THE EFFECT OF TESTOSTERONE ON VITAMIN B_{12} RETENTION AND RELATED FUNCTIONS IN THE RAT

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By

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AN ABSTRACT

Submitted to the College of Science and Arts, Michigan State University of Agriculture and Applied Science, in partial fulfillment of the requirements for the degree of

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With the demonstrated relationships existing between vitamin B_{12}, glutathione, carbohydrate metabolism, and the beta-cells of the pancreas, it became of interest to determine what factors might influence vitamin B_{12} retention. The effect of testosterone propionate on vitamin B_{12} retention and subsequent effects on related functions are the subjects for investigation in this paper.

Littermates, cast by mothers maintained on a vitamin B_{12}-deficient diet throughout pregnancy and lactation, were employed in all the experiments. The weaned animals (23 days) were likewise maintained on a vitamin B_{12}-deficient diet. From the day of birth, the littermates were divided into several groups. The control group received a daily subcutaneous injection of the placebo cottonseed oil while a test group received a daily injection of testosterone propionate; when approximately one month old, both groups received a subcutaneous injection of radioactive vitamin B_{12}. The radioactivity of the urinary excretion, collected at 24, 48, and 72 hour periods, was measured with the DS-1 directional scintillation detector. The count was interpreted as a measure of the excreted vitamin B_{12}. In all cases, but one, the urine count exhibited by the testosterone-treated animals was appreciably higher than their control littermates. A cross-comparison of litters shows that in 24 hours the controls excreted from 1.7 to 7.8 percent of the vitamin B_{12} while the testosterone-treated animals excreted from 4.4 to 15.5 percent. It appears, then, that least retention of the vitamin is found in the testosterone-treated animals.

In another part of the experiment, following the low radioactivity

exhibited by the 72-hour urine specimen, a large dose (16.6 micrograms) of vitamin B_{12} was given subcutaneously to both the control and the hormone-treated littermates. A 24-hour and a composite 48 to 72-hour urine specimen were collected and measured for radioactivity. A general "flush-out" phenomenon was observed as evidenced by the approximately 5-fold increase in the radioactive count. In all cases, but one, the urine specimen of the hormone-treated animals exhibited a count of more than 20 percent above that of their control littermates.

On the basis of the radioactive vitamin B_{12} excretion data, following the subcutaneously administered tagged vitamin, and on the "flush-out phenomenon" data a modus operandi for the observed testosterone action is proffered.

Another experiment employing littermates similarly weaned, as described above, was undertaken in an attempt to show an interrelationship between vitamin B_{12} and testosterone in regard to growth. At birth, the littermates were divided among four treatments where feasible: 1.) vitamin B_{12}-deficient diet; 2.) vitamin B_{12}-deficient diet plus subcutaneous administration of vitamin B_{12}; 3.) testosterone administration plus a vitamin B_{12}-deficient diet; 4.) testosterone administration plus subcutaneous administration of vitamin B_{12}. The actual vitamin B_{12} administration in groups 2 and 4 did not begin until the animals reached 30 or 32 days of age. Body weights, efficiency of food utilization, urinary nitrogen, and hair growth patterns were observed. Only suggestive results are reported in this part of the thesis. It is suggested that testosterone propionate administration is followed by a slight body growth-retardation and a decreased hair growth under the vitamin B_{12}-deficient conditions of this experiment. Upon vitamin B_{12} supplemen-

tation to the hormone-treated animals, both the body growth and hair growth appeared to be equivalent to that of the vitamin B_{12} supplemented control littermates.

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"I can show you the passes to understanding but you must climb them. I can lead you to worth while things but you yourself must unearth them and carry them away."

(Patten)

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INTRODUCTION

"In writing this monograph we are reminded of the parable of the Joyous Young Man who set out to conquer the world. As Time proceeded swiftly onwards, less and less of the far countries were included in his scope, even fewer and fewer of the outlying districts. He restricted his endeavors more and more, and finally learned that if he would hold his own in his own native district, that was as much as was permitted in his brief life-span to conquer." (Brooks and Brooks, 1944)

Similarly, in the selection of a topic of research, a vast field of interesting endeavor lay open. The factors operative in the etiology or exacerbation of diabetes mellitus have long attracted the attention of research men. This syndrome offers an interesting connection between metabolic and endocrine mechanisms. The production of the diabetic syndrome in animals upon injection of alloxan and the prevention of alloxan diabetes by the previous injection of glutathione, or some other sulfhydryl-containing compounds, appear to be well established. Lazarow (1949) believes, and Houssay (1950) agrees, that the beta-cells of the pancreas must be rich in sulfhydryl groups which are necessary for the synthesis of insulin. And Conn (1949) states that the beta-cells are more sensitive to injury by a decreased concentration of sulfhydryl-bearing compounds than are any other cells of the body. Alloxan, by combining with these sulfhydryl groups or by oxidizing them, inactivates many enzymes which require this group and in so doing is believed to depress insulin formation. In severe cases of human diabetes mellitus, a decrease of the sulfhydryl groups of many organs has been observed. According to Conn (1949), there is a "...consistent correlation between

loss of tolerance for carbohydrate...and depressed levels of blood glutathione."

Recently Ling and Chow (1951 and 1954) added an interesting observation. They demonstrated a relationship between the animal's vitamin B_{12} content and both the glutathione content and carbohydrate metabolism. They also showed that, if vitamin B_{12}-deficient animals are kept under a high carbohydrate-low fat regime, hyperglycemia occurs. Upon these observations, Ling and Chow suggest that vitamin B_{12}, via its maintenance of an adequate glutathione supply, plays a role in maintaining the activation of the sulfhydryl enzymes of the beta-cells in the pancreas.

To the fore comes the question: What factors influence the organism's retention of vitamin B_{12}? This is the problem examined in this dissertation. For reasons explained elsewhere, testosterone was believed and now had been indicated to be such a factor. This thesis further concerns itself with the effect of such a factor on growth. Parenthetically it might be mentioned that insulin is believed by many to be an important agent in growth.

The reader, now having explored with the author the seething possibilities for research, may now appreciate, as did the author, the parable of the Joyous Young Man.

The field was vast -- time was limited -- only a fraction of what the author desired to present could be explored -- a nook, to the distress and surprise of the experimenter, was chosen.

"He restricted his endeavors more and more, and finally learned that if he would hold his own in his native district, that was as much as was permitted in his brief life-span to conquer."

REVIEW OF LITERATURE

I. Testosterone

A. Testosterone and Growth

1. Growth and Nitrogen Metabolism

"Among the physiological processes most intimately linked to the phenomenon of growth are those of nitrogen metabolism and its control. This is so because true growth implies the accretion of tissue of composition similar to that of the original body. Addition of fat or water only is not growth, although these substances may accumulate; but retention of nitrogen in the form of protein is invariable. According to modern concepts of nitrogen metabolism, there must then be during growth an imbalance between catabolic and anabolic processes such that the latter predominate" (Russell, 1953). Consequently it is appropriate to recall some of the methods used to study the nitrogen balance of the body. These methods (Lukens, 1954) include the usual metabolic measurements, in which dietary, urinary, and fecal nitrogen are determined. Some other methods are: body growth as measured in gain in weight and/or length; weight changes under certain circumstances; alterations in carcass composition, with special interest to its protein or nitrogen content; changes in the rate of accumulation of blood nitrogen in nephrectomized animals; and, more recently, the retention of nitrogen has been followed by the incorporation of isotopically labeled amino acids.

From the value of extra nitrogen retained, the amount of tissue (protein) which may be added to the animal body, can be calculated by assuming that the tissue formed has 20 percent protein and that protein contains 16 percent nitrogen. Thus Kochakian et al. (1948) report that during initial spurt in body weight on castrated rats attributed to testosterone action, there was "...an extra nitrogen retention of 0.689 gms or the equivalent of 20.7 grams of tissue."

2. Effect of Castration on Body Weight

Data have been presented, at one time or another, which may be considered to demonstrate an anabolic action of testosterone. Information is available to the effect that male animals, castrated while young, are lighter when adult than their non-castrated littermates (Stotsenburg, 1909). Using rats castrated at weaning age, Van Wagenen (1928) found that the castrated animals did not attain the same weight as the non-castrated littermates, the retarding influence becoming first noticeable some 100 days later. Likewise, Rubinstein, Abarbanel, and Kurland (1939) observed that from the 40th day of life onward the castrated rats gained weight less rapidly than their intact fellows, but that at first their increase of weight was equal. An experiment performed by Commins (1932) may answer a question which may have occurred to the reader. Commins, using inbred rats kept on a stock diet, castrated some, operated on others as for castration but without removing the testicles, and left others intact. The body weights of these animals, when recorded at 165 days of age, indicated that the reduction in weight of the castrated animals was a result of the absence of the testis and not attributable to a retardation of nutrition resulting from the operation procedure.

Contrary to what happens in males, most observers have found that spayed females grow to a larger size than their intact sisters (Moore, 1922). The writer extends the hypothesis that the estrogen produced in the intact female inhibits to some extent the anabolic action of the naturally occurring androgens in the female; spaying may remove this antagonistic action. In support of this suggestion, Gley and Delor (1937) observed that daily doses of 1 milligram of oestradiol benzoate neutralized the stimulating activity of daily doses of 0.2 mg of testosterone propionate on the capon's comb. Using the capon comb growth response, Muhlbock (1938) found similar inhibition of androgenic action by estrogen. While 0.4 mg of testosterone applied to the capon's comb caused a significant growth, an inhibition of growth resulted when 0.5 mg of estrone or estradiol was applied simultaneously.

3. Effect of Testosterone Administration on Body Weight

The literature contains many instances which attest to testosterone's anabolic effect as well as its growth retarding effect. One of the major factors contributing to this enigmatic behavior of the hormone is the magnitude of the injected dose. To test the effect of this androgen on the rate of growth, Rubinstein, Kurland, and Goodwin (1939) administered 1 mg of testosterone propionate daily to rats from the 26th to the 76th day of age and observed that this treatment caused a reduction of their length and body weight. In 1940, Kochakian reported comparable results in young male mice which had been given 0.2 mg of testosterone propionate. Previously McEuen, Selye, and Collip (1937) had found no somatic growth inhibition in either young (36-38 days old at initiation of the experiment) male or female intact rats when

chronically treated with large doses of testosterone, although these same doses proved sufficient to inhibit gonad development in both sexes.

Further work by Rubinstein, et al. (1940) suggested that the check imposed on growth was attributable to the magnitude of the doses: 0.5 mg of testosterone propionate when given daily to 26 day old rats for periods varying from 26 to 80 days caused a significant increase in both body weight and length.

In this connection, outstanding experiments were performed by Kochakian and Murlin between 1935 and 1937. These workers demonstrated that urinary extracts containing androgenic material and also the pure compounds, androstene-dione and testosterone, induced a prompt and sustained decline in urinary nitrogen excretion accompanied by an increase in body weight in the castrated dog. This decline in urinary nitrogen was accounted for by a reduced urea excretion. The fecal nitrogen excretion remained unchanged and there was no elevation of the concentration of nitrogenous constituents in the blood. This indicated a true storage of nitrogen in the neighborhood of 0.05 gram of nitrogen per kilogram per day, probably in the form of protein during the period of androgen treatment. This value could not be exceeded by increasing or protracting the dosage of the androgen. On cessation of treatment, a rebound hyper-normal excretion of nitrogen could often be detected although the amount lost was only a small fraction of that retained. They 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." As a site of protein deposit, the genital accessories naturally came to mind. In 1936, however, Korenchevsky, Dennison, and

Broosin noted as had others the somewhat reduced weight of the castrate male rat and found that the heart, liver, and kidneys were smaller than those of the intact controls. Testosterone restored these organs of the castrate animals to normal and increased body weight. These experimenters spoke accordingly of an anabolic property of testosterone in a more general sense.

At about this time, Papanicolaou and Falk (1938) noticed that the temporal muscles of guinea pigs, castrated before puberty, remained small and similar to the muscles of the female. Furthermore, if testosterone propionate was administered repeatedly, a distinct hypertrophy of the temporal muscles and other skeletal muscles ensued in both the castrated male and the intact or spayed female. Recently (1950) the myotropic effect of testosterone in the rat has been demonstrated on the levator and muscle by Eisenberg et al.

A series of reports from Kenyon and his associates (1938, 1944) and McCullagh et al. (1941) appeared concerning the human. The clinical evidence, though not so convincing as controlled experiments performed in the laboratory, continued to suggest the anabolic action of testosterone. When eunuchoid individuals began receiving testosterone propionate intramuscularly, a progressive weight gain appeared usually within the first few weeks of treatment. This process, however, was self-limited since a plateau appeared in 40 to 70 days in spite of continued treatment. These experiments were carried out during constant diet and regulated activity. When the hormone treatment was discontinued the urinary excretion was increased, and for a short time exceeded the pre-treatment level.

Korenchevsky, et al. (1941) conducted experiments anent the effects

of testosterone on the weight and muscular power of the heart. Their observations were made on the isolated hearts of untreated normal or castrated rats, and of castrated rats which had been given repeated injections of testosterone propionate during the previous two months. They noticed that while castration caused a reduction in both the weight of the heart and its muscular activity, previous administration of testosterone prevented these losses.

Kochakian and van der Mark (1952) have suggested that the amount of nitrogen retained under the influence of an androgen is dependent, within limits, upon the protein content of the diet. However, they observed that in the castrate rat there was no change in nitrogen retention when the protein contents of the diet were set at 18 or 43 per cent. Thus, if the animal is provided with an adequate protein intake a further increase in this dietary constituent will not enhance the protein anabolic action of the hormone.

Kochakian (1952) claims that the protein anabolic action of testosterone propionate is a direct one and not mediated by its stimulation of the pituitary to produce the growth hormone. He finds that the androgen produces the typical anabolic effect in hypophysectomized-castrated dogs. "Furthermore, the administration of testosterone propionate at various dose levels to hypophysectomized male rats both castrated and non-castrated resulted in nitrogen retention and increase in body weight in the same manner but to a smaller degree than that observed in non-hypophysectomized animals except for two differences. Many of the hypophysectomized rats after a few days of administration of androgen decreased their food intake which in some instances was temporary; in other instances, however, the androgen had to be stopped

before restoration of appetite was obtained."

In the presence of starvation, administration of testosterone to rats still produces an increase in the weight of the accessories (Usuelli et al. 1949). The possibility that protein is diverted from other sites to the accessories, kidney and liver, is implied by the fact that when large doses of testosterone are given, the carcasses of the treated animals lose both protein and fat while the former mentioned organs continue to gain (Kochakian, 1950). The dosage factor is of importance, since Grayhack and Scott (1952) have demonstrated that while prostatic weight stimulation after testosterone administration is the same in partially starved as in normally fed rats, larger doses of testosterone cause a greater increase in prostatic weight in the well-nourished animals than in the starved controls.

The observations in the literature are not without conflict. While Kochakian reports a protein anabolic effect of testosterone propionate (employing dosages from 1.0-7.5 mg/day) on both castrated (1946) and normal male rats (1947), H. Turner et al. (1941) was unable to show a significant effect of the hormone regardless of the dosage used (0.25-2.0 mg per day), age of the rats at the time of the experiment, or length of treatment. The disagreement of these reports may lie in Kochakian's observation that the anabolic action of testosterone is very short in the rat so that approximately one week after the beginning of injections the nitrogen excretion gradually returns to normal despite continued treatment. Turner's observations on the rats were made not more frequently than once a week throughout the experimental period.

We should remember that the weight of an animal depends on several factors, among which are the size of the bones, the degree of muscular

development, and the amount of fat deposited in the tissues. After prolonged injections of various amounts of testosterone propionate, even when begun one day after birth, H. Turner, Lachmann and Hellbaum (1941) were unable to show significant alteration of skeletal maturation in the treated rats as compared to the controls. No difference in density or length of bones or in the degree of epiphyseal fusion was noticed.

What meager evidence there is on the effect of testosterone on fat metabolism suggests that fat is mobilized and utilized more rapidly under the influence of this hormone. J. C. Turner and Mulliken (1942) found that castrate mice metabolize more injected corn oil after treatment with testosterone than untreated control animals. Kinsell (1949) has shown that urinary ketones in a diabetic patient fell from an average value of 35 to as low as 8 mg per 100 cc. after administration of 50-150 mg of testosterone propionate per day. In another clinical case (1951) he was able to show a comparable effect. Kinsell believes that testosterone may affect fat catabolism. This would be consistent with the observation of Jones *et al.* (1941) that methyl testosterone causes a shift (lowering) in the respiratory quotient toward the type expected to exist when the metabolic mixture is high in fat.

Kochakian *et al.* (1950) carrying out experiments on rats fed *ad libitum* found that testosterone propionate initially produced a rapid increase in body weight which lasted for about 10 days after which there was a marked diminution in the rate of increase in body weight. After approximately three weeks the experimental animals not only weighed less than the controls but the control animals continued to gain in weight at a faster rate than the experimental animals. On

analyzing the tissues of both groups of animals, it was found that the smaller gain in body weight of the experimental animals was attributable to a very great loss in body fat. The liver, kidney, seminal vesicles, and prostate had increased in weight and also in protein content. Thus, Kochakian partially attributes testosterone's "wearing-off effect" in body weight to the hormone's ability to increase the utilization of body fat.

B. Effect of Testosterone on the Pituitary

Much experimental work (Moore and Price, 1932) has brought forward the suggested mechanism of reciprocal interactions between the gonads and the anterior pituitary. The oscillations in hormone secretion, granted other conditions are normal, appear to be regulated by the mutual interplay of these secretions on the gonads and pituitary.

It is well established that anterior pituitary secretions stimulate the gonads to function and that the presence of these secretions is necessary for the maintenance of the function; variability in production or release of pituitary secretions induces variabilities in gonad function. McCullagh and Walsh (1935) joined a number of pairs of male rats in parabiosis and castrated one of each pair. The excessive production of pituitary gonadotropic hormone of the castrated partner led to an increased output of gonadal hormones by the testis and a consequent hypertrophy of the prostate and seminal vesicles of the non-castrated partner. They found that this effect could be entirely prevented by giving subcutaneous injections of androsterone to the castrated rat. Hence it is not surprising that significant increases in

gonadotropic hormone content were found in the anterior pituitary (Leonard, 1937) of castrated animals. The work of McCullagh and Walsh illustrates the fact that an increase in the production or release of these gonadotropic hormones causes a corresponding increase in gonadal hormones; that the maintenance of the gonads is dependent upon the presence of adequate concentrations of the gonadotropic hormones.

