

# Studies on the Mechanism of Action of Estrogens<sup>1</sup>

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While the cells of a multicellular organism spring from a common heredity, the process of differentiation gives rise to specialization of function among segregated groups of daughter cells. It is indeed a remarkable feat of nature that cells with strikingly different attributes and functions can live together in a purposeful and cooperative manner. In this venture each cell is the pawn of an environment contributed by the other cells, yet it is important for its own contribution to the common medium. When this contribution, usually from a group of similar cells, is a transportable agent which produces a profound biological response in another group of susceptible cells or tissue, the agent is termed a hormone. Already this diverse class of substances includes proteins, peptides, steroids, unsaturated alcohols, synthetic polycyclic compounds, and factors which are yet to be identified. About the only thing they have in common is that they modify the biological function of a subordinate group of cells.

The response of the susceptible cells to the hormonal agent, however, appears to be largely an exaggerated version of the normal reaction of a cell to change in its environment, except that in the case of the hormone the effects of differentiation on the composition of the metabolic machinery of cells provide for a certain specificity of response. This is to say, the process of differentiation divides certain coupled functions between cell lines and thereby provides the opportunity for periodic interaction or physiologic control through their exchange of metabolic products.

Accordingly, when we seek to explain how a hormone acts we must search for the answer amid the framework of factors which account for biological function. Also, we are really posing a series of questions which involve the following points: the identity of the primary acceptor in the susceptible cell; the nature of the interaction of the hormone with the acceptor; the manner in which this primary effect is amplified and expressed in the over-all function of the cell; and, finally, a concept as to backreaction or limitation of

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this process. Of utmost importance are the concepts of time and of the relative stability of the biological state; some effects are instantaneous, whereas others are only accrued through time-consuming syntheses or degradations.

In this discussion we shall present some of our experimental attempts to decipher the mechanism of action of estrogens with particular reference to their uterotrophic activity. These studies result from an effort to utilize estrogens as a tool for unraveling the phenomenon of growth control generally and in cancer specifically. Accordingly this paper should not be considered as a review of all work in this area; for this purpose the reader is referred elsewhere (21, 24). But before we proceed to the experimental section, let us first consider certain cytophysiological factors which underlie any hormonal response.

### I. UNITS OF STRUCTURE AND FUNCTION

What is meant by the term, function? Actually function is something that has to be evaluated at every level of organization in the living host. Thus we have an over-all function which characterizes the organization of the animal; a cell; the subcellular particulate; the multiple enzyme system; the template in a polymeric synthesis; and the basic unit of catalysis, the enzyme. But, at any level, function can be measured only by the spectrum of products and the rate at which they are formed. This situation involves an expenditure of energy and therefore, directly or indirectly, requires the participation of nature's machine for facilitating the flow of energy in product formation, namely the enzyme.

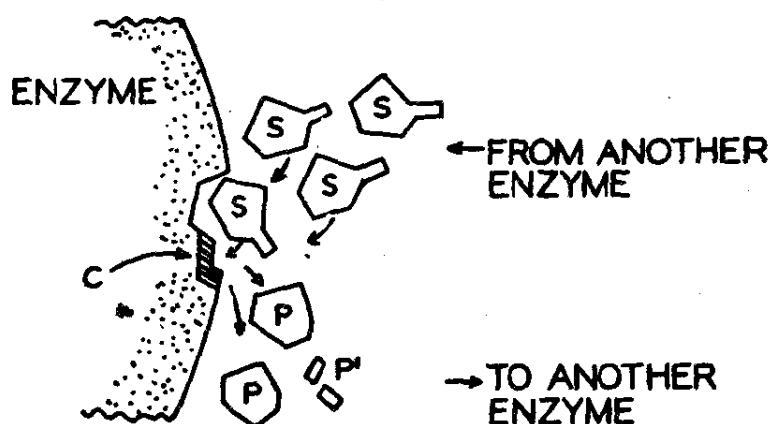


FIG. 1. A hypothetical surface of an enzyme. S = activated substrate molecules; P and P' = product molecules; C = coenzyme.

In Fig. 1, the enzyme is pictured as a protein carrier of a catalytic surface which is specifically complementary to the structure and configuration of a particular substrate molecule. Through the formation of an enzyme-substrate complex, reactions are catalyzed which are thermody-

namically possible. The over-all velocity of such a reaction is determined chiefly by the inherent catalytic properties of the enzyme, the amount of the enzyme, and the concentrations of the substrates and products. In the test tube the enzyme preparation may have free access to the reactants, but in life the enzymes are not free; instead, they exist amid the highly organized and labile compartmental structure of the cell.

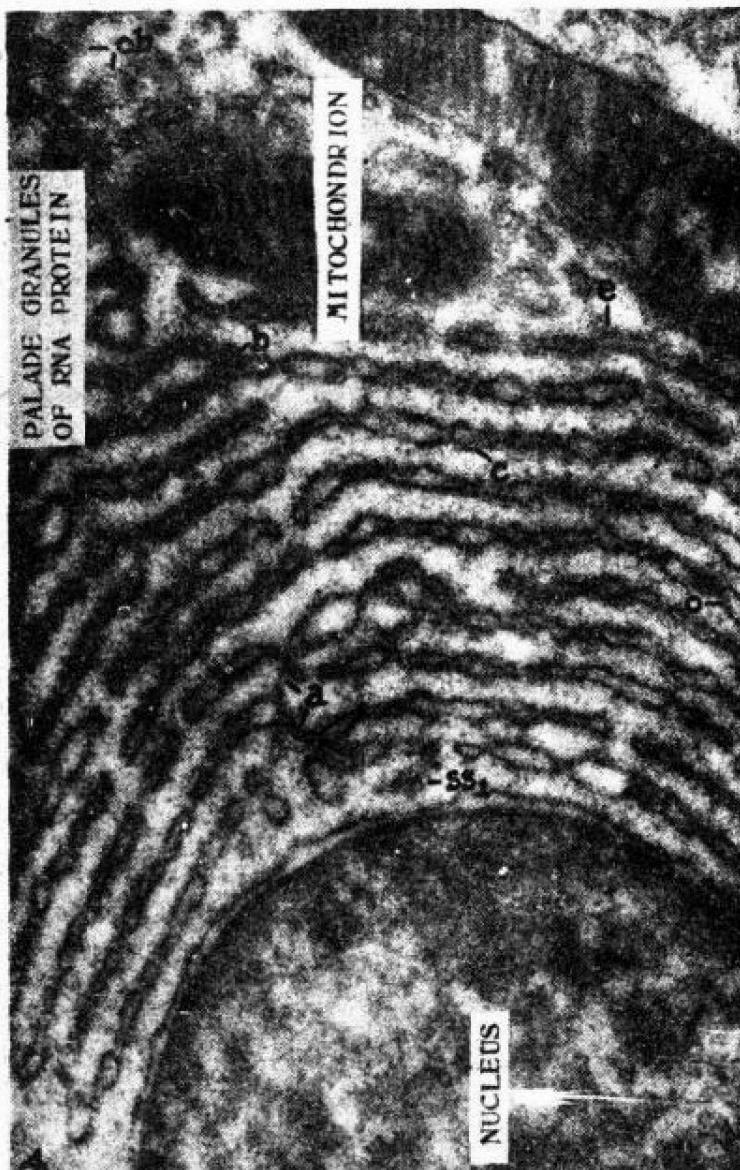


FIG. 2. An electron micrograph of a cell from a guinea pig pancreas. Field shows less than one-fifth of cell. Taken by Dr. George Palade of the Rockefeller Institute for Medical Research. Magnification:  $\times 40,000$ .

Some measure of the complexity of this organization is revealed in an electron micrograph of a pancreatic cell (Fig. 2) taken by Dr. George Palade of the Rockefeller Institute. Readily visible are the nucleus with its membranes, the mitochondria with their internal cristae structures, and the membranous endoplasmic reticulum with its affiliated Palade granules. Not shown are such structures as the cell membrane, the nucleolus, and certain

less well studied entities. Thus it is readily seen that the cell is made up of a tremendous number of compartments which have been shown by cell fractionation and cytochemical techniques to contain specific associations of enzymes. To mention a few, the tricarboxylic acid cycle-electron transport system is concentrated in the mitochondria; diphosphopyridine nucleotide synthesis and the pyrophosphorylation of uridine coenzymes are concentrated



FIG. 1. A photomicrograph of a cross section through the horn of a control uterus from an adult rat 6 weeks post ovariectomy. Section was stained with hematoxylin and eosin; magnified approximately 100 times. Prepared by Miss Ann Holton.

in nuclei; certain steps involved in protein and cholesterol synthesis are concentrated in the endoplasmic reticulum fraction; and glycolysis, amino acid activation, and many other functions are localized largely in the soluble fraction of the cell.

In each case the enzymes operate as members of a multiple enzyme system, issuing forth products in accordance with the principles of competition in such a system (8, 21). However, each compartment constitutes a state of semi-isolation, and products formed in one area have limited access to the enzyme systems located in another. As a result, gradients spring into

existence among the various semi-isolated multiple enzyme systems, which in turn determine the steady states of the respective areas. This complex interaction is operative throughout the entire cell structure and, in a modified version, between cells and tissues (cf. 21).

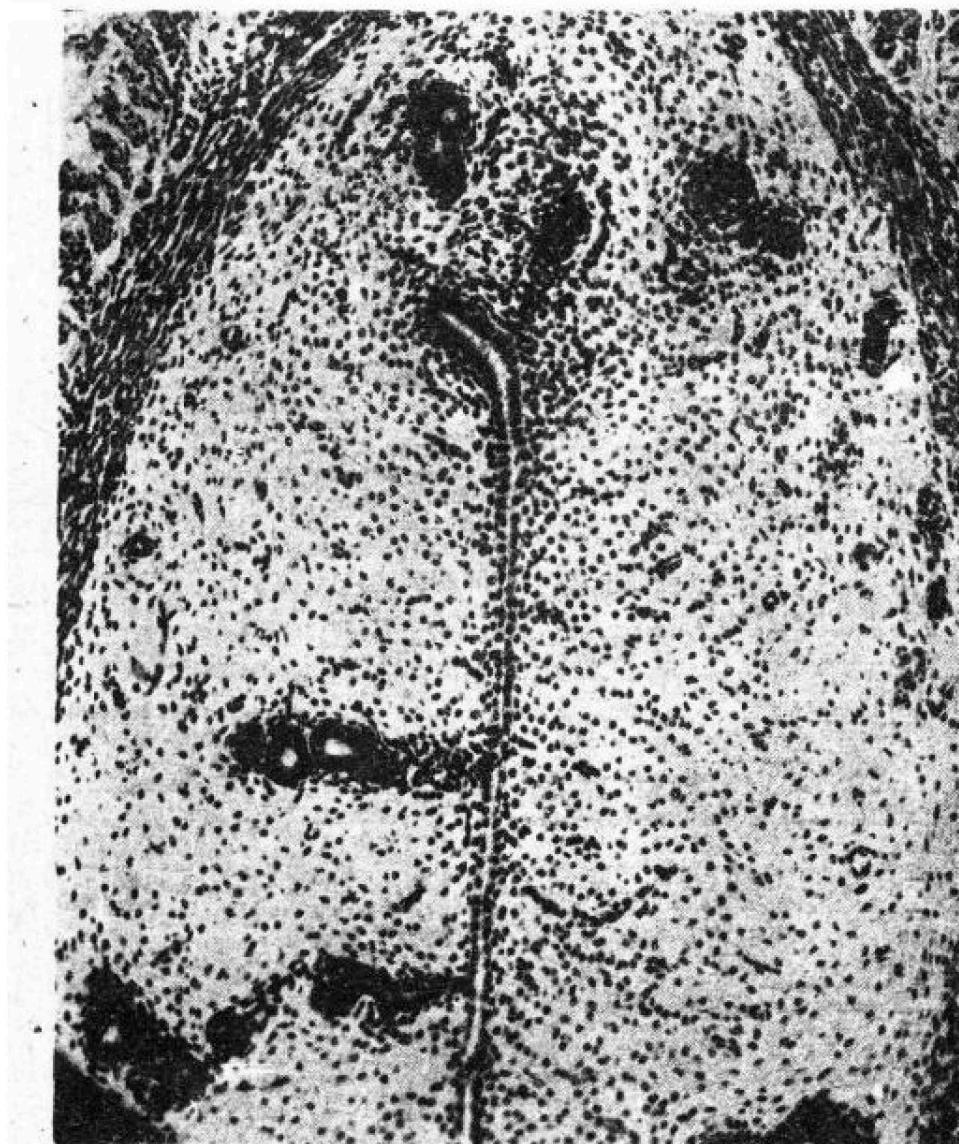


FIG. 4. A photomicrograph of a cross section of a uterine horn after 6 hours of treatment *in vivo* with 10 µg. of estradiol.

While this is a limited picture of the situation which prevails at any one instant, it must be taken into account that the cell is in a continuous state of flux. Being endowed with the capacity for polymeric syntheses, the cell is continually generating the membranous entities forming its compartmental structure, the protein catalysts, and the templates for protein synthesis. *In fact the cell's chief mode of expressing a response to a hormone lies in the cumulative effects of polymeric synthesis or degradation.*

## II. ALTERATIONS IN UTERINE METABOLISM IN RESPONSE TO ESTROGENS

1. *The Biological Response*

Among all the physiological effects of estrogens, the most striking is its action upon the uterus. In response to a single physiological dose of a natural estrogen, the atrophic uterus of the ovariectomized female rat is rapidly converted to an actively growing organ. As early as 1 hour after the

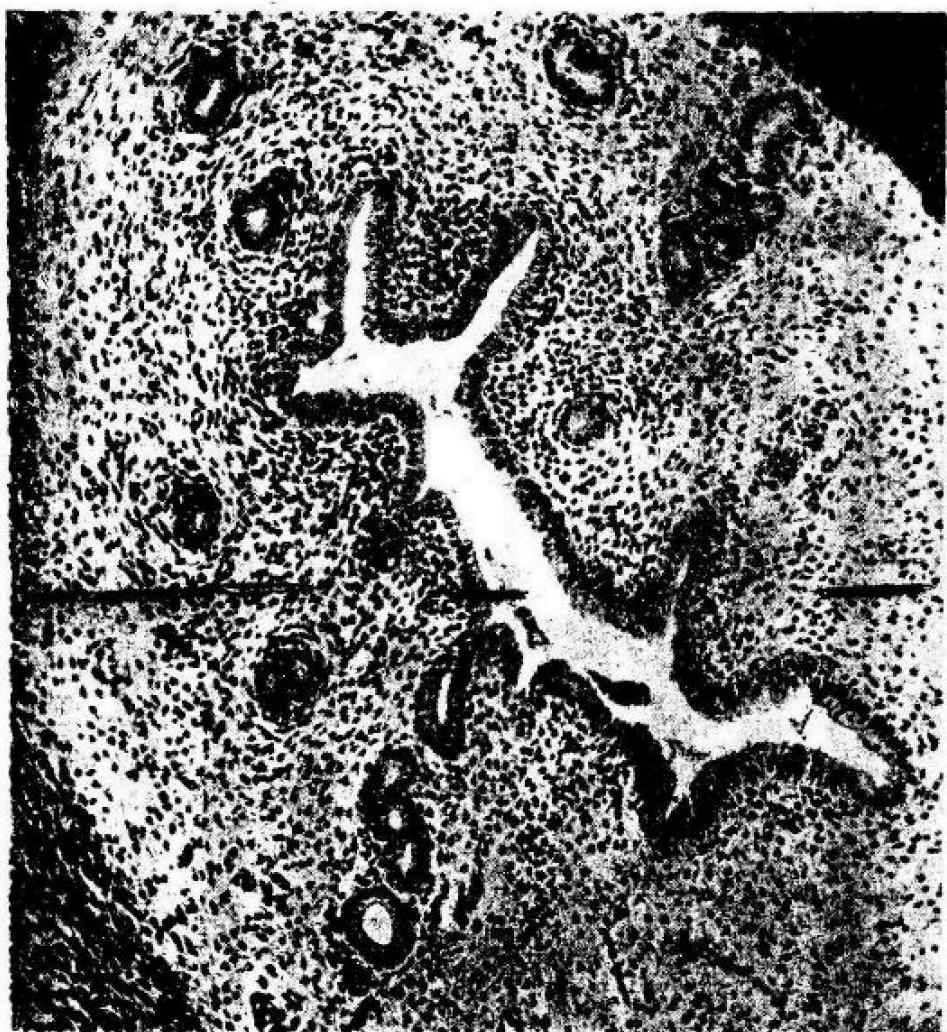


FIG. 5. A photomicrograph of a cross section of a uterine horn after 24 hours of treatment *in vivo* with 10 µg. of estradiol.

administration of the hormone there is a generalized hyperemia of the tissue (17), followed by an accumulation of fluid throughout the structure. This imbibition of water reaches a maximum after 4-6 hours of hormonal treatment (2, 24). At its peak the histological sections (Figs. 3 and 4) reveal a general increase in the spacing between nuclei, associated with an increase in the cell size. This response is evident throughout the uterine structure but appears most prominent in the endometrium (1). After 12 hours the dry weight begins to accumulate and is followed by a second cycle of water

TABLE I

*Effect of Estradiol on the Accumulation of Protein and Nucleic Acid in Rat Uteria* (13)

Hours of estradiol pretreatment	$\mu\text{Moles thymine}/$ $\text{flask}$	$\text{Mg. protein}/$ $\mu\text{mole thymine}$	$\mu\text{Moles uridine}/$ $\mu\text{mole thymine}$
Control	1.46	20.4	0.097
	1.43	19.5	0.102
6	1.19	20.4	0.099
	1.47	18.8	0.100
12	1.58	24.6	0.126
	1.51	23.0	0.135
18	1.45	24.6	0.167
	1.68	26.5	0.178
24	1.45	29.5	0.219
	1.48	30.1	0.222

<sup>a</sup> Ten micrograms of estradiol were given at zero time. DNA measured as micromoles thymine; RNA measured as micromoles uridine and compared as ratio: micromoles uridine per micromole thymine. Nucleic acid bases were isolated chromatographically.

