OPTIMUM CONDITIONS FOR THE LOCAL (INTRAVAGINAL) ACTION OF OESTROGENS

By J. D. Biggers* and P. J. Claringbold*

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Summary

The optimum conditions for the intravaginal administration of oestrogens are obtained by the use of 1 per cent. aqueous egg albumin as solvent, and two or four injections spread over 24-36 hr. The administration of oestrone, oestradiol-3,17β, and diethylstilboestrol in distilled water by four injections in 36 hr gives a close approximation to the optimum. Oestriol, however, has considerably reduced activity when administered under these conditions.

Relative activities of oestrone, oestradiol-3,17β, oestriol, equilin, equilenin, and diethylstilboestrol have been determined, using four injections in 36 hr, and 1 per cent. aqueous egg albumin as solvent. Under these conditions all have the same activity as oestrone, except oestradiol-3,17β, which is approximately 1.5 times as active.

The optimum conditions for intravaginal administration are similar to those for subcutaneous administration with respect to the period over which injections are spread. The two techniques, however, differ with respect to the frequency of injection; while repeated subdivision of dose in the subcutaneous method increases response there is a maximum response reached by four injections with the intravaginal method.

The relation of the above results to the quantitative study of oestrogen activity and the local action of oestrogens on the vaginal epithelium are discussed.

I. Introduction

Marrian and Parkes (1929) studied several variables in the subcutaneous Allen-Doisy technique for oestrogen assay. Using four injections they found that maximum sensitivity was elicited by spacing the injections over 24-36 hr, and that even greater sensitivity was obtained by administering the total dose in many injections (up to 20). Recently attention has been given to the technique of intravaginal assay. Mühlbock (1940) found, using oestrone, that three injections resulted in greater sensitivity than either one or two injections, while Emmens (1941), using oestrone, oestradiol-3,17β, oestriol, and diethylstilboestrol, showed that two injections in 24 hr resulted in greater sensitivity than a single injection. Since then, two injections in 24 hr have been accepted as standard (Emmens 1950*a*, 1950*b*; Biggers 1953*a*). Recently, however, Sulman (1952) has claimed that greatly increased activity is obtained if five injections are spread over 36 hr. In view of the incompleteness of the previous work on the intravaginal technique, systematic investigations of variations of technique were

* Department of Veterinary Physiology, University of Sydney.

undertaken, with the object of determining optimum conditions of administration. Experiments of factorial design which permit the study of factor interactions, and which also afford economy in animals and time, were employed where possible.

II. Materials and Methods

Four colonies, of 260, 360, 380, and 440 albino mice bred in this department, were ovariectomized. Their management was identical with that described by Biggers (1951), tests being carried out at 14-day intervals.

The oestrogens were administered either in distilled water or in 1 per cent. aqueous egg albumin solution. The solutions, with the exception of those used in experiments on the effect of volume, were prepared as described in earlier papers (Biggers 1951, 1953*b*) so that the volume of each injection was 0.01 ml. The oestrone, oestradiol-3,17β, and diethylstilboestrol were obtained from Organon Laboratories, the oestriol from Parke Davis & Co., and the equilin and equilenin from Ayerst, McKenna and Harrison Co.

III. Design and Analysis of Experiments

Past experience had indicated that under the various treatment combinations to be employed wide variations in the level of response would occur. Where necessary pilot experiments were used to locate the dose response lines, and on the basis of these results factorial experiments were designed (see Fisher 1949). In certain instances parallelogram designs have been adopted in order to avoid large regions of 0 and 100 per cent. response. All experiments have been analysed using the angular transformation and analysis of variance, according to the methods described by Claringbold, Biggers, and Emmens (1953).

Since several variables have been investigated simultaneously in each experiment, discussion has been facilitated by placing the design, results, and analyses of each experiment in an appendix.

In the text all expressions of the form "regression of response on . . ." should be taken to mean "regression of the angle of response on the logarithm of . . ." To facilitate computation the various experiments are calculated in the logarithmic base determined by the treatment levels, and in order to compare the various experiments the parameters concerned have been transformed into logarithms to base 10. Fiducial limits ($P = 0.05$), where required, have been placed between braces, after the estimates of a parameter; standard errors are indicated by placing them after the $\pm$ sign.

IV. Results

(*a*) *Total Volume of Injection* (*Table* 4)

Oestrone, administered in distilled water, has been used in this investigation. The full analysis of variance shows significant linear ($P < 0.001$) and quadratic ($0.05 > P > 0.01$) components of the regression of response on volume. A separate analysis of the first three points, however, shows no differences be-

tween them, and thus the regression is due to small responses obtained with the largest volumes. The effect of volume of injection is shown schematically in Figure 1, where it is seen that, provided the total volume of the two injections does not exceed 0.02 ml, the response is maximal. Volumes greater that this are highly inefficient, owing to leakage of the solution from the vagina. In all subsequent work a volume of 0.01 ml per injection has been employed. Both Mühlbock (1941) and Emmens (1950*b*) used this volume, while Sulman (1952) used a variable volume and found that in selected animals volumes of 0.02 ml may be used.

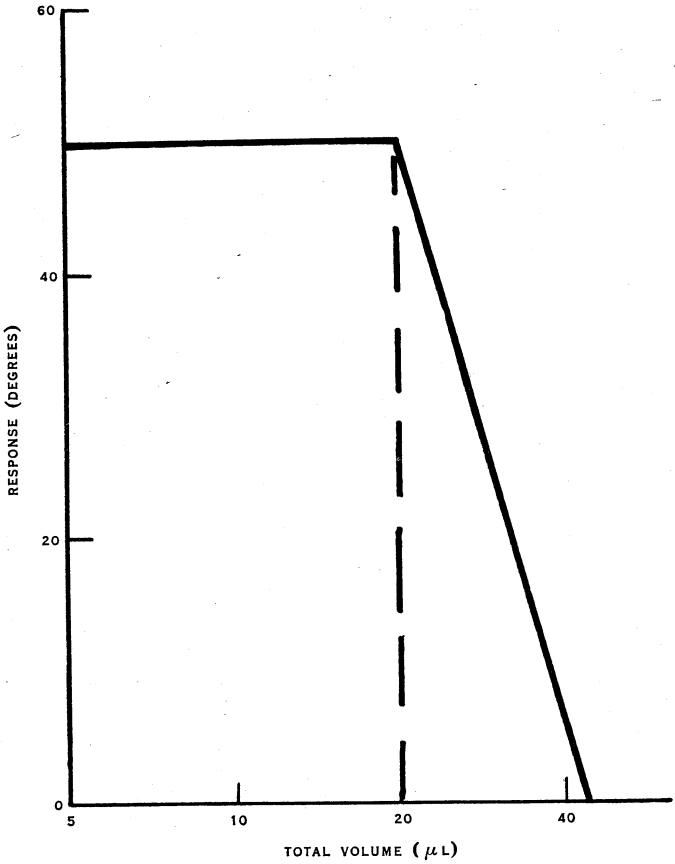

Fig. 1.—Effect of volume of injection on the response of mice to a dose of oestrone.