But what is the effect of administered androgen on the endogenously produced androgens? The exogenous androgen unquestionably stimulates the accessory reproductive organs but not so for the gonads themselves. Rather than acting as a stimulating agent on the gonad, injections of androgen in sufficient concentration are actually injurious to the gonad tissue present. Injections of estrogenic substance into normal female or male rats (Meyer et al. 1930) are positively injurious to both germ cell production and hormone secretion. Laqueur and Fluhmann (1942) giving daily injections of testosterone propionate to female rats for 16-21 days found a suppression of the pituitary gonadotropin content, particularly of LH. Likewise, Moore et al. (1933) observed that testis hormone injection into young, normal, sexually mature male rats suppressed the growth of the testis and caused visible injury to the seminiferous tubules; it probably lowers or abolishes hormone secretion, but this cannot be detected since the injection of the hormone more than counterbalances the loss of that produced by the testicles themselves. The administration of testosterone to immature male guinea pigs (Bottomley and Folley, 1938) is reflected in failure of gonadal development. This injurious action of the androgens on gonadal tissue is due to the decreased production of gonadotropic hormones by the pituitary when subjected to the inhibitory action of the

androgens. The latter observation was made by Bottomley and Folley when they noted that gonadal atrophy was prevented when gonadotropins were administered along with the daily 2.36 mg testosterone injection to the immature guinea pigs.

Cutuly and Cutuly (1938) performed an experiment which clearly shows the inhibitory effect of testosterone propionate on the gonadotropic functions of the pituitary. These investigators joined young male rats weighing between 50 and 150 grams parabiotically. One animal of each pair had its pituitary removed; in this rat testicular atrophy, retention of the testis within the abdomen, and involution of the accessory generative organs was invariably noted. If now the normal partner were castrated, the testis of the hypophysectomized male descended and along with the rest of the genital tract gradually resumed a normal state. This, then, showed that castration had led to a sufficient increase in the pituitary output of gonadotropin of the castrated rat to supply the deficit of this hormone in the partner whose pituitary had been removed. These reporters observed that when testosterone propionate was given to the castrated rat in daily doses ranging from 0.05 to 3.0 mg, the restoration of the reproductive organs of the hypophysectomized partner was prevented. Hellbaum and Greep (1943) concluded from their experiments that when testosterone propionate was administered in daily doses of 0.5 mg, the concentration of FSH was diminished in the blood of castrated rats.

On the basis of the above quoted work an investigator, in selecting a dosage level of testosterone to be administered daily, would do well to stop for a moment and consider. If his selected dosage level is below the normal physiological output of body androgens, he may in

effect not increase the total circulating androgen even after injecting the exogenous source (Selye and Friedman, 1941; Wells, 1943). Reason: the "feed back" system or the proposed reciprocal inhibition scheme allows that the sum total (endogenous and exogenous) of androgen present in the blood at any time acts back on the anterior pituitary to control the gonadotropic output. This scheme, then, postulates that the amount of stimulation received by the gonadal tissue would be reduced by an amount equivalent to the injected sub-physiological androgen dosage. Hence, it is within reason, that if the investigator is to observe the effects of an elevated androgen circulation, he should plan to administer a dose which is at once above the physiological secretion.

Approximately, then, what is the physiological secretion of testosterone? Although Alfred Novak (1951) in this laboratory found that daily administered dosages of testosterone propionate, even in the large range employed (0.05-0.3 mg), were unable to maintain the sex accessories of his young castrated albino mice, he notes that such had been attained on young rats by Greene and Burrill (1941). In 1942, Hooker castrated rats at birth and calculated the minimum dose of testosterone needed to stimulate their seminal vesicles at different ages. At this point it should be mentioned parenthetically that the prostate gland of the rat is stimulated by one-third to one-half the amount of hormone needed for the seminal vesicle (Callow and Deanesly, 1935). Hooker found the minimum effective dose of testosterone required to stimulate the seminal vesicles of castrated rats whose ages at the time of injection ranged between 10-80 days was 0.005-0.03 mg. Callow and Deanesly had found that 2 mg of androsterone were necessary to maintain the seminal vesicles of an adult castrated rat in an active

state. The International Standard male hormone unit is the activity equivalent of 0.1 mg of pure androsterone. By comparison of comb growth in the capon (Mieschner et al. 1936), testosterone propionate is five times as potent as androsterone, so that only 0.4 of testosterone propionate would be required for the maintenance of the seminal vesicle activity. If we consider this value to be rough approximation of the adult rat's androgen secretion then the data submitted by Hellbaum and Greep (1943), demonstrating that daily doses of 0.5 mg of the hormone caused inhibition of pituitary gonadotropic secretion, is consistent.

Having expressed the observation that exogenous gonad hormones (or for that matter, endogenous hormones) are not gonadal stimulants but rather are agents which automatically curtail the endogenous output of the hormone, let it suffice to mention here that the experiments reported in this paper employed androgen dosages in excess of that considered physiologic.

C. Effect of Testosterone on the Adrenal Cortex

1. Preliminary Remarks Concerning the Adrenal Cortex

The mammalian adrenal gland is a compound organ, consisting of the cortex which elaborates the cortical hormones and a medullary portion which secretes the hormones epinephrine and norepinephrine. The circulation to the two parts of the gland is common.

Conventionally agreed upon, the adrenal cortex consists of three distinguishable zones: the zona glomerulosa, a thin layer just beneath the capsule; the zona fasiculata, the widest portion; and the zona reticularis, the innermost zone. In the embryo and early postnatal life

many species contain a boundary zone between the cortex and the medulla which has been called the X-zone.

Evidence has been advanced (Dean, 1951), in support of Swann's original hypothesis (1940), that the glomerulosa is the source of the salt-regulating hormones. The fasciculata is believed to secrete the glucocorticoid hormones (11-oxysteroids). In short, the absence of an oxygen atom at C-11 in the molecular structure of these hormones is associated with virtual absence of effects upon carbohydrate metabolism but with most marked effects upon electrolytes and water. The notable exception to this general rule is the recently isolated aldosterone (electrocortin) which manifests both electrocorticoid and glucocorticoid properties. However, Simpson and Tait (1954) have shown that the amounts of this hormone which circulate normally (ca. 0.08 micrograms per 100 ml) are probably not sufficient to exert important glucocorticoid effects.

2. Factors Indicative of the State of Activity of the Adrenal Cortex

A variety of factors yield information as to the state of the adrenal cortex activity. Among these are: the content of the adrenal cortical steroid hormones in the blood of the adrenal vein; cortical hormone content of the urine; level of ascorbic acid or cholesterol in the gland; alteration in the number of circulating eosinophils or lymphocytes; and histological observations.

The histological approach has currently been receiving much attention. Alterations in such characteristics as the volume of cytoplasm and nucleus, the form and quantity of mitochondria and Golgi material and the nature and number of cytoplasmic inclusions have all been

found to correlate significantly with cellular activity. The nature of the cytoplasmic lipid droplets has been extensively employed by many workers since these lipid droplets are acetone-soluble, birefringent, autofluorescent (after formalin fixation they emit a yellowish or greenish fluorescence when examined in ultraviolet light), Schiff positive, and reactive with hydrazines. These properties collectively have been taken to indicate the presence of ketosteroids in the droplets, since this is said to be the only group of compounds known which is characterized by all these reactions (Dempsey, 1948; Deane and Greep, 1946). Hence it has been suggested (Greep and Deane, 1949) that these tests localize the sites of formation of the steroid hormones, although none of the reactions is specific for these compounds. These tests have been used, regardless of whether the substance giving the test is the hormone, precursor, or metabolically related compound, since the number, size, and reactivity of the droplets have been observed to change during induced activity or inactivity of the gland. "In both the glomerulosa and the fasciculata, the cells multiply and enlarge when stimulated. Their droplets become small and, with a moderate stress, increase in number. If the stress is more severe, they may disappear. On the other hand, when the cells are unstimulated, they shrink. The droplets at first enlarge in size but decrease in number; later they may disappear ... The subsequent interpretations of secretory activity...are based, therefore, on the appearance of the cells as well as on that of their lipid droplets" (Deane, 1951).

3. Secretory Activity of the Late Fetal and Postnatal Adrenal Cortex

Two groups of workers (Josimovich, Ladman, and Deane, 1954; Van

Dorp and Dean, 1950) have reported observations on the histology of the developing rat's adrenal cortex from the 17th fetal day to the 6th postnatal week. The histological approach concentrated on the cell volume (i.e. cell size) and the cytoplasmic lipid droplets. Cortical volume, as measured on serial sections, increased seven fold between the 17th day and the day of birth (hence paralleling total body growth), but during and following birth the cortical size declined 20-25 percent not resuming growth until the end of the second postnatal week. Thereafter enlargement was continuous, though at a gradually declining rate (Venning, in 1950, noted that after the second week of age there was a gradual increase in the glucocorticoids of the human infant). Hence an increase in secretory activity of the cortex took place during the observation period except for the two weeks immediately during and following birth at which time a depressed cortical activity (as shown by a shrinkage of the cells, lipid droplet enlargement, and increased cholesterol reactivity) was seen. The above is true for the zona fasciculata, whereas the glomerulosa showed no signs of depressed activity after birth. The fetal and neonatal reticularis seems to be only an inner region of the fasciculata; following birth the former zone degenerates, leaving a narrower cortex.

4. Adrenal Cortex-Pituitary Relationship

As with the gonads, the development and functional activity of the adrenal cortex are directly controlled through the agency of one or more of the anterior pituitary's adrenocorticotropins (Young, 1953). Davidson and Moon (1936; see also Davidson, 1937) demonstrated that adrenocorticotropic extract, free from gonadotropins or growth hormone,

caused enlargement of the adrenal cortex and accessory reproductive organs in rats whose testes and pituitaries had been removed. The adrenocorticotropin did not cause enlargement of the adrenal medulla. P. E. Smith first demonstrated in 1916 that removal of the hypophysis is followed by marked atrophy of the adrenal cortex. In rodents, the weight of the adrenal a few weeks after hypophysectomy is about one-half or one-third of the normal.

In short, the evidence (Ingle, 1951) which can be marshalled in support of the concept that the steroids produced by ACTH action on the adrenal cortex act back on the anterior pituitary to control the synthesis by the anterior pituitary is voluminous and persuasive. Then, on the strong evidence (Savard and Kolff, 1952; Bush, 1951) that hydrocortisone is the principle hormone secreted by the adrenal cortex, the "feed-back" scheme postulates that the amount of stimulation received by the adrenal cortex is coupled to the amount of hydrocortisone (believed to be secreted by the zona fasciculata) that is produced.

As mentioned before, Swann (1940) originally suggested that the zona glomerulosa which is responsible for the salt-water regulating principles is not under the influence of the anterior pituitary gland, but is under independent humoral control. The physiological conditions which alter the secretory activity of the glomerulosa suggest that its hormones affect chiefly the electrolyte balance of the body fluids and that the electrolytes, in turn, react upon the glomerulosa to affect its secretory rate. In support of this, it has been observed (Deane and Greep, 1946) that although in the two weeks following hypophysectomy the adrenal cortex shrinks to about one-half of its normal cross-sectional area, the glomerular zone exhibits little change in width

(actually increasing to some extent). The large fasciculata zone is most affected, frequently being reduced to only a fraction of its normal width and gradually loses all its reactive lipids and "keto-steroid" reaction.

5. Effect of Testosterone on the Adrenal Gland

Both an inhibitory and a stimulatory action on the adrenal cortex have been shown for testosterone. Selye (1940a) treated albino rats with the androgen and found a decrease in their adrenal weights when compared with their male controls. Korenchevsky et al. (1939) and Schilling et al. (1943) found a similar effect. Greep and Jones (1950) found, in gonadectomized animals, a consistent increase in the amount of sudanophilic, Schiff-positive, Schultz-positive, and birefringent material in the entire cortex with the increase being most strikingly apparent in the outer fasciculata. Such histological data indicate an increase in the lipid content of the cell and increased ketosteroids in the droplets; in other words, increased activity. On the other hand, the adrenal of the intact female after treatment with 0.1 mg of testosterone propionate daily for 45 days showed marked clumping of lipid droplets in scattered cells of the inner fasciculata, indicating an inactive secretory state.

However, Nathanson and Brues (1941) found an increased mitotic activity in the adrenals of immature female rats. Vidgoff (1940), using an extract of bull testes showed an increase in adrenal weight due to hypertrophy of the zonae fasciculata and reticularis.

II. Vitamin B_{12}

A. Vitamin B_{12} -- a Regulator of Enzyme Activity?

Metabolic reactions depend on the availability to the cells of the major foodstuffs and on the normal functioning of enzyme systems. Enzymes are proteins and are presumably themselves synthesized within the cell from the constituent amino acids. It has been shown in micro-organisms that the concentration of certain intracellular enzymes can be influenced by the nutritional substances available to the cells (Monod and Cohn, 1952). A fundamental fact is that the fate of a given chemical compound that is delivered to the cell is not determined solely by the nature of that compound. That is to say, compounds that enter the cells are not predestined to be used in a particular way. There are a number of possible alternative metabolic pathways open to them. Their fate is determined by the enzymes present in the cell. It is generally stated that all an enzyme does is to hasten the attainment of equilibrium in a chemical reaction. While this statement is true, it takes on added significance when it is realized that in many instances the rate is so low as to be zero for all practical purposes. A food molecule, then, may take a number of alternative pathways: be burned as fuel, used for growth or maintenance, or used as a building block for the synthesis of a special chemical product (e.g. a hormone);

some molecules of a given compound may take one alternative while other molecules of the same compound follow another pathway, depending upon the active enzymes they encounter. Many enzymes require, for their characteristic activity, the presence of a co-enzyme or prosthetic group which is not a protein and in many instances contain a vitamin as a part of the coenzyme molecule. In an organism which requires a particular vitamin, the level of the related co-enzyme can often be shown to be influenced by the amount of vitamin in the diet (Novelli, 1953). The molecular structure of the enzyme is another factor in consideration of its native activity; alterations of its constituent groups have been shown to decrease and even abolish its activity. In the most general sense, the ability of an enzyme to catalyze a given reaction is probably based not only upon the "fit" between enzyme and substrate but also on the ability of the enzyme to react with other molecules that restore it to its original form. It is the latter ability that makes it possible for one enzyme molecule to activate many substrate molecules successively. Perhaps certain enzymes in order to maintain their activity depend upon their sulfhydryl groups in the reduced state. It has been suggested (Lazarow, 1949) that glutathione may function in maintaining reduced sulfhydryl groups in certain enzyme systems.

Where might vitamin B_{12} fit into this general scheme? Anderson and Stekol (1953) have demonstrated a link between glutathione synthesis and vitamin B_{12}. Rats under a certain dietary condition (where the animal must rely upon the mechanisms which elaborate the necessary amino acids for the biosynthesis of glutathione) and in a vitamin B_{12} deficient state were shown to have a lowered elaboration of glutathione.

Dubnoff (1954), along a similar vein of thought, presents his thesis that vitamin B_{12} plays a role in maintaining the SH groups of enzymes. He points out that there are disulfide-reducing systems which require DPN or TPN in the presence of a DPN or TPN-reducing enzyme and an active sulfhydryl (glutathione). He states, "We have very direct evidence now that B_{12} will influence...the activation of the TPN-reducing enzyme. We can show an activation of an inactive sulfhydryl enzyme in the presence of B_{12} for example." The disulfide group of the inactive enzyme is reduced to the sulfhydryl group. He further states, "An inactive sulfhydryl enzyme is not necessarily a disulfide, but I think the evidence favors the view that it is a disulfide." In respect to the other suggested role of vitamin B_{12}, Chow (1952) discusses the possibility that vitamin B_{12} may act in some form as a coenzyme.

Returning once more to the theory of alternative metabolic pathways, the possibility exists that growth may be achieved not by the deletion of a catabolic enzyme but by the suppression of its activity or function. Such a mechanism could be just as effective in making strategic building blocks available for growth as the deletion of the appropriate enzyme, and in addition the change would not imply irreversibility. In other words, the balance between the catabolic and anabolic pathways need not be governed by the relative amounts of the corresponding enzymes; it is equally possible that the balance is governed by the regulation of the relative enzyme activities.

B. Vitamin B_{12}, Methionine, and Transmethylation

Rose (1938) showed by feeding known mixtures of amino acids that

certain amino acids derived from food sources are necessary for the growth of the rat. That is to say, these certain amino acids are not capable of being synthesized by the animal out of the materials ordinarily available at a speed commensurate with the demands for normal growth. Consequently, Rose (1949) calls these amino acids "essentials". Methionine is one of the essential amino acids needed in the biosynthesis of the characteristic cellular proteins; if withheld from the diet, it not only causes its characteristic metabolic derangements but also causes a marked decrease in the utilization of the other amino acids.

The presence of an ample supply of protein carbohydrate, fat, and mineral matter in an animal's diet, then, is not sufficient without qualification. Protein quantity is not an adequate criterion of diet -- protein quality must be taken into consideration. Osborne and Mendel (1917) were the first workers to show that raw soybeans when fed as the sole source of protein in the ration of the rat were unsatisfactory. Jackson and Block (1932) demonstrated that when methionine was added to the diet, rat growth was stimulated. Similarly, White and Beach (1937) demonstrated that when arachin (globulin from the peanut) serves as the chief source of nitrogen in an otherwise nutritionally adequate diet, this protein is incapable of supporting good growth in young rats. They found the nutritional inadequacy of arachin to be attributable to its low methionine content.

A major factor lending to the "essential" nature of methionine is its importance as a source of labile methyl groups. The methyl group may be transferred to other compounds (transmethylation) for the synthesis of choline or of creatine, for example.

In 1939 du Vigneaud's laboratory demonstrated that dietary methionine could be replaced by homocystine and an adequate methyl donor. Soon after the discovery of vitamin B_{12}, it became evident that this vitamin was concerned with enabling rats and chicks to respond to homocystine while on a methionine-deficient diet (Jukes et al. 1950). Davis and Mingioli (1950), studying mutants of Escherichia coli, demonstrated that vitamin B_{12} and methionine serve as alternate growth factors in certain mutants. They interpret this as meaning that vitamin B_{12} functions as a coenzyme in the synthesis or transfer of labile methyl groups, since all the vitamin B_{12}-requiring mutants are blocked between homocystine and methionine. Factors present in liver extract, among them vitamin B_{12}, have been shown to influence methyl group synthesis (Oginsky, 1950). The tissue synthesis of methyl groups from such precursors as formate, methanol, serine, glycine, and acetone is now also established (du Vigneaud et al. 1950). Hence, Bennett (1949, 1950) reports a slow but continuous growth of rats on so-called "methyl-free" diets (homocystine diets) when the diet is supplemented with vitamin B_{12} and folic acid. Similar reports have appeared (Dinning et al. 1951; Verly et al. 1952). Oginsky (1950), using liver homogenates from normal and vitamin B_{12}-deficient rats, studied the in vitro formation of methionine. He found that the deficiency state greatly reduced the ability of the liver homogenates to methylate homocystine. Likewise, Williams et al. (1953) has demonstrated that liver slices from vitamin B_{12}-deficient animals are low in betaine-homocystine transmethylase activity (betaine serves as a dietary source of preformed methyl groups). Whether this is a direct effect on labile methyl transfer or is a redox effect, principally exerted on enzyme systems

containing sulfhydryl groups is not certain. Dubnoff (1950) suggests that vitamin B_{12} activates the homocystine-methionine reaction by maintaining homocystine in a reduced state, thus allowing it to accept a methyl group from a suitable donor.

