A STUDY OF THE JOINT ACTION OF OESTRONE, OESTRADIOL-3,17β, AND OESTRIOL

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Summary

When administered by the intravaginal route oestrone, oestradiol-3,17β, and oestriol have a mutually antagonistic action on the vaginal response of the ovariectomized mouse. Using the subcutaneous route, these oestrogens are found to have an additive action, behaving simply as dilutions of one active substance.

It is suggested that these results support the view that following systemic administration the natural oestrogens are converted to a common form or mixture in the liver. This common form or mixture elicits responses in the target organs.

The present study confirms the earlier work on the slope and relative position of dose response lines obtained with oestrone, oestradiol-3,17β, and oestriol using either subcutaneous or intravaginal administration.

I. INTRODUCTION

Several investigations have indicated differences in the mode of action of oestrone, oestradiol-3,17β, and oestriol on the vaginal epithelium of the ovariectomized mouse. When administered intravaginally, oestrone and oestriol reach equal potency given optimum conditions of administration, while under these conditions oestradiol-3,17β is 1·5 times as active (Biggers and Claringbold 1954*a*). The slope of the dose response line depends on the oestrogen used to stimulate the epithelium locally. Oestrone elicits responses fitted by a line twice as steep as the dose response line for oestradiol-3,17β (Biggers and Claringbold 1953). In this work it was also demonstrated that the thyroid status influences responses to oestrone but not to oestradiol-3,17β. Claringbold (1953) found that while cyanide increased the percentage response to oestrone it was without effect on the response to oestradiol-3,17β.

It was thought that studies of the joint or simultaneous action of the three oestrogens would help to elucidate their mode of action. While Bliss (1939) first discussed some of the aspects of the study of the joint action of related compounds these ideas have been extended by Finney (1952) and Plackett and Hewlett (1951). Recently Claringbold (1954) suggested a new approach to the problem based on a special experimental design and in that paper details of the design and methods of analysis are fully given. Only a brief outline of the statistical methods will be given here.

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II. Materials and Methods

A colony of 400 albino ovariectomized mice bred in this Department were used. Before their use in tests the mice were "primed" by the subcutaneous injection of 1 μg of oestrone in peanut oil. Intravaginal injections were made in 1 per cent. aqueous egg albumin (Biggers 1953) while subcutaneous injec-

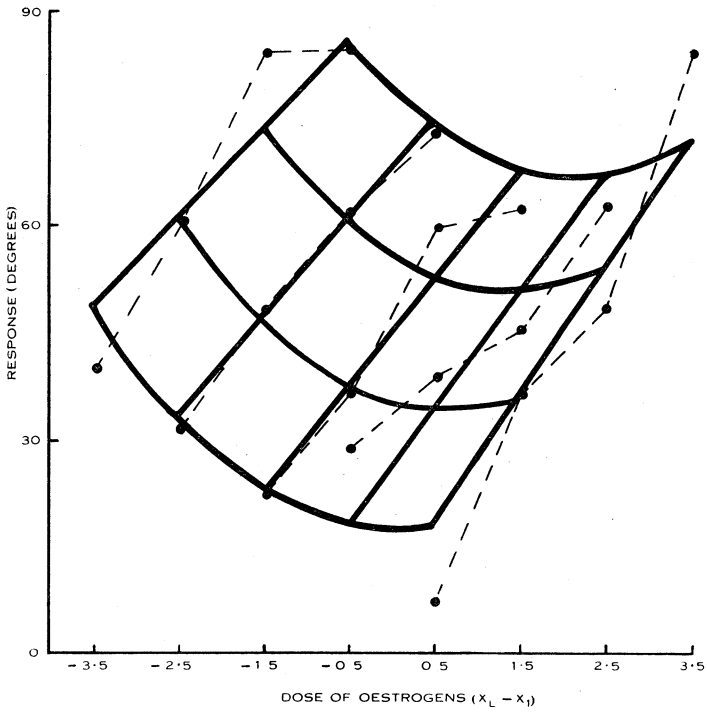

Fig. 1.—The following regression equation has been computed using the data of Table 1:

