OXYGEN CONSUMPTION OF BONE MARROW AND COMB TISSUE OF CAPONS AND NORMAL CHICKENS AND THE IN VITRO EFFECT OF ANDROGENS

by

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Androgens have a pronounced effect on both the comb and bone marrow of the capon. The comb responds to androgen stimulation by increased proliferation of cells and increased mucoid content which results in increased size and turgidity of the comb. Bone marrow responds to androgen stimulation by increased proliferation of erythroid elements with a resulting increase in blood values such as the number of erythrocytes and hemoglobin content.

Blacher (1926) first reported that there was a sex difference in chickens. Red blood cell counts and hemoglobin values were greater in the cock than in the hen. These differences were found, however, only after the chickens were three months of age. Chaudhuri (1926) confirmed these findings and, also, found that immature birds had values that were intermediate between the values of the adult cock and hen. Similar sex differences have been reported in many other animals. (Rat, Steinglass et al. 1941; mouse, Kamenoff, 1936; rabbit, Rosahn et al. 1934; cat, Lewis, 1941; pigeon, Riddle and Braucher, 1934; and in man, Ponder, 1934).

Juhn and Domm (1930) found that juvenile and gonadectomized birds of both sexes and mature birds had similar red blood cell counts until the juveniles reached sexual maturity, at which time the adult male values increased to about 30 percent over that of the adult females.

Evidence for the stimulation of the bone marrow by androgens with

resulting hyperplasia of the erythroid elements has come largely from the studies of Padoa (1931), Taber, et al. (1943), Domm, et al. (1943), Steinglass, et al. (1941), and McCullagh and Jones (1942).

Padoa (1931) showed that the increased proliferation of the testicular tissue of the ovo-testes after the removal of the left functional ovary of the hen was responsible for a rise in the red blood cell counts of these animals.

Domm, et al. (1943) found that androgens injected into capons and pullets caused an increase in the erythrocyte count of these birds to the level of normal cocks.

Similar results were obtained by Steinglass, et al. (1941) in castrated male rats, and McCullagh and Jones (1942) in eunuchoid humans.

Steinglass, et al. (1941) demonstrated that castration in rats results in a hypoplasia and vacuolization of the bone marrow with a corresponding decrease in the blood values (red blood cell count and percent hemoglobin). Testosterone induced a hyperplasia of the bone marrow with an increase in erythopoetic elements and a corresponding increase in the blood values. Estrogen injected into normal male rats caused an erythroid hypoplasia and in some, an increase in myeloid areas. In females, castration increased the blood values and estrogen administration decreased the values. These experiments would seem to indicate that erythopoesis is definitely controlled by variations in the amount of sex hormones.

Finkelstein, et al. (1944) found that although red cell production of the bone marrow of rats was stimulated by testosterone, hemoglobin

synthesis was inhibited. Her experiments were conducted to show the effect sex hormones had on the regeneration time from the anemia produced by hemorrhage.

Hemoglobin regeneration was less in bled male normals and in bled castrate females when testosterone was administered, even though red cell production was stimulated. In the normal female, however, testosterone accelerated hemoglobin regeneration. Estrogen was found to delay the regeneration of red cells in normal bled females. A combination of testosterone and cobalt was especially effective in accelerating regeneration in bled female normals.

Estrogen treatment results in a depression of the erythrocyte and hemoglobin level. It has been shown, however, that there is a definite species susceptibility in the response of the bone marrow to estrogens. Both natural and synthetic estrogens have been shown by Castrodale, et al. (1941), Crafts (1941), and Tyslowitz and Hartman (1941) to give different degrees of action on the bone marrow of different animals. Dogs were found to be more sensitive to estrogen treatment than were monkeys; monkeys were found to be more sensitive than rats.

In the majority of studies, estrogen treatment decreased the erythrocyte values and hemoglobin content of the blood. One study, however, (Davis and Boynton, 1941) reported that the bone marrow was stimulated and that the blood values increased when estrogens were administered. This has not been confirmed by other studies. Taber, et al. (1943), Crafts (1941) and Martin (1948) reported there was a consistent decrease in both the red blood cell count and hemoglobin level when estrogens were

administered.

Evidence for the direct antagonism by estrogens on the stimulation of the bone marrow by androgens has been shown by Domm, et al. (1943), and Taber, et al. (1943) who found that intersexes, produced by injecting estrogens into genetically determined males on the fourth day of incubation, had values as juveniles between that of the normal adult cocks and hens. As adults, however, the values approximated those of the mature female. Evidently, at time of sexual maturity erythropoesis in these birds was inhibited since the values dropped to that of the mature females. Taber, et al. (1943) attributed this to an antagonism between estrogen and the male sex hormone.

Estrogens injected into the adult male birds decreased the red blood cell count. In the female, estrogens also decreased the count but the decrease was much greater in males than in females. For this reason, and the fact that the clotting time of all estrogen treated birds was also decreased while the controls were not, Taber, et al. (1943) concluded that there was a direct inhibitory effect on erythropoesis and that the effects were not due to a neutralization of the stimulus initiated by androgens.

Simultaneous injections of estrogens and androgens were found by Domm, et al. (1943) to increase the number of red blood cells in normal birds. The mixtures that were injected never produced as great a response as when testosterone or stilbesterol were injected by itself. Tyslowitz and Dingemanse (1941) however, found a decrease when mixtures were given simultaneously in castrate dogs. Injections of testosterone did not pre-

vent the action of the estrogens.

Martin (1948) determined that simultaneous injections of sex hormones decreased the values of the normal cock and capon. The decrease was attributed to a slower action of the estrogen as compared with testosterone proprionate. Martin interpreted from his findings on the red blood cell counts and hemoglobin levels that estrogen acted only on the anterior lobe of the pituitary. The pituitary then was inhibited in its secretion of gonadotropins which control the testes in the production of testosterone which in turn has a direct effect on hemopoesis.

Hypophysectomy causes a marked vacuolization and progressive hypoplasia of rat bone marrow with a resulting anemia. (Vollmer, et al. 1939). After being hypophysectomized for two months the red blood cell values and hemoglobin values decrease to 60 - 70 percent of normal. It was shown that the resulting anemia was not due to the lack of food intake or surgery.

Vollmer and Gordon (1941) found that testosterone proprionate elevated the red blood cell counts of hypophysectomized animals to a high normal in both males and females. Estradiol on the other hand decreased the values and intensified the anemia. Pregnant mare serum was found to have the same effect as testosterone proprionate. From studies on the hypo and hyperplastic marrows of these animals, Vollmer and Gordon concluded that the effects were due to some action of the androgens and estrogens on the activity of the tissue.

Different modes of action have been advanced for the control of hemopoesis by endocrines. The main views are the following:

1. The hypophysis acts directly on the bone marrow through a hormone of the anterior pituitary. Vollmer and Gordon (1941) found that pregnant mare serum (gonadotropins) when administered to castrate rats had the same effect as testosterone proprionate on the bone marrow. Gordon and Charipper (1947) advanced the view that the pituitary was the initiating source for the stimulation of bone marrow but that the effects were caused by the effects of the anterior pituitary hormones on metabolism in general.

2. The hypophysis controls the secretion of other hormones which then act directly on the bone marrow. It would be expected according to this view that the removal of an endocrine gland would then cause an effect on hemopoesis. Castration, thyroidectomy, and hypophysectomy have all been shown in the studies quoted to affect the bone marrow. No studies, however, can be definitely said to contribute much information as to whether the hormones stimulated by the hypophysis act directly on the marrow or by influencing metabolism in general.

3. The hypophysis governs the action of other hormones which together with the hormones of the anterior pituitary are responsible for an effect on the bone marrow through increased metabolism.

Evidence for this view is based on the known effects of the anterior pituitary hormones on general metabolism (Riddle, et al. 1936), (Billingsley, et al. 1939) and the effects of other hormones controlled by the hypophysis on metabolism. According to this view sex hormones would exert their effect by increasing or decreasing the metabolic state of the animal. If this were true then the hormones should cause an in-

creased basal metabolic rate in castrate animals. Several reports have appeared to support this view. (McCullagh and Rossmiller (1941), Jones, et al. (1941), Kenyon, et al. (1940), Sandiford, et al. (1941). Meyer and Danon (1942), however, found that the basal metabolic rate of castrates was not increased by methyl testosterone.