Arnstein and Neuberger (1953) conducted experiments to show the relative efficiency of some methyl group precursors. They placed weanling rats on a synthetic diet containing pure amino acids in place of protein. In order to eliminate as far as possible preformed labile methyl groups, the amino acid mixture contained homocystine in place of methionine. When the diet was supplemented with vitamin B_{12}, small daily weight gains were noticed. When the diet contained suboptimal amounts of choline (a dietary source of preformed methyl groups) but no vitamin B_{12}, growth rates were minimal. Suboptimal choline supplementation in addition to vitamin B_{12} administration resulted in moderate growth. These experiments clearly demonstrate the beneficial effect of vitamin B_{12} when added to a ration containing suboptimal quantities of choline. Vitamin B_{12} supplementation was shown to be more effective than choline supplementation alone.

In another experiment by these investigators, the basal ration was supplemented with choline. Some of the rats on this basal ration received vitamin B_{12} but were restricted in food intake to the amounts consumed by the control animals receiving no added vitamin B_{12}. Under these conditions vitamin B_{12} did not exert a significant increase in growth rate. This experiment suggests a link between vitamin B_{12} and food consumption -- the beneficial effect of vitamin B_{12} supplementation in the above restricted diet only making its appearance when the animals were fed ad libitum. Nevertheless, addition of vitamin B_{12} to the

ration of pair-fed animals still caused a noticeable increase in the isotope content of methyl groups of methionine whether the precursor was C^{14} labeled glycine, serine, or formate.

The above evidence, in addition to Shive's (1950) observation that the inhibitory effect of sulfanilamide on the growth of E. coli was overcome either by vitamin B_{12} or by methionine (but not by homocystine), demonstrates a function of vitamin B_{12} in the formation of methionine from homocystine; provides evidence for the role of vitamin B_{12} and methionine in growth; and shows that vitamin B_{12} promotes the production and transfer of labile methyl groups.

C. Vitamin B_{12}, Nucleic Acids, and Protein Synthesis

Caspersson (1947), Hyden (1947), Thorell (1947) and many other investigators working along similar lines support the premise that the nucleic acid content of a cell is an indication of the protein metabolism within the cell. Caspersson has shown that relatively high concentrations of ribonucleic acid are found in the cytoplasm when the cell is active in the synthesis of protein. According to Haurowitz (1950), "Protein synthesis is particularly intense in those parts of the cell where ribonucleic acid is abundant." Consequently, any change in the ribonucleic acid content ought to be a reflection of the metabolic activity in that cell.

Both ribose and the desoxy-type of nucleic acid may be found in the same cell; the latter seems to be confined to the nucleus, whereas the ribose-type is found predominately in the cytoplasm, a smaller amount being present in the nucleolus and the nucleus of the cell

(Caspersson and Schultz, 1940). The "...major fraction of ribonucleic acid is bound to ultracentrifugable granules (microsomes)..." (Brachet, 1952).

It is a common observation that the viruses (nucleoprotein bodies) stimulate the cells to intense protein synthesis. Caldwell et al. (1950) have shown that the ribose-nucleic acid content is proportional to the rate at which the B. lactis aerogenes grow. Spiegelman and Kamen suggest that the nucleic acids control the metabolic energy in some manner so as to funnel it into protein synthesis at critical stages in the cell's development (cited by Davson, 1951).

With the previous paragraphs serving as an introduction to the nucleic acid-protein inter-relationship, let us now consider a proposed role of vitamin B_{12}: Vitamin B_{12} appears to catalyze the formation of pyrimidine bases which are the building blocks of nucleic acids (Roberts et al. 1949; Shive et al. 1948). Thus the desoxyriboside, thymidine, can replace vitamin B_{12} in the growth of the microorganisms lactobacillus leischmannii and streptococcus faecalis when purines are available (Shive et al. 1951). Thymidine (Hausmann, 1951) or its pyrimidine, thymine (Spies et al. 1946) will induce remissions in persons with pernicious anemia in relapse. In addition, the nucleic acids obtained from pernicious anemia bone marrow contain more thymine (a pyrmidine base of desoxynucleic acid) and less uracil (a pyrimidine base of ribonucleic acid) after the administration of vitamin B_{12} than before (Vilter et al. 1953). This observation links the vitamin to the formation of desoxy-type of nucleic acid and the degradation of ribose-type nucleic acid.

On the other hand, vitamin B_{12} has been shown to be important in

the maintenance of the ribose-type of nucleic acid. Experiments (Alexander and Backlar, 1951; Alexander, 1953) have shown a definite reduction in the ribose nucleic acid of nerve cells in B_{12} deficient animals and an increase in this nucleic acid in vitamin B_{12} treated animals. Stern and coworkers (1949, 1951) investigated the effect of the vitamin upon nucleic acids in rat liver tissue. It was found that rats were deficient in vitamin B_{12} had relatively little ribose nucleic acid content in the liver, but the ribose nucleic acid content was maintained in those rats receiving the vitamin.

Consequently it is probable that vitamin B_{12} is involved in the formation and degradation of both the ribose nucleic acid and the desoxyribonucleic acid. As Davson (1951) remarks, it would seem that the two types of nucleic acid are interconvertible; related enzymes probably determine in which direction these reactions go. So, in one tissue the emphasis may be placed on the formation of the desoxy-type nucleic acid and the degradation of the ribose nucleic acid, while in another tissue, the reverse effect may be observed.

D. Vitamin B_{12} and Growth

Many reports appear in the literature indicating that vitamin B_{12} is a factor in normal growth. For the most part these reports are based on experiments which first deplete, to variable degrees, the animal's supply of the vitamin. This is usually accomplished by the use of vegetable diets; vitamin-free casein is also occasionally used as a protein source. Likewise, use has been made of Ershoff's observation (1947) that administration of thyroxine increases vitamin

requirements. Once the animal has been made deficient, its growth rate may be compared to that of animals receiving a standard diet and/or a vitamin B_{12} supplemented diet. Some reports based on this and other experimental approaches follow:

Emerson (1949) maintained one group of mothers, during gestation and lactation, on a 60 percent soybean meal diet while another group received a vitamin B_{12} supplement (5 micrograms B_{12} daily). Emerson reported that "The size and birth weights of the litters cast by the rats in each group were the same." However, it is important to note that the weaning weights (28 days) of the young from mothers receiving vitamin B_{12} averaged 50 percent more than did the young from the untreated females. Cheng (1952), attempting to determine the vitamin B_{12} requirement, states "The presence of vitamin B_{12} in the diet is essential for supporting the growth of the rat." The data of McCollum *et al.* (1950) and Alexander (1953) further demonstrate this.

Johnson and Neumann (1949) showed that liver extract or vitamin B_{12} was required by baby pigs on a synthetic milk diet containing soybean protein. Similarly, Kline *et al.* (1954) demonstrated that addition of vitamin B_{12} to a diet previously deficient in the vitamin caused a significantly increased rate of weight gain.

Bosshardt *et al.* (1949) receiving similar results in the mouse, describe two methods for vitamin B_{12} bioassay: in one, only a deficient diet is used; in the other a thyroid preparation is included to exaggerate the vitamin B_{12} requirement.

It is important to note that very few experiments, if any, have shown a significant growth stimulating effect when vitamin B_{12} is added to the diet of animals that are not vitamin B_{12} deficient. Mirone and

Wade (1953) caution that "...vitamin B_{12} when added to a diet which contains the required nutrients for growth will elicit no further response." Larcomb et al. (1954) found no effect of vitamin B_{12} on the height or weight of normal children but found a significant increase in the weight of underweight children.

It should also be noted that vitamin B_{12} does not appear to increase the efficiency of food utilization. Chow and Barrows (1950) demonstrated that when B_{12}-deficient rats were given a restricted food intake (6-8 grams of diet/rat/day), supplementation of vitamin B_{12} did not increase the growth rate nor the nitrogen retention. "When the dietary allowance was increased by 50 percent, vitamin B_{12} brought about a greater rate of growth but no better protein utilization." Likewise, Rupp, Paschkis, and Cantarow (1951) observe that, since vitamin B_{12} fails to influence weight gain or nitrogen retention in rats fed a constant diet, the growth-enhancing effect in ad libitum fed animals may be a result of increased food intake and not increased utilization of food.

Although Black and Bratzler (1952) found that the "Efficiency of gains was of a much higher order on ad libitum feeding than on paired feeding, and the efficiency of gains was greater in the paired animals receiving the vitamin B_{12} supplement" they note that "...the rats receiving the supplemented ration ad libitum were consuming feed far in excess of their maintenance requirement...." The data presented show that the better growth of the rats receiving vitamin B_{12} is largely due to the increased food intake.

The appetite as well as the food intake are known to increase in animals treated with vitamin B_{12} (Meites and Ogle, 1951). The

inhibition of the growth retarding effects of cortisone under *ad libitum* conditions (Meites and Feng, 1954) is believed to be a result of the appetite stimulating effect of vitamin B_{12} and "...thereby enhancing the availability of carbohydrate or protein to the organism..." On the other hand, when the food intake is kept constant (Rupp and Paschkis, 1953), "The weight loss and the increase in urinary nitrogen excretion induced by cortisone were not influenced by vitamin B_{12}...."

It should be mentioned however that the reports on the lack of an effect of vitamin B_{12} on food utilization are not without contradiction in the literature. Cheng (1952) found that "Vitamin B_{12} was effective in increasing nitrogen retention in rats when they were fed rations containing moderate or large percentages of soybean oil meal provided the experimental period was proceded by a B_{12}-depletion period"; an increased "efficiency of feed utilization" was also reported. Feng's data (1954) also tend to suggest this; on evaluation, Meites, Feng, and Wilwerth (1955) report that "the effects of vitamin B_{12} on protein metabolism are relatively minor...."

E. Effect of Vitamin B_{12} on Metabolism of Protein, Carbohydrate, and Fat

"There is ample evidence that chicks raised on vegetable protein diets, especially when the protein level is higher than normal, require vitamin B_{12}" (Smith, 1951). That is to say, high levels of protein in vitamin B_{12}-deficient diets inhibit the rate of growth of chicks (Rubin and Bird, 1947) and rats (Cary *et al.* 1946). In 1950, Menge and Combs reported that the growth of vitamin B_{12}-deficient chicks was depressed by addition of high levels of glycine to their diets; vitamin B_{12}

supplementation largely overcame this growth depression. In 1952(b), Hsu and Combs found that leucine and zein had growth inhibitory effects which were likewise counteracted by subcutaneous injection of vitamin B_{12}. The growth-depressing action of zein was attributed to its leucine content.

Charkey et al. (1950) demonstrated that vitamin B_{12} increased the chick growth response and at the same time lowered the blood levels of certain amino acids. They concluded that the vitamin appeared to function by enhancing the utilization of circulating free amino acids for building fixed protein tissues. McGinnis et al. (1948) and Hsu and Combs (1952a) found that the blood nonprotein nitrogen level was higher in vitamin B_{12}-deficient chicks than in controls which received the vitamin. Zucker and Zucker (1948) had previously demonstrated this in the rat. In addition, Sahasrabudhe and Rao (1951) reported that vitamin B_{12} stimulated protein synthesis in rat livers and presumed that this was a result of increased amino acid utilization.

However, Ling and Chow (1952) presented data indicating that vitamin B_{12} "...plays a role in carbohydrate or fat metabolism rather than in protein metabolism." In support of their hypothesis they cite the work of Bosshardt (1950), Chow (1950), McCollum (1950), and Rupp (1951) and their associates. From their data, Ling and Chow state, "No change in protein content was observed during the deficiency or after vitamin B_{12} administration. If the utilization of proteins were improved as the result of administration of vitamin B_{12}, one might expect an increase in nitrogen retention. This phenomenon was not observed after injection of vitamin B_{12} into our deficient rats. Thus, under our experimental conditions the administration of vitamin B_{12} did

not in any of the ways measured here alter nitrogen retention."

The observed (Hsu and Combs, 1952a, 1952b) increase in the blood glucose level of chicks resulting from a vitamin B_{12} deficiency suggests that vitamin B_{12} is involved in glucose utilization. This is also indicated by the higher blood sugar levels noted during the glucose tolerance tests performed on vitamin B_{12}-deficient rats (Ling and Chow, 1954). Also employing the glucose tolerance test, Hsu and Combs (1952b) demonstrated a higher blood sugar level in vitamin B_{12}-deficient chicks as compared to the blood sugar level of those chicks which received the vitamin.

Ling and Chow showed that a B_{12} deficiency under a high carbohydrate-low fat regime was followed by hyperglycemia. Collins et al. (1953) were able to show that the feeding of lactose increased the requirement for vitamin B_{12}.

From their data on the composition of the carcass of rats, Ling and Chow (1952) conclude that "...on a percentage basis, animals with vitamin B_{12} deficiency have low fat, high water, and normal protein contents. The two abnormalities could be corrected by injection of vitamin B_{12}." Knoebel and Black (1952) reported that the increased weight gained by rats fed a 10 percent vegetable protein diet supplemented with vitamin B_{12} was mainly the result of extra fat deposition. Following their experiments on glucose tolerance and phospholipid content of the blood and tissues, Ling and Chow (1954) suggest that the vitamin B_{12} deficient rats lost part of their ability to transform carbohydrate to fat.

An indication that vitamin B_{12} may be involved in fat metabolism comes from the observation (Spivey et al. 1954) that the addition of

20 percent fat to the basal diet appeared to be most successful in the production of a B_{12} deficiency in the chick. The same effect was observed for an increment of 20 percent protein. The basal diet used "...contained approximately 20 percent protein and 2.5 to 4 percent fat, by calculation." The investigators state "...the effect of substituting either protein or fat for 20 percent corn in depressing growth in the absence of vitamin B_{12} is believed to be due to the added constituent and not to the lowered amount of corn or to the altered proportion of soybean oil meal to corn." However, Spivey et al. (1954) cite the contradictory data gathered by McCollum and Chow from experiments with the rat demonstrating that a better vitamin B_{12} deficiency is achieved when the diet is low in fat.

EXPERIMENTAL

Experiment I. Effect of Testosterone Propionate on Vitamin B_{12} Retention

A. Purpose

Recent reports (Anderson and Sketol, 1953; Register, 1954; Fraser, 1951; Ling and Chow, 1951) establish that vitamin B_{12} is associated with sulfhydryl compounds in metabolism. Ling and Chow (1951, 1952, 1953, and 1954) have demonstrated a relationship between the animal's vitamin B_{12} content and both the glutathione content and carbohydrate metabolism. They suggest, on the basis of their work and the work of others (Lazarow, 1946, 1949, 1954; Patterson and Lazarow, 1950; Sen and Bhattacharya, 1952), that vitamin B_{12}, via its maintenance of an adequate supply of glutathione in the blood and tissues, plays a role in maintaining the activation of the sulfhydryl enzymes of the beta-cells in the pancreas. Dubnoff (1954) states that his laboratory is able to show, by the presence of vitamin B_{12}, an activation of a previously inactive sulfhydryl enzyme.

The above mentioned reports in conjunction with the demonstration (Ling and Chow, 1954; Hsu and Combs, 1952) that a B_{12} deficiency under a high carbohydrate-low fat regime causes hyperglycemia, and the possible relationship of these observations to the metabolic syndrome -- diabetes mellitus, bring to the fore the question: What factors influence the organism's retention of vitamin B_{12}? Or, what factors

influence the excretion of vitamin B_{12}?

It has been adequately demonstrated (Wahlstrom and Johnson, 1951; Becker, Lang, and Chow, 1953; Feng, 1954) that cortisone increases the excretion of vitamin B_{12}. Numerous reports have appeared showing both a stimulative (Vidgoff, 1940; Nathanson and Brues, 1941; Zizine, 1953) and a depressive (Selye and Collip, 1937; Greep and Jones, 1950) effect of testosterone propionate on the adrenal cortex and its hormone elaboration. In this respect, it was of interest to ascertain whether testosterone is a possible factor in the excretion of vitamin B_{12}.

B. Effect of Testosterone Propionate on Vitamin B_{12} Excretion

1. Procedure

On the basis that there is transplacental transmission of vitamin B_{12} as well as transmission by way of the mother's milk supply (Chow, 1952), litters, born to mothers maintained on a vitamin B_{12} deficient diet during pregnancy and lactation, were used in this experiment. When the young were 23 days of age they were removed from the mother. The diet contained, as the protein constituent, soy bean meal; a description of its complete contents can be found in the appendix. The weanling rats were, in all cases, continued on this same B_{12} deficient basal ration throughout the experiment. Food and water were given ad libitum.

Three litters were employed, the litter size ranging from 7 to 10 animals per litter. No attempt was made to limit the litter size since the primary objective was the comparison of the vitamin B_{12} excretion of litter-mates. The number of treatments that these animals were

subsequently to receive, required large litters.

From the very day of birth until 24 days of age, each litter, after random selection and marking, received two types of treatment: the controls received a daily subcutaneous injection of 0.02 cc. of cottonseed oil, whereas, the other litter-mates received a subcutaneous injection of 1 mg. of testosterone propionate* (in cottonseed oil) daily. The concentration of testosterone propionate used was 50 mg./cc. In order to assure accuracy in the delivery of such a small volume, the syringe was controlled by a micrometer. Upon calibration, it was found that on moving the syringe plunger 80 micrometer units, the desired 0.02 cc. volume was delivered. When the testosterone treated rats reached 24 days of age, the hormone dosage was increased to 3 mg. per animal. The concentration of the testosterone preparation used at this time consisted of 25 mg. of testosterone propionate per cubic centimeter of cottonseed oil. Consequently, the litter-mate controls received an increased volume of the placebo (0.12 cc. cottonseed oil). Sites of injection were alternated. As stated previously, the animals received throughout this experiment a B_{12} deficient basal ration. This, then, constituted the preliminary preparation of the animals for the experiment.

When 30 or 32 days old, in order to determine whether testosterone propionate might be a factor in vitamin B_{12} excretion, both the controls and the hormone-treated animals were given a 0.1 cc. subcutaneous

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* Kindly supplied by Dr. G. Stocking of The Upjohn Company, Kalamazoo, Michigan.

injection of radioactive vitamin B_{12} (Co^{60} labeled)** in addition to a separate injection of 0.1 cc. of crystalline B_{12} in physiological saline. A parenteral route was chosen since oral administration is attended by many complications (see discussion and section on review of literature). The specific activity of the radioactive B_{12} preparation used was 1016 microcuries per milligram of vitamin B_{12}; and since the concentration was 1.0 microgram B_{12}/cc., the administered radioactive source contained 0.1 microgram of vitamin B_{12}. The concentration of the crystalline solution was 5 micrograms B_{12}/cc. Hence, on the 30th day, considering both the radioactive vitamin B_{12} and the carrier B_{12}, the rats received a total of 0.6 microgram of the vitamin. This amount was above the optimum vitamin B_{12} requirement (0.5 microgram B_{12}/120 gm. body weight; subcutaneously; daily) observed by Emerson (1949) in B_{12} deficient rats.

