1971

The biochemical effects of ovarian steroids

Albert Carl Weihl II
Yale University

THE BIOCHEMICAL EFFECTS OF OVARIAN STEROIDS

ALBERT CARL WEIHL II

1971

THE BIOCHEMICAL EFFECTS OF OVARIAN STEROIDS

ALBERT CARL WEIHL II

B. S., YALE UNIVERSITY, 1967

A THESIS

SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF MEDICINE

YALE UNIVERSITY SCHOOL OF MEDICINE

DEPARTMENT OF OBSTETRICS AND GYNECOLOGY

NEW HAVEN, CONNECTICUT

APRIL 1, 1971

ACKNOWLEDGEMENTS

I should like to thank Dr. John McClean Morris and Dr. Joseph McGuire for their advice and assistance in the design and conduct of the experiments described in this thesis. Without their continued initiative and support this work would neither have been begun nor have been pursued to a satisfactory conclusion.

In addition I would like to thank Dr. Mark Bitensky for his personal advice on details of the adenyl cyclase assay system.

TABLE OF CONTENTS

TABLE OF CONTENTS

Introduction

Current research in the field of endocrinology has yielded a considerable body of data on the cellular and intra-cellular mechanisms of action of various hormones. In the case of some hormones, the precise mechanisms of action have been elucidated. A good example of such a well understood hormone action is the effect of epinephrine on the adenyl cyclase system found in liver (54). However, in the case of the ovarian steroids, such an understanding of the fine points of hormone action has yet to be reached. An understanding of the precise control exerted by the reproductive hormones is greatly needed to permit development of safer and more practical methods of human birth control.

In an effort to gain further insight into the hormonal control of reproduction, the investigations to be discussed in this paper were undertaken.

The following effects of ovarian steroids were examined:

(1) the effect of progesterone and progesterone-estradiol combinations on a soluble TPNH-DPN transhydrogenase from human placenta,

(2) the effect of progesterone and estradiol on a soluble DPNH

oxidase from human placenta,

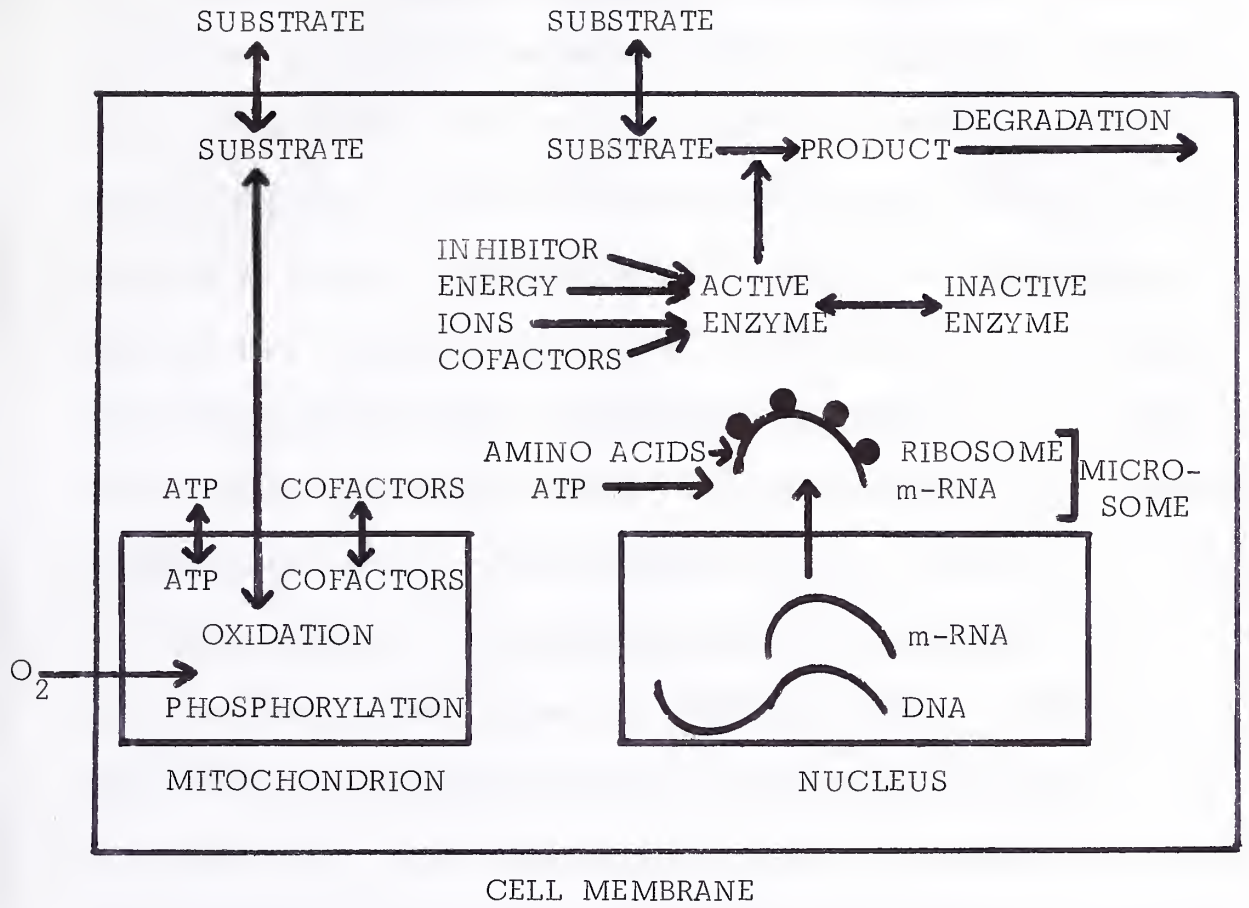
(3) alterations in glycogen-phosphorylase activity in the endometrium of pregnant rabbits,

