BIOASSAY OF OESTROGEN USING SHEEP

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Summary

The quantitative assay of oestrogen has been investigated using immature ewes and wethers and adult ovariectomized ewes. Stilboestrol dipropionate was administered at several dosage levels for varying periods, and uterine and cervical weights in the ewes and bulbo-urethral gland weights in the wethers were obtained at slaughter.

A 3-day injection period in ovariectomized ewes, using the uterine weight response, gave an assay of high precision and sensitivity.

I. INTRODUCTION

It is well established that numerous pasture species contain oestrogenic substances (Bennetts, Underwood, and Shier 1946; Bartlett *et al.* 1948; Curnow, Robinson, and Underwood 1948; Pope 1954; Pieterse and Andrews 1956; Bickoff *et al.* 1959). Affected pastures may interfere with reproduction in sheep (Bennetts 1946). The problem of oestrogenic pastures in Australia was first thought to be confined to the Dwalganup strain of subterranean clover (*Trifolium subterraneum* L.), grown principally in Western Australia, and, following the classic work of Bennetts and his co-workers, the disease of grazing sheep, commonly known as "clover disease", seemed to be well understood. However, it has become evident from recent surveys (Barrett *et al.* 1961; Moule 1961) that the syndrome of clover disease is more widespread in the eastern States than was previously thought. Thus the problem of oestrogenic pastures may be of greater significance to the sheep-breeding industry than was hitherto supposed.

As present chemical methods of detecting and measuring oestrogenic substances in pasture plants are time-consuming and costly, biological methods assume considerable importance. Because of the uncertainty of the nature and actions of the oestrogenic substances, qualitative as well as quantitative assay methods must be developed to a high degree of reliability. To date these methods have been investigated in laboratory animals such as the guinea pig (Alexander and Watson 1951) and mouse (Robinson 1949; Biggers and Curnow 1954; Kitts *et al.* 1959; Mumford and Flux 1961). The ewe has been used occasionally for qualitative assay (Sanger, Engle, and Bell 1958; Jennings and Dow 1959), but has not received as much attention as may be warranted, particularly in view of the fact that ingestion of pastures by the sheep is a mode of administration of which little is known.

This paper describes experiments designed to examine the use of sheep for quantitative bioassay, using the readily available synthetic oestrogen, stilboestrol, as the test substance. The choice of stilboestrol was prompted by the difficulty in

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obtaining genistein, coumoestrol, or biochanin A, by their relatively low potency, and by the fact that in laboratory animals stilboestrol has proved to be a useful standard when given orally.

II. Materials and Methods

(*a*) *Experiment* 1*A*

Twenty 16–17-month-old maiden Merino ewes were ovariectomized 9 days before receiving the first of a series of injections of stilboestrol dipropionate in peanut oil, in March 1960. Two dose levels were employed, 5 μg or 15 μg, given intramuscularly on Mondays, Wednesdays, and Fridays of each week for 5 weeks. Twenty Merino wethers of similar age received the same two dose levels for the same period.

Table 1

Treatment schedule for experiment 2

Group*	Total Dose (μg)	Daily Dose (μg)	No. of Consecutive Daily Injections
1	10	10	1
2	10	3·3	3
3	10	1·1	9
4	30	30	1
5	30	10	3
6	30	3·3	9
7	90	90	1
8	90	30	3
9	90	10	9

* Three classes of sheep: ewe lambs, wether lambs, or adult ovariectomized ewes. Three sheep of each class per group.

There were 10 sheep per group; all sheep were run together in pens for 1 week prior to commencement of injections and throughout the series of injections. They were fed a ration of cereal grain and oaten chaff. The sheep were killed 2 days after the final injection and uterine and cervical weights (weighed together) and bulbourethral gland weights were obtained immediately after slaughter.

(*b*) *Experiment* 1*B*

Groups of four Merino ewe lambs (37–55 lb wt.) and six ovariectomized adult Merino ewes (60–86 lb wt.) received six intramuscular injections of stilboestrol dipropionate in peanut oil, in February 1961. Doses and methods of administration were the same as for experiment 1A but the duration of the experiment was 2 weeks. The adult ewes had been spayed 2 months previously and were on pasture until 1 week before commencement of injections. The uterus and cervix were weighed separately.

