XLIX. THE ASSAY OF TESTICULAR HORMONE PREPARATIONS.

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(Received February 29th, 1932.)

Up to the present time the following tests have been used for the assay of testicular hormone.

The comb growth of capons is the test which has been most used [Dodds *et al.*, 1930; Freud, 1930; Funk and Harrow, 1929, 1930; Koch and Gallagher, 1929; Gallagher and Koch, 1930; Kabak, 1931; McGee, 1927; McGee, Juhn and Domm, 1928; Pezard, 1918]. This test, the details of which were worked out by Gallagher and Koch [1930] has many advantages.

(i) Only a very short time (5 days) is claimed to be necessary for its completion.

(ii) The results are accurately expressed in figures of increase of length and height of comb.

(iii) The same birds can be used for several assays, since after cessation of the injections the comb decreases again to its previous size.

The test has the following disadvantages.

(i) The comb, although differing in size and appearance, is an organ present in both sexes, and comb growth could, therefore, be obtained in birds of both sexes after injection of the hormone [Freud, 1930; Champy, 1931]. Injections of yolk and of ovarian extract give similar comb growth to that produced by testicular extract [Champy, 1931]. Therefore the hormone producing comb growth is considered by Champy to be specific for both sexes.

(ii) Sometimes an extract which is inactive on comb growth is active on growth of genital organs of castrated guinea-pigs and *vice versa* [Champy, 1931].

(iii) High doses of testicular preparations give exceedingly irregular results [Gallagher and Koch, 1930].

(iv) Although small doses give satisfactory results in assays, considerable variations are obtained, as is shown by the following experiments of Gallagher and Koch [1930, p. 318]. Forty-five capons were injected with the same dose of the same testicular preparation. In 2.2 % of them no comb growth was obtained; in 13.3 % 2 mm., in 15.5 % 3 mm., in 28.8 % 4 mm., in 28.8 % 5 mm., in 4.4 % 6 mm., and in 6.6 % 7 mm. increase in comb growth was obtained.

Still more striking variations were obtained by Kabak [1931], who, for example, on injecting 6 capons for 10 days, obtained the following increases in comb growth, 23, 13, 11, 9, 6, and 6 mm.

Tests on mammals. The following tests have been proposed.

On rats.

1. Sperm motility test [Moore, 1927, 1928].
2. Prostate cytology [Moore and Gallagher, 1929; Moore, Price and Gallagher, 1930].
3. Seminal vesicle cytology [Moore and Gallagher, 1929].
4. Vas deferens cytology [Vatna, 1930].
5. Cowper's gland cytology [Hiller, 1930].

On mice.

6. Seminal vesicle cytology (Loewe and Voss, 1930).

On guinea-pigs.

7. Electric ejaculation test [Moore and Gallagher, 1929].

All these tests are satisfactory, but Gallagher and Koch have objected to them, because either they take too long or lack quantitative precision in the end-point reading, or have both deficiencies. The necessity of operating on new animals for each assay is also a disadvantage in nearly all these tests.

Measurement of the size of the prostate, or of seminal vesicles, or of both, has also been used [Korenchevsky, 1928; Korenchevsky and Schultess-Young, 1928; Freud, 1930; Dodds *et al.* 1930]. This method, however, was not worked out as it was found by some authors (Freud, Dodds) to give contradictory results.

Weighing of prostate with seminal vesicles and of penis. I have, in previous publications [Korenchevsky, 1928, 1930; Korenchevsky and Schultess-Young, 1928], besides lineal measurements, used as a test of the activity of the extracts or for other purposes the increase in weight of the prostate with seminal vesicles, and of the penis of castrated rats. The aim of this paper is to give an accurate description of the method as it is now used.

THE PRESENT INVESTIGATION.

The lineal measurements of the sexual and other organs were found in our experiments to be unsuitable, since (1) it is not easy to make accurate measurements of organs of irregular shape and (2) the results have to be expressed in at least two figures, the length and width or height of the organ.

