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A STUDY OF THE IN VITRO INFLUENCE OF STEROIDS ON UTERINE ENZYME ACTIVITY IN RATS

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CHAPTER I

INTRODUCTION

Knauer, Hunter, Berthold, Brown-Séquard and others made investigations in the field of endocrinology during the late nineteenth and early twentieth century. Their studies indicated that the ovaries and the testes of mammals or birds were both capable of secreting hormones. Later, in 1929, Doisy and Butenandt isolated a crystalline estrogenic hormone from the blood and urine of pregnant animals. The progestational hormone was isolated from corpora lutea in 1934 by four independent groups of investigators. By administering the crystalline substances to animals lacking them, the early workers were able to study the outward effect of the hormones on the sexual and somatic development of the animals. Later, steroid hormones were found to have diverse effects on the metabolic actions, the skeletal system, and the retention of electrolytes and water in mammals.

Many workers wondered if there were some relationship between hormones and enzymes in these physiological actions and investigations were soon made into this matter. In the past few years

there have been volumes of papers and reports concerning the effect of various hormones on enzyme systems in vivo. In most instances the steroid sex hormones (androgens, estrogens and progestational) often have an activating effect on many enzyme systems studied in vivo in heart, liver, kidney and genital tissue. It has been shown that progesterone, when administered to castrated females, caused an increase in serum histaminase activity (Mueller, 1953), liver ATP-ase activity (Jones and Wade, 1953), and uterine lactic dehydrogenase activity (Bever et al., 1956). Estrogenic hormones have been shown to enhance the activity of uterine succinic dehydrogenase and cytochrome oxidase (Telfer, 1953), alkaline phosphatase (Atkinson and Elftman, 1947), lactic dehydrogenase (Bever et al., 1956), and β-glucuronidase (Fishman, 1944). If androgens are administered to castrated female rats, the enzyme systems of genital organs are activated as if an estrogen had been given (Knobil, 1952). This phenomenon is more understandable when one considers the work of Nathason and West (See Engel, 1957), which demonstrated a conversion of testosterone to an estrogen in human subjects. Adrenalectomized and castrated females made this conversion, showing that these particular endocrine organs are not concerned with the biosynthesis of estrogen from testosterone.

In view of the influence of steroid hormones on enzyme systems in vivo, many workers turned their attention to studying the effect of various steroid hormones on enzymes associated with the reproductive organs of both the male and female rat. One of the earliest observations of an effect of estrogenic hormones on the rat uterus in vivo was the large increase in uterine water content following the

administration of estrogenic hormones (Astwood, 1938). The water imbibition appeared to reach its maximum about six hours after the injection of estrogen. This phenomenon may have been the consequence of an estrogenic stimulation of an enzyme system.

There are also numerous instances in which hormones are mutually antagonistic to one another when administered simultaneously to an animal. The actual relationship between hormones and enzymes is still rather obscure and there has been no definite evidence that any enzyme is totally dependent on any hormone, steroid or other.

We know of more than half a dozen estrogenically active steroids which have been isolated from biological sources - mostly from domestic farm animals and human beings. As examples of this group, estradiol and estrone have been isolated from equine, bovine, porcine and human species, estriol only from higher primates, and equilin and equilinen from pregnant mare's urine (Pincus and Thiman, 1955). Each of these hormones is capable of influencing the activity of uterine enzyme systems in different hosts, even when the estrogen administered is not normally secreted by that host. This would indicate that tissues capable of being affected by estrogens apparently do not have a high degree of specificity for a particular estrogen. Many synthetic estrogens do not resemble natural estrogens in their chemical structure, but yet are capable of exerting effects in vivo which are identical to those produced by normal estrogens. These facts cause us to speculate on the mechanism of action between estrogenic compounds and the enzyme systems influenced by them.

The form in which estrogenic hormones are presented to

target organs is perhaps as baffling a problem as their mechanism of action. There is still a controversy as to whether the steroids are presented to their receptor sites in a free form or in a conjugated form (Szego and Roberts, 1946; Roberts and Szego, 1953). There is also the problem of whether the steroid is utilized *in toto* by the target organ or if the steroid might be first activated by the liver or the target organ to produce a more active steroid (Twombly and Taylor, 1942; Ryan and Engel, 1953). Sandberg and Slaunwhite (1951) have shown through *in vivo* and *in vitro* studies that in human beings the estrogens appear to be bound to the albumin portion of serum proteins (Cohn Fraction V), but still it is not known if the estrogen is presented to target cells in the free form or conjugated as a sulfate or glucuronide.

Another question is whether the hormone acts as a coenzyme to an enzyme system or if the hormone actually stimulates the formation of new enzyme molecules. If the latter is correct, by what mechanism does it act? Does it act through a direct stimulation of nucleic acid synthesis which might allow more enzyme protein to be formed, or through the activation of preformed enzyme precursors? These are a few of the questions confronting the biochemist concerning the mechanism of hormone action. An attempt will be made in this dissertation to answer some of these questions through data collected from *in vitro* studies.

Elucidation of the estrogenic mechanism of *in vivo* responses of enzymes to estrogens would be a difficult task and hence many workers in this field have turned to *in vitro* techniques in the hope of solving this baffling problem. *In vitro* methods, of course, remove

hormone and enzyme systems from a multitude of possible influencing factors.

Much of the in vitro work has been somewhat discouraging in that little or no enhancement of enzyme activity has been obtained. Hardy (1953) was able to demonstrate an estrogenic influence on the cornification of isolated mouse vagina and Mueller (1953) showed that rat uterine segments have a greater degree of amino acid incorporation if the rats were given estradiol six hours prior to sacrificing the animal. Although Mueller's work does not strictly show an effect of estrogens in vitro, it shows that once estradiol has instigated its action on uterine tissue, this effect can be continued in the presence of proper substrates. In both of these experiments enzyme systems were undoubtedly influenced by estrogens to bring about the end result.

By using animals which have been pretreated with an estrogen, some groups of investigators have been able to demonstrate increased protein and nucleic acid synthesis in surviving uterine segments (Mueller, 1953, 1957; Mueller and Herrman, 1953; Reddy, 1951).

The effect of estrone on the aerobic glycolysis of rat uterus was studied by Sweeney (1944), using the Warburg constant volume respirometer. She was unable to show any significant change in oxygen uptake by uterine tissue slices in the presence of estrone over a six hour period. She was able, however, to show an increase in oxygen uptake by uterine tissue when the animals had been treated with estrone twenty-four hours prior to the experiment. A thirty-five percent increase was obtained under these conditions.

Only recently has anyone been able to demonstrate what

may be a direct effect of a hormone on an enzyme system. Villee (1953, 1955) has shown an estradiol-induced stimulation of citrate utilization by a homogenate of human placenta at term. In this work the nonparticulate fraction of placental tissue homogenates showed an increased citrate utilization and increased alpha-ketoglutarate production in the presence of estradiol and DPN. This phenomenon could not be demonstrated in tissues other than human endometrium or term placenta. At this time there is apparently some doubt as to whether Villee's work shows a direct relationship between estrogen and enzyme at the estrogen-iso-citric dehydrogenase level (Talalay and Williams-Ashman, 1958).

No one except Villee has been able to obtain a direct in vitro effect of steroid hormones on the various enzymatic phenomena discussed above using either tissue segments or homogenates. When tissue homogenates are used there are actually many factors in addition to normal control mechanisms which alter enzyme activity. These factors include dilution of competing systems, liberation of autolytic enzymes, physical changes caused by surface phenomena, osmotic effects or specific ion effects. The normal mechanisms may be changed to such a degree in homogenates that there will be little or no enzyme activity when the cells are disrupted.

It was thought that by using tissue segments under proper conditions to study the influence of hormones on enzymes, the shortcomings of homogenate techniques might be overcome and at the same time a closer duplication of in vivo conditions of hormone-enzyme relationship might be obtained.

It is the purpose of this dissertation to present a new

approach for studying the effect *in vitro* of steroid hormones on a few of the enzymes found in the rat uterus. Results of the work *in vitro* will also be correlated with some work *in vivo* in an effort to help elucidate the mechanism of hormone action on enzyme systems.

CHAPTER II

EXPERIMENTAL METHODS AND RESULTS

Procedure for the Determination of Lactic Dehydrogenase Activity

The enzyme lactic dehydrogenase (LDH) acts reversibly in the reaction Lactate + $DPN^{+} \rightleftharpoons$ Pyruvate + DPNH + H^{+}. Since the coenzyme DPN is reduced during the formation of pyruvate, this offers a convenient method to follow the rate of the reaction. As DPN is reduced in this reaction, its absorbency at 340 mμ. is increased proportionally. Therefore, one unit of LDH activity is arbitrarily represented by a change of 0.001 in optical density of DPN at 340 mμ. per minute. The equilibrium of the above reaction is towards lactate; therefore, if KCN is added to the reaction, the pyruvate formed is trapped as pyruvate-cyanohydrin complex and the equilibrium is shifted to the right.

The determination of LDH activity throughout this investigation was made in the following manner. An assay medium was prepared containing five parts of 1.0 M. sodium lactate, pH 8.0; two parts of 1% KCN, pH 8.0; five parts of DPN (1 mg./ml.) in H_2O; and thirteen parts of 0.1 M. Sigma 7-9 Tris buffer, pH 8.0. Two and one-half ml. portions of this mixture were placed in several 15 ml. conical centrifuge tubes and incubated at 37° C for twenty-five to thirty minutes prior to their use. Tissue homogenates were prepared at concentrations of