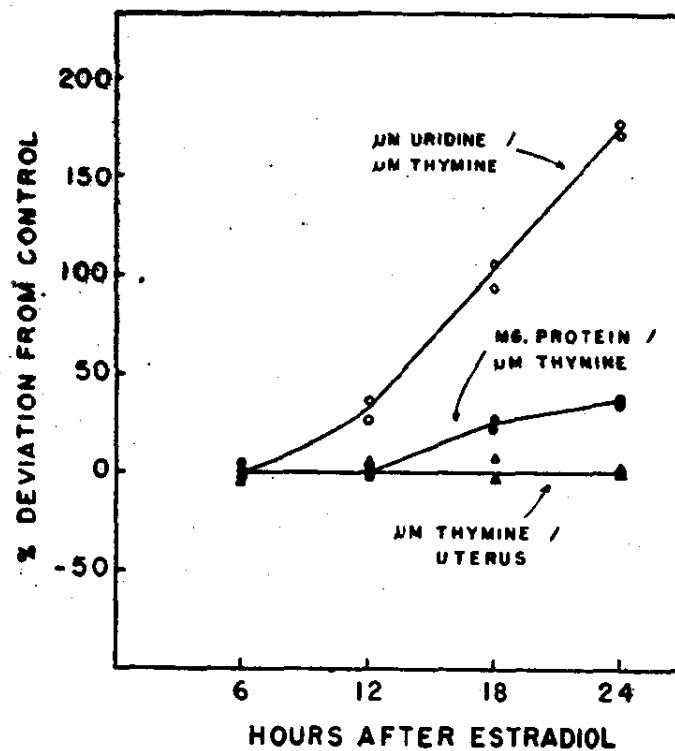


FIG. 6. Alterations in uterine composition following a single dose of estradiol. Ten  $\mu\text{g}$ . estradiol injected at zero time. DNA measures as micromoles thymine. RNA measured as micromoles of uridine and compared as ratio: micromoles uridine per micromole thymine. The data are expressed as per cent deviation from control during the first 24 hours following hormonal treatment (13).

imbibition (24) which has been correlated with the elevated uterine solids and evidence of true tissue growth by 24 hours (Fig. 5).

To better orient these responses for further discussion, charts of early alterations in uterine composition observed in these studies following a single intravenous dose of estradiol (10) have been prepared (Table I). In Fig. 6 the data are expressed as per cent deviation from the control level. The first 6 hours may be regarded as the *induction phase*; it is evident that while

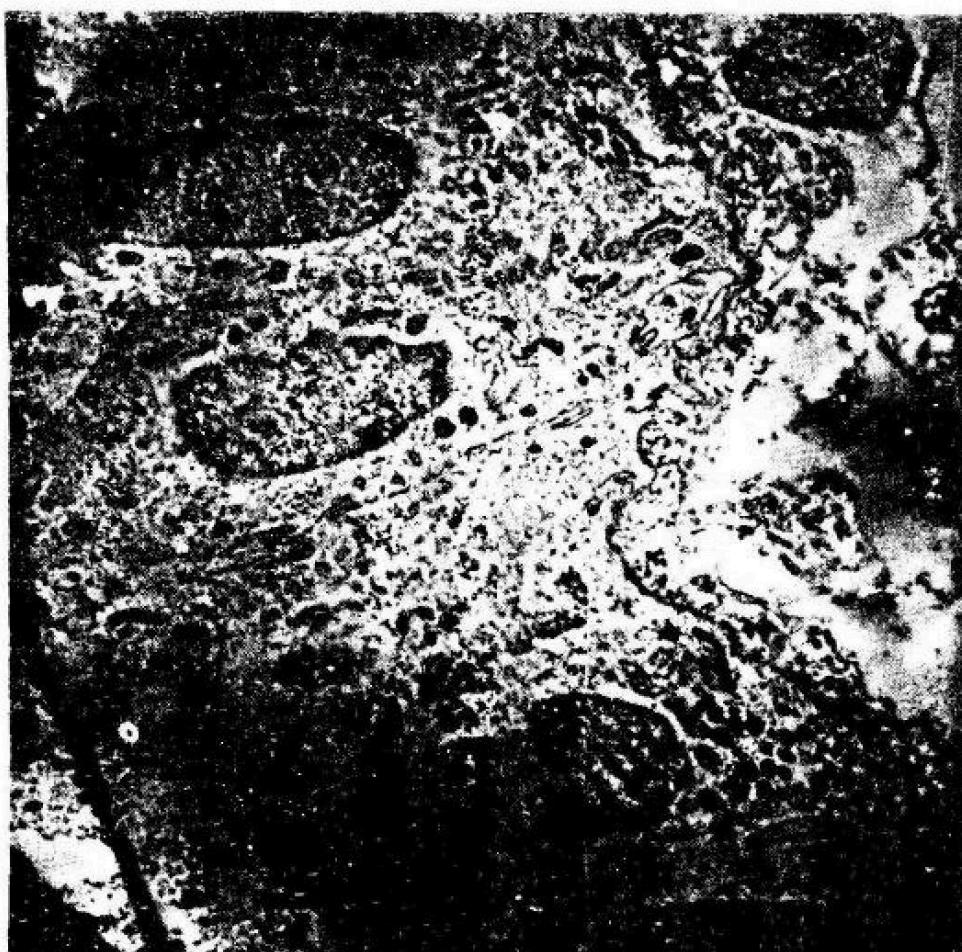


FIG. 7. An electron micrograph of the epithelial cell lining of the lumen of the uterine horn from a control rat. Tissue was fixed with buffered osmic acid. Magnified approximately 3800 times. The authors are very grateful to Miss Ann Holton for preparation of the electron micrographs of the rat uterus. The authors also wish to acknowledge the assistance of Dr. Hans Ris in the electron-microscopic studies.

there has been a dramatic change in water content of the tissue, little or no accumulation of protein, ribonucleic acid (RNA), or deoxyribonucleic acid (DNA) occurs during this period. The period from 6 to 24 hours is referred to as the *RNA-accumulation phase*, and it is evident that the protein accumulates in the wake of the rising RNA content of the uterus. These findings are in agreement with those of Telfer (27). While no increase in DNA occurs in the initial 24 hours of the response, if the hormonal stimulus is continued a *DNA-synthesis phase* does occur sometime between 40 and

72 hours (27). Thus the initial response is one of hypertrophy rather than hyperplasia—in fact the hypertrophy appears to be a necessary step in preparation for hyperplasia.

Evidence of this process is also revealed in a comparison of electron micrographs of the epithelial cells lining the uterine lumen after varying periods of hormone treatment (Figs. 7 and 8). In the period from 0 to 6 hours the cells become swollen, the mitochondria become vesiculated, and the endo-

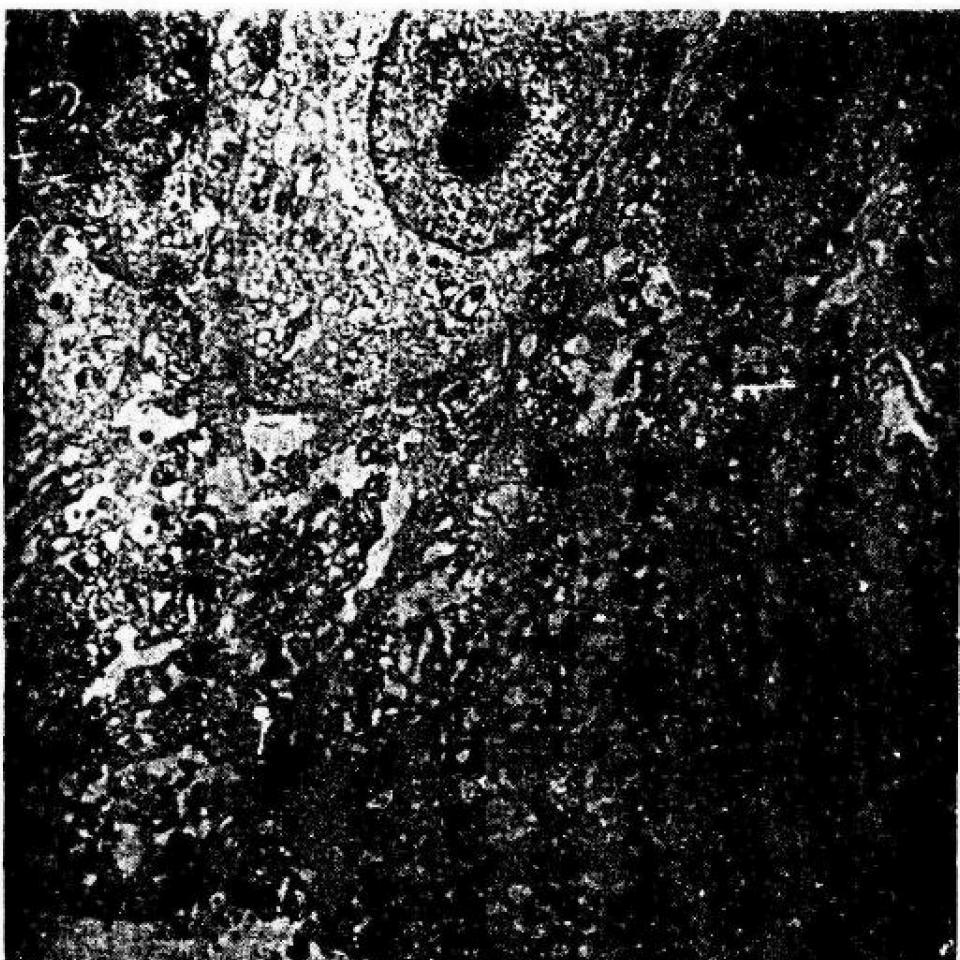


FIG. 8. An electron micrograph of the epithelial cell lining of a rat uterine horn 24 hours after a single 10- $\mu$ g. dose of estradiol. Magnification:  $\times 3800$ . In comparison with Fig. 7 note the increase in nucleolar size, amount and dilatation of endoplasmic reticulum, and cell enlargement.

plasmic reticulum spreads apart. Thereafter the endoplasmic reticulum becomes more abundant, and a striking increase in the size of the nucleolus occurs by 24 hours. These changes are in agreement with the changes in uterine composition just described, since the nucleolus and the granules of endoplasmic reticulum are known for their high RNA content.

## 2. General Procedure

In the sections which follow, evidence of early alterations of various areas of uterine metabolism by estrogenic hormones will be presented. For the

most part the data have been derived from experiments with isotopic precursors designed to show the sequence of changes in certain metabolic pathways with respect to time of estrogen treatment. In the procedure utilized in this laboratory, ovariectomized rats are injected with a single dose of estradiol via the tail vein. For this purpose the hormone is dissolved in a buffered saline solution (19). After varying periods the rats are sacrificed and the uterine horns are removed, incised longitudinally, and cut into small, equal segments. These segments are incubated at 37° C. in a glucose-balanced salt solution (19) containing a radioactive precursor in an atmosphere of oxygen. After incubation the enzymatic reactions are stopped with acid and the tissue is separated into the following fractions: acid-soluble, lipid, nucleic acids, and protein (22). Specific isolations are carried out on these fractions as will be described; in addition, carbon dioxide was collected whenever indicated. In certain experiments a tissue culture medium was used as the incubation mixture; these will be described separately.

### 3. Early Effects on Nucleic Acid Metabolism

Following ovariectomy, the RNA content of the rat uterus falls to a very low level, which is subject to rapid restoration on administration of estradiol (Fig. 6). This observation suggested that the level of the uterine RNA was uniquely dependent on estrogen levels in the animal and that some steps in nucleic acid synthesis might be highly susceptible to estrogen action. Accordingly a partial dissection of nucleic acid metabolism was undertaken with a variety of  $C^{14}$ -labeled precursors.

In Fig. 9 the results of a typical experiment are presented in which formate- $C^{14}$  and glycine-2- $C^{14}$  have been used as precursors for nucleic acid purine synthesis (22). For this purpose surviving uterine segments from control rats and rats pretreated for 6 hours with 10  $\mu$ g. of estradiol were incubated for 2 hours in a glucose-balanced salt medium containing the labeled precursors. The results demonstrate a striking increase in the incorporation of labeled formate into both adenine and guanine of the mixed nucleic acid fraction (i.e., fourfold increase). A similar situation was also observed with glycine-2- $C^{14}$ . Here, however, the inclusion of a pool of non-radioactive formate acted as an efficient trap for the one-carbon fragment and decreased the amount of labeling strikingly (i.e., positions 2 and 8). The residual incorporation is very probably due to the incorporation of the intact glycine molecule into positions 4, 5, and 7 of the purine ring. An estrogen effect on this incorporation is also evident. The experiments point out especially the high sensitivity of "one-carbon" metabolism to the influence of estrogen action.

This conclusion is further borne out in experiments (9) in which serine-3- $C^{14}$  was used as the labeled precursor (Fig. 10). After 6 hours of *in vivo*

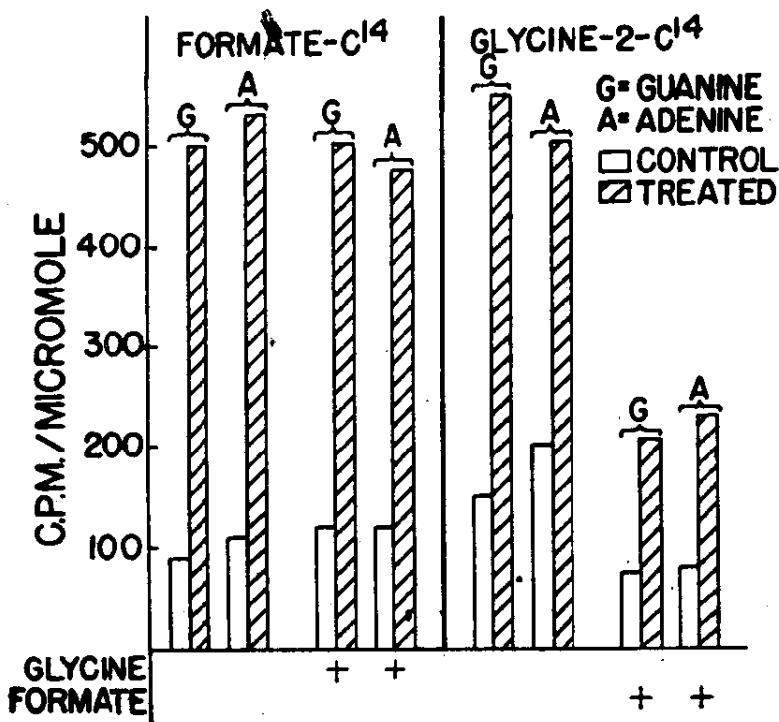


FIG. 9. The effect of pretreatment with estradiol on the incorporation of labeled formate and glycine into nucleic acid purines of surviving uterine segments. Uterine segments were from rats pretreated for 6 hours with a single 10- $\mu$ g. dose of estradiol. Incubation period was 2 hours with labeled formate or glycine. Data are expressed as counts per minute (C.P.M.) per micromole purine and taken from a publication by Mueller and Herranen (22).

