THE ASSAY OF PROGESTIN.

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MANY effects have been attributed to the active principle or principles of the corpus luteum. Hisaw [1927, 1929] described relaxation of the guinea-pig pubic symphysis following administration of an extract of the corpus luteum, but later, Fevold, Hisaw and Meyer [1930] showed that this reaction was not due to the important hormone of the corpus luteum discovered by Corner and Allen [1929], which causes progestational proliferation of the uterus. Other physiological effects described have been the sensitization to deciduomata formation of the uterus of castrate rats and guinea-pigs [Weichert, 1928; Goldstein and Tatelbaum 1929], the causation of mucification of the vaginal mucosa of mice [Harris and Newman, 1931] and the production of the premenstrual endometrium in the uterus of the monkey [Hisaw *et al.*, 1930]. These reactions, however, are not all due to the same active principle, as has been demonstrated by Robson [1931] and Meyer and Allen [1932]. Knaus [1930] showed that during the luteal phase of pregnancy and pseudo-pregnancy the excised rabbit uterus loses its *in vitro* response to pituitrin and suggested a method of assay based on this fact. De Fremery, Luchs and Tausk [1932], however, found that there was no direct correlation between the power of extracts to cause this reaction and to produce progestational proliferation of the uterus.

In connection with a chemical study of the progestational hormone of the corpus luteum at present being carried out in this laboratory by Dr R. K. Callow, it became necessary to examine methods of biological assay. The only two definite tests for this hormone, appropriately named progestin by Corner and his co-workers [Allen, 1930 *b*], are the causation of progestational proliferation of the rabbit uterus and the sensitization of the rat, mouse or guinea-pig uterus to the deciduomata reaction. Of the two, the former is almost certainly the more reliable, but there is no general agreement in the technique of employing this reaction in the

assay of progestin. Corner and Allen [1929] described their method in detail; it consisted of the use of adult œstrous animals, which were mated and then castrated about 18 hours later. The corpus luteum extract was then given subcutaneously daily for 5 days. Fevold and co-workers [1932] define a unit of the hormone as that amount which, when injected over a period of 4 days following ovariectomy of the œstrous rabbit, will cause complete progestational proliferation in the uterus. They do not state whether the animals used were mated. Allen [1930 a] studied the action of progestin on the immature rabbit, 570–1640 g., after initial treatment with œstrin and found that, as in the adult, progestational proliferation could be induced regularly, but he apparently never utilized such animals for assay purposes. Clauberg [1930 a, b, c, d, 1931], in a series of papers, records his method of testing progestin. Rabbits between 600–800 g. in weight were injected for eight successive days with 10 mouse units of œstrin and afterwards with five successive daily doses of the corpus luteum hormone. They were then killed and the uteri examined histologically. Although Clauberg states that the above method of testing is the most convenient known, he does not give exact details of his experiments.

The test on the immature rabbit is obviously the most convenient if it can be made quantitative, and the present paper records an examination of some of the chief variables in the assay of progestin on this animal.

Variables in the assay of progestin on immature rabbits.

The more obvious variables are (a) the size of the rabbit, (b) the amount of œstrin given to cause the initial growth of the uterus, and the duration of treatment, (c) the duration of administration of progestin, (d) the nature of the medium for injection and the site of injection, and (e) the extent of individual variation in response of immature rabbits. Of these, the weight of the animal was standardized as far as possible to be between 850–950 g., and both the œstrin and progestin were administered intramuscularly in oil solution. Both could have been prepared as emulsions suitable for subcutaneous injection, but since rabbits can easily be injected intramuscularly, it seemed preferable to administer the active substances in small volumes of oil by this route.

The conditions required to give the maximum efficiency of the test have been ascertained by administering:

(a) Different amounts of œstrin to immature rabbits over the same period of time.

(*b*) A constant dose of corpus luteum extract to rabbits sensitized with different amounts of œstrin.

(*c*) Different amounts of corpus luteum extract over the same time to animals sensitized by a standard œstrin treatment.

(*d*) The same amount of corpus luteum extract over different periods of time in animals sensitized by a standard œstrin treatment.

