That the acid itself has little or no toxicity was shown by intravenous and subcutaneous injection into white rats. A 2-p. c. aqueous solution of the sodium salt was used in doses of 250, 310 and 420 mg. per kilo body weight. No outward symptoms of toxicity could be detected.

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BIO-ASSAY OF PREPARATIONS OF OVARIAN FOLLICULAR HORMONE.*

BY E. P. BUGBEE AND A. E. SIMOND.

The Ovarian Follicular Hormone is the female sex hormone which is obtained from the fluid contained in ovarian follicles, from whole ovaries, placentas and other female tissues. In France and Germany it is known as Folliculin and in England as Estrin because it produces oestrus or heat in animals.

Preparations containing Ovarian Follicular Hormone are assayed for biological activity by injecting the solutions into white rats from which the ovaries have been completely removed. If adequate doses are injected the rats will come into oestrus and show the same evidences of heat that normal rats show when in that condition.

The most accurate way for the laboratory worker to tell when the rats are in heat is by examination of vaginal smears. For this method we are indebted to Stockard and Papanicolaou (1), who in 1917 discovered that in guinea-pigs the vaginal mucous membrane goes through the same growth changes as the uterine mucous membrane in each oestrous cycle. Long and Evans (2) soon confirmed this in their studies with rats and Allen (3) confirmed it in his studies with mice.

Small cotton swabs are inserted gently into the vaginas and then glass slides are smeared with the swabs. After drying, the slides are stained with haematoxylin and eosin to differentiate the cells. They are examined under the microscope to determine the types of cells present. Certain types characterize the 5 stages of the oestrus cycles in rats. These have been classified by Long and Evans (2) as follows:

Stage I—Pro-oestrus. Before heat begins. No sexual excitement. Vaginal smears show small epithelial cells with nuclei. No leucocytes.

Stage II—Oestrus. The heat period. Copulation accepted. Vaginal smears show large squamous cells without nuclei. No leucocytes.

Stage III—Late Oestrus. The heat period is over. No sexual excitement. Vaginal smears are thick and cheesy, containing clumps of large squamous cells without nuclei. Late in this stage there may be some large epithelial cells with nuclei. There are no leucocytes.

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Stage IV—Metoestrus or Postoestrus. The stage of degeneration and leucocytosis. Vaginal smears show moderate numbers of polymorphonuclear leucocytes, squamous cells and large epithelial cells.

Stage V—Anoestrus or Dioestrous Pause. The resting stage. Vaginal smears show polymorphonuclear leucocytes as the predominating cells and moderate numbers of large epithelial cells and strings of mucus.

A smear showing the cells characteristic of Stage 2, or oestrus, indicates that the rat is in heat. The smallest dose which will cause typical oestrus in a rat is called a Rat Unit.

The Rat Unit was first proposed and defined (4) by Dr. Edgar Allen, Dr. Edward A. Doisy and their associates in 1924. Their definition is as follows:

"Provisionally we wish to define a Rat Unit as the quantity of material necessary to induce oestrus as judged by the smear method in an ovariectomized sexually mature rat weighing 140 plus or minus 20 grams. For physiological reasons we generally make 3 injections at intervals of 4 hours. The Rat Unit then is the minimum amount so injected which produces full oestrus growth in the genital tract."

In recognition of the fundamental work which Allen and Doisy (5) did in gathering together the bits of information regarding the ovarian and placental extracts and adding their own observation that the ovarian follicular fluid would produce oestrus when injected into rats, it seems proper that the Allen and Doisy Rat Unit should be adopted as the standard of activity of preparations of the Ovarian Follicular Hormone.