(4) acute effects of intravenously administered estradiol and progesterone on the activity of glycogen-phosphorylase in normal rabbit endometrium,

(5) *in vitro* effect of progesterone on the adenyl cyclase system of rabbit endometrium.

To understand the nature of hormonal control and to serve as a basis for discussion of the actions of the ovarian steroids, the effects of hormones in general will be examined, with particular reference to the cellular processes susceptible to hormonal influences. Then a general examination will be made of the techniques by which the ovarian steroids might be studied, with emphasis placed on the usefulness and limitations of the various approaches. Finally a review will be made of the data gathered thus far on the biochemical effects of the ovarian steroids on cellular processes.

Cellular processes susceptible to hormonal control



In looking at the cellular and sub-cellular processes which may be readily influenced by hormones, it is useful to construct an idealized cell with easily identifiable compartments. The cell can be divided into nuclear and cytoplasmic compartments; the cytoplasm can be further sub-divided into a soluble phase and a particulate phase, the latter composed of mitochondrial and microsomal fractions. These divisions roughly correspond to the fractions into which a cell can be divided by differential centrifugation.

Within the nuclear compartment, cell functions which can be influenced by hormones are primarily those of cell division and of cell differentiation. In both cases the nucleus exerts a marked effect on the rest of the cell, but the nuclear events are the primary ones being altered, and the cytoplasmic events are influenced only secondarily. That the ovarian steroids influence both cell division and differentiation is best demonstrated by changes in the endometrium during the menstrual cycle. The early phases, before ovulation, during which estrogen is the dominant hormone, are marked by rapid cell proliferation; the later phases, during which progesterone acts on an estrogen-primed tissue, are marked by cellular differentiation, such that the endometrium becomes receptive to implantation of the fertilized ovum. Such effects of the steroids are difficult to examine in isolated or disrupted cells because these effects are heavily dependent upon observation of cytoplasmic changes in response to nuclear control. In fact, observation of the target tissue itself, in this case endometrium, is the best index of hormone action since the hormone response is an organized tissue response as well as a cellular one.

Another aspect of hormonal control is seen in the nuclear-cytoplasmic relationships, in which the nucleus undergoes no apparent change, but the cytoplasm is influenced in such a way as to

alter its metabolism or to synthesize a new substance. Such nuclear control of cytoplasmic function is mediated through production of messenger-RNA (m-RNA), which in turn leads to production of a new protein or enzyme in the cytoplasm. According to current theories, hormones influence the expression of a gene or group of genes, either by causing new m-RNA to be produced or by blocking the synthesis of m-RNA already being produced (71). This m-RNA in turn stimulates production of specific protein or proteins within the cytoplasm.