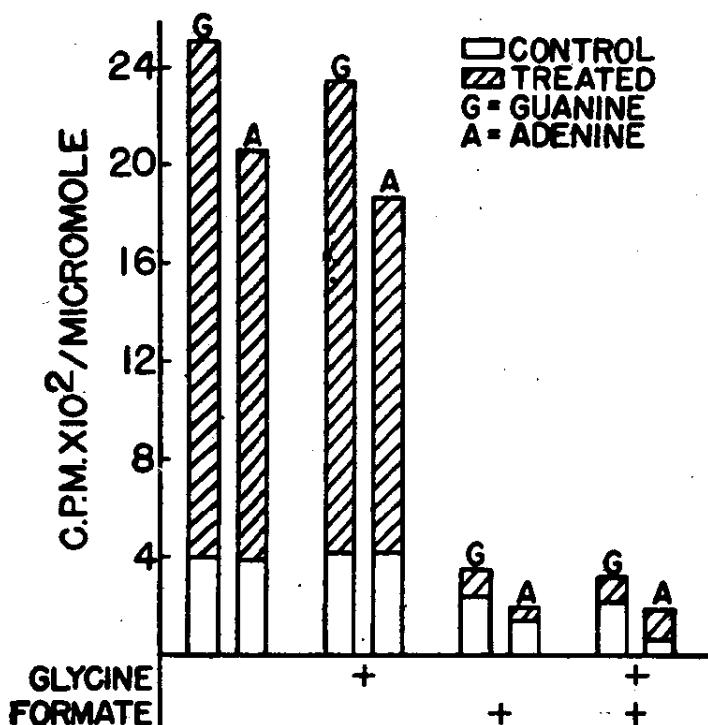


FIG. 10. The effect of pretreatment with estradiol on the incorporation of serine-3-C14 into nucleic acid purines of surviving uterine segments. Uterine segments were from animals pretreated for 6 hours with a single 10- $\mu$ g. dose of estradiol. Incubated with serine-3-C14 for 2 hours. Data, expressed as counts per minute per micromole purine, are taken from a publication by Herranen and Mueller (9).

hormonal treatment, the test segments incorporated 5—6 times as much labeled precursor into nucleic acid adenine and guanine as did the control segments. The inclusion of a nonradioactive pool of formate almost obliterated the labeling; thus the estrogen stimulation largely involved the formation of a "one-carbon" precursor from serine-3-C<sup>14</sup> and its subsequent incorporation into the purine structure. Carrier reisolation of formate pool from the reaction medium supported this conclusion in that the specific activity of the formate from the control flasks was 1310 c.p.m. (counts per minute) per micromole as compared with 2590 c.p.m. per micromole in the

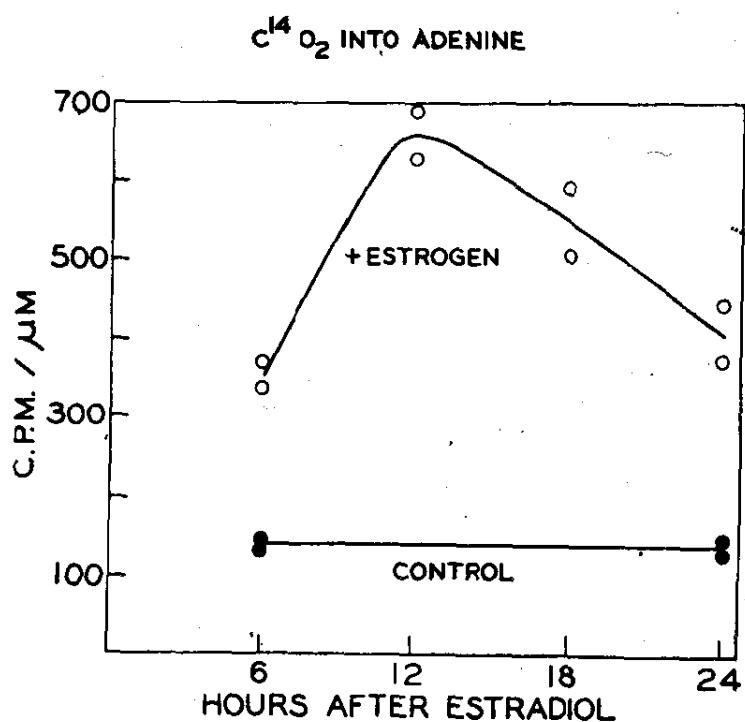


FIG. 11. The effect of pretreatment with estradiol on the incorporation of radioactive carbon dioxide into adenine of the mixed nucleic acids of surviving uterine segments. Ten  $\mu$ g. estradiol was injected at zero time; incubation period was 1 hour. Data are expressed as counts per minute per micromole adenine (13).

flasks containing the estrogen-treated segments. This difference, however, is not adequate to account for the observed influence of estradiol on purine labeling, and thus other steps or factors in purine synthesis must be susceptible to estrogen action.

The effect of estradiol treatment on the incorporation of radioactive carbon dioxide into nucleic acid adenine is shown in Fig. 11 and Table II. Again there is a marked stimulation of the labeling of adenine, which increases up to 12 hours and then declines. The reason for the decline is not completely established, but it has been demonstrated in other experiments that with a single 10- $\mu$ g. intravenous dose of estradiol, the level of active estrogen becomes limiting between 12 and 18 hours after the injection. Other factors,

however, may be operative here since the labeling is decreasing during a period when RNA is still accumulating rapidly. Results similar to those given for adenine also were obtained with guanine.

In similar experiments (Table II), uridine was isolated from the nucleic acid fraction. Surprisingly, no significant effect of estrogen was observed on the labeling of this pyrimidine base even during a period of rapid RNA-uridine accumulation (i.e., 12 hours). The labeling of thymine was also low; this was not surprising inasmuch as there was no demonstrable DNA accumulation at these early time periods.

TABLE II  
*Effect of Estradiol on Incorporation of C<sup>14</sup>O<sub>2</sub> into Uridine, Thymine, and Mixed Nucleic Acid Purines of Surviving Uterine Segments<sup>a</sup>* (13)

Estradiol pretreatment: Isolated component	6 Hours		12 Hours	
	Control	Estrogen	Control	Estrogen
Uridine	7500	7350	7850	8060
	7150	7250	8250	8100
Thymine	5	13	11	14
	11	14	12	16
Adenine	69	260	107	431
	66	228	96	472

<sup>a</sup> Uterine segments from rats pretreated 6 or 12 hours with 10 µg. of estradiol were incubated 1 hour with 5 µmoles of C<sup>14</sup>O<sub>2</sub>. Nucleic acid bases were isolated chromatographically. Data are expressed as counts per minute per micromole of nucleic acid base.

These results, indicating a lack of the hormonal (estrogen) effect on pyrimidine labeling, were supported by other studies with radioactive orotic acid (13) and suggested initially that the estrogen may have a specific action on purine metabolism. Accordingly it was decided to analyze the labeling of the acid-soluble pool in an effort to throw some light on this apparent segmental effect of the estrogen on nucleic acid metabolism. Initially, carrier experiments were conducted in which adenosine monophosphate (AMP), guanosine monophosphate (GMP) and uridine monophosphate (UMP) were added to aliquots of the acid-soluble fraction from a C<sup>14</sup>O<sub>2</sub> incorporation experiment and hydrolyzed to liberate the purine bases and UMP from the various nucleotide forms. The adenine, guanine, and UMP were then re-isolated and purified chromatographically to constant specific activity.

In Table III the effect of estradiol pretreatment on the total radioactivity found in the guanine of the acid-soluble fraction is shown. It is evident that even with 1 hour of *in vivo* pretreatment with estradiol the labeling of guanine of the acid-soluble nucleotides was enhanced; this effect was in-

creased to nearly a fourfold stimulation by 6 hours. Similar results were also obtained for adenine.

TABLE III  
*Effect of Estradiol on the Labeling of Guanine in the Acid-soluble Fraction<sup>a</sup>* (13)

<i>In vivo</i> pretreatment	Control	Plus estrogen
1 Hour	95	105
	84	100
3 Hours		263
		136
6 Hours	75	359
	78	368

<sup>a</sup> Ten micrograms estradiol injected intravenously at zero time. Segments from 3 uterine horns were incubated 1 hour with 5  $\mu$ moles  $\text{C}^{14}\text{O}_2$ ; 0.58  $\mu$ mole guanosine monophosphate carrier added to aliquot of acid-soluble fraction. Acid-soluble fraction was hydrolyzed 25 min. at 90°C. in 4% perchloric acid; guanine isolated chromatographically (13). Data expressed as total c.p.m. in guanine per micromole thymine.

In the carrier experiments for acid-soluble uridine-5'- $\text{PO}_4$  a surprising situation was encountered. In contrast to the lack of effect of estrogen on the labeling of uridine in the nucleic acid polymer, the total counts incorporated into the acid-soluble UMP were increased strikingly and progressively with time of estrogen pretreatment (Table IV). Thus, at the level of precursor nucleotides the hormonal effect was similar for both the purine and pyrimidine pathways of synthesis.

To explain the apparent discrepancy relative to the labeling of purines and

TABLE IV  
*Effect of Estradiol on the Labeling of Uridine-5'-Phosphate in the Acid-soluble Fraction<sup>a</sup>* (13)

<i>In vivo</i> pretreatment	Control	Plus estrogen
1 Hour	8,970	11,950
	9,550	9,100
3 Hours		12,500
		7,550
6 Hours	8,500	29,500
	10,400	27,400

<sup>a</sup> System as in Table III and 0.83  $\mu$ mole UMP added as carrier. After hydrolysis UMP was reisolated by ion exchange (Dowex 1, chloride form) and paper chromatography with *tert*-butanol-HCl as solvent system. Data expressed as total c.p.m. in UMP per micromole thymine.

pyrimidines in the nucleic acid fraction, it was necessary to determine the actual specific activity and pool size of the acid-soluble precursors from incorporation experiments with control estrogen-treated uteri. For this purpose it was necessary to combine the acid-soluble fractions from 12 uteri which had been incubated with  $C^{14}O_2$ ; the results of the isolation studies

TABLE V  
*Effect of Estrogen on Labeling of Acid-soluble Nucleotides and the Size of the Nucleotide Pools<sup>a</sup> (13)*

	Experiment I <sup>b</sup>		Experiment II <sup>c</sup>		Experiment III <sup>c</sup>	
	Adenine	UMP	Adenine		Adenine	UMP
$\mu$ Moles isolated	<i>C<sup>d</sup></i>	0.470 0.490	0.0060		0.238	0.0037
	<i>E<sup>e</sup></i>	0.565 0.540	0.011		0.282	0.024
Specific activity (c.p.m.: / $\mu$ mole)	<i>C</i>			2,465	2,270	30,600
	<i>E</i>			6,465	9,630	26,700
Total c.p.m. incorporated	<i>C</i>			1,080 945		141
	<i>E</i>			4,260 4,560		783

<sup>a</sup> Uterine segments incubated with  $NaHC^{14}O_3$  (except Exp. I). Acid-soluble fraction was hydrolyzed 25 min. at 90°C. in 4% perchloric acid. Adenine and UMP were isolated by ion exchange and paper chromatographic techniques. For the determination of total c.p.m., 1  $\mu$ mole each of AMP and UMP were added as carriers to an aliquot of the acid-soluble fraction. The data are expressed as micromoles adenine or UMP per micromole thymine, c.p.m. per micromole adenine or UMP, and total c.p.m. incorporated per micromole thymine.

<sup>b</sup> Experiment I: The data are obtained from 6 control and 6 estrogen-treated rats. No radioactivity measurements.

<sup>c</sup> Experiments II and III: Data obtained from 12 control and 12 estrogen-treated rats which had been incubated 1 hour with  $C^{14}O_2$ .

<sup>d</sup> C = controls.

<sup>e</sup> E = 6 hours pretreatment *in vivo* with 10  $\mu$ g. estradiol.

are shown in Table V. In experiment III it is demonstrated that the UMP pool increased strikingly with estrogen treatment, while that of the adenine nucleotides increased only slightly. In the case of the UMP this expansion of the pool size was just sufficient to compensate for the estrogen stimulation on the labeling process; the result was that the specific activities of the

UMP nucleotide precursors for nucleic acid synthesis were similar in control and estrogen-treated uteri. In the case of adenine, the specific activity of the pool increased and was reflected in the amount of radioactivity incorporated into the nucleic acid polymer. Thus an apparent discrepancy is explained and a new problem is introduced; for the questions now are why the UMP pool expands as an early response to estrogen treatment and what is the sense of the nucleotides.

In this connection it is interesting to note that the adenine nucleotide pool is approximately tenfold larger than the uridine pool; however, the estrogen-induced increase in each pool is in the same range. It seems likely, therefore, that the similar absolute expansions for both pools is more than coincidental. One explanation might be that the early action of the hormone is associated with some reshuffling of nucleic acid polymers (presumably RNA), during which process a certain amount of nucleic acid is broken down to the nucleotide level; this would be nonradioactive and could be anticipated to produce the observed effects. In the case of the adenine nucleotides this contribution would be superimposed on a relatively large pool of nucleotides, whereas the contribution to the acid-soluble uridine pool results in a major expansion and dilution of this nucleotide pool. This situation has its analogy in the fluctuating RNA content of bacteria during the conversion from a resting state to one of active growth (4, 30).

As a final consideration of nucleic metabolism in the rat uterus, the question was asked as to whether or not estrogen pretreatment influenced the utilization of preformed purines for nucleic acid synthesis. Other workers have demonstrated that they may be utilized in accordance with the diagram in Fig. 12. Accordingly, uterine segments were incubated with adenine-8-C<sup>14</sup>,

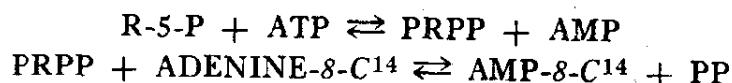


FIG. 12. The metabolic pathway for the utilization of preformed purines. R-5-P = ribose-5-phosphate; ATP = adenosine triphosphate; PRPP = phosphoribosyl pyrophosphate; AMP = adenosine monophosphate; PP = pyrophosphate.

and the results are expressed in Table VI. It is clearly demonstrated that pretreatment with the hormone also facilitated the utilization of adenine-8-C<sup>14</sup> for both adenine and guanine of the nucleic acid structure. As one might expect, owing to the intervention of metabolic pools, the nucleic acid adenine was much more radioactive than was the guanine formed indirectly from this precursor.

In a consideration of the over-all picture of the estrogen effect on nucleic acid metabolism in rat uteri, it can be concluded that the data depict a general stimulation of nucleotide synthesis and that the labeling of the nucleic

acid polymers reflects mainly the specific activity of the precursor pools of acid-soluble nucleotides. Thus the labeling of positions 2, 4, 5, 6, and 8 of the purine ring systems are all susceptible to the action of the hormones. This is further borne out by the stimulating effect of estradiol on pyrimidine nucleotide synthesis, which is apparent when one examines the acid-soluble pool.

TABLE VI  
*Effect of Estradiol on the in Vitro Labeling of Nucleic Acid Purines with Adenine-8-C<sup>14</sup>a*

	Control	Plus estrogen
Adenine	4,500	11,500
	4,840	9,950
	5,350	9,200
Guanine	313	1,145
	322	1,120
	350	1,095

<sup>a</sup> Six hours pretreatment with 10 µg. estradiol. Segments from 4 uterine horns, 1 µmole adenine-8-C<sup>14</sup> ( $3.7 \times 10^6$  c.p.m.) in 2.0 ml. Robinson medium containing 0.022 M glucose. Incubation time, 1 hour in oxygen atmosphere at 37°C. Mixed nucleic acid purines isolated according to the method of LePage (10). Data expressed as c.p.m. per micromole purine.

In order to establish which steps in the respective synthetic operations are rate-limiting and are directly or indirectly under the influence of the estrogenic hormone, it will be necessary to dissect the pathways in cell-free systems and to evaluate the levels of individual enzymes and their substrate levels. In this connection it is interesting to note that the formation of PRPP and the activation of carbon dioxide represent steps which are common to both purine and pyrimidine nucleotide synthesis. On the other hand, both pathways are generally dependent on the activation of carboxyl groups through reactions involving the availability of ATP. It is anticipated, for reasons to be cited later, that the observed stimulation of nucleotide synthesis will be accounted for by actual increases in the enzymes necessary for these synthetic reactions and particularly by increases in certain enzymes which operate at rate-limiting steps.

