THE EFFECT OF SIMULTANEOUS ADMINISTRATION OF TESTOSTERONE PROPIONATE AND THYROPROTEIN ON GROWTH AND ON THE MECHANISM OF PROTEIN METABOLISM IN GROWING MICE

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INTRODUCTION

The field of growth and organismic development has challenged the curiosity of man for many centuries. Historically, one aspect of growth, then another was developed and took precedence so that at one time the general nutritional factors in growth were the center of interest and effort, then came the era of calories and energy factors, followed by the era of vitamins. At a later date the trace elements and endocrine glands came to the forefront. Today, the relationships of the various glands to one another is receiving considerable attention along with the basic food substrate requirements of micro-organisms and of other organisms of comparatively simple nature. The latter field of study now occupies the center of the stage of the drama of growth both from the standpoint of maintenance and development of the individual and the prevention of abnormal growth. Basic research is going on in the area of biochemistry of growth where the underlying nutritional problems are not those of general protein, carbohydrate, and fat or water metabolism but of particular amino acid, glucose-6-phosphate, glyceric acid, vitamin B requirements, of enzyme activity, of gluconeogenesis, high phosphate bond energy transfer, carbon pool turnovers, etc. In addition, in the field of endocrinology, one sees a determined search for the play of forces of the various endocrine glands upon one another, upon the various tissues, organs, and systems of the body, and upon the various activities mentioned above. Among the endocrine glands that are known to play a role in growth are the anterior pituitary via its growth hormone, the pancreas via its hormone control of glycogen storage and glucose metabolism, the adrenals through growth-stimulating and glycogen-storing action of cortical hormones, and the thyroid and testes via

their hormones. It is evident from even cursory perusal of the literature that the interplay of scores of factors must be analyzed and interrelated if the growth process is to be fully understood.

This paper represents work carried out to determine the effects of various dosages of thyroidal substances and male hormones when administered simultaneously in immature male albino mice. The stimulus for this study came from the observations and experiments of the many workers concerned with the growth problem which indicated that the separate growth effects of the male hormone and thyroid hormone might produce synergistic effects when administered together.

HISTORICAL APPROACH TO THE PROBLEM

Growth Effects of Male Hormone

It is pertinent at this point to discuss the literature related to the problem. At the time of writing, no one has attempted direct studies on the simultaneous administration of testis hormone and of thyroidal substance but the field of endocrinology shows much basic work done by investigators interested solely in the effects on growth of thyroid or testis hormones.

Since ancient times differences in body weight and muscular development have been observed between the human male and female. This same relationship was noticed in many other mammals and even among birds so that without much understanding as to the basic principles involved, castration was practiced and the market value of pigs and chickens was raised considerably by this procedure.

Other historical evidence of the male hormone deprivation effects, although again understood only superficially, were observed in the classical eunuch. Here the body configuration of a young male eunuch was little changed from the boyhood softness and muscle distribution whereas castration of older males resulted in a regression from firm bodies to flabby, fleshy bodies with increased girdle fat, diminished genitalia and disproportionately long arms and legs.

A hazy concept of the anabolic effects of the testis may have been in the mind of Brown-Sequard who in 1889 at the age of 72 injected testis extracts into his own body and reported a general somatic invigoration as well as sexual stimulation. Here was a man of profound vision and courage notwithstanding the subjective nature of his inquiries. However, it wasn't until 1935 that a definite protein anabolic effect of

androgens was established by Kochakian and Murlin in rats. Soon thereafter, Kenyon and his group (1938) showed that the same effect could be produced in man. Viewed from another direction Richter (1933) noted that the removal of the testes produced a decrease of about four-fifths in the daily running activity of rats.

In 1922 Dr. Carl Moore at the University of Chicago noticed a decrease in the growth rate of castrate guinea pigs. Both the body weight and bone lengths were smaller in castrates than in normal males. Moore quotes Steinach as saying that "inasmuch as the male of both rats and guinea pigs is usually heavier than the female, the testis is responsible for an internal secretion that is liberated by the interstitial cells that promotes growth". This is the first evidence of clear-cut thinking concerning the question. Somewhat later Korenchevsky (1925) reported that in most cases, removal or hypofunction of the sexual glands in animals as well as human beings is followed by an increase in weight and an accumulation of fat. He called these the "fat" castrates (eunuchs). In approximately 40% of the cases, castration or hypofunction of the gonads did not produce the above effect. These he called the "thin" castrates (eunuchs). This apparent discrepancy in results of castration was conducive to further study. In 1932, Commins noted a retarding effect on weight accumulation after castration of male albino rats. In 1936, Lawless showed that castrate rats were shorter and weighed less than the controls. These results verified the ideas of Steinach and the work of Moore. Lawless also had a check on the thyroid weights of his animals in both groups and noted no definite differences although earlier workers had indicated contrary results. In the same year, Holt, et al, after a short period of study concluded

that the weights of male albino rats following castration varied and that these variations were of no significance and not predictable. He also found no significant difference in food consumption in castrates as compared to normals.

It was not clearly recognized at the time that a very high dose of a compound might have opposite effects to those of a small dose. Rubinstein, et al. (1939b) showed significant depression of body weight and length of male albino rats by injection of daily 1 mg. doses of testosterone propionate. Deansley and Parkes (1941) found growth inhibition with 20 mg. pellets of testosterone propionate in rats. McEuen, et al. (1937), however, showed no inhibition of somatic growth in the rat when treated with large doses of testosterone although these doses were sufficient to inhibit gonad development in both sexes. The inhibition of gonadal development can be explained through pituitary action; however, the discrepancy with Rubinstein's work is yet to be explained. Turner, et al. (1941), in agreement with McEuen's work, found no effect of testosterone propionate in dosages as high as 2 mg. daily on body weight increase or bone growth in castrated male rats even with prolonged injection from the day of birth. Rubinstein's group (1939a) found a significantly lesser final body weight in the castrate male rat and at the end of the experimental period the castrate group also showed a smaller size, averaging 194 mm. in length as compared to 205 mm. in the control group. It was assumed that the animals were of equal size at the start of the experiment and the rats were not measured; only final measurements were made. As indicated in a later part of this thesis, the procedure for measuring animal lengths especially highly active forms like rats and mice is subject to some variation and the

significance of the above measurements is to be questioned especially where the assumption is made that the animals are of equal lengths at the start of the experiment. After later adjustments of dosage levels, Rubinstein, et al. (1940) were able to stimulate the growth of castrate rats. The testosterone-treated castrate males showed an average difference of 12 mm. in body length above that of the castrate controls.

Kochakian (1941) in a study of rates of absorption of testosterone propionate pellets in mice, noticed that castrate controls accumulated large amounts of abdominal fat while the implanted animals had only traces. The early work of Kochakian and Murlin (1935) with human patients stimulated Kochakian to postulate protein anabolic effects of testosterone propionate as a probability. In a series of experiments, he found that the administration of testosterone propionate to castrated adult male rats produces a marked reduction in urinary nitrogen but in about a week after the beginning of the injections the nitrogen excretion gradually returns to normal in spite of repeated injections. After 30 days treatment with testosterone propionate, the mice average 3.4 gm. body weight more than that of the controls (Kochakian, 1944a).

The observations by early workers of increase in body weight following small doses of testosterone propionate provoked the question as to exactly what accounts for the increase. Is the increase in weight due to the known growth effects on the accessory sex organs, an increase in muscle mass, or an increase of all tissues generally? The question analyzes the mechanism and probes for the site of action. Kenyon, et al. (1940) state that the 102.7 to 252.2 gm. of protein estimated as being retained by their men patients is not accounted for by increases in the bulk of the genital tissue and represents deposits of new material

elsewhere in the body. Kochakian (1944c) showed a great increase in kidney size after steroid hormone treatment by pellet implantation for thirty days in mice and hamsters but not in guinea pigs. Wrete (1945) found no increase in rabbits, but Selye (1939) found kidney size increase in testosterone propionate-treated mice. Papanicolau and Falk in 1938 made the preliminary observation of pronounced increase in size of the temporal muscle of testosterone propionate-treated immature castrate male guinea pigs over that of immature male controls. Other muscles of the body responded in the same way but the temporal muscle alone was used quantitatively as indication of the response. The above facts provided good evidence that the protein anabolic effect of androgens was not entirely on the accessory organs in spite of the fact that great changes in weight do occur there. The evidence of temporal muscle effect was confirmed in 1948 by Kochakian, et al., working with short-haired English albino guinea pigs. Castration decreased the weight of the temporal muscles to less than one-third that of the normal animals. Testosterone propionate administered as a pellet restored the temporal muscles to about half normal size. The castrated guinea pigs did not gain as much weight as the normal controls. The testosterone propionate (12.5 mg./day) treated animals showed a 39% gain in body weight over that of the control animals. The slight restoration of temporal muscle indicated that maintenance rather than restoration would be the better technique in indicating androgenic effect. In a later experiment, these workers castrated another group of guinea pigs and immediately implanted them with pellets of testosterone propionate of the same dosage as was used in previous attempts at restoration. Thirty days later, the temporal muscles had been perfectly maintained.

These workers claim, however, that inasmuch as the steroid hormones used were unable to really simulate the normal myotrophic and androgenic status of the normal guinea pig, they are not "the hormone" of the guinea pig or mouse testes. The results to be described in the present report show complete seminal vesicle-ventral prostate recovery or development with a sufficient amount of the same hormone in mice.

Kochakian and Stettner (1948a), in work with mice, showed that the change in body weight was due in part to changes in weight of the accessory sex organs and kidneys but that the greatest synthesis of protein occurred in the carcass. The total amount of fat, however, decreased as the length of the treatment was increased showing an increased catabolism of fat.

While attempting to find whether or not testosterone propionate would hasten protein repletion after exhaustion of the reserve stores by complete removal of food from adult rats until shortly before death, Kochakian, et al. (1948) obtained supplementary evidence of the protein anabolic effect. Nine rats were castrated approximately 150 days before the beginning of the fasting period. At the end of 12 days, the five animals used were given food plus 2.5 mg. testosterone propionate daily as an injection while the remaining four animals had food alone. There was an initial spurt in body weight increase over that of the controls but by the 31st day the control rats had attained the same weight as the injected animals and continued to gain while the treated animals were no longer or only slightly increasing their body weight. During the initial spurt there was an extra nitrogen retention of 0.689 grams or the equivalent of 20.7 grams of tissue.

Some of the same activities of testosterone propionate have been shown in larger animals. In 1935, Kochakian, and Kochakian and Murlin "reported that 'male hormone' extracts prepared from medical student urine produced a marked reduction in the urinary excretion of 'thin' and 'fat' castrated dogs fed a constant diet" (cited by Kochakian, 1946b). In 1936, Kochakian and Murlin produced the same results as above with synthetic androstenedione. Thorne and Harrop (1937) noted decreased excretion of sodium and water in dogs following administration of testosterone. Thorne and Engel (1938) noted a decrease in urinary nitrogen in normal dogs given testosterone propionate. Kochakian (1935) determined the effect of testosterone on protein metabolism in castrate dogs and observed nitrogen retention in every animal. The nitrogen retention in each case was due to a decrease in urinary nitrogen and not in fecal nitrogen. The dogs also showed a definite and gradual increase in body weight which continued for a short period after injection and then gradually returned to basal. Gaebler and Tarnowski in 1943 found that nitrogen storage was induced in normal bitches by testosterone propionate. Overbeek and Tausk (1946) obtained regular increases in body weight of spayed monkeys with subsequent subcutaneous injection of 5 mg. testosterone propionate daily.

In the human being Sandiford and others (1941) found a significant increase in creatinine excretion following 22 days of testosterone propionate treatment. "They attributed this increase in creatinine excretion to be a reflection of the development of a greater muscular mass" (cited by Kochakian, 1946b). This diversion of protein, under the stimulus of the potent steroid hormones, from catabolic to anabolic processes deprives the organism of a small amount of energy. There is, however,

no decrease in basal metabolic rate. As a matter of fact, evidence points to an increase in the basal metabolic rate (Kenyon, 1938; McCullagh and Jones, 1942; and Bugbee and Simond, 1926). Kochakian (1946b) thinks that the energy to replace that lost to protein anabolism and the increase in energy when it occurred was obtained by the utilization of more fat. Thorne and Engel (1938) obtained an increase in nitrogen retention after treating a man having Addison's disease with 25 mg. testosterone propionate daily for seven days. Abels (1944) treated normal and gastric cancer patients with testosterone propionate and obtained both nitrogen retention and increased extracellular water.

The effects of male hormone in man have been deduced largely from cases in which it was apparently absent such as in eunuchs, accidental or therapeutic castration, or hypo-functional testicular conditions or other abnormalities. Gain in weight is found after treatment of eunuchoids with methyl testosterone (McCullagh and Rossmiller, 1941b). McCullagh and McGurl (1940) state that weight gain during treatment of eunuchoids with testosterone propionate is the rule. Growth is affected inasmuch as large doses of testosterone propionate can cause earlier epiphyseal union than normally. This can cause dwarfing. However, Finkler, et al. (1944) show that testosterone has a tendency to accelerate longitudinal bone growth in children without hastening epiphyseal union. The difference in results here may be due to the difference between children and eunuchoids or it may be due to the dosage differences. Dorff (1941) in working with dwarfed infantile identical twins showed a rapid growth in height and rapid gain in weight of one twin treated for a year with chorionic gonadotropin. The treated boy showed a linear growth spurt of 4 inches as compared to the identical control twin's growth of 1 1/4

inches. The treated twin gained 14 lbs. in one year as compared to a gain of 6 lbs. of the identical twin control. Here nature has set up the basis for a controlled experiment the likes of which are not found often in clinical medicine. Presumably the chorionic gonadotropin was low in follicle-stimulating hormone and high in luteinizing hormone and undoubtedly the amount of luteinizing hormone was enough to produce interstitial cell stimulation in the testes as a spurt in development of secondary sex characteristics was noticed in the treated twin. Browne and Ross added evidence to the above when they showed that the rate of growth of stunted children was more than doubled by treatment with testosterone propionate (1941).

Finkler, et al. (1942) treated 15 children with testosterone propionate and found an advance in growth rate of 13 of them. Webster and Hoskins (1940) administered 75 to 125 mg. testosterone to 8 hypogonadal boys between 9 and 13 years of age and produced an increase in average growth rate from 1.36 cm. to 3.6 cm. in 100 days.

In children suffering from Progeria, a disease with such strange symptoms as extreme immaturity upon which has descended the blight of premature senility including cessation of growth, Talbot, et al. (1945) gave daily intramuscular injections of testosterone propionate and evidenced a marked gain in body weight of 500 gm. per year. Closely akin to this, are the results of Kenyon, et al. (1942) who produced a maximum nitrogen retention in two 76-year-old men, approximating that of normal young men, by administration of 25 mg. testosterone propionate daily. The same group produced a uniform gain in body weight of from 1 to 2.6 kilograms per 10 day period when eunuchoids were treated with the same dosage as above. In accord with the above is the work of Wilkins and

Fleischman (1946a) who treated immature boys with testosterone and observed protein anabolic changes. Shelton, et al. (1947) set up an experiment with four sets of premature twins, where one of each set was treated with testosterone propionate intragluteally. A distinct shortening of time required to regain the birth weight and to gain 2500 grams was noted in the twins receiving treatment. Somewhat the same type of experiment as those indicated above "has been carried out by nature in cases of interstitial cell tumors of the testicle which cause marked somatic as well as sexual stimulation" (Albright, 1942-43). This is well illustrated in a case reported by Rolands, et al. (1929) of a boy at age nine who had the sexual and somatic development of an adult man. In a personal communication to Albright (1942) Browne made some observations on a patient with such an interstitial cell cancer with extensive metastases; the most striking findings were a 17-ketosteroid excretion of over 1000 mg./24 hours, and the failure of the patient right up to the time of death to lose weight or to become debilitated in spite of extensive metastases. The large 17-ketosteroid figure here would compare to large doses of male hormones given in previously described cases where a weight loss was the result - this discrepancy invites further question. Albright and his group carried out similar studies of the effect of testosterone propionate in dwarfs suffering from panhypopituitarism. The dwarfs grow in every aspect; not only do their muscles and epiphyseal cartilages grow, but the entire body grows. Albright believes that testosterone stimulates production of tissues rather than promoting the storage of nitrogen in some other form (e.g. deposit protein). He shows that from a clinical point of view the findings in the adreno-genital syndrome (associated

with a cancer or adenoma of one adrenal cortex or with hyperplasia of both adrenal cortices) are all consistent with the thesis that some hormone is being produced in excess which has an action very similar to testosterone propionate. The 17-ketosteroid excretions seem to be invariably high (60 mg./24 hours in many cases). Such patients show evidence of excessive somatic and sexual development in the male direction. Thus the evidence herein submitted leaves little doubt as to the protein anabolic action of the male hormone. The thyroid hormone likewise in proper dosage is shown to act in a similar manner.

Growth Effects of Thyroid Hormone

The growth effect of the thyroid hormone has been known for many years and extensive reviews in the literature make full discussion here unnecessary (Hoskins, 1916; Cameron and Carmicheal, 1920; Schneider, 1939; and Koger and Turner, 1943). Deprivation of thyroid hormone in immature animals by thyroidectomy or with thiouracil, the newer approach, results in growth stasis. Examination of bones shows delayed osseous development. In rabbits thyroidectomized at an early age, growth is definitely inhibited compared to that of litter mate controls. C.D. Turner shows pictures of such rabbits in his text (1949). The normal controls weighed 1600 grams compared to the thyroidectomized animals which weighed only 800 grams. Scow and Simpson (1945) in thyroidectomized newborn rats showed exceedingly slow but continuous increase in body weight and size of skeleton. Gonads and dependent accessories were markedly subnormal in development. The cretinous human being shows what happens to growth of an individual when the thyroid is not secreting sufficient quantities of its hormone. In addition, the

clinical evidence of thyroid treatment of young cretins is sufficient evidence of the anabolic effects of the thyroid hormone. Topper and Cohen (1928) obtained in a relatively short time an extraordinary increase in growth of four normal children 10 to 12 years old. Further evidence is presented by G. B. Dorff (1935) who stimulated the growth of small but apparently normal children 6 to 8 years of age by daily doses of from 1 to 5 grains of thyroid substance although Johnston (1941) states that more than one grain of Parke-Davis thyroid would be catabolic in its reaction.