It is at the level of protein synthesis under m-RNA control that another potential site for hormone influence exists. The protein synthetic apparatus must interact with the m-RNA and join the constituent amino acids to one another. Hormones may interfere with the participation of the m-RNA or amino acids in this process. Another mechanism of control over the protein synthetic process would be through availability of energy needed to couple the amino acids to specific transfer-RNA (t-RNA) prior to joining into peptide linkages.

Control over metabolic processes within the cytoplasm could be exerted through a great many different mechanisms. Current biochemical knowledge has provided well studied examples of control over single metabolic processes, but, unfortunately, understanding of the interrelationship of known examples of metabolic control is

incomplete. Nonetheless, it is useful to examine some of the types of metabolic control which may be influenced.

One of the basic mechanisms of metabolic control occurs through the activation or inhibition of a pre-existing enzyme. Similar effects can, of course, be produced through stimulation or inhibition of synthesis of the active enzyme through nuclear production of m-RNA. However, intra-cytoplasmic control can be exerted without addition or deletion of enzyme itself through direct activation or inhibition of the enzyme and also by hormonal influence on the presence of necessary co-factors, such as ions, ATP, DPN, and TPN. More indirect control over enzyme activity can be exerted through availability of substrates, either by increased or decreased production, or by transport across cell and mitochondrial membranes. The hormone may influence the rate of a specific process through control of the compartmentalization of a substrate involved; for example, substrate sequestration within a vacuole or passage across a cell membrane into a mitochondrion or lysosome may be influenced.

It is common to measure enzyme activity and to ascribe a change in activity to hormonal influence. However, it is most important that the conditions under which the activity change was observed be examined closely; the many ways by which a hormone may influence enzyme activity have vastly different implications in understanding hormone action.

If enzyme activity is observed in the intact animal, a change may represent transport from one body or organ site to another. In a cell capable of protein synthesis, the change may represent net synthesis of the active enzyme. Or, one may be observing activation of pre-existing enzyme, possibly by actual combination of hormone with enzyme through an allosteric effect (71). Change in enzyme activity may not represent activation but removal of an inhibitor otherwise present. Since most enzyme activities are followed indirectly through measurement of a product of the enzyme reaction, an apparent change in enzyme activity leading to production of a given substance may actually represent an inhibition of the degradative step which serves to remove the substance being measured. Furthermore, measurement of enzyme activity, usually done in homogenates of tissues, reveals only total activity, giving no information on the possibility of intracellular compartmentalization of enzyme, substrate or co-factors.

Methods of study

Having examined the various metabolic processes over which hormonal control can be exerted, the methods by which these metabolic processes can be studied will be discussed.

Research into the biochemical effects of ovarian steroids has involved the study of hormone action in the intact animal, in the isolated target site such as endometrium, and in fragmented tissue reduced to a particulate or soluble fraction by centrifugation techniques.

Studies with the intact animal can be carried out using normal pregnant and non-pregnant animals, influenced only by naturally occurring endogenous hormones. Since in most cases the identities and relative quantities of endogenous hormones are not entirely known, data obtained in this manner involves certain assumptions on the part of the investigator with regard to the etiology of a given observation. The need for such assumptions can be obviated in part by superimposing exogenous hormones upon the naturally present hormones, administered in physiologic or pharmacologic doses. Observed effects can then be attributed to the administered hormone with some degree of certainty. This degree of certainty can be improved upon

by removing endogenous hormones through castration, hypophysectomy, or adrenalectomy, followed by administration of exogenous hormones.