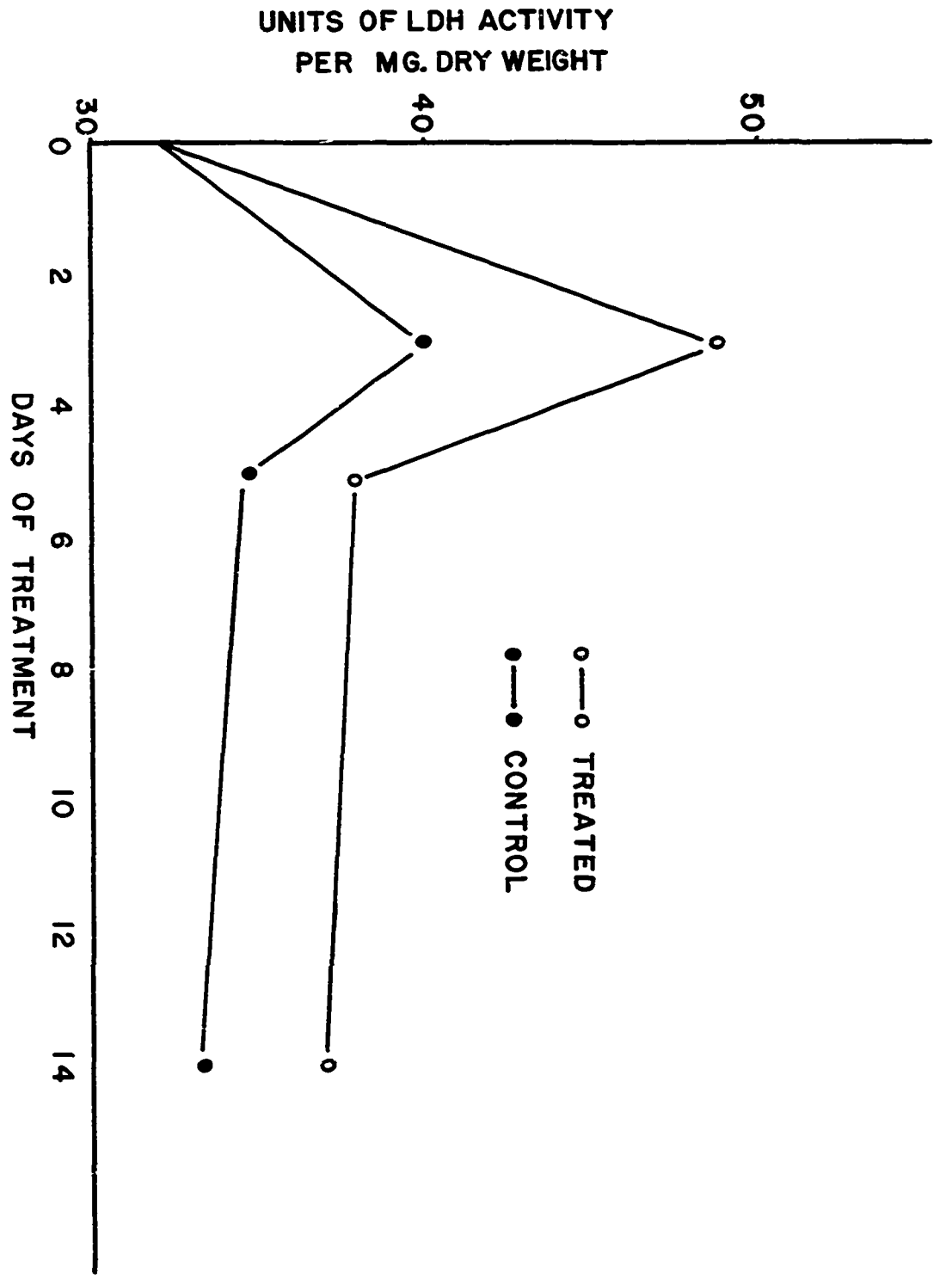
approximately 0.1 percent in 0.1 M. Tris buffer, pH 7.4, and placed in an ice bath until they were added to the assay mixture. For determination of LDH activity, the incubated assay mixture (2.5 ml.) was transferred to a cuvette (1 cm. by 1 cm.), 0.5 ml. of homogenate added rapidly, and the entire mixture inverted twice to insure adequate mixing. As the cuvettes were inverted a stop watch was started and optical density readings at 340 mμ. were taken at twenty second intervals, up to 140 or 160 seconds. A Beckman DU spectrophotometer was used for optical density determinations and every run was made in duplicate.

The reduction of DPN under these conditions was linear during the first two or three minutes. Therefore, the change in optical density during the first minute was used in determining the enzyme activities presented here.

Effects of Estrogens on Rat Uterine Homogenates

It has been shown that the administration of 17β-estradiol to castrated female rats causes the uterine enzyme activity of lactic dehydrogenase (Bever and Velardo, 1950) and β-glucuronidase (Fishman, 1944) to rise between one hundred and two hundred percent over the castrate level. The administration of estradiol benzoate to castrated female rats was shown to produce a similar effect on uterine LDH activity during early phases of this investigation. Figure 1 shows the type of response in LDH obtained from daily injections of one μg. of estradiol benzoate to castrated female rats of the Holtzman strain. The sharp rise in activity at the third day of injection is also characteristic of uterine succinic dehydrogenase, ATP-ase and glutamicoxalacetic trans-

Fig. 1. The effect of the daily administration of 1.0 µg. estradiol benzoate on the LDH activity in one hundred day old castrated rat uteri. The estradiol benzoate was given subcutaneously in 0.1 ml. sesame oil. Control rats received daily injections of 0.1 ml. sesame oil.


UNITS OF LDH ACTIVITY
PER MG. DRY WEIGHT
30
40
50
0
2
4
6
8
10
12
14
DAYS OF TREATMENT
o—o TREATED
●—● CONTROL

aminase, which were run simultaneously with the LDH. Since such a pronounced increase in uterine enzyme activity can be obtained by the administration of estrogen in vivo, it was tempting to try to duplicate these results using in vitro techniques.

Steroid hormones are often administered to rats in sesame oil, but since this oil is insoluble in aqueous solutions it is not desirable for in vitro studies. Possible solvents for steroids in studies in vitro were propylene glycol, ethylene glycol, glycerol, six percent bovine albumin and ethyl alcohol. Ethyl alcohol was eliminated as a steroid solvent because many investigators have reported a decrease in enzyme activity of tissue homogenates when it was added, even in low concentrations. This fact was verified, as shown in Table 1. The effect of the other solvents was tried on lactic dehydrogenase in uterine tissue homogenates. Table 1 shows the influence of these agents.

None of the agents in Table 1 gave any appreciable change in LDH activity. Also, none of the solvents are capable of acting as substrate under the conditions given. Propylene glycol was tried first as a steroid solvent because of its innocuous effect when administered in vivo and also because it is water soluble as well as a moderately good anti-fungal agent. Propylene glycol has been shown to be metabolized to pyruvic acid and acetic acid (Whitmore, 1937). There is apparently no conversion of this nature under the conditions used, but, if there is, the KCN must trap any pyruvic acid which is formed. The rate of the reaction would normally be reduced if an appreciable amount of pyruvic acid were being formed and not trapped. Work done by Katzberg (1956) has also shown that propylene glycol at low concentrations

had a beneficial effect on the growth of chick fibroblasts in tissue cultures. This would indicate that propylene glycol probably does not exert any detrimental effect on cellular growth, which in itself would be a reflection of general enzyme activity. Propylene glycol showed no lowering of LDH activity in tissue homogenates under the conditions described and was chosen as the routine steroid solvent for in vitro studies.

TABLE 1

EFFECT OF VARIOUS SOLVENTS ON RAT UTERINE LACTIC DEHYDROGENASE ACTIVITY

Solvent	Activity* With Substrate	Activity* With No Substrate
Control	80	0
Propylene glycol	80	0
Ethylene glycol	81	0
Glycerol	82	0
Bovine Albumin	82	0
95% Ethyl Alcohol	71	0

*One unit of activity equals the change in optical density at 340 mμ. x 10^3 per minute per mg. dry tissue weight.

The reaction mixture consisted of 0.5 ml. 1 M. sodium lactate, pH 8.0, 0.5 mg. DPN; 0.2 ml. 1% KCN; 0.8 ml. 0.1 M. Sigma 7-9 Tris buffer, pH 8.0; 0.5 ml. of a 3% uterine homogenate; 0.5 ml. of the solvent being tested, except only 0.2 ml. of ethyl alcohol was used. Total volume was brought to 3.0 ml.

Since an innocuous solvent for the steroid hormones had been chosen, an attempt was then made to determine if any steroid hormone might have an influence on the LDH activity of tissue homogenates. This had been tried by other workers with no change in enzyme activity

being noted. However, a different solvent was to be tried here and also a longer than usual incubation time was to be used.

A one percent homogenate was prepared in 0.1 M. Sigma 7-9 Tris buffer, pH 7.4 (Sigma Chemical Co.), using the uterus of a proestrus rat. To 12.5 ml. of this homogenate was added 0.5 ml. of propylene glycol containing one µg. estradiol. A control tube was set up having 12.5 ml. of homogenate and 0.5 ml. propylene glycol. These tubes were incubated at 37° C in a water bath and LDH activities determined on both the test and control tubes at 0, 15, 45, 95 and 125 minutes, as well as 31 and 49 hours. The LDH activities of both tubes were comparable and little if any difference was observed in their activities.

Another attempt was made to show an effect of estradiol benzoate on the uterine homogenate of a rat castrated seventeen days. For this determination, 0.5 ml. of propylene glycol containing one µg. estradiol benzoate was added to 5 ml. of homogenate. This gave a little higher concentration of estrogen per milliliter homogenate than in the previous run. After incubating at 37° C for 0, 15 and 30 minutes, the LDH activity of the test and of the control tubes were essentially the same. In neither this nor the previous determination was any activating influence of the estrogen on LDH noticed.

Other attempts were made to show some estrogenic influence on castrated rat uterine homogenates. No significant difference was ever observed between control and test runs when the concentration of estrogen was varied from 0.1 to 3.0 µg. per milliliter of reaction mixture and the time of incubation at 37° C extended from one to forty-eight hours. Prior incubation of estradiol with a liver homogenate,

followed by the addition of this mixture to a uterine homogenate, gave no significant change in LDH activity. Even though the estradiol may have been altered in some manner by the liver tissue, this gave no increase in enzyme activity. LDH activity has been shown to be, for the most part, in the supernatant fraction of uterine homogenates (LePage and Schneider, 1948), and has been confirmed as shown in Table 2, using both 0.22 M. sucrose and 0.1 M. Sigma 7-9 Tris buffer during this investigation. The supernatant fraction from centrifugation at 20,000 R.C.F.[1] for thirty minutes, was treated with estradiol as in prior runs and again no change in LDH activity was noticed.

TABLE 2

DISTRIBUTION OF LACTIC DEHYDROGENASE ACTIVITY IN RAT UTERINE HOMOGENATES

	Units of Activity per Min. per Mg. Dry Weight				
Run No.*	Whole Homogenate	Residue of 1,000 R.C.F.	Residue of 10,000 R.C.F.	Supernatant of 100,000 R.C.F.	Residue of 100,000 R.C.F.
1	68	5	6	52	7
2	93	15	17	84	(Includes residue)
3	40	1	1	56	(Includes residue)
4	35	1	1	52	(Includes residue)

*Runs 1 and 2 were made in 0.22 M. sucrose, pH 7.0, where as runs 3 and 4 were made in 0.1 M. Sigma 7-9 Tris buffer, pH 7.4.

It would appear from these data that no estrogenic influence could be induced on the lactic dehydrogenase of rat uterus under

[1] R.C.F. - Relative centrifugal force.

the conditions employed here. Perhaps the intact cell is necessary before the steroids are able to exert their influence. An examination of this possibility was the next phase of work undertaken in this investigation.

Method of Studying the Effect of Estrogenic Steroids on Surviving Rat Uterine Tissue

Since no promising results were obtained when studying the effect of estrogens on tissue homogenates, the next logical approach was to use tissue segments and thereby retain the intact tissue cells which might be capable of utilizing the steroids or otherwise be affected by them. The approach used follows the concept of tissue culture in that an attempt was made to maintain tissue integrity for as long a time as possible. The integrity of the tissue was determined by following the lactic dehydrogenase activity.