Evidence of overgrowth in cases of juvenile thyrotoxic patients is so clear and occurs with such remarkable regularity that Hertz and Galli-Mainini (1941) consider it as one of the cardinal symptomatic manifestations of the disease in this age group. "Its growth effect (thyroid) can be considered as truly synergistic to the normally existing factors for structural increase (substrate)". Smith (1933) reports the same. Koger, et al. (1942) show that thyroxine-treated female albino mice repeatedly gained an average of 28% more weight during a period of five weeks than did the controls. They reported that most investigators attribute a catabolic effect to thyroid treatment as indicated by body weight but that Moussu (1899) and Dott (1923) obtained acceleration of the growth rate in feeding small amounts of thyroid tissue to immature dogs. Schafer (1912) and Hoskins (1916) both reported increased rate of growth and feed intake in female rats fed small amounts of thyroid. Robertson (1928) reported results in mice which were substantiated by Koger, et al. above. The amounts of thyroxine used by Koger were very small - 0.015 to 0.04 mg. daily by subcutaneous injection. More recently Koger, Reineke and Turner (1943)

have reported the influence of thyroactive iodocasein on growth. The thyroactive iodocasein-fed mice gained from 16 to 23% more in weight and an average of 28% more in length than the untreated controls. Reineke, et al., (1948) showed definite growth stimulation of thyroprotein on several strains of pigs.

Evidence somewhat contradictory at first glance is shown in the early experiments of Gudernatsch (1914) where he found that rapid metamorphosis of frog tadpoles occurred when desiccated thyroid is sprinkled into the water. Although he says growth is retarded while differentiation is greatly accelerated it is clear that he was using the term growth in the limited sense referring to somatic growth. Two points occur to the reader here, however, (1) the developmental process unique to the frog is such that the loss of a long tail would hardly warrant growth (linear) effects, and (2), the excessive maturation is again not conducive to size gains. A similar situation is present in rapid cleavage divisions of fertilized mammalian eggs where size remains the same; the tadpole compares favorably with the embryonic stage of mammals. After the process of differentiation has slowed up, one would predict a rapid growth in thyroid-treated animals, however the facts reveal that tadpoles invariably die after this treatment.

Alexis Carrell (1913) was able to increase the growth of brain tissue several fold by the <u>in vitro</u> addition of thyroid substance, thus shedding a little light on the site of action of the thyroid anabolic effect. The exact site and mode of action of the thyroid hormone are still questions being energetically investigated and as will be seen, furnish indirect evidence to its anabolic effects. Leblond (1949) shows distribution of a physiological dose of radiothyroxine in the rat

to be concentrated mainly in liver, gastrointestinal tract, muscles and skin in studies made two hours after injection. Although great emphasis was placed on large concentrations in liver and gastrointestinal tract, little was made of the fact of heavy concentrations in the muscles; confirmatory evidence for anabolic activity. Dr. Albert Ritzmann (1949) in commenting on Leblond's paper presented to the New York Academy of Sciences, relates Leblond's findings with his own clinical work. Interpreted in one way "patients with marked hyperthyroidism, in diffuse toxic goiter, the abnormal or exaggerated findings of excess skin perspiration, irritable heart, diarrhea, altered liver function and aimless exaggerated muscle movements link up with those organs of tissue systems in which the thyroxine content is high. Interpreted in another way, Leblond's findings show the association of high thyroxine concentration in tissues or organs where functional need and cell metabolism are greatest". Addis, et al. (1938) show the protein content of heart, kidney and liver to increase in thyroxine-injected rats. This ties in very well with Ritzmann's results and agrees with his interpretation.

Research workers began inquiring into the relationships of the other endocrine glands to the testis after clear-cut castration effects were noticed in the pituitary. Castration causes vacuolization of basophil cells in the anterior pituitary. Andersen and Kennedy (1933) found atrophy of the thyroid three weeks after castration of a number of rats while consistent atrophy of the thyroid is found in all cases eight weeks after castration. Further evidence from Carriere, et al. and Nathanson, et al. (cited by Jones, et al. 1941) show that hypersecretion of the thyroids occurs with treatment with testosterone propionate.

The histologic changes in the thyroid include increase in cell height, increase in number of mitotic figures, assumption of a round shape by the nucleus, migration of the nucleus toward the base of the cell and finally vacuolization of the colloid.

Effects of Combinations of Male and Thyroid Hormones

At this date, no large scale direct study on the relationship of the two hormones has been undertaken nor has anyone done any quantitative work with testosterone on immature laboratory animals.

The relationship of testosterone propionate with the growth hormone of the pituitary has been studied in male rats by Simpson, et al. (1944) who found what they called a synergistic growth effect. This work was repeated by Kochakian and Stettner in 1948 (a) on male mice and the results of Simpson were corroborated. Thirty days after treatment with a 14 mg. pellet of testosterone, albino male mice gained on the average of 2 1/2 gm. more than the controls. Those animals treated with pituitary growth hormone gained 3 gm. more than the controls. The combination of pituitary growth hormone and testosterone propionate gave a 5 1/2 gm. increase over the controls - an exact summation effect. The authors state that "It is unlikely that these results are due to a further stimulation of the same intermediary metabolic processes for doubling the dose of testosterone does not produce a further increase in the body weight of the castrated mouse".

Five different groups of workers, however, have carried out indirectly some aspects of this experiment in their handling of clinical cases. Albright, et al. (1941) had a patient with panhypopituitarism on thyroid therapy for six years. The patient showed increased weight

and height. However, after testosterone propionate therapy in addition to the thyroid therapy, there was a marked weight and height increase for the next three years.

Eidelsberg and Ornstein (1940) noticed that the effect of administration of 3 to 5 grains daily of thyroid on the basal metabolic rate of human young males was more marked during the periods of administration of testosterone propionate. Also, Kinsel, et al. (1944) found that testosterone propionate in thyrotoxic patients induces a strongly positive nitrogen balance and gain in weight even when the caloric intake is less than the caloric expenditure - an action indicated a year earlier by the same group (Reifenstein, et al. 1943). Thyrotoxicosis is characterized by an increase in the urinary excretion of nitrogen and creatine and by a decrease in body weight. Testosterone propionate in small doses had the opposite effect on these three variables. Armstrong (1944) working with a patient with both diabetes mellitus and Addison's disease gave 0.032 gm. thyroid daily (about 1/2 grain) in addition to testosterone propionate for three successive days and noticed no change in body weight but he reported that the patient exhibited a remarkable increase in vigor, well-being and appetite. The data, however, are insufficient to show any real trend. Wilkins (1945) treated a 4 1/2 year old hypothyroid boy with thyroid, and later with thyroid plus methyl testosterone but showed no gain in weight in this one case.

From the facts disclosed in the foregoing review, it seemed obvious that both testis hormone and thyroid hormone will influence protein metabolism under certain conditions. Rather fragmentary clinical data indicate that there may be some relationship between the two hormones

in their effect on protein anabolism. No controlled experiments have been found bearing specifically on this problem. The experiments to be reported were designed to determine more fully, the effects of the two hormones given alone and in combination on growth and protein metabolism.

EXPERIMENTAL PROCEDURE AND RESULTS

Preliminary Investigation

In order to determine the amount of male hormone necessary to maintain the sex accessories in this particular strain of mice the following preliminary assay was carried out. Young male albino mice of the Rockland Strain and bred in our laboratories were divided into four groups of five mice per group (mice averaged 10 to 16 gms. weight). Fifteen animals were castrated and given subcutaneous injections of testosterone propionate in varying dosages in corn oil daily for 5 days. The results showed that none of the dosages used brought seminal vesicle and ventral prostate weights up to those of normal controls. The average combined weights of these organs are shown in Table I along with the particular dosage used. Evidence in the literature testifies to the fact that testosterone propionate even in tremendous dosage rarely maintains seminal vesicle weights up to normal although in pellet form the sex accessories were maintained up to normal with certain doses. The assay, however, primarily indicated the approximate amount of hormone to be used in the later phases of this study and to develop a range of dosages around this point for young animals. Greene and Burrill (1941) found the sex accessory response to be much less variable in young rats than in older ones.

Following this preliminary assay, the first of four major experiments was started. The first experiment was done to establish the procedure, to learn the techniques inherent in this type of procedure, to test the efficiency of pellets as compared to injections, to determine quantities of food and water used under the regime, and to evaluate

TABLE I

FIVE DAY PRELIMINARY ASSAY OF TESTOSTERONE PROPIONATE ON CASTRATE MALE MICE

Group	Daily Dosage Testosterone Propionate	Seminal Vesicle-Ventral Prostate Wt./25 Gm. Body Wt.
A	50 µg.	63.0 mg. ±3.1*
B	150 µg.	65.3 mg. ±3.9
C	300 µg.	76.4 mg. ±4.1
D	Normal littermate controls	119.2 mg. ±13.0

Five mice were used in each group.
*Standard error.

responses to the dosages of hormone used - in short, to orient the problem.

General Procedure

The albino mouse was used as the experimental animal because numerous investigations have shown it to respond favorably to suitable thyroid dosage. The mice were males of the Rockland Farm strain.

In each of the 4 experiments, the mice were castrated three days after weaning. All animals were fed a Purina diet[1] ad libitum which came as a pellet and was ground into a coarse powder. The food was placed in specially designed food boxes which kept food waste at a minimum. Wherever thyroprotein was part of the treatment, Protamone (an iodinated casein compound supplied by the Cerophyll Laboratories; prepared by the method of Reineke) was mixed thoroughly into the diet in the desired percentage. The synthesized compound resembles the thyroid hormone in its physiological activity and had both biological and chemical assays made on its thyroxine content as a check on its activity, uniformity and reliability[2].

In all cases where the animal was to receive testosterone propionate treatment by pellet technique, the pellet was implanted under the skin of the neck through a small incision. This was done shortly after

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[1]Purina Laboratory chow pellets consist of: Meat meal, dried skimmed milk, wheat germ, fish meal, liver meal, dried beet pulp, corn grits, oat middlings, soybean oil meal, dried alfalfa meal, molasses, riboflavin supplement, brewers dried yeast, thiamin, niacin, Vitamin A and D feeding oils, D activated plant sterol, 1% steamed bone meal, iodized salt and $MnSO_4$.

[2]See Vitamins and Hormones, Vol. IV. (1946) for full description of history and technique of Reineke's synthesis.

the castration operation. The pellet was pushed about an inch from the site of the incision with a very narrow forceps. A single stitch closed the incision and the area was painted with Mercresin. The particular form of male hormone used was testosterone propionate because of its efficiency of absorption in subcutaneous areas (Leathem, 1948) and its effectiveness. The results of Parkes (1936) showed testosterone propionate injections to be more effective than testosterone acetate which in turn was more effective than the free hormone. A year later, Deansely and Parkes found that pure crystalline androgens were extremely effective when implanted subcutaneously as tablets. They obtained the usual response - the enlargement of the prostates and seminal vesicles in castrate male rats. In 1941, Biskind and Meyer verified the above experiment and obtained effective androgenic response from immature castrate rats with pellets of testosterone of about 4 mg. implanted subcutaneously in the back of the animal. These workers showed that the hormone maintained the same degree of activity when compared to oil injections. "This uniform rate of absorption from pellets may permit the concentration of the androgen in the tissues to be maintained at a constant level, whereas with oil injections absorption may be rapid and uneven, producing variations in the concentration of the injected androgen."

Kochakian in 1943 used the pellet techniques extensively in his steroid hormone work on guinea pigs, rats, mice and men. Vests, et al. (1940) used the pellet technique successfully in the monkey and established average absorption rates on the basis of accessory organ weights. More recently Wills, et al. (1949) state that the variance of the assay decreases when you go from 0.2 cc. subcutaneous dose given once daily

to a 0.1 cc. subcutaneous dose given twice daily. This leads one to believe that greater accuracy is obtained from smaller uniform doses which would come from gradual pellet absorption.

The decision to use the pellet technique was made on the basis of the above advantages including the time-saving advantage of eliminating one or two daily injections into sixty animals for a period as long as 35 days. The technique also reduces the amount of handling of animals.

Regular records were taken of body weight, and food and water consumption during the course of each experiment. The animals were kept in cages on steel racks in an air-conditioned laboratory under uniform conditions of lighting and humidity. At the end of each experiment, the mice were sacrificed, body weights taken and in the first two experiments their final lengths determined. The seminal vesicles and ventral prostates were removed carefully from each mouse, trimmed of extraneous tissue, and weighed rapidly on a Roller-Smith torsion balance. These glands were then placed under the peritoneum and saved for later analysis. The animals treated with testosterone propionate pellets had their necks explored and the pellets recovered. These pellets were washed several times in distilled water and thoroughly dried in a desiccator to constant weight. The weights were recorded and compared to the original pellet weights in the particular animal. Each animal was placed in a specimen jar, sealed tightly and stored in a refrigerator in a frozen condition.

Experiment One

In the first experiment, testosterone propionate pellets were made by cutting large tablets into smaller ones by running a hot tungsten wire down through the tablet and then touching the hot wire to the edges

of the small pellet to smooth it into a sphere. These were then weighed and the most uniform pellets selected for use. The pellet weights from this method of manufacture ranged from 10 to 16 mg.

The mice of each group in the experiment were kept together in one large cage and individual mice were identified by the customary ear markings. The castrated animals had their testes removed through a small abdominal opening and the incision was closed with silk sutures. One stitch was taken in the peritoneum and one or two stitches in the muscle and skin depending on the size of the incision. The area was painted with Mercresin (a highly germicidal product of Upjohn Co.), body weights recorded, length measured and the animal was returned to the cage. Two measurements of length were made: one was from the tip of the nose to the end of the rump; the other was made from the tip of the nose to the end of the tail. While the animal was still under the effects of ether, the back of the animal was pressed down gently and the measurements of length made.

Those animals in the testosterone propionate treated group had pellets inserted in the nape of the neck. Two days were allowed for operative recovery. The animals were then placed in their proper groups and put on the desired food regime.

The mice in this experiment were grouped according to the outline indicated in Table II. The average gain in weight per week for each of five consecutive weeks is indicated in Table II. The average cumulative weight gains of the various groups are plotted against <u>time</u> as the abscissa and illustrated in Figures 1 and 2.

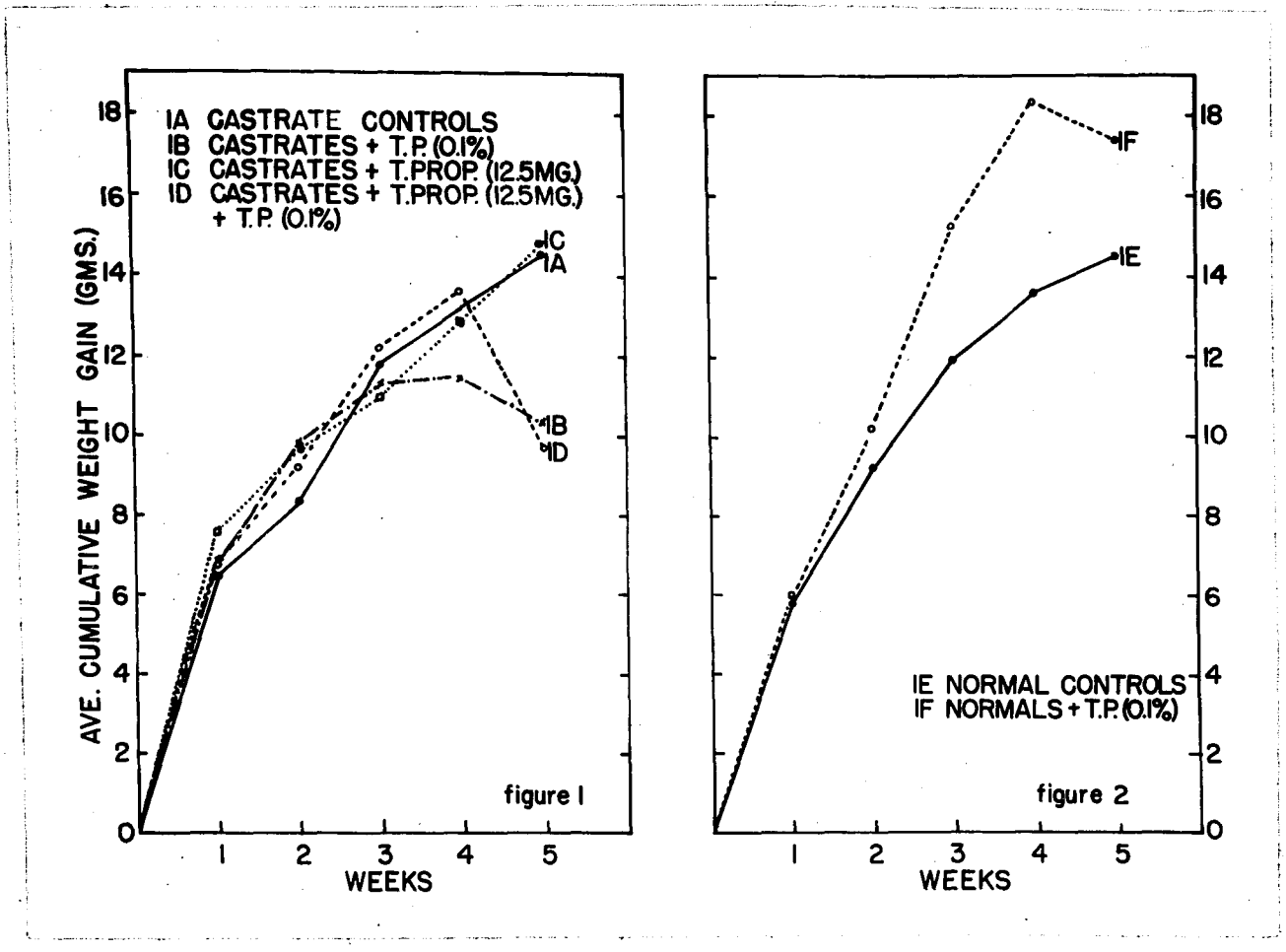
The first noticeable relationship is the effect of thyroprotein. Those groups which had thyroprotein in the feed showed definite drops in the growth curves between the 4th and 5th weeks of the experiment

TABLE II

AVERAGE CUMULATIVE WEIGHT GAINS OF MICE IN EXPERIMENT ONE

Experimental Plan			Gain by First Week in Grams	Gain by Second Week in Grams	Gain by Third Week in Grams	Gain by Fourth Week in Grams	Gain by Fifth Week in Grams
Group	No. Animals	Description					
1A	7	Castrate controls	6.5	8.3	11.8	13.2	14.5 ±1.0*
1B	8	Castrates plus thyroprotein (0.1%)	6.6	9.9	11.3	11.5	10.3 ±1.5
1C	8	Castrates plus testosterone propionate (12.5 mg.)	7.6	9.7	11.0	12.9	14.8 ±0.8
1D	8	Castrates plus thyroprotein (0.1%) plus testosterone propionate (12.5 mg.)	6.7	9.2	12.2	13.6	9.7 ±1.1
1E	6	Normal controls	5.7	9.2	11.9	13.6	14.5 ±2.5
1F	5	Normals plus thyroprotein (0.1%)	5.8	10.2	15.2	18.5	17.5 ±2.6

*Standard error.



Figures 1 and 2. Growth Curves of Mice in Experiment One Over a Five Week Period.