I have, therefore, chosen the weight of the organs as a criterion giving only one figure, and as being at the same time the most precise in end-point reading and a measurement of organs irrespective of their shape. Certain precautions, however, have to be taken to obtain accurate results. Some of these precautions have already been mentioned elsewhere [Korenchevsky, 1930].

1. *Feeding the animals.*

It is necessary, unless it is otherwise required, to feed all the animals used for the test on the same diet, which must be adequate in every respect. A synthetic diet in the form of a paste is used of the following composition:

Caseinogen	18	Dried yeast	15
Starch	50	Lemon juice	5
Butter	15	Water	64
Salt mixture	5		

In addition to this diet the animals receive daily 1 g. of wheat germ and 3 to 6 drops of cod-liver oil, for it is well known that for good development of the sexual organs nearly all the vitamins are of the greatest importance. A daily record of the amount of paste consumed by each rat is made. The object of giving this diet is first to supply the greatly altered organs of the castrated animals with all the factors necessary for a return to the normal condition, and, in addition, to measure the appetite of the injected animals, since testicular preparations frequently contain substances which depress the general functions and the appetite. If the appetite is decreased, the satisfactory regeneration of the atrophied organs of the castrated animals is difficult. Further, our experience has shown us that the decrease in appetite is one of the best and easiest tests for the demonstration of toxic depressing substances present in the testicular preparation, resulting from insufficient purification.

2. *Measurement of changes produced in organs.*

In castrated animals the following changes are observed [Korenchevsky, 1930]: atrophy of the prostate, seminal vesicles, penis and thyroids, hypertrophy of the adrenals and hypophysis, and increase in body fat. Under the influence of injections of testicular preparations, containing all the testicular hormones, these changes should return towards normal or even disappear.

Rats for examination are killed by coal gas. A quick and simple method for the accurate determination of fat deposition in the body of rats by means of the estimation of the weight of retroperitoneal fat has already been described [Korenchevsky, 1930]. The penis, after separation from the skin, is cut off at the base, where it is attached to the pelvic bones, and weighed, as is also the fat, without further treatment. Until weighed the fat and penis are kept in large covered crystallising dishes on two thicknesses of filter-paper moistened with saline solution. If the weighing has to be postponed the fat and penis can be kept in the dish in the cold store for several days without influencing the accuracy of the results. All the other organs are accurately but quickly separated from the body and immediately plunged for fixation and preservation in Allen's modification of Bouin's fixative. This contains saturated picric acid 75 cc., formalin 15 cc., glacial acetic acid 10 cc., urea 1 g. The seminal vesicles are always dissected as attached to prostate and the two weighed together, otherwise the secretion of the seminal vesicles would be lost. If necessary,

beside the organs mentioned above, pieces of other tissues are also cut off and plunged into the fixative.

Bouin's fixative, unlike others, does not change the weight of the organs appreciably, and in any case gives satisfactory results for comparative investigations.

After fixation is completed (2-3 days) the organs of the rats are placed in a large crystallising dish on two thicknesses of filter-paper moistened with saline solution, the organs of each rat being covered with a small Petri dish in order to prevent evaporation and consequent drying of the glands. It is possible in this way to place in one crystallising dish the organs of 7 rats, without any fear of mixing them. To prevent evaporation of the water from the filter-paper on which the 7 groups of organs are placed, the large dish in its turn is covered with a lid, which is only removed to take out or replace the gland. The weighing is made in a very small weighing-bottle on a chemical balance.

The treatment of the glands with Bouin's fixative was introduced for the following reasons: (1) the fixed glands are not affected so readily as fresh glands when exposed to the air or when kept, nor is their weight so easily changed; (2) after weighing, the organs can be used for histological investigation whilst, without fixation, artificial changes are produced during the somewhat lengthy process of dissection from the rat and subsequent weighing; (3) when weighed they do not stick to and do not soil the weighing-bottle, as do fresh glands; (4) it is very much easier to make the final separation of the fat from very atrophied prostate and seminal vesicles, if the organs are fixed. This is a matter of some importance since the prostate and seminal vesicles are the chief organs used in the assay.

