THE EFFECT OF METABOLIC INHIBITORS ON THE LOCAL ACTION OF OESTRONE AND OESTRADIOL-3,17β ON THE VAGINA OF OVARIECTOMIZED MICE

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Summary

In the intravaginal Allen-Doisy test for oestrogenic activity potassium cyanide produced a significant increase in response to oestrone, but not to oestradiol-3,17β.

Sodium azide, 2,4-dinitrophenol, and sodium monoiodoacetate caused a reduction in response to both oestrogens studied.

Inhibitory dose response lines obtained with sodium monoiodoacetate in mice receiving maximal doses of oestrone or oestradiol-3,17β indicate greater variability of response to oestradiol-3,17β than to oestrone.

I. INTRODUCTION

While it has been known for some years that vaginal cornification in the rodent may be caused by local administration of oestrogens (see Emmens 1950, for review of the literature), the actual site and mode of action of the oestrogen in the epithelium have been the subject of speculation. It was thought that the effect of metabolic inhibitors, administered intravaginally, might help to elucidate this problem.

II. MATERIALS AND METHODS

A colony of 350 albino mice bred in this department were ovariectomized. Their management and use in tests were as described by Biggers (1951), tests being carried out fortnightly, with two injections in 24 hr. The oestrogens and inhibitors were administered together by the intravaginal route in phosphate buffer solution (pH 7.0, 0.1M), so that the total volume of two injections was 0.02 ml. The oestrone and oestradiol-3,17β were obtained from Organon Laboratories.

Standard procedures for probit analysis, as described by Finney (1952) have been employed throughout. At the start of this work it was thought probable that significant slope differences would be found, particularly when different oestrogens were being compared (Biggers and Claringbold 1953; Biggers 1953*a*). The estimation of relative potency for the comparison of activity is only justified if the dose response lines are parallel and if this is not so the median effective dose (M.E.D.) ratio test must be used (Biggers 1951). If the final

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Table 1

EFFECT OF POTASSIUM CYANIDE ON THE DOSE RESPONSE LINES OBTAINED BY THE LOCAL ADMINISTRATION OF OESTRONE OR OESTRADIOL-3,17β

Oestrone

Test	Control Dose (10^{-4} μg)	Control Response	Treated Dose (10^{-4} μg)	Treated Response	M.E.D. Ratio
1	1·0	0 : 20	1·0	4 : 19	
	2·3	3 : 20	2·3	8 : 20	1·83
	5·3	12 : 20	5·3	16 : 20	{1·12-2·98}
	12·2	15 : 20	12·2	17 : 20	
2	1·0	1 : 20	1·0	7 : 20	
	2·3	4 : 20	2·3	9 : 20	2·49
	5·3	13 : 19	5·3	17 : 19	{1·47-4·24}
	12·2	15 : 20	12·2	17 : 20	
3	1·0	3 : 20	0·4	3 : 20	
	2·3	10 : 18	1·0	10 : 19	1·91
	5·3	12 : 20	2·3	17 : 19	{1·11-3·29}
	12·2	19 : 20	5·3	17 : 20	
Mean slopes	2·29±0·27		1·71±0·24		
Common mean slopes	1·96±0·18				

Partitioning of χ^2

Source of variation	D.F.	χ^2	P
Parallelism			
Between mean slopes	1	2·68	0·2-0·1
Within mean slopes	4	0·99	0·95-0·9
Heterogeneity	11	22·40	0·05-0·02

Common M.E.D. ratio 2·05 {1·24-3·38} $_{P=0\cdot001}$

Relative potency 2·28 {1·48-3·52} $_{P=0\cdot001}$

Oestradiol-3,17β

Test	Control Dose (10^{-4} μg)	Control Response	Treated Dose (10^{-4} μg)	Treated Response	M.E.D. Ratio
1	2·0	9 : 20	2·0	10 : 19	
	3·0	12 : 20	3·0	11 : 19	1·23
	4·5	13 : 20	4·5	14 : 20	{0·52-2·90}
	6·8	17 : 20	6·8	16 : 20	
2	1·0	4 : 20	1·0	5 : 20	
	2·3	11 : 20	2·3	12 : 20	1·25
	5·3	12 : 20	5·3	12 : 20	{0·58-2·69}
	12·2	13 : 18	12·2	15 : 19	
Mean slopes	1·37±0·34		1·27±0·34		
Common mean slopes	1·32±0·24				

Source of variation	D.F.	χ^2	P
Parallelism			
Between mean slopes	1	0·05	0·8-0·7
Within mean slopes	2	1·04	0·7-0·5
Heterogeneity	8	5·87	0·7-0·5

Common M.E.D. ratio 1·24 {0·70-2·20}

Relative potency 1·16 {0·64-2·11}

partitioning of χ^2 indicated no significant slope differences the relative potency estimate was computed, otherwise the more conservative M.E.D. ratio test was employed.