$$Y = 45{\cdot}1 + 15{\cdot}1\,X_L + 5{\cdot}6\,X_1 + 2{\cdot}7\,X_1^2 - 1{\cdot}4\,X_1X_L,$$

where Y = angle response, and X_L is the partition. The equation has been plotted using the difference $(X_L - X_1)$ of the independent variables as abscissa in order to reduce a three-dimensional relationship to a two-dimensional one. This places the dose response line obtained with oestradiol-3,17β alone to the extreme left and that with oestrone alone on the extreme right. The experimental points are indicated joined by thin, broken lines. The calculated surface is indicated in thick lines.

tions were made in peanut oil (Emmens 1950). For either route of administration, two injections were given 24 hr apart. The oestrone, oestradiol-3,17β, and oestriol were obtained from Organon Laboratories.

In tests involving intravaginal administration, vaginal smears were taken at 48, 56, and 72 hr after the first applications. With subcutaneous administration smears were taken 56, 72, and 80 hr after the first injection. Response in each animal was scored as positive if at least one vaginal smear contained cornified or nucleated cells or both in the absence of leucocytes.

III. Design and Results of Experiments

(*a*) *Joint Action of the Natural Oestrogens Administered Intravaginally*

The design of the first experiment involving the two oestrogens, oestrone, and oestradiol-3,17β, is indicated in Table 1. Four levels of total or joint dose entered the design, i.e. either 1, 2, 4, or 8×10^{-4} μg of a mixture of oestrone and

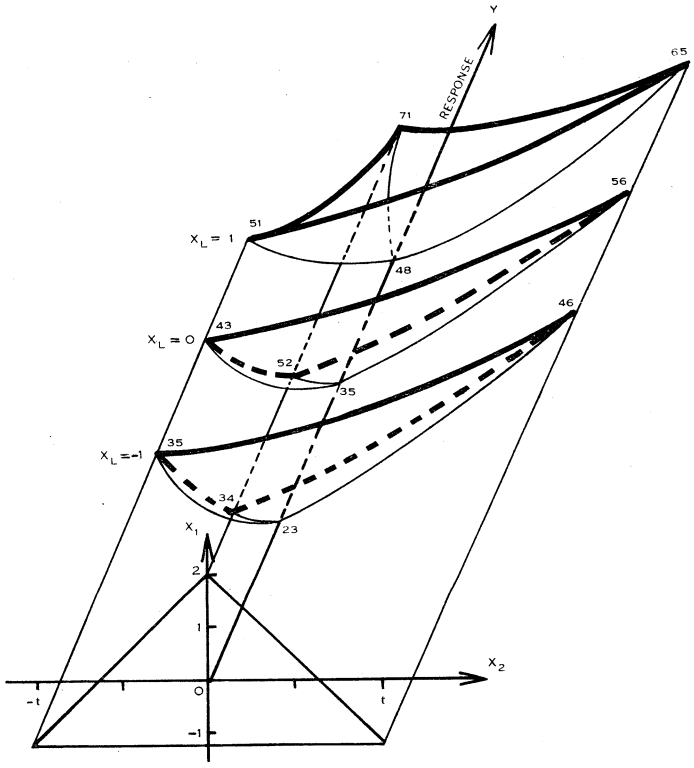

Fig. 2.—Representation of the regression surface estimated in the analysis summarized in Table 3. The two-dimensional partitioning of the dose governed by X_1 and X_2 is indicated in the base triangle. The dose response lines obtained with either oestrone, oestradiol-3,17β, or oestriol by themselves lie above the back, right-hand, and left-hand corners of this triangle. Response is plotted above the triangle, as on the sides of an equilateral triangular prism. Three levels of response corresponding to the three values of joint dose are plotted. The replication factor is ignored in this figure. The response coordinate (Y) runs through the centre of the three surfaces and the triangle and corresponds to a 0·33, 0·33, 0·33 mixture of the three oestrogens. The small figures at the corners and centre of the surfaces indicate the expected response at these points on the basis of the estimated equation (ignoring the replication coefficient).

oestradiol-3,17β was administered. At each level of joint dose, five different partitionings of this dose were made between the two oestrogens. These partitionings are in a linear order and were coded as indicated in Table 1.