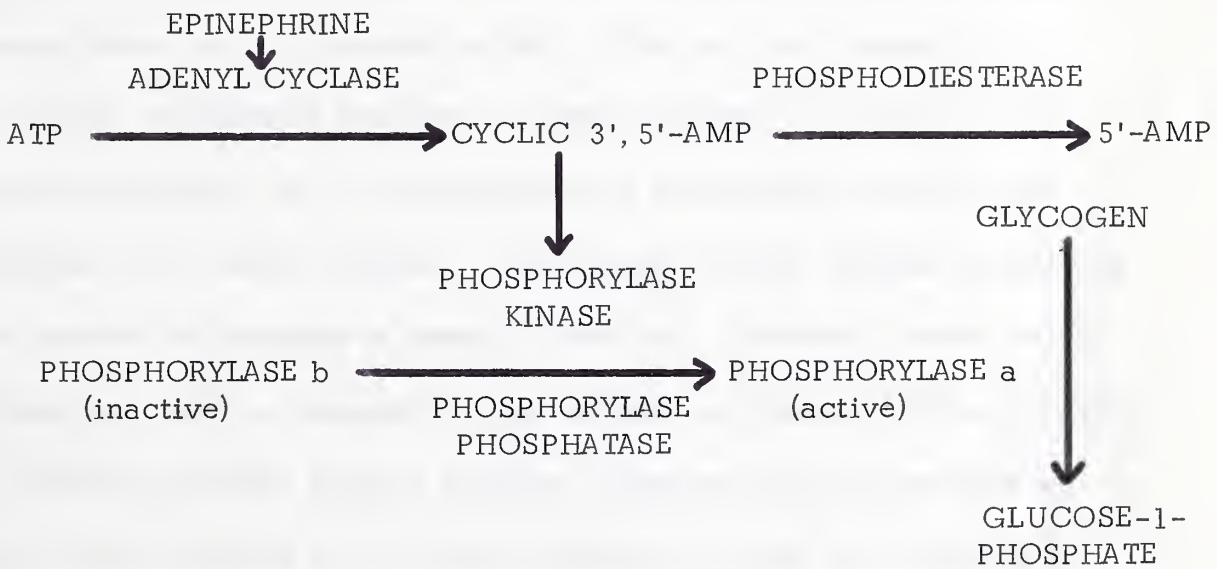
In any case, animals are biopsied or sacrificed at a specific time when they are under the regulatory influence of a specific hormone or group of hormones. Tissues are then analyzed for substrate concentration and enzyme activity. These studies make possible examination of effects which can be observed only in intact animals; however, the investigator has difficulty in determining at what level of control over metabolic processes the hormone is operating. Since total enzyme activity or substrate concentration is being measured, the intermediate steps leading to the final observation are not apparent. Frequently it is not possible to differentiate between enzyme synthesis and activation, or between increased rate of substrate production and decreased rate of breakdown. The cellular events involved may include cell division and differentiation, enzyme synthesis and activation, changes in blood supply, oxygen availability, energy supply, and compartmental shifts. Any one of these processes may produce an end result of increased enzyme activity or increased substrate concentration.

Studies of organs, tissues, and tissue homogenates in vitro allow greater control over experimental conditions so that a differentiation can be made among the many processes that could result in

the same observed effect. In vitro techniques permit the use of certain specific inhibitors of protein and RNA synthesis that cannot be used in the intact animal due to problems of toxicity. Tissue culture techniques allow precise definition of incubation conditions, without the variables of blood flow, innervation and extraneous hormones. Homogenization and centrifugation permit reactions to be studied in the absence of possible RNA or protein synthesis. Furthermore, studies of a process influenced by inhibitors of RNA and protein synthesis provide additional information on the mechanism of the process being observed.

With in vitro methods, samples can be taken from a homogenous tissue and cultured under well defined conditions in the presence or absence of a specific hormone. Alternatively, aliquots of a common homogenate, consisting of particulate or soluble fraction, can be compared under conditions that differ only in the addition of a hormone to one of the samples. These techniques permit fractionation and recombination of particulate and soluble fractions, producing effects in combination that neither will produce alone. Thus it is possible to investigate hormonal effects that can be attributed with reasonable certainty to changes in enzyme activity, whether due to allosteric effects, availability of co-factors, or changes in substrate concentration and energy supplies.

The prototype of such an investigation is the study of the glycogen phosphorylase-adenyl cyclase system carried out first by the Coris (10, 11) and later by Sutherland and Rall (43, 54). Successive investigations, both in vivo and in vitro, revealed an activation of glycogen phosphorylase by epinephrine. The mechanism of this activation was shown by Sutherland and Rall to proceed through stimulation of adenyl cyclase by epinephrine, producing cyclic-AMP which in turn stimulated dephosphophosphorylase kinase, converting inactive phosphorylase to its active form. This is diagrammed below:



Further studies by other investigators have revealed that the adenyl cyclase system is an intermediary step influenced by many other hormones (7). Cyclic-AMP has been shown to influence numerous enzyme reactions in addition to the dephosphophosphorylase kinase conversion of inactive phosphorylase to its active form. This function of cyclic-AMP as a "second messenger" will be referred to later in a discussion of the effects of ovarian steroids.