(*b*) *Frequency of Injection* (*Tables* 5-9)

The effect of frequency of injection (2, 4, and 8 injections) during various time intervals has been studied using oestrone, oestradiol-3,17β, and diethylstilboestrol, administered in distilled water. In the analysis of variance for all experiments no significant interactions were observed between frequency of injection and other variables, and hence the effect of frequency can be discussed independently.

Oestrone and oestradiol-3,17β.—For both the analysis of variance shows the linear components of the regressions of response on frequency as non-significant while the quadratic components are highly significant. This implies that there are no significant differences in response to two injections and eight injections, whereas four injections are more effective. The effect was consistent over all time intervals and doses studied.

Diethylstilboestrol.—Here the linear and quadratic components of the regression of response on frequency are both significant. Eight injections are much less effective than two injections, both being less effective than four injections. This relationship is found over all time intervals and doses studied.

Oestriol.—This oestrogen was found to behave in an analogous manner to oestrone and oestradiol-3,17β, in the restricted time interval (24 hr) studied. In terms of oestrone as standard, the relative activity of oestriol, when administered in distilled water by four injections in 24 hr (Table 2), shows that oestriol is considerably less active under these conditions than the three oestrogens mentioned above. Therefore no further investigations on oestriol in distilled water were undertaken.

(*c*) *Time Interval* (*Tables* 6-8, 10-12)

In all the following experiments distilled water was again the vehicle of administration.

Oestrone.—In an initial experiment with oestrone a two-injection technique was employed over time intervals ranging from 0.9 to 24 hr (Table 10), and a highly significant linear regression of response on time interval (b_1*) was observed. As the interval between the two injections was increased the response became greater. A factorial experiment was designed (Table 6) investigating time intervals of 16, 24, and 36 hr in conjunction with other variables, and the linear regression observed above was confirmed. In yet another experiment (Table 11) this effect has been confirmed over the time intervals of 13.5, 18, and 24 hr.

The various regressions, and their standard errors, have been converted to common logarithms and a homogeneity χ^2 test applied. The weighted mean regression was 42.7 ± 6.1 ($\chi^2_{[2]} = 3.20$, $0.3 > P > 0.2$).

Further investigation with four injections over the time intervals 24, 36, and 48 hr (Table 12) showed that 36 hr gave maximum activity. The collective information is shown schematically in Figure 2.

Oestradiol-3,17β.—A factorial experiment was designed on the basis of the results obtained with oestrone (Table 7). However, in contrast to these findings, no evidence of a regression of response on time interval (b_1*) was found with oestradiol-3,17β. In a further experiment (Table 11), using four injections, the responses over time intervals 13.5, 18, and 24 hr were studied, and no evidence of a linear regression was found in this range. The quadratic effect (Table 11) is difficult to explain and would require further work for its confirmation. The effect has not been examined further as it is of no particular

interest in the present work. The experiments reported, however, fully demonstrate a difference in the action of oestrone and oestradiol-3,17β. The data are illustrated in Figure 2.

Diethylstilboestrol.—Similar experiments to the above were designed for diethylstilboestrol with appropriate modifications in dosage (Tables 8 and 11). No significant differences in response were observed over the time intervals 16-36 hr, while in the 13.5-24 hr interval a steep linear regression (b_1*) was obtained. The linear regression coefficient, in common logarithms, is 190 ± 41. This is significantly greater than the corresponding regression coefficient for oestrone ($t_{[\infty]} = 3.61$, $P < 0.001$). The data are illustrated in Figure 2.

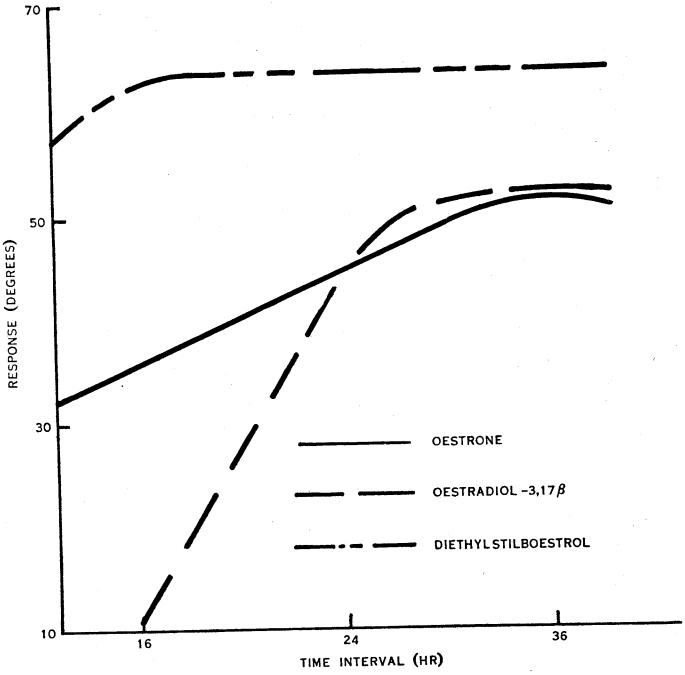

Fig. 2.—Effect of time interval over which the injections are spread on the response of mice to oestrone, oestradiol-3,17β, and diethylstilboestrol.

(*d*) *Unequally Divided Doses* (*Table* 13)

In the following experiment the effect of different dosage of oestrone *per injection* by a four-injection technique over 36 hr was examined. Four dose levels were investigated and at each dose level five different treatments were used. The design is illustrated in Figure 3. The treatments have been coded $(-2, -1, 0, 1, 2)$ in equal steps for the purposes of analysis, this being possible since the distribution of total dose fractions has been arranged in a linear order.

Treatment (0) thus represents the normal four-injection technique, i.e. four equal injections.

