CXVI. PROPERTIES OF THE COMB-GROWTH-PROMOTING SUBSTANCE OBTAINED FROM TESTES AND URINE.

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A series of publications has recently appeared concerning the preparation of comb-growth-promoting substances from testes and urine [Moore and McGee, 1928; McGee, Juhn and Domm, 1928; Moore, Gallagher and Koch, 1929; Funk, Harrow and Lejwa, 1930; Freud *et al.* 1930]. Similar work has been carried on in these laboratories and a method has been published [Dodds, Greenwood and Gallimore, 1930] for the preparation of the hormone in a water-soluble form. A study of the existing literature yields very little information concerning the properties of the active substance. The workers first mentioned proved that the active principle could be extracted from testes by means of benzene and that it was soluble in alcohol and ether. It is also stated that the active substance could be extracted by means of chloroform.

It was shown by us that the benzene-soluble material withstood saponification with barium hydroxide, and that after the insoluble barium soaps are removed the active substance is present in aqueous solution. In this communication the properties of this water-soluble material have been still further studied.

As in the previous communication the potency of the material has been tested on capons.

I. Method of testing for activity.

The earliest attempt to use the male castrated fowl as a material for the demonstration of the activity of testis extracts was made by Pézard [1911] who obtained the assumption of male characters and behaviour in a capon by injections of extracts from the testes of a cryptorchid pig. McGee, Domm and Juhn have employed the capon in an extensive assay of extracts of bull testis, the reaction taken as indicative of a positive result being the increased growth of the comb and wattles.

In the young cock, two types of growth in the head furnishings are found. In the first few weeks from hatching they increase in size at a rate relative to

increase in size of the body as a whole. Some time later, however, the rate of growth is suddenly accelerated and the comb and wattles become intensely red owing to the development and extension of the sub-epithelial vascular system. At this time the comb, which has previously been smooth, shows a roughened surface due to the projection from the surface of innumerable red papillae. The point at which this heterogonic growth occurs has been shown to be coincident with the onset of spermatogenetic activity in the testis. It varies considerably not only in different breeds but between different individuals of the same breed.

The extent to which this phenomenon is dependent on the presence of the reproductive glands is shown by the effect of prepubertal castration. Such early castration leads to the complete suppression of the second growth phase, although the first type of growth is not affected. In a complete castrate the comb becomes dry, scaly and anaemic, although it grows at a rate comparable to body growth as a whole and reaches its maximum about 30 weeks after hatching.

It is found that this second type of growth can be induced in castrated birds by injection of suitable preparations of mammalian testis.

Before proceeding to a description of the technique involved in the present series of experiments it is desirable to point out briefly certain precautions to be taken into consideration when using castrated fowls for such experiments.

If the birds are used before they reach the end of their growth period, then it is legitimate to accept as positive only those individuals in which the comb-growth after injection is such that it is thrown well outside the range of variation in comb size of control castrates of the same age.

Where extensive growth of the comb occurs a regression ought to ensue following cessation of the injections.

Increase in comb size should be coincident with the development of red papillae from the surface of the comb. These first appear at the base of the comb and spread upwards until even the points of the comb show a strong development of them.

From a review of castrated birds in the stock used for experimental purposes it was found desirable to discard any birds which at maturity exhibited a comb length greater than 5 cm., since in almost every case such birds had not been completely castrated but retained minute fragments of testis insufficient in amount at the time of *post mortem* examination to increase materially the size of the comb beyond that of the complete castrate, but it is always possible that further development of the testicular fragment some considerable time later may produce an increased comb growth. Many of these birds, while possessing the same size of comb as a complete capon, may yet be recognised by the tendency, more or less well expressed, to develop red papillae.

Since in the following experiments only a qualitative and not a quantitative result was sought, and further since the amount of extract available for use in

any one experiment was limited, only those birds were used which as adults possessed a comb falling within the previously defined limit of variation for castrates of the brown Leghorn stock used [Dodds, Greenwood and Gallimore, 1930]. The birds were bred at the department and castrated in the first few weeks after hatching (3–78 days). The combs were measured at fortnightly intervals during the development of the birds, and since the operation had never shown any development of red papillae characteristic of testicular activity, but were dry and scaly. As mature birds they had shown no signs of growth for several weeks prior to the experiments.