Urine collections were made 24, 48, and 72 hours after the administration of the radioactive plus carrier vitamin B_{12}. After filtering, a 2 cc. portion of the urine was pipetted into aluminum planchets; these samples were slowly evaporated to dryness under 250 watt (G.E.) infrared bulbs. The dried urine aliquots were then counted with the DS-1 directional scintillation detector, which in order to reduce the background count, was encased in a lead shield (Model 3036, Nuclear Instrument and Chemical Corporation). The counts, recorded on a scaling unit (Model 163, Nuclear), were all corrected for general background. Counts were made to either the 5 per cent or 10 per cent level of accuracy (Calvin, 1949) depending on feasibility.

** Purchased from Abbott Laboratories, Chicago, Illinois.

In order to determine, in micrograms, the amount of Vitamin B_{12} excreted, a standard was prepared: 0.05 cc. of the radioactive B_{12} preparation was pipetted, in duplicate, into the counting vessels; then, to each planchet, 2 cc. of non-radioactive rat urine was added. After slight agitation to assure even distribution of the isotope containing vitamin, the aliquots were dried and counted as described above.

Constant geometry was assured by the fixed distance maintained between the window of the scintillation detector tube mounted within the lead shield and the sample slide which fits into position in a machined slot in the lead shield; the planchets (Central Scientific Company aluminum dishes, with their central elevation flattened out) fitted snugly into the sample slide. Geometric reproducibility and reproducibility of the count were found to be highly satisfactory.

Urine collection was made possible by the use of round wire-mesh-glass funnel rat metabolism cages (Lazarow, 1954; a review). The funnels were cleaned each day.

1. Results

The values for the urinary excretion of vitamin B_{12}, as measured by the radioactivity in the 24-hour urinary specimen, may be found in Table I. The second day urinary collection showed a definite drop in the vitamin B_{12} content, seldom yielding more than 10 per cent of the amount excreted the first 24 hours. This is in agreement with the observation of Becker *et al.* (1953) that in human subjects, a 16-hour extension of the collection period past the first 8 hours yielded less than an additional 5 per cent vitamin B_{12}.

The first 24-hour urinary excretion data (Table I) suggest the

Table I

Effect of Testosterone on Urinary Excretion of Vitamin B_{12}

Following Subcutaneous Injection of Carrier plus Radio-B_{12}

Litter	Average wt. (gms)	Treatment	Vitamin B_{12} in the urine: cpm*/rat/24hrs.	micrograms of administered B_{12}/rat/24hrs.	% excreted of injected dose
I (10)	35.6	B_{12} deficient diet [3]	408	.148	24.6
	36.4	" " " plus testosterone [3]	650	.237	39.4
II (7)	36.2	B_{12} deficient diet [3]	210	.076	12.7
	37.5	" " " plus testosterone [2]	355	.129	21.4
III (7)	45.9	B_{12} deficient diet [2]	648	.235	39.1
	40.9	" " " plus testosterone [2]	429	.155	25.9

*counted to the 5% level of accuracy

* corrected for general background

[ ] number of animals in each group

( ) number of animals weaned in the litter

possibility that testosterone may tend to increase the excretion of vitamin B_{12}. Only litter-mates should be compared.

The scintillation tube and the scaler were able to detect, with the method of counting employed, 828 counts per minute (corrected for background) from the disintegrations emitted from the 0.05 microcurie prepared standard. Considering our counting efficiency, the administered 0.1 microcurie vitamin B_{12} is equivalent to 1656 counts per minute. A simple calculation, using the ratio

$$\frac{\text{total micrograms } B_{12} \text{ injected}}{\text{total micrograms } B_{12} \text{ excreted}} = \frac{\text{cpm equivalent to injected } B_{12}^{60}}{\text{cpm in excreted urine}},$$

will convert the cpm values found in Table I to micrograms B_{12}. We are further able to calculate the percent of vitamin B_{12} excreted during the 24-hour period by dividing the number of micrograms B_{12} excreted by the total number of micrograms of B_{12} administered. Since, as will be elaborated in the discussion section, the comparison of litter-mates and not a cross-comparison of animals in different litters was the primary concern, the figures reported in Table I are compared directly, without body weight corrections. It should be noticed that the weights of the individual animals in the same litter are very similar. From Table I, it may be observed that the per cent of the injected dose of vitamin B_{12} that was excreted during the first day ranged between 12 and 39 per cent.

2. Procedure

In order to obtain further information on the possible relationship of testosterone to vitamin B_{12} excretion, the experiment was

repeated with important modifications: a.) in an attempt to more markedly differentiate, by decreasing their subsequent level of saturation, the radioactive excretion resulting from the two treatments among the litter-mates, a much smaller dose of vitamin B_{12} (one-fourth of the previous dose) was administered; b.) injected by the same route, the 0.25 micrograms B_{12} consisted of cobalt labeled vitamin B_{12} only (0.25 uc.); c.) a larger number of litters were employed, with replication within the litters whenever possible; d.) so as to assure more complete absorption from the site of injection, the volume of testosterone propionate administered was decreased by the use of the more concentrated preparation (50 mg./cc.) throughout this part of the experiment.

The animals employed were, as previously described, born of mothers fed a vitamin B_{12} deficient diet from the time of breeding. Here, also, no attempt was made to limit the litter size. Aside from the important modifications mentioned above, the animals received the same diet and treatment as described in procedure A.

Upon subcutaneous injection of the radioactive vitamin B_{12}, urine collections were made at 24, 48, and 72-hour intervals. The glass collection funnels were thoroughly washed and dried twice a day to assure a minimum loss of urine by feces absorption. With the same purpose in mind, to reduce loss of urine, a buchner-funnel arrangement was employed for filtering the urine collections. The urinary aliquots were prepared for scintillation counting in the same manner as described earlier.

2. Results

All litters employed and the replicas within litters yielded consistent data: the testosterone-treated rats excreted significantly

more vitamin B_{12} than their control (maintained on a B_{12} deficient diet) litter-mates. The data are reported in Table II. A cross-comparison of litters shows that in 24 hours the controls excreted from 1.7 to 7.8 per cent of the B_{12} while the testosterone treated animals excreted from 4.4 to 15.5 per cent. The difference between treatments is significant at the 8 per cent level of probability (t-test).

By far the greatest excretion of vitamin B_{12} occurred during the first 24-hour collection period, with only an occasional excretion of more than 10 per cent of the original excretion occurring during the second or third days. This observation is similar to that mentioned in the results in section "A".

As might be expected, the rats receiving the larger quantity (0.5 microgram) of vitamin B_{12} excreted a considerably higher per cent of it than those animals receiving a smaller dose (0.2 microgram). Thus Table I shows that the controls excreted 12 to 39 per cent in the first 24 hours following the parenteral administration of the vitamin, whereas the controls receiving the lower dosage (Table II) excreted as little as 1.7 to 7.8 per cent. Similar results are obtained if the testosterone-treated animals of Table I are compared with the hormone-treated animals of Table II. This observation, that vitamin B_{12}, when administered parenterally, is excreted in the urine in amounts proportional to the size of the injected dose, is an agreement with other reports (Sokoloff *et al.* 1952; Chow *et al.* 1950; Lang *et al.* 1952).

Table II.

Effect of Testosterone on Urinary Excretion of Vitamin B_{12}
Following Subcutaneous injection of radioactive B_{12} only

Litter	Initial Average wt. (gm.)	Treatment	1st 24 hr. Excretion*					2nd 24 hr. Excretion**		3rd 24 hr. Excretion**	
			average volume (cc.)	cpm/2cc	cpm/rat/24hrs.	micrograms administered B_{12} per rat per 24 hrs.	% of dose excreted during 1st day	cpm/rat/24hrs.	% of dose excreted during 2nd day	cpm/rat/24hrs.	% of dose excreted during 3rd day
I (9)	66.8	B_{12} deficient diet [2]	7.5	49	184	.011	4.4	22	0.53	19	0.46
	56.4	" " " plus testosterone [2]	9.6	50	240	.015	5.8	20	0.48	23	0.56
II (10)	64.7	B_{12} deficient diet [2]	3.4	42	71	.004	1.7	14	0.34	14*	0.34
	51.2	" " " plus testosterone [2]	5.4	67	181	.011	4.4	24	0.58	13*	0.31
	49.3	" " " plus testosterone [2]	5.3	201	528	.032	12.8	22	0.53	17	0.41
III (12)	38.2	B_{12} deficient diet [2]	4.6	142	323	.020	7.8	59	1.43	11	0.27
	31.6	" " " plus testosterone [2]	6.3	196	613	.037	14.8	13*	0.31	12*	0.29
	35.8	" " " plus testosterone [3]	3.2	396	640	.039	15.5	13*	0.31	9*	0.22
IV (6)	64.3	B_{12} deficient diet [2]	5.4	69	178	.011	4.3	16*	0.39	14*	0.34
	53.9	" " " plus testosterone [2]	4.1	97	199	.012	4.8	14*	0.34	13*	0.31

* counted to the 5% level of accuracy
** counted to the 10% level of accuracy
* counted to less than 10% accuracy
[ ] number of animals in each group
( ) number of animals born in the litter and weaned

C. "Flush-out" Phenomenon

1. Procedure

To ascertain whether a difference existed in the nature of the radioactivity retained in the tissues (assuming this to be intact vitamin B_{12}) of the testosterone-treated rats as compared to their control litter-mates and to ascertain whether the excretion pattern, observed in section "B" above, would be repeated, a large dose (many times above Emerson's physiological requirement, 1949) of stable vitamin B_{12} was administered subcutaneously. This unlabeled vitamin B_{12} solution was prepared by the addition of crystalline B_{12} (containing mannitol, Merck) to distilled water (166 micrograms vitamin B_{12}/cc.). As a preservative, phenol crystals (Hartley, Stross, and Stuckey, 1950) were added to make a 0.5 per cent solution. A volume of 0.1 cc. of this unlabeled vitamin was injected into the already tagged rats, employed in section "B", on the 72nd hour after the radioactive dose was given and each day thereafter. It should be understood that the testosterone-treated animals continued to receive their subcutaneous injection of testosterone throughout this experiment. The vitamin was always injected in the dorsal region of the animal and the hormone was administered in the ventral region, care being taken always to alternate the sites of successive injections in their respective regions. A 24-hour urine collection and a composite (48 to 72-hour) collection were made. The methodology remaining the same, the urine samples were measured for radioactivity.

1. Results

A definite "flush-out" or increased excretion of the previously

Table III.

Effect of Testosterone on the "Flush-out" of labelled B_{12} by subsequent mass injection of stable B_{12}

Litter	Treatment	Vitamin B_{12} in the urine			
		1st 24 hr. Excretion*		Average of 48 hr. and 72 hr. Excretion**	
		cpm/rat	% of labelled B_{12} excreted	cpm/rat	% of labelled B_{12} excreted
I (9)	B_{12} deficient diet [2]	97	2.3	42	1.0
	" " plus testosterone [2]	118	2.9	57	1.4
II (10)	B_{12} deficient diet [2]	65	1.6	36	.87
	" " plus testosterone [2]	105	2.5	38	.92
	" " " [2]	85	2.1	31	.75
III (12)	B_{12} deficient diet [2]	54	1.3	29	.65
	" " plus testosterone [2]	72	1.7	27	.65
	" " " [3]	76	1.8	30	.72
IV (6)	B_{12} deficient diet [2]	110	2.7	48	1.2
	" " plus testosterone [2]	69	1.6	44	1.1

* counted to the 5% level of accuracy
* * counted to the 10% level of accuracy

administered tagged vitamin by the unlabeled vitamin may be observed by a comparison of the first 24-hour excretion in Table III with the third 24-hour excretion of Table II. In fact, in the case of every animal, its urinary excretion of the radioactive vitamin B_{12} increased more than 5-fold above the previous day's excretion (comparing Tables III and II) as a result of the unlabeled vitamin injection. From Table II we see that the urinary excretion of the labeled vitamin is markedly decreased subsequent to the first day's excretion; Table III also shows a marked decrease in the "flush-out" phenomenon subsequent to the first day's attempt.

But the importance of this experiment does not lie completely with these observations. Instead, it is to be observed from Table III that the testosterone-treated animals excreted consistently (except for litter IV) more of the radioactive vitamin than did their control littermates -- an interesting correlation with the similar excretion pattern shown in Table II.

D. Discussion

Before attempting to evaluate the data in this paper, it is relevant and pertinent to discuss the reasons for particular procedures followed and the reasons for the type of treatment of the data gathered.

A parenteral mode of administration of the radiovitamin was chosen for several reasons. Subcutaneous administration of tagged vitamin B_{12} makes more of the dose available to the animal and the excreted portion is found predominantly in the urine. Neither of these points (Rosenblum *et al.* 1952), is true when the radiovitamin is given orally: on *per os*

administration 82 per cent of the radioactivity (81 per cent via the feces; 1 per cent via the urine) is excreted at the end of four days; on subcutaneous administration 56 per cent of the radioactivity (50 per cent via the urine; 6 per cent via the feces) is voided in the same time period. Consequently the parenteral route is more economical. In addition, while the excretion of the vitamin was to be determined by the observed radioactivity, the Co^{60} elimination in the feces is not associated with the original vitamin, whereas the radioactivity elimination in the urine (as a result of parenteral administration) is associated with the intact vitamin (Rosenblum et al. 1952).

Exogenous gonad hormones are known to curtail the endogenous output of the hormone (Hellbaum and Greep, 1943; Selye and Freedman, 1941). In order, then, to observe the effects of an elevated androgen circulation, the level of testosterone propionate administered was at once above the physiological secretion (further discussion on this topic may be found in the section reviewing the literature).

Since it is known that vitamin B_{12} is stored in various tissues of the body (Rosenblum et al. 1952) and that the amount of excretion of this vitamin depends on the relative amount of reserve, it was necessary to use vitamin B_{12} deficient animals in these experiments. Had both the testosterone-treated animals and the controls been in a saturated state, on administration of radioactive B_{12}, little of the radioactivity might be expected to be retained by the body; if this were the case, it would have been difficult to differentiate between the excretions of the treated animals as compared to the controls.

In regard to the treatment of the data, it was deemed preferable to report the radioactive excretion of the vitamin in terms of "per

animal" rather than in terms of "100 grams body weight". There is little reason, if any, to assume that vitamin excretion increases or decreases with the weight raised to the first power. It is certainly more reasonable to assume that the vitamin excretion varies with another reference base -- Brody's (1945) (See also Miner, 1954) "physiological weight" (i.e. body weight raised to the 0.7 power) -- as does the basal metabolism and endogenous protein metabolism. This means that increasing the body weight by 1 per cent might alter the radioactive excretion not by 1 per cent but only by 0.7 per cent. Again, and more significantly, it has been noticed that the excretion values have been treated as if they bore a direct relationship to body weight. An inverse relationship is much more likely. Lang and Chow (1952) reported that older rats excreted greater amounts of tagged vitamin B_{12} than young mature animals. Although age is probably an important factor, the animals with the greater active protoplasmic mass may be expected to excrete less of the cobalt labeled vitamin. Comparison (Table II) of the urinary excretion of the radiovitamin among similarly treated animals of the same age seems to support this supposition. It is to be noted that Lang and Chow (1952), expressing their results in micrograms per animal, were not able to show an altered excretion of the vitamin in animals that were twice the weight of other mature animals, yet acknowledging this they continue to re-calculate their data in terms of micrograms B_{12} per 100 gram body weight. In short, on the basis of Lang's and Chow's report (1952) and the data presented in Table II of this paper, the writer believes that, at most, the radiovitamin excretion may be inversely proportional to the animals active protoplasmic mass or that, at least, no sharp difference exists. If this be the

case, it is a fallacy to cloak the vitamin excretion with the "micrograms per 100 grams body weight" treatment. Likewise the expression of the excretion in terms of an individual milliliter is improper since, as table II shows, the volume of urine excreted by each rat is as variable as is the specific activity.

It has been demonstrated (Tables I and II) that testosterone propionate, administered from the day of birth, causes an increased excretion of the radiovitamin B_{12} when the latter is administered to B_{12} deficient rats (30 or 32 days of age). An extensive review of the literature reveals only one suggestion that testosterone may cause an increased excretion of this vitamin (Becker, Lang, and Chow, 1953). Becker *et al.* state, in regard to the radioactive urinary B_{12} excretion, that "the testosterone-injected animals, however, showed no significant difference from the controls under these experimental conditions". Nevertheless, they were able to show, on the basis of tissue analysis, that "to some extent those receiving testosterone retained less radioactivity than saline-injected controls."

It is altogether possible that Becker *et al.* did not get a definite clear-cut differentiation between the two types of treatments for several reasons. In all, four controls and four treated animals were employed. It is understood that the animals were healthy and on a normal diet. Animals are capable of storing vitamin B_{12} and actually do have a vitamin B_{12} reserve in their tissues (Chow, 1952). Under these conditions, the administration of a hormone, with intent to observe whether the hormone alters the uptake of the subsequent injection of tagged vitamin B_{12}, is under most adverse conditions. It is to be expected that the B_{12} adequate control will retain little of the

administered radioactivity (Rosenblum et al. 1952); if testosterone is a factor which allows little retention of the administered radioactivity it is conceivable, since the excretions of both the controls and treated animals vary in the same direction, that such an experiment might demonstrate little or even no differentiation.

With this in mind, the experiments reported in this thesis were all conducted on diets deficient in vitamin B_{12}. Here the deficient control animals might be expected to retain a larger portion of the radiovitamin (than would B_{12} sufficient control animals), whereas the testosterone-treated animals would excrete a greater amount of the radiovitamin. This, in effect, is supported by the data in Tables I and II. An interesting observation, in addition, is that comparison of the radiovitamin excretion within litters (Tables I, II, and III) shows the greatest differentiation in the larger litters, as if a more limited supply of nutrition per animal from the mother had incurred a greater B_{12} deficiency among the members of these particular litters. If this supposition is correct, the greater B_{12} deficiency incurred by the control members of the larger litters, offered, by retaining an increased portion of the radioactive B_{12}, a still greater contrast to their testosterone-treated litter-mates (who excreted some of the radioactive vitamin as a result of the hormone treatment).

We should be aware of the fact that the expression "vitamin deficiency state" implies a relative rather than an absolute situation. Hence, if we are to compare animals, it is of utmost importance to have similar nutritional sources available to them. In order to approximate this equality of dietary treatment, these experiments were designed to allow the comparison of litter-mates. While it is evident, then from

such a comparison that the hormone treatment resulted in an increased vitamin B_{12} excretion, cross-comparison of litters also shows a similar trend: the controls excreted from 1.7 to 7.8 per cent during the first 24-hour period; the testosterone-treated animals excreted from 4.4 to 15.5 per cent.

