THE MECHANISM OF *IN VIVO* VAGINAL TTC REDUCTION STIMULATED BY ESTROGEN IN SPAYED MICE

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It is well known that tetrazolium salts, because of their low redox potential, are sensitive reagents for the reducing enzyme system. The ability of various tissues to reduce tetrazolium salts has been utilized as a parameter for the estimation of metabolic activity of the tissues responding to steroid hormones (Martin, 1960; Rosenkrantz *et al.*, 1961; Schultze, 1964). Among these, it should be noticed that *in vivo* reduction of 2,3,5-triphenyltetrazolium chloride (TTC) in the vagina of ovariectomized mice, as described by Martin (1960), is a highly sensitive and specific index for the action of estrogen applied not only locally (Martin, 1960) but also systemically (Hori, 1964a), and thus considered as a useful tool for biochemical approach to an earlier reaction of the tissue to estrogen or anti-estrogen.

However, it is still uncertain as to whether the increase of vaginal TTC reduction reflects any physiological or biochemical changes taking place in the vagina after estrogen stimulation. After our previous short communication (Hori and Miyake, 1964), further study was undertaken in order to clarify the mechanism of *in vivo* vaginal TTC reduction stimulated by estrogen and to discuss its biological significance in relation to the action of estrogens or anti-estrogens.

MATERIALS AND METHODS

Animals

Female albino mice of our ddS colony were used through all the experiments. Vaginal smears of intact mice about 40 days of age were examined daily for more than 5 days. Immature mice were used at about 23 days of age. In the experiments on spayed mice, the mice ovariectomized at 30 to 32 days of age were used 10 days after the castration, since the mice under this condition were the most sensitive to estrogen as previously reported (Hori, 1964a).

Estrogen administration

Estradiol-17 β dissolved in sesame oil was administered subcutaneously at the dosage of 0.03 μ g per mouse in a volume of 0.1 ml into the immature mice or the spayed mice 10 days after the castration. Except in the cases noticed, animals were sacrificed 27 hrs. after the injection and vaginae were dissected for the measurements described below. The mice receiving the same volume of vehicle alone were used as the control.

TTC reduction in vivo

Animals receiving an intravaginal micro-injection of TTC 1 mg dissolved in a volume of

0.02 ml of distilled water were sacrificed exactly 30 mins. after the TTC injection. The vaginae were immediately dissected, quickly rinsed with cold water and placed in 10% formalin in order to stop the reaction. The time needed from autopsy to stopping the vaginal TTC reduction is a critical factor as described in the next chapter, so that much attention was paid to perform the above procedure within 30 secs. Formazan produced in the vagina was extracted with 1.5 ml of 3:1 ethanol-tetrachlorethylene and measured at 490 $m\mu$ with a Coleman spectrophotometer Junior 6C. The reading for each tube was converted to μg of formazan by an empirical constant obtained from a standard curve of formazan.

Oxygen uptake

For the determination of oxygen uptake of vaginal tissue, each of the dissected vaginae was put into a Warburg flask containing 2.0 ml of Krebs-Ringer phosphate buffer (pH 7.4). KOH was center well and the gas phase was air. After 5 mins. of preincubation, the oxygen consumption was determined at 37°C for 1 hr. Then each tissue was dried overnight at 90 to 100°C in the vacuum drying oven.

TTC reduction in vaginal homogenate

Vaginal tissues of a group of ten animals were pooled and homogenized in 4.0 ml of 0.1 M phosphate buffer (pH 7.4) in a glass homogenizer after being ground in a mortar. The homogenate was put into two Thumberg tubes, each of which contained 1 ml of 0.5% TTC solution in the side arm. One was heated at 70°C for 10 mins., and the other was not. Both tubes were incubated for 60 mins. at 37°C under anaerobic conditions. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid, and the formazan produced was extracted with 5 ml of ethyl acetate and estimated colorimetrically. In the determination of reduction rate of TTC using NADH as hydrogen-donor, the crude homogenate in 10 ml of 0.04 M phosphate buffer (pH 8.2) was prepared from pooled vaginal tissues of 20 mice, and centrifuged at 2000 r.p.m. for 5 mins. The supernatant was bubbled with oxygen at 30°C for 30 mins. in order to reduce the endogeneous activity. An aliquot of the homogenate, one-tenth of the original volume, was put into a Thumberg tube, the side arm of which contained 0.5 ml of 0.04 M phosphate buffer with 5×10^{-4} mole of NADH and 7.5×10^{-4} mole of TTC. A mixture of the homogenate, NADH and TTC in a total volume of 1.5 ml was incubated at 35°C under anaerobic condition. Amount of proteins in the homogenate were measured by the method of Lowry *et al.* (1951).