In all these experiments, individual variation has been investigated, and allowed for by the use of a group of rabbits for each test.

I. METHODS AND MATERIAL.

Animals. All animals were supplied from the Institute's Farm Laboratories at Mill Hill. No discrimination was made between different breeds of rabbits, except that unduly light and heavy breeds were avoided. The animals selected were as uniform in weight as possible. The extreme weights in the series of 106 animals are 700 and 1120 g., the average being 877 g.

Extracts. The œstrin used in this work was œstrone (keto-hydroxy-œstrin) supplied by the British Drug Houses, to whom my thanks are due. It was administered in the form of an oil solution containing 1000 International Units (0·1 mg.) per c.c. All quantities of œstrone in the following paper are expressed in International Units (I.U.). The progestin was obtained in oil solution from Løvens Kemiske Fabrik, Copenhagen. With only one exception, indicated below, the same batch of material was used in all experiments—"Lutex Leo, No. 336." The preparation was said to contain 1 rabbit unit of progestin per c.c. To distinguish this original standardization of the material, these units are referred to as "Løvens units."

Injections. All injections were made into the hindleg muscles, the two legs being used alternately. Œstrin injections were made every other day, and for descriptive purposes one injection is considered as treatment for 2 days. Corpus luteum injections were made each day. All animals were killed on the day following the last one of treatment.

Histology. The mammary glands of all animals were examined, and preserved whenever it was thought desirable. The ovaries, Fallopian tubes, uteri and a small portion of the vagina were removed and fixed in Bouin's fluid. After fixation the tissue was moved to 70 p.c. alcohol, and at this stage the organs were dissected and weighed. The weights given below are those of pairs; when only one organ was weighed its weight has been doubled for comparative purposes. A distal, median and proximal

segment

segment was cut from each uterine cornu and embedded, so that sections
of six distinct regions could be cut simultaneously. Sections were cut at
10μ and stained with hæmatoxylin and eosin.

In two groups of animals sections were cut of the Fallopian tubes. In
these same groups weights were recorded of the adrenal and thyroid
glands.

Evaluation of proliferation. To evaluate the degree of proliferation of
the uterus a method was used similar to that described by Allen [1930a].
Five uteri showing graduated degrees of response from the œstrous condi-
tion to that characteristic of full pseudo-pregnancy were carefully
selected and designated respectively as 0, 1, 2, 3 and 4 reactions. These
are shown in Plate I.

It will be seen that these type specimens show definite morphological
stages, in so far as it is possible in what is really a continuous proliferation.
Proliferation 0 is the œstrous condition, in which the endometrium is
compact and the comparatively few glands are evenly distributed
throughout the stroma. The epithelial lining of the lumen is almost
continuous, being only rarely broken by the mouths of glands (Plate I,
fig. 2). During the early phases of progestational proliferation glands
begin to develop round the lumen, but they are quite short and the deeper
stroma is unaffected (Proliferation 1, Plate I, fig. 3). As a development
proceeds, the glands increase in number and length. At this and the
previous stage the darker staining glandular tissue appears to form a
border to the lumen, while the deeper stroma is still unbroken and lightly
staining (Proliferation 2, Plate I, fig. 4). Further development results in
the glands extending and penetrating deeper into the stroma, which in
cross-section is becoming much reduced in amount. The glands are still
narrow. The glandular tissue has lost its appearance of being a border
round the lumen (Proliferation 3, Plate I, fig. 5). Finally, the glands
penetrate nearly to the myometrium, and in section the stroma is re-
stricted to the connective tissue cores of the endometrial folds. This final
stage is especially characterized by distention of the glands, which gives
a broken-up appearance to the endometrium (Proliferation 4, Plate I,
fig. 6).