T. P. = thyroprotein.

T. PROP. = testosterone propionate.

compared to the other groups. This indicated that the level of thyroprotein in the food was too high and was causing a marked hyperthyroid condition with the concomitant loss in weight. The indication was to lower the thyroprotein level for the next experiment. In the uncastrate animals receiving thyroprotein however, the early increase in body weight was so large, that at the end of the fifth week, the body weights were still significantly higher ($F = 2.37$, see methods in Table 1 of the Appendix) than those of the normal controls in spite of the drop in the last week. Group 1D (castrates treated with both testosterone propionate and thyroprotein) was off to a slow start but finally rose above the other castrate groups. The rise was above that of group 1C (castrates plus testosterone propionate alone) but the amount was not significantly different. At the end of the fourth week, the order of body weight increase from highest to lowest was: 1D, 1C, 1A and 1B. From the known growth effects of thyroprotein it was clear that a lowered thyroprotein dosage would have reversed the positions of 1A and 1B. This was verified in the second experiment.

A study of the daily consumption of food and water was made for each group and the results from the data are illustrated in Table III following. The results reveal increased food consumption by the thyroprotein groups over and above that of the other groups. The normal castrate controls (1A) averaged 3.8 gms. of food per mouse per day whereas the castrates plus thyroprotein animals (1B) averaged 4.7 grams. The castrates plus testosterone propionate (1C) averaged about 4 gms. daily while the castrates plus testosterone propionate plus thyroprotein (1D) ate about 5 gms. each. It appears that 0.1% thyroprotein in the feed increased daily food consumption by roughly 1 gm. per mouse daily.

There were no significant differences in the food consumption of the respective groups of mice that can be attributed to the treatment with testosterone propionate. Among the uncastrated control animals, the normal controls (1F) again consumed about a gram less food than the normal animals with thyroprotein.

The consumption of water (Table III) follows the same pattern as the consumption of food in the various groups. Castrate controls drink[1] on the average of 7.1 gms. water per mouse daily while the castrates plus thyroprotein drink 9.4 gms. - a difference of 2.3 gms. per mouse per day. This greater consumption of water is found wherever thyroprotein (0.1%) is contained in the diet.

The average mouse consumed a total amount of food and water for the five week period as indicated in Table IV. The food intake per unit weight gain is a criterion of "efficiency"; the term as used in this paper refers to body economics and not to energy as used by Brody (1945) and others. The last two columns of Table IV show this "efficiency" for food and water respectively. Analysis of the data shows groups A and C as the most "efficient" groups of animals, that is, the least amount of food was consumed per unit weight gain. The groups receiving thyroprotein at the level indicated, i.e., groups B, D and F were the least "efficient". The administration of testosterone propionate apparently has no effect on food intake except perhaps a slight

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[1]The normal castrated and uncastrated animals follow Adolph's (1949) rate of water intake to body weight equation: $I = aB^k$
I = rate of water intake in gms./hr.
a = constant 0.01
B = body weight in gms.
k = exponent 0.88

TABLE III

AVERAGE WATER AND FOOD CONSUMPTION/MOUSE/DAY

	1A	1B	1C	1D	1E	1F
Week	Castrate Controls	Castrates + Thyro-protein (0.1%)	Castrates + Test. Prop. (12.5 mg.)	Castrates + Thyro-protein (0.1%) + Test. Prop. (12.5 mg.)	Normal Controls	Normals + Thyro-protein (0.1%)
Water Consumption (in Grams)						
1	5.8	7.1	6.6	7.6	7.6	7.9
2	6.9	10.1	8.6	10.2	9.2	11.0
3	7.2	10.6	7.8	9.7	8.8	12.6
4	7.9	9.9	8.1	10.5	9.2	13.8
5	7.7	9.2	8.4	11.1	9.1	13.1
Ave.	7.1	9.4	7.9	9.6	8.9	11.7
Food Consumption (in Grams)						
1	3.2	3.6	3.2	3.8	3.9	3.4
2	3.7	4.9	4.0	4.8	4.6	5.0
3	3.9	5.0	3.8	5.2	4.9	7.2
4	4.3	5.0	4.2	5.7	4.4	6.3
5	4.1	4.9	4.2	5.0	5.0	6.6
Ave.	3.8	4.7	3.9	4.9	4.6	5.7

TABLE IV

AVERAGE FOOD AND WATER-BODY WEIGHT GAIN RELATIONSHIPS PER MOUSE
(EXPERIMENT ONE)

Group	Description	Five Weeks Total Food in Grams	Five Weeks Total Water in Grams	Average Five Wk. Gain in Body Wt. in Grams	Food Intake Per Unit Wt. Gain in Grams	Water Intake Per Unit Wt. Gain in Grams
1A	Castrate controls	133.9	249.5	14.5	9.2	18.3
1B	Castrates plus thyroprotein (0.1%)	163.6	329.6	10.3	15.9	32.0
1C	Castrates plus testosterone propionate (12.5 mg.)	135.5	277.1	14.8	9.2	18.7
1D	Castrates plus thyroprotein (0.1%) plus testosterone propionate (12.5 mg.)	171.7	343.9	9.0	19.8	38.2
1E	Normal controls	160.0	307.6	14.5	11.0	21.2
1F	Normals plus thyroprotein (0.1%)	200.7	410.9	17.5	11.6	23.5

synergistic effect when administered simultaneously with thyroprotein. Group D consumed more food per unit weight gain than group B. This is not shown by the uncastrated controls (1F) receiving thyroprotein.

The water consumption per unit weight gain shows exactly the same picture as in food consumption. Again 1A and 1C are about equivalent and more "efficient" than the other groups. Group 1D is slightly higher than 1B, again indicating the synergistic action mentioned above. The groups receiving thyroprotein were in every case higher than the other animals. The parallelism of food and water consumption lends credence to the data obtained. The castrate animals treated with testosterone propionate (1C) gained more weight with less food and water than the normal controls (1E).

The data on body lengths included measurements from nose to rump and from nose to end of tail. These growth changes are presented in Table V below. Considering the evidence of McMeekan (1940) and Wallace (1948), and following the formulations of Huxley concerning the existence of growth gradients, one can analyze the growth data from the gradient aspect. Thus the ratio of nose to rump increase over nose to end of tail increase is used with the data. The changes in length shown took place over the period of the experiment (35 days). Maroney and Johnston (1939) consider height data reliable only if taken over long periods of time. It is to be noted that groups B and D with thyroprotein treatment have lower ratios as indicated in the table which shows greater effect of thyroprotein on tail growth than on "body" growth. This can be understood when we recognize that the posterior parts of a growing organism are in a less differentiated state and therefore more plastic. These results are in agreement with McMeekan

TABLE V

FIVE WEEK INCREASE IN BODY LENGTH AND SEX ACCESSORIES
(EXPERIMENT ONE)

Group	Description	(A) Average Increase Nose to Rump	(B) Average Increase Nose to End of Tail	Ratio $\frac{A}{B}$	Ave. Wt. Sem. Ves.-Ventral Prostate	Ave. Wt. Sem. Ves.-Ventral Prostate Per 25 Gm. Body Wt.
1A	Castrate controls	27 mm. ±1*	51 mm. ±2	0.53	14.2 mg. ±4.9	12.8
1B	Castrates plus thyroprotein (0.1%)	25 ±1	51 ±2	0.50	5.0 ±2.4	5.3
1C	Castrates plus testosterone propionate (12.5 mg.)	29 ±1	52 ±2	0.56	196.4 ±16.3	171.1
1D	Castrates plus thyroprotein (0.1%) plus testosterone propionate (12.5 mg.)	26 ±1	47 ±2	0.55	146.3 ±7.3	152.4
1E	Normal controls	24 ±3	45 ±7	0.53	165.0 ±11.4	125.0
1F	Normals plus thyroprotein (0.1%)	32 ±4	56 ±9	0.57	104.5 ±13.4	82.4

*Standard error.

(1940) who found that the sacral bones of the vertebral column in pigs show the greatest growth from birth to 28 weeks of age and that the lumbar, thoracic, cervical and skull bones follow in order.

Inasmuch as a criterion is needed to substantiate the androgenic effect, the sex accessories serve in this capacity here and so the seminal vesicles and ventral prostates were carefully removed at the end of the experimental period and their weights recorded (Korenchevsky, et al., 1932, and Price, 1944). In the event that a particular animal treated with testosterone propionate had only negligible seminal vesicle and prostate weights, the animal was considered as not under the effect of the hormone and stricken from the records. In the few cases where this was found, a check revealed that no pellet was recovered from the animal. The pellets evidently slipped out of the incision and were <u>not</u> in the animal during the experimental period. The averages of the accessory sex organ weights are shown in Table V for each of the groups. Figures on the basis of 25 gm. body weight are also given. Deansley and Parkes (1933) find high correlation of body weight to sex accessories in the 19 to 24 gm. body weight range in mice.

The data indicate typical castration effects in groups A and B where no androgenic hormone was given to the mice. Groups C and D showed typical maintenance of the sex accessories. In castrate mice treated with testosterone propionate and thyroprotein and in the normal animals treated with thyroprotein there is apparently a detrimental effect of the thyroprotein on the sex accessories. This adds weight to the hypothesis stated earlier that the level of thyroprotein was too high in this first experiment and that it was having a definite catabolic action throughout the body. The greater average weights of the

sex accessories in group 1C compared to the normal controls (1E) is either due to the wide range of the accessory weights even in normal animals, the few animals used in this first experiment, or damaging dosage of testosterone propionate.

The results of this first experiment seem to indicate then, that the dosage levels of both testosterone propionate and thyroprotein are above that which could be expected to give anabolic effects. Having roughly established the above, the next phase of the research was set up with more appropriate dosage levels and with larger numbers of animals. The results of the first experiment must be considered tentative and of orientation value.

Experiment Two

The second experiment of this problem followed the first closely in time. Slightly older mice were used here as compared to the first experiment. The average starting weight of the mice was 18.5 gms. The mice were relegated to the various groups so as to make fairly uniform experimental conditions. The experimental plan is shown in Table VI.

The castrations of groups 2A, 2B, 2C, and 2D were done over a period of 48 hours and in the same manner as in experiment one. The male hormone pellets were placed in the nape of the neck on the day following castration. Two days were allowed for operative recovery and the animals were placed, one group to a large cage, with proper food and water. In this experiment the thyroprotein-treated mice had Protamone mixed in their feed on the basis of 0.05% of the feed. The experiment was carried out for a five week period during which time daily records of food and water consumption were made. The weights of

the animals were taken weekly. A measurement of length was made on the first day of the experiment and on the 35th day. At the end of the experiment, the animals were treated as previously indicated in experiment one.

The results of analysis of the comparative gain in weight of the various groups of animals is compiled in Table VI below.

Translation of the data in Table VI into curves appears in Figures 3, 4, 5, and 6. In Figure 3, one notices the growth effect of thyroprotein at the 0.05% level on castrate mice as compared to the castrate controls. Likewise, in Figure 4, a comparison of normal controls with normal animals treated with thyroprotein shows a definite growth stimulus of thyroprotein. Analysis of Figure 5 reveals that the addition of testosterone propionate has little effect on the growth rate at the level of hormone used here (10.2 mg. pellet). Apparently the dosage of testosterone propionate is still too high (the pellets averaged 12.5 mg. in the first experiment) and is having a detrimental effect on the animals. Figure 6 shows that the additional treatment with testosterone propionate of castrate animals receiving thyroprotein has a slight growth-inhibiting effect. A number of workers (Korenchevsky, et al., 1937) have shown that there is a body weight-depressing action of large quantities of testosterone propionate.

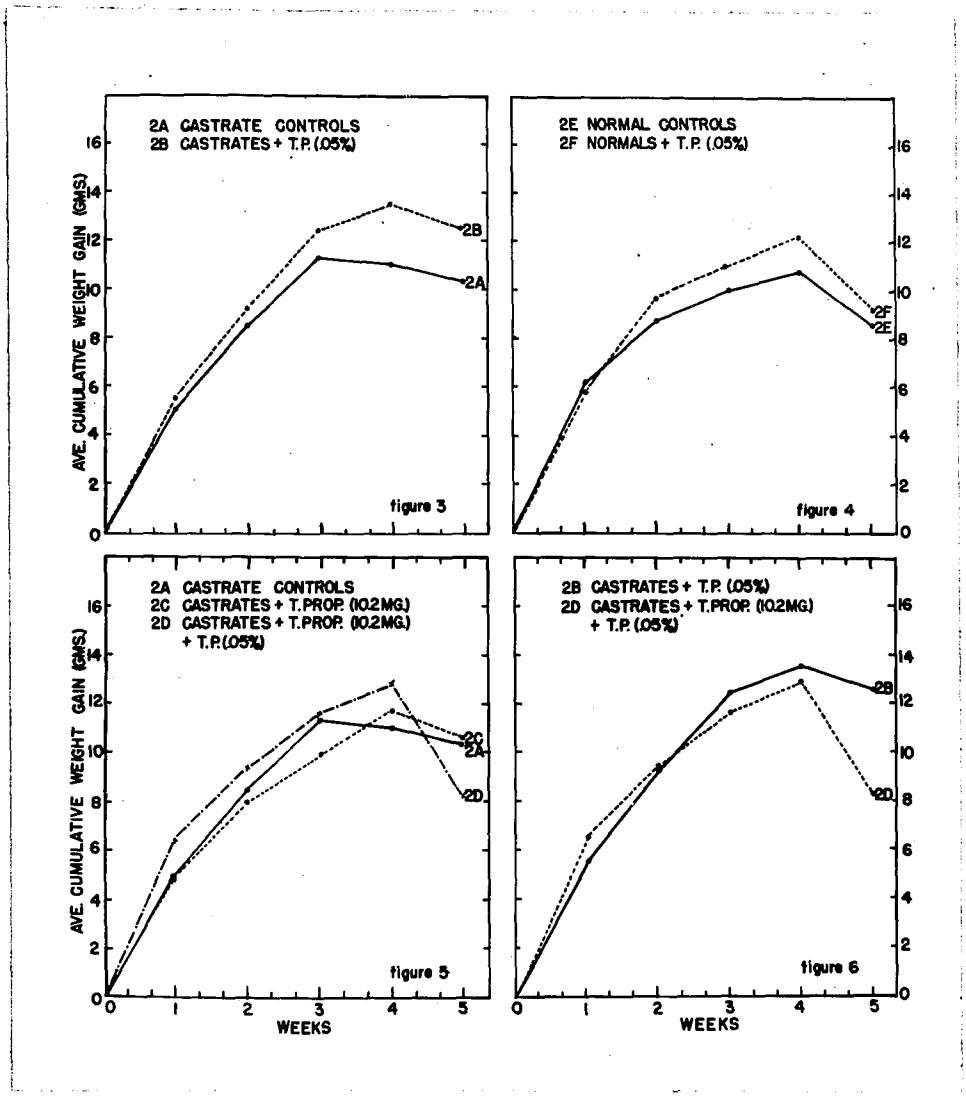
Every one of the curves shows a drop in weight during the fifth week of the experiment - a result which cannot as yet be accounted for in the experimental conditions. For this reason, cumulative weight gains at the end of four weeks should be examined and compared. The prediction after the first experiment that a lowered thyroprotein level would result in better weight gains is verified here. The level of thyroprotein in experiment two was half of that used in experiment one.

TABLE VI

AVERAGE CUMULATIVE WEIGHT GAINS OF MICE IN EXPERIMENT TWO

Experimental Plan			Gain by First Week in Grams	Gain by Second Week in Grams	Gain by Third Week in Grams	Gain by Fourth Week in Grams	Gain by Fifth Week in Grams
Group	No. Animals	Description					
2A	15	Castrate controls	5.0	8.5	11.3	11.0	10.3 ±.7*
2B	14	Castrates plus thyroprotein (0.05%)	5.5	9.2	12.4	13.5	12.5 ±.8
2C	14	Castrates plus testosterone propionate (10.2 mg.)	5.0	8.0	9.9	11.7	10.6 ±1.1
2D	14	Castrates plus thyroprotein (0.05%) plus testosterone propionate (10.2 mg.)	6.5	9.4	11.6	12.8	8.2 ±.8
2E	16	Normal controls	6.2	8.8	10.0	10.8	8.6 ±.5
2F	14	Normals plus thyroprotein (0.05%)	5.9	9.7	11.0	12.2	9.3 ±.7

*Standard error.



Figures 3, 4, 5 and 6. Growth Curves of Mice in Experiment Two Over a Five Week Period.

A comparison of the daily consumption of food and water for the various groups reveals further points of interest. The results are expressed in Table VII.

The effect of thyroprotein in the diet is again clearly indicated in the daily food consumption of groups 2B, 2D, and 2F. The thyroprotein treatment caused an increased food consumption of 1 gm./day more than in those animals not receiving thyroprotein. As in experiment one, the animals treated with testosterone propionate show no significant difference in food consumption from that of the castrate controls. The water consumption follows the pattern of food consumption. Large amounts of water are consumed by the groups which had Protamone added to the feed. See Table VII.

In every case, water and food consumption increased slowly with maturity as would be expected. This same trend was seen in experiment one.

