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THE USE OF 2-3-5-TRIPHENYLTETRAZOLIUM CHLORIDE IN THE BIOLOGICAL ASSAY OF OESTROGENS

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SUMMARY

The reduction of 2-3-5-triphenyltetrazolium chloride in the vaginal epithelium of the mouse is shown to form a suitable basis for a highly sensitive assay of oestrogens. The method is as sensitive and precise as those utilizing mitosis and epithelial thickness, with the added advantage of extreme simplicity.

The earliest morphological response of the vaginal epithelium of the mouse, following a single intravaginal injection of oestrogen, is an increase in cell size and rate of cell division 16–18 hr later [Biggers & Claringbold, 1955]. These responses have been utilized in a highly sensitive assay for oestrogens [Martin & Claringbold, 1958, 1960].

Little is known of the biochemical reorganization that must precede and accompany these changes. While Jeener [1948] observed an increase in vaginal ribonucleic acid and alkaline phosphatase content 24 hr after injection of a massive dose of oestradiol-3:17-β, most other studies of vaginal metabolism have been concerned with processes associated with cornification [Kamell & Atkinson, 1948; Ring, 1950; Biggers, 1953; Rosa & Velardo, 1954; Balmain, Biggers & Claringbold, 1956].

Preliminary manometric studies in this laboratory suggested that vaginal respiration is elevated 24 hr after treatment with oestrogen, although it was impossible to distinguish between epithelial respiration and that of the organ as a whole. These methods were found to be too insensitive and unwieldy to be of use in the routine assay of oestrogens. It was thought that 2-3-5-triphenyltetrazolium chloride might afford a simple method of following respiratory changes in the vaginal epithelium following oestrogenic stimulation.

2-3-5-Triphenyltetrazolium chloride is a pale yellow, non-toxic, water-soluble compound which interacts with a number of intracellular reductases as a hydrogen acceptor. Under these conditions it is reduced to a stable, water-insoluble, deep red pigment which is precipitated at the site of reaction. The reduction product, formazan, is soluble in a number of organic solvents, and may be extracted from tissues and estimated colorimetrically. The extreme simplicity of the method has led to its use in the cytological localization of enzymes [Farber, Sternberg & Dunlap, 1954; Jardetsky & Glick, 1956; Glick & Nayyar, 1956], the determination of seed viability [Lakon, 1939, 1942] and the identification of actively growing tissues [Doerr, 1950].

In the present investigation, triphenyltetrazolium is used only as an index of change in biochemical organization of the vaginal epithelium following oestrogenic stimulation.

MATERIALS AND METHODS

(*a*) *Animals*

Randomly bred, adult, ovariectomized, albino mice of the Sydney White strain were used in these experiments. The mice were used 7–10 days after ovariectomy and priming with oestrone was omitted.

In all experiments, individual animals were alloted to the experimental groups with tables of random numbers. Ovariectomy was carried out by a modification of the method of Emmens [1950], as described by Martin & Claringbold [1960].

(*b*) *Solutions*

Stock solutions of steroids were prepared at a concentration of 1·0 mg/ml. in ethanol, and were stored in a refrigerator at 5° C. Immediately before each experiment, aliquots were diluted with distilled water.

Tetrazolium was prepared as an aqueous solution of 25 mg/ml. shortly before killing the animals.

(*c*) *Biological assay*

Mice received a single intravaginal injection of oestrogen in 0·01 ml. distilled water. With the exception of Expt. 1, they were killed 24 hr later. Except in cases where the dose and time of injection of tetrazolium were varied, mice received 0·5 mg tetrazolium in 0·02 ml. water intravaginally 30 min prior to killing. The vaginae were dissected out, cut open and washed in distilled water to remove excess tetrazolium chloride, dried on filter-paper, and placed in 1·0 ml. 3:1 ethanol-tetrachlorethylene [Jardetsky & Glick, 1956]. The amount of formazan was estimated colorimetrically, using a Uvispek spectrophotometer at 500 mμ. Since the relationship between optical density (O.D.) and weight of formazan is linear in the range 0–40 μg/ml. (Fig. 1), it was not considered necessary to convert O.D.'s to μg of formazan. For analysis, O.D.'s were therefore converted direct to logarithms.