TABLE 1

PERCENTAGE RESPONSE OF GROUPS OF 18 OVARIECTOMIZED MICE TO 20 DIFFERENT DOSES OF BOTH OESTRONE AND OESTRADIOL-3,17β

In order to save space the partitioning of the lowest joint dose is indicated in full. This set was coded $X_L = -1{\cdot}5$. Successive doses are obtained by repeated doubling of these doses and correspond to the cases where $X_L = -0{\cdot}5$, $0{\cdot}5$, and $1{\cdot}5$. Thus when $X_L = -0{\cdot}5$ and $X_1 = 0$, 1×10^{-4} μg of both oestrone and oestradiol-3,17 β were administered

Joint Dose		Response (%)				
Dose Oestrone (10^{-4} μg)	Dose Oestradiol-3,17 β (10^{-4} μg)	X_1	X_L			
			$-1{\cdot}5$	$-0{\cdot}5$	$0{\cdot}5$	$1{\cdot}5$
1·00	0·00	−2	0	33	55	100
0·75	0·25	−1	28	42	50	78
0·50	0·50	0	11	33	72	70
0·25	0·75	1	28	55	78	89
0·00	1·00	2	40	78	100	100

Analysis of variance (Table 2) of the percentage data following the empirical angular transformation (Claringbold, Biggers, and Emmens 1953) indicates that the five dose response lines are linear. It also indicates that mixtures of the two oestrogens are considerably less potent than predicted on the basis of additivity of the activities of the components. The difference in slope be-

TABLE 2

ANALYSIS OF VARIANCE OF THE DATA OF TABLE 1 EMPLOYING THE EMPIRICAL ANGULAR TRANSFORMATION (CLARINGBOLD, BIGGERS, AND EMMENS 1953)

Source of Variation	D.F.	Mean Square	F
Doses (X_L)	(3)		
Linear	1	5730	118·1***
Quadratic	1	48	1·0
Cubic	1	1	<1
Partition (X_1)	(4)		
Linear	1	1232	25·4***
Quadratic	1	402	8·3**
Cubic	1	17	<1
Quartic	1	46	<1
Doses × partition	(12)		
Linear × linear	1	98·0	2·0
Remainder	11	76·4	1·6
Theoretical variance	∞	48·5	

**$0{\cdot}01 > P > 0{\cdot}001$.
***$P < 0{\cdot}001$.

tween the dose response line obtained with oestrone and that obtained with oestradiol-3,17β is of the same order as that reported by Biggers and Claringbold (1953), but fails to reach significance owing to the small size and nature of the design of the experiment. As a result the interaction term has been included in the graphical representation of the experimental results in Figure 1. It would appear that the two oestrogens exhibit a mutually antagonistic action.

TABLE 3

PERCENTAGE RESPONSE OF GROUPS OF 12 OVARIECTOMIZED MICE TO DIIFFERENT COMBINATIONS OF DOSES OF OESTRONE, OESTRADIOL-3,17β, AND OESTRIOL

The partitioning at the lowest joint dose ($X_L = -1$) is shown in full in each replicate. Subsequent levels of joint dose are obtained by repeated doubling and correspond to $X_L = 0$ and 1