The "efficiency" of the groups of mice in converting food into body weight is again of special interest. The food intake per unit weight gain of the average mouse of each group at the end of the five week period is illustrated in Table VIII. The castrate controls and castrate animals treated with testosterone propionate appear to be the most "efficient" groups for both food and water consumption. These groups require less food and water per gram of body weight gain. As found in experiment one, the castrate mice treated with both testosterone propionate and thyroprotein are the least "efficient", consuming more food per unit gain in weight. This substantiates the hypothesis that the male hormone used has a synergistic action with thyroprotein. The action is apparently on the process of metabolism and the two

TABLE VII

AVERAGE FOOD AND WATER CONSUMPTION/MOUSE/DAY
(EXPERIMENT TWO)

	2A	2B	2C	2D	2E	2F
Week	Castrate Controls	Castrates + Thyro-protein (0.05%)	Castrates + Test. Prop. (10.2 mg.)	Castrates + Thyro-protein (0.05%) + Test. Prop. (10.2 mg.)	Normal Controls	Normals + Thyro-protein (0.05%)
Water Consumption (in Grams)						
1	7.8	11.2	7.5	13.2	7.7	9.6
2	8.1	14.1	8.2	15.6	8.7	11.6
3	9.4	15.9	8.6	12.2	9.9	11.9
4	8.8	14.9	9.3	12.1	10.3	12.0
5	8.7	16.4	10.2	14.5	11.3	14.6
Ave.	8.5	14.5	8.8	13.5	9.6	11.9
Food Consumption (in Grams)						
1	3.8	4.2	3.7	4.5	4.3	4.3
2	4.1	5.4	4.0	5.3	4.5	5.5
3	4.2	6.3	4.3	5.4	4.7	6.0
4	4.2	6.2	4.4	6.1	4.9	6.1
5	4.1	6.2	4.6	5.5	4.7	6.0
Ave.	4.1	5.6	4.2	5.3	4.6	5.6

TABLE VIII

AVERAGE FOOD AND WATER-BODY WEIGHT GAIN RELATIONSHIPS PER MOUSE
(EXPERIMENT TWO)

Group	Description	Five Weeks Total Food in Grams	Five Weeks Total Water in Grams	Average Five Wk. Gain in Body Wt. in Grams	Food Intake Per Unit Wt. Gain in Grams	Water Intake Per Unit Wt. Gain in Grams
2A	Castrate controls	143.4	299.6	10.3	13.9	29.1
2B	Castrates plus thyroprotein (0.05%)	199.0	508.5	12.5	15.9	40.7
2C	Castrates plus testosterone propionate (10.2 mg.)	148.2	306.7	10.6	14.0	28.9
2D	Castrates plus thyroprotein (0.05%) plus testosterone propionate (10.2 mg.)	187.3	473.9	8.2	22.8	57.7
2E	Normal controls	161.9	336.4	8.6	18.8	39.1
2F	Normals plus thyroprotein (0.05%)	195.9	418.2	9.3	21.2	45.0

substances together raise the rate of "utilization" of energy above the threshold of the anabolic effect. An alternative hypothesis might be that the two substances are acting together in reducing the absorption of food and water from the digestive tract. The singular similarity of food and water intake per unit weight gain for castrate controls and for castrate animals treated with testosterone propionate shows the lack of male hormone effect when unassociated with thyroprotein treatment. Also, the close correlation of water "efficiency" with food "efficiency" for each group lends greater reliance to the data. The data also show the normal controls to be not as "efficient" in gaining weight as are the castrate controls, the castrates receiving thyroprotein, or the castrates treated with testosterone propionate.

In this second experiment, an attempt was again made to obtain reliable data on body length increase as an adjunct to the body weight increase in evaluating the growth effects of the hormones. The following figure illustrates the linear relationships among the groups (Figure 7). The millimeter increase in body length shows the thyroprotein-treated castrate mice to have a two-millimeter lead over the castrate controls. Inasmuch as the groups dropped off in body weight in the fifth week of the experiment and inasmuch as the body length would not be as flexible as the body weight, greater significance might lie in the length measurements as a growth criterion. The parallelism between length data of experiment one and experiment two is fairly close. Again more evidence of synergistic action of the two hormones arises from the data. Group 2D shows the same catabolic action of simultaneous administration of the hormones, i.e., at these high dosage levels as is shown in the food consumption pattern.

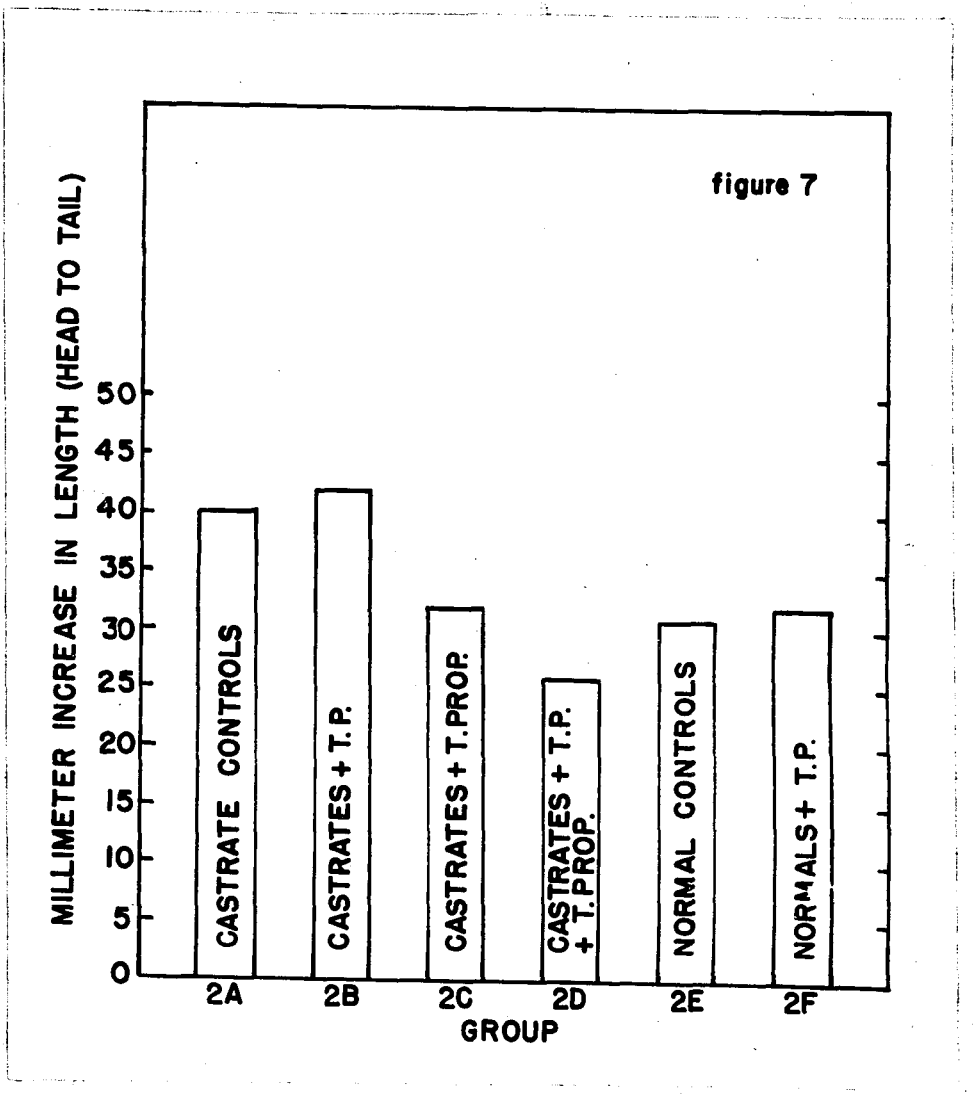


Figure 7. Increase in Length of Mice in Experiment Two Over a Five Week Period. Thyroprotein Dosage at .05% Level of Feed and Testosterone Propionate Dosage as 10.2 mg. Pellet.

The data on seminal vesicle-ventral prostate weight shows, like in experiment one, that the dosage of testosterone propionate was still too high; evidence indicated also by body weight data. These results were used to establish the male hormone dosage in the third experiment where dilution of the pellet with cholesterol reduced the dosage by one-half. The weights of the sex accessories are indicated in Table IX and pictorially in Figure 8. On the basis of equivalent body weight, the average weight of the sex accessories of group 2C is greater than that of group 2E. The addition of thyroprotein to the animals' diet results in an increase in seminal vesicle-ventral prostate weights. Caridroit and Arvy (1942) and Masson (1947) found the same effect of thyroprotein on the sex accessory weights.

Experiment Three

The third experiment was established in somewhat the same manner as the previous experiments but with the following differences: The first and main change was made in the testosterone propionate dosage. Dr. Kochakian while at the Steroid Hormone meetings in Madison, Wis., September, 1948, suggested the method he used successfully in reducing the absorption rate of the male hormone. This method was used previously by Shimkin and White in 1941. Cholesterol is mixed with the testosterone propionate at various percentages and pellets are made from the mixture. In the method used here, cholesterol (from the spinal cord of cattle) was ground up finely in an agate mortar with an equal weight of the male hormone to make a 50% mixture (Kochakian, 1946a). This mixture was placed in an oven and brought up to 143° C. In this melted state, the mixture was taken up in a warmed pipette and released drop by drop into

TABLE IX

AVERAGE WEIGHTS OF SEX ACCESSORIES AT END OF FIVE WEEKS
(EXPERIMENT TWO)

Group	Description	Average Body Weight	Average Seminal Vesicle-Ventral Prostate Weight Per 25 Gm. Body Weight
2A	Castrate Controls	28.9 gm.	Negligible
2B	Castrates plus thyroprotein (0.05%)	31.0 gm.	Negligible
2C	Castrates plus testosterone propionate (10.2 mg.)	28.8 gm.	202 mg. ±9*
2D	Castrates plus thyroprotein (0.05%) plus testosterone propionate (10.2 mg.)	26.7	215 mg. ±17
2E	Normal controls	27.8	142 mg. ±11
2F	Normals plus thyroprotein (0.05%)	28.0	149 mg. ±9

*Standard error.

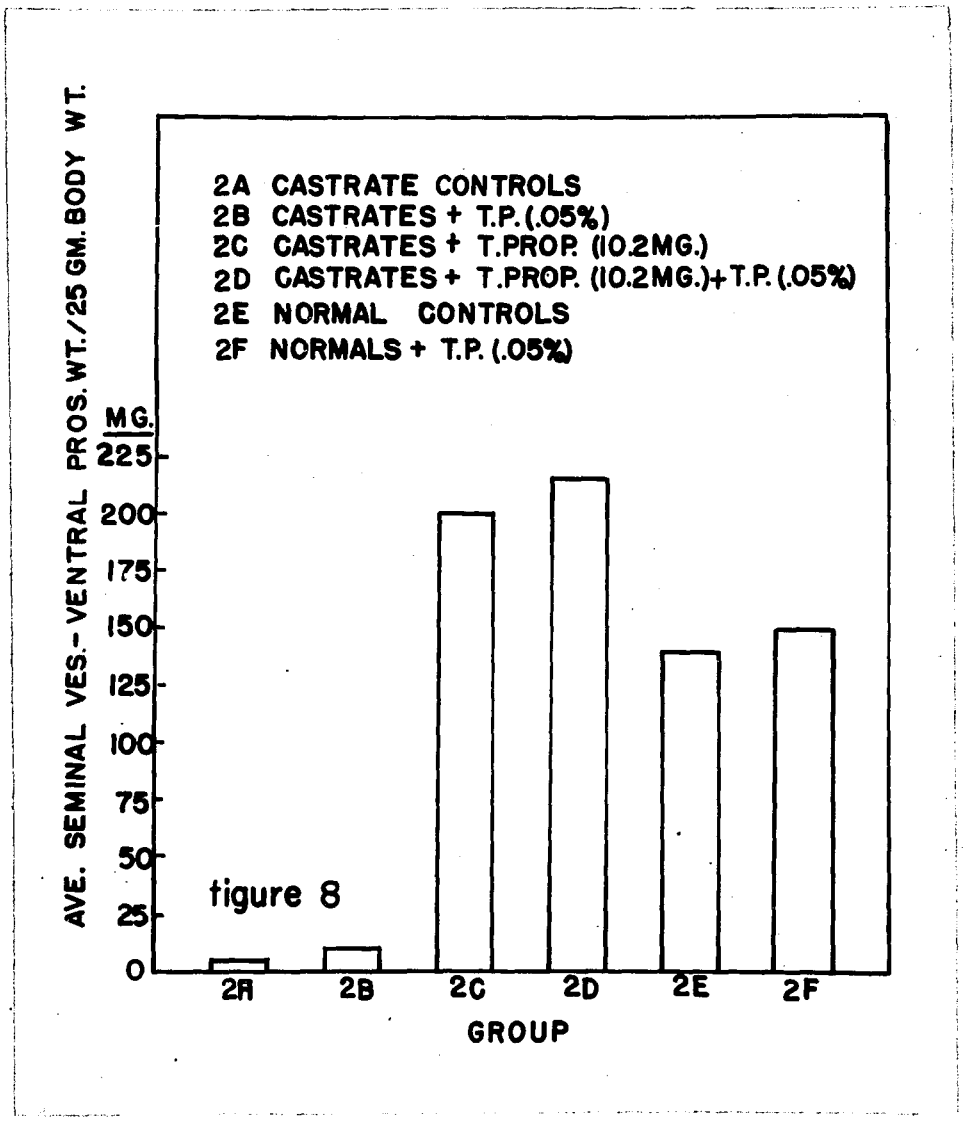


Figure 8. Relationship of Sex Accessory Response to Various Treatments of Testosterone Propionate and Thyroprotein.

distilled ice water. As the drops struck the cold water, they solidified and dropped to the bottom. They were left in the beaker and placed in a refrigerator for 24 hours. They were then removed and dried in a desiccator to constant weight. The pellets were stored in a cool place until used. The average weight of the pellets formed in this manner was 10 mg, thus the pellet contained 5 mg. of testosterone propionate. Pellets were also made with 75% cholesterol and 25% testosterone propionate thus containing 2.5 mg. male hormone in a 10 mg. pellet.

A second change made in the third experiment was the use of individual cages for the mice rather than keeping them in a large cage. For this purpose, a large battery of cages was purchased which housed 140 mice individually. A photograph of the whole battery and a photograph of an individual cage is shown below. The cages were 7 inches long, 5 inches high, and 5 inches wide. The sides and back of the cages were solid metal; front and bottom were made of 1/4 inch galvanized wire mesh. The change from group caging to individualized caging was made primarily to obtain statistically treatable data on food and water consumption. Its use resulted in certain other advantages however:

1. No necessity for marking animals.
2. Animals going off feed can be detected quickly and eliminated from the records.
3. Less aggressive animals have a chance at food and water.
4. Certain animals (food wasters) can be detected and eliminated from the records.

5. Spreading of disease is minimized and diseased animals can be detected more easily.

Dr. Wolterink of the Department of Physiology and Pharmacology points out however, that some workers say individualized cages offer some disadvantages over group caging in that there is supposed to be lessened food and water consumption with individualized caging. Gregariousness is supposed to stimulate better feeding. These observations are not borne out by the results of the experiment reported here. There is actually a small, but statistically insignificant, increase in food consumption.

Another change from the previous two experiments was made in the addition of another group. A group of 15 mice were treated with subcutaneous injections of testosterone propionate in oil three times weekly. This was used as an added control for the pellet-treated group inasmuch as it is clearly established that subcutaneous injections are absorbed at a very uniform rate (Bulbring and Burn, 1935). A fourth change from the earlier experiments was the reduction of the experimental period from five weeks to four weeks.

The mice arrived from Rockland Farms at an average weight of about 16 grams and were grouped according to the scheme indicated in Table XI. Groups 3B to 3F and 3I were castrated on June 8th by the same techniques previously described. Testosterone propionate-cholesterol pellets were inserted into the nape of the neck of the animals shortly after castration. Two days were allowed for operative recovery. On June 11th, the mice were distributed into their individual cages and placed on their requisite food regime. Food and water consumption for each mouse was obtained every other day and averaged for daily values. Body weight

records were made every fourth day rather than weekly. This provided more points to the growth curve and quicker indication of the effects of various treatments. Group I was injected thrice weekly with 0.47 mg. testosterone propionate in corn oil. Kochakian showed in 1940, that subcutaneous injections in mice given three times per week of testosterone propionate in oil showed no different results from daily injections.

After computing body weight gains on June 30th, it was noticed that the animals treated with thyroprotein were beginning to lose weight and so these animals were returned to normal Purina diet on July 1st. Since chronic thyroidal stimulation does not stop immediately with the cessation of treatment, but follows a characteristic decay curve extending over about four weeks (W. T. Salter, 1940), it was expected that the effect of the early stimulation would carry over through the fourth week. The next weighing period showed the weights going up again. Group I were on normal diet for the first two weeks, then were placed on Protamone for one week and returned to normal diet during the fourth week.

At the end of the experimental period, the animals were weighed and sacrificed. The pellets were recovered in the usual manner. Seminal vesicle-ventral prostate glands were removed and weighed immediately.

The average gain in body weight of the animals in the different groups at the end of four weeks is shown in Table X. The same data translated into curves is illustrated in Figures 9 to 15.

Figure 9 shows the cumulative weight gain of the castrate controls (3A) as compared to the thyroprotein-treated castrates (3B). The curve for the latter group is above that for the castrate controls, showing

TABLE X

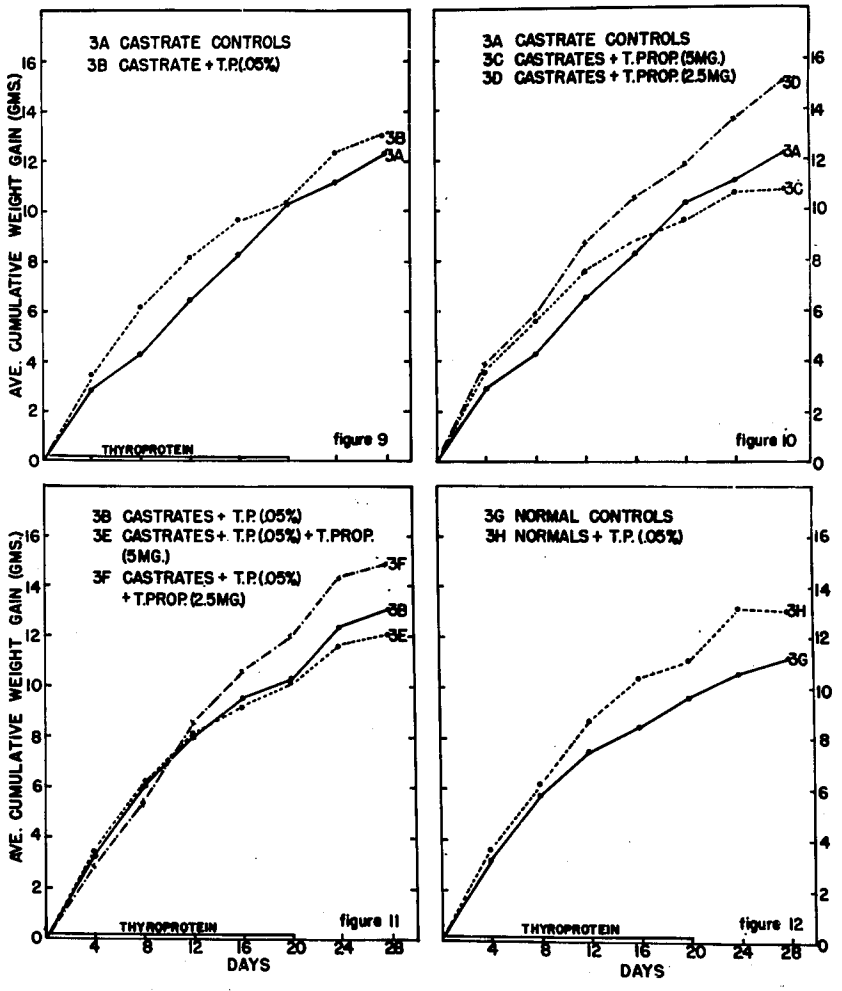
AVERAGE CUMULATIVE WEIGHT GAINS (IN GRAMS) OF MICE IN EXPERIMENT THREE

Experimental Plan			Gain by 4th Day	Gain by 8th Day	Gain by 12th Day	Gain by 16th Day	Gain by 20th Day	Gain by 24th Day	Gain by 28th Day
Group	No.	Description							
3A	13	Castrate controls	2.9	4.3	6.5	8.3	10.3	11.2	12.3 ±.8*
3B	11	Castrates plus thyroprotein (0.05%)	3.4	6.2	8.1	9.6	10.3	12.4	13.1 ±.9
3C	9	Castrates plus testosterone propionate (5 mg.)	3.6	5.6	7.6	8.8	9.6	10.7	10.8 ±.8
3D	11	Castrates plus testosterone propionate (2.5 mg.)	3.9	5.8	8.7	10.5	11.8	13.6	15.1 ±.9
3E	11	Castrates plus thyroprotein (0.05%) plus testosterone propionate (5 mg.)	3.6	6.2	8.2	9.3	10.1	11.7	12.1 ±.6
3F	7	Castrates plus thyroprotein (0.05%) plus testosterone propionate (2.5 mg.)	3.0	5.5	8.6	10.6	12.0	14.4	14.9 ±.8
3G	11	Normal controls	3.2	5.8	7.5	8.5	9.7	10.6	11.2 ±.5
3H	10	Normals plus thyroprotein (0.05%)	3.6	6.2	8.7	10.4	11.1	13.2	13.1 ±.5
3I	12	Castrates plus thyroprotein (0.05%) third week plus testosterone propionate (0.47 mg. in oil 3/week)	3.7	5.9	7.7	9.1	10.4	12.2	13.0 ±.6

