the war and its aftermath have recorded that famine edema is rare in persons under 15 years of age (46).

It is interesting to note that, in the description of pellagra in native children in Africa, several authors (209, 245, 248) have mentioned edema. It is almost certain that, in addition to niacin deficiency (*q.v.*), the infants were also suffering from protein deficiency.

Premature infants may suffer from an insufficient supply of protein. Miller (47) maintains that in the premature infant the alimentary tract is immature and that the development of the gastric mucous membrane bears a direct relationship to the birth weight. Crosse (48) believes that the premature infant has difficulty in digesting the protein of cow's milk while Levine (261-263) has observed that the metabolism of certain amino acids which is incomplete in some premature infants, is completed by an increased vitamin C intake (*q.v.*). This subject has been discussed at considerable length by Mitchell (49).

Good growth response has been obtained by several workers who have given premature infants additional protein (50, 51).

IV. WATER

An adequate intake of water is essential in infancy, not only for the satisfactory metabolism of the various nutrients, but also for life itself.

1. Physiology

The water content of the individual varies with age. In the month-old fetus it represents about 90% of the weight, at birth it has fallen to between 75 and 80% (2, 37b), while in the adult it comprises 72% of the body weight (37b). The water within the body is divided between the blood stream, the cellular tissue and the intracellular spaces. The ratio of the quantities in each of these varies at different ages. The following are the ratios (from Stearns (36)):

Age	Water Percentage Distribution	
	Intracellular	Extra-cellular
24 Weeks fetus	27.5	58
32 Weeks fetus	29	51
Birth	31	43
Adult	41	20

Stearns (36) has pointed out that "whereas in the adult the intracellular water of musculature accounts for nearly half the total body water, at birth only 18% of the water content is so found."

The importance of adequate supplies of water in the diet of an infant is shown by the results of metabolism experiments, which demonstrated that a 5 kg. infant who gains 25 g. per day does so by the addition of 3.15 g. protein, 3.0 g. fat and 18.0 g. of water (52).

2. Sources and Requirements

The infant is dependent on others for its water supply, and Marriott (53) is of the opinion that the newborn breast fed infant needs fluid in addition to that supplied in breast milk. Feldman (52) considers that infants up to 3 months of age require 3 ozs. of water per lb. of body weight, those 3 to 6 months require $2\frac{1}{2}$ ozs., and those 6 to 12 months require 2 ozs. per lb. of body weight.

3. Pathology of Deficiency States

Insufficient consumption of water or excessive fluid loss will result in anhydremia. This subject has been fully reviewed by Marriott (53) and by Rowntree (35). Anhydremia may develop relatively slowly as the result of water starvation, or rapidly following upon sudden loss of fluid. In either case the most important change in the body is hemoconcentration. This is characterized by a rise in blood solids, in serum protein and in red blood cells, and in hemoglobin value (54).

Wiley and Wiley (55) have studied the salt balance in the body during dehydration and found that in slight cases (with losses up to 1.5% of body weight) no changes occurred. More marked dehydration was accompanied by a loss of potassium, sodium and chlorides.

Associated with all these changes is a decrease in blood volume due mostly to a reduction in plasma. This is followed by a compensating constriction of the finer blood vessels with an attendant diminution in blood flow through the extremities (53).

4. Clinical Manifestations of Deficiency

Mild degrees of inadequate consumption of water—a comparatively common occurrence during hot weather in young infants, many of whom reject water when it is offered to them—are characterized by restlessness and constipation. A depressed fontanelle is a frequent observation in these cases.

In the newborn infant inadequate water intake, especially in hot weather, may lead to the development of high fever (called 'dehydration fever' by Bakwin (56)). The temperature falls after the administration of small amounts of fluid (53).

Increased restlessness, sleeplessness and drying of the mucous membranes are the first signs following large water losses or a markedly inadequate intake (57). Untreated, the child's condition deteriorates, the skin becomes dry, takes on a grey wrinkled appearance and loses its elasticity, the pulse becomes rapid and small, and ultimately death may supervene.

V. VITAMIN A

The physiological effect of vitamin A is brought about by vitamin A and at least nine other compounds known as provitamins A. All these compounds are soluble in fat. The chemistry of these compounds has been reviewed by Rosenberg (58a).

1. Physiology

The infant obtains its vitamin A requirements as both carotene and vitamin A. Bile is essential for absorption of carotene (59), which is probably transferred across the wall of the gut as a water-soluble diffusible compound (60), and carried to the liver where it is first stored (61) and then converted into vitamin A under the action of an enzyme, carotenase (62).

The extent to which carotene is capable of meeting the vitamin A requirements of human beings is at present under investigation by a committee of the Medical Research Council of Great Britain. "Children apparently utilize carotene very badly" (63).

Vitamin A is absorbed in the intestine and passes through the gut wall in

the form of the alcohol and, at least for infants and children, bile is essential for the absorption (64).

Vitamin A is stored in the liver and the level of storage is dependent upon the previous diet and the absence of disease (65). The stores in young infants are low (66). Both the hepatic cells and Kupffer cells store vitamin A (66, 67).

Ellison and Moore (65) found definite increase in liver stores between the 5 weeks to 3 months period and the 4 to 8 months period, and they consider that the reserve reaches a more or less static level during the later stages of lactation after vegetable purées have been added to the diet. In the normal healthy man vitamin A is never excreted (68), but it may appear in the urine in disease conditions. Both carotene and vitamin A are transported through the body by the blood, where the level is fairly constant, only temporary rises occurring following absorption from the intestines.

May *et al.* (69) have developed a reliable method for the determination of vitamin A and carotenoids in small amounts of blood. Normal well-nourished infants were found by these workers to have levels of vitamin A in their blood ranging from 47.0 to 164.7 International Units per 100 cc.* The values for carotenoids were influenced by the addition to the diet of foods rich in carotene. Lewis (70) found that the vitamin A content of the blood of 64 infants who had received an average diet lay between 45 and 141 International Units per 100 cc., whereas that of 62 infants who had received in addition a daily supplement of 17,000 International Units of vitamin A lay between 50 and 141 International Units per 100 cc. These results suggest that the addition of supplements of vitamin A to the diet of normal healthy infants does not influence the vitamin A concentration in the blood.

In contrast to these findings are the observations of Henley *et al.* (71), who showed that the vitamin A content of the plasma of premature infants at 3 weeks of age was related to the intake of vitamin A during the preceding 3 weeks and was unrelated to birth weight. The average value for those who had not received any supplement of vitamin A was 68 International Units per 100 cc. of blood plasma.

Lewis *et al.* (72) found a sharp drop (down to 42 International Units per 100 cc.) in the blood level of the vitamin during the first 48 hours of life and a rise to normal level (*i.e.*, about 68 International Units per 100 cc. of plasma) on the fourth day. They suggest that both failure of the liver to mobilize adequate quantities of the vitamin and the low intake were the salient factors responsible for this drop.

The function of vitamin A within the body is unknown, but it has been

* I have converted the figures given by the authors into International Units by the formula used by Henley *et al.* (71).

suggested that, because of its chemical structure, with five unsaturated bonds, it plays the part of an oxidation-reduction catalyst (73a).

2. Sources of Vitamin A in Infancy

During uterine life the fetus obtains supplies of vitamin A from the mother and comparatively large amounts have been found in the fetal liver, especially in the early months of pregnancy (74, 75, 76, 77). Some evidence has been produced which shows that the placenta can act as both a super-filter and a reservoir for vitamin A and carotene. High values have been found for the vitamin A and carotene content of the placenta, especially when the dietary level has been high (78), while several observers have recorded that both the carotene and the vitamin A content of umbilical cord blood was lower than the concentrations in maternal blood drawn at the same time (79, 80).

From its intrauterine existence, the newborn infant brings appreciable quantities of vitamin A stored in the liver. Amounts recorded extend from 25 to 1040 International Units per gram (70, 81, 82, 83, 84).

Henley and his co-workers (71) studied the vitamin A content of the liver of mature infants and of 16 premature infants all of whom died within 24 hours of birth from causes thought not to have influenced the vitamin A content of the liver. The vitamin A content of the liver from the former ranged from 195 to 419 International Units per gram of liver with an average of 303, and that of the latter from 44 to 376 International Units per gram with an average of 138. From these figures they calculated that the average vitamin A content of the whole liver in the mature infants was 39,994 International Units and in the premature infants 7,260 International Units. In general, the more premature the infant the lower the total supply of vitamin A in the liver.

Lund and Kimble (85) found that in the newborn infant the vitamin A content of the plasma was independent of the maternal vitamin A metabolism. Low maternal levels were not reflected by the fetus. Administration of excessive amounts to the mother elevated the maternal blood levels but did not affect the fetal blood values. The range of fetal plasma vitamin A was 24 to 79 International Units per 100 cc. (mean 49). The plasma carotene value of cord blood was found to be very low, the mean for 149 normal infants being 23 γ per 100 cc. (range 9 to 75 γ). Fetal plasma carotene was found to vary regularly with maternal plasma carotene. In general the former is $\frac{1}{10}$ th of the latter when it is average. This intimate relationship suggests the small but regular transfer of carotene from the mother to the fetus, and this the infant synthesizes into vitamin A.

Human milk is a good source of vitamin A which occurs both in the pro-vitamin form and as vitamin A. Portes and Varangot (86) found the range

of carotene and vitamin A values for the milk from women collected on the 5th to 8th day to be 38 to 165 γ (mean 78) and 125 to 220 International Units per 100 cc. (mean 174) respectively. Hrubetz and her co-workers (87) obtained an average of 331 International Units of preformed vitamin A per 100 cc., or 424 International Units when the activity of carotene was included, for milk obtained during the 2nd to 10th day of lactation. In later periods the total vitamin A averaged 270 International Units per 100 cc. of milk. Dann (88) did not find the human colostrum (632 International Units per 100 cc.) particularly rich in vitamin A, being only twice as rich as milk (346 International Units per 100 cc.), whereas colostrum of cow's milk is from 10 to 100 times richer than milk (89). The vitamin A content of colostrum was not increased in Dann's group of subjects by regular ingestion of cod liver oil during pregnancy.

From these figures it is apparent that human milk will provide from 2400 to 4000 International Units of vitamin A per day, dependent upon the milk consumption.

Fridericksen and With (90) investigated the effect of high maternal intakes of carotene and vitamin A upon the carotene and vitamin A content of the milk. Daily additions to the diet of 2 to 10 mg. of carotene were followed by increases in serum carotene, but there was no increase in the carotene or vitamin A content of the milk. A large dose of vitamin A, 120,000 International Units, did not affect the vitamin A of the serum but was followed by a significant rise in the vitamin A content of the milk. Results of a similar nature have been obtained by Hrubetz (87). This observation supports the suggestion that additional vitamin A in the diet in pregnancy should be as vitamin A and not as carotene.

The vitamin A potency of cow's milk is affected by the diet; pasture fed cattle yield a milk higher in vitamin A than stall fed cattle. The Medical Research Council of Great Britain (91) has accepted the following values—summertime, 140 International Units per 100 g.; wintertime, 70 International Units per 100 g. The vitamin A value of cow's milk has been discussed in a recent review (92). Thus the vitamin A potency of cow's milk in the diet of an artificially fed infant will vary from 420 to 1400 International Units, depending upon the formula and the age of the infant.

3. Pathology of Deficiency States

The pathological anatomy of vitamin A deficiency is essentially the same in man and in experimental animals. Eddy and Dalldorf (93a) have reviewed the pathological findings in *post mortem* examinations on 26 autopsied cases of vitamin A deficiency in infants.

Of special interest are two cases among those described by Frazier *et al.* (94), who traced the changes in the skin in cases of vitamin A deficiency in

subjects of various ages. One of their cases was an infant 69 days old whose skin showed slight hyperkeratosis of the surface epithelium. The epidermis in general looked to be atrophic although there were areas of beginning hyperplasia. One hair follicle contained a keratotic spine which extended beyond the mouth of the follicle. Other follicles showed considerable hyperkeratosis without spine formation. The sebaceous glands showed evidence of atrophy. The hypertrophy of the epidermal cells usually seen in adults in the same stage of the disease was not present in this case. Another case, aged 16 months showed well defined intrafollicular hyperkeratosis with distension of the follicles.

These changes in young children did not exhibit any perifollicular infiltration—the pathology responsible for the follicular eruption which apparently occurs only in persons who have attained sexual maturity.

Vitamin A and Vision. Vitamin A is essential for vision in dim light as it is necessary for the regeneration of the bleached pigments of the retina back to the original visual purple. For a full description of these changes see Wald (95, 95a), Hecht (96), Edmund and Clemmesen (97).

4. Biochemical Pathology of Deficiency States

In an attempt to find a biochemical test for the early diagnosis of vitamin A deficiency states, May *et al.* (69) studied the blood levels of both carotene and vitamin A in a series of infants who had had a very restricted consumption of food, with extremely low intakes of vitamin A. They found the carotenoids disappeared from the blood first; presumably these were withdrawn from the blood to the liver for conversion into vitamin A. A fall in the vitamin A level occurred sometime later. In their experience the earliest clinical sign of established deficiency of vitamin A—namely, cornified epithelial cells—did not appear until the bodily stores had been completely exhausted and the vitamin A had disappeared from the blood.

When vitamin A therapy was instituted, the level of vitamin A in the blood returned to normal in 10 days.

5. Clinical Manifestations of Deficiency

A review of the literature relating to clinical signs reveals two outstanding facts, namely, that the classical signs of xerophthalmia and keratomalacia are comparatively late manifestations of the disease and that these signs, particularly the latter, occur in subjects whose diet has been badly balanced, being deficient not only in vitamin A but in several other nutrients.

Deficiency of vitamin A produces changes in epithelial tissues, including the skin, mucous membranes of nasal and respiratory passages, urinary system, *etc.*, as well as in the conjunctiva and cornea. In infants and

children, while the first changes consist of epithelial metaplasia of the mucous membrane of the respiratory tract, patients are invariably brought for medical advice because of the ocular signs. Furthermore, Blegvad (98) observed that, whereas xerosis of the conjunctiva with hemeralopia most frequently occur in older children, keratomalacia is distinctly a disease of infancy and young children, and that affection of the cornea with xerophthalmia is very seldom seen in adults and the typical sudden necrosis of the whole cornea is observed only in infants and young children.

a. The General Signs. Most workers have reported wasting as a prominent feature of the disease (98, 99, 100, 101), but, as Mackay (102) has observed, it is impossible to determine whether this is due to the specific deficiency or to the generally bad diet. Blegvad (98) recorded a loss or a standstill in weight for some time prior to the appearance of the ocular signs in six infants who developed keratomalacia during a stay in hospital. This confirmed the observations of Ross (100) that a loss of weight preceded the objective signs.

b. Changes in the Eyes. In adults and older children night blindness precedes the objective signs (41a). A satisfactory technique has not been developed to record changes in dark adaptation in children under 2 years of age, so it is not known whether the same sequence of events occurs in infants as in adults.

In infants the eyelids are frequently swollen and infected (101), while some have a copious sticky discharge from the eyes when first seen. Photophobia has been reported as well as itching and burning and asthenopia (93a).

The first change in the bulbar tissue is irregular dryness (xerophthalmia) and loss of luster revealed when the lids are held open for a few moments. The diagnosis can be established at this stage by the discovery of keratinous cells in scrapings taken gently from the bulbar conjunctiva (101), or by examination of the cornea with the biomicroscope (103).

Parallel with these changes is the appearance of Bitôt's spots—first described in 1863 (104). The character of these spots would seem to be determined by the severity of the disease (105). It would appear that in acute cases they consist of spots, usually triangular in shape, though sometimes round or oval, in the palpebral fissure generally lateral to the cornea. Occasionally spots may occur on the inner side. They are usually described as resembling soap foam.

"In the babies observed by Forest and Wolff (106), Bitôt's spots appeared 10 to 15 days after the first signs of dryness of the conjunctiva" (102). Nicholls and Nimalasuriya (105) described the changes in the bulbar conjunctiva in several hundred children in Ceylon. In their cases the spots began as a "slight thickening and pigmentation of the conjunctiva of the

sclerotic" which progressed to a "heaped up accumulation of epithelial cells." "They resemble a piece of chalk paste striated with a pin." These authors found that these changes were very chronic but that they responded to large doses of vitamin A. No indication is given of the time taken for the development of these spots in these chronic cases.

As a sequence to the xerophthalmia when the deficiency is severe, changes develop in the deeper layers of the cornea. In young infants these changes may parallel the xerosis. Clinically, there develops in the cornea, cloudiness which may rapidly increase until the whole structure is quite opaque and soft (keratomalacia). This frequently happens in infants under 1 year (98). Ulceration of the cornea may be coincident with this change. The preliminary haziness and softness may rapidly lead to perforation of the cornea with all its sequelae. This is often the picture in infants under 3 months of age who develop eye lesions.

A high percentage of infants who develop keratomalacia are subsequently blind. Blegvad (98) found that of the infants who had keratomalacia in Denmark in the years 1909 to 1920 and survived, (21% died), 27% were totally blind and 24% had vision greatly impaired. De Haas (107) noted in Batavia that 20 to 30% of infants and children became totally blind and 10 to 20% blind in one eye.

In his extensive review, Blegvad (98) found that in older infants (*i.e.*, 12 to 24 months of age), death occurred in a shorter time after the appearance of keratomalacia than in younger ones (under 6 months). He concluded that the infection of the cornea in a younger child appears earlier in the disease than in older ones, and goes on to point out that vulnerability of the cornea declines with increasing age. This is supported by a general observation that keratomalacia is rare in adults.

c. *Changes in the Skin.* Phrynodermia, or follicular hyperkeratosis, has been described in adults as one of the characteristic signs of vitamin A deficiency (94, 108, 109, 110). Frazier (94) and May (111) have described skin changes in infants, the former in two cases, the latter in one. The skin was dry and scaly over the shoulders in an infant 55 days old, while in one 16 months old it was dry, loose and scaly, with many sharply pointed follicular papules on the upper extremities, the trunk and in the axillary folds (see pathology section).

d. *Other Systems.* Many infants when brought for medical advice have severe diarrhea—this may be a complication of the disease or it may be a manifestation of niacin deficiency (*q.v.*). Suffice it to say that dysentery and bronchopneumonia are the principal causes of death in infants with vitamin A deficiency (98, 107, 112, 113, 114).

Various aspects of the clinical features of vitamin A deficiency, mostly as they are seen in adults, have been described by Jolliffe and Most (115).

Although *pyelitis* has been reported in older children with vitamin A deficiency (112, 116, 117), it has not been recorded in infants or children under 2 years of age.

6. Relationship of Vitamin A Deficiency to Local Infections

An antiinfective function was attributed to vitamin A by Green and Mellanby (118, 119). The common experience of pediatricians is that children with clinically recognizable vitamin A deficiency are an easy prey to infections of the mucous membranes affected by the deficiency (see previous section). This aspect of the subject has been reviewed by Heilbron, Jones and Bacharach (92) who concluded "the importance of vitamin A supplies for the maintenance of normal mucosa, 'the first line of defense,' of the body against invasion of microorganisms, seems to be almost universally accepted."

The observations of Barenberg and Lewis (114, 120) demonstrate that, whatever may be the part played by vitamin A in preventing local infection by the maintenance of healthy mucosa, doses above that necessary to do this will not affect the incidence of local infections.

VI. THIAMINE

Thiamine, as the pyrophosphoric acid ester (cocarboxylase) is intimately concerned with carbohydrate metabolism, especially with the degradation of pyruvic acid.

1. Physiology

Thiamine is absorbed easily from the small intestine and probably also from the large intestine. The human body is not able to store thiamine in any appreciable quantity so requirements must be met by a constant supply in the food.

Thiamine is present in the blood chiefly in the form of cocarboxylase. The range of values for normal adults varies from 4.5 to 12.0 γ per 100 cc. (121-126). Knott (434) obtained an average of 5 γ per 100 cc. for the cocarboxylase value of blood in infants.

Thiamine is excreted in the urine and feces. Borsook and his co-workers (127) found that, after injecting 16 mg. of radioactive thiamine into a normal subject, 61% appeared in the urine and 11% in the feces six days after injection. Thus some thiamine is secreted by the intestinal mucosa or its accessory organs. The work of Najjar and Holt (128) indicates that, in some individuals, thiamine which is synthesized by microorganisms in the large intestine is absorbed. At the present time it is not possible to say how much of the thiamine in the feces is the unabsorbed portion from the

food, how much has been excreted into the bowel or how much has been synthesized in the bowel.

The range of daily excretion of thiamine in the urine of normal subjects, as observed by various workers, extends from 53 to 500 γ per 24 hours (73b). These figures were obtained for children and adults, and Knott (434, 435) obtained similar results for infants.

2. Sources of Thiamine in Infancy

Little information is available from which conclusions can be drawn concerning the stocks of thiamine in the newly born infant. Neuweiler (129) observed that the thiamine content of venous blood in the umbilical cord was higher than that of arterial blood, which supports the suggestion that the fetus obtains supplies through the placenta. The further observation by this investigator that, in many cases, the thiamine content of maternal venous blood and that of placenta and venous cord blood was similar, suggests that the placenta acts as a superfilter (1) for the transmission of thiamine and does not concentrate it as it may do for other vitamins.

Various estimations have been made of the thiamine content of human milk. Widenbauer and Heckler (130) used the thiochrome method to measure the free thiamine and obtained values ranging from 2 to 36 γ per 100 cc. (average 10) and Neuweiler (131) also used the thiochrome method to measure the total unphosphorylated thiamine and found values between 5 and 13 γ per 100 cc. Knott (132) obtained an average of 20 γ per 100 cc. for the thiamine content of the milk of 50 women.

The most extensive work in this field has been carried out by Slater and Rial (133) who found that the total thiamine content of normal human milk increased from 9.5 γ per 100 cc. in the third week of lactation to a maximum of 14.8 γ per 100 cc. in the twentieth week. They also found particularly low values, in the vicinity of 4 γ per 100 cc., for milk on the first to the seventh day of lactation. These workers found that the thiamine content of human milk is dependent upon the thiamine intake of the subject, but that it was not possible to raise it beyond 20.7 γ per 100 cc. by administering large quantities of thiamine.

A breast fed infant will thus obtain from 45 to 150 γ of thiamine daily, depending upon its age and the value of the maternal milk.

Cow's milk is richer in thiamine than human milk and Clements (134) has shown that a fully artificially fed infant may receive a diet much richer in thiamine than a breast fed infant of the same age.

Requirements of Infants. As a result of their determinations, Slater and Rial (133) suggested that the marginal requirement of the breast fed infant for thiamine is 0.36 γ for each non-fat calorie and that the maximum intake would be in the vicinity of 0.62 γ for each non-fat calorie. Thus an infant

who consumed 600 cc. of human milk would obtain a maximum of about 124 γ of thiamine, whereas the table of recommended allowances of the National Research Council of America (183) puts the requirements of infants at 400 γ per day.

3. Pathology of Deficiency States

The gross morbid anatomy of infants who have died from beri-beri is one of congestion, anasarca and heart failure (93b). The most characteristic changes are to be found in the heart. It is greatly dilated with but slight compensatory hypertrophy. The enlargement is most pronounced on the right side. The right auricle is huge with paper thin walls which are easily torn. The conus arteriosus is greatly dilated and Wenckebach (135) considers this pathognomonic of the disease.

4. Biochemical Pathology of Deficiency States

In the normal subject, glucose is indirectly oxidized to carbon dioxide and water through a number of stages. Two intermediate products in this process are lactic and pyruvic acid. In the normal individual, pyruvic acid is broken down under the catalytic action of cocarboxylase (136-139). In subjects with beri-beri this process is apparently retarded, for it has been shown (140-141) that pyruvic acid accumulates in the blood in these cases.

In beri-beri two biochemical changes occur: glucose is not completely combusted in all cells of the body and there is an accumulation of pyruvic and lactic acids. Sufficient evidence is not available to allow of a choice between these two happenings as the specific factors of changes in tissues in beri-beri.

In 1934 Takamatsu (142) extracted from the milk of women whom he considered to be suffering from thiamine deficiency a substance which Sato (143) subsequently showed to be chiefly pyruvaldehyde (the aldehyde of pyruvic acid). Platt and Lu (140) examined the milk of 5 women whose infants had had beri-beri and found an increase in the quantity of bisulphite binding substances—of which pyruvaldehyde is one.

Fehily (144-147) considers that the symptoms in cases of infantile beri-beri can be separated into two groups—those due to lack of thiamine and those due to the effects of toxic materials in the maternal milk. The characteristic heart lesion is placed by some workers in the latter category. This whole problem has been reviewed by Stannus (148).

Attempts have been made to use the level of excretion of thiamine in a 24 hour specimen of urine as a measure of thiamine deficiency, especially of subclinical states. Earlier workers considered excretions of less than 30 γ per 24 hours a sign of thiamine deficiency, more recently the figure has been set at 100 γ. In cases of beri-beri, figures in the vicinity of 10 γ and under have been obtained (73b).

Several tests, based upon the amount of thiamine excreted in a 24 hour specimen of urine or on the effect of a measured dose of thiamine on the level of urinary excretion (149-154, 435, 436), have been developed to assess the degree of tissue saturation. With one exception (435) all these tests have been applied to children and adults with the object of discovering subclinical stages of the disease. It is doubtful whether they will be widely applied to infant nutrition.

5. Clinical Manifestations of Deficiency

Thiamine deficiency in infants may occur as a partial deficiency or as frank infantile beri-beri.

a. Partial Deficiency of Thiamine. Clements (134) has described a condition of partial thiamine deficiency in breast fed infants due to suboptimal quantities of thiamine in the maternal milk, which in turn is due to insufficient thiamine in the diet of the mother. In a series of 150 cases studied at an infant welfare center some 8% showed evidence of partial thiamine deficiency. The majority of the infants commenced to develop signs at about 14 weeks of age.

The three symptoms characteristic of the condition are failure to gain in weight at a normal rate, constipation and vomiting. It is admitted that these symptoms are common occurrences in infancy but where they are due to partial thiamine deficiency, improvement occurs within a few days upon the administration of thiamine either to the mother or the infant. Coupled with these symptoms is a low thiamine content of the maternal milk, in the vicinity of 5.0 γ per 100 cc. (normal 14.8 γ per 100 cc.).

Cow's milk is approximately three times richer in thiamine than human milk, hence partial thiamine deficiency does not occur in artificially fed infants. Should the diet be generally inadequate, signs due to undernutrition (*q.v.*) will tend to overshadow any due to vitamin deficiency.

b. Infantile Beri-beri. Infantile beri-beri was first described by Hirota (155) in 1898 and since then has been reviewed and discussed by a number of observers (156-163, 144-147, 115). The following description is drawn partly from the works of these workers and partly from cases seen and treated by the author in New Guinea.

Infantile beri-beri occurs in infants who are breast fed or who have been breast fed to within a week or so of the onset of the disease. It can be shown that the diet of the mother is very low in thiamine and in many instances it has been reported that the mother herself has chronic adult beri-beri, although it is by no means always the case. The age of onset is between 3 and 5 months of age.

Bray (158) has described three forms of the disease, the acute, the chronic and the insidious form. A close scrutiny of Bray's case records did not reveal any justification for these three subdivisions. The first two types appear to

be stages in the development of the disease, while it is doubtful if the third form is truly beri-beri. The disease is relatively uncommon among western peoples and most descriptions have been of cases in Asiatics, who are reluctant to bring a child for medical attention unless it is seriously ill. Thus it is that prodromal signs or signs of the developing disease, are not seen. This, I am sure, explains the usual description given of the disease, which shows it to be an acute illness rapidly becoming fatal unless quickly treated.

The prodromal signs include a disinclination for food, peevishness, intermittent crying, restlessness extending to sleeplessness, associated with intermittent vomiting. During this stage the mother will often observe swelling of the abdomen. All these signs, at first mild in character, become progressively worse until the crying has changed to screaming and the vomiting has become persistent. The prodromal period lasts from 2 to 3 weeks. By the time the vomiting is persistent, edema has appeared, giving the child a plumpness. During the early stages of the disease the infant may actually gain weight owing to the developing edema.

Concurrent with the development of the edema is the appearance of dyspnea and cyanosis. The heart rate accelerates to 180 to 200 beats per minute with an increase in the respiratory rate as high as 60 per minute. During this time the edema has increased and has extended to the various serous cavities, with enlargement of the liver, thus placing a considerable burden upon the heart.

At about this time dilatation of the right side of the heart may be demonstrated by percussion and, because of the dilatation, the heart is twisted on its axis and the apex beat becomes diffuse and even disappears, being replaced by a curious wriggling, fluttering motion of the heart muscle (135). Wenckebach (135) has reported that "even in the most serious cases there is never any irregularity of the heart, no extra systoles, flutter or fibrillation." This is contrary to the observations of others (158) who noted irregularity in infants.

Fehily (147) believes the vomiting, restlessness, cyanosis, dyspnea, running pulse (last three signs associated with cardiac failure) are due to intoxication with pyruvaldehyde and its associated products while the anorexia, retarded growth, loss of weight and constipation are due to deficiency of thiamine.

As the disease progresses the screaming is replaced by aphonia and a peculiar cry which is considered characteristic of the disease. Alcantara (164) has examined infants by laryngoscopy and found the 'cry' was due to paralysis of the vocal cords, usually the left. This is the stage in which the mother frequently brings the child for medical attention. The congestion quickly spreads to the brain and a bulging fontanelle indicates increased intracranial pressure which soon makes itself manifest by retraction of the

head, general rigidity and muscular twitchings. Drowsiness develops and passes into coma and finally death. The picture in death is one of congestion, anasarca and heart failure.

Treatment introduced at any stage will produce spectacular results, a very sick infant can be transformed almost overnight by the injection of thiamine.

The outstanding difference between infantile beri-beri and the adult form is the prominent place occupied by the cardiac signs and the almost complete absence of involvement of the nervous system in the former.

6. Radiographic Appearance of the Heart in Beri-beri

Wenckebach (135) has described the appearance in a radiographic study of the heart. "X-Rays show not only an enlargement to the right but also a bulging of the left auricle in the upper left border of the heart. The left ventricle is larger than normal." Raschoff (165) observed this picture in an infant 4 months old.

7. Electrocardiograph Tracings

Raschoff (165) reported infantile beri-beri in an infant 4 months old. The electrocardiographic tracing did not show any abnormalities in conduction or in the contour of the individual waves.

VII. RIBOFLAVIN

Prior to 1938 no definite set of signs had been ascribed to deficiency of riboflavin in the diet. In that year Sebrell and Butler (166) published their findings of experimental ariboflavinosis produced in a number of women on a diet deficient in riboflavin. After from 94 to 130 days, 10 of the 18 women developed lesions which were cured by the administration of riboflavin.

Since that time a number of investigators (167-175) have reported the cure of typical lesions with pure riboflavin. However, little or no work has been carried out on riboflavin metabolism in infants, nor have many observations been made of deficiency states in infants.

1. Physiology

Riboflavin is widely distributed in animal and plant tissues and is probably present in all human tissue cells where it takes part in a number of enzyme systems (Rosenberg (58b) gives 13) associated with the intermediate metabolism of food, particularly carbohydrates.

The most important food sources of riboflavin are milk, yeast and meat products, especially liver. Riboflavin occurs in food as both a free and a chemically bound form, the latter occurring predominantly in vegetables and seeds.

The naturally occurring free forms are easily absorbed from the small intestine and excretion is through the urine, only the unabsorbed portion of a high intake appearing in the feces.

The riboflavin content of tissues is reasonably constant, for under conditions of optimal intake "saturation" is attained, and even in deficiency states there is not a marked departure from normal (see section 4).

The suggestion has been made that riboflavin plays an important role in the metabolism of the eye, especially of the cornea (73c). Bessey and Lawry (176) have investigated the riboflavin content of the two layers of the cornea of normal and riboflavin deficient rats. The figures they obtained for the cellular portion, which is the richer of the two, are of the same order as the riboflavin content of muscle (177).

2. Sources of Riboflavin in Infancy

Human milk contains appreciable quantities of riboflavin. Williams and his co-workers (178) obtained values ranging from 33 to 44 γ per 100 cc. for 7 samples from 3 subjects. Francis (179), at this Institute, recorded values ranging from 14 to 27 γ per 100 cc., while the average for 1500 samples analyzed by Kon and his colleagues (180) was 25 γ per 100 cc. Thus, it is apparent that an infant who consumed 600 cc. of milk would obtain from 78 to 468 γ (Francis' figures).

A number of observations have been made on the riboflavin content of cow's milk. Holmes and Holmes (181) obtained values ranging from 1.13 to 1.75 mg. per liter, while Kon (182) quotes values of 1.5 mg. per liter for pasture fed and 1.0 mg. per liter for stall fed animals. Thus, an artificially fed infant who consumed 600 cc. of cow's milk would obtain from 0.6 mg. to 1.05 mg. per day.

Requirements of Riboflavin in Infancy. The table of recommended allowances of the National Research Council of America (183) puts the requirements of infants at 0.6 mg. per day. The indications are that the average breast fed infant does not obtain anything like this amount, whereas it is probable that artificially fed infants do receive it.

3. Pathology of Deficiency States

Histological studies of the changes in arriboflavinosis have not been reported, although Wolbach and Bessey (184) have reviewed the changes in experimental animals, which are probably not dissimilar to those occurring in man.

4. Biochemical Pathology of Deficiency States

The urinary excretion of riboflavin has been studied in normal and riboflavin-deficient subjects. The urinary output of riboflavin in a normal person has been found to vary, figures of the following order having been obtained: 819 to 1250 γ per day (185), 320 to 350 γ per day (186), 500 to 800 γ

per day (187). An increased intake is followed by an increased excretion and *vice versa*. It has been suggested that the level of riboflavin excreted in the urine can be used to indicate deficiency. Fedder (188) claims that the amount of riboflavin per milliliter of urine in the fasting morning specimen is a reliable guide to the state of nutrition.

Several 'tests' have been developed in which the amount excreted in the urine after a 'test dose' of from 1 to 10 mg. is used to measure the degree of deficiency (189-191).

The human body has no special organs in which riboflavin is stored; comparatively large amounts are, however, found in the human liver and kidney, 16 γ per gram and 20 to 25 γ per gram respectively (177). Although the concentration in muscle is much less, about 2.0 γ per gram, it represents a high percentage of the body total. In experimental animals, where death occurred from riboflavin deficiency, the quantity present in the liver, kidney and heart was found to be about one-third of that present in the controls (192).

Human blood contains about 0.5 γ per cc. (187), and it has been shown that in cases of riboflavin deficiency, the riboflavin content of muscles and blood was within the normal range (189). All these observations and tests have been made on adults and I was unable to find any results from investigations upon infants.

5. Clinical Manifestations of Ariboflavinosis

The clinical manifestations of ariboflavinosis in human beings have been recognized for only some 6 or 7 years. They are, in general, mild chronic changes which, although they produce some local disturbances and annoyance, do not as a rule incapacitate the subject nor, of themselves, result in hospitalization. As yet, recognition of the signs has been limited to children and adults. However from Kark's (248) description of pellagra it is probable that some, at least, of his cases were suffering from ariboflavinosis as well. Under these circumstances it is desirable to describe the clinical manifestations as reported in children and adults.

The riboflavin deficiency syndrome are lesions of the lips, tongue and skin of the face and eyes.

Sebrell and Butler (166) who first described the mouth signs gave the name cheiosis to the condition of the lips which commences as a pallor of the vermillion of the lip followed by maceration. The angles of the mouth appear to be more frequently involved than the medial portion and to this stage the name angular stomatitis has been given. These cracks in the skin spread outward from the corner of the mouth following the lines of skin cleavage. They remain moist and become covered with a honey colored nonadherent crust.

A seborrheic dermatitis develops in the nasolabial folds (168, 170, 171,

193). It commences as a fine, scaly desquamation superimposed on an erythematous patch. It appears in the nasolabial folds and extends round into the mucous membranes of the nose. Cases have been described in which these patches also became covered with honey colored nonadherent crusts (173).

Sydenstricker and his colleagues (168) have described a specific type of glossitis in which the tongue was a characteristic magenta color, the surface smooth and the papillae flattened and mushroom shaped and slightly edematous. The subjects frequently complain of pain which varies from a tingling sensation to a raw and burning feeling.

To these signs and symptoms Kruse *et al.* (194) have added a series of ocular manifestations. These consist of an itching burning sensation associated with a feeling of roughness of the insides of the lids, complicated in China by phlyctenular conjunctivitis (170, 171). It seems that many of the subjects with these symptoms also complained of dimness of vision and photophobia.

In their report in 1940, Kruse and his colleagues (194), in addition to the above detailed signs and symptoms, described circumcorneal injection and corneal vascularization in cases of ariboflavinosis, and American nutrition surveys (195, 196) have used it as an index of the state of riboflavin nutrition. This procedure has been hotly contested in both England and America where there has been, during the last 3 years, an intense study of the blood vascular pattern of the circumcorneal tissue and of the cornea itself, in both apparently normal subjects and in those with other signs of ariboflavinosis (197-207). In some of these investigations patients with corneal vascularization have been given comparatively large doses of riboflavin, *i.e.*, up to 10 mg. daily, and only a percentage showed improvement (206, 207). Following upon their study by photography and the slit lamp, on subjects with corneal vascularization who had been given 9.9 mg. riboflavin, McCreary and his co-workers (208) concluded that once blood vessels had developed in the cornea they were not reabsorbed and that it is possible that corneal vascularization is a sign that the subject has been the victim of ariboflavinosis at some time in the past if not at the time of examination.

The subject has been reviewed by a number of authors during the last 2 years (209, 210) and it seems that the present position is that corneal vascularization is a clinically recognizable pathological condition and that ariboflavinosis is one cause, but that a number of other factors will produce the condition. Recent work suggests that tryptophan deficiency is another factor (211).

Despite the absence of recorded cases of ariboflavinosis in infants, it is obvious from the descriptions of Kark and others (248) in Africa that many of the infants in their series of cases of pellagra were also suffering from ariboflavinosis.

Some of Kark's cases had stomatitis which involved the lips and buccal mucosa. The lesion often started in the angle of the mouth which was sudden, with flaming red lips. There was, in addition, redness of mouth and throat in these cases, with atrophic glossitis involving the whole of the dorsum of the tongue.

VIII. NIACIN

By successfully treating pellagra with niacin, Fouts and his co-workers (212) in 1937 first demonstrated that deficiency of this vitamin in the diet was one etiological factor in a disease that was first described by Casal in 1725.

There is now much evidence that the disease known as pellagra is the result of multiple deficiencies (213, 214). Thus, while the administration of niacin will generally bring about recovery, it will not always do so (215). Sydenstricker (214) has reported the almost spectacularly successful treatment of severely ill cases of pellagra with desiccated, defatted preparation of pig's gastric mucosa. Similar results were obtained with ventriculin together with liver extracts. This experience has led Sydenstricker to postulate that continued deprivation of the essential vitamin or vitamins leads to atrophy of the gastric mucosa and changes in the liver. Added to this is the recent experience of Gillman and his co-workers (215) in South Africa, in which they succeeded in arresting the downward progress of cases of severe malnutrition in infants with signs of pellagra by the administration of ventriculin.

Thus, while niacin deficiency is a prime factor in the etiology of the disease, it appears that certain other factors must also be considered.

1. Physiology

Niacin is present in all living cells, where it forms part of the enzyme systems associated with the intermediate metabolism of foodstuffs. In particular it enters into the formation of coenzymes I and II which play a vital role in biological oxidation. If insufficient niacin is available the processes of oxidation and reduction in the tissue cells will be impaired.

Niacin is normally absorbed from the intestine. Goldberger (216) found that gastrointestinal disease interfered with absorption and precipitated pathological changes in the tissues.

The concentration of niacin in the blood of adults has been determined by a number of workers and the following ranges have been recorded: 0.30 to 0.50 mg. per 100 cc. (217), 0.62 to 0.80 mg. per 100 cc. (218), 0.40 to 0.80 mg. per 100 cc. (219), 0.54 to 0.83 mg. per 100 cc. (220). The greater part of the niacin is in the blood corpuscles (221).

Niacin is present in the tissues mainly as the coenzyme and the content of normal tissues is fairly uniform; the concentration, however, is higher in the liver and the kidney, while the muscles contain more than the brain, the ductless glands and the viscera, except the heart. Because of their relative bulk, the muscles contain a high percentage of the total niacin of the body (177). Niacin and some of its metabolites, nicotinuric acid, trigonelline and N-methylnicotinamide, are excreted in the urine. There is some evidence that, in the normal individual, the ratio of these four substances is fairly constant, but that the nature of the ratio is dependent upon the form in which the niacin is taken—*i.e.*, whether as niacin or the amide—and the degree of tissue saturation and physical activity of the subject.

2. Sources of Niacin in Infancy

So far as I have been able to discover, only one group of workers has compared the niacin content of human fetal tissue with that of maternal (222). Lwoff and his fellow workers (222) concluded the human fetus has no reserve of niacin and all its organs, except the heart, have a lower content than the corresponding organs of the mother. This work needs confirmation, for it indicates that the human fetus has to depend from birth on its food for niacin supplies, that is, if it does not secure its requirements by biosynthesis in its own intestine (223).

Lwoff and his colleagues (224-226) used the Proteus technique to study the niacin content of human milk. They found that the colostrum contained about 0.16 mg. per 100 cc. of niacin, but, as the quantity of milk increased, the niacin content fell, reaching a minimum of 0.05 to 0.09 mg. per 100 cc. between the 2nd and the 9th day. This was followed by a rise to a maximum of from 0.15 to 0.34 mg. per 100 cc. between the 9th and 16th days, and this level was maintained for six months. They also observed that the injection of 1 g. per day of nicotinamide caused an immediate rise in the nicotinamide content of milk, especially when it was low. The rise occurred within two hours. These observations were made on three subjects who recorded 0.54, 0.28 and 0.33 mg. per 100 cc. (227). Williams and his co-workers (178), also, made a limited number of observations of the niacin content of human milk, using *Lactobacillus arabinosis*. The nine samples in their series were collected from five subjects. These workers obtained values for niacin ranging from 1.2 to 2.2 γ per cc. The level of the vitamin (namely 1.4 and 1.33 γ per cc.) was fairly uniform in two normal subjects, while a third had 2.2 γ per cc. These figures are of the same order as some of those quoted by Lwoff.

It is apparent that more extensive data on the niacin content of human milk are required. It is known that at least two groups of investigators have

this matter in hand and it is probable that their reports will be released before this review is published.

If the mean value obtained by Williams, namely 1.6 γ per cc. is used as a basis for calculation, it is apparent that breast fed infants will obtain from 0.96 mg. daily in the first week of life to 1.4 mg. daily at 3 months of age.

The niacin content of cow's milk varies from 19 to 120 γ per 100 cc. (178), 100 to 500 γ per 100 cc. (228), 10 to 50 γ per 100 cc. (229). Assuming an average value of 1.02 γ per cc., it is obvious that an infant fed on cow's milk will obtain from 0.3 mg. daily at a week old to 0.6 mg. daily at 3 months old.

Intakes of this order are far below the amounts usually considered necessary to prevent the onset of pellagra, and this fact focuses attention upon the possibility of infants obtaining their requirements from bacterial action in the intestine. This is discussed later.

Requirements of Niacin in Infancy. No quantitative study has been carried out to determine the human requirement of niacin. Kodicek (230) has calculated the niacin content of the average prewar middle class diet of an adult in England to be 12.3 mg. daily and of the wartime rationed diet to be 12.25 mg. daily. On the same figures a vegetarian would obtain about 8.2 mg. daily. Kodicek computed that the average daily intake of niacin by an adult in England in wartime was between 8 and 12 mg. Using Kodicek's figures, it can be shown that an infant 12 to 14 months old would have a maximum intake of about 3.1 mg. daily*. Several intensive nutritional surveys have been made in England in wartime by competent observers (231) without the discovery of a case of infantile pellagra. This is, however, not sufficient ground for concluding that the requirements of niacin in infancy are in the vicinity of 3.0 mg. daily. At this stage in our knowledge it must be confessed that it is not possible to state what the requirements are.

3. Pathology of Infantile Pellagra

As has been said, deficiency of niacin in the diet is a prime, if not the only, etiological factor in the development of pellagra. So far as I have been able to discover, the pathological changes in infantile pellagra have not been described. Moreover the *post mortem* appearance in the adult is frequently complicated by intercurrent and terminal infections. For this reason, pathologists consider that, in pellagra, the tissues of only three systems are altered—the skin, the gastrointestinal tract and the nervous system. The pathological changes in the adult were originally described by Denton (232) and later by Dalldorf (93c).

* This figure is calculated on the following diet, the niacin value of the quantity (mg.) being shown in brackets. Milk 1000 cc. (0.8); Meat 28 g. (1.2); Oatmeal 10 g. (0.3). (Kodicek's figures for niacin).

4. Biochemical Pathology in Infantile Pellagra

The blood contains some niacin, 0.5 mg. % (217), of which about 85% is as coenzymes in the blood cells; that portion in the plasma is in the form of free niacin or the amide.

Axelrod and his co-workers (233) have demonstrated that, although the coenzyme I content of the blood may fall in pellagra, it still lies within the normal range. Niacin therapy will raise the coenzyme I and II in the blood but not significantly (234, 235). Therefore, measurements of the niacin content of blood are of little or no diagnostic value.

As mentioned earlier, niacin and some of its products—namely nicotinuric acid, trigonelline and N-methylnicotinamide—are excreted in the urine and the amount of each excreted is dependent upon a number of factors.

The 'test dose' technique has been applied to the diagnosis of pellagra (236, 237) but it would seem that standards must be worked out before it can be applied clinically.

5. Clinical Manifestations of Infantile Pellagra

From the descriptions that have been given of infantile pellagra it is apparent that the disease does not follow the same course as in adults.

Eddy and Dalldorf (93c) point out that the disease was first reported in infants in 1794 by one Strambio.

a. *Age Incidence.* Dodd (238) is not satisfied that pellagra can occur at birth, although cases have been described in infants 2 months old. The age distribution of the reported cases in infants under 12 months is as follows:

Age in Months	Number of Cases
2	2 (239, 240)
3	1 (239)
4	2 (239)
5	3 (239, 241, 242)
6	1 (243)
7	2 (239)
10	2 (238, 239)
11	1 (244)
12	3 (243)
Age not given	5 (244)

Gillman and his co-workers (215) reported 300 cases of malnutrition in infants and children of which 60% showed signs of pellagra. The ages of these cases are not given.

b. *Prodromal Signs.* Most of the younger infants reported to be victims of pellagra were breast fed at least for a time and in almost every instance the mother was a pellagrin. However, Voegtlín and Harries (242) reported

the case of an infant who developed the classical signs of pellagra although the mother remained free of symptoms.

Trowell (243, 245) has provided a very full description of infantile pellagra as it occurs in West Africa. Although in most cases the characteristic signs of dermatitis, stomatitis and diarrhea were evident on presentation of the child at hospital, it is apparent from the description given that prodromal signs occurred in all cases. In Trowell's experience these usually last about one month, the duration depending, of course, on the age of the child, being shorter in younger children. During this time the infant is wretched and irritable, has photophobia and anorexia, and shows signs of malnutrition. In most cases reported, diarrhea appears to be sometimes the only sign (240, 244, 246). The diarrhea may exhibit spontaneous remissions and exacerbations (243), and during the latter blood may appear (238, 243). Stomatitis, is of course present in most cases, though generally it is not a source of complaint in older children.

c. *Skin Manifestations.* Smith and Ruffin (247) from a study of 465 cases in adults concluded that trauma was the deciding factor in the location and even the intensity of the skin lesions in pellagra. Trauma may result from exposure to sunlight, but irradiation from an electric heater may be just as effective; local trauma may likewise result from tight clothes as, for example, in the diaper area. Intercurrent dermatitis may also be a factor.

It is possible that young infants who have the gastrointestinal manifestations of the disease may not be subjected to sun exposure and for this reason may not develop the typical skin lesions. Physicians usually require the presence of skin signs before accepting the diagnosis of infantile pellagra. In the opinion of several groups of workers in the Southern States of America it is possible that many cases of pellagra escape diagnosis for this reason (238, 244).

The eruption usually appears on the face either over the nose or at the corners of the mouth (238), *i.e.*, that portion of an infant likely to be exposed to sunlight in a pram or when carried. The lesion first appears as an erythema. In native races, the lesion commences as a slightly raised pigmented area of hyperkeratosis (243). In the white skinned infant, the erythematous patch turns dark and thick and becomes rough. Sometimes cracks appear along the lines of skin cleavage and these invade the subcutaneous layer without obvious inflammation (243). The pigmented patches frequently peel, leaving pale areas. This not uncommonly happens where discharges favor exfoliation, as, for example, at the corner of the mouth and the nose.

Where the legs and arms have been exposed, the dermatitis spreads to these areas also and in very severe cases to the whole body. Trowell (243) and Kark (248) reported that the scrotum and napkin area are frequently involved, (areas of local pressure). Where the whole body of the infant may

be exposed to sunlight as in the tropics, the whole body may become pale owing to desquamation. Kark (248) compared the skin lesions of adults with those usually seen in infants and came to the conclusion that they were essentially the same in character, being modified by local conditions at each age.

Trowell has reported changes in the hair and nails. There was, in his cases, marked loss of hair and the nails became very thin.

d. Nervous Signs. In adult pellagrins mental symptoms develop in one-third to one-quarter of the cases. The mental features have been studied by Frostig and Spies (249). Infants with pellagra are frequently described as irritable and restless, while in children there are frequently complaints of weakness, dizziness, lassitude and fatigue. It is possible that these are symptoms of thiamine deficiency.

It would seem that the mental and nervous signs characteristic of adult pellagra have not been described in infants.

IX. ASCORBIC ACID

Ascorbic acid is widely distributed in animal and plant tissues. In animal, and probably also in plant tissues, ascorbic acid occurs probably in equilibrium with the dehydro form (58c).

1. Physiology

Ascorbic acid is absorbed from the intestinal tract, chiefly from the small intestine (250), and conveyed to the tissues in the blood stream. Plasma ascorbic acid levels will be discussed under the heading 'Biochemical Pathology.'

While this vitamin is present in most tissues and no special organ acts as a reservoir, some tissues, notably those with a high metabolic activity, including the adrenals, are richer in it than others (73d).

A certain amount of ascorbic acid is stored in the body, for it has been shown that human scurvy takes months to develop after the vitamin has been withdrawn from the diet (251-254).

Ascorbic acid is excreted in the urine and feces, but by far the larger amount through the kidneys. The amount excreted depends upon the intake. Harris and his colleagues (255) consider that a daily urinary excretion by adults of 13 mg. is borderline, and that an excretion of 20 mg. represents a moderately low intake, and of 40 mg. a liberal intake. Denoyelle and Sirand (256) have observed that the excretion in infants is frequently less than 5 mg. daily.

It has been conclusively shown that ascorbic acid is essential for the formation of the intercellular material in cartilage and bone and the collagen of all fibrous tissue (251-253, 257). Bicknell and Prescott (73d) have

suggested that ascorbic acid "appears to be necessary for the proper functioning of the osteoblasts which in its absence revert to their prototype."

The Relationship of Ascorbic Acid to Amino Acid Metabolism. Dann (258) made an interesting observation on 22 premature babies who, as soon as they were able to take a full diet of 120 to 125 calories per kg., were given either boiled breast milk or diluted cow's milk mixtures. Each was given 800 to 900 mg. of ascorbic acid for 4 to 5 days, then no dose for 5 to 8 days. The breast fed infants retained a larger part of the test dose in their tissues, which suggests that the vitamin C requirement of artificially fed children is higher than that of breast fed children. Dann suggests that the higher protein content of cow's milk mixtures demands more ascorbic acid; a view which is in accord with the suggestion that ascorbic acid is concerned with the intermediary metabolism of the aromatic amino acids (259-263).

Levine and his co-workers (262) found that, when premature infants were given a diet relatively high in protein (*e.g.*, 5 g. or more per kg. per day), they exhibited spontaneous defects in metabolism of tyrosine and phenylalanine as revealed by abnormal excretion products. The defect could be eradicated by the administration of ascorbic acid (263). Full term infants showed the same reaction when fed pure tyrosine and phenylalanine (263).

2. Sources of Ascorbic Acid in Infancy

There is ample evidence that the newly born infant has acquired appreciable stores of ascorbic acid from its mother. A number of workers have demonstrated that the concentration of ascorbic acid in blood plasma drawn from the umbilical cord is from 1 to 4 times greater than the concentration in maternal blood immediately after delivery (264-269). Comparisons of this nature must take into account the fact that the maternal plasma ascorbic acid values fall abruptly during labor with a rise immediately after (268, 270), and that values obtained for cord blood are higher than those occurring in peripheral blood drawn at the same time (265). The ascorbic acid content of the maternal diet affects the plasma ascorbic acid values of both maternal and cord blood (270). The difference between maternal and cord blood levels suggests that the placenta is capable of concentrating ascorbic acid (265), a function not attributed to it by the biologists (1).

It is probable that the fetal liver carries rich stores of ascorbic acid but, although at least two surveys have been made of the ascorbic acid concentration in the liver of infants, it is doubtful whether the results are of any great significance. Toverud (271) found the average for full term infants at birth was 7.01 mg. per 100 g. (range 2.7 to 10.4 mg. per 100 g.), whereas Ingalls (272) found values ranging from 20 to 75 mg. per 100 g. with marked declines during the first month. Both these results were obtained by titra-

tion methods and it is certain that substances other than ascorbic acid would reduce the reagent. This undoubtedly explains the wide range in figures and renders valueless figures obtained for infants who died after living for a period.

Braestrup (273) has demonstrated a sharp fall in plasma ascorbic acid values in the first 24 hours after birth, with a further fall by the 5th day. Several investigators (268, 269, 274) have shown that the level of plasma ascorbic acid after the first week of life is dependent upon the method of feeding, higher values being obtained in breast fed infants than in those fed cow's milk mixtures.

Human breast milk has been shown to contain from 2 to 6 mg. per 100 ml. ascorbic acid, (see Braestrup (273)) and the diet of the mother has a marked influence upon the actual level. Maternal diets rich in ascorbic acid yield a milk up to 6 times richer in ascorbic acid than do diets with low intakes (270). When liberal supplies of ascorbic acid are given to the mother, the ascorbic acid content of the milk is raised; in one set of observations (275) an average of 7.3 mg. per 100 cc. was obtained (prefeeding average 4.5 mg.), while in another set a maximum of 8 mg. per 100 cc. was obtained (276). It would appear that under the most satisfactory conditions 8 mg. per 100 cc. is the maximum value for breast milk.

On the basis of these results, breast fed infants will receive from 10 to 40 mg. of ascorbic acid daily, depending on the age of the infant and the concentration in the milk. The lowest intakes will of course occur in premature and young infants whose consumption of milk is low.

The ascorbic acid intake of artificially fed children will depend almost entirely upon the amount supplied as a supplement, either as fruit juice or as the synthetic product.

The premature infant calls for special reference. It is now recognized that he enters this world with poor reserves of some nutrients, but I was unable to find any reliable data which demonstrated that the premature infant had less ascorbic acid per kilogram of body weight than the full term infant. Toverud's (271) finding that the concentration of ascorbic acid in the liver of the premature infant was less than that in the liver of full term infants must be viewed with suspicion.

Ascorbic Acid Requirements of Infants. It has been shown that breast fed infants may receive up to 40 mg. per day and that average intakes are in the vicinity of 20 mg. Macy and her co-workers (277), as a result of observations on 427 infants, considered that the minimal protective dose of ascorbic acid for the average healthy infant was 10 mg. daily.

The recommended daily allowances for specific nutrients prepared by the National Research Council of America (183) provide for infants under 1 year and for those in their second year, 30 mg. and 35 mg. of ascorbic acid, respectively.

3. Pathology of Deficiency States

The characteristic lesions in human scurvy are the hemorrhages and the bone defects. Petechial hemorrhages that occur in the skin about the hair follicles do not show any characteristic histological changes.

It has been suggested that it is lack of extracellular material in or around the capillaries which leads to the development of petechial hemorrhages in the skin and mucous membranes, and weakens the union between periosteum and bone allowing the former to be easily raised by escaping blood (93d, 184).

4. Biochemical Pathology of Deficiency States

a. Plasma Ascorbic Acid. A number of techniques have been developed for the determination of plasma ascorbic acid (278-281), and since 1936 several investigations have been made of the plasma ascorbic acid levels of infants. Despite the early hopes of a satisfactory diagnostic aid which this procedure engendered, an isolated determination is now recognized to be of no value (73d, 268, 282). However, a number of interesting facts have emerged from the assembled data.

The plasma ascorbic acid is high immediately after birth and it falls appreciably during the first 10 days, from 0.69 mg. per 100 cc. to 0.27 mg. (273).

The plasma ascorbic acid is higher in breast fed infants than in artificially fed infants of the same age. Mindlin (283) obtained an average of 1.0 mg.% for the former and 0.3 mg.% for the latter; both groups were 13 to 14 days old. Even with supplements of ascorbic acid of 75 mg. daily, 4 artificially fed infants had values of 0.7, 0.6, 0.8 and 0.7 mg.% for plasma ascorbic acid. Other workers have obtained similar results (274). Thus, the plasma ascorbic acid level of normal infants has been found to lie within the range 0.05 to 1.6 mg. per 100 cc., and Holmes, Cullen and Nelson (284) have observed infants with low levels which persisted for several months.

Ingalls (285) records the plasma ascorbic acid values in 15 cases of infantile scurvy, as follows: 5 cases—nil; 2 cases 0 to 0.05 mg.; 3 cases 0.06 to 0.1 mg.; 3 cases 0.11 to 0.15 mg.; 2 cases 0.16 mg. Similar results were obtained by Snelling (274).

b. Serum Phosphatase in Scurvy. Schwachman (286) has recorded a fall in serum phosphatase in 18 cases of untreated scurvy.

c. Serum Protein in Scurvy. Rosenkranz (287) determined the serum protein in 6 cases of infantile scurvy and found the total protein diminished, especially in severe cases, with the albumin affected more than the globulin. In milder cases the albumin alone was affected. During treatment, values gradually returned to normal in from 5 to 10 weeks. Dodd and Minot (40) have reported several cases of general starvation accompanied by scurvy in which the total plasma protein was low, with a very low albumin figure.

It is hard to visualize the nature of the pathological changes responsible for the figures obtained by Rosenkranz and it is obvious that more observations of this nature are needed before any conclusions can be drawn.

5. Clinical Manifestations of Deficiency

The infantile scurvy originally described by Thomas Barlow (288-290) is now recognized to be an advanced form of the disease and, while cases of this are still seen in large metropolitan hospitals, it is not so common as the milder forms nor as frequent as latent scurvy.

a. Age Incidence. Five cases of fetal and congenital scurvy in infants aged from 7 days to 2 months have been reported (291-294). All these cases were reviewed by Jackson and Park (294) when they reported their case which was a 20 days old infant of a mother with scurvy. They cast some doubt on the diagnosis of the other cases and also on whether the diagnosis of congenital scurvy is warranted in an infant 2 months old. The fact remains that, although congenital scurvy is very rare, it can occur and should not be overlooked in infants who present vague swellings of the legs.

While the disease may apparently occur at any age, infants are more susceptible between 7 and 12 months of age (295). This corresponds to the end of lactation and the establishment of the infant on a fully artificial diet.

b. Subclinical Scurvy. Hess (296) was the first to draw attention to the sub-clinical forms of the disease and since then numerous workers have described cases.

The first indication that all is not well with the nutrition of the infant is the weight curve. The infant ceases to gain weight, or gains at a rate less than normal, and is irritable and peevish and the mother reports that the appetite is poor. The onset of these signs and symptoms is insidious and it is only when the infant has failed to gain weight for 3 or 4 weeks that the diagnosis can be made. The dietary pattern is constant, for in our series all infants who exhibited these features were found to have been artificially fed on cow's milk mixtures for periods varying from 3 to 5 months, but without any supplement of ascorbic acid. The usual story is that orange juice was offered but the child rejected it or it caused him to vomit and mother had not persevered.

In this form of the disease there are seldom any other clinical features and the radiographs are not diagnostic. The diagnosis is generally made and confirmed by the effect of ascorbic acid therapy.

c. Clinical Scurvy. Hess (297) observed that the next stage of the disease is reached in the same insidious manner as the subclinical. It is characterized by vague tenderness of the lower thighs which is difficult to elicit with certainty. Some cases exhibited edema over the crest of the tibia. Hess coupled slight hemorrhage of the gums with these limb changes.

This phase passes imperceptibly into the advanced stage, the essential characteristic of which is hemorrhage into tissues at various sites. Harris (298b) quotes observations made by Reyher of the sites at which hemorrhages occur. This list is informative and stresses the importance of the limb lesions in infants and the lesser significance of mouth changes.

	<i>Per Cent</i>
Osseous system	93.3
Intestinal tract	60.0
Gums	43.3
Skin	43.3
Eyelids	36.6
Intestinal mucous membrane	13.3
Nasal mucous membrane	3.3
Palatal mucous membrane	3.3
Margin of the tongue	3.3

d. Limbs. The signs in the limbs are associated with hemorrhages beneath the periosteum which itself is not normal and is consequently insecurely attached to the shaft of bone, so that it is readily stripped off by hemorrhage (297). In a review of 64 cases Still (299) found swelling and tenderness in the legs only in 47 cases, in the arms and legs in 10, and in the arm only in 1.

The essential sign is tenderness and swelling of the affected limb, this is sometimes so slight that it may be missed unless the limb is carefully palpated. The swelling extends along the shaft and is not limited to the epiphysis. The infant will not move the limb because of the pain involved and cries piteously whenever anyone approaches the bed.

e. The Ribs. Barlow (289) first described the lesions of the ribs. They have also been well described by Hess (297) and by Still (299) and consist of backward displacement of the costal cartilage so that the end of the costal cartilage and the bony rib are not exactly on the same plane. This sign occurred in 35 out of 40 of Still's cases.

f. Hemorrhages. Hemorrhages do not usually occur in the gums unless the teeth have erupted, although Still has observed them where the teeth are not actually through but are close to the surface. The commonest site of the hemorrhage is around the upper incisors, but they may occur around the molar or canine teeth (297). At first the gums may be merely deep red or bluish red, but later they become spongy with bleeding points, particularly along the edges through which the teeth have erupted. Still described hemorrhage into the middle part of the hard palate. Petechial hemorrhages occur in the skin especially in areas of pressure or at the site of another skin lesion.

Mucous membranes are also the site of hemorrhages but there are differences of opinion as to how frequently these occur.

g. Cardiorespiratory Sign. In 1917 Hess (300) described enlargement of the heart, and more especially of the right side, as a frequent accompaniment of infantile scurvy. He pointed out that the enlargement could be recognized clinically by the appropriate physical signs and by X-ray. The heart-beat was found to be greatly increased, even up to 200 beats per minute, with corresponding rapid respirations. A sharp drop in the pulse and in the respiratory rates occurred when orange juice was given. The clinical picture and the radiograph in these cases bears a close relationship to the cardiac signs described by Wenckebach (135) in cases of beri-beri (cf.).

h. Anemia in Scurvy. Anemia has been recorded in cases of infantile scurvy. It is not a constant feature, however, nor, it would seem, a necessary accompaniment of human scurvy (301). Anemia is a common occurrence in infants in the second half of the first year of life, (see section on iron deficiency) so that it is not surprising that cases of scurvy have been recorded in infants with anemia. Despite this we have the observation of Kenney and Rapoport (302) that the administration of ascorbic acid alone in cases of infantile scurvy caused a rise in the red blood cells, the reticulocytes and the hemoglobin value.

6. The Relationship of Ascorbic Acid Deficiency to other Diseases

a. Wound Repair. The now classical experiment of Crandon (251-253) on himself and the experiment by Hunt (257) have conclusively demonstrated that an adequate supply of ascorbic acid in the body is essential for a wound to heal. Frankly scurvy wounds will not heal. For satisfactory wound repair the formation of intercellular material is necessary and this will not take place in the absence of ascorbic acid (303, 304).

Thus while other materials, *e.g.*, protein and the other vitamins, are essential for satisfactory wound healing, the breakdown of an operation site on or about the 10th day might quite easily be a sign of latent scurvy. This possibility should be sufficient justification, if such is needed, to ensure that all surgical patients are both pre- and postoperatively saturated with ascorbic acid (305-308).

b. Union of Fractures. It has been demonstrated in experimental animals (309, 310) that ascorbic acid is essential for the formation of callus in the union of fractured bones. It is the personal experience of Stirling (311) that clinical union of fractures is achieved sooner if ascorbic acid is given as a routine, even in cases where an ascorbic acid deficiency is not proved.

c. Infections. Bicknell and Prescott (73d) have assembled and discussed the now considerable volume of evidence relating to the relationship of ascorbic acid to infection. It is evident that ascorbic acid requirements are increased in infections and that the peak demand occurs in the active stages of the disease. Hess (297) pointed out that cases of latent scurvy were frequently precipitated into the frank form of the disease by the onset of an

infectious disease. Thus it is quite possible for scurvy to appear as a complication of an infectious disease.

While the administration of liberal quantities of ascorbic acid to infants suffering from pneumonia, diphtheria, etc., is desirable as a routine procedure, the evidence that ascorbic acid has a specific therapeutic effect on the diseases, thus altering their course, is conflicting. A full discussion of this aspect of the physiological action of ascorbic acid is beyond the scope of this review and the interested reader is referred to the article by Bicknell and Prescott (73d).

7. Radiographic Appearance of Bones in Scurvy

Hess (297) has reviewed the original observations of Hart and Lessing on the radiographic appearance of the bones in scurvy. These were later discussed by Schwartz (312), while Pelkan (313) and Park (314) have outlined the radiographic appearance of very early scurvy.

X. VITAMIN D

Deficiency of vitamin D in the body of the infant and the young child will lead to the development of rickets, principally through interference with calcium metabolism. The essential physiological abnormality in older infants and children affected with rickets is diminished absorption of calcium and phosphorus from the intestinal tract (315, 316), while there is some evidence to suggest that in the young infant the level of calcium in the prenatal maternal diet may be the prime etiological factor. Before proceeding to the consideration of vitamin D deficiency, some aspects of calcium metabolism will be reviewed.

1. Relevant Features of Calcium Metabolism

In the fetus, bone develops in preexisting cartilage by deposition of calcium salts (298a). Growth in length and thickness of bone in both prenatal and postnatal life occurs by deposition of calcium salts in newly formed cartilage at the growing points (184, 298a, 317). In his review of the chemistry of calcification Logan (318) has outlined the method by which calcium salts are deposited to form true bone. On this point there seems to be agreement, but much uncertainty exists regarding the source of these calcium salts during the first six months of life. It is accepted that in older children they are provided by the diet. Leitch (319), in an extensive review of the calcium requirements of man, drew attention to the fact that the amount of calcium absorbed from breast milk during the first 3 to 4 months of life was insufficient to maintain the skeleton at the same standard of calcification as that at birth, and, because of this, decalcification of already formed bone is a normal physiological procedure.

However, another explanation of this problem has been advanced. In

1922 Hamilton (320) advanced the hypothesis that the long bones of the fetus acted as a reservoir for the storage of calcium laid down during pregnancy. Coons and Blunt (321) showed that nearly three times as much calcium is deposited in the fetus in the last 2 months of pregnancy as is deposited in the first 7. Eliot and Park (322) have expressed an opinion similar to that advanced by Hamilton; this was elaborated by Clements (323) and further support was given to it by the experimental work of Wake (324). This alternative theory is that during intrauterine life, the fetus whose mother has had an adequate diet lays up in the compact bone a reserve of calcium above its immediate requirements. This reserve supply is used during postnatal life to augment that obtained from breast milk. Additional support for this theory is to be found in the figures quoted by Hamilton (320) of the range of calcium content of the infant at birth, namely from 5.01 g. per kg. to 10.27 g. per kg. of body weight. If calcium deposition in the fetus were always at a maximum level, as the all-or-none phenomenon of Needham (1) would suggest, the range of concentration in the newborn infant would not extend over 100 %. Likewise of interest is the observation of numerous workers that, in many infants, bones that were firm at birth became soft during the first few months of life (325-328).

2. The Sources of Vitamin D in Infancy

I have been unable to find any references to the amount of vitamin D that the fetus carries over into postnatal life. Needham (1) in his comprehensive review of placental transmission makes no reference to the ability of vitamin D to pass through the human placenta.

Drummond and his co-workers (329) investigated the amount of vitamin D in human milk and showed that the mean value of 26 samples was about 6 International Units per 100 cc., but that the range was from 2 to 18 International Units. Thus an infant consuming 600 cc. of milk would obtain from 12 to 108 International Units per day. The vitamin D content of milk of women who received, in addition to the vitamin D in their diet, 1000 International Units daily as halibut oil, ranged from 4 to 9 International Units per 100 cc.

Liu *et al.* (330) demonstrated in four cases that the administration of large doses of vitamin D to the mother greatly benefited the infant, bringing about a higher retention of calcium from the maternal milk. Several workers (331-336) have demonstrated that vitamin D in milk, either from a natural source or as an addition, is more effective per unit dosage than given as a concentrate, owing, it is suggested by Supplee (333) to the formation of a compound with the lactalbumin. Barnes *et al.* (337) found that the milk of a mother who was on an adequate diet, fortified with milk containing 300 International Units of vitamin D, maintained her baby free from rickets

but did not cure rickets in another infant. Of course there are several explanations for this—the therapeutic dosage of a vitamin is higher than the prophylactic dosage; the development of rickets in the first few months of life may not be associated with the vitamin D supplies alone.