The method used for studying the in vitro effects of estrogenic steroids on rat uterine enzymes necessitated adopting certain techniques used in standard tissue culture work. For example, all equipment used in setting up the tissue segments was autoclaved at fifteen pounds pressure for twenty minutes or sterilized in dry heat at 180° to 200° C for two hours. A separate, sterile working area was maintained in a draft-free area of the laboratory. Aseptic technique was used throughout the entire procedure of removing the uterus from the rat until the tissue segments were in their incubating flasks. In the first days of this investigation female rats castrated for seven to twenty days were used routinely. It was found later that the uterus of intact rats in diestrus or proestrus responded to estrogen treatment

in vitro equally as well as the castrated rats. "Neutraglas" lyopholyzing bottles of twenty-five milliliter capacity were used in this procedure as incubation flasks, thereby minimizing any chance of change in pH due to the presence of sodium or other metals in the glass. Each flask was prepared for incubation by pipetting into it 4.0 ml. of a medium containing seven parts Tyrode's solution, pH 7.6, six parts 0.1 M. Sigma 7-9 Tris buffer, pH 7.6, four parts of human male ascitic fluid, and one part of Terramycin (400 µg./ml.) in 0.1 M. Sigma 7-9 Tris buffer. In the first extensive series of enzyme activity determinations in surviving rat uteri, no antibiotic was used and the amount of Tris buffer was actually eight parts rather than seven parts per unit of medium. Smears of both the nutrient medium and of the tissues themselves showed no sign of bacterial growth when stained with Gram's stain. After preliminary studies indicated that Terramycin at a level of 20-30 µg. per ml. of medium caused no deleterious effect to the tissue, Terramycin at a concentration of 22 µg. per ml. was routinely used (Metzgler, 1954). To the 4.0 ml. of the above nutrient medium, 0.5 ml. of propylene glycol was usually added with or without an added steroid hormone. To allow a free exchange of gases in the flasks, gauze plugs wrapped with thread were used to plug the flasks. Bovine amniotic fluid was substituted for the human ascitic fluid because it was perhaps more nutritious. The bovine amniotic fluid was obtained from a local packing house, filtered through an ultrafine sintered glass filter and used in the nutrient medium in place of the ascitic fluid. No difference in enzyme activity was noticed when either of the fluids were used. The use of ascitic fluid was therefore continued in this investigation.

The tissue was prepared by first removing the uterus from the rat as aseptically as possible and transferring the uterus to a sterile moistened filter paper on a watchglass. One horn of the uterus was stretched lengthwise and segments approximately one mm. thick were cut with a sterile double-edged razor blade. After one entire horn had been cut into segments, six to ten pieces were picked from different areas of the uterus and transferred to the incubation flasks and incubated at 37° C for the desired length of time. This process was completed as rapidly as possible in order to prevent the tissue from drying out.

In the initial phases of this investigation the small tissue segments were floated on a piece of lens paper 1.5 cm. square, having a silicone coating on the bottom side. When six to eight tissue fragments were placed on a piece of this lens paper, more often than not the paper would sink to the bottom of the flask. It was found that uterine tissue fragments which remained submerged in the incubation media for even two to three hours retained little or no LDH activity. Therefore, a variation in utilization of the lens paper technique was tried with good success. The squares of lens paper were placed on the inside wall of the incubation flasks so that about one-half of the paper was below the surface and one-half of the paper was above the surface of the incubation medium. The tissue fragments were then placed on this paper in a horizontal row immediately above the surface of the medium. After standing for forty-five to sixty minutes the tissue adhered well enough to the paper so that one could tip the flasks and bathe the tissue in the medium without the tissues falling off the paper. During the incubation period

the flasks were occasionally tipped so that the tissue was bathed in fresh medium. It was felt that by placing the tissue right above the surface of the medium, enough nutrient material would be able to diffuse up through the paper into the segments to maintain their viability and at the same time the metabolic products should be able to diffuse out of the tissue and into the medium.

For the determination of LDH activity the tissue segments were removed from the incubation flasks and blotted three or four times between filter papers in order to remove all free liquid. The tissue was weighed to the nearest 0.1 mg. on a Roller-Smith balance and homogenized in a Potter-Elvehjem type tissue homogenizer. Enough cold 0.1 M. Sigma 7-9 Tris buffer, pH 7.4, was used to give a tissue concentration of 7 to 10 mg./ml. of homogenate. The LDH activity was then determined on the Beckman DU spectrophotometer at 340 mμ. All determinations were run in duplicate.

This method was adequate to the extent that tissues maintained a detectable amount of LDH activity after seventy-two and, occasionally, ninety-six hours of incubation. The activity after twelve hours of incubation was usually about sixty to seventy percent that of the activity at zero time. Table 3 shows the average LDH activity of both control and test tissues when using estradiol at a level of one μg. per flask.

The amount of enzyme activity was based on wet weights of the tissue rather than on dry weight or nitrogen content. This was because of the relatively small amounts of homogenate which could be prepared from each flask of tissue and because accurate wet weights of

tissue were easily obtained using the Roller-Smith type balance. These two facts were thought to justify expressing enzyme activity on a wet weight basis.

TABLE 3

LACTIC DEHYDROGENASE ACTIVITY OF ESTRADIOL TREATED RAT UTERINE TISSUE SEGMENTS

Time	Control*	Treated*
Hours	Units Activity per Mg. Wet Weight of Tissue	
0	22.6	22.6
12	13.3	15.3
24	9.1	11.3
48	5.8	7.5
72	negligible	negligible

*Each group represents the average value of five separate determinations. Reaction mixture consisted of 0.5 ml. of 1 mg. DPN/ml., 0.5 ml. of 1 M. Sodium lactate, pH 8.0; 0.2 ml. of 1% KCN, pH 8.0; 0.5 ml. of tissue homogenate; and 1.3 ml. of 0.1 M. Sigma 7-9 Tris buffer, pH 8.0. Estradiol concentration is 10^{-9} M. Activity is based on change in optical density x 10^{3} at 340 mμ. per minute per mg. wet tissue.

To establish further if a wet weight basis for enzyme activity is acceptable under the conditions of this test, both castrated and proestrus rat uteri were incubated in the presence and the absence of estradiol and estriol. Because in vivo data show that the earliest observable effect of estrogens is to cause water imbibition in the rat uterus (Astwood, 1938), a similar effect might occur in vitro which would result in even a greater difference in enzyme activity between treated and untreated tissues.

To study the effect of estrogens on uterine water imbibition, segments of uterine tissue at various phases of estrous were incubated in the same medium as used to study the effect of estrogens on enzyme activity. The tissue was either placed on lens paper as described earlier, or submerged in the medium. Table 4 shows the percentage of change in tissue weight when incubated either with estradiol or estriol under the conditions given. There was no statistical difference in water imbibition between the control tissues and the treated tissues. ($P = 0.35$).

TABLE 4

WATER IMBIBITION BY RAT UTERINE TISSUE

Test No.	% Increase over Initial Weight		Steroid Used	Placement of Tissue
	Control	Test		
A	32	27	Estradiol	On paper
B	30	44	Estriol	Submerged
C	31	32	Estriol	On paper
D	49	47	Estradiol	Submerged
E	28	27	Estriol	Submerged
F	33	46	Estriol	On paper
Average	33.8	37.0	(P = 0.35)	

Further investigation showed that the relative difference in activity between the test and the control surviving tissues was in the same range, whether the activity was based on wet tissue weight or dry weight. Protein determinations were also run on four groups of cultures, using the method of Lowry et al. (1951). Again comparable activities with those based on wet weight values were obtained.

It was therefore condluded that under the conditions of this procedure, enzyme activity based on initial wet tissue weight was reliable.

Effect of Various Estrogens on Lactic Dehydrogenase Activity in Surviving Rat Uterine Tissue

Estradiol proved to have a significant influence on the LDH activity of the surviving uterine tissue; therefore, other estrogenic steroids were tried to determine what effect they would give. Estrone, estriol and estradiol benzoate were also tested, as well as estradiol in combination with progesterone.

In the early phases of this investigation the tissue segments were incubated at 37° C for periods as long as seventy-two hours. Usually, after seventy-two hours of incubation the enzyme activity per milligram wet weight was so low that reliable activity values were not obtainable. The incubations were therefore terminated at forty-eight hours.

The results of the study using estrogen-treated tissue segments are shown in Table 5 and Figure 2. The curves in Figure 2 represent the net differences in activity between test and control tissues. It will be noticed that the four estrogenic compounds elicit an increase in LDH activity which reaches a maximum after twelve to twenty-four hours of incubation. Although estriol has been shown to have much less activity than estradiol or estrone by the vaginal smear test (Szego, 1950), slightly more LDH activity is obtained in the surviving uterine tissue from the use of estriol rather than estradiol or estrone. Szego (1950) also demonstrated that the injection of estriol in saline into

TABLE 5

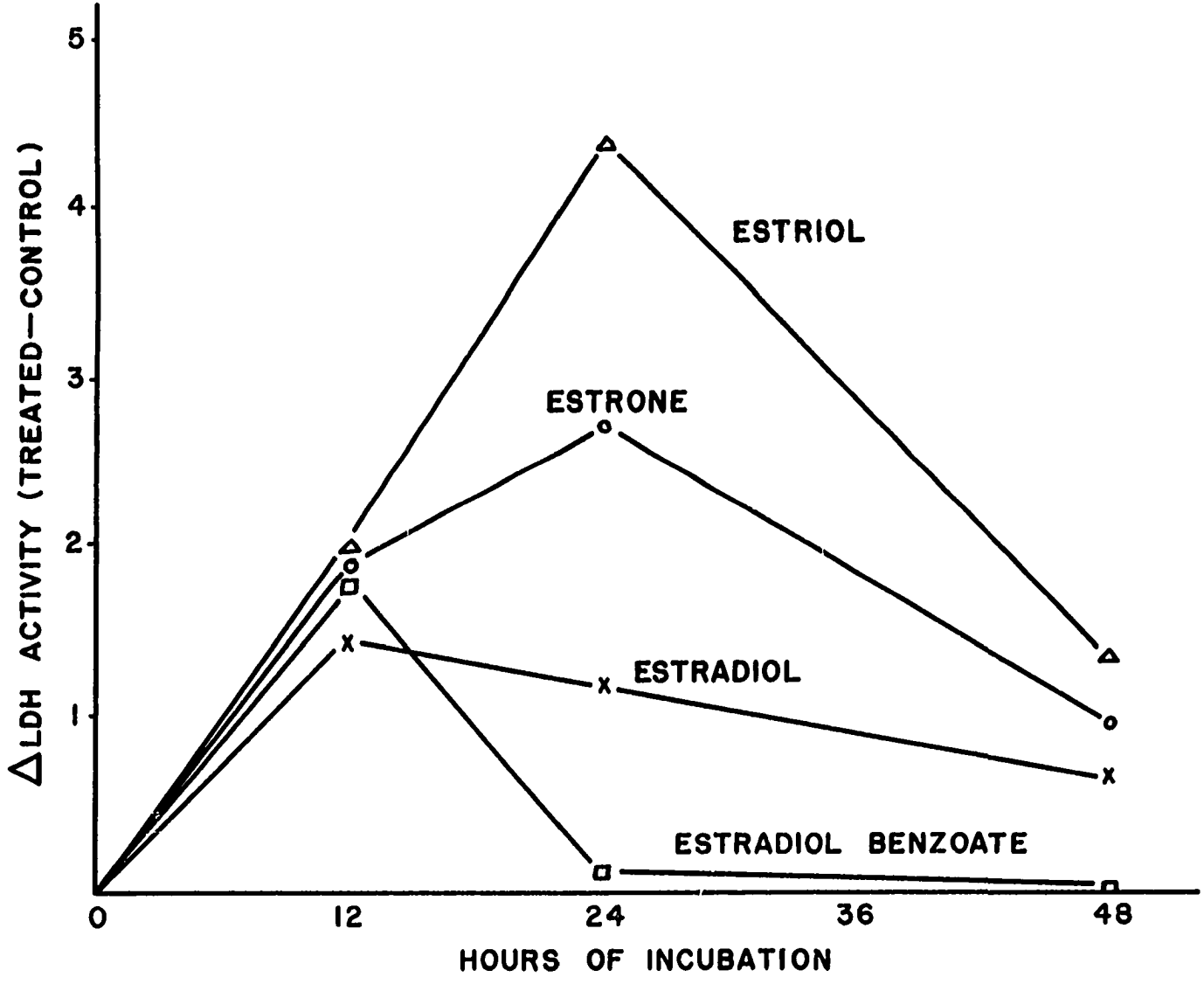
LACTIC DEHYDROGENASE ACTIVITY IN SURVIVING RAT UTERINE TISSUE UNDER INFLUENCE OF VARIOUS ESTROGENS