Response is recorded in the tables as a ratio of the number of animals positive to the number of animals per group. Standard errors of mean values follow a ± sign; fiducial limits of error ($P = 0.05$, except where otherwise indicated by subscript) have been enclosed in braces following the appropriate estimate; and M.E.D. ratios and relative potencies are given in their arithmetic form to avoid constant reference to the logarithmic base adopted in a particular test.

III. Results

(*a*) *Potassium Cyanide* (*Table* 1)

In the five tests reported 24 μg of potassium cyanide were injected with the oestrogen in buffer solution, three tests being carried out with oestrone and two with oestradiol-3,17β. Where oestrone was the oestrogen under test, a significant increase in response occurred on all occasions. The partitioning of χ^2 gives no evidence of any significant slope differences, and while the data obtained with oestrone show evidence of heterogeneity, this has been ignored since the total χ^2 for heterogeneity over all the tests reported in this paper is well within the range of random sampling variation ($\chi^2_{(54)} = 50.67$, $P = 0.59$).

(*b*) *Sodium Monoiodoacetate* (*Tables* 2 *and* 3)

The tests shown in Table 2 were based on the results of preliminary investigations and were designed for the purpose of comparing the inhibition produced by monoiodoacetate in mice receiving a maximal dose of (1) oestrone and (2) oestradiol-3,17β. The dose response lines have negative slopes and may be used to estimate the median inhibitory dose (M.I.D.), i.e. the dose required to prevent cornification in 50 per cent. of animals. Although equal amounts of oestrone and oestradiol-3,17β were given to both groups (20×10^{-4} μg), the expected response in the absence of inhibition is in each approximately 98 per cent. Under all conditions of administration oestradiol-3,17β has been found more variable in action than oestrone (Biggers and Claringbold 1953), with the result that the expected responses to doses of approximately 20×10^{-4} μg are equal (cf. Biggers and Claringbold 1953, Fig. 3).

Analysis of the results of Table 2 indicates that in order to produce 50 per cent. inhibition significantly more monoiodoacetate is required with oestradiol-3,17β than with oestrone. Partitioning of χ^2 indicates that the slope of the inhibitory dose response line for the latter is significantly steeper than for the former.

A simple factorial experiment (Table 3) was used to test the hypothesis that the activities of oestrone and monoiodoacetate are additive, i.e. to test whether the variability of response to oestrone is independent of the level of monoiodoacetate used. The results of the experiment have been fitted by means of a probit plane (Finney 1952), the regression equation involving the probit of response, the log dose of oestrone, the log dose of monoiodoacetate, and a constant. The goodness of fit of this equation was found to be satisfactory ($\chi^2_{(7)} = 2.68$, $0.95 > P > 0.9$). There is thus no evidence of departure from additivity and it may be concluded that the variability of response to oestrone is independent of the level of monoiodoacetate.

TABLE 2

EFFECT OF FOUR CONCENTRATIONS OF MONOIODOACETATE ON MAXIMAL CORNIFICATION PRODUCED BY 20×10^{-4} μg OF OESTRONE OR OESTRADIOL-3,17β

Twenty animals per group

Mono-iodoacetate Dose (μg)	Animals Positive			
	Test 1		Test 2	
	Oestrone	Oestradiol-3,17β	Oestrone	Oestradiol-3,17β
10	17	11	18	13
20	14	7	11	10
40	3	6	7	5
80	0	4	6	5
*M.I.D. ratio:	2·00 {0·72-5·42}		1·71 {0·85-3·45}	

Common M.I.D. ratio	1·80 {1·02-3·19}	
Mean slopes:		
Oestrone	$-2{\cdot}47 \pm 0{\cdot}39$	
Oestradiol-3,17β	$-1{\cdot}15 \pm 0{\cdot}31$	
Common mean slope		$-1{\cdot}67 \pm 0{\cdot}24$

Partitioning of χ^2

Source of variation	D.f.	χ^2	P
Parallelism			
Between mean slopes	1	7·09	0·02-0·01
Within mean slopes	2	5·75	0·1 -0·05
Heterogeneity	7	6·40	0·5 -0·3

* Median inhibitory dose.

The data of this test have been analysed by alternative methods for purposes of comparison; the results of this investigation are published elsewhere (Claringbold, Biggers, and Emmens 1953). With these alternative procedures the same conclusions have been reached.