It is apparent that, unless deliberate steps are taken to expose young infants to sunlight, it is doubtful if they obtain more than a small percentage of their requirements of vitamin D unless given a preparation containing vitamin D.

Requirements of Vitamin D in Infancy. Breast fed infants whose mothers have an adequate diet fortified with additional vitamin D can obtain up to a maximum of 100 International Units per day.

A Sub-committee of the British Pediatric Association under the chairmanship of Wilfred Sheldon (338) drew up a report which, after considering the evidence available, recommended 700 International Units per day for full term infants and children up to 5 years of age and 1400 Units for premature infants under $5\frac{1}{2}$ lbs. in weight.

3. Pathology of Deficiency States

a. Bone. In 1942 Wolbach and Bessey (184) summarized the pathological changes in rickets as:

- (a) Failure of calcification of the cartilage columns in the so-called zone of provisional calcification and failure of calcification of osteoid;
- (b) Continued growth and consequent increase in thickness of the diaphyseal cartilage and osteoid;
- (c) Lack of vascular growth into cartilage;
- (d) Resorption of bones formed before the deficiency.

b. Teeth. The relationship of vitamin D to dental caries has been discussed by Bicknell and Prescott (73e) and they summarize the existing evidence with the statement: "Vitamin D decreases caries to some extent in some children but . . . caries is not dependent on a deficiency acting directly on the teeth themselves. It appears most likely that vitamin D improves caries only indirectly by improving the general health and nutrition of the child."

4. Biochemical Pathology of Deficiency States

a. Serum Calcium. The serum calcium is usually normal in rickets (10 to 11 mg. per 100 cc.) but in tetany it is lower. In uncomplicated rickets the distribution of the various fractions, ionized and non-ionized, is unaltered (37c).

In cases of fetal rickets diagnosed by radiography Maxwell (339, 340) obtained figures for the serum calcium content of cord blood ranging from 5.6 to 10.6 mg. per 100 cc. The values for 2 infants, one of which died in

utero just before birth, and the other was still-born, were 5.6 mg. and 8.83 mg. per 100 cc., respectively.

Garrahan and Thomas (341) investigated the serum calcium level in six cases of spasmophilia, the diagnosis of which was based on clinical and chemical examinations and on electrical stimulation (Erbs Test). They found the diffusible calcium was normal, sometimes above normal, 4.8 to 6.5 mg. per 100 cc., while the non-diffusible calcium was greatly reduced, ranging from 0.7 to 2.2 mg. per 100 cc. (their figures for normal—5.2 mg. per 100 cc.).

Shohl (37c) believes latent tetany may be present when the serum calcium is about 8 mg. per 100 cc., and tetany becomes clinically manifest when the level reaches 4.5 to 6 mg. per 100 cc.

b. *Serum Phosphate*. The serum inorganic phosphate, usually 5 to 6 mg. per 100 cc. is sometimes reduced to as low as 0.2 mg. per 100 cc. in rickets.

Serum inorganic phosphate is generally raised in cases of tetany, values ranging from 6.2 to 8.4 mg. per 100 cc. having been obtained (342, 343). However, Shohl (37c) makes the point that infantile tetany can occur with a variable serum phosphate of 3 to 8 mg. per 100 cc.

c. *Serum Magnesium*. Kruger (344) has shown that, whereas the serum magnesium of 11 normal healthy infants was within the range 1.92 to 2.5 mg. per 100 cc., that of 10 infants with active rickets was from 0.81 to 1.14 mg. per 100 cc. Normal serum magnesium values for infants with tetany have been reported (344, 345).

d. *Serum Phosphatase*. The relationship of serum phosphatase to bone metabolism has been recently reviewed (346). Several workers have studied the level of this enzyme in the blood in cases of rickets. Some (347-352) have recorded high values in a number of subjects many of whom were subsequently shown by radiographs to have developed rickets. Morris (349) quotes an interesting group of cases which had high serum phosphatase values but normal radiographs, who, when investigated from 4 to 6 months later, were found still to have high serum phosphatase and also radiographic evidence of rickets. In general the height of serum phosphatase values bears a rough correlation to the severity of the clinical symptoms of rickets. In cases of clinically manifest rickets which were allowed to run their course, the values of serum phosphatase rose as the disease became worse and fell when improvement occurred. After administration of vitamin D, the values return to normal within 2 or 3 weeks—the rate of fall depends on the dosage of vitamin D, being more rapid with large doses (349). This is consistent with the function of phosphatase, for, when healing commences, the deposition of calcium is vigorously resumed to bring about the calcification of the excess osteoid tissue and more than normal amounts of phosphatase enzyme are required.

5. Clinical Manifestations of Deficiency

Rickets occurs only during the period of actual growth and healing may actually occur during periods of undernutrition. The stunted semi-starved child is seldom the victim of rickets. Although rickets may occur at any time during the growing period (353) by far the highest incidence is in the first two years of life. It is frequently stated in text books that fetal rickets does not occur, but the work of Maxwell and his co-workers (339, 340) in China has demonstrated that this is not so, for they have reported several cases in infants, the offspring of women with osteomalacia.

Numerous investigators during the last 10 years have reported that rickets, at least in the mild form, is common, ranging from 20 to 40% (354-359, 323).

It is now well established that premature babies are very susceptible to rickets (320, 323, 355, 360-362). They suffer from two disabilities; they have very inadequate supplies of calcium, having been deprived of the large amounts normally obtained in the last month of pregnancy, and they grow more rapidly than normal infants.

The nature of the manifestations will depend entirely upon the degree to which the disease has advanced. The florid form of rickets was undoubtedly extremely common in England during the 17th, 18th and 19th centuries (363) but is now rare. Clinicians now recognize that the disease may exist in a form that can be diagnosed only by X-ray or blood examination.

a. General Signs. In the mild form there may be complete absence of clinical signs (323). If this form continues for several months, in the advanced stages, catarrh of mucous membranes, muscular weakness, increased nervous irritability (299) and marked restlessness, especially at night (73e), have been reported. The rachitic infant is frequently stated to be fat and flabby. It is necessary to endeavor to separate those conditions due to deranged calcium-phosphorus metabolism from those due to deficiency of other nutrients, which frequently occurs in the diet of the rachitic infant. It is possible that the restlessness may be a sign of a mild form of spasmophilia (see a later section) and thus a complication of vitamin D deficiency, but more work is necessary to demonstrate that the accumulation of soft tissue, the catarrh of the mucous membranes and the muscular weakness are due to uncomplicated vitamin D deficiency.

b. Bony Changes. The earliest signs, and frequently the only ones recognizable in the mild form of the disease, are three in number:

- (a) Barlow recognized a softening of the free margins of the flat bones of the skull at the border of the anterior fontanelle. In our experience this is the first sign of unsatisfactory calcification. It can be recognized at 3 months of age.

- (b) Thickening of the junction of the ribs with the costal cartilage (Beading—rickety rosary) is recognized by many authors to be diagnostic of rickets (364, 73e). Bicknell and Prescott (73e) make the point that some slight swelling occurs in normal infants.
- (c) Craniotabes—thinning to parchment-like texture of portions of the parietal and occipital bones was, according to Barlow (364), an early sign. Youmans (41b) has pointed out that the condition may occur in osteogenesis imperfecta, in hydrocephalus and even in normal (especially premature) infants. As most premature infants develop rickets it is possible that Youmans' cases had rickets.

In the later stages and in the advanced form of the disease other changes occur in the shape and structure of bones:

- (a) Bosses may develop in the frontal and parietal bones, in front of and behind the fontanelle. This thickening is due to new bone laid down by the action of the periosteum and occurs in the center of each bone (41b).
- (b) Some writers have placed importance upon late closure of the anterior fontanelle. This is undoubtedly so in severe cases, but it is doubtful if the sign is of much value in the mild form.
- (c) Because of its structure the contour of the chest is affected by rickets. In the early stages of the disease the site of insertion of the diaphragm along the ribs dips inward markedly with each inspiration. As the disease progresses this becomes a fixed groove, known as Harrison's sulcus. Pigeon chest has often been attributed to rickets, but Barlow, in 1889 (364), cast doubt on this. We have investigated the family history of a considerable number of children with a pigeon chest and found strong support for the suggestion that it is a familial trait.
- (d) Barlow (364) described clinically obvious thickening of the lower end of the ulna and radius and Bicknell and Prescott (73e) have printed an illustration showing this. This is a manifestation of the advanced form of the disease.
- (e) Inward bowing of the lower third of the tibia together with swelling of the epiphysis has been described (364, 73e). However some bowing of the lower third of the tibia is a common observation in young infants. From a study of radiographs, Sheldon (365) has pointed out that any curvature is more apparent than real. Bowing of the legs is found in some cases of rickets, but in addition to *genu valgum* there is both inward and backward bowing of the lower third of the tibia.
- (f) Knock knees have been attributed to rickets (73e).
- (g) The advanced rickets of a former century was undoubtedly accompanied by gross structural changes in the vertebrae and pelvis.

c. *Nervous Disturbances.* "Sooner or later marked nervous disturbances occur in most cases of severe rickets and in many cases of moderate rickets. Perhaps the earliest and one of the most constant of these is the undue irritability and restlessness at night during which rachitic children throw off their bedclothes." So wrote Barlow (364) more than half a century ago.

Irritability of the nervous system, called spasmophilia, which sometimes accompanies the bony changes of rickets, may extend from irritability and restlessness of the infant to attacks of prolonged tetany or generalized convulsions. Tetany is associated with a reduction in blood calcium. It has been shown that rarely is the serum calcium affected in uncomplicated rickets. For this reason a variety of opinion exists as to the relationship of tetany to rickets, for it has been observed (73e) that the proportion of rachitic infants with tetany varies greatly in different countries.

Fu Tang Chu and Chieh Sung (366) have described the development of tetany in infants in China. The characteristic features of their cases were:

- (1) Many occurred in the first 3 months of life;
- (2) 80% of the mothers had impaired calcium metabolism during pregnancy and lactation;
- (3) Many of the patients showed rickets during the first 3 months after birth.

In the opinion of these authors the three factors are related and prove that congenital lack of vitamin D and calcium are the cause of infantile tetany.

Capper (367) has recently reviewed the clinical features and laboratory findings of "true" infantile tetany and, as well as the above points, he stresses the occurrence of a lowered blood calcium and a raised blood phosphorus.

6. Radiographic Appearance of Bones in Rickets

In the infant with rickets the pathological changes in the bones occur at all growing points and are usually most noticeable at the epiphysis of the long bones. The growth pattern of the long bones is constant, the end of the bone opposite to the end to which the nutrient artery is directed, grows more than the other end (298). Thus the changes in rickets are more easily detected in the wrist and ankle joints.

Lindblom (368) has described the radiographic appearance of the minute changes that occur in the mild forms of rickets. These are decalcification of the bony tissue immediately beneath the calcifying zone of the diaphysis—that is the metaphysis. However, Eliot and Park (322) have described the changes found in cases of moderate and severe rickets and their description has been used by numerous workers as a guide. The changes consist of splaying out and hollowing of the ends of the long bones, particularly well seen in the ulna. As the disease advances, so does the degree of cupping and the splaying out.

XI. VITAMIN E

As a result of numerous investigations during the last 17 years, commencing with those of Evans and Burr in 1928 (369), it is now recognized that those substances classified under the term 'vitamin E' are essential for the metabolism of skeletal muscle. In 1931 Goettsch and Pappenheimer (370) produced lesions in rabbits and guinea pigs by using a diet deficient in vitamin E and named the condition 'nutritional muscular dystrophy.' Similar conditions have been produced in sheep and goats (371). Pappenheimer (372) considered that the pathological changes obtained in laboratory animals are identical with those seen in the advanced stages of pseudohypertrophic muscular dystrophy of humans.

The various aspects of the muscular disorders in animals associated with vitamin E deficiency have been covered in previous reviews (41c, 73f, 184, 373, 374, 375).

Bicknell (376) and Stone (377, 378) claim that cases of muscular dystrophy in man slowly improve when treated for some considerable time, even years, with preparations possessing vitamin E activity. Many other workers have failed to confirm their observations. Thus, while muscular atrophy in certain animals may be attributed to vitamin E deficiency, the evidence that the muscular dystrophy of man is a deficiency disease is extremely confusing.

Pseudohypertrophic muscular dystrophy does not occur before the age of 5 years, so that the controversy on the use of vitamin E in therapy lies outside the scope of this review. However, the possible relation of vitamin E to this disease raises the need for demonstrating conclusively that deficiency of vitamin E does not occur in infancy.

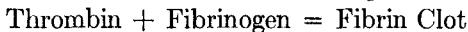
XII. VITAMIN K

Vitamin K is among the latest vitamins that have been shown to be essential for human nutrition. Details of its discovery, isolation and the synthesis of compounds with similar physiological properties, are to be found in several recent reviews (58d, 379).

1. Physiology

So far, vitamin K has been demonstrated to have only one function in the body, namely, it is associated with the formation of prothrombin, one of the substances concerned with the clotting of blood (380). When tissues are injured, thromboplastin is liberated into the surrounding space and interacts with the calcium ions and prothrombin normally present in the blood to liberate thrombin. Thrombin then converts the soluble plasma protein fibrinogen into insoluble fibrin which is the basis of the clot.

Blood clotting, therefore, takes place in two stages:



Prothrombin is produced in the liver (380, 381) and, for this, adequate supplies of vitamin K are necessary. During vitamin K deficiency, the prothrombin concentration in the blood is diminished (382-384). However, vitamin K is not prothrombin (385) nor does it enter into the formation of the prothrombin molecule (381). Liver damage by disease will considerably reduce the production of prothrombin even in the presence of apparently adequate supplies of vitamin K in the diet.

Vitamin K deficiency results in a drop in blood prothrombin.

During the last few years, numerous studies have been made of the prothrombin level in the blood of the newborn infant. The results of six of these have been critically reviewed by Smith and Warner (386). They consider the investigations fall into two groups. Most studies report that in "normal" infants the level of prothrombin on the first day after birth is high, with a rapid fall on the second, to a lower level on the third and fourth, followed by a recovery by the sixth day. Other studies have found the level low on the first and second days with a slow but progressive rise to a satisfactory level by the sixth day. Smith and Warner accept the fact that the method of analysis may be a factor in the production of these two types of curves, but are satisfied that the class of patient and season of the year are also important items. These latter affect the diet of the mother and, hence, the vitamin K intake. It should be noted that these authors believe that exogenous sources are the most important sources of vitamin K for the mother. They also point out that improvement in the low levels of prothrombin found in some infants occurs as soon as the full intake of milk is established.

Naturally occurring vitamin K is fat soluble and, hence, bile salts are essential for its absorption from the intestinal tract. Thus, disease of the biliary tract which interferes with the secretion of bile into the intestines will result in a low blood prothrombin (387, 388).

Vitamin K is only found in very small amounts in the blood, and it is suggested that this occurs only during transport (58d). Larger amounts are found in the liver, but these are not great because fatal hypoprothrombinemia can occur in a week (58d).

Vitamin K is found in the feces but whether any of this has been excreted by the intestine or all is due to bacterial action is not known.

2. Sources of Vitamin K in Infancy

Two schools of thought exist on the question of the sources of vitamin K in infancy. The arguments of one school have been set out in a number of

recent reviews (41c, 73g, 58d). Briefly, they are that the newly born infant obtains a supply of vitamin K from its mother during intrauterine life and that this normally is sufficient to meet the demands of the infant until the flora in its intestinal tract commence the production of vitamin K. These arguments are based upon the assumption that the vitamin K produced by microorganisms in the intestinal tract of man is the principal source of the vitamin for both adults and infants. So far as I have been able to discover, it has not been proven that any of the vitamin K produced in this way is available to man, or that it contributes appreciable quantities to his requirements.

The opinions of the other school have been very ably expressed by Smith and Warner (386) of Iowa. They have brought forward substantial arguments which suggest that the quantity of vitamin K obtained by the infant from its mother during its intrauterine life is influenced strongly, if not entirely, by the vitamin K in the mother's food. When the diet of the mother contains reasonable quantities of green vegetables, the newly born infant is generally adequately provided with vitamin K and *vice versa*. The Iowa group (389) have been able to show that minute quantities of vitamin K, of the order of 1 γ per day, when given daily from birth, are sufficient to meet the full demands of the infant. These workers have shown that a reasonable intake of milk, 30 to 60 cc., does contain adequate amounts of vitamin K to meet this minimal requirement.

Requirements of Vitamin K in Infancy. So far as I have been able to discover, only one attempt has been made to study the vitamin K requirements of the newly born infant. The work of Sells and his co-workers (389) has been referred to earlier; they have demonstrated that doses of the order of 1 γ daily are sufficient to meet requirements. Beyond this age the human requirements of vitamin K are unknown.

3. Pathology of Deficiency States

It has already been shown that vitamin K is essential for the production of prothrombin and that, when the vitamin K supplies in the body of the newborn infant are low, there is a corresponding fall in the blood prothrombin level sometime during the first 7 days of the infant's life. While it has been demonstrated that the prophylactic administration of vitamin K to pregnant women just prior to delivery has reduced the incidence of hemorrhagic disease of the newborn, investigators have not yet conclusively proven that this disease is due to hypoprothrombinemia alone. Scobie (390) has confirmed the findings of others, namely, that prolongation of the clotting time is found in only a proportion of cases of hemorrhagic disease of the newborn. She did not, moreover, find that the prothrombin index in every case of hemorrhagic disease was lower than in the

physiological hypoprothrombinemia, nor was it relatively lower in the severe than in the mild cases.

Some writers (387) however, are confident that the prothrombin level is abnormally low in infants with hemorrhagic disease of the newborn, and Kark and Souter (391) recognize two stages in the fall of blood prothrombin—the stage of latent hemorrhages, when the blood prothrombin level has fallen to about 35% of normal, and the stage of spontaneous hemorrhages when the prothrombin level is 15 to 20% of normal.

No explanation has been advanced linking a low prothrombin value with the initiation of a hemorrhage, but attempts have been made to explain the commencement of the hemorrhage on other grounds. Thus Clifford (392), Snedeker (393), and Ross and Malloy (394) have drawn attention to the pathological changes brought about in blood vessels by *asphyxia neonatorum*. Clifford (392) studied these changes in a number of infants who died from asphyxia due to a *placenta praevia* and the resultant uterine hemorrhage. All infants had been delivered by caesarean section and so were saved excessive trauma. There was congestion of the blood vessels extending down to the final capillaries, associated with petechial and small frank hemorrhages into most organs, including those commonly the site of hemorrhage in cases of hemorrhagic disease of the newborn. The prophylactic use of vitamin K in cases of *asphyxia neonatorum* has been suggested (394).

Recently, interest has centered on the occurrence of abnormal capillary fragility in the newborn (395, 396). Moloney (396) studied the incidence of capillary resistance in 55 newborn infants, only 22 of whom were considered by the author to be free of capillary fragility; 9 showed slight, 12 moderately severe, 8 severe, and 4 very severe, reduction of capillary resistance. In this series the duration of labor in the infants with severe and very severe capillary fragility averaged 14 hours, while the duration of labor for the moderately severe, the slight and the normal group averaged 5 hours.

The failure of escaping blood to coagulate has been explained by the presence of a low level of prothrombin but it would seem work is still necessary to determine the factors responsible for commencement of the hemorrhage.

4. Clinical Manifestations of Deficiency

While Scobbie (390) has defined hemorrhagic disease of the new born as "spontaneous internal and external hemorrhage in the absence of trauma, infection or other definite disease," other authors have not limited the definition to such narrow bounds and have included cases with intracranial hemorrhage. The difference in definition is seen in the types of cases reported by the various authors. Although a classification has been produced

by Quick (397), it would seem that more work is necessary to develop a classification which will give full weight to all the etiological factors.

The recorded incidence of the disease lies between 1% and 4% (389-401). In a disease of this nature, in which the essential pathology is one of degree of deficiency of a substance, there will be a range in clinical manifestations extending from minor departures from normal to gross lesions leading rapidly to death unless controlled by immediate therapy. This factor must influence both the incidence of the disease and the response to prophylactic administration of vitamin K to the mother or to the infant.

The hemorrhage usually occurs between the 2nd and the 6th day after birth (398). However, instances have been described in which the hemorrhage presumably commenced *in utero* (402) and quite a few cases have occurred in the first day of life (403).

The commonest site of bleeding is from the alimentary tract and this is frequently the first sign. In a series of 146 cases Scobie (390) noted either melena or hematemesis in 135 infants. The bleeding may vary from small amounts to large quantities of bright red blood that exsanguinate the infant and, if not treated, lead to death. Other sites of hemorrhage are the cord, which may be the initial site, the nose, the vagina and into the palate.

Retinal hemorrhages have been recorded in infants whose prothrombin values were low and it has been shown that the incidence can be reduced by the administration of vitamin K to the mother (404, 405).

Intracranial hemorrhage is a not uncommon occurrence in newborn infants and much interest has centered around the relationship of this condition to vitamin K deficiency. Salomonsen (398) observed that, whereas there were 66 cases of spontaneous hemorrhage in 9748 live infants, 84 cases of cerebral hemorrhage occurred in the same series; 68 of these happened on the first day of life, and did not exhibit hemorrhage in any other site. Of the remaining 16, only one showed signs of cerebral hemorrhage on the first day, 12 developed hemorrhage in other sites and in all these the coagulation time was increased appreciably.

It is the experience of most workers in this field that the administration of vitamin K to the mother before or during labor reduces the risk of spontaneous hemorrhage. In one series the mortality among infants of the treated group was 1.9% and that among those of the control series 3.9% (404); and, in another series, spontaneous hemorrhage occurred in 2% of untreated cases and in 0.5% of treated (399). Results consistent with these have been obtained by other investigators (406-408). The observations of Parks and Sweet (400) and Sanford (409), however, do not support this and will be discussed later.

That spontaneous hemorrhages respond to treatment with vitamin K is the experience of many workers (394, 410-412).

The observations of two groups of workers must receive comment. Parks and Sweet (400) in Washington, and Sanford and his co-workers (409) in Chicago, have found the administration of vitamin K to the mother did not reduce the incidence of neonatal hemorrhage. The nature of the cases included in their series is important in this discussion. The approximate percentage distribution of Sanford's cases was: conjunctival 32%, vaginal 17%, cephalhematoma 10%, petechial and ecchymosis 10%, cerebral 9%, hematemesis 9%, umbilical 9%, circumcision 4%, melena 4%. This is in marked contrast to most other workers who have found hemorrhage from the bowel in upwards of 80% of the cases.

This immediately raises the question whether these two groups of workers have used the same definition as others and whether their results are comparable, and emphasizes the need for more work on the etiological factors of hemorrhagic disease of the newborn in order to establish a satisfactory classification.

XIII. IRON

In all higher forms of life, including man, iron is an integral part of the substance required for the transfer of oxygen from the lungs to the tissues. In man this substance is hemoglobin which, in the adult, contains about 60% of the iron; in the newborn infant the percentage is much higher.

1. Physiology

Little is known of the internal metabolism of iron. It is absorbed through the intestine in small amounts and carried in the plasma to the bone marrow where it is built into the complex hemoglobin molecule. In addition to the iron in hemoglobin, some is permanently fixed in all intracellular material, taking part in the internal economy of the cells. Any reserve supplies are stored in the liver.

Iron is not destroyed or used up by the body but is conserved. In the healthy individual iron is not excreted in appreciable quantities by either the kidney or the intestinal tract. It has, for this reason been called a "one-way substance."

2. Sources of Iron in Infancy

The newborn infant brings considerable quantities of iron with it from its fetal existence. It has been computed that the newborn full term infant of a healthy mother has in its body from 294 to 392 mg. of iron. It was formerly thought that a high percentage of this was stored in the liver of the newborn infant (413), but it is now recognized that at birth most of this iron is in the red blood cells, and that not more than 60 mg. are in the liver (414).

The iron brought with the infant has to fulfil its requirements until it is

able to eat a mixed diet, for both human and cow's milk are poor sources of iron (415, 416).

3. The Development of Nutritional Anemia in Infants

It is now well recognized that the hemoglobin level of normal full term infants which is very high at birth (15 to 20 g. per 100 cc. is the range usually recorded (36)) falls progressively after birth to a figure between 10 and 12 g. per 100 cc. (417, 418). The procedure in premature infants is similar, for the hemoglobin level at birth is generally within the range observed for mature infants (418-420), but the fall is more rapid and generally to a lower level (418).

The lowest levels are reached at 2 to 4 months of age, from which point the value slowly increases until the adult level of 14 g. is reached in early adolescence. An infant is considered to be suffering from nutritional anemia when the hemoglobin level of the blood is below the normal (415) and levels as low as 7 g. have been considered to be pathological (421).

Nutritional anemia of infancy makes itself manifest in the second half of the 1st year, earlier in premature infants and twins—when growth makes heavy demands on the iron supplies.

Fullerton (422) has discussed the possible etiological factors under 4 headings:

- (a) Type of feeding,
- (b) Effects of maternal iron deficiency,
- (c) Birth weight, and
- (d) Effect of infection,

all of which may play a part under various circumstances. Copper is essential (423) for hemoglobin synthesis but either the liver has adequate stores, or sufficient is obtained in the diet, for it was the experience of Mackay that additional copper does not produce better results than iron alone (424). Other workers (425, 426) have obtained beneficial results from the use of copper. Various aspects of the etiology of the condition have been covered by previous reviews (414, 415, 424, 427).

4. Biochemical Pathology of Infantile Anemia

Nutritional anemia is characterized by a normal, high or low erythrocyte count, a low hemoglobin and marked microcytosis and hypochromia. The microcytosis appears to develop first and to be the most important sign heralding the development of the anemia (428).

5. Prevalence of Infantile Anemia

Mild forms of nutritional anemia of infancy are common (423). As much as 42% of breast fed and 70% of artificially fed infants at 12 to 13 months

have been found to be anemic (415). Severe forms are not common and generally indicate a very bad standard of infant feeding and are almost always accompanied by signs of other deficiencies, particularly of protein (437).

6. Clinical Manifestations of Deficiency

Nutritional anemia is insidious in its onset. Pallor is the most constant sign (41, 415, 429), but pallor is often deceptive, for infants with a normal hemoglobin are often pale. It would seem that, in the mild forms, the condition does not progress beyond pallor. When the level of hemoglobin is reduced to 5 or 6 g., or when the mild form has persisted for several months, observers have reported loss of appetite, fatigue and flabby musculature (429). Mackay (415) has stressed the susceptibility of anemic infants to infection, pointing out that their resistance to infection is lowered. In view of the fact that the severe forms are frequently the sequel of bad feeding, it is difficult to determine how many of the signs are due to deficiency of protein and vitamins and how many are due to deficiency of iron.

Although the blood changes in infantile anemia have been recognized for many years the clinical effects of these are still not clearly defined.

XIV. IODINE

Iodine forms an integral part of the molecule of thyroxine—the secretion of the thyroid gland (430). Lack or shortage of iodine in the food or water will result in the development of hypothyroidism, with or without enlargement of the thyroid gland. Hypothyroidism in infancy leads to cretinism, which may be sporadic or endemic. Endemic cretins also usually have a goiter, while the sporadic cretin does not (41f). The former are the progeny of a long line of goitrous forebears in an iodine-deficient geographical area. The cause of sporadic cretinism has not been fully determined (431).

Infants born with athyreosis (sporadic cretinism), are normal at birth but soon show signs of the deficiency (432), likewise "the endemic cretinous child, even when the thyroid of the mother as well as that of the child is plainly below normal, is perfectly developed at birth" (413). Kerley (433) has reported the physical signs in a cretin 21 months old, while Wolff (431) and Youmans (41f) have given a full description of the condition.

XV. CONCLUDING REMARKS

The data discussed in this paper demonstrate that, in infants, departures from normal health due to an inadequate intake of one or more nutrients occur in three sets of circumstances:

- (a) a poorly balanced maternal diet during pregnancy may affect the supply of some of the nutrients brought by the infant from its

- intrauterine life and so it commences life impoverished nutritionally;
- (b) a poorly balanced maternal diet during lactation can significantly affect the quantity of some of the nutrients supplied by the maternal milk, and these can in some circumstances be so inadequate that the infant becomes the victim of a deficiency disease;
 - (c) a poorly balanced artificial diet fed to the infant frequently leads to the development of deficiency diseases.

A review of each deficiency disease shows that most of the clinical manifestations had been recognized prior to this century. During the last 20 years research has been concentrated upon the identification of the etiological factors and of the biochemical changes associated with each disease. The details enumerated in this review are evidence that much has been achieved. However, much yet remains to be done—for instance, our knowledge of the requirements of infants for each of the nutrients is imperfect.

Despite the shortcomings in our scientific knowledge, much has already been accomplished in Western countries in this century in the application of this knowledge to the problems of child health. Perhaps the most outstanding advance has been the marked improvement in the standard of mothercraft, which is now, more than ever, based upon scientific principles. This improved standard has contributed largely to the reduction in the incidence and severity of deficiency diseases in infancy.

In the field of preventive medicine the present indications are that the application of even existing knowledge to the level of maternal diet during pregnancy will bring about a marked improvement in maternal health, fetal nutrition and infant health.

For progress to be maintained, the next decade must see an intense study of the prenatal phase. Although much evidence has been assembled concerning the relationship of maternal diet during pregnancy to infant health, there is scope for further scientific work.

This, it seems, is the task for the coming decade.

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Effect of B Vitamins on the Endocrinological Aspects of Reproduction

By ROY HERTZ

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I. INTRODUCTION

The relationship between diet and reproductive function has been appreciated from empirical observation for many years. In fact, there has developed a very considerable body of folk belief relating certain foods to fertility, *libido* and potency. For example, eggs and fish are regarded very widely as particularly good sources of whatever it is that is required for optimal sexual function. Conversely, the addition of certain nitrate salts to the diet is thought by many to have a markedly depressant effect upon the *libido*. The waters of the legendary fountain of youth have been heralded for centuries as a beverage capable of inducing complete rejuvenation. Several dietary articles, such as the pomegranate, and innumerable decoctions and potions have been held to promote fertility. Thus, it is clear that man's interest in the effect of ingested materials upon his reproductive capacity is both universal and ancient.

In contrast with this backlog of notion and belief, a modest beginning has been made in the application of scientific nutritional data to the problems of human and animal reproduction. It is aimed in this review to present a critical, rather than an exhaustive survey of the significant contributions to the more restricted problem of the effects of the B vitamins on the endocrinological aspects of reproduction. The clinical observations in this field will be reviewed in another chapter (p.147) in this volume.

The physiology of reproduction is considered to include not only those processes directly concerned with gametogenesis and embryonic development but also such accessory functions as lactation, mating behavior and the ordered succession of estrus cycles in the female.

II. EFFECTS OF FOOD RESTRICTION ON GONADAL FUNCTION

Evans and Bishop (1-4), in their early studies relating diet to reproductive function, were the first to emphasize that a dietary regimen which is adequate for body growth may be grossly deficient for normal reproduction. They showed that simple restriction in food intake on a grossly adequate diet led to impairment of ovarian function. In addition, the amount of yeast supplement necessary for maintenance of normal estrus cycles was shown to be greater than that required for normal body growth in both preventive and curative experiments. The further studies of these authors on the dietary induction of sterility led to their identification of the sterility syndrome attributable to vitamin E deficiency. Hence, the dietary factors in dried yeast which had so critically affected the reproductive cycles of their rats received only secondary consideration.

Mulinos and Pomerantz and co-workers (5-7) presented an extended series of observations concerning the effects of inanition upon the endocrine status of the rat. They concluded that chronic inanition leads to a physiological state somewhat comparable with that following hypophysectomy. They termed this condition "pseudohypophysectomy." Pseudohypophysectomized animals exhibited a marked atrophy of the entire genital tract in both males and females, as well as atrophic changes in thyroid, adrenal cortex, thymus and pituitary. The diet employed in these studies was a natural grain diet supplemented with meat scraps, fish oil and salts. This diet may be regarded as adequate empirically. Nevertheless, it is not unlikely that restriction of food intake by 50% on such a diet may have led to deprivation of specific dietary factors rather than to simple caloric restriction, as implied by the authors. Additional studies of the effects of simple caloric restriction with the intake of at least the known dietary factors properly controlled are clearly indicated.

Numerous studies have shown that the atrophic changes in the genital tracts of B-deficient and chronically underfed animals are correctable by the administration of gonadal or gonadotropic hormones. Moore and Samuels (8) reported that the atrophic accessory sex glands of the B-deficient rat could be stimulated to secretory activity by the administration of either testis hormone preparations or chorionic gonadotropin. Estrus may be restored and ovarian hypertrophy induced by pituitary hormone preparations in underfed rats (9-10). This indicates that the tissues themselves have not become unresponsive to stimulating hormones, but that the dietary inadequacy has resulted in at least a quantitative failure of endogenous hormone production. This failure of hormone production upon the part of the anterior pituitary and the gonads is so readily produced by dietary change as to suggest the existence of some specific supporting factors in natural diets. In terms of available techniques of modern nutritional

experimentation, the existing data represent only a lead in an unexplored field.

Much has been written concerning the relationship between seasonal breeding and the seasonal variation in food supply. Brody (76) has summarized some of the available data. It should be pointed out, however, that animals with distinctive seasonal breeding activity and food habits would probably be extremely useful subjects for experimentation in vitamin-hormone relationships.

III. RELATIONSHIP OF SPECIFIC B COMPLEX FACTORS TO GONADAL FUNCTION AND ESTROGEN METABOLISM*

Well-controlled observations were presented by Drill and Burrill, who reported that inanition as well as B-deficiency leads to a failure of ovarian function in spite of the daily oral administration of large amounts of all of the known B complex factors (except folic acid). They were also successful in stimulating an ovarian response in the deficient animals with gonadotrophic hormone (11).

More recently Hertz and Sebrell studied the effect of various specific dietary restrictions upon the quantitative response to stilbestrol stimulation in the chick oviduct (12-13). It was found that chicks which had been maintained on a diet deficient in folic acid (one of the more recently identified factors of the B complex (14-15)) exhibited only slight estrogen response to maximal doses of stilbestrol. Moreover, as folic acid was added to the diet in increasing amounts, progressive increments in estrogen response were observed. The daily injection of 20 γ of crystalline folic acid per chick per day raised the increment in oviduct weight following stilbestrol administration from three-fold to thirty-fold. Chicks suffering from riboflavin, pyridoxine and pantothenate deficiencies all showed adequate oviduct growth following similar stilbestrol treatment.

Thus a highly specific dietary factor may be shown to be both quantitatively and qualitatively involved in a hormonal response resulting in new tissue formation. The situation is somewhat analogous to the necessity for adequate iodine ingestion for normal thyroid function.

Zondek (16) and Heller (17) demonstrated that liver tissue *in vitro* inactivates estrone. Moreover, Biskind *et al.* (18-19) demonstrated that in rats estrogen pellets implanted in the spleen are not biologically effective, whereas the same pellets implanted subcutaneously, or into the subcutaneously transplanted spleen, are fully active. Direct absorption into the hepatic circulation and immediate inactivation by the liver is considered to be responsible for the ineffectiveness of the intrasplenic implants. Support is given this thesis by the fact that rats bearing intrasplenic pellets but rendered B complex-deficient show a full estrogenic response which dis-

*See also review by Biskind (p. 147).

appears when these animals are returned to an adequate diet. It is considered that the capacity of the liver of the B complex-deficient animal for estrone inactivation is markedly impaired, thus allowing the intrasplenic estrogen pellet to exhibit its characteristic effect of cornification of the vaginal epithelium.

Segaloff and Segaloff (20-21) confirmed and extended these interesting observations. They showed that estrone, α -estradiol and several synthetic estrogenic compounds are relatively ineffective when administered intrasplenically to rats maintained on an adequate diet. However, such intrasplenically administered estrogens had the expected potency in animals maintained on a purified diet free of B complex factors, although such deficient animals showed a reduced responsiveness to the same estrogen injected subcutaneously. The addition of thiamine or riboflavin to the deficient diet restored the animals' capacity to inactivate estrone and α -estradiol, but choline, calcium pantothenate and pyridoxine were without effect. In the case of diethylstilbestrol, however, none of these elements of the B complex exhibited a restorative effect. Moreover, inanition *per se* was without effect upon the inactivation of intrasplenically administered estrone and estradiol but exerted an intermediate effect upon the inactivation of diethylstilbestrol.

It is regrettable that these highly significant studies of Biskind *et al.* and of the Segaloffs were based on a qualitative response to estrogen, namely, vaginal cornification. This response is notably variable from animal to animal and in the same animal at different times. Coward and Burn (22) and D'Amour and Gustavson (23) dealt with the statistical aspects of this variability in vaginal cornification following a given estrogen treatment. Comparative data employing uterine weight as a quantitative measure of the effectiveness of estrogenic substances under varying dietary conditions would afford more decisive information concerning the role of the liver and of the diet in estrogen metabolism.

Shipley and Gyorgy (24) more recently presented data indicating that liver injury in animals maintained either on a cirrhosis-producing diet or on a B complex-deficient diet results in a failure to inactivate intrasplenic estrone. They noted that the addition of whole yeast was more effective than the addition of the known vitamins of the B complex in restoring normal estrogen inactivation. It should be pointed out that neither biotin nor folic acid was included in the supplement employed.

Singher *et al.* (25) showed that liver tissue from riboflavin- and thiamine-deficient rats is incapable of inactivating estradiol *in vitro*. The livers of animals which were deficient in pyridoxine, pantothenate or biotin continued to show a normal degree of liver inactivation under identical conditions. Moreover, a definite level of thiamine and riboflavin content in the

livers of the depleted animals proved critical for estradiol inactivation. The significance of the data is obscured by the fact that these authors did not present any data permitting comparison of the estradiol inactivation with any basal tissue function, such as oxygen consumption. However, the general statement was made that "only those experiments which showed actively respiring tissue were included in the final results."

It is interesting that in experiments designed for another purpose, Warkany *et al.* (26) observed that in about one-half of their rats fed a purified diet supplemented with five members of the B complex but lacking riboflavin there was a cessation of estrus cycles. These animals could be restored to fertility by periodic feeding of riboflavin in amounts sufficient to cause a substantial gain in weight. Similar data were also presented in the case of both thiamine- and riboflavin-deficient rats by Coward *et al.* (27).

In view of the growing information relating the B complex factors to estrogen metabolism, an earlier observation of Van Horn's (28) becomes of renewed interest. He observed that in the thyroid-fed rat, estrone had a markedly reduced biological potency. Drill (29-30) presented extensive data indicating that the thyroid-fed rat has an increased requirement for thiamine, pyridoxine and pantothenic acid. Additional studies are desirable to determine whether the thyrotoxic rat's failure to utilize estrogen optimally is related to its relative deficiency in the B complex factors. The clinical implications of such information are apparent when one considers the marked effect of thyrotoxicosis on menses in women (31).

From the foregoing discussion, it may be noted that some of the available data indicate a reduced sensitivity to estrogenic hormone in B complex-deficient animals (12-13). Conversely, the studies describing the activity of intrasplenically administered estrogen suggest an increased efficiency of the hormone resulting from the failure of hepatic inactivation (19, 31, 24). These observations are made on different species and for different factors of the B complex. They should, therefore, not be regarded as necessarily in conflict with each other.

Kennedy and Palmer (32) showed that biotin deficiency in the rat induced by feeding 30% egg albumen in a purified diet does not interfere with conception but leads to frequent fetal resorption and impaired lactation. Neither the feeding of 6 γ of biotin daily nor the reduction of the egg-white content of the diet to 15% improved these reproductive functions. The data show that such egg white-intoxicated animals lack some factor other than biotin itself. Similarly, hamsters were shown to require biotin as well as *p*-aminobenzoic acid and inositol for growth and reproduction (33).

Hertz, Fraps and Sebrell (34-35) showed that avidin, the antibiotin factor in egg white, is a secretory product of the albumen-secreting portion of the hen's oviduct and that avidin formation may be induced in the oviduct

of the young chick by administering stilbestrol plus progesterone. Extensive efforts to demonstrate the presence of avidin in mucosal secretions of the fallopian tube of the pig, cow and guinea pig have failed (36).

The significance of avidin and the avidin-biotin complex for reproduction remains an enigma, but the appearance of this specific material under hormonal control in the genital tract of birds and amphibia may be expected to have some bearing upon the normal reproductive physiology.

IV. B COMPLEX FACTORS AND LACTATION

There is little agreement regarding the B factors required for lactation. Evans and Burr (37) showed that thiamine was required for lactation in the rat at a level about five times that necessary for body growth. Sure (38) also presented quantitative data concerning the rat's requirements during lactation and indicated the necessity for choline for adequate growth and lactation in this species (39). Jukes (40) reported adequate reproductive performance in the rat on a purified diet supplemented with the known factors of the B complex. Three additional laboratories succeeded in raising rats through three generations on a similar diet (41-44). They noted, however, that weight of the young at weaning was distinctly below that observed in animals on the stock diet. Richardson *et al.* also agreed that lactation is suboptimal on a purified diet unless it is supplemented with a liver extract (45).

Nakahara *et al.* (46, 47) concluded from indecisive studies employing a diet of polished rice, fish protein and salt that specific substances contained in yeast and liver which they termed L₁ and L₂ are essential for normal lactation in the rat. Similarly, Sure (48) indicated that a purified diet containing six of the elements of the B complex plus generous amounts of "factor W" was inadequate for lactation in the rat and that *p*-aminobenzoic acid and inositol are also required. Subsequently, he extended his observations and concluded that *p*-aminobenzoic acid "has a markedly favorable influence on lactation," whereas inositol was found to have a deleterious effect which could be reversed by *p*-aminobenzoic acid (49). Climenko and McChesney concluded that inositol rather than *p*-aminobenzoic acid is critical for lactation although they found the latter exerted some favorable effect (50).

Conversely, Foster *et al.* and Troescher-Elam and Evans raised mice on purified diets including *p*-aminobenzoic acid but reported subnormal growth and a high mortality during the suckling period (51-52). Rogers *et al.* reported a marked difference in reproductive efficiency between two strains of mice fed a purified diet supplemented with eight crystalline factors and a rice-polish concentrate (53). Ball and Barnes (54) found that a purified diet supplemented with 8% yeast proved inadequate for lactation in the mouse,

but lactation was improved upon the addition of dried grass and wheat bran to the diet. Folley *et al.* (55) reported that although rats on a highly purified diet exhibited "subnormal lactation," they still successfully reared three successive generations on such diets.

Vinson and Cerecedo (56) developed a synthetic ration capable of maintaining good body growth in rats, but they noted a considerable weight loss in the lactating mother fed such a diet. The addition of biotin, *p*-aminobenzoic acid, inositol and a yeast nucleic acid concentrate did not protect the nursing animals from loss of weight, but brewer's yeast at the level of 0.5 g. daily was completely protective. Their subsequent studies indicated that the addition of either a folic acid concentrate or crystalline *L. casei* factor increased the average size of the litters and the proportion of young weaned, and protected the nursing mother from weight loss (57, 58). Similarly, mice showed an improvement in lactation following the addition of a folic acid concentrate from a plant source to a highly purified diet containing thiamine, riboflavin, pyridoxine, pantothenate and choline but excluding both *p*-aminobenzoic acid and inositol (59).

An interesting dietary study on lactating pigs and rats was reported by Ross *et al.* (60). They found that on a diet consisting of yellow corn, soybean oil, iodized salt and calcium carbonate a supplement of 5% of alfalfa did not support lactation whereas a 15% alfalfa supplement was adequate for optimal lactation (60). It is pertinent that alfalfa is one of the best natural sources of folic acid (61).

It is apparent that the B complex requirements of the lactating individual have not been completely clarified. Numerous possible reasons for the discrepancies between the reports cited may be suggested. Genetic differences between the various strains studied are presumably a factor. Trace contamination of one investigator's diet with such potent factors as folic acid and *p*-aminobenzoic acid could affect the results very materially. Also the criteria for adequacy of lactation are not clearly defined, thus altering the interpretation of experimental data.

Moreover, since lactation is hormonally controlled, the nutritional conditions essential for this important process may be regarded also as critical for normal hormonal function. Thus, optimal lactation implies adequate function of at least the ovaries as sources of estrogen and progesterone and the anterior pituitary which secretes the lactogenic and gonadotrophic factors (62, 63). From the foregoing, we have seen the profound effects of dietary changes on endocrine function as well as on lactation itself. This relationship opens an extensive field for experimentation aimed at segregating the dietary and hormonal factors operating in much of the work reported herein.

V. EFFECTS OF B-COMPLEX CONTENT OF THE MATERNAL DIET ON THE YOUNG

Warkany and his associates reported a distinguished series of studies of the effects of diet upon the incidence of congenital malformations in the rat (26, 64-67a). Their early observations had established the fact that a maternal diet of yellow corn meal, wheat gluten, salts and vitamin D led to a relatively high incidence of a specific pattern of deformity in the offspring. This pattern of deformity was characterized by "shortening of the tibia, mandible, fibula, radius and ulna, fusion of ribs, fingers and toes, and cleft palate." Genetic factors were ruled out by similar findings on three widely different strains, as well as by studies on the comparative incidence of abnormal young delivered by the same animal when fed the experimental and a control diet. The protective factor was first observed to be present in pig liver and in an alcoholic liver extract. Supplementation of the deficient diet with a combination of riboflavin, thiamine, niacin, pyridoxine and pantothenate afforded complete protection. Neither thiamine nor niacin alone, nor a combination of pyridoxine plus pantothenate or a further combination of these with thiamine and niacin, proved effective. Riboflavin alone proved highly protective and was regarded by the authors as the essential causal factor lacking in the experimental diet.

By careful adjustment of the riboflavin intake in a purified diet, the yield of abnormal young was materially increased. It is concluded that the level of riboflavin in the maternal blood is critical in determining whether a given embryo will die, will develop normally or will be delivered with the deformities described.

Earlier studies by Lepkovsky *et al.* indicated that the riboflavin content of the maternal diet played a critical role in the maintenance of optimum hatchability of hen's eggs (68). Restoration of adequate riboflavin to the diet of the hen promptly led to a return to normal riboflavin content in the egg albumen and to a higher hatchability. Death of the embryos was accompanied by such defects as edema, clubbed down and anemia. Moreover, chicks hatched from riboflavin-deficient eggs showed a degenerative lesion at the junction of the hard and soft portions of the beak, a condition referred to by the authors as "notched beak." The possible relationship between this defect and the nasopharyngeal defects described by Warkany (65) in the rat warrants consideration.

Bauernfeind and Norris (69-70) presented evidence indicating that both pantothenic acid and an additional unidentified water-soluble factor were required for optimal hatchability in hen's eggs. Cravens *et al.* subsequently demonstrated that hatchability, as well as egg production, was reduced when hens were maintained on a diet lacking a corrective factor contained in a Norit eluate fraction from liver (71).

Snell *et al.* (72) concluded that the pantothenic acid content of an egg is quantitatively determined by the dietary intake of the hen. Snell and Quarles (73) studied the effect of incubation on the B complex content of hen's eggs. They found that during the course of incubation there developed no material change in the content of pantothenic acid, riboflavin and biotin. However, niacin and inositol were formed in considerable amounts. The authors point out that this fact demonstrates synthesis of these two factors in a bacteria-free system.

These determinations were carried out on filtrates from autolyzates which had been precipitated with glacial acetic acid. Subsequent studies (74) have shown that such procedures are not reliable for the complete liberation of the tissue content of several of these factors. It would, therefore, be desirable to reinvestigate the effect of incubation upon the B complex content of the egg, employing the more recently developed methods of tissue digestion.

VI. GENERAL CONSIDERATIONS CONCERNING VITAMIN-HORMONE INTERRELATIONSHIPS

Our survey of the growing body of data concerning the interrelationship between the factors of the B complex and certain hormonally controlled reproductive processes leads to a few general considerations.

First, what are the possible points of interaction between a vitamin and a hormone? The hormones for the most part stimulate either new tissue formation or qualitative alteration in the constitution of preexisting tissue. These effects naturally create a demand for the essential constituents of such tissue substance, and the absence of a readily available reserve of such elements seriously impairs the hormonal response. The vitamins of the B complex may be regarded as such critical building blocks and thus a great deal of the data previously outlined may be readily rationalized.

This may, however, represent an oversimplification of what is really a much more complex interrelationship. Practically nothing is known of the intracellular state, in which the steroid and protein hormones function in nature, and very little information is available regarding the actual mechanism of action of many of the factors of the B complex. It would, therefore, be difficult to attempt explanation of the functional relationships between these two highly potent classes of compounds.

This unsatisfactory state of our knowledge has led many investigators to regard the type of study reviewed herein as a mere juggling of crude variables leading to nonspecific effects which can be secured by any of several experimental procedures. One of the first points raised concerning a phenomenon of vitamin-hormone interaction is the specificity of the effect described for the particular vitamin in question. Any lack of specificity is com-

monly thought to reduce the significance of the effect observed. Thus, the failure of estrus cycles which may follow reduced caloric intake (6), reduced riboflavin intake (26) or reduced thiamine intake (11) is generally regarded as an occurrence of little experimental consequence. On the contrary, there is presumably a highly significant common factor operating under these three diverse circumstances to effect a failure of estrus, and the experimental utilization of the factors already recognized may lead to its ultimate elucidation.

The difficulties of endocrinological research are multiplied in the investigation of a vitamin-hormone relationship. The factor of food intake must be given full consideration (11). In addition, the accessory effects of the vitamin deficiencies, such as reduced resistance to intercurrent infection, must be kept in mind in evaluating results. Possible retardation of absorption of orally or parenterally administered hormones and vitamins in animals debilitated by the several deficiencies is also a potentially significant experimental factor. Too often these considerations are overlooked in evaluating the significance of the data.

The stimulating effect of some of the hormones on new tissue formation may be experimentally employed to demonstrate specific nutrients required for the rapid and excessive growth of specific tissues. The excessive folic acid requirement for optimal oviduct response to stilbestrol in the chick is a case in point (13). Similar data have recently been published indicating an increased vitamin A requirement in rats stimulated to rapid growth by pituitary growth hormone (75). Thus, one can readily visualize the manifestation of otherwise obscure quantitative and qualitative nutritional requirements by means of the hormonal demand for excessive and rapid tissue growth. This promises to be a useful tool in nutritional research.

The disturbance of a normal hormonal mechanism in association with a vitamin deficiency may represent either failure in hormone production, a loss of responsiveness on the part of the tissues involved or decreased hormone destruction. Thus, the anestrus resulting from underfeeding (9, 11) is demonstrated to be a failure of gonadotrophic hormone output by the pituitary, whereas the impairment of stilbestrol response in the folic acid-deficient chick (13) is clearly an inability to respond to the hormone administered. In the case of the persistent estrus observed in B complex-deficient rats to which estrogens have been intrasplenically administered (18, 19), the normal processes of hormone inactivation are at fault. Although the end result in all three instances is a dysfunction, the respective mechanisms are different. Extensive experimentation is indicated in order to determine in which of these categories one should place many of the nutritionally induced endocrine failures described.

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Nutritional Therapy of Endocrine Disturbances

By MORTON S. BISKIND

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I. INTRODUCTION

Although direct nutritional therapy of endocrine disturbances is of very recent origin, numerous clinical observations during the past half century, viewed in retrospect, reveal surprising correlations between nutritional and endocrine disturbances. And scattered animal investigations during the past thirty years have indicated a more direct relation between nutritional deficiency and changes in endocrine function.

Among these older studies were the use of liver and liver extracts in diabetes (Gilbert and Carnot, 1896). Stockton (1908) described the characteristic appearance of the tongue in diabetes as "large, red, 'beefy,' and bordered with a fissured margin . . .," a description now accepted as classical of pellagra. Functional changes in the central nervous system in diabetes that we now recognize as characteristic of thiamine and niacinamide deficiency were described with great accuracy by Futcher in 1907. The occurrence of hyperglycemia in pigeons on a "vitamin-free" diet was reported by Funk and von Schoenborn in 1914; this observation was followed up by other workers but its clinical implications were long neglected.

In chlorosis, long recognized as a nutritional disorder, menometrorrhagia and other menstrual disturbances were known to be characteristic (Cabot, 1908), and the occurrence of excessive uterine bleeding associated with cirrhosis of the liver, was equally well known three or four decades ago (Kelly, 1908; Rolleston, 1912). Goldberger subsequently observed menorrhagia in pellagra.

Plaut (1923) first reported hypertrophy of the adrenals in avitaminosis in animals.

Testicular atrophy occurring secondary to "malnutrition" was mentioned by Reynolds and Macomber in 1921, and both testicular atrophy and gynecomastia have been known for some time to be associated with hepatic cirrhosis (cited by Glass *et al.*, 1940).

More direct evidence of a relation between nutrition and endocrine function was provided by Evans and Bishop (1922); they reported that rats on a vitamin B free diet became anestrous. This phenomenon was later shown to be due to the suppression of the anterior pituitary (Parkes, 1928; Marrian and Parkes, 1929).

Despite these and other accumulated data, the concomitant appearance of nutritional and endocrine disturbances in the human being was considered to be accidental. Recent investigations have shown the nutritional defect, in many instances, to be etiologic in relation to the glandular dyscrasias. This will be discussed in the subsequent pages.

II. SYNDROMES RELATED TO EXCESS ESTROGEN

1. Relation of Nutritional Deficiency to Inactivation of Estrogen in the Liver

Soon after the isolation of theelin (Doisy, Veler and Thayer, 1929), it became apparent that this and related steroids are rapidly inactivated in the body (Frank, Goldberger and Spielman, 1932; Zondek, 1934). One earlier clue pointed to the possibility that estrogen is destroyed somewhere in the portal circulation. Evans and Burr (1926) had observed that estrogenic preparations were more effective when given subcutaneously than when administered intraperitoneally. The first suggestion that the liver might be a site of inactivation appears to have been made by Silberstein, Molnar and Engel (1933), although as Zondek (1935) pointed out, the method employed by these workers was not adequate to establish this conclusively. It was Zondek (1934) who first reported an extensive investigation of this problem. He demonstrated that the liver is capable of inactivating from 80 to 90 % of added estrogen *in vitro*. This function of the liver was subsequently confirmed in another way; circulation of estrone through a heart-lung perfusion system did not result in destruction of the estrogen but circulation through a heart-lung-liver system led to rapid inactivation (Israel, Meranze and Johnston, 1937). Estrus did not occur in animals in which the ovaries had been transplanted to the mesentery (in the portal circulation); regular estrous cycles did occur in rats having ovarian transplants in the axillae (Golden and Sevringshaus, 1938).

Employing the method of pellet implantation (Shelesnyak and Engle,

1932; Deanesly and Parkes, 1937), G. R. Biskind devised the technic of inserting pellets of crystalline steroids into the spleens of castrate male and female rats. With the spleens containing pellets in the normal situation in the portal circulation, no estrogenic or androgenic effect occurred with estrone, estradiol, estradiol benzoate, or with testosterone, testosterone propionate or methyl testosterone (G. R. Biskind and Mark, 1939; G. R. Biskind, 1940, 1941, 1942). Inactivation of both estrogens and androgens occurred in the livers of both male and female rats. If the spleen containing the pellet of steroid was subsequently transplanted subcutaneously and, after establishment of a collateral circulation, the pedicle was ligated, the specific estrogenic or androgenic effect became evident (G. R. Biskind and Mark, 1939).

On the basis of the latter investigations, and others implicating nutritional defects in alterations of hepatic morphology (Patek, 1937; Ando, 1938; Rhoads *et al.*, 1938, 1940; Sebrell and Onstatt, 1938; Nakahara *et al.*, 1939; György and Goldblatt, 1939, 1940; Rich and Hamilton, 1940) (*cf.* also: Blumberg and McCollum, 1941; Daft *et al.*, 1941; Earle *et al.*, 1941, 1942; Lillie *et al.*, 1941, 1942; Lowry *et al.*, 1941; Patek and Post, 1941; Broun and Muether, 1942), M. S. and G. R. Biskind (1941), utilizing the method of splenic implantation, found that, while castrate female rats with pellets of estrone in their spleens remained anestrous when on a normal diet, they went into continuous estrus when the diet was depleted in B complex vitamins, thus demonstrating that, in this type of nutritional deficiency, the liver loses its ability to inactivate estrogen. Addition of brewers yeast, or a mixture of crystalline thiamine, riboflavin, pyridoxine and calcium pantothenate, to the diet restored the anestrous state and subsequent depletion again led to continuous estrus (M. S. and G. R. Biskind, 1942; M. S. Biskind, 1943). Thus, the flow of estrogen through the liver could be controlled at will by withholding the B vitamins or restoring them to the diet. Impairment of the estrogen-inactivating mechanism of the liver occurred in the absence of detectable morphologic change in this organ (Fig. 1) (M. S. and G. R. Biskind, 1942); conversely, inactivation of estrogen can occur in livers which are the site of severe necrosis and fat infiltration, induced by a B complex-free diet supplemented with thiamine, riboflavin, pyridoxine and calcium pantothenate (Fig. 2) (M. S. Biskind, 1944). The functional and morphologic changes in the liver thus bear no necessary relation to each other.

Subsequent investigation of the problem of estrogen inactivation in the liver has shown that, *in the rat*, thiamine and riboflavin alone among the B vitamins are adequate to permit hepatic destruction of estrogen (Singher, Taylor *et al.*, 1944; Singher, Kensler *et al.*, 1944; Segaloff and Segaloff, 1944:

Shipley and György, 1944) and that the presence of methionine is essential to this function (Unna, Singher *et al.*, 1944; György and Goldblatt, 1945).*

* While this review was in press, Drill and Pfeiffer (1946) reported experiments from which they conclude, in contrast to the results obtained by the other investigators in this field, that "deficiency of the whole vitamin B complex affects the inactivation of estrogen only through the concomitant inanition produced," and that "supplements of methionine were without effect."

While it is important that the role of all nutrients in a given phenomenon be adequately assessed, in actual practice the tendency to dismiss the effects of the various vitamins as due to the associated inanition, has acted in the past greatly to retard proper evaluation of experimental data and to delay their application to human nutrition. The problems involved are complicated and the greatest care is necessary both in the performance of the investigations and in their interpretation. Reference may be made, for instance, to the observation of Funk and von Schoenborn (1914) on experimental diabetes and the subsequent work of Collazo (1922) and others, reviewed at length by Sherman and Smith (1931) and discussed briefly in Section V. Mason (1939) has reviewed the literature relating to the differential aspects of vitamin depletion and inanition on the reproductive apparatus, and Lepkovsky (1942) has pointed out (with specific reference to the effect of pantothenic acid on the testis) a serious pitfall common to virtually all paired feeding experiments used for separating the effects of specific vitamin deficiencies from those of the associated cachexia.

With specific reference to the aforementioned report of Drill and Pfeiffer, important discrepancies and omissions appear in relation to previously reported investigations in this field: In the experiments described by M. S. and G. R. Biskind (1941, 1942), estrual reactions occurred in some castrate animals with pellets of estrone in their spleens, as early as 2 or 3 days after being placed on a vitamin B complex-free diet, long before inanition could possibly be a factor. Shipley and György (1944) in observations on the same strain of rats, reported, "All became positive [estrous] within 5 to 14 days, and before any serious ill effects from the diet were evident." Estrual reactions as early as the third day of depletion also occurred in the experiments of Biskind and Shelesnyak (1942), in which one ovary was transplanted to the spleen and the other was removed. In addition, restoration of the B vitamins to the diet in severely depleted castrate animals with splenic pellets, led to resumption of the hepatic estrogen-inactivating function in as little as 2 to 4 days, before the cachectic state of the animals was significantly altered. Shipley and György observed this reversal under similar conditions, in as little as 3 or 4 days. Obviously, with respect to hepatic estrogen-inactivation in the animal with an intact gonad, inanition cannot be of practical importance, since, as is well known, ovarian estrogen secretion ceases during starvation, whereas Biskind and Shelesnyak showed that impairment of estrogen inactivation occurs long before ovarian function is depressed.

The prolonged period required to induce estrus in the experiments of Drill and Pfeiffer (20 to 30 days), compared to the experience of M. S. and G. R. Biskind and Shipley and György with the Sherman strain of rats (2 to 23 days in the former, 5 to 14 days in the latter) suggests that Drill and Pfeiffer may have used a relatively resistant strain (Shipley and György showed the Sherman variety to be the most susceptible to nutritional impairment of hepatic function of 3 strains tested). Unless the animals receiving a vitamin B complex-free diet are individually fed daily with fresh food (which was done in the original experiments of M. S. and G. R. Biskind), they stop eating long before they otherwise would. Because the utilization of

In connection with the previous study on the relation of B complex deficiency to the inactivation of estrogens, M. S. and G. R. Biskind (1943) investigated the effect of dietary depletion of the B vitamins on the inactivation of androgen in the liver. Unlike the estrogens, there was no *significant* impairment of the ability of the liver to destroy androgen in B complex deficiency. Thus, a serious alteration of the estrogen-androgen equilibrium must result. This has important clinical implications which will be discussed subsequently.

In view of the observation by Evans and Bishop (1922) that in vitamin B deficiency, rats become anestrous, M. S. Biskind and Shelesnyak (1942) studied the effect of vitamin B complex deficiency on rats in which one ovary had been removed and the other transplanted to the spleen. On a complete diet such animals remained anestrous; on a B complex-free diet all showed estrual reactions. Thus, it became apparent that the estrogen-inactivating mechanism of the liver can be impaired at a time when the ovary is still functional. This, too, has important clinical implications.

The observation by Plaut (1923), previously mentioned, that the adrenal cortex is hypertrophic in avitaminosis B in rats, is undoubtedly related to failure of inactivation of estrogen in the liver. Excess estrogen regularly produces this phenomenon (Korenchevsky and Dennison, 1935), and it was observed by the author in vitamin B deficient castrate animals with a pellet of estrogen or an ovary implanted in the spleen, showing protracted estrual reactions as a result of impaired inactivation in the liver.

thiamine and riboflavin is related to the caloric intake, failure to take this precaution may lead to inanition before the reserves of the B vitamins are depleted. It is not clear whether this precaution was taken by Drill and Pfeiffer. These workers kept their animals in the pre-depletion control period, not on a full diet, but on a purified diet plus A, D and 9 B factors; loss of other nutrients thus occurred even before intentional depletion (see Section VI).

Unna, Singher *et al.* (1944), György and Goldblatt (1945) and György (1945) have shown that, in the absence of an adequate amount of protein, the B vitamins cannot maintain estrogen-inactivation in the liver. Hence, restriction of diet as performed in the paired feeding experiments of Drill and Pfeiffer could easily have reduced the protein intake below the critical level, and the apparent failure of methionine in their experiments, in contrast to the positive effect reported by Unna *et al.* and György, may have been due (assuming the possibility that other factors were equal) to differences in the basic protein intake.

From unpublished experiments of G. R. and M. S. Biskind (interrupted by wartime exigencies) other factors in addition to thiamine, riboflavin and methionine appear to be involved in the estrogen-inactivating mechanism, and it appears that inanition can *also* disturb it, even though this has little practical significance. As discussed subsequently, in the clinical syndromes in which nutritional deficiency is associated with excess estrogen, the vast majority of the subjects are extremely well fed; the latter respond dramatically to administration of the whole B complex.

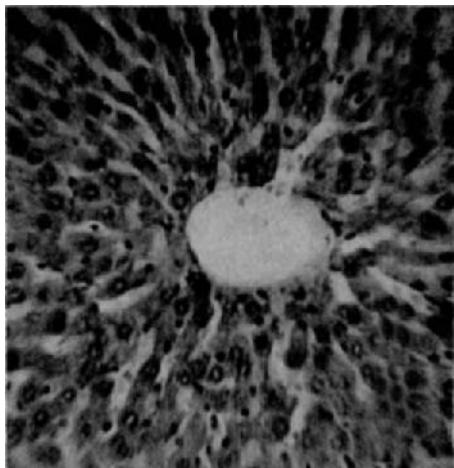


Fig. 1. Photomicrograph of liver of castrate female rat with pellet of estrone in the spleen, after depletion on a vitamin B complex-free diet. The morphology is normal although the liver had lost its estrogen inactivating function.

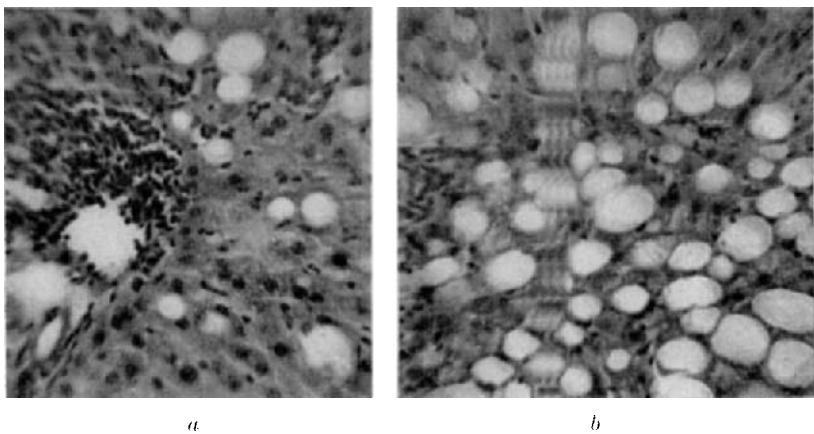


Fig. 2, *a* and *b*, Photomicrographs of livers of rats similar to animal represented in Fig. 1, except that thiamine, riboflavin, pyridoxine and pantothenic acid had been added to the vitamin B complex-free diet. The livers had regained their ability to inactivate estrone but are morphologically seriously damaged.

2. "Functional" Uterine Bleeding, Cystic Mastitis, Premenstrual Tension

Evidence relating the occurrence of certain forms of pathologic uterine bleeding and of premenstrual tension, chronic cystic mastitis and other disturbances to an excess of estrogen was provided originally by the fundamental work of R. T. Frank and his collaborators (Frank, 1931; Frank, Goldberger and Spielman, 1934; Frank, 1935; 1940). Ehrlich (1941) has

reported that the endometrium of patients with menorrhagia or metrorrhagia contains thrombotic phenomena similar to those illustrated by Zuckerman (1937) in the endometrium of castrated monkeys treated with estrogen.

The occurrence of excess estrogen in the clinical syndromes mentioned was thought to be due to excessive secretion by the ovaries (Frank, 1935). However, the observation that the liver fails to inactivate estrogen in vitamin B complex deficiency suggested another explanation (M. S. and G. R. Biskind, 1941, 1942, 1943; Biskind and Shelesnyak, 1942), and the relation of these syndromes to nutritional deficiency was therefore investigated (M. S. Biskind, 1943; M. S., G. R. and L. H. Biskind, 1943, 1944).

In three series of patients, involving at the time of writing, a total of more than 450 cases, a striking correlation was found between signs and symptoms of nutritional deficiency and the occurrence of syndromes related to excess estrogen. In these series, every patient, without exception, who had excessive uterine bleeding, cystic mastitis or premenstrual tension also had definite and usually severe objective and subjective indications of nutritional deficiency. Treatment with vitamin B complex orally, or orally and parenterally, produced rapid and dramatic improvement in the endocrine disturbances, along with healing of the avitaminotic lesions (see Figs. 3-6 and 8-10).

Among the patients with functional uterine bleeding, every endometrium examined showed evidence of estrogenic proliferation and the absence of a progesterone effect. The histologic appearance of the endometrium varied from that occurring in the early proliferative phase to cystic glandular hyperplasia and adenomyosis. Figs. 4, 5 and 6 illustrate the correlation of the lesions of nutritional deficiency and the estrogenic proliferation of the endometrium.

It has long been known that menorrhagia and metrorrhagia may occur early in the course of cirrhosis of the liver (Kelly, 1908; Rolleston, 1912). Excessive uterine bleeding has been reported also in intoxication with a number of liver poisons, such as lead, benzene, carbon disulfide and tetryl. Cirrhosis of the liver is now known, from the work of György and Goldblatt (1939, 1940, 1942) and others, to result from nutritional deficiency. Sources of the vitamin B complex have been shown to protect the liver against a variety of agents (such as lead, arsenic, carbon tetrachloride and dimethylaminoazobenzene) which cause functional and morphologic damage to this organ (Forbes and Neale, 1936; Forbes and McConnell, 1937; Von Glahn and Flinn, 1939; Ando, 1938; Nakahara *et al.*, 1939; Kensler *et al.*, 1940, 1941; Rhoads, 1940; Sugiura and Rhoads, 1941). In addition to this evidence, Goldberger (Goldberger and Sebrell, 1932) has reported that menorrhagia may occur in pellagra.

Glass, Edmondson and Soll (1940, 1944) have demonstrated in male patients with cirrhosis of the liver that the estrogen in the urine is increased and appears in active (uncombined) form, while the urinary androgen is somewhat reduced in amount and all of it continues to be excreted in combined form. These patients had gynecomastia, testicular atrophy, or both. These results agree precisely with the observation by M. S. and G. R. Biskind (1942, 1943) already mentioned, that in vitamin B complex deficiency the liver loses its ability to inactivate estrogen while it continues to inactivate androgen. Wu (1942) has observed histologic changes in the prostate glands of patients with cirrhosis of the liver, indicative of stimulation by estrogen. A survey of necropsy records of female patients with cirrhosis of the liver (M. S. and L. H. Biskind, 1943) showed evidence of excess estrogen in every case in which data were available on the pelvic organs.

