LXXX. RELATIVE DURATION OF ACTION OF VARIOUS ESTERS OF OESTRONE, OESTRADIOL AND OESTRIOL

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INTRODUCTION

Effects of esterification. Allen and Doisy and their co-workers [1924] in their early work on ovarian extracts found that a given amount of active substance was much more efficient when administered as a series of small doses than when given in one injection. With the advent of more purified preparations and of aqueous solutions, divided dosage became even more advantageous. Thus Laqueur & de Jongh [1929] found that more than 30 times the total amount of extract was required to give the same effect when the whole dose was given in one injection instead of in six separate injections over 36 hours. These results were confirmed by Dodds and his co-workers [Allan *et al.* 1928] and by Marrian & Parkes [1929], and could obviously be explained by supposing that a single dose, especially in aqueous solution, was quickly absorbed and excreted or destroyed, and that the duration of threshold concentration in the blood necessary for a positive response could only be achieved either by a massive single dose or by a series of injections.

Soon afterwards it was discovered by Butenandt [1930] that oestrone benzoate had a prolonged action, a finding afterwards confirmed by David *et al.* [1935] for oestradiol monobenzoate. In the meantime Zondek [1934] had found, as might be expected from Butenandt's results, that the benzoate did not disappear from the body so rapidly after injection as the free hormone. Sub-division of the total dose is thus not necessary for achieving the maximum effectiveness of the benzoate as it is in the case of the free hormone. It follows from this that extraordinary differences in the apparent relative activities are obtained according to the method of test used for comparing the free hormone and the benzoate. Other esters of the oestrone series which have been examined include oestriol triacetate [Marrian, 1930], which in the ordinary routine test was about two-thirds as active as oestriol, and oestradiol diacetate and dibenzoate which were found by David *et al.* [1935] to show much less intense, but more prolonged actions than the free hormone, the minimum effective dose of the dibenzoate being 170 times greater than that of the free hormone.

This work on the effect of mode of administration on the efficiency of oestrone and oestradiol has been paralleled remarkably closely by recent tests on the male hormones. Both androsterone and testosterone are rapidly excreted or destroyed after subcutaneous injection. An extraordinary increase in effectiveness of a given dose can be induced if absorption is delayed by subdividing the daily dose, increasing the volume of oil in which the hormone is dissolved, adding fatty acid to the solution or esterifying the hormone [see Miescher *et al.* 1936, and Parkes, 1936].

Physiology of feather development. The prolongation of action by esterification in the oestrone series suggested that results of interest would be obtained on the feathers of the Brown Leghorn capon.

Brown Leghorns show extreme sex dimorphism in the plumage; in particular the ventral feathers are solid black in the cock and fawn in the hen. Castration does not affect the colour of the cock plumage, but complete ovariectomy results in reversal of the plumage to the male type at the first moult after operation or after plucking. The male plumage is therefore the asexual or neutral type, while the female plumage results from the action of the ovarian hormone on this neutral type. The effect can be reproduced by the injection of oestrone into the capon while feathers are growing; the black ventral feathers grow brown so long as an effective concentration of oestrone is present in the circulation. Existing plumage is not, of course, affected. The physiology of this feather response has been fully dealt with by Juhn & Gustavson [1930], Juhn *et al.* [1931] and Lillie & Juhn [1932]. Their chief findings relevant to the present work are:

(a) The feathers of the different tracts grow at different rates and have correspondingly different sensitivities to oestrogenic substance; thus the saddle hackle grows much more slowly than the breast feather and is much more sensitive. The rate of feather growth can easily be determined and with it the duration of action of a dose or a course of doses of oestrone.

Fig. 1. (a)-(d) Diagram of types of response of breast feathers of Brown Leghorn capons to various types and doses of oestrogens.

(b) The apical-basal rate of growth of the feather is approximately constant so that the sensitivity of the feather is similar at different points along its length. The barbules, however, show a growth-rate gradient, the growth of the tips being faster than the subsequent growth of the base, and as a result the tips are less sensitive to oestrone.

It is thus possible to put a transverse or a longitudinal bar in the feather by appropriate dosage with oestrone. Fig. 1 illustrates some of the extreme types of response observed in the present work. In the following description a stimulus of "full effective intensity" means one sufficient to influence the barbs at any stage of their growth, while a stimulus of "minimum effective intensity" means one which will influence only the more sensitive bases of the barbs.

(a) *Rachis spot*. This type of response results from a stimulus of minimum effective intensity and short duration, such as is provided by a single small dose of a compound readily available to the organism.

(b) *Narrow complete bar*. This type of response results from a stimulus which is of short duration, but of full effective intensity during the time. A large dose of a readily available compound gives a response of this type.

(c) *Long rachis stripe*. This type of response results from a minimum stimulus maintained at a constant level for a long period. Such a response can only be obtained by a carefully adjusted daily dosage or by a single very large dose of a compound which becomes available to the bird very slowly but continuously.