	Hours of Incubation		
0	12	24	48
Estradiol			
31.2	14.5 (13.8)*	3.7 (3.9)	0 (0)
26.2	15.9 (13.2)	11.8 (11.7)	4.4 (4.7)
18.1	14.7 (14.1)	7.7 (7.4)	5.7 (4.5)
19.1	12.9 (12.3)	12.9 (11.2)	10.6 (10.2)
20.7	15.8 (14.7)	13.5 (9.5)	5.6 (3.6)
	P=<0.06&>0.05	P=<0.12&>0.11	P=<0.05&>0.04
Estrone			
31.2	14.4 (13.8)	- (3.9)	0 (0)
16.5	10.5 (4.6)	6.8 (2.6)	3.2 (0)
26.2	14.2 (13.2)	12.6 (11.7)	4.1 (4.7)
18.1	15.9 (14.1)	10.5 (11.2)	5.1 (4.5)
30.4	14.0 (13.1)	9.9 (6.1)	9.8 (0)
	P=<0.12&>0.11	P=<0.09&>0.08	P=<1.0&>0.9
Estriol			
16.5	6.2 (4.6)	8.0 (2.6)	0 (0)
26.2	16.6 (13.2)	14.0 (11.7)	2.4 (4.7)
31.2	15.5 (13.8)	0 (3.9)	0 (0)
18.1	15.0 (14.1)	9.1 (9.4)	9.1 (4.5)
23.5	19.6 (17.3)	20.1 (14.3)	9.1 (7.5)
	P=<0.02&>0.01	P=<0.16&>0.15	P=<1.0&>0.9
Estradiol Benzoate			
16.5	7.4 (4.6)	4.7 (2.6)	0 (0)
26.2	15.1 (13.2)	12.1 (11.7)	- (4.7)
18.1	14.8 (14.1)	7.5 (9.4)	4.4 (4.5)
31.2	16.4 (13.8)	3.5 (3.9)	0 (0)
	P=<0.05&>0.04	P=<1.0&>0.9	P=<1.0&>0.9

*Control values in parentheses.

Units activity expressed as change in optical density x 10^3 per minute per mg. wet weight of tissue.

Fig. 2. The in vitro influence of estrogens on LDH activity in surviving rat uterine tissue. The ordinate represents the net difference between the specific activity of the treated and control tissue segments. Each estrogen was present at a 10^{-9} M. concentration. LDH activity was determined as given in Table 3.


ΔLDH ACTIVITY (TREATED—CONTROL)
5
4
3
2
1
0
12
24
36
48
HOURS OF INCUBATION
ESTRIOL
ESTRONE
ESTRADIOL
ESTRADIOL BENZOATE

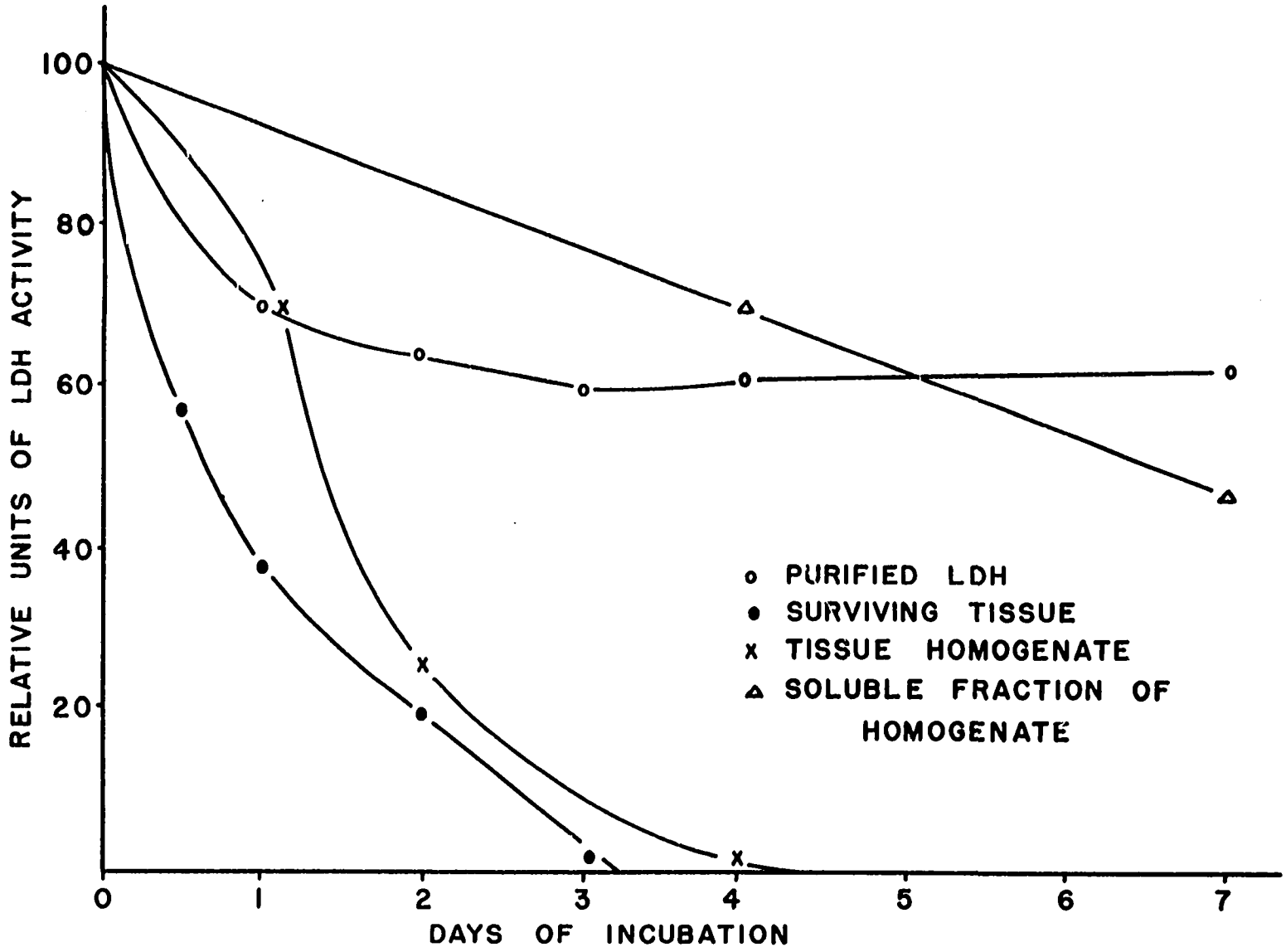
immature rats had five times the activity of estradiol and forty times the activity of estrone - using uterine wet weights as the criterion of effectiveness. It is debatable exactly why this phenomenon occurs, but this same type of effect has been shown from in vivo work by Bever et al. (1956) in which estriol gave approximately twenty-five percent more LDH activity than a corresponding dose of estradiol. Both estrogens were given at the level of 1.0 µg./day for three days. Estriol has not been shown to be present normally in rats and apparently this estrogen causes a greater LDH response in the rat uterus than do naturally occurring estrogens of the rat.

It will be noticed that in the case of estradiol benzoate, the LDH activity of the tissue dropped to the level of the controls after twelve hours of incubation. One explanation for this might be that the benzoate moiety of the hormone was hydrolyzed from the steroid molecule and the benzoic acid itself was toxic to the tissue. In vivo, the benzoic acid could be converted to hippuric acid in the liver and excreted in the urine.

Comparison of LDH Activity in Aged Tissue Homogenates, Cultured Tissue Segments and a Purified Enzyme Preparation

Since the surviving uterine tissue appeared to maintain LDH activity for only forty-eight to seventy-two hours, the duration of LDH activity was determined for both a uterine homogenate and a purified LDH preparation. This was done to determine if the LDH, per se, in the cultured tissue segment was losing activity of its own accord, or if some other factor was responsible for its destruction. As shown by Figure 3, the purified enzyme preparation in Tris buffer maintained its

Fig. 3. A comparison of LDH activity in various tissue preparations under prolonged incubation at 37° C. Initial activity for the purified LDH was 430 units per min. per mg. protein; for the surviving tissue, 22.6 units per min. per mg. tissue; for the tissue homogenate, 10.1 units per min. per mg. tissue, and for the soluble fraction of a homogenate, 12.8 units per min. per mg. tissue. The homogenates were prepared from the uteri of castrated rats and the purified LDH was obtained from Worthington Biochemical Corp.


100
80
60
40
20
RELATIVE UNITS OF LDH ACTIVITY
0
1
2
3
4
5
6
7
DAYS OF INCUBATION
o PURIFIED LDH
• SURVIVING TISSUE
x TISSUE HOMOGENATE
△ SOLUBLE FRACTION OF HOMOGENATE

activity relatively well through seven days of incubation at 37° C, while a uterine homogenate in 0.1 M. Sigma 7-9 Tris buffer, pH 7.4, maintained a detectable amount of activity for only ninety-six hours. A second uterine homogenate prepared in the same manner was centrifuged at 25,000 R.C.F. for thirty minutes to obtain the combined supernatant and microsomal homogenate fraction. This fraction maintained a proportionately high amount of its original activity after seven days of incubation at 37° C.