Experiment 2

Adult ewes, ovariectomized 3 weeks prior to experiment, and ewe and wether lambs were divided at random into nine groups each of three animals for each of the three classes. Stilboestrol dipropionate in propylene glycol was given intramuscularly according to the schedule as set out in Table 1. The experiment was a 3×3^2 factorial design consisting of three classes of sheep and three total dosage levels of oestrogen given over three periods of time. There were thus 27 groups each of three animals.

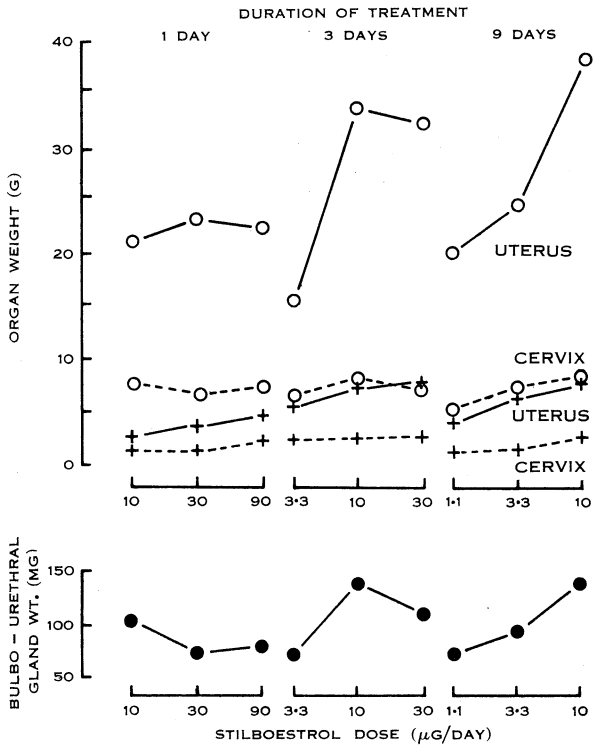

Fig. 1.—Mean responses to stilboestrol administration in ovariectomized ewes (○), ewe lambs (+), and wether lambs (●) for experiment 2.

The injections were given in such a way that all sheep received their final injections within a period of 24 hr. They were slaughtered 1 day after the final injection. Uterine and cervical weights were obtained separately. Bulbo-urethral glands were dissected free of overlying muscle and fascia and each pair of glands weighed. All organs were weighed in the fresh state, as before.

III. Results

The results are set out in Table 2 (expt. 1) and Table 3 (expt. 2). In Table 2 the group means and variances together with standard errors of the slopes are presented. The group means for experiment 2 are not included in Table 3 but are shown graphically in Figure 1. In each table is included an estimate of the value

TABLE 2

LOG DOSE–RESPONSE DATA FOR EXPERIMENTS 1A AND 1B

Expt. No.		Mean Response		D.F.	Variance of Response (V_x)	Slope (b)	S.E. of Slope (S.E.$_b$)	Index of Precision $\lambda \pm$ S.E.
		5 μg	15 μg					
1A	Ovariectomized ewes							
	Uterus plus cervix (g)	14·53	20·28	18	6·40	12·05	2·37	0·21±0·05
	Wethers							
	Bulbo-urethral glands (mg)	350	920	18	16,700	1195	120	0·11±0·02
1B	Ewe lambs							
	Uterus (g)	5·90	7·07	6	1·06	2·43	1·53	0·42±0·29
	Cervix (g)	1·82	2·46	6	0·44	1·32	0·99	0·50±0·40
	Ovariectomized ewes							
	Uterus (g)	8·74	13·27	10	6·63	9·50	3·11	0·27±0·11
	Cervix (g)	2·85	4·02	10	0·30	2·45	0·67	0·22±0·08