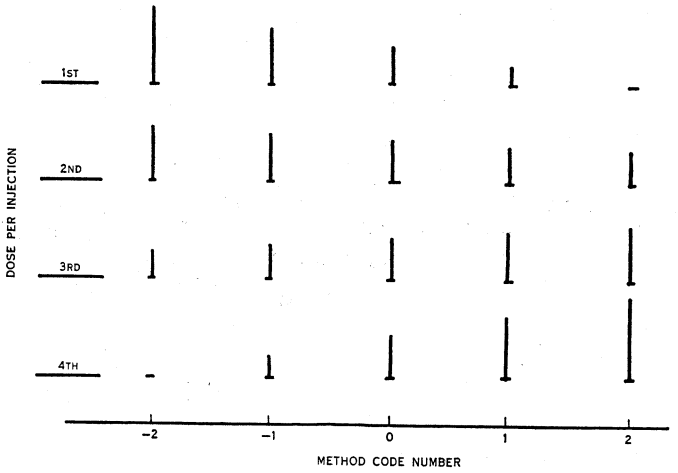

Fig. 3.—Scheme of doses used in the experiment designed to investigate the effect of unequal division of total dosage.

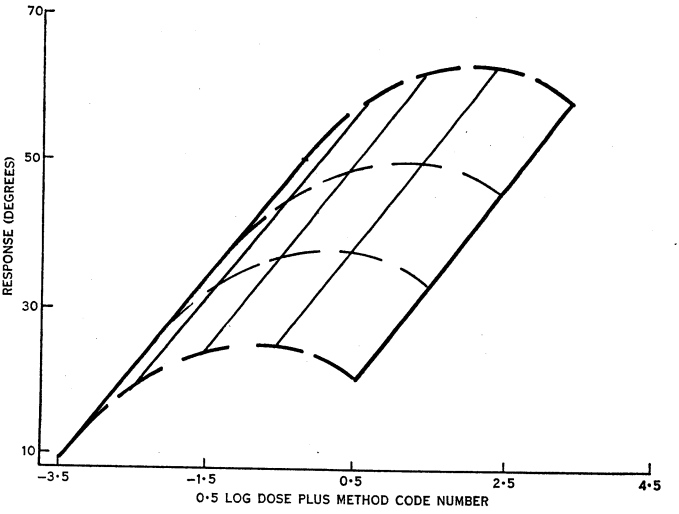

Fig. 4.—Effect of unequal doses *per injection* on the response of mice to oestrone. (See Finney (1952) for explanation of this method of plotting results.)

The results of the experiments are shown in Table 13. The analysis of variance shows that both linear and quadratic ($0.01 > P > 0.001$) components of regression of response on treatments are significant. Figure 4 has been plotted from the calculated regression coefficients. Inspection of the figure shows that treatment (1) was slightly more effective than any other, indicat-

ing that a moderately increasing series of doses is more effective than equal doses, and considerably more effective than any other treatment.

(*e*) *Studies with Egg Albumin*

Previous studies have shown that with a two-injection technique the potency of oestrogens is enhanced when administered in 1 per cent. aqueous egg albumin solution (Biggers 1951, 1953*b*). It was suggested that the protein bound the oestrogen, and this prevented its rapid removal from the vagina by absorption into the blood stream. The results presented above show that four injections in 36 hr is the technique by which oestrogens should be administered for optimum activity. This fact may be dependent upon the normal rate of utilization of oestrogen, and under these conditions losses may be expected to be minimal. If these hypotheses are true we may predict that egg albumin as a solvent will have little effect in enhancing the activity of oestrogens administered over 36 hr. The experiment shown in Table 12 was designed to test this prediction. Oestrone and a four-injection technique were used over the time intervals 26, 36, and 49 hr.

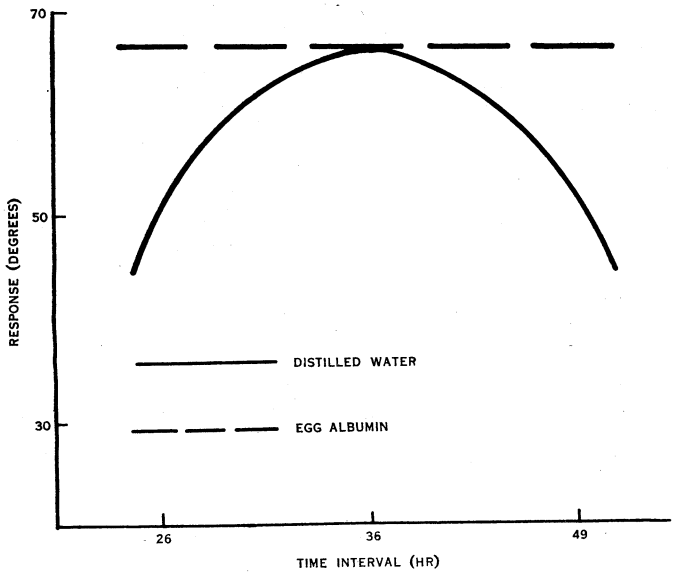

Fig. 5.—Effect of distilled water and 1 per cent. aqueous egg albumin as solvents, at various time intervals over which the injections are spread, on the response of mice to oestrone.

The analysis of variance shows a significant *vehicle* × *time interval* interaction. On partitioning of the sums of squares the quadratic component only is significant ($0.05 > P > 0.01$). Further analysis shows no linear or quadratic regression of response on time interval in the egg albumin data. Both the above quadratic effects are therefore due to the small responses at 26 and 49 hr, with

a clear optimum at 36 hr. A comparison of the pooled median effective doses (M.E.D.) of the three egg albumin dose response lines and the M.E.D. of the dose response line obtained with distilled water at 36 hr shows no difference between them ($t_{[1]} = 0.02$, $P > 0.05$). The results are illustrated in Figure 5.

Relative activity of oestrone in distilled water and in 1 per cent. aqueous egg albumin by a one-injection technique.—Table 1 shows the M.E.D. ratios obtained with oestrone administered by one injection in distilled water and in 1 per cent. aqueous egg albumin. The M.E.D. of each line shows how profoundly the albumin enhances the activity of the oestrogen. These two lines were determined on different occasions, but the effect has not been examined more critically by simultaneous determinations since the difference is far greater than the secular shifts in sensitivity normally observed in the colonies (Biggers 1953*a*). The ineffectiveness of oestrone by a one-injection technique in distilled water is in agreement with the insensitivity observed earlier by Emmens (1941), using 50 per cent. aqueous glycerol as solvent.

TABLE 1

MEDIAN EFFECTIVE DOSES ($10^{-4}\mu g$) AND THEIR RATIOS FOR TWO TECHNIQUES OF ADMINISTRATION

Technique	Test	Solvent		Ratio and its Fiducial Limits ($P = 0{\cdot}05$)
		Distilled Water	1% Aqueous Egg Albumin	
One injection	1	178	25·8	6·90 {3·91-12·20}
Four injections in 36 hr	1	1·50	1·74	0·86 {0·39-1·94}
	2	1·95	1·38	1·41 {0·93-2·13}
	3	3·31	3·40	0·97 {0·66-1·44}
Combined data: weighted mean ratio 1·12 {0·86-1·46}				

Relative activity of oestrone in distilled water and in 1 per cent. aqueous egg albumin by a four-injection technique.—Table 1 shows the results of three tests comparing the relative activity of oestrone in distilled water given by four injections in 36 hr and in 1 per cent. aqueous egg albumin by a similar technique. No difference in activity was observed between the two solvents, indicating that the protein exerted no effect under these conditions.