#### 4. Early Effects on Protein Synthesis

The effect of a single injection of estradiol (10 µg.) on the rate of incorporation of glycine-2-C<sup>14</sup> into the proteins of surviving segments is shown in Fig. 13. It should be noted that with estradiol the rate of incorporation of glycine was accelerated in a linear manner over the first 12 hours; thereafter the stimulatory process gradually subsided and reached a peak incorporation rate at 20 hours. At this time an estradiol deficiency appeared to

exist, since a second injection of the hormone given at 16 hours promoted the acceleration in a linear manner well beyond this point (19). Similarly, if the second injection was made at 40 hours (on the descending limb of the curve) a complete reversion was obtained which resembled the initial response of the system to the hormone.

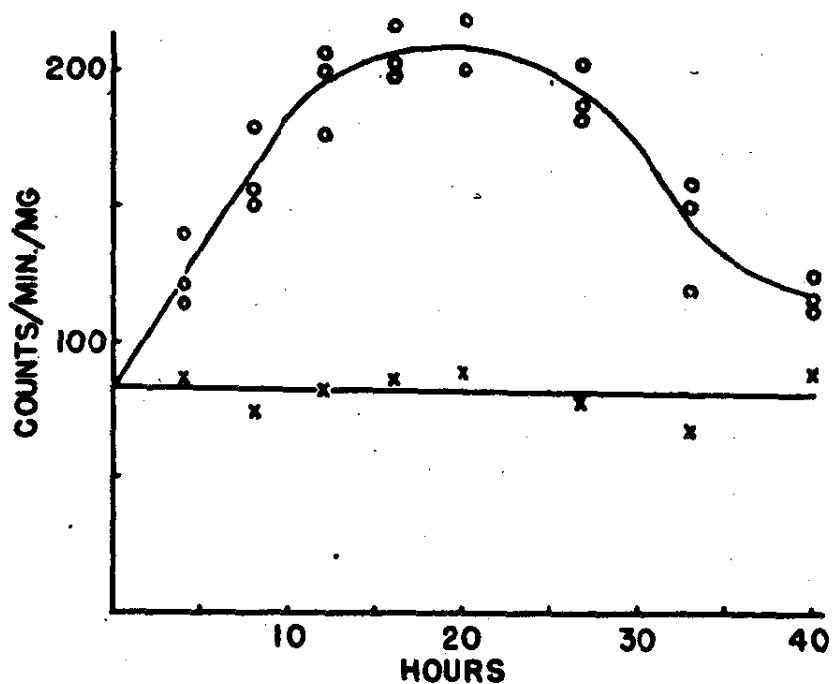


FIG. 13. The effect of a single dose of estradiol on the rate of incorporation of glycine-2-C<sup>14</sup> into protein of surviving uterine segments. Ten µg. estradiol injected intravenously at zero time. At the indicated times uterine segments were incubated with glycine-2-C<sup>14</sup> for 2 hours. Data are presented in counts per minute per milligram protein and are taken from a publication by Mueller (19).

The stimulation of amino acid incorporation into the protein of surviving uterine segments by *in vivo* pretreatment with estradiol occurred with all amino acids tested; the data for tryptophan, lysine, serine, and formate are given in Table VII and Figs. 14 and 15. It was observed that in general the stimulation was 70–100% after 6 hours of pretreatment with estrogen. In the case of formate, which was incorporated into serine prior to incorporation into protein, the stimulation was considerably greater, thus pointing to an additional estrogen-sensitive step in this reaction sequence.

These results suggested an estrogen-induced protein anabolism in general, and the question arose as to what part of the pathway for protein synthesis was influenced by the hormone. To investigate this area it was necessary to study the reactions of protein synthesis in cell-free systems. From the work of Zamecnik (32, 33), Hoagland (12), DeMoss (6), Berg (3), and others (5, 14, 15), a number of reactions involved in protein synthesis have been

demonstrated; these are presented diagrammatically in Fig. 16. The initial step in protein synthesis involves an activation of the carboxyl group of the amino acid with energy from ATP; the activated amino acid is next transferred as a highly active compound to some position amid the soluble RNA of the cell; in the presence of microsomes and GTP (guanosine triphosphate), the activated, RNA-bound amino acid is incorporated into protein via a true peptide linkage.

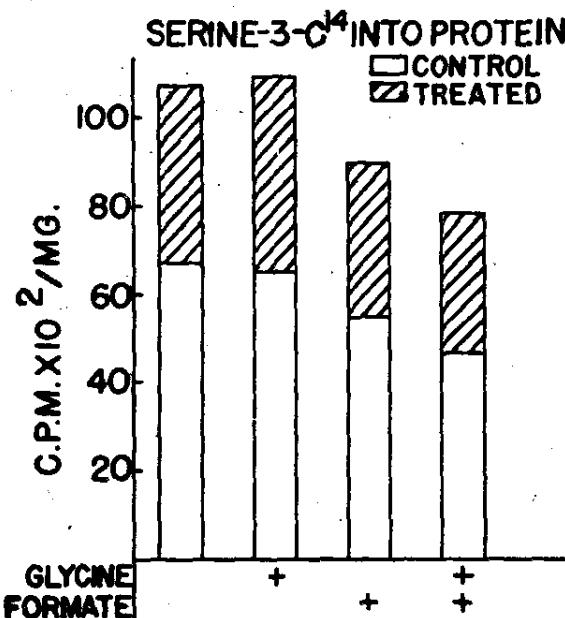


FIG. 14. Effect of estradiol pretreatment on the incorporation of serine-3-C<sup>14</sup> into protein of surviving rat uteri. Rats injected with 10 µg. of estradiol 6 hours prior to a 2-hour incubation study *in vitro*. Data are expressed as counts per minute per milligram of protein and are taken from a publication by Herranen and Mueller (9).

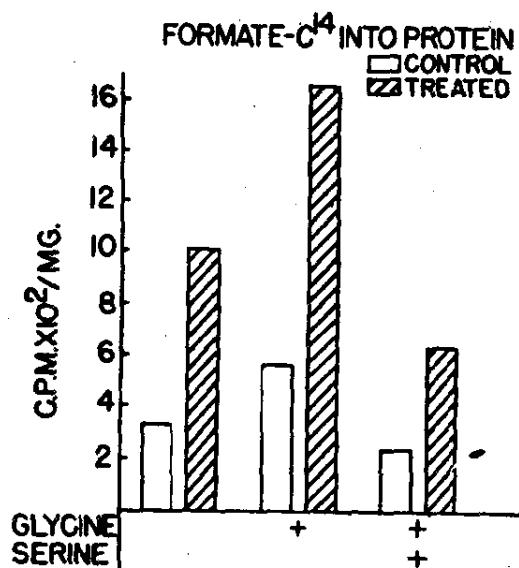


FIG. 15. Effect of estradiol on incorporation of radioactive formate into protein of surviving uterine segments. Rats were pretreated with 10 µg. of estradiol 6 hours prior to a 2-hour incorporation study. Data are expressed as counts per minute per milligram of protein and are taken from a publication by Mueller and Herranen (22).

TABLE VII  
*Effect of Pretreatment with Estradiol on the Incorporation of Amino Acids into Protein of Surviving Uterine Segments*

Amino acid <sup>a</sup>	C.p.m./flask	Molarity	C.p.m./mg. protein	
			Control	Estradiol-treated
Glycine-2-C <sup>14</sup>	2.15 × 10 <sup>5</sup>	1.0 × 10 <sup>-2</sup>	39	83
Tryptophan-β-C <sup>14</sup>	1.0 × 10 <sup>5</sup>	2.4 × 10 <sup>-4</sup>	134	234
Lysine-2-C <sup>14</sup>	1.1 × 10 <sup>6</sup>	1.3 × 10 <sup>-2</sup>	63	97

<sup>a</sup> In glycine and tryptophan experiments, rats were pretreated with 10 µg. estradiol 8 hours; 6 hours pretreatment was used in lysine experiment. Segments from 2 uterine horns were incubated in Robinson medium containing 0.022 M glucose and the labeled amino acid for a 2-hour period at 37.5°C. in an atmosphere of oxygen (19).

Since the initial step in the amino acid activation is reversible, it was possible to assay the enzyme involved in terms of the amino acid-dependent exchange of P<sup>32</sup>-pyrophosphate with ATP (5, 6, 12). For this purpose homogenates of control and estrogen-treated uteri were prepared in 0.05 M KCl and sedimented at 105,000g. The results of a representative experiment are shown in Fig. 17; individual amino acids were tested for their ability to catalyze the exchange reaction in supernatants from homogenates of control and estrogen-treated (12 hours) uteri. Of 20 amino acids tested only the 7 listed in this figure gave significant stimulation of the exchange reaction. It was also demonstrated that pretreatment with estradiol increased the level of activity for these enzymes; however, it is important to note that enzymes for the various amino acids responded independently.

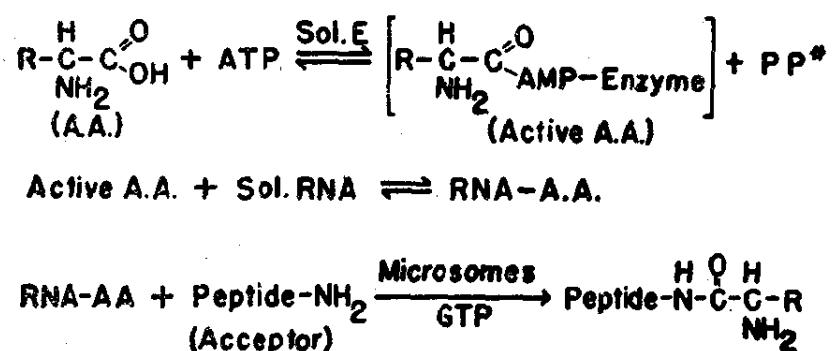


FIG. 16. Diagram of reactions involved in protein synthesis. PP\* = radioactive pyrophosphate (PP<sup>32</sup>).

When various mixtures of the 7 amino acids active in amino acid exchange were tested, it was found that their combined effects on the incorporation of P<sup>32</sup>-pyrophosphate into ATP was nearly equivalent to that expected from adding the activities of the individual amino acids (Table VIII). Thus it

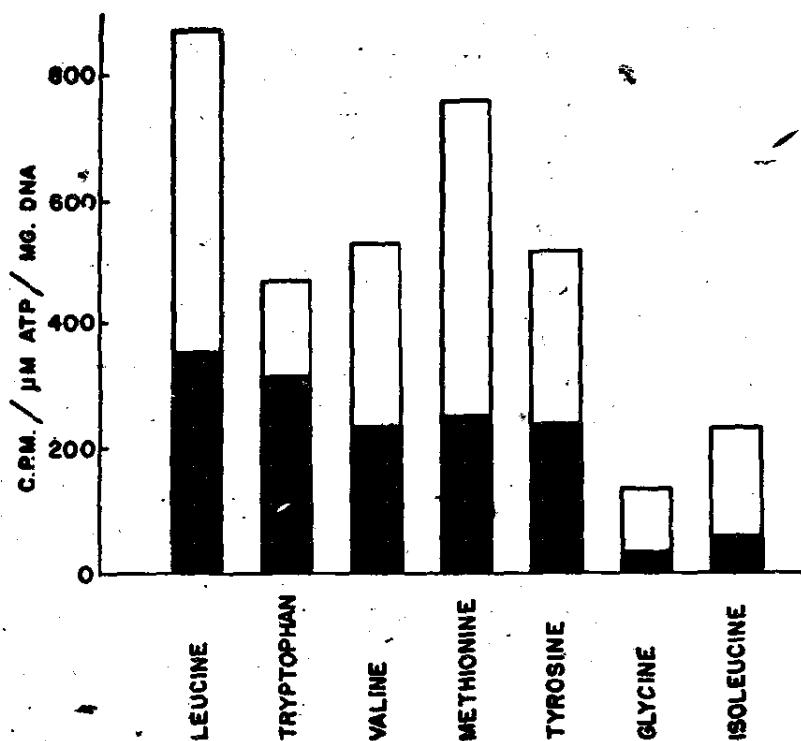


FIG. 17. Effect of *in vivo* estrogen treatment on the level of amino acid activating enzymes in the rat uterus. Ten micrograms of estradiol injected 12 hours prior to preparation of the soluble enzyme fraction. Data are expressed as counts per minute per micromole of ATP incorporated from PP<sup>32</sup> into ATP over a 15-min. incubation period by the enzyme derived from an amount of tissue containing 1.0 mg. of DNA. The dark bar represents the amino acid-dependent exchange in the enzyme preparation from control uteri; the open bar expresses the increment with estrogen treatment. The 7 amino acids which were active in promoting the PP<sup>32</sup>-ATP exchange were tested independently (16).

TABLE VIII  
Additivity of Individual Amino Acid Activities in the PP-ATP Exchange Reaction<sup>a</sup>

Amino acid tested	C.p.m./μmole ATP	
	Observed	Expected
Leucine	168	
Tryptophan	149	
Valine	112	
Tyrosine	111	
Methionine	119	
Glycine	11	
Isoleucine	29	
Leu + Try + Val + Tyr		543
Met + Gly + Ileu	150	159
All 7 amino acids	621	702

<sup>a</sup> 0.5 Ml. of soluble enzyme fraction from rat uteri was incubated 15 min. with 5 μmoles ATP, 1 μmole PP<sup>32</sup> [19,100 c.p.m. per micromole], 2 μmoles MgCl<sub>2</sub>, 5 μmoles Tris buffer (pH 7.6), and 5 μmoles of the indicated amino acid (16). Enzyme preparation contained 3.3 mg. protein per milliliter.

appears that a specific enzyme is involved in the activation of each amino acid; this finding is in agreement with the data of other workers (6,12). In addition, these enzymes are completely specific for the L-amino acid isomer, and no exchange was observed when P<sup>32</sup>-inorganic phosphate (Table IX) was substituted for pyrophosphate.

TABLE IX  
*Substrate Specificity for the PP-ATP Exchange Reaction<sup>a</sup>*

	Amino acid added	C.p.m./μmole ATP
E + PP <sup>32</sup>	None	820
E + PP <sup>32</sup>	L-leucine	1704
E + PP <sup>32</sup>	D-leucine	726
E + PP <sup>32</sup>	L-tryptophan	1322
E + PP <sup>32</sup>	D-tryptophan	666
E + PP <sup>32</sup>	L-valine	969
E + PP <sup>32</sup>	D-valine	790
E + PP <sup>32</sup>	None	8
E + PP <sup>32</sup>	L-leucine	11

<sup>a</sup> 0.5 ml. of soluble enzyme fraction from rat uteri was incubated 15 min. with 5 μmoles ATP, 1 μmole PP<sup>32</sup> [19,100 c.p.m. per micromole], 2 μmoles MgCl<sub>2</sub>, 5 μmoles Tris buffer (pH 7.6), and 5 μmoles of the indicated amino acid (16). Enzyme preparation contained 3.3 mg. protein per milliliter.