It seems likely that dietary estrogen, which normally is destroyed in the liver, would largely or entirely escape inactivation in vitamin B complex deficiency and, added to endogenous estrogen, already in excess, would exert a further deleterious effect. That failure of inactivation of dietary estrogen occurs in cirrhosis of the liver is suggested in a case (for the report of which I am indebted to Dr. G. R. Biskind) of a woman aged 70, who died of hepatic cirrhosis; the endometrium showed extreme active cystic hyperplasia.

Bean (1942, 1943) has found that the cutaneous vascular spiders and palmar erythema formerly associated mainly with cirrhosis of the liver, occur also in nutritional deficiency and at the period in pregnancy when estrogen increases significantly. Administration of estrogen to patients with the cutaneous phenomena led to the appearance of new lesions and exacerbation of those already present; withdrawal of the estrogen caused regression of the vascular disturbances. Bean has correlated his observations with experimental studies by Reynolds and Foster (1940), who showed that estrogen causes dilatation of the minute vessels in the ears of the castrated rabbit, and he has shown that the cutaneous vascular spiders are histologically similar to the spiral arteries of the endometrium observed by Bartelmez and Markee (*cf.* Bartelmez, 1942) and by Jones and Brewer (1939). Perera (1942) has also reported the appearance of palmar erythema in nutritional deficiency. He noted regression of this lesion in two cases of hepatic cirrhosis under dietary and vitamin therapy.

A considerable proportion of patients who have lesions of nutritional deficiency associated with functional uterine bleeding, cystic mastitis and premenstrual tension also have the cutaneous vascular spiders and palmar erythema noted by Bean and by Perera. In addition, a tendency to develop petechial hemorrhages from relatively minor bruises and an increased tend-

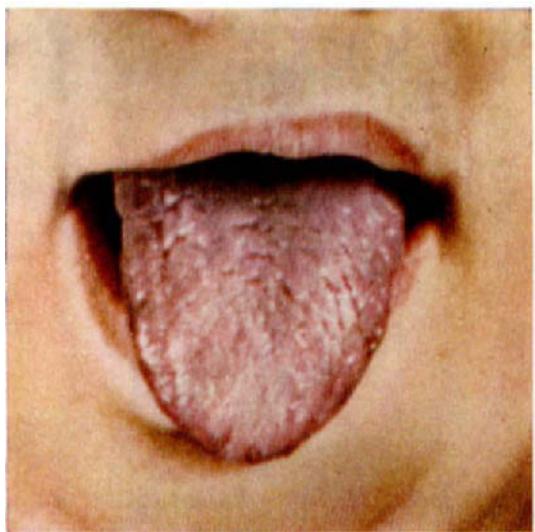


FIG. 3 *a*



FIG. 4 *a*

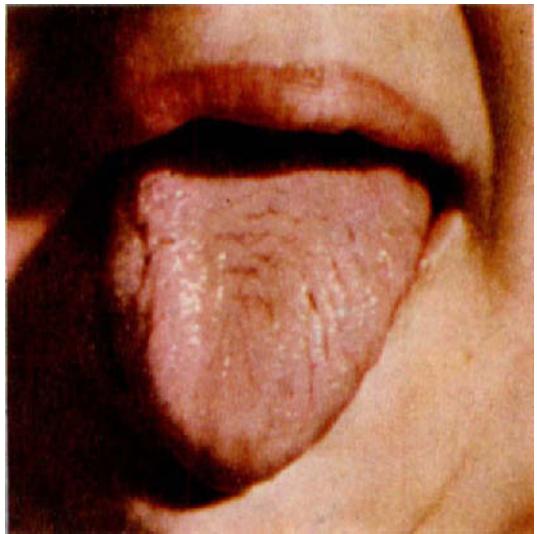


FIG. 3 *b*



FIG. 4 *b*

Fig. 3. *a*, Atrophic glossitis in a patient with cystic mastitis and premenstrual tension; following a pregnancy two years previously she had had subinvolution of the uterus and menometrorrhagia. *b*, Healing of the glossitis after treatment with vitamin B complex; the cystic mastitis and premenstrual tension completely cleared up. Both the glossitis and the premenstrual symptoms recurred as soon as dosage of the B complex was reduced below the maintenance level.

Fig. 4. *a*, Atrophic glossitis observed in a patient immediately following hysterectomy for uterine myomas and menometrorrhagia. *b*, Cystic hyperplasia of the endometrium in hysterectomy specimen.

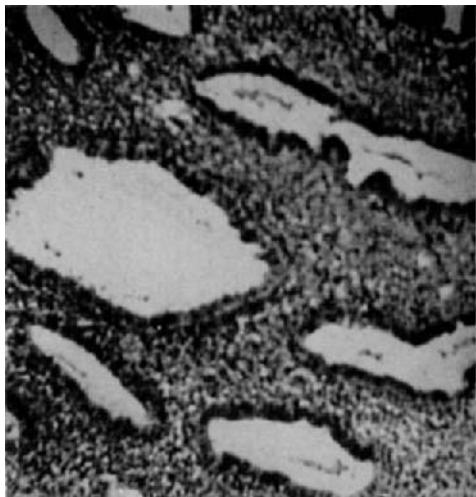
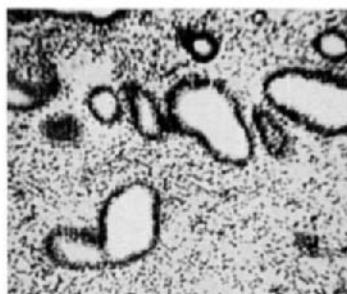
FIG. 5 *b*FIG. 6 *a*FIG. 6 *b*

Fig. 5. *a*, Atrophic glossitis in a patient with menometrorrhagia. *b*, Glandular cystic hyperplasia of the endometrium in the same patient. The menometrorrhagia recurred after curettage; it responded promptly to vitamin B complex therapy.

Fig. 6. *a*, Atrophic glossitis in a patient with severe menometrorrhagia which recurred after curettage but responded promptly to vitamin B complex. *b*, Cystic hyperplasia of the endometrium in the same patient.

Fig. 7. Severe cheilosis and glossitis produced by administration of estrogen to a patient in tenuous nutritional equilibrium as the result of hyperthyroidism which had been treated by roentgen irradiation.



FIG. 5 a



FIG. 7

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ency to bleed, are extremely common among these patients. This clears up rapidly on vitamin B complex therapy (M. S. Biskind, 1943; M. S., G. R. and L. H. Biskind, 1944; M. S. Biskind, unpublished), indicating that this phenomenon is related to dilatation of cutaneous vessels under the influence of estrogen and is not due to vitamin K deficiency, which has been thought to bear a relation to menorrhagia (Gubner and Ungerleider, 1944). However, in the patients who have prominent cutaneous vascular spiders, these rarely show more than slight or moderate regression under treatment.* The palmar erythema, however, occasionally shows definite diminution after prolonged and intensive nutritional therapy (M. S. Biskind, unpublished).



FIG. 8 a



FIG. 8 b

Fig. 8. a. Central and marginal papillary atrophy of the tongue in a patient with menorrhagia, cystic mastitis and premenstrual tension. b. The effect of intensive, protracted therapy on the glossitis; the menstrual symptoms responded promptly to vitamin B complex, orally and parenterally. The glossitis healed slowly.

Deficiency of the vitamin B complex sets up a vicious cycle. For not only does vitamin B deprivation impair the inactivation of estrogen in the liver, but estrogen may cause vitamin B deficiency. Spontaneous exacerbations of signs of B deficiency occur during the menstrual cycle in relation to cyclic changes in body estrogen (Ashworth and Sutton, 1942; M. S. and G. R. Biskind, 1943; M. S. Biskind, 1943), and administration of estrogen leads to exacerbation of lesions already present. Fig. 7 shows the effect of administration of estrogen to a patient in tenuous nutritional equilibrium.

Heilig and Kantiengar (1942) found that in women in whom there is a relatively low liver function (as measured by the ability to convert benzoic to hippuric acid) on the 13th or 14th day of the menstrual cycle, there is a

* In a recent more extensive review, Bean (1945) has discussed factors, other than nutritional deficiency and the concomitant disturbance in estrogen metabolism, that may be involved in the production of the vascular spiders.

further diminution in liver function on the first day of menstruation. This suggests that in women who have impaired liver function as a result of vitamin B complex deficiency, there is further impairment during the period of the cycle when the highest level of estrogen occurs. A similar diminution in liver function occurs during the latter part of pregnancy (at the time when body estrogen rises to a high level) as measured by the excretion of administered bilirubin (Sullivan, Tew and Watson, 1934) or by the conversion of benzoic to hippuric acid (Hirsheimer, 1935). The latter observations are the more significant in that nutritional deficiency commonly



FIG. 9 a



FIG. 9 b

Fig. 9. a. Cheilosis, glossitis and characteristic "muddy" complexion in a patient who had had menorrhagia and severe premenstrual tension for 4 years. b. Effect of vitamin B complex therapy, oral and parenteral, on the avitaminotic lesions. Note partial healing of cheilosis and healing of glossitis and clearing of complexion. This patient had no menorrhagia or premenstrual tension for more than a year, so long as she continued intensive B complex therapy; these promptly recurred on reduction of dosage.

occurs in pregnancy, owing to the metabolic demands of the fetus which are usually not compensated by adequate fortification of the mother's diet (Tompkins, 1941; Williams and Fralin, 1942; Burke *et al.*, 1943). And menometrorrhagia, cystic mastitis and premenstrual tension frequently follow a pregnancy (M. S. Biskind, 1943).

Among the observations made in patients receiving vitamin B complex is that a number of patients who flowed usually for 5 or 6 days and had not considered this abnormal, subsequently flowed for not more than 3 or 4 days under therapy. This indicates that it may be necessary to revise our concept of the normal range in the duration of the menstrual flow.

Characteristically, patients with only mild or moderate signs or symptoms of vitamin B deficiency, who had become accustomed to increased nervous tension, insomnia, tenderness of the breasts, a feeling of "fullness" or "puffiness," lumbar backache, headache, increased fatigability, lower abdominal cramps and the like, as premonitory indications of impending

menstruation, reported after the first or second subsequent period while on B complex therapy, that the flow came on completely "without warning." Conversely, other patients, especially those who had signs of a more severe and more protracted deficiency and who were suffering from menorrhagia, reported that there had been dysmenorrhea for the first time while on vitamin B therapy, or that the pain had become more severe than before (this also occurred in one of the cases in which there were uterine myomas). In virtually every case in which this occurred there was little or no pain during subsequent periods while the nutritional therapy was continued.

The findings in animals indicating that the estrogen-androgen equilibrium is altered in B complex deficiency, is of especial interest in view of the response of the clinical conditions here discussed to treatment with androgens.



FIG. 10a



FIG. 10b

Fig. 10. *a*, Atrophic glossitis in a girl, aged 13½, with severe puberal bleeding since the menarche; bleeding occurred every 21 days, lasted 9 to 10 days. *b*, After one week on oral B complex therapy. A normal period occurred after 3 weeks of therapy. Normal periods then occurred every 27 days during the year subsequent that the patient was on therapy, under observation.

The therapeutic effect observed with the androgens is undoubtedly due to re-establishment of the estrogen-androgen equilibrium at a higher absolute level. Administration of the B complex, in contrast, reduces the body estrogen to the normal range and thus re-establishes the equilibrium at a physiologic level.

The use of estrogen in the (more or less successful) treatment of menorrhagia and of cystic mastitis appears paradoxical with reference to the observations indicating that these syndromes are caused by excess estrogen and respond to therapy which reduces the estrogen level. The occasionally successful use of estrogen in these cases does not alter the fact that this therapy is unphysiologic, accomplishing its end in menometrorrhagia probably by depressing or abolishing the cycle and maintaining a proliferative endometrium (which, in the absence of a cycle, may bleed little or not at

all). With reference to mastitis, Gardner (1941) has shown that while moderate doses of estrogen in castrated animals stimulate mammary growth, larger doses depress it. In any event, administration of estrogen in these cases not only does not cure the basic defect but actually makes it worse.

3. Postpartum Subinvolution of the Uterus

The generally accepted view with regard to postpartum subinvolution of the uterus, as expressed in current textbooks on obstetrics and gynecology, is that this condition results from local pathologic conditions in this



Fig. 11.

Atrophic glossitis in a patient with postpartum subinvolution of the uterus.

organ rather than from any systemic physiologic disturbance. The occurrence of incomplete uterine involution among patients having menometrorrhagia, cystic mastitis and premenstrual tension, associated with lesions of nutritional deficiency (see Fig. 11), suggested that the contrary might be the case (M. S. Biskind, 1943). On nutritional therapy in these patients, the uterus involuted rapidly and the endocrine disturbances cleared up promptly.

As postpartum uterine subinvolution appeared to be related to excess estrogen, owing to failure of destruction in the liver, L. H. and M. S. Biskind (1944) studied two groups of pregnant women. One was maintained on an average diet; the other received substantial supplements of vitamin B complex during pregnancy. All the patients were examined 6 weeks postpartum for evidence of uterine subinvolution. In the control group of 107, 6 patients had poor involution, in 23 it was fair, in 78 good and in none could it be called excellent. In the group of 76 that received B complex, none had poor involution; in 3 involution was fair, in 56 good and in 17 excellent,

—or satisfactory involution in approximately 96% of the women receiving B complex as against 73% in the control group. Thus, the rate of involution was definitely enhanced in the group receiving B complex.

Recent reports emphasize the inadequacies of American diets during pregnancy (Tompkins, 1941; Williams and Fralin, 1942; Burke, Beal *et al.*, 1943; Ebbs, 1943; Lockhart, Kirkwood and Harris, 1943; Bean, Spies and Blankenhorn, 1944). As L. H. and M. S. Biskind have pointed out, at times and in places that people subsisted largely on whole grains (Drummond and Wilbraham, 1939) they customarily required shorter periods of postpartum rest than our own usual minimum of ten days (Charles White, 1773; Goodell, 1875; Küstner, 1899—cited by Williams, 1927). This study provides further evidence for the need of greatly increased intake of vitamin B complex during pregnancy and the puerperium.

4. Diminished Libido and Impotence in the Male

As already mentioned, a number of isolated observations have appeared in the literature on impairment of gonadal function in the male, in conditions which we now know to be related, directly or indirectly, to nutritional deficiency, and in which disturbance of liver function occurs. For instance, in diabetes, T. B. Futcher wrote in 1907, "Loss of sexual desire and power in men is common, and may be an early feature." In intoxication with carbon disulfide, a liver poison, Edsall, writing the same year, pointed out that partial or complete impotence usually supervened. Cirrhosis of the liver has long been known to lead to testicular atrophy (cited by Glass *et al.*, 1940). Reynolds and Macomber (1921) noted the occurrence of testicular atrophy in "malnutrition."

In view of the alteration of the estrogen-androgen equilibrium which occurs through failure of inactivation of estrogen in the liver in vitamin B complex deficiency (M. S. and G. R. Biskind, 1943), (*cf.* also the observations of Glass *et al.*, and of Wu cited on page 154), it appeared likely that many cases of diminished libido and impotence in the male might be associated with nutritional deficiency, similar to the condition in the female. This was found to be the case (M. S. Biskind, 1944). There was a striking correlation between the occurrence of indications of nutritional deficiency (atrophic glossitis, cheilosis, gingivitis, seborrhea alae nasae, keratosis of the lower eyelids, conjunctival injection, cutaneous vascular spiders, emotional instability, insomnia, rapid fatigability, peripheral neuritis, *etc.*) and the presence of testicular softening and atrophy. Gynecomastia occurred occasionally. Not infrequently in these cases, the liver was large and tender. Under intensive vitamin B complex therapy, not only did the lesions of nutritional deficiency clear up (with rapid diminution in the size of the liver when this was enlarged) but there was rapid and dramatic restoration

of libido and potency. This was especially striking in cases of diabetes (Biskind and Schreier, 1945), in which diminished gonadal function in the male has long been considered almost invariable (*cf.* Root and Bailey, 1945).

Restoration of function in cases in which impotence is associated with nutritional deficiency is usually rapid and complete (of course, cases purely psychogenic in origin are excluded) with restoration of normal testicular texture but rarely of the original size (M. S. Biskind; Biskind and Schreier, 1945).

p-Aminobenzoic acid occasionally has been noted to produce marked increase in libido and potency (reviewed in Vol. II of *Vitamins and Hormones* by Ansbacher).

5. Implications for Industrial Toxicology

Numerous studies already cited have established that cirrhosis of the liver can be produced by nutritional deficiency or nutritional imbalance and that the B vitamins (especially choline) and the protein content of the diet play a major rôle in this phenomenon. In addition to the lesion just mentioned, other disturbances have been produced in the liver, both functional and morphological, by vitamin B deficiency (Rhoads and Miller, 1938; Sebrell and Onstatt, 1938). György and Goldblatt (1939, 1940, 1942) have shown that, in experimental cirrhosis of the liver in rats, the lesions may be prevented by the administration of brewer's yeast, yeast extract or choline. Subsequently, Blumberg and McCollum (1941) also reported prevention of dietary cirrhosis with choline, and Lowry and his co-workers (1941) have reported successful treatment of this condition with choline and casein. Patek (1937) and Patek and Post (1941), have noted amelioration of clinical hepatic cirrhosis with dietary therapy, mainly with sources of the vitamin B complex. Choline has also been found to be useful in clinical cirrhosis (Broun and Muether, 1941).

It has been demonstrated that the lesions produced by certain liver poisons resemble, at least in part, those which occur in nutritional deficiency (György and Goldblatt, 1939). Liver damage produced by carbon tetrachloride or chloroform may be prevented by liver extract (Forbes and Neale, 1936; Forbes and McConnell, 1937); that induced by lead arsenate may be prevented by brewer's yeast (Von Glahn and Flinn, 1939). Cirrhosis and carcinoma of the liver caused by dimethylaminoazobenzene ('butter yellow') may be prevented, in part or entirely, by liver extract, brewer's yeast, yeast extract, rice bran extract or by riboflavin and casein (Ando, 1938; Nakahara *et al.*, 1939; Rhoads, Kensler, Sugiura *et al.*, 1940, 1941; see also Burk and Winzler, 1944). Rhoads (1940, 1942) has pointed out that intoxication with butter yellow produces a secondary or 'conditioned' deficiency

of factors of the B complex. Rhoads and his co-workers have shown that breakdown products of butter yellow inhibit the cozymase system in very minute amounts.

Talbot (1939) extended the observation of Golden and Sevringshaus (1938) on destruction of endogenous estrogen by demonstrating that a liver poison, carbon tetrachloride, can impair the estrogen-inactivating mechanism. Administration of carbon tetrachloride to rats from 21 to 25 days old with intact ovaries led to a definite increase in the weight of the uterus over that of control animals. This effect did not occur in animals previously castrated. Pincus and Martin (1940) confirmed the observation that administration of carbon tetrachloride impairs the estrogen-inactivating system; the effectiveness of a given dose of estrone was thus increased about 80%.

These results are in agreement with the observations of Glass, Edmondson and Soll (Edmondson *et al.*, 1939; Glass *et al.*, 1940), already mentioned, on the excretion of free and combined estrogens and androgens in the urine of male patients with cirrhosis of the liver, all of whom had testicular atrophy, gynecomastia or both. They found that while all, or almost all, of the estrogen was excreted in active, uncombined form in these patients, free androgen was not found. Not only was there an absolute increase in the excretion of estrogen in these cases over the values for normal men but, in most of the cases, there was also a diminution in the excretion of androgen. The latter may have been due, at least in part, to depression of the gonadotropic function of the pituitary by the excess of free estrogen.

In view of the relation of nutritional deficiency to failure of inactivation of estrogen in the liver, discussed previously, and the extensive evidence that a variety of liver poisons act to produce a secondary deficiency of factors of the B complex, it might be expected that exposure to liver poisons in industry might lead to syndromes related to excess estrogen (M. S. and G. R. Biskind, 1942, 1943). Unfortunately, these syndromes have rarely been noted and, so far as the author can determine, have never been specifically investigated in relation to industrial toxicology. But in every case in which casual mention has been made of the menstrual function or of sexual potency in the male in relation to intoxication by liver poisons, the expected relationship appears. Thus, menorrhagia and metrorrhagia have been reported to occur in intoxication with lead (Edsall, 1907; Sollmann, 1942), benzene (Hamilton, 1926; International Labour Office, 1934), carbon disulfide (Edsall, 1907), tetryl ("nitramine") (Witkowski *et al.*, 1942); while exposure to lead (Sollmann, 1942) and carbon disulfide (Edsall, 1907; Braceland, 1942) are said also to cause loss of libido and impotence.

Another substance widely used in industry, furfural, was at one time sold as a nostrum for the treatment of amenorrhea ("Anogen," Notice of Judg-

ment, No. 27226, F. D. A., 1937). In rats on a polished rice diet Nakahara and Mori (1941) found that furfural produced hepatic cirrhosis. Feeding of furfural to castrate female rats with a pellet of estrone in the spleen, led to impairment of the estrogen-inactivating function of the liver in some animals even when the rats were maintained on a full diet (M. S. Biskind, unpublished).

Thus, the phenomenon of impaired inactivation of estrogen in the liver, whether induced by primary nutritional deficiency, by exposure to hepatotoxic agents or by other conditioning factors, appears to have extensive implications for industrial toxicology and for application of modern knowledge of nutrition among industrial workers. This problem requires extensive further investigation.

6. Prevention and Treatment of Neoplasms in Tissues Responsive to Estrogen

Investigations by many workers have shown that estrogen may be etiologically involved in the production of a variety of neoplasms in tissues responsive to estrogen, notably in the breast and uterus. The literature has been reviewed at length by Allen, Hisaw and Gardner (1939).

Clinical observations already described indicate a definite correlation between the occurrence of lesions of nutritional deficiency with impairment of the estrogen-inactivating function of the liver, and the incidence of lesions of the breast and myomas of the uterus (M. S. Biskind, 1943; M. S., G. R. and L. H. Biskind, 1944). Nutritional therapy has resulted not only in improvement in the functional conditions related to excess estrogen and in striking alleviation of cystic mastitis (considered by some investigators a pre-neoplastic lesion) but also in some cases in actual regression of fibroadenomas of the breast and myomas of the uterus (M. S., G. R. and L. H. Biskind, 1944; M. S. Biskind, unpublished).

Failure of the liver to inactivate estrogen in a deficiency of the vitamin B complex while this organ continues to inactivate androgen must seriously disturb the estrogen-androgen equilibrium. One possible consequence of such an alteration is indicated by the work of Lipschütz and his collaborators (1939-1944; cf. also Vargas, 1942; Marx *et al.*, 1942; Dosne, 1944; Iglesias *et al.*, 1944). Lipschütz *et al.* have shown that subserous fibroids can be produced by the continuous (but not by the intermittent) action of estrogen, not only in the uterus but also in other abdominal organs and in the abdominal wall. Fibroids thus produced can be prevented by the simultaneous administration of testosterone (or of progesterone or other steroids having the androstene nucleus).

When viewed in the light of present knowledge on the relation of the B vitamins to inactivation of estrogen in the liver, a number of otherwise

puzzling facts, especially in regard to tumors of the breast, become explicable. It is well known that the incidence of cancer of the breast is higher in obese women than in those of more nearly average proportions. This applies also to the occurrence of menorrhagia and other disturbances related to excess estrogen (M. S. Biskind, 1943). Loeb, Suntzeff *et al.* (1942) have shown that there is a definite direct correlation between body weight and the incidence of spontaneous mammary cancers in mice. The converse has been demonstrated by Tannenbaum (1940; 1942), who showed that caloric restriction diminished the incidence of mammary carcinomas.

As the need for thiamine and riboflavin is directly related to the caloric intake of carbohydrate, in a diet in which there is less than the minimal amount of these factors (and this is especially true of the present average American diet as well as that elsewhere) (Drummond and Wilbraham, 1939; Stiebeling, 1941, 1943; Jolliffe, 1943; Adamson *et al.*, 1945), the greater the caloric intake, especially of carbohydrate, the greater the vitamin deficit. Clinically, obese patients virtually always show signs of avitaminosis unless they have had a nutritional supplement.

The fact that diabetics show an incidence of cancer six times that of the general population as a whole (Ellinger and Landsman, 1944) is quite in keeping with the etiologic relationship of nutritional deficiency to diabetes (see Section V).

Minot (1938) has cited the classic case of a man who developed polyneuritis after gaining much weight on a high carbohydrate diet; the neuritis disappeared on dietary restriction without specific therapy. Biskind (1943) has cited a similar case of a woman who had 3 spells of compulsive overeating. Each time, she gained about 50 pounds and promptly developed metrorrhagia. On dietary restriction the metrorrhagia disappeared only to recur at the next bout of overeating.

It seems likely that regressions in the growth of mammary carcinomas in animals, observed following administration of yeast extract or yeast extract and riboflavin (Lewisohn *et al.*, 1941, 1942) may have been mediated through enhancement of the estrogen-inactivating function of the liver.

Since, however, the development of a malignant tumor represents a qualitative and, for the most part, irreversible change in the tissue affected, nutritional therapy would in practice be of value mainly in the prevention of pre-neoplastic lesions. As related to those produced by excess estrogen, the maintenance of normal hepatic function appears to be paramount.*

* While this review was in press, Ayre and Bauld (1946) published a report which provides further confirmation of the relation of nutritional deficiency to syndromes caused by failure of inactivation of estrogen in the liver. Thiamine deficiency (as measured by retention of a test dose) was found to occur regularly in patients with menorrhagia and with cancer of the uterus, associated with estrogenic changes in the

III. INFERTILITY

Nutritional deficiency affects the reproductive function in two ways: (1) by a direct effect on cellular metabolism of the reproductive tissues, thus affecting their responsiveness to endocrine principles (*cf.* Hertz and Sebrell, 1944; Hertz, 1945), as well as other functions, and (2) by secondary systemic alterations of endocrine function. Undoubtedly, in a given case, both operate simultaneously and are inseparable. The general literature on this subject has been reviewed by K. E. Mason (1939) and the specific effects of vitamin E in this respect are covered by Mason in Vol. II of *Vitamins and Hormones* (1944).

This discussion is concerned mainly with diminished fertility occasioned by secondary endocrine disturbances and the effects of nutritional therapy. The disturbance which appears to have the most far-reaching effect in this instance appears again to be the rise in active estrogen occasioned by failure of inactivation in the liver. As mentioned on page 153, endometriums obtained from patients during functional uterine bleeding (associated with nutritional deficiency) show a more or less pronounced estrogen effect and little or no secretory effect, suggesting a deficiency or absence of progesterone.

This, of course, may be an etiologic factor in female infertility, and a number of apparent restorations of fertility in such patients have been occasioned by intensive vitamin B complex therapy (M. S. Biskind, unpublished) although the problem has not yet been sufficiently studied. Peters and Footer (1945) have reported that, in patients who bleed excessively owing to the persistence of an acyclic estrogenic endometrium, the endometrium becomes cyclic on vitamin B complex therapy and, in confirmation of the studies described in Section II, the menstrual flow becomes normal.

In the male, not only is spermatogenesis and spermic function affected directly by nutritional deficiency (vitamin A, vitamin E, [*cf.* Mason, 1939], riboflavin [MacLeod, 1942] and undoubtedly other factors) but the rise in body estrogen which results from failure of inactivation in the liver secondarily affects spermatogenesis. Biskind and Falk (1943) studied the effect of therapy with vitamin B complex (in some cases with addition of vitamin E) and found definite increases in sperm counts and in motility, and diminu-

cervical smear. While the method employed by these investigators places emphasis mainly on lack of thiamine (and deficiency of this factor alone would be *sufficient* to impair hepatic estrogen inactivation), it should be remembered that it is virtually impossible for an uncomplicated clinical deficiency of a single factor ever to occur. From the practical standpoint this is extremely important, as administration of thiamine alone is usually of little therapeutic benefit, whereas *complete* therapy (with all the known vitamin B factors together with an adequate natural source) is dramatically effective (see Section VI).

tion in the percentage of abnormal forms, with apparent restoration of fertility in eight of thirteen cases. However, in infertility resulting from vitamin E deficiency (even though addition of vitamin E to the B complex therapy produced striking effects on spermic motility) there was no evidence that this defect was other than irreversible (*cf.* Mason, 1939).

Subsequent experience with vitamin B complex in male infertility has borne out the original experience and Pool (1945) has briefly reported similar favorable results. That the vitamins are not the only nutritional factors of possible clinical importance in spermatogenesis is indicated in a study by Holt *et al.* (1942), on the deleterious effects of deficiency of certain amino acids, especially arginine, on production of spermia in human subjects—a factor of especial importance during wartime restrictions on protein foods. In the treatment of these patients it is important to take into consideration the frequent occurrence of multiple nutritional deficiencies (see Section VI). Nutritional therapy, at least of the type at present available, nevertheless fails to affect fertility in a significant number of patients. These cases also do not respond to direct endocrine therapy of various types—steroids, thyroid, gonadotropins.

IV. THYROID DISTURBANCES AND THYROID THERAPY

The majority of the patients with syndromes related to excess estrogen studied by M. S., G. R. and L. H. Biskind (1944) had a low basal metabolic rate. This was especially true of the patients with signs of severe or moderately severe nutritional deficiency. Administration of thyroid to these patients, in the absence of a vitamin B supplement, usually caused exacerbation of the signs and symptoms of the deficiency without significant change in the metabolic rate. The low metabolic rate in these patients may be the expression of a safety mechanism; the rise in body estrogen resulting from failure of inactivation in the liver depresses the pituitary with diminution in secretion of the thyrotropic principle.

In several patients with enlarged thyroids who were treated nutritionally for syndromes related to excess estrogen (M. S., G. R. and L. H. Biskind, 1944; M. S. Biskind, unpublished), a definite diminution in the size of this gland was observed after several months of therapy with vitamin B complex; this was sufficiently striking to be noticed spontaneously by members of their families. In none of these cases, however, did the goiter completely regress during the period of observation. This is of interest in view of the well-known observation that patients with goiters show periodic further enlargement of the gland during the latter part of the intermenstruum, when the body estrogen rises, and that administration of estrogen to some patients with hyperthyroidism leads to diminution in the basal metabolic rate (Goldman *et al.*, 1940). (Because the administered estrogen causes

further exacerbation of an already tenuous nutritional equilibrium produced by the hyperthyroidism [see Fig. 7] it would seem inadvisable to use estrogen therapy for this purpose unless the nutritional defect were simultaneously corrected.)

Williams and Kendall (1943) have reported that administered thyroid is "less effective in promoting metabolic activity . . . in a state of thiamine deficiency than it is when the intake of thiamine is adequate."

The observations described clarify numerous problems of thyroid therapy that have formerly been both confusing and frustrating. Thyroid is not only one of the most valuable of endocrine substances but it is also one of the most misused. Until the relation of thyroid function (and the physiologic activity of thyroxine) to nutritional status had been clarified, it was impossible to evaluate a number of clinical phenomena related to thyroid function and the metabolic effects of administered thyroid. Among these are: (1) the significance of the basal metabolic rate as an indication for thyroid therapy; (2) the frequent failure of administered thyroid to affect the basal metabolic rate, although side actions such as tachycardia and nervousness may be prominent; (3) the development of endocrine complications (*e.g.*, menorrhagia, cystic mastitis) following the use of thyroid in the treatment of obesity.

It is notable that both the nutritional factors affecting thyroid function operate in the same direction. Failure of inactivation of estrogen in the liver leads to a consistently high blood estrogen which depresses the thyrogenic function of the anterior pituitary, and thiamine deficiency (the effect of other factors has not yet been reported) prevents the development of the normal metabolic effects of thyroxine, whether endogenous or administered. Thus, in the presence of nutritional deficiency, a low basal metabolic rate might be expected and little effect on the metabolic rate would be derived from ingestion of thyroid. Precisely this occurs. In such cases, both the usual assumption that a low basal metabolic rate is a necessary indication for thyroid, and that this can be remedied by giving thyroid, are fallacious. Actually the administration of thyroid in the presence of nutritional deficiency is sharply contraindicated; the sole effect of thyroid therapy in such cases is to cause an exacerbation of the nutritional deficiency, although, probably because of the protective mechanisms discussed, this exacerbation is often not as striking as that produced by estrogen.

In evaluating the basal metabolic rate, it is necessary first to keep in mind the fact that the range of normality is now considered to extend from plus 5 to minus 20% (DuBois and Chambers, 1943) and that both hypothyroidism and hyperthyroidism can occur within this range. Therefore, the clinical status of the patient must be considered in relation to the basal metabolic rate. Often enough, signs usually considered referable to hypo-

thyroidism disappear promptly, solely by correcting the accompanying nutritional defect. The author makes it a practice always first to correct the avitaminosis and, when the lesions are healed, then, and then only, to administer thyroid if this is required, continuing the vitamin therapy at the same time.

Two factors especially have operated to distort the significance of the basal metabolic rate as it is now calculated:—(1) Calculation of the rate to the body surface. (2) Representation of the rate in terms of percentage above and below a theoretically average normal. As Norman Wetzel (1933) has pointed out, heat production is more appropriately considered a function of body mass; in the early work on metabolism, it was related to body surface only as a stratagem to reduce the apparent range of normal variability.

The fallacy of relating the metabolic rate to the body surface is readily seen in cases of extreme obesity. In such cases body weight increases greatly while only a fraction of this increase occurs in body surface. It is common to obtain a basal metabolic rate of plus 10 or plus 20% in a patient with extreme obesity, even when clinical signs point to hypothyroidism. If the oxygen consumption were calculated to the body weight, the metabolic rate would more nearly agree with the clinical condition of the patient.

The practice of calculating the metabolic rate in percentages above and below the "normal" zero serves little purpose but confusion. The fallacious position even of the zero point is indicated by the current shift in the "normal" range proposed by DuBois, already mentioned. Representation of basal metabolic rate in terms of calories per kilogram of body weight per day, even though this provides figures having a wider divergence in the normal range than present practice, would at least give the physician a truer indication of caloric output. In addition this method of calculation would be as useful in obesity as in persons of more nearly average weight.

Thus it is evident that the basal metabolic rate, especially considering the difficulties inherent in the present method of calculation, is not by itself adequate for determining the need for thyroid administration and that estimation of the nutritional status of the patient is of great importance for rational therapy of thyroid disturbances.

Berman (1945) has observed regression in clinical hyperthyroidism under therapy with *p*-aminobenzoic acid (see also the review of Ansbacher, 1944).

V. DIABETES*

The association of deficiencies in accessory dietary factors with certain defects in carbohydrate metabolism has been the subject of numerous

* See also the review of Houssay on the Thyroid and Diabetes in this volume. Ed.

studies. Among substances known to be necessary for utilization of carbohydrate are thiamine, riboflavin, niacin amide, pantothenic acid, ascorbic acid, vitamin A and vitamin D, although the rôle of the latter three is not as well understood as that of the B vitamins (the literature is cited by Rosenberg, 1942). Many investigations have also been made on nutritional deficiency in diabetes and on the effects of nutritional supplements. For the most part, however, these have been concerned with the treatment of specific avitaminotic lesions rather than with any fundamental defect of the disease itself. Duncan (1943), however, has made passing mention of the fact that he had observed an apparent economy of insulin in certain patients on vitamin B complex therapy.

Soskin and his collaborators (Soskin *et al.*, 1934, 1935, 1938, 1939, 1941, 1944), in a series of fundamental investigations, have demonstrated the basic rôle of the liver in maintaining normal carbohydrate balance. They have shown that:—

- (1) In pancreatectomized animals receiving a constant intravenous injection of insulin just sufficient to maintain normal blood sugar, administration of dextrose yields *normal* dextrose tolerance curves.
- (2) In hepatectomized animals with intact pancreas, receiving a constant intravenous injection of dextrose just sufficient to maintain normal blood sugar, administration of additional dextrose yields *diabetic* dextrose tolerance curves.
- (3) When the liver is damaged by a toxic agent, the diabetic type of dextrose tolerance curve is obtained.

C. H. Best (1935) has emphasized the fact that insulin is secreted into the portal circulation. As Waters and Best (1942) have pointed out, "If one were obliged to name the organ in which insulin exerts the most potent influence, there would be little hesitation in selecting the liver."

It has become customary to think of diabetes mainly in terms of insulin deficiency (*cf.* Root and Bailey, 1945). On the basis of an investigation into the nutritional aspects of diabetes, Biskind and Schreier (1945) have suggested an alternative explanation, namely that, owing to impaired function of the liver, the latter organ is no longer able to respond to endogenous insulin, which need not be deficient. Carbohydrate balance could then be restored in two ways, by administration of additional insulin, which (if the functional defect is not too great) forces the recalcitrant liver to behave, or by restoring normal hepatic function so that the liver can respond to pancreatic insulin. Thus the concept of insulin resistance, now restricted to cases in which there is failure of response to exogenous insulin, might be extended to include many more (and perhaps most) cases of diabetes, which they believe are caused by the fact that the liver becomes resistant to the action of endogenous insulin. Depending on the severity

of the functional liver defect, the hepatic response to additional (exogenous) insulin would vary accordingly. Biskind and Schreier believe that impairment of the ability of the liver to maintain carbohydrate balance occurs as a result of nutritional deficiency, and they have shown that intensive nutritional therapy can partly or entirely restore this function.

In a group of 94 diabetics studied by Biskind and Schreier, every one showed signs and symptoms of deficiency of factors of the vitamin B complex. Glossitis occurred in almost all the patients (*cf.* Fig. 12). Cheilosis, nasolabial seborrhea, keratosis of the lower eyelids, splitting of the fingernails in layers, clouding of consciousness, nervousness, insomnia, impairment of memory for recent events, precordial pain or distress, gastrointestinal



FIG. 12 *a*



FIG. 12 *b*

Fig. 12. *a.* Atrophic glossitis in an aged male diabetic, resistant to large doses of insulin, and with severe ketonuria. *b.* After 19 days on intensive oral and parenteral vitamin B complex therapy, during which responsiveness to insulin was restored, and the diabetes could be completely controlled on reduced dosage of insulin; the ketonuria disappeared.

disturbances and polyneuritis were noted frequently. Syndromes related to excess estrogen occurred concomitantly. Among the premenopausal women there was an almost invariable association of menstrual disturbances with the diabetes and the lesions of avitaminosis; among the male patients there was the characteristic lack of libido and potency, usually associated with testicular atrophy. For the most part, all these conditions showed a more or less prompt response to intensive nutritional therapy. Associated with the improvement in the avitaminotic lesions, marked improvement occurred in carbohydrate metabolism. In some cases the insulin requirement could be reduced; in others insulin could be eliminated altogether. Improvement in general health was usually striking.

Fourteen patients required an average daily dose of 41 units of insulin

(range 25 to 80 units) before nutritional therapy and were able to maintain themselves free of glycosuria after vitamin therapy on an average of 18 units (range 10 to 40 units). Sixteen patients previously on insulin required no insulin at all after the nutritional deficiency had been controlled with B complex. Thirty-seven patients, who were all continued for the most part on the same dose of insulin, showed a variety of favorable responses to nutritional therapy:—striking improvement of general health was invariable; there was less tendency to glycosuria; in some there was marked reduction in blood cholesterol levels, improvement in cephalin flocculation tests and other changes indicating improved liver function. Especially striking was the diminished tendency to insulin reactions in patients in whom this had previously been a troublesome factor.

Twenty-five patients who had had diabetes for periods ranging from 3 months to 20 years (average duration 5.9 years), showed striking reductions in fasting blood sugar from an average of 270 mg. per 100 cc. (range 180 mg. to 325 mg.) to an average of 123 mg. (range 100 mg. to 219 mg.) on vitamin therapy alone. Of the series of 94 cases, 2 patients proved refractory to intensive and protracted nutritional therapy, showing no improvement in carbohydrate metabolism and only slight improvement in avitaminotic lesions. Both these patients had very severe indications of avitaminosis B of many years' duration. The failure of response no doubt represents either irreversible tissue changes or failure to supply as yet unrecognized nutritional factors. Significantly, both these patients failed completely to respond to large doses of insulin.

Except in a few cases in which this was not feasible, the factor of diet in the cases reported by Biskind and Schreier was maintained as nearly constant as possible before and during nutritional therapy, until the effect of the latter on carbohydrate balance could be ascertained. Thereafter, whenever possible, the diet was liberalized.

In 1896 Gilbert and Carnot reported that liver had a beneficial but variable effect on diabetes. Following their initial publication numerous French investigators studied the effects of various liver extracts with similar variable results. In 1922 Levine, in this country, reported improvement in 3 of 4 diabetics treated with a special liver extract. And several years later Blotner and Murphy (Murphy and Blotner, 1927; Blotner and Murphy, 1929, 1930) conducted an extremely well-controlled investigation which demonstrated conclusively in patients, that the feeding of raw liver (or of liver fractions other than those containing the antipernicious anemia factor) had a definite effect in lowering the blood sugar of diabetics. Blotner and Murphy, as well as previous workers, thought of this phenomenon as representing the presence in liver of a blood sugar-reducing substance and they even indicated an equivalence of liver by weight with a definite unitage of insulin.

It is, of course, now well known that liver is an excellent source of the B complex and that these factors appear in greatest concentration precisely in the fraction of liver found by Blotner and Murphy to be effective in diabetes; the search for an insulin-like substance in liver was doomed to failure and when De Pencier, Soskin and Best (1934) investigated the effect of liver as a substitute for insulin on nondepleted pancreatectomized dogs, negative results were obtained.

The contrary occurred in other investigations of a similar nature in which depleted animals were used. Martin (1937) observed that the insulin requirement of depancreatized dogs increased steadily during ingestion of a vitamin-deficient diet and that restoration of the vitamins to the diet could restore the responsiveness to insulin provided the deficiency had not progressed too far. Gaebler and Ciszewski (1945) found in 3 of 4 pancreatectomized dogs kept on a maintenance dosage of insulin, that withdrawal of the B vitamins from the diet caused exacerbation of the diabetic state and increased the insulin requirement by 50%. Using as a source of the B vitamins, either yeast (which has also been credited in the past with containing a blood sugar-reducing substance [*cf.* Dubin and Corbitt, 1923], and which has a history of use in diabetes almost as long as that of liver), or a mixture of thiamine, riboflavin, nicotinic acid, inositol, pyridoxine, pantothenic acid and *p*-aminobenzoic acid, they were able to produce prompt amelioration of the diabetic state and to reduce the insulin requirement to its former level. Richter *et al.* (1945) have shown that partially depancreatized rats, given free choice of various nutrients, consumed more protein, more fat, much less carbohydrate and more of the components of the vitamin B complex—thiamine, riboflavin, pantothenic acid, pyridoxine and choline—than normal animals given a similar choice. So long as the depancreatized animals remained on the high protein, high fat, high vitamin diet of their own selection, they showed no symptoms of diabetes; on a stock diet symptoms of diabetes appeared promptly.

Ten years before the experiment just cited, Best (1935) pointed out, "Evidence is accumulating that certain accessory food factors may exert an appreciable effect on the intensity of glycosuria in pancreatic diabetes." But that nutritional deficiency has an etiologic relation to diabetes mellitus was actually shown twenty years before that by Casimir Funk and von Schoenborn (1914). They reported that pigeons kept on a "vitamin-free artificial diet" showed disappearance of glycogen from the liver and marked rise in blood sugar. This phenomenon was interpreted by Collazo (1922) as representing the effect of inanition; however, a later experiment by Funk and Corbitt (1923) showed that a vitamin B extract of yeast could reduce the blood sugar and restore liver glycogen. Eggleton and Gross (1925) subsequently showed that, in vitamin B deficiency in rats, the blood sugar is above normal until terminal deficiency occurs, whereupon

the blood sugar diminishes (at the time when liver glycogen has been depleted). This is analogous to the situation which often occurs in the human diabetic, as Soskin (1944) has pointed out, when the diabetic state apparently improves but at the expense of liver depletion.

When examined in the light of the thesis presented by Biskind and Schreier, that diabetes represents an end result of protracted nutritional deficiency and that impairment of hepatic function which thus results leads to failure of hepatic response to endogenous insulin, many previously puzzling facts about diabetes become clear and take their places in a logical system.

Taub *et al.* (1945), in independent studies, have also implicated dysfunction of the liver in many cases of diabetes and have demonstrated favorable results from nutritional therapy.

VI. ON THE TECHNIC OF NUTRITIONAL THERAPY

For adequate nutritional therapy in the human being there are three requisites: (1) Complete therapy; (2) Intensive dosage by routes assuring adequate utilization; (3) Protracted administration.

Most failures, especially in treatment with the vitamin B complex, result from disregard of one or more of these principles (*cf.* Spies, 1943). The use of single drugs has a strong modern tradition in medicine and, as a consequence, the tendency to administer thiamine or riboflavin or niacinamide alone, in an effort to correct lesions showing a predominant deficiency of one of these factors, is widespread. But clinically, single uncomplicated deficiencies rarely, if ever, occur, and considering the difficulties involved in producing such deficiencies in animals under strict laboratory control, it would be extremely surprising to find a deficiency of a single vitamin in a human being even with the most esoteric dietary habits. The tendency to administer mixtures of the known crystalline B vitamins, while an improvement over the use of single factors, produces clinical effects only slightly better. The addition to these mixtures of adequate quantities of yeast, yeast extracts, rice bran extract, suitable liver extracts or whole liver, produces dramatically superior results. For the purpose of supplying necessary nutritional factors that are as yet unidentified, liver is far superior to yeast or rice bran, the most suitable products being the 80% alcohol-insoluble fraction or whole desiccated liver (in doses supplying the equivalent of at least two or three ounces of liver a day).

The fallacy of administering mixtures of crystalline vitamins alone in nutritional deficiency is illustrated by the experiments of M. S. and G. R. Biskind (1942, 1944) illustrated in Figs. 1 and 2 (p. 152). In these experiments it was possible, by producing deficiency of all the factors of the B complex, to impair the estrogen-inactivating function of rat livers that

appeared perfectly normal histologically. However, by administering a mixture of thiamine, riboflavin, pyridoxine and calcium pantothenate as the sole source of B vitamins, the estrogen-inactivating function could be restored but these rats all developed fatty livers containing focal areas of necrosis. This principle is further illustrated by a recent clinical report of T. and J. Gillman (1945) who studied liver biopsies in infantile pellagrins before and during nutritional therapy. The use of a mixture of thiamine, niacin and ascorbic acid or of riboflavin and niacin in these patients not only failed to effect histologic improvement in the fatty livers (which resembled morphologically the rat livers illustrated in Fig. 2 of this review) but caused actual aggravation of the hepatic lesions. T. and J. Gillman demonstrated that "crude" parenteral antianemic liver extract was only moderately superior to the crystalline vitamins used. However, so compelling is the prevailing view that aqueous extracts of liver represent all the activity of whole liver that these workers turned, for an adequate source of essential nutritional factors, not to desiccated whole liver but to desiccated stomach, which they found to be superior to the parenteral liver extract previously used.

It is unfortunate, from the standpoint of nutritional therapy, that liver extracts concentrated mainly with a view to increasing their antianemic potency, were for a long time virtually the only ones available; and only a small fraction of such extracts currently produced are used for the purpose for which they were originally intended—pernicious anemia. Most of them are employed for treatment of conditions for which they are ill-adapted—mainly nutritional deficiency. This is the more regrettable as the whole liver from which the extracts are derived would, if ingested as food, produce dramatically superior results.

An investigation of the effectiveness of whole liver and of various liver fractions in the treatment of avitaminosis was carried out by M. S. Biskind (1944), with a view to elucidating the factors involved in the refractoriness of certain lesions of avitaminosis B, especially certain types of atrophic glossitis. He found that although the great majority of patients had an excellent response to commercial preparations of the vitamin B complex, and the ameliorative effect persisted indefinitely as long as maintenance therapy was continued, in a few of them the atrophic glossitis showed only a temporary response to the B complex (even though other signs subsided), and this lesion then recurred in a more refractory form. Subsequent intensive administration of aqueous liver extracts (together with the crystalline B factors) orally and parenterally, usually failed to have more than a slight effect on this type of glossitis. Addition to this regimen of the known liposoluble vitamins (A, D, E and K) was equally ineffective. However, the ingestion of cooked whole liver in an amount much less than

that from which the ineffective extracts were derived, caused a rapid and complete healing of the tongue. If the ingestion of whole liver was discontinued, the glossitis recurred in a few days. The lesion could again be healed on resuming this therapy and the tongue could be maintained in the normal state indefinitely as long as liver was ingested at least several times a week. But whole liver alone could not control the associated signs and symptoms of nutritional deficiency; these responded to intensive therapy with the water-soluble B factors.

Among the factors missing from the ineffective liver extracts is biotin, which is bound to the protein in liver. In view of the report by Sydenstricker *et al.* (1942) that atrophic glossitis may occur as a result of biotin deficiency in man, a biotin concentrate was administered parenterally in one patient, in a dose providing 50 γ per day; no perceptible effect occurred.

Accordingly, Biskind further investigated the effect of different liver extracts in maintaining or restoring the estrogen-inactivating function of the liver in rats (utilizing the technic of G. R. Biskind and Mark, 1939), when these extracts were added to a vitamin B complex-free diet. The first preparation was an antianemic extract soluble in 70% alcohol; the second was a nonsaponifiable liposoluble extract originally described by Wiles and Maurer (1939), obtained from the portion of liver remaining after the antianemic fraction is separated.

The nonsaponifiable lipoid extract of liver had a definite, but quite limited, effect in preventing impairment of the estrogen-inactivating function of the liver in animals on a vitamin B complex-free diet and in restoring this function after it had been impaired by vitamin B complex deficiency. The antianemic extract had only a slight effect in restoring the mechanism, although this extract could to a certain extent maintain the estrogen-inactivating function in animals not previously depleted. Another aqueous antianemic liver extract, more highly purified by additional alcohol precipitation, was even less effective.

The lipoid extract could neither restore the body weight of animals depleted in the B complex nor maintain it in nondepleted animals. The antianemic fraction likewise could not restore the body weight in depleted animals but could maintain it (and actually permit a further gain) in rats not previously depleted.

In contrast to the limited effects of either extract alone, *a mixture of the water-soluble and liposoluble fractions*, in proportions representing equal amounts of fresh liver, had a striking effect in restoring the estrogen-inactivating function of the liver and in maintaining it. In addition this mixture caused rapid gains in body weight in animals previously depleted.

This evidence provides experimental confirmation for the existence of factors essential to nutrition in the lipoid fraction of liver and suggests

the advisability of combining these liver factors with those now employed in manufacturing commercial preparations for nutritional therapy. Turner and Miller (1943) have obtained from liver lipoids two substances that stimulate production of white blood cells.

The simplest method of administering a combination of aqueous and lipid fractions of liver is to use the whole desiccated unfractionated liver substance. This, as already indicated, has been found to be extremely effective as a source of accessory nutritional factors in the nutritional therapy of syndromes related to excess estrogen by M. S. Biskind (1944) and of diabetes by Biskind and Schreier (1945). Cooperman *et al.* (1945) have found whole desiccated liver to contain a factor or factors (not present in aqueous liver extracts tested) which are essential to nutrition of the monkey.

Failure of absorption is common in severe deficiencies, as changes take place in the gastro-intestinal tract rather early. Many patients therefore require parenteral therapy. However, as the available *parenteral* liver extracts lack essential nutritional factors, it is not as yet possible to administer complete B complex therapy by the parenteral route. Therefore, mixtures of crystalline B vitamins are thus used along with oral administration of the more nearly complete preparations.

The following is the therapeutic regime employed by Biskind and Schreier (1945) in the nutritional therapy of diabetes: The nutritional factors given orally are usually administered in the following daily amounts, in divided doses after meals: 36 to 45 mg. thiamine, 21 to 36 mg. riboflavin, 12 to 27 mg. calcium pantothenate, 200 mg. niacinamide (occasionally increased to 500 mg.), 3 mg. pyridoxine, 210 mg. choline, 27 to 150 mg. inositol, and 60 to 280 γ *L. casei* factor (folic acid). These vitamins were derived in part from crystalline material and in part from brewers' yeast extract, 80 % alcohol-insoluble liver extract, desiccated whole liver or combinations of these (the inositol and folic acid were derived solely from the natural sources); 300 mg. ascorbic acid was often included.

In addition, in cases in which parenteral therapy was required, from 20 to 60 mg. thiamine, 5 to 10 mg. riboflavin, 50 to 250 mg. niacin amide, 5 to 10 mg. pyridoxine and 5 to 50 mg. calcium pantothenate, was given intramuscularly every day or every other day.

Although many patients respond rapidly and dramatically to therapy with the vitamin B factors, not a few have severe lesions of nutritional deficiency which respond slowly despite intensive therapy (*cf.* Kruse, 1942, 1943). Sometimes rather sudden improvement occurs following protracted intensive therapy, as in some of the cases of diabetes observed by Biskind and Schreier (1945). Perseverance is therefore important. And, as already indicated, the importance of including in the nutritional regime

adequate amounts of accessory B complex factors (preferably in the form of suitable liver fractions or desiccated liver or combinations of these) cannot be too strongly stressed. Few patients respond satisfactorily to mixtures of crystalline B factors alone or to those containing, as sources of accessory factors, a few grains of brewers' yeast.

As in other conditions related to nutritional deficiency, large doses of the B complex factors must be administered indefinitely even after all morphological defects have healed, the minimum maintenance dosage at this stage being at least five to ten times the maintenance amounts for normal persons (*cf.* Martin and Koop, 1942) and often much more.

A clue to the greatly increased vitamin requirement of the patient whose avitaminotic lesions have been healed, as compared with the maintenance requirement of the nondepleted person, may perhaps be found in a study by Bessey and Lowry (1944) on factors influencing the riboflavin content of the rat cornea. These investigators found that the level of riboflavin in the cornea reflects the riboflavin intake but that, once the animal has been depleted, administration of riboflavin fails to increase the flavin content of the cornea to the level originally present, suggesting a qualitative tissue change.

This problem requires extensive further investigation, but the mass of evidence points to the occurrence in human nutritional deficiency of the phenomenon noted by Bessey and Lowry. For the depleted individual to utilize the nutritional factors at all, they must apparently be present in high concentration in the body fluids; and concentrations higher than are necessary to the nondepleted person must be maintained indefinitely. The author has observed numerous cases in which diminution of vitamin intake led to rapid recrudescence of the deficiency lesions even years after the original deficiency was apparently "cured."

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The Thyroid and Diabetes

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I. RELATIONSHIP BETWEEN THE THYROID AND THE INTESTINAL ABSORPTION OF SUGARS

Althausen and Stockholm (1938) and Althausen (1943) have shown that the thyroid hormone both selectively and specifically increases the intestinal rate of absorption of dextrose, galactose, starch and oleic acid. Injection of thyroxine produces the same effect in thyroidectomized, in hypophysectomized and in normal white rats. Conversely, thyroidectomy decreases the intestinal rate of absorption of glucose and galactose. According to Althausen, thyroxine increases the rate of intestinal absorption of substances which are selectively absorbed by a phosphorylation process. Increased galactose absorption does not always take place in hyperthyroidized dogs (Grauer *et al.*, 1942; Brignone, 1944).

The increased rate of intestinal absorption of galactose has been indirectly demonstrated in human hyperthyroidism by giving 40 g. of galactose *per os* and studying the concentration of this sugar in the blood at various intervals. Blood galactose increases three times more in hyperthyroid cases than in the non-hyperthyroid (Althausen *et al.*, 1940). Of 304 hyperthyroid patients studied by different authors, 92% showed a post absorptive increase in the blood galactose level far above that of normal subjects, (Schneeberg *et al.*, 1943). Althausen and his co-workers (1940) state that the rate of absorption returns to normal after surgical treatment of hyperthyroidism, although Schneeberg *et al.* (1943) say that this is not always the case. If galactose is administered intravenously, hyperthyroid cases and normal persons show no difference in their blood-galactose curves (Althausen *et al.*, 1940). An increased absorption rate is not specific for hyperthyroidism, as it can also occur in other diseases. If absent, the diagnosis of hyperthyroidism can be excluded with a fair degree of probability; on the other hand, an increase in blood galactose absorption is no absolute sign of hyperthyroidism (Schneeberg *et al.*, 1943). In myxedema, the rate of intestinal absorption of galactose is decreased (Althausen *et al.* 1940).

II. CARBOHYDRATE METABOLISM IN HYPERTHYROIDISM

1. Blood Sugar

In hyperthyroid cases, the fasting blood sugar level is often within normal limits, although values of 120–140 mg.% may sometimes be found without a coexisting diabetes. The average blood sugar values in hyperthyroidism are slightly higher than in normal subjects (Sanger and Hun, 1933; Yriart and Gotta, 1933).

An intravenous injection of thyroxine or thyrotropic hormone induces an immediate and transitory (few hours) increase of blood sugar in dogs (Zunz and La Barre, 1932-1935) and sheep (Bodansky, 1922).

We have administered thyroid powder (0.5 to 4 g./kg./day, during 6 to 30 days) to 76 dogs. In most cases there was an increase in blood sugar, which reached its peak between the 3rd and 12th day, and returned to normal or subnormal at the 14th to 16th day. The blood sugar never surpassed 149 mg.% and diabetes did not occur in any of the animals. In a large number of rats treated with doses from 4 to 100 mg./kg./day, blood sugar levels increased in some, but never beyond 135 mg.% and no diabetes appeared. After a few days, subnormal fasting values were frequently found but later these become normal. In sheep (Bodansky, 1922-1924) and rabbits (Mark, 1925) blood sugar increases at the beginning of the treatment and decreases later, the rabbits sometimes dying in hypoglycemic convulsions.

2. Tolerance Tests

There is an increase in blood sugar after oral administration of glucose and, at the end of the first half hour, levels are higher in hyperthyroid cases than in normal subjects. The return to normal blood sugar is slower in hyperthyroidism, though faster than in diabetes. In hyperthyroidism blood sugar becomes normal two hours after administration of the sugar. This higher hyperglycemic curve has been observed in 50% (Althausen *et al.*, 1940), 66% (Yriart and Gotta, 1933) and 80% (Popper and Hirschhorn, 1941) of hyperthyroid cases. After thyroidectomy, the tolerance curve improved in 38% of non-diabetic and in 50% of diabetic hyperthyroid cases (John, 1942). This higher curve can also be seen in hyperthyroid cases if glucose is given in two separate doses (Wilder, 1940). In these cases there is also a lower tolerance to glucose given by continuous intravenous infusion (Wilder and Sansum, 1917).

In dogs treated with thyroid gland, the hyperglycemic curves caused by ingestion of glucose are higher than normal (Mark, 1926), although the blood sugar curve following intravenous injection of glucose is not significantly modified (Yriart, 1930; Brignone, 1944). In rabbits treated with thyroid, the intravenous administration of glucose gives rise to a curve similar to that obtained with the controls, but after 2-3 hours there is a secondary increase greater than the first. When the thyroid treatment was both intense and prolonged, glucose injection caused hyperglycemia followed by a secondary severe and fatal hypoglycemia (Burn and Marks, 1925; Marks, 1925).

3. Glycosuria

Slight spontaneous glycosuria is frequently found in hyperthyroidism. In a series of 500 cases, glycosuria was observed in 38% of those with

Graves' disease, in 27.7% of adenomas with hyperthyroidism and in 14.7% of non-toxic goiters (Joslin, 1940). The statistical percentages of glycosuria in hyperthyroid cases vary, according to the method used in its detection, from 18% (John, 1938-1940) to 65% (Sattler, 1909).

Glycosuria can often be observed after a meal or following ingestion of glucose; this occurs in 25% (Schulze, 1922) or 81% (John, 1927) of hyperthyroids. Galactose in the urine, following administration of this sugar to hyperthyroid cases, has been found in 33 to 78% of patients.

Thyroid therapy can increase an existing glycosuria in hyperthyroids, but rarely causes it to appear.

4. Glycogen

Administration of thyroid gland or injection of thyroxine produces a marked decrease of glycogen in numerous species of animals but no data are available concerning man. The hormone acts first on liver glycogen, then on that of the heart, and finally on the most resistant of all, muscle glycogen. At the beginning of the treatment, liver glycogen increases normally after a meal but decreases in a few hours. As the treatment progresses there is little increase in liver glycogen following ingestion of food or glucose. This means that, at the beginning, glycogen is imperfectly stored and later, defectively formed. In the muscles the glycogen content is at first normal and only decreases after intense and prolonged treatment (Brignone, 1944); it is very slowly re-synthesized after exercise (Dambrosi, 1933). This effect is conditioned by the dose, length of treatment, animal species and diet. The decrease occurs rapidly, before the consumption of oxygen rises (Cramer and McCall, 1917; Abelin, 1930). The glycogen content becomes normal a few days after treatment is suspended. A certain degree of tolerance to thyroid treatment may develop. The injection of hypophysial thyrotropic hormone decreases liver glycogen when the animal still has its thyroid, but not after thyroidectomy.

The action of the thyroid gland or of thyroxine can be counteracted, to a varying degree, by several substances; these include the suprarenal hormones (Abelin and Althaus, 1942), corpus luteum (Murao, 1930), anterior pituitary extract (Magistris, 1932), the proteins, casein or ovalbumin (Abelin, 1930), fats (Abelin, Knochel and Spichtin, 1930), which are supposed to act through the pituitary gland (Magistris, 1932), substances rich in vitamins (Abelin, Knochel and Spichtin, 1930), and several vitamins, especially vitamin A (Drill, 1943). Moreover, there is a greater need of vitamins in hyperthyroidism.

5. Mechanism of the Alterations Observed

The higher postprandial rise of the blood sugar in hyperthyroid cases has been attributed to several factors. Althausen considers it due to the in-

creased absorption rate of glucose or galactose produced by the thyroid hormone. For a long time it was attributed to a decreased capacity of the liver to store the excess blood sugar; MacLagan (1941) and others still believe that this is so. However, many think that both factors are responsible (Schneeberg *et al.*, 1943).

6. Glucose Consumption

Glucose administration increases the respiratory quotient in rabbits (Parhon, 1913) and rats (Cramer and McCall, 1916-17) treated with thyroid gland. In human hyperthyroidism, the basal respiratory quotient is subnormal, but it rises more than in normal subjects after ingestion of glucose. Within 2 hours, normal subjects burn 18% of the glucose given and hyperthyroids 38%; in the latter, this figure drops to 19% after an extensive thyroidectomy (Sanger and Hun, 1933; Yriart and Gotta, 1933). In hyperthyroidism, the difference between arterial and venous blood sugar is normal or high, but it is found to be diminished in diabetics (Trumper and Cantarow, 1932; John, 1938). During the rise in blood sugar there is a fall in inorganic phosphate of plasma in hyperthyroid cases.

The extra-hepatic consumption of glucose has been measured in eviscerated animals. It was found to be high in rabbits (Mirsky and Broh-Kahn, 1936) and normal, though slightly increased, in hyperthyroid dogs (Housay, Dosne and Foglia, 1944).

III. CARBOHYDRATE METABOLISM IN HYPOTHYROIDISM

1. Blood Sugar

In severe thyroid deficiency in certain animals and man, fasting blood sugar is usually normal or slightly low (Geyelin, 1915; Gilligan *et al.*, 1934; Schnitker *et al.*, 1936). After oral administration of glucose or galactose, the blood sugar curve of hypothyroids usually rises less than that of the controls; still, intravenous injection of these sugars in dogs establishes no divergence from the normal (Yriart, 1930).

2. Glucose Consumption

Thyroidectomized rats have a normal respiratory quotient; this quotient rises when glucose is administered, but the decreased absorption rate of the sugar brings about a smaller hourly consumption than in the controls (Russell, 1942). In thyroidectomized dogs, parenteral administration of glucose is followed by normal consumption (Underhill and Hilditch, 1909).

3. Glycogen in Hypothyroidism

In thyroid deficiency, the liver and muscle glycogen is normal or slightly low. When the deficiency is severe or there is cachexia, the amount of stored glycogen decreases still further. Oral administration of glucose to thy-

roidectomized rats gives rise to a subnormal increase of glycogen due to the lower rate of intestinal absorption (Russell, 1943).

After the injection of adrenalin, glycogen mobilization is slower in thyroidectomized animals than in the controls. On the other hand, the injection of insulin in young rabbits increases the liver glycogen, though this does not take place if the animals have been thyroidectomized (Goldblatt, 1936).

In thyroidectomized dogs, anterior pituitary extract favors the deposit of glycogen (Houssay, Biasotti and Dambrosi, 1936). Diethylstilbestrol causes a supernormal increase of glycogen in thyroidectomized rats (Janes, 1943).

IV. SENSITIVITY TO ADRENALIN

Doses of adrenalin incapable of producing glycosuria in normal subjects do so in 85% of cases of Graves' disease (Schulze, 1922). In rabbits treated with thyroid, the hyperglycemic action of adrenalin is increased at first, but it becomes subnormal as treatment continues and is followed by a fatal secondary hypoglycemia (Burn and Marks, 1925; Marks, 1925). In dogs and cats, the intense reaction to adrenalin persists (Abbot and van Buskirk, 1931). In thyroidectomized rabbits, adrenalin hyperglycemia is less and of shorter duration, perhaps due to slower liver glycogenesis.

V. SENSITIVITY TO INSULIN

There is an increased sensitivity to insulin in cases of thyroid deficiency, but it is less marked than in pituitary deficiency. In hyperthyroidism there is usually an increased resistance to the drug, though this may be diminished in advanced stages of the condition.

VI. DIABETOGENIC ACTION OF THE THYROID GLAND

1. Animals with Whole Pancreas

In animals with intact and healthy pancreas, thyroid administration does not produce diabetes. Many rats and 76 dogs were given large doses (0.5 to 4 g./kg./day in dogs) over a period of several weeks. Fasting blood sugar increased slightly in the first few days, the highest figure in dogs being 149 mg.%, and in rats 135 mg.%; after one or two weeks it tended to become normal or subnormal. Transient glycosuria has appeared in men undergoing thyroid treatment (Muller, 1906; Wilder, 1940).

The administration of thyroid can produce diabetes in dogs, previously treated with alloxan, which had undergone a transitory increase in blood sugar with a later return to normal. Alloxan weakens the islet cells and renders them sensitive to thyroid action (Carrasco-Formiguera, 1945; unpublished results obtained in the author's institute).

2. Animals with Partial Pancreatectomy

Oral administration of thyroid can produce diabetes in dogs from which a large portion of the pancreas had been removed. The smaller the amount of pancreas left, the easier it is to obtain this result. The species is important. It is easy in the dog, provided only $\frac{1}{7}$ to $\frac{1}{8}$ of the pancreas has been left. The diet is also important, large amounts of carbohydrates favoring the production of diabetes.

Dogs with a surgically reduced pancreas, when submitted to thyroid treatment show an increased blood sugar, glycosuria and ketonuria (Shpiner, 1927-1930; Yriart, 1930; De Finis and Houssay, 1943). If thyroid treatment is continued long enough, then, when the treatment is suspended, the animal remains with a permanent diabetes (Baronoff, 1928; Shpiner, 1930; De Finis and Houssay, 1943; Houssay, 1944). Controls which were not given thyroid had a normal blood sugar, (9 were followed for 5 to 9 months, 8 for 18 to 21 months).

Thyroid diabetes is the name given to the transitory diabetes which appears during thyroid treatment and disappears a few days after the administration has ceased. The damage to the β -cells of the islets is not permanent but tends to disappear and finally they become completely normal.

Metathyroid diabetes is the name given to that condition which not only remains permanent, even after treatment has been suspended, but also becomes increasingly worse. The β -cells undergo irreversible changes and are slowly destroyed, thus giving rise to an atrophy of most of the islets. It is a diabetes due to a deficiency of the β -cells, the insulin-producing cells of the islets, and it has been named metathyroid because it is caused—though not maintained—by thyroid administration.

It is easy to obtain a thyroid diabetes when the resistance of the islets has been lowered: a) due to the short time elapsed after the operation, or b) by recent treatment with anterior pituitary lobe or alloxan.

Sugar administration favors the appearance of diabetes or increases its severity. On the other hand, insulin treatment may cure a metathyroid diabetes. Thus, of 6 dogs which were given insulin 6 days after their blood sugar had increased 180 to 240 mg.%, diabetes disappeared in 4; in 5 untreated ones, the disease persisted and became worse (Houssay, 1945). If the diabetic condition has lasted for 2 or more weeks, with hyperglycemia as high as 250 mg.%, insulin treatment reduces the blood sugar and glycosuria but, when insulin treatment is suspended, the diabetes returns to its initial intensity because of permanent damage to the islets.

Metathyroid diabetes was obtained in 19 dogs which were given daily doses of thyroid, beginning with 0.5 g./kg./day and increasing the dose, when necessary, to 1 or 2 g./kg./day. Permanent diabetes was not produced in 7 dogs, while 2 were completely resistant to thyroid action.

Thyroid and metathyroid diabetes were caused in animals deprived of various organs: thyroid, testicles, ovaries, suprarenal medulla. These types of diabetes did not appear in hypophysectomized dogs nor in adrenalectomized dogs treated with sodium chloride and desoxycorticosterone; these animals became very sensitive to the toxic action of thyroid, which produced hypoglycemia and death in a few days.