The hypophysis is generally conceded to be controlled in its secretion of gonadotropins by the level of the sex hormones. Another view, however, is that anoxia or the decreased oxygen capacity of the blood is responsible for hypophyseal secretion. Evidence for this view comes from the study of Gordon, et al. (1943) who found that a lowered barometric pressure affected not only the hypophysis, but the thyroid, gonads, and adrenals as well. The stimulus of anoxia or a lowered oxygen capacity of the blood generally increases the erythroid elements of the bone marrow.

In-vitro studies of the respiration of bone marrow of animals subjected to lowered oxygen tensions were made by Warren (1941) in which he found that the respiration of the bone marrow of such treated animals increased slightly and that the increase corresponded exactly to the respiration of erythroid hyperplastic marrows that were induced by hemorrhage. In-vitro, lowered oxygen tension did not affect the respiration until quite low values were obtained. Warren concluded that the rate of respiration of bone marrow is determined by the composition of the marrow rather than the means used to obtain the changes.

Meyer, et al. (1940) studied erythropoesis after the animals had been exposed to lowered tensions. Hypophysectomized animals could not respond with increased erythropoesis from the stimulus of anoxia. Landau and

Gordon (1952) studied the respiration of hypophysectomized rats. The respiration of these animals was found to be similar to normals. In another form of anoxia, hemorrhage, the hypophysectomized rat exhibits a delayed red blood cell regeneration. (Vollmer, et al. 1939).

In a later study, Warren (1942) attributed the effect of oxygen tension on tissue in-vitro to the Pasteur Effect which is a shift at a critical oxygen tension from anaerobic oxidations to aerobic oxidations. Bone marrow was found to have a low critical oxygen tension of 0.8 percent.

Since anoxia will not result in stimulation in hypophysectomized animals and also since gonadotropins stimulate the bone marrow of castrate animals, it is possible that the gonadotropins or androgens are responsible for the stimulation of bone marrow by the acceleration or inhibition of cellular enzymes.

In the only in-vitro study of the effect of androgens on marrow Yagi (1937) found that spleen and bone marrow was stimulated in tissue cultures by androgens, while estrogens inhibited the growth of bone marrow, spleen and dental pulp. His experiments were conducted using Enarmon, a male sex hormone preparation, in a base of mazola oil. Mazola oil by itself also had the power of stimulating the growth of bone marrow.

If a direct effect on the oxygen uptake of the bone marrow were observable then more evidence would be available for the direct action by the gonads. It should be emphasized, however, that negative results in a study of this sort cannot be judged as contributing materially to the evidence against the theory of the direct action by the gonads since

there are many variables to be considered before a tissue can be judged as exhibiting normal respiration and normal response to a stimulus. The principle factors are:

a. The separation of cells from the close proximity that they occupy in-vivo.

b. The removal and separation of nutrients and ions from the close proximity and higher concentration they have in-vivo.

c. The change from an essentially anaerobic media to an aerobic media with resulting changes in concentration of oxygen and carbon dioxide.

d. The change from a constant temperature in-vivo to a chilled state and then a return to a temperature that may not be optimum for bone marrow or comb tissue.

e. The influence of the added hormones on enzyme systems.

Review of the Literature on Comb Tissue

Although the capon's comb has been used for many years for the biological assay of androgens, there has been little evidence for the assumption that androgens act directly on the tissue.

When the normal cock is castrated, the comb regresses and becomes pale and dry. The degree of regression however, depends on previous fibrous tissue development. (Pezard 1926)

When androgens are administered, there is an increased proliferation of fibroblasts that parallels the deposition of mucoid material in the

intermediate zone of the comb, (Ludwig and Boas, 1950). Champy and Kritch (1926), Hardesty (1931), and Ludwig and Boas (1950) have demonstrated that the secretion of this mucoid material is due to androgen stimulation. No direct evidence could be ascribed by Ludwig and Boas, however, for the fibroblasts being responsible for the secretion since the fibroblasts were increased in all layers of the comb.

When androgens were administered to normal cocks, no effect was noted until massive amounts were used. (Parkes and Emmens, 1944). Only 12 micrograms are necessary to promote measurable growth of the comb of the capon. (Walter, 1950) Estrogens applied to the capon's comb do not have any stimulating effect. When applied to the cock's comb, estrogen causes a regression in size. (Morato-Manaro, et al., 1938). The combs become pale and dry and are similar to the combs of castrates. Gley and Delor (1937) found a decreased response to testosterone when estradiol was also given.

Kosin and Munro (1942) studied the effect of dosage of sex hormones on the water content of combs of male chicks. No synergism or antagonism was found but the water content of the tissue was greatly increased when the comb was stimulated by testosterone proprionate.

As evidence for the direct inhibition by estrogens on the comb the studies of Morato-Manaro, et al. (1935), and Gley and Delor (1937) could be cited. There are two general views as to how estrogen causes the regression of comb tissue:

1. by a direct action on the comb,
2. by an inhibiting action on the pituitary.

The relationship of the comb to other endocrines is not clear. The relationship of the pituitary - testes - comb can be illustrated most easily by the diagram:

Pituitary ⟷ testes ⟶ comb

Zawadowsky (1936) ground up cocks combs and fed them to young male rats. The testes, seminal vesicles, and prostate glands of these rats were smaller than normal. When combs are removed from cocks the testes increase in size. (Buckner et al., 1933). Zawadowsky (1936 a) postulated a reciprocal action between the testes and comb:

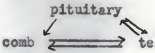
Pituitary ⟷ testes ⟷ comb

Hoskins and Koch (1939) confirmed the increase of testes weight following comb removal. Landauer (1942), however, suggested that there was no evidence for a specific effect by the comb on the testes and that the reason for the slight increase found in his experiments was the trauma involved in the operation.

If there is a reciprocal relationship between the comb, the testes, and the pituitary it is effective only over a considerable period of time. Hoskins and Koch found no effect on testes weight at 3 months after comb removal but a definite effect at 7 months. This has also been confirmed by Taibel (1949) who found a slight effect at 5 months.

Marlow (1950) injected a saline extract of cock pituitaries into castrate chicks. A significant increase in weight and comb growth was found. Bovine pituitary extracts did not have any effect, neither did adrenocorticotropic hormone. Marlow concluded that there was a definite relationship between the pituitary and the comb and could be representated

as:



The normal development of the comb depends on the thyroid gland. When the thyroid is removed the comb regresses and testosterone proprionate will only partially restore the comb. When thyroxin is given simultaneously, however, the comb response is augmented (Caridroit, 1942). Thyroxin was believed to intensify the metabolism of the comb tissue and, therefore, increase its reaction. Caridroit (1942) found that posterior pituitary extracts did not augment the action of testosterone on thyroidectomized birds. Thyroid extracts when injected with testosterone proprionate promoted growth when the amount of testosterone proprionate was ineffective by itself.

Regnier and Caridroit (1943) found that the combs of cocks and capons had similar concentrations of total lipids. Capon combs, however, had more fatty acids than the cock; while the cock had more cholesterol. In the hen's comb there was a higher lipid content.

If the metabolism of the comb can be studied by in-vitro methods then perhaps the relationship of the comb to the hormones involved can be elucidated.

METHODS AND APPARATUS

These experiments were conducted over a period of 18 weeks. Single comb, White Leghorn cockerals were used for all experiments. The animals were obtained as chicks and were raised under constant laboratory conditions. Feed and water were given ad libitum. The feed was

a standard chicken mash with a corn supplement and good gain in weight was noted. As soon as the chicks reached approximately four weeks of age, they were placed in larger cages so that crowding would not take place. No weight groupings or blood work was done since the small number of birds used in the experiments did not appear to justify such groupings. Birds that were droopy, considerably below the weight of the average birds or that had possible infections or broken bones were disposed of. As far as is known, the birds used in the study were all in a good state of health. Two groups of birds were received, 36 in the first group and 11 in the second group. High mortality occurred in transit in the second group. Of the initial group of 36 birds, 12 were used as normals in the comb experiments, eight were implanted with diethylstilbesterol and used in the comb experiments, five were used as normal controls in the marrow experiments, and seven were surgically castrated for use in the comb experiments. Of the second group of 11 birds, two were used as normal controls for marrow studies and four were implanted with diethylstilbesterol for use as capons in the marrow experiments. A full 30-day period was allowed for the birds used in the comb experiments to recover from the partial removal of comb tissue, after which some of them were used as normals and capons in the marrow experiments. For the first week after the combs had been cut these birds were kept separate. Inspection of the combs revealed that all of the combs healed rapidly and that no infections had developed. Thirty days after being surgically castrated the experiments were begun. Evidence of the completeness of castration came from the comparison of the combs of castrated birds with normal birds.