RESULTS

(*a*) *Tetrazolium reduction in vagina at various times after a single intravaginal dose of oestrone*

The first experiment (Table 1, Fig. 2) was a factorial in which mice were killed at various intervals after a single intravaginal injection of oestrone. Thirty min prior to killing, each animal received 0·5 mg 2-3-5-triphenyltetrazolium chloride as a single intravaginal injection in 0·02 ml. distilled water. Three replicates were carried out, using eight, six and six mice per group. In this experiment, organ weight was measured after extraction of formazan. Each tissue was placed on a filter-paper to remove excess fluid, then rapidly weighed on a torsion balance. The wet weight of the vagina (mg) was used in the calculations.

The correlation of tetrazolium reduction (Y_1) with organ weight was small ($r = 0{\cdot}2178$; $P = 0{\cdot}001$). Correction of tetrazolium colour for concomitant variation

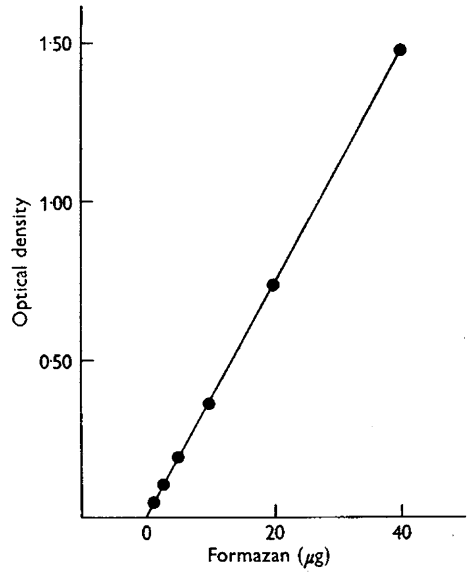

Fig. 1. Relationship between weight of formazan and optical density.

Table 1. *Reduction of 2-3-5-triphenyltetrazolium chloride in vaginal epithelium of mouse, at various times after a single injection of oestrone*

(No. of animals/group (20) is constant.)

Dose of oestrone ($\mu g \times 10^{-4}$)	Mean optical density (Y_1)					
	0 hr	6 hr	12 hr	18 hr	24 hr	30 hr
0·04	0·046	0·050	0·071	0·070	0·064	0·091
1·00	0·042	0·070	0·142	0·255	0·417	0·532
Slope	−4·1	11·0	25·0	40·9	57·9	56·5

Dose of oestrone ($\mu g \times 10^{-4}$)	Mean organ weight (mg) (Y_2)					
	0 hr	6 hr	12 hr	18 hr	24 hr	30 hr
0·04	12·5	14·1	14·3	15·1	14·6	15·8
1·00	15·0	15·3	16·5	16·3	18·2	18·1
Slope	1·8	0·9	1·6	0·9	2·6	1·6

Analysis of variance

Source of variation	D.F.	Y_1 Mean square	Y_2 Mean square	Y_1Y_2 Mean product
Time				
Linear	1	188,798***	32,784***	78,673
Residual	4	1,254	171	113
Dose	1	139,572***	34,827***	69,720
Replicates	2	3,953***	91,379***	18,069
Time × dose				
Linear	1	75,110***	649	6,983
Residual	4	679	837	176
Time × replicate	10	620*	495	140
Dose × replicate	2	264	543	197
Residual	10	693*	674	254
Error	204	314	556	91

*** $P < 0{\cdot}001$. * $0{\cdot}01 < P < 0{\cdot}05$.

in organ weight is invalid, as organ weight is dependent on treatment. Since the analyses of variances showed similarities, the possibility of using a combined score was investigated by multivariate analysis. Since two variates are measured, two scores are possible. Such scores may be linearly defined as

$$S_1 = b_1 Y_1 + b_2 Y_2,$$
$$S_2 = b_1' Y_1 + b_2' Y_2.$$

The weights b_1, b_2 and b_1', b_2' are evaluated by the method of Rao [1952] which involves the solution of the determinential equation

$$[A - \lambda B] = 0,$$

where A and B are, respectively, the dispersion matrices within and between treatment populations. The latent roots ($\lambda_1 \lambda_2$) are proportional to the between-treatment variances of the corrresponding linear functions.