An important aspect of this study is the demonstration that estrogen treatment increased the levels of a number of amino acid-activating enzymes. A plot of this response with varying periods of hormonal treatment is given in Fig. 18. It is of interest that the enzyme activity rose very rapidly in the first 6 hours, at a time when actual protein and RNA accumulation were not yet measurable. Thus the acceleration of amino acid incorporation in surviving-uterine segments by estrogens is explained in part, if not entirely, by a rapid rise in the level of the soluble amino acid-activating enzymes per cell. The presence of possible insoluble or particulate forms of the amino acid-activating enzymes could not be tested for in this manner owing to interfering reactions.<sup>4</sup>

##### 5. Effect of Pretreatment with Estradiol on the Serine Aldolase Activity in the Uterus

As cited under heading II, 3; evidence was obtained for an early estrogen effect on both the cleavage of serine to a "one-carbon" compound plus glycine and the synthesis of serine from formate and glycine. Since this is

<sup>4</sup> The amino acid activation studies were carried out in collaboration with Dr. D. J. McCorquodale who held a post-doctorate fellowship from the American Cancer Society (16).

a reversible reaction it was decided to assay for the level of serine aldolase which catalyzes this process; the scheme for this reaction is shown in Fig. 19. All attempts to use radioactive formate as the "one-carbon" precursor were unsuccessful, owing to a failure of the homogenate system to activate this

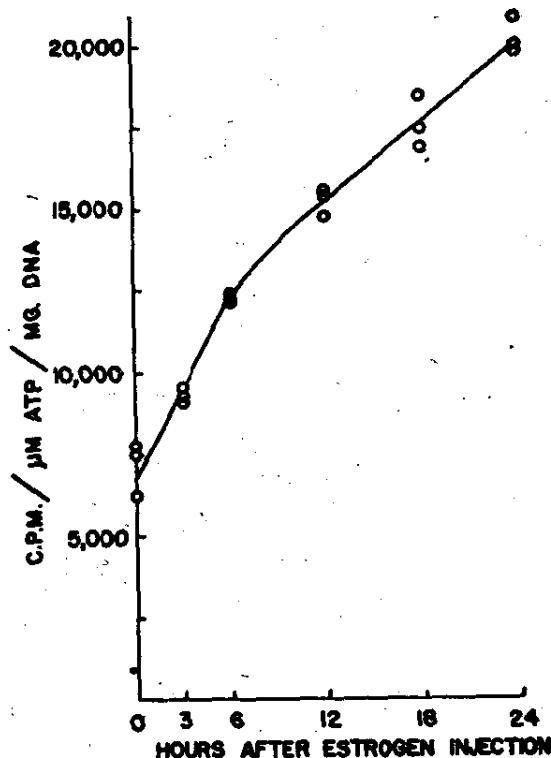


FIG. 18. Time curve on the combined response of 4 amino acid-activating enzymes following a single 10-μg. dose of estradiol. A mixture of leucine, methionine, tryptophan, and valine were used as substrates for the PP<sup>32</sup>-ATP exchange system. Data are expressed as counts per minute per micromole of ATP by the enzyme from an amount of tissue containing 1.0 mg. of DNA (16).

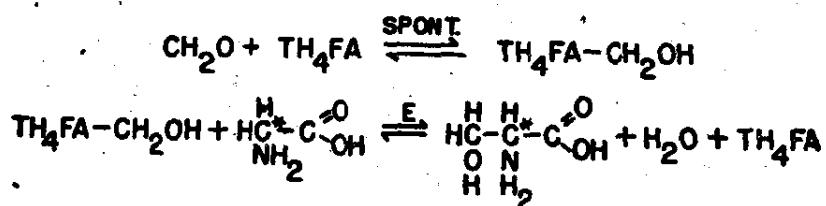


FIG. 19. A diagram of the serine aldolase system. First reaction proceeds spontaneously; second reaction catalyzed by serine aldolase (E).

compound. However, formaldehyde reacts spontaneously with tetrahydrofolic acid as shown in the first reaction of the diagram, and the resulting hydroxymethyl derivative donates the one-carbon fragment to serine synthesis very effectively in the presence of glycine-2-C<sup>14</sup> and the enzyme, serine aldolase. The activity of the latter enzyme can thus be assayed by the amount of radioactive serine formed per unit time under controlled conditions.

The effect of varying times of estrogen pretreatment on the level of serine aldolase activity is observed to rise at a linear rate without apparent lag (Fig. 20); thus again one of the biological responses to the hormone observed in intact uterine segments can be explained at least in part by an increase in activity of the enzyme which catalyzes the specific reaction (10).

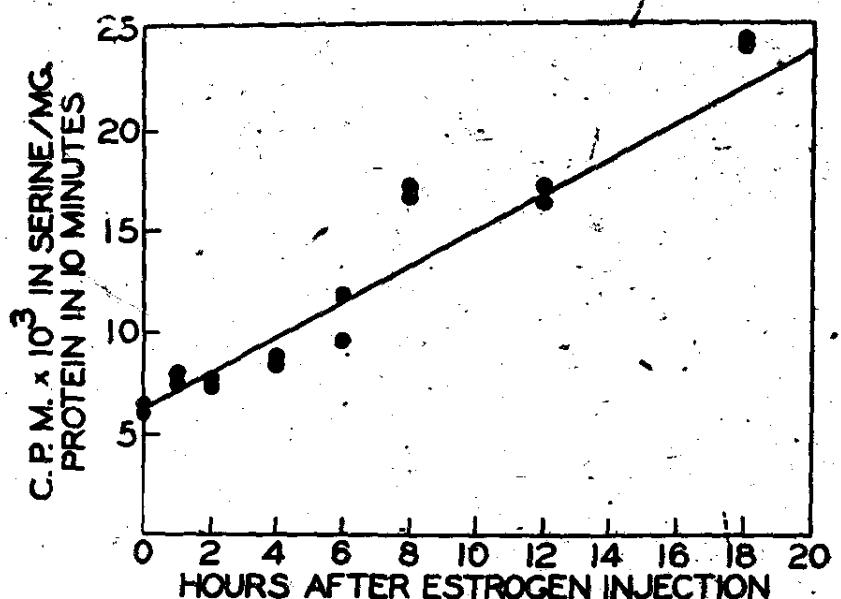


FIG. 20. Effect of estradiol pretreatment on the levels of serine aldolase in the rat uterus. Ten per cent homogenates were prepared from uterine horns of rats pretreated with 10  $\mu$ g. estradiol for the indicated time. Data are expressed as counts per minute in serine per milligram protein residue of enzyme aliquot and are taken from a publication by Herranen and Mueller (10).

Whether or not all metabolic responses so far observed with intact segments can be explained in terms of increases in enzyme activities will have to await enzymatic analysis of the individual responses; while pool sizes and substrate availability may play tricks on the analysts from time to time, it is anticipated that the major responses will be accompanied by alterations in enzyme activities.

### III. THE *in Vitro* INDUCTION OF CERTAIN ANABOLIC PROCESSES IN SURVIVING UTERINE SEGMENTS

The above observations introduced an important question: Do the observed increases in enzyme activity in response to estrogen result from an activation of some pre-existing protein (i.e., a pro-enzyme), or do they indicate *de novo* synthesis of new enzyme protein?

To answer this question in part, experimental efforts were directed toward testing for an *in vitro* activation of serine aldolase in uterine homogenates with a variety of estrogens. Despite fortification with a variety of cofactors (i.e., diphosphopyridine nucleotide, triphosphopyridine nucleotide, nicotina-

mide, adenosine triphosphate, creatine phosphate with creatine transphosphorylase, pyridoxal phosphate, ascorbic acid, cysteine, reduced glutathione, divalent ions, amino acid mixtures, and folic coenzymes) in various combinations, only marginal activation effects were observed; not every time, but fairly routinely, a 15—20% increase of serine aldolase activity was obtained on preincubation of uterine homogenates with the 4-hydroxy derivative of estradiol. Other estrogens were even less effective.

While suggestive, these results did not yield a definitive answer to the fundamental question posed. Therefore, it was decided to establish first the optimum conditions for the *in vitro* activation of surviving uterine segments with estrogens before undertaking further study in cell-free systems. This venture was encouraged by the limited success experienced earlier (20) with the activation of formate incorporation into protein by the 2- and 4-hydroxy derivatives of estradiol (Table X). Since these incubations were carried out

TABLE X  
*In Vitro Stimulation of Formate Incorporation into Protein in Surviving Uterine Segments with Hydroxylated Estradiols (20)*

Hormone added <sup>a</sup>	C.p.m./mg. protein
None	227
Estradiol-17 $\beta$	241
Estradiol	222
Estrone	200
2-Hydroxyestradiol	365
4-Hydroxyestradiol	356

<sup>a</sup> Ten micrograms of a hormone were added per flask. Segments were incubated for 3 hours at 37.5°C. in O<sub>2</sub>: (19, 20).

in a balanced salt medium containing glucose without further fortification with essential nutrients, uterine horn segments from ovariectomized rats were now incubated in Eagle's tissue culture medium (7) containing 10% bovine serum in the presence and absence of estrogenic hormones. After varying periods of incubation in Eagle's medium (HeLa) the segments were tested for their ability to incorporate glycine-2-C<sup>14</sup> into protein and, in addition, representative segments were homogenized and assayed for serine aldolase activity (10).

Contrary to expectations, the presence of estrogen in the medium produced little or no effect on the incorporation of glycine into protein or on the level of serine aldolase as compared with the controls. A surprising situation presented itself: incubation of the segments in tissue culture medium alone for a period of 18 hours resulted in a striking increase in the activity of both test systems (Table XI). This activation appeared to be dependent on keeping metabolic processes intact during the preincubation period. As

shown in Fig. 21, incubation at zero degrees did not yield an activated system; an oxygen atmosphere was also necessary. Similarly the presence of cyanide in the medium completely abolished the activation process.

TABLE XI  
*Effect of Incubation of Uterine Segments in Tissue Culture Media on Glycine Incorporation and Serine Aldolase Activities<sup>a</sup>* (11).

	Glycine incorporation (c.p.m./mg. protein)	Serine aldolase (c.p.m. in serine per aliquot)
Zero-time control	495	134
	620	135
18-Hour incubation	1230	257
	1350	242

<sup>a</sup> Conditions as in Fig. 21.

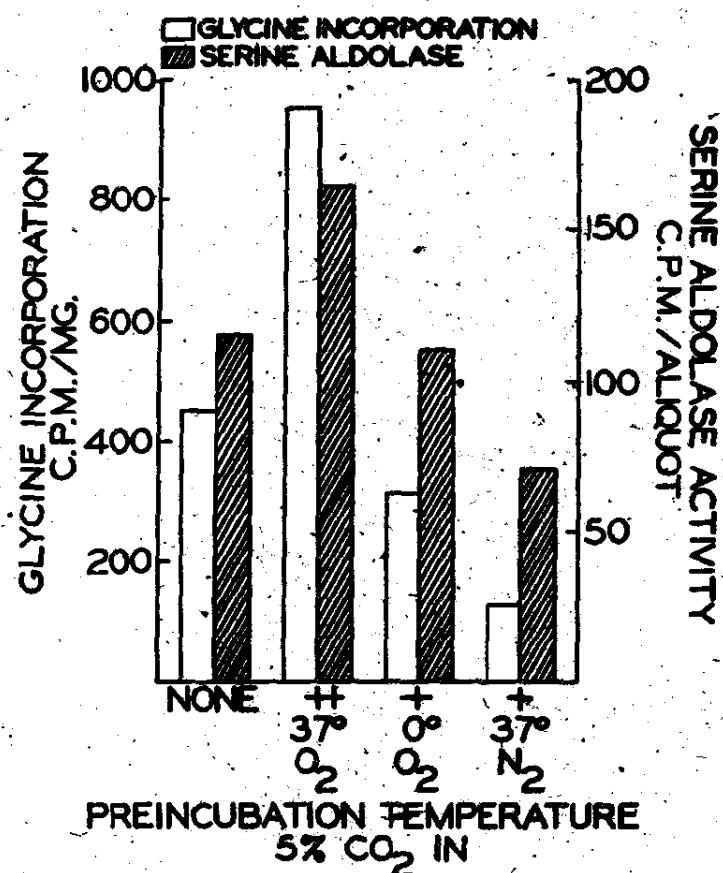


FIG. 21. The induction of anabolic effects on incubation of uterine segments in Eagle's tissue culture medium. Uterine horn segments from ovariectomized rats were incubated for 18 hours under the indicated conditions. Representative segments were homogenized and assayed for the level of serine aldolase activity. The ability of similar segments to incorporate glycine into protein was determined by the addition of 0.005 M glycine-2-C<sup>14</sup> to the reaction mixture after the preincubation period. The incorporation was followed over a 2-hour period (10). Data are expressed as counts per minute per milligram protein and counts per minute incorporated into serine per milligram of protein in a uterine homogenate (11).

Experiments in which various components of the incubation medium were omitted demonstrated that the activation process was partially dependent on the presence of the amino acids in Eagle's medium and on glucose, but that it probably did not require the presence of vitamins, serum, or glutamine (Table XII). It is important to keep in mind that for the relatively short period of these incubations (18 hours) some of these agents may be contained in adequate levels within the tissue itself. In addition, the replacement of bovine serum with serum from ovariectomized rats was also without effect, and from the data at hand it appears that the activation process does not require the presence of exogenous estrogen.

TABLE XII

*Effect of Medium Composition on the Activation of Glycine Incorporation in Vitro (11)*

Description	C.p.m./mg. protein
Eagle's medium, zero time control	336
	286
Eagle's medium, 18-hour incubation	845
	915
Minus amino acids <sup>a</sup>	525
	703
Minus glutamine <sup>a</sup>	880
	866
Minus vitamins <sup>a</sup>	974
	870
Minus serum <sup>a</sup>	864
	964
Minus glucose <sup>a</sup>	770
	803

<sup>a</sup> Uterine segments were preincubated for 18 hours in Eagle's medium from which the indicated constituents were omitted. Incorporation of glycine-2-C<sup>14</sup> (0.005 M) was followed over a 2-hour period.

Since both systems tested initially concerned the metabolism of glycine, the incubated segments were tested for their ability to incorporate leucine-2-C<sup>14</sup> into protein. Incubation of the segments in tissue culture medium also enhanced the incorporation of this amino acid into protein (Fig. 22).

Isolation of the purine bases from the mixed nucleic acid fractions (RNA + DNA) from uterine segments which have been incubated for varying periods of time in tissue culture medium prior to a 2-hour incorporation period with glycine-2-C<sup>14</sup> are given in Fig. 23. It was demonstrated that the ability of uterine segments to label both adenine and guanine of the nucleic

acid fraction increased rapidly during the first 12 hours of preincubation. The specific activity of the guanine was much higher than that of adenine, but this appeared to be owing in part to differences in the size of the respective acid-soluble nucleotide pools.

The *in vitro* induction without estrogens of metabolic changes which resemble the early *in vivo* action of estradiol on uterine metabolism introduced an important question: To what extent are these two anabolic states equivalent? While this question has not been settled entirely, a significant perspec-

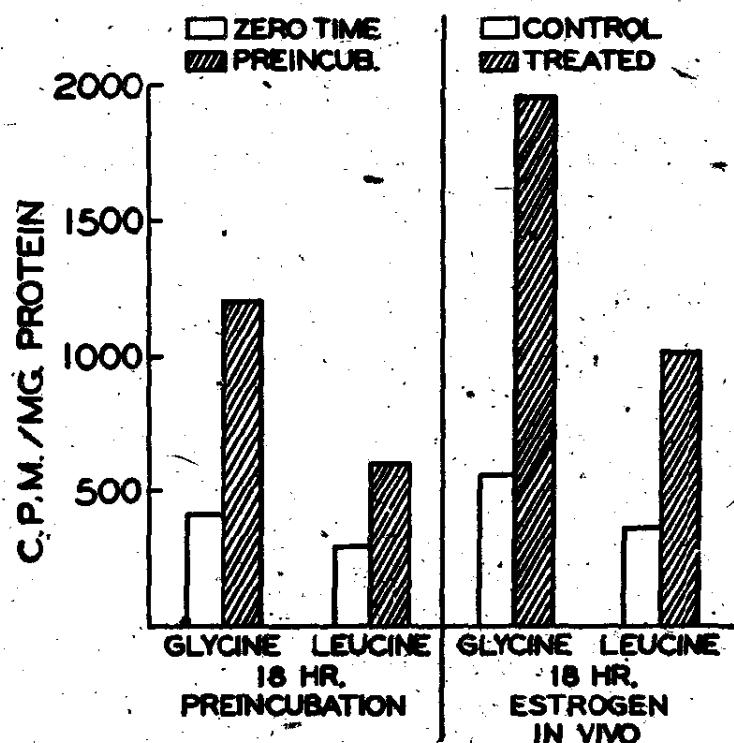


FIG. 22. Effect of incubation of uterine segments in Eagle's medium on the incorporation of leucine-2-C<sup>14</sup> into protein. Leucine (0.005 M) was added to the incubation flasks after 18 hours of preincubation and the incorporation of radioactivity was followed over a 2-hour period. Similar data are given for glycine. Also shown in the figure for comparison is the effect of 18 hours' pretreatment *in vivo* with a single 10- $\mu$ g. dose of estradiol on the incorporation of leucine and glycine into uterine segments (i.e., no incubation in Eagle's medium). Data are expressed as counts per minute per milligram protein (11).

tive was attained through testing the effect of varying periods of estradiol pretreatment *in vivo* on the response of surviving uterine segments to incubation in the tissue culture medium. The results of such an experiment are shown in Fig. 24.