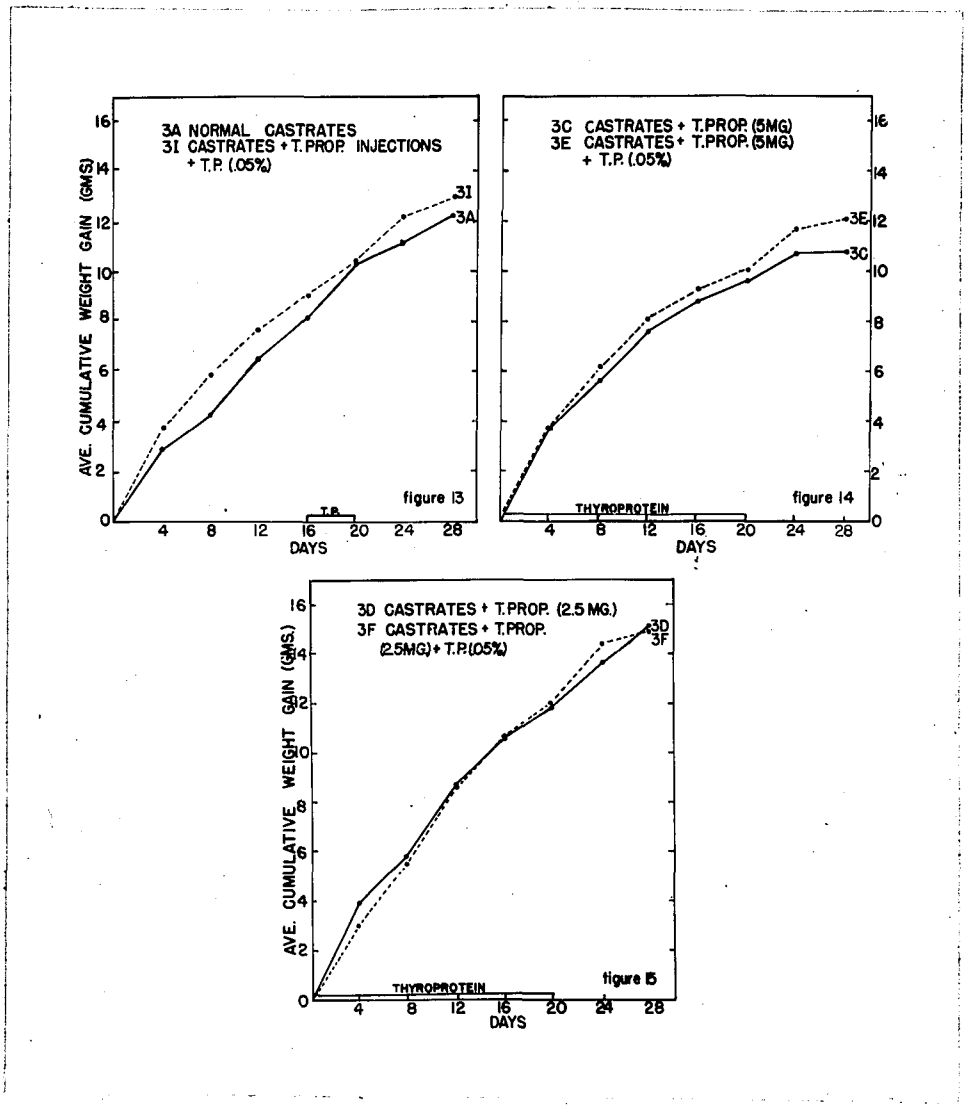
*Standard error.

the anabolic effect of thyroprotein on the animals. On the 20th day, the curve begins to level off indicating the disappearance of the anabolic action. This same condition was found in every group on thyroprotein at this time while it was not found in the other groups (see Figure 11). The natural assumption to be made was that the level of thyroprotein was still too high. The thyroprotein feed was immediately replaced with normal feed and the next ten days showed a spurt in the growth curves of these groups. The remarkable similarity of the change in growth curves at the 20th day is noted in Figure 11. Except for the slight dip in the growth curve of group 3B during the 16th to 20th days, as seen in Figure 9, the curves of both groups, i.e., 3A and 3B would be parallel with about a 1 gram advantage to the group on thyroprotein.

Figure 10 shows the effects of two different dosage levels of testosterone propionate on the growth of mice. For the first two weeks both groups on testosterone propionate are above the castrate control animals. At about 14 days, the group on the 5 mg. pellet does not show as great a gain in body weight as does the castrate control group. Again this indicates that the dosage here is too high and after an initial accumulation begins to have a catabolic effect. Growth of the castrates receiving 2.5 mg. testosterone propionate is rapid with steady climb and at the end of the experimental period each mouse averaged almost three gm. more in weight gain than the castrate control animals. The same pattern is shown in Figure 11, where the groups were treated simultaneously with thyroprotein and male hormone. The addition of the thyroprotein raised the level of the three curves; compare, with castrate controls and castrates plus testosterone propionate alone.



Figures 9, 10, 11 and 12. Growth Curves of Mice in Experiment Three Over a Twenty-Eight Day Period.



Figures 13, 14 and 15. Growth Curves of Mice in Experiment Three Over a Twenty-Eight Day Period.

Figure 14 shows the growth of group 3E (castrates plus testosterone propionate 5 mg. plus thyroprotein) as compared to the growth of group 3C (castrates plus testosterone propionate 5 mg.). Figure 9 was compared previously and shows the same features as Figure 13. Figure 15 indicates that the anabolic action of testosterone propionate may have allowed little room for the action of the thyroprotein addition. Either the physiological limit to the stimulation of the growth is reached or the high dosage of thyroprotein is preventing an additive effect. From the tendency of group 3F's growth curve to move slightly upwards when the thyroprotein was withdrawn on day 20, apparently the latter hypothesis was the correct one.

The analysis of the food consumption data shows that again groups treated with thyroprotein are consuming more food and water than the groups not so treated. Table XI below indicates the relationships. The thyroprotein treatment increases food consumption by about 1 1/4 gm. daily while the water consumption is increased by about 1 1/2 gm. The animals treated with male hormone show no apparent difference in food and water consumption from that of control animals; however, the food consumption per unit weight gain is smaller. See Table XII. In each case those groups treated with thyroprotein show a higher food consumption/weight gain ratio than the comparable group. Apparently the combination of thyroprotein and testosterone propionate at the 2.5 mg. level produces the greatest growth at low level food consumption. The group injected with testosterone propionate also shows good weight gain at relatively low food intake.

TABLE XI

AVERAGE DAILY CONSUMPTION OF FOOD AND WATER/MOUSE
(EXPERIMENT THREE)

Group	Description	Food Intake	Water Intake	Food Intake/Unit Wt. Gain
3A	Castrate controls	5.5 gm. ±.2*	8.5 gm. ±.5	$\frac{151.4}{12.3}$ = 12.3 gm.
3B	Castrates plus thyroprotein (0.05%)	6.7 gm. ±.3	10.5 gm. ±.5	$\frac{175.2}{13.1}$ = 13.3 gm.
3C	Castrates plus testosterone propionate (5 mg.)	5.1 gm. ±.3	8.0 gm. ±.3	$\frac{154.2}{10.8}$ = 13.9 gm.
3D	Castrates plus testosterone propionate (2.5 mg.)	6.0 gm. ±.2	7.5 gm. ±.4	$\frac{156.5}{15.1}$ = 10.4 gm.
3E	Castrates plus testosterone propionate (5 mg.) plus thyroprotein (0.05%)	7.1 gm. ±.2	9.5 gm. ±.4	$\frac{183.9}{12.1}$ = 15.2 gm.
3F	Castrates plus testosterone propionate (2.5 mg.) plus thyroprotein (0.05%)	7.0 gm. ±.2	8.0 gm. ±.4	$\frac{182.1}{14.9}$ = 12.2 gm.
3G	Normal controls	6.1 gm. ±.2	7.5 gm. ±.4	$\frac{138.2}{11.2}$ = 12.3 gm.
3H	Normals plus thyroprotein (0.05%)	6.9 gm. ±.2	10.0 gm. ±.6	$\frac{180.6}{13.1}$ = 13.6 gm.
3I	Castrates plus testosterone propionate (0.47 mg. in oil 3/week) plus thyroprotein (0.05%) third week	5.8 gm. ±.1	7.5 gm. ±.4	$\frac{144.7}{13.0}$ = 11.1 gm.

*Standard error.

Experiment Four

The results of the experiment above give good evidence of the growth effect of thyroprotein given as 0.05% of the feed and of the growth effect of the 2.5 mg. dosage of testosterone propionate. The nature of the experiment suggested the idea that perhaps the dosage levels of both hormones could be lowered to the point where no response in growth would result from the administration of either hormone alone but that a synergistic action if present would show up upon simultaneous administration of thyroprotein and testosterone propionate. The idea was also considered as to the possibility of obtaining a growth effect with a dosage of the male hormone so small as to produce no androgenic effect.[1] Experiment four was established to test the above hypotheses. In this phase, the thyroprotein level was reduced to 0.025% of the feed. Testosterone propionate was administered at two different levels: one at 2.5 mg. in an attempt to duplicate part of experiment three, and the other at the 1.5 mg. level to test the hypothesis above.

The experimental plan for experiment four was as follows:

Group	Description
4A	Castrate controls
4B	Castrates plus thyroprotein (0.025%)
4C	Castrates plus testosterone prop. (2.5 mg.)
4D	Castrates plus testosterone prop. (1.5 mg.)

[1]George W. Thorn of the Department of Medicine at Harvard asked at one of the Conferences on Metabolic Aspects of Steroid Compounds, if clinically useful protein anabolism could be induced by amounts of testosterone propionate insufficient to induce masculinization.

Group	Description
4E	Castrates plus testosterone prop. (2.5 mg.) plus thyroprotein (0.025%)
4F	Castrates plus testosterone prop. (1.5 mg.) plus thyroprotein (0.025%)
4G	Normal controls
4H	Normal controls plus thyroprotein (0.025%)

One hundred and forty male albino mice were received from Rockland Farms at an average weight of 11 gm. One hundred nineteen mice were castrated and the incisions closed by surgical clips rather than with silk sutures as was done in previous experiments. The animals were treated aseptically during the operation and dusted with sulfa powder[1] after the incision was closed. Immediately following the castrations, the animals were placed in the individual cages provided for them. After a lapse of one day, 60 mice had testosterone propionate-cholesterol pellets implanted into the nape of the neck. Thirty of these mice received a single 10 mg. pellet containing 2.5 mg. testosterone propionate and the other 30 received a single pellet containing 1.5 mg. of testosterone propionate. The remainder of the pellet in each case was made up of cholesterol. The operated animals were allowed one day for operative recovery and were then weighed and placed on the desired food and water regime. The battery of individual cages and the laboratory conditions under which the animals were placed was the same as in the previous experiment except that there was question as to whether the air-conditioning system would maintain temperature under conditions of summer weather outside. Daily temperature records of the animal laboratory revealed a variation no greater than 2 degrees on either side of the mean temperature which was 74°.

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[1]Containing urea, sulfanilamide and sulfathiozole.

The animals were weighed every 4th day and a record of food and water consumption was taken every other day for the length of the experiment (4 weeks). At the end of the experimental period, the animals were weighed and sacrificed. The stomach and intestines were removed, washed clean with distilled water and replaced into the abdomen. The animals' seminal vesicles and ventral prostate glands were dissected out, weighed and returned to the animal. Testosterone propionate pellets were recovered and treated as described previously. Data from the earlier experiments indicated that some of the pellets weighed more at the end of the experimental period than at the start. This was again verified here and search of the literature revealed the phenomenon as one not uncommon to pellet usage. Folley (1942 and 1943) reports the formation of "ghost" materials around implanted tablets of synthetic oestrogens. However, from the size of sex accessories, no apparent inhibition resulted here from these scleroprotein "ghosts".

After cleaning the digestive tract, weighing sex accessories and removing pellets, the carcass[1] was weighed and recorded as carcass weight. Each animal was placed in a tared, labelled specimen bottle and placed in the deep freeze (minus 5 degrees C.) for a period of one week. The bottles were then transferred to a drying oven (90° C.) for another week. This period was found satisfactory in a pilot study carried out previously. At the end of this time the animals were at constant weight. The animals were chopped crudely in the dried state

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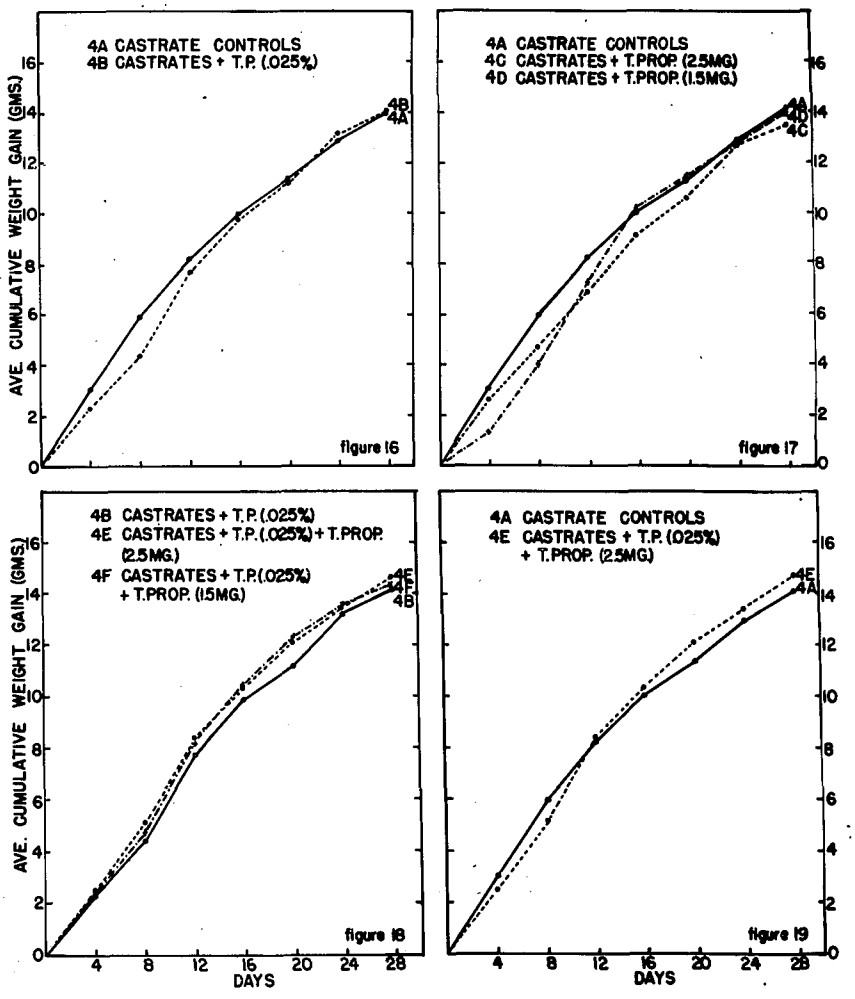
[1]The term carcass as used here includes the total animal body minus contents of the digestive tract in contrast to the term as used by some authors to mean the animal body minus internal organs.

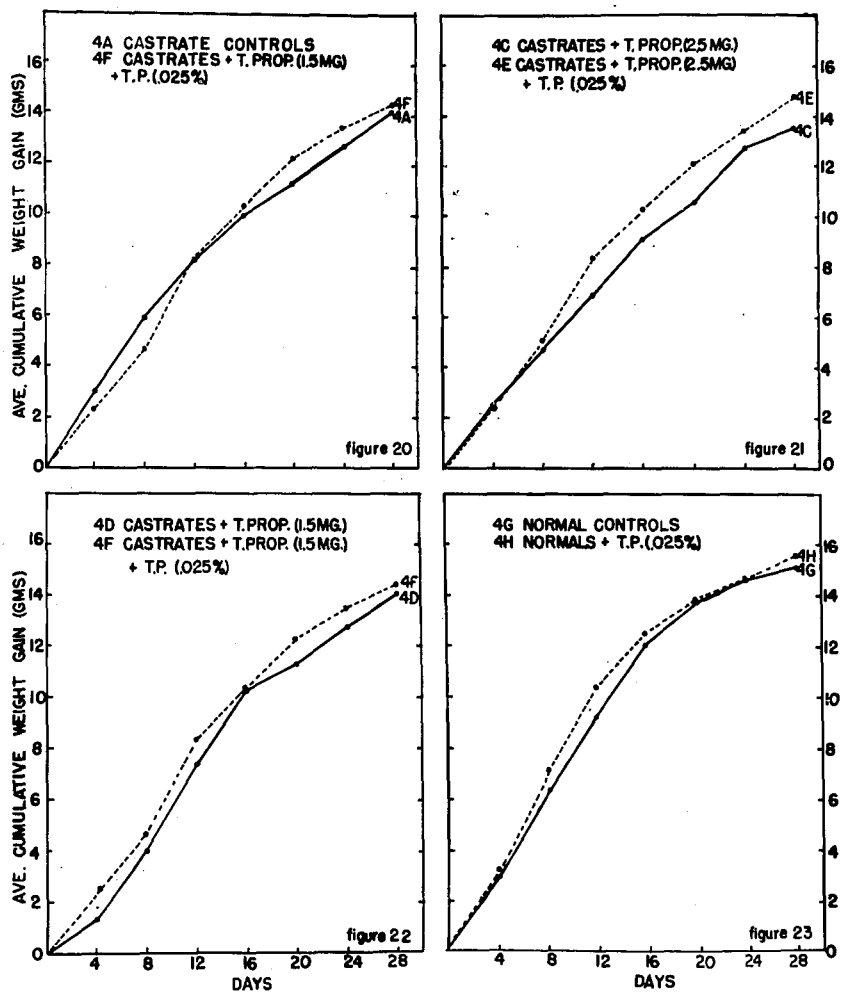
in a Waring blender for one-half minute. They were stored in this form in a dry oven at 60° C. These total mice were analyzed later for fat, protein and ash in Dr. F. L. Wynd's Laboratory of Plant Physiology.