Only the 4 degrees of freedom (D.F.) associated with significant effects of time and dose were included in the analysis, these being combined into a single addition matrix B. A is the within group covariance matrix with 204 D.F.

$$B_{(4)} = \begin{bmatrix} 101230 & — \\ 38651 & 17168 \end{bmatrix}$$

$$A_{(204)} = \begin{bmatrix} 314 & — \\ 91 & 556 \end{bmatrix}$$

The latent roots and their test of significance [Claringbold, 1958] are as follows:

Latent root	D.F.	χ^2	P
$\lambda_1 = 5{\cdot}4000$	5	436·0	$P < 0{\cdot}001$
$\lambda_2 = 0{\cdot}0755$	3	17·1	$0{\cdot}001 < P < 0{\cdot}01$

Although both roots are significant, it is clear that only the linear function corresponding to λ_1 is of importance. This function is defined as follows:

$$S_1 = 0{\cdot}998 Y_1 + 0{\cdot}056 Y_2.$$

The amount of information obtained from organ weights is small compared with that obtained from tetrazolium; accordingly organ weights were ignored in subsequent experiments since the small gain in precision was offset by the amount of labour involved.

Tetrazolium reduction increases linearly after the local application of oestrone. The highly significant dose-response line at 6 hr shows that absorption of oestrogen and subsequent biochemical reorganization in the epithelium proceed rapidly after injection of the hormone. Gross morphological changes are not observed for at least another 12 hr [Biggers & Claringbold, 1955; Martin & Claringbold, 1960]. The cause of increased tetrazolium reduction is not clear, and may result from greater permeability of the cell to the compound, or to elevated concentrations of intracellular reductases and their substrates. Such increases have been observed in the lactic dehydrogenase activity of uterine tissue shortly after oestrogen administration [Mueller, Herranen & Jervell, 1958]. The slope of the dose-response line increases linearly with time, reaching a maximum at 24 hr. There is no significant change in slope between 24 and 30 hr. In all routine assays mice were killed 24 hr after the injection of oestrone.

(*b*) *Site of reduction of tetrazolium*

Frozen sections were cut from vaginae 24 hr after injection of 1×10^{-4} μg oestrone: 0·5 mg tetrazolium was injected intravaginally 30 min before killing the mice. A heavy deposit of formazan crystals was present in the epithelium, but not in the underlying tissues. The basement membrane was quite clearly marked by formazan crystals. The superficial cells of the epithelium appeared to contain a heavier deposit of

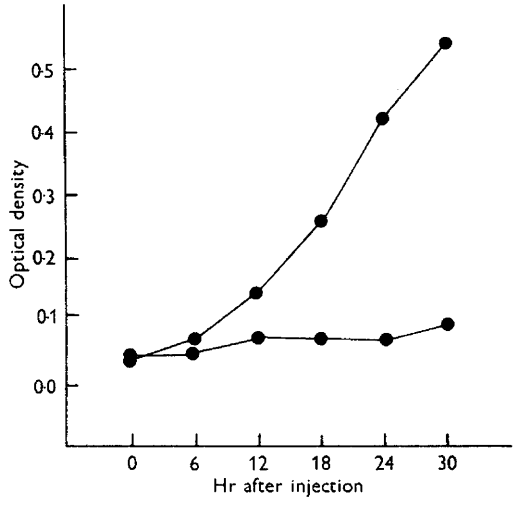

Fig. 2. Increase in tetrazolium reduction by vaginal epithelium of mouse at various times after a single injection of oestrone. Top line: 1×10^{-4} μg; bottom line: 4×10^{-6} μg.

Table 2. *Effects of differing times of injection of tetrazolium on the amount of reduction (Expt. 2)*

(No. of animals/group (6) is constant. Results are expressed as the mean O.D.'s for each treatment group).

Time of injection (min)	Dose of oestrone (μg $\times 10^{-4}$)		
	0·04	1·0	Slope
2	0·079	0·173	27·4
4	0·087	0·023	26·8
8	0·099	0·267	30·7
16	0·091	0·354	44·2
32	0·091	0·340	45·1
64	0·033	0·291	65·4

Error mean square (logs) 364

formazan than other parts of the epithelium, but this may have resulted from the method of administration of the tetrazolium. In sections of vaginae which had not received oestrogen, only scattered small crystals of formazan were observed in the epithelium.