It was observed that uterine segments from rat pretreated for 18 hours with a single dose of estradiol (10  $\mu$ g.) were highly activated for glycine incorporation as expected and as shown by the zero time incorporation studies. These segments, however, exhibited a very limited capacity for further activa-

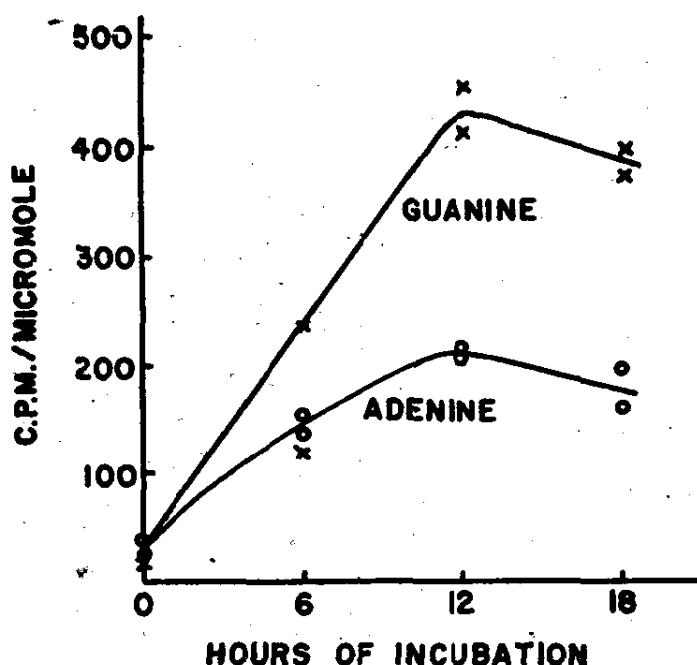


FIG. 23. The effect of preincubation of surviving uterine segments in Eagle's medium on the incorporation of glycine-2- $C^{14}$  into nucleic acid purines. Uterine segments were incubated for 6, 12, or 18 hours in Eagle's medium; at the specified interval 0.005 M glycine-2- $C^{14}$  was added to the incubation flasks and the incorporation into nucleic acid purines measured for a 2-hour period. Data are expressed as counts per minute per micromole of purine (11).

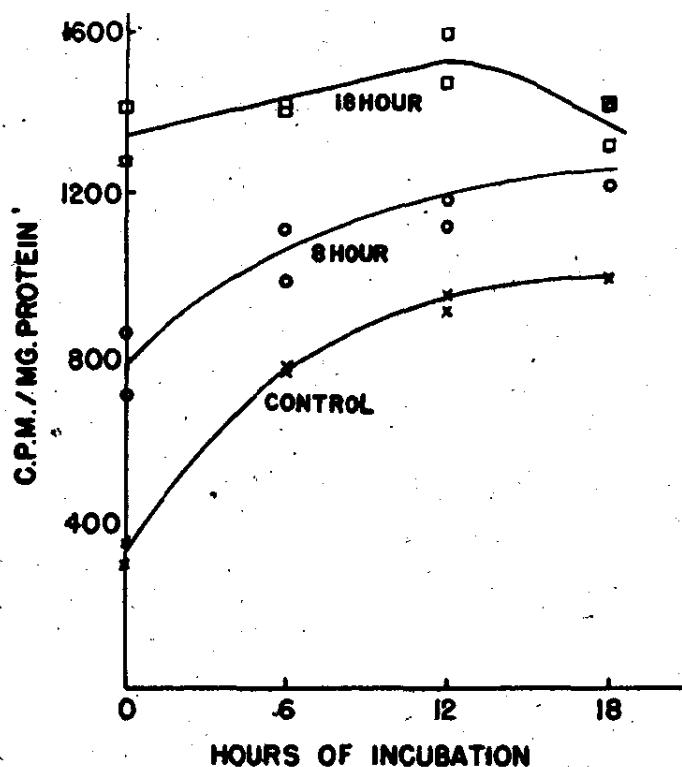


FIG. 24. The effect of estradiol pretreatment *in vivo* on the ability of uterine segments to be activated on incubation *in vitro* in Eagle's tissue culture medium. Uterine segments from rats pretreated for 8 and 18 hours with estradiol were incubated for indicated periods *in vitro* and tested for ability to incorporate 0.005 M glycine-2- $C^{14}$  into protein over a 2-hour period. Data are expressed as counts per minute per milligram of protein (11).

tion on incubation *in vitro* in the tissue culture fluid. With 8 hours and 12 hours (not shown) of estrogen pretreatment, intermediate responses were obtained. Thus, estrogen pretreatment *in vivo* activated the systems incorporating glycine into protein at the expense of the competency of the segments to respond anabolically to incubation *in vitro*.

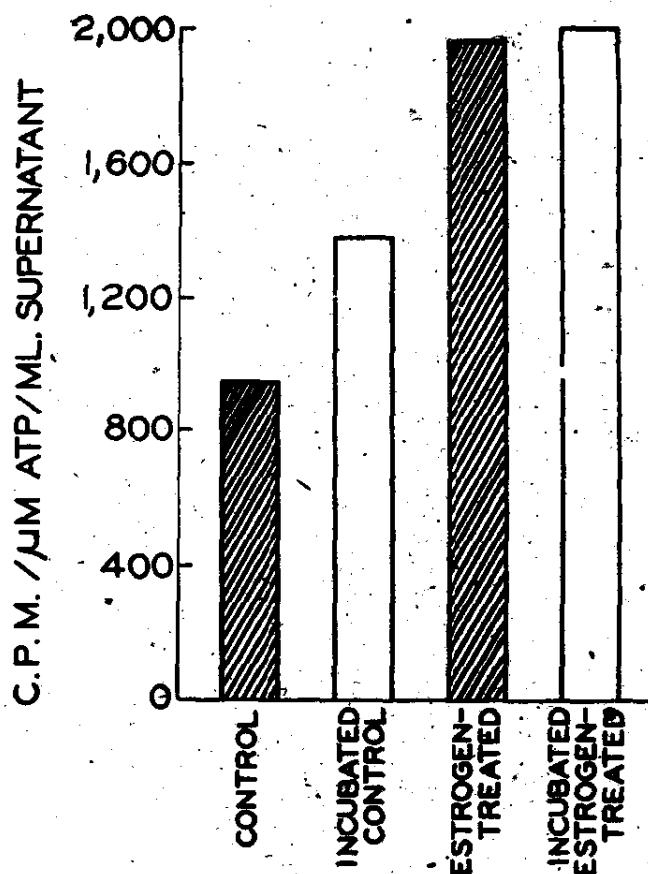


FIG. 25. The effect of estradiol pretreatment *in vivo* on the ability of uterine segments to increase the level of amino acid-activating enzymes on incubation *in vitro* in Eagle's medium. Pre-incubation period was 18 hours in Eagle's medium. Where indicated, estrogen treatment was a single 10- $\mu\text{g}$ . dose 18 hours prior to incubation studies. Segments were homogenized in 0.05  $M$  KCl and centrifuged 30 min. at 105,000 $\times g$  to obtain soluble protein fraction, which was assayed for amino acid-dependent  $\text{PP}^{32}$ -ATP exchange. A mixture of leucine, valine, tryptophan, tyrosine, methionine, isoleucine, and glycine was used as substrate in the exchange reaction. Data are expressed as counts per minute incorporated into 1  $\mu\text{mole}$  ATP per milliliter of supernatant enzyme solution (16).

A similar situation was observed when the levels of amino acid-activating enzymes were studied under comparable conditions. It was observed that incubation of control segments for 18 hours in Eagle's medium increased the combined activities of six soluble amino acid-activating enzymes by approximately 45%; in contrast, the *in vivo* activated segments from rats pretreated for 18 hours with 10  $\mu\text{g}$ . of estradiol failed to exhibit any further elevation of the activities of these enzymes on incubation *in vitro* (Fig. 25).

From these two aspects it would thus appear that the incubation of the segments in tissue culture medium alone accomplished much the same effect that the estrogen was able to do *in vivo*. This is *not* to say that the activated state was achieved through the same steps; rather it seems that the specialized hormonal mechanism for inducing an anabolic response in the uterus was bypassed by metabolic alterations which are even more fundamental to the induction of growth among cells in general.

Histological comparison of the tissue after 18 hours of incubation in tissue culture medium revealed a number of shrunken nuclei and some loss of surface epithelium (Figs. 26 and 27), but in many respects the picture is very similar to that in rat uteri following 6 hours of *in vivo* treatment with 10 µg. of estradiol (Fig. 4) in that an edematous state prevails throughout the endometrium.

#### IV. COMMENTS ON HORMONE ACTION AND ANABOLIC RESPONSES

In this study of estrogen action, an attempt has been made to dissect the sequence of metabolic alterations induced by the hormone. This work has been executed with the philosophy that such a characterization would point out the site and specific mechanism of action of this hormone as well as lend some understanding to the phenomenon of growth regulation in general. While the picture is far from being complete, several aspects begin to emerge.

The observations that the early estrogen-induced alterations in two pathways (i.e., serine synthesis and protein synthesis) can be accounted for by increases in the amounts of participating enzymes suggest the possibility that other early metabolic alterations also result from increases in the levels of certain rate-limiting enzymes. Such increases would require either the activation of existing proenzymes or the *de novo* synthesis of new enzyme protein. Since in the present study the increases in enzymes lead in a continuous manner into a phase of RNA and protein accumulation, it is suggested that both phenomena occur. The interaction of the estrogen with the cell appears to set in operation a process leading initially to the release (i.e., unmasking or activation) of a number of proenzymes; this is followed in turn by a period of enzyme production. Thus estrogen action may be regarded as a problem in the regulation of polymer syntheses.

In Fig. 28 a scheme is presented for the induced biosynthesis of macromolecules (i.e., nucleic acids and protein) in which three potential sites of estrogen intervention are indicated. In this scheme, *T* represents the template and is considered to consist in part of nucleic acid (RNA especially); *E* represents the active or inactive enzyme protein. It is visualized that in the resting cell some of the templates, possibly covered with their own specific proteins, reside within barriers which prevent their participation in

polymeric syntheses. Such barriers may be membranous in nature and consist of lipids, polysaccharides, fibrous proteins, or involve ionic gradients (i.e., chelates); they may also make up the compartmental nature of the cell discussed previously (22). It is visualized that the barriers are relatively unspecific in their relationship to any one template and that each barrier

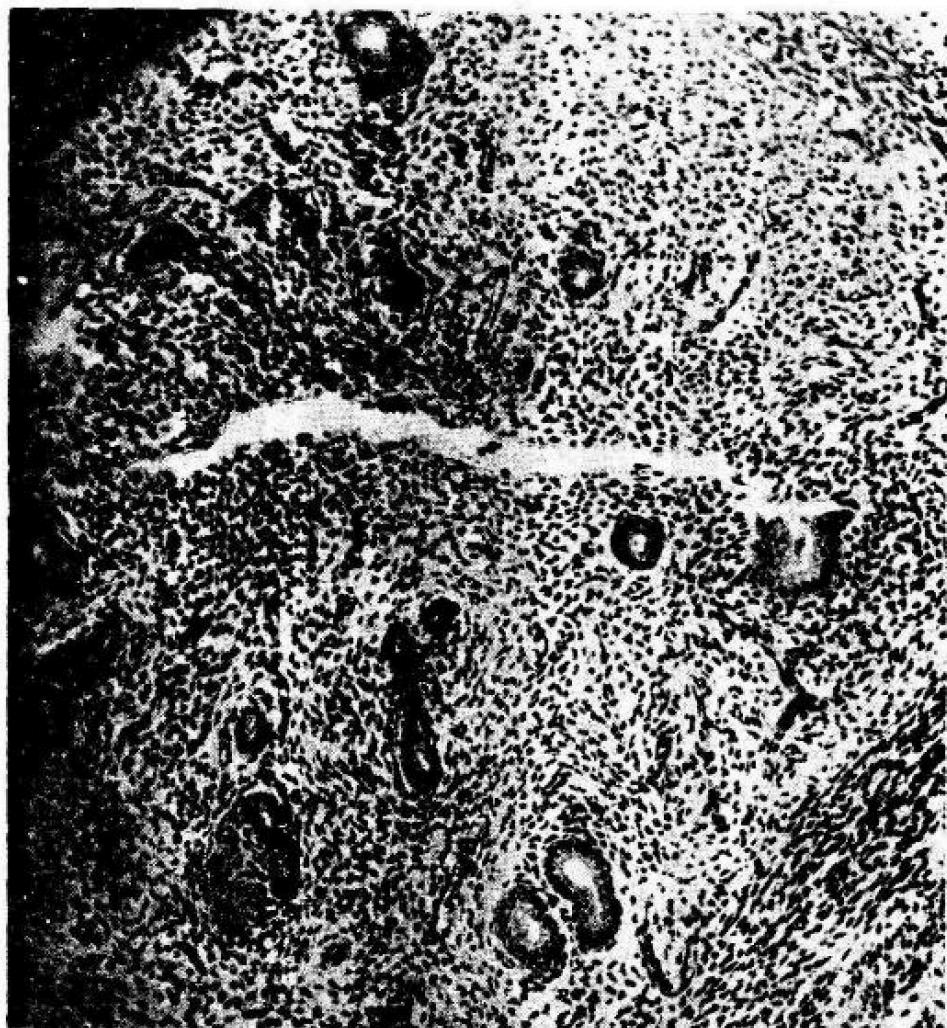


FIG. 26. A photomicrograph of a cross section through the horn of a control segment which has been cut open just prior to incubation in Eagle's medium. The epithelial cells have been largely lost in the processing. Section was stained with hematoxylin and eosin; magnification:  $\times 100$ .

probably contains one or more particles composed of a series of templates. When such a barrier is disrupted the contained templates are rendered available for active participation in the formation of new enzyme protein and more templates. The particulate nature of such concerted synthetic operations appears fundamental to the maintenance of the functional organization of differentiated cells.

The breaking down of such a barrier, "unmasking," constitutes a potential site of hormone action (site No. 1, Fig. 28); this could be enzymatic or non-

enzymatic. If enzymatic, one would expect the hormone to activate or inactivate a specific enzyme concerned directly or indirectly with the maintenance of the barriers. In the case of a nonenzymatic process the hormone might act by altering energy transmission throughout the barrier and its environment in accordance with the principles recently proposed by Szent-

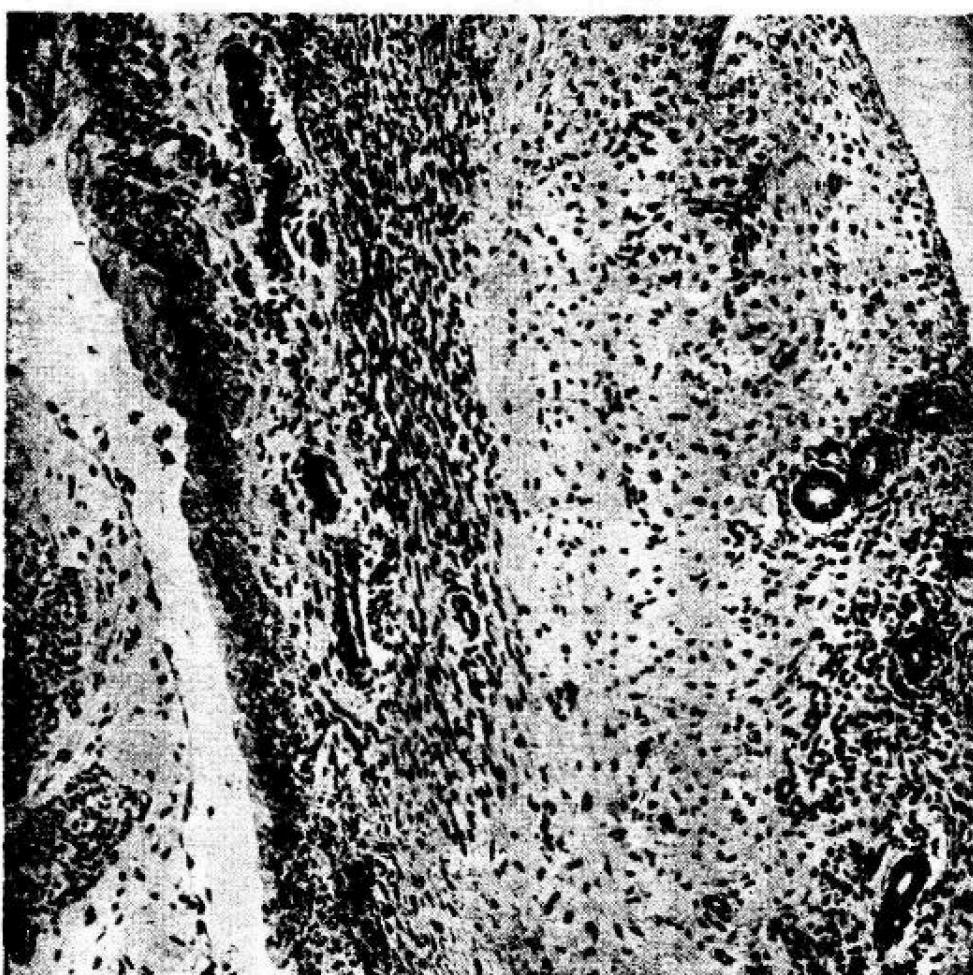


FIG. 27. A photomicrograph of a cross section of a uterine segment which had been incubated for 18 hours in Eagle's medium. Note the spacing between nuclei; compare with Fig. 4.

