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A CONSIDERATION OF THE METHODS AND RESULTS IN THE STANDARDIZATION OF THE OVARIAN HORMONE.

BY HOWARD T. GRABER.

In view of the increasing interest in the estrus-producing principle and of the fact that the subject has been considered from various standpoints, it seems advisable to consider, in a collective way, or to analyze the present methods used in the physiological standardization of this remarkable substance. Prior to 1917 it was difficult by external observations to determine the stage of the estrus cycle of either rats, mice or guinea-pigs.

In 1912 Adler (1) stated that by injecting watery extracts of ovaries into animals he was able to produce all the symptoms of estrus. Later, Iscovesco (2), Fellner (3) and co-workers, and then Herman and Frankel (4) carried on this line of work.

In 1917 Stockard and Papanicolaou (5) showed that it was possible to follow the cycle in the guinea-pig by microscopical examination of the cells contained in the vaginal smear.

In 1922 Long and Evans (6) applied the principle to a study of the conditions in the rat. In the same year Allen (7) studied those in the mouse.

In 1923 Hartman (8) applied the same principle to the conditions in the opossum. This same year, Allen and Doisy (9) obtained a solution containing the active principle from the follicular fluid obtained from pig ovaries.

Parkes (10) applied the principle to the study of the conditions in the mouse in 1926.

There have been many other workers in this field notable among whom should be mentioned Laqueur (11) Dodds, Dickens (12) and co-workers, and Zondek and Ascheim (13).

As our experience with different products has advanced, naturally there have been developments in different laboratories which undoubtedly are an advantage. This, however, we feel would be lost unless these differences in technique and procedure are reconciled with one another. In other words, in order for the findings in this laboratory to be compared with those of another there must be some common ground upon which they stand.

With this in mind, it seems advisable to set down, as concisely as possible, a statement about each of the current procedures used in the evaluation of products containing the estrus-producing principle.

First of all, all of these methods are based fundamentally on the work of Long and Evans who demonstrated the fact, in 1922, that the estrus cycle in the rat could be followed by the vaginal smear method.

There has been quite general agreement regarding the weight and conditions of the animals used in these experiments—white Albino rats ranging in weight from 120 to 160 Gm. are ovariectomized and allowed to recover. Two weeks after the operation they are studied for a certain period, usually seven days, for the microscopical evidences which are used to determine the various stages of estrus, and thereafter if they have been proven to be in the "resting" stage they receive subcutaneous injections of the product it is desired to study, and the microscopical examinations of the vaginal smears are continued.

Right here comes the principle variation in the methods of the investigators, and it is because of these differences and their possible importance that this matter has been given consideration.

Since the reaction of an animal to a given physiological stimulus depends not only upon the stimulant, but also upon the responsiveness of the animal to that stimulant, naturally the time of the injections, their relation to one another as well as the dose must be taken into consideration.

Doisy (14) has suggested the following:

> Vaginal smears of female rats weighing from 90 to 110 Gm. at an age of 7–8 weeks were made daily for a period of at least two weeks to determine the regularity of the estrus cycle. Only those animals having regular cycles are used. The ovariectomy was performed under deep ether anæsthesia, aseptic precautions being observed. Daily vaginal smears were made for two weeks following ovariectomy to determine whether all ovarian tissue had been removed. Negative smears were obtained in every case, indicating that the ovariectomy had been complete.

Injections.—In earlier work it was attempted to simulate the ovarian activity by injecting preparations of the hormone in three portions given at four-hour intervals.

Their test animals are used once a week thus corresponding roughly to the length of the normal ovarian cycle. Prime the animals with two R. U.

One week later, test the reaction of each animal by injecting 1.3 R. U. If the smears are negative discard the animal.

A week later, test the reaction to 0.70 R. U.; if positive smears occur discard the animal.

Use each animal for no longer than four months.

In attempting accurate assays, prime all animals by injecting 1.5 R. U., if the reaction of the preceding week was negative.

Use a sufficient number of animals. If 75 per cent of the animals injected with the same volume give a positive reaction, consider that the amount injected contained 1 R. U. A rat unit being defined as the minimum amount of hormone that will induce full estrus growth, as judged by the smear method, in the genital tract of a spayed adult white rat weighing about 140 Gm. forty-eight hours after the first of *three* one cc. injections given at intervals of from four to six hours.

Allen, Dickens, Dodds and Howitt, on the other hand, observe the following technique:

Method.—All standardizations have been performed upon ovariectomized rats. When a weight of 140 Gm. is reached the ovaries are removed and vaginal smears are taken for 2 weeks. If no signs of cyclical activity are observed, the animals are then put into use. *Six* doses of 0.2 cc. each are injected into the animals, the preparations being diluted to contain approximately 1 unit in 1.2 cc. The injections are made as follows: first day, 9 A.M. and 6 P.M.; second day, 9 A.M., 3 P.M. and 9 P.M.; third day, 9 A.M., only. Smears are taken at 9 A.M. on the first and second days; at 9 A.M. and 6 P.M. on the third day; and again at 9 A.M. and 6 P.M. on the fourth day. It is rare for a rat to attain estrus after the evening of the fourth day, and often signs of a return to the diestrus condition are already present in the sixth smear. A minimum of 20 rats and, where possible, 100 rats are used for each batch. Smears have been stained with hæmatoxylin and eosin, since this enables a permanent record to be kept of the response elicited by various preparations.

Interpretation.—One unit of activity is that quantity of material required to produce a full estrus response in 50% of the rats used (Coward and Burn). Estrus has been taken to be present when cornified cells only are shown in the vaginal smear.