Slips that occurred were not disposed of, since, if there was enough residual or accessory tissue available to maintain the comb, then the comb was considered as representative of a normal comb. Slips were not used in the marrow experiments since the marrow might be more sensitive to variable amounts of hormone than the comb tissue. Chemical caponization was instigated by implanting two pellets of 15 mg. of diethylstilbesterol subdermally in the neck region. All chemically caponized birds used in the marrow experiments were used only after being implanted for a full 30-day period.

The apparatus that was common to both the comb tissue and bone marrow experiments included: the Warburg constant volume respirometer, an analytical balance, and similar types of pipettes and reagents. The Warburg instrument, (Plates I and II) although bearing the name of Warburg, evolved from the blood gas manometers described by Barcroft and Haldane (1902) and Brodie (1910). Warburg, however, used the instrument exclusively for tissue studies and modified it to its present form. The Warburg assembly consists of:

(a) Reaction flasks to hold the respiring samples of tissue and substrate.

(b) Manometers to measure the pressure of gas in the reaction flasks.

(c) A constant temperature water bath to hold the temperature constant in the flasks so that the manometer readings are due to changes in the concentration of gas.

(d) A shaking apparatus to promote the absorption and the release of gases in the suspending media in the flasks.

EXPLANATION OF PLATE I

Photograph of the Warburg assembly showing seven manometers attached to the shaking apparatus mounting. Attached to the arms of the manometers are flasks that are immersed in the constant temperature water bath.

The controls for regulating the temperature of the water bath and the speed of the shaking apparatus can be seen behind the upper portions of the manometers and at the lower side of the assembly.

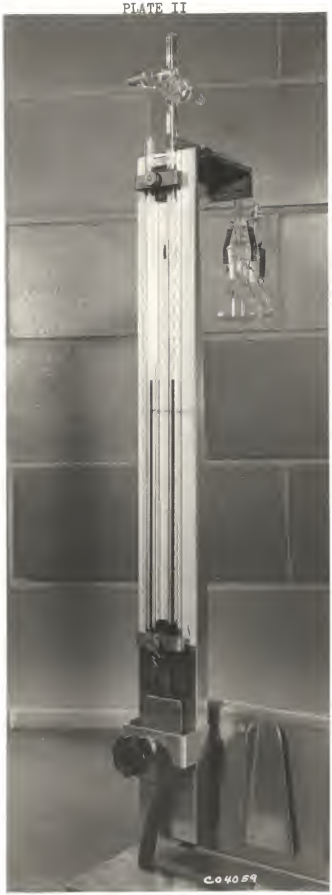
PLATE I

EXPLANATION OF PLATE II

Photograph of a Warburg manometer and reaction flask. The reaction flask is attached with a ground glass joint and tension springs to the manometer by a T-connection, one end of which is connected to a three-way stopcock to allow the contents to come to equilibrium and the other end is fused to the manometer arm. The side bulb and vent, which were not used in the experiments, can be seen on the far side of the reaction flask proper. The center well or alkali cup can be seen within the flask.

At the lower portion of the manometer, the manometer fluid reservoir and clamp can be seen. The manometer fluid extends to the middle of the manometer arms, where the fine calibration of the manometer can also be seen.

PLATE II



The reaction flasks used in the experiments were the "B" type of 12 to 17 milliliter capacity with side bulb attached. This type of flask has a curved base that prevents the accumulation of tissue in the corners of the flask. A center well, or alkali cup, of approximately 0.5 ml. was sealed in the center of the flask as a container for a gas absorbing agent. The following procedure was found to be the most satisfactory for cleaning the reaction flasks and other glassware:

1. Stopcock grease was removed with gasoline on a cotton swab.
2. Flasks and glassware were then immersed in a detergent solution and gently boiled for ten minutes.
3. Flasks and glassware were then rinsed six times with tap water and three times with distilled water.
4. Flasks and glassware were then inverted and allowed to air dry or were placed in a drying oven.

The flasks used in these experiments had a side bulb and vent to allow gassing of the flask and tipping in of reagents. This side bulb and vent was not used in the experiments to be described. Before reagents or tissue were added to the flasks, the top of the alkali cup was rimmed with a thin layer of stopcock grease since Umbreit, et al. (1949, p. 11) pointed out that alkali may creep out over the top of the cup and contaminate the tissue. The reaction flasks were connected to a manometer which consisted of a U-tube of uniform bore, (an internal cross-section of about 1 square mm.), about 30 cm. in length and graduated from 1 mm. to 300 mm. One arm was open to the atmosphere and the other was connected to a T-tube to which was attached a reaction flask with a ground joint and small tension springs to hold it in place. The

other end of the T-tube was connected to the air by a three-way stopcock. At the base of the U-tube there was attached a manometer fluid reservoir with a screw clamp which allowed the level of the manometer fluid to be altered. In use, the level was set at a "zero" point of 150 mm. with the stopcock leading to the atmosphere open. After an equilibration period of 15 minutes the stopcock was closed and measurements were begun. After respiring for a set time, the fluid, which rises in the tube when a gas is being evolved, was corrected to the zero point and the rise or fall of the second arm was recorded. These readings were taken to the nearest one-fourth of a division. It was found that if dust and grease accumulated on the inside of the manometer arms the meniscus of the manometer fluid was interfered with and readings were not accurate. Because of this the manometers were kept covered with a sheet of plastic material. The manometer fluid used in the manometers was Brodie's fluid. This fluid consisted of 23 grams of sodium chloride and 5 grams of sodium choleate in 500 ml. of water and colored with Evan's blue. The density of this fluid was 1.030.

The balance used in the experiments had an estimated precision of $\pm$ 0.1 milligram. The comb slices varied in their dry weight from 10 to 30 milligrams and the error of measurement was, therefore, higher when fewer slices were used than when more slices were used (1% to 0.3% error in the Q_{O_2} value). The comb dry weights were obtained using the multiple swing method of weighing. The marrow wet weights were determined to only $\pm$ 1 milligram and the single swing method of weighing was used. Samples of comb tissue were weighed in small vials of about four to six grams.

The same set of weights was used throughout and the weights were considered as being interchangeable. The pipettes used in the reagent preparations and in placing the reagents into the flasks were of "exax" quality.

The combination constant temperature bath and shaking apparatus used was the 10-manometer instrument manufactured by the Precision Scientific Instrument Company. The water bath consisted of a tank of about 10-gallon capacity with a mixer that allowed vigorous stirring of the water to give equal warming to all flasks. Heating coils were controlled by a thermostat by which it was possible to hold the temperature to ± 0.01° C. The temperature selected for all experiments was 37.5° C. The shaking assembly consisted of separate brackets for each manometer arm which moved in a back and forth motion by an arm connected to a gear drive that could be set for varied speeds. The shaking speed used for all experiments was 120 to 130 complete strokes per minute.

Calibration of the Flasks and Manometers

All of the flasks and manometers used in this study were calibrated so that the flasks would be interchangeable with one another. Calibration was accomplished by Grisolia's modification of Schale's method (Umbreit, 1949). The steps involved in the calibration are given in detail by Umbreit, et al. (1944) and consist essentially of adding clean mercury to a flask and seating it firmly on each manometer ground glass joint so that the level of the mercury coincided with a mark previously

placed on the manometer arm. By weighing this mercury and dividing the weight obtained by the density of the mercury at the temperature at which it was weighed, the volume of the flask up to the mark was determined. In a similar fashion the volume from the mark to some reading on the manometer was determined, as well as the volume between two divisions of the manometer, (modification of Loomis, 1949). The volume from the 150 mm. reading to the mark was then constant for all flasks and the volume of each flask to the mark was all that needed to be determined to give the total volume of the entire system to the "zero" setting.