In comparing the LDH activities of the four preparations, the results suggest that some metabolic products or proteolytic enzymes in both the surviving tissue preparations and the whole homogenate may be lowering the LDH activity. Both the supernatant fraction of this homogenate and the purified LDH preparation show a considerably greater degree of activity after even longer periods of incubation. In these latter cases the presence of proteolytic enzymes or metabolic products would certainly be at a minimum if they are present at all. This would allow a greater survival time for LDH enzyme activity.

These data lend support to the working hypothesis that either toxic metabolic products, proteolytic enzymes, or both, were destroying LDH activity in the incubated uterine tissue.

Effect of Varying Concentration of Estrogenic Hormones on Enzyme Activity of Surviving Tissue

The effect of varying the concentration of estrogens in the incubation medium was also studied. Levels of 0.5, 1.0, 5.0 and 10.0 µg. of estradiol, estrone, estriol and estradiol benzoate were introduced into the medium in 0.5 ml. of propylene glycol. These

amounts gave final concentrations of approximately 5×10^{-10}, 1.0×10^{-9}, 5×10^{-9} and 1.0×10^{-8} M. of estrogen, respectively.

It was found that the 5×10^{-10} M. level gave little or no detectable change in enzyme activity over the control, while the 1×10^{-9} M. level appeared to be the optimum working level. At 5×10^{-9} and 1×10^{-8} M. concentrations there was generally an equal, or sometimes a lower, enzyme activity than given by the 1×10^{-9} M. level. This comparison was made at incubation periods varying from twelve to twenty-four hours and held true for all four estrogenic compounds tested. Table 6 gives a comparison of activities for this group of studies.

It will be noted that 0.5 µg. of estrone was apparently enough to maintain a significantly higher level of LDH than its control. The fact that the 5.0 and 10.0 µg. level of estrogens gave lower activities than a 1.0 µg. level may be attributed to the fact that either the point of maximum enzyme activity had already been passed or the steroids may have been toxic to the tissue at the higher level.

Effect of Various Non-Estrogenic Steroid Hormones on Surviving Tissue Enzyme Activity

Since estrogenic hormones definitely were shown to maintain LDH activity in surviving rat uterine tissue, the effects of other various steroid compounds were studied. This was in the nature of a screening procedure and therefore a standard quantity of 1.0 µg. of steroid in 0.5 ml. propylene glycol was routinely introduced into the culture media. This gave approximately 1×10^{-9} molar concentration of each hormone since the molecular weight of each was close to three hundred. Also, since optimum LDH activity occurred between

TABLE 6

COMPARISON OF VARYING ESTROGEN CONCENTRATIONS ON LACTIC DEHYDROGENASE ACTIVITY

Estrogen and Level (µg.)	Hours of Incubation	Unit Activity per Min. per Mg. Wet Weight
Estradiol		
0	12	- -
0.5	12	16.4
1.0	12	18.1
5.0	12	13.3
10.0	12	13.1
Estrone		
0	12	11.1
0.5	12	14.0
1.0	12	12.3
5.0	12	12.2
10.0	12	14.0
Estriol		
0	26	14.3
0.5	26	15.0
1.0	26	20.1
5.0	26	10.0
10.0	26	10.1
Estradiol Benzoate		
0	19	9.5
0.5	19	11.7
1.0	19	12.9
5.0	19	11.7
10.0	19	12.5

twelve and twenty-four hours of incubation with the estrogenic steroids, an eighteen or nineteen hour incubation period was selected for studying this group of steroids. The same experimental procedure was used for preparing the uterine tissue and the same nutrient media was used in this experiment as was used in the estrogenic study.

First cholesterol was selected for study because of its having the same cyclopentanophenanthrene nucleus as the estrogens. It was felt this steroid might also serve as a control type compound for this investigation. This supposition proved to be correct as the results show in Table 7. Surviving tissue incubated with cholesterol was also checked at six, twenty-four, forty-eight and ninety-six hour intervals for lactic dehydrogenase and β-glucuronidase activity. No statistical difference was observed between the test and control tissues.

β-Glucuronidase was found to be concentrated in the soluble fraction of tissue homogenates, as was the LDH. By following the activity of two different enzymes, it was thought that a better check might be obtained of the influence of the various steroids. The glucuronidase activity was determined by the method of Fishman and Fishman (1944), using phenolphthalein glucuronic acid as substrate.

Since the estrogens contain an unsaturated ring with a hydroxyl group, as shown in Figure 4, it was thought that this phenolic-type arrangement within the molecule might be of significance in its actions. Phenol and the ortho, meta and para isomers of cresol were added to incubating tissue segments to determine if phenolic compounds could influence LDH activity as did the estrogens. The four compounds were added to give a final concentration of 2×10^{-7} and 1×10^{-6} M. in

TABLE 7

THE EFFECT OF VARIOUS AGENTS ON LACTIC DEHYDROGENASE AND β-GLUCURONIDASE ACTIVITIES IN SURVIVING RAT UTERINE TISSUE

Agent	Molar Concentration	B-Glucuronidase Activity*	No. Animals Used	LDH Activity*	No. Animals Used
Cholesterol	10^{-9}	5.0 (5.0)	4	4.8 (4.6)	6
Phenol	10^{-6}	4.4 (4.9)	1	6.8 (8.3)	1
	10^{-7}	4.6 (4.9)	1	7.9 (8.3)	1
o-Cresol	10^{-6}	4.6 (4.9)	1	8.4 (8.3)	1
	10^{-7}	4.5 (4.9)	1	7.9 (8.3)	1
m-Cresol	10^{-6}	4.7 (4.5)	1	8.2 (6.8)	1
	10^{-7}	5.0 (4.5)	1	6.6 (6.8)	1
p-Cresol	10^{-6}	4.5 (4.5)	1	5.3 (6.8)	1
	10^{-7}	4.6 (4.5)	1	6.5 (6.8)	1
Progesterone	10^{-9}	3.6 (3.2)	4	4.9 (3.9)	4
Diethyl-Stilbesterol	10^{-9}	6.2 (5.4)	3	8.1 (7.2)	3
Testosterone	10^{-9}	3.8 (3.2)	2	4.8 (3.8)	3
Hydrocortisone	10^{-9}	4.0 (4.0)	3	4.8 (4.0)	6
Cortisone	10^{-9}	3.5 (3.4)	3	4.4 (4.1)	4
Prednisolone	10^{-9}	3.4 (3.5)	3	4.1 (4.1)	4
Estradiol	10^{-9}	5.2 (4.5)	2	5.9 (5.1)	5
Estriol	10^{-9}	4.8 (4.1)	2	7.7 (6.6)	7
Estrone	10^{-9}	6.4 (4.8)	2	8.7 (7.2)	4

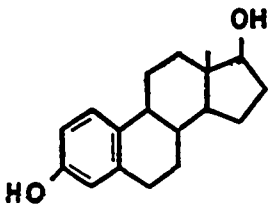
*Units of enzyme activity per mg. wet weight.

Enzyme activities were all run 18 to 20 hours after sacrificing the animal.

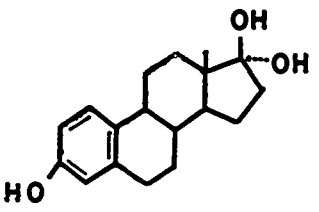
Control values in parentheses.

FIGURE 4

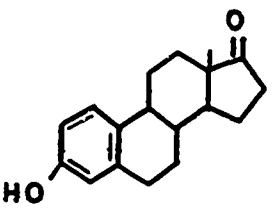
STRUCTURAL RELATIONSHIP OF SELECTED COMPOUNDS



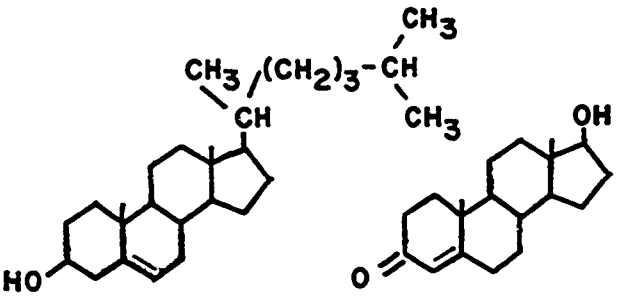
ESTRADIOL



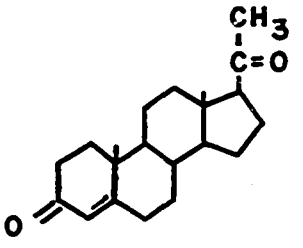
ESTRIOL



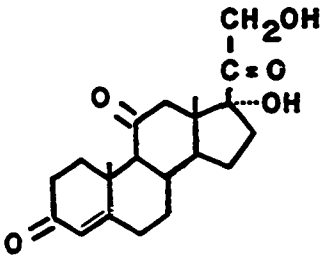
ESTRONE



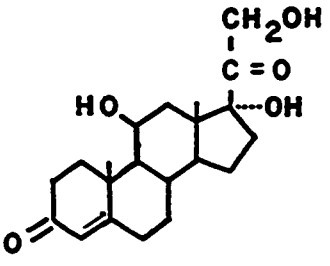
CHOLESTEROL TESTOSTERONE



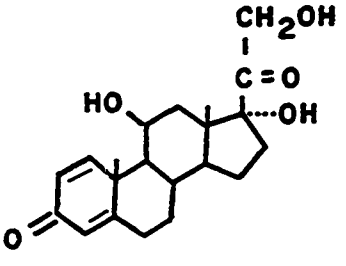
PROGESTERONE



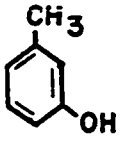
CORTISONE



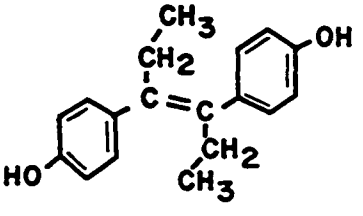
HYDROCORTISONE



PREDNISOLONE



m-CRESOL



DIETHYLSTILBESTEROL

the nutrient media. There was no appreciable difference in activity between the test and control tissue in any case except for the LDH activity when using m-cresol at 10^{-6} M. concentration. This was not checked further.

Progesterone at a 10^{-9} M. concentration (1 µg./4 ml.) gave a twenty percent increase in LDH activity over the control, while the β-glucuronidase activity rose only ten percent with the same treatment. Progesterone was shown by Bever et al. (1953) to raise LDH activity of uterine tissue in vivo, and the effect can be duplicated in vitro, as shown here.

The action of stilbesterol in giving increased activity for both LDH and β-glucuronidase in these surviving tissues might be expected since estradiol gave increased activity. It is unknown whether or not diethylstilbesterol is converted in vivo to a more active compound before it exerts its estrogenic action. Since there is an apparent difference between the activities of the control and test samples, this would suggest that the structure of diethylstilbesterol is responsible for its estrogenic activity, or the compound is altered to a more active estrogen by the uterine tissue itself.