3. *Fluctuations in weight of organs of non-injected castrated rats.*

Before considering the changes in weight of the prostate with seminal vesicles, penis, thyroids, adrenals, hypophysis and retroperitoneal fat of castrated rats as tests of the influence of testicular injections it is necessary to ascertain what are the normal fluctuations in weight of these organs in non-injected castrated rats.

For this purpose I have used 25 pairs of castrated rats, each pair having served for different experiments as the controls to their litter-mates injected with different preparations. The weights of the organs were calculated per 200 g. of rat weight. In each pair the average weight of each organ was found and the individual deviation from this average calculated as a percentage for each pair. For example, in litter 1497, the weights of prostate with seminal vesicles in the two rats were 0.208 g. and 0.195 g. Average weight 0.2015 g. Deviation from the average ± 0.0065 g. or ± 3.2 %. These deviations expressed as % for each organ for each pair are collected in Table I, arranged in ascending order of magnitude.

The difference in weight of the organs of the two rats of the pair was also calculated in a different manner. Taking the same example as mentioned above,

Table I. *Percentage deviations of weight of organs from their average weight in each pair of castrated uninjected rats of the same litter.*

Prostate with seminal vesicles	1.1, 1.8, 2.1, 3.2, 3.6, 3.6, 4.0, 4.1, 4.2, 5.1, 5.1, 5.9, 6.1, 6.3, 6.6, 7.2, 7.7, 8.9, 9.4, 9.4, 10.3, 10.9, 13.2, 13.3, 16.8
	Average = 6.8
Penis	0, 1.5, 2.1, 2.5, 2.8, 3.7, 4.8, 5.0, 5.6, 5.8, 8.6, 8.8, 10.1, 10.2, 10.6, 12.0, 12.0, 12.3, 12.4, 12.4, 13.4, 13.8, 16.1, 16.6, 17.7
	Average = 8.8
Adrenals	0.4, 0.8, 1.7, 2.1, 3.2, 3.7, 3.8, 4.2, 4.2, 4.7, 4.7, 6.0, 6.4, 8.1, 12.9, 13.0, 13.2, 15.4, 16.1, 16.7, 17.1, 17.4, 17.4, 23.3, 26.9
	Average = 9.7
Thyroid	0, 0.5, 0.5, 2.0, 2.3, 2.7, 3.6, 3.9, 4.1, 4.3, 4.8, 5.0, 6.0, 6.8, 7.7, 8.6, 9.7, 11.4, 14.1, 15.2, 15.7, 17.7, 17.9, 18.0
	Average = 7.6
Hypophysis	0, 1.2, 1.4, 2.3, 2.6, 2.7, 3.5, 3.8, 5.3, 5.4, 5.7, 6.7, 7.4, 7.5, 7.6, 8.4, 8.5, 8.7, 9.9, 10.6, 15.5, 18.5, 20.7
	Average = 6.9
Retropertitoneal fat	0.1, 0.3, 1.5, 3.5, 3.9, 4.8, 4.9, 4.9, 5.7, 7.1, 9.2, 9.5, 10.7, 10.9, 12.0, 12.1, 14.0, 21.2, 21.8, 25.8, 29.0, 29.8, 35.0, 37.0, 59.0
	Average = 14.9

litter 1497, the difference in weight of the prostate with seminal vesicles of the two rats is 0.013 g. (0.208 g–0.195 g.) which is 6.7 % of 0.195. In these calculations the percentage was taken with reference to the lower of the two weights referred to, the aim being to obtain the greatest difference of the figures. From the percentage difference in weight of the organs of the two rats of a pair, an average figure was obtained for each gland. All these average figures and the maximum and minimum percentage differences are given in Table II.