Allen and Doisy based their Rat Unit upon a rather small rat weighing about 140 Gm. In our laboratory the rats weigh considerably more than that soon after reaching maturity. We (6) have found that the dosage of the hormone required to cause oestrus in a rat varies directly with the body weight of the rat.

$$\text{Dose} = \text{U (weight or volume of 1 Rat Unit)} \times \frac{\text{W (weight of rat)}}{140}$$

In testing an unknown solution it is injected into a series of rats to determine the smallest dose which causes typical oestrus. This minimum effective dose may be called Q. Had a smaller rat of 140 Gm. been used, then instead of Q, the minimum effective dose would have been only $\frac{Q \times 140}{W}$. To find the number of Rat Units per cc. divide 1 by this quantity.

$$\text{Rat Units per cc.} = \frac{1}{Q \times \frac{140}{W}} = \frac{W}{140\,Q}$$

Allen and Doisy specified that the rats should be given 3 injections at intervals of 4 hours. They employed oil solutions which are absorbed slowly. When aqueous colloid solutions, instead of oil solutions, were employed in our laboratory, it was found that 8 injections, 4 on each of two successive days, gave better results than the 3 originally specified by Allen and Doisy.

In a recent experiment two series of rats were injected with the same preparation, a colloid solution containing the Ovarian Follicular Hormone. One series was given 3 injections at 4-hour intervals and the other series was given 8 injections, 4 on each of two successive days. The results with the series receiving 8 injections

indicated that the preparation had a potency of 34 Rat Units per cc. while the series receiving 3 injections indicated a potency of less than 17 Rat Units per cc. Attention is particularly called to the importance of the number of doses given, for this is probably the source of the greatest variation in the technic of the workers in the different laboratories in this country. It is well known that two laboratories may find widely different values for the potency of the same product.

There is some individual variation among rats given the same dosage in regard to their oestrous reactions. For this reason a fairly large number of rats should be used in standardizing a preparation. At least 12 rats should be used and 3 should be given the same dosage. The smallest dose which will cause 2 out of 3 rats to show *typical oestrus* may be taken as the threshold dose. Oestrus is called typical when the Stage 2 smear shows only squamous cells, not a single leucocyte being found in the whole slide. If leucocytes are present in the Stage 2 smear, the reaction is called *atypical* oestrus and indicates that an inadequate amount of the Ovarian Follicular Hormone was injected. This end-point for determining whether a rat really shows oestrus or not is that originally proposed by Allen and Doisy (4), (5). It is a more severe criterion than that used by several European investigators (Laqueur (7), Loewe (8) and others) who base their end-point on the finding of a pro-oestrus condition in the vaginal smear. This important feature of the bio-assay method is discussed more completely in the excellent article by Allen and Doisy in *Physiological Reviews* (9).

Another important point concerns the time at which vaginal smears are taken after the injections have been given. Often typical oestrus cell forms, with no leucocytes present, are found in the vaginal smears for only a few hours. For that reason we take several smears at intervals as follows: one on the first day of injection, one on the second day of the test, three on the third day and three on the fourth. In terms of hours these intervals may be stated as 0, 24, 48, 52, 56, 72, 76 and 80 hours. The oestrus condition comes on at an interval which varies according to the size of the dosage injected. With very large dosage it comes on quickly and lasts for many hours. With the small dosages used in bio-assay the oestrus smear is usually found about noon of the third day of the experiment, that is in the 52-hour smear and lasts through the 56-hour smear and in some cases even until the 72-hour smear. Usually, however, either Stage 3 or Stage 4 is found in the 72-hour smear.

We have noted that some preparations of Ovarian Follicular Hormone produce a very transient oestrus. The typical oestrus may be found in only one smear. Stage 3 also may be very short. Apparently the material which causes this transient oestrus is unstable, for it deteriorates rapidly. One half of the activity may be lost in a month.

In regard to deterioration there seems to be great variation among several preparations. The best preparations lose very little, if any, activity in 4 to 6 months, while poor preparations may lose $^2/_3$ of their activity in this length of time.

The rats used in the bio-assay should be between 3 and 11 months of age and should have had regular oestrous cycles every 4 to 6 days before ovariectomy. The completeness of the ovariectomy should be proved by daily examination of vaginal smears to make sure the rats do not have natural oestrous cycles. The rats should never go more than two weeks without being injected with enough Ovarian Follic-

ular Hormone to cause an oestrous cycle, otherwise the uteri may atrophy and fail to respond to a unit dose. The rats should be kept under proper living conditions so as to be in good health. They must be fed a diet adequate in amount and containing the proper constituents. It is essential to have included sufficient vitamins. We find the simple Sherman (10) rat ration given in U. S. P. X, page 469 quite satisfactory. This consists of whole wheat flour 66%, whole milk powder 33% and sodium chloride 1%.