The data in Table III further show that a difference exists, in respect to the vitamin uptake, between the androgen-treated animals and their litter-mate controls. Whereas Table II illustrates a differential vitamin B_{12} uptake, Table III suggests a different distribution for the acquired vitamin. The latter is assumed since the data show a greater availability for exchange of the radiovitamin in the hormone-treated animals. The greater radio-B_{12} excretion is associated with a greater availability of the radiovitamin to exchange with the injected stable vitamin. It is likely that an equilibrium exists in the distribution of vitamin B_{12} and that the administered hormone altered this equilibrium. Diagram A illustrates the possible situation existing in the control B_{12}-deficient animal. Here the radioactive vitamin B_{12} distributes itself in a manner abiding by "necessity" and equilibrium factors. Three "spaces" are diagramatically shown -- the cell space, the intercellular space, and the blood space. The cell space is, so to speak, at liberty to take the amount of the administered radioactive B_{12} it "needs" from the fluids that bathe its outer walls. And it is likely that the cell does just this and soon the labeled vitamin B_{12} is distributed according to equilibrium factors between the three "spaces". Upon administration of the stable vitamin B_{12}, it is expected that in a dynamic system the stable B_{12} will exchange with that portion of the radioactive B_{12} which is not at the moment combined in the performance

of some function. Since a large amount of stable vitamin B_{12} was administered we would expect much of the stable vitamin and, as a result of exchange in the tissues, some of the radioactive vitamin to be excreted. This exchange of the radiovitamin with some of the stable vitamin and the appearance of the former in the urine has been termed a "flush-out" phenomenon.

It is now theorized, on the basis of the data obtained, that the resulting decrease in radiovitamin uptake by the testosterone-treated animal resulted from a decreased uptake by the individual cells; that testosterone in some manner decreased the ability of the cell space to take up a "needed amount" of B_{12} from the surrounding environment (Diagram B). Then, since the uptake and exchange by the cell of vitamin B_{12} is greatly reduced, only two significant spaces exist in the androgen-treated animal -- the intercellular space and the blood space. Upon stable vitamin B_{12} administration, the greater flush-out effect exhibited in the testosterone-treated animal is interpreted to indicate the presence of a greater exchangeable pool of the vitamin than exists in the control animal. This might, indeed, be the case if in the androgen-treated animal the administered B_{12} isotope being somewhat prevented from entering the cell, distributed itself according to the equilibrium factors between the intercellular space and the blood.

On the other hand, the cell spaces of the control animals are able to receive their "needed" requirement of the labeled vitamin B_{12}; the labeled B_{12} once entering the heretofore deficient cell performs its metabolic role and in performing its role is less available for exchange with the subsequent stable B_{12} than the isotope B_{12} situated in the blood and intercellular spaces. Hence, we might expect that the control-

animals would excrete less radiovitamin than the treated animals. The data in Table III shows this to be the case. This, then, may explain why more radio-B_{12} was available for exchange in the androgen-treated animal than in its control brother or sister.

On review of the literature it was found that, on a different theoretical basis, Glass in 1954 postulated that a fraction of the B_{12} is in a bound form during its passage through the intestinal mucosa. This assumption of the existence of a B_{12} acceptor is based on the fact that the efficiency of B_{12} retention is greatest in, perhaps, what one might call the "physiological range." Glass observes that when the administered dose of B_{12} is increased the efficiency of retention decreases rapidly as if some mechanism existed for its binding. As noted previously in this thesis, a small dose of administered radio-B_{12} was followed by a larger percent of retention than that following the administration of a larger dose of radioactive B_{12}. The marked decrease in the urinary excretion of the tagged vitamin (Table II) subsequent to the first day's excretion and the marked decrease in the flush-out phenomenon (Table III) subsequent to the first day's attempt, also suggest the existence of a bound state for the vitamin.

Working with the observation that androgen-treated animals have a greater radio-B_{12} flush-out effect and assuming that this indicates that the radio-B_{12} is more available for exchange, the question arises "what makes the radio-B_{12} more available for exchange?" It is postulated that testosterone in some manner decreases the ability of B_{12} to enter the cell and hence the site of metabolic activity. Although the mechanism is not established it is known (Chow, 1952) that vitamin B_{12} plays a role in carbohydrate metabolism. Chow (1952) suggests it plays a role

as a coenzyme. It is conceivable that vitamin B_{12} may be "actively" transported into the cell. That is to say, that as vitamin B_{12} performs its role in the cell and is thereby combined in a complex or "used up", the concentration of the B_{12} in the medium surrounding the cell becomes relatively greater than its counterpart inside the cell and the former B_{12} is consequently "actively" transported into the cell along a concentration gradient. A substance which would alter carbohydrate metabolism within the cell would simultaneously alter the demand for the vitamin B_{12} concerned in that metabolism. Such circumstances might certainly decrease the amount of radiovitamin which would actively be "drawn" into the cell, and thus impart to whatever may be called the membrane of the cell space a relative appearance of impermeability. If testosterone, directly or indirectly, were the substance capable of decreasing the "need" or rate of entrance of vitamin B_{12} into the metabolic apparatus of the cell space, more radiovitamin might be located in the blood or intercellular space, and our question "what makes radio-B_{12} more available for exchange?" would be at least partially answered.

It is of interest to note that cortisone is well known to cause a lesion in the carbohydrate metabolic scheme; that cortisone increases vitamin B_{12} excretion (Wahlstrom et al. 1951; Becker et al. 1953; Feng, 1954); that a protein rich diet has been shown to cause adrenal hypertrophy and increased adrenal function (Tepperman, Engel, and Long, 1943) and observed to cause a vitamin B_{12} deficiency (Spivey et al. 1954). Is it possible, then, that testosterone may either potentiate cortisone or in some manner give rise to a cortisone-like compound? In respect to body growth and hair patterns, it is known (Meites et al. 1954; Meites et al. 1955; Meites, 1956) that under the proper conditions

vitamin B_{12} will counteract the cortisone effect; the experimental work in this paper suggests that large amounts of vitamin B_{12} will counteract the effects of testosterone.

Is it a coincidence that testosterone (under the conditions of this experiment) caused a suggestive decrease in body weight and a scanty hair pattern, similar to the effects of cortisone administration, and that the testosterone effect was counteracted by vitamin B_{12} administration, similar again to the counteraction by vitamin B_{12} of the cortisone effect? This will be referred to later.

Since it has been demonstrated that administration of cortisone will increase the urinary excretion of a dose of radioactive B_{12}, it is conceivable that testosterone, acting through the adrenals, in a manner to increase the adrenal cortical hormone secretion, could increase the radiovitamin excretion. Nathanson and Drues (1941) found an increased mitotic activity in the adrenals of immature female rats. Vidgoff (1940), using an extract of bull testes, showed an increase in adrenal weight due to hypertrophy and hyperplasia in the zonae fasiculata and reticularis. It is known that the adrenal cortex atrophies as a result of hypophysectomy. Numerous investigators (Cutuly, Cutuly, and McCullagh, 1938; Leonard, 1944; Zizine, 1953) have found that testosterone propionate and other androgenic substances maintain the adrenal cortex following hypophysectomy. Considering adrenal activity, Li (1953) states "...thymus reduction is a very good index of adrenal activity, because it measures the functional activity of the adrenal." Dorfman and Shipley (1956) suggest that thymus involution following testosterone administration may indicate that the effect is operative through the adrenal cortex. They base this suggestion on the observation

that no thymus involution can be demonstrated in the castrated-adrenalectomized mouse after testosterone administration. Although it is a consistent observation that estrogens, when administered in moderate-sized doses, produce striking hypertrophy of the adrenals, similar reports concerning androgens are not as numerous. Hence, Bottomley and Folley (1938), employing immature male guinea pigs averaging 170-190 grams body weight at the beginning of the experiment, were unable to demonstrate alteration in the size of the adrenals following a month's daily injections of 2 mg. of testosterone. Mazer and Mazer (1939) describe atrophy in mature and immature female rats while Selye (1940) in a series of injections paralleling the procedure employed in this experiment also demonstrated adrenal atrophy in immature rats. As a result of the inconsistency of results regarding the effect of testosterone on adrenal size, a new experiment is in progress to ascertain the effect of the androgen on adrenal size under our experimental conditions.

Even considering, for the moment, that testosterone might cause a loss in adrenal weight it is to be emphasized that adrenal atrophy from such treatment has been observed to be concerned with involution of the glomerulosa. Selye (1940a) reports that "...the entire glomerulosa region is substituted by a dense connective tissue scar" and that these histological changes "...differed significantly from those observed after hypophysectomy." It appears possible, then, that a decreased secretion of cortical hormones arising from the glomerulosa might release an amount of anterior pituitary inhibition and cause a corresponding increase of an ACTH hormone which could further increase the fasiculata secretion. If this were the case, the increased glucocorticoid

secretion might cause a greater excretion of the cobalt-labeled vitamin B_{12}.

Rennels (1952), employing immature rats, demonstrated that "In all animals given testosterone or its propionate an almost complete loss of cholesterol from the cells of the zona fasiculata occurs." Since cholesterol has been shown to be a precursor of cortical hormones (Hechter and Pincus, 1954), this is an indication of increased hormone production. However, Rennels cautions that this increased production of the hormone does not imply an increased release of the hormone. He suggests that of the cytoplasmic bodies the spheroid complexes are the locus for the synthesis of cortical hormones and that the discharge bodies which represent transformed spheroid complexes are concerned with the mechanism for the release of the hormone into the circulation. Whereas "proof of this is completely lacking", Rennels believes that the lack of discharge bodies in the adrenals of testosterone-treated rats may be indicative that hormone release is inoperative.

The possibility, considering the large doses of testosterone propionate administered, that the androgen may be converted by the body into a substance that either resembles in action a cortical hormone or acts upon the adrenal cortex to secrete an adrenal cortical-like hormone should not be overlooked. The conversion *in vivo* of testosterone to estrogens is now a well known fact. Steinach and Kun (1937) demonstrated an increased estrogen excretion in men subsequent to testosterone propionate administration. In fact, in one case they were able to show an increase in estrogenic material from 36 to 1200 R.U. per liter after the administration of 1 gram of testosterone propionate. The work has been confirmed by Nathanson *et al.* (1951); Baggett *et al.* (1955)

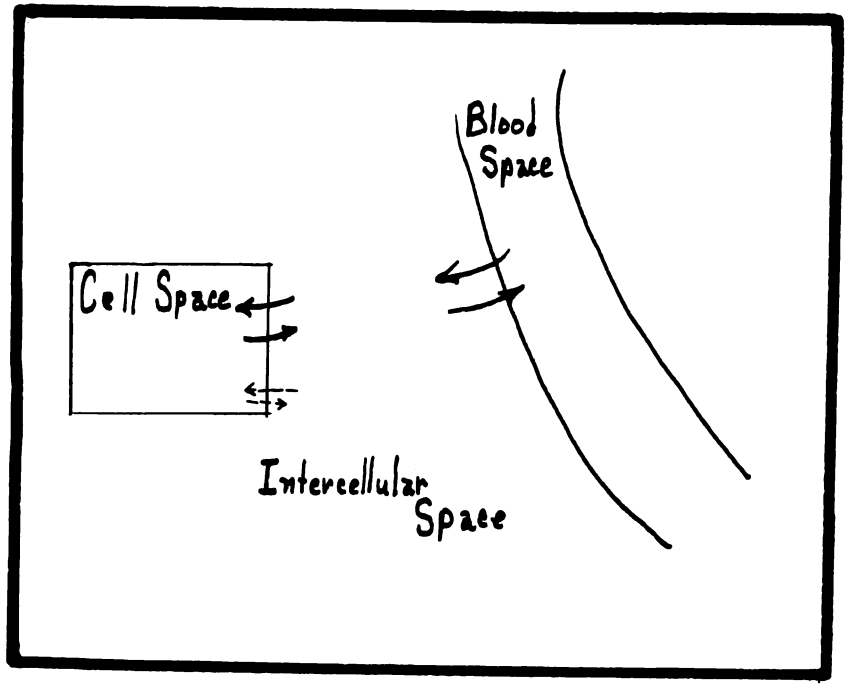
have further established this by the use of labelled testosterone. It has been amply demonstrated that administration of estrogens will cause hypertrophy of the adrenal cortex and a subsequent increase in cortical secretion (Samuels, 1951). Although not established, it is possible, considering a common origin of testosterone (Dorfman and Shipley, 1956) and the corticoids (Dorfman and Shipley, 1956; Hechter and Pincus, 1954) from cholesterol, that testosterone might be chemically altered en route through the adrenals, the liver, or other peripheral tissue to a substance resembling in action a glucocorticoid. Indeed, Hechter (1953) perfused a randomly labeled C_{19} steroid (dehydroepiandrosterone-C^{14}) through a beef adrenal and, after repeated paper chromatography, showed that hydrocortisone was produced. At the moment (Dorfman and Shipley, 1956), there is no decisive evidence of the presence of androgens in the bile which would indicate "the possibility of the hormone being reabsorbed through the gut and recirculated, as has been demonstrated for the estrogens."

Further indication that administered testosterone may bring about a cortisone effect may be had by assessing the physiological state of the animal treated with testosterone propionate. Although certainly not conclusive, it was observed that the androgen treated animals exhibited a small but consistent loss of weight while their littermates treated with androgen and vitamin B_{12} showed weight gains similar to the controls. The data for this experiment is reported in the latter part of this thesis. It should be observed that the loss of weight resultant from androgen treatment resembles a cortisone effect and that the B_{12} counteraction of the androgen effect noted under these experimental conditions is strikingly similar to the well established vitamin

B_{12} counteraction of cortisone-induced effects. Another point of interest is that the animals receiving the androgen showed scanty hair patterns. This is reminiscent of a cortisone effect. And again similar to observations made with cortisone, when large amounts of vitamin B_{12} were administered in addition to testosterone the hair patterns were observed to be normal.

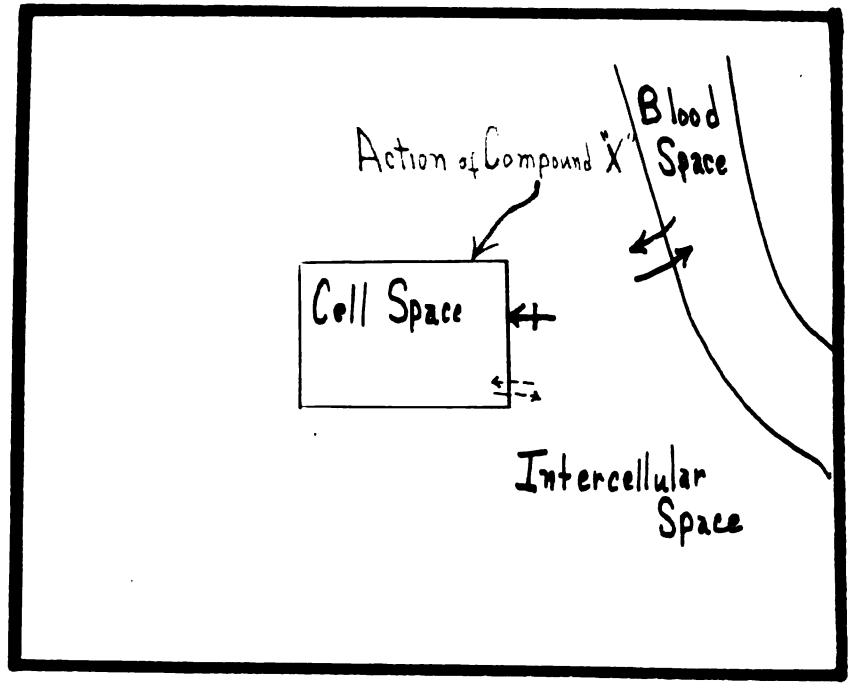
To avoid further circumlocution, let us summarize what has been suggested here concerning the effect of testosterone and the adrenal cortex. It has been suggested that testosterone administration results in an increased circulating level of glucocorticoid or corticoid-like substance. Whether this effect might be mediated either through the adrenal cortex or some other organ such as the liver, is further conjecture. The evidence available at this time indicates that testosterone causes the observed increment in radio-B_{12} excretion as a result of an increased circulating level of corticoid-like substance or a potentiating effect on cortisone action. Dorfman and Shipley (1956) cite recent data indicating that testosterone may be a potentiator of cortisone. It is to be observed that the possibility that testosterone acts directly to increase vitamin B_{12} excretion has not been ruled out. An experiment designed to evaluate this possibility would encounter many difficulties in interpretation, since adrenalectomy alone would answer only part of the question. It must also be noted that the data reported in this paper has been interpreted on the basis of the present day understanding that the radioactivity found in the urine, subsequent to the parenteral administration of labeled vitamin B_{12}, is primarily the result of the excreted intact vitamin. A good correlation exists between the microbiological assay and the radioactivity measurement of

Diagramatic Representation of the "Spaces" Available for Vitamin B_{12} Distribution



Proposed schema operating in control animal
(Solid arrows at cell space indicate "active transport;
broken arrows indicate "passive" situation)

Diagram A



Proposed site of defect in vitamin B_{12} retention
in the testosterone-treated animal.
(Note that the "Cell Space" is less available)

Diagram B

Mechanism* by Which the Proposed Compound "X" Might Decrease Permeability

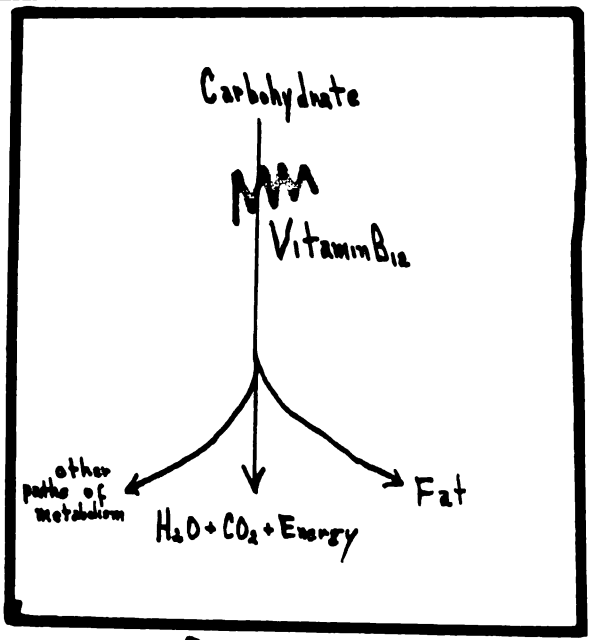


Diagram C

A lesion (marked by the red line) in the path of carbohydrate utilization would stop the "cellular pump" or the active transport of vitamin B_{12} into the cell. If this were the case, only the limited "passive" transfer would take place.

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* Evidence in support of the mechanism:

1. a.) Vitamin B_{12} is believed (Hsu and Combs, 1952a) to be involved in carbohydrate utilization: a B_{12} deficient state is followed by elevated blood glucose level.
   b.) If carbohydrate metabolism were to become defective, we might expect that vitamin B_{12} would "lose" a job, and so become more available for excretion or exchange.
   Cortisone is known to cause a lesion in carbohydrate metabolism; the hormone administration is followed by increased vitamin B_{12} excretion and tissue depletion (Becker et al., 1955).