Györgyi (25). Also the possible surface action of some hormones has to be considered.

It is proposed that after liberation of the templates, their participation in polymeric syntheses is controlled by the balance between inducers and anti-inducers (i.e., suppressors) of enzyme synthesis in accordance with the principles which have evolved from the study of bacterial systems (18, 23, 29, 31). In this process it is proposed that the inducer exhibits a complementary surface to the template, the protein, or both, and acts primarily by facilitating the dissociation of the newly formed protein from the template surface. The anti-inducer acts competitively to inhibit the dissociation phenomenon;

it need not, however, compete with the inducer for the same geographical site. Once dissociated, the template is free to guide the synthesis of more protein and, as the case may be, to enter into the synthesis of more templates composed of nucleic acids. In this cyclic operation the hormone, the estrogen in particular, may itself act as an inducer or anti-inducer (site No. 2).

The third potential site of hormone action is the newly formed enzyme (site No. 3). Through the formation of a complex of the hormone with the enzyme, the latter may be either activated or inactivated and thereby modify the rate of certain catalytic processes in which the products feed back into the synthesis of the barriers, into the pool of inducers or anti-inducers, or into the pool of precursors needed for the synthesis of proteins and nucleic acids. It can readily be seen that positive or negative feed-back are integral

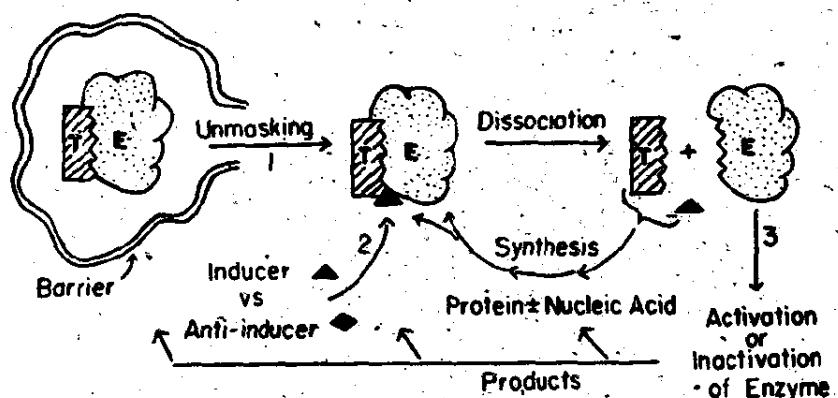


FIG. 28. A scheme depicting possible sites of hormonal regulation of induced biosynthesis.  $T$  = template;  $E$  = enzyme; 1, 2, or 3 = possible sites of hormone action.

parts of this scheme. It should also be apparent that considerable overlap is likely to occur with such an operational sequence.

In consideration of the over-all response of the uterine tissue to the action of the estrogen, it seems certain that some such cyclic process must be re-enacted many times in response to a primary interaction with the hormone. While the nature of this primary interaction still eludes us, the specific association of the estrogen with proteins intrigues us. The beautiful work of Villee and Gordon (28) and Talalay (26) prompts the consideration that certain proteins may be the receptors within the cell and that on activation these proteins amplify the hormonal signal through catalyzing reactions which lead to a change in the environment controlling the activity of the enzyme-forming systems.

While the mechanism of estrogen action is not yet solved, we are encouraged that certain parameters of the hormone response are yielding rapidly to scientific inquiry. With the definition of these parameters there also comes insight into the problems of general biological regulation, cell

physiology, and the action of other hormones in health and disease. The on-rushing tide of scientific achievements in the realms of biochemistry, virology, genetics, and surface chemistry assures an optimistic future.

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## DISCUSSION

**L. L. Engel:** It is always a very exciting experience for me to hear Dr. Mueller's story unfold as it increases in detail and as he sets forth his beautiful biochemical dissection of the events which occur after the rat uterus is stimulated with estrogen. There are so many things that are worthy of discussion, so many questions which one can ask, that I would just like to limit myself to a very few comments and perhaps one or two questions. In Dr. Mueller's last slide, allusion was made to Dr. Villee's estrogen-sensitive enzyme system in which diphosphopyridine nucleotide is the coenzyme. We have been interested in the interaction of steroid hormones, not only estrogens, but also the neutral steroid hormones, with purine and pyrimidine nucleotides including those substances which have a coenzyme function. It is interesting that, in the first place, as far as we have been able to detect, the pyrimidine nucleotides form no complexes with the steroid hormones, whereas with these hormones, purine nucleotides, nucleosides, and purine bases as well will form complexes of varying degrees of stability. I think here it is possible to speculate that perhaps some of the effects of the steroid hormone upon enzyme systems may be mediated through the coenzyme as well as through the apoenzyme.

One of the most important questions which must be considered in the interpretation of Dr. Mueller's work is whether the biochemical changes which he observes are specifically characteristic of hormonally stimulated growth or are phenomena which define a particular phase of the growth process regardless of the stimulus which initiates the growth. I wonder if Dr. Mueller would care to elaborate on this point.

I think the experiments on the stimulatory effects of the 2- and 4-hydroxyestradiols is particularly interesting in view of the recent isolation by Dr. Gallagher and his associates, and also in our own laboratories, of 2-methoxyestrone as a metabolite in the human of administered estradiol.

Finally, I wonder if Dr. Mueller has any information on the fate of the estrogen in his system and on whether any residual estrogen or metabolites can be detected in the tissue at the time when he carries out his biochemical experiments.

**G. C. Mueller:** I have been very interested in Dr. Engel's results on the formation of complexes between steroids and nucleotides; I think this is an intriguing situation. At our state of development it is too early to eliminate from consideration any type of complex without further experimental work. In fact, it seems likely that this type of work will ultimately lead to important findings on the mechanism of hormone action. Dr. Engel's work is particularly interesting in light of recent work on vitamin D from Dr. Steenbock's laboratory at the University of Wisconsin. As I understand, they have evidence that vitamin D affects the activity of the TPN (triphosphopyridine nucleotide) specific isocitric dehydrogenases, in which course the ability of TPN to associate with the enzyme is altered. In this regard Dr. Villee's experiments with a DPN (diphosphopyridine nucleotide) specific isocitric dehydrogenase from placenta also suggest a similar situation; in order to get his best effects with the addition of estradiol to the enzyme preparation, he uses a low substrate concentration (isocitric acid). This may thus indicate a steroid-induced alteration of the surface of the enzyme which reacts with substrates, among which are the coenzymes.

Relative to whether or not the responses herein studied represent a general phenomenon or are specific for estrogens, we must state that we have worked almost entirely with estrogen-activated tissues. Very early we did a little work with the effect of testosterone on glycine incorporation using similar experimental conditions, and I believe Dr. Frieden has also studied this response. In these experiments a stimulatory effect of testosterone

on glycine incorporation in surviving urine segments was observed, but the response was separate and additive to the response due to estrogen pretreatment.

When I said that I think that an anabolic response to a hormone represents a rather general reaction of the cell to its environment, I meant that there are probably many mechanisms for initiating an anabolic response but that they all end up activating common mechanisms by which the cell exhibits accumulative growth. In the case of the hormone a certain specificity of response is built into the tissue by the processes of differentiation. That is to say that certain cells possess either a primary acceptor for the hormone or possibly a certain composition of cell machinery which effects an amplification of the initial signal of hormone interaction with the cell and in this manner triggers or promotes the enzyme-forming systems to a state of activity characteristic of a particular hyperplastic or hypertrophic response. In this respect I would like to add that many hormonal responses are hypertrophic in nature and that all may involve mechanisms for activating certain enzyme-forming systems leading to the attainment of a particular biological state.

The philosophy of our approach has been to accept the biological picture and to attempt to dissect it in terms of early alterations of biochemical processes. In this effort it has been necessary to begin with some gross evidence of the hypertrophic action of the estrogen and to attempt to work back in the sequence of biochemical changes toward the initial site of the hormone action. Although we have a fair understanding as to the nature of this sequence, the primary action of the estrogen still eludes us. I look with very great interest upon the studies of Dr. Villee, Dr. Talalay; and the structural studies of Dr. Huggins for possible models of the end point in this sequential analysis of the hormonal response. While the future looks very bright, more work is necessary to relate these observations.

In regard to the formation of the 2-methoxyestrone observed by Dr. Gailagher, I must say that having originally synthesized the 2-hydroxyestradiol used in some of this work, we have been very interested in this area. In addition, we have worked with the 4-hydroxy derivative of estradiol as shown on one of the slides. I have not had time to discuss the metabolic fate of the hormone in the biological systems described; this represents quite another side of our approach. However, I can relate briefly that studies of Dr. Gideon Rumney and Dr. Ilse Riegel from our group with *in vitro* systems have shown that estradiol-16-C<sup>14</sup> is metabolized by practically all tissues. In addition to the phenomenon of protein-binding which has been described, the disappearance of the estrogen is associated with the production of oxygenated metabolites, among which the main one is 6 $\beta$ -hydroxyestradiol. The expected derivatives involving the oxidation and reduction of positions 6 and 17 were also present in the reaction mixtures.

In addition there are some unidentified compounds which have not been characterized as yet. The most recent compound that we have encountered is the paraquinol derivative of estradiol; this corresponds to a 10-hydroxy-3-keto compound. However, let me emphasize that the role of these metabolites of estradiol to the biological responses discussed is unclear.

**E. H. Frieden:** I was especially struck by Dr. Mueller's experiments with long-term incubation in Eagle's medium. As I interpreted these slides, these tissues were not treated with estrogen. Is that correct?

**G. C. Mueller:** You are right; these tissues were not treated with estrogen in most of the experiments. However, in the experiments where the tissues were from animals pretreated with estrogen you observed that the anabolic response on incubation

was eliminated or decreased by previous hormonal treatment. With 18 hours' pretreatment the tissues practically failed to respond with the usual increased anabolism arising with the *in vitro* incubation.

**E. H. Frieden:** The interpretation that presented itself as I looked at these slides was that you are doing essentially the same thing by treatment with estrogen that you do in making available to the isolated tissue an especially favorable environment with growth factors, amino acids, and other metabolites—perhaps more readily available than exist in the intact organism. One could speculate a little bit further in this respect and reach the conclusion that the action of these steroids is to make more readily available certain metabolites that are required, which brings us back again to the possible interaction of steroid growth promoters with what someone has called a cytostructural interface. Some earlier experiments of ours seemed to indicate that this was a very promising lead; unfortunately later experiments seemed to have denied this. I would like to ask Dr. Mueller one question which is perhaps not unrelated and that is: In our very early experiments, and I think in some of his as well, incorporation of alanine is not stimulated by prior treatment with estrogen; do you think this is simply a consequence of the fact that alanine can be diluted in the metabolic pool in so many different ways whereas other amino acids perhaps cannot.

**G. C. Mueller:** The evidence is in favor of the dilution concept, Dr. Frieden. If one uses a different precursor for alanine, such as radioactive pyruvate or glucose in which alanine is made *in situ*, the biological action of estrogen is now evident as increased amounts of radioactivity which can be isolated as alanine from the tissue. Therefore, I think that the results we have observed are due to variations in pool size among the intermediates which equilibrate readily with endogenous alanine. Relative to the proposition of barriers and the possibility of increasing more nutrients to contained systems, certainly I agree that this is a part of the over-all picture. In considering the action of the hormone, it is difficult to be sure at this point which effects are primary and which are secondary. I am optimistic, however, that an interpretation is forthcoming.

**N. T. Werthessen:** While Dr. Mueller was speaking, I realized that some aspects of the work we have been doing may be applicable to this problem. We have been working with lipids in the aorta. We have found that the organ, and of course its cells, responds to estrogen in one way, testosterone in another, hydrocortisone in a third. The response lies in the alteration of the rate of enzyme action. Of significance to the work that you have been doing, the observation is that in our system the cell is stimulated after it has been placed in the *in vitro* situation. Thus we find ourselves in complete agreement with your findings that the cell can respond to the hormone by changing the enzyme systems that are contained within it. An important point, as you have found, is the length of time which the cell has to respond to the agent. In one of our studies the response to the cells of the aorta to estrone is the increased incorporation of C<sup>14</sup> from acetate into cholesterol. It takes 72 hours' *in vitro* maintenance to show this response. On the other hand, studying other lipids such as phospholipids, the response can be observed within 6 to 8 hours. Another point which I think it is important to bear in mind here is that *p*-aminobenzoic acid was able in that case to fully duplicate and exaggerate the effect of estrone. Thus these studies, if they show that the time course and the possibility of the hormone acting through or in conjunction with a vitamin, must be considered.

**G. C. Mueller:** Such a situation is completely possible, Dr. Werthessen. However,

let me first ask you a question: In the system in which you tested the effects of folic acid did you also test aminopterin?

N. T. Werthessen: No, we have not as yet.

G. C. Mueller: Similarly, we have not tested folic acid. However, strongly influenced by the results of Drs. Hertz and Tullner and also by the results of Dr. Klein on the inhibitory effects of aminopterin on estrogen-induced growth of uteri, we were prompted to test this compound in our system. Administering a lethal dose of aminopterin (200 µg.) to adult female rats over a course of 3 days; we observed that the early activation of amino acid incorporation into protein by a single dose of estradiol was not limited by the aminopterin as was expected. It was concluded that aminopterin toxicity or the observed antagonism of the estrogen response did not involve the initial steps of estrogen action leading to induced growth of the uterus. It was also observed that aminopterin given in an acute dose, 6 hours before the amino acid incorporations study, acted somewhat like an estrogen in the sense that it stimulated glycine incorporation into the protein of surviving uterine segments.

In regard to the rest of Dr. Werthessen's comment, I should state that our philosophy is that the closer that you get to the actual hormonal response in the time sequence, the easier it will be to identify the primary response; one must, however, consider possibilities of limitation in transport of the hormone.

G. Rumney: Dr. Mueller mentioned that a search has been made for the 2-hydroxy- and 4-hydroxyestradiol (2,3,17 $\beta$ -trihydroxyestratriene and 3,4,17 $\beta$ -trihydroxyestratriene) as metabolites of estradiol-16-C<sup>14</sup> incubated in a fortified mouse liver homogenate. These compounds were not found in significant quantities, but as I understand he has recently been able to identify from incubation (with a simplified liver enzyme system) the paraquinol, 10-hydroxy-3-ketone derivative of estradiol.

I would like to know whether he has tried this compound, as a possible "activated" estrogen, in the *in vitro* system incorporating formate-C<sup>14</sup> into protein by surviving uterine segments—the same system in which he found an estrogen-like action with both the 2-hydroxy- and 4-hydroxyestradiol.

G. C. Mueller: We have not tested this compound in the experimental systems described.\* One reason for not having done so is that the free paraquinol exhibited less than 1% of the biological activity of estradiol in the 3-day immature mouse uterine growth test. The 10,17-diacetoxy derivative which is obtained as an intermediate in the course of the synthesis is, however, 10% as active as the parent compound, estradiol. Since the diacetoxy compound rearomatizes quite readily, it is possible that the results reflect this conversion in the animal body also. Accordingly, we have not been too excited by the observed biological activities of either substance. These are, however, very recent results and deserve more exploration.

U. Westphal: I would like to supplement part of Dr. Engel's remarks and mention a few results which may perhaps be of interest in connection with Dr. Mueller's last slide. We have also taken up the problem of interaction of the neutral steroid hormones with nucleotides. First, we confirmed Dr. Engel's work on testosterone and found the interactions as he described them. Then we were interested in studying progesterone, deoxycorticosterone, and cortisol in order to have a series of steroid hormones with a

\* Estraquinol (3-keto-10,17 $\beta$ -dihydroxyestra-1,3,5(10)-trien-17 $\beta$ -ol) was synthesized by Dr. Erich Hecker during the tenure of a post-doctorate fellowship from the McArdle Memorial Laboratory.

varying number of polar groups. We found that adenine interacts comparatively strongly with these, adenosine somewhat less. The adenosine phosphoric acids also interact; with ATP this interaction is relatively stronger for cortisol than for the less polar progesterone, whereas the opposite relationship exists in the interaction with adenine. Well, this is not unusual. We have the same difference in relative strength of interaction if we compare the binding of the three steroid hormones to albumin with that to chymotrypsinogen, lysozyme, or certain other proteins. Furthermore, we found that guanosine and cytidine showed interaction; uridine, UMP, UDP, UTP gave practically no interaction. Now, I cannot give any rhyme or reason for these differences; these are just the results we have obtained so far. We were also interested in the behavior of nucleic acids; we found that ribonucleic acid (commercial yeast preparation) interacted with testosterone and cortisone as well as with the three other steroid hormones that I named. However, deoxyribonucleic acid (highly polymerized preparation) showed almost no interaction.