Figure 6 summarizes the results presented in this section together with the earlier results of Biggers (1951). It is seen that optimum activity is approached either by dividing the dose or by using egg albumin solution as a solvent. However, the two methods converge and when optimum conditions are reached they do not potentiate each other.

(*f*) *Relative Activities of Oestrogens under Optimum Conditions.*

In view of the results presented above, the relative activities of oestradiol-3,17β, oestriol, and diethylstilboestrol in terms of oestrone were determined.

The oestrogens were administered in distilled water by four injections in 36 hr, and for comparison the same technique was applied with 1 per cent. aqueous egg albumin. In addition, the activities of equilin and equilenin were determined in egg albumin solution only. The results have been expressed as

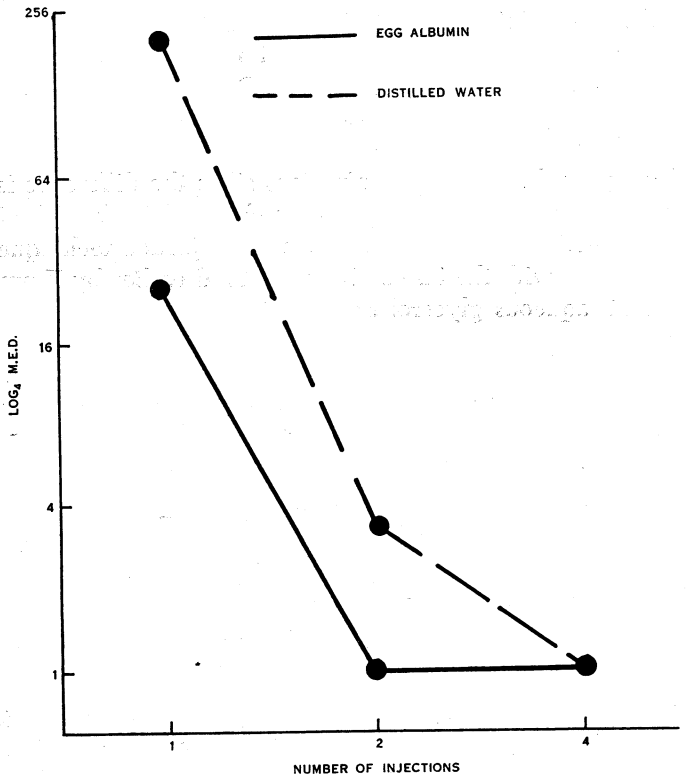

Fig. 6.—Effect of a single injection, two injections in 24 hr, and four injections in 36 hr on the response of mice to oestrone, using distilled water and 1 per cent. aqueous egg albumin as solvents.

M.E.D. ratios (see Biggers 1951) since relative potency estimates are invalid where the slopes of the dose response lines are not parallel. This was demonstrated with oestrone and oestradiol-3,17β by Biggers and Claringbold (1953) and by Claringbold (1953), and with oestrone and oestriol in the present work. The M.E.D. and its variance have been computed for each line, using individual estimates of slope instead of a pooled estimate, since earlier work (Biggers 1953*a*) and the present work have shown pooled estimates to be sometimes heterogeneous. Thus the relative activities have been calculated assuming the worst conditions and therefore any differences observed will be highly significant. The results are shown in Table 2.

Oestradiol-3,17β under all conditions of administration (Table 2) is significantly more active than oestrone (approximately 1.5 times as active), while

diethylstilboestrol has the same activity as oestrone. Oestriol, however, behaves differently according to the vehicle of administration. In the presence of egg albumin oestriol has the same activity as oestrone while in distilled water its

TABLE 2

RELATIVE ACTIVITIES OF OESTRADIOL-3,17β, OESTRIOL, DIETHYLSTILBOESTROL, EQUILIN, AND EQUILENIN IN COMPARISON WITH OESTRONE UNDER VARIOUS CONDITIONS OF ADMINISTRATION

	Present Work (optimal)			Earlier Work*
	Weighted M.E.D. Ratio and Fiducial Limits ($P = 0{\cdot}05$)	No. of Tests	No. of Animals	
Distilled water				
Oestradiol-3,17β	1·37 {1·04-1·81}	4	504	1·52 {1·18-2·02}†
Oestriol	0·09 {0·04-0·21}	1	108	0·46 {0·32-0·62}†
Diethylstilboestrol	0·76 {0·51-1·39}	2	380	—
1% Egg albumin				
Oestradiol-3,17β	1·57 {1·03-2·37}	4	520	1·75 {1·15-2·66}‡
Oestriol	1·13 {0·72-1·76}	3	412	1·09 {0·70-1·70}‡
Diethylstilboestrol	0·82 {0·57-1·19}	3	374	—
Equilin	0·84 {0·60-1·20}	1	180	—
Equilenin	0·65 {0·37-1·15}	1	180	—

* Relative potencies and their fiducial limits ($P = 0{\cdot}05$).

† Two-injection technique over 24 hr. Vehicle: 50 per cent. aqueous glycerol (Emmens 1950).

‡ Two-injection technique over 24 hr. Vehicle: 1 per cent. aqueous egg albumin (Biggers 1951).

activity is considerably reduced; mere subdivision of the dose does not attain maximum activity. In this respect oestriol differs from the other oestrogens studied. It is possible that the different behaviour of oestriol is related to its water solubility. Table 3 shows the solubilities in water of various oestrogens,

TABLE 3

SOLUBILITIES OF OESTROGENS IN WATER

Oestrogen	Solubility (μg/ml)	References
Oestrone	1·6	Jewitt, quoted by Heard (1949)
	2·1	Butenandt and Westphal (1934)
Oestradiol-3,17β	2·7	Bischoff and Pilhorn (1948)
Oestriol	27·0	Jewitt, quoted by Heard (1949)

where it is seen that oestriol is considerably more soluble. The high solubility may favour its loss into the blood stream, and mere subdivision of the dose may fail to overcome this loss. The egg albumin, however, may hold the oestriol in the vagina and allow it to exert its maximal effect.

V. Discussion

(*a*) *The Quantitative Study of Oestrogen Activity*

The optimum conditions for the intravaginal administration of oestrone, oestradiol-3,17β, oestriol, and diethylstilboestrol are attained by two or four injections in 36 hr, using 1 per cent. aqueous egg albumin as solvent. Except with oestriol these conditions may be closely approximated by four injections over 36 hr, using distilled water as solvent. The use of four unequal injections in an increasing series provides the best approximation to the optimum with oestrone.