2. If carbohydrate metabolism is accelerated, we might expect from the proposed mechanism, an increased vitamin B_{12} retention. This, Meites and Feng (Fed. Proc., 1955) have shown to be true: "Insulin injection greatly reduced the urinary excretion of tracer doses of radioactive B_{12} in normal, alloxanized, and cortisone treated rats ...."

3. Large amounts of vitamin B_{12} counteract the cortisone-catabolic effects (Meites, 1954). Here we may suppose that the large doses of B_{12} entered the cell (and the subsequent site of action) due to its quantity (i.e. along a concentration gradient) -- once the B_{12} was at its site of action, it was able to perform its role.

4. High protein diet has been shown (Topperman, Engle, and Long, 1943) to cause adrenal cortex hypertrophy; likewise the high protein diet has been shown (Spivey et al., 1954) to cause vitamin B_{12} deficiency.

the vitamin (Chow, 1953 and 1956).

At this point, with the permission of the reader, the author would like to illustrate a possible mechanism by which testosterone might reduce the permeability of a cell. Since it cannot be stated with certainty that testosterone in some manner causes an increased cortisone effect, we will call the proposed specific agent that interferes with carbohydrate metabolism compound X. The suggested mechanism is shown in diagram C.

Before closing the discussion it should be noted that the term "cell-space" is indeed a general one. Davies (1954) states, on the basis of work that he has done, that "... the whole cell is not the simplest unit which is able to maintain active transport. It is now known that the mitochondria, which are the structures responsible for virtually all the respiratory mechanisms of cells, are able both to secrete and to accumulate a variety of inorganic and organic cations and anions." Rosenberg (1954) says that "certain observations (Lehninger, 1951) indicate that very similar phenomena of transport across membranes also occur in intracellular particles, especially mitochondria." Danielli (1954) adds that although some work has been done "... it has not so far been possible to relate the physiological function of mitochondria to their structure and enzyme organization, so far as the field of active transport is concerned." When referring to the term "active transport", the author has meant, as Rosenberg (1954) states, "transport of substances across one or more cell membranes which is influenced not only by the force responsible for passive diffusion, but also by other forces which are maintained and regulated by the metabolism of the cell."

Experiment II. Some Effects and Interrelationships of Testosterone and Vitamin B_{12} on Growth

A. Purpose

The literature is replete with observations that attest to both testosterone's anabolic function and its growth retarding effect. In essence, the anabolic effect of testosterone is observed as a result of the re-establishment of the normal hormone level in such cases where the hormonal balance is disturbed (e.g. hypogonadism, castration). Even, Kochakian, a proponent for the anabolic effect, states that "The presence of the functioning gonads in man as in the dog makes the subject 'resistant' to the metabolic effects of testosterone propionate" (1946). Likewise (Anderson, 1953; Sleeth et al. 1953) no anabolic effect of injected testosterone could be demonstrated in normal sheep, pigs, or pullets. H. Turner et al. (1941) and McEuen et al. (1937) found no effect of testosterone propionate in castrated male rats, even when injected from the day of birth. And, furthermore, Rubinstein et al. (1939) reported a significant depression of body growth on testosterone administration to the normal male albino rat; a similar retardation of body growth in young male mice of the dba strain was reported by Kochakian (1940).

The question naturally arises as to a possible method by which testosterone, when administered to a normal animal, might cause a

retardation of growth. In this respect, it is of interest to note that many reports have appeared suggesting that vitamin B_{12} may be a growth factor (Emerson, 1949; Chow and Barrows, 1950; Meites, 1951; Venkataraman, Dubin, and Friedell, 1954; Kline and Kastelic, 1954).

With the demonstration in Experiment I that testosterone may increase vitamin B_{12} excretion, the problem arose whether the interrelationship existing between these factors might influence growth. Does testosterone cause a retardation of body growth, and if so, will vitamin B_{12} supplementation aid in the return of the growth rate?

B. Some Observations on Body Weight, Efficiency of Food Utilization and Hair Patterns

1. Procedure

It is understood that growth is a general term encompassing many things and expressed in numerous ways. In this experiment, body growth was measured as gain or loss in body weight; an attempt to gain in insight as to the extent of protein anabolism or catabolism was made by determining the urinary nitrogen at various points of the experiment.

The animals used in the corresponding part of experiment I were employed. Following birth, their body weights were recorded at regular 4-day intervals. Let us briefly recall the experimental conditions -- these animals had been born of mothers kept on a vitamin B_{12} deficient diet; from weaning age, both the control and testosterone-treated animals were likewise maintained on this deficient diet. When 30 or 32 days old, each animal received a radioactive B_{12} injection and was observed for the next three days, whereupon experiment I terminated. But, in order to ascertain the effect of vitamin B_{12} on body growth, further

procedures were initiated at this time. For the sake of clarity, the body growth measurements and urinary nitrogen determinations have been designated as Experiment II.

Following the termination of experiment I, both the B_{12}-deficient controls and the B_{12}-deficient testosterone-treated animals were given a daily subcutaneous injection of 5 micrograms of stable B_{12}. The B_{12} solution, prepared in physiological saline, contained phenol as a preservative. It should be understood that the testosterone-treated animals continued to receive the hormone throughout the experiment whereas the other animals received the cottonseed oil placebo. Food was given ad libitum.

In addition to the recording of the weight gains occurring in each 4-day period, the weight gain, food consumption, and total urinary nitrogen were recorded during a specific period prior to the radioactive B_{12}-treatment and after the radio-vitamin treatment.

1. Results

The lack of sufficient data in this part of the experiment permits only suggestive remarks. The slope of each curve in figures 1, 2, and 3 appreciably increases following the vitamin B_{12} supplementation. Likewise, Table 4 shows that, except for one instance, there was a large increase in the weight gained by the animals during the "Post-B_{12}^{60} Treatment" period as compared with their weight gain during the "Pre-B_{12}^{60} Treatment" period. In regard to efficiency of food utilization, Table 4 tends to show an increase following the initial administration of the radioactive plus stable vitamin B_{12}. There is also suggestive evidence that the initial vitamin B_{12} injection to the animals maintained

Fig. 1. Growth curves of the young rat under the specified conditions.

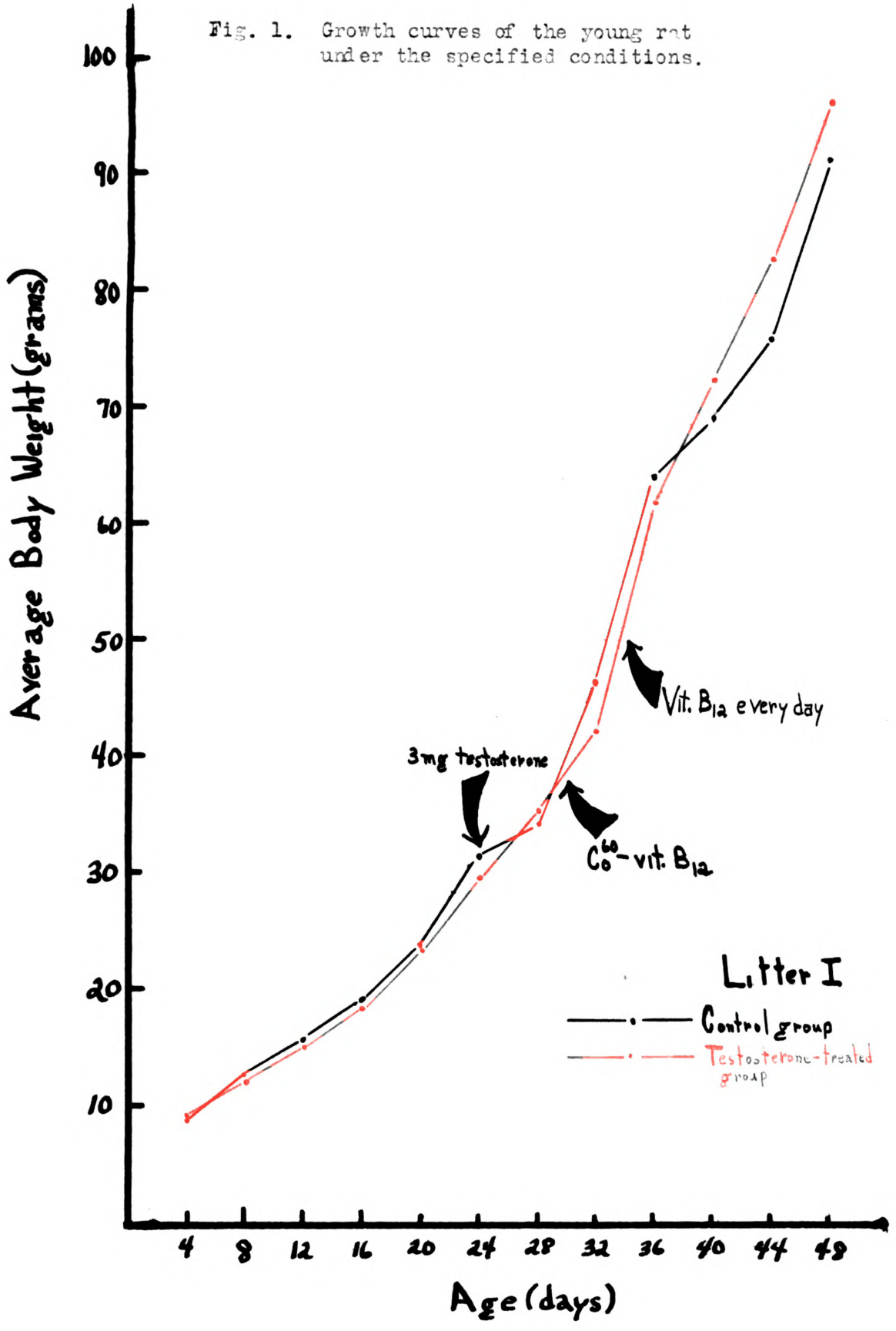


Fig. 2. Growth curves of the young rat under the specified conditions.

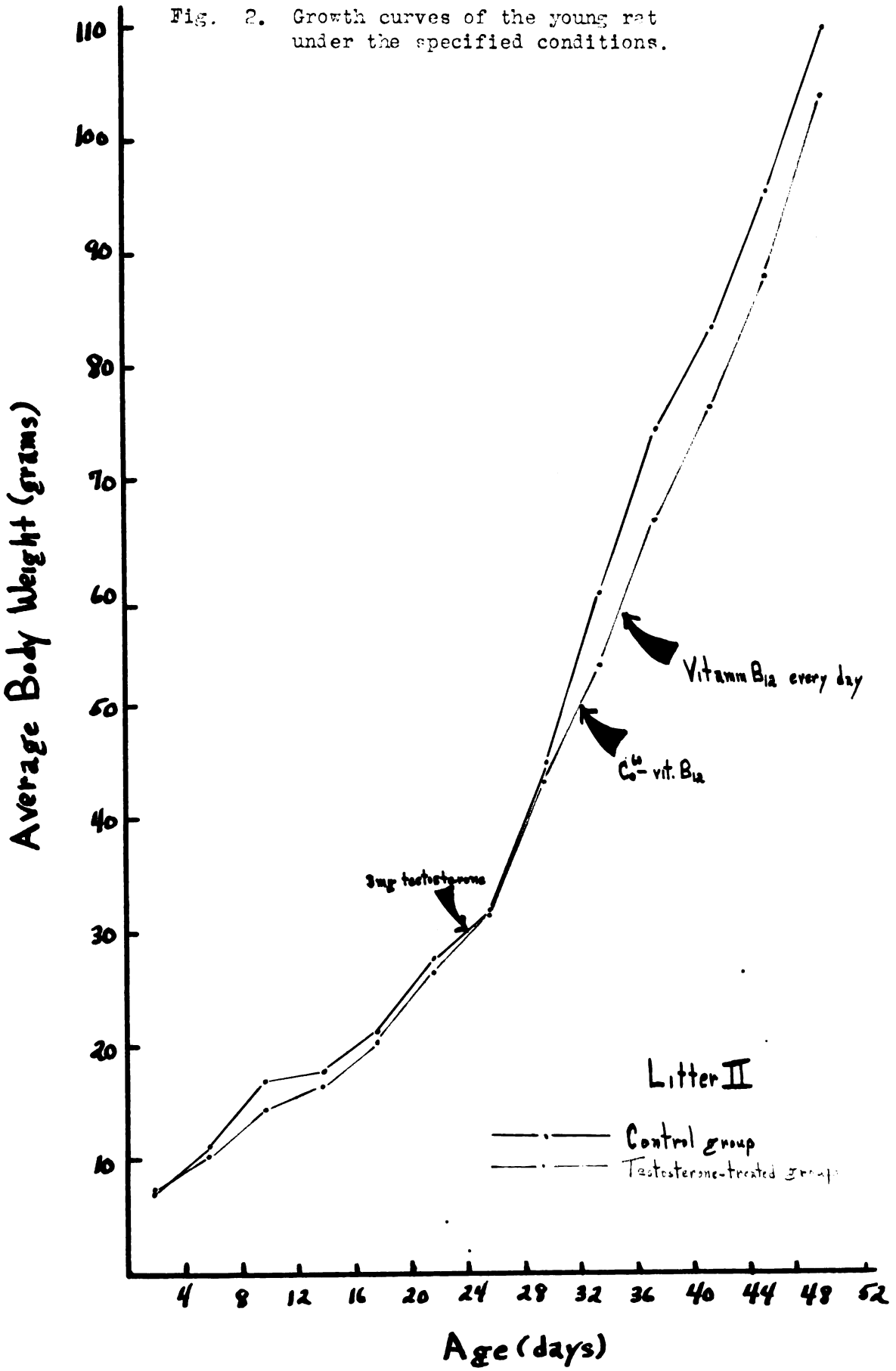


Fig. 3. Growth curves of the young rat under the specified conditions.

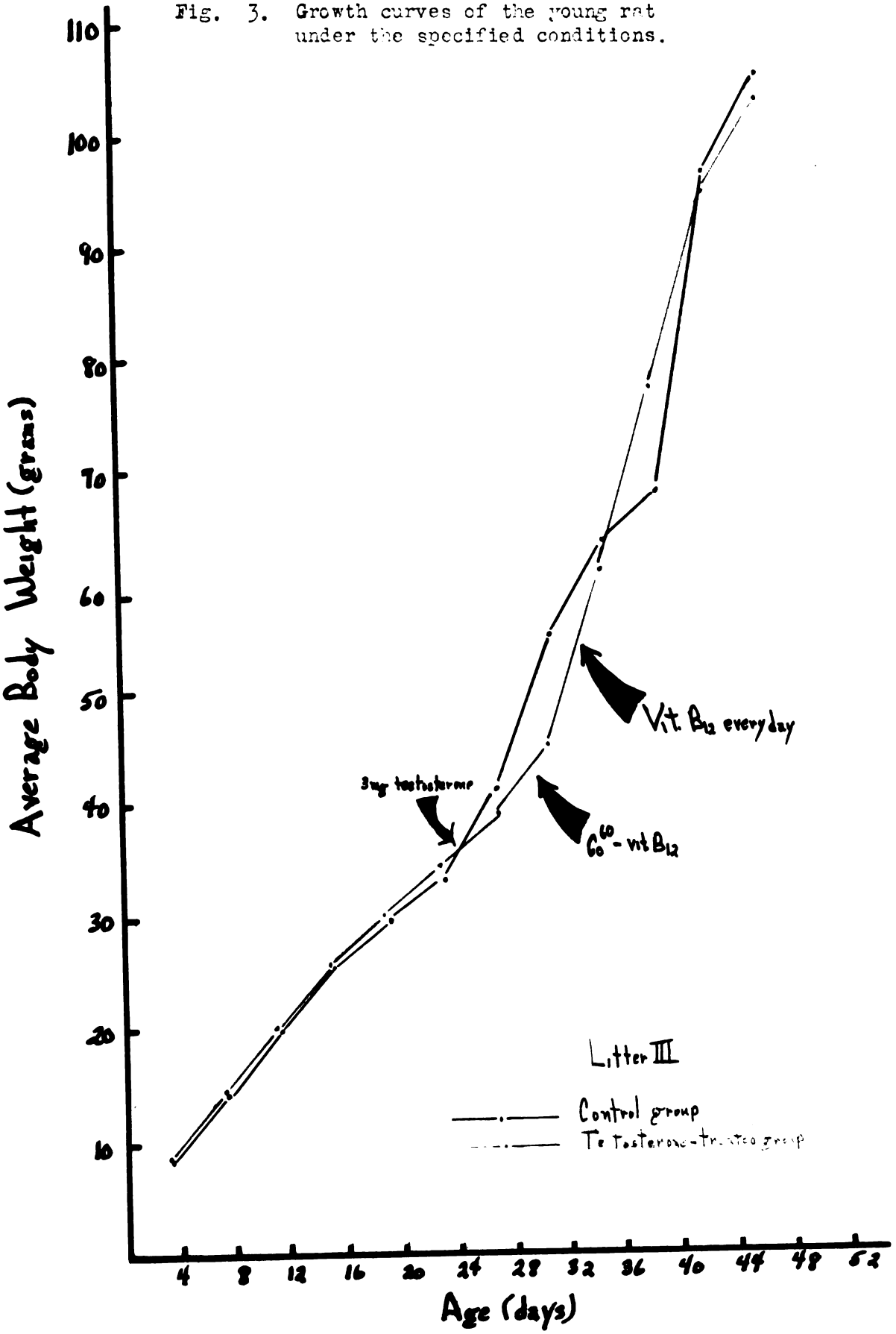


Table IV.

Effect of Vitamin B_{12} on Efficiency of Food Utilization and Urinary Nitrogen Excretion

Litter	Animal	Pre-B_{12}^{Co} Treatment*				Post-B_{12}^{Co} Treatment**				
		Average+ Weight Gain (gm.)	Average+ Food Consumed (gm.)	Food Utilization Efficiency	Average+ Total Urinary Nitrogen mgN/100gm body wt.	Average+ Weight Gain (gm.)	Average Food Consumed (gm.)	Food Utilization Efficiency	Average+ Total Urinary Nitrogen 1st Day	Average+ Total Urinary Nitrogen 2nd Day
I	B_{12} deficient [3]	0.8	2.9	27.6	108	4.2	6.8	61.8	182	82
	B_{12} deficient plus testosterone [3]	1.4	3.1	45.2	103	4.3	6.4	67.2	187	87
II	B_{12} deficient [3]	0.4	0.7	57.1	—	4.4	5.4	83.0	—	—
	B_{12} deficient plus testosterone [3]	0.8	2.0	40.0	—	3.0 [2]	3.5	85.7	—	—
III	B_{12} deficient [2]	2.7	3.8	71.0	118	1.8	2.5	72.0	156	78
	B_{12} deficient plus testosterone [2]	1.2	3.3	36.4	70	3.0	4.5	66.6	184	81

[ ] number of animals in group

\+ all averages are in terms of "per animal per day"

* the length of the time period during which the data was recorded varied as follows:

3 day period for Litter I (from 23rd to 26th day of age)
2 day period for Litter II (from 24th to 26th day of age)
2 day period for Litter III (" " " " " ")

** the length of the time period during which this data was recorded varied as follows:

4 day period for Litter I (from 30th to 34th day of age)
2 day period for Litter II (from 28th to 30th " " ")
4 day period for Litter III (from 30th to 34th " " ")

on the vitamin B_{12}-deficient diet is followed by a brief elevation in the urinary nitrogen. However, the urinary nitrogen determined on the second day subsequent to the administered radioactive plus stable vitamin B_{12} showed a marked decrease.