(*c*) *Optimum conditions of administration of tetrazolium*

In preliminary experiments 0·5 mg tetrazolium was chosen arbitrarily as a suitable quantity for injection, and 30 min as a period in which adequate reduction might be expected to occur. Surprisingly, both conditions were found to be near optimal.

In the experiment of Table 2, mice were injected with two doses of oestrone

(4×10^{-6} μg and 1×10^{-4} μg) and killed 24 hr later. For each dose of oestrone 0·5 mg tetrazolium was injected intravaginally at various times prior to killing. In the maximally stimulated vagina the rate of tetrazolium reduction by the epithelium is approximately linear for the first 16 min, dropping abruptly to zero at this time (Fig. 3). This fall in rate is due probably to inactivation of enzymes by the intracellular deposition of formazan crystals. After 16 min there is a slow loss of formazan from the epithelium, perhaps due to further metabolism to colourless compounds. A similar decrease is seen in the unstimulated epithelium. The initial rate of reduction of tetrazolium in the unstimulated epithelium appears to be lower than that in the stimulated epithelium. Moreover, the rate falls off rapidly to zero 8 min after injection and at a level of formazan far lower than that of the unstimulated epithelium.

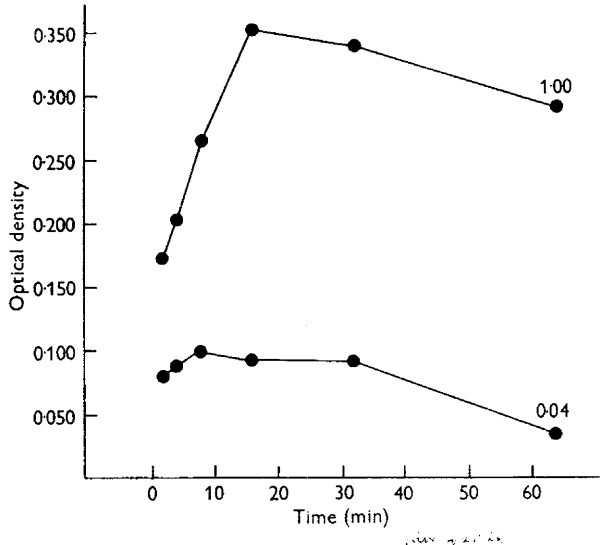

Fig. 3. Effect of time of injection of tetrazolium on tetrazolium reduction by vagina stimulated with 1·0 and $0{\cdot}04 \times 10^{-4}$ μg oestrone.

It is clear that oestrogen not only increases enzyme activity at certain foci, but also the number of foci. This agrees with the observations of Martin & Claringbold [1960] that the epithelial-cell volume is increased eightfold 24 hr after a maximal dose of oestrone.

Since colour development and slope are near maximal at any time from 16 to 64 min, 30 min was retained as a convenient period of colour development in succeeding experiments.

It was known that only a small proportion of injected tetrazolium was actually reduced. Accordingly, two experiments were carried out to determine the relationship between the amount injected to the amount reduced. In both experiments mice received 1×10^{-5} μg or 1×10^{-4} μg oestrone intravaginally, and were killed 24 hr later. Tetrazolium was injected intravaginally in 0·02 ml. distilled water, 30 min prior to killing. The results are summarized in Table 3 and Fig. 4.

In the first experiment the doses of tetrazolium used ranged by fivefold intervals from 0·5 to 0·004 mg. Reduction was almost non-existent at doses below 0·5 mg. In the second experiment, therefore, doses were used in the range 1·0 mg decreasing

by twofold intervals to 0·125 mg. It can be seen that, below 0·5 mg tetrazolium, reduction decreases rapidly with decreasing dose of tetrazolium. This discrepancy between the amount injected and the amount reduced probably results from the relatively small proportion of tetrazolium actually coming into contact with the

Table 3. *Effect of dose of tetrazolium on reduction of tetrazolium*

(Results are expressed as mean O.D.'s for each treatment group. Nos. of animals/group in the two experiments were 6 and 5 respectively.)