Testosterone exerts a slight effect towards increasing enzyme activity in these preparations. Bever et al. (1953) were able to demonstrate a two hundred percent increase in uterine LDH activity over the castrate level of female rats by the in vivo administration of large daily doses of testosterone (75 mg./day). Engel (1957) has demonstrated that testosterone is capable of being metabolized to estrogenic compounds in vivo by tissue other than that in genital or adrenal glands.

Perhaps the uterus is capable of performing this conversion.

Hydrocortisone, cortisone and prednisolone all have questionable action on the surviving tissue. None of them had much influence on the β-glucuronidase enzyme activity and only hydrocortisone and prednisolone had any effect on LDH activity - the latter compound being the more active. Hydrocortisone has recently been shown to enhance the aerobic glycolysis of cultured cells (Grassfield, 1958); therefore, in this connection LDH might be altered since it is more or less sandwiched in between the aerobic and anaerobic phases of carbohydrate metabolism. Rosen et al. (1958) suggest an enzymatic mechanism for the gluconeogenic action of hydrocortisone in which glycogen and lactic acid are formed as the end products. It is possible that lactic acid production, under the influence of hydrocortisone or other glucocorticoid type hormones, might be a stimulus for increased LDH activity.

Study of Estrogen Conversion by Surviving Rat Uterine Tissue

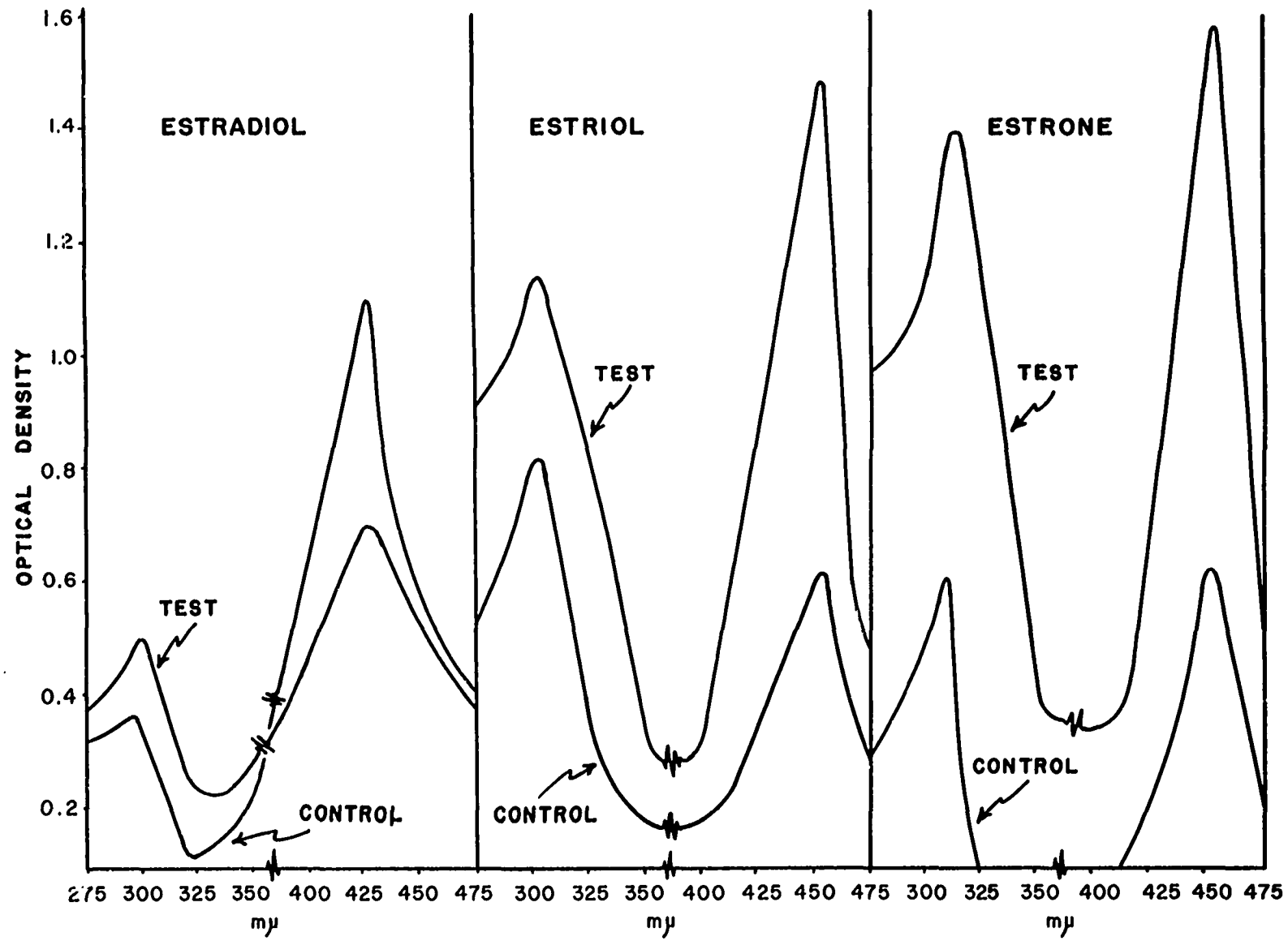
It has been shown that uterine tissue is capable of metabolizing estrone to a twenty-fold more potent estrogenic compound (Heller, 1940). Other workers have shown a conversion of estradiol to estrone by uterine tissue (Ryan et al., 1953; Bischoff et al., 1953). Since this phenomenon may play an important part in the mechanism of estrogenic action, a study was made of such conversion or metabolism of estrogens as might be associated with surviving rat uterine tissue. The routine nutrient medium for the tissue was prepared and 100 µg. of estrogen added in distilled water. It was found that by introducing the steroid in propylene glycol, the glycol interfered with the quantitative

estimation of the steroid. The presence of ascitic fluid in the medium may have helped in solubilizing the steroid.

After the nutrient medium, containing 100 µg. of the appropriate estrogen, was added to an incubating flask, tissue segments were placed on lens paper, as previously described. Since there was a hundred-fold increase in the concentration of estrogen per flask, the amount of tissue per flask was increased two to three-fold to give adequate opportunity for conversion. After twenty-two hours of incubation at 37° C the nutrient medium of each flask was transferred to a 15 ml. conical centrifuge tube, 6 to 7 ml. of ethyl ether added, and the contents shaken vigorously for ten to fifteen seconds. The tubes were centrifuged (500 - 600 R.C.F.) for five minutes and approximately 5 ml. of ether drawn off with a pipette and transferred to a second conical centrifuge tube. The ether was evaporated off and 5 ml. of concentrated H_2SO_4 added. The tubes were allowed to set overnight at room temperature for development of the color. An ultraviolet absorption spectra from 280 to 500 mµ. was run on each preparation, using the Beckman DU spectrophotometer. This method of steroid treatment is a modification of that used by Axelrod (1953) in the qualitative assay of estrogens. Figure 5 shows the spectra of each estrogen after incubation and its corresponding control.

To determine if the surviving tissue was capable of transforming the estrogens, a method similar to that suggested by Umberger and Curtis (1949) was tried for the qualitative estimation of estrogenic steroids. These workers identified estrogenic steroids by their ratio of light absorption at wavelengths of 430, 450, 480 and 520

Fig. 5. Absorption spectra of estradiol, estriol and estrone, having been incubated in the presence of surviving rat uterine tissue. Concentration of each estrogen was 33 μg. per ml.


ESTRADIOL
ESTRIOL
ESTRONE
TEST
TEST
TEST
CONTROL
CONTROL
CONTROL
OPTICAL DENSITY
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
275 300 325 350 400 425 450 475 300 325 350 400 425 450 475 300 325 350 400 425 450 475
mμ
mμ
mμ

mμ. Estradiol, estriol and estrone each gave only one absorption peak in this range of light. By adopting a similar method in this investigation a qualitative change could be detected in the steroid being studied. The absorption spectrum in the range 280 to 500 mμ. was determined for each of the above three estrogens. This range included enough of the ultraviolet spectrum to show a peak in the range 295 to 305 mμ. as well as the peak in the visible light range around 450 mμ. The absorbency values for the two peaks of any of the estrogens were different enough to give a ratio of optical densities at 300 mμ./450 mμ., which was indicative of the individual estrogen being studied. As seen in Table 8, estrone has a 300 mμ./450 mμ. ratio of approximately 1, while estradiol and estriol have significantly lower and higher ratios than this, respectively. Any deviation from these ratios after an estrogen had been incubated with uterine tissue would indicate some metabolism or alteration of the estrogen.

TABLE 8

ABSORPTION RATIOS OF ESTRADIOL, ESTRIOL AND ESTRONE

Ratio of Absorbency of Peak at 300 mμ. vs. Peak at 450 mμ.		
	Control (No Tissue)	Incubated (With Tissue)
Estradiol (First Run)	0.22	0.42
(Second Run)	0.20	0.55
Estrone	0.97	0.87
Estriol	1.35	0.80

It is quite evident that the peaks in the absorbency curves

of each of the three estrogens are changed to a significant degree after twenty-two hours of incubation with itssue. No attempt was made here to identify the conversion products for any of the three estrogens. Previaous workers have shown that a reversible conversion of estrone and estradiol occurs in uterine tissue (Ryan and Engel, 1953; Twombly and Taylor, 1942) and it is possible that in this study estradiol and estrone are being converted to one another. Estradiol could be converted to either estrone or estriol because its absorbency ratio is increased from its control level during incubation with uterine tissue. However, it is possible that the estradiol may be converted to a metabolite such as 2-methoxyestrone, as shown by the work of Kraychy and Gallagher (1957). It is not known if uterine tissue is capable of making this conversion. The estrone, however, presumably could be going to estradiol since the absorbency ratio after incubation is decreased. An increase in the ratio of estrone would suggest a conversion to estriol. No one has shown that estriol is capable of being converted to one of the other two estrogenic steroids, but the changes in absorbency ratios in this study indicate that some transformation of metabolism is occurring.

Effect of Estradiol and Estriol on Oxygen Uptake of Surviving Rat Uterine Tissue

In the initial phases of this study, an attempt was made to demonstrate an effect of an estrogen *in vitro* on the respiration of rat uterine tissue in the Warburg constant volume respirometer. No estrogenic influence was detectable when the estrogens were added at the one µg. level, using 95% ethyl alcohol as a solvent. There actually may have been more contributing factors to account for the negative

results than were first apparent. For example, uterine tissue homogenates were used as well as submerged pieces of tissue, in an attempt to demonstrate an estrogenic effect on oxygen uptake. The concentration of the tissue homogenates was not sufficient to give measurable values for oxygen utilization and the submerged tissue did not have a significant oxygen uptake after two or three hours. These discrepancies were corrected in later work. Sweeney (1944) studied the effect of estrone on the anaerobic glycolysis of rat uterine tissue slices and was unable to show any definite influence on oxygen uptake when the estrone was introduced to the tissue in vitro. It was thought that perhaps her studies were not observed over a long enough period of time, i.e., the effect of the estrone in the medium had not had time enough to cause any alteration in respiration. The work of Telfer (1953) would indicate that probably enzymatic activity is not influenced in uterine tissue until about thirteen to fifteen hours after the administration of estrogen to a castrated animal. Thus, if an estrogenic influence on uterine enzyme systems were to occur in surviving tissues, at least thirteen hours and even more likely, fifteen to twenty hours, might be required before the effect would be observed. The longer length of time required for in vitro effects would be due to the lack of a continuous blood supply to the tissues and, hence, only a slow diffusion of the nutrient media into the tissue slices probably would occur.