The most satisfactory measurement of comb size would be a volumetric determination, but, while such a method has been evolved, the great care necessary to obtain an accurate result and the time involved makes it impracticable when dealing with numerous experimental individuals. Moreover, in the case of small increments in comb size the chance of error is great. McGee, Domm and Juhn used the product of length and breadth as an expression of comb size and obtained an index of growth by dividing the initial product into the length-height products obtained during the course of the experiment. This method was rejected in the present series of experiments in favour of a simple measure of maximum length as an indication of increase in comb size because in the first place only qualitative results were desired

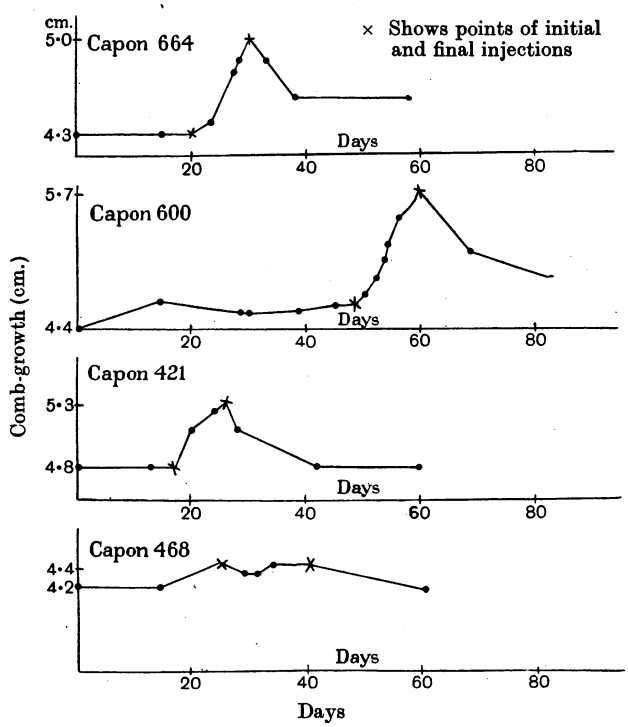

Fig. 1. Specimen charts showing record of comb-growth.

and it was found that increase in comb length was invariably accompanied by increases in height of the comb. Secondly, because, although two well-defined points are available for the accurate measurement of comb length, the measurement of height presents only one such well-defined point at the apex of the comb and an indefinite measuring point where the comb merges into the skin of the head. It was thought, therefore, that less error would be obtained where the single measurement was employed in view of the fact that relatively small increases in size were to be expected from the amount of extract at disposal for injection (Fig. 1).

The second criterion of activity of a given extract was the development of scarlet papillations on the otherwise dry scaly comb and the simultaneous sloughing off of the dry scales in these areas.

It was felt that any attempt at a statistical experiment to show the relationship between comb-growth and the amount injected had better be postponed until a highly active substance could be obtained. With crude extracts the rate of absorption, etc. might vary the response in a similar manner to the effects of crude oily preparations of the oestrus-producing hormone on rats.

II. Chemical properties.

The following experiments were designed to show the general properties of the active substance as prepared by the method already described [Dodds, Greenwood and Gallimore, 1930]. The extract used was water-soluble and prepared by the barium hydroxide method of hydrolysis either from bulls' testes or from concentrated male urine (solution A). In each experiment the starting material was divided into two portions, one of which was subjected to the treatment described, whilst the other was used as a control. In the case of the enzyme experiments the control was treated with a boiled solution of the enzyme. Both of the final solutions were injected into at least two capons and the result was recorded as to whether a definite sudden increase in comb-growth was produced. In all the experiments recorded the control gave positive results.

The action of mineral acids.

Sufficient HCl was added to solution A to make the concentration $N/10$. The resulting solution was boiled for 1 hour under reflux, cooled and neutralised. The control of solution A was boiled for 1 hour in neutral solution. Injection into the capon proved that the activity was not destroyed.

The action of trypsin.

An active solution of trypsin was prepared of which 0·2 cc. digested 10 cc. of 0·1 % alkaline caseinogen solution in 40 minutes (method of Gross, 1907). Two portions of 50 cc. each of solution A were taken and the reaction of both was adjusted so that it was just alkaline to phenolphthalein. One portion was incubated for 2 hours at 40° with 10 cc. of the active trypsin solution, whilst

the control quantity of 50 cc. was incubated with 10 cc. of the same trypsin solution which had been previously boiled for a sufficient length of time to destroy its activity. After the incubation both solutions were neutralised. The test showed that the potency was destroyed by trypsin under these conditions.

The action of pepsin.