TTC absorption

The vagina isolated from the mice receiving TTC 30 mins. prior to autopsy was rinsed in cold water and ground in a mortar with 0.2 ml of 0.05% sodium hydrosulfite in 1% sodium bicarbonate solution. Total amount of formazan produced was extracted with 3 ml of ethyl acetate and estimated colorimetrically.

TTC reduction in vitro

The dissected vagina was incubated in the test tube containing 0.5% TTC in 4 ml of Krebs-Ringer phosphate buffer at 37°C for 30 mins. under air. The reaction was stopped by the addition of trichloroacetic acid and the amount of formazan produced was determined.

Histological examination

The isolated vaginae of a group of 5 to 10 mice were fixed in 10% formalin, and embedded in paraffin, sectioned at 5 μ in thickness, and stained by hematoxylin-eosin. In histochemical observations of vaginal TTC reduction, much attention was paid to isolate the vaginae exactly 30 mins. after the TTC injection, and to stop the reaction by putting the tissue into cold 10% formalin solution within 30 secs. Frozen sections of the vaginae were prepared in the cryostat at -15°C and embedded in Apathy solution.

RESULTS

1) *Vaginal TTC reduction in vivo of intact, immature and spayed mice*

One mg of TTC was administered intravaginally to intact adult, intact immature, and spayed adult mice, receiving estrogen or vehicle alone, and the amount of formazan produced in the vagina for 30 mins. was determined. The relation of vaginal TTC reducing activity *in vivo* of the intact adult mice to their estrus cycles is presented in the upper part of Table 1, showing that formazan formation stays relatively low during the stage of diestrus to proestrus, and it drastically falls at the stage of estrus. The data indicate that almost no detectable activity of vaginal TTC reduction is obtained at the stage of estrus and no significant increase takes place at the stage of proestrus to estrus in spite of the elevation of uterine weight, which may be resulted from the increase of endogenous estrogen secretion.

The data on intact immature and spayed mice treated with estrogen are shown in the middle and lower part of Table 1. Both immature and spayed mice receiving the vehicle alone produced a minute quantity of formazan, the amount of which is somewhat less than that of intact adult mice in the diestrus stage. When the mice are treated with a single injection of $0.03 \mu\text{g}$ estradiol-17 β , a marked increase of formazan production, the grade of which is about ten fold over the control level, is obtained either in intact immature or in spayed adult mice. In the simultaneous measurement of uterine weight, however, only a two fold increase is produced by the estrogen administration. On the contrary, a

Table 1. Vaginal TTC reducing activity *in vivo* of intact, immature and spayed mice with and without estrogen treatment

Treatment	No. of mice	Uterine weight (mg)	<i>In vivo</i> formazan formation ($\mu\text{g}/30 \text{ mins./vagina}$)
Adult mice a)			
Diestrus	7	$22.5 \pm 2.3^*$	$1.8 \pm 0.3^*$
Proestrus	6	18.2 ± 4.1	2.5 ± 0.3
Estrus	7	55.9 ± 10.6	0.6 ± 0.2
Metestrus	7	34.4 ± 2.9	3.7 ± 0.5
Immature mice b)			
Sesame oil	10	7.2 ± 0.9	1.5 ± 0.6
Estradiol-17 β $0.03 \mu\text{g}$	9	14.0 ± 1.3	10.4 ± 1.6
Spayed mice c)			
Sesame oil	10	11.4 ± 0.8	1.2 ± 0.2
Estradiol-17 β $0.03 \mu\text{g}$	10	23.1 ± 2.0	10.1 ± 0.8
Estradiol-17 β $0.03 \mu\text{g}$ daily for 3 days	10	46.1 ± 2.0	0.2 ± 0.1

* Mean $\pm$ S.E.

a) Around 40 days of age

b) Around 23 days of age

c) Ovariectomized at 32 days of age

multiple injection of estradiol-17 β , 0.03 μ g a day for 3 days, rather causes a decrease of formazan production in the vagina, whereas a four fold increase is obtained in the uterine weight.