Expt. 1	Dose of tetrazolium (μg)			
Dose of oestrone ($\mu g \times 10^{-4}$)	4	20	100	500
1·0	0·015	0·038	0·124	0·668
0·1	0·014	0·025	0·069	0·161
Slope (logs)	0	18·5	23·3	64·5

Error mean square (logs) 190

Expt. 2	Dose of tetrazolium (μg)			
Dose of oestrone ($\mu g \times 10^{-4}$)	125	250	500	1000
1·0	0·241	0·360	0·738	0·723
0·1	0·076	0·130	0·134	0·201
Slope (logs)	47·8	42·8	78·0	57·4

Error mean square (logs) 288

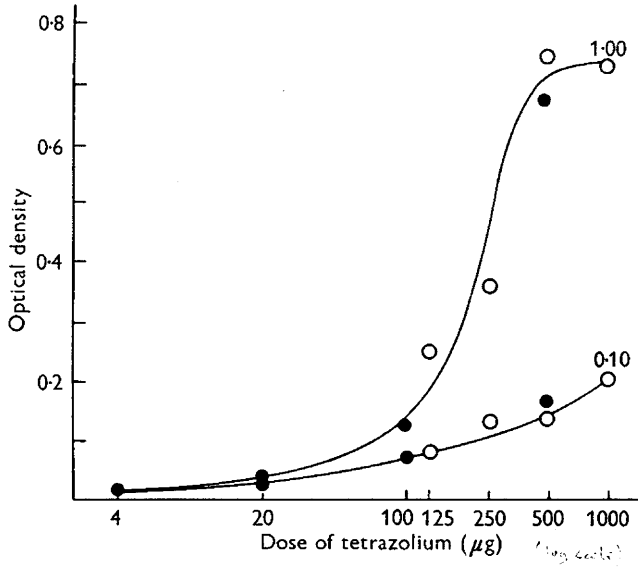

Fig. 4. Effect of dose of tetrazolium on reduction of tetrazolium after stimulation with 1·0 and 0·1 × 10^{-4} μg oestrone. ●, First experiment; ○, second experiment.

epithelium. Since slope and total reduction are near maximal at dose level of 0·5 mg, this quantity was used in all succeeding experiments.

(d) Effect of vehicle of administration

Since in previous experiment [Martin & Claringbold, 1960] propylene glycol was found to exert toxic effects on the vaginal epithelium, it was of interest to discover whether such effects were reflected in tests involving tetrazolium reduction. In a

preliminary experiment, oestrone was injected intravaginally in 0·01 ml. of various concentrations of propylene glycol in water; the mice were killed 24 hr later. Tetrazolium reduction decreased linearly with increasing concentrations of propylene glycol. It was not clear whether this resulted from inhibition of the oestrogenic response, or from a direct action of residual propylene glycol on the actual processes associated with tetrazolium reduction. In the next experiment, therefore, groups of mice were injected as follows:

Table 4. *Effect of propylene glycol injected at different times*

(Results are expressed as mean O.D.'s for the treatment groups each of which contained 6 animals. All animals received a single intravaginal injection of 1×10^{-4} μg oestrone. The various treatments are explained in the text.)

	Concn. of propylene glycol (%)				
Treatment	0	25	50	100	Slope (logs)
A	0·419	0·328	0·102	0·026	−41·0
B	0·553	0·578	0·376	0·425	− 4·5
C	0·343	0·249	0·246	0·143	−12·2

Analysis of variance

Source of variation	D.F.	Mean square	*F* ratio
Time (*T*)			
Linear	1	6,030	15·9***
Quadratic	1	30,742	81·1***
Dose (*D*)			
Linear	1	33,814	89·2***
Residual	2	840	2·2
T × *D*			
Linear × linear	1	12,877	34·0***
Quadratic × linear	1	10,035	26·5***
Residual	4	708	1·9
Error mean square	60	379·0	379·0

*** $P < 0·001$.

(A) Oestrone injected intravaginally in 0·01 ml. of various concentrations of propylene glycol.

(B) Oestrone injected intravaginally in 0·01 ml. distilled water: 0·01 ml. of the various propylene glycol solutions were injected intravaginally 5 hr before killing the animals.

(C) As for B, but propylene glycol injected immediately before injecting the tetrazolium.

Groups A and B were injected with 0·01 ml. distilled water, intravaginally, immediately before injection of tetrazolium, while groups A and C received a similar injection 5 hr before this. The results are summarized in Table 4.

Although propylene glycol interferes directly with tetrazolium reduction (group C), this does not account for the much larger effect when it is administered jointly with the hormone (group A). The lack of inhibition when propylene glycol was injected 5 hr beforehand, indicated that the bulk of it disappears from the vagina in this period, and that the inhibition of oestrogen is not directly associated with irreversible damage to the systems involved in reduction of tetrazolium.