Analysis of the growth data reveals that the earlier hypothesis concerning the synergistic action of the two hormones in question is still tenable although statistical treatment shows the differences as not significant. The direction of the data, however, is so similar among the various comparisons that it cannot be ruled out. The growth curves of the various groups are compared among one another in Figures 16 to 23.

In comparing the growth curves of group 4A and 4B of Figure 16, it is seen that the curves are almost superimposed upon one another indicating that the level of thyroprotein used gave little if any additional growth response to castrated mice. Figure 17 reveals that the levels of testosterone propionate also caused no growth stimulation above that of the castrate controls. The growth curve of the group treated with 2.5 mg. testosterone propionate is contrary to the results of the same dosage used on slightly heavier animals in experiment three where good growth response was obtained. Except for the weight difference (starting weight for experiment three was on average of 16 gms. compared to starting weight of 11 gms. in experiment four) there is no other clue as to the reason for the discrepancy. The curves of Figure 18 are almost identical in shape to those in Figure 17 except that they are higher, indicating that thyroprotein has boosted each of the groups somewhat above its paired group, i.e., 4E above 4C, and 4F above 4D. The mice treated with both hormones simultaneously show an increased growth rate above the castrate controls; a phenomenon not evident when each hormone is used alone. This slight anabolic action although statistically



Figures 16, 17, 18 and 19. Growth Curves of Mice in Experiment Four Over a Twenty-Eight Day Period.



Figures 20, 21, 22 and 23. Growth Curves of Mice in Experiment Four Over a Twenty-Eight Day Period.

insignificant is shown in both Figures 19 and 20 where in each case the treated groups are above the untreated castrate controls. Again the duplicated parallels evince a "feeling" that the results show something in spite of the cold calculating facts of statistics.

Groups 4G and 4H in Figure 23 again show that the level of thyroprotein used had no great growth effect. There is a slight increase in favor of the thyroprotein-treated group.

As in the previous experiments, the sex accessories are used as criteria for effectiveness or the "take" of the male hormone pellet. The sex accessory weights indicated that the pellets containing 2.5 mg. testosterone propionate were having 71.0% of the action of normal testicular secretion assuming that group 4G exhibited normal comparative values for accessory gland weights. See Table XII below. The groups treated with pellets containing 1.5 mg. testosterone propionate showed seminal vesicle-ventral prostate effect of 51.0% of that of full normal testicular response (group 4G). The thyroprotein showed a slight effect on uncastrated animals but no effect on castrated forms.

Individual records of food and water consumption reveal further information. Although the growth effects of thyroprotein treatment was negligible, the animals showed an increase in both food and water consumption although not to the same extent as at the higher dosage levels. The data of experiment three show that thyroprotein treatment at the level of 0.05% of the feed had an effect on appetite so as to increase food consumption by 1.2 grams over that of the controls in the castrate groups while the uncastrated animals on thyroprotein (0.05%) showed 0.8 gm. more food consumed daily than the normal controls. In experiment four there is an increased daily food consumption of 1.0 gm. in the

TABLE XII

AVERAGE WEIGHTS OF SEX ACCESSORIES AT END OF FOUR WEEKS
(EXPERIMENT FOUR)

Group	Description	Average Weight Seminal Vesicle-Ventral Prostate/ 25 Gm. Body Weight	Ratio: Experimental Wts. / Control Wts.
4A	Castrate controls	Negligible	---
4B	Castrates plus thyroprotein (0.025%)	Negligible	---
4C	Castrates plus testosterone propionate (2.5 mg.)	127.5 mg. ±19.2*	0.78
4D	Castrates plus testosterone propionate (1.5 mg.)	85.4 mg. ±8.8	0.52
4E	Castrates plus testosterone propionate (2.5 mg.) plus thyroprotein (0.025%)	108.5 mg. ±18.3	0.66
4F	Castrates plus testosterone propionate (1.5 mg.) plus thyroprotein (0.025%)	84.5 mg. ±8.4	0.51
4G	Normal controls	163.3 mg. ±15.0	1.00
4H	Normals plus thyroprotein (0.025%)	181.8 mg. ±12.5	1.11

*Standard error.

thyroprotein-treated (0.025%) castrates over that of the castrate controls and 0.6 gm. increase in daily food consumption of uncastrated animals fed thyroprotein (0.025%) over that of the normal controls. The consumption of water followed the same pattern.

The evidence shows an apparent lag in growth following thyroprotein treatment. Low doses in the range used in this experiment result in increased food and water consumption with no noticeable anabolic action. Slightly higher doses show anabolic effects with increased food and water consumption. Still higher doses show excessively high food and water intake with catabolic effects on the body. Presumably the thyroprotein is acting on two functions, one on the appetite, and the other on tissue metabolism, and it appears that these two actions are not directly dependent on each other. If the food and water intake were a straight line function of metabolism, then at middle range dosages the food and water should be adequate to the metabolic needs of the body rather than excessive so that the total effect is an anabolic one, and at the higher range dosages the food and water intake on ad libitum diet should be adequate instead of being insufficient so that the total effect is a catabolic one. Apparently then, there is a greater effect on the appetite in the middle dosage range than there is either in the lower or higher dosage range so that more food is consumed than is utilized resulting in a net profit. At the higher range, a large amount of food is taken in but the high metabolic rate causes utilization of all ingested food plus animal body food stores resulting in a net loss.

The actual average daily food and water intake is tabulated in the table below (Table XIII). The high levels of food consumption following high doses of thyroprotein evidently do not contain sufficiently large

TABLE XIII

AVERAGE DAILY FOOD AND WATER CONSUMPTION/MOUSE
(EXPERIMENT FOUR)

Group	Description	Food Intake	Water Intake
4A	Castrate controls	5.4 gm. ±.2*	6.5 gm. ±.3
4B	Castrates plus thyroprotein (0.025%)	6.4 gm. ±.2	8.7 gm. ±.6
4C	Castrates plus testosterone propionate (2.5 mg.)	4.9 gm. ±.2	5.6 gm. ±.3
4D	Castrates plus testosterone propionate (1.5 mg.)	5.0 gm. ±.3	6.2 gm. ±.4
4E	Castrates plus testosterone propionate (2.5 mg.) plus thyroprotein (0.025%)	6.2 gm. ±.3	7.8 gm. ±.4
4F	Castrates plus testosterone propionate (1.5 mg.) plus thyroprotein (0.025%)	6.5 gm. ±.4	8.0 gm. ±.8
4G	Normal controls	6.0 gm. ±.3	7.0 gm. ±.4
4H	Normals plus thyroprotein (0.025%)	6.6 gm. ±.2	9.6 gm. ±.5

*Standard error.

quantities of the vitamins necessary to certain metabolic activities in nutrition. Drill (1943) reports increased need for vitamins A, B complex, C and probably D with hyperthyroidism. Another explanation for the net loss incurred with high dosage of thyroprotein might be that the absorptive process in the digestive tract cannot keep pace with either the food intake or the energy requirements.

In the search for a synergistic anabolic growth effect, it became apparent that any gross anabolic action noted would prove valuable only if the quality of the gain in weight was known. With this end in mind, analysis of some 82 mice ranging from 7 to 14 in a group was made for protein, fat, water content, ash and carbohydrate (by difference). Particular advantage was obtained here inasmuch as the analyzed mice were all approximately the same weight, i.e., at the end of the 28 day growth period studied, no great gains in growth occurred in any one group.

The dehydrated, crudely chopped animals were taken from their storage place and analyzed first for total "fat" (i.e., anything in animal carcass soluble in ether would come out as "fat"). A battery of 12 Goldfish Fat Extractors greatly facilitated the whole procedure. By this method, ether drips continuously through the mouse tissue for a four hour period. A total mouse of known weight in the crudely chopped state was placed in a tall, thin, porous thimble of ceramic. This thimble is placed in the Goldfish Apparatus and is enclosed by a tared Goldfish beaker containing 30 ml. of ether. The beaker is sealed tightly into the system by means of cork gaskets and lock rings. Individual electric heaters evaporate the ether which rises in the apparatus, condenses, drops down into the thimble and seeps through the contents.

All ether-soluble substances are dissolved out and drip back into the Goldfish beaker. The cyclic evaporation and condensation continues in this way for 4 hours. At the end of this time, the thimble and contents were removed and a glass thimble substituted for the ceramic one. Continued evaporation of the ether and condensation traps it in the glass thimble and most of the ether is thus freed from the contents of the beaker. The beakers were removed and warmed gently to remove the last traces of ether and then placed in a drying oven at 100° C. for 30 minutes and transferred to a desiccator for 12 hours. The beakers and contents were weighed to the closest 10 thousandth of a gram. The fat-extracted tissue was put into large vials and stored in a dry oven. The average amount of total "fat" in the various groups is expressed as percentages of fresh and dry carcass and can be seen in Tables XXX and XXXI respectively. The amount of total "fat" in each animal is shown in Tables XIV to XXIX.

Upon completion of the fat extraction, the remaining carcass was in a less gummy form and was readily ground into a fine powder in a powerful Wiley mill. The resulting powdered carcass was mixed thoroughly and returned to individual vials for further analysis.

The protein analysis was made by characteristic determination of total nitrogen present as $-NH_2$, -NH, and -NO (Triebold, 1946; Bradstreet, 1940). The analysis also includes of course ammonium compounds, amino acids, urea and the more complex breakdown products of protein metabolism. A modified macro-Kjeldahl method was used as follows. The first phase of wet oxidation was carried out on a 0.15 gm. sample from each powdered carcass weighed and wrapped in Wattman #1, size 9 filter paper. This sample was placed in a large Kjeldahl flask to which 25 ml.

TABLE XIV

BODY COMPOSITION OF CASTRATE MICE EXPRESSED AS PERCENT OF DRY WEIGHT (GROUP 4A)

No.	Proteins	Fats	Carbohydrates	Ash
1	69.23	16.28	0.69	13.80
2	68.81	12.51	4.24	14.44
3	58.14	20.26	8.54	13.06
4	70.81	12.92	2.85	13.42
7	59.69	26.45	2.68	11.18
9	67.88	14.69	3.44	13.99
10	62.75	21.78	4.32	11.15
11	62.63	22.03	3.20	12.14
12	72.44	10.09	3.28	14.19
13	64.44	19.23	4.17	12.16
14	58.50	25.99	4.12	11.39
Ave.	65.03	18.38	3.78	12.81

TABLE XV

BODY COMPOSITION OF THYROPROTEIN-TREATED (.025%) CASTRATE MICE EXPRESSED AS PERCENT OF DRY WEIGHT (GROUP 4B)

No.	Proteins	Fats	Carbohydrates	Ash
1	63.63	22.40	1.92	12.05
2	71.50	11.58	3.11	13.81
4	72.50	11.26	2.28	13.96
5	62.63	16.01	8.43	12.93
7	64.81	20.54	2.31	12.34
10	71.56	13.39	2.45	12.60
11	67.63	14.28	4.04	14.05
12	68.25	12.54	4.84	14.37
13	63.81	19.98	4.19	12.02
14	70.63	13.00	1.42	14.95
Ave.	67.70	15.50	3.49	13.31

TABLE XVI

BODY COMPOSITION OF TESTOSTERONE PROPIONATE-TREATED (2.5 MG.) CASTRATE MICE EXPRESSED AS PERCENT OF DRY WEIGHT (GROUP 4C)

No.	Proteins	Fats	Carbohydrates	Ash
2	66.38	19.61	1.44	12.57
3	72.13	15.09	----	13.04
6	69.44	14.34	2.48	13.74
8	57.50	26.66	3.71	12.13
12	60.13	29.74	----	10.99
14	64.63	19.17	3.94	12.26
15	66.13	20.56	0.58	12.73
Ave.	65.19	20.74	1.58	12.49

TABLE XVII

BODY COMPOSITION OF TESTOSTERONE PROPIONATE-TREATED (1.5 MG.) CASTRATE MICE EXPRESSED AS PERCENT OF DRY WEIGHT (GROUP 4D)

No.	Proteins	Fats	Carbohydrates	Ash
1	71.13	15.93	1.29	11.65
4	54.88	29.88	4.03	11.21
5	67.69	15.79	4.05	12.47
6	66.44	19.62	2.87	11.07
7	69.56	12.09	6.43	11.92
8	73.38	6.52	8.04	12.06
9	70.56	10.91	6.44	12.09
13	71.44	11.79	3.92	12.85
14	65.25	17.09	4.97	12.69
Ave.	67.81	15.51	4.68	12.00

TABLE XVIII

BODY COMPOSITION OF CASTRATE MICE TREATED WITH TESTOSTERONE PROPIONATE (2.5 MG.) AND THYROPROTEIN (.025%) EXPRESSED AS PERCENT OF DRY WEIGHT (GROUP 4E)

No.	Proteins	Fats	Carbohydrates	Ash
1	76.94	8.69	----	15.42
2	65.69	17.13	3.43	13.75
3	59.94	28.68	1.99	9.39
4	73.50	11.27	2.32	12.91
5	69.56	10.01	4.21	16.22
6	48.94	35.73	6.16	9.17
7	68.63	15.33	3.50	12.54
8	69.06	12.26	3.90	14.78
10	70.06	13.25	3.22	13.47
13	60.00	26.26	4.45	9.29
14	68.69	16.94	3.39	10.98
15	71.56	12.12	3.08	13.24
Ave.	66.88	17.31	3.26	12.59

TABLE XIX

BODY COMPOSITION OF CASTRATE MICE TREATED WITH TESTOSTERONE PROPIONATE (1.5 MG.) AND THYROPROTEIN (.025%) EXPRESSED AS PERCENT OF DRY WEIGHT (GROUP 4G)

No.	Proteins	Fats	Carbohydrates	Ash
1	71.19	8.20	5.71	14.90
2	73.56	12.37	1.84	12.23
3	73.44	6.37	3.98	16.21
8	71.25	11.01	4.04	13.70
9	74.44	11.75	1.72	12.09
10	72.50	8.39	5.93	13.18
12	58.13	30.50	1.13	10.24
13	59.63	24.97	4.45	10.95
14	63.75	19.37	4.50	12.38
Ave.	68.65	14.77	3.70	12.88

TABLE XX

BODY COMPOSITION OF NORMAL MICE EXPRESSED AS PERCENT OF DRY WEIGHT (GROUP 4G)

No.	Proteins	Fats	Carbohydrates	Ash
1	69.50	12.24	3.50	14.76
2	67.69	13.92	6.78	11.61
3	65.49	21.94	----	13.08
4	72.50	8.93	4.75	13.82
5	62.63	24.08	3.36	9.93
6	54.81	29.80	5.16	10.23
8	72.88	17.67	----	16.27
9	62.31	24.35	2.96	10.38
10	73.13	8.03	6.05	12.79
11	59.88	22.24	6.28	11.50
13	61.69	21.44	6.49	10.38
14	59.00	28.34	1.72	10.94
15	69.81	13.52	4.57	12.10
16	60.63	25.38	2.50	11.49
Ave.	65.14	19.42	3.35	12.09

TABLE XXI

BODY COMPOSITION OF THYROPROTEIN-TREATED (.025%) MICE EXPRESSED AS PERCENT OF DRY WEIGHT (GROUP 4H)

No.	Proteins	Fats	Carbohydrates	Ash
1	61.13	18.99	8.43	11.45
2	67.88	13.76	5.00	13.36
3	63.13	18.05	6.41	12.41
5	65.63	16.22	4.88	13.27
6	69.25	13.95	4.14	12.66
7	68.63	9.41	7.59	14.37
9	72.38	5.73	8.31	13.58
10	73.44	6.02	5.79	14.75
15	62.88	21.81	3.20	12.11
16	69.31	13.44	3.56	13.69
Ave.	67.37	13.74	5.72	13.17

TABLE XXII

BODY COMPOSITION OF CASTRATE MICE EXPRESSED AS PERCENT OF WET WEIGHT (GROUP 4A)

No.	Moisture	Proteins	Fats	Carbohydrates	Ash
1	74.67	17.54	4.12	0.17	3.50
2	73.33	18.35	3.34	1.13	3.85
3	69.51	17.73	6.16	2.62	3.98
4	72.60	19.40	3.54	0.78	3.68
7	67.19	19.58	8.69	0.87	3.67
9	74.78	17.12	3.70	0.87	3.53
10	73.31	16.75	5.81	1.15	2.98
11	71.10	18.10	6.37	0.92	3.51
12	73.33	19.32	2.69	0.88	3.78
13	72.12	17.97	5.36	1.16	3.39
14	68.62	18.36	8.16	1.27	3.59
Ave.	71.87	18.20	5.26	1.09	3.58

TABLE XXIII

BODY COMPOSITION OF THYROPROTEIN-TREATED (.025%) CASTRATE MICE EXPRESSED AS PERCENT OF WET WEIGHT (GROUP 4B)

No.	Moisture	Proteins	Fats	Carbohydrates	Ash
1	70.81	18.57	6.54	0.56	3.52
2	74.60	18.16	2.94	0.79	3.51
4	71.11	20.95	3.25	0.66	4.03
5	73.22	16.77	4.29	2.26	3.46
7	69.95	19.48	6.17	0.64	3.76
10	71.37	20.49	3.83	0.70	3.61
11	70.27	20.11	4.25	1.19	4.18
12	73.17	18.31	3.36	1.30	3.86
13	70.55	18.79	5.88	1.24	3.54
14	72.31	19.56	3.60	0.39	4.14
Ave.	71.74	19.12	4.41	0.97	3.76

TABLE XXIV

BODY COMPOSITION OF TESTOSTERONE PROPIONATE-TREATED (2.5 MG.) CASTRATE MICE EXPRESSED AS PERCENT OF WET WEIGHT (GROUP 4C)

No.	Moisture	Proteins	Fats	Carbohydrates	Ash
2	68.46	20.93	6.19	1.00	3.96
3	71.84	20.31	4.25	----	3.67
6	71.99	19.45	4.02	0.69	3.85
8	67.50	18.69	8.66	1.21	3.94
12	66.07	20.40	10.09	----	3.73
14	71.83	18.21	5.40	1.11	3.45
15	68.84	20.61	6.41	0.17	3.97
Ave.	69.50	19.80	6.43	0.48	3.79