Table II. *Maximum, minimum and average percentage differences in weight of organs in each pair of uninjected castrated rats.*

Organs	Average difference	Maximum difference	Minimum difference
Prostate with seminal vesicles	15.3	34.3	3.6
Penis	19.1	41.8	0.0
Adrenals	22.3	73.7	1.7
Thyroid	17.1	43.5	0.0
Hypophysis	19.2	88.9	1.2
Retropertitoneal fat	29.7	117.2	0.2

To economise space the detailed figures are not given since the percentage figures of the differences of the weights of the glands of two rats can be roughly estimated as a little more than twice the respective figures given in Table I.

Examination of the figures given in Tables I and II shows that the weights of the prostate with seminal vesicles, of the penis and of the thyroid of castrated rats belonging to the same litter do not vary very considerably. The

maximum percentage deviation from the average weight of these respective organs in two litter-mate rats was 16.8, 17.7 and 18.0 % (Table I). The average of the percentage deviations was much less, being 6.8, 8.8 and 7.6 for these organs (Table I). At the same time the maximum percentage differences of the weights of these organs in the pairs of rats are 34.3, 41.8 and 43.5 (Table II).

It, therefore, seems reasonable to infer the positive influence of a testicular extract on each of the mentioned organs if, in a litter of at least four rats, in the two injected rats these organs have increased on an average by at least 40 %, as compared with those of the two uninjected rats, left as controls.

4. *Fluctuations in weight of organs of the castrated rats injected with testicular hormone preparations.*

It is important, in order to estimate the value of this method of assay, to ascertain whether the variations in weight of the injected castrated rats are similar to those of their uninjected litter-mates. For this purpose the percentage deviations from the average weight of each organ in each pair of injected rats is given (Table III), *i.e.* the table is constructed in the same way as Table I. Table IV is constructed in the same way as Table II, *i.e.* it contains the average figures of the percentage difference in weight between the organs of two rats in each pair. (These are the same pairs as used for Table III.)

Altogether 31 pairs of rats were used. The two rats of each pair belonged to the same litter and were injected with the same amount of testicular hormone

Table III. *Percentage deviations of weight of organs from their average weight in each pair of castrated injected rats of the same litter.*

Prostate with seminal vesicles	0.4, 0.7, 0.8, 1.3, 1.5, 1.9, 2.1, 2.5, 2.6, 2.6, 3.6, 3.8, 4.5, 4.7, 4.8, 5.3, 5.7, 5.9, 7.0, 7.4, 7.8, 7.9, 8.0, 11.6, 12.1, 13.7, 15.5, 16.1, 20.0, 29.2, 38.3
	Average = 8.04
Penis	0, 0, 0.6, 0.8, 0.8, 1.1, 1.5, 1.7, 1.9, 2.6, 2.8, 2.9, 3.4, 3.5, 4.5, 4.7, 5.1, 5.2, 5.6, 7.5, 8.2, 8.3, 8.4, 9.1, 9.3, 10.1, 12.6, 12.9, 14.8, 19.8, 32.1
	Average = 6.5
Adrenals	0.3, 0.7, 0.8, 0.9, 1.4, 1.6, 1.9, 1.9, 1.9, 2.5, 2.8, 2.8, 2.8, 3.0, 3.1, 3.8, 3.8, 4.2, 4.2, 4.4, 4.7, 5.2, 5.8, 5.9, 7.6, 7.7, 8.7, 9.1, 10.0, 11.7, 14.5
	Average = 4.5
Thyroid	0.2, 0.5, 0.7, 1.8, 1.9, 2.0, 2.6, 2.8, 3.4, 3.7, 3.8, 4.1, 4.5, 4.6, 5.4, 5.9, 6.6, 6.6, 6.7, 6.8, 7.2, 9.9, 11.0, 11.9, 12.4, 12.5, 13.0, 14.0, 14.7, 15.7, 15.7
	Average = 6.9
Hypophysis	0.4, 0.4, 0.5, 0.5, 2.3, 2.8, 2.9, 3.1, 3.6, 3.6, 3.9, 4.6, 4.7, 4.8, 5.0, 6.0, 6.0, 6.1, 6.2, 7.2, 8.0, 8.3, 8.9, 10.6, 11.4, 12.2, 13.6, 18.7
	Average = 5.6
Retroperitoneal fat	0.5, 0.8, 1.0, 2.0, 2.4, 3.9, 5.4, 5.5, 6.6, 7.0, 7.8, 8.2, 8.3, 8.6, 9.1, 10.2, 10.2, 10.3, 12.4, 13.8, 15.0, 15.2, 18.6, 19.0, 19.6, 23.3, 30.1, 34.6, 35.8, 40.6, 56.4
	Average = 14.3