SUMMARY.

I. We propose the adoption as the standard unit in the bio-assay of preparations of the Ovarian Follicular Hormone, the Rat Unit, originated and defined by Allen, Doisy and associates in 1924 as follows:

"Provisionally we wish to define a Rat Unit as the quantity of material necessary to induce oestrus as judged by the smear method in an ovariectomized sexually mature rat weighing 140 plus or minus 20 grams."

II. We propose the adoption of a standard technic for the bio-assay of preparations of the Ovarian Follicular Hormone. The technic to be as follows:

1. Select normal healthy female white rats between 3 and 11 months of age, keep them in well-ventilated, clean, dry quarters in which the temperature is maintained constant, between 70° and 76° F.

2. Feed the rats an adequate amount of a well-balanced diet containing sufficient vitamins.

3. Examine vaginal smears daily for a period of two weeks to determine if the oestrous cycles are regular.

4. Perform complete double ovariectomy on those rats which have regular oestrous cycles at intervals of 4 to 6 days.

5. If any doubt exists as to the completeness of the ovariectomy, take vaginal smears daily to make sure there are no spontaneous oestrous cycles.

6. Prevent atrophy of the uteri by the injection of sufficient Ovarian Follicular Hormone every week or every two weeks to cause oestrous growth of the uteri.

7. After ovariectomy allow one week for recovery from the operation before making use of the rats for testing preparations.

8. In testing oil solutions of the Ovarian Follicular Hormone give 3 subcutaneous injections at 4-hour intervals in one day.

9. In testing aqueous colloid solutions and true aqueous solutions give 8 subcutaneous injections, 4 on each of two successive days.

10. Take vaginal smears at intervals of approximately 48, 52, 56, 72, 76 and 80 hours after the first injection.

11. The reaction caused by the Ovarian Follicular Hormone is the production of *typical oestrus* in which the Stage 2 vaginal smear contains only squamous cells.

12. This reaction should occur in 48 to 52 hours after the first injection and should last several hours.

13. Use at least 12 rats in testing a preparation, 3 to be given the same dosage.

14. The minimal effective dosage is that in which 2 out of 3 rats given the same dosage show *typical oestrus*.

15. Consider the standard rat to be one weighing 140 ± 20 Gm.

16. When rats weighing more than this standard are employed reduce the results to the 140-Gm. standard by the use of the formula,

$$\text{Rat Units per cc.} = \frac{W}{140\,Q}$$

in which W is the weight of the rat in Gm. and Q is the volume in cc. of the minimum dosage required to cause *typical oestrus* in 2 out of 3 rats given the same dosage.

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THE STABILITY OF ANÆSTHETIC ETHER.*

BY F. W. NITARDY AND M. W. TAPLEY.

The causes responsible for the development of traces of peroxide in anæsthetic or U. S. P. ether have long been the subject of scientific research. Of recent years, added stimulus has been given to this work by reports appearing in the medical press indicating that respiratory and nervous complications observed in certain cases after ether anæsthesia occurred simultaneously with the presence of peroxides in ether. While it is manifestly difficult to demonstrate by clinical experiment or physiological test that the trace of peroxides often present in ordinary anæsthetic ether is responsible for the adverse reactions reported, the evidence is such as to make freedom from peroxides in anæsthetic ether a highly desirable condition.

During the last five years, a number of articles have appeared in the scientific press discussing methods of measuring the quantity of peroxides present and suggesting means for their removal as well as giving methods for treating ether to prevent their formation. None of these methods, however, have proved of value in eliminating peroxides or even controlling their formation. The problem of isolating ether peroxides is not simple, because of their highly explosive character in pure form, hence their constitution is not fully determined at present. While an explanation of the causes and nature of the reactions involved in peroxide formation is yet to be formulated, intensive work has revealed that peroxides form in pure anæsthetic ether more readily than was formerly believed. In fact, peroxides can be shown to develop in practically 100% of all containers of anæsthetic ether

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