2) *Oxygen uptake of the isolated vagina of spayed mice treated with estradiol-17 β*

Endogeneous oxygen uptake of vagina isolated from spayed mice with and without estrogen stimulation is presented in Table 2. The data indicate that either single or multiple injections of 0.03 μ g of estradiol-17 β causes apparent increase of oxygen uptake over the control of ovariectomy alone in terms of μ l oxygen per whole vagina per hr. but this is not true in respect of Q_{O_2} , that is the oxygen consumption per hr. per mg of dry vaginal tissue.

Table 2. Oxygen uptake of the vaginal tissue in vitro

Vagina obtained after	No. of mice	Tissue weight (mg)		Oxygen uptake	
		wet	dry	μ l/vagina/hr.	Q_{O_2}
Ovariectomy	12	16.0 $\pm$ 0.8*	2.9 $\pm$ 0.0*	12.0 $\pm$ 1.0*	4.26 $\pm$ 0.92*
Ovariectomy+estradiol-17 β 0.03 μ g a)	12	25.9 $\pm$ 1.6	4.0 $\pm$ 0.1	20.5 $\pm$ 1.1	5.19 $\pm$ 0.27
Ovariectomy+estradiol-17 β 0.03 μ g $\times$ 3 b)	9	39.3 $\pm$ 2.0	8.2 $\pm$ 0.5	31.2 $\pm$ 1.2	3.86 $\pm$ 0.02

The dissected vaginae were incubated at 37°C for 60 mins. in Warburg flask containing 2.0 ml of Krebs-Ringer phosphate buffer.

* Mean $\pm$ S.E.

a) Single subcutaneous injection

b) Multiple injection, once daily for 3 days

3) *Endogeneous reduction of TTC by the vaginal homogenate*

Pooled vaginal tissues of either group of ovariectomized mice receiving oil or estrogen are homogenized with phosphate buffer (pH 7.4) and incubated with

Table 3. In vitro TTC reduction by vaginal tissue homogenate of spayed mice treated with estradiol-17 β

Vagina obtained after	Average wet weight of vaginal tissue (mg)	Formazan formed	
		μ g/animal	μ g/mg tissue
Ovariectomy	22.0	13.7	0.62
Ovariectomy+estradiol-17 β 0.03 μ g a)	33.2	19.4	0.58
Ovariectomy+estradiol-17 β 0.03 μ g $\times$ 3 b)	50.6	28.9	0.57

Ten individual vaginal tissue of a group of animals were homogenized together in 4 ml of phosphate buffer (pH 7.4). The homogenate was divided into two Thunberg tubes which contained 1 ml of 0.5% TTC solution in the side arm. Each tube was incubated for 60 mins. at 37°C under anaerobic condition.

a) Single subcutaneous injection

b) Multiple injection, once daily for 3 days

TTC at 37°C for 60 mins. under anaerobic condition.

The results are presented in Table 3. Endogeneous reduction of TTC, is increased 1.4 fold by the single injection of 0.03 μ g estradiol-17 β and 2.1 fold by the multiple injection once daily for 3 days, when the activity is represented as the amount of formazan produced per vagina per 60 mins. However, there is no significant difference between the ovariectomized control and the estrogen treated group in terms of formazan production per mg of wet tissue. These results suggest that estrogen does not increase endogeneous substrate which is able to couple with the reduction of tetrazolium salt in whole vaginal tissue. In addition, the formazan production in vaginal tissue is completely destroyed by heating at 70°C for 10 mins., suggesting that the reduction of TTC in these preparations is an enzymatic reaction.