The greatly increased sensitivity claimed by Sulman (1952), who used a five-injection technique, has not been substantiated. His comparisons with the results of Emmens (1950*a*) are invalid for three reasons:

(1) Sulman's criterion of positivity—being positive when " . . . the number of leucocytes does not exceed the number of epithelial cells"—is highly subjective and obviously more sensitive than the criterion of cornified and nucleated cells in the absence of mucin and leucocytes used by Emmens;

(2) Emmens' figures are median effective doses while Sulman's figures are minimal effective doses; and

(3) Responses of the order quoted by Sulman are by no means exceptional in certain individual animals in our colonies; however, the median effective dose is a far more efficient statistic than the minimal effective dose in describing the sensitivity to oestrogen.

In their study of the subcutaneous technique Marrian and Parkes (1929), although using crude extracts, found the optimum conditions to be many injections (up to 20 over 24-36 hr). Thus while the two routes resemble each other with respect to time interval they are quite dissimilar with respect to subdivision of dose.

The results demonstrate the extent to which quantitative studies on the relative activity of oestrogens may be influenced by technique. Studies using the subcutaneous route are quite unreliable as indicators of relative activity at the cellular level, the results being severely biased by the effects of passage through the body, involving such factors as differential absorption and blood transport, and differential excretion and transformation to other forms. Recent studies (Biggers, Claringbold, and Emmens 1954) showed no evidence of a correlation between the sensitivity of the vagina to local and systemic administration. From the clinical point of view, information obtained by systemic administration is valuable, especially if tested on the species concerned. The recent assays of oestrogens in women by Bishop (1950) are examples of this. The nearest approach to *in vivo* assays on the cells responding is the intravaginal method, and here variations of technique can severely affect results. This is well illustrated by the low activity of oestriol in distilled water and high activity in egg albumin solution. A comparison of relative activities would seem to be meaningful only under optimum conditions of administration, and unless these are attained the results cannot readily be appraised. It seems almost certain that similar situations exist in the study of other hormones and pharmacological substances.

(*b*) *Local Action of Oestrogens*

(i) *Probability Model.*—In this work we are concerned with the probability of response and *not* with the degree of response of any one individual. For simplicity we can consider dose as a constant, e.g. the M.E.D. at the optimum conditions. The probability model then becomes a bivariate distribution with its peak at four injections and 36 hr. This means that, given the optimum conditions of administration, the probability of response is maximal.

(ii) *Uptake by the Epithelium.*—All the results presented indicate that an eight-injection technique is less effective than the four-injection technique. The smaller aliquots of oestrogen in the former are insufficient to stimulate the cellular processes, whereas the larger aliquots in the latter are more likely to produce the response, although in both instances the total dose is the same. It is necessary, therefore, to postulate a minimum dose of oestrogen which, following uptake by the epithelium, will stimulate a response.

The relative ineffectiveness of two injections in comparison with four injections is also of importance in that still larger aliquots are less effective. The larger quantity of oestrogen seems to saturate the epithelium and also leave an excess, which may be lost to the blood stream and from the vagina.

The work with 1 per cent. aqueous egg albumin supports the above theory fully. Using this solvent optimum conditions are obtained over a wider range of treatments than with distilled water. The egg albumin binds with the oestrogen and holds the hormone near its site of action, and there is thus a competition between the cells of the epithelium and the protein. Strong evidence for this view is furnished by the earlier observations of Biggers (1953*b*) with bovine plasma albumin as solvent. This protein in 0.1 per cent. solution binds to such an extent that the response is highly variable while in 0.01 per cent. solution the binding is still sufficient to increase variability. With the weakly binding egg albumin molecule no increase in variability is observed, suggesting that the epithelium may absorb the oestrogen from the protein as it is utilized with little loss into the blood stream.

(iii) *The Problem of Structure and Function.*—A large number of substances possessing oestrogenic activity are now known, and several theories as to the cause of this activity have been put forward (see Jacques 1949 for a full review of this subject). Most of these theories are based upon misleading evidence obtained from poorly conducted subcutaneous tests. The work of Emmens (1941, 1942) indicated two major groups of oestrogenic molecules—the *true oestrogens* and the *pro-oestrogens,* the latter acquiring their activity following transformation in the body to a true oestrogen. A pro-oestrogen is best defined as a substance which may be transformed into a true oestrogenic molecule, this transformation taking place at a site other than that of local action. Some of the theories, namely those based on active groups and spatial molecular configurations, completely fail to take account of this distinction, with the result that specific groups or physical chemical measurements are correlated with both pro-oestrogenic and true oestrogenic activity. It seems that

in order to clarify the field much of the earlier work should be repeated to identify those substances which are true oestrogens. These substances should then be studied at a cellular level with techniques which will ensure optimum activity.

In all the above work oestradiol-3,17β stands apart from the others and it is also the most active substance at optimum conditions, all others being of the same lower activity. Although no direct evidence can be produced to show that oestradiol-3,17β is the substance to which the others are converted in the epithelium such a process seems a distinct possibility. Evidence for the intracellular conversion of oestrogens is provided by the work of Biggers and Claringbold (1953) and Claringbold (1953) on the effect of thyroid status and cyanide administration on oestrogenic activity. Much work needs to be done before any definite statements on this problem can be made.

VI. Acknowledgments

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VII. References

Biggers, J. D. (1951).—*J. Endocrinol.* **7**: 163.

Biggers, J. D. (1953*a*).—*J. Endocrinol.* **9**: 145.

Biggers, J. D. (1953*b*).—*J. Endocrinol.* **9**: 136.

Biggers, J. D., and Claringbold, P. J. (1953).—*Aust. J. Biol. Sci.* **6**: 305.

Biggers, J. D., Claringbold, P. J., and Emmens, C. W. (1954).—In press.

Bischoff, F., and Pilhorn, H. (1948).—*J. Biol. Chem.* **174**: 663.

Bishop, P. M. F. (1950).—*Lancet* **258** (i): 848.

Butenandt, A., and Westphal, V. (1934).—*Hoppe-Seyl. Z.* **223**: 147.

Claringbold, P. J. (1953).—*Aust. J. Biol. Sci.* **6**: 657.

Claringbold, P. J., Biggers, J. D., and Emmens, C. W. (1953).—*Biometrics* **9** (4).

Emmens, C. W. (1941).—*J. Endocrinol.* **2**: 444.

Emmens, C. W. (1942).—*J. Endocrinol.* **3**: 168.