TABLE XXV

BODY COMPOSITION OF TESTOSTERONE PROPIONATE-TREATED (1.5 MG.) CASTRATE MICE EXPRESSED AS PERCENT OF WET WEIGHT (GROUP 4D)

No.	Moisture	Proteins	Fats	Carbohydrates	Ash
1	71.91	19.98	4.48	0.36	3.27
4	67.08	18.07	9.84	1.32	3.69
5	71.50	19.29	4.50	1.16	3.55
6	70.24	19.77	5.84	0.86	3.29
7	73.10	18.71	3.25	1.73	3.21
8	73.20	19.66	1.75	2.16	3.23
9	74.08	18.29	2.83	1.67	3.13
13	71.81	20.14	3.32	1.11	3.62
14	71.56	18.56	4.86	1.41	3.61
Ave.	71.61	19.16	4.52	1.31	3.40

TABLE XXVI

BODY COMPOSITION OF CASTRATE MICE TREATED WITH TESTOSTERONE PROPIONATE (2.5 MG.) AND THYROPROTEIN (.025%) EXPRESSED AS PERCENT OF WET WEIGHT (GROUP 4E)

No.	Moisture	Proteins	Fats	Carbohydrates	Ash
1	70.31	22.84	2.58	----	4.58
2	71.49	18.73	4.88	0.98	3.92
3	70.30	17.80	8.52	0.62	2.76
4	74.35	18.85	2.89	0.60	3.31
5	72.90	18.85	2.71	1.14	4.40
6	65.00	17.13	12.51	2.15	3.21
7	71.99	18.24	4.29	1.96	3.52
8	72.51	18.98	3.37	1.08	4.06
10	72.21	19.47	3.68	0.90	3.74
13	68.80	18.80	8.19	1.30	2.91
14	71.56	19.54	4.82	0.96	3.12
15	72.16	19.92	3.37	0.86	3.69
Ave.	71.13	19.10	5.15	1.02	3.60

TABLE XXVII

BODY COMPOSITION OF CASTRATE MICE TREATED WITH TESTOSTERONE PROPIONATE (1.5 MG.) AND THYROPROTEIN (.025%) EXPRESSED AS PERCENT OF WET WEIGHT (GROUP 4F)

No.	Moisture	Proteins	Fats	Carbohydrates	Ash
1	74.45	18.19	2.10	1.45	3.81
2	73.30	19.64	3.30	0.49	3.27
3	75.60	17.92	1.55	0.97	3.96
8	74.15	18.42	2.85	1.04	3.54
9	72.01	20.84	3.29	0.48	3.38
10	74.52	17.98	2.14	2.00	3.36
12	70.33	17.25	9.05	0.33	3.04
13	65.57	20.53	8.60	1.53	3.77
14	70.73	18.66	5.67	1.32	3.62
Ave.	72.30	18.82	4.28	1.07	3.53

TABLE XXVIII

BODY COMPOSITION OF NORMAL MICE EXPRESSED AS PERCENT OF WET WEIGHT (GROUP 4G)

No.	Moisture	Proteins	Fats	Carbohydrates	Ash
1	71.05	20.12	3.54	1.02	4.27
2	72.22	18.92	3.87	1.76	3.23
3	67.49	21.27	7.13	----	4.26
4	74.05	18.93	2.32	1.12	3.58
5	62.63	23.40	9.00	1.26	3.71
6	73.44	14.56	7.91	1.37	2.72
8	72.64	19.93	4.83	----	4.45
9	69.05	19.28	7.54	0.92	3.21
10	73.71	19.23	2.11	1.59	3.36
11	69.50	18.25	6.78	1.94	3.53
13	70.00	18.42	6.43	2.04	3.11
14	67.66	19.00	9.17	0.63	3.54
15	72.73	19.05	3.69	1.23	3.30
16	71.06	17.55	7.34	0.72	3.33
Ave.	70.52	19.14	5.83	0.97	3.54

TABLE XXIX

BODY COMPOSITION OF THYROPROTEIN-TREATED (.025%) MICE EXPRESSED AS PERCENT OF WET WEIGHT (GROUP 4H)

No.	Moisture	Proteins	Fats	Carbohydrates	Ash
1	70.99	17.82	5.51	2.36	3.32
2	72.15	19.02	3.83	1.28	3.72
3	71.48	17.83	5.15	2.00	3.54
5	71.62	18.63	4.60	1.38	3.77
6	72.65	19.01	3.82	1.06	3.46
7	73.74	18.14	2.47	1.87	3.78
9	74.42	18.95	1.47	1.69	3.47
10	73.68	19.34	1.58	1.52	3.88
15	70.39	18.62	6.46	0.94	3.59
16	70.89	20.18	3.91	1.03	3.99
Ave.	72.20	18.75	3.88	1.52	3.65

TABLE XXX

SUMMARY OF DATA ON BODY COMPOSITION OF TREATED AND CONTROL GROUPS OF MICE EXPRESSED AS PERCENT OF WET WEIGHT

Group 4	No. Animals	Moisture	Proteins	Fats	Carbohydrates	Ash
4A Castrate controls	11	71.87 ±.74*	18.20 ±.28	5.26 ±.60	1.09 ±.18	3.58 ±.08
4B Castrates plus thyroprotein	10	71.74 ±.50	19.12 ±.39	4.41 ±.41	0.97 ±.17	3.76 ±.08
4C Castrates plus testosterone propionate (2.5 mg.)	7	69.50 ±.91	19.80 ±.85	6.43 ±.39	0.48 ±.21	3.79 ±.07
4D Castrates plus testosterone propionate (1.5 mg.)	9	71.61 ±.67	19.16 ±.26	4.52 ±.78	1.31 ±.17	3.40 ±.07
4E Castrates plus testosterone propionate (2.5 mg.) plus thyroprotein	12	71.13 ±.69	19.10 ±.41	5.15 ±1.00	1.02 ±.18	3.60 ±.17
4F Castrates plus testosterone propionate (1.5 mg.) plus thyroprotein	9	72.30 ±1.14	18.82 ±.47	4.28 ±1.07	1.07 ±.21	3.53 ±.11
4G Normal controls	14	70.52 ±.61	19.14 ±.51	5.83 ±.64	0.97 ±.17	3.54 ±.13
4H Normal controls plus thyroprotein	10	72.20 ±.44	18.75 ±.23	3.88 ±.51	1.52 ±.15	3.65 ±.07

*Standard error.

TABLE XXXI

SUMMARY OF DATA ON BODY COMPOSITION OF TREATED AND CONTROL GROUPS OF MICE EXPRESSED AS PERCENT OF DRY WEIGHT

Group 4	No. Animals	Moisture	Proteins	Fats	Carbohydrates	Ash
4A Castrate controls	11	----	65.03 ±1.53*	18.38 ±1.66	3.78 ±.57	12.81 ±.35
4B Castrates plus thyroprotein	10	----	67.70 ±1.19	15.50 ±1.28	3.49 ±.65	13.31 ±.33
4C Castrates plus testosterone propionate (2.5 mg.)	7	----	65.19 ±1.91	20.74 ±2.14	1.58 ±.63	12.49 ±.32
4D Castrates plus testosterone propionate (1.5 mg.)	9	----	67.81 ±1.12	15.51 ±1.55	4.68 ±.69	12.00 ±.21
4E Castrates plus testosterone propionate (2.5 mg.) plus thyroprotein	12	----	66.88 ±2.16	17.31 ±2.44	3.22 ±.43	12.59 ±.69
4F Castrates plus testosterone propionate (1.5 mg.) plus thyroprotein	9	----	68.65 ±2.12	14.77 ±2.78	3.70 ±.64	12.88 ±.62
4G Normal controls	14	----	65.14 ±1.55	19.42 ±1.88	3.35 ±.62	12.09 ±.50
4H Normal controls plus thyroprotein	10	----	67.37 ±1.29	13.74 ±1.70	5.72 ±.60	13.17 ±.31

*Standard error.

of Kjeldahl Digestion mixture for Total Nitrogen[1] was added. The contents of the flask were well shaken to get good wetting and 10 gm. of powdered potassium sulfate was added to increase the boiling point and speed up the oxidation process. Five gm. of powdered sodium thiosulfate was next added and the flask gently warmed on the digestion apparatus. When frothing ceased, full heat was applied to the electric burners. The flasks were rotated regularly throughout the first 15 minutes and occasionally during the next hour. The heating and digestion was continued for a period of two hours at which time the contents appeared as a clear solution. Twelve Kjeldahls were run at a time including one blank as a reagent control. The flasks were allowed to cool and were removed to storage racks.

In the second phase, the ammonium sulfate formed in the process above was decomposed with strong alkali and the ammonia distilled off into boric acid. In this phase, 200 ml. of distilled water was first added to the cooled digested mixture and mixed completely. After cooling again, 50 ml. of concentrated sodium hydroxide (50%) was added slowly beneath surface of the flask contents. A piece of mossy zinc was added to reduce bumping and to react to form a zincate (sodium zincate) and hydrogen which keeps the solution well stirred. Approximately 150 ml. of the mixture was distilled into an Ehrlenmeyer flask containing 50 ml. of Kjeldahl Boric Acid (indicator added)[2]. This

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[1]Made up of $CuSO_4$ (15 gm.), conc. H_2SO_4 (8 lbs.), Salicylic Acid (125 gm.), Water (25 ml.).

[2]Indicator consists of: 5 parts 0.1% Bromcresol Green in 95% ethanol, 1 part 0.1% Methyl Red in 95% ethanol. 10 ml. of indicator added to each liter boric acid.

boric acid modification (Meeker and Wagner, 1933) somewhat simplifies the method inasmuch as it eliminates the use of standard base. The boric acid reacts with and holds the ammonia which is titrated against a standard acid in the next phase.

In the third phase of protein determination, a simple titration was made of the ammonia in the boric acid solution against 0.0714 N HCl. This normality is useful in that each milliliter of acid used corresponds to one milligram of nitrogen and the burette reading automatically gives the nitrogen equivalent. A duplicate was run for each mouse carcass and after subtracting the amount of nitrogen in the blank, the two values were averaged, and this average value multiplied by 6.25 (the protein conversion factor for total mouse tissue). The amount of protein per "computed weight" of sample gave the percentage of the protein in the sample. The "computed weight" in each case was the actual sample weight corrected for the weight of the fat which was removed so that the "computed weight" acts as a true sample weight (as if fat were still in it). The amount of protein in <u>each mouse</u> can be seen in Tables XIV to XXIX, while the average protein in <u>each group</u> is shown in Tables XXX and XXXI.

The next step in the analysis was determination of ash content. After review of the literature on ashing techniques a method was arbitrarily chosen based on certain of the precautions necessary to obtain accurate and verifiable results. The process of ashing tissue, being a very complex one, lends itself to the establishment of certain arbitrary techniques which are maintained throughout the ashing procedure. Ashing methods are numerous and the common aspects of these methods were incorporated into the procedure used here. In the main, A.O.A.C.

Recommendations are followed. Previous workers have found the following factors to be the critical ones:

1) Rate of incineration.

2) Temperature of incineration.

3) Incineration time.

4) State of sample.

5) Volume of sample.

Ashing was started in a cold muffle furnace. A 1 gm. sample was enclosed in ashless filter paper (average ash per circle was 0.000064 gm.) and put into a platinum crucible. Twelve crucibles were placed at one time in the furnace where the temperature was brought gradually to about 200° C. and maintained at that temperature for one-half hour. This first gradual ignition prevents mechanical loss of ash which occurs with rapid incineration.

The temperature of incineration which is probably the most critical factor in ashing tissues was fixed at 650° C. Very high temperatures, i.e., in the vicinity of 800°, cause volatilization of certain elements such as K, Na, Cl, and P. It also causes fusion of the mineral matter which then prevents oxidation of the interior of the fused mass. The water of hydration is lost in certain compounds at one temperature and in other compounds at another temperature. Ashing temperature also effects the decomposition of carbonates to oxides. The following carbonates decompose at the designated temperatures:

$MgCO_3$ between 300-400 degrees C.[1]
$CaCO_3$ between 600-650 degrees C. (as precipitate)
K_2CO_3 between 700-800 degrees C.

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[1] Triebold, 1946.

The ashing temperature was set at 650°. At this temperature some of the carbonates were decomposed to the oxide form and addition of saturated ammonium carbonate at the end of the incineration period restored the oxides to the carbonate form. At a lower ashing temperature, the time of ashing was extended beyond practical laboratory handling. The temperature was maintained by an electronically controlled thermostat.

The total time of incineration was set at 4 hours including the half hour ignition time; the tissue was reduced to a fine greyish white powder in this time. A shorter period of time resulted in incomplete oxidation and a dark ash.

The state of the material to be ashed here was such as to make no precautions necessary. The finely ground sample includes fairly large amounts of bone, hair, nails, etc. which allows for sufficient permeability of the air and more uniform oxidation. If sample is too compact, sand is ground into the tissue so as to spread the material and provide more porous ash. This was not necessary here. Also a large mass of the sample prevents diffusion of air into the more central portions and results in less oxidation and higher ash weights. In the experiment here, a one gram sample was large enough for easy determination of ash weight and small enough to allow thorough oxidation.

After addition of saturated ammonium carbonate to the cooled ash, the ammonia and water were evaporated off gently in a drying oven at 100° C. and the crucibles transferred immediately to an oven at 200° C. for two hours. At the end of this time, the crucibles were removed to a desiccator for four hours and then weighed. The percentage of ash in <u>each mouse</u> is shown in Tables XIV to XXIX and the average ash weights for <u>each group</u> is shown in Tables XXX and XXXI.

The above analyses account for water content, protein, fat and ash; by standard practice, carbohydrates were determined by difference.

The data were scanned carefully for extremes and on this basis one animal was eliminated from the calculations. The basis for judging the extremes was the statistical basis for extreme variates where any animal showing variance less than three standard deviations from the mean was considered within the normal range of variability. The criterion of three standard deviations from the mean was used because of the comparatively small number of animals in each group.

None of the differences between groups of protein, fat, water, ash or carbohydrate content were statistically significant when ordinary methods of statistics are used. By finer statistical manipulations, i.e., by methods of analysis of variance, the results were still shown to be on the borderline of significance. By this method, the "t" values of protein, fat, and ash were 0.737, 1.21, and 0.92 respectively[1]. However, even though the statistical analysis is highly reliable, one may take the liberty of assuming that the correlation of "directions" makes interpretation of the data necessary.

In each pair of experiments, i.e., 4A versus 4B, 4C versus 4E, 4D versus 4F, and 4G versus 4H, the addition of thyroprotein to the feed has raised the nitrogen storage of the group (see Table XXXI). In each case, this increased protein percentage has been at the expense of fats. An early study by Cameron and Carmicheal (1920) showed disappearance of fats in rats fed thyroid. McKay and Sherrill (1941) reported a large decrease in fat in thyroid-treated animals on a high fat diet as compared

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[1]See Appendix for statistical analysis.

to the controls on a high fat diet. In one case, it may also have been at the expense of carbohydrates. Compare 4D with 4F where carbohydrates is reduced from 4.68% to 3.70%. In every case, there is an increase in ash content of those groups treated with thyroprotein over and above their "paired" group, as indicated in the following table (Table XXXII).

Group 4C at the 2.5 mg. level of testosterone propionate did not show protein anabolism or gain in weight over that of the castrate controls. Group 4B with 0.025% thyroprotein did show protein anabolism with no gain in weight over that of the castrate controls. This indicates a shifting around of metabolites by thyroprotein but not by the 2.5 mg. dosage level of male hormone. However, the 1.5 mg. level of testosterone propionate does show a protein anabolic action in tissue percentage at least but no gain in weight over the castrate controls. This is in agreement with previous work that shows higher levels of male hormone acting in a definitely catabolic or even at equilibrium state.

The question can be justly asked at this point as to why in experiment three, the level of 2.5 mg. testosterone propionate produced an anabolic growth effect whereas in experiment four the same dosage level showed no such activity. The only known difference between the experiments was that the animals in the last experiment were about 10 days younger (about 5 gm. less in weight). The difference in effect might be explained by this age difference in that the younger animals may be more sensitive to the dosage used than the older animals. Pharmacologists find wide ranges of sensitivity with age variations and a ten day difference among mice might be equivalent to perhaps six months difference in children. Other explanations are not attempted here.

TABLE XXXII

AVERAGE ASH CONTENT/MOUSE FOR VARIOUS GROUPS (EXPERIMENT FOUR)

Group	Ash (% Dry Carcass)
4A	12.81
4B*	13.31
4C	12.49
4E*	12.59
4D	12.00
4F*	12.88
4G	12.09
4H*	13.17

*Thyroprotein-treated group.

The combinations of thyroprotein with testosterone propionate at either level of dosage (groups 4E and 4F) and with the normal male hormone secretion (group 4H) show in each case a reduction in amount of fat in the animals. The increase in protein of group 4B over that of group 4A is 2.67 percentage units. The increase of protein in the 2.5 mg. testosterone propionate group over that of castrate controls is 0.16. On the basis of one of the earlier hypotheses the combination of the two hormones ought to produce an additive effect (i.e., 2.67 plus 0.16 equals 2.83). The protein difference between group 4E and 4A is actually only 1.85. The increase of protein in the 1.5 mg. testosterone propionate group is 2.78 percentage units more than that of the castrate controls. In combination with thyroprotein one might expect a total change of 5.45 percentage units (2.78 plus 2.67). Actually the combination produces a 3.62 change. Here is a synergistic effect, at least from one pharmacodynamic point of view which defines a synergistic effect as one which results when the total effect of two agents is greater than the effect of each agent taken individually. This is in contrast to another pharmacological definition, one by Sollman (1948) where synergism is a simple algebraic summation. Webster's synergism has the total effect greater than the sum of the two effects taken independently. Sollman says "if the action of two agents is really identical, their mixture would be equivalent to increasing the concentration of either, and would therefore follow the Weber-Fechner law of diminishing returns and appear deficient".

Evidence from Kinsel, Hertz and Reifenstein (1944) apparently lends credence to the hypothesis that both thyroprotein and testosterone propionate act on the same metabolic intermediary, where testosterone

propionate induced a strongly positive nitrogen balance and gain in weight in thyrotoxic patients characterized by increased urinary nitrogen and creatine excretion and by decrease in body weight. Interpreted from another point of view, it is difficult to believe that the two hormones are working on the same metabolic intermediary if in thyrotoxic patients where the thyroid hormone is present in large quantity and available to the metabolic intermediary, the addition of another substance acting on that intermediary should act any differently than more of the same substance. The thyroid hormone affects so many of the endocrine glands that a simple interpretation may not be warranted. Thyroprotein stimulates the adrenal cortex to additional production of steroid hormones which may have an additive or antagonistic effect depending on the quantities involved. Certainly sufficient evidence exists as to adrenal stimulation by thyroidal substances (Wallach and Reineke, 1949). Kenyon, et al. (1943b) show no reason to adduce the adrenal cortex as a necessary intermediary in the metabolic response of men to testosterone propionate inasmuch as the concentration of serum electrolytes was unaltered.