Reagents

The reagents that were employed for both types of tissue were Krebs-Ringer phosphate buffer pH 7.4, normal saline, 0.01 M glucose, 20 percent potassium hydroxide, and suspensions of testosterone proprionate and methyl testosterone. The Krebs-Ringer phosphate buffer was prepared as follows:

a. 100 parts of 0.154 M sodium chloride
b. 4 parts of 0.154 M potassium chloride
c. 3 parts of 0.11 M calcium chloride
d. 1 part of 0.154 M potassium dihydrogen phosphate, KH_2PO_4
e. 1 part of 0.154 M magnesium sulphate, $MgSO_4 . 7\ H_2O$
f. 12 parts of 0.1 M phosphate buffer prepared by diluting 17.8 grams of disodium monohydrogen phosphate $Na_2HPO_4 . 2\ H_2O$ plus

20 ml. of 1.0 N. hydrochloric acid (HC_1) to one liter. The completed buffer was then aerated by shaking it in a 250 ml. flask for five minutes. Stock solutions of reagents a. through e. were made five times the concentration of the working solutions.

These concentrated solutions, according to Umbreit et al. (1949) are stable for months. No information was found on the stability of the working buffer solution. It was assumed that the buffer with re-aeration before use would be stable for at least 48 hours. Usually, however, a fresh buffer solution was made up for each experiment.

The normal saline solution was prepared from the same stock solution of sodium chloride as the buffer. The glucose solutions used in the experiments varied in their concentration of glucose. For the comb experiments a 0.06 Molar solution was prepared so that when 0.5 ml. was added to the other flask contents the concentration would be 0.01 Molar. For the bone marrow experiments a 0.04 Molar solution of glucose was prepared so that 0.5 ml. of the solution in a total volume of 2.0 ml. gave a final concentration of 0.01 Molar. Fresh glucose solutions were made up weekly. All of the solutions except the potassium hydroxide were refrigerated. The suspension of testosterone proprionate was prepared by weighing crystals of the hormone and suspending them in distilled water. A 4.36×10^{-4} Molar solution was prepared and aliquots were made from this solution so that the flasks had concentrations ranging from 1.45×10^{-4} M to 4.36×10^{-4} M. The suspension of testosterone proprionate was not very stable but it was found that if the hormone suspension was shaken vigorously as is done in the Klein test then the suspension was stable enough

for pipetting. The methyl testosterone suspension seemed to be much more stable. Tablets were used which contained 10 mg. of methyl testosterone with a filler of "polyhydrol". No information was obtainable on the composition of the filler; however, a fresh suspension of the hormone when added to flasks without tissue and read over a three-hour period gave readings that were within the range of errors from the readings themselves. A tuberculin syringe of 2.0 ml. capacity was used with some experiments to obtain a more even suspension of hormone. This syringe with a long needle was also used to dispense the hormone into the flasks. A 6.6×10^{-3} M solution of methyl testosterone was prepared and aliquots were made from this solution so that the flasks had concentrations of 4.4×10^{-4} M to 1.1×10^{-3} M. Only methyl testosterone was used in the bone marrow experiments. For these experiments aliquots of the 6.6×10^{-3} M stock solution of methyl testosterone were made so that the flasks varied in concentration from 3.3×10^{-4} M to 1.6×10^{-3} M.

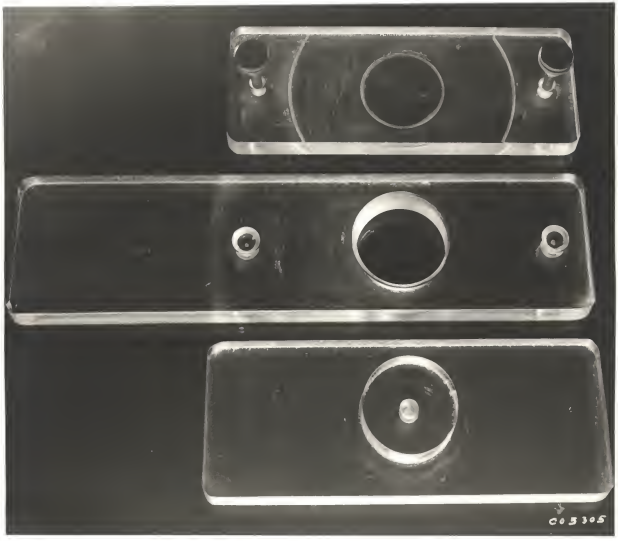
Apparatus Used in the Comb Experiments

The apparatus that was used only in the comb experiments and not previously described included a Stadie-Riggs slicing microtome and a drying oven. The slicer is illustrated in Plate III. The microtome consisted of three sections of plastic, a blade, and a holder. The top of the microtome was held to the mid-piece by means of two milled head bolts. A depression in the top section was machine ground to a certain depth so that all slices were constant. The mid-section had a hole approx-

EXPLANATION OF PLATE III

Photograph of the Stadie-Riggs slicing microtome showing the base portion, (bottom figure), with raised portion that fits into the mid-piece (middle figure), and presses the tissue to be sliced against the upper portion, (top figure). Two screws, shown seated in the upper portion, connect the two portions, upper and mid, with each other and the space between, seen as a larger circle in the upper figure, is where the blade is inserted.

PLATE III



imately four cm. in diameter that corresponded closely to the diameter of a raised portion of the base section. A round piece of filter paper, slightly less in diameter than the raised portion of the base, was moistened with cold saline or buffer and then placed on the pedestal of the base. The comb tissue to be sliced was then placed on the filter paper. The blade, by means of its holder, was then slipped between the two upper sections. The upper sections of the microtome were then gently pressed down on the tissue to bring the tissue up flush with the depression in the upper section. The blade was then drawn through the tissue in a to-and-fro motion. This left the tissue slice within the small depression of the upper section. The tissue slice was then placed in a small beaker of buffer set in ice, by means of small glass rods drawn out and curved at the ends. The drying oven used for the drying of these slices was thermostatically controlled and set to 105° C. ± 5. Samples to be dried to constant weight were placed in the oven for 2 hours at this temperature. To evaluate the completeness of the drying procedure, the slices from the first experiments were removed at 2, 2½, and 3 hours drying time and reweighed. The weight was found to remain constant within the error of the scales themselves.

Apparatus Used in the Bone Marrow Experiments

Exclusive of the instruments previously discussed, additional apparatus that was used only for the marrow experiments included also a syringe and filter assembly. The syringe used was a 10 ml. Luer-Lok

type connected to a Swinney B-D syringe type filter. Plate IV is a composite diagram of the filter adapter with the portions in line as they are when the parts are connected together. Essentially, the adapter is placed between the syringe tip and the needle. A circular metal screen with a diameter of 13 mm. and pores of 1 mm. by 1 mm. is set inside the adapter and locked in place by means of a rubber washer. Further description of the use of the filter assembly will be covered under the preparation of tissue for analysis.

Preparation of Comb Tissue for Analysis

Combs from three types of birds were used in these experiments. The three types correspond to the three conditions; normal, implanted with estrogen, and surgically castrated. The three types were different in the amount and size of the sample of tissue that could be obtained for analysis.

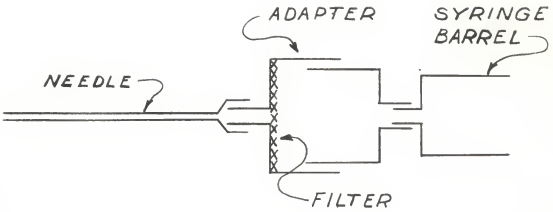
The combs varied in size from the largest, normals; the next largest, implants; and the smallest, the surgical capons. So that representative slices would be obtained from each type a cross-section slice of the broadest portion of the last point of each comb was taken for analysis. One full slice from the normals and implants and three full slices from castrates were used in each flask for analysis.

Previous to excision of tissue, the birds were immobilized by straps placed around the legs and wings. The comb was then cleaned with tap water and a cotton sponge. Surgical castrates and some of the normal

EXPLANATION OF PLATE IV

Diagrammatic illustration of the Swinney B-D syringe type filter adapter. The filter has a diameter of 13 mm. and pores of 1 mm. by 1 mm.