4) *Reduction rate of TTC in the vaginal homogenate added with NADH as the substrate*

Using NADH as the hydrogen donor and TTC as the hydrogen acceptor, the rate of TTC reduction in vaginal homogenate is determined in order to clarify

Fig. 1. Rate of TTC reduction in the vaginal homogenate, added NADH, of spayed mice treated with and without estradiol-17 β

A pooled vaginal tissue of 20 mice was homogenized with 10 ml of phosphate buffer. After preincubation, an aliquot of homogenate, one-tenth the volume of original, was incubated under anaerobic condition at 35°C. Incubation mixture: 5×10^{-4} mole of NADH and 7.5×10^{-4} mole of TTC in phosphate buffer. Total volume: 1.5 ml

whether or not estrogen causes any change of enzyme activity related to reduction of TTC.

Twenty-seven hrs. after the injection of oil or estradiol-17 β , pooled vaginal tissues of a group of 20 mice were homogenized with 10 ml of phosphate buffer (pH 8.2) and centrifuged. In order to diminish the endogeneous activity, the supernatant was preincubated with oxygen bubbling at 30°C for 30 mins. Under the conditions described, the preparation shows a negligible amount of formazan production when NADH is omitted or NAD is added instead of the NADH.

When TTC and NADH are added, the formazan production goes up linearly in the time course of 15 to 60 mins., as demonstrated in Figure 1. Calculating from the data of Figure 1, the TTC reducing activity, represented by the formazan production μ g per hr. per mouse, is 17.0 for the ovariectomized control and 31.9 for the estradiol treated group. However, the specific activity represented by the formazan production equivalent to mg protein is 30.9 μ g per hr. for the control and 34.9 μ g per hr. for the estradiol treated group. Therefore, estradiol causes only a 1.9 fold increase in the total activity, while it does not produce any increase at all in the specific activity.

5) *Effect of estrogen on TTC absorption into the vaginal tissue*

It is possible to consider that the increase of TTC reduction caused by estrogen results from the augmentation of TTC-penetration through the vaginal wall. Therefore, the amount of TTC absorbed into the vaginal tissue within 30 mins. is measured after the dissection of vagina followed by the addition of hydrosulfite, a reducing agent. As shown in Table 4, the mean value of hydrosulfite-reducible TTC is only 1.3 μ g per vagina in spayed mice receiving vehicle alone. This correlates well with the data in Table 1 showing that the formazan production of spayed mice receiving sesame oil is equal to 1.2 μ g per 30 mins. per vagina. A single injection of estradiol causes ten fold increase in TTC content, whereas the multiple injection of estradiol for 3 days rather causes a decrease of TTC absorption. This suggests that permeability of the vaginal wall to TTC may be stimulated by a single dose of estrogen, and it may be prevented by the multiple treatment of estrogen causing vaginal cornification. However, other evidence

Table 4. Amount of TTC absorbed into the vaginal tissue of spayed mice treated with estradiol-17 β

Estradiol-17 β treatment	No. of mice	Amount of TTC absorbed into the vagina (μ g/animal) ^{c)}
None	10	1.3 $\pm$ 0.2*
0.03 μ g ^{a)}	10	12.6 $\pm$ 1.2
0.03 μ g $\times$ 3 ^{b)}	10	0.7 $\pm$ 0.2

* Mean $\pm$ S.E.

^{a)} Single injection

^{b)} Multiple injection, once daily for 3 days

^{c)} Represented by the amount of formazan

described below denies the possibility that the permeability of vaginal wall is a factor influencing *in vivo* TTC reduction.

6) *Vaginal TTC reduction in vitro of spayed mice treated with estradiol-17 β*

The dissected vaginae obtained from the mice receiving oil or estrogen were incubated with TTC, and the *in vitro* TTC reducing activity was estimated. As shown in Table 5, intense activity of formazan production is observed even in non-stimulated mice and only a slight increase is obtained by the estrogen treatment. The differences among the three groups, however, are insignificant when the activity is represented by the equivalent per mg wet tissue.