Looking again at the in vitro study of hormone effects, whereby a given effect may be ascribed with a high degree of certainty to a specific hormone, difficulty arises in determining the biological significance of the observed effect. The fact that steroids in a cell-free supernatant fraction may affect certain co-factors gives little information on the significance of the observed effect when applied to an intact animal. The in vitro system utilizes conditions to isolate and magnify a specific reaction. However, these conditions may bear no resemblance to cellular or tissue conditions; within a cell the specific enzyme reaction measured may not proceed at all or may proceed in a direction opposite to the one observed in the cell-free system. Also, the magnitude of the observed effect may be so small under existing intra-cellular conditions as to be of no significance in vivo. The addition of ions and co-factors, the adjustment of pH, and the concentration of substrate in a cell-free

system may potentiate an effect that is of no significance or may produce an effect that does not exist at all under normal physiologic conditions.

Thus, although observation of effects in vivo are subject to uncertainty as to their precise causes, their significance can usually be well defined. On the other hand, observations in vitro can be well defined with respect to cause but are associated with considerable uncertainty with respect to significance.

Observed biochemical effects of steroids

It is now appropriate to review the available information on the biochemical effects of ovarian steroids. There exists an enormous body of descriptive data based on observations of reproductive anatomy and behavior in both animals and man. This data has been gathered over a period of many years, some of the most significant findings dating to the 1920's and 1930's. No attempt will be made to review or summarize this body of descriptive data. Rather, attention will be focused on data, gathered mostly since 1950, that concerns the primary biochemical influences of ovarian steroids.

Research in the last twenty years has shifted emphasis from anatomical and behavioral investigation to studies of the activity of various enzymes, the concentration of substrates and nucleic acids, and the way in which these parameters are influenced by ovarian steroids. Data has been collected based on studies of normal and treated animals, of segments of reproductive tract incubated in tissue culture, and of homogenates of reproductive tissue. These three types of systems have been examined with respect to specific alterations in function as a response to identifiable hormone influences. In reviewing the data that has been collected, an attempt will be

made to approach each set of experiments with a view to determining how the information gained aids in understanding the precise intracellular site of hormone action. The data will be viewed in the framework discussed in the preceding section on sites of hormone action.

In reviewing the available data, studies will be placed into three main groups: (1) data obtained by measurement of enzyme activity and concentration of various substances in reproductive tract tissue from normal animals and from those treated with hormones *in vivo*; (2) data obtained on enzyme activity in tissue slices and homogenates incubated *in vitro* in the presence and absence of hormone; and (3) data obtained through the use of inhibitors of RNA and protein synthesis. Naturally there are some experiments which fall into more than one of these groups, as *in vivo* observations have been subsequently studied in *in vitro* systems.

Some of the early data available on changes in nucleic acid content and in enzyme activity during the reproductive cycle is found in the work of Stuermer and Stein (50, 51). In one of their earlier papers these investigators measured RNA and DNA content of human endometrium as a function of the phase of the menstrual cycle, dividing the cycle into early and late proliferative, and early and late secretory phases (50). Their data, shown in Figure 1, shows a