	Joint Dose			Coordinates		Response (%)		
						$X_L =$		
	Dose Oestrone (10^{-4} μg)	Dose Oestradiol-3,17 β (10^{-4} μg)	Dose Oestriol (10^{-4} μg)	X_1	X_2	-1	0	1
First replicate $X_R = -1$	1·00	0·00	0·00	2	0	17	42	83
	0·67	0·33	0·00	1	$t/3$*	0	33	75
	0·33	0·67	0·00	0	$2t/3$	33	33	75
	0·00	1·00	0·00	-1	t	58	58	100
	0·00	0·67	0·33	-1	$t/3$	17	33	67
	0·00	0·33	0·67	-1	$-t/3$	33	33	58
	0·00	0·00	1·00	-1	$-t$	25	50	42
	0·33	0·00	0·67	0	$-2t/3$	25	42	42
	0·67	0·00	0·33	1	$-t/3$	0	25	75
	0·33	0·33	0·33	0	0	17	25	58
Second replicate $X_R = 1$	1·00	0·00	0·00	2	0	42	50	75
	0·50	0·50	0·00	1/2	$t/2$	17	33	83
	0·00	1·00	0·00	-1	t	75	67	83
	0·00	0·50	0·50	-1	0	33	42	67
	0·00	0·00	1·00	-1	$-t$	50	42	100
	0·50	0·00	0·50	1/2	$-t/2$	17	42	58
	0·67	0·17	0·17	1	0	33	33	58
	0·17	0·67	0·17	$-1/2$	$t/2$	50	50	58
	0·17	0·17	0·67	$-1/2$	$-t/2$	33	33	50
	0·33	0·33	0·33	0	0	17	42	42

* $t = \sqrt{3}$.

A further experiment was carried out employing oestrone, oestradiol-3,17β, and oestriol. The experimental design employed in this work was specially devised for this study and has been described fully elsewhere (Claringbold 1954). Three levels of joint dose were chosen for study, namely 0·75, 1·5, and 3×10^{-4} μg of mixtures of the three oestrogens. The experiment was car-

ried out in two replicates one week apart. In each replicate 10 different methods of partitioning the joint dose amongst the three oestrogens were chosen. These are given in Table 3 together with the mathematical coding of the replicates, levels, and partitions. Two coordinates are required to describe the partitionings since the three doses of the oestrogens at each point sum up to a constant joint dose (within each level).

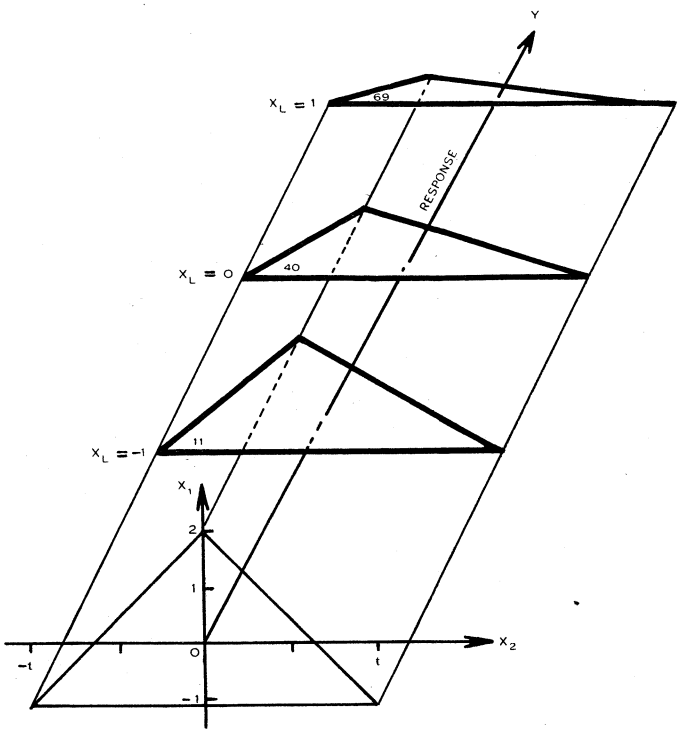

Fig. 3.—Representation of the response surface estimated in the analysis summarized in Table 6. It is plotted in an analogous manner to Figure 2. Response is linearly related to the logarithm of the joint dose (X_L) and its internal coordinates (X_1 X_2). The three levels are thus parallel, equidistant, horizontal cuts in the triangular prism resting on the base triangle. The estimated response at each level (ignoring all regression coefficients except the mean and that on X_L) is shown in small figures.