These type specimens were used throughout as standards for compari-
son. Sections of the uteri of all animals were studied through a projection
microscope in such a manner that the image of the uterus under observa-
tion was thrown on to a table at the same magnification as the photo-
graphs of the standards which were kept alongside for comparison. In
estimating the degree of proliferation, the six sections of each uterus
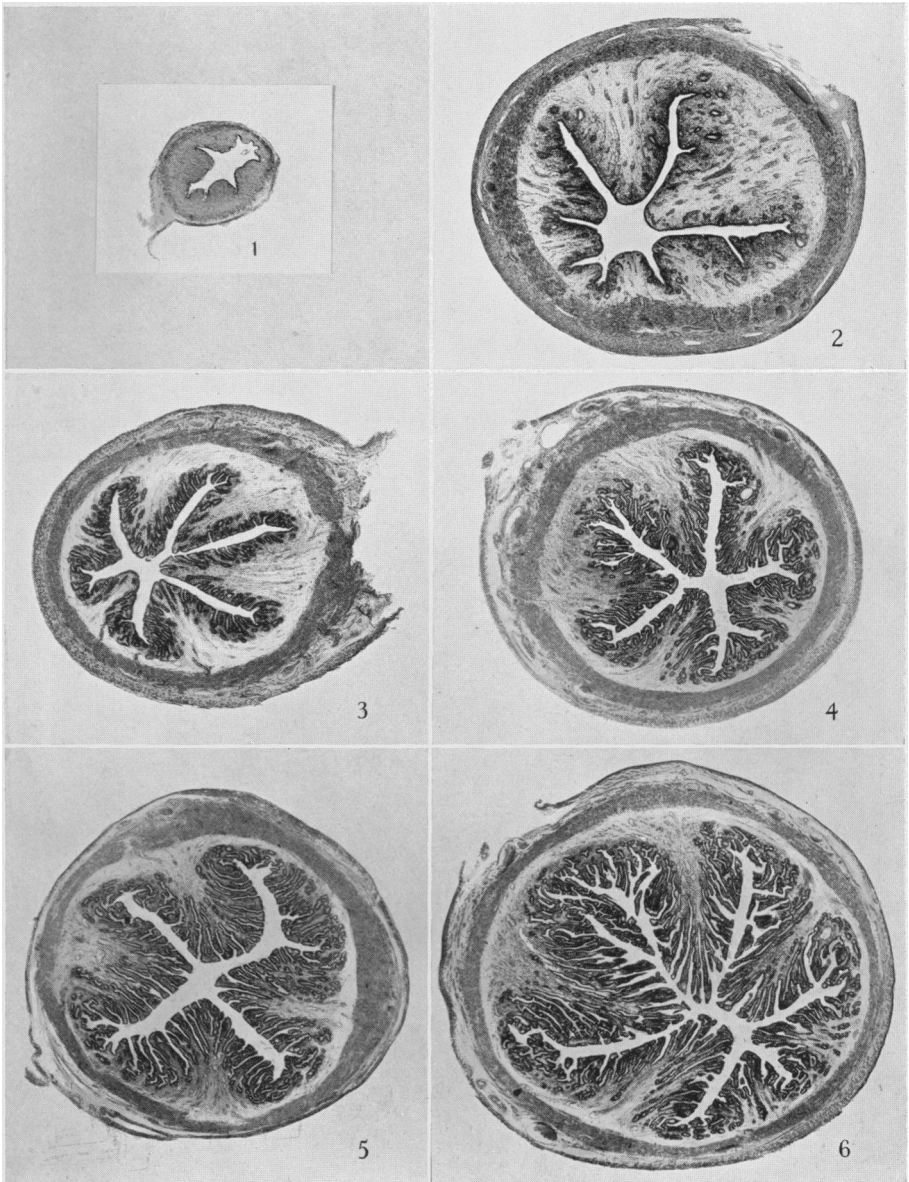
Uteri of immature rabbits, showing the standard scale of progestational proliferation (× 17). Fig. 1, no treatment; fig. 2, œstrin only, reaction 0; figs. 3–6, œstrin followed by progestin, reactions 1, 2, 3 and 4 respectively.

were examined and the average proliferation, judged as accurately as possible, taken as the result. Variation in different parts of the same uterus often exceeded one stage. When it was considered that the average reaction of the six sections lay between two of the standards the result was estimated to the nearest half, *e.g.* if it was between 2 and 3 the result was recorded as 2·5. The average of all reactions in a group of animals is recorded below as the "proliferation index."