Emmens, C. W. (1950*a*).—*J. Endocrinol.* **6**: 302.

Emmens, C. W. (1950*b*).—"Hormone Assay." Ch. XVI. (Academic Press: New York.)

Finney, D. J. (1952).—"Probit Analysis. A Statistical Treatment of the Sigmoid Response Curve." 2nd Ed. (Cambridge University Press.)

Fisher, R. A. (1949).—"Design of Experiments." 3rd Ed. (Oliver and Boyd: Edinburgh.)

Heard, R. D. H. (1949).—*Recent Progr. Hormone Res.* **4**: 25.

Jacques, J. (1949).—*Bull. Soc. Chim. Paris* **16**: 411.

Marrian, G. F., and Parkes, A. S. (1929).—*J. Physiol.* **67**: 389.

Mühlbock, O. (1940).—*Acta Brev. Neerl. Physiol.* **10**: 42.

Sulman, F. G. (1952).—*Endocrinology* **50**: 61.

Appendix I

Design, results, and analyses of the factorial experiments

To facilitate description of the factorial experiments the following conventions have been adopted:

(1) Since the levels of the independent variables were in equal steps on some scale (usually logarithmic) simple coding has been used, and is indicated by subscripts in parentheses.

(2) In each experiment the number of animals per group is constant over all treatment combinations and is indicated in the title. With the exception of Tables 9-11 the percentage response is tabulated in the appropriate cell of the design. In Tables 9-11, however, where the dosage varies in each cell, a matrix of doses is given, and this matrix is in one-to-one correspondence with the matrix of responses shown alongside.

(3) Where parallelogram designs have been employed (Tables 6-11) the analysis is carried out following a linear transformation of an independent variable. The linear regression coefficient on this variable which is obtained from the analysis must be modified allowing for this transformation, and this new coefficient is indicated at the foot of the analysis of variance by b_i^*. For further details see Claringbold, Biggers, and Emmens (1953).

Table 4

EFFECT OF TOTAL VOLUME OF INJECTION WITH A TWO-INJECTION TECHNIQUE AND LEVEL OF DOSAGE OF OESTRONE (18 ANIMALS PER GROUP)

Total Volume (ml)	Dose (10^{-4} µg)	
	$1{\cdot}5_{(-1)}$	$6{\cdot}0_{(1)}$
$0{\cdot}005_{(-2)}$	22·2	72·2
$0{\cdot}01_{(-1)}$	27·8	77·8
$0{\cdot}02_{(0)}$ (normal technique)	11·1	88·9
$0{\cdot}04_{(1)}$	5·6	50·0
$0{\cdot}08_{(2)}$	0·0	22·2

TABLE 5

EFFECT OF THREE LEVELS OF OESTRONE WHEN ADMINISTERED IN TWO, FOUR, OR EIGHT INJECTIONS OVER 24 HR (18 ANIMALS PER GROUP)

	Frequency of Injection	Dose Oestrone (10^{-4} μg)		
		$2_{(-1)}$	$4_{(0)}$	$8_{(+1)}$
Rep. I	$2_{(-1)}$	38·9	55·6	66·7
	$4_{(0)}$	21·8	55·6	72·0
	$8_{(+1)}$	16·7	33·3	50·0
Rep. II	$2_{(-1)}$	44·4	88·9	94·4
	$4_{(0)}$	94·4	83·3	88·9
	$8_{(+1)}$	66·7	88·9	94·4
Rep. III	$2_{(-1)}$	33·3	61·1	66·7
	$4_{(0)}$	66·7	66·7	72·2
	$8_{(+1)}$	16·7	38·9	61·1

Analysis of Variance

Source of Variation	D.f.	Sum of Squares	Mean Square	*F*	*P*
Replications	(2)	(2994·9)	(1497·5)		
Linear	1		103·1	2·1	>0·05
Quadratic	1		2891·8	59·6	<0·001
Frequency of injection	(2)	(538·3)	(269·2)		
Linear	1		168·7	3·5	>0·05
Quadratic	1		369·6	7·6	0·01-0·001
Doses	(2)	(1678·3)	(839·2)		
Linear	1		1636·5	33·7	<0·001
Quadratic	1		41·8	0·8	>0·05
Interactions	20	1136·0	56·8	1·2	>0·05
Theoretical variance	∞		48·5		

TABLE 6

EFFECT OF TIME INTERVAL (X_1), DOSE OF OESTRONE (X_2), AND FREQUENCY OF INJECTION (12 ANIMALS PER GROUP

Time Interval	Frequency of Injection	Dose Oestrone ($10^{-4}\mu$g)		
		$2{\cdot}6_{(0{\cdot}415)}$	$5{\cdot}3_{(0{\cdot}585)}$	$10{\cdot}5_{(1{\cdot}585)}$
$16_{(-1)}$	$2_{(-1)}$	25·0	50·0	75·0
	$4_{(0)}$	41·7	66·7	91·7
	$8_{(1)}$	33·3	58·3	66·7
		$1{\cdot}8_{(-1)}$	$3{\cdot}5_{(0)}$	$7{\cdot}0_{(1)}$
$24_{(0)}$	2	8·3	50·0	66·7
	4	33·0	58·3	91·7
	8	25·0	50·0	83·3
		$1{\cdot}2_{(-1{\cdot}585)}$	$2{\cdot}3_{(-0{\cdot}585)}$	$4{\cdot}7_{(0{\cdot}415)}$
$36_{(1)}$	2	41·7	58·3	66·7
	4	50·0	83·3	83·3
	8	58·3	58·3	83·3

Analysis of Variance

Transformation: $X_2 = X_2{}^* + 0{\cdot}585\,X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Doses	(2)	(3368·4)	(1684·2)		
Linear	1		3362·0	44·6	<0·001
Quadratic	1		6·4	0·2	>0·05
Frequency	(2)	(637·3)	(318·7)		
Linear	1		138·0	1·8	>0·05
Quadratic	1		499·3	6·6	0·05-0·01
Time Interval (X_1)	(2)	(287·5)	(143·8)		
Linear	1		107·3	1·4	>0·05
Quadratic	1		180·2	2·4	>0·05
Interactions	20	629·0	31·5	0·4	0·05-0·01
Theoretical variance	∞		75·3		

Significance of linear regression coefficient of X_1: $b_1{}^* = 10{\cdot}44$, $s_{b1}{}^* = 2{\cdot}37$; $t_{[\infty]} = 4{\cdot}40$, $P<0{\cdot}001$.