1. Discussion

The data do not permit conclusions. Suggestive evidence is presented, in accord with the literature (Emerson, 1949; Kline and Kastelic, 1954) that vitamin B_{12} enhances growth. This action of vitamin B_{12} is indicated by the increased slope observed for each growth curve (Figures 1, 2, and 3) subsequent to vitamin B_{12} supplementation. In addition Table 4 shows in most instances a many fold increase in the weight gained during the period at which vitamin B_{12} was given in addition to the basal diet. These observations further indicate that the experimental conditions employed, previous to the vitamin B_{12} supplementation, were sufficient to produce a vitamin B_{12} deficiency in the animals.

Following vitamin B_{12} administration there is a trend suggesting an increased feed utilization and a decreased urinary nitrogen excretion. It should also be observed that vitamin B_{12} increased the food intake.

Figures 1, 2, and 3 clearly show, on comparison of the first thirty day growth performance, that testosterone administration did not increase the growth rate; in fact, the testosterone-treated animals seem not to have increased their weight as well as did the controls. Following the vitamin B_{12} supplementation to both the hormone-treated and control animals, there was an increased performance by both the

testosterone-treated animals and the controls. Since the data does neither allow nor refute the statement that vitamin B_{12} may counteract any retarding effects of testosterone on growth, another experiment was undertaken. The latter experiment follows.

2. Procedure

In order to more finely determine whether an interrelationship existed between vitamin B_{12} and testosterone on body growth, it became necessary to compare the B_{12}-deficient control animals against their B_{12}-deficient testosterone-treated litter mates as well as to compare the B_{12}-supplemented control rats against their B_{12}-supplemented testosterone-treated litter mates. To this effect, when the animals employed in experiment I, part C, reached 30 days of age, each litter was divided, in a random fashion, into the following groups where possible:

1. B_{12}-deficient
2. B_{12}-supplemented

] Control animals

3. B_{12}-deficient plus testosterone
4. B_{12}-supplemented plus testosterone

] Test animals

On the 31st day of age, the appropriate animals received their first vitamin B_{12} injection (0.25 microgram of radio-B_{12}) while their littermates received an equal volume of placebo (distilled water). Following the period necessary in experiment I, for the collection of the radioactive urine, (i.e. on the 34th day) daily doses of 16.6 micrograms of stable vitamin B_{12} were subcutaneously administered to the 2nd and 4th groups of littermates. As noted earlier, the testosterone-treated animals continued to receive 3 mg of the hormone while the control animals received the cottonseed oil placebo. Prior to the vitamin B_{12}

Fig. 4. Growth curves of the young rat under the specified conditions.

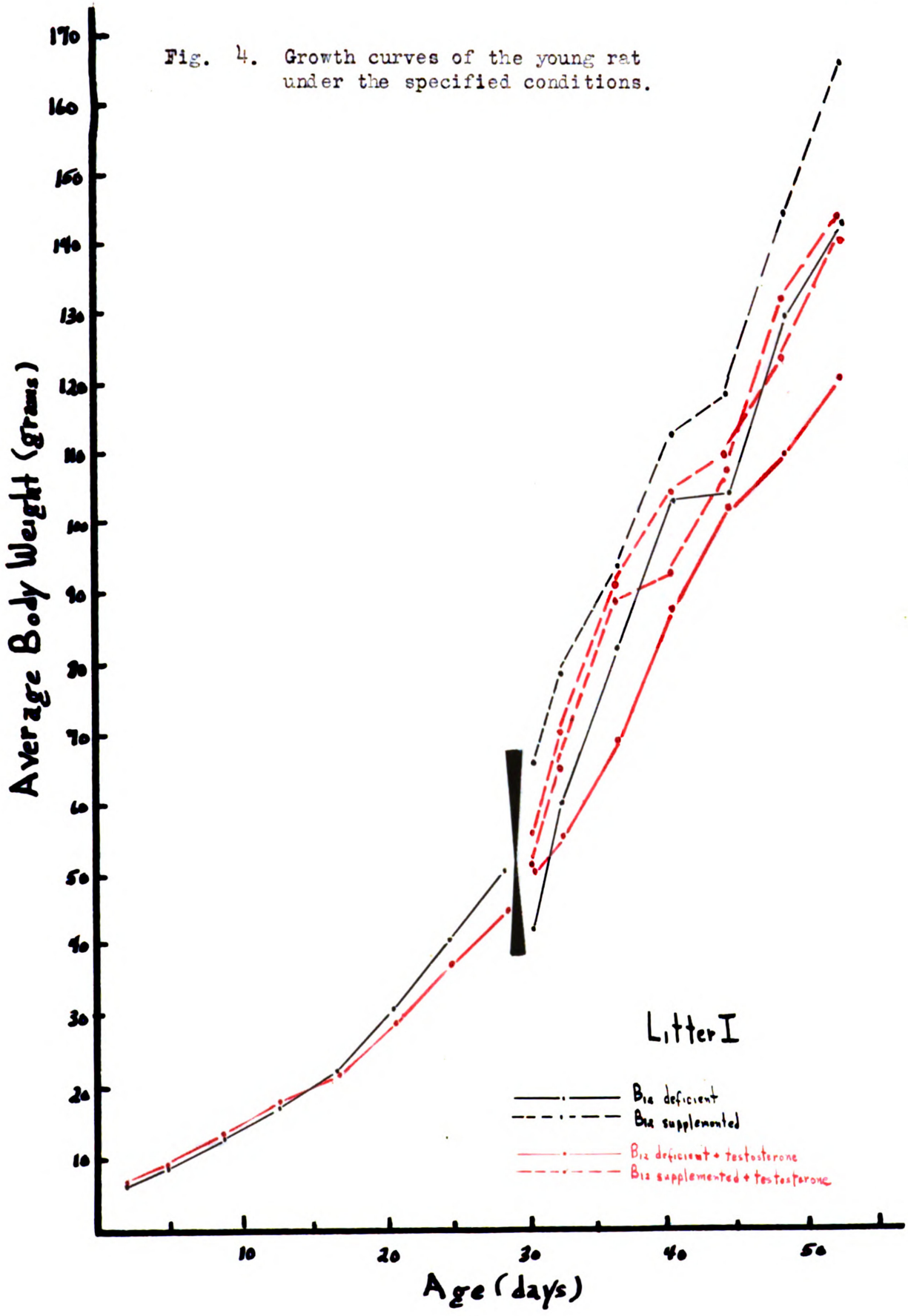


Fig. 5. Growth curves of the young rat under the specified conditions.

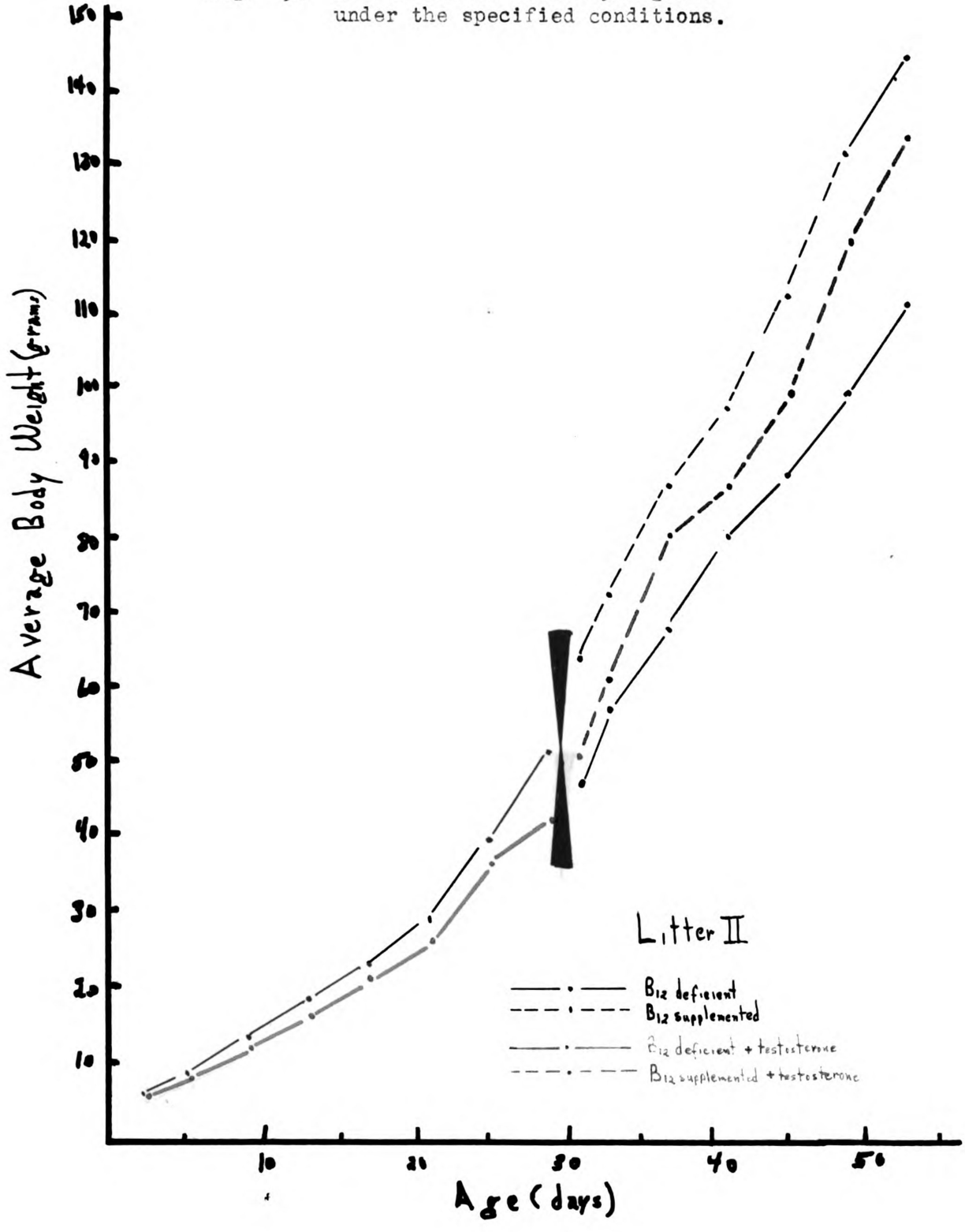


Fig. 6. Growth curves of the young rat under the specified conditions.

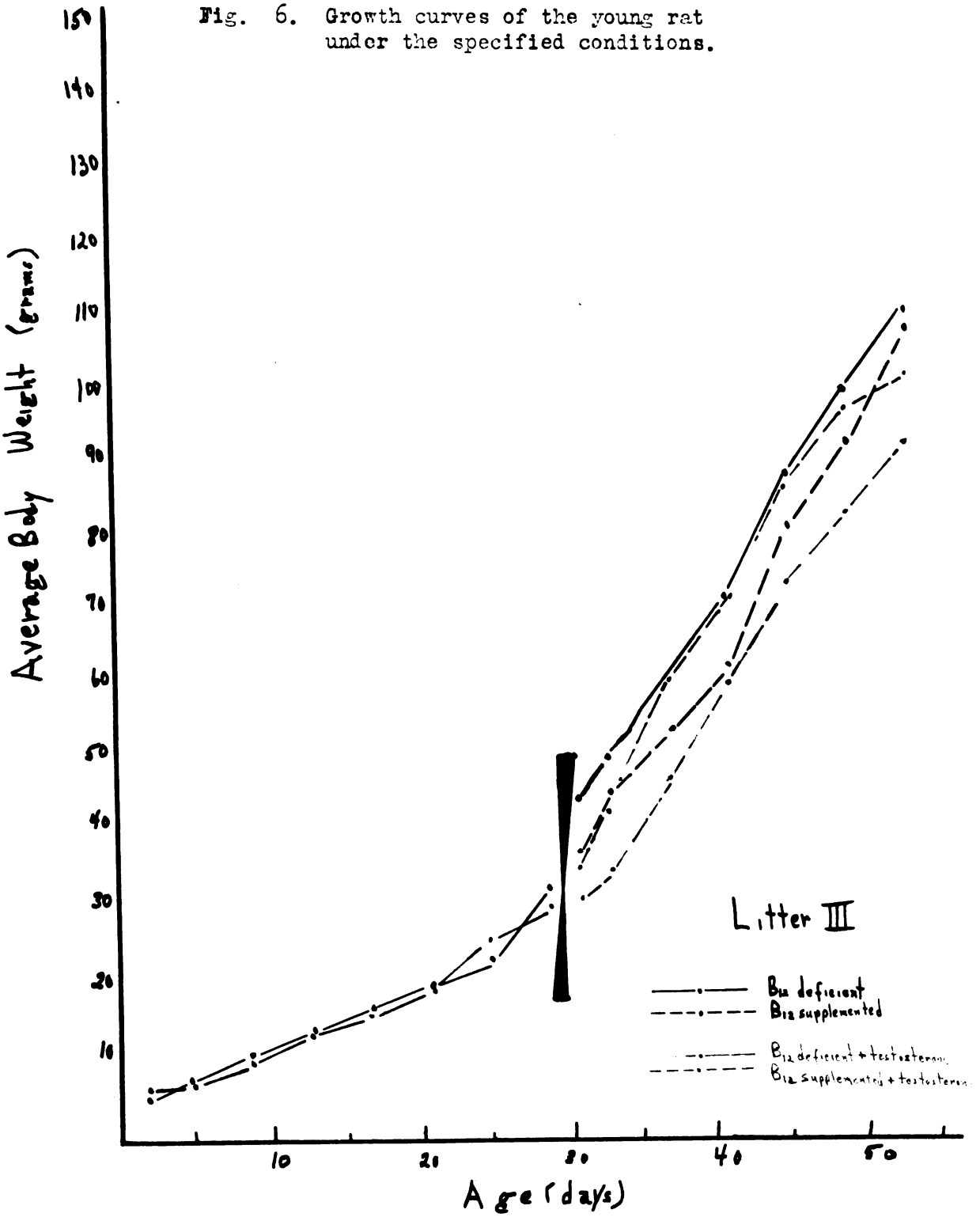
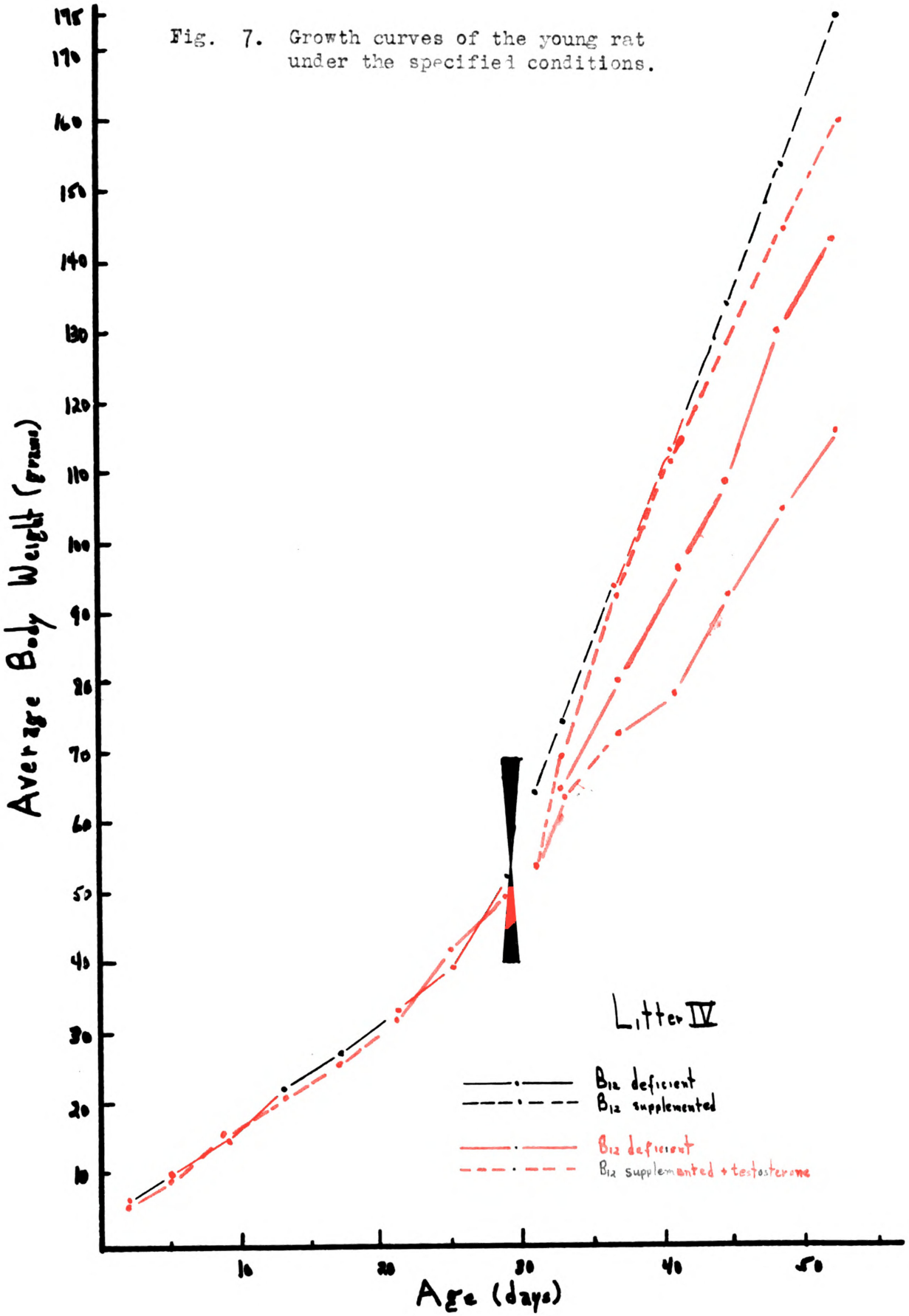


Fig. 7. Growth curves of the young rat under the specified conditions.



administration, the average weight gain during each 4-day period was recorded; at the time of and following the vitamin B_{12} administration, the weight gain during the determined intervals was recorded for each animal. In addition the average weight gains, average food consumption, and average total urinary nitrogen were determined for each of the four litter subdivisions, both prior to and following the radio-vitamin administration.