(c) *Sodium Azide* (*Table* 4) *and 2,4-Dinitrophenol* (*Table* 5)

In the tests reported 60 μg of sodium azide or 600 μg 2,4-dinitrophenol were injected with the oestrogen in buffer solution. The inhibitors always caused a significant lowering of response to the oestrogens. The partitionings of χ^2 show no significant slope differences.

TABLE 3

EFFECT OF COMBINATIONS OF LOCALLY ADMINISTERED DOSES OF MONOIODOACETATE AND OESTRONE

Twenty animals per group is constant; number positive tabulated

Oestrone Dose (10^{-4} μg)	Monoiodoacetate Dose (μg)			
	12·5	25	50	100
2	5	3	3	0
4	5	3	4	1
8	9	7	3	3
16	14	10	7	4

Mean slopes:

Oestrone: $1 \cdot 17 \pm 0 \cdot 24$
Monoiodoacetate: $-1 \cdot 19 \pm 0 \cdot 24$

IV. Discussion

Following the intravaginal administration of a dose of oestrogen, the changes which take place may be divided into two stages. In the first stage the oestrogen is absorbed by the epithelium and transferred to the final site of action, perhaps undergoing transformation on the way. If the dose is sufficient the second stage begins, involving morphological changes associated with greatly increased mitotic rate in the cells of the stratum germinativum. Metabolic inhibitors or activators may act on either of these stages; if acting at the first stage their effect may depend on the oestrogen used, whereas if acting at the second, their effect should be independent of the oestrogen used.

Evidence for the local conversion of oestrone to oestradiol-3,17β has been presented in an earlier paper by Biggers and Claringbold (1953), who have also pointed out the fundamental difference in variability of response to the local administration of these oestrogens. The differential action of cyanide with regard to oestrone and oestradiol-3,17β cannot be explained simply in terms of a local metabolic action taking place in the second stage, but must indicate that oestrone and oestradiol-3,17β undergo different changes in reaching their site of action.

Bullough and Johnson (1951) have studied the effect of various metabolic inhibitors on mitotic activity in adult mouse epidermis and found that cyanide, azide, monoiodoacetate, and 2, 4-dinitrophenol are powerful mitotic inhibitors acting in the antephase. The histological and cytological aspects of the vaginal

Table 4

EFFECT OF SODIUM AZIDE ON THE DOSE RESPONSE LINES OBTAINED BY THE LOCAL ADMINISTRATION OF OESTRONE OR OESTRADIOL-3,17β

Oestrone

Test	Control Dose (10^{-4} μg)	Control Response	Treated Dose (10^{-4} μg)	Treated Response	M.E.D. Ratio
1	1·0	2 : 20	1·0	1 : 20	
	2·3	6 : 20	2·3	4 : 20	0·47
	5·3	13 : 20	5·3	7 : 20	{0·25-0·91}
	12·2	16 : 20	12·2	12 : 20	
2	1·0	2 : 20	1·0	1 : 20	
	2·3	2 : 20	2·3	3 : 20	0·56
	5·3	14 : 20	5·3	6 : 20	{0·37-0·83}
	12·2	14 : 20	12·2	12 : 20	
Mean slopes	2·02±0·30		1·70±0·31		
Common mean slope	1·86±0·22				

Partitioning of χ^2

Source of variation	D.f.	χ^2	P
Parallelism			
Between mean slopes	1	0·55	0·5-0·3
Within mean slopes	2	2·22	0·5-0·3
Heterogeneity	8	4·38	0·9-0·8

Common M.E.D. ratio 0·53 {0·38-0·75}

Relative potency 0·49 {0·33-0·72}

Oestradiol-3,17β

Test	Control Dose (10^{-4} μg)	Control Response	Treated Dose (10^{-4} μg)	Treated Response	M.E.D. Ratio
1*			1·0	4 : 20	
			2·3	4 : 20	
	1·0	7 : 20	5·3	9 : 20	
	2·3	9 : 20	12·2	11 : 20	0·24
	5·3	13 : 20			{0·09-0·67}
	12·2	16 : 20	1·0	6 : 20	
			2·3	4 : 20	
			5·3	8 : 20	
			12·2	10 : 20	
Mean slopes	1·15±0·37		0·80±0·26		
Common mean slope	0·92±0·24				

Source of variation	D.f.	χ^2	P
Parallelism			
Between mean slopes	1	0·56	0·5-0·3
Within mean slopes	1	2·15	0·2-0·1
Heterogeneity	6	1·13	0·98-0·95

Common M.E.D. ratio 0·24 {0·09-0·67}

Relative potency 0·23 {0·08-0·68}

* A common M.E.D. for the two treated groups was obtained and tested against the control M.E.D.