An active solution of pepsin was prepared of such a strength that 0·005 cc. just digested 2 cc. of 0·1 % edestin solution in 30 minutes (method of Fuld, 1907). Two quantities of solution A were treated with the pepsin solution; in one case the activity had been destroyed by boiling in exactly the same manner as described above, with the exception that the reaction was adjusted to the acid side of Congo red. On injection it was found that the activity was partially destroyed.

The action of oxidising agents.

Hydrogen peroxide was added to solution A in sufficient concentration to render it 1·7 *N* (estimated by permanganate titration). The solution was shaken vigorously for 1 hour and the excess of hydrogen peroxide was removed by boiling with sodium hydroxide. The control amount of solution A was treated with the same quantity of sodium hydroxide and was boiled, but no hydrogen peroxide was added. Injection showed that the activity was destroyed.

The solubility of the hormone in alcohol, benzene and light petroleum.

A series of experiments was performed in which attempts were made to extract the hormone from its solution by solvents. The result is expressed in Table I. From this it can be seen that the hormone is freely soluble in benzene, and that the benzene solution may be washed with *N* sodium carbonate, *N* HCl and 10 % potassium hydroxide solutions without any loss of potency in so far as it is possible to tell by the method of testing. The weight is considerably reduced by this treatment. Also, when dissolved in 50 % alcohol the active substance can be completely extracted with light petroleum.

Table I.

Urine 30 litres
| Evaporated to about 2 litres
| Acidified and extracted with benzene in the warm
Benzene-soluble 5500 mg.
| Benzene solution washed with *N* Na_2CO_3 solution
Benzene-soluble ——— Na_2CO_3 solution
255 mg.
| Dissolved in absolute alcohol, water added to make the alcohol 50 %, and extracted with light petroleum
Light petroleum-soluble ——— Alcohol-soluble
81 mg.
| Dissolved in benzene, washed with *N* HCl and then washed with 10 % KOH
Benzene-soluble ——— Alkali-soluble
16 mg.

All the extracts on the right of the table were inactive and the final product (16 mg.) when dissolved in sesame oil and injected into two capons for 10 days gave good comb-growth.

The above may be used as a basis for the preparation of the hormone.

Distillation of the active principle.

For these experiments a triple stage Gaede mercury-vapour pump was employed. The distillation apparatus was made of glass in one piece and was jointed directly to the top of the pump (Fig. 2). The material to be distilled was poured into the bulb and after very careful drying over P_2O_5 was heated in an oil-bath. The side-tube was cooled with a freezing mixture. The vacuum obtained was less than 0·1 mm. It was found impossible to distil the crude benzene extract of testes or concentrated urine as excessive frothing occurred accompanied by the evolution of benzoic acid in the case of the latter. Successful results, however, were obtained with purified material. Solution A was evaporated to dryness and the residue was dissolved in alcohol. The alcoholic solution was transferred to the flask of the distillation apparatus and the alcohol was evaporated off. After careful drying distillation was carried out by the method described. Distillation began at 90° when a small amount of the material came over, but the greater part came over between 140° and 160°. The distillate was found to consist of a mixture of crystals and pale yellow oil and was readily soluble in water. On injection the material was found to be active. A similar distillation experiment was performed with the material purified by light petroleum extraction. It was found that the maximum distillation occurred between 140° and 160° and the distillate in this case consisted of a dark oil insoluble in water; it was dissolved in sesame oil and was found to be active. The temperature was raised to 200° and it was found that the residue was inactive.

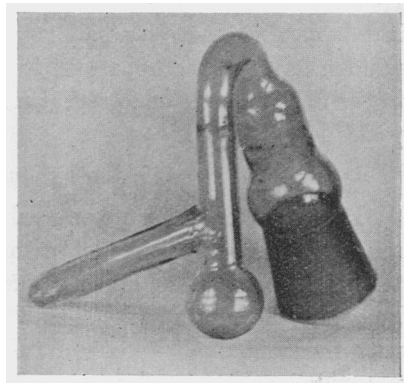

Fig. 2.

OTHER PHYSIOLOGICAL PROPERTIES.

Effect on seminal vesicles.

In these experiments rats and mice were used. Difficulty was experienced in obtaining consistent results, until it was realised that there is a great variation in the size of the seminal vesicles of normal rats. Moreover, the rate of atrophy of these organs after castration is very variable, and may in some cases be extremely slow. It has, therefore, been found advisable to allow a period of about 6 months to elapse after castration before using the animals

for testing purposes, and in addition we have found it advisable to perform exploratory laparotomy some few days prior to the actual test. In this way the seminal vesicles can be inspected and the animals destined for injections can be compared with the controls. Experiments on mice showed a similar variability in the size of the seminal vesicles and in the rate of atrophy after castration.