Lukens and Dohan (1942) were unable to produce glycosuria in partially pancreatectomized cats treated with thyroxine or thyrotropic hormone. These experiments in cats, however, should be repeated making a larger pancreatic resection, coupled with stronger and more prolonged thyroid treatment. Rats submitted to subtotal pancreatectomy (95%) were fed with thyroid powder (unpublished experiments by Houssay, Foglia and Martinez). Daily doses of 4 to 20 mg./100. g./day at first caused an increase in blood sugar; then, after a period during which the blood sugar was normal, diabetes appeared to a lesser extent than in controls.

3. Action on Animals Previously Diabetic

The administration of thyroid gland increases diabetic symptoms (Allen, 1922; Lukens and Dohan, 1942; De Finis and Houssay, 1943; Houssay, 1944). Dogs with metahypophysial, metathyroid and Sandmeyer's diabetes (the latter being caused by a very large removal of the pancreas), when treated with thyroid showed depression, weakness, rapid loss of weight, diarrhea and a dirty aspect. The blood sugar rose if below or near 200 mg.%, but remained unchanged if in the neighborhood of 300 mg.%. Polyuria, glycosuria and, especially, ketonuria increases and survival is shortened. The severe condition of the dogs remained unchanged when thyroid treatment was suspended, and insulin could not always improve it.

Uninterrupted administration of thyroid powder to diabetic rats (4 mg./100 g./day) caused a transitory improvement of the diabetes, but later most of the animals became normoglycemic (Martinez, 1946). High doses (50 mg./100 g./ day) caused increasing anorexia, loss of weight and a fall in glycosuria and glycemia. The latter conditions were due to the anorexia, because if the animal is forcibly fed through a gastric catheter, thyroid administration greatly increases the urinary excretion of glucose and nitrogen.

4. Thyroid and Anterior Pituitary Association

Joint administration of thyroid gland and anterior pituitary lobe to subtotally pancreatectomized dogs was followed by a greater diabetogenic effect than when each gland was given separately (De Finis and Houssay, 1943; Houssay, 1944). When dogs with whole pancreas were given large doses of both glands for a short time the diabetogenic effect was less pro-

nounced than when anterior pituitary lobe alone was given (De Finis and Houssay, 1943). This anomalous result might be explained by the fact that the animals ate different amounts, since the food given was not weighed. Both substances, either together or alternately, were given over a long period of time to several dogs with whole pancreas. This association was too toxic for them and most of the animals died, although some remained with a permanent diabetes.

5. Thyroid Action on Langerhans' Islets

Increase in size and number of the Langerhans' islets following thyroid treatment has been observed in several species (Farrant, 1913; Herring, 1917; Watrin and Florentin, 1929-1931; Hess, 1942.) Glaser (1926) alone has described alterations in the islet cells of mice treated with thyroxine.

The following lesions of the β -cells of the Langerhans' islets were found by De Finis and Houssay (1943) and Houssay (1944) in the pancreas of dogs with thyroid diabetes:—disappearance of granulations, increase in size, vacuolization, pycnosis and disintegration. These alterations are discrete and transitory in thyroid diabetes. In metathyroid diabetes they are severe and irreversible, with gradual disappearance of the β -cells, atrophy of the islets and increase in fibrous tissue.

6. Insulin Concentration in the Pancreas

In normal rats, thyroxine in small doses increased the insulin concentration of the pancreas. In hypophysectomized rats, however, it had the opposite effect (Fraenkel-Conrat, Herring, Simpson and Evans, 1942).

7. Insulin Secretion

According to Zunz and La Barre, (1932) intravenous injection of strong doses of thyroxine increases the secretion of insulin because of its action on the islets, while smaller doses do so through the stimulation of adrenalin secretion. The same authors (1935) also claim that the thyrotropic hormone has a similar effect.

By means of a pancreatic graft in the neck of a diabetic dog, it is possible to find out whether that pancreas secretes insulin and if it can correct hyperglycemic diabetes. Applying this method to 8 dogs with whole pancreas and treated with thyroid, it was found that the insulin secretion was diminished in 5 (slightly in 3 and markedly in 2). The whole pancreas of dogs with metathyroid diabetes showed no insulin secretion (De Finis and Houssay, 1943; Houssay, 1944; 1945).

8. Characteristic Features of Thyroid and Metathyroid Diabetes

Thyroid diabetes, as compared with pancreatic diabetes of a similar degree, presents the following special characteristic features:—(1) greater

polyuria, (2) a more marked loss of weight, and (3) the need of higher doses of insulin to check the hyperglycemia and glycosuria.

Metathyroid diabetes becomes steadily worse, finally reaching an intensity corresponding to that following total pancreatectomy. The glycemia varies between 300 and 360 mg.%; glycosuria between 2 and 5 g./kg./day (more often 3.5 to 4 g./kg./day); the D:N ratio is between 2 and 2.9; and ketonic bodies rise from 4-10 mg./kg./day (normal value) to 100-200 mg./kg./day on certain days. There is a marked decrease in hepatic glycogen, and a lesser one in muscle glycogen. The tolerance curve is diabetic. Basal metabolic rate is high. Insulin sensitivity is that usually found in diabetes. As in other diabetes, hepatectomy produces a rapid fall in the blood sugar (Houssay, 1944).

9. Mechanism of Thyroid and Metathyroid Diabetes

Under these conditions, there is an initial extrapancreatic phase followed by a pancreatic phase. The extrapancreatic disorders produced by the thyroid are:—(a) an increase in intestinal absorption rate of glucose and, consequently, higher hyperglycemia during the absorption period, (b) a decrease of hepatic glycogen, (c) in a more advanced stage a decrease in muscle glycogen, accompanied by a slower rate of re-synthesis after exercise, (d) at first an increased resistance to insulin, and later a subnormal resistance, (e) high basal metabolism, (f) increased liability to ketonemia and ketonuria.

At a later stage, lesions of the pancreatic islets appear; these are at first reversible but finally become irreversible. They are apparently due to three factors:—(a) hyperglycemia, (b) diminished pancreatic resistance, and (c) toxic thyroid action.

Postprandial hyperglycemia gradually increases in height and duration. Probably this is mainly due to a more rapid intestinal absorption; it is not sufficiently well known whether hepatic homeostasis is impaired or not. These large amounts of sugar flooding the organism require a greater insulin secretion and so exhaust and damage the β -cells. The part played by carbohydrate ingestion is confirmed by two facts:—(1) a diet rich in sugar favors the production of diabetes, (2) early insulin therapy, commenced while the β -cell impairment is still reversible, can cure the diabetes.

The pancreatic resistance must be diminished for the thyroid to produce diabetes. This may be brought about either by reducing the amount of pancreatic tissue by surgical methods, or by a previous treatment with anterior hypophysial extract, alloxan or thyroid gland. Hyperthyroidism probably only produces diabetes in man when the pancreas is already suffering from a hereditary or acquired weakness and the individual has reached the age at which it becomes manifest.

The toxic effect of the thyroid is made manifest by lesions or functional changes in the liver, heart, digestive tract and other organs, which may end in death. When its physiological or metabolic mechanisms are better known the vague term "toxic action" will be dropped in favor of a correct name and explanation.

10. Sensitivity to Alloxan

Hyperthyroidism first increases sensitivity to the toxic and diabetogenic action of alloxan injected intravenously or intraperitoneally, in the rat, but resistance becomes supernormal later (Martinez, 1946).

TABLE I
Incidence of Diabetes in Hyperthyroid Patients

	Hyperthyroidism		Diabetes		Diabetes Per cent of Population
	Total Number of Cases	Per cent Dia- betics	Total Number of cases	Per cent Hyper- thyroids	
<i>Foster, D. P., and Lowrie, W. L., 1938.</i>	1607	2.43	1616	2.41	
<i>John, H. J., 1938.</i>		2.3			1 (1)
<i>Joslin, E. P., Root, H. F., White, P., and Marble, A., 1940</i>	3869	3.13	15601	1.15	0.5 (2)
<i>Wilder, R. M., 1940</i>	(1935- 1938) 1882	3.3 (3)			1.8

(1) In a total of 20,325 cases under observation.

(2) On the basis of 660,000 diabetic cases per 140,000,000 inhabitants.

(3) Mayo Clinic patients in 1937; 5.6% of the patients with adenomatous goiter and hyperthyroidism, 1.7% of cases of Graves' disease and 1.67% of goiters without hyperthyroidism were diabetic.

VII. DIABETES AND HYPERTHYROIDISM IN MAN

1. Incidence of Hyperthyroidism in Diabetic Cases

Hyperthyroidism is no more frequent among diabetic patients than in the rest of the population. Where endemic goiter exists, iodine prophylaxis reduces it equally in diabetic and non-diabetic cases. (Foster and Lowrie, 1938; Wilder, 1940).

2. Incidence of Diabetes in Cases of Hyperthyroidism

Diabetes is not very frequently found in cases of hyperthyroidism, but its occurrence is greater with such than with patients having no hyperthyroidism, being 2 or 3 times more frequent. (See Table I.) The proportion

is higher in adenomatous goiter with hyperthyroidism, probably because the patients are more elderly and their disease is of longer standing (Wilder, 1940).

3. Diagnosis

An incomplete clinical examination may induce a mistaken diagnosis of diabetes in some hyperthyroid patients, chiefly because they show the following symptoms: polyphagia, polyuria, spontaneous or alimentary glycosuria, loss of weight, muscular weakness, a high and somewhat prolonged alimentary hyperglycemia curve. These symptoms are quite frequent and are the cause of mistaken diagnosis which can lead to restriction of carbohydrates in the patients' diet and insulin therapy, both of which are usually harmful in hyperthyroid cases.

A patient whose fasting blood sugar is 130 mg.% or more, and in whom this figure rises to 170 mg.% after a meal or from 30 to 60 minutes after receiving glucose (1 g./kg. weight), is usually considered a diabetic subject. However, in hyperthyroid cases the blood sugar is expected to be higher, and diabetes is not diagnosed unless glycemia findings are 150 mg.% or higher before meals and 200 mg.% after meals or following administration of glucose (Foster and Lowrie, 1938; Joslin *et al.*, 1940; Wilder, 1940). Some prefer the Exton-Rose test, *i.e.*, they administer two doses of 50 g. of glucose (20% concentration) with a half hour interval, and measure the blood sugar at the outset and an hour later (Wilder, 1940).

It has been unanimously established that diabetes and hyperthyroidism have reciprocal bad effects on each condition. Before present-day methods of treatment of hyperthyroidism and diabetes were known, this coincidence used to be catastrophic (Joslin *et al.*, 1940). Hyperthyroidism or the administration of thyroid gland increases the severity of a pre-existing diabetes and may bring about its onset in predisposed subjects (Wilder 1940). The symptoms become more marked, insulin exerts a decreasing efficiency and it becomes necessary to increase the dose, while the tendency to acidosis is increased. The lower liver glycogen makes hyperthyroid diabetics more sensitive to toxic substances and reduces the resistance to hypoglycemia, thus rendering insulin hypoglycemic accidents more serious. It is not always an easy matter to determine which of the two diseases precedes the other, though the anamnesis shows that hyperthyroidism precedes diabetes in 62 to 85% of the cases.

4. Pancreatic Lesions

Usually there are no thyroid lesions in diabetes (Holst, 1921). The pancreas has been studied in a small number of cases of both diabetic and non-diabetic cases of hyperthyroidism (Holst, 1923; Warren, 1938). Holst

observed a reduction in the number of islets and loss of pancreatic weight in six fatal cases and no change in four.

5. Thyroid Administration

Thyroid administration usually increases glycosuria in diabetes, or gives rise to it if there is no glycosuria; there is a tendency to increase ketonuria and the required dose of insulin becomes larger (Wilder, 1940). When the thyroid treatment is discontinued the disorders take a long time to disappear and often make insulin therapy essential.

6. Treatment

Hyperthyroidism requires urgent attention:—(a) to reduce diabetes, (b) forestall acidosis, and (c) avoid organic damage of important organs, especially the liver and heart. If there was a diabetic condition, treatment of hyperthyroidism often improved it. Treatment may be surgical or medical (iodine is efficacious; thiouracil is now being studied).

Preoperative dietetic and iodine therapy reduced the operative mortality to 3.4% in 168 cases; this percentage dropped to 2.6% in a later series (Joslin *et al.*, 1940). The pre- and post-operative rules to be followed have been given by Joslin, Wilder and especially by Haines *et al.* (1941) and McDonough *et al.* (1941). The diet must be high in calories of a suitable composition and rich in vitamins, particularly in the B complex.

Thyroidectomy does not cure diabetes, but sometimes brings about an immediate improvement and a reduction of its intensity (Joslin *et al.*, 1940). Glycosuria disappears in some cases but, when tests are made, the tolerance curve is found to be abnormal. Some cases show only a slight improvement and others none at all. The blood sugar often decreases (Foster and Lowrie, 138) and in most cases there is a reduction in the amount of insulin requirement (Wilder, 1940; McDonough *et al.*, 1941). The sugar tolerance curve became better in 55% of patients, worse in 30% and remained stationary in 5% (John, 1938). Diabetes sometimes appears in patients who have undergone an extensive thyroidectomy (Wilder, 1940; Fongi, 1942).

Hyperthyroid diabetic cases, who have been submitted to surgical treatment for their hyperthyroidism, have the same ulterior course and survival as the other diabetic patients (Wilder, 1940).

VIII. THYROID INSUFFICIENCY AND PANCREATIC DIABETES

1. Dogs

The older experiments gave inaccurate results, mainly because of sepsis, anorexia and short survival after pancreatectomy. Frequently, removal of

the parathyroid glands caused tetany, or else thyroidectomy was incomplete. Many of these old experiments are, therefore, of little value (see bibliography in Houssay, 1945).

Since the discovery of insulin, studies have been made on pancreatectomized animals after the operative wounds were healed and when they were in good condition. Thyroidectomy did not improve the existing diabetes (Wolfson, 1927; Baronoff, 1928; Yriart, 1930; etc.). Yriart, (1930) and De Finis and Houssay, (1943) have made many experiments in the author's Institute; 21 dogs were used for this purpose, 7 with total pancreatectomy, 5 with Sandmeyer's diabetes, 6 with metathyroid diabetes and 3 with metapituitary diabetes. The thyroid gland was removed in all, leaving the parathyroids. The operation was followed by an increase in the diabetic symptoms during 3 to 7 days, after which the usual course and length of survival was observed. None of the animals showed any improvement. The dogs survived for 12 to 32 days after the thyroidectomy; blood sugar was 280 to 360 mg.%; glycosuria varied between 5 and 9%, and was generally in the neighborhood of 4 g./kg./day; urinary nitrogen oscillated between 1 and 1.7 g./kg./day and the D:N ratio between 2 and 2.9; ketonuria increased progressively from 10-20 mg./kg./day to 100-200 mg./kg./day.

Thyroid deficiency is always less severe in dogs than in any other animal species. It is not true that dogs do not undergo any changes when deprived of this gland; growth is retarded in puppies, and adults show signs of deficiency, such as a drop of nearly 24% in their basal metabolism. The minimum nitrogen during fasting is also subnormal (0.25 g./kg./day as opposed to 0.36 g./kg./day in controls), and phagocytosis is below normal, etc.

2. Cats

The pancreatic diabetes of the cat is not affected by thyroidectomy (Lukens and Dohan, 1942). In thyroidectomized cats, pancreatectomy causes a small but significant decrease in the urinary excretion of glucose, of nitrogen and sometimes of ketonic bodies (Lukens and Dohan, 1940).

3. Rats

Early thyroidectomy in rats, deprived of 95% of their pancreas, has a definite preventive action on the onset of diabetes. Conversely, thyroidectomy has no effect on incipient or manifest diabetes (Houssay, Foglia, Prieto Díaz and Sara, 1945).

In animals followed for 1 year, several interesting features were observed. In rats with extensive pancreatectomy (removal of 95% of the pancreas) diabetes appeared in 20 out of 21 males (95%) and in 9 out of 19 females

(52%). When 95% of the pancreas and all of the thyroid gland were removed, diabetes was seen in 2 out of 12 males (17%) and 3 out of 13 females (24%); the remaining 83% of males and 76% of females showed no diabetes. With the exception of one case in 25 animals, diabetes did not occur if it had not appeared within the first 3 months following the operation. In thyroidectomized pancreatectomized rats, there were no changes in the islets or in the β -cells, while those deprived of only their pancreas showed atrophy of many islets, and in the remaining ones there were a decrease in the number and marked alterations in the β -cells, such as enlargement, vacuolization, loss of granules, etc.

The protection against diabetes afforded by thyroidectomy was removed when the thyroid deficiency was treated by means of thyroid administration (4 mg./100 g./day) if this was started from the moment the thyroidectomy and pancreatectomy were performed. Normal body growth was observed, and within 6 months, 10 of 12 males (83%) and 8 of 12 females (66%) had diabetes.

The evolution of the incipient diabetes of 11 rats, already having glycosuria but in which the blood sugar (after 7 hours of fasting) was not higher than 150 mg.% was not impaired by a thyroidectomy carried out 3 months after the subtotal pancreatectomy. Neither did thyroidectomy cure diabetes. Of 8 rats with extensive thyroidectomies and blood sugar between 200 and 350 mg.% (7 hours after the last meal), thyroid removal produced only a transitory improvement in 6, no change in 1 and only one was cured.

The protective action of thyroidectomy shown by the normal blood sugar and the good condition of the islets, may be attributed to the following factors:

- (1) Decrease of the functional effort of the islets, thus preventing their exhaustion and damage. This could be due to:
 - a. the slower intestinal absorption in the thyroidectomized animals, which would diminish the postprandial hyperglycemic excess and, therefore, remove the cause of exhaustion and damage of the islets,
 - b. the fall in the metabolic rate, thus calling for a smaller insulin secretion,
 - c. the reduced body growth, requiring less insulin.
- (2) Anatomical or chemical changes in the islets which make them more resistant to the diabetogenic action. This interpretation is supported by the greater resistance to the diabetogenic action of alloxan after thyroidectomy.
- (3) An undemonstrated increase in the insulin secretion.
- (4) An increased sensitivity of the tissues of thyroidectomized animals to normal amounts of insulin.

The experiments described above show that thyroidectomy prevents, but does not cure, diabetes in rats with subtotal pancreatectomy.

4. Action of Thiouracil

Martinez (1945) administered 20–40 mg. of thiouracil/100 g./day to rats with subtotal pancreatectomy (removal of 95% of the pancreas). A similar number of pancreatectomized rats were used as controls. Observations during 6 months showed that thiouracil greatly delayed the onset of diabetes. These results are similar to those of early thyroidectomy; with thiouracil they are less marked because this drug only diminishes the production and secretion of the thyroid hormone, while thyroidectomy suppresses it completely.

IX. PHLORIZIN DIABETES IN THYROIDECTOMIZED ANIMALS

1. Dogs

During fasting, phlorizin diabetes gives rise in thyroidectomized dogs to a glycosuria similar to that of the controls (1.91 g./kg./day and 2 g./kg./day respectively), although the former eliminate less nitrogen (thyroidectomized 0.63 g./kg./day; controls: 0.80 g./kg./day) (Biasotti and Houssay, 1932).

2. Rats

Wells and Kendall (1940) and Wells and Chapman (1940) state that both the adrenals and the thyroid play a part in glucose formation in the fasting phlorizinated rat. After thyroidectomy, the urinary excretion of glucose and nitrogen undergoes a 15% drop; after adrenalectomy there is a further drop, and a still greater one (79%) following hypophysectomy. The latter operation seems to act through simultaneous deficiency of the thyroid and adrenals, since it gives results similar to those obtained by the removal of both glands. An injection of thyroxine or thyrotropic hormone increases the excretion of glucose and nitrogen in the phlorizinated normal rat, and an injection of thyroxine alone returns it to normal levels in the phlorizinated thyroidectomized animal. On the other hand, simultaneous injection of 17-hydroxy-11-dehydrocorticosterone and thyroxine gives rise to a maximum increase of glucose and nitrogen excretion in (1) the hypophysectomized, (2) the adrenalectomized-thyroidectomized and (3) the control animals. The authors conclude that the adrenals, together with the thyroid, influence the rate of glucose formation in the phlorizinated rat.

X. ALLOXAN DIABETES IN THE THYROIDECTOMIZED RAT

The diabetogenic and toxic actions of alloxan administered either by the intraperitoneal route (Houssay and Sara, 1945), or intravenously (Martí-

nez, 1945), are decreased by thyroidectomy and even more so by thiouracil (Martinez, 1946). In the dog, thyroidectomy does not modify diabetes produced by alloxan.

XI. THYROID DEFICIENCY IN HUMAN DIABETES

1. Total Thyroidectomy

Complete removal of the thyroid gland has been carried out in at least 5 cases of diabetes, with the object of either improving the disease or treating circulatory disorders.

Wilder, Foster and Pemberton (1934) removed the thyroid in a 26 year old man with a diabetes of 11 years standing. His basal metabolism fell below -30%, carbohydrate tolerance increased, and the need for insulin dropped from 45 units daily to only 8-12. The diet had to be controlled and insulin was still required. The sensation of cold and lack of strength made the patient very uncomfortable, and the onset of myxedema called for small doses of thyroxine.

After thyroid removal, Rudy, Blumgart and Berlin (1935) observed a reduction of the basal metabolism and of insulin requirement, together with an increase in body weight. But as the patient became weak and drowsy on the 82nd. day after the operation, the myxedema was treated with thyroid, which increased metabolism and the dose of insulin required.

Gilligan and his co-workers (1934) saw that in a diabetic with heart lesions, total thyroidectomy improved the hyperglycemic curve produced by the ingestion of 100 g. of glucose. Schnitker *et al.* (1936) also found that in 2 cases of mild diabetes, there was an increased tolerance to glucose when myxedema appeared.

These cases are interesting from the theoretical standpoint, but total thyroidectomy is not an advisable method for treatment of diabetes as, in order to attain results equal to those gained by diet and insulin, patients develop a disease (myxedema) even more unpleasant than the diabetes itself.

2. Myxedema and Diabetes

Of 70,000 clinical cases studied by Shepardson and Wever (1934) only 34 had myxedema (0.04%) and 1,120 had diabetes (1.6%). Both diseases were present at the same time in only one case, which was the expected figure in view of the incidence of each disease. Weinstein (1932) only saw 2 cases of co-existence of the diseases in 3,000 diabetic and 22 myxedematous patients.

In a survey of the published cases of co-existence of these diseases Shepardson and Wever mention 12 cases beside the one of their own;

Fongi's survey gives 28 cases and one of his own, but of these only 20 appear to be certain. In their respective books on diabetes, Falta records having seen only 1 case, Wilder 1 and Joslin 5. In most observations, diabetes appeared first and myxedema later. However, in the cases of Holst (1921) and Fongi (1942), and in an unpublished one of Gotta and Yriart, diabetes appeared in a patient with post-operative myxedema and the disease followed a mild course.

The onset of myxedema frequently improves the diabetic condition; glycosuria disappears, tolerance to ingested sugar improves and there is a reduction in the insulin requirement (Wilder, 1940). In most cases, the diabetic condition grew worse under thyroid administration, but in 2 of Gordon and 1 of Falta and Höglér (Fongi, *loc. cit.*) thyroid medication improved both the myxedema and the diabetes.

Rohdenburg's case (1922) is often quoted. In a 30 year old diabetic woman, sugar disappeared from the urine at the age of 42; she became myxedematous at 43 and died 2 years later. The *post mortem* examination showed a destruction of the thyroid alveoli and the pancreas had the aspect mentioned below.

Wilder's case (1926) is very illustrative. A baby became diabetic when 16 months old and glycosuria was controlled by a very strict diet. At the age of 3, the child developed hypothyroid symptoms and the sugar tolerance rose to the point where even with the usual diet there was no sugar excretion. Thyroid extract was given, but had to be suspended owing to a severe glycosuria. When 7 years old, there was myxedema, 120 mg.% blood sugar and no glycosuria. Thyroid was administered again, and in spite of the dietetic restrictions, glycosuria appeared when the basal metabolism rose from -45% to -25%.

According to Fongi, only 5 *post mortem* results have been published; these were by Rohdenburgh; Falta and Höglér; Weinstein; Joslin (Warren, 1938); Carey, Arey and Norris. All of them discovered severe alterations in the thyroid gland, together with the following pancreatic lesions:—fibrosis of the head of the pancreas and giant islets in the body (Rohdenburg); pancreatic arteriosclerosis (Falta); hyaline degeneration in most islets (Weinstein); some islets enlarged and others with a moderate degree of hyalinization (Warren).

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Thyroactive Iodinated Proteins

By E. P. REINEKE

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I. INTRODUCTION

Investigations on the iodination of proteins date back to the latter part of the nineteenth century following Baumann's (14) discovery of iodine in

organic combination in the thyroid gland. Further research, particularly by Hutcheson (67) and Oswald (108), indicated that the greater part of the iodine of the thyroid occurs in combination with thyroglobulin, thus establishing the fact that the active substance is actually an iodoprotein.

In addition to giving much impetus to investigations on the natural thyroid hormone, these early discoveries led to a number of attempts to duplicate the active substance artificially by the iodination of ordinary proteins. In fact, some early claims for the formation of active iodinated proteins were made (21, 22) but these were subsequently withdrawn when the results could not be confirmed. Attempts to increase the activity of thyroid protein by further iodination (68) were also unsuccessful. However, a long series of investigations on the chemistry of the iodination process, inaugurated by the pioneer researches of Hofmeister, Oswald, and Blum, and continuing over a period of more than thirty years, provided the basis for much of our present knowledge of the groups affected by iodination within the protein molecule.

In the meantime, the classical experiments of Kendall (76) resulted in the isolation of crystalline thyroxine in 1915. As a result of a series of brilliant investigations, the chemical configuration of thyroxine was deduced by Harington (50) in 1926. Shortly thereafter *dl*-thyroxine was synthesized by Harington and Barger (54).

Parallel experiments on artificially iodinated proteins finally resulted in isolation of crystalline thyroxine by Ludwig and von Mutzenbecher (92, 93). Subsequent research has been directed principally toward elucidation of the mechanism involved in the formation of thyroxine by this means, the improvement of the iodination method to form products with greater thyroidal activity and higher thyroxine content, and determination of the effects of active iodinated proteins on various physiological processes.

II. THE IODINATION OF PROTEINS

1. *Iodination Methods*

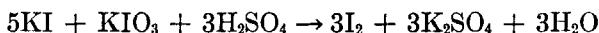
Proteins may be iodinated in neutral, basic or acid media, though the iodine content and the reactive protein groups affected, will vary according to the conditions employed. Although it has long been known that iodine would combine with proteins (27), the awakened interest occasioned by the discovery of iodine in the thyroid led to intensive investigations of this reaction.

Liebricht (90) combined iodine with casein by mixing the two substances together in the proportion of 20 g. of iodine to 80 g. of casein, and stirring at 100°C. The brown powder obtained was extracted repeatedly with ether to remove excess iodine. This product, designated as *periodocasein* contained

17.8% iodine, of which a large proportion was in loose combination. The loosely combined iodine could be removed by treatment with alkaline bisulfite, resulting in a preparation containing 5.7% iodine, and termed *iodocasein*. Brief digestion of *periodocasein* with 10% sulfuric acid produced *caseoiodine*, a split-product containing 8.5 to 9.3% iodine and having many chemical properties in common with the *iodothyryin* of Baumann.

It was discovered shortly afterwards, by Blum and Vaubel (26), that hydriodic acid liberated as a side product prevented the reaction of iodine with protein from going to completion. To overcome this they conducted the iodination in a solution buffered with sodium bicarbonate. By this means the hydriodic acid is neutralized continuously, thus permitting the reaction to proceed. It is of considerable interest that more than forty years later this method, with but slight modifications, has been found to provide optimal conditions for the formation of iodinated proteins with high thyroxine content and marked thyroidal properties.

Proteins were also iodinated (62, 84) by the addition of potassium iodide and iodate together with sufficient sulfuric acid to liberate iodine by the well known equation:



Iodinated egg albumin formed by this method contained from 7 to 12% iodine, the amount combined depending on the excess of iodine added, the temperature, and the time permitted for the reaction to go to completion.

Iodination in an ammoniacal medium (25) was believed to produce less alteration in the protein than did the bicarbonate method. In the formation of thyroidally active iodinated proteins, to be reviewed in later sections, both of these methods have been employed.

2. Iodine-binding Groups in the Protein Molecule

With the discovery of iodination methods, attention naturally turned to investigations on the mode of combination of iodine with proteins. It was noted (62, 84) that iodination caused both the Millon and Adamkiewicz reaction to become negative, indicating alteration of both the tyrosine and tryptophane. Evidence for the oxidation of sulfur was presented (62). Iodination of oxyhemoglobin (85) caused an increase in the ratio of carbon to nitrogen, leading to the assumption that an albumose-like fraction was split off in the process.

The isolation of iodogorgonic acid by Drechsel (37) in 1896 from a hydrolyzate of gorgonin, a protein derived from the axial skeleton of the coral, and its identification as diiodotyrosine by Wheeler and Jamieson (162), and Henze (59) drew attention to tyrosine as an iodine-binding group.

Final proof of the iodination of tyrosine in proteins was provided by

Oswald when he isolated this compound from the hydrolytic products of iodoalbumin (109), iodogliadin (110) and iodocasein (111). It was not until twenty years later that Harington and Randall (57) succeeded in isolating the same compound from a hydrolyzate of the thyroid gland.

From the known tyrosine composition of a number of proteins it could be calculated that more iodine was combined than could be accounted for by substitution of tyrosine. The key to the solution of this problem was provided by Pauly (114) with the discovery that iodine can be substituted on the imidazole ring of histidine. Imidazole itself was shown to substitute one atom of iodine on the imino group and three atoms on carbon. Since one carbon position is blocked in histidine by the side chain, two carbons and the imino nitrogen would be available for the substitution of iodine in this amino acid. The amount of iodine that will actually combine with histidine appears to depend in large measure on the conditions used. Pauly found that imidazole will take up iodine more readily the more alkaline the solution.

TABLE I
Iodine Content of Some Artificial Iodoproteins

Preparation	"A" substance	"B" substance	"C" substance	Ratio of Nitrogen-I to Carbon-I
Iodoovalbumin	7.55	5.12	4.91	1:2
Iodoserumalbumin	8.96	6.73	6.70	1:3
Iodoserumglobulin	8.30	6.64	—	1:4
Iodothyroglobulin	6.14	4.88	4.96	1:4
Iodocasein	7.51	7.51	7.51	0:2

(From *Z. physiol. Chem.* (24).)

Though he was not able to iodinate free histidine directly, benzoyldiiodohistidine and *p*-nitrobenzoyldiiodohistidine, which served as structural analogues, were formed. The iodine taken up by carbon was in firm combination, but that attached to nitrogen could be removed easily with bisulfite.

Blum and Strauss (24) suggested that the extent to which iodine can be substituted on the imino group or on the unsaturated carbon atoms in histidine varies with different proteins according to the accessibility of these groups within the protein molecule. Various proteins iodinated by the bicarbonate method yielded characteristic iodine numbers which varied with the protein used. The fully iodinated protein was designated as substance A. Removal of the nitrogen-bound iodine by treatment with bisulfite resulted in a product with iodine bound only to carbon, and designated as substance B. Rapid iodination over a short period produced a C-substance with an iodine content similar to that of B, as shown in Table I.

In proteins treated with the maximum amount of iodine a number of side

reactions were shown to take place. Negative tests for tryptophane indicated oxidation of this compound. Negative tests for reduced sulfur, and the formation of iodoform indicated the oxidation of cystine. The hydriodic acid formed was more than four times the amount that would be expected from substitution alone. It was pointed out that oxidation of the sulfur from one molecule of cysteine to cysteic acid ($R-SO_3H$) would result in the formation of six molecules of hydriodic acid. In summary, the reaction of iodine with proteins was pictured by Blum and Strauss as follows:

- | | |
|--|---|
| I. Main reactions <ul style="list-style-type: none">a. Full carbon iodination (negative Millon reaction)b. Destruction of $\frac{1}{2}$ the groups giving the biuret reactionc. HI formation from substitution | II. Side reactions <ul style="list-style-type: none">a. N-iodinationb. Oxidation of cystine and tryptophane. Splitting off of sulfurc. Iodoform formationd. Further HI formation from oxidation |
|--|---|

By virtue of its high histidine and low tyrosine content the iodination of globin is of particular interest in demonstrating the mode of substitution of histidine. Maximally iodinated globin (153) contained 11.4% of iodine; upon removing the N-iodine by treatment with bisulfite 7.6% of iodine remained bound to carbon. Re-iodination of this preparation restored the iodine content to 11.4%. Globin was shown (10) to combine with twice as much iodine as could be accounted for by substitution on the tyrosine alone, the remainder being accounted for by substitution on histidine. Nitroglobin (12) combined with only three-fourths of the theoretical amount of iodine because one of the reactive carbon atoms of tyrosine was already occupied by an NO_2 group. On the basis of quantitative analyses it was calculated (11) that globin would have six possible iodine numbers depending upon the extent of iodination of the histidine. By variation of the iodination method it was shown that all six possible steps can be brought about in stoichiometrically exact and reproducible proportions.

III. THYROIDAL ACTIVITY OF IODINATED PROTEINS

With the awakened interest in iodinated proteins resulting from the discoveries on the nature of the thyroid hormone in the 1890's, claims for the formation of thyroidally active preparations were made from time to time. These were subsequently abandoned, apparently due to lack of confirmation. In the light of present knowledge of the many conditions affecting the formation of such substances this is not surprising. Biological tests which would indicate thyroidal activity were made on only a few iodo-proteins, with little regard for the method of preparation.

1. Early Evidence of Thyroidal Activity

Blum (21) asserted that his iodinated albumin produced the same effects in myxedema as thyroid substance. In a later report (23) this claim of specific thyroidal action was apparently withdrawn. The *caseoiodine* of Liebricht (90) was stated (167) to be wholly ineffective when tested on thyroidectomized dogs. Since the function of the parathyroid was unknown at that time, and the criterion of thyroidal activity used was the prevention of tetany and final death, this result is inconclusive.

The specific effect of thyroid substance in stimulating the metamorphosis of frog tadpoles was first reported by Gudernatsch (47, 48) in 1913. In the next year Morse (103) reported that comparable effects were produced by iodinated proteins. Lenhart (89) tested a commercial preparation of iodoalbumin containing 21% iodine, much of it loosely combined, on tadpoles. Even though stimulation of metamorphosis was observed, toxic side effects were also noted and, therefore, the results were not accepted as establishing a thyroid-like action. From tests on a similar preparation, Rogoff and Marine (139) concluded that iodinated albumin has a thyroid-like action on tadpoles, but that the effect develops more slowly than with thyroid substance. Further tests on a series of iodinated proteins (140) showed evidence of some activity for all of them. Alkaline hydrolysis was reported to destroy the activity of all these preparations, however. Unfortunately no confirmatory assays on other types of test animals were conducted, with the result that these early findings came to be regarded merely as indicating a special action of such preparations on tadpoles.

2. Hydrolysis and Concentration of the Active Substance

The successful hydrolysis and concentration of a physiologically active substance from iodinated protein was first reported by Brandt, Mattis and Nolte (29). Ordinary iodinated proteins were stated to have no effect on the metamorphosis of tadpoles when fed for as long as four weeks. The acid-insoluble precipitate obtained after hydrolysis of the iodoprotein with barium hydroxide, however, exerted a metamorphosis-stimulating effect similar to that of thyroid substance.

The concentration of a thyroidally active substance from hydrolyzates of iodinated proteins was investigated extensively by Abelin and coworkers, their initial report appearing in 1933. The acid-insoluble substance obtained after hydrolysis with alkali was designated as *homothyroxine* (5). Physiological and chemical tests of this substance showed that, qualitatively, it possessed many of the properties of thyroxine. When given in high dosage, homothyroxine caused moulting and the appearance of white feathers in black chickens (1). It also counteracted the decrease in body temperature

ordinarily caused in guinea pigs by the injection of novocaine. Both of these effects can be duplicated by administering thyroxine. An active concentrate containing more than 26% of iodine and capable of producing pronounced stimulation of metabolism in the rat was prepared (2). Further purification (3) resulted in the isolation of a crystalline compound similar to thyroxine in microscopic appearance, and possessing high thyroidal activity. Complete identification of the compound was not made, however.

Finally, the isolation from iodinated proteins of thyroxine in crystalline form was reported by Ludwig and von Mutzenbecher (92, 93), proving that the active principle formed in the iodination of ordinary proteins under certain conditions is identical with that of the thyroid gland.

3. Formation of Iodinated Proteins Which are Effective Without Hydrolysis

In the experiments leading to the isolation of thyroxine the idea was advanced by Brandt, Mattis and Nolte (29) that hydrolysis of the protein was required to obtain an active product. Huge doses of iodinated protein given orally to a rat had no effect on its metabolism; small amounts of a hydrolysate produced a pronounced increase (Abelin, 2). On the other hand, Kaer (74) reported that a commercial iodinated protein containing 5% of iodine produced thyroidal effects when fed to both tadpoles and guinea pigs. Both iodinated serum proteins and their degradation products were reported by Lerman and Salter (91) to be effective in the relief of myxedema in man. The iodinated casein prepared by Harington and Pitt Rivers (55) for the isolation of thyroxine was stated to increase the metabolism of rats when administered orally.

Iodinated proteins prepared in the author's laboratory (124) consistently produced thyroidal effects when given orally to either normal or thyroidectomized animals. Iodinated protein differed from thyroid or thyroxine (137) in that no response was produced in tadpoles placed in a solution containing the material in the form of a suspension. Pronounced metamorphosis was induced, however, when iodinated casein was given either orally or by intraperitoneal injection.

In view of the crude nature of this material, the marked thyroidal response, and the lack of toxic side effects when it was injected without a preliminary hydrolysis were quite surprising. Subsequent investigations revealed that guinea pigs (126) and mice and rats (82) responded similarly when injected with thyroactive iodinated casein. Discovery of the effectiveness of these products when given parenterally greatly facilitated the quantitative assay of experimental preparations, since injected iodoprotein could be compared directly with injected thyroxine, thus avoiding the differences in digestion and absorption which would be encountered in comparisons made by oral administration.

4. Methods of Forming Highly Active Iodinated Protein

In view of the contradictory information as to the possible thyroidal nature of various iodinated proteins, and also the lack of knowledge of the influence of variables in the iodination process on the amount of thyroidal substance formed, a series of investigations were undertaken by the author in collaboration with Dr. C. W. Turner and others in order to determine the factors influencing this reaction. The general iodination method was similar to that devised by Blum and Vaubel (26) and used subsequently (92, 93, 55) in preparing iodinated proteins for the isolation of thyroxine.

In this recent work the principal departures from earlier procedures have been (a) limitation of the iodine to the optimal level established for thyroxine formation, and (b) incubation of the iodinated protein at 60 to 70°C. The general procedure is as follows:

Twenty g. of casein is placed in 700 ml. of distilled water containing 5 g. of sodium bicarbonate, and is dissolved by stirring. The mixture is then placed in a water bath held at 38 to 40° C., and a total of 3.7 g. of finely powdered iodine is added in small portions over a period of 3 to 4 hours, the solution meanwhile being agitated vigorously with a mechanical stirrer. When the requisite amount of iodine has been added the solution is incubated at 70°C., with vigorous stirring, for 18 to 20 hours. After dialysis, the iodinated protein is recovered by isoelectric precipitation, dried and ground to a fine powder.

Individual factors in this basic procedure were varied singly to determine their effect on the end product. The thyroidal potency of the iodinated protein was determined by biological assays on tadpoles and guinea pigs (126) and more recently by chemical determination of its thyroxine content (135).

a. Effect of Extent of Iodination. The necessity of following rather closely defined limits of iodination for the formation of iodinated proteins capable of yielding thyroxine on hydrolysis was stressed by Ludwig and von Mutzenbecher (93), but no information was available as to the effect of varying degrees of iodination on the thyroidal activity of the iodinated protein itself. Muus *et al.* (105) reported that when serum albumin in an ammoniacal medium was treated with progressively increasing amounts of iodine, thyroidal activity, as determined by tests on myxedematous patients, did not begin until 6% of iodine or the equivalent of 2 atoms per mole of tyrosine in the protein had been bound. With increasing iodination the thyroidal activity increased until 3 to 4 atoms had been combined per mole of tyrosine, and thereafter remained at a relatively constant level.

A distinctly different picture, probably because of differences in the medium used, was obtained by Reineke *et al.* (137) when both casein and the mixed proteins of skim milk were iodinated progressively in the sodium bicarbonate medium already described. Beginning at a low level, the thy-

roidal activity of successive preparations increased with increasing iodination until 4.5 to 5.0 atoms had been added per mole of tyrosine in the protein. If it is assumed that one-half the iodine is used in the formation of hydriodic acid and the remainder for substitution, this would be just sufficient for the substitution of 2 atoms per mole of tyrosine in the protein. Iodination beyond this point resulted in a rapid decline in activity.

With the discovery (126) that the formation of active thyroidal substance is increased markedly by incubation of the iodinated protein at an elevated temperature, the effect of progressively increasing increments of iodine on the activity of iodinated casein and soybean protein when incubated at

TABLE II
Effect of Progressive Iodination and High Temperature Incubation on Thyroidal Activity of Iodinated Protein

Series No.	Preparation No.	Iodine added per 100 g. protein	Iodine added per mole tyrosine atoms	Iodine combined per cent	Iodine combined per mole tyrosine atoms	Thyroxine content per cent	Tadpole responses per cent	Per cent of thyroxine response	P*
		g.							
I. Iodinated casein									
	1	7.5	1.89	4.11	1.08	0.67	16.2	4.17	1
	2	12.5	3.16	5.93	1.59	1.31	27.2	5.83	1
	3	19.0	4.80	7.55	2.06	1.85	34.9	8.50	5
	4	25.0	6.31	8.19	2.25	1.72	21.6	5.07	1
	5	32.5	8.21	8.60	2.38	1.35	11.8	3.42	1
	6	38.0	9.60	9.13	2.54	1.04	4.6	2.40	5
II. Iodinated soy bean protein									
	1	6.0	2.03	3.21	1.12		14.2	3.82	
	2	11.5	3.88	5.20	1.85		21.5	5.07	5
	3	17.5	6.35	6.15	2.22		22.9	5.25	5
	4	23.5	8.95	6.51	2.35		7.7	2.92	1
	5	30.0	10.14	7.40	2.70		2.9	2.35	1
	6	36.0	12.17	7.78	2.85		1.6	2.32	5

*P—the *per cent* probability that the difference from the preceding member of the series is due to chance variation.

(From the *J. Biol. Chem.* (136), with thyroxine analyses (135) added.)

70°C. for 18 to 20 hours was investigated (136). At all levels of iodination the actual thyroidal potency was considerably higher than that of similar preparations incubated at a lower temperature. Just as in the earlier series, however, the thyroidal activity rose to a maximum (Table II) when sufficient iodine had been added to substitute 2 atoms per mole of tyrosine in the protein. Further iodination resulted in a pronounced decline in activity of both the iodinated casein and the soybean protein.

For reasons which will be discussed later, the thyroxine content of iodinated proteins as determined by chemical analysis is considerably lower than that indicated by the tadpole assays. However, the two measures fol-

low a parallel course with respect to the relative potency of succeeding preparations in the iodinated casein series.

Approximately one-half the iodine used is combined with the protein until sufficient has been added to substitute 2 atoms per mole of tyrosine. At this point the Millon reaction becomes negative, indicating full substitution on the 2 carbon atoms *ortho* to the phenolic hydroxyl group of tyrosine.

It is thus apparent that, under the conditions employed, iodine is taken up principally by tyrosine according to the equation,



After the tyrosine has been fully substituted iodine is combined less readily, with the result that progressively smaller increments are taken up as more is added. The best evidence available indicates that the formation of thyroxine in iodinated proteins is effected by the oxidative coupling of two molecules of diiodotyrosine, with the elimination of one side chain. The addition of iodine in excessive amounts apparently causes further oxidations which result in inactivation of the compound.

b. Relation of pH and Extent of Iodination to Formation of Active Substance. The pH of the medium appears to have a decided influence on the relative reactivity of the iodine-binding groups in proteins, and this is also reflected in the amount of thyroxine formed under various conditions. Investigation of this problem is complicated by the fact that the continuous formation of hydriodic acid with increasing iodination depresses the pH of the medium (137) unless a considerable excess of buffer substance is present.

The effect of the pH on the thyroidal activity of skim milk proteins was studied (126) by making up a series of preparations in which the iodine input was held constant, and the amount of sodium bicarbonate added was increased in progressive order in succeeding samples. As indicated by assays on tadpoles, the formation of active substance was markedly retarded when the amount of buffer present was insufficient to hold the pH of the reaction medium at a value of approximately 7.0 or above. Excess of sodium bicarbonate beyond this amount appeared to have no effect on the result. From the fact that normal amounts of iodine were combined by the protein at all pH values covered, it is believed that substitution of iodine on the tyrosine occurred as usual, but conditions were not such as to permit the formation of appreciable amounts of thyroxine at the lower pH levels.

In further experiments (132) it was found that when the sodium bicarbonate concentration of the medium was increased concurrently with the iodine input in order to prevent decline in pH with excessive iodination, considerably more iodine could be added before the usual decline in thyroxine content occurred. Under these conditions, thyroxine formation increased until 6 to 7 atoms of iodine were added per mole of tyrosine in the

protein. Further addition of iodine caused a decline in thyroxine content even though the pH of the solution was not depressed. The thyroxine content at the optimum level of iodination was also somewhat higher than under the former conditions.

c. Relation between Iodination and Incubation Temperature. In all the earlier experiments on formation of thyroidally-active iodinated proteins the reactions were conducted, where possible, at approximately 38°C., presumably in the expectation that thyroxine formation would be favored at physiological temperatures. von Mutzenbecher (106) reported that when casein was iodinated in ammonia solution in the cold it showed little or no thyroidal activity. Re-suspension of the iodinated casein in sodium bicarbonate solution, and incubation at 37°C., with stirring, for 2 to 3 days resulted in appreciable increases in activity as indicated by tests on guinea pigs. From this it might be expected that the potency of the iodinated protein would be a function of the time of incubation.

In order to determine the effect of long-continued incubation on the formation of thyroidal substance, casein was iodinated by the usual procedure (126), and then placed in a water bath at 37° to 38°C., with continuous stirring, for varying periods up to 39 hours (Table III). When a uniform dosage of iodinated protein from the various lots was injected into groups of tadpoles there was no significant difference in the stimulation of metamorphosis, as indicated in the column headed "Per cent response." This experiment, with slight variations, was repeated several times with essentially the same result. Thus, it was concluded that little or no increase in thyroidal potency could be obtained by use of a long incubation period under the conditions employed.

However, it was discovered (126) that a pronounced increase in thyroidal potency of iodinated protein could be obtained by holding the reaction mixture at the elevated temperature of 60° to 70°C., beginning either before the iodination step or subsequent to it. In two groups of preparations (Table IV), incubation at 39°C. was continued for 28 hours without a demonstrable increase in the thyroidal activity as determined by injection in tadpoles. When the temperature was increased to 65°C. during the last 18 hours of incubation there was a large increase in potency.

When preparations were incubated at various temperatures from 30° to 97°C. the thyroidal potency remained at a uniform level over the range of 30° to 45°C. There was a pronounced rise in activity at 60°C., with the maximum occurring at 70°C. Further increase in the temperature of incubation to 97°C. resulted in a considerable decline in activity of the resulting product.

In all of these experiments the iodinated protein was incubated at the elevated temperature for 18 to 20 hours subsequent to iodination. The

effect of longer incubation periods under the given conditions has not been reported. In further investigations by the author (unpublished) it has been observed that the thyroxine content of iodinated casein increases progressively with increasing length of incubation up to 24 hours. At this point

TABLE III
The Effect of Length of Incubation Period at 37° to 38°C. on Thyroidal Potency of Iodinated Casein

Preparation No.	No. of Tadpoles	Per Cent Response	Hours Incubated
AB26-1 (in glass)	9	12.6	None
2	4	12.5	5.0
3	9	12.1	16.0
4	7	13.8	21.0
5	7	9.7	39.0
AB27-1 (brass stirrer)	6	13.6	None
2	6	16.6	5.0
3	7	17.3	16.0
4	8	17.1	21.0
5	7	14.0	39.0

Dosage 0.025 γ per tadpole.

(From *Agr. Exp. Sta. Mo., Res. Bull.* **355** (126).)

TABLE IV
The Effect on Thyroidal Activity of Elevating the Incubation Temperature Subsequent to Iodination

Preparation No.	No. of Tadpoles	Per Cent Response	Hours Incubated	Temperature °C.
AB47-1 (in glass)	10	1.20	4	39
2	10	4.37	16	39
3	8	5.56	28	39
4	6	12.10	46	65
AB48-1 (brass stirrer)	9	12.40	4	39
2	7	10.80	16	39
3	8	6.20	28	39
4	10	19.20	46	65

Dosage 20 γ per tadpole.

(From *Agr. Exp. Sta. Mo., Res. Bull.* **355** (126).)

thyroxine formation appears to continue, but at a constantly diminishing rate.

d. Catalysis of Thyroxine Formation by Manganese Compounds. During the course of the work just described, it was observed that iodinated proteins made up in the presence of a common brass stirrer rather consistently possessed greater thyroidal properties than those prepared exclusively in

TABLE V

Showing the Effect of the Incubation Temperature, Manganese Compounds and Amount of Agitation on the Formation of Thyroxine in Iodinated Protein

Catalyst	Stirring	Thyroxine Content per cent
I. Skim milk Proteins Iodinated and Incubated at 37°C.		
None	Very gentle	0.33
None	Very gentle	0.26
None	Very gentle	0.27
	Average	0.29
II. Casein Iodinated at 38-40°C. and incubated at 70°C.		
None	300 RPM	1.67
None	600 RPM	1.73
None	600 RPM	1.80
None	600 RPM	1.75
None	600 RPM	1.84
	Average	1.76
Mn ₃ O ₄	300 RPM	1.94
Mn ₃ O ₄	300 RPM	1.99
	Average	1.96
Mn ₃ O ₄	600 RPM	2.72
Mn ₃ O ₄	600 RPM	2.93
Mn ₃ O ₄	600 RPM	3.03
Mn ₃ O ₄	600 RPM	2.78
Mn ₃ O ₄	600 RPM	2.80
Mn ₃ O ₄	600 RPM	3.04
	Average	2.88
Oxides from reduction of KMnO ₄	600 RPM	2.97
	600 RPM	2.96
	600 RPM	2.60
	Average	2.84
MnO ₂	600 RPM	2.16
MnO ₂	600 RPM	2.19
	Average	2.17
Mn ₂ O ₃	600 RPM	2.26
Mn ₂ O ₃	600 RPM	2.33
	Average	2.30
MnSO ₄	600 RPM	2.00
MnSO ₄	600 RPM	2.13
	Average	2.07

(From the *J. Biol. Chem.* (132).)

glass equipment. This led to the supposition that one of the metals contained in brass, or perhaps the combination of materials present, catalyzed the formation of thyroxine. In further experiments no augmentation of the thyroidal potency of iodinated proteins prepared in the presence of salts or oxides of copper, iron or cerium was observed. Thyroxine formation was uniformly increased, however, upon the addition of small amounts of manganese compounds (132), including manganese sulfate and a series of manganese oxides.

The effects on thyroxine formation of the incubation temperature, amount of agitation and various manganese compounds are summarized in Table V. It will be noted that the rate of stirring during the incubation period is a factor in the formation of thyroxine. From results obtained in the incubation of diiodotyrosine (133) this is believed to be due to incorporation of atmospheric oxygen in the solution.

With all other factors held constant, the thyroxine content of iodinated casein was increased markedly by incubation in the presence of a small amount of manganese tetroxide (Mn_3O_4). Similar results were obtained by use of the mixed oxides resulting from the reduction of potassium permanganate with glucose. Definite increases in thyroxine formation, but of a smaller magnitude, were obtained with the other manganese compounds tested.

The thyroxine content of a series of preparations formed by combining progressively increasing amounts of iodine with casein in the presence of manganese tetroxide is compared graphically in Fig. 1 with that of a control series prepared without the catalyst. The procedure differed from that reviewed previously in that the amount of sodium bicarbonate added was increased proportionately with the iodine. Under these conditions thyroxine formation continued to increase in the control series until slightly more than 7 atoms of iodine had been added per mol. of tyrosine in the casein. In the presence of manganese oxide approximately 6 atoms of iodine per mol. of tyrosine were required for maximum thyroxine formation. More iodine was required for optimal results than under the former conditions of bicarbonate concentration, but the maximum thyroxine content was also higher. By addition of the catalyst the thyroxine content at the level of optimum iodination increased from 2.8 to 3.37%. The formation of thyroxine from diiodotyrosine was catalyzed by manganese oxide (133) in a similar manner.

In view of the many factors now known to influence the formation of thyroxine in iodinated proteins, it is not surprising that many of the early reports in this field were negative or contradictory. The reaction appears to be quite specific, depending for its successful completion upon the proper balance of the various factors reviewed in this discussion.

Assuming the tyrosine content of casein to be 5.65%, the theoretical yield of thyroxine would be 10.6%. The maximum thyroxine content of

3.37% (Fig. 1) represents slightly more than 30% of the theoretical. In instances where proper attention has been given to all factors now known to influence this reaction, iodinated casein containing 4% or more of

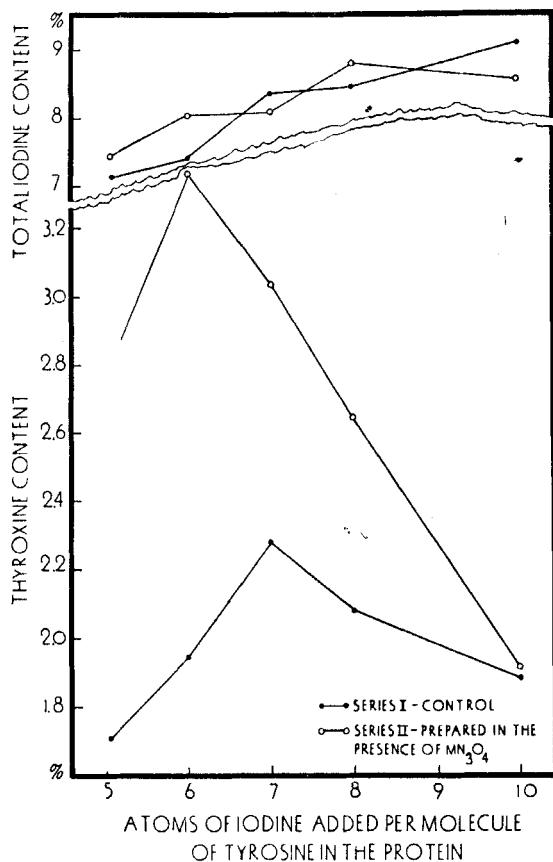


Fig. 1

The Effect of Progressive Iodination in the Presence of Excess Bicarbonate and Manganese Oxide on the Thyroxine Content of Iodinated Casein
(From the *J. Biol. Chem.* (132))

thyroxine, as determined by both biological assay and chemical analysis, has been reported (135).

5. Proteins Suitable for Iodination

Of the many proteins that might be considered for the formation of thyroactively active substances, casein has been most widely used, probably because of its ready availability and the ease with which it can be manipulated. It is well established that by means of the iodination and subsequent

treatment thyroxine is formed from the tyrosine originally present in the protein. Thus, it might be expected that any protein containing tyrosine might be suitable for this purpose unless the position of this amino acid was such in certain proteins as to interfere with the coupling reaction involved in the formation of thyroxine. Although the number of proteins studied from this point of view has been limited, no instance has come to the attention of the author wherein a protein which contains tyrosine has failed to form a thyroidally active substance when iodinated under suitable conditions.

Ludwig and von Mutzenbecher (93) reported that crystalline thyroxine was obtained by hydrolysis in alkali of iodinated casein, iodinated serum albumin, iodinated serum globulin, iodinated silk fibroin, and iodinated edestin. It was believed that all proteins containing tyrosine could be used successfully, but that the best conditions must be established for each protein.

Abelin and Neftel (6) reported that the formation of thyroidally active iodinated proteins depended upon both the type of protein used and the iodination method. Iodination of peptones failed to produce an active product. Because of the paucity of information available at that time on the other factors affecting this reaction, however, the interpretation of these results is doubtful.

Hypothyroidism was corrected by the administration of acid-insoluble substance obtained from hydrolyzates of blood serum proteins (143), by iodinated serum proteins given as such (91), and by iodinated serum albumin (105).

A relatively low order of thyroidal potency was reported by Blaxter (17) for iodinated blood proteins. Higher activity was observed with iodinated ardein, while the highest potencies were obtained with iodinated casein. Data on the preparation of these products were not given, however.

Highly active iodinated proteins were prepared from casein, egg albumin and soybean proteins by Reineke and Turner (126). In comparison with iodinated casein prepared under the same conditions, iodinated soybean protein showed lower thyroidal potency, proportionate with its lower original tyrosine content.

With the factors influencing the formation of iodinated protein now worked out sufficiently well to permit standardization of procedures, it would be of considerable interest to extend these observations to a series of proteins of widely varying tyrosine content.

IV. THE ISOLATION OF THYROXINE FROM IODINATED PROTEIN

1. Isolation of *dl*-Thyroxine

Once an iodinated protein has been prepared under the proper conditions for thyroxine formation, the pure amino acid can be isolated readily

by use of the principles first established by Kendall (77) and Harington (49) in the isolation of thyroxine from thyroid substance.

The isolation of crystalline thyroxine from iodinated proteins was first reported by Ludwig and von Mutzenbecher (92) in 1936. In a later report (93), details of the isolation procedure used for the recovery of thyroxine, diiodotyrosine and monoiodotyrosine were presented. The iodinated protein was first hydrolyzed in boiling 40% barium hydroxide solution for 20 hours to liberate the thyroxine. The sandy precipitate of barium salts which formed was recovered by filtering the hot solution, and then decomposed with hydrochloric acid to obtain an acid-insoluble precipitate of high iodine content. A second portion of acid-insoluble substance was obtained by acidification of the liquid portion of the hydrolysate after removing the excess barium hydroxide which crystallized when the solution was cooled. The last traces of barium were removed from the combined acid-insoluble precipitates by treatment with sodium sulfate in boiling N/10 sodium hydroxide solution, the barium sulfate formed being removed by centrifuging. After precipitation from the hot sodium hydroxide solution by acidifying with dilute sulfuric acid solution, and washing with dilute acetic acid, the acid-insoluble substance was dissolved in a minimum of boiling N/10 potassium carbonate solution. The mono-potassium salt of thyroxine crystallized from this solution when cooled to 0°C. After purifying the compound by recrystallization as the mono-potassium salt, it was dissolved in 70% alkaline alcohol. Upon acidifying the boiling solution with glacial acetic acid, free thyroxine crystallized in the characteristic bundles of microscopic needles. The yield of purified thyroxine obtained amounted to approximately 0.1% of the iodinated casein hydrolyzed. Harington and Pitt Rivers (55) reported that a similar yield of thyroxine was obtained when casein was iodinated and treated in a manner identical with that described by Ludwig and von Mutzenbecher.

By using iodinated casein with high initial thyroidal activity, and a similar isolation procedure, Reineke and Turner (127) obtained a yield of 0.424% of crystalline thyroxine. The increased yield of thyroxine obtained by isolation thus supported the evidence of increased thyroidal activity indicated by biological assays. The actual yield, however, was only 28% of the figure indicated biologically.

The thyroxine obtained from iodinated proteins has been found to be identical with synthetic thyroxine in every respect. Iodine contents ranging from 63.6 to 65.0% (theoretical 65.4%) have been reported (93, 127). When the iodine was removed by reduction (93), thyronine was obtained. The spectrographic absorption curve of thyroxine isolated from iodinated protein is identical with that of synthetic thyroxine (Fig. 2).

Metabolic stimulation equal to that produced by the synthetic compound was observed (127) when thyroxine obtained from iodinated protein was

administered to guinea pigs, thus providing biological proof of its identity. Because of racemization occurring during hydrolysis in alkali, the thyroxine obtained by the barium hydroxide procedure is a racemic mixture.

2. Isolation of l-Thyroxine

From theoretical considerations on the mode of its formation, the thyroxine in iodinated proteins would be expected to be the natural levorota-

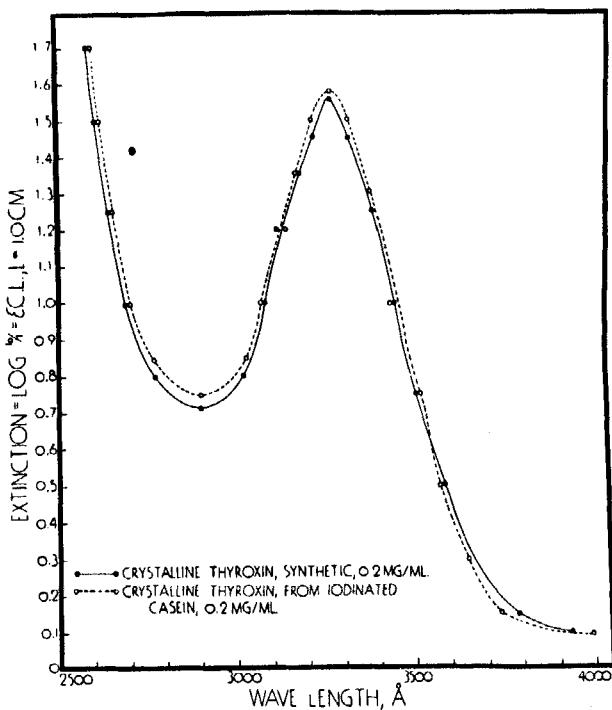


Fig. 2

Spectrographic Absorption Curves of Synthetic Thyroxine and Thyroxine Isolated from a Barium Hydroxide Hydrolyzate of Iodinated Casein
(From the *J. Biol. Chem.* (127))

tory isomer. Optically active amino acids are ordinarily obtained from proteins by hydrolyzing with acids to avoid racemization. Early attempts to concentrate the active principle of thyroid substance after hydrolysis with acid as reviewed by Kendall (78) and Harington (52) resulted in failure, presumably due to destruction of the thyroxine by the acid. Minute yields of *l*-thyroxine were obtained from thyroid by Harington and Salter (58) and Foster *et al.* (41) after hydrolysis with proteolytic enzymes.

Abelin (3) reported that hydrolysis of iodinated casein in 10% sulfuric acid solution failed to yield a thyroidally active product, either in the acid-insoluble portion or in a *n*-butanol extract. Ludwig and von Mutzenbecher (93) stated that attempts to isolate thyroxine from iodinated casein, hydrolyzed with either sulfuric acid or proteolytic enzymes, were unsuccessful. Although Lerman and Salter (91) were able to separate iodinated serum protein into thyroxine and diiodotyrosine fractions by stepwise hydrolysis with enzymes, attempts to isolate thyroxine resulted in failure.

The isolation of pure *l*-thyroxine from iodinated casein was finally accomplished by Reineke and Turner (128). To obtain this result, advantage was taken of the fact that thyroxine is soluble in *n*-butanol even in strongly acid solution. When iodinated casein was hydrolyzed in a mixture consisting of equal parts by volume of 32% sulfuric acid and *n*-butanol, crystalline *l*-thyroxine was obtained on the first attempt. A greatly diminished yield of thyroxine was also recovered after hydrolysis in a *n*-butanol-hydrochloric acid mixture.

Isolation of the thyroxine from the acid hydrolyzate required a procedure modified considerably from that used previously with barium hydroxide hydrolyzates. After 13 hours heating under reflux in a boiling water bath, the *n*-butanol-sulfuric acid digest of the iodinated protein was diluted with 6 volumes of distilled water, whereupon the *n*-butanol, with the dissolved hydrolytic products, formed a separate layer. A considerable amount of dark colored impurity was removed from the *n*-butanol extract by several extractions with 4 *N* sodium hydroxide solution containing 5% sodium carbonate. Removal of the *n*-butanol by vacuum distillation left a residue which still contained considerable tarry material that remained with the acid-insoluble portion despite repeated dissolution and precipitation. This was removed rather easily, however, by dissolving the precipitate in distilled water with the aid of ammonia, heating to 60°C., and adding a slight excess of warm barium hydroxide solution. The barium salts of the tarry substances formed a flocculent precipitate, leaving most of the thyroxine in solution. When acidified, this solution yielded a light-colored precipitate of greatly improved appearance. The traces of barium remaining were removed by centrifuging, after dissolving the precipitate with the aid of ammonium hydroxide and adding ammonium sulfate to the boiling solution. The thyroxine concentrate was recovered by acidifying the hot solution with dilute sulfuric acid, and was finally dissolved in a minimum of boiling sodium carbonate solution. Upon chilling this solution a heavy white precipitate of the monosodium salt of thyroxine settled out. After several recrystallizations from sodium carbonate solution, the thyroxine was dissolved in alkaline 70% alcohol. The free amino acid (Fig. 3) crystallized immediately when a few drops of acetic acid were added to the boiling solu-

tion. The yield of crystalline material was approximately 0.1%, as compared to 0.421% of *dl*-thyroxine that was obtained from the same lot of iodinated casein. The iodine content of the purified *l*-thyroxine was 65.1%, and the

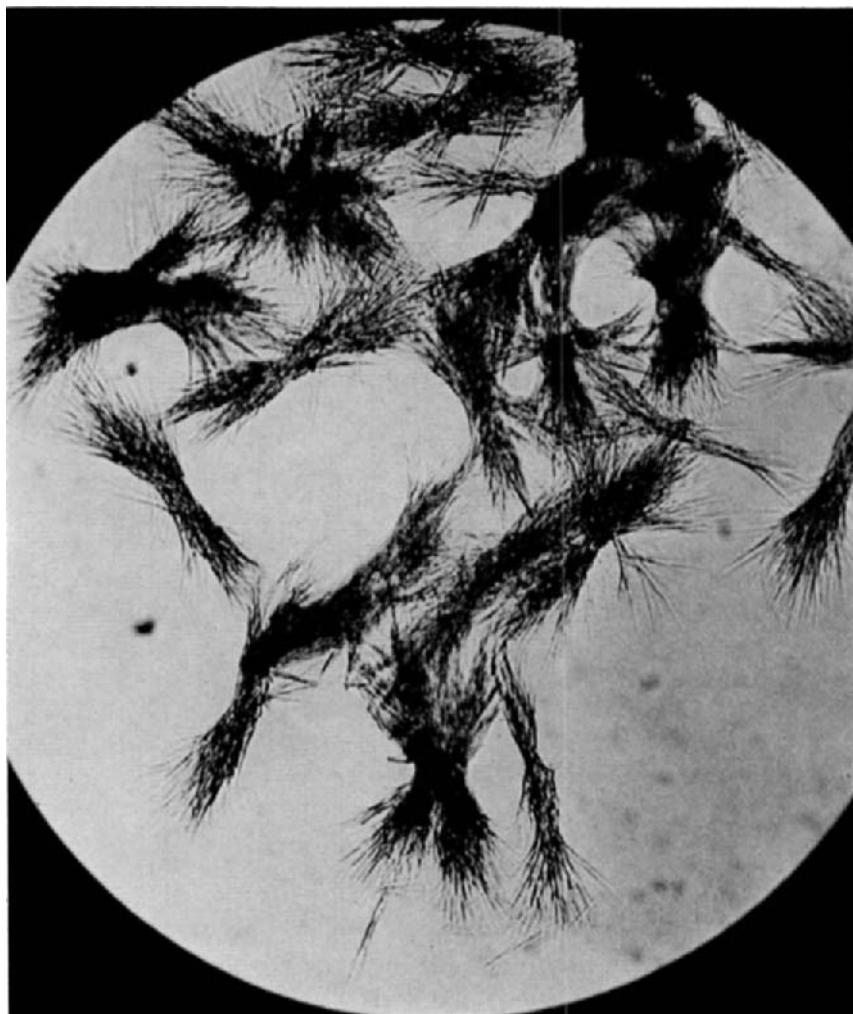


Fig. 3

Crystalline *l* Thyroxine Isolated from an Acid Hydrolysate of Iodinated Casein ($\times 400$)

melting point was 236–238°C., as compared to a melting point of 230–232°C. obtained with *dl*-thyroxine. The specific rotation was $[\alpha]_D = -4.2$. Metabolic tests on guinea pigs indicated that *l*-thyroxine possesses twice the thyroidal potency of a *dl*-mixture, a point which has an important bearing on the interpretation of biological assays of iodinated proteins to be discussed later.

V. THE QUANTITATIVE ASSAY OF THYROXINE IN THYROACTIVE IODINATED PROTEINS

1. Biological Assays

The biological methods developed for the assay of thyroid are all adaptable, with slight modifications in some cases, for estimation of the thyroidal activity of iodinated proteins. While no attempt will be made to cover the extensive literature in this field, the results obtained by various assay methods when applied to iodinated proteins will be reviewed.