(*e*) *Dose-response line at 24 hr*

The 24 hr dose-response line for oestrone was investigated more fully in a series of experiments listed in Table 5. In all cases, the dose-response relationship was linear in the dose range 1×10^{-5} μg to 1×10^{-4} μg of oestrone, while in three cases, the linear segment extended above this upper limit. The linear segment of the oestradiol-3:17β dose-response line appears to lie within a similar dose range. The slopes of the dose-response line for oestrone over a number of experiments formed a homogeneous group [$\chi^2_8 = 12{\cdot}8$; $0{\cdot}2 > P > 0{\cdot}1$] (see Table 6).

Table 5. *Dose-response line at 24 hr*

Expt.	Substance	Dose ($\mu g \times 10^{-4}$)	No. of animals	Mean O.D.	*b*
1	Oestrone	5·0	7	0·652	47·9 ± 5·92
		1·0	7	0·446	
		0·2	7	0·177	
		0·04	7	0·071	
2	Oestrone	1·0	5	0·235	49·1 ± 7·92
		0·5	4	0·218	
		0·25	5	0·166	
		0·125	4	0·097	
		0·0625	5	0·065	
3	Oestrone	3·0	5	0·459	74·4 ± 11·92
		1·0	5	0·566	
		0·33	5	0·263	
		0·11	5	0·118	
		0·037	5	0·094	
		0·012	5	0·104	
	Oestradiol-3:17β	3·0	5	0·612	58·9 ± 11·92
		1·0	5	0·622	
		0·33	5	0·304	
		0·11	5	0·189	
		0·037	5	0·191	
		0·012	5	0·133	
4	Oestrone	3·0	6	0·893	57·4 ± 7·46
		1·0	6	0·834	
		0·33	6	0·288	
		0·11	6	0·178	

(*f*) *Relative potency of certain oestrogens* (*Table* 7)

The data of Expt. 3 (Table 5) were analysed as a six-point assay with doses of 10×10^{-4} μg, $0{\cdot}33 \times 10^{-3}$ μg. The relative potency of oestradiol-3:17β in terms of oestrone was 1·4 {0·79 – 2·48; $P = 0{\cdot}05$}. The relative potency of diethylstilboestrol in terms of oestrone was 2·8{2·57 – 3·05; $P = 0{\cdot}05$} (Table 7). The estimate for oestradiol-3:17β agrees with previous estimates obtained using mitosis, epithelial thickness [Martin & Claringbold, 1960] and vaginal cornification [Biggers & Claringbold, 1954]. The slope of oestradiol-3:17β, although lower than that for oestrone, was not significantly so. Similarly, diethylstilboestrol and oestrone did not differ in slope. The estimate of potency of diethylstilboestrol was significantly higher than estimates obtained previously. This particular experiment was unusual in its low variance (see Table 6). No explanation can be given regarding either phenomenon.

(g) Specificity

In contradistinction to the three oestrogens tested, progesterone, testosterone and cortisol (Table 8) did not increase tetrazolium reduction at dose levels 1000 times that of oestrone, a not unexpected finding in view of their lack of activity in the mitosis and epithelial thickness assays [Martin & Claringbold, 1960].

Table 6. *Secular variation in slope and variance*

(Slopes and variances were calculated from transformed data.)

Expt.	b	s_b	Variance
1	57·9	± 4·0	314
2	45·1	± 11·0	364
3	64·5	± 7·7	190
4	78·0	± 8·8	288
5	55·2	± 7·4	384
6	—	—	379
7	47·9	± 5·9	602
8	49·1	± 7·9	284
9	74·4	± 11·9	325
10	57·4	± 7·5	380
11	45·9	± 6·1	90

$\chi^2_9 = 15{\cdot}258$ $(0{\cdot}05 < P < 0{\cdot}1)$. $\chi^2_{10} = 24{\cdot}96$ $(0{\cdot}001 < P < 0{\cdot}01)$.

Significant heterogeneity of variance is dependent primarily on the unusually low variance for Expt. 11. If this is omitted in Bartlett's test, the data form a homogeneous group $\chi^2_9 = 12{\cdot}7$ $(0{\cdot}1 < P < 0{\cdot}2)$: $\bar{b} = 55{\cdot}7$; $\bar{v} = 336{\cdot}3$.