TABLE 7

EFFECT OF TIME INTERVAL (X_1), DOSE OF OESTRADIOL-3,17β (X_2), AND FREQUENCY OF INJECTION (12 ANIMALS PER GROUP)

Time Interval (hr)	Frequency of Injection	Dose Oestradiol-3,17β (10^{-4} μg)		
		$2{\cdot}6_{(0{\cdot}415)}$	$5{\cdot}3_{(0{\cdot}585)}$	$10{\cdot}5_{(1{\cdot}585)}$
$16_{(-1)}$	$2_{(-1)}$	50·0	66·7	75·0
	$4_{(0)}$	83·3	100·0	100·0
	$8_{(1)}$	58·3	66·7	83·3
		$1{\cdot}8_{(-1)}$	$3{\cdot}5_{(0)}$	$7{\cdot}0_{(1)}$
$24_{(0)}$	2	50·0	75·0	75·0
	4	66·7	66·7	100·0
	8	41·7	75·0	83·3
		$1{\cdot}2_{(-1{\cdot}585)}$	$2{\cdot}3_{(-0{\cdot}585)}$	$4{\cdot}7_{(0{\cdot}415)}$
$36_{(1)}$	2	16·7	41·7	58·3
	4	33·3	91·7	83·3
	8	41·7	50·0	83·3

Analysis of Variance

Transformation: $X_2^* = X_2 + 0{\cdot}585\ X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Doses	(2)	(2195·5)	(1097·8)		
Linear	1		2146·0	28·5	<0·001
Quadratic	1		49·5	0·7	>0·05
Frequency	(2)	(1414·3)	(707·2)		
Linear	1		132·0	1·8	>0·05
Quadratic	1		1282·3	17·0	<0·001
Time interval (X_1)	(2)	(804·8)	(402·4)		
Linear	1		796·5	10·6	0·05-0·01
Quadratic	1		38·3	0·5	>0·05
Interactions	20	1025·3	51·3	0·7	>0·05
Theoretical variance	∞		75·3		

Significance of linear regression coefficient of X_1: $b_1^* = -0{\cdot}27$, $s_{b1}^* = 2{\cdot}37$; $t_{[\infty]} = 0{\cdot}11$, $P>0{\cdot}05$.

TABLE 8

EFFECT OF TIME INTERVAL (X_1), DOSE OF DIETHYLSTILBOESTROL (X_2), AND FREQUENCY OF INJECTION (12 ANIMALS PER GROUP)

Time Interval (hr)	Frequency of Injection	Dose Diethylstilboestrol (10^{-4} μg)		
		$6{\cdot}0_{(-0{\cdot}415)}$	$12{\cdot}0_{(0{\cdot}585)}$	$24{\cdot}0_{(1{\cdot}585)}$
$16_{(-1)}$	$2_{(-1)}$	75·0	58·3	100·0
	$4_{(0)}$	66·7	83·3	91·7
	$8_{(1)}$	41·7	58·3	66·7
		$4{\cdot}0_{(-1)}$	$8{\cdot}0_{(0)}$	$16{\cdot}0_{(1)}$
$24_{(0)}$	2	50·0	100·0	83·3
	4	75·0	83·3	100·0
	8	25·0	58·3	66·7
		$2{\cdot}7_{(-1{\cdot}585)}$	$5{\cdot}3_{(-0{\cdot}585)}$	$10{\cdot}7_{(0{\cdot}415)}$
$36_{(1)}$	2	33·3	50·0	75·0
	4	33·3	66·7	91·7
	8	8·3	50·0	75·0

Analysis of Variance

Transformation: $X_2^* = X_2 + 0{\cdot}585\ X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Doses	(2)	(2911·7)	(1455·9)		
Linear	1		2892·1	38·4	<0·001
Quadratic	1		19·6	0·3	>0·05
Frequency	(2)	(1616·4)	(808·2)		
Linear	1		844·1	11·2	<0·001
Quadratic	1		772·3	10·3	0·01-0·001
Time interval (X_1)	(2)	(851·9)	(421·0)		
Linear	1		611·3	8·1	0·01-0·001
Quadratic	1		240·6	3·2	0·1-0·05
Interaction	20	1351·4	67·57	0·9	>0·05
Theoretical variance	∞		75·3		

Significance of linear regression coefficient of X_1: $b_1^* = 1{\cdot}59$, $s_{b1}^* = 2{\cdot}37$; $t_{[\infty]} = 0{\cdot}67$; $P>0{\cdot}05$.

TABLE 9

EFFECT OF FREQUENCY OF INJECTION IN 24 HR (X_3) AND DOSE OF OESTRIOL (X_2) (18 ANIMALS PER GROUP)

Frequency of Injection	Dose Oestriol (10^{-4} μg)			Response		
$2_{(-1)}$	$128_{(2)}$	$256_{(3)}$	$512_{(4)}$	33·3	55·6	66·7
$4_{(0)}$	$16_{(-1)}$	$32_{(0)}$	$64_{(1)}$	50·0	83·3	88·9
$8_{(1)}$	$2_{(-4)}$	$4_{(-3)}$	$8_{(-2)}$	0·0	11·1	11·1

Analysis of Variance

Transformation: $X_2^* = X_2 + 3X_3$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Doses	(2)	(625·2)	(312·6)		
Linear	1		555·8	11·5	<0·001
Quadratic	1		69·4	1·4	>0·05
Frequency (X_3)	(2)	(3205·6)	(1602·8)		
Linear	1		1427·0	29·4	<0·001
Quadratic	1		1778·6	36·7	<0·001
Interactions	4	49·52	12·38	0·3	>0·05
Theoretical variance	∞		48·5		

Significance of linear regression coefficient of X_3: $b_3^* = 13{\cdot}45$, $s_{b3}^* = 8{\cdot}99$; $t_{[\infty]} = 1{\cdot}50$, $P>0{\cdot}05$.

TABLE 10

EFFECT OF TIME INTERVAL (X_1) AND DOSE OF OESTRONE (X_2) (20 ANIMALS PER GROUP)

Time Interval (hr)	Dose Oestrone (10^{-4} μg)				Response			
$0{\cdot}9_{(-3)}$	$64_{(3)}$	$128_{(5)}$	$256_{(7)}$	$512_{(9)}$	25	30	60	85
$2{\cdot}7_{(-1)}$	$16_{(-1)}$	$32_{(1)}$	$64_{(3)}$	$128_{(5)}$	30	55	60	70
$8{\cdot}0_{(1)}$	$4_{(-5)}$	$8_{(-3)}$	$16_{(-1)}$	$32_{(1)}$	15	30	35	60
$24{\cdot}0_{(3)}$	$1_{(-9)}$	$2_{(-7)}$	$4_{(-3)}$	$8_{(-3)}$	20	45	50	70

Analysis of Variance

Transformation: $X_2^* = X_2 + 2X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Doses	(3)	(1949·0)	(649·7)		
Linear	1		1922·7	46·9	<0·001
Quadratic	1		3·8	0·3	>0·05
Cubic	1		22·5	0·5	>0·05
Time interval	3	260·3	86·8	2·0	>0·05
Interactions	9	202·5	22·5	0·5	>0·05
Theoretical variance	∞		43·3		

Significance of linear regression coefficient of X_1: $b_1^* = 17{\cdot}74$, $s_b^* = 3{\cdot}29$; $t_{[\infty]} = 5{\cdot}38$; $P<0{\cdot}001$.