TABLE 3

ANALYSIS OF LOG DOSE–RESPONSE DATA FOR EXPERIMENT 2

Symbols used in table headings are defined in Table 2

	Single Injection				Three Injections				Nine Injections			
	V_x*	b†	S.E.$_b$	$\lambda \pm$S.E.	V_x	b	S.E.$_b$	$\lambda \pm$S.E.	V_x	b	S.E.$_b$	$\lambda \pm$S.E.
Ewe lambs												
Uterus (g)	1·38	2·77	1·76	0·37±0·24	1·38	3·27	1·76	0·31±0·17	0·43	5·03	1·76	0·20±0·08
Cervix (g)	0·21	—	—	—	0·30	—	—	—	0·01	1·56	0·19	0·07±0·01
Ovariectomized ewes												
Uterus (g)	12·39	—	—	—	29·92	38·5	7·71	0·12±0·03	18·57	28·2	7·71	0·16±0·05
Cervix (g)	0·96	—	—	—	1·37	—	—	—	2·79	4·34	2·24	0·30±0·16
Wether lambs												
Bulbo-urethral glands (mg)	634	—	—	—	1751	—	—	—	792	95·7	53·4	0·32±0·19

* Where variances for responses within-classes are homogeneous the test for linearity (slope) was made using the pooled error (within-group) variance. The standard error of the slope and the index of precision were similarly calculated from the pooled variance.

† Slopes were calculated from the doses giving the greatest increment in response (see Fig. 1).

of each assay for quantitative studies, the index of precision λ. This is calculated from the formula

$$\lambda = \frac{\text{standard deviation of response}}{\text{slope}} = \frac{s}{b}.$$

The standard error of λ is given by the equations

$$\text{S.E.}_\lambda = [b^{-2}(V_s + \lambda^2 V_b)]^{\frac{1}{2}},$$

where

$$V_s = \frac{\text{variance of response } (V_x)}{2 \times \text{degrees of freedom}}.$$

There was no relationship between responses and body weights in either the mature or immature animals.

Within-group variances for particular classes of sheep and for the separate responses were examined for heterogeneity. It is often found that variability in response increases with dose but in these experiments there was no evidence of heterogeneity, except in the ewe lamb cervical weights in experiment 2. In the latter instance the average variance of the 9-day treatment was low; this variance and not the pooled average for lamb cervical weights was used in calculation of the standard error of the slope and the index of precision. Compared to the other assays using this response, these values seem extremely low, and may be fortuitously so.

In certain cases in experiment 2 the mean square for slope was not significantly different from the error mean square and hence no estimates of slope were possible.

IV. Discussion

The main factors which determine the choice of an optimum assay are as follows:

(1) Precision—the index of precision gives a useful, broad comparison between assays.

(2) Sensitivity—this is an extremely important factor in assaying pasture oestrogens since those identified so far are of low potency and are present in variable concentrations.

(3) Reliability—it is important that the assay method measures what the experimenter is looking for. This infers that the assay must be accurate and specific—it was this consideration which prompted the examination of the sheep for bioassay (Moule, Lamond, and Braden, unpublished data, 1962).

(4) Simplicity and cost—assays which are time-consuming and require considerable technical skill are not easily adapted to routine use.

On the basis of precision, three assays stand out. They are all uterine weight assays and include 9-day injections to ewe lambs and 3-day and 9-day injections using ovariectomized ewes. Nine-day cervical weight assays gave reasonable results but bulbo-urethral glands were not satisfactory except after administration of hormones for some weeks.

The most sensitive uterine weight assay was the 3-day method in ovariectomized ewes. Uterine weight increases in ovariectomized animals generally result from oestrogen action whereas animals in which the ovaries are intact may respond to gonadotrophic substances. Old ewes are readily obtained and ovariectomy can be carried out rapidly, particularly when a laparotomy cradle is used (Lamond and Urquhart 1961). From these considerations it seems reasonable to recommend the use of short-term (3-day) assay using uterine weights in ovariectomized ewes.

In maiden ewes ovariectomized some months previously (expt. 1B) uterine weights of uninjected ewes averaged approximately 6 g. In experiment 2 the ewes were not virgin and had been spayed only 3 weeks. It is not known to what extent the uteri regressed in these ewes but it does seem reasonable to suggest that the greater sensitivity was related to the fact that the uteri were in a partly primed state, not unlike the oestrogen priming given to mice prior to Allen–Doisy assays. Robinson (1955) has shown the importance of progesterone priming in the spayed ewe in the oestrous response to injected oestrogen. It is possible therefore that progesterone or oestrogen priming or both might improve the assay.

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