PLATE IV



birds were decapitated and the entire head and comb was placed on a section of ice for selection and removal of a representative section of tissue. For most of the normal birds and implants used in this study, however, a sharp razor was used to remove the appropriate area of comb tissue. The comb tissue or the entire head was then placed on ice and the portion of comb selected was trimmed into a block of tissue approximately 1 cm. by 1 cm. This block of tissue was then placed on the moistened filter paper on the raised portion of the base of the Stadie-Riggs microtome. The mid and upper sections of the microtome were then placed over the block of tissue and gentle pressure was applied. With practice it was found that slices were most uniform when only enough pressure was applied to allow the tissue to flatten itself against the upper section of the microtome. Eight or nine slices were made depending on the type of comb. Each slice was removed from the microtome by slipping a glass rod, previously drawn out and curved at the end, under the slice after which the slice was removed to a small beaker of cold buffer placed in an ice bath. Timing of the entire slicing procedure revealed that an average of 10 minutes was required from the initial removal of the comb until the beginning of the equilibrium period, the maximum time required was 15 minutes. The slices in ice cold buffer were then removed with the glass rods a slice at a time to previously prepared flasks. The flasks had previously been prepared by following the steps outlined below and in the order given:

(a) The center walls were rimmed with stopcock grease.

(b) 0.2 ml. of 20 percent KOH was placed in each center well except

the flask set aside for the thermobarometer.

(c) 2.0 ml. of buffer was added to each flask.

(d) Hormone additions of 0.5 ml. hormone suspension to 0 ml. were made.

(e) Saline was then added to correct all values to 3.0 ml.

(f) The tissue sample was then added. 1 slice approximately 1 cm. by 1 cm. or when surgical castrate combs were used, 2 slices of .5 cm. by .5 cm.

(g) Filter paper strips 1 cm. by 1 cm., or 1 cm. by 1.5 cm., folded accordion fahsion were then placed in the alkali cup with the aid of forceps. Because the flasks varied in the depth of the center cup, the strips were selected so that a uniform amount of filter paper extended upwards from the cup in order to expose a uniform amount of surface to the gas environment of the flask.

The flasks were then connected to the manometers, after sealing the ground glass joints, and secured with small, tension springs. The manometers, with open stopcocks and the manometer fluid set to 150 mm., were then connected to the bracket of the shaking apparatus in which position the flasks were immersed in the water bath. The shaking apparatus was then turned on and timing begun. During the 15 minute equilibration period the setting of the manometer fluid was rechecked and the stopcocks were closed. The flasks with the attached manometers then constituted a closed system. Readings were then made every ten minutes for the first hour and hourly thereafter for a total of three hours. At the completion

of this period the stopcocks were then opened to the atmosphere, the manometer and flask assemblies removed from the shaking bracket, and the flask disconnected from the manometer. A curved glass rod was then used to remove the slices which were first placed on filter paper to absorb excess water and then placed in vials that had been previously weighed and numbered. The vials were weighed and then placed in the drying oven for two hours at 105° C., after which they were re-weighed and by subtraction of the initial weight of the dry vials the dry weight was determined.

Preparation of Bone Marrow for Analysis

The birds used for the bone marrow experiments were not immobilized as with the comb experiments. After all flask and weighing preparations had been made, the bird selected for examination was quickly decapitated, and both the posterior appendages were removed in their entirety. The outer skin was then stripped down over the feet. A pair of fine pointed scissors was then inserted between the gastrocnemius muscle and the tibia, and the tendons and musculature were removed. The bones were then scraped free of the periosteum covering. A heavy pair of scissors was then used to cut through the bone at the point of the insertion of the fibula to the tibia, and at a point approximately 1½ to 2 cm. below the reference point. A forceps was then used to pick away from the bone sections any fragments of bone after which both bone sections were placed in a previously weighed 50 ml. beaker and the first weighing

was obtained (A). One of the sections of bone was then removed and a hollow probe with an outside diameter of approximately 0.2 cm. was inserted carefully into one end of the bone section. By gradually inserting the probe it was found that the whole core of bone marrow could be removed with little disruption of the tissue. The bone marrow was allowed to fall directly into a test tube that contained 3.0 ml. of phosphate buffer and that was set in an ice bath. The first bone section, minus the marrow, was then returned to the beaker and the second section was handled the same way. Both sections of bone, minus the marrow, were then reweighed to obtain (B). (A-B) then equaled the wet weight of the marrow in the 3.0 ml. of buffer. The amount of buffer that remained to be added to make a 10 percent by weight suspension was calculated by multiplying the wet weight of the tissue by 9 and subtracting the 3.0 ml. already in the test tube. After this amount of buffer was added, the contents of the test tube, usually between 6 to 8 ml., was transferred to the barrel of a 10 ml. syringe fitted with the Swinney filter. The plunger was then inserted and gentle pressure was applied. Only two runs were found necessary to achieve an even suspension of cells. This technique is in contrast to other studies on bone marrow using suspension techniques. Von Breza (1926) teased rabbit bone marrow into a suspension and measured the oxygen uptake. Fujita (1928) formed a suspension of cells by shaking the marrow in ringer-phosphate solution. Using this technique the uptake fell to zero in 3 hours. Orr and Strickland (1938) also used a shaking technique and found that in contrast to Fugita's work respiration proceeded linearily and was not unstable as Fugita maintained. Goldringer, et al. (1947) compared the respiration of bone marrow using ground

tissue, teased and shaking procedure, and slices. Grinding the tissue in Ringers phosphate buffer decreased the respiration by one-half from that found when serum was used in place of buffer. Teasing the tissue and shaking it further decreased the rate. Teased preparations also were not constant after the first half-hour, whereas slices remained constant throughout the two hours during which it was measured. Glucose additions up to 0.01 Molar did not affect respiration. This was also confirmed by Evans and Bird (1949). Glucose is utilized, however, as was shown by Bird, et al. (1949) and the aerobic glucose can be accounted for by the oxygen consumption. Landau and Gordon (1952) used a syringe and a 20 gauge needle for preparing marrow for respiration studies. All of these techniques were attempted in deciding what method to use for the preparation of bone marrow. Teasing and shaking the marrow left the suspension in large clumps. Landau and Gordon's method of using a syringe produced an even suspension that could be easily pipetted. Extreme force was necessary, however, to expel and draw the tissue through the syringe. By using the syringe filter adapter, however, a minimum of force was required and a suspension was produced that was free of large clumps of tissue and did not separate readily.

After preparing the suspension, it was pipetted into the reaction flasks. A total volume of 2.2 ml. was used with the marrow experiments. The flasks were prepared as follows, with the components listed in the order in which they were added.

(a) The alkali cups of the flasks were rimmed with stopcock grease.

(b) 0.2 ml. of 20 percent potassium hydroxide was placed in the alkali cups.

(c) 0.5 ml. of 0.04 Molar glucose was added to the flask.

(d) Hormone additions were made from 0.0 ml. to 0.5 ml. which corresponded to additions of from 0 to 1.1 X 10^{-3} Molar.

(e) Saline was then added to correct all flasks to exactly 1.0 ml.

(f) 1.0 ml. of the bone marrow suspension was then added to the flasks.

(g) From this step on the treatment of the flasks corresponded with that of the comb tissue experiments except that at the end of the 3-hour period the flasks were checked only for hemolysis and then rinsed out.

Theory of the Warburg Constant Volume Respirometer

The term "respiration" was used originally for the actual uptake of gaseous oxygen. Oxidations can occur, however, without the actual uptake of oxygen, (by removal of hydrogen or electrons). The term respiration is still used, however, for both methods of obtaining energy. In this paper respiration is taken as meaning the actual uptake of gaseous oxygen.

Utilization of oxygen results, in most tissues, in the simultaneous release of carbon dioxide. When tissue slices or suspensions of cells are placed in a flask, carbon dioxide is produced as the oxygen in the flask is absorbed.

If a method is available for the removal of one of the gases then the concentration of the other gas can be measured by a manometer, pro-

viding only these two gases are changing. With a closed system such as the Warburg apparatus alkali is commonly used to absorb the carbon dioxide produced so that manometer readings are then due to the change in the concentration of oxygen. When used with alkali to absorb the evolved carbon dioxide and when another flask and manometer with only a slight amount of water is used as a thermobarometer, the method is called the "Direct Method" of determining oxygen uptake. Not only the amount of oxygen but the rate at which the oxygen is taken up may be determined by this method.