preparation. Different pairs, however, were injected with different preparations and in varying doses. Only those pairs were taken for the Table which showed a minimum increase of 40 % in the weight of prostate with seminal vesicles as compared with that of the controls of the same litter. The period of injection was from 6 to 14 days. A full account of these experiments will be given elsewhere. In this paper are discussed only the variations in the weights of the organs of the injected rats of the pair, for which purpose further details of the experiments are unnecessary. As can be seen from Table III, the averages of the percentage deviations of weights of organs of injected rats are sufficiently close to those of uninjected rats (Table I). The percentage differences of the weights of organs in the rats of pairs of injected rats are also similar to those of uninjected rats (compare Tables II and IV) with the exception of the high maximum percentage difference in the weight of prostate with seminal vesicles, penis and fat which, however, occurs in a few cases in injected pairs. This can be explained by the fact that the influence of testicular extract is exceptionally strong in a few rats because of their sensitivity to hormonal influence. In addition injections were often made with crude material, insufficiently purified from depressing substances which depress the appetite and therefore the nutrition of the more susceptible animals. Decrease of appetite may occur for example in only one rat of the pair as the result of the injection, or the decrease may be greater in one than in the other with consequent check of the return towards normal of the changes produced. However, as the figures show, this happens in only a few cases (Tables III and IV).

Table IV. *Maximum, minimum and average percentage differences in weight of organs in each pair of injected castrated rats.*

Organs	Average difference	Maximum difference	Minimum difference
Prostate and seminal vesicles	20.8	124.0	0.9
Penis	15.6	95.9	0.0
Adrenals	9.8	34.4	0.7
Thyroid	15.5	38.2	0.4
Hypophysis	13.7	48.1	0.8
Retroperitoneal fat	41.9	259.1	1.1

5. *The test.*

The data from all four tables show, therefore, that for the simple assay of testicular hormones two rats as controls and two rats for injection are sufficient to obtain accurate information with regard to the positive influence of the extract.

For the usual tests, especially when only a small amount of extract is available, injections should be continued for 6 or 7 days. I propose to use the increase in weight of prostate with seminal vesicles as the test of potency. As a rule the weight of the penis is also simultaneously increased, thus corroborating the results obtained on prostate and seminal vesicles, but the penis

responds more slowly to the injections than does the prostate and seminal vesicles, as we shall show in a later paper.

An average increase of 40 % in the weight of the prostate with seminal vesicles of the injected pair, as compared with the control pair, was suggested above as a quite definite proof of the activity of testicular extracts and perhaps as a basic figure for one rat unit. The figure 40 % is a preliminary one, since experiments, which are now in progress, may decrease it.

The figures for percentage deviation from their average weight (Table I) and percentage difference in weights of organs in each pair (Table II) are very close in the case of penis and thyroid to those for prostate and seminal vesicles. Therefore by the same argument one would suggest that an increase by 40 % in the weight of these organs in the injected pair will also clearly prove the activity of the extract on these organs. In the case of the hypophysis and the adrenals the maximum percentage differences in weight of the organs in a pair (Table II) are larger. Perhaps it would, therefore, be advisable for these glands to take a higher figure than 40 % decrease in weight as a definite proof of the activity of testicular extracts, and for the same reason a still higher figure would have to be taken for assays from changes in the weight of retroperitoneal fat under the influence of injections. In this paper a value for these figures is not suggested as has been done for prostate, penis and thyroids, since preliminary work on the influence of injections for a very much longer period than was usual in our experiments would be necessary.