Bugbee and co-workers observe entirely different details of injections as follows: In carrying out the standardization, ovariectomized rats are used. *Eight* injections are given to the animals, four daily for two days, estrus being determined by examining the cell forms found in vaginal smears taken from the rats on the third and fourth days of the test. One rat unit is defined as the minimum amount of extract which will produce estrus in the ovariectomized animal of 140 Gm. weight.

Laqueur prefers the mouse as a test animal to the white Albino rat, others check the reaction in the monkey.

Now while the difference between these procedures may not be great, there is a difference and because of this it seems that some effort should be made to standardize the standardization, and make it possible for those who are interested, both in the manufacture as well as the standardization of these products, to make satisfactory comparisons of their physiological activity.

Bear in mind that if a product is standardized in harmony with, shall we say, the Allen and Doisy method the result will probably vary from the results of the apparently similar, but really considerably different, method of Dodds and the results reported by the Dodds' method will no doubt produce a higher rat unit and therefore a lower dosage.

For instance, it is claimed that the same relative dose given in three injections during the period of 48 hours has an apparent potency much less than the same dose given in eight injections over the same period of time.

Clinical use of this ovarian hormone has shown that it is necessary to give relatively large doses in order to get results, and a reported potency of 25 rat units obtained by distributing the dose over eight injections would in reality be reported much lower than when tested by the method which distributes the dose over three injections.

Take for example the standardization of certain biochemical substances like ferments; much misunderstanding has already been brought about by the differences of procedure in applying what is presumed to be the same method. In biochemistry where physiology is involved, as in the standardization of a

preparation by animal experiments, other factors enter in which have to be appreciated.

It is for this reason that it becomes necessary for all standardizing this principle to adopt a uniform procedure in the assay of this ovarian hormone.

I am not in agreement with Bugbee (15) in his suggestion for testing aqueous colloid solutions and true aqueous solutions by giving eight subcutaneous injections, 4 on each of two successive days, because of what has already been recorded.

In view of what has been said, I propose the following procedure for routine adoption:

1. Select normal healthy white rats of about 100 Gm. in weight.
2. Determine that the animal has a normal cycle, before spaying, by examining the vaginal smears daily for a period of two weeks.
3. Perform complete double ovariectomy and examine the vaginal smears daily to determine that the animal continues in the diestrus or resting stage.
4. Allow one week for recovery from the operation.
5. The food of the rats should be well balanced and contain a sufficient amount of vitamines.
6. For aqueous colloid or true aqueous solutions, three equal injections are given at intervals of four hours. The total amount injected should be about 1 cc. As far as I know there is no ovarian hormone to-day which is dissolved in oil, so it seems unnecessary to consider oil-soluble products.
7. Take vaginal smears 36 and 48 hours after the first injection.
8. The rat should be as near 120 to 160 Gm. as possible, if over or under weight, the correction of Bugbee (15) for weight should be applied.
9. Be sure the rats are primed up. They should be injected at frequent intervals, when not under test, to be sure they remain responsive to the material.
10. Rats should not be used for a longer period than four months. Rats over weight should be used for the rough preliminary assay, checking finally against a series of rats of approximately 140 Gm.
11. In interpreting the smears the reaction is to be classified as positive when the bulk of the smear consists of cornified cells.
12. Use from 9 to 12 rats for a test and give three the same dosage.
13. Two out of three rats given the same dosage should show a positive reaction.
14. Do not use the same rat more often than once every seven days.

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A METABOLIC CAGE FOR DOGS.*

BY A. RICHARD BLISS, JR.[1]

The cages, described herein and used in the laboratories of the University of Tennessee Colleges of Medicine and Dentistry and School of Pharmacy at Memphis during the past college session, have proved very satisfactory. Long experiences with stock cages and with cages constructed in the Shop of the University of Tennessee, all of which showed some marked disadvantages from the standpoints of animal comfort, care, accurate work and costs, resulted in the invention of the cages under discussion.

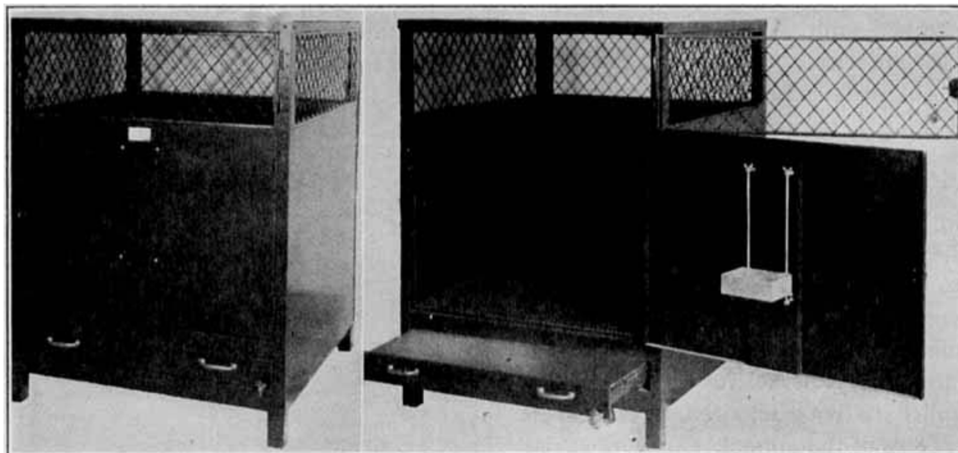

Fig. 1. Fig. 2.

These cages, which may be built to any desired dimensions, provide isolating compartments for dogs of any size, cats, rabbits, monkeys or other small animals. They are constructed so as to furnish maximum ventilation and visibility, while affording complete comfort to caged animals. The high degree of comfort provided is conclusively shown by the fact that large dogs, which have been confined by the author in cages of this type 3 ft. x 3 ft. x 3 ft., have given none of

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* Scientific Section, A. PH. A., Portland meeting, 1928.

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