(d) *Wide complete bar*. This results from a stimulus of full effective intensity and long duration, and is obtained with adequate daily dosage or with one large dose of a compound which becomes available at an optimum rate.

Material

Greenwood & Blyth [1935] have dealt with individual variation in birds, and with certain factors affecting it, of which the most important appears to be age.

The birds used in the present study comprised four batches of capons hatched in 1935. Each batch was divided into a number of groups of, usually, 5 birds, and so far as possible results are compared only on groups of the same batch injected at the same time. For each test, the whole of one side of the breast was plucked and regeneration was allowed to proceed for 10–17 days before injection was made. When the feathers were fully grown, the inside rows of the tract were sampled in the region of the crop. Under these conditions, variation in response among different feathers from this small area of one bird was slight, while that from bird to bird was not serious and actually appeared to be less than that found with many biological tests.

The dose of hormone was dissolved in a fairly large volume of arachis oil, usually 5 ml. No other solvent was used in these experiments. A definitive experiment failed to show any influence of the volume of oil on the duration of the response, but other experiments, not included here, show a very clear influence of the nature of the solvent. The total dose was administered as a single intramuscular injection.

Dosage of the esters was always calculated in terms of free hormone. Thus 1.38 mg. oestradiol benzoate $\equiv$ 1.0 mg. oestradiol etc.

Feather growth rate

In order to translate "width of brown bar" into "days of action of the hormone" it is necessary to know the feather growth rate. This was found for the stock of birds in question by measuring the distance from the tip of the feather to the top of the bar at the rachis and then plotting this against the time between the plucking of the previous feathers and the injection. As the injection does not take effect instantaneously the figures for growth rate obtained by this method are slightly on the high side, but the data given by Lillie & Juhn [1932] suggest that the error is negligible.

The results are shown in Fig. 2, from which it will be seen that 3–5 days elapse after plucking before a new feather blade is laid down, and that subsequent growth is nearly linear (as shown by Lillie) and averages about 2 mm.

per day. This figure has therefore been used as the basis of calculation of duration of hormone action.

Fig. 2. Length of breast feather blade of Brown Leghorn capons at various times after plucking of the previous feathers. (Each point is the average for a group of 3-5 birds.)

Action of free hormones

Oestrone. Three different doses of oestrone were used, 100 γ , 250 γ and 4 mg. Eight birds received 100 γ ; three showed no response, three showed good rachis spots and one a faint one, while the last had a narrow incomplete bar. Four received 250 γ ; one had a faint rachis mark only, while the other three had complete narrow bars. All the five birds on 4 mg. showed complete bars, averaging 5 mm. in width at the rachis.

Oestradiol. Twenty-five birds received 100 γ oestradiol, of which two showed good rachis spots, three faint rachis spots and three faint incomplete bars, while seventeen gave no response. Six birds received 250 γ free oestradiol, of which two showed no response, two showed rachis spots and two narrow bars. Four received 1 mg., three of which showed bars and one a rachis spot. Fourteen receiving 4 mg. all showed a well-developed bar (Fig. 3 and Plate IV, fig. 1).

Oestriol. Five birds receiving 250 γ oestriol all failed to show a response.

The free hormones therefore show the following descending order of effectiveness by this test: oestrone, oestradiol, oestriol. It appears to be impossible to obtain a prolonged effect with any of these free hormones in oil solution even if the dose is increased to twenty times the minimum effective level. About 3 days is the longest period of activity obtained.

Effect of acetylation

Oestrone acetate. Four birds received 100 γ oestrone as acetate; two of these showed no response, while the other two showed rachis spots. Five received 250 γ ; two showed faint rachis spots, two narrow complete bars and one a wider bar. Three birds receiving 1 mg. all showed a well-developed bar, averaging about 4 mm. in width (Plate IV, fig. 3). Comparison of these results with those obtained with free oestrone shows that acetylation in position 3 has little effect on the duration of action.

Oestradiol diacetate. Five birds were given 100γ ; three showed heavy rachis spots and two complete bars. Nine receiving 250γ all showed complete bars, of which one was nearly 10 mm. wide. Three receiving 1 mg. all showed wide bars (average 11.6 mm.). Of three receiving 4 mg. two showed wide bars, and one a much less wide bar (average 13.3 mm.). Comparing these results with those given by the oestrone acetate it is evident that acetylation in position 17 has a very decided effect on duration of action as compared with acetylation in position 3.

Oestriol triacetate. Five birds receiving 100γ and five 250γ gave no response. Of five receiving 1 mg. one showed a narrow bar, three good rachis spots, and one a faint rachis spot. Five receiving 4 mg. all showed complete bars, average width 4 mm. From these results it is clear that oestriol triacetate is an ineffective diacetate.

Effect of benzoylation

Oestrone benzoate. This proved to be a highly effective compound. 100γ produced good bars in three out of five birds, and heavy rachis spots in the other two. Amounts of 250γ , 1 mg. and 4 mg. each given to five birds produced increasingly wide bars, the heaviest dose being capable of feminizing almost the whole feather (see Fig. 3).