Table 5. *In vitro* TTC reducing activity of the vagina isolated from spayed mice treated with estradiol-17 β

Vagina obtained after	No. of mice	Vaginal weight (mg)	Formazan produced (μ g/vagina/30 mins.)
Ovariectomy	10	20.4 $\pm$ 2.3*	19.4 $\pm$ 2.0*
Ovariectomy+estradiol-17 β 0.03 μ g a)	10	29.9 $\pm$ 2.0	27.3 $\pm$ 4.1
Ovariectomy+estradiol-17 β 0.03 μ g $\times$ 3 b)	10	41.6 $\pm$ 3.1	32.5 $\pm$ 1.8

* Mean $\pm$ S.E.

a) Single subcutaneous injection

b) Multiple injection, once daily for 3 days

7) *TTC reduction in the vagina of dead mice*

Spayed mice treated with estradiol-17 β and untreated controls were sacrificed immediately after the intravaginal injection of TTC, and the carcasses were incubated for 30 mins. at 37°C. The vaginal tissues were then isolated and the amount of formazan produced in the tissue was determined. The results are illustrated in the middle of Figure 2, showing that the amount of formazan produced in the postmortal vagina is equal to 18.4 μ g per animal for the spayed control, 29.7 μ g for the group treated with a single injection of estradiol, and 1.1 μ g per mouse for the group receiving the 3-day treatment of estrogen. In comparison with the above results, the data on the living spayed mice and on the *in vitro* experiment of vaginal TTC reduction are transferred into Figure 2 from Tables 1 and 5, respectively. It is noted that there are great differences between the data on living animals and those on dead animals in spite of the same route of TTC application. Namely, the estrogen-dependent activity is demonstrated very clearly in the vagina of living mice while it is diluted by the estrogen-independent activity proceeding in the vagina of dead animals as well as in the isolated vagina. The authors are interested in the fact that vagina of the spayed mouse has almost no activity to reduce TTC *in vivo* without estrogen stimulation, whereas it is able to reduce a considerable amount of TTC after death. The pattern of formazan production progressing after the intravaginal injection of TTC is further investigated either on the living mice or on the dead mice with

Fig. 2. Comparison of formazan production in the vagina of living mice and carcasses, and isolated vagina

Fig. 3. Pattern of in vivo TTC reduction in the vagina of spayed mice treated with estradiol-17β

Each plot represents a mean value in the group consisting of 9 mice, respectively.

Fig. 4. Pattern of postmortal TTC reduction in the vagina of spayed mice treated with estradiol-17 β

Each plot represents a mean value in the group consisting of 10 mice, respectively.

and without estradiol treatment as described previously. The results are presented in Figures 3 and 4. The rate of formazan production in the estrogen-stimulated vagina of living mice goes up immediately after the injection of TTC and reaches a plateau within 20 mins., whereas extremely low level of the formazan production and no significant increase of it is demonstrated in the non-estrogen-stimulated vagina (Fig. 3). In contrast with the data on living mice, the rate of vaginal TTC reduction in the carcasses increases linearly according to the time during the period of investigation either in the estrogen-stimulated or in the non-stimulated vagina, and the amount of formazan produced in a period of more than 20 mins. after the TTC injection are much greater than those in the living mice, resulting in an inconspicuous effect of estradiol on the vaginal TTC reduction (Fig. 4).

These results that TTC may be rapidly decomposed to a non-reducible form when absorbed once into the vaginal tissue having intact blood circulation. It is also substantiated that practically no occurrence of *in vivo* TTC reduction in the vagina of spayed mice receiving no estrogen is not due to the lack of TTC penetration into the vagina, and that the penetration of TTC is not a factor regulating the *in vivo* TTC reduction of the vagina even in the estrogen-stimulated spayed mice. It is demonstrated, however, that the well cornified vagina caused

by the multiple injections of estrogen shows the most potent activity of TTC reduction *in vitro*, but hardly any activity is obtained when TTC was applied intravaginally either to living mice or to postmortal mice. It is concluded, therefore, that cornification of vaginal epithelium markedly prevents the penetration of TTC into the tissue.

8) *Site of reduction of TTC*

Histochemical observations were carried out in order to clarify the site of TTC reduction of vaginal tissue. Thirty mins. after intravaginal injection of TTC, the mice were killed, and vaginae were immediately dissected and put into 10% cold formalin solution in order to stop the reaction. The time required for attaining to this step was limited to less than 30 secs. in every experiment. Under these conditions, almost no detectable activity is manifested in the frozen section of non-stimulated vagina in spayed mice (Fig. 5). In a period of 27 hrs. after the single injection of 0.03 μ g of estradiol, a marked increase of formazan production in the vagina is demonstrated by rich distribution of the formazan particles, which are located only on the layer of superficial cells (Fig. 6). It is noticed that a sharp decline of TTC reducing activity is evident in the underlying layers, stratum granulosum and stratum spinosum, and it is quite absent in the stratum basale.