Table V. *Influence of the testicular extracts A and B prepared by two different methods from the same batch of bulls' testes.*

2.5 mg. of the extract were injected twice a day subcutaneously for 7 days. Weights of organs per 200 g. of rat's weight.

Nos. of rats	Injections	Final wt. of rats, g.	Retroperi- toneal fat g.	Prostate + sem. ves. mg.	Penis mg.	Adrenals mg.	Thyroids mg.	Hypo- physis mg.
1769 } 1773 }	Controls (uninjected)	161 159	3.03 1.46	99 91	111 92	740 734	187 189	129 134
	Average...	160	2.25	95	102	737	188	132
1770 } 1771 }	Extract A	165 164	2.35 2.10	138 134	128 131	573 577	181 183	103 110
	Average...	165	2.23	136	130	575	182	107
1772 } 1774 }	Extract B	160 149	2.61 1.78	140 142	136 164	588 622	228 165	93 117
	Average...	155	2.20	141	150	605	197	105

The importance of using rats of the same litter, kept on the same diet, for the different tests, has already been emphasised in previous papers [see, especially, Korenchevsky, 1930].

For an absolutely accurate assay a standard preparation of testicular hormone would have to be used, with which the preparation to be tested would have to be compared. In this case it would be necessary to use a litter of six

castrated rats, two as controls, uninjected, two injected with the standard preparation and two with the new hormone preparation to be tested.

Since a standard preparation is not at present available the following procedure is suggested as the most satisfactory method for ascertaining the strength of the preparation to be tested.

For comparison of the results it is necessary to use rats in litters of the same age and at the same date after castration, belonging to the same stock, reared on the same diet and kept during the experiment on a special ration of the complete synthetic diet mentioned above.

It is preferable to use litters of six castrated rats since they can then be divided into three pairs, one for the controls, uninjected, the other two for the injections of two different doses, or for the comparison of a new preparation with that previously prepared (a substitute for a standard preparation).

As the test of the activity and strength of the preparation a 40 % increase in weight of the prostate with seminal vesicles should be obtained, for which result 6 days of injections should give accurate data, as will be shown in a later paper. For the comparison the weights of the organs calculated per the same unit of rat weight must be used, not the actual figures, which may differ much more according to the differing weights of the rats. An example of the test of two preparations is given in Table V, showing them to be of about the same strength.

SUMMARY.

1. Twenty-five litter-mate pairs of uninjected castrated rats, and thirty-one litter-mate pairs of castrated rats injected with testicular hormone preparations were used.

2. All the rats used for this investigation belonged to the same stock, were reared on the same diet and kept during the experiment on the same paste; but the pairs differed in age and in the period of time since castration.

3. A special method of preparing the organs for accurate weighing was described, by which it was possible to use them afterwards for histological investigation, if necessary.

4. The percentage deviation of weight of prostate with seminal vesicles, adrenals, penis, thyroids, hypophysis and of retroperitoneal fat, from their average weight in each pair, and the percentage differences in weight of these organs in each pair were ascertained.

5. On the average these deviations and differences are not very great and, therefore, the changes in weight of special organs of the pairs of injected rats, as compared with the weight of the same organs of control uninjected rats are suggested for studying the activity and strength of testicular preparations.

6. Since after castration there is a decrease in weight of prostate, seminal vesicles, penis and thyroids and an increase in weight of hypophysis, adrenals and body fat, it is necessary in order to demonstrate the complete activity of

the testicular hormone, or hormones, to obtain a return towards the normal of these organs.

7. However, as a quick, simple and sensitive test, we suggest an increase in the weight of the prostate with seminal vesicles of the injected castrated rats, by at least 40 %, as compared with that of uninjected control rats.

8. For comparison the weights of organs should be used only as calculated per the same unit of rat weight.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled me to carry out this work and to them my thanks are due.

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