Previous work indicated that submerged tissues did not maintain their enzymatic activity for any appreciable length of time and therefore means were taken to keep the tissue segments exposed to air and still be bathed by nutrient medium. This was done by crinkling a

one by seven and one-half inch strip of lens paper and placing it in the bottom of a Warburg flask. Such flasks were autoclaved after adding the lens paper in order to keep bacterial contamination at a minimum. Three ml. of the routinely used nutrient medium (containing Terramycin) were then added to the flasks. The only substrate present in the nutrient medium was the glucose in the Tyrode's media. The estrogens were added in 0.5 ml. of propylene glycol. Uterine tissue segments were cut approximately one mm. thick with a razor blade and between 30 and 50 mg. of the pieces were placed on the lens paper protruding above the surface of the medium. The Warburg flasks each contained 0.2 ml. of 2N. NaOH in their center well to absorb carbon dioxide in the system. Oxygen uptake was then followed for seventy-two hours. Table 9 shows that estradiol and estriol, at a concentration of 3×10^{-9} M., give a significant rise in oxygen uptake during the period of six to seventy-two hours. Comparison of oxygen uptake after only five to six hours of incubation showed no statistical difference between the control and test samples.

The values in Table 9 show fairly low oxygen uptake values for the uterine tissue but this is apparently normal since uterine tissue does not have nearly the quantity of oxidative enzyme activity found in most other tissues. The rat uterus used in studying estradiol effect on oxygen uptake was close to full estrus - judging from the amount of water imbibition and increased vascularity. The animals were not smeared to determine the stage of estrus for these determinations because of the chance of washing bacteria into the uterus.

The rat used to study the effect of estriol on oxygen

uptake was in diestrus as judged by appearance of the uterus. Slightly more oxygen was consumed after estriol treatment than after the estradiol (comparing the control with the treated tissue), but this may have been due to the difference in stage of estrus of the two rats. In another determination using estradiol and estriol on the same rat uterus (again approximately in diestrus) results indicated that both estriol and estradiol gave similar degrees of oxygen utilization by the uterine tissue.

TABLE 9

OXYGEN UPTAKE OF UTERINE TISSUE UNDER INFLUENCE OF ESTROGEN

	Microliters Oxygen/Mg. Tissue (Wet Weight)			
Time (Hrs.)	Estradiol (3×10^{-9} M.)	Estradiol Control	Estriol (3×10^{-9} M.)	Estriol Control
5-6	1.5 ± 0.2*	1.3 ± 0.2	1.0 ± 0.1	0.6 ± 0.2
11-12	2.2 ± 0.15	1.8 ± 0.1	1.4 ± 0.3	0.8 ± 0.2
30	3.3 ± 0.6	2.2 ± 0.4	2.3 ± 0.3	1.2 ± 0.3
48-50	3.9 ± 0.8	2.4 ± 0.4	2.5 ± 0.3	1.4 ± 0.2
72	5.9 ± 0.4	3.7 ± 0.4	3.4 ± 0.4	1.9 ± 0.1

*Standard deviation.

Sulfhydryl Group Levels in Normal and Surviving Rat Uterine Tissues

This investigation has shown a definite effect of estrogenic hormones in vitro on the lactic dehydrogenase activity of rat uterine tissue. The question naturally arose as to whether the increase in LDH activity is due to the formation of new enzyme molecules or merely due to the activation of existing enzyme precursors. It was

thought that perhaps a functional group of the LDH molecule might be followed to determine if there had been an alteration in LDH content. Recently Neilands (1954) was able to demonstrate the presence of sulfhydryl (SH) groups on LDH from heart tissue. Until this time no one had been able to associate these groups with LDH. The sulfhydryl group therefore seemed to be a good functional group of the enzyme to assay along with enzyme activity, assuming, of course, that uterine LDH also contained SH groups.

To determine whether or not the sulfhydryl groups in uterine tissue were detectable by the commonly used ferricyanide reduction method of Anson (1940), normal and castrated rat uteri were homogenized in distilled water and subjected to the determination. As seen in Table 10, there is a significant difference between the sulfhydryl content of castrated and normal uterine tissue, both being based on dry weight measurements. The sulfhydryl group content of tissue is expressed as equivalents of cysteine because cysteine has one SH group per molecule and is a good standard for this determination due to its ease in reducing ferricyanide. An approximation of the apparent number of SH groups in the tissue protein can also be made by comparing the results with cysteine, for which a known number of SH groups can be calculated.

The normal rat uterus in estrus showed almost a three-fold increase in sulfhydryl group content over the castrated uterus, while the proestrus uterus showed slightly less than a two-fold increase. Similar results were seen when using surviving tissues incubated for twenty hours, except that the increase in sulfhydryl level of the estrogen-

treated tissue was increased only fifty percent over the control tissue. It appears that optimum survival conditions are still unknown for the uterine tissue because the sulfhydryl level could be raised to only one-sixth that of estrogen-treated animals. The same relationship held true for LDH activity in that it could not be increased as much in surviving tissue as it could in the intact animal, using an estrogen to stimulate enzyme activity in both cases.

TABLE 10

SULFHYDRYL GROUP LEVEL IN NORMAL AND SURVIVING RAT UTERINE TISSUE

Tissue	Equivalent μg. of cysteine/ mg. dry wt. of tissue
Normal Rat (Estrus) (2 rats)	46.0 ± 1.0
Normal Rat (Proestrus) (1 rat)	30.2
Castrated Rat (5 rats)	16.0 ± 3.0
Estrogen-Treated Tissue (4 runs) (1×10^{-9} M. Estriol, 20 hr. incubation)	23.9 ± 2.1
Control for Treated Tissue (4 runs)	14.8 ± 1.5
LDH Preparation (crystallized two-fold)*	7.7 ± 1.2

*Worthington Biochemical Corp., prepared from skeletal muscle.

The values for the equivalent of cysteine in the purified LDH preparation and the tissue preparations differ from two to six-fold (Table 10). This indicates that there must be proteins other than LDH in the tissues which contain sulfhydryl groups. There are certainly a variety of oxidative enzymes present in the uterus which would

also be contributors of sulfhydryl groups. Since LDH does possess SH groups it would seem only likely that it is one of the contributors to the total sulfhydryl content of the tissue. This assumption is supported by the data which show a significant rise in LDH activity upon the administration of estrogens to either castrated animals or to the surviving uterine tissue.

Ribonucleic Acid Content of Surviving Rat Uterine Tissue

It has been shown by Telfer (1953) that the ribonucleic acid (RNA) content of rat uterus varies during the estrus cycle. She has also shown that the administration of estrogen to a castrated rat gives an increased level of RNA per unit of tissue while the deoxyribonucleic acid (DNA) level changes little, if any. Mueller (1957) has shown that the administration of estrogens to castrated rats causes a greatly increased formation of RNA and a small increase in DNA in surviving uterine tissue slices. Mueller used the uptake of formate and glycine, and their conversion into adenine and guanine, as evidence for nucleic acid synthesis. Since the current concept of protein (or enzyme) synthesis is based on the assumption that RNA is the template for the formation of the protein, an attempt was made in this investigation to study RNA levels in surviving rat uterine tissues. In this manner the enzyme activity of both the control and estrogen-treated tissue might be shown to be related to the RNA level as well as to the sulfhydryl group content mentioned earlier.

The colorimetric method of Webb (1956) was used for the quantitative determination of RNA. This determination basically

consists of hydrolyzing the nucleic acids in trichloracetic acid, conversion of liberated ribose to furfural and a colorimetric determination of the furfural made by using p-bromophenylhydrazine to develop the color. The tissue homogenates which were used for determining some of the enzyme activities given in Table 7 were also used for determination of RNA levels.

Table 11 shows the levels of RNA encountered in the surviving uterine tissue as well as the RNA level of castrated and estriol-treated castrated rats. Surviving tissue was incubated in the routine nutrient medium and contained 0.5 ml. propylene glycol per flask with estradiol being used at a level of 10^{-9} M. for the treated tissues.

Table 11 also shows that estradiol-treated tissue maintained higher levels of RNA than did the corresponding control. The administration of estriol in vivo showed about a fifty percent increase in RNA, which was comparable to the percentage increase in surviving uterine tissue incubated for twenty-four hours.

It was interesting to note that the RNA content of estrogen-treated tissue in two separate instances was lower than the corresponding control. In both cases the determination was made at less than twenty-four hours of incubation. These results seemed to be in disagreement with the RNA content of tissues incubated slightly longer. Re-examination of the data published by Telfer (1953) indicated that there was also a corresponding drop in the RNA level of uterine tissue about six hours after administering estradiol to castrated rats. The zero time level was higher than the six hour level, and at forty-eight hours the level had risen nearly twice as high as it was at zero time. This

slight drop in RNA content, which occurs after the administration of estrogen, leaves room for speculation as to why this phenomenon occurs and also whether it is related in any manner with protein synthesis or enzyme activity.

TABLE 11

RIBONUCLEIC ACID IN RAT UTERINE TISSUES

Tissue	µg. RNA/mg. Wet Wt.
1. Surviving Tissues	
20-24 Hour Incubation	
Estradiol-Treated	7.70 ± 2.30*
Control	4.45 ± 0.40
Estriol-Treated	5.6 ± 0.80
Control	4.7
Testosterone-Treated	3.2
Control	2.6
2. Surviving Tissue	
11 Hour Incubation	
Estradiol-Treated	2.29 ± 0.39
Control	2.85 ± 0.25
16 Hour Incubation	
Estradiol-Treated	2.32 ± 0.08
Control	2.61 ± 0.01
3. Castrated Rats	
1 ug. Estriol for 3 days	33.7 ± 2.3
Control	23.5 ± 1.3

*Standard deviation.