Table 7. *Relative potency of oestrone and diethylstilboestrol*

(Diethylstilboestrol:oestrone; six animals per group.)

	Dose ($\mu g \times 10^{-4}$)	Mean O.D.	b
Oestrone	1·0	0·343	45·75
	0·125	0·131	
Diethylstilboestrol	1·19	0·600	46·12
	0·149	0·231	

Table 8. *Effects of testosterone, progesterone and cortisol on tetrazolium reduction in vaginal epithelium of the mouse*

(No. of animals per group (6) is constant.)

Compound	Dose (μg)	Mean O.D.
—	—	0·093
Testosterone	1×10^{-1}	0·106
	1×10^{-2}	0·076
Cortisol	1×10^{-1}	0·080
	1×10^{-2}	0·097
Progesterone	1×10^{-1}	0·068
	1×10^{-2}	0·093

DISCUSSION

The reduction of tetrazolium by the vaginal epithelium 24 hr after administration of oestrogens parallels cell growth and division in most respects, and may be regarded as an index of such changes. At this time the method is comparable in sensitivity

and accuracy to assays involving mitosis and epithelial thickness, with the added advantage of extreme simplicity.

Although tetrazolium reduction is increased only 6 hr after the injection of oestrogens, thus preceding morphological responses by at least 12 hr, the slope of the dose-response line at that time is so low as to exclude the possibility of using such an early response in the routine assay of oestrogens. It does, however, indicate that extensive biochemical reorganization is taking place in the epithelium at that time, and opens up the possibility of using other earlier metabolic responses in such assays. As was discussed by Martin & Claringbold [1960], it might be expected that the earlier the response the more information will be gained regarding the mode of action of oestrogens.

The recent work of Mueller *et al.* [1958] and Kalman & Lowenstein [1958] on early responses in the uterus, suggests that the intravaginal application of radio-isotopes may be important in the development of such short-term assays.

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REFERENCES

Balmain, J. H., Biggers, J. D. & Claringbold, P. J. [1956]. *Aust. J. Biol. Sci.* **9**, 147.
Biggers, J. D. [1953]. *J. Anat. Lond.*, **87**, 327.
Biggers, J. D. & Claringbold, P. J. [1954]. *Aust. J. Biol. Sci.* **7**, 118.
Biggers J. D. & Claringbold, P. J. [1955]. *J. Anat. Lond.*, **89**, 124.
Claringbold, P. J. [1958]. *J. roy. Stat. Soc.* B, **20**.
Doerr, W. [1950]. *Frankfurt. Z. Path.* **61**, 557.
Emmens, C. W. [1950]. *Hormone assay*, p. 391. New York: Academic Press.
Farber, E., Sternberg, W. H. & Dunlap, C. E. [1954]. *Amer. J. Path.* **30**, 616.
Glick, D. & Nayyar, S. N. [1956]. *J. Histochem. Cytochem.* **4**, 389.
Jardetsky, C. D. & Glick, D. [1956]. *J. biol. Chem.* **218**, 283.
Jeener, R. [1948]. *Biochim. Biophys. Acta*, **2**, 439.
Kalman, S. M. & Lowenstein, J. M. [1958]. *J. Pharmacol.* **122**, 163.
Kamell, S. A. & Atkinson, W. B. [1948]. *Proc. Soc. exp. Biol., N.Y.*, **68**, 537.
Lakon, D. T. [1939]. *Ber. Dtsch. bot. Ges.* **57**, 191.
Lakon, D. T. [1942]. *Ber. Dtsch. bot. Ges.* **60**, 299.
Martin, L. & Claringbold, P. J. [1958]. *Nature, Lond.*, **181**, 620.
Martin, L. & Claringbold, P. J. [1960]. *J. Endocrin.* **20**, 173.
Mueller, G. G., Herranen, A. M. & Jervell, K. F. [1958]. *Recent Progr. Hormone Res.* **14**, 95.
Rao, C. R. [1952]. *Advanced statistical methods in biometric research.* New York: Wiley.
Ring, J. R. [1950]. *Anat. Rec.* **107**, 121.
Rosa, C. G. & Velardo, J. T. [1954]. *Anat. Rec.* **120**, 771.