The empirical angular response has been related by a regression analysis to 10 functions of the coordinates of the design (Table 4). This analysis indicates that the oestrogens have a mutually antagonistic action. It also confirms the earlier work indicating that oestradiol-3,17β is more potent and with oestriol elicits responses fitted by a flatter dose response line than oestrone. The response surface is illustrated in Figure 2.

(*b*) *Joint Action of the Natural Oestrogens Administered Subcutaneously*

The design of the experiment in this case is complicated by the fact that the three oestrogens studied have markedly different activities when adminis-

tered by this route. In this more general case a mathematical approach is as follows. Suppose θ_1, θ_2, and θ_3 are approximately equivalent doses of oestrone, oestradiol-3,17β, and oestriol. Then a joint dose (D) may be defined

$$D = a_1\theta_1 + a_2\theta_2 + a_3\theta_3,\ 0 \leqslant a_1, a_2, a_3 \leqslant 1, \quad .. \quad (1)$$

where

$$a_1 + a_2 + a_3 = 1.$$

Since we are interested in the relationship of angle response to log dose this equation is an unsuitable form since log D is not a linear function of the multipliers a_1, a_2, and a_3. By means of transformations (Claringbold 1954) equation (**1**) may be written

$$\log D = \beta_1 \log\theta_1 + \beta_2 \log\theta_2 + \beta_3 \log\theta_3,\ 0 \leqslant \beta_1, \beta_2, \beta_3 \leqslant 1,$$

where

$$\beta_1 + \beta_2 + \beta_3 = 1.$$

The transformations allow sets of a to be determined corresponding to sets of β, and are derived in short below. Briefly, equation (**1**) may be written

$$\begin{aligned} D &= p\{p^1\theta_1 + (1-p^1)\theta_2\} + (1-p)\theta_3,\ 0 \leqslant p, p^1 \leqslant 1 \\ &= p\,b + (1-p)\,\theta_3, \\ \log D &= q\,q^1 \log\theta_1 + q\,(1-q^1)\log\theta_2 + (1-q)\log\theta_3 \\ &= \beta_1\log\theta_1 + \beta_2\log\theta_2 + \beta_3\log\theta_3, \end{aligned}$$

where

$$p^1 = (r_1^{(1-q^1)} - r_1)/(1-r_1);\ r_1 = a_2/a_1,$$

and

$$p = (r_2^{(1-q)} - r_2)/(1-r_2);\ r_2 = a_3/b.$$

The experimental design adopted in this study was identical to the one used in the previous experiment, the doses administered being determined by the above transformation. The experiment likewise was in two replicates one week apart. In the first replicate many responses near 0 and 100 per cent. were observed and in the second replicate the logarithmic interval was reduced. The doses administered and their coding is indicated in Table 5, together with the experimental results. The regression analysis is given in Table 6. This analysis indicates that all partitionings at the one level and in the same replicate are equivalent in their effect on percentage response. There is no evidence of any antagonistic action for this route of administration (compare Fig. 3 with Fig. 2), and response is linearly related to the logarithm of the dose of each oestrogen separately or in mixtures.

IV. Discussion

Comparison of the median effective dose (M.E.D.) obtained by the subcutaneous administration of a true oestrogen with the M.E.D. obtained by intravaginal administration of the same oestrogen indicates that less than 1 per cent. of the dose administered subcutaneously finally reaches the vaginal epithelium to initiate a response. Presumably following subcutaneous administration the oestrogen is absorbed from the subcutaneous depot and transported via the blood stream to the target organs. The factors underlying the high systemic/local M.E.D. ratio may be divided into two groups: (i) factors governing the amount of oestrogen utilized or held in other target organs, and (ii)

factors governing the rapid destruction and excretion of oestrogens. The amount of oestrogen utilized in the other target organs such as the uterus and mammary gland is unknown, but since these organs are considerably larger than the vaginal epithelium it would be expected that each would utilize more oestrogen than the vaginal epithelium. Studies with radioactive compounds related to the oestrogens (Twombley 1951) have indicated that oestrogens are rapidly excreted in the bile; up to 80 per cent. of products of the injected oestrogen are found in the bile 5-6 hr after administration. An enterohepatic circulation of oestrogen is set up which probably maintains the level of circulating oestrogen.