Knowing the volumes of the flask and manometer arms to the zero mark, the temperature of operation, the density of the manometer fluid, and the solubilities of the gases exchanged in the suspending media, it is possible to calculate a flask constant. Manometer readings, when corrected by the readings of the thermobarometer, can then be multiplied by this constant to give the amount of oxygen absorbed directly. The formula used for the calculation of this constant is:

$$X = h\left[\frac{V_g \frac{273}{T} + V_f A}{P_o}\right]$$

Where:

X = the amount of oxygen taken up by the flask contents in mm^3 at standard temperature and pressure;

h = the manometer reading in mm. minus the manometer reading of the thermobarometer;

V_g = the volume of the flask and manometer arm to the zero point in mm^3;

V_f = the volume of all the fluid used in the flasks including the hydroxide used to absorb the carbon dioxide and is expressed in mm^3;

T = the temperature at which the apparatus was maintained and is expressed in degrees absolute;

P_o = the standard pressure expressed in terms of the manometer fluid. Brodie's fluid has a density of 1.033 so that

$$P_o = \frac{\text{density of mercury}}{\text{density of Brodie's fluid}} = 10{,}000.$$

A = the solubility of oxygen in the suspending media and is expressed in mls. of oxygen per ml. of fluid. "A" is sometimes referred to as the Bunsen Coefficient.

The temperature selected for measurement of the tissue samples was 37.5° C.

The solubility of oxygen is dependent on the temperature and on the presence of dissolved solids in the fluid. As the temperature rises, the solubility of oxygen decreases. Dissolved solids cause a decrease in the solubility of oxygen in the fluid.

No complete charts are available for determining the solubility of oxygen in buffers that contain many different substances. From a study of the tables in Umbreit, et al. (1949, p.45) the solubility was taken as 0.027.

In these experiments the uptake of oxygen is reported in mm^3 and the rate is expressed as Q_{O_2}. According to convention, Q_{O_2} refers to the uptake per unit of dry weight per hour. The Q_{O_2} of comb tissue in

these experiments is then in terms of the uptake of oxygen in $mm^3/1.0$ mg. of dry weight/hour. Bone marrow respiration according to convention should be listed as $Q_{O_2}^{air}$ • suspension. In these experiments, however, the rates will be identified only by the designation Q_{O_2} and will refer to the uptake of oxygen in $mm^3/1.0$ ml. of 10 percent suspension of bone marrow cells/hour.

For the measured respiration to be representative of the true tissue values there are three main criteria that must be carefully controlled to prevent them from becoming limiting in the reaction. These factors are: a. the rate of diffusion of the oxygen into the suspending media; b. the rate of absorption of carbon dioxide; and c. the diffusion of oxygen into the tissue, which is dependent on the thickness of the tissue slice.

The rate of diffusion of oxygen into the suspending media will become limiting if: a. too much tissue is taken for analysis so that the oxygen content of the media is rapidly diminished; and b. if there is not a constant and fairly rapid change in the surface of the media so that diffusion of the oxygen molecules into the media can take place. Between 100 and 200 mgs. of tissue (wet weight) was taken for analysis. This amount is well within the limits for the apparatus. The second limiting factor, that of change in the surface of the media, is due primarily to the speed at which the flask is agitated by the shaking apparatus. 120 - 130 strokes per minute was the speed used for these experiments and is slightly higher than most tissue experiments.

The rate of absorption of carbon dioxide must be rapid when tissue

respiration is measured. The method ob absorbing carbon dioxide in these experiments allowed absorption to take place up to 1000 mm^3/ hour, which is far over the amount produced by comb tissue.

The third main criteria for the accurate determination of tissue respiration is that the thickness of the tissue must not be great enough to limit the diffusion of the oxygen from the media into the center of the tissue slice. Warburg (1923) devised the following formula to calculate the maximum thickness of tissue slices that would still allow representative respiration to occur.

$$d' = \sqrt{8\, C_o\, \frac{D}{A}}$$

Where:

- d = the thickness of the tissue slice in cm. at which the oxygen concentration at the center layer of the slice is just zero,
- C_o = the concentration of oxygen immediately outside the tissue slice expressed in atmospheres,
- D = the diffusion constant of oxygen in the tissue in cm^3 of oxygen (at S.T.P.) per cm^2 per minute when the pressure gradient is 1 atmosphere per cm.,
- A = the rate of respiration of the tissue expressed as cm^3 per minute per cm^3 of tissue.

D is equal to 1.4×10^{-5} at 38° C. C_o is equal to 0.2 when air is used. (Dixon, 1951, p. 57) The volumes of representative samples were determined by spreading the tissue slice on a sheet of hard graph paper and lightly outlining the slice. A Planimeter was then used to measure

the areas representated by these tracings. A number of measurements were made using the fine scale of a microscope to determine the depth of the depression of the Stadie-Riggs slicer more accurately. The depth was found to vary somewhat but averaged about .4 mm. (measurements ranged from .3 to .5 mm.). Only tissues without hormone added to the flasks were used in the calculation of the allowable thickness. The d' value found was .52 mm. so that air was permissible for these tissues. To make absolutely certain that the thickness of the tissue was not limiting, the speed at which the apparatus was shaken was increased from the normal 110-115 strokes per minute to 120-130 strokes per minute.

RESULTS AND DISCUSSION

The results of the experiments are listed according to groups in Tables 1 through 7. In each table are listed the oxygen quotient found at the 120-minute and the 180-minute periods. Only the values at the 180-minute period were analyzed statistically. The statistical analysis consisted of analysis of variance as illustrated by Snedecor (1946).

In both the comb and the bone marrow there were wide variations in values of duplicates, values obtained from different dosages, and variations of response among birds. Relatively low values were obtained for the rates of respiration of comb slices. The average value for slices of normal cocks combs was 1.16 (Table 4). Castrate combs were 0.79 (Tables 2 and 3) and estrogen treated birds were 0.73 (Table 2). The difference in the rate of respiration of normal and castrate, and normal and estrogen-

treated birds was significant. The difference between castrates and estrogen-treated birds had only slight significance.

The rates of respiration of comb slices from normal cocks, with and without flask treatment with methyl testosterone are listed in Table 1. Analysis of this group indicated that there were no significant treatment differences. The birds themselves are significantly different. The mean of this group of birds without flask treatment was 1.05. Since methyl testosterone did cause significant differences in other groups, the failure of normal comb tissue to respond to treatment indicates a refractory state of the tissue to androgen.

The rates of comb slices of castrate cocks and estrogen-treated cocks with and without flask treatment with methyl testosterone are listed in Table 2. Analysis of these groups indicated that the rates of comb slices from castrate cocks were not significantly different from the rates of comb slices of estrogen-treated cocks. The mean of the rates of comb slices from castrate combs without additions of methyl testosterone in Table 2 and those of Table 3 was 0.79. Methyl testosterone additions (Table 2) significantly increased the rates of respiration of these birds.

Different patterns of response occured in the values from estrogen-treated cocks combs. At the 9-day period the mean of tissue with no hormone added to flasks was 0.92. Additions of methyl testosterone increased the rate of oxygen consumption to 1.70. At the 10 and 11 day period the values either did not show any great deviation from the untreated slices or the values decreased. At the 15-day period the flask treatment again caused a high increase in the rates.

Table 1. Rates of oxygen uptake of combs from normal cocks with and without flask treatment with methyl testosterone expressed as Q_{O_2} (mm^3 oxygen/1 mg. dry weight/hour).

Bird number	Treatment Level*					
	(0)	(1)	(2)	(3)	(4)	(5)
120 minute period						
21	1.05	.63	.57	.48	.44	.53
22	.92	1.42	.92	.58	1.17	.91
40	.91	1.38	.77	1.00	1.66	1.29
41	.90	1.60	1.32	2.50	2.35	2.74
Means	.94	1.26	.90	1.14	1.40	1.39
180 minute period						
21	1.04	.50	.57	.52	.48	.60
22	.98	1.33	.95	.91	1.28	1.06
40	.91	1.20	.71	.86	1.86	1.04
41	1.26	1.83	1.56	2.94	2.86	3.12
Means	1.05	1.22	.95	1.31	1.62	1.46

* Treatment levels:

(0) No Hormone added to flasks;
(1) 2.2×10^{-4} Molar
(2) 4.4×10^{-4} Molar
(3) 6.6×10^{-4} Molar
(4) 8.8×10^{-4} Molar
(5) 1.1×10^{-3} Molar

Table 2. Rates of oxygen uptake of combs of castrate and estrogen-treated birds with and without flask treatment with methyl testosterone, expressed as Q_{O_2} (mm^3 oxygen/1 mg. dry weight/hour).