In contrast with the picture on living mice, the vaginae obtained from the mice incubated at 37°C for 30 mins. after sacrifice following TTC injection have a heavy formazan deposit not only in the superficial cells but also in the basal cells, as shown in Figure 7.

From these observations, it is concluded that *in vivo* TTC reduction occurs only in superficial cells, and the increase of *in vivo* TTC reduction in the vagina caused by estrogen administration does not reflect the metabolic change in the whole epithelial tissue but reflects some biochemical change of superficial cells.

Subsequently, the histology of the vaginal wall of spayed mice before and after the estradiol treatment was investigated by ordinary hematoxylin-eosin staining. The histology of non-estrogen-stimulated vagina of spayed mice shows atrophied epithelium which consists of a single layer of superficial cells and a non-differentiated basal cell layer as shown in Figure 8. At a period of 12 hrs. after the single injection of 0.03 μ g of estradiol, hypertrophy of basal cells without proliferation in epithelial tissue occurs, and no significant change takes place in superficial cells (Fig. 9). At a period of 18 hrs. after the injection, remarkable changes take place at the epithelial tissue; namely, thickened epithelium accompanied with proliferated basal cells is observed, and the superficial cells are clearly distinguished from the underlying cell layers. At a period of 24 to 30 hrs. after the injection, superficial cells show slight swelling and partial vacuolization and stand out in bold relief against the underlying layer of stratum granulosum (Fig. 10). At a period of 40 hrs. after the injection, most of the superficial cells are desquamated from the epithelium (Fig. 11).

It was reported (Hori, 1964a) that *in vivo* TTC reduction in the vagina increases at least 18 hrs. after the subcutaneous injection of estrogen and the reaction reaches the maximum in a period of 27 to 30 hrs. after the estrogen admin-

Plate 1

Fig. 5. A frozen section of vagina obtained from the ovariectomized mice, showing almost no deposit of formazan in epithelial cells

TTC was injected intravaginally 30 mins. prior to autopsy. $\times 100$

Fig. 6. A frozen section of vagina obtained from the ovariectomized and estradiol-17 β treated mice, showing a heavy formazan deposit in superficial cells and a sharp decline of formazan deposit in the intermediate cell layers

Intavaginal TTC injection 30 mins. prior to autopsy was made 27 hrs. after a single subcutaneous injection of 0.03 μ g of estradiol-17 β . $\times 100$

Plate 2

Fig. 7. A frozen section of vagina obtained from the ovariectomized and estradiol- 17β treated mice which were incubated at 37°C for 30 mins. immediately after sacrifice

A heavy formazan deposit is observed not only in the superficial cells but also in the basal cell layer. TTC was injected intravaginally just before sacrifice.

$\times 100$

Fig. 8. A section of vagina obtained from the ovariectomized mice, showing atrophy of epithelium which consist of single layer of superficial cells and non-differentiated basal cells (hematoxylin-eosin staining)

$\times 100$

Plate 3

Fig. 9. A section of vagina obtained from the ovariectomized mice at 12 hrs. after administration of estradiol- 17β showing hypertrophy of basal cell layer without proliferation (hematoxylin-eosin staining) $\times 100$

Fig. 10. A section of vagina obtained from the ovariectomized mice at 27 hrs. after administration of estradiol- 17β showing typical differentiated structure associated with proliferation of basal cells

Superficial cells show slight swelling and partial vacuolization (hematoxylin-eosin staining). $\times 100$

Plate 4

Fig. 11. A section of vagina obtained from the ovariectomized mice at 40 hrs. after administration of estradiol-17 β

Superficial cells begin falling off (hematoxylin-eosin staining). $\times 100$

Fig. 12. A frozen section of vagina obtained from the ovariectomized mice receiving 0.03 μ g estradiol-17 β once daily for 3 days showing hardly any formazan deposition