**G. C. Mueller:** Dr. Westphal, your comments are particularly interesting in light of a paper which has been published by Agrell and Persson. They have isolated deoxyribonucleoproteins and incubated them with deoxyribonuclease in the presence and absence of estradiol. In the presence of the hormone an increased rate of breakdown of the DNA protein complex occurred. This would seem to provide an interesting counterpart to the picture you describe for the corticoids.

**U. Westphal:** Unfortunately, so far we don't know anything about the biological significance of these interactions; we just have these interactions, and we'll have to learn to understand the biological meaning of them.

**I. Mills:** I have been interested in another aspect of estrogen activity. In patients maintained on constant doses of cortisone acetate intramuscularly, when estrogen is given to them, the plasma hydrocortisone level is raised and the metabolites appearing in the urine are decreased. I wondered whether this activity of estrogen in interfering with the metabolism of corticoids could in any way facilitate the action of estrogen on the end organ.

**G. C. Mueller:** I do not know the answer to your question. However, several years ago Drs. Szego and Roberts presented a paper at this session in which certain corticoids were found to inhibit the response of the uterus to estrogens. I know of no other evidence along this particular line. In their hands, as I recall, compound F was very active in blocking the initial water uptake by the uterus but was relatively ineffective in blocking the true anabolic response measured at 20 hours after the injection of the estrogen. This whole area of study, involving the antagonism of various steroids, should be investigated in the systems described here this evening. Unfortunately, we have not been able to pursue this line of study with our limited group.

**A. White:** I would like to return, if I may, to two comments made by Dr. Engel and one by Dr. Frieden. I believe one of Dr. Engel's points should be emphasized, namely, are the data which you present, Dr. Mueller, with respect to alterations in the nucleic acid and protein and certain of the enzyme systems in the uterine tissue during growth stimulation produced by estradiol administration, phenomena which are characteristic of growth in general, or is this something specific and unique for the uterus, and are we overlooking what is perhaps the first phenomenon underlying this whole series of reactions, which may be of greater importance, namely, why the specific response—if it is specific—of this particular tissue to estradiol? In connection with Dr. Engel's comments on the combination of certain of the neutral steroids with nucleotides, nucleosides, and purines but not with pyrimidines, Dr. Jedeikin in our laboratory has demonstrated

an inhibition of glucose and succinate oxidation and of over-all respiration of normal lymphocyte suspensions *in vitro* by the addition of estradiol. In relation to Dr. Frieden's comment concerning the 18-hour incubation experiments, I wonder, in view of the suggestion—I think in your remarks, Dr. Mueller—that there may be a growth factor in the incubation medium, whether we are to infer that under these circumstances there is actually a net increase in total protein and total nucleic acid, or whether this is a turnover phenomenon which is seen in other nonproliferating cells. For example, coming back again to the lymphocyte, under circumstances *in vitro* in which the cell is not proliferating, there is a rapid incorporation of radioactivity from glycine-1-C<sup>14</sup> into the total nucleic acids and into the total protein, but we do not infer that this is an actual net increase in total nucleic acid and total protein. I could not judge from your histological section whether you felt that there was proliferation of cells during the 18-hour incubation or whether you merely inferred that there was a turnover rather than a net increase of protein and nucleic acid.

**G. C. Mueller:** When a person begins talking about turnover in this kind of a system it amounts to a delicate subject. We do not have any method of telling whether or not the isotope incorporation into surviving segments is due to turnover or synthesis in most cases.

**A. White:** How do you use the term incorporation?

**G. C. Mueller:** We always define incorporation as the amount of radioactive counts incorporated into the end product under a defined set of experimental conditions. In some of the studies presented here, there was actual evidence of more enzyme activity; this I do not think is primarily a matter of turnover; rather, I think, it must result from net protein synthesis or possibly the activation of some pre-existing proenzyme. In attempting to answer this aged question, I look forward to the work of those people who are trained in specific protein analysis by immunological techniques and to further resolution of the enzymatic mechanisms of protein syntheses.

Now, may I ask you one question with reference to the addition of estrogen to the lymphocytes system? How much hormone do you add to your flask; what is the final concentration?

**A. White:** The effects which we observed have been with concentrations of estradiol of the order of 30 µg. per 3 ml. of total incubation medium. I would like to add, also, that an increase in enzyme activity does not establish unequivocally that there is an increment in total enzyme protein.

**G. C. Mueller:** With that level of estradiol we have also observed an inhibition of glycine incorporation into segments of uterine tissue. In those cases where we have observed stimulation (and this is not a predictable feat), it has occurred at levels of 0.1—1.0 µg. of estradiol per 2 ml. of reactions. If one calculates the amount of estrogen necessary to get a maximal response in the rat, it comes out to something like 10<sup>-9</sup> molar concentration. Accordingly, I do not think that the observed inhibitions reflect the physiological response unless it is being pushed so far that we don't recognize it any longer. Does that answer your questions, Dr. White?

**A. White:** Thank you, yes, unless you would like to comment on whether the estradiol effects are a reflection of what might be seen in any tissue in which growth was stimulated.

**G. C. Mueller:** Oh yes, the question of whether or not this is a picture of a hormone-specific or a general response. I feel that the response that we have been studying here involves a sequence of reactions which may take place in any general

hypertrophic response. I say hypertrophy specifically since there is actually no hyperplasia in these systems in the first 24-hour period. The first 40 hours involved primarily a hypertrophy in which total protein and RNA accumulate in the tissue. DNA synthesis does not become significant until sometime between 40 and 72 hours after the initiation of the growth processes by estrogen. The specific role of the hormones is something we know all too little about as yet. In other words, in terms of total protein and total RNA which is accumulating in the system. I feel that this is a general reaction rather than the specific part, because the specific part is something that we do not know anything about yet; in other words, we had set out to try to find out how estrogens work and we have only learned a little bit about the tail; maybe we can sort of sneak up and get a hold of the front pretty soon.

**G. Cohn:** A few years ago Drs. Melvin Simpson and McLean and I studied the *in vivo* incorporation of L-leucine-C<sup>14</sup> into skeletal muscle, and we found that within 5 minutes the most rapid incorporation took place into the mitochondria and that microsomes were very low in incorporation. Using an *in vitro* system we found that the incorporation could take place if we had a system which produced energy for this incorporation. Do you have any information, relative to uterine muscle, where this incorporation takes place? Do you know whether it is in the mitochondria or in the microsomes? I think that this might give some insight as to where the reaction is taking place. I would like to speculate that you'll find increased incorporation into the mitochondria.

**G. C. Mueller:** So far we have only studied the amino acid activation step in cell-free systems; therefore, I am afraid that I am unable to answer your question. Some of these studies are projected for this fall, and we hope that radioautographs will provide some of the information you request.

We have not fractionated our systems any further since the uterine tissue is a tough tissue to work with; when you grind it up and attempt to fractionate it centrifugally according to procedures which have been successful with other tissues, many complications arise. In this connection one difficulty concerns the tendency for the uterine particulate to aggregate.

**V. Hollander:** We have been interested in studying the estradiol-sensitive isocitric dehydrogenase system described by Dr. Villee in placenta. Our observations were limited to human breast and breast cancer. We have observed many examples of surgical specimens of human breast and breast cancer which show prompt increase in isocitrate utilization when stimulated with levels of estradiol of 1 µg. per milliliter. The system is effective in the soluble fraction of human breast homogenate. There seems to be little relation with age or menstrual status of patient. The striking thing to us after we got used to this effect was that so many of the breasts would fail to show this enzymatic reaction. Approximately half of our surgical material, regardless of the care with which we would acquire it from the operating room failed to show *in vitro* sensitivity to estradiol. I wonder if in Dr. Mueller's homogenate system of serine aldolase, the aldolase shows frequent negative assays and whether this would imply some missing cofactor.

**G. C. Mueller:** To the contrary, the results we have reported here are very reproducible, considering the type of systems we are concerned with. I have been interested in your work, Dr. Hollander, as well as being acquainted with the work of Dr. Villee. We have also wondered whether or not the DPN-specific isocitric dehydrogenase is demonstrable in our uterine specimens; however, we have not tested this point yet. It would be of special interest to us if it could be demonstrated.

I have been intrigued with the possibility that this enzyme might be involved in the transport of divalent ions. In this manner it might be effective in changing the environment for the enzyme-forming systems we have discussed. This idea occurs to me since the enzyme catalyzes the conversion of isocitric acid to oxalosuccinic acid, which would be expected to have different chelating abilities for the divalent ions.

**G. Pincus:** I think that the demonstration by Dr. Mueller of an increase in at least two enzyme systems as a result of the action of estrogen is obviously reminiscent of many other situations described in the literature of the increase of enzyme activity in various tissues as a result of steroid action. This morning I described briefly the increased carbonic anhydrase activity in the uterine endometrium as a result of progesterone action. For Dr. Mueller's edification and possible explanation, if you measure carbonic anhydrase in the ventral prostate following testosterone administration, you get an increase, but if you measure it in the seminal vesicles, you get a decrease. If you measure carbonic anhydrase in the uterus of the rat when progesterone is administered, you get no change; when you give estrogen, you get a very marked decrease. Here, then, is a situation where an enzyme under the influence of steroids in one instance increases in amount and in other cases decreases in amount. Why is this, Professor?

**G. C. Mueller:** Dr. Pincus, I think you have very interesting problems with the variation of biological responses in different tissues to one type of hormone. While I do not know the answer to your primary question, it does seem to me that the carbonic anhydrase is quite a long way from the initial effects of progesterone or related compounds that you have tested. In other words, alterations of this enzyme are induced or suppressed indirectly. Referring to the final slide on macromolecular synthesis (Fig. 28) it seems possible that your variations revolve around position No. 2 of that diagram. The ratios of anti-inducers and inducers may be varying in different directions in the individual tissues in response to your pregestational hormones. This is a hypothetical explanation which might be worthy of further investigation.

**L. T. Samuels:** The point which Dr. White brought up about the question of specificity is of course a very important one. This can be either a question of entry of the material into the cell or of its action on the system. I judge that you have not yet tried testosterone in your uterine system, but this would be very important, it seems to me, in regard to this question. The other thing that I wanted to ask is: Have you tried systems in which the activation of the amino acids would be the major action? In other words, have you tried systems where activation of subsequent synthesis could be ruled out as primary effect?

**G. C. Mueller:** I don't quite understand your last point. Are you referring to the *in vitro* incubations?

**L. T. Samuels:** Yes, I understand that you had a mixed system in these, a crude homogenate.

**G. C. Mueller:** We included all the amino acids present in Eagle's medium; as a group they constitute the essential amino acids in nutrition. If the amino acids are omitted from the incubation mixture, the biological response obtained on incubation is decreased to approximately one-half of the optimal value. In a single experiment in which ethionine and thionylalanine were added to the incubation medium, an inhibition of the response was also observed. This situation requires further testing since the experiment was carried out without omitting methionine from the Eagle's medium.

With the limited facts at hand it appears probable that the initiation of the anabolic response involves a freeing of competent template sites for macromolecular synthesis.

Whether or not the initiation of such synthesis leads to accumulation of macromolecular products is very likely one of whether or not the cellular environment will continue to support such syntheses. Thus, if the environment fails as it does when cyanide or some other inhibitor is added to the system, little or no accumulative synthesis will be observed.

**E. Frieden:** At the risk of introducing a polemic, I wonder whether it is wise always to conclude that increased enzyme activity necessarily means increased concentration of the enzyme per se.

**G. C. Mueller:** Before the measurements were made, conditions were established for determining enzyme activity by kinetic measurements. The protein content and enzyme activities were related to DNA content of the uterine homogenates and thus the data correspond to a measurement of enzyme activity per cell. On this basis we have evidence for more biological activity—that is, more enzyme. Whether this increase in activity springs from activation of a proenzyme, from *de novo* synthesis of new enzyme protein, or from both situations cannot be established at this particular time. I actually favor the existence of both processes. In this interest we have experiments planned which will test for possible activation of proenzymes in cell-free preparations. A negative or a positive answer from these experiments will facilitate our thinking.

**E. Knobil:** The work of Dr. Villee and its implication in the mechanism of action of estrogens has been referred to repeatedly this evening. You will remember that in his system, estradiol was active *in vitro*. Quite surprisingly, stilbestrol, a perfectly sound estrogen from the systemic point of view, had no effect at all on the system and even inhibited the action of estradiol. I was wondering what your comments are on this and whether or not you have had similar experiences in your systems.

**G. C. Mueller:** We have not carried out any appreciable amount of work with synthetic estrogens. Initially I was also perplexed by the results of Dr. Villee with synthetic estrogens. He accounts for his results with the explanation that while synthetic estrogens are not as effective in activation of the DPN-specific isocitric dehydrogenase, they may be retained within the *in vivo* system for a longer period of time than the natural estrogens and thus perpetuate the activated state over a greater period of time. Dr. Villee has commented recently that he does not know the exact relationship of his enzyme activation process to the biological role of estrogens in the intact animal. It is principally the unusual specificity which the enzyme exhibits toward natural estrogens and some of the results with antagonists which prompt one to consider it as candidate for the primary site of hormone action *in vivo*. It is quite possible that there are other enzymes of similar responsiveness. When one looks at the different levels of organization in cellular processes, it is intriguing to consider the possibilities of different degrees of specificity amid responding systems. In the experiments which were reported this morning by Dr. Huggins, it was demonstrated that a variety of molecular structures would effect similar results in the intact animal. Thus, one wonders whether or not intermediate degrees of specificity may be involved in the interaction of steroids with biologically active proteins. That is to say that actually a number of enzymes may present surfaces which are complementary with a group of steroids. Varying degrees of efficiency may be observed for such interactions.

**R. Kroc:** I hesitate to introduce additional "triggers," but Dr. Steinert and Miss Beach of our laboratory have confirmed and extended the observation of Dr. Zarrow that relaxin markedly and rapidly stimulates an increase *in*-water content of the estrogen-primed rat uterus. Our group also finds significant increases in uterine glycogen and nitrogen. This is obtained with both high- and low-potent relaxin preparations.

**G. C. Mueller:** I have followed your work in this area and have wondered whether or not relaxin would have any effect on the systems we have studied. While I believe that Dr. Frieden has had some experience along this line, we have not.

**E. Frieden:** One of the things which may bear to some extent on this is our work some time ago on the action of relaxin on incorporation, upon endogenous respiration, and so on of the connective tissue of the primary target of its action in the guinea pig—the symphysis pubis. Here we found that relaxin had an effect on amino acid incorporation and other physiological phenomena quite apart from its estrogen dependence. In other words, while the guinea pig will not relax under the influence of relaxin without prior estrogen, one can demonstrate other effects upon symphyseal connective tissue. However, hard as we have looked for effects of relaxin upon other kinds of connective tissue and other sorts of tissue of various animals, we have been unable to find it.

**R. Hertz:** I would like to offer you an experimental tool which may be of some use to you. If you take the sexually immature rat which has a uterus of about 20 mg. and give a maximally effective dose of estrogen, that uterus will weigh around 80 mg. at the end of 3 days. As we previously described, a folic acid antagonist—aminopterin or any of the others—will keep this reaction down to slightly above the control value. Dr. Bruzzoune showed that again after 3 weeks' time the previously inhibited uterus will then give an almost maximal response. In other words, this uterine tissue has learned how to get around the folic acid antagonist in some way and has become re-educated in its response to the administered estrogen. It appeared to me that in your biochemical analyses this re-educated or reconditioned uterus might prove to be of considerable value in pointing up to you some of the more specific rate-limiting steps which you are seeking in your studies.

**G. C. Mueller:** Dr. Hertz, this is a very interesting suggestion you have made. If successful one might bypass a part of the response which has been associated with the sensitivity of one-carbon metabolism to the action of estrogen.