Type of comb and bird no.		Treatment level* (0)	(1)	(2)	(3)	(4)	(5)
120 minute period							
Castrate	35	.25	.38	.29	.89	.84	1.45
Castrate	36	.76	.74	1.50	1.52	.31	5.04
Means		.50	.56	.90	1.20	.58	32.4
9 day							
Estrogen	29	.67	.82	1.45	1.69	1.69	1.56
Treatment	30	.54	.34	.48	.61	.81	1.64
10 day							
Estrogen	23	.29	.50	.13	.46	.62	.31
Treatment	24	.64	.51	.58	.46	.37	.24
11 day							
Estrogen	25	.78	.41	.52	.26	.18	.28
Treatment	26	.51	.84	.59	.95	-	.24
15 day							
Estrogen	31	.52	.66	1.44	.53	1.34	2.87
Treatment	32	.50	1.23	2.46	1.17	1.44	1.88
Means		.56	.66	.96	.77	1.08	1.13
180 minute period							
Castrate	35	.78	.78	1.38	1.46	.25	4.81
Castrate	36	.54	.61	.40	.98	1.04	1.68
Means		.66	.70	.89	1.22	.64	3.24
9 day							
Estrogen	29	1.29	1.07	1.96	2.38	2.37	1.95
Treatment	30	.55	.36	.44	.74	.80	1.44
10 day							
Estrogen	23	.30	.41	.10	.38	.59	.30
Treatment	24	.62	.50	.53	.49	.38	.33
11 day							
Estrogen	25	1.08	.43	.47	.24	.30	.36
Treatment	26	.56	.80	.60	.86	-	.26
15 day							
Estrogen	31	.70	.69	1.29	1.64	1.48	2.99
Treatment	32	.74	1.28	2.57	1.23	1.56	2.01
Means		.73	.69	1.00	1.00	1.09	1.20

* Treatment levels:

(0) No hormone added to flasks
(1) 2.2 X 10-4 Molar
(2) 4.4 X 10-4 Molar
(3) 6.6 X 10-4 Molar
(4) 8.8 X 10-4 Molar
(5) 1.1 X 10-3 Molar

Table 3. Rates of oxygen uptake of castrate combs with and without flask treatment with testosterone proprionate, expressed as Q_{O_2} (mm3 oxygen/1 mg. dry weight/hour).

Bird Number	Treatment level*			
	(0)	(1)	(2)	(3)
120 minute period				
18	.64	.44	.25	.83
	1.60	.77	.66	.64
19	1.08	1.98	.87	1.00
	1.67	1.69	.95	1.12
20	.64	1.14	.72	.95
	.68	.86	.84	1.78
Means	1.05	1.15	.72	1.05
180 minute period				
18	.67	.63	.23	.71
	1.01	.66	.82	.54
19	1.07	1.58	.78	1.00
	1.42	1.61	.89	1.16
20	.45	1.15	.72	.98
	.69	.86	.92	1.59
Means	.88	1.08	.73	1.00

* Treatment levels:
(0) No hormone added to flasks
(1) 1.45×10^{-4} Molar
(2) 2.9×10^{-4} Molar
(3) 4.4×10^{-4} Molar

Table 3 lists the rates of comb slices from castrates with and without flask treatment with testosterone proprionate. Treatment with testosterone proprionate caused slight differences which had only borderline significance. This slight difference is in contrast to the effect methyl testosterone had on respiration (Table 2).

In Table 4 are listed only the rates of respiration of comb slices from normal cocks. Analysis of this group of birds revealed that the birds themselves were not significantly different from each other. The mean of this group at the 180-minute period was 1.16.

Table 4. Rates of oxygen uptake of combs from normal cocks; no flask treatment with hormone, expressed as Q_{O_2} (mm^3 oxygen/1 mg. dry weight/hour).

Period	Bird Number				
	12	14	16	38	13
120 minute period	1.06	.92	1.46	.81	1.41
	1.74	1.00	1.06	1.16	1.52
					1.23
					.66
					1.12
					1.22
Means	1.40	.96	1.26	.98	1.19
180 minute period	1.00	1.10	1.52	.91	1.25
	1.64	1.04	1.07	1.14	1.22
					1.09
					.65
					1.20
					1.12
Means	1.32	1.07	1.30	1.02	1.09

Kosin and Munro (1942) found that the growth of the comb of chicks under the stimulation of testosterone proprionate was accompanied by an increase in moisture content. Testosterone treatment increased the ratio of wet to dry weight from 4.0 : 1 (normal untreated chick combs) to 7.1 : 1. Estradiol did not cause any significant change in the moisture content. In this study the combs from normal adult cocks had a ratio of 12.7 : 1. The combs from estrogen-treated cocks had a ratio of 5.3 : 1 after nine days of estrogen treatment and 7.0 :1 after 15 days of estrogen treatment. Combs from castrate cocks had a ratio of 4.1 : 1.

Table 7 lists the values found for bone marrow suspensions from nor-

mal cocks. Analysis of this group indicated that there was a highly significant interaction between birds and treatments. The pattern of response of the birds to the different treatments was not consistent.

Tables 5 and 6 list the values found for bone marrow suspensions for two groups of estrogen-treated cocks. Table 5 lists those for cocks of 6 months of age and Table 6 lists those for cocks of 3 months of age. The values when no hormone (methyl testosterone) was added to the flasks are similar for the normal cocks and the 3 and 6 month old groups. There was a significant interaction between birds and treatments in the normal cocks and the 3 month old estrogen-treated group. The older birds, however, showed a highly significant response to treatment (+ 40.1 over the mean of the flasks without hormone).

SUMMARY

1. The Q_{O_2} of comb slices from normal cocks was found to be 1.16, from castrate birds 0.79, and from estrogen-treated cocks 0.73.
2. The rate of respiration of comb slices from castrate cocks was increased by additions of methyl testosterone in the range 4.4×10^{-4} M. to 1.1×10^{-3} M., but not by testosterone proprionate up to 4.4×10^{-4} M.
3. Methyl testosterone did not affect the Q_{O_2} of slices from combs of normal animals.
4. The period of time that cocks were treated with estrogens caused different patterns of response of comb slices to flask additions of methyl testosterone. Nine-day implants were increased by flask

Table 5. Rates of oxygen uptake of bone marrow suspensions from estrogen-treated birds (6 months of age) with and without flask treatment with methyl testosterone, expressed as Q_{O_2} (mm^3 oxygen/1 ml. of 10 percent suspension/hour).

Bird Number	Treatment level*		
	(0)	(1)	(2)
120 minute period			
42	21.4	27.3	20.2
	33.1	36.2	-
43	30.1	24.6	60.5
	24.4	25.0	35.2
45	17.4	35.9	80.6
	51.1	32.0	78.6
47	6.6	24.0	59.4
	10.4	22.7	56.0
Means	24.3	28.5	55.8
180 minute period			
42	24.3	39.0	49.5
	34.8	44.5	50.0
43	35.8	38.0	83.3
	29.5	38.7	58.5
45	16.8	45.7	88.0
	64.7	37.1	91.0
47	7.9	26.2	63.5
	9.3	24.0	60.5
Means	27.9	36.6	68.0

* Treatment levels:
(0) No hormone added to flasks
(1) 6.6×10^{-4} Molar
(2) 3.3×10^{-3} Molar

Table 6. Rates of oxygen uptake of bone marrow suspensions from estrogen-treated birds (3 months of age) with and without flask treatment with methyl testosterone expressed as Q_{O_2} (mm^3 oxygen/1 ml. of 10 percent suspension/hour).