II. AMOUNT OF ŒSTRIN REQUIRED TO CAUSE ŒSTROUS DEVELOPMENT OF THE IMMATURE RABBIT UTERUS.

Five groups of animals were given various amounts of œstrin; the total being given in three equal doses on alternate days and the animals killed on the seventh day. The results are shown in Table I, from which it

TABLE I. The effect of different doses of œstrin given over 6 days.

No. of animals used	Amount of œstrin I.U.	Av. body wt. g.	Av. ovary wt. g.	Av. uterus wt. g.	Proliferation index
4	50	814	0·021	0·34	0
5	150	855	0·043	1·23	0
4	600	840	0·030	1·49	0
4	1500	799	0·046	0·98	0
4	3000	837	0·025	1·11	0

will be seen (see also Plate I, fig. 2) that, while very considerable growth of the uterus has taken place, no pseudo-pregnant proliferation of the endometrium has occurred. That œstrin does not cause this reaction either in the immature or adult female rabbit had been shown early by Courrier and Potvin (1926), and has been confirmed many times since [Courrier and Masse, 1928; Corner and Allen, 1929]. The increases in the uterus weights indicate that there is no significant difference in the growth of the uteri of animals receiving 3000 I.U. of œstrin compared with those receiving 150 I.U., though all the animals in these groups showed greater uterine growth than those in the group receiving only 50 I.U. This result is borne out by histological examination. In none of the animals is the epithelium of the uterus broken regularly by glands (see Plate I, fig. 2), but in those which received 150–3000 units it is generally much more vascular and more highly developed than in those receiving 50 units.

Since the full œstrous condition could be produced in 6 days with a comparatively small dose of œstrin, it seemed worth while to find out if the necessary growth of the uterus could be produced in a shorter time by

larger amounts of œstrin. Four small groups of animals were injected, two groups receiving one single injection and the other groups two injections (Table II). Both the weight and histological appearance of the uteri of

TABLE II. The effect of different doses of œstrin given over different times.

No. of animals used	Amount of œstrin I.U.	Days of treat-ment	Av. body wt. g.	Av. ovary wt. g.	Av. uterus wt. g.	Proli-feration index
3	500	2	953	0·043	0·372	0
3	1000	2	935	0·042	0·455	0
3	1000	4	953	0·042	0·627	0
3	2000	4	947	0·040	0·483	0

these animals showed that the response lay approximately between that obtained with 50 and 150 units over 6 days (see Table I). In view of this fact, and especially since these animals were rather heavier than those used in the first experiment, it appeared unlikely that adequate growth of the uteri could be induced in 4 days even by large amounts of œstrin. A 6-day period of œstrin treatment seemed therefore necessary, and over this period, as already shown, it was improbable that any advantage would be obtained by giving more than 150 I.U. œstrin before a progestin test. It still remained, however, to show that the sensitization to the action of progestin was actually efficient with this comparatively small dose.

III. Amount of œstrin necessary to sensitize the immature rabbit uterus to the action of progestin.

Five groups of rabbits were given 2 Løvens units of progestin over 5 days after sensitization with various amounts of œstrin. The results are given in Table III and Fig. 1.

TABLE III. Effect of a constant amount of progestin (2 Løvens units) given over 5 days after sensitization with different amounts of œstrin.

No. of animals used	Amount of œstrin I.U.	Av. body wt. g.	Av. ovary wt. g.	Av. uterus wt. g.	Proli-feration index
5	50	941	0·034	1·21	2·5
5	150	972	0·037	1·83	3·0
5	600	933	0·036	1·92	3·2
5	1500	892	0·027	2·26	3·0
5	3000	974	0·047	2·00	2·8

There is an appreciable increase in the uterine weight and response in animals receiving 150 I.U. of œstrin and upwards over those getting only 50 I.U. There is a slight falling off in the reaction (see "proliferation

index") after sensitization with 3000 I.U. Such large amounts may still exert their effect after the end of œstrin treatment, and cause a diminution in the response to progestin. The antagonistic effect of œstrin to the action of progestin is well known [Allen, 1932].

TABLE IV. Effect of a constant amount of progestin (1 Løvens unit) given over 5 days after sensitization with different amounts of œstrin.