The correction of myxedema and elevation of the metabolic rate in man was used by Salter and associates (143, 91, 105) to measure the potency of iodinated serum proteins. For laboratory investigations methods based on the acceleration of metamorphosis in frog tadpoles or elevation of the metabolic rate of small animals such as guinea pigs are the most suitable.

a. *Stimulation of Metamorphosis in Frog Tadpoles.* The sudden and dramatic metamorphosis of amphibian larvae when fed a small amount of thyroid substance was first described by Gudernatsch (47, 48) in 1913. It was found that frog tadpoles exposed to thyroid, either by feeding or as a solution in the water surrounding the tadpole, showed rapid and precocious differentiation, but no further body growth. These findings were confirmed and extended by Lenhart (89), Romeis (141), Kahn (75), Rogoff (138) and others. Allen (7, 8) and Hoskins and Hoskins (63, 64) reported that when thyroidectomized at an early stage, tadpoles would not metamorphose unless fed thyroid substances.

Gaddum (42) reported that the decrease in body length of tadpoles in response to thyroxine administration was roughly proportional to the dose. Wokes (165) showed that the percentage decrease in body length of tadpoles given thyroid substance bore a straight line relationship to the log of the dosage, and described a detailed procedure for the use of this measure in the assay of thyroid preparations.

As already discussed, the early evidence of the stimulation of metamorphosis by iodinated proteins was discounted as a non-specific response, not indicating true thyroidal properties. However, little or no metamorphosis is induced by the administration of non-thyroxine iodine compounds such as diiodotyrosine or potassium iodide (42, 87, 126).

Application of the tadpole method to the quantitative assay of the thyroidal activity of iodinated proteins has been reported by Reineke and Turner (126). Tadpoles will respond to active iodinated protein when it is fed or when a small amount in solution is injected into the body cavity. This material differs from thyroid substance or thyroxine in that it is not absorbed when placed in solution in the water surrounding the tadpole.

Of the many endpoints which could be taken as measures of metamorphosis, such as the decrease in body weight, rate of growth of the limbs and

the time of emergence of the left front limb bud, the percentage decrease in body length is the most convenient measure, and shows the best proportionality with the dosage. *Rana pipiens* larvae were the most satisfactory of the species tested.

The sensitivity of tadpoles to thyroidal stimulation depends upon the species, their stage of development, the environmental temperature and probably other factors. For this reason the best comparisons are obtained by assaying a large number of preparations concurrently on tadpoles of uniform size and development. Comparative assays can be obtained by simply injecting a uniform dosage of each preparation into groups of tadpoles, and taking the average percentage decrease in body length produced by each preparation as a relative measure of its potency. For quantitative results it is necessary to set up a graded dosage series, using thyroxine or a standard preparation in order to establish a response curve from which the potency of the unknowns can be estimated.

Large *Rana pipiens* tadpoles collected in nature and injected at about the 60 mm. stage show pronounced metamorphosis within 4 days after a single injection of iodinated protein (Fig. 4). Laboratory-reared tadpoles obtained by the method of Rugh (142) require from 6 to 10 days after injection to show sufficient response for satisfactory measurement.

When assayed by injection in tadpoles, iodinated casein shows about 2.7 times the thyroidal potency, expressed in terms of a thyroxine standard, indicated by oral assays on guinea pigs (126). While a part of this discrepancy can undoubtedly be explained by the difference in the route of administration, further work should be done to establish more fully the reasons for this difference in response.

b. Assays Based on Elevation of the Metabolic Rate and Decrease in Body Weight. Of the common laboratory animals, the guinea pig is very suitable for thyroidal assays (163, 38, 126) because of its sensitivity to this type of stimulation. The increase in carbon dioxide production of mice in response to thyroid administration was used by Morch (102) and Gaddum (44) in the assay of thyroid preparations. While the elevation of the oxygen consumption of normal rats in response to thyroidal stimulation has been used (43) in studies on thyroxine and related compounds, thyroidectomy has been reported by Meyer and Wertz (100) to increase the sensitivity of rats to such stimulation 25- to 30-fold.

The method of Kreitmair (83), based on the percentage weight loss of guinea pigs induced by administration of the test substance for 6 days was used (93) in developing the procedure for the isolation of thyroxine. This method was compared (126) with a procedure based on the elevation, due to thyroidal stimulation, of the oxygen consumption of guinea pigs. The weight loss method can be criticized seriously for a lack of specificity. However, the results agree fairly well with values obtained by the metabolic

method. A group of iodinated proteins assayed by this method showed thyroidal potencies equivalent to 1.0 to 4.0% of the activity of *dl*-thyroxine, the value obtained depending on the method of formation of the iodinated

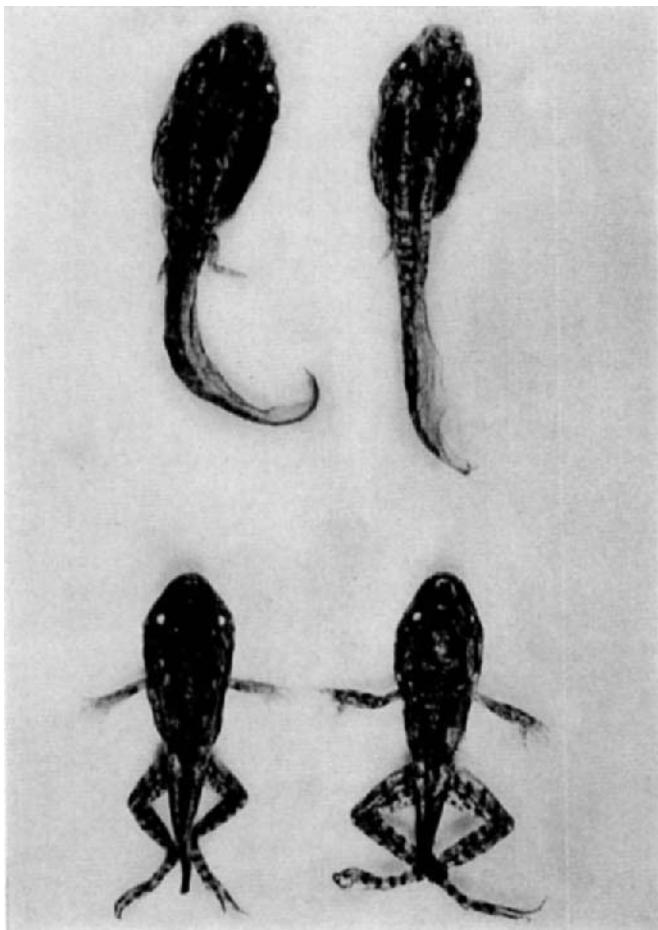


Fig. 4

The Response of Large Frog Tadpoles (60 mm. size) to the Injection of Artificial Thyroproteins

The tadpoles at the top are normal controls. The two at the bottom illustrate the striking degree of metamorphosis occurring within four days after the injection of 0.1 mg. of iodinated casein. (From *Agr. Exp. Sta. Mo., Res. Bull.* **355** (126).)

protein. Estimates of the thyroxine content of iodinated proteins as indicated by biological assay in guinea pigs show excellent agreement with chemical analyses for thyroxine (135).

A recently developed assay method based on the ability of thyroidal substances to reduce the enlarged thyroids of thiouracil-treated chicks (101) and rats (123) may be useful in the biological assay of thyroidally active iodinated proteins. The percentage reduction in thyroid weight of thiouracil-treated animals given graded amounts of thyroxine shows good proportionality with dosage, and agrees well with the results of metabolism measurements. Considerably less labor is involved than in the metabolic procedures. Preliminary experiments by Reineke and Turner (134) indicate that iodinated proteins assayed in chicks by this method produce results which are proportionate with the values obtained by the other biological procedures. The exact quantitative relationship between this and other assay methods, however, remains to be established.

In most of the experiments conducted by the author, the iodinated protein has been administered by either subcutaneous or intraperitoneal injection instead of orally, because it was desired to avoid possible differences in the relative absorption of the various test materials from the gastrointestinal tract. By use of this technique, processes for the formation of iodinated proteins of exceptionally high thyroidal potency when assayed by injection have been developed. These materials are also effective orally, but little information is available on their relative effectiveness by the various routes of administration. Further research on these points is needed to provide the basis for practical use of thyroactive iodinated proteins by oral administration.

c. *The Relative Thyroidal Potency of l- and dl-Thyroxine.* Experiments dealing with the question as to whether all of the physiological activity of *dl*-thyroxine resides in the natural levorotatory component, or whether the dextrorotatory isomer also contributes some activity will be reviewed at this point because this question has an important bearing on the interpretation of chemical and biological estimates of thyroxine content.

Both *d*- and *l*-thyroxine obtained by Harington (51) by resolution of the racemic mixture were assayed by Gaddum, who reported that in both tadpoles (42) and rats (43) the *d*-form showed about one-third the potency of the *l*-compound. Salter, Lerman and Means (144) reported that no difference in the activity of the two compounds could be discerned as judged by their effects on myxedema in man. Foster, Palmer and Leland (41) reported that *l*-thyroxine obtained by the enzymatic hydrolysis of thyroid substance exerted twice the calorogenic effect of a racemic mixture when administered to guinea pigs. Similarly, *l*-thyroxine isolated from an acid hydrolysate of iodinated casein was reported by Reineke and Turner (128) to produce twice the metabolic stimulation of a racemic mixture when tested on guinea pigs. These observations on the same compounds were extended to three additional species (131). As determined by its ability to reduce the weights

of the thyroids of thiouracil-treated chicks (Fig. 5), *l*-thyroxine again showed fully twice the potency of a *dl*-mixture. Closely similar results were obtained with tadpoles and thiouracil-treated rats. From the close agreement of results in four species of animals it was concluded that all of the activity of racemic thyroxine can be accounted for by its *l*-component and that the *d*-compound must have little or no activity. From the fact that the

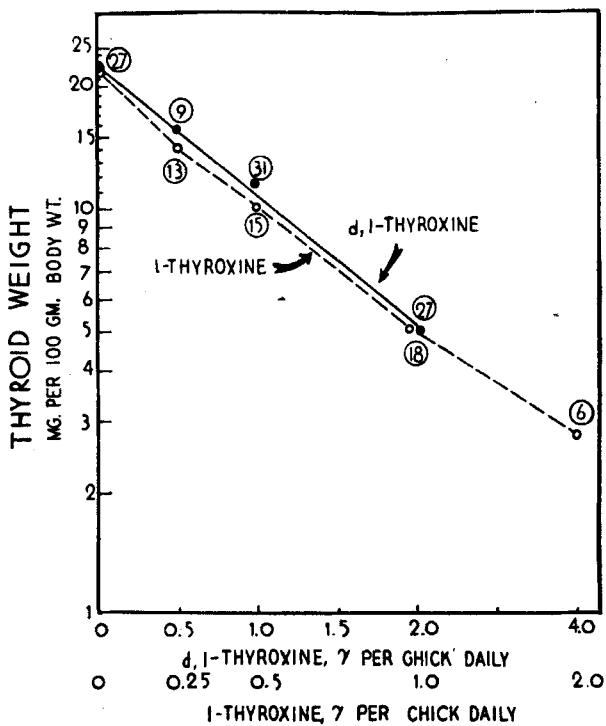


Fig. 5

The Relative Potency of *l*- and *dl*-Thyroxine in Reducing the Thyroid Weight of Thiouracil-Treated Male Chicks

The encircled numerals indicate the number of animals per dosage group. (From Endocrinology, 131.)

l-thyroxine isolated for the more recent assays (41, 128, 13) showed a higher specific rotation than did the compounds separated by Harington (51) it appears that the discrepancy in the biological results can be explained by somewhat incomplete resolution of the latter compounds. This is a possibility which was recognized by Harington in his original report.

In comparing biological assay data with chemical determinations of

thyroxine content it is necessary to take into account the fact that the thyroxine formed in the iodinated proteins is the pure *l*-compound, which has twice the potency of the racemic mixture ordinarily used as a standard.

2. Chemical Determination of the Thyroxine Content of Thyroactive Iodinated Proteins

Although a number of chemical methods have been devised for the estimation of thyroxine in thyroid substance, none of these methods has been

TABLE VI

Data Demonstrating the Correlation Between the Chemical and Biological Assay Methods for Thyroxine

Preparation No.	Iodine Added per Mole Tyrosine in Protein atoms	Iodinated Protein Injected ($\gamma/100$ g. body wt.)	Increase in CO_2 Output per cent	Thyroxine Found		Difference per cent
				Bioassay* per cent	Chemical Analysis per cent	
1		223	25.4	2.46	2.69	- 8.6
2		138	24.7	3.80	3.91	- 2.8
3		176	20.8	2.46	3.06	-19.6
4		300	27.6	2.01	2.06	- 2.4
5		150	20.9	2.90	3.88	-25.3
6		145	25.1	3.71	3.73	- 0.5
6		161	25.0	3.31	3.73	-11.3
6		161	25.1	3.34	3.73	-10.5
7	4.51	243	21.7	1.86	2.21	-15.8
8	5.01	198	23.4	2.50	2.71	- 7.7
9	5.51	201	26.8	2.90	2.69	+ 7.8
10	6.01	190	23.0	2.55	2.83	- 9.9
11	6.51	175	23.1	2.78	3.09	-10.0
12	7.01	174	22.2	2.67	3.11	-14.1
13	8.01	191	26.4	2.98	2.83	+ 5.3
14	9.01	194	23.2	2.53	2.78	- 9.0
15	10.01	209	25.8	2.66	2.58	+ 3.0
Weighted average				2.79	3.04	- 8.1

* Estimated from standard response curve for intraperitoneally injected *l*-thyroxine. (From the *J. Biol. Chem.* (135).)

used with iodinated proteins until quite recently because of serious questions as to their specificity when applied to such materials. Ludwig and von Mutzenbecher (93) expressed doubt that the iodine of the acid-insoluble substance obtained after alkaline hydrolysis of iodinated protein could all be considered to be thyroxine iodine as in the method of Harington and Randall (56). It was concluded by Abelin and Neftel (6) that the method of Leland and Foster (88) yielded only qualitative results when applied to iodinated proteins because a direct relation between the thyroxine iodine as determined by this method and the physiological effects produced could not be demonstrated.

Excellent agreement between the results of biological assays and a chemical extraction procedure for thyroxine modified from the method of Blau (15, 16) have been obtained recently by Reineke *et al.* (135). Preliminary work indicated that Blau's method as described for thyroid substance yielded values which were considerably too high when compared with biological assays on the same preparations. The method also appeared to be non-specific when applied to preparations that had been iodinated excessively. By hydrolyzing the iodinated casein in 40% barium hydroxide

TABLE VII

Data Showing the Thyroxine Content of Samples of Iodinated Casein Carried through Different Stages of the Chemical Assay Procedure

Hydrolysate Injected* ($\gamma/100$ g. body weight)	Increase in CO_2 Output per cent	Thyroxine found Bioassay** per cent	Chemical Analysis per cent	Difference per cent
I. Acid <i>n</i>-butanol extract				
500	24.0	2.04	2.06	- 1.0
150	14.8	3.93	3.88	+ 1.3
II. Acid butanol extract after washing with alkali				
360	23.5	2.76	3.00	- 8.0
412	22.3	2.28	2.62	--13.0
545	23.9	1.88	1.98	- 5.1
294	24.6	3.57	3.67	- 2.7
Average		2.74	2.87	- 4.36

* The figures given indicate the amount of original iodinated casein represented.

** Estimated from standard response curve for intraperitoneally injected *d,l*-thyroxine. (From the *J. Biol. Chem.* (135).)

instead of in the 8% solution employed in the original method, values agreeing well with the biological data were obtained. Apparently the more drastic hydrolysis liberates the thyroxine from combination with non-thyroxine compounds otherwise carried through with the thyroxine in the extraction with *n*-butanol.

In the modified method the iodinated casein is first hydrolyzed with 40% barium hydroxide by heating in a boiling water bath for 18 to 20 hours. After dilution of the hydrolysates and decomposition of the barium salts, aliquots are acidified with dilute hydrochloric acid and then extracted with an equal volume of *n*-butanol. The *n*-butanol extract is washed in turn with an equal volume and a half-volume of 4 N sodium hydroxide, containing 5% sodium carbonate. Finally the *n*-butanol is removed by evaporation, and the iodine content of the residue determined.

For comparison with the chemical extraction values, the metabolic stimulation produced by a group of iodinated proteins when administered by intraperitoneal injection was determined, and the apparent thyroxine content estimated from a standard response curve based on *l*-thyroxine.

In a group of 15 iodinated casein preparations formed under varying conditions, the guinea pig assays indicated an average thyroxine content of 2.79% as compared to 3.03% thyroxine obtained by the chemical method (Table VI).

Biological assays of the *n*-butanol extract at two stages in the procedure (Table VII) showed that all of the thyroidally active substance of the original iodinated protein is recovered by the extraction. By comparison of the chemical and biological data, it is evident that if an active compound other than thyroxine is present it must be very similar to thyroxine in both iodine content and thyroidal potency.

From these results it is believed that the chemical method as modified for iodinated protein is highly specific for thyroxine, and it supports the early evidence of high thyroxine content provided principally by biological assays. As indicated earlier, however, further research is needed on the relative utilization of the thyroxine in iodinated proteins when administered orally.

VI. THE FORMATION OF THYROIDINE FROM DIIODOTYROSINE

The formation of thyroxine directly from diiodotyrosine was first reported by von Mutzenbecher (106). By incubating diiodotyrosine in mildly alkaline solution at 37°C. for a period of two weeks, a yield of crystalline thyroxine equivalent to about 0.1% of the diiodotyrosine taken initially was obtained. By use of the same incubation procedure, Block (20) formed thyroxine from synthetic diiodotyrosine, thus ruling out the possibility that the thyroxine might have had its origin from preformed thyronine occurring in tyrosine obtained from natural sources. By use of the same conditions very similar yields of thyroxine were obtained by Johnson and Tewkesbury (73) and Barkdoll and Ross (13). Harington (53) stated that a slight increase in thyroxine formation was obtained by oxidation with hydrogen peroxide at 37°C. By the addition of hydrogen peroxide at steam bath temperatures, the solution meanwhile being shaken constantly with *n*-butanol to extract the thyroxine as it was formed, a yield of 1.36% thyroxine was obtained.

The formation of thyroxine from diiodotyrosine has been found by Reincke and Turner (133) to be influenced by the same conditions established previously for iodinated proteins. Diiodotyrosine dissolved in *N*/10 sodium hydroxide at a pH of approximately 9.5 was incubated for 18 to 20 hours under various conditions. The yield of thyroxine obtained

by isolation, after incubation at a given temperature, was increased by either stirring or aeration, or by the addition of manganese oxide as a catalyst (Table VIII). The catalyst was ineffective, however, in the absence of added oxygen introduced either by stirring or aeration.

The temperature of incubation is highly critical (Fig. 7), having its optimum at 60°C. instead of at 37°C., the temperature employed in the earlier investigations. Manganese oxide increases the amount of thyroxine formation throughout the effective temperature range. With all conditions optimum an overall yield of 0.85% and a net yield of 2.8% of crystalline thyroxine was obtained.

TABLE VIII

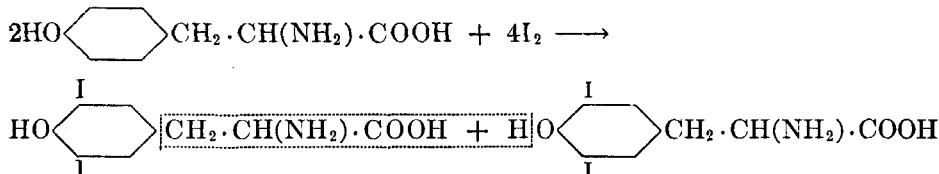
The Effect of Stirring and Aeration on the Formation of Thyroxine from Diiodotyrosine

Incubation Temperature °C.	Thyroxine yield per cent	Treatment
40	0.04	Mn ₃ O ₄ , 2 g.; stirred at 600 RPM
40	0.04	Mn ₃ O ₄ , 2 g.; aerated vigorously
50	0.38	Mn ₃ O ₄ , 2 g.; stirred at 600 RPM
50	0.36	Mn ₃ O ₄ , 2 g.; aerated vigorously
60	0.85	Mn ₃ O ₄ , 2 g.; stirred at 600 RPM
60	0.52	No catalyst; stirred at 600 RPM
60	0.02	Mn ₃ O ₄ , 2 g.; no stirring or aeration
70	0.27	No catalyst; stirred at 600 RPM
70	0.01	No catalyst; no stirring or aeration

(From the *J. Biol. Chem.* (133).)

VII. MECHANISM OF THYROXINE FORMATION

In connection with their classical experiments on the constitution and synthesis of thyroxine (54), Harington and Barger first advanced the theory that thyroxine is synthesized biologically in the thyroid by iodination of tyrosine, followed by oxidative coupling of two molecules of diiodotyrosine, and the elimination of one side chain, as shown below.



With the discovery that thyroxine can be isolated from proteins iodinated artificially under the proper conditions, this oxidative mechanism appeared to provide the most plausible explanation for formation of the compound in

such materials (93, 55). Because of the complexity of protein systems, however, it was necessary to turn to experiments with diiodotyrosine itself for further development of the theoretical background.

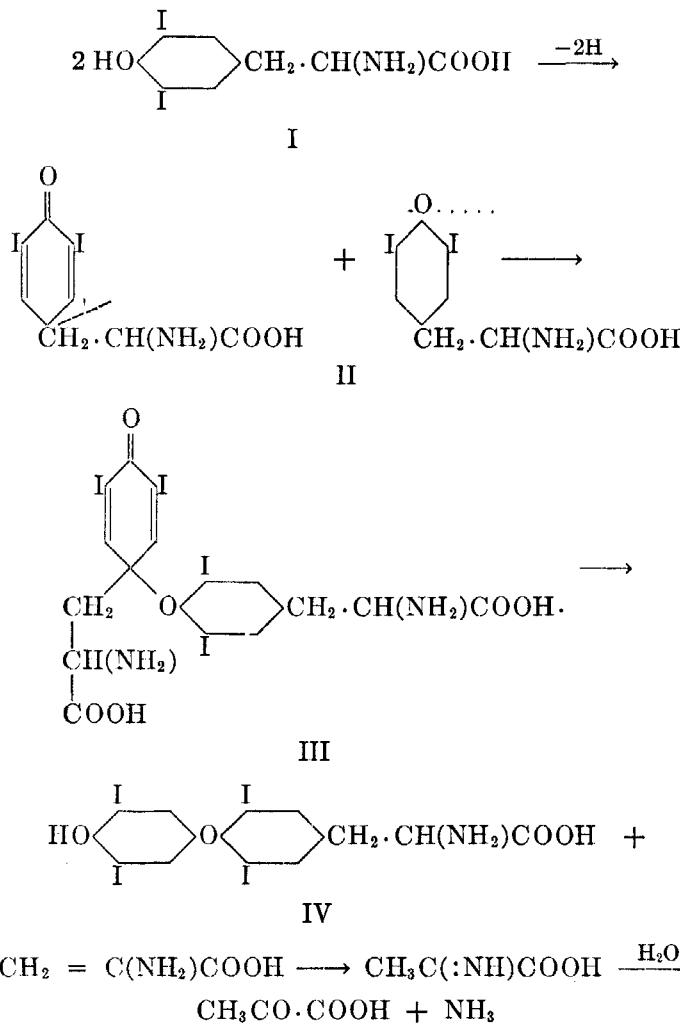


Fig. 6.

A Mechanism for the Conversion of Diiodotyrosine to Thyroxine
(From *Proc. Nat. Acad. Sci., U. S.* (73))

By analogy with the results reported by Pummerer *et al.* (115) on the oxidation of *p*-cresol with potassium ferricyanide, Johnson and Tewkesbury (73) proposed a detailed reaction mechanism (Fig. 6) which would account

for the oxidative formation of thyroxine from diiodotyrosine. The first steps in the reaction would result in the oxidative coupling of two molecules of diiodotyrosine to form the intermediate compound **III**. Compound **III** must follow one of two courses, namely, (a) molecular dissociation with loss of one alanine side chain and formation of thyroxine, **IV**, and imino-pyruvic acid, or (b) hydrolysis, with production of serine.

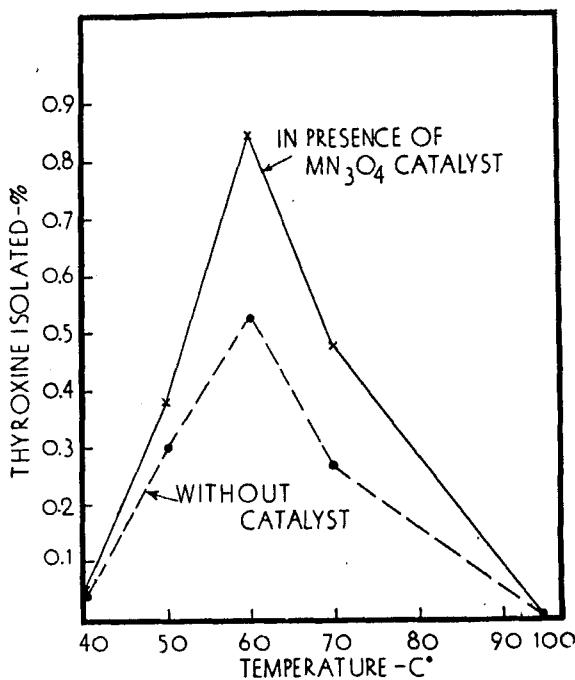


Fig. 7

Gross Yield of Thyroxine Isolated after Incubation of Diiodotyrosine at Various Temperatures

(From the *J. Biol. Chem.* (133))

In support of the theoretical considerations, pyruvic acid and ammonia, but not serine, were identified as secondary products in the reaction mixture. The results support the view that the formation of thyroxine is an oxidative process. In fact, it was stated that the yield of thyroxine obtained was increased slightly by adding hypoiodous acid to the reaction mixture in theoretical amounts. Harington (53) concurred with the general outline of the proposed reaction mechanism, and developed the theoretical background supporting it still further.

It has generally been assumed that the coupling reaction believed to be

involved in the formation of thyroxine in iodinated proteins, as well as from diiodotyrosine, is brought about by the mild oxidative action of hypoiodite (93, 55, 106, 73, 53). It has been reported by Barkdoll and Ross (13), however, that diiodotyrosine incubated in an oxygen-free system yielded no thyroxine and, conversely, the yield of thyroxine was increased by bubbling air through the solution. In recent experiments by Reineke and Turner (133), greatly increased yields of thyroxine were obtained by stirring or aeration of the solutions. Manganese oxide was effective as a catalyst in the presence, but not in the absence, of added oxygen introduced either by stirring or aeration, suggesting that manganese can act as a carrier for oxygen involved in the coupling reaction (See Fig. 7). From these results it appears that the oxidation previously attributed to hypoiodite is actually brought about by atmospheric oxidation.

It is of considerable interest that the results obtained with iodinated proteins *in vitro* support and supplement the recent investigations on the mechanism of thyroxine formation *in vivo*.

Chapman (32) reported that extra iodine administered to rats on a low-iodine diet produced thyroidal effects in thyroidectomized but not in intact animals. It was concluded that iodine may play a role in body metabolism in the absence of the thyroid, possibly by production of a thyroxine-like substance in the tissues. Morton *et al.* (104) administered radioactive iodine to thyroidectomized rats, and separated the thyroxine-like and diiodotyrosine-like iodine from hydrolysates of the tissues, by extraction with *n*-butanol. It was reported that 96 hours after its injection 30% of the radioiodine obtained from the liver and small intestines was organically bound, 20% as diiodotyrosine and as much as 8% as thyroxine.

Analyses of purified thyroglobulin (31) indicated that the protein from goitrous thyroids contained less thyroxine and non-thyroxine iodine than that from normal glands. Colloid from goitrous glands, although otherwise quite constant in amino acid composition was deficient in the iodine-containing amino acids; the tyrosine content showed a proportionate increase (Cavett, 30). McClendon, Foster and Cavett (94) reported that the thyroglobulin from colloid goiters was subnormal in both its thyroxine content and calorogenic effect. This was believed to indicate that thyroglobulin is first secreted as a colloid, and that the thyroxine radicle may then be synthesized within the protein molecule in a manner similar to that occurring in iodinated protein *in vitro*.

Mann *et al.* (95) injected radioiodine into dogs and compared the activity of thyroid iodine fractions. The data supported the belief that diiodotyrosine is the natural precursor of thyroxine and, further, that the iodination of tyrosine occurs outside of the thyroid cell. Further studies of histological sections of thyroid glands by Leblonde (86) after the administration of trace

doses of radioiodine indicated that the iodine is located almost exclusively in the colloid.

Salter and McKay (145) reported that in thyroids in which the formation of hormone was inhibited by the administration of thiouracil or thiocyanate the synthesis of thyroid protein can proceed independently of endocrine potency. All of these findings are in harmony with the idea that thyroxine is formed within the thyroglobulin molecule by an iodination process.

The influence of anaerobiosis and enzyme inhibitors on the formation of thyroxine and diiodotyrosine was studied by Schachner *et al.* (146) by means of radioiodine. Under the conditions cited, the formation of both substances was inhibited leading to the conclusion that the formation of both thyroxine and diiodotyrosine by the thyroid gland is linked with aerobic oxidations in which the cytochrome-cytochrome-oxidase system is involved.

Paschkis *et al.* (113) reported that the oxidase activity of thyroid tissue is decreased by adding thiouracil; inhibition of oxidase may be a factor, therefore, in the suppression of thyroid function by this drug.

Ray and Deysach (117) reported that the thyroid has a special capacity for the storage of manganese and, further, that the injection of small amounts of manganese chloride caused an increase in the oxygen consumption of guinea pigs. This, together with the finding that manganese catalyzes the formation of thyroxine in iodinated proteins and also from diiodotyrosine, prompted Reineke and Turner (132) to suggest that manganese may act *in vivo* as well as *in vitro* in promoting the oxidative formation of thyroxine.

VIII. THE EFFECT OF IODINATION ON PHYSICO-CHEMICAL PROPERTIES OF PROTEIN

1. Spectrographic Absorption

Most proteins exhibit specific light absorption in the ultraviolet region between 2500 and 3000 Ångström units. This property is due to the presence in proteins of the aromatic amino acids. In common with other cyclic compounds thyroxine shows a typical absorption curve, with a maximum at approximately 3250 Å (Fig. 2). Tyrosine, phenylalanine, tryptophane and indole show maxima in the range of about 2600 to 2900 Å (150, 39). Spectrographic measurements on proteins are thus complicated by the fact that the influence of the various amino acids cannot be differentiated because of their overlapping spectra. Certain changes due to iodination have been reported, however.

By measurements on thyroxine and related compounds, Marenzi and Villalonga (96) determined that iodination of the phenolic nucleus ortho to the hydroxyl group shifts the absorption maximum toward slightly longer wave lengths and also increased the difference in molecular extinction be-

tween the minimum and maximum. Iodinated casein had its absorption maximum shifted toward longer wave lengths as compared with normal casein (97).

Progressive iodination of casein (126) caused a shift in the absorption maxima to longer wave lengths and a simultaneous increase in the intensity of absorption which, in the earlier samples of the series, appeared to be correlated with the increase in thyroidal activity. The trend toward high intensities continued in excessively iodinated preparations reaching the maximum value in samples of declining thyroidal potency. This was believed to indicate the formation of a compound with excessive iodination, physiologically inert but thyroxine-like in structure and absorptive properties.

2. X-ray Diffraction Pattern of Iodinated Amino Acids

By study of the X-ray diffraction patterns of tyrosine, diiodotyrosine and thyroxine, Spiegel-Adolph *et al.* (152) concluded that iodination causes some structural rearrangement of amino acids. No difference was found in the X-ray diffraction pattern of thyroglobulin preparations of varying thyroxine content. Likewise, the diffraction pattern of iodinated casein was the same as that of the iodine-free protein.

3. The Effect of Iodination on the Dissociation Constant of Tyrosine

Cohn (33) pointed out that tyrosine has three pK values, of which two are due to the dissociation of the amino and carboxyl groups, and the third to the phenolic hydroxyl group. Iodination of tyrosine increased the dissociation of the phenolic hydroxyl group about a thousand-fold, and the amino group also dissociated at a somewhat more acid reaction.

When iodine was added to zein in an amount sufficient to iodinate completely the tyrosine present (107), the total acid- and base-binding capacity of the iodozein was identical with that of the original protein. However, the portion of the curve corresponding to the titration of the phenolic group of tyrosine was shifted to a lower pH range, as expected from the lower pK (OH) value of diiodotyrosine. Cohn, Salter and Ferry (34) reported that when iodine sufficient to iodinate the tyrosine radicals was combined with globin the base combined was diminished by an amount approximately equivalent to the amount of iodine taken up. In other words, the phenolic hydroxyl groups apparently disappeared completely from the reaction. It is interesting to note that this would be expected with the formation of the intermediate compounds postulated in the mechanism for thyroxine formation (Fig. 6) proposed by Johnson and Tewkesbury (73). •

IX. EFFECT OF THYROACTIVE IODINATED PROTEINS ON PHYSIOLOGICAL PROCESSES OF DOMESTIC ANIMALS

With a large supply of thyroidally active material now potentially available by means of controlled methods of iodination, investigations have been inaugurated to determine the possibility of influencing various productive processes in domestic animals by the administration of these substances. Although this is a comparatively new field of study, a number of discoveries of considerable physiological interest and some of possible economic importance have been made. Experiments of this type are based on the concept that through its influence on general metabolism, and possibly on endocrine systems interrelated with the thyroid, the administration of small amounts of thyroidal substance will favorably influence certain physiological functions such as milk production, egg production, or growth. Obviously, regulation of the dosage to avoid overstimulation is an all-important factor.

1. Effect on Milk Secretion

The fact that the administration of thyroid substance (45) or thyroxine (46) to normal cows will cause a rapid increase in both milk yield and the percentage of fat in the milk was first reported by Graham in 1934. A greater effect was observed on milk fat production than on milk yield. This increase in both milk yield and milk fat production has been confirmed by all of the subsequent investigators. A less consistent effect has been observed on the constituents of milk other than the fat.

Slight increases in the solids-not-fat content of milk following the administration of thyroid powder or thyroxine for varying periods of approximately three days to four weeks were reported by Herman *et al.* (60, 61), Folley and White (40) and Ralston *et al.* (116). No change was observed in the solids-not-fat fraction by Jack and Bechdel (71) and Smith and Dastur (151), although the usual rise in milk yield and fat percentage occurred. Jones (72) reported that when eight cows were injected with 10 mg. of thyroxine daily for 14 days, the milk yield and pulse rate increased 28%, and 23%, respectively. The blood sugar increased 10% and then returned to normal, while the lactose content of the milk increased an average of 9%. Sharp increases of as much as 38% in milk and 60% in fat production were reported by Hurst (65, 66) when 10 to 15 mg. of thyroxine were injected daily in lactating cows for a period of 4 weeks. In one instance the persistency of production was increased during a 9 month injection period.

In most of these experiments the dosage given was 1 to 2 oz. of desiccated thyroid or 10 to 15 mg. of thyroxine daily. Although quite variable, the average increase in milk production was approximately 13 to 18%, with

an increase in the yield of milk fat of 22 to 24% during limited periods of thyroidal stimulation.

Turner (155) called attention to the possibility of stimulating milk secretion in dairy cattle by the use of thyroactive iodinated proteins. Data on the actual stimulation of lactation by feeding iodinated proteins were first reported by Reineke and Turner (125). In 14 individual feeding trials in which active iodinated protein was fed to cows in advanced lactation for a 3 day period a rise in milk production ranging from 6 to 22% was observed in all except two cases. Due to the increase in fat percentage of the milk, the total yield of milk fat increased as much as 28%. Similar results were obtained in experiments with goats.

Further investigation resulted in the development of methods for preparing iodinated proteins of greatly increased thyroidal potency (120, 121). The administration of these preparations to lactating cows at the rate of 1.5 to 2.5 g. per 100 lbs. body weight daily resulted in an average increase of 18.6% in milk production in a group of 27 animals. Increases in the milk fat yield of more than 50% occurred due to the concurrent increase in milk fat percentage. After the initial rise in milk production, lactation again declined, but at a retarded rate as compared with the normal.

Van Landingham *et al.* (160, 161) in similar experiments reported that the feeding of 15 g. of iodinated casein daily to lactating cows caused an increase of 5 to 20% in milk yield and 25 to 50% in the yield of milk fat. The solids-not-fat of the milk increased slightly, but there was a 33% decrease in the ascorbic acid content.

Most of the results obtained by the administration of thyroidal substances to dairy cows indicate that there is a greater percentage increase in the milk fat percentage than in milk yield. With high dosages the increase is obtained at the expense of some loss in body weight. Reece (118) reported that when a moderate amount of iodinated casein was fed, the milk fat percentage increased from 3.6 to 4.1%, with little increase in milk yield, and only slight losses in body weight. The continuous feeding of thyrooidally active iodinated protein for periods of 3 to 16 months (116) resulted in substantial increases in total production of milk and milk fat, with no harmful effects on the cows being noted.

The effects of a broad range of iodinated protein dosage on the milk production and physiologic well-being of dairy cows was investigated by Reineke *et al.* (122). Expressed in terms of an iodinated protein standardized to 3% thyroxine content as determined by chemical analysis, a dosage of 0.5 g. per 100 lbs. body weight daily produced a slight, but questionable rise in milk fat test with little or no effect on milk yield. A dosage of 1.0 to 1.5 g. caused an average increase over paired controls of about 10% in milk yield and 15 to 25% in milk fat yield, the increase being maintained for five

months, while the treatment was continued. Higher dosages, up to 4.75 g. per 100 lbs. body weight daily (Fig. 8) caused a higher initial rise in production, but this was accompanied by severe losses in body weight, and elevation of the pulse rate and body temperature.

Although some increase in milk production and milk fat test were noted when iodinated casein was fed, Seath *et al.* (148, 149) questioned the advisability of its use under Louisiana conditions because of possible injury

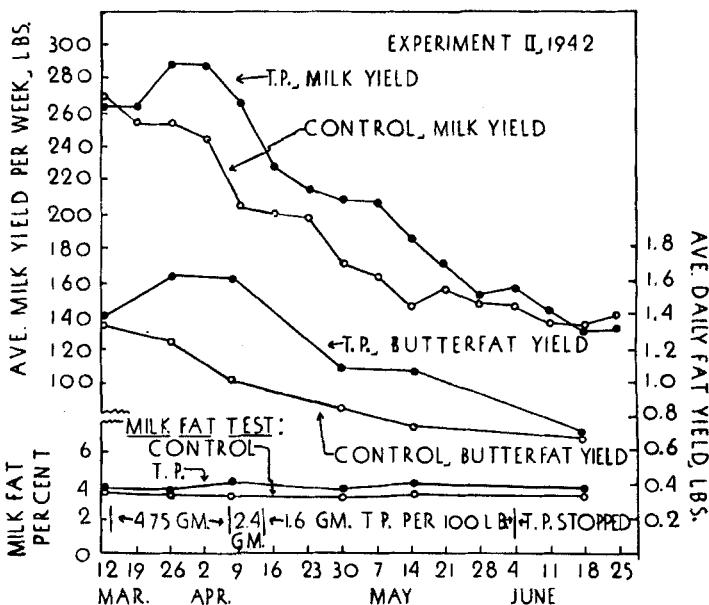


Fig. 8

The Lactational Response of Cows when Fed Heavy to Moderate Amounts of Thyroidally Active Iodinated Casein

The letters T.P. refer to the treatment with this material. The records of four experimental and three control cows are averaged in data for the respective groups.

to the cows due to slight losses of body weight and increases in body temperature during hot weather.

The results of extensive experiments conducted in England on the feeding of iodinated proteins to lactating cows have been reported by Blaxter (17). Iodinated casein, whole blood protein and ardein were all effective in stimulating milk production. Fifteen grams of iodinated casein daily increased production by about 16%, and 30 g. daily caused an increase of 33%, indicating that within this range the response is directly proportionate to the dosage. The heart rate increase was nearly trebled by doubling the

dosage, which was believed to indicate that at low dose levels the increased metabolism is probably not reflected by an increased heart rate. The increase in pounds of milk per day was greater the higher the initial milk production, but the percentage response declined with increasing initial yield. A favorable outlook for increasing milk production under practical conditions by feeding iodinated protein is given in the recent report by Blaxter (18).

In view of the possibility of feeding thyroidally active substances to increase milk production it is important to know whether or not some of this material would be secreted in the milk. No clear-cut evidence for the detection of thyroid hormone in milk could be found in the literature (129), although a number of reports to the contrary have appeared. In further experiments on this question, guinea pigs were given daily approximately 100 ml. of milk obtained from cows receiving a high dosage of active iodinated protein. Frequent determinations of their metabolism during this period failed to show any significant differences between these animals and paired controls receiving a milk diet, or normal guinea pigs on a stock diet. Similar results were obtained in trials with thyroidectomized goats. Thus, the amount of thyroid hormone passing into the milk, if this occurs at all, is too small to be detected by the biological methods used.

Comparisons of the amount of thyroidally active iodinated protein required to produce a lactation response in dairy cattle with the dosage of thyroxine required to produce the same effect when injected, indicate that iodinated protein is utilized quite inefficiently in ruminants. As judged by the dosage of iodinated protein required to cause a standard weight reduction in sheep (159) only 5% as much iodinated protein was required by subcutaneous injection as when given orally. When the iodinated protein was placed directly in the abomasum through a permanent cannula in order to bypass the rumen, no increase in utilization was observed. Thus the rumen was ruled out as a possible site of inactivation of the active principle. The oral utilization was increased by preliminary hydrolysis of the iodinated protein with acid, indicating that the poor utilization obtained with the whole protein may be due to incomplete digestion.

2. Effect on Body Growth

It is well established that hypothyroidism, whether induced or spontaneous, is detrimental to growth. Complete thyroidectomy in immature animals results in growth stasis and symptoms of cretinism; replacement therapy with thyroidal substance corrects these deficiencies.

The oral administration of graded doses of thyroidally active iodinated protein to young thyroidectomized goats arrested the symptoms of cretinism, and stimulated growth approaching the normal (124). Within the

range covered, the growth was roughly proportional to the dosage. When the iodinated protein therapy was begun immediately after thyroidectomy, and the dosage was gradually increased to keep pace with increasing body size, young goats developed normally in every respect during nearly a year of treatment (126). An animal in which pronounced cretinism was allowed to develop made a complete recovery when given iodinated protein.

From the marked improvement in growth which results from the administration of small amounts of thyroidal substance to hypothyroid individuals, it might be suspected that the induction of a slightly hyperthyroid condition would cause some acceleration of the growth rate above the normal. Although the literature on this subject, as reviewed by Turner and Koger (82), is quite controversial, there is some evidence that a properly regulated dosage of thyroid substance will cause an increase in the growth rate, at least in some species.

Parker (112) reported that Rhode Island Red chicks raised to the age of twelve weeks on diets containing graduated dosage levels from 0.025 to 0.2% of active iodinated protein made slightly greater gains in body weight than did the control chicks. A slight increase in the body weight of White Plymouth Rock chicks when fed a ration containing 0.08% of thyroactive iodinated casein was reported by Irwin *et al.* (70). Lower dosages had no effect on body weight, while amounts greater than 0.08% of the ration caused retardation of growth. The iodinated casein used in this instance showed 3.1% of the potency of *dl*-thyroxine by the guinea pig assay method. The same preparation fed at the level of 0.1% of the ration caused a slight decrease in growth, to twelve weeks of age, of Barred Plymouth Rock cockerels (156). Experiments by Schultze and Turner (147) indicate that a ration containing 0.009% of an iodinated casein preparation of a potency similar to that used in the work cited above will replace the thyroid hormone secretion of thiouracil-treated chicks. The most favorable effects on growth could be expected to occur with amounts slightly above this figure. The broad tolerance range to this type of treatment is indicated by the fact that chickens receiving iodinated protein equivalent to ten times the normal thyroid hormone secretion rate showed little growth retardation.

A significant increase in body weight gains of growing mice injected with small amounts of thyroxine was reported by Koger *et al.* (80). The carcasses of the experimental mice contained more protein and water but less fat than those of the control mice. The total energy stored by the two groups was the same. A similar increase in body weight gains and also in skeletal growth was observed in mice fed suitable amounts of thyroactive iodocasein (81). Oral administration of iodinated casein over broad ranges of dosage produced no acceleration of the growth rate of rats, rabbits or guinea pigs (82),

except for slight increase in growth of female rats of the Missouri strain. The growth rate of mice was increased by iodinated casein administered either orally or by injection.

3. Effect on Feather Growth

The administration of large doses of thyroid substance or thyroxine to mature chickens (168-173, 98, 69) causes abrupt moulting of old feathers, and depigmentation of the new feathers which appear.

When more nearly physiologic doses of thyroactive iodinated protein were given continuously to growing chicks (112, 70, 156) there was a significant stimulation of feather growth above that of normal controls. The rate of feathering in various groups was in direct proportion to the dosage of iodinated protein, the most rapid feather growth being obtained with dosage levels high enough to depress the growth rate. Quite pronounced stimulation of feather growth (Fig. 9) was obtained on a dosage which permitted approximately normal gains in body weight.

Further evidence that the thyroid is concerned with feathering is provided by the reports that feather development is retarded by thyroidectomy (19) or thiouracil administration (36).

4. Effect on Egg Production

In a famous paper published in 1925, Crew (35) reported the rejuvenation of aged fowls by the administration of desiccated thyroid. In addition to the development of a younger type of plumage in both hens and cocks, there was an increase in egg production. Zawadowsky *et al.* (171) claimed that the egg production of certain hens was increased when 0.01 to 0.05 g. of desiccated thyroid was fed daily. Asmundson and Pinsky (9) found no increase in the egg production of hens fed 0.33 mg. of desiccated thyroid daily, although some changes in egg composition were reported.

Egg production was reduced markedly by thyroidectomy (154, 164).

It is known that egg production of hens is at a maximum during their first years of life, and declines thereafter at a rate of approximately 15% per year. It was believed possible that this decrease in production might be due to a diminishing rate of thyroid hormone secretion. In an attempt to arrest the decline in egg production with increasing age, Turner *et al.* (157) fed rations containing 5, 10 and 20 g. of thyroactive iodinated protein per 100 lbs. of feed to White Leghorn hens in their second year of production. On the 5 and 10 g. levels there was some increase in egg production above that of controls. Of particular interest, however, was the fact that the egg production of the experimental groups was maintained at the winter level during the hot weather of summer, while the controls showed the usual seasonal decline. Rhode Island Red pullets, in their first year of egg pro-

duction, receiving the optimal dosage of iodinated protein showed no increase above the controls during the winter (158). With the onset of hot



Fig. 9

The Effect of Thyroactive Iodinated Protein on Feathering in Young Chickens

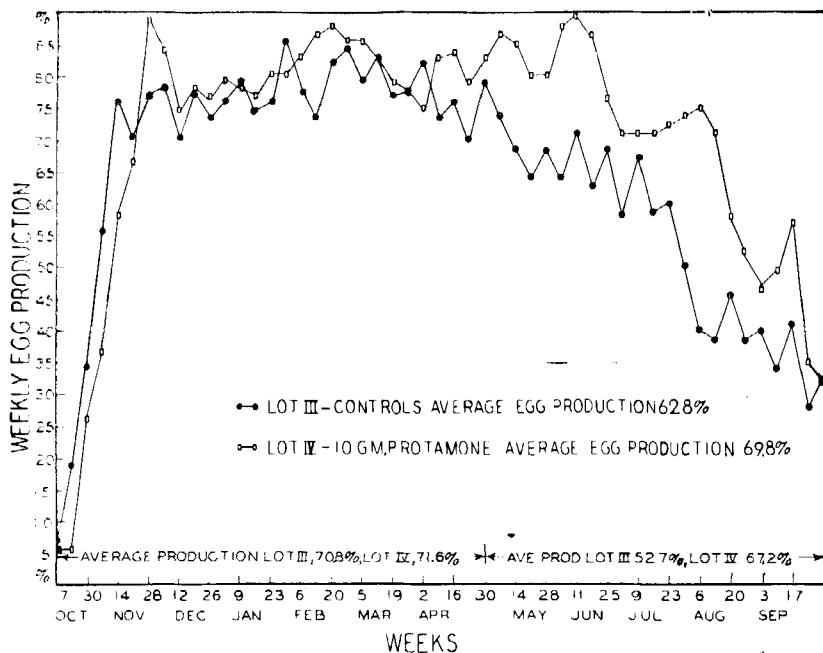
Upper: Normal controls.

Lower: White Plymouth Rock chicks receiving 0.1% iodinated protein in their ration at the age of six weeks.

weather, the egg production of the controls declined while that of the experimental group was maintained for a time (Fig. 10).

The seasonal decline in normal egg production is believed to be associated with a diminished rate of thyroid hormone secretion during the summer

months. While seasonal variations in thyroid hormone output of hens have not actually been determined, a seasonal rhythm in the thyroid secretion rate of chicks has been observed (130).



analysis or biological assay (135). Although further investigation is needed to establish unequivocally that all of the material indicated by these measures is actually thyroxine, comparisons of results obtained by the two methods indicate a very close similarity to thyroxine in both chemical characteristics and biological activity. Subsequent to hydrolysis of thyroactive iodinated protein, yields of crystalline thyroxine far in excess of that actually present in U.S.P. thyroid can be obtained.

These artificial preparations are active when administered either orally or parenterally. No protein sensitization has been observed after parenteral administration of these nondescript iodinated proteins, probably because iodination causes a loss of antigenic specificity (166, 28, 79). Research is much needed on the digestion and absorption of these preparations, and also on their metabolism in the body tissues.

Although Abelin (4) as recently as 1942 still denied that intact iodinated proteins possessed complete thyroid activity, the bulk of the evidence now available indicates that preparations formed under properly controlled conditions will provide full replacement for the thyroid.

With the exception of the early work by Lerman and Salter (91, 143) no reports on the use of thyroactive iodinated proteins in clinical therapy have appeared. Clinical investigations with the more active products now available would be of great interest. In fact, such preparations have been reported to be free of the heart-stimulating factor claimed to be present in thyroid (99), and in this respect might afford some advantages over thyroid substance.

Only a few of the possible applications of thyroactive iodinated proteins to some of the problems of agriculture have been indicated in this review. It is fully established that lactation can be stimulated by the administration of a properly regulated amount of such material. The growth rate in certain species of animals is accelerated slightly, and the rate of feather growth in chickens is stimulated markedly by such treatment. Although the results still remain to be confirmed by other laboratories, it appears that the summer decline in egg production can be prevented in large part by feeding optimal amounts of iodinated protein. Although at present only in its beginning, the application of the thyroactive iodinated protein now available to the problems of animal physiology promises to be a profitable field for future investigation.

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The Protein Anabolic Effects of Steroid Hormones

BY CHARLES D. KOCHAKIAN^{1,2}

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I. INTRODUCTION

It is now about 10 years since Kochakian and Murlin (1935) first reported that androgens had a pronounced and unquestioned effect on protein anabolism. The extension of this initial observation to the crystalline steroid compounds was followed shortly by a series of reports from Kenyon and his associates, who conclusively demonstrated that the same phenomenon occurred in man. Furthermore, they showed that testosterone propionate stimulated the organism to retain not only nitrogen but also the other elements which are essential for the formation of tissue. The androgens thus became a potential tool for the stimulation of protein anabolic processes which provoked many laboratories to investigate their use for the replenishment of protein tissue and the stimulation of growth in many and varied disorders.

In recent years several laboratories (Kochakian, Albright, Wilkins) have entertained the hope of finding a protein anabolic steroid without any, or with only minor, sexual effects. These studies have received special impetus and encouragement from the observation of Kochakian that certain steroids have greater renotrophic (anabolic?) than androgenic effects.

The detailed analyses of the effect of the steroids on various tissues and

their enzyme contents are beginning to throw some light on the mechanism of action of this group of hormones.

In this review special emphasis has been placed on the studies concerned with nitrogen-retaining properties of the steroids. These data are purposely segregated and treated by species, steroid and status of the animal or individual to focus attention on the many ramifications of these topics.

II. NOMENCLATURE AND FORMULAE OF STEROID HORMONES

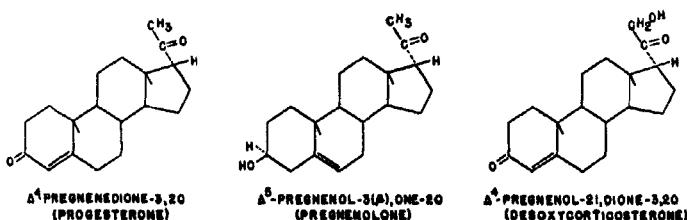
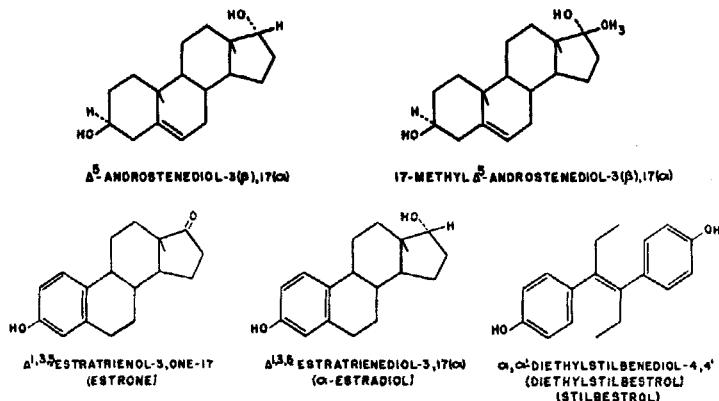
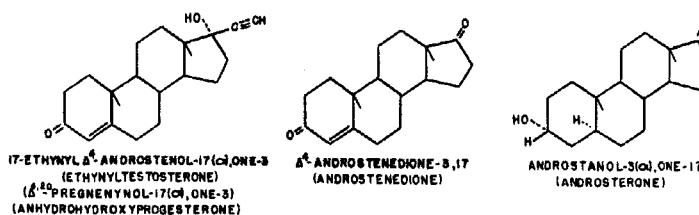
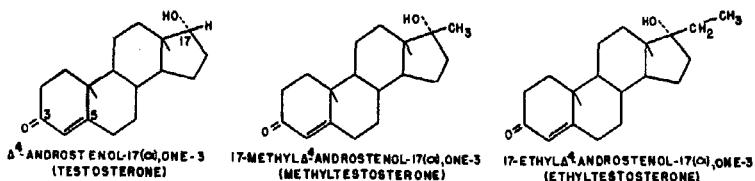
Since this review involves the consideration of a large number of closely related steroid hormones, it seems pertinent to present the structural formulae and nomenclature of these compounds. The ring structure is presented as a planar projection of a spatial model taken from Strain (1943) (*cf.* Crowfoot, 1944) and the substituent groups are drawn to show their spatial arrangement according to the method used by Reichstein and Shoppee (1943). Broken lines indicate projection back and solid lines in front of the plane of the page. Formulae for esters of the steroids are not presented here, since they can be readily visualized from the free steroids.

Each compound is given its systematic chemical name and its common name, if available. The Greek letters α - and β - are used to indicate the spatial position of the attached groups in accordance with the now generally accepted suggestion of Fieser (1936) for the 3 position and as extended to the other positions by Marker, Reichstein and others (*cf.* Reichstein and Shoppee, 1943; Callow, 1939). Whenever possible the common names of the steroids will be used in the text in preference to their systematic names because of their general use.

III. EARLY EXPERIMENTS WITH CRUDE EXTRACTS OF TESTES

It is of historical interest that Bogrov as early as 1891, stimulated by the remarkable invigorating effects claimed by Brown-Sequard (1889) on the administration of glycerine extracts of testes to himself and others, injected a similar emulsion of rabbit testes into two patients and claimed a slight decrease in urea excretion. Later Korenchevsky, after studying the effect of castration on the nitrogen and energy metabolism of dogs and rabbits (Korenchevsky, 1925a),³ attempted to demonstrate the presence of a hormone (or hormones) in the testes and prostate by the administration of emulsions (Korenchevsky, 1925b; Korenchevsky and Carr, 1925a), "insulin-like" extracts (Korenchevsky and Carr, 1925b; Korenchevsky and Schultess-Young, 1928) and lipoïd extracts (Korenchevsky, 1928) of these organs alone and together into normal, castrated, thyroidectomized and castrated and thyroidectomized rabbits and also dogs. In no instance

³ This article contains a critical and comprehensive review of early work concerning gonadectomy and metabolism.



was he able to obtain a satisfactory demonstration of a metabolic effect by these extracts. Furthermore, these preparations did not stimulate the accessory sex organs of normal or castrated rats.

IV. THE DEMONSTRATION THAT "MALE HORMONE" EXTRACTS OF URINE CAUSE NITROGEN RETENTION

Shortly after the demonstration by Loewe, Voss *et al.* (1928) and Funk, Harrow and Lejwa (1930) of the presence of a factor(s) in male urine with comb-growth-promoting properties similar to that of the cell-free extract prepared from bulls' testes by McGee (1927), Kochakian (1935) and Kochakian and Murlin (1935) reported that "male hormone" extracts prepared from medical student urine produced a marked reduction in the urinary nitrogen excretion of "thin" and "fat" castrated dogs fed a constant diet (Fig. 1). A similar decrease in urinary nitrogen was produced by small frequent injections or a single large injection. The maximum rate of retention was attained in 2 to 3 days and continued injections or increased dosage did not further increase the rate of retention. The maximum rate of retention was found to be directly proportional to the mass of the dog at 0.05 to 0.06 g. nitrogen per kg. body weight per day. Cessation of injections always resulted in a loss of some of the nitrogen—a "rebound"—by the "fat" dog but only in one experiment by the "thin" dog. The amount lost, however, was only a small fraction of that retained. It was assumed that the retained nitrogen had been incorporated into permanent tissue structures while the nitrogen lost had not been incorporated as yet into such tissue and probably was present in the body as reserve protein.

Extra nitrogen intake in one experiment (Kochakian and Murlin, 1935) did not favor a greater nitrogen retention. Similar results (Kochakian, 1944a) have been obtained in rats. This is of special interest because Gaebler (1933) noted that a crude growth hormone preparation of the anterior pituitary did not produce nitrogen retention in dogs unless the nitrogen intake was rather great (10.35 g./day for a 15 kg. dog) and then it was very marked and much greater than that observed with androgens in castrated dogs by Kochakian and Murlin (1935).

V. THE EFFECT OF STEROID HORMONES ON NITROGEN EXCRETION IN URINE

1. *Experiments in Dogs*

a. Δ^4 -Androstenedione- $\beta,17$. The synthesis of androsterone from cholesterol (Ruzicka, Goldberg *et al.*, 1934) followed rapidly by the synthesis of testosterone (Butenandt and Hanisch, 1935), Ruzicka and Wettstein, 1935b) before it was actually isolated from bulls' testes (David, 1935 and

David, Dingemanse *et al.*, 1935) and the synthesis of androstenedione because it was thought that it might be the testis hormone, provided a means of preparing crystalline hormones for comparing the protein anabolic property of the various steroids. Therefore, since androstenedione was intermediate in chemical structure and androgenic activity between androsterone,

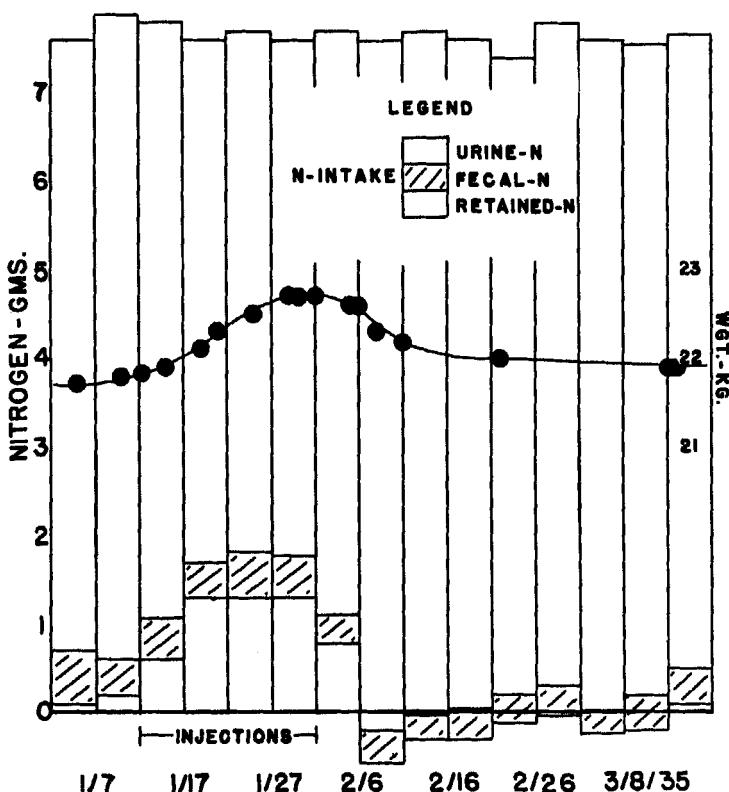


FIG. 1

The Effect of "Male Hormone" Urine Extracts on the Nitrogen Metabolism and Body Weight of the Castrated Dog.

Redrawn from Kochakian and Murlin; *J. Nutrition* 10, 437 (1935). The dose, 133 I.U./day, has been converted to international units (1 I.U. = 0.1 mg. androsterone) on the basis of subsequent assays by standardized methods.

which was assumed to be the protein anabolic substance in the urine extracts, and testosterone, it was synthesized by Kochakian by the identical procedures of Ruzicka and Wettstein (1935a) and Butenandt and Kudszus (1935) and studied for its protein anabolic properties by Kochakian and Murlin (1936). When this substance was given in doses of 20-60 mg./day

for 3 successive days to a "thin" and a "fat" castrated dog an effect identical with that of the urine extracts was obtained. The treatment of a normal dog in the same manner as the castrated dogs resulted in no effect. Thorn and Engel (1938) were also unable to show any change in the nitrogen excretion of a normal dog after the injection of 40 mg. of androstenedione.

b. *Testosterone, Testosterone Acetate and Testosterone Propionate.* Since testosterone was presumed to be the male hormone because of its isolation from bulls' testes as the most potent naturally occurring androgen, the above studies were extended by Kochakian (1937) to include observations on this compound and its more efficacious ester, testosterone acetate. The administration of these compounds at 20 mg./day for 3 days to the fat castrated dog and testosterone acetate at 25 mg./day for 3 days to the thin castrated dog produced effects on urinary nitrogen excretion identical with those observed in the previous two studies. When testosterone was administered to the thin castrated dog at 15 mg./day for 3 days, there was only a small but definite decrease in nitrogen excretion.

Thorn and Engel (1938), while studying the effect of various steroids on electrolyte balance, were unable to demonstrate any significant effect on the nitrogen excretion of normal dogs injected with a single dose of 25 mg. of testosterone propionate and only a slight decrease after administration of a large single dose of 125 mg. They reported, however, a remarkable decrease in the nitrogen excretion of a normal dog injected with 25 mg./day for 7 successive days. The decrease was approximately seven times that previously noted by Kochakian and Murlin in castrated dogs and curiously enough did not appear until the three-day period after cessation of injections. This experiment is unique and needs confirmation, especially since Gaebler and Tarnowski (1943) injected 25 mg./day of this same steroid into normal and depancreatized bitches maintained on a constant diet with a large protein intake, 15-20 g./day, and obtained decreases in nitrogen excretion comparable to those obtained in castrated dogs.

Thorn and Engel (1938) attempted to study the effect of testosterone propionate, 25 mg./day, in an adrenalectomized dog. The experiment, however, had to be terminated because of the rapid appearance of adrenal cortical deficiency. This effect is not surprising as Spurr and Kochakian (1939) demonstrated that various androgens, in spite of their close chemical relationship to the adrenal cortical steroids (Reichstein and Shoppee, 1943) and their ability to cause retention of minerals and water (Thorn and Harrop, 1937), are not able to maintain the life of adrenalectomized rats. Indeed, they proved to be toxic. A striking clinical example of the inability of androgens to substitute for adrenal cortical steroids is provided by the report of Wilkins, Fleischmann and Howard (1940) of a boy who died in adrenal cortical deficiency despite the fact that a tumor of the adrenal

cortex was producing excessive amounts of steroids with androgenic activity but had destroyed all normal adrenal cortical tissue.

There is preliminary evidence that the pituitary is not essential for the protein anabolic activity of the steroids. Kochakian (unpublished) administered 25 mg./day of testosterone propionate to an hypophysectomized-castrated dog and obtained a decrease in urinary nitrogen excretion similar to that in castrated dogs.

c. Δ^5 -Androstanediol- $3(\beta),17(\alpha)$. Thorn and Engel (1938) were unable to detect any change in the urinary nitrogen of normal dogs injected subcutaneously with 40 mg. of Δ^5 -androstanediol- $3(\beta),17(\alpha)$. The following compounds were also found to be inactive: 50 mg. of 1,2-cyclopentenophenanthrene; 200 mg. of cholesterol (purified through the dibromide) and 500 mg. of 7-keto-cholesteryl acetate.

d. *Estrogens and Progesterone*. There are a few experiments which indicate that estrogens are also able to decrease the nitrogen excretion in dogs. Thorn and Engel (1938) reported a "marked" decrease in the nitrogen excretion of 5 normal dogs after a single subcutaneous injection of 5 mg. of α -estradiol, 15 mg. of estrone, or 40,000–100,000 I.U. of "amniotin" but no effect after 20 mg. of progesterone. Gaebler and Tarnowski (1943) observed a barely significant decrease in the urine nitrogen after administering 10 mg./day of estrone for 5 and 10 days to two depancreatized bitches maintained with insulin.

2. Experiments in Rats

a. *Testosterone Propionate*. The administration of testosterone propionate (Kochakian, 1944a) to castrated adult male rats produces an effect on urinary nitrogen excretion similar to that observed in castrated dogs except that, about one week after the beginning of the injections, the nitrogen excretion gradually returns to normal in spite of continued injections (Fig. 2). On cessation of injections there is a negative nitrogen balance followed by a strong positive balance. Similar effects were obtained at various dose levels—1.0, 1.25, 2.5, 5.0 and 7.5 mg./day of testosterone propionate, while the rats were on either a fox chow diet or a synthetic diet containing casein as the source of protein, on a higher (30%) protein intake, and after supplementing the synthetic and the commercial diet with $\frac{1}{2}\%$ of cystine. The maximum rate of nitrogen retention is about 5 times greater, 250–300 mg./kg./day, in the rat than in the dog or man. A comparison of the protein anabolic properties of a large number of steroids is now being made with castrated rats in the author's laboratory.

Testosterone propionate will hasten the replenishment of protein in starved rats. Kochakian (unpublished) fasted adult castrated male rats for 12 days, then placed the animals on a constant diet and injected half of the

group with 2.5 mg./day of testosterone propionate. The treated rats retained more nitrogen than the untreated during the period of replenishment. When the body weight was restored, the nitrogen retention of the treated animals decreased to that of the controls in spite of continued injections.

3. Experiments in Man

When the presumed hormone of the testes became available commercially, it provided a means of extending to man the studies of Kochakian

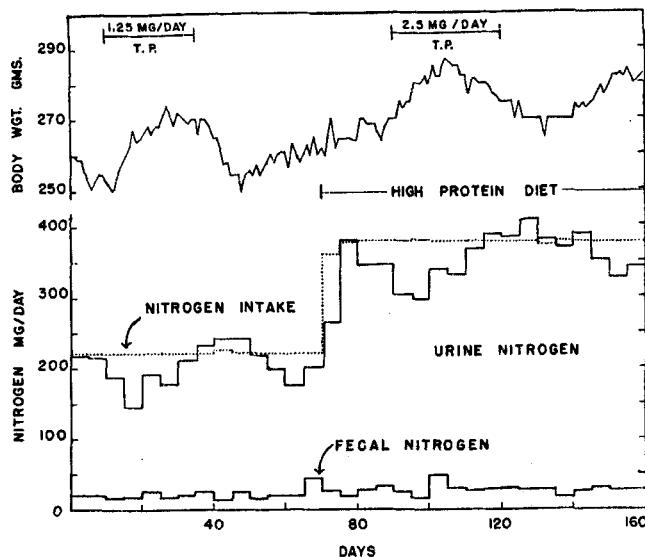


FIG. 2

The Effect of Testosterone Propionate on the Nitrogen Balance and Body Weight of the Castrated Male Rat While on a Constant Diet of "Normal Protein" (18% Casein) and "High Protein" (30% Casein) Content.

(Kochakian, *Macy Conf. Metabol. Aspects Convalescence* 7, 97 (1944)).

and Murlin in the dog. This was quickly accomplished by Kenyon (1944a) and his associates (Kenyon, Knowlton and Sandiford, 1944) at Chicago with apparently the same results in eunuchoids as in the castrated dog. This study was followed by a series of other studies from the Chicago group. In more recent years many laboratories have delved into the clinical potentialities of the protein anabolic properties of not only testosterone propionate but also a number of related steroids which have been made available for comparative study largely through the generous cooperation of the research staff of Ciba Pharmaceutical Products, Inc. These various compounds will be considered separately.

a. *Testosterone Propionate. Hypogonadism.* Kenyon, Sandiford *et al.* (1938) demonstrated conclusively that the injection of 25 mg./day of testosterone propionate decreased the urinary nitrogen excretion of four eunuchoid patients, 26–51 years old. The effect was precisely the same as that observed in castrated dogs by Kochakian (1935, 1937) and Kochakian and Murlin (1935, 1936), except for one patient with a suprasellar cyst which, at autopsy, proved to have largely destroyed the pituitary. The response in this patient was qualitatively the same but quantitatively somewhat less than that observed in the three other subjects.