TABLE 4

REGRESSION ANALYSIS OF EMPIRICAL ANGULAR RESPONSE ON 10 FUNCTIONS OF THE COORDINATES OF THE DESIGN

Coordinate Function	Regression Coefficient	$t_{(\infty)}$
Constant	35·39±1·81	
X_R	2·61±1·12	2·3*
X_1	1·93±1·78	<1
X_2	2·84±1·69	1·7
X_L	12·30±1·37	9***
X_1X_2	−0·90±1·96	<1
X_1X_L	3·12±1·41	2·21*
X_2X_L	0·54±1·41	<1
X_1^2	3·20±1·32	2·42**
X_2^2	4·21±1·32	3·19**

Deviations from regression: $\chi^2_{(50)} = 49·7$

* $0·05>P>0·01$.
** $0·01>P>0·001$.
*** $P<0·001$.

Studies of the optimum conditions of administration of oestrogens (Biggers and Claringbold 1954*a*) have indicated that an effective dose of oestrogen must be in contact with the vaginal epithelium for 36-48 hr in order to elicit a response. The finding is supported by studies of Biggers and Claringbold (1954*b*) who found that the number of mitoses in the vaginal epithelium arrested by colchicine was greatly increased in the period. The work supports the view that the level of circulating oestrogen remains raised for 36-48 hr after a subcutaneous injection and the view that, although subcutaneously administered oestrogen rapidly appears in the bile, it is still available to the target organs via the blood stream in a modified form. Biggers and Claringbold (1954*b*) found that following one maximal dose of oestrone given intravaginally, mitotic activity did not begin to increase until 18 hr had passed. Thus if most oestrogen is lost in the bile in 5-6 hr and the circulating oestrogen practically

disappears, it would be expected that mitosis would begin immediately following injection of oestrogen rather than after a delay. The work suggested that it is the oestrogen from the enterohepatic circulation which finally acts on the

TABLE 5

PERCENTAGE RESPONSE OF GROUPS OF 12 OVARIECTOMIZED MICE TO DIFFERENT COMBINATIONS OF DOSES OF OESTRONE, OESTRADIOL-3,17β, AND OESTRIOL

The partitioning of the middle joint dose ($X_L = 0$) is shown in full for each replicate. In the first replicate the other levels of X_L are -1 and 1, and the corresponding doses are obtained by respective division and multiplication by 2. The other levels of X_L in the second replicate are $X_L = -0{\cdot}49$ and $0{\cdot}49$. The appropriate doses are obtained by successive division and multiplication by $1{\cdot}4$

	Joint Dose			Coordinates		Response (%)		
	Dose Oestrone (10^{-2} µg)	Dose Oestradiol-3,17 β (10^{-2} µg)	Dose Oestriol (10^{-2} µg)	X_1	X_2	$X_L =$		
						-1	0	1
First replicate $X_R = -1$	8·00	0·00	0·00	2	0	8	58	92
	4·70	1·64	0·00	1	$t/3$*	8	17	83
	2·08	2·96	0·00	0	$2t/3$	0	50	92
	0·00	4·00	0·00	-1	t	8	67	75
	0·00	3·34	3·92	-1	$t/3$	0	58	100
	0·00	2·16	11·06	-1	$-t/3$	25	33	92
	0·00	0·00	24·00	-1	$-t$	8	50	83
	3·68	0·00	12·96	0	$-2t/3$	8	50	83
	6·22	0·00	5·30	1	$-t/3$	25	42	92
	2·68	1·90	4·60	0	0	8	75	100
						$-0{\cdot}49$	0	0·49
Second replicate $X_R = 1$	8·00	0·00	0·00	2	0	8	33	67
	3·31	2·34	0·00	1/2	$t/2$	17	42	75
	0·00	4·00	0·00	-1	t	8	33	58
	0·00	2·84	6·96	-1	0	17	25	83
	0·00	0·00	24·00	-1	$-t$	8	25	42
	5·07	0·00	8·78	1/2	$-t/2$	0	33	50
	3·82	0·66	6·19	1	0	8	42	67
	1·11	3·14	1·82	$-1/2$	$t/2$	8	33	83
	1·66	1·17	12·00	$-1/2$	$-t/2$	17	42	67
	2·68	1·90	4·61	0	0	17	25	58