The difficulty in regard to the slow rate of atrophy can be avoided by operating on the animals before the vesicles have reached any considerable size. Since rats and mice may be operated on at 3 weeks of age without any appreciable operative mortality, suitable material for the test may easily be obtained. It would appear that very young animals require more of the extract to bring about observable growth of the seminal vesicles than fully grown ones. This may be comparable to the fact observed by Parkes and Bellerby [1926] that the new-born female rat or mouse does not respond to oestrin.

Table II.

Animal	No. of injected animals	No. of control animals	Age	Time interval between experiments and castration	Injections	Result on seminal vesicles
Rat	3	1	—	5 months	Twice daily for 5 days	Positive
Rat	4	2	16 days	Not castrated	Twice daily for 4 days	Negative
Rat	3	1	—	7 months	Twice daily for 6 days	Positive (Fig. 3*a*)
Rat	3	1	—	? months	Once daily for 15 days	Strongly positive
Mouse	4	2	4 weeks	1 day	Twice daily for 8 days	Negative
Mouse	4	2	4 weeks	9 days	Once daily for 2 days	Negative
Mouse	1	1	6 weeks	2 weeks	Once daily for 10 days	Strongly positive
	3				Once daily for 22 days	„ (Fig. 3*b*)
Mouse	4	1	28 days	5 days	Twice daily for 4 days	Positive

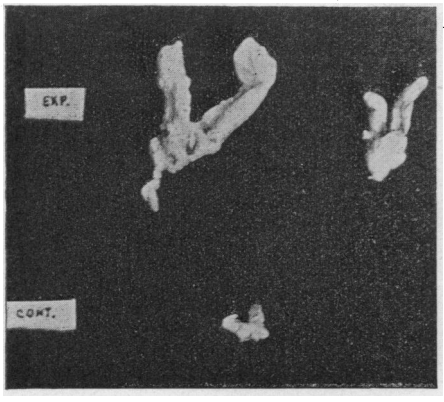

Fig. 3*a*.

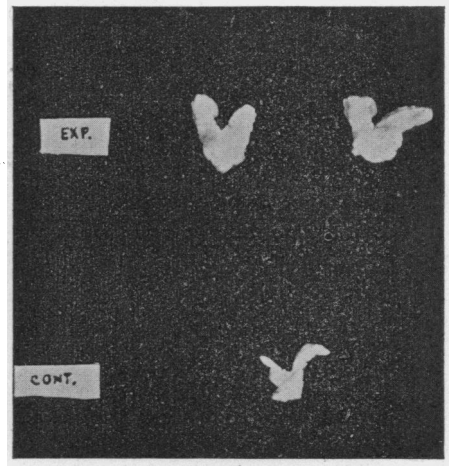

Fig. 3*b*.

The extracts from testes do not produce oestrus when injected into ovariectomised rats.

SUMMARY.

1. The preparation and properties of a comb-growth-promoting factor, and a qualitative examination of its activity on capons, are described.

2. The activity of this factor is destroyed by pepsin, trypsin and oxidation, but survives boiling with acids and alkalis.

3. The benzene solution containing the active substance can be washed with strong acids and alkalis, and the active substance can be extracted from a 50 % alcohol-water solution with light petroleum. These properties have been utilised in a provisional method for the extraction and purification of the active principle from male urine.

4. The hormone can be distilled in a high vacuum, but only after previous purification and drying.

5. When injected into male castrated rats or mice the atrophied seminal vesicles regain their normal size. The extracts from testes do not produce oestrus.

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REFERENCES.

Dodds, Greenwood and Gallimore (1930). *Lancet*, i, 683.
Freud, de Jongh, Laqueur and Münch (1930). *Klin. Woch.* **9**, 772.
Funk, Harrow and Lejwa (1930). *Amer. J. Physiol.* **92**, 440.
McGee, Juhn and Domm (1928). *Amer. J. Physiol.* **87**, 406.
Moore, Gallagher and Koch (1929). *Endocrinol.* **13**, 367.
—— and McGee (1928). *Amer. J. Physiol.* **87**, 436.
Parkes and Bellerby (1926). *J. Physiol.* **62**, 145.
Pézard (1911). *Compt. Rend. Acad. Sci.* **153**, 1027.