In subsequent studies these investigators confirmed the above observations in the same subjects as well as two more eunuchoids and a hypogonadal woman, and also investigated the effect of dose on maximum response. They found that, as in the castrated dog, there was no further response in the maximum daily nitrogen retention when the dose was increased to 25 mg. twice a day (Kenyon, Knowlton *et al.*, 1940) or to a single injection of 50 mg./day (Knowlton, Kenyon *et al.*, 1942). Reduction of the dose to 5 mg./day (Sandiford, Knowlton and Kenyon, 1941; Knowlton, Kenyon *et al.*, 1942) gave approximately half the response obtained with 25 mg./day. A dose of 10 mg./day (Sandiford, Knowlton and Kenyon, 1941) produced an intermediate response (Fig. 3).

Eidelsberg, Bruger and Lipkin (1942) injected a 21-year-old eunuchoid with 25 mg./day of testosterone propionate and obtained a similar nitrogen retention. Bassett, Keutmann and Kochakian (1944, 1945) confirmed the nitrogen-retaining effect of 25 mg./day of testosterone propionate in a 21-year-old eunuchoid. They observed further that the maximum rate of nitrogen retention per day was not attained until after the sixth day of treatment which is three to four days longer than that noted in the castrated dogs injected with urinary extracts, androstenedione or testosterone. This difference is probably a manifestation of some influence exerted by the apparently non-functioning testes of the eunuchoids. It is unfortunate that there are no experiments on castrated men to provide a direct comparison with eunuchoids.

Normal Individuals. The presence of the functioning gonads in man as in the dog makes the subject "resistant" to the metabolic effects of testosterone propionate. Kenyon, Knowlton *et al.* (1940) observed a maximum daily nitrogen retention in two normal young men of 19 and 21 years of half that obtained in eunuchoids. Thorn and Engel (1938) reported a nitrogen-retaining effect in a normal man treated for 7 days with 25 mg./day of testosterone propionate. Bassett, Keutmann and Kochakian (1943b) obtained similar results in two young men of 24 and 31 years and also observed that the time of attainment of the maximum daily nitrogen retention was extended from about 6 days for eunuchoids to about 12 days for normal

men. This latter fact is also evident but not noted in the report of the Chicago group.

The administration of 25 mg./day of testosterone propionate to two aged men (76 years) produced a maximum nitrogen retention approximating that of normal young men (Kenyon, Knowlton, *et al.*, 1942a). Albright (1942-43) obtained a similar response in an aged man suffering from senile osteoporosis.

The administration of 25 mg./day of testosterone propionate to two normal young women (24 and 31 years) produced in one a response in the

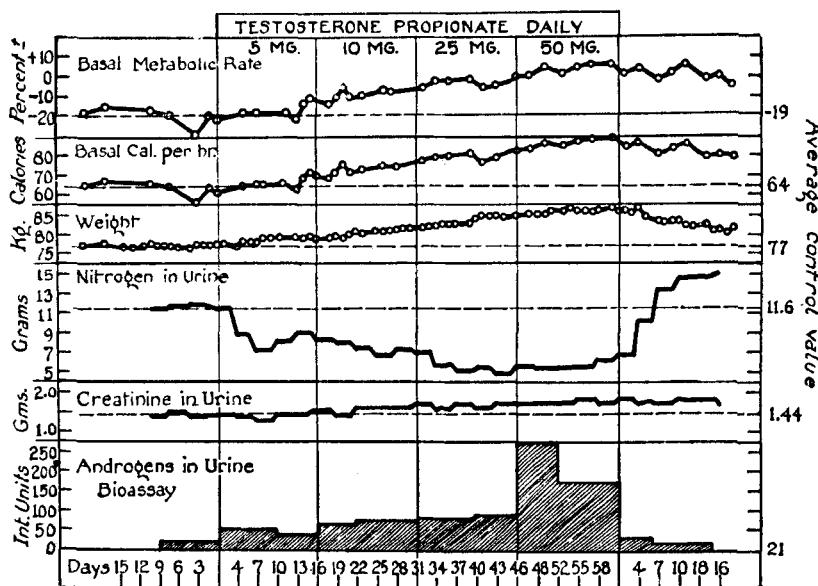


FIG. 3

Effects of Varying Dosages of Testosterone Propionate on Basal Heat Production, Body Weight, Urinary Nitrogen, Creatinine and Androgens in the Eunuchoid J. K.

(Sandiford, Knowlton and Kenyon, *J. Clin. Endocrinology* 1, 931 (1941).)

nitrogen retention intermediate between that of eunuchoids and normal men and a questionable slight response in the other (Kenyon, Knowlton *et al.*, 1940).

Adrenal Cortical Dysfunction. Thorn and Engel (1938) administered 25 mg./day of testosterone propionate for 7 days to a man with Addison's disease and obtained a decrease in nitrogen excretion during the period of treatment and the following 3 days. Kenyon, Knowlton *et al.* (1943) in an effort to evaluate the possible role of the adrenal cortex in the metabolic effects of testosterone propionate, injected a 39 year old man and a woman

of the same age with 25 mg./day of the androgen for 6 days. To prevent adrenal cortical crisis the man was given 1 mg./day and the woman 1.25 mg./day of desoxycorticosterone acetate⁴ and a constant salt intake. Both subjects gave a metabolic response similar to that observed in young normal men and women and aged men. The maximum nitrogen retained/kg. body weight/day was 0.043 for the man and 0.039 for the woman. Williams, Whittenberger *et al.* (1945) obtained variable and unexplainable results in similar patients treated with 25 mg./day of testosterone propionate for 8 days. The nitrogen excretion decreased in the urine of a 42 year old woman with panhypopituitarism and a 43 year old woman with Addison's disease. The diagnosis of the disease of the former was confirmed later at autopsy when the pituitary proved to be small and composed almost entirely of fibrous tissue. In contrast to these two patients, a 42 year old man with Simmond's disease and a 36 year old woman with Addison's disease, responded to the androgen with an increase in urinary nitrogen excretion. These two experiments are the first and only instances of such an effect. Talbot, Butler and MacLachlan (1943) obtained very marked nitrogen retention, 0.115 g. nitrogen/kg. body weight/day in an eight year old girl with severe Addison's disease injected with 25 mg./day of testosterone propionate and maintained on sodium chloride and 3 mg./day of desoxycorticosterone acetate.⁴ The same effect was obtained when the desoxycorticosterone acetate was omitted and 50 mg./day of testosterone propionate was injected. Indeed, the patient was reported to be relieved of all signs and symptoms of acute adrenal cortical insufficiency.

Albright, Parsons and Bloomberg (1941) and Albright (1942-1943) have interpreted the effects of Cushing's syndrome as due to excess production of adrenal cortical compounds concerned with conversion of protein to carbohydrate (Long, Katzin and Fry, 1940) and not enough of the protein anabolic hormones;⁵ the latter are considered to be produced by the adrenal cortex as well as the testes, as indicated by the precocious muscular development and masculinization of patients with tumors of the adrenal cortex (adrenal genital syndrome, *cf.* Kenyon, 1944b), and the isolation of androgenic substances from the normal adrenal cortex (*cf.* Reichstein and Shoppee, 1943). Therefore, they felt that administration of a protein anabolic stimulant like testosterone propionate would rebuild the degenerated skin, muscle, blood vessels and matrix of the skeleton. Treatment of 3 cases of this disease resulted in a prompt decrease in the excretion of the

⁴ Desoxycorticosterone acetate does not affect protein metabolism (Thorn, Howard and Emerson, 1939, Talbot, Butler and MacLachlan, 1943).

⁵ Albright (1942-1943) has suggested the general terms "N-Hormone" for all the protein anabolic steroids and "S-Hormone" for the protein catabolic steroids.

urinary nitrogen similar to that occurring in eunuchoid men and a general improvement in the condition of the patients. There was a suggestion of diminution of response by one patient on prolonged treatment even though the dose had been raised to 50 mg./day. This last phenomenon was clearly recognized by Bassett, Keutmann and Kochakian (1943a) in their study of a 15 year old girl with Cushing's syndrome. The decrease in response probably would have appeared after 30 days but at this point the dose was increased to 50 mg./day for 10 days. When the dose was dropped to 10 mg./day the nitrogen excretion promptly returned to normal which remained at this point even when the dose was raised to the initial value of 25 mg./day. A further increase of the dose to 50 mg./day resulted in a lowering of the nitrogen excretion, but only to one-half that observed on initiation of the experiment. After a lapse of testosterone propionate treatment for 3 months, the patient again gave the normal response to 25 mg./day of the steroid. Similar results were observed simultaneously by Perloff, Rose and Sunderman (1943). The nitrogen retention induced in a 30 year old woman with Cushing's syndrome by testosterone propionate became less marked when large doses of Vitamin D₂ were given. In subsequent therapy with testosterone propionate, vitamin D₂ and calcium gluconate, the characteristic response was obtained but on prolongation of therapy the nitrogen excretion returned to normal. In view of the observations of Bassett, Keutmann and Kochakian, the "wearing off" effect observed in the above experiment probably would have occurred without the vitamin D₂ treatment. Deakins, Friedgood and Ferrebee (1944) treated a 15 year old girl with 25 mg./day of testosterone propionate for a shorter period of time, 17 days, and obtained the characteristic effect in nitrogen excretion. The period of treatment was not long enough to show the gradual return to normal.

Pituitary Hypofunction in Children. Testosterone propionate is effective in decreasing the urinary nitrogen excretion of children suffering from pituitary hypofunction. Kenyon, Knowlton *et al.* (1942b) obtained a response in a hypopituitary 13 year old boy similar to that found in eunuchoids. They observed further that a daily dose of 750-1500 I.U. of chorionic gonadotropin could induce a nitrogen retention equivalent to that induced in eunuchoids by the injection of 25 mg./day of testosterone propionate. Wilkins and Fleischmann (in press) observed that administration of 25 mg./day of testosterone propionate to sexually immature male and female dwarfs caused a maximum nitrogen retention/kg./day of 0.057-0.107 g. (av. 0.076 g.). In a previous report, Wilkins, Fleischmann and Howard (1941) were unable to observe nitrogen retention in a 17 year old dwarf boy treated with increasing doses of 5-25 mg./day of testosterone

propionate. Talbot, Butler *et al.* (1945) found that a child with progeria⁶ showed a prompt retention of nitrogen when injected with 25 mg./day of testosterone propionate. A further increase in nitrogen retention was obtained on increasing the caloric intake.

Thyroid Dysfunction. Kinsell, Hertz and Reifenstein (1944) treated 3 patients suffering from thyrotoxicosis with testosterone propionate. There was the usual response of decrease in nitrogen excretion even when the caloric intake of the patients was less than that of their expenditure. The injections of 50 mg./day or 100 mg./day gave only a slightly greater increase in nitrogen retention than 25 mg./day. The slightly greater effect, however, may have been due to the fact that the larger doses were begun before the smaller dose had had an opportunity to induce its maximum daily response.

Conditions Inducing Protein Catabolism. Browne and Schenker (1942) in the course of their extensive studies on damaged patients noted that an ordinary cold decreased the strongly positive nitrogen balance induced by 50 mg./day of testosterone propionate in a normal young man. The nitrogen balance became slightly negative at the height of the infection then gradually returned to its previous strongly positive value as the patient recovered. Kenyon and Knowlton (1942a) noted a similar incident while treating a chronically undernourished 47 year old man with 25 mg./day of testosterone propionate. At the height of maximum nitrogen retention the subject suffered a brisk atypical pneumonia with increase in body temperature. The positive nitrogen balance decreased to normal but returned gradually to its previous positive state as the infection waned. It is impossible to state whether the infection counteracted the nitrogen-retaining effect of the androgen or that the effect of the androgen continued during the course of the infection and the loss in nitrogen was due to some unrelated process. In any event, the patients were in a more favorable nitrogen balance with the androgen than they would have been without it. Browne and Schenker (1942) noted that 25 mg./day of testosterone propionate decreased the protein catabolism caused by burns and fracture. Kenyon and Knowlton (1944) observed nitrogen retention when 25 mg./day of testosterone propionate was administered to a patient with progressive muscular dystrophy.

Protein Repletion in Debilitated Patients. The rapid protein anabolism which occurs in patients recovering from chronic undernutrition may be further speeded up by the administration of testosterone propionate. Kenyon and Knowlton (1942b) administered 25 mg./day of testosterone propionate to a 25 year old man reduced to 92 pounds by severe chronic rheumatoid arthritis and observed a nitrogen retention similar to that ob-

⁶ Although this condition is not, or not directly, a dysfunction of the pituitary, it is included arbitrarily in this group because, like hypofunction of the pituitary, it prevents growth.

tained in normal young men. A similar effect was obtained in a 47 year old man whose weight had decreased to 114 lbs. as the result of a "capricious and fastidious" appetite. They were unable to show any significant effect in a 55 year old man who had lost 80 lbs. in the preceding two years as a result of the obstruction of the common bile duct and was in a positive nitrogen balance from dietary repletion. In a subsequent study Knowlton

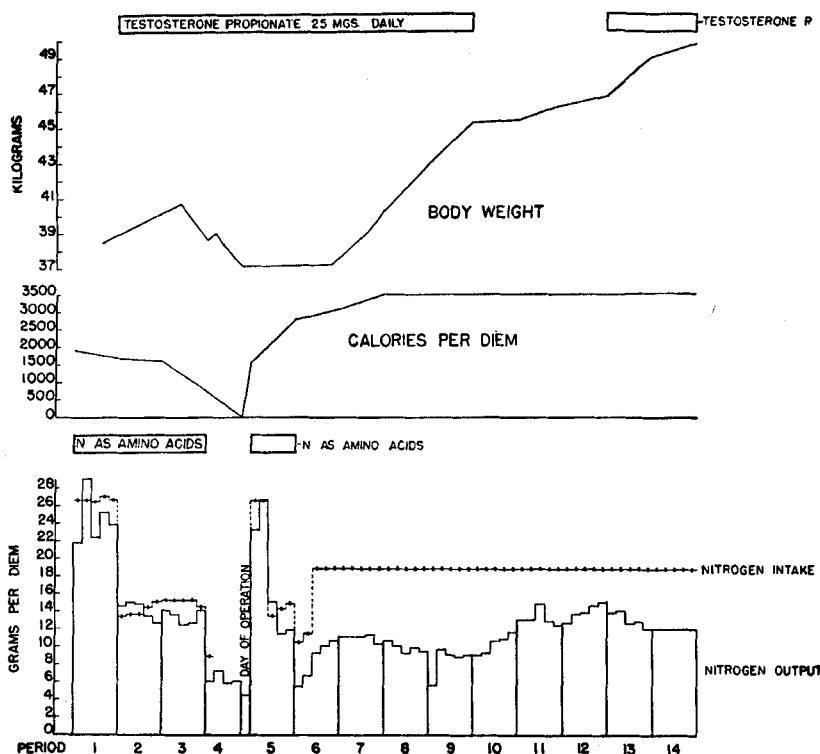


FIG. 4

The Effect of Testosterone Propionate on the Rate of Recovery of an Emaciated Patient after Surgical Removal of an Intestinal Obstruction.
Note change in rate of nitrogen retention and increase in body weight on cessation and renewal of androgen administration.

(Bassett, *Macy Conf. Metabol. Aspects Convalescence* 4, 126 (1943).)

and Kenyon (1943) found that androgen treatment could produce an increase in nitrogen retention over and above that already occurring as a result of dietary repletion in a 34 year old man recovering from a severe and acute non-specific ulcerative colitis. Bassett (1943) noted a similar effect in an extraordinarily emaciated young man recovering from the surgical resection of an intestinal obstruction (Fig. 4). Abels, Young and

Taylor (1944) observed that 50 mg./day of testosterone propionate for 3, 6 and 15 days caused a retention of nitrogen in three men with gastric cancer. Bassett, Keutmann and Kochakian (1943d) treated a young male subject suffering from nephrosis with 25 mg./day and 50 mg./day for 85 days. There was a remarkably good conversion of dietary protein to body protein; half of the newly formed protein was excreted in the urine and half was retained to build body tissue.

Kenyon, in an experiment on himself, (Kenyon and Knowlton, 1943b) found a decrease in the effectiveness of testosterone propionate on nitrogen excretion as he decreased the nitrogen and caloric intake from an adequate level of 13.5 g./day of nitrogen and 2816 calories/day to 4.6 g. nitrogen and 2251 cal., and then to 1.7 g. nitrogen and 1589 cal. The nitrogen excretion, however, was always less during testosterone propionate treatment than without treatment.

Effect of Changes in Protein and Caloric Intake. Bassett, Keutmann and Kochakian (1942, 1943c) found that 150 g./day of extra carbohydrate to a 15 year old girl being treated with 25 mg./day of testosterone propionate caused a further decrease beyond the maximum in nitrogen excretion. Kenyon and Knowlton (1943a) found that removal of 100 g. of carbohydrate and 20 g. of fat per day from the diet of a patient being treated with testosterone propionate elevated the nitrogen excretion 1 g./day by the 4th to the 6th day. Restoration of the removed food resulted in a return to the previous level of nitrogen retention. Albright (1942-1943) found that 50 mg./day of testosterone propionate reduced to normal the strongly negative nitrogen balance in an obese man on a low caloric intake. When injections were stopped the negative nitrogen balance recurred. Bassett (1945) injected 25 mg./day of testosterone propionate into a woman on a low nitrogen (3.09 g./day) intake and excreting only 2.03 g./day nitrogen in the urine and obtained a further decrease in the urinary nitrogen to 1.39 g./day, a value similar to that reported in the literature for minimum endogenous nitrogen metabolism. Butler, Talbot *et al.* (1945) found that injection of testosterone propionate decreased the urea output in the urine of male subjects on a total dietary fast.

It is evident that testosterone propionate not only decreases catabolism of exogenous but also endogenous protein.

b. Testosterone. As testosterone propionate has proven to be more efficacious than the free compound, it has received preference for clinical trial and experimentation. Consequently, there have been only a few experiments with the unesterified compound. Eidelsberg, Bruger and Lipkin (1942) made the interesting observation that a subcutaneous implant of 450 mg. of testosterone as pellets initiated and maintained a decrease in urinary nitrogen excretion. The maximum nitrogen retention was of the order of that observed by injection of testosterone propionate.

Abels, Young and Taylor (1944) injected two normal young men with testosterone. One individual received 50 mg./day of testosterone for 15 days with a prompt decrease in urinary nitrogen excretion which continued for the duration of treatment. The other individual was injected with 90 mg./day for 50 days. The urinary nitrogen excretion decreased until about the 16th day, then gradually increased until, by the 45th to 50th day of treatment, it had returned to normal. There was, however, a rebound in nitrogen excretion when the injections were stopped. Wilkins and Fleischmann (in press, cf. 1945) injected 2 sexually immature dwarfs with 20 mg./day of testosterone and obtained a response in nitrogen retention similar to that observed with testosterone propionate.

Kenyon, Knowlton *et al.* (1940) found that injection of 25 mg./day of testosterone produced a definite decrease in urinary nitrogen of a eunuchoid. Elevations of the dose to 50 mg./day produced a further decrease.

Deakins, Friedgood and Ferrebee (1944) found no change in the nitrogen excretion of a 15 year old girl with Cushing's syndrome given 40 mg./day of testosterone by mouth.

c. *17-Methyltestosterone*. The discovery that the introduction of a methyl group into the 17 position of testosterone made the compound orally effective provided a simpler method of administering androgens. Consequently, the oral administration of this synthetic compound has been investigated for its metabolic as well as clinical effects.

Hypogonadism. Jones, McCullagh *et al.* (1941) found a prompt decrease in the excretion of urine nitrogen in three eunuchoids receiving massive doses, 200 and 500 mg./day, of methyltestosterone. Bassett, Keutmann and Kochakian (1945) in a more carefully conducted experiment found that 60 mg./day of methyltestosterone by mouth for 20 days decreased the nitrogen excretion of a Chinese eunuchoid about two-thirds as much as 25 mg./day of testosterone propionate by injection.

Normal Men. Samuels, Henschel and Keys (1942) gave 50 mg./day of methyltestosterone to 4 normal young medical students and found no effect on the nitrogen excretion during strenuous work.

Adrenal Cortical Dysfunction. Talbot, Butler and MacLachlan (1943) gave 90 mg./day of methyltestosterone to an 8 year old girl with Addison's disease and obtained a retention of nitrogen equivalent to that obtained with 25 mg./day of testosterone propionate. The effect was evident when the patient was or was not maintained on 3 mg./day of desoxycorticosterone acetate⁴. Williams, Whittenberger *et al.* (1945) studied a 43 year old woman with Addison's disease maintained on 5 g./day of extra sodium chloride and 6 mg./day of desoxycorticosterone. Administration of 60 mg./day of methyltestosterone resulted in a prompt decrease in urinary nitrogen excretion which returned to normal when the dose was lowered to 30 mg./day. Werner and West (1943) studied two cases of Simmond's disease due

to destruction of the hypophysis by intrasellar tumors, a girl of 20 years and a man of 31. They administered 20 mg. of methyltestosterone 5 times/day. In both instances there was a prompt decrease in the excretion of the urinary nitrogen which began to return towards normal after about 7 days of treatment. This "wearing off" effect was more pronounced in the male subject.

Albright (1942-1943) obtained a marked decrease of urinary nitrogen excretion on administering 50 mg./day of methyltestosterone to an 11 year old girl with Cushing's syndrome. The effect of the steroid was maintained for about 18 days and then it began to decrease even though the tissues of the patient seemed to be still depleted. The decrease in response was not prevented (Reifenstein, 1944) by increasing the dose of the steroid or the administration of extra methionine. Deakins, Friedgood and Ferrebee (1944) obtained a similar prompt and sustained decrease in urinary nitrogen on giving 40 mg./day of methyltestosterone to a 15 year old girl with Cushing's syndrome. It is possible that if treatment had been continued for longer than 16 days, they would have seen the "wearing off" phenomenon.

Pituitary hypofunction in Children. Wilkins, Fleischmann and Howard (1941) administered orally 25 mg./day of methyltestosterone to 7 (5 boys and 2 girls) sexually immature dwarfs and observed a retention as long as 5 months after beginning the therapy. It should be noted that these patients were brought in for metabolic studies for 20 days at the beginning and end of the experiments. The *ad libitum* diet may be a factor in the continued response over the long period. In a subsequent study, Wilkins and Fleischmann (in press, cf. 1945) administered 10-30 mg./day of methyltestosterone to 5 sexually immature dwarf boys for varying periods of time and obtained a prompt decrease in nitrogen excretion which showed a tendency to return to normal on prolonged treatment. A similar response was obtained in one subject injected with 20 mg./day of the steroid which was followed for 3 days with the same amount by mouth. The duration of treatment unfortunately was too short to permit a comparison of the efficacy of the two routes of administration.

Talbot, Butler *et al.* (1945) obtained a relatively large positive nitrogen balance in a boy suffering from progeria⁶ after administering 50 mg./day of methyltestosterone for 5 days.

Thyroid Dysfunction. Kinsell, Hertz and Reifenstein (1944) gave 100 mg./day of methyltestosterone to 3 patients with hyperthyroidism and obtained a prompt and substantial decrease in nitrogen excretion which, however, began returning to normal within a few days. Howard, Wilkins and Fleischmann (1942) induced nitrogen retention in a 27 year old cretin treated with 25 mg./day of methyltestosterone by mouth.

Protein Repletion in Debilitated Patients. Kenyon and Knowlton (1943) administered 30 mg./day of methyltestosterone to a patient in strong (5.68 g./day) nitrogen balance and obtained a prompt further decrease in nitrogen excretion which showed a tendency to return to the previous level after the first day. No greater decrease was obtained by increasing the dose to 60 mg./day for 3 days or giving a combination of 30 mg./day of methyltestosterone and 25 mg./day of testosterone propionate. The greater dose of methyltestosterone, however, seemed to inhibit the rapid return of nitrogen excretion to normal.

d. 17-Ethyltestosterone. Pituitary Hypofunction in Children. Wilkins and Fleischmann (in press, cf. 1945) have administered ethyltestosterone in daily doses of 20 to 40 mg. by oral and intramuscular injection into sexually immature dwarf boys without demonstrating any definite decrease in urinary nitrogen.

e. 17-Ethynodiol- Δ -Diol (anhydrohydroxyprogesterone, pregnenolone). Pituitary Hypofunction in Children. Howard, Wilkins and Fleischmann (1942) were unable to find a definite nitrogen retention in sexually immature dwarf boys treated with 40 mg./day of ethynodiol- Δ -diol by mouth for two weeks.

Adrenal Cortical Deficiency. Talbot, Butler and MacLachlan (1943) found that 90 mg./day of ethynodiol- Δ -diol by mouth to an 8 year old girl with Addison's disease reduced the urinary nitrogen excretion to about the same extent as that obtained with a similar dose of methyltestosterone. Reifenstein and Albright (1943), on the other hand, were unable to obtain any effect on nitrogen excretion on administering 120 mg./day by mouth for 18 days to a female patient aged 50 with Cushing's syndrome.

f. Δ^4 -Androstenedione- β ,17. Pituitary Hypofunction in Children. Howard, Wilkins and Fleischmann (1942) were unable to induce nitrogen retention in sexually immature dwarfs by the injection of 20 mg./day of androstenedione for two weeks.

Normal Women. Kenyon and Knowlton (1945) also found no significant nitrogen retention in a 44 year old woman after injections of 15 mg. daily for 3 days and 30 mg. daily for 8 days. However, in a second study on a 23 year old woman definite nitrogen retention was obtained when the dose was raised to 45 and 60 mg./day.

g. Androsterone. Pituitary Hypofunction in Children. Howard, Wilkins and Fleischmann (1942) reported no change in nitrogen excretion in the urine of sexually immature dwarfs injected with 20 mg./day of androsterone for two weeks.

Cushing's Syndrome. Bassett, Keutmann and Kochakian (1943c) were unable to find any nitrogen retention on injection of a 15 year old girl with Cushing's syndrome for 20 days with 10 to 25 mg./day of androsterone.

h. Dehydroisoandrosterone and Dehydroisoandrosterone Acetate. Pituitary Hypofunction. Howard, Wilkins and Fleischmann (1942) found no change in nitrogen excretion in sexually immature dwarfs on injection of 40 mg./day of dehydroisoandrosterone. The acetate injected at 10-40 mg./day for 13 and 18 days also proved ineffective (Wilkins and Fleischmann, 1945).

Mason and Kepler (1945) injected a 30 year old man, who had a pituitary tumor removed 5 years previously, with 25 mg. twice/day of dehydroisoandrosterone acetate for 7 days followed by 50 mg. twice/day for 9 days without any effect on nitrogen excretion.

Cushing's Syndrome. Bassett, Keutmann and Kochakian (1943c) administered 25 mg./day of dehydroisoandrosterone for 10 days to a 15 year old girl with Cushing's syndrome and found no change in nitrogen excretion. Similarly, Albright (1942-1943) found that 25 mg./day of dehydroisoandrosterone acetate for 25 days did not inhibit the "rebound" in nitrogen on omitting testosterone propionate injections in a patient with Cushing's syndrome.

i. Δ^4 -Androstenediol- $3(\beta),17(\alpha)$ and Δ^5 -Androstenediol- $3(\beta),17(\alpha)$ -diacetate- $3, 17$. Senile Osteoporosis. Albright (1942-1943) obtained a possible slight nitrogen retention in a man with senile osteoporosis on injecting 60 mg./day of Δ^5 -androstenediol- $3(\beta),17(\alpha)$ for 10 days.

Cushing's Syndrome. Shorr (1944) obtained no change in a 58 year old woman with Cushing's syndrome on injecting 30 mg./day of Δ^5 -androstenediol- $3(\beta),17(\alpha)$ for 6 days but on increasing the dose to 45 mg./day for the next 9 days, observed a small nitrogen retention of 0.010 g./kg./day.

Pituitary Hypofunction in Children. Wilkins and Fleischmann (in press) obtained a questionable slight decrease of urinary nitrogen in a male sexually immature dwarf injected with 10 mg./day of Δ^5 -androstenediol- $3(\beta),17(\alpha)$ for 15 days. The injection of 30 mg./day for 8 days followed by 45 mg./day for 7 days of the diacetate of this steroid produced a small decrease in nitrogen retention. A third subject was injected with 100 mg./day of the diacetate for 10 days. There was a slight retention of nitrogen which continued for 15 days after cessation of injections. This delayed effect probably was due to the extremely low solubility of this steroid in tissue fluids (*cf.* Kochakian, 1944b).

j. 17-Methyl- Δ^5 -Androstenediol- $3(\beta),17(\alpha)$. Adrenal Cortical Dysfunction. Talbot, Butler and MacLachlan (1943) found a nitrogen retention of 0.052 g./kg./day after administering 50 mg./day of 17-methyl- Δ^5 -androstenediol- $3(\beta),17(\alpha)$ to an 8 year old girl with Addison's disease who was maintained on desoxycorticosterone acetate and sodium chloride. They noted that the substance was very irritating to the mouth and gastrointestinal tract. The patient developed sores in her mouth and excreted considerable mucus in the feces.

Pituitary Hypofunction in Children. Wilkins and Fleischmann (in press) have studied the effect of 17-methyl- Δ^5 -androstenediol-3(β),17(α) in sexually immature dwarf boys by mouth, intramuscular injection and simultaneously by both routes in doses of 10 to 50 mg./day with suggestive but variable decreases in nitrogen excretion. No irritating effect of the compound was reported.

k. Androstanediol-3(α),17(α) and androstanediol-3(α),17(α)-diacetate-3,17. The observation by Kochakian (1944b) that androstanediol-3(α),17(α) and its 17-methyl derivative had a greater renotrophic (protein anabolic?) than androgenic effect has stimulated an interest in the protein anabolic properties of these and related compounds.

Normal Man. Bassett, Keutmann and Kochakian (1943e) observed a small but definite decrease in nitrogen excretion on injecting 10 mg./day of androstanediol-3(α),17(α) for 8 days in a normal young man. The same treatment by mouth showed a questionable slight decrease.

Adrenal Cortical Dysfunction. Albright (1942-1943) injected 10.7 mg./day of androstanediol-3(α),17(α) into a girl with Cushing's syndrome and noted a definite decrease in nitrogen excretion. Williams, Whittenberger *et al.* (1945), on the other hand, obtained an increase in urinary nitrogen excretion after administration (by mouth?) of 20 mg. four times a day of the same steroid for 8 days to a 44 year old man with Addison's disease. A similar effect was obtained by giving 10 mg. four times daily for 16 days to a 36 year old woman with Addison's disease. If this contrary effect is real, it may be assumed that the route of administration has drastically altered the protein anabolic effect of this steroid. Other possibilities may also be considered but further more careful studies are needed.

Pituitary Hypofunction in Children. Wilkins and Fleischmann (in press) injected 4 sexually immature boys with 30-100 mg./day of androstanediol-3(α),17(α) diacetate and obtained moderate to definite nitrogen retention. There was continued retention for at least 15 days after discontinuing injections with the 100 mg./day dose. The prolongation of effect very likely was due to the very low solubility of this material in tissue fluids.⁷

l. 17-Methandrostanediol-3(α),17(α). Hypogonadism. Bassett, Keutmann and Kochakian (1944) administered 60 mg./day of 17-methyl-androstanediol-3(α),17(α) by mouth to a Chinese eunuchoid and found a prompt decrease in urinary nitrogen excretion which reached a maximum on the third day and was maintained at this point for 7 days. Then the nitrogen excretion began to increase until it returned to normal by the 17th day of treatment. An effect similar to that obtained with methyltestosterone. The maximum nitrogen retention per day was approximately one-half

⁷ The diester is much more soluble in vegetable oils (*e.g.*, sesame) than the free compound but it is much less soluble in the tissue fluids (Kochakian, 1944b).

that obtained on later treatment by injection of the same patient with 25 mg./day of testosterone propionate.

Adrenal Cortical Dysfunction. Williams, Whittenberger *et al.* (1945) obtained a slight retention in nitrogen in a 43 year old woman with Addison's disease on giving 10 mg. four times per day for 16 days. Another patient, a 36 year old woman with the same disease, showed no change in nitrogen excretion on taking the same steroid by mouth at 10 mg. four times per day for 8 days followed by 20 mg./day four times per day for another 8 days.

Pituitary Hypofunction in Children. Wilkins and Fleischmann (in press cf. 1945) gave 30-50 mg./day of 17-methylandrostanediol-3(α), 17(α) to two preadolescent boys and obtained a moderate but questionable retention of nitrogen. The experimental conditions were not too satisfactory in these studies.

m. Estrone. Normal Girls. Johnston (1941) administered estrone in doses of 12,000 and 36,000 "units" for 6-18 days to 8 apparently normal girls at puberty and found an increase in the excretion of urinary nitrogen in 3 of the girls and no effect or a possible slight retention in the other girls. He recognized and emphasized that since the girls were normal, the effects obtained were a measure of excessive amounts of the estrogen and not of a substitution therapy.

n. α -Estradiol and α -Estradiol Benzoate. Hypogonadism. Knowlton, Kenyon *et al.* (1942) administered 5 mg./day of α -estradiol benzoate to 2 eunuchoids and 1 hypogonad woman for 4-20 days and noted a decrease in nitrogen excretion comparable to that obtained with 5 mg./day of testosterone propionate.

Normal Woman. Knowlton, Kenyon *et al.* (1942) injected 5 mg./day of estradiol benzoate into a 19 year old normal girl and obtained the characteristic decrease in urine nitrogen.

Adrenal-Cortical Dysfunction. Knowlton, Kenyon *et al.* (1942) in contrast to their previous experiments were unable to induce nitrogen retention in a masculinized girl by injecting 5 mg./day of α -estradiol benzoate in two experiments of 5 and 6 days each.

Thorn and Engel (1938) gave a subcutaneous injection of 17 mg. of α -estradiol to a woman with Addison's disease who was maintained up to the time of the experiment on whole adrenal cortical extract. They observed a maximum nitrogen retention of 2.52 g. on about the third day after the injection.

Albright (1942-1943) administered 1.66 mg./day of α -estradiol benzoate to patients with Cushing's syndrome who already were in maximum positive nitrogen balance induced by testosterone propionate and observed no significant changes. Parloff, Rose and Sunderman (1943), however,

obtained a definite nitrogen retention in a similar patient with the same dose before treatment with testosterone propionate.

o. Diethylstilbestrol and Its Dipalmitate. Adrenal Cortical Dysfunction. Deakins, Friedgood and Ferrebee (1944) injected a 15 year old girl with Cushing's syndrome with successively increased doses of 2, 5 and 10 mg./day of diethylstilbestrol. There was a suggestion of a decrease in nitrogen excretion. Perloff, Rose and Sunderman (1943) were able to obtain a definite decrease on administering 1-2 mg./day of the same compound to a 30 year old woman with a similar affliction. Williams, Whittenberger *et al.* (1945) injected a 36 year old woman with Addison's disease with 5 mg./day of stilbestrol dipalmitate for 6 days and obtained an *increased* excretion of nitrogen in the urine. This contrary effect, however, probably was due to the anorexia, nausea and pronounced weakness suffered by the patient two days after initiation of therapy.

Reifenstein (1942) has cited a number of other patients treated with different estrogens by various investigators. These data, however, are not sufficient to evaluate completely.

p. Progesterone. Hypogonadism. Knowlton, Kenyon *et al.* (1942) did not find any change in the nitrogen excretion of a hypogonadal woman injected for three days with 15 mg./day of progesterone while in nitrogen equilibrium, or later while in positive nitrogen balance as a result of α -estradiol benzoate treatment.

Adrenal Cortical Dysfunction. Albright, Parsons and Bloomberg (1941) obtained a small but definite nitrogen retention on giving 25 mg./day of progesterone to a 43 year old woman with Cushing's syndrome. The retention became evident on about the 5th day, continued for the 25 days of treatment and was only about one fifth that induced by subsequent treatment with 25 mg./day of testosterone propionate.

q. Δ^5 -Pregnenol- β (β)-one-20. Normal Man. Abels (1944) injected 100 mg./day of Δ^5 -pregnenol- β (β)-one-20 for 36 days to a normal man and obtained a positive nitrogen balance during the next 18 days and a negative nitrogen balance on the last 9 days. Part of the nitrogen retained was accounted for by extra circulating protein.

VI. THE EFFECT OF STEROID HORMONES ON THE NITROGEN CONSTITUENTS OF URINE AND BLOOD

Urea and Non-Protein Nitrogen

a. Dog. Kochakian and Murlin (1935) demonstrated that the changes in total nitrogen excretion obtained during and after the injection of castrated dogs with "male hormone" urine extracts were paralleled by comparable changes in the urea of the urine without any significant changes

in the ammonia (Fig. 5). This observation was confirmed in their experiments (Kochakian and Murlin, 1936) with androstanedione, and at the same time they observed that the urea and N.P.N. of the blood did not increase but, in fact, decreased. The results of Gaebler and Tarnowski (1943) on the blood N.P.N. of normal and depancreatized bitches injected

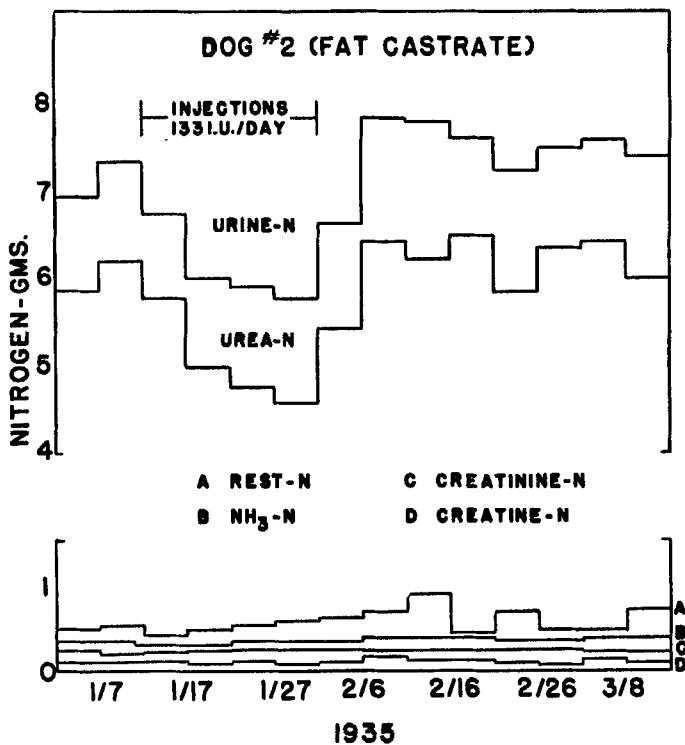


FIG. 5

The Effect of "Male Hormone" Urine Extract on the Various Nitrogen Constituents in the Urine of the Castrated Dog.

Note parallelism between total urine nitrogen and urea nitrogen.

with testosterone propionate and also estrone were in complete agreement with those of Kochakian and Murlin in the castrated dog.

b. *Man.* The urea in the urine has been determined in only two of the many studies in man. Kenyon, Knowlton *et al.* (1938) demonstrated that the urea nitrogen paralleled the changes in total urinary nitrogen of one of their eunuchoid subjects injected with testosterone propionate. Similar

changes in the urinary urea of a girl with Addison's disease were observed by Talbot, Butler and MacLachlan (1943) after testosterone propionate and methyltestosterone treatment. No changes occurred with desoxycorticosterone acetate.

The observations of Kochakian and Murlin on the urea and N.P.N. of the blood have been confirmed in several experiments in man. Kenyon, Knowlton *et al.* (1938); Kenyon, Knowlton *et al.* (1940) and Knowlton, Kenyon *et al.* (1942), in eunuchoidism; Kenyon, Knowlton *et al.* (1940), in normal men and women; and Kenyon, Knowlton *et al.* (1942a), in aged men treated with testosterone propionate, found no change or a decrease in N.P.N. and urea. Similar results were obtained by Knowlton, Kenyon, *et al.* (1942) with α -estradiol benzoate but not with progesterone. Williams, Whittenberger *et al.* (1945) determined the blood N.P.N. of a woman with Addison's disease treated successively with testosterone propionate, stilbestrol dipalmitate, androstanediol-3(α), 17(α) and 17-methylandrostanediol-3(α), 17(α) but came to no conclusions. The figures presented suggest a decrease after testosterone propionate injections and no changes after the other treatments.

Butler, Talbot *et al.* (1945) found a decrease in the excretion of urea and a slight increase in the excretion of ammonia on injecting a fasting man with testosterone propionate. The increase in ammonia was accompanied by a large increase in organic acids and acetone bodies. The administration of glucose inhibited all three effects. Amino acid excretion was not significantly altered.

Werner and West (1943) found no consistent changes in the uric acid excretion of a man and a woman with Simmond's disease on treatment with methyltestosterone.

2. Protein

To obtain information concerning the protein fabrication processes stimulated by the steroid hormones, studies have been made on the protein constituents of blood. Kenyon and his associates found that 25 mg./day of testosterone propionate did not change the plasma proteins of three eunuchoids (Kenyon, Sandiford *et al.* (1938), Kenyon, Knowlton *et al.*, 1940). Williams, Whittenberger *et al.* (1945) found no changes in the serum protein of a 36 year old woman with Addison's disease after treatment with 25 mg./day of testosterone propionate, 5 mg./day of stilbestrol dipalmitate, androstanediol-3(α), 17(α) or 17-methylandrostanediol-3(α), 17(α). Bassett, Keutmann, and Kochakian (1943d), on the other hand, found a small increase in the serum protein and an appreciable increase in the proteinuria of a 24 year old man with nephrosis on injection with 25 to 50 mg./day of testosterone propionate. The greatest increase occurred in

the couple of periods following cessation of injections, during which there is a rebound in the urinary nitrogen. Abels (1944) and Abels, Young and Taylor (1944) found that the injection of 90 mg./day of testosterone or 100 mg./day of Δ^6 -pregnenol-3 β , one-20 to a normal male first decreased and then increased the serum protein, while 50 mg./day of testosterone propionate to 2 normal males and 3 patients with gastric cancer produced a decrease with a subsequent return only to the original values during injections. They concluded that there is at first a large stimulus for the synthesis of tissue protein which is carried on partly at the expense of the serum protein.

3. Creatine-Creatinine

The accumulated evidence (*cf.* Hunter (1928), Rose (1935) and Wang (1939)) that there probably is a connection between sexual activity and creatine metabolism prompted further investigation of this problem in many species with the hormones of the gonads as soon as they became available.

a. Dog. Kochakian and Murlin (1935) noted that the creatine excretion of castrated dogs paralleled the changes in total and urea nitrogen after a single injection (777 I.U.) of "male hormone" urine extracts. This effect was present but not sufficiently definite on injection of smaller (45 to 133 I.U.) repeated doses (Fig. 4). The creatinine excretion remained unchanged. These investigators also noted that normal dogs while on the same diet as the castrated dogs exhibited a similar creatinuria. When the diet was made creatine-free, the creatinuria disappeared. It seemed, therefore, that the castrated dogs were retaining exogenous creatine along with the increased protein anabolism induced by the androgen preparation. Furthermore, the demonstration of an exogenous origin of the creatinuria provided an explanation for the inability of Shen (1925) to confirm the creatinuria observed in Chinese eunuchs by Read (1921). Later Nitzescu and Gontzea (1937a) in their studies on the role of the liver in creatine metabolism also noted that dog urine normally contained creatine, which disappeared when meat was omitted from the diet. Buhler (1935) extended his previous studies in man (Buhler, 1933) to similar investigations in the dog. He found that normal littermate control and castrated male dogs exhibited the same degree of creatinuria even after parenteral administration of creatine. In both instances the creatinuria was decreased by the injection of "Proviron", a "male hormone" extract prepared from urine. In addition, the creatinuria of normal dogs, but not of castrated or immature dogs, was decreased by "Prolan", a gonadotrophin preparation. Thus, the testes of the normal but not the immature dog was stimulated to produce androgens which exhibited the same effect on the creatinuria as the injected material.

b. *Rabbit.* Schrire and Zwarenstein (1932a) reported that castration of adult male rabbits caused a 25–50% increase in creatinine excretion which fell to the normal level on transplantation of testes and reappeared when the testes grafts were removed. In subsequent studies they reported that the increased creatinine excretion produced by castration was decreased by injection of a suspension of testes, testicular extracts (Schrire and Zwarenstein, 1932b) and lipoid extracts of the testes (Cheetham and Zwarenstein, 1938). When this last preparation was injected into normal and recently castrated male adult rabbits, there was a reverse effect—an increase in the urinary excretion of creatinine. Furthermore, it had no effect in castrated rabbits that had not shown the expected increase in creatinine excretion after castration. These results were similar to those previously obtained (Schrire and Zwarenstein (1933)) with "antuitrin" and extracts of the anterior pituitary. The injection of an extract of adult male urine caused a fall, followed by a rise above normal, and then a return to normal in creatinine excretion. Buhler (1935) found that castration not only increased the excretion of creatinine but also of creatine by rabbits. The creatinuria could be diminished in both castrated and normal rabbits by the injection of estrogen (Progynon) or androgen (Proviron). The creatinine, however, in contrast to the results of Schrire and Zwarenstein was not affected by the injection of either hormone. Seghini (1937) found that the intense creatinuria of recently castrated rabbits spontaneously disappeared one month after castration and could also be decreased by the injection of testicular extract. On cessation of injections, the creatinuria reappeared. More recently Williamson and Gulick (1941) showed that 2.5 mg./day of testosterone propionate decreased within 4 days the creatinuria of normal male rabbits with or without the administration of creatine. The retained creatine apparently was diverted to the muscle which showed an increase in creatine without any change in the blood. The blood and urine creatinine were not affected.

c. *Rat.* Kun and Peczenik (1935) reported that the injection of "male sex hormone" prevented the creatinuria induced in castrated male rats by feeding creatine. They suggested that there is a sex specificity regarding the effect of sex hormones in creatine metabolism since they found that estrogens induced endogenous creatinuria in normal female and castrated male rats. Beard and his associates in a series of papers (Pizzolato and Beard (1939), Koven and Beard (1939), Beard, Espenan *et al.* (1941) and Beard and Jacob (1940)) reported that "sex hormones" (estrone, progesterone and testosterone propionate) injected into normal rats produced a creatinuria which was intensified by simultaneous administration of creatinine. These effects were further intensified by castration but disappeared 90 days later due probably to secondary changes in other glands (*cf.* Parkes (1945)). Since saline alone increased creatinuria, Beard and his

associates suggested that the sex hormones were assisting in the hydrolytic transformation of creatinine to creatine through their salt- and water-retaining effects (Thorn and Harrop 1937).

The apparently anomalous results of Beard and his associates become somewhat clearer after considering the study of Coffman and Koch (1940). They found no indication of a greater creatinuria in rats two months after castration; furthermore, both the normal and castrated rats showed a similar intense creatinuria on ingestion of 40 mg. of creatine/kg. body weight/day. The induced creatinuria was diminished in both groups of rats by the injection of 0.9 mg./day of testosterone propionate. The creatinuria of the castrated rats, however, was decreased more than that of the normal rats. After about the 7th day of injections the creatinuria began to increase again in spite of continued injections. On cessation of androgen and creatine treatments, the creatine content of the urine returned to its normal low level. There is one noteworthy and fundamental difference between the experiments of Beard and associates and those of Coffman and Koch. The former not only treated their rats for a longer period of time but also determined the creatine on the pooled urine of the entire period, while Coffman and Koch fortunately made daily analyses of the urine. On the basis of these facts the results of Beard and associates may be construed to reflect the second or the increased creatinuria phase which in their experiments was apparently large enough to overshadow the initial decrease in creatine observed by the Chicago investigators.

After completing their experiment on the rats, Coffman and Koch placed these same rats on a similar experiment with a stock diet for 19 days and determined the creatine of the gastrocnemius muscle and found no changes between controls and treated castrated and normal animals. It seems that either testosterone propionate had not diverted any of the retained creatine to the muscle or that the creatinuria obtained on further treatment was due, at least in part, to loss of the initially retained creatine. The latter possibility is supported by the observations of Williamson and Gulick (1941) in the rabbit. It would be extremely valuable to follow muscle creatine simultaneously with urine creatine. Coffman and Koch observed no effect by testosterone propionate in creatinine excretion but it is noteworthy that the ingestion of creatine alone increased the excretion of creatinine by both normal and castrated rats.

d. Monkey. Buhler (1935) found that the creatinuria of normal or recently (2-3 weeks) castrated monkeys was not affected but that of 2½ months castrated monkeys was decreased by the injection of "Proviron", an androgen extract of urine, but an estrogen, "Progynon", had no effect. Jailer (1940) observed that 5 mg./day of testosterone propionate decreased and, in some instances, completely abolished in 2 to 5 days the creatinuria of immature and castrated mature male monkeys. The testosterone pro-

pionate also restored the capacity to retain administered creatine. Follutein (chorionic gonadotrophin) 400 R.U. on alternate days caused the complete disappearance of creatinuria in an immature monkey. α -Estradiol benzoate, on the other hand, was ineffective in both immature and mature monkeys at 100 and 200 I.U. per day, respectively. The creatinine excretion was not affected by the above treatments. In a later study, Jailer (1941) demonstrated that 10 mg./day of testosterone propionate was able to decrease, and in some instances abolish, within 3 days the intense creatinuria produced in monkeys by the injection of 0.75 or 1.0 mg./day of thyroxin.

e. Man. The spontaneous creatinuria of children has been investigated by a number of workers. Both Fasold (1932) and Buhler (1933) were unable to find any effect on the creatinuria of prepubertal children treated with large doses of estrogens. A lack of effect was also noted by Buhler (1933) after the injection of the androgen preparation "Proviron". More recently Duckworth (1942) reported that neither 25 mg. of testosterone propionate three times per week nor Follutein, chorionic gonadotrophin, affected the creatinuria (83 to 577 mg./24 hrs.) of prepubertal children. Androgen treatment of a 9 year old boy with Frohlich's syndrome did decrease the creatinuria. Wilkins, Fleischmann and Howard (1941) also were unable to find any change in the creatine (45 mg./24 hrs.) or creatinine excretion of a 17 year old boy treated with 5 to 25 mg./day of testosterone propionate while on a creatine-free diet. They also found no nitrogen retention. In contrast to the observations of Duckworth (1942) and Wilkins, Fleischmann and Howard (1941), Hoagland, Gilder and Shank (1945) observed that 20 mg./day of testosterone propionate produced a sharp drop in the creatinuria (about 600 mg./24 hrs.) of normal children within 72 hours following the administration of the androgen. The decrease persisted as long as the hormone was given. On cessation of injections, a marked creatinuria ensued for several days. In patients with progressive muscular dystrophy, there was no decrease in the creatinuria. But on withdrawal of the hormone there was a marked increase similar to that observed in normal patients. The androgen had no significant effect on the glycoccyamine or creatinine excretion in either series of patients. The interesting report of Whitelaw (1944) that 25 mg. of testosterone propionate three times per week produced a creatinuria accompanied by a slight increase in creatinine and a creatinemia is reminiscent of the results of Coffmann and Koch (1940) in rats. Unfortunately, the data are not too well substantiated; a single set of control determinations are compared with another set made after prolonged treatment. Nitzescu and Gontzea (1937b) have proposed the interesting hypothesis that since the creatinuria produced by the growth hormone was nullified by sex hormones, the creatinuria of childhood is due to the growth hormone.

Wilkins, Fleischmann and Howard (1941) made the surprising and

valuable discovery that the synthetic androgen, methyltestosterone, produced an intense creatinuria in dwarfed boys and girls. Wilkins and Fleischmann (1945) extended this observation by further experiments in these same subjects, while on a relatively creatine-free diet, with a number of other steroids. They found that this effect is not produced by any of the non-methylated steroids in the doses used: androsterone, 20 mg./day for 10 days; dehydroisoandrosterone acetate, 10-40 mg./day for 13 and 18 days; Δ^4 -androstenedione-3,17, 20 mg./day for 12 days; testosterone, 20 mg./day for 16 and 18 days; testosterone propionate, 25 mg./day for 15 or more days; Δ^5 -androstenediol-3(β),17(α), 10 mg./day for 13 days; its diacetate 45 mg./day for 16 and 23 days; androstanediol-3(α),17(α) diacetate-3,17,20-40 mg./day for 16 to 24 days; and 17-ethyltestosterone, 40 mg./day for 20 days. All of the 17-methylated compounds tried were effective. Thus, 17-methyltestosterone, 25 mg./day by mouth, was effective in all of the 15 cases tried and also effective when given intramuscularly at 20 mg./day for 16-18 days, indicating that the route of administration was not involved; 17-methyl- Δ^5 -androstenediol-3(β),17(α) was effective in 5 cases when given in oral doses of 30-50 mg./day and by intramuscular injections of 10 mg./day for 16-45 days; 17-methylandrostanediol-3(α),17(α), 50 mg./day by mouth, was effective in one case but not in another. The increased creatinuria produced by the 17-methylated steroids did not become apparent until after a latent period of 4 to 16 days and progressively increased until the third to sixth months, after which the excretion was maintained at the high level as long as the androgen was administered. Creatinine excretion followed a similar pattern. On cessation of treatment there was a further increase or release of the stored creatine. This effect was also observed after cessation of testosterone propionate injections. The authors believe that the 17-methylated compounds stimulate both synthesis and storage while testosterone propionate does only the latter.

Since testosterone propionate and methyltestosterone have opposite effects on creatine excretion, Wilkins and Fleischmann (1945) tried to inhibit the intense creatinuria produced in one patient by the oral administration of 25 mg./day of methyltestosterone for 36 days by the injection of an equal amount of testosterone propionate. They found no change in the intense creatinuria or the increased excretion of creatinine. Hoagland, Gilder and Shank (1945) found that normal children and children with progressive muscular dystrophy also responded to methyltestosterone treatment, 20 mg./day for 10 days, with an intense creatinuria but they did not show a further increase on the few days after withdrawal of the androgen or any changes in the excretion of creatinine. The failure to observe these effects, however, may have been due to the short period of treatment. These investigators made the added important observation that

the glycoccyamine excretion changed in parallel with the creatine. The administration of 5 and 10 mg. of desoxycorticosterone acetate three times weekly for 40 days to two patients with moderately severe muscular dystrophy produced no changes in the excretion of creatine or creatinine.

There are many studies on the effect of steroid hormones on the excretion of creatine and creatinine by normal men and women and also those suffering from various afflictions. The early experiments with potent urine extracts of the sex hormones forecast the later results obtained with the crystalline steroids. Buhler (1933) reported that "Proviron" and "Progynon" were sex-specific in decreasing the creatinuria induced by the administration of 500 mg. of creatine to old men and women with hypogonadal or hypothyroid function and with muscular dystrophy. Schittenhelm and Buhler (1935) confirmed the effect of "Proviron" in men with anatomical or functional gonadal insufficiency. Usui, Miwa and Aoki (1935) also found that male hormone urine extract, "Enarmon", caused a "remarkable" decrease in the urinary excretion of creatine in old men and patients with Addison's disease. In contrast to the observations of Buhler (1933), Paschkis and Schworer (1936) reported that the sex hormone preparations were not sex-specific in decreasing the creatinuria of old men and women after the administration of creatine or glycine.

The studies on the nitrogen-retaining properties of the pure steroid hormones were accompanied in many instances by determinations of the excretion of creatine and creatinine in the urine. Kenyon, Sandiford *et al.* (1938) and Kenyon, Knowlton *et al.* (1940) found that the creatinine and slight creatine excretion of eunuchoids was not affected by the injection of 25 mg./day of testosterone propionate. Similar treatment also failed to affect the creatinuria, 200 mg./day, of an aged man (Kenyon, Knowlton *et al.*, 1942a). A smaller creatinuria (100–189 mg./day) of 2 normal women, however, was decreased by identical treatment (Kenyon, Knowlton *et al.*, 1940). Furthermore testosterone propionate at 25 mg./day or 5 mg./day decreased the marked creatinuria (0.844 and 1.5 mg./day) of eunuchoids while on a mixed diet (Kenyon, Sandiford *et al.*, 1938), or induced by the oral administration of 1 g./day of creatine (Kenyon, Knowlton *et al.*, 1940) and the dietary creatinuria of a hypogonad woman (Knowlton, Kenyon *et al.*, 1942). On cessation of injections, the creatinuria increased above that of the control period.

At first Kenyon, Sandiford *et al.* (1938) and Kenyon, Knowlton *et al.* (1940) found no change or only the suggestion of a slight decrease in creatinine excretion on injection of testosterone propionate. In a later study, however, with more prolonged treatment Sandiford, Knowlton, and Kenyon (1941) found a significant increase which first became apparent about the twenty-second day (cf. Fig. 6). They attributed this increase

in creatinine excretion to be a reflection of the development of a greater muscular mass.

The decrease in spontaneous (dietary) creatinuria and increase in creatinine excretion after injection of testosterone propionate has also been observed by Deakins, Friedgood and Ferrebee (1944) in a 15 year old girl with Cushing's syndrome, by Kinsell, Hertz and Reifenstein (1944) in three patients with thyrotoxicosis and by Keutmann, Bassett and Kochakian (1944) in a patient with Cushing's syndrome, a young man recovering from an emaciated condition, a man with nephrotic syndrome and two normal young men. The last investigators also demonstrated that the increased excretion of creatine on cessation of injections was more than the amount actually retained and concluded that testosterone propionate not only favored storage of creatine but also stimulated synthesis of this substance.

Williams, Whittenberger *et al.* (1945) found no change in the slight creatinuria of a male patient with Addison's disease but a decrease in two women suffering from the same disease and with a greater initial creatinuria. The creatinine showed no changes in any of their patients. Fleischmann (1941) was unable to change the creatine or creatinine excretion of patients with amyotonia congenita and progressive muscular dystrophy by the injection of moderate doses, 25-50 mg. twice per week for two weeks, of testosterone propionate. Butler, Talbot *et al.* (1945) found that testosterone propionate decreased the creatine and increased the creatinine excretions of young men during starvation.

At about the same time that Wilkins, Fleischmann and Howard (1941) observed the production of intense creatinuria in dwarfed children by the administration of methyltestosterone, Samuels, Henschel and Keys (1942) noted, also unexpectedly, that an identical phenomenon occurred in normal young men. Tager (1943) observed that eunuchoids showed a similar response to this compound if the dose was 40 mg. or more per day, but when it was lowered to 20 mg., or less, there was no excessive creatinuria. He suggested that the large doses of the steroid act as methylating agents. This hypothesis, while enticing, is not tenable on a quantitative basis. There are not sufficient methyl groups provided by the steroid to account for the number necessary in the synthesis of the creatine excreted. Werner and West (1943) obtained both creatinuria and increased creatinine excretion in 2 patients with Simmond's disease. The results of Williams, Whittenberger *et al.* (1945), however, do not show a definite creatinuria in either a woman with Simmond's disease or another woman with Addison's disease. In both instances there was an increase in creatinine on administration of methyltestosterone at 60 and 50-25 mg./day. Deakins, Friedgood and Ferrebee (1944) obtained an initial short decrease followed by an intensive creatinuria in a young girl with Cushing's syndrome on adminis-

tering 40 mg./day of methyltestosterone. The creatinuria showed a further increase before decreasing on withdrawal of treatment. There was no significant effect on the creatinine. A similar dose of testosterone by mouth had no effect. Kinsell, Hertz and Reifenstein (1944) showed that methyltestosterone produced an intense creatinuria and an increased creatinine excretion in male and female patients with thyrotoxicosis. On subsequent injection with testosterone propionate, the creatinuria was decreased or even abolished.

The only other 17-methyl steroid, 17-methylandrostanediol-3(α), 17(α), studied in adults was shown by Keutmann, Bassett and Kochakian (1944) to have an effect on the creatinuria and creatinine excretion of a eunuchoid patient identical to that obtained with methyltestosterone. There was an initial brief decrease followed by a progressive increase which became even greater for several days after cessation of treatment. Williams, Whittenberger *et al.* (1945), on the other hand, found that the same steroid decreased the creatinuria of a female patient with Addison's disease.

Knowlton, Kenyon *et al.* (1942) found that 5 mg./day of α -estradiol benzoate produced a slight or variable decrease in the creatinuria of eunuchoids, a definite and prolonged decrease in an hypogonad woman and the reverse effect, a definite increase, in a masculinized girl. The effects of diethylstilbestrol (Deakins, Friedgood and Ferrebee, 1944), and stilbestrol dipalmitate (Williams, Whittenberger *et al.*, 1945) are difficult to evaluate because of the conditions of these experiments.

VII. THE LACK OF EFFECT OF STEROID HORMONES ON FECAL NITROGEN EXCRETION

The fecal nitrogen has been studied in only surprisingly few of the many investigations. Kochakian and Murlin (1935) in their original studies definitely demonstrated that "male hormone" urine extracts had no effect on the output of nitrogen in the feces of dogs either during or after treatment. Similar results have been obtained in extensive studies in rats (Kochakian, 1944a) and in the few cases studied in man. Knowlton, Kenyon *et al.* (1942) found no effect in the fecal nitrogen of two eunuchoids treated with testosterone propionate; of the same eunuchoids, one hypogonad woman, and one normal woman after treatment with α -estradiol benzoate and one hypogonad woman treated with progesterone. Perloff, Rose and Sunderman (1943) obtained no change in fecal nitrogen on treating a 30 year old woman suffering from pituitary basophilism (Cushing's syndrome) with α -estradiol benzoate, testosterone propionate or stilbestrol. Bassett, Keutmann and Kochakian found no significant changes in the fecal nitrogen of a male nephrotic (1943d), a girl with Cushing's syndrome (1943c), 2 normal males (1943b) and a eunuchoid (1944) injected with testosterone propionate. The same eunuchoid showed no change in

fecal nitrogen excretion on oral treatment with 17-methyltestosterone and 17-methylandrostanediol-3(α),17(α) (1944). Talbot, Butler and MacLachlan (1943) found the fecal nitrogen output of an 8-year old girl with Addison's disease treated with methyltestosterone to be constant and at 8% of the nitrogen intake during the first five 3 day periods. Therefore, a fecal nitrogen value of 8% was assumed for subsequent periods. In a later study Talbot, Butler *et al.* (1945) found by chemical analyses that the fecal nitrogen of a child suffering with progeria was not affected by successive treatments with methyltestosterone, testosterone propionate, testosterone propionate plus α -estradiol benzoate and testosterone propionate. The average fecal nitrogen output was 6.8% and 6.3% at food intakes of 8.29 and 11.14 g. of nitrogen/day, respectively. Albright, Bloomberg and Parsons (1941) in their study on Cushing's syndrome assumed a fecal nitrogen value of 10% of the intake. In a later study from the same laboratory, on patients with hyperthyroidism, Kinsell, Hertz and Reifenstein (1944) found this assumption to be essentially correct by analysis in one instance while the subject was in strong positive nitrogen balance as a result of testosterone propionate injections. A more comprehensive study (Albright and Reifenstein (1944)) in which the food and feces nitrogen values were obtained by chemical analyses showed that the 10% value was true only for average food intakes, e.g., 12 to 13 g. nitrogen/day. There were wide discrepancies when the food intake varied from the average.

No chemical determinations of fecal nitrogen were made in the many other studies cited. The nitrogen balances were obtained by noting the changes in urinary nitrogen excretion in previously established control periods.

It should be mentioned at this time that the nitrogen intake of the subjects in the above studies were calculated from figures given in standard tables. Those investigators, however, who determined the fecal nitrogen always made chemical analyses on aliquots of the food eaten by their subjects also. The latter procedure of complete analysis of intake and output provides absolute values for quantitative comparison and calculations.

VIII. THE EFFECT OF STEROID HORMONES ON ELECTROLYTE⁸ AND WATER METABOLISM

1. Dog

Thorn and Harrop (1937), impressed by the close chemical relationship between the sex hormones and the hormones of the adrenal cortex (*cf.*

⁸ The reader is referred to Gardner and Pfeiffer (1942) and Albright (1942-1943) for information concerning the effect of steroid hormones on calcium and phosphorus metabolism.

Reichstein and Shoppee (1943)) and by the prolonged survival of animals adrenalectomized during estrus or pregnancy, felt that the steroid hormones from different organs might have one, or more, common physiological property. Therefore, they investigated the effect of various "sex hormones" on sodium and water excretion in a 24 hour test on normal male and female dogs and compared the response to that obtained with a standardized adrenal cortical extract. On comparing the effect of 10 mg. of each of the steroids, they obtained the following relative potency with respect to ability to retain sodium: α -estradiol 700, progesterone 400, estrone 200+, pregnanediol 140, testosterone 80 and testosterone propionate 25+. The effect of α -estradiol, in contrast to the adrenal cortical extract, was found to be prolonged. Along with the retention of the sodium, there was a retention of water. As the effect of the hormone wore off, a sodium and water diuresis occurred. In a later study, Thorn and Engel (1938) extended these observations to adrenalectomized dogs and included observations on chloride, potassium and inorganic phosphorus. Progesterone, in single subcutaneous injections of 1 to 5 mg., had no effect on the renal excretion of electrolytes by normal male dogs. A single injection of 5 to 20 mg., however, caused a decrease in sodium, chloride and water and a slight increase in potassium excretion in the 24 hour period following the injection. Then the values returned to normal without any "rebound". The injection of 20 mg. of progesterone into an adrenalectomized dog prevented the weight loss and sodium and chloride and water diuresis that occurs immediately on withdrawal of adrenal cortical extract treatment (Thorn, Engel and Eisenberg, 1938, and Thorn, Howard and Emerson, 1939. In normal dogs the single subcutaneous injection of 5 mg. of α -estradiol, 15 mg. of estrone or 40,000 to 100,000 I.U. "Amniotin" resulted in a marked decrease in the renal excretion of water, sodium chloride and inorganic phosphorus; potassium excretion was slightly increased. A reverse effect usually occurred after the period of retention which was maintained for 24 to 72 hours. The injection of 20,000 to 100,000 I.U. of "Amniotin" into an adrenalectomized male dog partially prevented the effects of adrenal cortical insufficiency. Testosterone propionate proved to be relatively ineffective. The single subcutaneous injection of 25 mg. of testosterone propionate in three normal dogs had no appreciable effect. A large dose of 125 mg. had a slight effect on sodium excretion, but decreased the urine volume and the excretion of chloride and phosphorus. Repeated injections of 25 mg./day for 7 days produced a decreased excretion of potassium and phosphorus which did not become evident, however, until the three day period following cessation of injections. The sodium, chloride and urine volumes were not altered. The substitution of 25 mg./day of testosterone propionate for adrenal cortical extract treatment in an adrenalectomized dog did not prevent any of the changes due to adrenal cortical insufficiency. The injection of

40 mg. of Δ^4 -androstenediol- $3(\beta),17(\alpha)$ or 40 mg. of androstenedione had no effect on the electrolyte excretion of normal dogs.

2. Rat

Selye and Dosne (1941) found that injections of 1 mg./day of testosterone for 10 days decreased the blood chloride of rats but not as effectively as similar treatment with desoxycorticosterone. Both of these steroids inhibited the increase in blood chloride obtained by injections of α -estradiol. Progesterone was ineffective. Miller (1943), however, observed no changes in the chloride, sodium or potassium in the serum of rats injected for 14–26 days with 1 mg./day of testosterone propionate, 0.080 to 0.310 mg./day of α -estradiol benzoate or 1 mg./day of progesterone. The α -estradiol benzoate and progesterone, however, decreased the potassium but not the chloride, sodium or phosphorus content of muscle. On extension of the injections for 6 months the estrogen caused a further decrease in the muscle potassium and the testosterone propionate showed a decrease similar to that obtained with estrogen and progesterone in the shorter periods of injection. The sodium and chloride were not affected.

3. Rabbit

Fichera and Catania (1938) reported that 1 mg. of testosterone per kg. of bodyweight increased the blood potassium of rabbits up to 12%.

4. Mouse

A small increase in the serum potassium of castrated mice implanted with pellets of testosterone propionate was observed by Kochakian (1941).