In comparing the castrate groups receiving testosterone propionate at levels of 2.5 mg. and 1.5 mg. with the castrate controls, it looks as if the 2.5 mg. level has no nitrogen retentive ability in very young mice but apparently assists in converting carbohydrates to fats, while the 1.5 mg. level has nitrogen retentive ability at the expense of the pool of substrates which would have gone into the fats. This latter effect was postulated by Kochakian some years back. Kenyon (1944) maintained glycosuria in human patients and was not able to

promote conversion of the carbohydrates to anything else with testosterone propionate treatment.

The discrepancies here, however, indicate needed investigation as to the differences between "fat" and "thin" castrates among dogs and man because the same apparent discrepancies occur there.

DISCUSSION

The relationships of the thyroid and testes hormones to the growth and metabolism complex are examined in this dissertation from four aspects: growth, tissue analysis, enzymes, and metabolic rate.

In specific dosages, the hormones used in this particular study were shown by previous workers to increase body weight, to stimulate nitrogen retention, and to increase water content of the body. Assuming these facts to be correct and capable of verification, it is of great interest to pursue the mechanism of the reaction. If the mechanism of action of one hormone is the same as that of another hormone, then when maximum anabolic results are produced by a certain level of each hormone acting alone, a combination of the two hormones should produce no greater results than each one alone. If no greater result is produced, then it can be assumed as stated above that the mechanisms are the same unless one interjects the plaguing thought that there is a limit to "how far an organism can be pushed". It is no doubt true that there are certain physiological limits established by the hereditary pattern of the species beyond which the organism cannot be made to go.

If, however, the gross growth data do not reveal a synergistic effect which would allow for different mechanisms of action, a careful detailed analysis of the carcass under treatment with the different hormones and combinations thereof, might reveal information of value in understanding a little more of the complex Metabolism-Energy-Enzyme system.

In understanding the process, it is necessary to scan the total mechanism of energy supply. First, a certain quantity X of food and water is ingested. The food contains water, proteins, fats, carbohydrates, vitamins and minerals. Assuming normal digestion and absorption[1], the food is broken down into the typical products of the major constituents of the food and "taken into the body". Let us also assume here that a greater intake of food and water (quantity Y) results in an increase in body weight. Now, if the percentage of proteins, fats, carbohydrates, vitamins, minerals and water in the carcass remains the same as at the lower body weight, then the metabolic picture has not changed. However, if the percentage composition of animal carcass Y has changed from the picture in animal X, it can be assumed that

1) the quality of the absorption in animal Y has changed so that at increased food intake, certain components are more readily absorbed in relation to the other components, or
2) the digestive products in animal Y are absorbed at the same rate as in animal X but the products are thrown into the energy pool, i.e., the glycerols as alpha-glycerophosphate work their way into carbohydrate metabolism, as do the carbohydrates themselves which all leads toward protein synthesis, or
3) water is absorbed in animal Y at a greater rate than in animal X, resulting in a uniform decrease in the percentage

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[1]The assumption may not be justified in view of the evidence that thyroid hormone causes increased absorption of carbohydrates. (Althausen, 1940; Althausen and Stockholm, 1938.)

of other components based on wet weight but in an overall increase in body weight.

Some speculation on locus of action of testosterone propionate in inducing protein anabolism in patients with hyperthyroidism was made by Albright (1943b) as follows:

"1. That testosterone propionate causes such a demand for amino acids that none are available for gluconeogenesis regardless of the partition of carbohydrates and fats in the metabolic picture.
2. Testosterone propionate may produce some alterations in the metabolism so that it is no longer necessary to have as high a ratio of carbohydrates being burned to fat being burned.
3. Testosterone propionate may facilitate the combustion of fat.
4. Testosterone propionate may convert fat into carbohydrate."

A number of hypotheses concerning the mechanism were laid down as follows and examined in the light of the evidence available:

Hypothesis A - The gain in weight would be proportionate among the major components of the animal body, i.e., there would be an increase in absolute weights of protein, fat, carbohydrate, water and minerals.

Hypothesis B - The gain in weight would be due to a greater protein anabolism and would show up on a percentage basis at the expense of the other components.

Hypothesis C - The gain in weight would be due to greater protein anabolism, but at the expense of fats with no change in percentage of the other components.

Hypothesis D - The gain in weight would be due simply to an increase in amount of water and the dry carcass would reveal no differences in the major components.

Hypothesis E - The gain in weight would be due to an increased amount of fat at the expense of the other components.

Hypothesis F - The gain in weight would be due to conversion of fat and protein into common substrates with subsequent conversion to carbohydrates.

Hypothesis G - The gain in weight would be due to both increased protein and water at the expense of the other components.

Support for Hypothesis B comes from various sources. Protein anabolic effects of the male hormone have been shown by Kochakian in increased muscular mass in treated mice. Verification of greater nitrogen retention is reported by Johnston (1941) with children and Terroine and Babad (1939) with rats.

Kenyon and Knowlton (1944) have "attempted to secure some information as to the role of muscle in the initial deposit of nitrogen in man" after treatment with testosterone propionate of a patient with progressive muscular dystrophy. The nitrogen retention was distinct but not spectacular and the experiment did not elucidate the site of the new tissue deposit. They state:

> "We do not understand now why the organism dissipates so much feed nitrogen during recovery from protein losses and is hence so dependent upon greatly augmented appetite during convalescence.
>
> 1. The need of the body for the energy derived from protein for ordinary maintenance needs does not seem to be of major importance.
> 2. The failure of ordinary food to meet the precise amino acid, and if you wish, vitamin requirements of the particular state of the animal may well be of importance, but neither the composition of food nor the manner of ingress of protein fragments precludes intensification of nitrogen retention when a suitable stimulus to protein anabolism is given.
> 3. It has not, however, been shown that such stimuli as have been administered precisely meet the requirements of the organism during protein depletion. Until this is done, we cannot assume that the body has omitted wise synthetic measures from its economy or that it has been unable to use those it has.

"4. It is possible that we have focussed too much attention on nitrogen itself, and that the carbon chains derived from the amino acids are of much more importance for obscure reasons during recovery than are the intact amino acids. Such a purpose has been sought for the mechanism of nitrogen loss after fractures described by Cuthbertson and by Howard, et al. Such carbon chains may serve tissues in the course of repair after fractures, and may serve a similar purpose in recovery from protein losses."

The importance of vitamins mentioned in point two above is brought out by Peters and Rossiter (1939) who find that administration of thyroid or thyroxine increases the requirement of vitamin B. See also Drill (1943). The vitamin B requirements vary with the caloric output, which is raised by thyroxine. The loss of weight accompanying high thyroid administration is lessened by adequate intake of vitamin B.

Kenyon, et al. (1943a) after a study of anorexia in man concluded that when anorexia limits the food intake to amounts insufficient to favor material storage of protein, then the androgen will induce such storage. Here there is more efficient usage of available proteins or stimulus to conversion of other types of food to protein or perhaps protein is shifted into storage form whereas under the conditions of anorexia, it might have been utilized for energy.

The concept of a dynamic equilibrium implies the presence of a large protein pool in tissues which contributes to the plasma protein and the latter contributes in turn to the cells and tissues. There are evidently little or no reserves of free essential amino acids and in a deficiency of any one, cannot obtain it from the protein stores (Cannon, 1949). Thus with the known anabolic effect of testosterone propionate and the known appetite-stimulating effect of thyroprotein, the combination ought to resolve itself into sufficient amino acid supply through increased food intake to promote substantial growth effects.

"Fat" eunuchs lend support to Hypothesis E where there is direct evidence of increased fat storage beneath the skin, however, the "thin" eunuchs show the opposite effect. The well known action of thyroid in "reducing tablets" on the fat stores of the body is also evidence.

Hypothesis G is supported by data of Kenyon, et al. (1940) who postulate the gain in weight of man after treatment with testosterone propionate to be due largely to water held in association with the retained salts and protein. Koger, Hurst and Turner (1942) determined the composition of thyroxin-injected female mice and compared the data of the analysis with control mice. "The fresh carcasses contained a greater percentage of nitrogen and water, and a lesser percentage of fat than did their controls". A resume of their data follows:

Composition of Dry Carcass	Controls	28 Day Thyroxine-treated
Protein %	54.0	59.6
Fat %	30.4	26.1
Ash %	10.6	10.3
Water %	63.7	65.7

Since steroids from both the testes and adrenal cortex (Thorne and Eisenberg, 1939) influence the growth process, it is pertinent to more complete understanding of the problem to study the role of enzymes. Corticosterone prevents hypoglycemia of Addison's disease either by facilitating the formation of sugar from protein or by inhibition of sugar utilization by the tissues (Talbot, et al. 1943). Gluconeogenesis is also stimulated by thyroxine. Alanine, glycerose and pyruvic acid are transformed to glycogen slowly. The three-carbon keto acid fragments of amino acids are important in gluconeogenesis, thus glycine and the three-carbon acids are converted quantitatively while the other amino acids have parts convertible.

Inasmuch as the enzyme phosphatase functions in carbohydrate metabolism in several places such as phosphocreatine to creatine conversion, phosphoglycerate to pyruvic acid conversion, and adenosine triphosphate change to adenylic acid, the variation of quantity of the enzyme is of importance. Although the liver is of key importance other organs have been examined also for changes in phosphatase. In an extensive study of the normal mouse kidney Kochakian (1947) reports that:

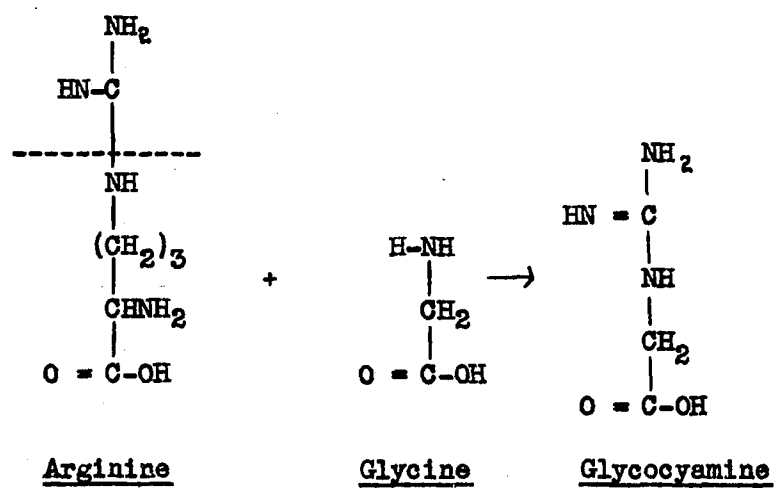
> "The normal mouse kidney shows alkaline phosphatase in the cells of the proximal convoluted but not the distal tubules. It is present at the turn of Henle's loop and in the capsule about the glomerulus. In the castrated mouse the cells of the kidney decrease in size with a resulting greater concentration of the enzyme in the cells. After testosterone treatment there is an apparent 'washing out' of the 'alkaline' phosphatase in the distal end of the proximal convoluted tubules with a somewhat greater concentration in the portion nearest the glomerulus. This shift in the concentration of the enzyme suggests that the kidney is striving to recover substances (phosphates?) as quickly as possible from the glomerular filtrate."

Androgens also affect the secretion of acid phosphatase by the prostate gland. The prostate gland of children contains little of this enzyme and administration of testosterone propionate results in a small increase. Much evidence is available of the effects of the male hormone on serum alkaline phosphatase where an increase is noticed after treatment (Kochakian and Vail, 1947; Humm, et al. 1948, Kochakian, et al. 1948; and Buchwald and Hudson, 1944).

The thyroid hormone also affects the serum phosphatase. In infants and children suffering from hypothyroidism, the serum phosphatase was found to be abnormally low and adequate thyroid therapy restored the phosphatase to normal (Talbot, et al. 1941). Wilkins and Fleischmann (1946b) report that the thyroid influences conversion of creatine into

creatinine directly or through an effect upon the enzymes controlling the creatine-phosphocreatine cycle. Sure, et al. (1941) report marked creatinuria with a reduction in preformed creatinine in hyperthyroid rats.

Concerning arginase, Kochakian (1947) reports that the addition of 0.5 mg. testosterone to samples of incubation mixture containing homogenisate of kidney did not produce any significant change in arginase. Kidney arginase increases with testosterone propionate treatment (Kochakian, 1945). Since arginase occurs in the proximal convoluted tubules, Kochakian assumes that the cells in this tissue are stimulated to recover for protein fabrication more of those materials that ordinarily would be excreted. Kochakian in 1943 postulated that arginase has a greater function than in the urea cycle in the liver. S. Edlbacher (1938) as a result of extensive studies postulated that one of the functions of arginase may be to provide suitable nitrogen forms for protein synthesis. Borsook and Dubnoff, (1943) provide evidence for one synthetic process, the formation of glycocyamine, in the kidney by the transfer of the amidine group of arginine to glycine as follows:



Arginine Glycine Glycocyamine

The glycocyamine is then methylated in the liver to form creatine. In order to explain the above, one must assume that arginase promotes glycinolysis of arginine in the kidney instead of hydrolysis as occurs in the liver. This is entirely plausible. DuVigneaud (1942) showed the significance of labile methyl groups in the diet and their relation to transmethylation. Isotopic methyl groups made their appearance in the creatinine of the urine and in the choline of the blood.

No increase in arginase in the liver was produced with treatment of mice with testosterone propionate while the expected anabolic effect, however, was in evidence. This is in good agreement with the above postulations.

Finally, inasmuch as both the thyroid and testes hormones affect the metabolism of the organism it is pertinent to question the relationships of the mechanisms. Sandiford, et al. (1941) find that the basal metabolic rate of 5 eunuchoids and 1 eunuch was increased by 10 points each by testosterone propionate treatment. They point out, however, that the action is not similar to that occurring via the thyroid as the pulse rate remained the same. McCullagh and Rossmiller (1941a) get the same effect with methyl testosterone and they also remark as to the difference of effect from that of the thyroid. They say that the clinical picture does not resemble hyperthyroidism even when the B.M.R. is raised as high as 38%. Jones, et al. (1941) from their results postulate the effect of the male hormone to be directly on the tissues or intermediately through some other gland. In <u>man</u>, the hypermetabolism whenever it appeared following steroid hormone treatment differed from that occurring as a result of hyperthyroidism. It was not accompanied by tachycardia, palpitation, hyperpiesis or increased sweating. A

qualitative as well as quantitative effect on metabolism was suggested by the fall in respiratory quotient of more than half the subjects. In some cases the R.Q. dropped to 0.690 (Chambers, et al. 1942).

In general, the evidence presented above indicates definite differences in the mechanism whereby an anabolic effect is produced by testosterone propionate and by thyroprotein.

SUMMARY AND CONCLUSIONS

The growth-stimulating effect of thyroprotein is substantiated. The dosage of thyroprotein having the greatest anabolic effect on the immature albino male mice used in this experiment is .05% of the feed. The growth-stimulating effect of testosterone propionate is substantiated. The dosage of testosterone propionate having the greatest anabolic effect on the immature albino male mice is 2.5 mg. in pellet form under the skin. A slight synergistic effect on growth although statistically not significant is obtained when thyroprotein (.025%) is administered simultaneously with testosterone propionate at the 2.5 mg. level in pellet form under the skin.

Food and water consumption increases with increasing dosage of thyroprotein. No such effect is noticeable with testosterone propionate treatment.

Analysis of the carcasses of eight groups of mice under various experimental conditions indicates an increase in protein in the groups treated with thyroprotein (.025%) which is apparently at the expense of fats. The testosterone propionate-treated group (1.5 mg.) shows higher protein content than the controls. The combination of testosterone propionate (1.5 mg.) and thyroprotein (.025%) shows a synergistic protein anabolic action. This is viewed in the light of the known weight depressing effect of larger dosages of each of the hormones. The data herein described lend weight to the hypothesis that these hormones are acting on different mechanisms of protein and fat metabolism.

The relationship of these hormones to the enzyme-substrate picture of carbohydrate, protein and fat metabolism is now being investigated energetically by a number of workers and the next few years should reveal fundamental links in the chain of events of tissue metabolism between enzymes, substrates and hormones.

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APPENDIX

TABLE I

METHOD OF ANALYSIS OF VARIANCE

Group	Group A	Group B	Group C	Group D	Group Total
INDIVIDUAL DATA	x_1	x_1	x_1	x_1	
	x_2	x_2	x_2	x_2	
	x_3	x_3	x_3	x_3	
	.	.	.	.	
	.	.	.	.	
	.	.	.	.	
Σx	g_A	g_B	g_C	g_D	$= T$
$\Sigma x^2 =$	$x_1{}^2+x_2{}^2+x_3{}^2.$				All items Σx^2

n = Tot. No. Animals in Group N = Tot. No. of Animals

$$C.T. = \frac{T^2}{N}$$

$$A = \underset{\Sigma x^2}{\text{All items}} - \frac{T^2}{N} = \text{Total Sum of Squares}$$

$$B = \left(\frac{g_A{}^2}{n_A} + \frac{g_B{}^2}{n_B} + \frac{g_C{}^2}{n_C} + \ldots\ldots\ldots \right) - C.T.$$

= Between Group Sum of Squares

ANALYSIS OF VARIANCE OF MEASUREMENT				
Source of Variation	Degrees of Freedom	Sum of Squares	M_1 Sq.	F (Snedecor Table)
Total		A =		
Between Groups	F_B	B =	$\frac{B}{F_B} = W$	$\frac{W}{w} =$
Within Groups - Error	F_E	E=A-B=	$\frac{E}{F_E} = w$	

For Significance Comparison Between Any Two Groups:

$$s = \sqrt{w}$$

$$\sigma_{m_A} = \frac{\sqrt{w}}{\sqrt{n_A}} \; ; \quad \sigma_{m_B} = \frac{\sqrt{w}}{\sqrt{n_B}} \; ; \quad \sigma_{m_C} = \frac{\sqrt{w}}{\sqrt{n_C}} \; ; \ldots\ldots$$

$$\sigma_{(m_A - m_B)} = \sqrt{\sigma_{m_A}^2 + \sigma_{m_B}^2}$$

$$t = \frac{M_A - M_B}{\sigma_{(m_A - m_B)}}$$

The "t" value was also computed on the basis of the following equation:

$$t = \frac{M_1 - M_2}{s\sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

where:

t = "t" value and is compared with "t" tables for significance at 5% or 10% probability.

M = mean

s = ungrouped standard deviation = $\sqrt{\frac{\Sigma (v-M)^2}{N-1}}$

N = number of animals.

The standard errors were computed on the basis of Bessel's formula:

$$S.E. \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$