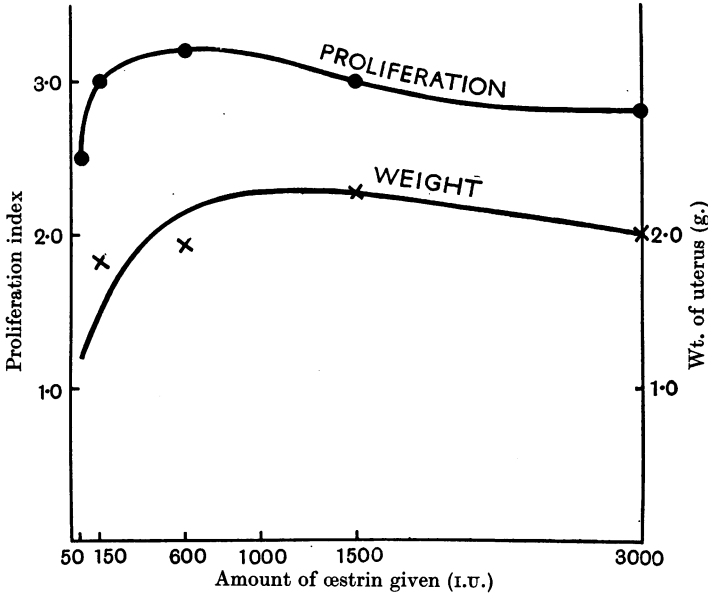| No. of animals used	Amount of œstrin I.U.	Av. body wt. g.	Av. ovary wt. g.	Av. uterus wt. g.	Proliferation index
2	50	1025	0·032	0·87	0·5
1	150	1040	0·032	1·12	3·0
1	600	835	0·029	1·01	2·5
2	1500	950	0·035	1·17	0·7
1	3000	1060	0·093	1·90	2·5

Fig. 1. Proliferation index and weight of the uterus in rabbits receiving 2 Løvens units of progestin over 5 days after sensitization with different amounts of œstrin.

Further groups of animals were given only 1 Løvens unit of progestin following various amounts of œstrin. Unfortunately, the progestin was of a different batch from that used in all the other experiments. The results, summarized in Table IV, cannot be considered as satisfactory owing to the few animals employed. They at least indicate the necessity for the use of groups of animals for each test.

The experiments described in this section show that 150 I.U. of œstrin given over 6 days is as efficient in sensitizing the uterus to the action of progestin as are much larger doses, and this amount has therefore been adopted as the standard preliminary treatment in the assay of progestin.

The next point to investigate was the optimum time over which to distribute the dose of progestin.

IV. EFFECT OF DURATION OF PROGESTIN TREATMENT.

Two sets of experiments were carried out. In the first, the injection of 1 Løvens unit of progestin was divided over 5 days and 10 days; in the second 2 units were given over 3, 5, 7 and 10 days. In both series the animals were previously sensitized with 150 I.U. of œstrin. Details are given in Tables V and VI and Fig. 2.

TABLE V. The effect of a constant amount of progestin (1 Løvens unit) given over 5 and 10 days.

No. of animals used	No. of days progestin given	Av. body wt. g.	Av. ovary wt. g.	Av. uterus wt. g.	Proli- feration index
6	5	819	0·036	1·54	2·3
5	10	811	0·042	0·31	0

TABLE VI. The effect of a constant amount of progestin (2 Løvens units) given over different periods of time.

No. of animals used	No. of days progestin given	Av. body wt. g.	Av. ovary wt. g.	Av. uterus wt. g.	Proli- feration index
5	3	886	0·047	2·10	2·9
5	5	850	0·043	1·91	3·1
5	5	972	0·037	1·83	3·0
5	7	1070	0·094	0·92	2·1
5	10	812	0·050	0·52	0·3