5. Man

Torok and Neufeld (1934) noted that the administration of an estrogen extract of urine or ovarian and testicular extracts caused a retention of chloride in 12 children 6–10 years old. Cessation of injections resulted in an excessive excretion. Thorn and Engel (1938) extended their experiments on dogs to patients with Addison's disease. The injection of 17 mg. of α -estradiol into a female patient with Addison's disease resulted in a marked and prolonged retention of sodium, chloride and inorganic phosphorus. The potassium and water excretion was maintained at the same level as when the patient was receiving adrenal cortical extract. The injection of 25 mg./day testosterone propionate for 7 days into a male subject with Addison's disease produced a decrease in the excretion of water, sodium, chloride and potassium. While Harrop and Thorn (1937) were carrying out their experiments on the effect of steroid hormones on renal electrolyte excretion, Kenyon, (1938) noted that eunuchoids treated for long periods

of time with testosterone propionate exhibited edema, indicating a gain in body water. The analysis of the urine of 4 of the patients while on a metabolic regime (Kenyon, Sandiford *et al.* (1938) showed a reduction in the excretion of sodium and chloride during injections with testosterone propionate followed by a diuresis on cessation of injections. These changes were paralleled by similar changes in the urine volume. The potassium excretion was not affected. In a later and better regulated study, Kenyon, Knowlton *et al.* (1940) were able to demonstrate a convincing decrease in the excretion of potassium as well as sodium, chloride and phosphorus. On cessation of injections there was a diuresis of all three elements but the potassium was not as rapidly or as completely discharged as the sodium and chloride. This difference was attributed to the extracellular nature of the sodium and chloride and the intracellular nature of the potassium. The fluid retained apparently was distributed between intracellular and extracellular extravascular compartments. There was no evidence of blood dilution as indicated by the constancy of the plasma protein concentration, red blood cell count and cell volume. The sodium, chloride and potassium of the serum were unchanged by testosterone propionate treatment but the inorganic phosphorus declined slightly during treatment and returned to normal on cessation of injections. Normal men and women responded in a comparable manner to the eunuchoids in the changes in electrolyte excretion after testosterone propionate injections. In subsequent studies not only these effects but also similar effects on sulfur metabolism were demonstrated in two eunuchoids and a hypogonad woman (Knowlton, Kenyon *et al.*, 1942), a short boy (Kenyon, Knowlton *et al.*, 1942b), two aged men (Kenyon, Knowlton *et al.*, 1942a), and a man and woman with Addison's disease (Kenyon, Knowlton *et al.*, 1943). These effects have been confirmed in whole or in part in a girl with Addison's disease (Talbot, Butler and MacLachlan, 1943), in women with Addison's disease (Williams, Whittenberger *et al.*, 1945), in progeria (Talbot, Butler *et al.*, 1945), and in fasting men (Butler, Talbot *et al.*, 1945).

Several other steroid compounds have been shown to have an effect on mineral and water metabolism similar to that of testosterone propionate. Eidelsberg, Bruger and Lipkin (1942) found that testosterone implants decreased the urinary excretion of chloride and phosphates by eunuchoids. The oral administration of methyltestosterone has been shown to be effective in patients with Simmond's disease (Williams, Whittenberger *et al.*, 1945), in a girl with Addison's disease (Talbot, Butler and MacLachlan, 1943), in a patient with both Addison's disease and *diabetes mellitus* (Armstrong, 1944), in a boy with progeria (Talbot, Butler *et al.*, 1945) and in a eunuchoid (Bassett, 1945). In the latter case there was an apparent difference between the action of methyltestosterone and testosterone

propionate. The retention of chloride after administering the first compound was delayed and, on cessation of treatment, was excreted immediately. Testosterone propionate, on the other hand, caused an immediate retention of the chloride followed by a gradual and extended loss on cessation of injections. The oral administration of androstanediol-3(α),17(α) and its 17-methyl derivative to two women with Addison's disease produced changes in the electrolyte balance similar to that reported for the testosterone derivatives (Williams, Whittenberger *et al.*, 1945).

Knowlton, Kenyon *et al.* (1942) have found that 5 mg./day of α -estradiol benzoate also produces changes in renal excretion of electrolytes in eunuchoids, a hypogonad and a normal woman but not in a masculinized girl. Williams, Whittenberger *et al.* (1945) on the other hand found an increased excretion of sodium, chloride and potassium on administration of stilbestrol dipalmitate to a woman with Addison's disease. Talbot, Butler and MacLachlan (1943) found that α -estradiol benzoate reduced the strongly positive balance in electrolytes produced by testosterone propionate in a girl with Addison's disease.

Kenyon, Knowlton *et al.* (1940) were unable to demonstrate any significant effect on the sodium, chloride or potassium of the serum by the injections of testosterone propionate for about one week in two eunuchoids. Butler, Talbot *et al.* (1942) by longer treatment with testosterone propionate and 17-methyltestosterone were able to demonstrate an almost complete disappearance of potassium from the serum of a girl with Addison's disease, 2 dwarf boys and a female suffering from hyperthyroidism. In the extension of their study (Talbot, Butler and MacLachlan, 1943) on the girl with Addison's disease, they found no changes in the serum sodium. Werner and West (1943) were also unable to obtain any change in the serum sodium of two patients with Simmond's disease treated with methyl-testosterone.

IX. THE EFFECT OF STEROID HORMONES ON ENERGY METABOLISM

The diverting of protein, under the stimulus of the potent steroid hormones, from catabolic to anabolic processes deprives the organism of a small amount of energy. There is, however, no decrease in the basal metabolism. In fact, there is an increase under certain circumstances.

1. Dog ~

Kochakian and Murlin (1935) noted that on prolonged treatment with "male hormone" urine extracts a "fat" castrated, but not a "thin" castrated, dog showed a small increase of 10% in the basal metabolism and the R.Q. tended to decrease. In no instance, however, was there an effect immediately after injections of "male hormone" urine extract, androstenedi-

one, testosterone or testosterone acetate. The energy to replace that lost to protein anabolism and the increase in energy when it occurred was obtained by the utilization of more fat (Fig. 6).

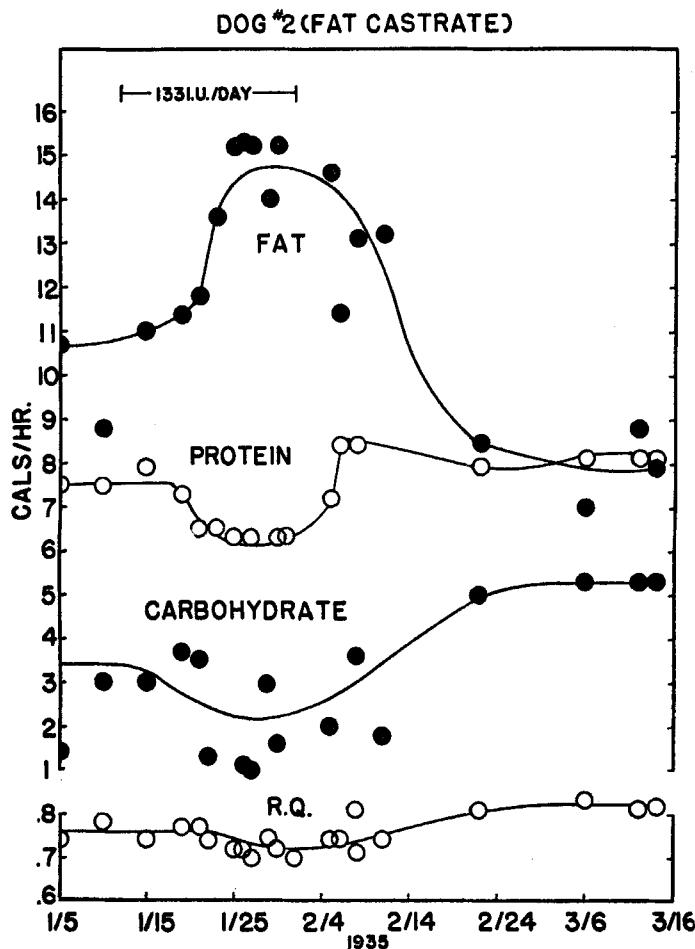


FIG. 6

The Effect of "Male Hormone" Urine Extract on the Partition of the Basal Caloric Requirements of the Castrated Dog.

(Kochakian and Murlin, *J. Nutrition* 10, 437 (1935).)

2. Rat

Neither testosterone propionate (Koch, 1937) nor methyltestosterone (Meyer and Danow, 1942) influence the basal metabolism of castrated

rats. The latter compound in daily doses of 0.5 mg. however, produces a moderate increase in the more sensitive thyroidectomized-castrated rats.

3. Man

Kenyon, Sandiford *et al.* (1938) and Kenyon, Knowlton *et al.* (1940) noted an increase of 8 to 14% in four eunuchoid patients on injection with testosterone propionate for 9 to 14 days. Another subject with a gross demonstrable pituitary lesion showed no change. A fifth eunuchoid showed no change during the first 10 days of treatment, but by the 40th day his energy metabolism had increased 19% (Kenyon, 1938), which was in agreement with the increase reported by Thompson and Heckel (1939) in a eunuchoid subject after two months' treatment. In a later study, Sandiford, Knowlton and Kenyon (1941) found variable but definite increases in the basal metabolism of six eunuchoids and one castrated man after prolonged treatment with testosterone propionate (Fig. 7; also cf. Fig. 3). The maximum effects obtained varied from 5 to 34%. The R.Q. was not altered. These same investigators did not observe any changes in the energy metabolism of normal men or women (Kenyon, Knowlton *et al.*, 1940), aged men (Kenyon, Knowlton *et al.*, 1942a) or a man and woman with Addison's disease (Kenyon, Knowlton *et al.*, 1943) after treatment for 6-10 days even though the other metabolic responses were observed. Galli (1938) found no significant increase in the basal metabolism of normal subjects treated for 6 days with testosterone propionate. Eidelberg and Ornstein (1940) did not find a significant increase in the basal metabolism of eunuchoids by administration of testosterone propionate but found that their patients became more sensitive to thyroid treatment. McCullagh and Rossmiller (1941) noted an increase from -27 to -17% in the basal metabolism of a eunuch on administering 25-50 mg./day of testosterone propionate. Bassett, Friedman and Kochakian (1942) found a restoration toward normal of the very low basal metabolic rate of a girl with Cushing's syndrome after administration of testosterone propionate injections. On prolonged treatment, however, the increase became erratic before it had reached normal. Escamillo and Lisser (1942) were not able to detect any significant increase in the energy metabolism of patients with Simmond's disease after prolonged treatment with 25 mg. of testosterone propionate three times per week. Kinsell, Hertz and Reifenstein (1944) found no change in the heightened energy metabolism of patients with thyrotoxicosis after the administration of 25, 50 or 100 mg./day of testosterone propionate.

McCullagh and Rossmiller (1941) observed striking and immediate increases of 12 to 54% in the basal metabolism of one eunuch and 9 eunuchoids on oral administration of 50 to 300 mg. of methyltestosterone.

In a subsequent study, Jones, McCullagh *et al.* (1941) confirmed these results in 5 of the previous patients and 6 others including a normal man and a man with heart disease. The hypermetabolic rate in these subjects was shown to be accompanied by a lowering of the R.Q. Byron and Katsen (1941) found an increase in the basal metabolism of two of three eunuchoids

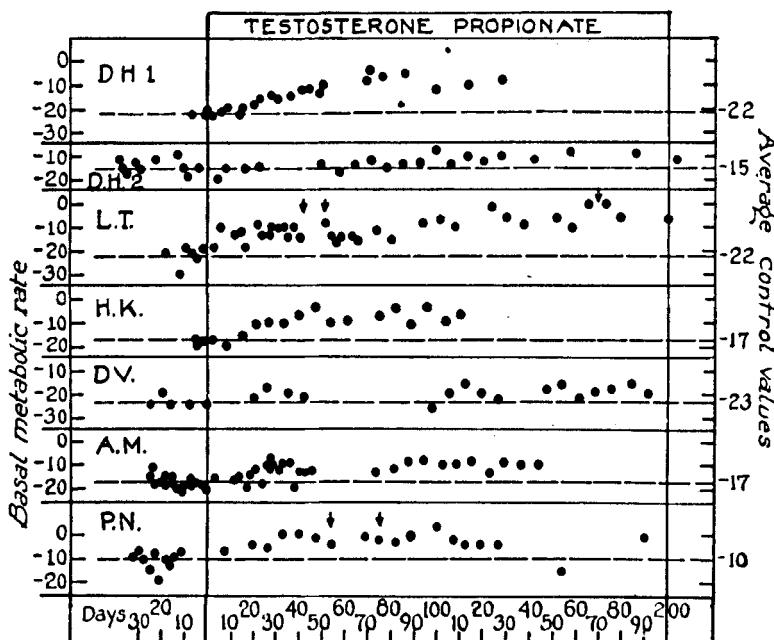


FIG. 7

Effect of Protracted Treatment with Testosterone Propionate on B.M.R. of Hypogonadal Men.

P. N. is a eunuch, the others eunuchoids. Testosterone propionate was given intramuscularly 3 times weekly with the following exceptions:—L. T. received no treatment between the first 2 arrows and 25 mg. 4 times weekly after the third arrow; P. N. received 50 mg. 3 times weekly between the first 2 arrows and 25 mg. twice weekly after the 2nd arrow.

(Sandiford, Knowlton and Kenyon, *J. Clin. Endocrinology* 1, 931 (1941).)

treated for long periods of time with methyltestosterone. Similar observations have been made by Wilkins, Fleischmann and Howard (1941), Howard, Wilkins and Fleischman (1942) and Wilkins and Fleischmann (1945) in pituitary dwarfs treated with daily oral doses of 25 mg./day of methyltestosterone. There were, however, irregular and unexplained large fluctuations so that one day a B.M.R. of +30% might be followed on the

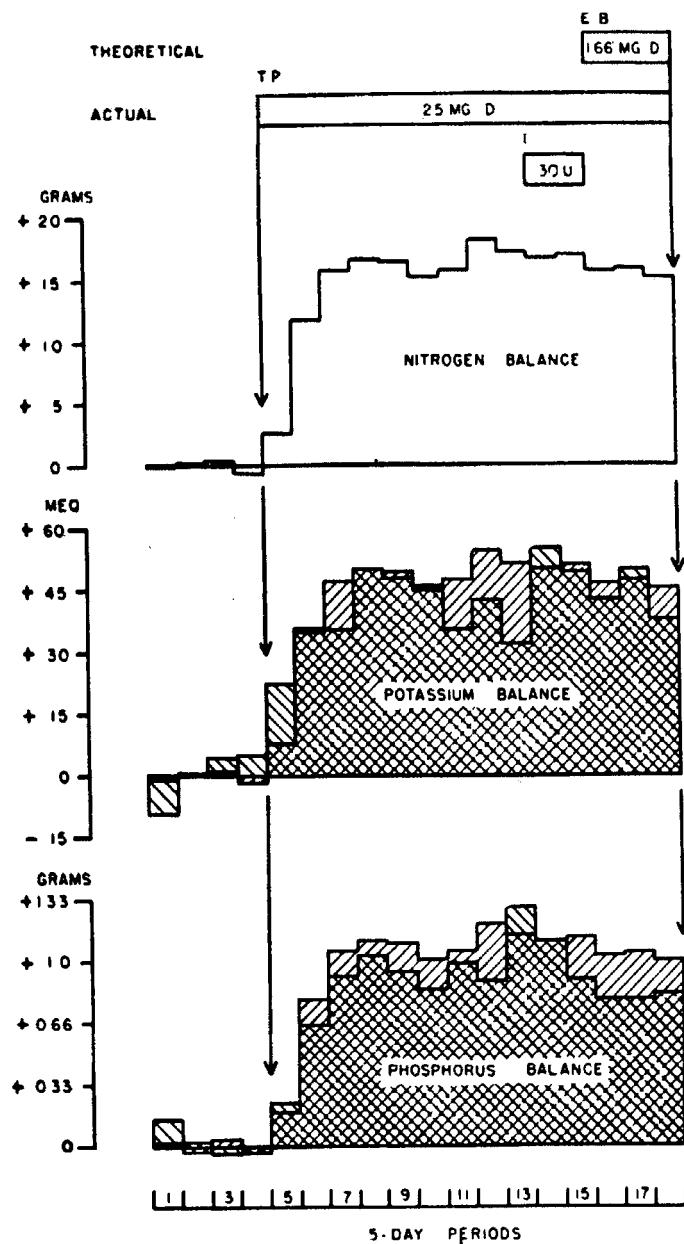


FIG. 8

next day by a normal value. Werner and West (1943) reported that the basal metabolism of a man and a young woman with Simmond's disease tended to rise after the oral administration of 100 mg./day of methyltestosterone. Kinsell, Hertz and Reifenstein (1944) found that methyltestosterone, in contrast to testosterone propionate, further increased the elevated basal metabolism of hyperthyroid patients.

Attempts to associate the hypermetabolic effect of methyltestosterone with stimulation of the thyroid gland by the above investigators has been unsuccessful. The changes in cholesterol and blood iodine are not indicative of such an effect.

X. THE EFFECT OF STEROID HORMONES ON TISSUE FORMATION

1. Body Weight⁹

The changes in nitrogen and electrolytes induced by the steroid hormones are paralleled by changes in body weight (Figs. 1, 2, 4, 6, 7). The changes in the various constituents are in the proportion in which they exist in tissue as shown by the calculations of Albright (1942-1943) (Fig. 8), Bassett, Keutmann and Kochakian (1943d), Talbot, Butler and MacLachlan (1943),

⁹ There have been many clinical reports without metabolic studies in which not only increases in body weight but also increases in height have been induced for as long as 2 years on the administration of testosterone propionate, methyltestosterone and also gonadotrophin to underdeveloped (dwarf) children by Moricard and Bize (1937), Webster and Hoskins (1940), Browne and Ross (1941), Dorf (1941), Gordon and Fields (1942), Finkler, Furst and Cohn (1942) and Albright (1942-1943); to eunuchoids by Usui, Ito *et al.* (1943), Foss (1937), Kenyon (1938), Rapfogel (1940), McCullagh and Rossmiller (1941), Escamillo and Lisser (1941) and Grauer and Alexander (1942); to persons with Simmond's disease by Escamillo and Lisser (1942) and to a patient with muscular dystrophy by Bock (1941).

FIG. 8

Analysis of Nitrogen, Potassium and Phosphorus Balances in Patient B. V., M.G.H. #74372, Suffering from Cushing's Syndrome, While Under Testosterone Propionate and Other Therapy.

The data are expressed as g./5 day period. T.P. = testosterone propionate; I = Insul'n; E.B. = α -estradiol benzoate; D = dosage per day. The three base lines represent the average values during the 4 five-day control periods. The data for potassium are based on urinary excretions alone. It will be noted that the potassium retention corresponds with the nitrogen retention almost exactly if, as is the case here, one calculates the theoretical potassium retention on the basis of the N/K ratio in tissue. It will be further noted that the phosphorus balance likewise corresponds fairly accurately with the nitrogen balance if one takes as the basis of correspondence the thesis that the phosphorus balance which cannot be explained by the calcium balance ($\text{Ca}/\text{P} = 2.0$) represents the phosphorus in tissue ($\text{N}/\text{P} = \text{circa } 15/1$).

(Albright; *Harvey Lecture Series* 38, 123 (1942-1943).)

Talbot, Butler *et al.* (1945) and Bassett (1945). The anabolism of protein, however, is not confined to one type of tissue but to many different parts of the body.

2. Accessory Sex Organs

The accessory sex organs lay claim to some of the retained elements. The increase in weight of these organs, however, only accounts for a fraction of the change in body weight, as indicated by calculation (Kenyon, Sandiford *et al.*, 1938) or direct analysis of the rat and mouse (Kochakian, unpublished). These organs, nevertheless, have first claim on the anabolic processes stimulated by the steroid hormones. Pazos and Huggins (1945) have shown that fasted dogs demonstrate normal prostatic secretion when injected with androgens. In a more objective experiment, Kochakian (1945a) has found that many different androgens produce as great an increase in the seminal vesicles and prostates of underfed as normally fed mice. Furthermore, Kochakian (unpublished) finds that these organs of the rat continue to increase in size on continued injections with testosterone propionate even though the body weight and nitrogen retention have returned to or are less than normal (*cf.* Fig. 2).

3. Kidney and Other Organs

Korenchevsky early recognized the possibility that changes in the weight of internal organs might be used as indicators of metabolic effects of hormones of the gonads. Korenchevsky (1930), and later Korenchevsky and Dennison (1934), reported that castration produced a small but consistent decrease in the weight of the kidney, liver and heart. These organs were restored to normal by injection of purified "male hormone" extract from urine (Korenchevsky, Dennison and Kohn-Speyer, 1933), androsterone, androstanediol-3(α),17(α) and the lithium salts of their succinates (Korenchevsky, Dennison and Simpson, 1935), dehydroisoandrosterone (Korenchevsky and Dennison, 1936), and testosterone propionate (Korenchevsky, Dennison and Hall, 1935). These effects have been reviewed by Korenchevsky (1939), Korenchevsky, Hall, Burbank and Cohen (1941) for the liver and heart and Korenchevsky and Ross (1940) for the kidney.

In recent years the initial observations of Korenchevsky and his associates on the kidney have been extended by a large number of investigators with variable results. Selye (1940) obtained a small increase in the kidney weight of male and female rats treated with testosterone propionate. In a subsequent experiment, Selye and Albert (1942) found no effect. Pronounced effects, however, were obtained in hypophysectomized rats (Selye, 1941). Ludden, Krueger and Wright (1941) found that the increase occurred only at an optimum dose of testosterone propionate. MacKay (1940) ob-

served that testosterone propionate did not increase the kidney size of the normal rat but produced a further increase in renal hypertrophy after unilateral nephrectomy. A similar observation in unilaterally nephrectomized rats and dogs was made by Lattimer (1942). Lecompte (1944) found greater kidney enlargement in rats treated simultaneously with testosterone propionate and a rabbit antiserum than those treated with the androgen alone. Kochakian (unpublished) did not observe definite kidney enlarge-

ANDROSTANEDIOL- $3\alpha, 17\alpha$ AND ORGAN WEIGHTS

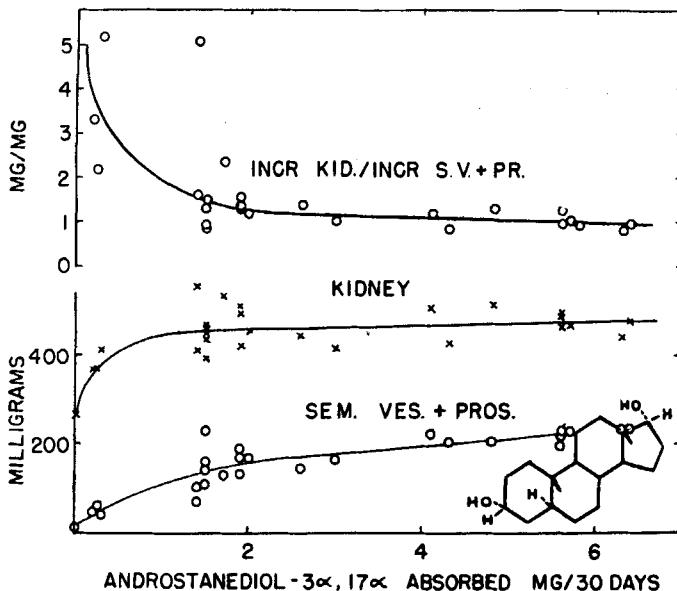


FIG. 9

A Comparison of the Renotrophic and Androgenic Activities of Androstanediol- $3(\alpha), 17(\alpha)$ at Different Doses.
(Kochakian, *Am. J. Physiol.* 145: 549 (1946).)

ment in female or castrated male rats treated with testosterone propionate but definite and very marked enlargement on prolonged treatment in castrated male rats maintained on a prepared diet. Small and variable increases in kidney size of the rat after testosterone or testosterone propionate treatment were reported by Shay, Gershon-Cohen *et al.* (1941), Mark and Biskind (1941), Ratschow (1942) and Miller (1943).

Lattimer (1942) and Blackman, Thomas and Howard (1944) report that testosterone propionate increases the kidney weight of dogs and puppies respectively. The latter group also found an increase in the liver.

A more clear-cut and unquestionable effect of the androgens on the

kidney was reported in the mouse by Selye (1939a, 1939b) and soon after by Pfeiffer, Emmel and Gardner (1940), Kochakian (1941), Crabtree (1941) and Feyel (1942, 1943). The kidney of the mouse has been stimulated to more than double its size in a uniform manner (Kochakian, 1941). Kochakian (1944b) demonstrated, and Beland, Masson and Selye (1944) confirmed, that the renotrophic effect of steroid hormones did not parallel the androgenic effect (Table I). Androstanediol-3(α), 17(α) and its 17-methyl derivative had a greater renotrophic than androgenic effect which became more pronounced at lower and more efficacious doses (Fig. 9).

TABLE I.

The Renotrophic-Androgenic Ratio of Various Steroids

Single pellets (14 ± 1 mg.) of the respective steroids were implanted subcutaneously into dba mice one month after castration which was performed at 16-19 g. body weight. From Kochakian, C.D., *Am. J. Physiol.* **142**, 319 (1944).

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		

(Kochakian, 1946). The more favorable renotrophic effect was due to a decrease in androgenic activity rather than a further increase in renotrophic property. Of particular interest was the observation that those steroids that had the greatest renotrophic effect were also the ones that were known to have the greatest nitrogen-retaining properties. The active steroids increased the kidney size of underfed mice but not nearly as greatly as in normally fed animals.

4. *Skeletal Muscle*

Papanicolaou and Falk (1938) made the important observation that testosterone propionate caused a generalized hypertrophy of the skeletal muscles of castrated male and normal female guinea pigs similar to that

originally observed in female guinea pigs injected with "Follutein." Estrogen and progesterone had no effect in either male or female guinea pigs. This phenomenon has been confirmed by Kochakian (1943a) and has been extended to a number of other steroids.

XI. THE MECHANISM OF ACTION OF THE ANABOLIC STEROID HORMONES

In an attempt to elucidate the mechanism of the anabolic effects of steroid hormones, Kochakian has initiated a study of the enzymes in the kidney, liver and intestine. This subject has been recently reviewed in detail by Kochakian (1945c) and will be treated only briefly here. Kochakian and Fox (1944) found that testosterone propionate decreased the "alkaline" and increased the "acid" phosphatases of the kidneys of castrated and normal mice but did not affect the same enzymes of the liver or intestine. Kochakian (1945b) extended these observations to many other steroids and noted that the changes paralleled the increase in size of the kidney irrespective of the steroid used. In contrast to the mice, both castrated (Kochakian, unpublished) and adrenalectomized (Kochakian and Vail, 1944) rats showed an increase in "alkaline" phosphatase on injection with testosterone propionate which was not altered by simultaneous injections with adrenal cortical extract. Furthermore, testosterone propionate did not affect the increase in "alkaline" phosphatase obtained in the liver of adrenalectomized rats after injections with adrenal cortical extracts.

Clark, Kochakian and Fox (1943) found that testosterone propionate increased the *d*-amino acid oxidase of the kidney of the mouse.

On treatment of castrated mice for 30 days with implants of pellets of various steroids, Kochakian (1944c) found that many of the compounds produced large increases in the arginase content of the kidney but not of the liver or intestine. The order of change, in *per cent* difference per g. of kidney tissue, was as follows: Methyltestosterone 632, testosterone 584, testosterone propionate 308, 17-methylandrostanediol-3(α),17(α) 269, androstanol-17(α)-one-3 135, α -estradiol 88, androstanediol-3(α),17(α) 71, 17-vinyltestosterone 55, testosterone acetate-3, propionate-17 35. Eighteen other compounds had no effect and several caused decreases in the enzyme. In a subsequent study, Kochakian (1945a) found that the changes in arginase content of the kidney were not directly related to the increase in size of the kidney but to the dose of steroid administered. The increase in arginase activity continued even after the kidney had attained its maximum size (Figs. 10 and 11). Furthermore, the 17-methyl group, *e.g.*, methyltestosterone (Fig. 11) and 17-methylandrostanediol-3(α),17(α) hastened the occurrence of the increase in the arginase activity. Both castrated (Kochakian, unpublished) and adrenalectomized (Kochakian and Vail, 1944) rats show a similar, but not as great, response. Kochakian sug-

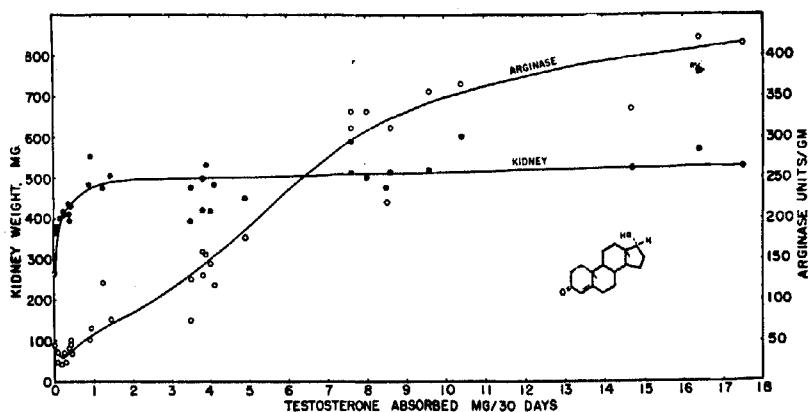


FIG. 10

The Effect of Dose of Testosterone on the Arginase Content of the Kidney of the Castrated Mouse.

The kidney of the normal mouse weighs 414 and has 27 units/g.
(Kochakian, *J. Biol. Chem.* **161**, 115 (1945).)

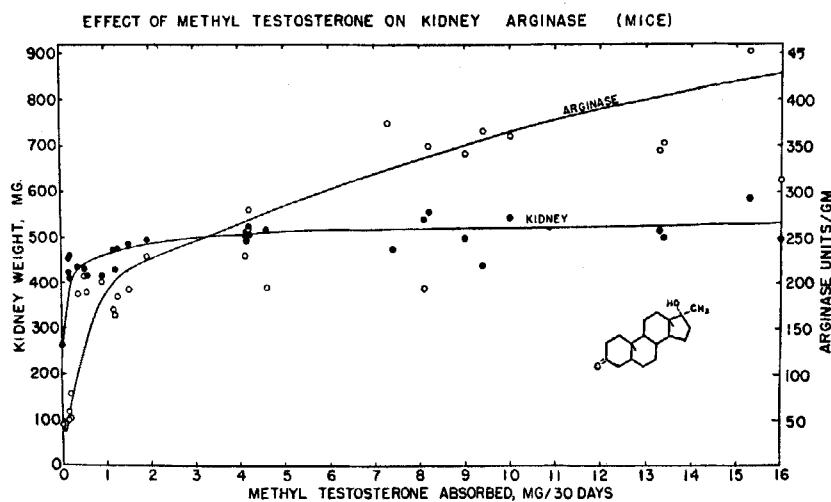


FIG. 11

The Effect of Dose of Methyltestosterone on the Kidney Arginase of the Castrated Mouse.

Note the rapid increase in arginase activity as compared to that observed with testosterone (Fig. 10).

(Kochakian, *J. Biol. Chem.* **161**, 115 (1945).)

gested that these changes may be associated with the protein anabolic properties of the steroids.

XII. DISCUSSION AND SUMMARY

The state of gonadal function of the individual definitely modifies the effectiveness of the protein anabolic properties of the steroid hormones. The castrated dog—man has not been studied—gives a maximum response in a very short time, 2 to 3 days. The presence of the apparently non-functional gonad, *e.g.*, the eunuchoid, does not prevent the maximum response but delays its appearance by about 4 days. The normally functioning testis on the other hand not only decreases the maximum attainable response but also further delays its appearance by about two weeks. If the individual is producing an excess amount of protein anabolic steroids as in the adreno-genital syndrome, then the parenterally administered substances are ineffective. The many other conditions in which the steroids have been demonstrated to be effective may be considered to be hypogonadal states. The endocrine or nutritive status of these individuals is not conducive to the production of protein anabolic steroid hormones. It is conceivable that these individuals may be more responsive to the steroids because of their tissue- as well as hormone-depleted condition. It becomes apparent, therefore, that the individual will respond to a protein anabolic stimulus from a steroid only if it has "room" for that particular stimulus. This does not mean, however, that sparing of nitrogen cannot be induced by other means while the steroid effect is at a maximum. Bassett, Keutmann and Kochakian (1943c) and Talbot, Butler and MacLachlan (1943) caused further nitrogen retention by administering extra carbohydrate to a patient with Cushing's syndrome and extra calories to a girl with Addison's disease while they were responding maximally to testosterone propionate treatment.

Although a large number of steroids have been studied for their protein anabolic effects, not one has shown as great an efficacy as the original urine extracts of Kochakian and Murlin. The minimum amount of this preparation necessary to produce maximum nitrogen retention in the castrated dog was about 40 mg./day which contained the equivalent of 4.4 mg. of androsterone, the most potent known androgen in normal male urine, or 0.9 mg. testosterone, as determined by the capon assay method and by isolation studies (Kochakian, unpublished *cf.* 1943b). This value is in agreement with the figures in the literature (*cf.* Pincus and Pearlman, 1943). Thus the urine extract is at least 10 times as effective as testosterone or its acetate in promoting nitrogen retention. Since androsterone and also dehydroisoandrosterone have very little, if any, protein anabolic properties and are both relatively weak in the other physiological effects (*cf.* Koch, 1937; Parkes and Emmons, 1944 and Kochakian, 1944a, b), the activity of the urine extracts must be due to an hitherto unrecognized factor or combination of factors. Furthermore, the factor(s) does not have the same

chemical properties as testosterone, for it is stable to alkali while testosterone is destroyed by boiling with 20% sodium hydroxide (Gallagher and Koch, 1934). The isolation of the active principle is now under investigation in the author's laboratory.

Only 17-methylated steroids are effective by the oral route but in using this means of administration about 75% of the efficacy of the steroid is lost. As yet, methyltestosterone is the most potent orally administered steroid. 17-Methylandrostanediol-3(α), 17(α) is about two thirds as effective as methyltestosterone. The most potent parenterally administered steroid is testosterone propionate. Methyltestosterone and testosterone have not been adequately quantitated.¹⁰ Of the other compounds studied only androstenedione and androstanediol-3(α), 17(α) have been shown to be effective by at least two different laboratories. Many of the other compounds probably will prove to have definite activity when they have been studied at higher dose levels.

It seems to be fairly well established that the estrogens, too, have protein-anabolic properties. These effects bring to mind the nitrogen retention that proceeds during pregnancy (*cf.* Murlin, 1910; Rowe, Gallivan and Mathews, 1930; and Sandiford, Wheeler and Boothby, 1931).

A comparison of the known metabolic effects of the two most studied compounds, testosterone propionate and methyltestosterone, seems to demonstrate at first glance a marked dissimilarity in their actions. On further study, however, one is led to wonder whether these dissimilarities are more apparent than real. All of the "contrary" effects of methyltestosterone, *e.g.*, reversal in the increase in body weight, "wearing off" effect in nitrogen retention, excessive creatinuria, increases in B.M.R. and more rapid increase in kidney arginase are also produced by testosterone propionate if treatment is continued for a sufficient period. It would seem, therefore, that the real difference between these two steroids, or one might say class of steroids since the other potent 17-methyl steroids behave like methyltestosterone, is that the introduction of the 17-methyl group hastens these effects. Furthermore, the "contrary" effects probably would not appear if minimal doses were administered and the patients were on an *ad libitum* diet. The doses used in all of the experimental studies in which these "contrary" effects have been attained were amounts that produced a maximum rate of response while on a fixed intake. On the other hand, growth and gains in weight have been attained in dwarfed children for as long as two years but when these dwarfs are placed on a fixed diet for metabolic studies they begin to show a "wearing off" effect.

¹⁰ A comparison of the protein anabolic properties of many steroids is underway in the author's laboratory.

The suggestion of Shay, Gershon-Cohen *et al.* (1941) that the growth-promoting and nitrogen-retaining properties of the androgens are mediated through the growth hormone of the anterior pituitary hardly seems tenable on the basis of the available evidence. The experiment by Kochakian in the castrated-hypophysectomized dog, the many clinical experiments on patients with destroyed anterior pituitaries, the inability of androgens, in contrast to the growth hormone, to decrease the liver arginase of mice and rats, and the osteogenic and the synergistic effect of testosterone propionate with growth hormone on the growth of the hypophysectomized rat (Simpson, Marx *et al.* 1944) indicate that the androgens do not bring about their anabolic effects through the anterior pituitary. The experiments of Gaebler and Tarnowski in the depancreatized bitch have ruled out the pancreas as an intermediary. The many clinical experiments on patients with Addison's and Simmond's disease suggest that the adrenal cortex is not involved but this needs confirmation in the experimental animal with complete surgical extirpation of these glands.

The fact that many tissues other than the secondary sex organs are stimulated by the anabolic steroid hormones definitely establishes these compounds as growth factors. The androgenic function, however, seems to have priority over all of the other functions and the renotrophic is next. In the partially or totally fasted organism, the administration of androgens brings about maximum response in the accessory sex organs. The kidneys also are stimulated to increase in size but not maximally. These effects apparently take place at the expense of other processes.

Kochakian has tentatively interpreted the renal changes (size and enzyme activities), without any similar changes in the liver or intestine, as an indication that the anabolic properties of the androgens are mediated, at least in part, through the kidney.

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Methods of Bioassay of Animal Hormones

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I. INTRODUCTION

The following article does not attempt in any way to review the methods that are used to assay all the hormones. Rather, it will be limited to consideration of the principles which should govern biological methods and a brief discussion of the methods employed in the assay of the anterior pituitary and the adrenal cortical hormones.

The progress of work in the isolation of vitamins or hormones from natural sources is markedly dependent on biological assays. It is necessary to conduct fractionations on a large scale since the substance sought may be present in relatively low concentrations. It is obvious, therefore, that the rate of progress is dependent upon an adequate, accurate, and relatively non-laborious method of assay.

The assay work done in our laboratory started with the assay of the estrogens. We believe that Edgar Allen's (1923) introduction of the vaginal smear method for detection of the "ovarian follicular hormone" was an important contribution which has had a profound effect on the subsequent developments and advances in the hormone field. Although Allen's original procedure has been modified in many respects (Thayer, Doisy, Jr., *et al.*, 1944), the basic principle remains unchanged.

The work of Coward and Burn (1927), Kahnt and Doisy (1928), Marrian and Parkes (1929), Allen, Dickens *et al.* (1930), de Jongh, Laqueur *et al.* (1932), Marrian (1933), D'Amour and Gustavson (1936), Emmens (1939) and Pederson-Bjergaard (1939), has had a profound effect on the assay of other hormones and has been an invaluable aid to the development

of assay of natural products. The above authors have adequately reviewed the estrogen field, and the following authors:—Gallagher and Koch (1935), Greenwood, Blyth *et al.* (1935), Miescher, Wettstein *et al.* (1936), and others, have reviewed the androgen field. The estrogens and androgens will, therefore, be omitted from this article.

It is the purpose of this paper to emphasize the principles which govern biological methods and to review the assay procedures which are used to standardize extracts which contain two or more active principles, such as the anterior pituitary and adrenal cortical extracts.

II. PRINCIPLES WHICH SHOULD GOVERN BIOLOGICAL METHODS

Before quantitative measurements may be made, qualitative studies of the active principle must be made to determine the effect upon animal tissue. Knowing the qualitative responses produced by a product, the bioassayist endeavors to develop procedures having the greatest quantitative reliability. For this purpose each of the qualitative reactions is studied carefully. It is not necessary to use a bioassay technique which duplicates the clinical action, although there are many theoretical advantages in following such a procedure.

In considering the fundamental principles underlying bioassays, many important factors arise (Burn, 1930):—(1) The product; (2) Animal variation; (3) Choice of suitable standard; (4) Response; and (5) Definition of units.

1. *The Product*

Bioassay measurements are comparative. This necessitates preparation of a reference standard. The standard should resemble the unknown substance as closely as practicable. For example, in 1932 an international conference on the standardization of sex hormones was held in London at which estrone was chosen as the international standard. Recognizing the great inherent variability in the bioassay of the estrogens, the conference adopted regulations covering the proper application of the international standard (1935).

In view of the evidence before them as to the varying ratios between the activity of estrone and that of other natural estrogens as measured by different biological tests, the members of the conference were unable to recommend the determination of the activity of other forms in terms of International Units. Many investigators, however, attempted to assay other forms of estrogens and express the activity in terms of estrone international units. In consequence of this, a second conference on the standardization of sex hormones was held in 1935, and a second international standard, the so-called benzoate standard, was adopted for use in the assay.

of preparations of estradiol monobenzoate (1935). It is now apparent that the previous confusion was due to attempts to compare substances which are not susceptible of comparison.

2. The Determination of Animal Variation

The great progress which has been made in biological methods during the last twenty years has come largely from recognition of the fact of animal variation. de Lind van Wijngaarden (1926) published results of experiments on 573 cats in which he showed that there was a continuous variation in the sensitiveness of cats to digitalis. This work showed that the figure obtained for the lethal dose in one animal has little significance, and that the true lethal dose is the mean value determined in large numbers of animals.

The next advance was that made by Trevan (1927) who published an investigation of toxicity determination. He showed that when each dose is injected into a sufficiently large group of animals, the irregular results disappear. In other words, no exact meaning can be attached to the term "minimum effective dose." This point has been emphasized again and again in the assay of estrone and other substances (Coward and Burn, 1927).

3. Choice of Suitable Standard

Another important principle is that the standard of reference chosen for use in biological test must owe its activity to the active principle for which a preparation is to be assayed. In most instances this principle has been followed. However, in some cases it is necessary to substitute a compound that has similar properties, such as that used by Olson *et al.* (1944).

4. Response

The degree of response desired depends on the nature of the excitant. In certain types of assay the "all or none law" may be employed; this is particularly true in determining toxicity. In tests upon a single animal a given dose succeeds, or fails, in producing death. This type of response is subject to great variations. Hence, most bioassays are developed with the intention of relating dosage to a graded effect (Trevan, 1929; Burn, 1930).

5. Units

The ideal unit may be defined as the amount of activity contained in a given weight of the standard of reference. This has been accomplished for many of the hormones. This procedure has not been entirely satisfactory as the results of comparison by different methods will not always give the same comparison.

When a new active substance is described, there is always a period in which no international standard is available. This is a period during which an animal unit may be used. This question is important to manufacturers who are making preparations for clinical use. The clinician is interested in knowing the potency of the preparation and, therefore, the manufacturer can compare each new preparation directly with the previous one to insure that the product does not vary in potency.

III. STATISTICAL ANALYSIS OF DATA

1. Accuracy of Results

Biometry, or the treatment of animal variation in accordance with statistical principles, is proving to be of considerable value to the biochemist in the evaluation of biological assays (Trevar, 1927; Burn, 1930; Bliss, 1941). Research reports sometimes give a detailed description of the statistical treatment used, and in some cases one obtains the impression that such a description is included to impress the reader with the high quality of the experimental procedure. Statistical treatment does not add value to the observations. Statistics rarely constitute proof and they are merely a tool whereby the observer interprets his data. An understanding of the significant test will enable the analyst to see more clearly what his observations mean, but his conclusions are his own responsibility. Luykx (1944) suggests, "that when the nature of the conclusion depends to a large extent on the statistical analysis, this analysis be appended to a report for the benefit of those who wish to have it, but so that it will not detract from the value of the description for those who are not equipped to follow such an exposition."

In biological assays the results of experimentation are expressed in percentages to be compared with each other. For example, to assay a preparation, it is administered to a group of animals, A, and the known preparation or standard is given to a second group, B. If groups A and B give a similar response, then the preparations are considered equal in potency. In many assays, however, the response expressed in percentage is not the same as the standard and it is necessary to evaluate the results. In plotting doses of abscissae, and the percentage of test animals showing positive responses as ordinates, the usual relation resembles an S-curve (Burn, 1930). The degree of response taken as the end-point will lead to widely divergent results unless an adequate number of test animals are used. Conversely, an unknown and a standard may show different ratios of potency at different intensities of response. Since the inflection point of an S-curve is found in the neighborhood of the 50% response for test animals, this has led to the use of this percentage response as the more desirable for comparison. The magnitude of variable errors tend to follow a normal frequency curve

when a sufficient number of observations are made. The extent of the variable error of a set of observations may be determined from several values, most commonly the standard deviation or the probable error.

2. Standard Deviation

If d be used to indicate the difference or deviation of any figure in a series from the mean (irrespective of whether it is plus or minus), and Σd to indicate the sum of these deviations, and n the number of figures in the series, then the mean deviation is $\Sigma d/n$. But this is not the usual method of expressing the spread of the figures. For relatively small numbers of animals it is obtained from the formula:

$$\sigma = \sqrt{\frac{\Sigma d^2}{(n - 1)}}$$

The deviations from the mean are squared, the sum of the squares is found and divided by $(n - 1)$, and the square root of the quotient is called the standard deviation (single determination).

If we are interested in the accuracy of the mean, which increases in proportion to the square root of the number of animals used, then

$$\sqrt{\frac{\Sigma d^2}{n(n - 1)}}$$

is the standard deviation of the mean or "standard error."

3. Significant Difference

To determine whether two results differ significantly or whether the difference between them is due to the error of sampling, the formula

$$\frac{m_1 - m_2}{\sqrt{E_1^2 + E_2^2}}$$

is used, when m_1 and m_2 are the two mean results and E_1 and E_2 are their respective standard errors.

Each worker must decide what difference he proposes to consider significant, but most investigators insist that the figure derived from the above formula should be two or more. The method given for determining a significant difference is satisfactory when the standard error of the two means is small compared with the means themselves. If the standard error is greater than 15%, it is better to use logarithms.

4. The Equation to the Regression Line

It is commonly found that when a graph is drawn relating the logarithm of the dose to the mean effect of the dose, the points obtained lie on a

straight line which is called a regression line. Examples of a straight line relation between logarithm of dose and the mean effect are provided in the estimation of estrogenic, thyrotropic, androgenic and many other hormones.

A formula for a straight line of this kind is given by the equation (Fisher, 1932);

$$y = y^- + b(x - x^-)$$

where y^- = mean value of y and x^- = mean value of x (log dose) and $b = \frac{\Sigma y(x - x^-)}{\Sigma(x - x^-)^2}$. Finally, to determine the absolute precision of a given assay involving the comparison of an unknown with a standard, it is necessary to determine the quantity S_m , the standard error of the logarithm of the ratio of potencies (Bliss, 1941), identical with the λ of Gaddum (1933) and called by him the sample of error of the result. The formula for the computation of this factor, S_m , is derived under conditions in which the dosage of the unknown and the standard have been so adjusted that the mean responses of the animals to them are the same. Then, so far as can be predicted from the linear dosage-response curve, the standard error of the logarithm of the ratio of the potencies, S_m , is given by the equation:

$$S_m = \frac{\sigma_w}{b} \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

where n_1 is the number of animals injected with the unknown; n_2 is the number of animals injected with the standard; b is the slope of the regression line for both; σ_w is the weighted standard deviation calculated as follows:

$$\sigma_w = \frac{n_1 \sigma_1 + n_2 \sigma_2}{n_1 + n_2}$$

where n_1 and n_2 are as above; σ_1 is the standard deviation of a single result from the mean obtained in the assay of the unknown; σ_2 is the standard deviation of a single result from the mean obtained in the assay of the standard.

In concluding this part of the article, I would like to quote the following points considered by Bliss (1941) to be the marks of a valid quantitative bioassay method:—

“(1) Different samples of the same drug must show the same relative potencies in biological assay as under clinical test.

“(2) On the coordinates used for biological assay, the curve relating response to log dose should be a straight line and relatively steep when compared with the variation around the line. Either the curve should have been shown to have a constant, known slope by repeated test over a

considerable period of time or the slope should be determined as an integral part of each assay; assumed relations between dose and effect are to be avoided.

"(3) The potency of the unknown should be determined by comparative test with a stable reference standard and expressed in units of this standard.

"(4) The living material exposed to different doses of standard and unknown must be as nearly equivalent as it can be made.

"(5) A determination of potency should always include an estimate of its error, computed as an integral part of the assay. No assay with an indeterminate error can be considered as satisfactory."

IV. THE GONADOTROPIC HORMONES

At least three gonadotropins have been isolated from crude pituitary extracts. They are the follicle-stimulating hormone (FSH), the luteinizing hormone (LH), and the lactogenic hormone (PH). The complete assay of the pituitary gonadotrophic extracts presents quite a problem. In addition to containing these several factors, it has been observed that the proportion of FSH and LH present in the extract will affect the response qualitatively and quantitatively (Fevold, 1937). Not only do the two hormones work synergistically, but also when one of the hormones is present in much larger amounts than the other, it may mask the characteristic effect of the second. This is particularly true if a large amount of FSH is present in an extract with small amounts of LH. Consequently, a quantitative assay of the preparation gives very little information concerning the amount of each substance present unless it is also accompanied by a careful qualitative analysis.

Aschheim and Zondek (1927) found gonadotropic activity in a non-pituitary source, human pregnancy urine. Since that time, several additional non-hypophyseal sources of gonadotropic activity have been discovered. On the basis of their physiological properties, the gonadotropins from sources other than the pituitary can be subdivided into the following three categories (Levin, 1944) based on physiological properties: (1) Human chorionic gonadotropins (PU) include the substances present in the blood, urine and tissues of pregnant women and of men and women suffering from some malignant genital tumors; (2) Human nonchorionic gonadotropins (CU); the active principle of the blood and urine of ovariectomized and post-menopausal women; (3) Equine gonadotropins (PMS); the substances present in the blood and placental tissues of the pregnant mare. Although the members of these groups have many common properties, they also display such marked differences that it is impossible to consider them as being physiologically identical.

In a discussion of this complex problem of the assay of the gonadotropic factors, it is probably the best procedure to discuss the assay of each of the above preparations separately.

1. Assay of Anterior Pituitary Gland Extracts

The induction of ovulation in the unmated estrus rabbit by the administration of anterior pituitary extracts was first demonstrated by Bellerby (1929). Much of the early work done on the rabbit ovulation test has been in connection with pregnancy diagnosis.

Friedman (1932, 1939) recommended the *post partum* rabbit for the assay of gonadotropic materials because of the great regularity of the response and the rapidity with which the results were obtained.

Hill, Parkes *et al.* (1934) were the first investigators to consider individual variation in response or other variables. These authors observed that the *post partum* rabbits are no more reliable than ordinary non-pregnant animals isolated for three weeks.

Groups of about 20 rabbits were injected with extract at intervals of three weeks, this being the time required for them to return to estrus after a previous ovulation. Ovulation was determined by laparotomy. Standardization curves were constructed and the unit was defined as the ovulation-producing activity required to cause ovulation in 50% of a group of not less than ten estrus rabbits, the extract being administered as a single intravenous injection. Both hormones are said to be necessary to produce ovulation and, therefore, the rabbit test is not satisfactory unless one is interested in the FSH and LH present in the extract.

A great majority of the assays have been carried out by subcutaneous injection into immature female rats, immature female mice or male rats. The following criteria of activity have been used: (1) The resulting increase in ovarian weight (Wallen-Lawrence and Van Dyke, 1931); (2) Uterine weight (Smith and Engle, 1927-28); (3) The proportional incidence of vaginal cornification (Wallen-Lawrence and Van Dyke, 1931); (4) Vaginal opening (Evans, Meyer *et al.*, 1932); (5) The formation of *corpora lutea* (Janssen and Loeser, 1930); (6) Changes in the histology of the ovary, vagina and uterus in the female rats and mice (Smith and Engle, 1927-28); (7) The increase in weight of seminal vesicles or the combined weight of the seminal vesicles and prostate gland in male rats (Wallen-Lawrence and Van Dyke, 1931); or (8) The increase in the weight of the testes of ring doves (Riddle and Flemion, 1928).

One of the common methods for standardizing pituitary gonadotropic extracts is to inject them into immature female rats for a certain period of time and determine the increase in weight of the ovaries (72-100 hours after the last injection) over those of untreated controls (Fevold, 1939; Wallen-

Lawrence, 1934; D'Amour and D'Amour, 1938; Aschheim and Zondek, 1927). The results so obtained give very little information with regard to the proportion of the two hormones in the extract unless certain other factors are known and taken into consideration. According to Fevold (1939), it is possible for a dosage of an extract containing much LH and little FSH to produce the same size ovaries as a dosage of another extract containing much FSH and less LH. Another factor which must be considered is the length of the treatment. When a combination of the two hormones is given, the first response is the growth of the follicles, but luteinization does not occur until the follicles have undergone considerable development. If the ovaries are examined after three days of treatment, only follicles may be present with very little or no luteinization even though very appreciable quantities of LH may be present in the preparation. However, after five days of injection with the extract, luteinization is far advanced. If no luteinization is apparent at this time, it is quite safe to conclude that the extract contains little, if any, LH. To determine the approximate amount of the two factors in pituitary tissue or unfractionated extract, it is necessary to compare their relative activities by the ovarian and seminal vesicle responses. Fevold and Hisaw (1934) have shown that the ovarian response is governed largely by the amount of FSH, while the seminal vesicle response is determined largely by LH (Greep, Fevold *et al.*, 1936; Deanesly, 1939) and even though the response in each case is increased by the presence of the other factor, the results give a fair idea of the relative amounts of FSH and LH present.

To determine the approximate amounts of two factors in pituitary tissue or unfractionated extracts, Fevold (1939) compared their relative activities by the ovarian and seminal vesicle responses.

The ovarian test for unfractionated extracts (Fevold, 1939) is carried out on rats twenty-two days old which weigh from 35–40 g. at the beginning of the experiment. They are injected subcutaneously morning and evening with 0.25 cc. of the solution per injection. The animals are then autopsied and the ovaries weighed and examined the morning of the fifth or sixth day. The seminal vesicle test is performed on twenty-two day old rats in the same manner, with the exception that it is always continued for five days and the seminal vesicles together with the coagulation gland weighed the morning of the sixth day. It is apparently impossible to standardize a pituitary gonadotrophic extract quantitatively for one of the two factors or when the other is present. It must be emphasized that the values obtained by this method do not represent absolute units of either FSH or LH in an unfractionated extract, but they give approximate proportions of the two.

Heller, Lauson *et al.* (1938) have made a study of the various methods used for the assay of gonadotrophic content of pituitary glands. They found

that the curves of uterine weight (minus fluid) and vaginal weight reveal a close correlation. Since the curves rise rapidly to a maximum and very slowly recede, only a small portion, at the lowest dose levels, is useful for assay purposes. Vaginal opening was the least dependable, while uterine weight was considered the most satisfactory gonadotropic criterion.

D'Amour and D'Amour (1938) made a study of the assay methods and rated them according to objectivity, simplicity and sensitivity. Of the three factors on which they have based their rating, they consider objectivity the most important. Therefore, based on their experience with some 1600 animals, they consider the ovarian weight method of assay preferable.

Noble, Rowlands *et al.* (1939), who have recently compared the effects of different gonadotropic extracts in normal and hypophysectomized rats, concluded that the primary action of the injected gonadotropin on the ovary probably leads to a secondary stimulation of the pituitary gland, which secretes additional amounts of hormone and, therefore, must be regarded as a potential source of complication in the assay results. Smith (1930) found that the difference in response of normal and hypophysectomized rats to injection of gonadotropic extracts of the pituitary gland was quantitative rather than qualitative. It is evident, therefore, that in such tests, carried out on normal immature rats, endogenous gonadotropic hormones contribute to the stimulation of the gonads.

If hypophysectomized rats are available the detection of LH becomes more exact. As there are no secondary effects due to the action of estrone on the pituitary to complicate the results, FSH may be injected for long periods. The animals will remain in estrus and the ovaries will contain only follicles with no lutein tissue, provided the FSH is free of LH.

It is obvious from our discussion that the complete assay of pituitary extracts is a difficult problem, and it is impossible to assay quantitatively a mixture such as a pituitary gland extract which contains substances exhibiting qualitative physiological differences in the test animal.

2. Assay of Gonadotropic Substance of Pregnancy Urine (PU)

Aschheim and Zondek (1927) first demonstrated that within two weeks after the first missed menstrual period, that is, about four weeks after fertilization of the ovum, relatively tremendous quantities of gonad-stimulating activity may be demonstrated in the blood and urine. This high rate of excretion is sustained for a short time only, decreasing within the next 30 to 40 days to much lower values which are maintained until parturition. Within a week after expulsion of the fetus and placental tissue the gonadotropins fall to the very low prepregnancy level.

Early observations of the effects produced by human chorionic gonadotropin in intact test animals were widely misinterpreted because of failure

to take into account the possible participation of the animal's own pituitary gland. A great deal of confusion has been cleared away by the use of hypophysectomized animals. We know today that PU never produces follicular growth and maturation of itself. The follicular stimulation seen in intact immature rats treated with the gonadotropin is undoubtedly due to a synergism between this substance and the animal's own gonadotropin or to an induced hypersecretion of FSH by the animal's anterior hypophysis. When administered to hypophysectomized rats whose ovaries lack mature follicles, the chief effect of PU is a conversion of the ovarian thecal and interstitial cells into lutein-like tissue (Collip, Selye *et al.*, 1933; Leonard and Smith, 1934). The entire ovary assumes a luteoid structure. Estrogen secretion, presumably from the stimulated interstitial tissue, is markedly enhanced. The weight of the ovary is definitely increased, although with continued injections this weight increase is not maintained.

When administered to hypophysectomized rats whose ovaries contain mature follicles, PU is able to convert the follicles to *corpora lutea* (Leonard and Smith, 1934).

A very remarkable property of human chorionic gonadotropin is the synergistic effect it exerts when combined with certain other gonadotropins. Although PU of itself increases ovarian weight to only a limited extent, it is well known that when combined with other gonadotropins which stimulate follicular development, the resultant weight increase is far beyond the additive effects of the two substances. This phenomenon, which has been termed "augmentation," may be obtained without extensive lutein changes.

A number of the responses elicited in immature female rats and mice by pregnancy urine have been applied to the quantitative assay of this type of extract. The production of follicular enlargement, luteinization, "blood point formation," increase in ovarian weight, precocious vaginal canalization and induction of estrus as judged by the vaginal smear have all been used, singly or in combination, as indicators of the gonadotropic potency of the injected material.

It is obvious that, when any of the above criteria are used to assay the potency of a PU preparation, one must be certain that the extract is free from estrogenic substance; if present, it would render the results inaccurate. Levin and Tyndale (1937), and Katzman and Doisy (1932), using a similar method of preparation, have found little, if any, estrogenic potency in castrate or pregnancy urine extracts. It is, therefore, quite certain that if reasonable care is taken to remove estrone which may originally be present there is little likelihood of error from this source.

Because various investigators have assayed PU preparations for gonadotropic potency using different criteria for testing potency, there are many different units. International assay on a broad basis may therefore be said to be most desirable.

The Third International Conference on the Standardization of Hormones, meeting at Geneva in August 1938, under the auspices of the Health Organization of the League of Nations (Emmens, 1938), recommended the establishment of an international standard preparation of gonadotropic substance from human pregnancy urine. The preparations which had been offered as contributions to the standard were in the form of dry powders consisting either of undiluted urine extract or of such extract diluted with various amounts of lactose and sodium chloride. Samples of these preparations, labelled PU 1, 2, 3, 4, 5, 8, and 10, had been circulated to a number of laboratories in different countries so that their potency and suitability for inclusion in the final standard preparation could be determined on a wide basis. The conference decided from the data then available to elaborate the standard preparation by mixing all the contributions except PU 4 which was found by several investigators to give atypical results.

It was further decided by the conference that the international unit should be defined,—subject to confirmation after practical tests by members of the conference,—as the specific gonadotropic activity of 0.1 mg. of the standard so prepared, and that it should be used for recording the activities of all preparations of human pregnancy urine, but only of such. Details of the recommendation are contained in the report of the Third International Conference on the Standardization of Hormones, *Quart. Bull. Health Organization League Nations* (1938).

The great majority of the assays examined in the above report have been carried out by subcutaneous injection into immature female rats. Three were carried out on immature male rats, three on immature mice, two on rabbits, and one on ring doves. Activity has been estimated by the different investigators by observation of the following different kinds of changes: (1) the resulting increase in ovarian weight; (2) uterine weight; (3) the proportional incidence of vaginal cornification; (4) of vaginal opening; (5) the formation of *corpora lutea*; (6) changes in the histology of the ovary, vagina and uterus in female rats and mice; (7) the increase in weight of the seminal vesicles or the combined weight of the seminal vesicles and prostate gland in male rats; (8) the increase in the weight of the testes of the ring dove; or (9) the production of ovulation on intravenous injection into female rabbits. Tables I to V give the results of the assays by the different methods. Activity has been expressed in terms of the number of mg. of each preparation necessary to equal the activity of 1 mg. of PU 5, a procedure which avoids an index of less than unity, and which facilitates tabulation and compilation. In each table, the geometric mean of the estimates is given for each preparation, all computations having been carried out logarithmically together with the variance of the logs of the estimates in that table.

The vaginal cornification technique is, therefore, recommended as a test

method. It is the most accurate, inexpensive and simple method of assay. It also has the advantage that the animals need not be killed after the test, and may be used for other purposes later. On the other hand, if they are killed, the added test of uterine weight is available as a check on the findings

TABLE I

*The Number of Mg. of Each PU Preparation Required to Equal 1 Mg. of PU 5
When Tested by Increase in Ovarian Weight*

Animal	PU 1	PU 2	PU 3	PU 4	PU 8	PU 10
Rat	—	3.0	9.0	60	17	60
"	3.3	2.8	5.0	30	10	20
"	21.0	3.0	9.0	38	—	—
"	* 8.0	3.0	12.0	36	8	21
"	—	3.7	8.3	83	33	—
"	6.7	2.5	6.7	10	10	20
"	13.0	2.7	5.9	35	9	—
Geometric mean	8.65	2.94	7.69	35.5	12.6	26.6
Logarithmic variance	0.10650	0.00317	0.01650	0.08267	0.05440	0.05567

After C. W. Emmens, Bulletin of the Health Organization of the League of Nations, 8, Extract No. 15 (1938).

TABLE II

*The Number of Mg. of Each PU Preparation Required to Equal 1 Mg. of PU 5
When Tested by Increase in Uterine Weight*

Animal	PU 1	PU 2	PU 3	PU 4	PU 8	PU 10
Rat	—	3.2	14.9	108	—	28
"	10	3.3	8.0	80	8.0	14
"	—	5.1	12.1	38	18.0	—
"	21	3.5	10.0	44	—	—
"	6.1	3.0	9.0	30	8.4	25
Mouse	—	1.0	1.5	3	8.0	—
Rat	—	2.6	10.0	54	—	—
Geometric mean	10.86	2.84	7.91	35.4	9.92	21.4
Logarithmic variance	0.07300	0.04833	0.10917	0.25800	0.02967	0.02600

After C. W. Emmens, Bulletin of the Health Organization of the League of Nations, 8, Extract No. 15 (1938).

by vaginal cornification. There is one important drawback to the test in that its accuracy depends on the absence of estrogenic material from the preparations tested. This must be known or ascertained by a parallel test on ovariectomized animals. Tests by ovarian weight or *corpus luteum* formation are independent of the presence of small amounts of estrogenic material and, in view of their reasonably high accuracy, may be preferred for this reason.

The action of PU preparations in hypophysectomized rats shows that they do not contain follicle stimulating substance (Hamburger and Peder-

TABLE III

The Number of Mg. of Each PU Preparation Required to Equal 1 Mg. of PU 5 When Tested by the Vaginal Cornification Technique

Animal	PU 1	PU 2	PU 3	PU 4	PU 8	PU 10
Rat	9.8	2.8	7.7	39	20	12
"	—	2.3	6.3	25	—	35
"	7.9	3.0	8.6	47	20	19
"	11.0	2.5	7.9	34	16	40
"	6.7	4.0	7.7	80	9	18
"	—	3.0	9.0	78	—	—
"	9.3	4.0	10.0	(>20)	13	23
"	7.6	3.3	10.0	35	—	32
"	—	3.0	9.0	36	—	—
"	13.0	3.0	9.0	30	—	27
"	—	3.0	7.3	8	—	—
"	—	3.3	13.0	46	—	—
"	23.0	3.0	9.0	10	11	14
Geometric mean	10.24	3.06	8.68	32.4	14.2	22.7
Logarithmic variance	0.02843	0.00458	0.00583	0.09118	0.02020	0.03238

After C. W. Emmens, Bulletin of the Health Organization of the League of Nations, 8, Extract No. 15 (1938).

TABLE IV

The Number of Mg. of Each PU Preparation Required to Equal 1 Mg. of PU 5 when Tested by the Formation of Corpora Lutea

Animal	PU 1	PU 2	PU 3	PU 4	PU 8	PU 10
Rat	—	5.3	6.7	20	—	37
Mouse	6.0	3.0	—	62	8.0	28
Rat	8.0	3.0	9.0	28	—	30
"	4.5	3.0	13.0	35	—	—
Mouse	6.3	2.0	7.0	13	9.5	22
Rat	—	1.7	10.0	33	3.3	—
Mouse	—	6.4	9.6	80	8.0	—
Rat	—	4.7	12.8	83	33.0	—
"	12.7	5.1	14.0	—	7.6	—
"	17.3	6.6	16.2	102	—	34
Geometric mean	8.18	3.71	10.48	41.3	8.92	29.7
Logarithmic variance	0.04800	0.04300	0.01775	0.09438	0.10340	0.00775

After C. W. Emmens, Bulletin of the Health Organization of the League of Nations, 8, Extract No. 15 (1938).

sen-Bjergaard, 1937) and it should be emphasized that with other types of gonadotropic extracts, the relative accuracy of the methods may not be the same. Urine preparations containing luteinizing substance must, it seems,

produce their effects in immature intact rats and mice partly in a secondary manner, presumably by stimulation of the animal's own pituitary gland. None of the test methods employed can, therefore, be regarded as involving

TABLE V

The Number of Mg. of Each PU Preparation Required to Equal 1 Mg. of PU 5 when Tested by Various Other Methods

Method	Animal	PU 1	PU 2	PU 3	PU 4	PU 8	PU 10
Vaginal opening	Rat	—	1.5	12.0	42	20	—
	"	—	3.3	—	33	3.3	—
	"	—	2.0	7.0	(>92)	5	—
Histology of ovary, uterus and vagina	"	—	5.6	14.0	63	—	—
Prostate and seminal vesicle weight	"	19.0	3.0	8.0	15	15	30
Weight of seminal vesicles	"	—	4.4	20.0	80	32	—
Increase in testis weight	Ring dove	5.2	2.2	6.2	20	15	25
Ovulation	Rabbit	3.0	5.0	4.0	138	27	—
	"	1.6	1.2	1.1	4.2	3.4	5.5
Geometric mean		—	—	—	—	—	—
Logarithmic variance		4.67	2.77	6.68	31.5	11.6	16.0
		0.21300	0.05513	0.13475	0.20000	0.14500	0.16400

After C. W. Emmens, Bulletin of the Health Organization of the League of Nations, 8, Extract No. 15 (1938).

solely the observations of a direct gonadotrophic effect, uncomplicated by the action of the animal's own pituitary gland.

3. Equine Gonadotropins (PMS)

Although the gonadotrophic material of pregnant mare serum is thought to be of chorionic origin, it may be stated with certainty that it is not identical with the analogous human chorionic substances. Neither is it identical with the non-chorionic gonad-stimulating substances found in human urine, nor with the pure hormones obtained from the hypophysis itself. In many ways, the equine hormone closely resembles an artificial mixture of human chorionic and non-chorionic gonadotropins.

In intact immature female rodents, PMS causes greater ovarian enlargement than any other gonadotropin. The increased size is due to the growth of many follicles and their transformation into *corpora lutea*. The structural effects produced in the ovary of the hypophysectomized immature female rat are to a large extent dependent on the dose of active substance administered. All doses cause luteinization of the thecal cells. PMS thus differs from PU, which does not cause follicular growth in the hypophysectomized rat, and from castrate urine which stimulates follicular growth without thecal luteinization.

Cole, Guilbert *et al.* (1932) found the immature rat to be the most convenient test animal for the assay of the gonad-stimulating hormone in mare serum. They use rats 25 days old on the day of injection (one injection) and autopsy them five days later at 30 days of age. They define a rat unit of the gonad-stimulating hormone of mare serum as the ". . . amount which will produce, in a group of six rats, an average of from three to ten mature follicles or corpora for each immature female rat tested and autopsied five days after the injection and half of which amount will fail consistently to produce a vaginal smear of oestrum in another group of six rats." These authors believe that the vaginal smear should be considered in the definition of a rat unit because it is the most sensitive means for detecting a small amount of the hormone. These authors further state, "if one-half the amount necessary to produce an average of from three to ten corpora or mature follicles fail to produce a vaginal smear of oestrum in all of six rats, one is assured that the end point is being approached at which ovarian changes will be produced."

Independently of each other, Hamburger and Pedersen-Bjergaard (1937) compared the assays of the same preparations and showed that assays carried out at two different laboratories produce approximately identical standardization curves for gonadotropic hormones with immature females or female rats as test animals. The preparations were tested for their effect on the weight of the ovary, formation of *corpora lutea*, weight of uterus and vaginal smear. In their opinion a comparison between standard curves for PMS from rats and from mice clearly demonstrates the disadvantages of measuring gonadotropic hormone in "animal units." If the unit were based upon uterine response, the mice would be found to be more sensitive than the rats, but should the unit involve a 50% increase in the ovarian weight, the rat is the more sensitive test animal. When a unit based upon doubling of the weight of the ovaries is used, the mice are the more sensitive animals. Thus, it is not possible to lay down general rules as to the relative sensitivity of rats and mice to mare serum hormone. PMS was also assayed in both laboratories on castrated immature rats and mice; but no effect on the weight of the uterus could be detected. These investigators found that

the determination of the ovarian weight curves was the best method of standardization for mare's serum hormone.

Effect of Divided Dosage. Catchpole, Cole *et al.* (1935) studied the rate of disappearance and fate of mare gonadotropic hormone following intravenous injection. Their experiments confirm the differences already noticed between pregnant mare hormone and PU. Whereas the latter is rapidly excreted in the urine, the former remains in the blood stream until it is destroyed. This is undoubtedly an explanation of the finding of Cole, Guilbert *et al.* (1932) "that single doses of the hormones are as efficacious as split doses in the rat." The experiments of Catchpole, Cole *et al.* (1935) indicate that the animal body is capable of metabolizing and destroying this hormone promptly, at a rate depending roughly on the concentration of hormone in the blood stream, and on the animal species used. The metabolism of gonadotropins and their disappearance from the blood stream have been discussed in detail by Zondek and Sulman (1945).