Bird Number	Treatment level		
	(0)	(1)	(2)
120 minute period			
55	31.4	28.6	-
	-	43.2	80.6
57	22.1	-	84.6
	43.6	25.8	141.8
58	25.6	22.2	15.4
	47.3	20.2	21.9
59	28.5	16.3	13.9
	20.9	22.0	11.1
Means	33.3	25.5	52.8
180 minute period			
55	30.7	27.5	79.7
	30.0	36.5	80.0
57	16.4	32.4	59.3
	27.0	32.0	109.1
58	24.9	20.2	15.3
	47.6	18.5	19.2
59	29.0	17.1	18.2
	21.7	27.1	13.2
Means	28.4	26.4	49.2

* Treatment levels:
(0) No hormone added to flasks
(1) 6.6×10^{-4} Molar
(2) 3.3×10^{-3} Molar

Table 7. Rates of oxygen uptake of bone marrow suspensions from normal cocks with and without flask treatment with methyl testosterone, expressed as Q_{O_2} (mm^3 oxygen/1 ml. of 10 percent suspension/hour).

Bird Number	Treatment level*		
	(0)	(1)	(2)
120 minute period			
44	17.4	30.8	93.8
	7.0	35.2	114.5
49	34.2	-	18.8
	66.4	44.2	66.0
50	3.3	2.6	-
	16.9	1.6	3.1
52	11.7	15.3	11.4
	28.4	21.8	17.2
60	42.9	9.3	15.4
	30.4	17.3	4.8
Means	25.8	19.8	38.3
180 minute period			
44	15.1	33.7	103.0
	7.5	34.0	127.0
49	23.9	3.7	14.3
	59.0	40.3	59.9
50	4.4	3.2	.7
	17.5	2.8	4.6
52	11.3	12.5	10.8
	27.0	18.8	13.9
60	42.5	7.7	14.6
	26.7	13.7	4.6
Means	23.6	17.0	35.3

* Treatment levels:

(0) No hormone added to flasks
(1) 6.6 X 10-4 Molar
(2) 3.3 X 10-3 Molar

treatments, 10 and 11-day implants either showed no increase or a slight decrease, and 15-day implants were increased by additions of methyl testosterone.

5. The Q_{O_2} of comb slices from castrates was similar to that of estrogen-treated cocks.

6. The ratio of wet to dry weight of combs from normal cocks was 12.7 : 1, from castrates 4.1 : 1, from 9-day estrogen-treated cocks 5.3 : 1, and from 15-day estrogen-treated cocks 7.0 : 1.

7. Similar rates of respiration of bone marrow suspensions were found for normal, three and six month old estrogen-treated cocks with no methyl testosterone added to the flask.

8. The rate of respiration of bone marrow from 6 month-old, estrogen-treated cocks was significantly increased by additions of methyl testosterone. The Q_{O_2}'s of bone marrow from 3 month-old, estrogen-treated cocks were only slightly increased and normal cocks were not affected.

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OXYGEN CONSUMPTION OF BONE MARROW AND COMB TISSUE OF CAPONS AND NORMAL CHICKENS AND THE IN VITRO EFFECT OF ANDROGENS

by

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There have been numerous investigations on the action of androgens on bone marrow and comb tissue. Bone marrow responds to stimulation by androgens with hyperplasia of erythroid elements and a resulting increase in erythrocyte number and hemoglobin content in the circulating blood. Comb tissue responds to stimulation by androgens with increased water content and proliferation of cells.

Numerous theories have been advanced to explain the effects of androgens on the tissue elements. Most of the evidence points to the following modes of action as the most probable:

1. The androgens have a direct effect on the tissue elements of the comb and bone marrow.

2. The effects of androgens are a result of a general increased metabolic rate, that results from androgen administration.

3. The effects of androgens are a result of both a direct and an indirect effect. This indirect effect may come from an increased metabolic rate due to androgens or, the androgens may act on the thyroid, adrenal, and hypophysis, which then increases the metabolic rate and so affects the bone marrow and comb tissue.

These experiments were conducted to determine whether androgens had an in-vitro effect on the respiration of bone marrow suspensions and comb tissue slices. The bone marrow values are not comparable to other studies since respiration was based on mm3 oxygen per 1.0 ml of a 10 percent by weight suspension of cells per hour. Most bone marrow respiration rates are reported in terms of fat-free-dry weight, cellular composition, or nitrogen content. The rate of respiration, Q_{O_2}, of comb

tissue was calculated in terms of mm3 oxygen consumed per 1.0 mg. dry weight per hour.

Normal cocks, castrate cocks, and estrogen-treated cocks were used in the experiments. The combs of the estrogen-treated cocks were partially removed following 9, 10, 11, and 15 days of estrogen treatment (30 mg. diethylstilbesterol implanted subdermally in the neck region). Thirty days after the partial removal of the comb these birds were again used for the bone marrow experiments. The normal cocks were also re-used for the marrow experiments after a 30-day recovery period. The castrate birds were not re-used since the entire comb was taken for slicing and although the birds probably would have survived, there is evidence that abnormal effects would occur. The bone marrow was taken from the combined tibias of the cocks at a point where red bone marrow predominated.

The average Q_{O_2} of comb slices was 1.16 from normal cocks, 0.79 from castrate cocks, and 0.73 from estrogen-treated cocks. The difference between normal cocks and castrates and between normal cocks and estrogen-treated cocks, however, was not significant. Slices of comb tissue from normal cocks did not respond to methyl testosterone additions from 2.2×10^{-4} M. to 1.1×10^{-3} M. (Molar). Castrate cocks did not respond to testosterone propionate from 1.4×10^{-4} M. to 4.4×10^{-4} M., but did show a response to methyl testosterone from 4.4×10^{-4} M. to 1.1×10^{-3} M. The responses of comb slices and bone marrow suspensions were not linear responses.

The failure of normal cocks comb tissue to respond was interpreted as being due to a refractory state of the normal comb. A similar condi-

tion is seen in-vivo.

Different patterns of response occured in the values from estrogen-treated cocks combs. At the 9-day period the mean of tissue, with no hormone added to flasks, was 0.92. Additions of methyl testosterone increased the rate of oxygen consumption to 1.70. At the 10 and 11 day period the values either did not show any great deviation from the untreated slices or the values decreased. At the 15-day period the flask treatment again caused a high increase in the rates.

Treatment of the White Leghorn chick with testosterone proprionate increases the ratio of wet to dry weight of tissue from 4.0 : 1 to 7.1 : 1. The combs of normal adult cocks used in this study had a ratio of 12.7 : 1. Castrate cocks combs had a ratio of 4.1 : 1. Estrogen-treatment decreased the ratio from 12.7 : 1 to 5.3 : 1 at 9 days, and 7.0 : 1 at 15 days.

The pattern of response to methyl testosterone by bone marrow from normal combs and from estrogen-treated cocks 3 months of age varied and was not consistent. The values with no additions were similar in all the groups, normal cocks, 3 month old estrogen-treated cocks, and 6 month old estrogen-treated cocks; 6.6×10^{-4} M. additions of methyl testosterone did not significantly affect any of the groups; additions of 3.3×10^{-3} M. however, markedly increased the respiration of the six month old estrogen-treated cocks, had no significant effect on the normal cocks and slightly increased the rate of the 3 month old group of estrogen-treated cocks.

SUMMARY

1. The Q_{O_2} of comb slices from normal cocks was found to be 1.16, from castrate birds 0.79, and from estrogen-treated cocks 0.73.
2. The rate of respiration of comb slices from castrate cocks was increased by additions of methyl testosterone in the range 4.4×10^{-4} M. to 1.1×10^{-3} M., but not by testosterone proprionate up to 4.4×10^{-4} M.
3. Methyl testosterone did not affect the Q_{O_2} of slices from combs of normal animals.
4. The period of time that cocks were treated with estrogens caused different patterns of response of comb slices to flask additions of methyl testosterone. Nine-day implants were increased by flask treatments, 10 and 11 day implants either showed no increase or a slight decrease, and 15-day implants were increased by additions of methyl testosterone.
5. The Q_{O_2} of comb slices from castrates was similar to that of estrogen-treated cocks.
6. The ratio of wet to dry weight of combs of normal cocks was 12.7 :1, from castrates 4.1 : 1, from 9-day estrogen-treated cocks 5.3 : 1, and from 15-day estrogen-treated cocks 7.0 : 1.
7. Similar rates of respiration of bone marrow suspensions were found for normal, three and six month old estrogen-treated cocks with no methyl testosterone added to the flask.

8. The rate of respiration of bone marrow from 6 month-old, estrogen-treated cocks was significantly increased by additions of methyl testosterone. The Q_{O_2}'s of bone marrow from 3 month-old, estrogen-treated cocks were only slightly increased and normal cocks were not affected.