TTC was injected intravaginally 30 mins. prior to autopsy. $\times 100$

istration, from when it gradually decreases thereafter according to the progress of cornification. This was confirmed by histochemical observations of vaginal TTC reduction of spayed mice which were made every 6 hrs. after the estrogen injection. Namely, formazan deposit is seen only in superficial cells of the vaginal epithelial tissue at least 18 hrs. after the estrogen injection. Weak activity is occasionally found in the outer side of stratum granulosum after desquamation of the superficial cells. However, hardly any formazan deposition is shown in the well cornified vagina obtained from the mice receiving estrogen for 3 consecutive days (Fig. 12).

DISCUSSION

The present experiments demonstrated that the basal cells of vagina showed hardly any activity to reduce TTC administered locally when the mice were kept alive, but a remarkable activity of TTC reduction in the vaginal basal cells was shown when the mice were sacrificed immediately after TTC injection and kept at 37°C for a while. These results together with the fact that *in vitro* tetrazolium reduction is depressed with oxygen (Kun and Abood, 1949) suggest that the oxygen tension in the tissue is a critical factor to influence the vaginal TTC reduction *in vivo*. It was further demonstrated that the increased TTC reduction *in vivo* caused by estrogen reflects neither the change of permeability of vaginal wall nor the metabolic change of whole vaginal tissue but some biochemical change in superficial cells at the stage of development of stratum granulosum. It is most reasonable to conclude, therefore, that the increased TTC reduction in the superficial cells may result from the anaerobic condition induced by the differentiation of epithelial tissue, because a close correlation is obtained between the development of stratum granulosum and the TTC reduction in superficial cells. As previously reported (Martin, 1960; Hori, 1964b), vaginal TTC reduction is a specific and reliable parameter of estrogen bioassay, and a number of anti-estrogenic compounds interfere with this reaction when administered simultaneously with estrogen. In addition, it was also found that compounds which inhibit estrogen-stimulated vaginal TTC reduction produce a simultaneous inhibitory effect on the development of stratum granulosum stimulated by estrogen (Hori, unpublished). It is considered, therefore, that the *in vivo* TTC reduction in vagina may be used as a parameter showing the differentiation of epithelium occurring as an earlier transient phenomenon in the process of vaginal cornification, explaining the biological significance of the vaginal tetrazolium test standardized as a bioassay method for estrogens and anti-estrogens (Hori, 1964a and b).

It should be noticed, however, that the present findings are somewhat different from the original results of Martin (1960), who indicated that the whole epithelial tissue of the vagina participates in the TTC reduction. Martin (1964) recently reported in detail the technique of tetrazolium test for local assay of estrogen, and pointed out many factors influencing sensitivity and reliability of the test. It is not yet known, however, whether the difference between the authors and Martin (1960) in the conclusion for the localization of vaginal TTC reduction is merely due to the difference in the strain of mice used.

A few questions which may be raised from the present investigations as to whether superficial cells play any physiological role on the vaginal response to estrogen and whether the cells directly respond to estrogen must await further experimentation.

SUMMARY

It was demonstrated that neither the change of TTC penetration through the vaginal epithelium nor the metabolic changes of whole vaginal tissue participated in the estrogen-stimulation of vaginal TTC reduction, and that the vaginal TTC reduction *in vivo* was not a reaction occurred in the whole vaginal epithelium but a reaction localized in the superficial cells affected by estrogen administration, suggesting that the vaginal TTC reduction *in vivo* increased by estrogen reflects some biochemical changes in superficial cells. It was further demonstrated that the vaginal TTC reduction was histochemically localized in the superficial cells only when the reaction occurred in the vagina having intact blood circulation, whereas it was spread over the whole epithelium including basal cells when the mice were killed and the reaction occurred in the vagina without blood supply. Furthermore, a close correlation was obtained between the TTC reduction and the development of stratum granulosum in the vagina.

It is concluded, therefore, that the estrogen-stimulated TTC reduction in vagina is an earlier transient phenomenon in the process of vaginal cornification, in which the superficial cells are brought into the anaerobic condition resulting from the rapid differentiation of epithelium.

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