Leatham (1941) studied the ovarian response elicited in hypophysectomized immature female rats by the administration of the same total dosage of equine gonadotropin in five different ways. A single injection, regardless of the route of administration, exhibited more potency than divided subcutaneous injection. However, the greatest average ovarian response was produced by the daily intraperitoneal method.

This property of the hormone (of remaining in the blood stream until it is destroyed) may render it of particular importance therapeutically, since the persistence of the hormone for some time in the blood stream allows its action on the gonads to approximate normal conditions more closely.

V. GROWTH HORMONE

Acromegaly, dwarfism and gigantism are generally attributed to pituitary dysfunction. Hypophysectomy is followed by drawfism, while treatment with alkaline extracts of the pituitary leads to gigantism, according to Evans and Long (1921).

The most persistent studies attempting to purify and isolate the growth hormone have been conducted in the last twelve years by Evans and his colleagues (1933). Within the last five years, the California group has achieved notable progress in their investigations which culminated in the isolation of the hormone (Li and Evans, 1944). It was found that 0.01 mg. of hormone daily caused an increase of 10 g. in body weight of hypophysectomized female rats (27 days of age). A total dose of 5.0 mg. of the product did not show lactogenic, thyrotropic, adrenotropic, follicle-stimulating or interstitial cell-stimulating activities.

The most widely employed assay methods are: (a) the gain in weight produced in "plateaued" female rats, and (b) the resumption of growth

and subsequent gain in weight of hypophysectomized rats. These methods have been used most extensively in the laboratory of Evans at the University of California (1942). Most investigators who have employed these methods have carried out the injections of test material over periods varying from seven to 20 days. In most laboratories the unit of activity has been defined as that quantity of material that will produce an average gain of one gram a day for the injection period chosen. Evans and his collaborators have shown that normal rats ("plateaued" females) can be used quite as successfully as hypophysectomized animals and it seems that the normal rat would be less affected by small amounts of other pituitary hormones.

Freud, Levie *et al.* (1939) have observed that growth of tail vertebrae in the rat ceases after hypophysectomy, but can be caused to resume growth by injecting growth hormone. Treatment with growth hormone is started soon after operation, otherwise the epiphyses will be closed and no response can then be obtained. The measurement of the tail length, preferably in skiagrams, is used as an index of growth promotion. Evans has pointed out, however, that tail growth continues for some time after hypophysectomy in young rats and that it is questionable whether measurement of tail length is an accurate index of increase measurement of body weight.

Evans and his collaborators (1943) have recently described a new method for the bioassay of the pituitary growth hormone. This method is based on the fact that hypophysectomy causes regressive changes at the proximal end of the tibia in the immature rat which can be repaired with growth hormone. The increase in width of the cartilage observed during the administration of growth hormone is employed as the criterion of assay. A straight line relationship, within a limited range of doses, has been established between the response of the cartilage and the logarithm of the dose. The bone test is stated to be approximately three times as sensitive as body weight methods on the basis of daily dose, and requires one-fourth as much time and approximately one-tenth the quantity of hormone necessary for a corresponding response in the body weight method.

A variety of other procedures have been suggested as a measure of growth-promoting activity. However, the value of these methods is questionable, inasmuch as they are for the most part based upon measurements of alterations in nitrogen metabolism and it is not yet clearly established that the same agent may be responsible for both growth stimulation and changes in nitrogen metabolism. It should be pointed out that several other pituitary hormones, namely, the thyrotropic and adrenotropic hormones also markedly influence protein metabolism.

It is doubtful whether any of the suggested methods of assay is completely unaffected by the simultaneous presence of other pituitary hormones. Therefore, it is probable that, while certain procedures have proved useful

for following the chemical fractionation of the growth hormone, these methods have yielded only qualitative data which indicated the distribution of active substances rather than presenting a true index of the actual quantity of hormone present.

VI. ADRENOTROPIC HORMONE

It is well known that the cortex of the adrenal glands undergoes a pronounced atrophy after hypophysectomy, whereas the medulla is affected scarcely at all. Extracts of the anterior pituitary affect the cortex chiefly, if not entirely.

The effects of extracts in normal animals are, of course, difficult to evaluate accurately because the pituitary is intact.

Three methods have been employed for the assay of the adrenotropic hormone activity of various pituitary preparations.

1. Adrenal Hypertrophy of Intact Immature Rat

The procedure is essentially that described by Moon (1937) with several modifications. Twenty-one day old male rats were used. A solution of the material to be assayed is injected intraperitoneally three times daily for three days, the volume of each injection being 0.25 to 0.50 ml. Sixteen hours after the last injection, the animals are killed with illuminating gas, and the adrenals dissected carefully and weighed to the nearest tenth of a mg. with a torsion balance. A unit of adrenotropic activity is defined as the total amount of material which, when injected as described, will produce a 50% increase in adrenal weight over that of control animals injected with water. Adrenal weight is expressed as mg. per 100 g. of body weight at the beginning of the injection period. This precludes the possibility that any change in body weight, occurring during hormone treatment, may influence the results.

*2. Assay of Adrenotropic Hormone in Hypophysectomized rat**

Purified adrenotropic hormone preparations have been assayed on the basis of their ability (a) to repair the atrophied adrenals, and (b) to maintain the adrenals of the hypophysectomized rat.

a. Repair of Adrenals of Hypophysectomized Rat. Male rats approximately 45 days of age are hypophysectomized and, beginning on the tenth day after hypophysectomy, the material to be assayed is injected intraperitoneally three times daily for three days. Sixteen hours after the last injection, the animals are killed with illuminating gas and the adrenals, testes, thyroid and thymus weighed accurately on a torsion balance.

b. Maintenance of Adrenals of Hypophysectomized Rat. Male rats approxi-

* Sayers, White *et al.*, 1943; Li, Evans *et al.*, 1943.

mately 45 days of age are hypophysectomized and, beginning on the first day after hypophysectomy, are injected intraperitoneally with the material to be assayed once daily for fourteen days. Twenty-four hours after the last injections, they are killed as in procedure (a) and the various glands weighed.

The method of assay of adrenotropic hormone preparation based upon the repair of the atrophied adrenals in hypophysectomized rats has yielded useful data. Unfortunately, it is not possible to define clearly an adrenotropic hormone unit in terms of this method, inasmuch as investigators have chosen varying intervals of time postoperatively at which to begin hormone injections and have varied the length of the period of treatment. It is possible, however, in a single laboratory to differentiate the activity of various preparations. This method also requires large amounts of a preparation to repair atrophied adrenals in the hypophysectomized rat.

In the experience of White (1944), the methods (Sayers, White *et al.*, 1943; Li, Evans *et al.*, 1943) are the most sensitive, satisfactory and accurate for the assay of adrenotropic hormone activity. These authors have used the maintenance of adrenal size in the hypophysectomized rat as the criterion of potency of the extracts.

It cannot be too strongly emphasized that in the bioassay of adrenotropic hormones, great precaution must be used in adopting clearly defined and consistent conditions for conducting the assay. This is particularly true with respect to the strain of rats employed.

VII. THYROTROPIC HORMONE

One of the effects of hypophysectomy in mammals is a marked fall in the rate at which heat is produced. This change is due principally to inadequate function of the thyroid gland and can be correlated with morphological changes in the thyroid. A specific substance called the thyrotropic hormone is secreted only by the anterior pituitary; it is responsible for the maintenance of normal thyroid function and may be important in disorders attributed to deficient or excessive thyroid secretion.

The most active preparation of thyrotropic hormone (White, 1944) gives the usual qualitative protein color tests. The purified products have been examined in the Tiselius apparatus for homogeneity. Throughout the course of the experiment there was no evidence of the appearance of a second protein component in the electrophoresis cell. The approximate molecular weight of a pure preparation was 10,000.

The biological methods available depend upon either the direct effect of the thyrotropic hormone on the thyroid gland or indirect reactions caused by increased secretion of thyroid hormone by the stimulated thyroid gland.

The direct methods have as their criteria of activity alterations in histo-

logical appearance of the thyroid glands, changes in the iodine content of the thyroid and increases in weight of the thyroid upon stimulation by the thyrotropic hormone.

The indirect methods of assay involve measurements of effects produced through the mediation of the hyperactive thyroid gland. The changes which may be included are increased metabolic rate of the experimental animal, increased iodine content of the blood, decreased liver glycogen and induction of precocious metamorphosis in the larvae of amphibia. It is not easy to evaluate the various preparations of thyrotropic hormone which have been described in the literature, particularly in view of the widely different assay methods employed by different investigators.

Probably the criteria most widely employed for the assay of pituitary thyrotropic hormone have been proposed by Smelser (1938) who has found thyroid weights in day-old chicks and the examination of the histological changes occurring in the immature thyroid of day-old chicks, a convenient index for the assay. Both of these methods have also been described using the guinea pig as the test animal.

The chick thyroid is an exceedingly sensitive test for this material, reacting to one-tenth the amount required to affect the weight of guinea pig thyroids. Chick thyroid weight increases with the amount of hormone injected over a wide range of dosages, whereas the guinea pig thyroid weight increases over a narrow range. Administration of the total dose of thyrotropic extract in multiple injections greatly increases the response. Apparently, thyroid weight of normal guinea pigs is exceedingly variable, even when considered in terms of body weight, thus requiring that animals used for assay be within a narrow weight range.

Ratios for the relative sensitivity of the chick and guinea pig 'thyroid weight' methods were given by Smelser (1938) as 1:10, by Kabak and Liapin (1938) as 1:4, by Cope (1938) as 1:3, and by Bergman and Turner (1939) as 1:4. Such ratios are of some practical value but their absolute magnitude is of no great significance when one realizes that chicks and guinea pigs as well as other laboratory animals may be expected to show similar differences; however, the chick requires less thyrotropin in all cases.

Bates, Riddle *et al.* (1941) found that white carneau pigeons are unsuitable for the bioassay of thyrotropin, whereas in their earlier work they gave evidence of suitability. These investigators found that chicks from various sources require different amounts of thyrotropin to produce an equal amount of stimulation. This is not an unusual finding, and emphasizes that, irrespective of the animal employed, quantitative assays of thyrotropin require the use of parallel tests with a standard preparation of thyrotropin. Even under these circumstances, assay by more than one method is advisable in view of the varying responses observed in animals of different strains and sources.

VIII. LACTOGENIC HORMONE (PROLACTIN)

Prolactin is a hormone produced in the anterior lobe of the pituitary, probably by the eosinophilic cells. It was early found to stimulate lactation in the mammary gland and proliferation of the mucosa of the crop-sacs of doves and pigeons.

For assay purposes a direct action of a hormone is, in general, more suitable than an indirect or secondary action. That prolactin acts directly on the mucosa of the crop-sac is shown by its effectiveness in the hypophysectomized, adrenalectomized, thyroidectomized and castrate pigeons, and especially by the local crop-sac tests (Schooley, Riddle *et al.*, 1937).

Present methods for quantitative assay of prolactin fall into two groups: (1) crop-sac methods; and (2) lactation or mammary gland methods.

1. Crop-Gland Methods

a. Weight Method. The weight method is an objective method of assay depending on the combined weight of the two excised crop-sacs (with crop-milk removed) after a uniform period of injection under stated conditions.

For their standard method, Riddle, Bates *et al.* (1933) injected three months old birds intramuscularly once daily for four days, and autopsied them about 96 hours after first injection. Under these conditions, they found that a linear relation exists between the crop weight and the log of the dosage over a considerable range of dosage (see Fig. 1).

Their unit was the extrapolated value for the threshold dose. The slope (Fig. 1) is proportional to the body weight and in all their experiments has been found to be about 750 per 150 g. body weight (*i.e.*, 750 mg. increase in crop weight per 10 times increase in dose.) For practical purposes, a large group of birds is injected.

b. Minimum Stimulation Method. This is a subjective method of assay in which the excised distended crop-sac is examined by transmitted light. Positive stimulation is indicated by the presence of typical parallel strands of thickened mucosa. The "50% response" method of Trevan was applied to this minimum crop-sac stimulation by McShan and Turner (1936) who propose as a pigeon unit "the total amount of hormone injected during a period of 4 days which will cause a minimum but definite proliferation of the crop-glands of $50 \pm 11\%$ of 20 common pigeons weighing 300 ± 40 gms." (See Fig. 2). Injections are made into the pectoral muscles. These authors claim that this method is more accurate than the weight method.

c. Local Stimulation Method. Lyons and Page (1935) first suggested injecting lactogenic extracts intradermally over each crop gland. They recommend a single injection with autopsy after 48 hours. By this technique it was possible to detect small amounts of hormone due to a "local" reaction at the site of injection. A further advantage of this method lay in one's ability to compare two preparations in the same pigeon.

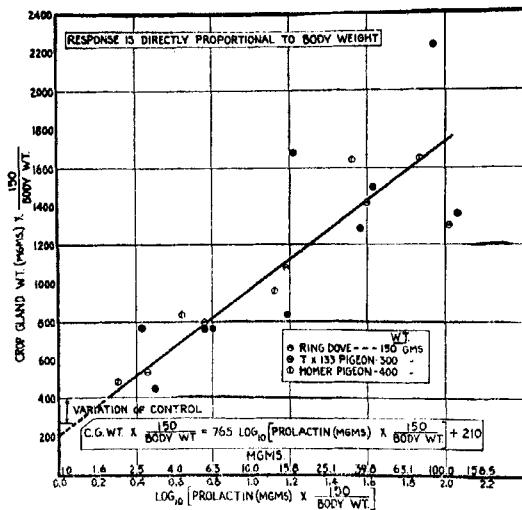


Fig. 1

Individual Response of Immature Doves and Pigeons to Prolactin #65
After Riddle, Bates and Dykshorn, *Am. J. Physiol.* 105, 213 (1933)

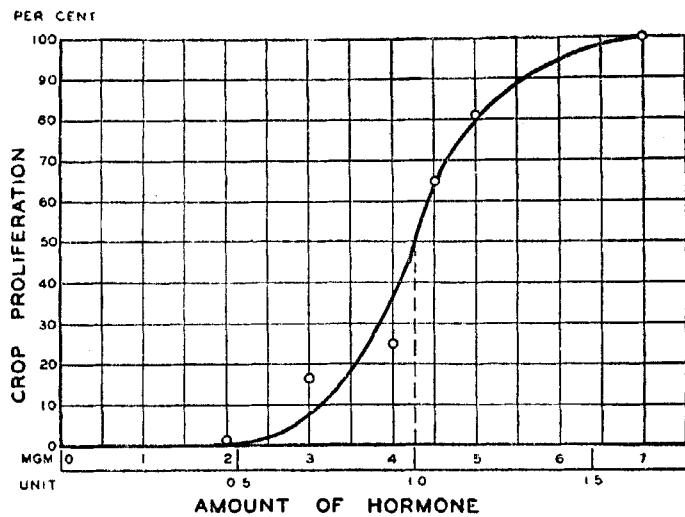


FIG. 1.

Fig. 2

Relation between Amount of Hormone Injected and Percentage of Pigeons
Showing Crop Gland Proliferation

Relation is described by a characteristic sigmoid curve which indicates extreme sensitivity to small amount of hormone in region of 50% response. The pigeon unit is based upon this curve, one unit of the hormone being the amount required to give a 50% response.

After McShan and Turner, *Proc. Soc. Exptl. Biol. Med.* 34, 50 (1936)

Reece and Turner (1937) have used this method and Bergman, Meites *et al.* (1940) have modified the method to include a large number of pigeons. These authors defined the unit as that amount of hormone which, when injected intradermally over the crop gland of 20 common pigeons, will elicit a minimum response in $50 \pm 11\%$ of the pigeons. The crop glands were examined and rated when viewed by transmitted light.

2. Mammary Gland Method

Since prolactin stimulates prepared mammary tissue to milk secretion, the criterion of prolactin action on the mammary glands is the initiation of milk secretion. Though qualitatively lactogenesis had been induced by prolactin in numerous species, only the pseudopregnant rabbit and the hysterectomized pregnant guinea pig have been sufficiently studied from the standpoint of bioassay.

Gardner and Turner (1933) proposed a method of assay using pseudopregnant rabbits while Nelson (1934) employed pregnant guinea pigs, hysterectomized between the fortieth and fifty-fifth days of pregnancy, and if milk can be expressed from the nipples during this test period the result is positive. Milk usually appeared in less than 48 hours. Nelson found that it required two and one-half times as much prolactin to induce lactation in the normal adult female guinea pig as in the hysterectomized pregnant guinea pig.

Most investigators in the field have concluded that lactation provides a less accurate assay method than the crop-sac weight method.

Since the International Standard lactogenic hormone became available (1938), Lyons (1940) has recently reported that 80 I.U. are required to induce lactation in 50% of 20 estrus guinea pigs weighing from 650-1000 gms.

Meites, Bergman *et al.* (1941), using three methods of assay, found that by the subcutaneous route of administration, 0.1 mg. of the International Standard was required which is equal to one I.U. The shallow intrapectoral method requires 1.25 I.U., and the intradermal (micro) unit required 1/160 of an I.U.

IX. THE BIOASSAY OF ADRENAL CORTICAL HORMONES

1. Introduction

The large number of physiological effects produced by the compounds extracted from the adrenal cortex and, in particular, the sharp separation of the qualitative effects of the various compounds emphasize the necessity of using suitable criteria for assay of these substances. Assay based on a single type of response is not satisfactory.

For the purpose of assay, the hormones of the adrenal cortex can be classified into two groups:—1, Those closely allied to desoxycorticosterone; and, 2, the steroids which have an oxygen at the C-11 position, either in keto- or hydroxy-form. The nomenclature of the latter type compounds is based on their relation to corticosterone. The physiological activity of these latter hormones, while showing quantitative differences, is essentially similar and seems primarily concerned in the regulation of some aspects of organic metabolism as opposed to inorganic.

Desoxycorticosterone is apparently the most potent of all known cortical hormones in maintaining the life of adrenalectomized animals and in the control of certain phases of electrolyte metabolism.

17-Hydroxycorticosterone has been found the most active in effecting some aspects of organic metabolism.

Many methods have been suggested for assay. Some of these will be reviewed briefly and the methods which have been most widely used will be discussed in detail in the latter part of the paper.

a. *Survival.* (Rogoff and Stewart, 1928; Hartman, 1927; Swingle and Pfiffner, 1930).—The criterion first suggested and used for the assay of cortical principles was the survival of adrenalectomized rats, cats, dogs and guinea pigs. This is useful to demonstrate the presence of compounds with cortical activity. In the use of these criterion it is essential to control the intake of sodium chloride and potassium ions.

b. *Growth of Young Rats.* (Hartman and Thorn, 1930).—For highly purified preparations of the amorphous fraction, the influence on the rate of growth of young rats is significant, but the directly antagonistic effect of corticosterone and 17-hydroxy-11-dehydrocorticosterone, which may cause not only retardation in growth but actual loss in weight, indicates that it is difficult to use growth of young rats as a general criterion for the assay of extracts of the adrenal cortex (Ingle and Kendall, 1940; Wells and Kendall, 1940). In a recent paper, Kuizenga, Nelson *et al.* (1943) found that 17-hydroxy-11-dehydrocorticosterone will maintain the young four-week old adrenalectomized rats and support growth at dosage levels of 1/8 to 1 mg. These authors state, "The reason for the discrepancy in the results obtained earlier by one of the authors using rats of 180 g. weight, and the results of the present experiments using rats of 50 to 60 g. weight, is not understood."

c. *Survival of Adrenalectomized Rats in a Low Environmental Temperature* (Selye and Schenker, 1938).—This is probably the most sensitive criterion for the determination of adrenal cortical activity; however, it is non-specific.

d. *Maintenance of a Normal Condition in Adrenalectomized Dogs.* The dog method of assay, (Pfiffner, Swingle *et al.*, 1934) consists essentially in the

determination of the minimum amount of a preparation necessary to maintain the adrenalectomized animal in a normal state. Although this method is expensive and time consuming, it is the most trustworthy method available for standardization of the active substances of the adrenal cortex in relation to renal function.

e. Sodium Retention. Hartman, Lewis *et al.* (1941) have elaborated a method in which the capacity of the extract to improve tubular resorption of sodium ions in the kidney is measured. A sodium retention unit is defined as one-tenth the amount of material which will cause the same sodium retention as 0.7 mg. of desoxycorticosterone in the same dog.

f. Deposition of Glycogen in Fasting Adrenalectomized Rats. (Long, Katzin, *et al.*, 1940; Reinecke and Kendall, 1942; Olson, Jacobs *et al.*, 1944.)—The glycogen in the liver of a fasting adrenalectomized rat is rapidly depleted, and after eighteen to twenty-four hours the concentration is close to 0.10%. The administration of corticosterone and related compounds will cause deposition of glycogen. Desoxycorticosterone and other substances of the adrenal cortex which do not possess an atom of oxygen at the C-11 position do not increase the concentration of glycogen.

g. Long Stimulation of Muscle. Ingle (1936, 1944) devised a muscle work test for the assay of adrenal extracts. It was found that the adrenalectomized nephrectomized rat is a satisfactory test animal. The experimental conditions have been standardized, the sensitivity of the test studied and a unit of activity defined. This method is a sensitive and reliable method for the detection and quantitative estimation of the biological activity characteristic of C₁₁ oxygenated cortical steroids.

From the survey of the above catalogue of bioassay procedures, it can be seen that the results of investigations have been difficult to interpret because, until quite recently, the qualitatively different physiological effects of the hormones of the adrenal cortex have not been recognized by many and attempts to formulate the function of the adrenal cortex by a single theory which included the influence on the distribution and excretion of inorganic ions as well as that on carbohydrate metabolism have not been successful. It immediately becomes evident that the adrenal cortex has more than one function and, therefore, more than one criterion must be used to assay adrenal cortical extracts.

2. Deposition of Glycogen in Fasting Adrenalectomized Rats

In our laboratory (Olson, Jacobs *et al.*, 1944; Olson, Thayer *et al.*, 1944), we made a quantitative biological comparison of certain extracts of the adrenal cortex and six crystalline adrenal cortical compounds. The methods that we used were the following: (a) the test of growth and survival in immature adrenalectomized rats as described by Grollman (1941); (b) the

test of renal function in adrenalectomized dogs as proposed by Pfiffner, Swingle *et al.* (1934); (c) the test of sodium retention in normal dogs as devised by Hartman, Lewis *et al.* (1941); and (d) a test of glycogen deposition in fasted, adrenalectomized rats based upon principles recommended by Britton and Silvert (1932), developed by Reinecke and Kendall (1942), and modified by Olson, Jacobs *et al.* (1944).

Olson, Jacobs *et al.* (1944) observed that under high protein regime a strictly linear relationship over a wide range of doses exists between the log of the total dose of adrenal cortical extract injected into a fasted test rat over a period of six hours and the mean amount of glycogen found in its liver two hours after the last injection. Numerous control experiments have pointed to the necessity of using rats at an age of 60–75 days. The rate of glycogen deposition in our procedure is such that maximum liver glycogen levels are attained 2–4 hours after the last injection. It is of considerable importance to restrict the fluid intake during the assay period, and to administer the hormones parenterally in saline or 10% alcohol since the use of oil as a vehicle results in the apparent recovery of only 50% of the activity. Careful attention to the maintenance of these conditions resulted in improvements in both the total response of the animal to a given dose of cortical extract and the linearity of curve in which the logarithm of the dose is plotted against the response.

a. Experimental Animals. Male albino rats from the highly inbred colony of this laboratory were employed in these experiments. In the course of these studies they were selected in groups at two age levels, 30–31 days and 60–75 days. Only those animals which weighed 60–90 g. in the first instance and 145–180 g. in the second were considered acceptable.

Diets. Several diets were employed in the course of this study. The so-called low protein rations were: (a) the growth assay ration of Grollman (1941); (b) Purina dog chow; and (c) G-2, an artificial ration employed by Mulford and Griffith (1942) in the study of choline deficiency. It contained: casein 18, dried brewer's yeast 6, sucrose 48.7, lard 19, Hawk-Oser (1931) salt mixture 4, calcium carbonate 1, codliver oil 1, cereal cellulose 2, cystine 0.3, and choline chloride 2 mg. per g. of food. The protein content of these diets ranged from 21–23%. The high protein diet containing approximately 58% protein was designated OG-2. It was produced by increasing the casein content of G-2 to 55% at the expense of carbohydrate. Gram for gram replacement of carbohydrate by protein lowered the carbohydrate content of OG-2 to 11.7%. All the diets were of sufficient biological value to support good growth in immature rats.

Final Assay Procedure. Male albino rats in groups of 20–30 animals ranging in age from 60–75 days and in weight from 145–185 g. were removed biweekly from their stock fare of Purina dog chow, anesthetized with ether,

bilaterally adrenalectomized by the lumbar route according to Grollman (1941) and then placed in raised bottom cages and given Diet OG-2 and tap water containing 1% sodium chloride until the morning of the fourth postoperative day. They were then fasted until the morning of the fifth postoperative day at which time their drinking water was removed and the cortical injections begun. Each rat was given a fixed dose of extract either in saline or 10% alcohol in four equally divided doses at two hour intervals. It was the usual practice to assay two extracts per day, arranging the injections so that each extract was given to groups of 4-6 rats at three dose levels. Two hours after the last injection, the animals were narcotized in batches of four with 1.5 cc. of 1% sodium amyta! intraperitoneally, the abdomens opened, and approximately one-half of the left lateral lobe of each liver dropped into a tared, tapered 50 cc. centrifuge tube containing 2.0 cc. of cold 30% KOH (Cori, 1932). The samples were quickly weighed on a damped chainomatic balance and transferred to a boiling water bath until dissolution of the liver tissue became complete. One hour sufficed to extirpate, weigh, and dissolve the livers from such a group. The precipitation and hydrolysis of the glycogen was then carried out according to the directions of Good, Kramer *et al.* (1933). Total reducing substances in the neutralized diluted filtrates were determined by the method of Shaffer and Somogyi (1933) using reagent 50 containing 1.0 g. of KI per liter. All glycogen values have been expressed in terms of their glucose equivalents. Control experiments testing the recovery of glycogen added to alkaline digests of liver tissue, the rate of glycogenolysis in sectioned, anesthetized liver tissue *in situ*, and the distribution of glycogen in the various lobes of the same liver, showed that the errors associated with these variables were much less than the mean biological error inherent in the assay response. Losses of weight up to 15% of the initial body weight in the five day period following adrenalectomy could be sustained without interference with the liver response. Only those animals which were moribund after the twenty-four hour fast were discarded. Diarrhea was an inconstant finding in our rats, occurring, without apparent effect upon the liver glycogen deposition, in about 5% of our animals. Mortality from adrenal insufficiency in the post-operative period ranged from 5-10%.

Extracts. All of the samples used for assay were kept at 4°C. No deterioration in the biological potency of any of them was noticed over a period of eighteen months. These extracts are referred to by different Roman numerals in the text and charts.

Standard. It is suggested that corticosterone be adopted as the reference standard for the glycogenic assay. This C₁₁-hydroxy steroid is highly active in inducing glycogen deposition in fasted, adrenalectomized rats as well as being very active in other biological tests of cortical activity (Reichstein

and Shoppee, 1943). We have defined a unit of glycogenic activity as the potency of 1 γ of corticosterone administered to a fasted, adrenalectomized rat in four divided doses at two hour intervals. Since this amount of corticosterone is actually below the threshold of response in our rats, various multiples of 1 γ , equivalent, then, to as many units, were administered in order to get measurable depositions of glycogen. All expressions of glycogenic potency in our experiments have been made with reference to corticosterone. The potency of the cortical extracts assayed by us is recorded in terms of units per cc. and per g. equivalent of fresh adrenal tissue.

b. The Comparative Activity of 7 Extracts of the Adrenal Cortex. Five cortical extracts of beef and two of hog adrenals were tested for their glycogenic activity by our standard procedure. All injections were made subcutaneously into the nape with either saline or 10% alcohol as the vehicle. Each extract was assayed at three, and commonly four or five, dose levels in order to define the position of its regression line with precision. Both the slope and the displacement of the individual regression lines from that of corticosterone were found to be of characteristic importance. The divergence in the slope of some of the regression lines from that of corticosterone made it necessary to select a level of glycogen deposition for the potency comparisons. A level of 1.00% liver glycogen was arbitrarily chosen. This level was considered optimum for our colony of rats since it fell well beneath the ceiling of response (3.00%) and at the same time was well above the fasting control base-line of $0.032 \pm .003\%$ obtained in 56 rats. By application of the method of least squares (Dunn, 1929) to the glycogen deposition values obtained in individual rats injected with various doses of each extract, the formulae for the respective regression lines were calculated and appear in Table VI. Application of the same method to the glycogen deposition data obtained in 23 rats injected with doses of corticosterone varying from 0.4–1.2 mg. revealed the equation of the standard regression line to be:

$$y = 2.06 \pm 0.29 \text{ (logarithm dose mg.)} + 1.24 \pm 0.05$$

where y is the quantity of liver glycogen in terms of its per cent of fresh liver weight (Olson *et al.*, 1944). By solving this deposition equation for 1.00% liver glycogen, the mean dose required to deposit this level of glycogen was found to be 0.766 ± 0.039 mg. Since the unit of glycogenic activity, as defined, is contained in 1 γ of corticosterone, it follows that 766 ± 39 units are required to secure a level of 1.00% liver glycogen in the test rat, and this number of units will be contained in the amount of extract which will deposit 1.00% liver glycogen in the test rat. These amounts, for all extracts tested, were calculated from their respective regression line equations and are found in Table VII together with their potencies in terms of glycogenic units per cc. and per g. equivalent of fresh adrenal tissue.

Since there is good evidence at present for strain differences in the reactivity of rats to injected cortical hormones, it may happen that all laboratories engaged in the glycogenic assay of cortical extracts will be unable to compare them at the same level of glycogen in the liver. The use of a reference standard and the calculation of regression line formulae for both standard and unknown in the manner outlined precludes any great discrepancy in the unitage reported for a given extract regardless of the sensitivity of the particular strain. The relationships of the regression lines for seven extracts to each other and to the regression line of corticosterone are shown graphically in Fig. 3.

There is a large variation in both the slope and position of the regression lines for the extracts tested (Tables VI, VII). Extract VI from hog adrenals is by far the most potent per g. of fresh adrenal tissue. Extracts I and IV from beef adrenals are approximately one-half as potent as Extract VI per g. of fresh adrenal tissue. All three of these extracts possess regression lines of high slope approximating that of the C₁₁-oxygen-containing standard. The remaining Extracts II, III, and V from beef adrenals and Extract VII from hog adrenals are less potent and have regression lines of significantly smaller slopes. In spite of differences in the slope of their regression lines, Extracts I and III are of comparable potency per g. equivalent of fresh adrenal tissue at a liver glycogen deposition level of 1.00%.

These changes in slope and potency would seem to indicate not only a quantitative loss of important hormones in the preparation of some of these extracts, but qualitative alteration in their composition as well. It is surprising that two extracts of hog adrenal tissue (VI and VII) could differ so markedly both in potency and in the slope of their regression lines.

3. The Test of Renal Function in Adrenalectomized Dogs

The second method of assay adopted by us for evaluation of the selected extracts was the real function test of Pfiffner, Swingle *et al.* (1934). Since the effects of adrenal cortical extract on renal function and carbohydrate metabolism seem to be the result of stimulation of independent physiological mechanisms which are controlled by separate fractions of the extract, a careful comparison of assay results by the two methods was particularly desirable.

a. Methods. Adult male dogs were adrenalectomized in two stages, placed in individual metabolism cages, and maintained upon an exclusive diet of Purina dog chow (1.25% NaCl) fed to them in the early part of the afternoon. The daily food intake of these animals was approximately 300 g. although borderline deficiency states induced in certain phases of the tests resulted in poorer appetites and lower food intakes. The animals were allowed to drink water *ad libitum* and exercise in a pen three times a day.

TABLE VI
Glycogen Depositions Obtained with Seven Extracts of the Adrenal Cortex

Extract No.	Rats No.	Total dose cc.	Mean glycogen deposition per cent	Regression line coefficients*	
				$b \pm \sigma_b$ †	$a \pm \sigma_a$
I	18	1.00	0.82 ± .05†		
	24	2.00	1.45 ± .09		
	4	4.00	1.88 ± .15	1.92 ± .25	0.84 ± .05
II	46	1.00	0.31 ± .02	1.50 ± .10	0.31 ± .02
	68	2.00	0.76 ± .03		
	37	4.00	1.22 ± .06		
III	20	1.00	0.42 ± .04		
	28	2.00	0.88 ± .04		
	4	4.00	1.20 ± .10	1.41 ± .17	0.44 ± .03
IV	6	0.50	0.30 ± .06		
	22	1.00	0.83 ± .05		
	13	2.00	1.53 ± .12		
	6	4.00	2.14 ± .07	2.15 ± .15	0.88 ± .04
V	4	1.00	0.09 ± .02		
	10	2.00	0.39 ± .08		
	5	4.00	0.89 ± .13		
	2	8.00*	1.25 ± .07	1.35 ± .20	0.03 ± .05
VI	9	0.25	0.33 ± .05		
	21	0.50	0.87 ± .07		
	37	1.00	1.57 ± .06		
	17	2.00	2.21 ± .10		
	6	4.00	2.93 ± .18	2.20 ± .08	1.57 ± .04
VII	4	1.00	0.09 ± .04		
	14	2.00	0.30 ± .05		
	7	4.00	0.30 ± .03	0.31 ± .19	0.16 ± .03

* The regression line coefficients are for the equation from which glycogen in per cent fresh liver weight may be predicted from the log of the dose (Bliss, 1943)
 $y = b (\log \text{dose mg.}) + a$.

† The standard errors of the regression line coefficients are calculated according to Dunn (1929).

‡ The deviations are expressed in terms of the standard error of the mean.

* In this experiment the quantity of extract was lyophilized to dryness and made up to 4.00 cc. with water before injection.

After R. E. Olson, F. A. Jacobs, D. Richert, S. A. Thayer, L. J. Kopp, and N. J. Wade, *Endocrinology* 35, 430 (1944).

Urea nitrogen in whole blood drawn from the saphenous vein in oxalated syringes was photometrically determined (Hoffman and Osgood, 1940) at intervals of from 5–7 days.

At the beginning of each assay period the adrenalectomized dogs were receiving more than enough cortical extract or desoxycorticosterone acetate (DCA) to maintain them in a normal condition. The cortical substances were administered daily in two equally divided subcutaneous doses. The cortical extract was given in saline and the DCA in 30% alcohol. As the assay progressed, the dosage of cortical substance was reduced stepwise, in

TABLE VII
Glycogenic Potency of Seven Extracts of the Adrenal Cortex in Terms of Corticosterone

Extract No.	Rats No.	Amount required to deposit 1.00% liver glycogen cc.	Potency per cc. (mg.)† U.	Potency per gram fresh adrenal tissue U.
I*	46	1.21 ± 0.07 *	635 ± 55 *	8.5 ± .7
II**	151	2.86 ± 0.10	269 ± 21	6.7 ± .5
III**	52	2.49 ± 0.13	308 ± 23	7.7 ± .5
IV*	47	1.13 ± 0.05	682 ± 49	9.1 ± .7
V**	21	5.43 ± 0.47	142 ± 13	3.6 ± .3
VI*	90	0.55 ± 0.02	1400 ± 116	18.7 ± 1.6
VII**	25	59.4 ± 14.7 ^a	7 ± 4	0.2 ± .1
Cortico-sterone†	23	0.77 ± 0.04 mg.†	1000.	

† (mg.) refers to the standard, corticosterone, only.

* The hormonal content of these extracts was equivalent to 75 g. of fresh adrenal tissue per cc.

** The hormonal content of these extracts was equivalent to 40 g. of fresh adrenal tissue per cc.

* The standard errors of the deposition equivalents and of the potency ratios are calculated according to the method of Bliss and Marks (1939).

^a Because of the extremely low potency of Extract VII, comparisons were made at 0.40% liver glycogen deposition instead of 1.00%.

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5-7 day periods, until urea retention was observed and a rise in blood urea-N of 100% was considered the end-point.

The unit of cortical activity in the adrenalectomized dog has been defined by Pfiffner, Swingle *et al.* (1934) as the "minimum daily kilogram dose of cortical hormone necessary to maintain normal physiological conditions in the adrenalectomized dog for a period of 7-10 days; the two criteria being the maintenance of normal weight and blood level of urea nitrogen." Instead of employing the "Pfiffner unit," which is a function of individual dog sensitivity to cortical hormones, we thought it would be preferable to adopt a unit based upon a reference standard. Since Reichstein and Shoppee (1943), Thorn and Eisenberg (1939), Cleghorn, Fowler *et al.* and Remington, Parkins *et al.* (1941) have found desoxycorticosterone

acetate (DCA) capable of supporting life in adrenalectomized dogs, and as our experiments fully corroborate theirs, we suggest that DCA be adopted as the reference standard for this assay. We have defined the unit as the anti-urea retention activity of 1 γ of DCA in the bilaterally adrenalectomized dog. The minimum amount of DCA which will maintain the

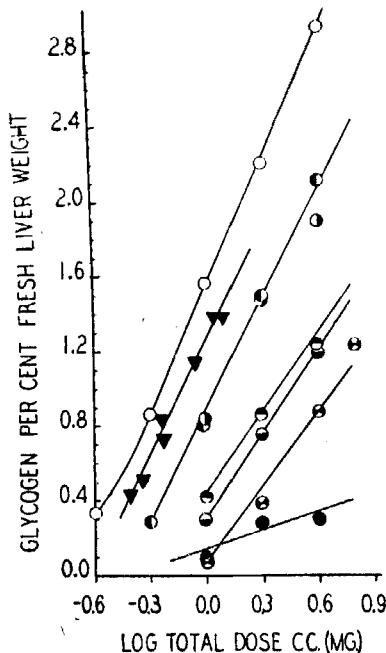


Fig. 3
Logarithm Dose-Response Regression Lines for 7 Extracts of the Adrenal Cortex and for Corticosterone

● Extract I; ○ Extract II; ● Extract III; ◑ Extract IV; ● Extract V; ○ Extract VI; ● Extract VII; ▼ Corticosterone. The abscissa units refer to the logarithm of the total dose of cortical extract in cc. in all cases but that of corticosterone in which the units refer to the logarithm of the total dose in milligrams.

After Olson, Jacobs, Richert, Thayer, Kopp and Wade. *Endocrinology* 35, 430 (1944)

blood urea nitrogen within the physiological span of 14–18 mg. % in a given dog is the protective dose of standard for that dog; the minimum amount of extract which will maintain blood urea nitrogen levels within the same span is the protective dose of extract for that dog. The potency of the extract is then obtained by substituting the following formula:

$$\frac{(\text{protective dose of DCA in mg.} \times 1000)}{(\text{protective dose of extract in cc.})} = \text{DCA units per cc.}$$

Since a lag is sometimes noticed when dogs are transferred from DCA to cortical extract, it is suggested that the first period in the assay of extract be eleven days instead of seven. The augmentatory effect of the previous treatment with DCA seems to be neutralized in four days.

b. Results. The results of preliminary experiments with desoxycorticosterone acetate in three adrenalectomized dogs are shown in Table VIII. The mean values for each dog were obtained by averaging several determinations made during its maintenance on a given dose of DCA. The minimum and maximum blood urea nitrogen values obtained in these serial

TABLE VIII
Blood Urea-Nitrogen Values Obtained in Adrenalectomized Dogs Maintained with Desoxycorticosterone Acetate

Dog No.	1.5*	Blood urea nitrogen in mg. %		
		1.0*	0.7*	0.5*
A. min.	8.0	18.2	14.5	28.5
	max.	15.5	20.2	32.9
	mean	11.4	19.2	30.7
B. min.	11.5	10.3	21.0	22.7
	max.	18.5	13.9	30.6
	mean	15.1	11.9	26.6
C. min.	10.0	10.3	19.0	
	max.	17.2	19.2	29.0
	mean	14.3	15.0	31.6**

* Dose of DCA per dog per day in mg.

** Each dog was bled 3-5 times on each dose level with the exception of Dog C on 0.5 mg. of DCA per day.

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analyses are also included for each dog on each dose. It may be seen that the protective dose of DCA for dog A is less than 1.5 mg. and greater than 1.0 mg.; for dogs B and C, it is approximately 1.0 mg. The protective dose of standard was determined for all of the animals used in these assays. The potency of the various extracts in controlling renal function as indicated by this method are listed in terms of DCA units per g. of fresh adrenal tissue in Table IX. The feature of these results is their average uniformity.

c. Discussion. The results of our assays by this method are in good agreement with those of Thorn and Eisenberg (1939), Cleghorn, Fowler *et al.* (1941), and others using diets of relatively low sodium chloride content. The lower maintenance doses which have been reported by Pfiffner, Swingle *et al.* (1934), Cleghorn, Fowler *et al.* (1941), and Remington, Parkins *et al.* (1941) for both desoxycorticosterone acetate and extract

have been found in dogs fed higher amounts of NaCl. The effects of differences both in animal sensitivity and dietary salt upon the assay results tend to be minimized by the use of a reference standard. In spite of carefully controlled standardizations, however, the variation in the assay results obtained for a single extract tested in several dogs may be excessive (Table IX). It would appear that a larger number of dogs is required for assays of statistical significance.

It may be concluded that, in spite of the difficulties which have been enumerated, this assay procedure for the measurement of the factors of the cortex which influence renal function serves to reveal a type of adrenal

TABLE IX
*The Renal Function Potency of Seven Extracts of the Adrenal Cortex in
DCA Units*

Extract No.	DCA units per gram equivalent of fresh adrenal tissue				
	Dog A	Dog B	Dog C	Dog D	Dog E
I	5.5 ± 1*	5 ± 1			
II	4 ± 1	4 ± 1	4 ± 1	4 ± 1	3 ± 1
III	4 ± 1	4 ± 1	4 ± 1	3 ± 0.5	4 ± 1
IV	4 ± 1	4 ± 1			
V	7 ± 2	7 ± 2		3 ± 0.5	4 ± 1
VI	6 ± 1	4 ± 1			
VII	5 ± 1				

* The standard error of the mean calculated for small groups according to Burn (1937) on the basis of at least three responses per dog per extract.

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cortical activity. The adrenalectomized dog is particularly sensitive to compounds related to desoxycorticosterone.

4. Sodium Retention in Normal Dogs

The method of Hartman, Lewis *et al.* (1941), in which the effect of cortical extract upon the excretion of sodium in dogs is measured, was selected for the evaluation of those factors of the cortex which affect inorganic metabolism.

a. *Methods.* Both male and female dogs ranging in weight from 8–15 kg. were used. After preliminary control experiments testing their degree of constancy and sensitivity in the assay had been conducted, four males and three females were selected from a group of ten males and ten females. The males were segregated from the females to avoid excitement. The females were fed the beef heart mixture of Hartman, Lewis *et al.* (1941) while the males were maintained upon Purina dog chow; both consumed their daily food in a period of fifteen minutes. The bioassays were con-

ducted according to the recommendations of the authors (Hartman, Lewis *et al.*, 1941) in both hot and cold weather and the results compared. Desoxycorticosterone acetate was employed as the standard instead of desoxycorticosterone and administered to the dogs in a solution containing 10% alcohol, 10% propylene glycol and 80% water. The solvent was found to exert no influence on sodium excretion. Urinary sodium was determined by the method of Butler and Tuthill (1931).

b. Results. In control experiments on normal dogs over a period of eight months, the degree of variation in sodium excretion under physiological conditions was determined. Typical of the results obtained are the following two examples: Dog LBM showed a mean sodium excretion of 14.3 ± 1.9 mE of Na in a series of twelve tests on different days in the summer, and an excretion of 17.6 ± 2.3 mE of Na in a similar series in the winter. Dog BM excreted 23.7 ± 1.9 mE of Na in the summer series and 30.0 ± 1.1 mE of Na in the winter series. Both dogs were males. Although the mean values are significantly different in the second case, the degree of variation as revealed by their standard errors was not appreciably altered by the hot weather in either case. Indifference to the season was also seen in experiments in which the degree of sodium retention induced by standard amounts of DCA was measured. Retention of sodium by Dog LBM per 0.7 mg. of DCA in the summer was $40.1 \pm 4.1\%$ in four tests; retention by the same dog per 0.7 mg. of DCA in the winter was $45.7 \pm 2.6\%$ in five tests. In similar tests of Dog BM the retention of sodium in the summer was $48.4 \pm 3.1\%$ while retention of sodium in the winter was $51.7 \pm 3.6\%$. The mean retention of sodium per 0.7 mg. of DCA in sixteen assays in both the winter and the summer on six dogs was $51.0 \pm 1.4\%$. The extracts were run in series with DCA on each dog after a "rest" of two days. Only those runs in which 0.7 mg. of DCA induced a percentile retention of sodium within the "sensitive range" of 35-65% were considered satisfactory for the calculation of the potency of an unknown extract. It was occasionally found that the same dose of DCA on successive runs would cause percentile retentions of sodium which fell both inside and outside the sensitive range.

The results of the assays of four extracts are listed in Table X. The potency of these extracts is expressed in Hartman DCA units per cc. of extract. The variation is presented in terms of the standard error of the mean calculated by orthodox methods (Dunn, 1929). Even though the experiments here reported are too few to be properly subject to statistical analysis (see *t* values, Table X), it is probable that Extracts II and III are more potent in sodium-retaining substances per g. equivalent of fresh adrenal tissue than are Extracts I and IV. Our data indicate that only slight changes in sodium-retaining potency are effected by different methods of preparation of extracts in four cases.

c. *Discussion.* The control observations on sodium excretion in selected assay dogs during the summer months showed that, although a slightly diminished excretion occurred in the summer, the variability in the results was approximately the same. Males and females showed no significant differences. The sodium retention responses to DCA were constant to the degree claimed by Hartman, Lewis *et al.* (1941). The retention responses to extract, however, although reasonably constant in a particular dog were highly variable in different dogs. Apparent differences in different dogs persisted in spite of standardizations with DCA in each case. The statistical significance of the differences is questionable in view of the small number of tests.

TABLE X
The Sodium-Retaining Potency of Four Extracts of the Adrenal Cortex in DCA Units

Extract No.	Assays No.	Dogs No.	Potency per cc.* U.	Potency per gram of fresh adrenal tissue U.	Significance of difference **
I	12	6	1.53 ± .11 ^a	0.021 ± .002	0.36
II	9	4	1.58 ± .16	0.040 ± .004	3.28
III	3	2	1.42 ± .31	0.036 ± .008	1.80
IV	2	2	1.42 ± .33	0.019 ± .005	0.00

* The unitage per cc. is calculated by the formula of Hartman *et al.* (1941):

$$\text{U./cc.} = \frac{\% \text{ retention by unknown}}{\% \text{ retention by } 0.7 \text{ mg. DCA}} \times \frac{10}{\text{volume of extract injected}}$$

** The *t* factors are calculated with respect to the least potent extract, IV.

^a The deviations are expressed in terms of the standard error of the mean.

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On the basis of the evidence presented it would appear that the Na-retaining factor of Hartman and Spoor (1940) is present in four adrenal cortical extracts in approximately the same amounts.

In view of the separation of the Na-retaining factor from the "essential" cortin fraction by Hartman and Spoor (1940), it is doubtful that one is able to predict the replacement value of a given adrenal cortical extract on the basis of its sodium retaining power in the normal dog.

5. *Growth and Survival in Immature Adrenalectomized Rats*

To test the effect of the selected adrenal cortical extracts upon the growth and survival of immature adrenalectomized rats, the procedure of Grollman (1941) was followed without modification.

a. *Methods.* Young male rats were weaned at the age of 20 days and placed upon Grollman's diet for 10 days. At the end of this time the animals weigh-

ing less than 50 g. were rejected and the others bilaterally adrenalectomized according to the technique of Grollman (1941). A few hours after the operation the animals were weighed and then injected subcutaneously with graded quantities of adrenal cortical extract or desoxycorticosterone acetate. The extracts were dissolved in either saline or 10% alcohol while the DCA vehicle contained 10% alcohol, 20% propylene glycol and 80% water. On each of the six following days the injections were repeated and on the seventh day the final weight recorded. The mean weight gain was calculated from the initial and final weights of the individuals in the assay group and plotted against the log of the daily dose of extract given. All animals surviving more than two weeks after the cessation of injections were not considered in the calculations of potency. Adrenal cortical remnants and accessory tissue were found in such surviving rats. In several experiments other diets than Grollman's were tested, but none permitted the development of the deficiency state after withdrawal of extract as quickly as did Grollman's diet.

Calculations of potency were made in terms of desoxycorticosterone acetate which was arbitrarily assigned a standard potency of 1000 growth units per mg.

b. Results. The growth responses of young adrenalectomized rats given adrenal cortical hormones parenterally in this procedure were extremely variable. Table XI contains a summary of growth tests conducted on 370 30-day adrenalectomized rats over a period of eight months. There was no noticeable seasonal change in the sensitivity or the variability of our animals in these assays. The mean growth responses were plotted against the log of the daily doses of extract or DCA given, and are shown in Fig. 4. The regression lines were approximated by standard methods (Dunn, 1929) and drawn. The equation found for that of the standard DCA was:

$$y = 30.8 \pm 4.4 (\log \text{dose } \gamma) - 47 \pm 1$$

where y is the mean growth in g. of body weight per seven days.

Because of the marked differences in the slopes of the regression lines for the standard and for the unknown extracts, it was arbitrarily decided to make potency comparisons at a growth level of 10 g. in seven days. By substituting 10 for y in the above equation, it may be shown that $70 \pm 8 \gamma$ of DCA were required to induce the standard weight gain in our rats. Since DCA has been assigned a potency of 1000 growth units per mg., it follows that the minimum amount of extract required to induce a weight gain of 10 g. in 7 days in our 30-day adrenalectomized rats would contain 70 ± 8 growth units. Calculations on this basis have been made for each extract and the potency values per cc. of extract and per g. equivalent of fresh adrenal tissue recorded in Table XII.

TABLE XI

Growth Stimulation Obtained with Five Extracts of the Adrenal Cortex and with Desoxycorticosterone Acetate in Immature Adrenalectomized Rats

Extract No.	Rats No.	Daily dose cc.	Mean growth g.
I	5	0.1	3.9 ± 2.1*
	14	0.2	1.5 ± 1.4
	16	0.3	5.1 ± 1.7
	12	0.4	4.5 ± 1.4
	8	0.5	8.1 ± 2.4
II	14	0.1	3.1 ± 1.3
	7	0.15	4.1 ± 2.0
	23	0.2	1.3 ± 1.1
	13	0.3	8.2 ± 1.7
	20	0.4	6.9 ± 0.9
	56	0.5	5.7 ± 0.7
	14	0.6	12.2 ± 0.9
	39	1.0	7.8 ± 0.7
III	15	0.4	3.8 ± 1.8
	17	0.5	5.1 ± 1.1
	15	0.6	3.9 ± 1.0
	16	1.0	6.7 ± 0.9
IV	24	0.3	1.8 ± 1.1
	27	0.7	2.8 ± 1.0
V	7	0.5	2.7 ± 2.0
	8	1.0	4.4 ± 1.4
DCA	6	0.04**	All died
	6	0.06	3.8 ± 2.6*
	8	0.10	17.4 ± 2.3
	9	0.15	21.6 ± 1.2
	6	0.25	24.8 ± 1.8

* The deviation is the standard error of the mean calculated according to Burn (1937).

** The dosage of desoxycorticosterone acetate is expressed in terms of mg. per day.

* One rat died.

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These results indicate that the selected extracts occupy the following sequence in order of potency per g. equivalent of fresh adrenal tissue: extracts II, I, III, V, and IV. Because of the large variability in the re-

TABLE XII

The Potency of Five Extracts of the Adrenal Cortex in Stimulating Growth in Immature Adrenalectomized Rats

Extract No.	Rats No.	Potency per cc. U.	Potency per g. of fresh adrenal tissue U.	Significance of <i>t</i> *
I	55	58 ± 22**	0.77 ± 0.30	2.07
II	186	66 ± 20	1.70 ± 0.50	3.12
III	63	20 ± 8	0.50 ± 0.20	1.75
IV	51	10 ± 5	0.13 ± 0.07	0.00
V	15	12 ± 8	0.30 ± 0.20	0.80

* The standard error of the potency calculated by the method of Burn (1937).

** *t* of Fisher (1932) is calculated with respect to the least potent extract, IV.

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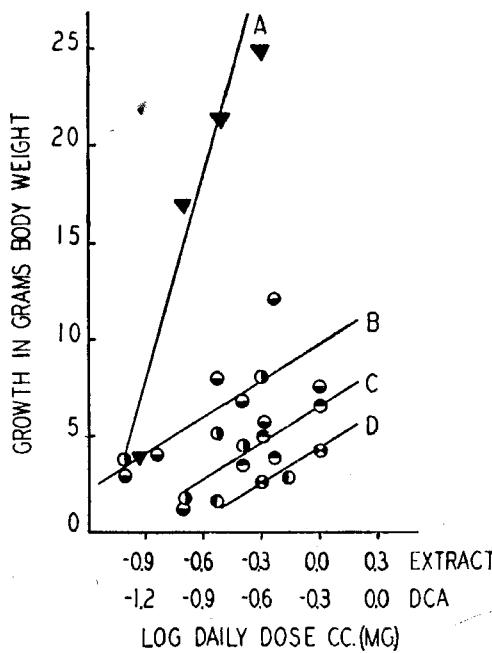


Fig. 4

Logarithm Dose-Response Regression Lines for Five Extracts of the Adrenal Cortex and for Desoxycorticosterone in Measurements of Growth in Immature Adrenalectomized Rats

● Extract I; ○ Extract II; ◉ Extract III; ▲ Extract IV; ▽ Extract V; ▼ Desoxycorticosterone acetate; A is the regression line for desoxycorticosterone acetate, B the most representative line for Extract I and II, C the same for Extract III, and D the same for Extracts IV and V. The abscissa units refer to the log of the daily dose in cc. for the extracts and to the log of the daily dose in mg. for desoxycorticosterone acetate.

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sponses of the animals, however, these mean potency values are subject to some qualification. It may be said that at least a one-fold difference in potency exists between extracts II and V, between extracts II and IV, and between extracts I and IV. Extracts I and II are only questionably different ($t = 1.5$) on the basis of equivalent adrenal tissue weights, while extracts III, IV, and V are indistinguishable.

c. *Discussion.* The large variation in the growth responses of similarly conditioned animals to the same dose of adrenal cortical extract or DCA significantly reduces the accuracy of this method. Although correlation coefficients in the neighborhood of 0.7 were obtained for the dose-response relationship when the mean values of Table XI were used, analyses of individual growth responses showed values nearer 0.2 except in the case of DCA in which the value was 0.8. The scatter of responses around the calculated regression lines in Fig. 4 and the magnitude of the standard errors recorded in Table XII are further statistical evidence for this large variability.

In addition, it must be pointed out that DCA has certain limitations as a reference standard for this method. For appropriate comparisons of the potency of an unknown with the standard, the slopes of the respective regression lines should be approximately the same (Bliss and Cattell, 1943). This is not so for DCA and the extracts in this procedure (see Fig. 4). It was also found, as would be expected from the position of the regression line, that amounts of DCA which would give growth responses comparable to those obtained with extract were barely sufficient to keep the assay animals alive.

Kuizenga, Nelson *et al.* (1940) found that approximately 20 γ of DCA per day induced weight gains of 1 g. per day over twenty day periods and insured the survival of 80% of their immature adrenalectomized rats. Grollman (1941) on the other hand, has reported that while 200 γ of DCA per day would give weight gains of 2 g. per day and permit the survival of approximately 80% of his young adrenalectomized rats, 1 mg. per day was required to permit complete survival and normal growth. Our data indicate that $67 \pm 8 \gamma$ of DCA daily will support a growth rate of 1 g. per day and protect 80% of our immature, adrenalectomized rats, but that 100 γ daily are required to permit normal growth and complete survival.

One extract (IV), found to be very active in the glycogenic assay, and of comparable activity to other extracts in tests of renal function, was practically inactive in stimulating growth in young adrenalectomized rats. The relative inadequacy of the C₁₁-substituted glycogenic steroids, or extracts containing them in large preponderance, in supporting growth in young adrenalectomized rats has already been reported (Grollman, 1939; Wells and Kendall, 1940).

*6. Comparisons of the Adrenal Cortical Potency of Seven extracts
Determined by Four Methods*

A summary of the results obtained with the four methods of assay expressed in terms of their respective units per g. equivalent of fresh adrenal tissue is given in Table XIII. It may be seen that, in spite of the large variation in the glycogenic potency of the seven extracts, ranging from 18.7 ± 1.6 to 0.2 ± 0.1 units per g. equivalent of tissue, the potency in units of renal

TABLE XIII

*The Comparative Activity of Seven Adrenal Cortical Extracts in Terms of Two Standards
in Four Methods of Assay*

Standard Extract No.	Corticosterone Glycogenic potency per g.* U. $\times 10$	DCA Renal function potency per g.* U.	DCA Potency in stimulating growth per g.* U. $\times 10$	DCA Sodium retaining potency per g.* U. $\times 1000$
VI**	$187 \pm 16\ddagger$	5 ± 2		
IV**	91 ± 7	5 ± 2	2 ± 1	19 ± 5
I**	85 ± 7	5 ± 2	8 ± 3	21 ± 2
III#	77 ± 5	4 ± 1	5 ± 3	36 ± 8
II#	67 ± 5	4 ± 1	17 ± 5	40 ± 4
V#	36 ± 3	5 ± 2	3 ± 2	
VII#	2 ± 1	5 ± 1		

* Per g. equivalent of fresh adrenal tissue.

** The hormonal content of these extracts was equivalent to 75 g. of fresh adrenal tissue per cc.

† The deviation is expressed in terms of the standard error.

The hormonal content of these extracts was equivalent to 40 g. of fresh adrenal issue per cc.

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function is practically constant. Extracts I and IV, the most potent glycogenic extracts of beef glands, are relatively poor in growth and sodium-retaining potency. Extracts II and III which are slightly poorer in glycogenic potency and regression line slope show better sodium-retaining power. Extract II is the most potent in stimulating growth in immature adrenalectomized rats. It is unfortunate that circumstances did not permit the assay of extracts VI and VII in all respects.

In general, the extracts higher in glycogenic potency, *i.e.*, those extracts containing preponderance of C₁₁-oxygen-containing steroids, are lower in growth and sodium-retaining factors. Extracts lower in glycogenic potency are generally higher in growth and sodium-retaining potency. This reciprocal relationship between glycogenic potency and the growth and sodium-retaining potency of these extracts parallels a similar reciprocal relationship in the physiological properties of desoxycorticosterone and the 17-hydroxy-

corticosterones (Thorn, Engel *et al.*, 1941). Our results with the extracts are anomalous insofar as the renal function potency did not more closely parallel the growth and sodium-retaining potencies.

In spite of the variation in the factors affecting renal function, growth and sodium retention recorded in Table XIII, it may be seen that these factors are retained by all methods of preparation to a much more uniform extent than are the factors concerned with carbohydrate metabolism. It would appear to us, therefore, that the biological examination of all extracts for their content of glycogenic factors, which appear to be largely, if not solely, the C₁₁-oxygen-substituted steroids (Olson, Jacobs *et al.*, 1944) is important.

These studies, as well as others (Swingle and Remington, 1944), seem to indicate that there are two major "types" of activity in adrenal cortical extracts. The influence of cortical extract upon renal function and inorganic metabolism appears to be due to cortical steroids which are completely reduced at C₁₁, *i.e.*, the desoxycorticosterones. The influence of cortical extract upon gluconeogenesis and other organic reactions, on the other hand, appears to be due to steroids which are partially oxidized at C₁₁, *i.e.*, the corticosterones. Since these activities of cortical extracts are more or less independent, it follows that at least two methods of bioassay are required to properly characterize their physiological potency. The results of Olson, Thayer *et al.* (1944) indicate that the test of glycogen deposition is fasted, adrenalectomized rats is satisfactory for the detection of the activity of the corticosterones. They indicate, further, that the tests of renal function in the adrenalectomized dog as indicated by an improvement in urea clearance, and in the normal dog as indicated by a retention of sodium, though less specific (Allers and Kendall, 1937; Thorn and Harrop, 1937), are suitable for the determination of the activity of the desoxycorticosterones. We feel that the former method is to be preferred because of its use of an adrenalectomized animal. Our objections to the test of growth and survival in young adrenalectomized rats are based chiefly on the large variability in the growth responses of these animals to injected cortical extracts. In addition to this, growth *per se* is a highly cumulative assay end-point upon which desoxycorticosterone and the corticosterones may exert opposite and neutralizing effects (Wells and Kendall, 1940).

It is highly questionable, in view of this division in the effects of adrenal cortical hormones in the animal body, that a single criterion of total adrenal cortical activity will be found.

7. Assay of Six Crystalline Hormones of the Adrenal Cortex

Six crystalline hormones of the cortex were tested for glycogenic potency (Olson, Thayer *et al.*, 1944). The steroids, with the exception of desoxy-

TABLE XIV

Glycogen Depositions Obtained with Six Crystalline Steroids of the Adrenal Cortex in Fasted Adrenalectomized Rats

Steroid	Total dose mg.	Rats No.	Mean glycogen deposition per cent	Regression line coefficients*	Significance of differences in b <i>t</i> ‡
				a ± r _a § b ± r _b	
Corticosterone	0.10	8	0.11 ± 0.01†		
	0.19	5	0.16 ± 0.03		
	0.23	7	0.21 ± 0.04		
	0.39	4	0.44 ± 0.08		
	0.47	4	0.52 ± 0.10‡‡		
	0.58	4	0.84 ± 0.06		
	0.62	3	0.72 ± 0.11		
	0.94	4	1.15 ± 0.09		
	1.15	4	1.40 ± 0.17		
	1.24	4	1.39 ± 0.19	1.24 ± 0.05 2.06 ± 0.29† 0.00	
11-Dehydro-corticosterone	0.39	5	0.66 ± 0.09		
	0.42	5	0.47 ± 0.05		
	0.79	6	1.00 ± 0.09		
	0.87	9	1.29 ± 0.08		
	1.16	5	1.40 ± 0.18		
	1.74	7	1.98 ± 0.16	1.37 ± 0.05 2.14 ± 0.21 0.23	
11-Dehydro-17-hydroxycorticosterone	0.33	4	0.79 ± 0.13		
	0.39	6	0.61 ± 0.12		
	0.39	5	0.61 ± 0.08		
	0.66	5	1.10 ± 0.15		
	0.77	6	1.05 ± 0.10		
	0.79	5	1.31 ± 0.13		
	1.27	5	1.72 ± 0.16	1.46 ± 0.05 1.91 ± 0.27 0.37	
17-Hydroxy-corticosterone	0.44	4	0.73 ± 0.07		
	0.54	4	1.22 ± 0.05		
	0.72	4	1.28 ± 0.14		
	0.88	4	1.80 ± 0.10		
	0.93	6	1.85 ± 0.16		
	1.08	4	2.09 ± 0.17	1.95 ± 0.05 3.29 ± 0.41 2.45	
11-Desoxycorticosterone acetate	1.16	4	0.03 ± 0.01		
	1.01	4	0.02 ± 0.01	0.00 0.00	—
Allopregnane-pentol-3, 11, 17, 20, 21	1.49	4	0.03 ± 0.01	0.00 0.00	—

* The regression line constants are for the equation from which glycogen deposition in per cent of the fresh liver weight may be predicted from the log of the dose; i.e., $y = b(\log \text{dose mg.}) + a$.

† The deviations are expressed in terms of the standard error of the mean.

‡ The standard errors of the regression line coefficients are calculated according to Dunn (1929).

§ *t* is computed according to Burn (1937) and evaluated according to Fisher (1932).

‡‡ Only those experiments in which the mean glycogen deposition was over 0.45% were used in the calculation of the regression line coefficients.

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corticosterone acetate which was given in 25% alcohol, were administered subcutaneously in 10% alcohol in four equally divided doses over a period of six hours as prescribed in the standard procedure. In order accurately to

define the slope and position of the regression line in each case, the active compounds were assayed at from six to eight dose levels as shown in Table XIV. The regression line constants, together with their standard errors as obtained in a statistical analysis of the deposition data by standard methods (Dunn, 1929), are also included in Table XIV. The amount of each steroid required for deposition of 1.00% liver glycogen in our rats calculated from its regression line formula, the potency of the steroid in glycogenic units per mg., and the significance (Fisher, 1932) of the observed differences are included in Table XV.

TABLE XV

The Glycogenic Potency of Six Crystalline Hormones of the Adrenal Cortex in Terms of Corticosterone

Steroid	Rats No.	Range of doses mg.	Amount required to deposit 1% liver glycogen γ	Significance of differences t	Potency in glycogenic U./mg.*
Corticosterone	23	0.4-1.2	766 \pm 39	0.00	1000
11-Dehydrocorticosterone	37	0.4-1.7	671 \pm 33	1.86	1143 \pm 90**
11-Dehydro-17-OH-corti- costerone	36	0.4-1.3	574 \pm 33	3.77	1335 \pm 112
17-Hydroxycorticosterone	26	0.3-1.1	514 \pm 19	5.84	1491 \pm 92
11-Desoxycorticosterone acetate	8	1.2		INACTIVE	
Allopregnaneptol-3, 11, 17, 20, 21	4	1.5		INACTIVE	

* A glycogenic unit has been defined by Olson *et al.* (1944) as the glycogenic activity of one γ of corticosterone administered to a fasted adrenalectomized rat in four divided doses at two hour intervals.

** The standard errors of the deposition equivalents and of the potency ratios are calculated according to the method of Bliss (1939).

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In agreement with Reinecke and Kendall (1942), we have found that for all practical purposes the corticosterones, *i.e.*, corticosterone and its 11-dehydro derivative, are of identical potency and regression line slope. We have found, on the other hand, that the 17-hydroxycorticosterones, *i.e.*, 17-hydroxycorticosterone and its 11-hydro derivative are markedly different. At a liver glycogen deposition level of 1.00%, 17-hydroxycorticosterone exhibits a regression line having a slope over 1.5 times that of corticosterone and a potency 1.49 ± 0.09 times that of corticosterone. In contrast, 11-dehydro-17-hydroxycorticosterone has a regression line of standard slope and a potency only slightly greater than that of corticosterone at that level of liver glycogen deposition. This is very interesting in view of the fact that the sole difference between the pairs of substances studied, *i.e.*, each C₁₁-

hydroxy-steroid and its 11-dehydro derivative, is two hydrogen atoms at C₁₁. It would appear that in the C₂₁O₅ series but not in the C₂₁O₄ series of cortical steroids, the state of oxidation of the C₁₁-carbon is an important determinant of the glycogenic activity. Wintersteiner's Compound A, an allopregnaneptenol containing a completely saturated ring A was totally

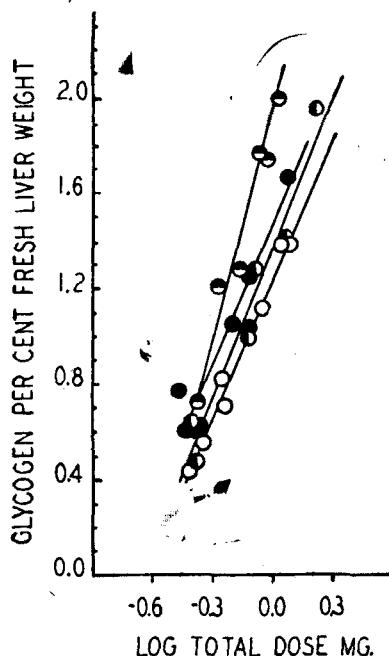


Fig. 5

Logarithm Dose-Response Regression Lines for Four Crystalline Steroids of the Adrenal Cortex

○ Corticosterone; ● 11-Dehydrocorticosterone; ◑ 17-Hydroxycorticosterone;
● 11-Dehydro-17-hydroxycorticosterone.

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inactive at a level of 1.5 mg. as was desoxycorticosterone acetate at a level of 1.2 mg. The relative positions of the regression lines for these compounds are shown graphically in Fig. 5.

Discussion. Our assays show that while corticosterone and its 11-dehydro derivative are of comparable potency in inducing glycogen deposition in the fasted adrenalectomized rat, 17-hydroxycorticosterone and its 11-dehydro derivative are markedly different. Ingle (1936) has found 17-hydroxycorticosterone to be superior to other C₁₁-oxygen-substituted steroids of the

adrenal cortex in promoting work tolerance in adrenalectomized nephrectomized rats (Ingle, 1936). 17-Hydroxycorticosterone has also been reported by Grattan and Jensen (1940) to be more active than corticosterone in preventing insulin hypoglycemia and in storing liver glycogen in young mice. Ingle and Kuizenga (1936, 1945) made a study of the relative potency of some adrenal cortical steroids in the muscle work test. From their present study they make the tentative generalization that the addition of a hydroxyl group in the C-17 position enhances the effect of the C₁₁ oxygenated compounds on the metabolism of carbohydrate and related functions which are involved in maintaining the ability of the adrenalectomized rats to work.

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