2. Results

The graphs (Figures 4, 5, and 6) illustrating the growth of the various sub-litter groups indicate in each case that on the 29th day of age the B_{12}-deficient controls had gained more weight than their B_{12}-deficient testosterone-treated litter mates. Unfortunately, as a result of the limitation (by death) of the size of the litters, only litter I (Figure 4) allows us a further comparison at a later date of group 1 with group 3. It may be seen that the growth of the testosterone-treated B_{12}-deficient animal is markedly retarded when compared to its B_{12}-deficient sister.

At the same time it may be seen in litter I that vitamin B_{12} supplementation to the other testosterone-treated sisters caused a growth rate which paralleled very closely the growth of the B_{12} supplemented controls. Again this is clearly indicated in litters IV and II; less suggestive in litter III.

On examination of Table 5, it will be seen that the testosterone-treated animals, observed during the 6-day "Pre-B_{12}^{60} Treatment" period, gained less weight than their control littermates. Likewise, the former consistently consumed less food and tended to utilize the food

Table V.

Effect of Vitamin B_{12} on Efficiency of Food Utilization and Urinary Nitrogen Excretion

Litter	Pre-B_{12}^{Co} Treatment*					Post-B_{12}^{Co} Treatment**					During Flush-out Treatment***			
	Animal	Average† Weight Gain (gm.)	Average† Food Consumed (gm.)	Food Utilization Efficiency (%)	Average† Total Urinary Nitrogen (mg. N/100 gm. body wt.)	Treatment	Average† Weight Gain (gm.)	Average† Food Consumed (gm.)	Food Utilization Efficiency	Average† Total Urinary Nitrogen (mg. N/100 gm. body wt.)	Average† Weight Gain (gm.)	Average† Food Consumed (gm.)	Food Utilization Efficiency	Average† Total Urinary Nitrogen (mg. N/100 gm. body wt.)
I	B_{12} deficient [3]	3.6	6.5	55.4	176	no B_{12} [1]	6.6	9.7	67.9	199	6.4	15.0	43.2	194
	" " plus testosterone [2]	3.6	6.5	55.6	204	plus B_{12} [2]	5.8	13.2	44.3	398	4.2	15.0	28.2	228
	" " " " [2]	1.4	4.2	33.3	166	plus B_{12} [3]	5.9	10.0	58.7	314	5.0	14.5	34.7	200
	" " " " [2]	2.8	5.2	53.8	165	no B_{12} [1] no B_{12} [1]	— —	—	—	284	5.0	10.0	51.5	249
II	B_{12} deficient [3]	2.5	5.8	43.5	139	plus B_{12} [3]	5.2	10.0	51.7	309	3.2	12.5	25.6	188
	" " plus testosterone [2]	2.6	5.3	47.8	168	no B_{12} [1] plus B_{12} [2]	3.0 3.9	6.3 13.6	52.3 26.4	208 253	3.6 4.5	10.5 13.0	34.3 34.6	162 162
	" " " " [2]	1.5	4.4	34.3	214	plus B_{12} [3]	4.3	8.7	49.4	178	4.8	12.0	40.0	173
						plus B_{12} [3]	2.1	6.8	30.9	283	[1]			210
III	B_{12} deficient [3]	1.9	4.2	45.2	143	no B_{12} [1] plus B_{12} [2]	2.7 3.1	6.0 7.8	45.0 39.7	142	3.4 3.0	10.8 8.8	31.5 34.1	97 136
	" " plus testosterone [2]	0.8	4.0	20.0	178	no B_{12} [2]	0.6	3.3	18.2	130				
	" " " " [2]	1.0	2.8	35.7	179	plus B_{12} [2]	1.0	4.3	23.2	152	4.5	8.5	52.9	188
	" " " " [3]	1.5	3.3	45.4	172	plus B_{12} [3]	2.3	5.7	40.0	185	6.0	15.2	39.5	193
IV	B_{12} deficient [2]	3.8	5.6	67.8	142	plus B_{12}	3.9	11.0	35.4	191	5.5	14.0	39.3	169
	" " plus testosterone [2]	1.9	4.4	43.2	135	no B_{12}	3.5	8.3	42.1	126	3.0	7.8	38.4	114
	" " " [2]	2.6	5.3	49.0	139	plus B_{12}	6.2	8.8	70.4	115	7.1	14.3	49.6	135

• B_{12}^{Co} is used instead of the phrase "Co-60 labeled vitamin B_{12}"

* covering the period between the 24th and 30th day of age

** " " " " " 31st " 34th " " "

*** " " " " " 34th " 38th " " "

† all averages are in terms of "per animal per day."

Table VI. Effect of Testosterone on Hair Growth

Treatment			Hair rating*
Control animals	Vitamin B_{12} deficient diet	[3]	normal
	Vitamin B_{12} deficient diet plus subcu. injection of vitamin B_{12}	[8]	normal
Hormone-treated animals	Vitamin B_{12} deficient diet plus testosterone injection	[3]	sparse
	Vitamin B_{12} deficient diet plus testosterone injection plus subcu. injection of vitamin B_{12}	[14]	normal

[ ] designates the number of animals in each group

* only two ratings were employed: normal or sparse

consumed less efficiently than the control animals. There appears to be no significant difference among the total urinary nitrogen values.

It may be observed (Table 5) that upon vitamin B_{12} supplementation during the "Flush-out Treatment" the testosterone-treated animals did not show the consistent trend that they had shown during the period prior to the administration of vitamin B_{12}. That is to say, grouping the animals treated in a similar manner shows, as far as weight gain and food consumption are concerned, a closer approximation of the testosterone-treated animals to the control animals.

Comparison of the food utilization of nearly each group in the "Pre-B_{12} Treatment" category with its own efficiency during the "Flush-out Treatment" does not show an increase in food utilization efficiency.

Although not originally scheduled for observation during the course of the experiment, a noticeable difference in the thickness of the hair distribution was observed among the animals when they reached 45 to 55 days of age. This difference in hair distribution was manifest mainly on the back of the animal. Only two ratings were used: 1.) normal distribution, and 2.) sparse distribution. Utilizing only the two ratings, Table 6 shows that the control animals had normal hair distribution. Among the testosterone-treated animals, the vitamin B_{12}-supplemented group had a normal hair distribution while the vitamin B_{12}-deficient animals showed a sparse hair pattern.

2. Discussion

The data suggest an interrelationship between testosterone, vitamin B_{12}, and growth. Upon testosterone administration, there is a consistent tendency for the animals to gain less weight than their controls during

the first 29 days of age. Comparing the growth of the testosterone-treated B_{12}-deficient rat with the control B_{12}-deficient litter mate (Figure 4), subsequent to the 29th day of age, indicates a further retardation of growth. However, when the testosterone-treated animals were supplemented with vitamin B_{12} (group 4) their growth rate approximated the growth rate of the control B_{12}-supplemented animals (group 2). This might indicate a counteraction by vitamin B_{12} of the suggested growth retardation observed when testosterone is administered by itself to vitamin B_{12}-deficient animals.

The observation of the differential hair growth may impart further information on a subject soon to be discussed. Hair growth exhibited by all the control animals and group 4 of the test animals was judged to be normal. However, a marked difference existed in the B_{12}-deficient testosterone-treated animals (group 3). A sparse hair distribution existed on the back of the latter animals. As noted previously, when testosterone-treated litter mates were supplemented with vitamin B_{12} the hair distribution was equivalent to that of the vitamin B_{12}-supplemented controls.

These suggestive observations on the body growth rate and hair growth recall data reported by Meites *et al.* (1954 and 1955) concerning the interactions of cortisone and vitamin B_{12}. Meites was able to demonstrate, under *ad libitum* feeding conditions, a counteraction by vitamin B_{12} of the cortisone-inhibiting effect on body growth and hair growth. The similar counteraction of testosterone by vitamin B_{12} suggested in this thesis arouses one's curiosity. Might these observations be related?

Further suggestive evidence that vitamin B_{12} may counteract the

testosterone effect may be obtained from Table 5. Here B_{12}-deficient testosterone-treated animals showed lower body weight gains and depressed food intake when compared to controls. Upon vitamin B_{12} supplementation, a closer approximation was seen to exist when similarly treated animals were considered as a group.

CONCLUSIONS

It has been demonstrated that testosterone propionate may be a factor influencing the retention of vitamin B_{12}. Upon subcutaneous injection of cobalt-labeled vitamin B_{12} into both control and testosterone-treated littermates, a consistently higher excretion of the labeled vitamin was observed in the hormone-treated littermates. In an attempt to "flush-out" the labeled vitamin with stable vitamin B_{12}, a greater excretion of the tagged B_{12} was again observed in those animals treated with testosterone. On the basis of these data, a modus operandi is proposed for the observed action of testosterone.

Data suggesting that testosterone, under vitamin B_{12}-deficient conditions, may decrease the rate of body weight gain and alter the hair pattern is presented; it is also suggested that large doses of vitamin B_{12} counteract this effect of testosterone.

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A P P E N D I X

4 Kilogram Stock Soybean Meal Diet

Yellow corn meal (Thoman)	1400 gm.
Ground whole wheat (Thoman)	1000 gm.
Alfalfa leaf meal (Thoman)	240 gm.
Brewer's yeast (Strain G) (A. Busch)	120 gm.
Iodized salt	40 gm.
Soybean meal (low fiber, solvent extracted, containing 50% protein, Archer-Daniels-Midland)	1200 gm.

Total Urinary Nitrogen Determination

Modification of the Koch and McMeekin Method (Hawk et al., 1951)

1. Measure volume of urine.

2. Dilute urine 1:40.

3. Pipette 1.0 ml of the diluted urine into a microdigestion flask and add 1.0 ml of 50% (by volume) sulfuric acid.

4. Add a glass bead and boil vigorously over a micro burner until dense white fumes appear. Remove from the flame and add a drop of 30% hydrogen peroxide. Heat gently until the digest clears up.

5. Rinse the digest quantitatively with distilled water into a graduated cylinder and make up to 170 ml and mix.

6. Pipette 17 ml of this mixture into a test tube, add 3 ml of Nessler's reagent and mix. Place a portion of the colored solution in a cuvette and determine the light transmission in the Fisher electrophotometer, using a blue filter (425 mu. wavelength) and scale setting B.

   The blank tube should contain a mixture made up of 16.9 ml distilled water 0.1 ml acid digestion mixture and 3 ml Nessler's solution.

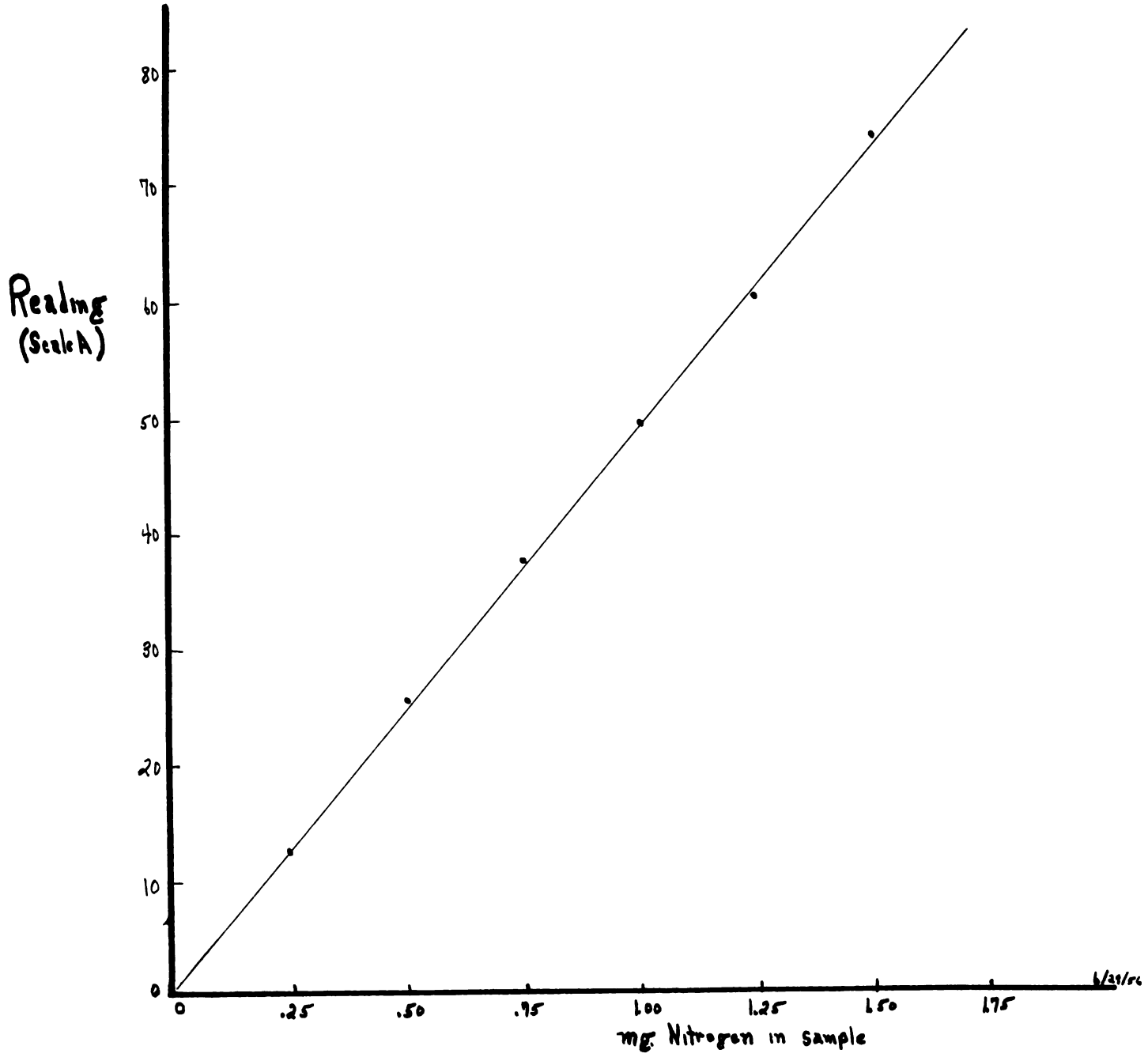
Calculation:

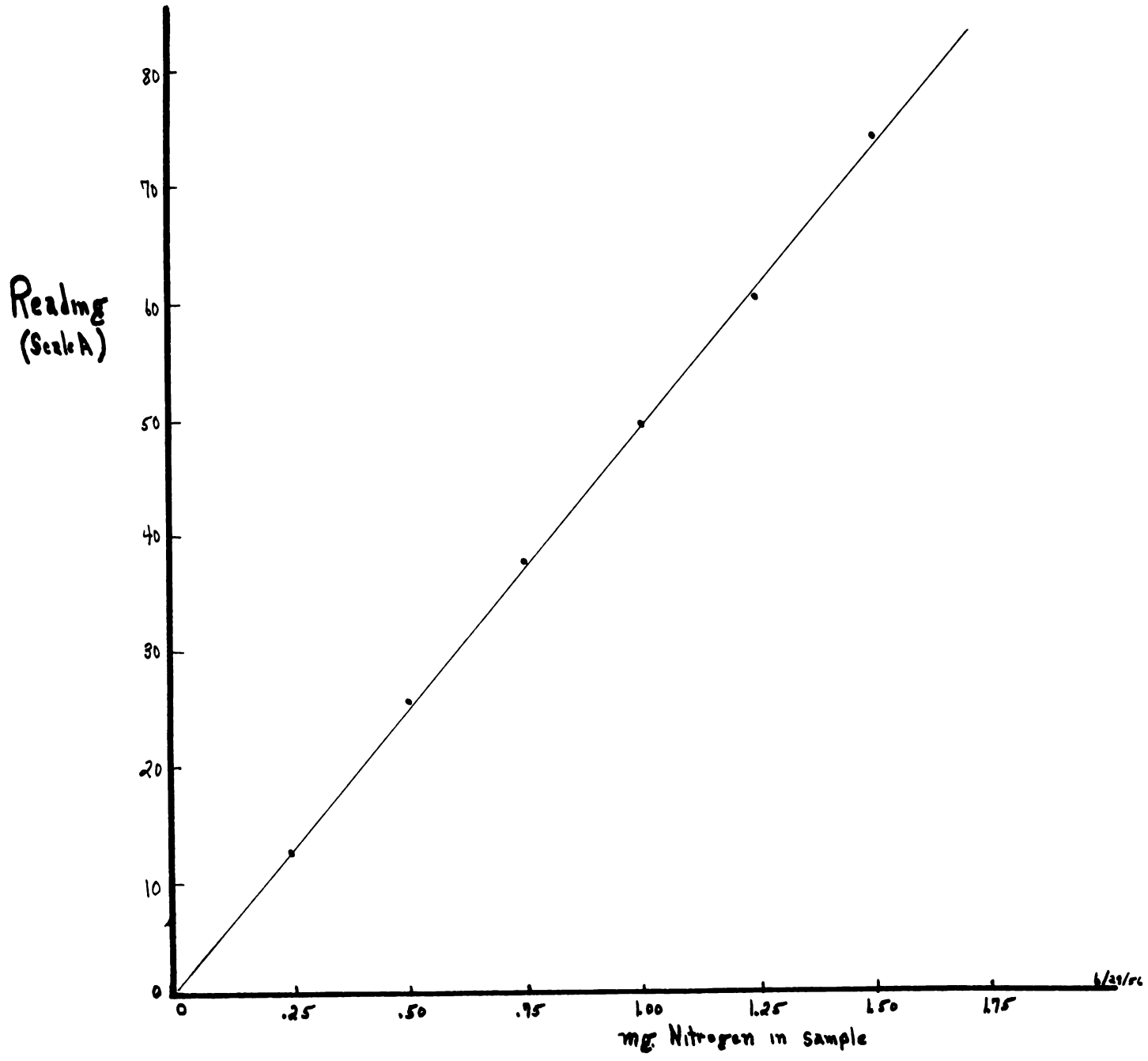
The total urinary nitrogen is equivalent to the mg nitrogen in the sample (read from standard curve) multiplied by the ml of diluted urine.

To get calibration calibration curve:

Ammonium sulfate solution (0.2357gm/liter)	mg. N	Distilled Water	Scale A Reading
0.5 ml	0.025	16.4 ml	12.50
1.0	0.050	15.9	25.25
1.5	0.075	15.4	37.50
2.0	0.100	14.9	49.25
2.5	0.125	14.4	60.00
3.0	0.150	13.9	74.00
Blank --	--	16.9	

To each sample add 0.1 ml 50% sulfuric acid
and 3.0 ml Nessler's solution;
mix, transfer to cuvettes , and read.


Reading
(Scale A)
80
70
60
50
40
30
20
10
0
0
.25
.50
.75
1.00
1.25
1.50
1.75
mg. Nitrogen in sample


Realmg
(Scale A)
80
70
60
50
40
30
20
10
0
0
.25
.50
.75
1.00
1.25
1.50
1.75
mg. Nitrogen in sample

Date Due