TABLE 11

EFFECT OF TIME INTERVAL (X_1) AND OESTROGEN (X_2) (12 ANIMALS PER GROUP)

Oestrogen	Time Interval	Dose (10^{-4} μg)			Response		
Oestrone	13·5$_{(-1)}$	6$_{(0)}$	12$_{(1)}$	24$_{(2)}$	47·7	50·0	66·7
	18·0$_{(0)}$	3$_{(-1)}$	6$_{(0)}$	12$_{(1)}$	50·0	58·3	83·3
	24·0$_{(1)}$	1·5$_{(-2)}$	3$_{(-1)}$	6$_{(0)}$	41·7	33·3	83·3
Oestradiol-3,17β	13·5	4$_{(0)}$	8$_{(1)}$	16$_{(2)}$	66·7	58·3	75·0
	18·0	2$_{(-1)}$	4$_{(0)}$	8$_{(1)}$	75·0	91·7	100·0
	24·0	1$_{(-2)}$	2$_{(-1)}$	4$_{(0)}$	33·3	75·0	66·7
Diethylstilboestrol	13·5	18$_{(0)}$	36$_{(1)}$	72$_{(2)}$	41·7	33·3	66·7
	18·0	9$_{(-1)}$	18$_{(0)}$	36$_{(1)}$	50·0	50·0	91·7
	24·0	4·5$_{(-2)}$	9$_{(-1)}$	18$_{(0)}$	33·3	83·3	100·0

Analysis of Variance

Although all parts of the experiment above were performed simultaneously the analysis has been conducted in three parts for simplicity. Transformation: $X_2^* = X_2 + X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
A. Oestrone					
Doses	(2)	(771·6)	(385·8)		
Linear	1		621·8	8·3	0·01-0·001
Quadratic	1		149·8	2·0	>0·05
Time interval (X_1)	2	89·2	44·6	0·6	>0·05
Interactions	4	118·3	29·6	0·4	>0·05

Significance of linear regression coefficient of X_1: $b_1^* = 10{\cdot}41$, $s_{b1}^* = 5{\cdot}02$; $t_{[\infty]} = 2{\cdot}07$; $0{\cdot}05> P>0{\cdot}02$.

TABLE 11 (*Continued*)

Source of Variation	D.f.	Sum of Squares	Mean Square	*F*	*P*
B. Oestradiol-3,17β					
Doses	(2)	(381·1)	(190·6)		
Linear	1		359·8	4·8	0·05-0·01
Quadratic	1		21·3	0·4	>0·05
Time interval (X_1)	(2)	(774·8)	(387·4)		
Linear	1		35·2	0·5	>0·05
Quadratic	1		739·6	9·8	0·01-0·001
Interactions	4	250·9	62·7	0·8	>0·05

Significance of linear regression coefficient of X_1: $b_1^* = 5{\cdot}32$, $s_{b1}^* = 5{\cdot}02$; $t_{[\infty]} = 1{\cdot}05$; $P > 0{\cdot}05$.

C. Diethylstilboestrol					
Doses	(2)	(1407·9)	(704·0)		
Linear	1		1327·9	17·6	<0·001
Quadratic	1		80·0	1·1	>0·05
Time interval (X_1)	(2)	(471·2)	(235·6)		
Linear	1		461·0	6·1	0·05-0·01
Quadratic	1		10·2	0·1	>0·05
Interactions	4	446·2	111·5	1·5	>0·05

Significance of linear regression coefficient of X_1: $b_1^* = 23{\cdot}64$, $s_{b1}^* = 5{\cdot}02$; $t_{[\infty]} = 4{\cdot}71$; $P < 0{\cdot}001$. Theoretical variance 75·3, D.f. ∞.

TABLE 12

EFFECT OF TIME INTERVAL, VEHICLE OF ADMINISTRATION, AND DOSE OF OESTRONE (18 ANIMALS PER GROUP)

Vehicle	Time Interval (hr)	Dose of Oestrone (10^{-4} μg)	
		$1{\cdot}4_{(-1)}$	$5{\cdot}6_{(1)}$
1·0% Egg albumin	$26_{(-1)}$	66·7	94·4
	$36_{(0)}$	72·2	94·4
	$49_{(1)}$	55·6	100·0
Distilled water	26	33·3	66·7
	36	66·7	100·0
	49	38·9	72·2

Analysis of Variance

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Vehicle	1	435·0	435·0	9·0	0·01-0·001
Time interval	(2)	(387·8)	(193·9)		
Linear	1		6·5	0·1	>0·05
Quadratic	1		381·4	7·9	0·01-0·001
Doses	1	1686·7	1686·7	34·8	<0·001
Dose × time interval	(2)	(290·4)	(145·2)		
Departures from parallelism	1		5·1	0·1	>0·05
Opposed curvature	1		285·3	5·9	0·05-0·01
Other interactions	5	110·3	22·1	0·5	>0·05
Theoretical variance	∞		48·5		

TABLE 13

EFFECT OF FOUR INJECTIONS IN 36 HR WITH UNEQUALLY DIVIDED DOSES, OESTRONE BEING ADMINISTERED IN DISTILLED WATER

A. Design

Groups	Dose Oestrone per Injection (10^{-4} μg)				Total Dose
	1st	2nd	3rd	4th	
−2	4	2·67	1·33	0	$8_{(+3)}$*
−1	3	2·33	1·67	1	8
0	2	2	2	2	8
1	1	1·67	2·33	3	8
2	0	1·33	2·67	4	8

* Four levels of total dose were used, i.e., 1, 2, 4, and 8 × 10^{-4} μg, coded −3, −1, 1, 3. Only the doses for the highest group are shown above, the others being obtained by successive division by 2.

B. Results (18 animals per group)

Group	Total Dose (×10^{-4} μg)			
	$1_{(-3)}$	$2_{(-1)}$	$4_{(1)}$	$8_{(3)}$
−2	0	16·7	33·3	50·0
−1	5·6	33·3	33·3	61·1
0	16·7	38·9	61·1	77·8
1	11·1	50·0	55·6	88·9
2	5·6	38·9	55·6	55·6