Since the RNA level in surviving tissue had been shown to be influenced by estrogens, more extensive studies were undertaken in this area. It might be possible to influence protein synthesis in tissues by introducing the basic building blocks or precursors of RNA (orotic acid, adenine, guanine, ribose, etc.) or the nucleotides of RNA,

or even RNA itself. Work by Gale (1956) has shown that disrupted cells of Staphylococcus aureus were able to increase β-galactosidase activity by the addition of fragments of DNA or RNA isolated from the same bacteria. Added fragments of RNA isolated from yeast also caused an increase in enzyme activity. By the same token, it was thought that some influence of RNA on enzyme activity might be observed in surviving uterine tissues. The addition of yeast RNA, or a mixture of adenine, guanine, uracil, d-ribose and adenosinetriphosphate as RNA precursors, to incubating tissue slices, gave no significant change in LDH activity. No change in activity was observed from using orotic acid at a level of 57 µg. per ml. of nutrient medium. Yeast RNA (1 mg./ml.) was incubated with ribonuclease (0.5 mg./ml.) to give nucleotides which might be utilized by the surviving tissue. After incubation, the mixture of yeast and RNA and ribonuclease was placed in a boiling water bath for ten minutes to denature the ribonuclease and thereby prevent destruction of RNA in the surviving tissue. Again, no noticeable difference was seen in LDH activity when 0.5 mg. and 1.0 mg. of the hydrolyzed RNA were added to separate flasks of surviving tissue.

Next, the RNA fraction from fifty uteri of one hundred day old female rats of the Holtzman strain was isolated and purified by the method of Anson (1940). In order to obtain maximum RNA from the uterine tissue the rats were killed when they appeared to be in estrus or proestrus, as determined by the smear technique. At full estrus the uterus has a maximum content of RNA. The uteri were frozen as the animals were killed over a period of four days and then they were combined and processed. After final purification of RNA, 24.0 mg.

were recovered from 58.5 gm. of uterine tissue. This represents about a forty percent recovery of the total RNA originally present, assuming there were 58 to 60 mg. RNA present initially (see below).

It was thought that if RNA from the same source as the tissues were added to the surviving tissue, it would have a better chance of influencing protein synthesis. Therefore, 0.3 and 1.0 mg. of uterine RNA were added to the routine incubation medium. No change from the control LDH activity was noticed at these levels. Telfer (1953) found a maximum of 1.16 mg. RNA per uterus from castrated rats receiving estradiol. The RNA level of a normal estrus rat would be expected to be in this range also. Therefore, in the above tissue study, for 40 mg. of uterine tissue (about one-sixth the weight of a whole diestrus uterus) there would have been about two to seven times the normal concentration of RNA available to the tissue.

Another portion of the extracted uterine RNA was incubated with ribonuclease (from bovine pancreas) for four hours at 37° C. The incubated mixture was boiled gently for one minute to denature the ribonuclease. Approximately 0.5 mg. and 1.0 mg. of the fragmented RNA were added to separate flasks of surviving uterine tissue. No change in LDH activity was noticed after twenty hours of incubation. The procedures above were repeated with the addition of 1.0 µg. estriol to each flask. Again, no change was seen in enzyme activity.

CHAPTER III

DISCUSSION

This investigation was undertaken to devise a method to demonstrate an effect of steroid hormones *in vitro* on surviving rat uterine tissue. The method utilized has been outlined along with the results obtained from studying its various ramifications. Although the method of studying surviving uterine slices *in vitro* gives results qualitatively comparable to those obtained from work *in vivo*, the effectiveness of steroid influence on the uterine enzyme activity does not equal that of estrogen administration *in vivo*. It appears that the decreased steroid effectiveness *in vitro* is due to one of two things. Either the tissue metabolites are not removed as efficiently as they would be *in vivo*, or, more likely, proteolytic enzymes are causing enzyme inactivation within the tissue. The latter reason seems more plausible when the data obtained from the supernatant fraction of a uterine homogenate is considered. This fraction was able to retain LDH activity more than twice as long as the unfractionated homogenate. Thus, the proteolytic enzyme activity would be confined to the cell membrane, the nucleus or the mitochondria. The mitochondria might well be responsible for the loss of enzyme activity because of the enormous number of catabolic and anabolic processes occurring there.

The LDH activity of both the control and estrogen-treated tissues fall off almost logarithmically, as can be calculated from the data in Table 5. From this it is not possible to say whether the presence of estrogen in the nutrient medium of the surviving tissue is causing the formation of new enzyme molecules, or if it is acting as a protective agent for the existing enzyme molecules. If the estrogen were acting as a protective agent for the LDH, among other enzymes, then the presence of estrogen in a forty-eight hour old tissue homogenate preparation should also have given some protection. This was not the case. As a rule, estrogens are considered to be anabolic in nature and therefore could be involved in the formation of new enzyme protein within the intact cells of the surviving tissue. The amount of ascitic fluid used in the nutrient medium should have been sufficient to provide an adequate source of amino acids for forming new protein. It would be assumed in this case that the uterine tissue would be capable of breaking down the protein in the ascitic fluid into peptide fragments or free amino acids which would be available for new protein synthesis.

If new protein were not being formed by the uterine tissue when treated with estrogen, the existing proteins must undergo a molecular rearrangement to form new proteins because of the increase in number of sulfhydryl groups found in the estrogen-treated tissue. However, the increase in sulfhydryl groups could be an indication of either new protein synthesis or free glutathione or cysteine as well. There is no way that the interconversion of proteins could be determined in this case other than by determining the change in content of reactive groups such as SH groups, or by the titration of amino groups by use of the

formaldehyde titration technique.

Considering the facts that both SH content and LDH activity can be influenced in the same direction, either by administration of estrogen *in vitro* or *in vivo*, and that LDH has been shown to contain SH groups (Neilands, 1954), it appears that LDH may actually be synthesized under the influence of estrogen. The estrogenic influence *in vivo* is more efficient and dramatic than the response *in vitro*, but with further refinements of the method instituted here, duplication of responses *in vivo* should be approached.

Comparing the effect of the three major estrogenic compounds - estradiol, estrone and estriol - the results obtained during this investigation indicate that estriol has a greater influence on certain enzyme activities than either estrone or estriol. These results are in agreement with work of Szego and Roberts (1946), in which they showed a greater increase in uterine fresh weight with the administration of estriol to castrated rats than with estrone or estradiol. Bever *et al.* (1956) also demonstrated that the administration of estriol to castrated rats caused a greater increase in LDH activity per unit of estrogen than did estrone or estradiol. It was mentioned earlier that since estriol is known not to be a naturally occurring estrogen in the rat, it may exert a more potent estrogenic effect toward uterine enzymes in the rat than its natural estrogens. In women, estriol has the least estrogenic activity of these three steroids and this same relationship holds true in the mouse vagina assay for estrogens.

The spectrophotometric study of the effect of incubating estrogens with uterine tissue under the conditions outlined was found to

be quite interesting. The most encouraging data were obtained from the incubation of estriol. As shown in Figure 5, the absorption spectrum, after incubation with surviving uterine tissues, was changed to a greater extent than was the spectrum of either estrone or estradiol. This correlates nicely with the fact that estriol caused a higher level of LDH activity in surviving tissues than did estrone or estradiol. The metabolism or alteration of this steroid is apparently coincidental with its estrogenic activity. No one has shown that estriol is capable of being enzymatically converted to estrone, estradiol or any other metabolic product, so it is difficult to predict what conversion took place in the estriol molecule to give such a change in its spectral curve.

From the data presented here it appears that various steroid hormones have varying degrees of influence on the LDH activity of surviving uterine tissue. The fact that cholesterol does not exert any influence on enzyme activity indicates that something more than the presence of a steroid nucleus is necessary to influence enzyme activity. It may be that the side chain on cholesterol actually inhibits any stimulation to enzyme activity it would otherwise be able to impart. Since the estrogens have a phenolic type structure in ring A of the molecule, it is possible that the phenolic grouping might have been responsible for influencing enzyme activity. This was not the case. Phenol itself was added to incubating tissues in place of estrogens and no change in LDH activity was observed. o-Cresol, m-cresol and p-cresol were substituted for the estrogens in surviving tissue studies and again no change was detected in LDH activity. The cresols were used because of their phenolic type configuration and because they had an additional group on

the molecule which might give a different reaction than would phenol alone.

Cortisone, hydrocortisone and prednisolone gave little, if any, increase in LDH activity and no change in β-glucuronidase activity when each of these three glucocorticoid type steroids were added to surviving tissues in vitro. The fact that neither the LDH or β-glucuronidase activity was increased with treatment of these steroids in vitro could be interpreted as meaning that these glucocorticoids have no marked estrogenic qualities. Therefore, they do not cause a rise in β-glucuronidase activity, an enzymic action intimately associated with estrogenic action in the uterus. Fishman's (1951) work would indicate that β-glucuronidase is concerned with allowing the entrance of estrogens into the tissue cells. His theory is based on the assumption that the estrogens presented to the target organs are conjugated as glucuronides.

The ribonucleic acid aspect of this study adds further support to show that the addition of estrogen in vitro to surviving uterine tissue can influence enzyme activity in the same direction as it does in vivo. The data in Table 11 show that the RNA level in estrogen-treated surviving tissue is significantly higher than that of the controls. This pattern is also seen in intact rats in estrus as well as in castrated rats. In two separate instances, the RNA content of the surviving tissue was by necessity determined after a reduced incubation period. The RNA level per mg. wet weight of tissue was lower for the estrogen-treated tissue than it was for the control. At first this appeared to be a contradiction to existing data, but as mentioned earlier, re-examination of Telfer's (1953) work indicated that the same thing occurred in

vivo. The observation that estrogen causes a slight drop in RNA content of tissue before a rise occurs, might be an indication that RNA is being broken down and then reformed in a different pattern. This would allow more of a new type of protein to be formed, as dictated by the higher level of new RNA in the cells. Dounce (1952) has suggested that cellular RNA can be thought of as a template for the formation of protein. This theory would fit ideally into the above discussion.

CHAPTER IV

SUMMARY

1. A method has been presented which indicates that estrogens are able to exert a stimulatory effect *in vitro* on rat uterine enzyme activity.
2. The effects exerted *in vitro* by the estrogens are similar to those observed from the administration of estrogens *in vivo*.
3. Various other steroids and related compounds were studied for their effects on enzyme activity in surviving tissue.
4. The lactic dehydrogenase and β-glucuronidase activities have been increased over control levels in estrogen-treated tissues. The rise and fall of lactic dehydrogenase activity *in vitro* parallel those observed *in vivo*, but the actual increase is much greater *in vivo*.
5. Under the influence of estrogens *in vitro*, the ribonucleic acid level and sulfhydryl group content of the estrogen-treated tissues were also shown to be increased in the same direction as enzyme activity.
6. The influence of an estrogen on surviving uterine tissue is apparently linked to the simultaneous alteration of its molecule. Estriol was shown to enhance enzyme activity to the greatest extent and its absorption spectrum was also altered to a greater extent than either estradiol or estrone.

7. Under the conditions outlined there is apparently an active formation or alteration of enzyme molecules which is brought about by the action of estrogen on ribonucleic acid, which in turn is involved in protein synthesis.
8. Under the conditions used in this investigation, intact tissue cells are necessary before any estrogenic influence on enzyme activity is detectable. This is evidenced by the results obtained from both the homogenate and surviving tissue data.

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