Fig. 3. Dose-response curves for duration of action of various esters of oestrone and oestradiol.

Oestradiol 3-monobenzoate. This compound studied on a total of fifty birds proved to have a prolonged action, though slightly less in the higher doses than that of oestrone benzoate. (Fig. 3 and Plate IV, fig. 2). Furthermore the compound showed a more intense action than the free hormone, 50γ producing rachis spots in two, and narrow bars in three, out of five birds.

Oestradiol dibenzoate. The results of acetylation suggested that esterification in position 17 had more effect in prolonging the action than had esterification in

position 3. It thus seemed that oestradiol dibenzoate might have an extremely prolonged action, in which case the minimum effective dose might be expected to be high. In practice, this turned out to be so. Doses of 250 γ , 1 mg. and 4 mg. evoked no response at all in thirteen birds. 10 mg. to each of seven birds produced the most varied effects. Two birds showed a rachis stripe down the whole length of the feather, three showed a curious brown mottling of the feather, quite unlike the usual clear-cut response, one gave no response, while one gave the full normal response for so long a period that new feathers growing six weeks after injection were still appearing brown.

Oestradiol 3-benzoate-17-acetate. The above results show that 3-benzoylation and also 17-acetylation are beneficial to lengthening activity. The combination of both to produce a double ester gave the longest-acting compound so far examined (Fig. 3 and Plate IV, fig. 3). 1 mg. produced an effect as prolonged as that of 4 mg. oestrone benzoate. This compound, however, was found to have a higher minimum effective dose, only one of the five birds given 100 γ showing even a rachis spot.

Other compounds. Five birds receiving 100 γ oestrone methyl ether gave three negative responses, one rachis spot and one bar. Of three birds given 17-methyloestradiol one only showed a faint rachis spot. Four birds receiving 1 mg. 3-methoxy-17:17-dimethyloestratetraene all failed to produce a response.

DISCUSSION

The results described above show that the growing feather of the Brown Leghorn capon is an excellent test object for determining the duration of action of oestrogenic compounds. This duration of action clearly depends on (a) the threshold value for the feminization effect and (b) the extent to which the bird can conserve hormone present in excess of the threshold requirement. It is evident that the bird, like the mammal, is unable to retain excess of free oestrone or oestradiol in the body and that a large dose is mostly wasted by excretion or destruction and will produce only an evanescent effect. From the nature of the results it is evident that esterification may greatly reduce the availability of the hormone to the bird, so that a single dose may be liberated over a long period and may therefore exert a prolonged effect if the amount of free hormone in the circulation is continuously above the threshold value. Esterification in position 17 decreases availability more than esterification in position 3 and benzoylation more than acetylation. The most efficient compound so far examined, oestradiol 3-benzoate-17-acetate, in a dose of 250 γ , gave an effect lasting more than 10 days, so that the necessary threshold value can apparently be maintained by the liberation daily of not more than 25 γ of free hormone. Probably the minimum figure is much less. Since 4 mg. free oestradiol give an effect for three days only, more than 98 % of this dose is wasted. We have so far no evidence as to whether the excess material is destroyed or excreted, but we hope to ascertain this by metabolism experiments, for which the fowl is well suited. It may be noted here that in view of Marrian's work [Cohen & Marrian, 1936] it is likely that the injected hormone is excreted as a water-soluble ester. In this case one might expect a marked difference in the behaviours of oestrone benzoate and oestradiol 3-benzoate, since the former could only be esterified for excretion after hydrolysis, whereas the latter could be made into a 17-glucuronide which might be excretable without hydrolysis. The fact that there is no great difference between the two esters suggests that they are both hydrolysed before excretion. More recent experiments show that hydrolysis is rapid once the compound reaches the blood

Fig. 1. Effect of oestradiol: (left to right) 100 γ , 250 γ , 1 mg., 4 mg.

Fig. 2. Effect of oestradiol monobenzoate: (left to right) 50 γ , 100 γ , 250 γ , 1 mg., 4 mg.

Fig. 3. (Left to right). Effect of 1 mg. oestrone as acetate, 1 mg. oestradiol as benzoate, and 1 mg. oestradiol as 3-benzoate-17-acetate.

stream and that the prolonged action of the esters is due mainly to delay in absorption from the site of injection. This aspect of the problem will be dealt with in a separate communication.

SUMMARY

1. Feminization of the growing plumage of Brown Leghorn capons has been used to determine the duration of action of various esters of oestrone, oestradiol and oestriol.

2. Even massive doses of the free hormones, given as one injection, have only a transient effect. Oestrone acetate is scarcely more effective. Oestradiol diacetate, oestradiol monobenzoate, oestrone benzoate, in that order, show increasingly prolonged activities without loss of intensity (i.e. without increase of the minimum effective dose).

3. Oestradiol 3-benzoate-17-acetate shows prolonged activity with a slight loss of intensity. Oestradiol dibenzoate has a very low intensity, but large doses may have a very prolonged action. Oestriol triacetate has a low intensity and apparently only a transient effect.

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