* $t = \sqrt{3}$.

target organs. Unfortunately, in the mouse, little is known of the constituents of the blood and urinary oestrogens and the nature of the circulating oestrogen cannot be stated or deduced.*

* *Note added in Proof.*—In a recent publication (Stimmel 1955) the metabolism of oestradiol-16-^{14}C and oestrone-16-^{14}C in dogs has been studied. Following subcutaneous

The present work has indicated differences between the subcutaneous and intravaginal actions of natural oestrogens. Using the subcutaneous route these oestrogens behave simply as dilutions of oestradiol-3,17β, the most potent under these conditions. All dose response lines of mixtures had similar slopes and the effect of joint dose was equal to that predicted on the basis of additivity of its component oestrogens. With the intravaginal route, however, the findings are completely reversed. The oestrogens are mutually antagonistic in their action and the dose response slopes depend on the oestrogen studied.

TABLE 6

REGRESSION ANALYSIS OF THE DATA OF TABLE 5

Coordinate Function	Regression Coefficient	$t_{(\infty)}$
Constant	$40{\cdot}25 \pm 1{\cdot}12$	
X_R	$-4{\cdot}38 \pm 1{\cdot}12$	$3{\cdot}9$***
X_1	$-0{\cdot}05 \pm 1{\cdot}15$	<1
X_2	$0{\cdot}72 \pm 1{\cdot}15$	<1
X_L ($\log_{10}$ dose)	97 ± 6	17***

Deviations from regression: $\chi^2_{(55)} = 43{\cdot}5$

*** $P<0{\cdot}001$.

If it is assumed that under the same conditions of administration the slope of the dose response line is a characteristic feature of the response of the vaginal epithelium to different oestrogens, it follows that the oestrogen or mixture of oestrogens finally reaching the vaginal epithelium after subcutaneous administration does not depend on the natural oestrogen administered subcutaneously. In particular the dose response line obtained by the intravaginal administration of oestradiol-3,17β is the flattest of all natural oestrogens, being roughly one-half of the slope obtained with oestrone. If, following subcutaneous administration of oestrone or oestradiol-3,17β, only the oestrogen administered finally reached the vaginal epithelium, different dose response slopes are expected and joint doses would have an antagonistic action. This is not the case, the simplest explanation being that the oestrogens are converted by the liver to a common form or mixture which is either excreted in the bile or passes through the blood stream to the target organs.

Following the administration of an effective dose of true oestrogen by the intravaginal route, it acts directly on the cells concerned (Robson and Adler 1940; Emmens 1942; Hardy, Biggers, and Claringbold 1953). The possibility that conversion to oestradiol-3,17β takes place in the epithelium was suggested

administration of either oestrogen the label is found in the urine as a mixture of oestrone, oestradiol-3,17β, and oestriol in approximately equal quantities. This is strong evidence that the circulating oestrogen is a mixture derived by metabolism of injected oestrogen as suggested above.

by Biggers and Claringbold (1953). The antagonistic action of the natural oestrogens could be explained in many ways. If interconversions, partial or full, take place, the administration of the final products of this conversion together with the stimulating oestrogen would tend to reduce the amount of final product formed by a mass action effect. Alternatively some process of differential transport or differential utilization may be suggested. The question cannot be fully answered on the basis of present knowledge although the present study strongly indicates that oestrogens undergo changes in the vaginal epithelium.

V. Acknowledgments

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