TABLE 5

EFFECT OF 2, 4-DINITROPHENOL ON THE DOSE RESPONSE LINES OBTAINED BY THE LOCAL ADMINISTRATION OF OESTRONE OR OESTRADIOL-3,17β

Oestrone

Test	Control Dose (10^{-4} μg)	Control Response	Treated Dose (10^{-4} μg)	Treated Response	M.E.D. Ratio
1	1·0	3 : 20	1·0	0 : 20	
	2·3	7 : 20	2·3	3 : 20	0·41
	5·3	12 : 20	5·3	8 : 20	{0·23-0·69}
	12·2	17 : 20	12·2	11 : 20	
2	1·0	2 : 15	1·0	0 : 15	
	2·3	4 : 19	2·3	2 : 20	0·48
	5·3	9 : 20	5·3	6 : 20	{0·24-0·94}
	12·2	16 : 20	12·2	10 : 20	
Mean slopes	1·91±0·31		1·98±0·36		
Common mean slope	1·94±0·23				

Partitioning of χ^2

Source of variation	D.f.	χ^2	P
Parallelism			
Between mean slopes	1	0·01	0·95-0·90
Within mean slopes	2	0·01	0·98-0·95
Heterogeneity	6	3·41	0·8-0·7

Common M.E.D. ratio 0·44 {0·28-0·69}

Relative potency 0·44 {0·29-0·66}

Oestradiol-3,17β

Test	Control Dose (10^{-4} μg)	Control Response	Treated Dose (10^{-4} μg)	Treated Response	M.E.D. Ratio
1	1·0	6 : 20	1·0	5 : 20	
	2·3	6 : 20	2·3	2 : 20	0·50
	5·3	12 : 20	5·3	9 : 20	{0·21-1·03}
	12·2	15 : 20	12·2	13 : 20	
2	1·0	7 : 20	1·0	2 : 20	
	2·3	9 : 20	2·3	5 : 20	0·32
	5·3	13 : 20	5·3	10 : 20	{0·13-0·78}
	12·2	16 : 20	12·2	11 : 20	
Mean slopes	1·18±0·26		1·25±0·27		
Common mean slope	1·22±0·19				

Source of variation	D.f.	χ^2	P
Parallelism			
Between mean slopes	1	0·03	0·9-0·8
Within mean slopes	2	0·05	0·98-0·95
Heterogeneity	8	7·08	0·7-0·5

Common M.E.D. ratio 0·40 {0·21-0·76}

Relative potency 0·41 {0·22-0·74}

response to oestrogens in mice have been discussed by Allen (1922) and Biggers (1953*b*), the first sign of oestrogen activity being division of the cells of the stratum germinativum. In the present work it would appear that azide, monoiodoacetate, and 2, 4-dinitrophenol are exerting their characteristic metabolic effect (inhibition of respiration in the first case, inhibition of glycolysis in the last two cases), resulting in inhibition of the morphological response. Bullough and Johnson (1951), however, found that cyanide acted similarly to azide whereas in the work described in this paper no inhibition occurred with the level of dosage used. In the present work the action of cyanide may be a specific enzymic effect. Heller (1940) showed that incubation of oestrone with liver mince resulted in inactivation of the oestrone unless cyanide was added, when oestrone was converted to oestradiol-3,17β with resultant increase in activity.

Further evidence has been presented as to the fundamental differences in activity and variability of oestrone and oestradiol-3,17β when administered by the intravaginal route. The slopes of the inhibitory dose response lines reflect the variability in response to the oestrogens, and indicate that oestradiol-3,17β elicits the more variable response. The fact that more inhibitor is required to produce 50 per cent. inhibition with oestradiol-3,17β than with oestrone demonstrates the greater relative activity of oestradiol-3,17β.

The present work, taken in conjunction with that of Bullough and Johnson (1951), indicates that oestrogens stimulate mitosis in the cells of the stratum germinativum, thus initiating the morphological process. Much remains to be done before a full understanding of the events leading up to this is possible.

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VI. References

Allen, E. (1922).—*Amer. J. Anat.* **30**: 297.

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

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

Biggers, J. D. (1953*b*).—*J. Anat.* (*Lond.*) **87** (in press).

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

Bullough, W. S., and Johnson, M. (1951).—*Proc. Roy. Soc.* B **138**: 562.

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

Emmens, C. W. (1950).—"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.)

Heller, C. G. (1940).—*Endocrinology* **26**: 619.