The results indicate clearly that following the initial treatment with œstrin, the greatest reaction is obtained when the dose of progestin is given over 3 or 5 days. It is unlikely that a greater response would be obtained earlier than 3 days, but it may be that 4 days of treatment would give a slightly better reaction than 5 days. This point was not investigated. The reason for the sharp decrease in response when the dose is divided over 7 or 10 days is not obvious, but it might be due to one of three factors: (1) that the effect of œstrin wears off after 5 days and the uterus is no longer capable of responding to progestin, (2) that the amount of

extract given is not concentrated enough to be effective when given over this time, or (3) that the immature rabbit uterus becomes insensitive to treatment after approximately 5 days. Since the reaction obtained with 1 Løvens unit given over 5 days is far better than that obtained with 2 units over 10 days, whereas theoretically they might be expected to be the same, the first of these three possibilities is the most likely.
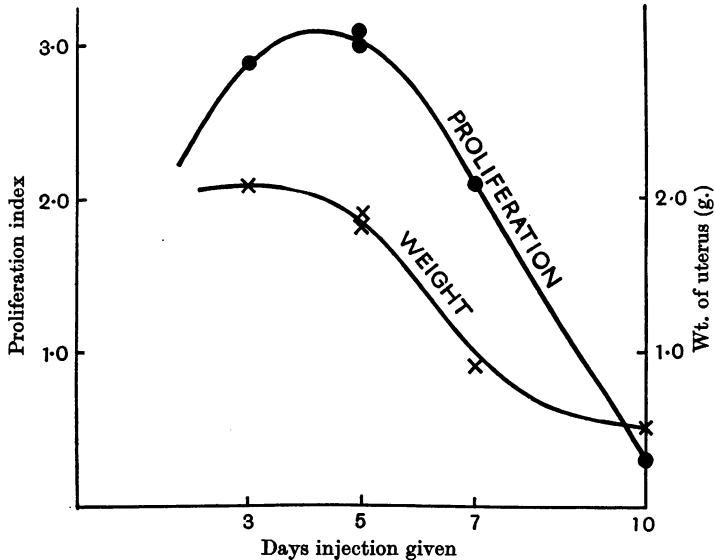

Fig. 2. Proliferation index and weight of the uterus of rabbits receiving 2 Løvens units of progestin over different periods of time.

From these results, a 3-day period of progestin injection might be adequate, but a 5-day period is probably safer, as well as more in keeping with current practice, and has been adopted in the routine testing of extracts.

V. RESPONSE TO DIFFERENT AMOUNTS OF PROGESTIN
AND DEFINITION OF A UNIT.

Three groups of rabbits receiving different amounts of progestin over 5 days after the standard sensitization with œstrin have been tested. The results are given in Table VII, and show a marked increase in response with increasing dose. Fig. 3 shows the proliferation index plotted against the dose, and obviously gives the rudiments of a standardization curve. The fact that the assessment of the reaction is inevitably rather approximate makes it difficult to put such a curve on a statistical basis, but it

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would appear that to cause a complete response in each rabbit of a group
would require an amount of progestin large in comparison with that
necessary to cause an easily assessed proliferation amounting to about
half the maximum.

TABLE VII. The effect of different amounts of progestin given over 5 days
after sensitization with 150 I.U. of œstrin.

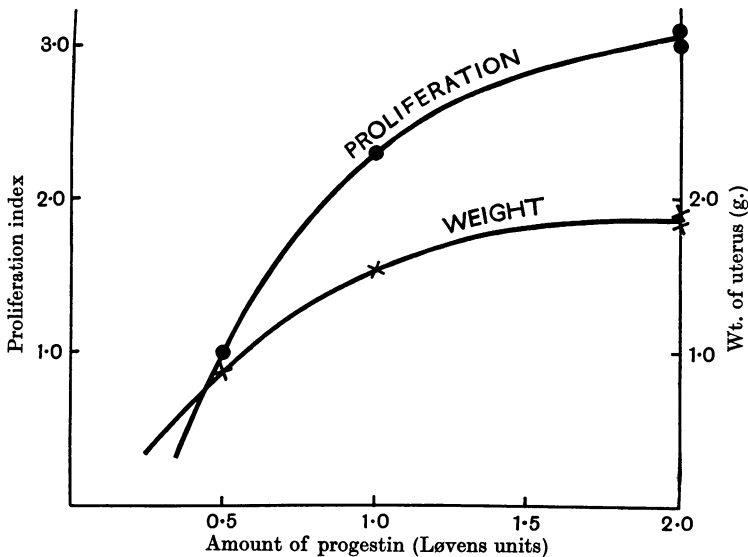| No. of animals used	Amount of progestin (Løvens units)	Av. body wt. g.	Av. ovary wt. g.	Av. uterus wt. g.	Proliferation index
6	0·5	764	0·036	0·87	1·0
6	1·0	819	0·036	1·54	2·3
5	2·0	850	0·043	1·91	3·1
5	2·0	972	0·037	1·83	3·0

Fig. 3. Proliferation index and weight of the uterus of rabbits receiving different amounts
of progestin over 5 days.

Definition of a unit of progestin. It is usually unsound to define a
biological unit of a substance as the amount required to produce a
maximum response. This fact is well illustrated by the work on progestin.
In this instance the objection is twofold—(a) the amount required to
produce a maximum reaction is large in comparison with that required
to produce a definite and readily detectable reaction, and (b) if a maximum
reaction is insisted on in every case, the whole object of using groups of
animals to increase the accuracy is lost—in fact the larger the group the

more chance of including less sensitive animals and the greater will be the unit. For these reasons it is proposed provisionally to adopt as a unit, for use in this laboratory, that amount of progestin which administered intramuscularly in oil over 5 days, following the injection of 150 I.U. of œstrin over 6 days, will cause an average proliferation of 2, on the scale here defined, in a group of immature rabbits (750–950 g.)

Size of the test group. Considerable individual variation was observed in these experiments. Where the average proliferation in a group of five rabbits was 2, individuals might vary from 1 to 3, and variation of this magnitude was found in most groups. It might therefore be desirable to use larger groups than 5, but owing to the variation found in different parts of the same uterus and to the personal factor in assessing the proliferation, it is unlikely that it will be possible to put the test on a really quantitative basis, and even groups of five animals involve considerable labour in routine assay.

VI. EFFECT OF PROGESTIN ON OTHER ORGANS.

Ovary. Ovarian weights were recorded in all the animals used. Sections were not cut, but all ovaries were examined macroscopically for signs of premature development. Clauberg [1930 d] showed that the ovary of the immature rabbit is insensitive to progestin, and this is clearly confirmed by the present data. There is no correlation between the degree of uterine development induced and the size of the ovary.

Fallopian tube. Fallopian tubes were sectioned from all animals recorded in Tables II, III and IV, *i.e.* from the animals of a group receiving œstrin alone and from two groups receiving œstrin *plus* progestin. No distinction was made between the different regions of the Fallopian tubes. No appreciable difference could be detected in the sections from twelve animals which received œstrin alone from the thirty-two treated with œstrin plus progestin.

Mammary gland. Œstrin treatment of the immature rabbit is known to cause proliferation of the mammary ducts to the slight extent found in the rabbit at its first œstrus [Parkes, 1930]. The great majority of the animals discussed above, however, showed little or no growth of the mammary gland, probably owing to the comparatively short period of injection. Corner [1930] appears to have shown conclusively that progestin will not induce the typical pseudo-pregnant development of the mammary gland, and so far as it goes, the present material confirms this conclusion.

Adrenal and thyroid glands. The adrenal and thyroid glands were also removed from the animals recorded in Tables II, III and IV and weighed.

No significant difference was found in the weights of the organs from animals treated with œstrin alone compared with those receiving œstrin and progestin.

VII. SUMMARY.

1. 106 immature rabbits were used in a study of the assay of progestin, the progestational hormone of the corpus luteum.

2. The reactions of the uterus are described (*a*) to amounts of œstrin ranging from 50–3000 I.U., (*b*) to varying doses of œstrin followed by a standard amount of progestin, (*c*) to varying doses of progestin following a standard amount of œstrin, and (*d*) to a standard dose of progestin administered over different periods following a standard amount of œstrin.

3. No significant effect of progestin was obtained on the mammary gland, ovary or Fallopian tube nor on the adrenal and thyroid glands.

4. A provisional unit of progestin has been defined for the laboratory.

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