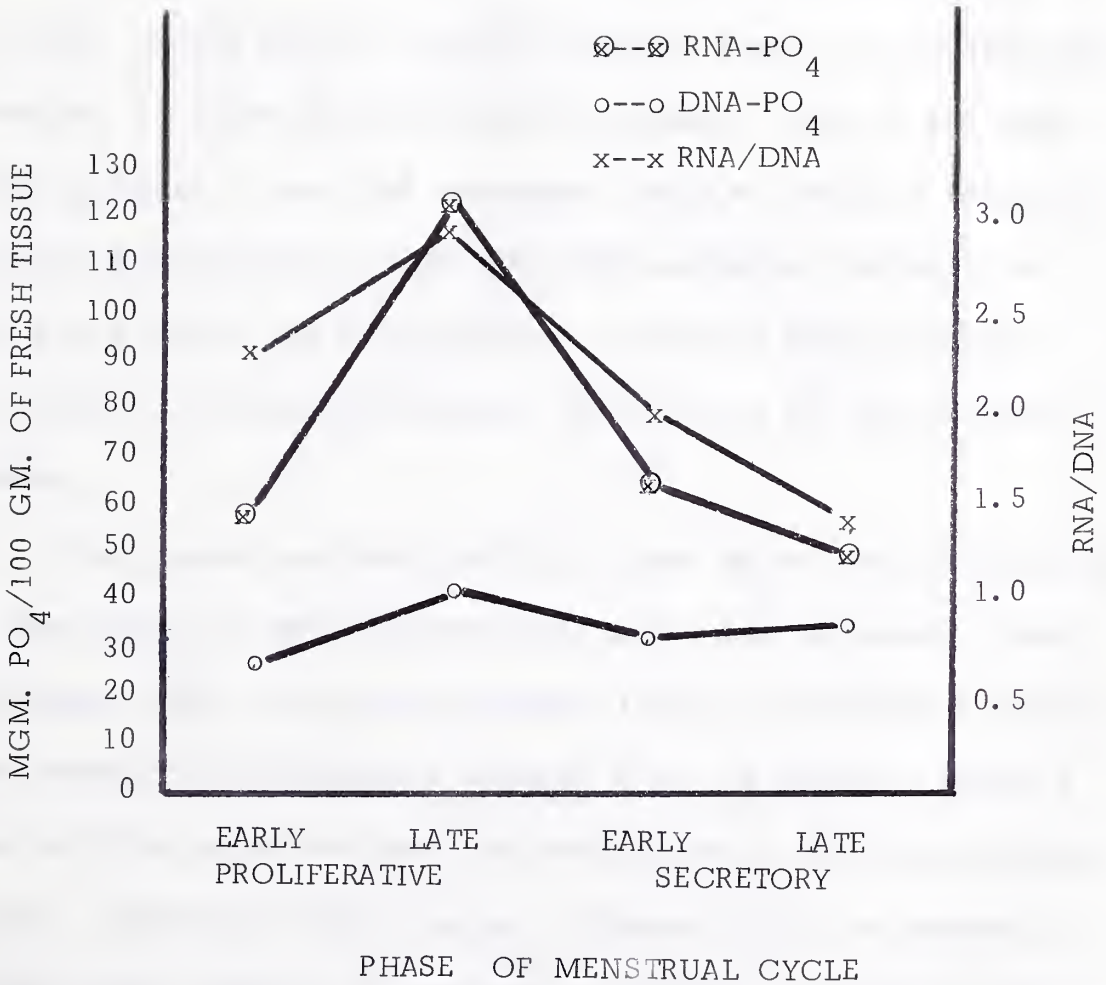


Fig. 1 Cyclic variations in RNA and DNA content of endometrium during the menstrual cycle. (from Stein and Stuermer(50)).

sharp increase in both RNA and DNA content during the proliferative phase of the menstrual cycle, with a peak immediately prior to, or at the time of, ovulation. In the human the endometrium is influenced solely by estrogens prior to ovulation.

Also evident from the data in Figure 1 is the fact that the increase in RNA content is proportionately greater than the increase in DNA. It can thus be seen that estrogen seems to accelerate cell division, as shown by increasing DNA content, while at the same time producing a rapid RNA synthesis. Such a finding of RNA production proportionately greater than DNA production indicates that rapid cell growth and differentiation, associated with an increase in activity of the protein synthetic machinery of the cell is taking place.

This protein synthetic activity is seen in the data presented in a later paper, in which measurements were made of succinic dehydrogenase (SDH), malic dehydrogenase (MDH), cytochrome-C oxidase, and adenosine triphosphatase (ATPase) (51). As shown in Figure 2, the activities of all enzymes measured peaked in the early secretory phase, immediately after ovulation. Whether this is a function of rapid protein synthesis in response to estrogens, of further synthesis in response to progestogens, or of activation of a pre-existing enzyme apparatus cannot be determined. In all probability each of these

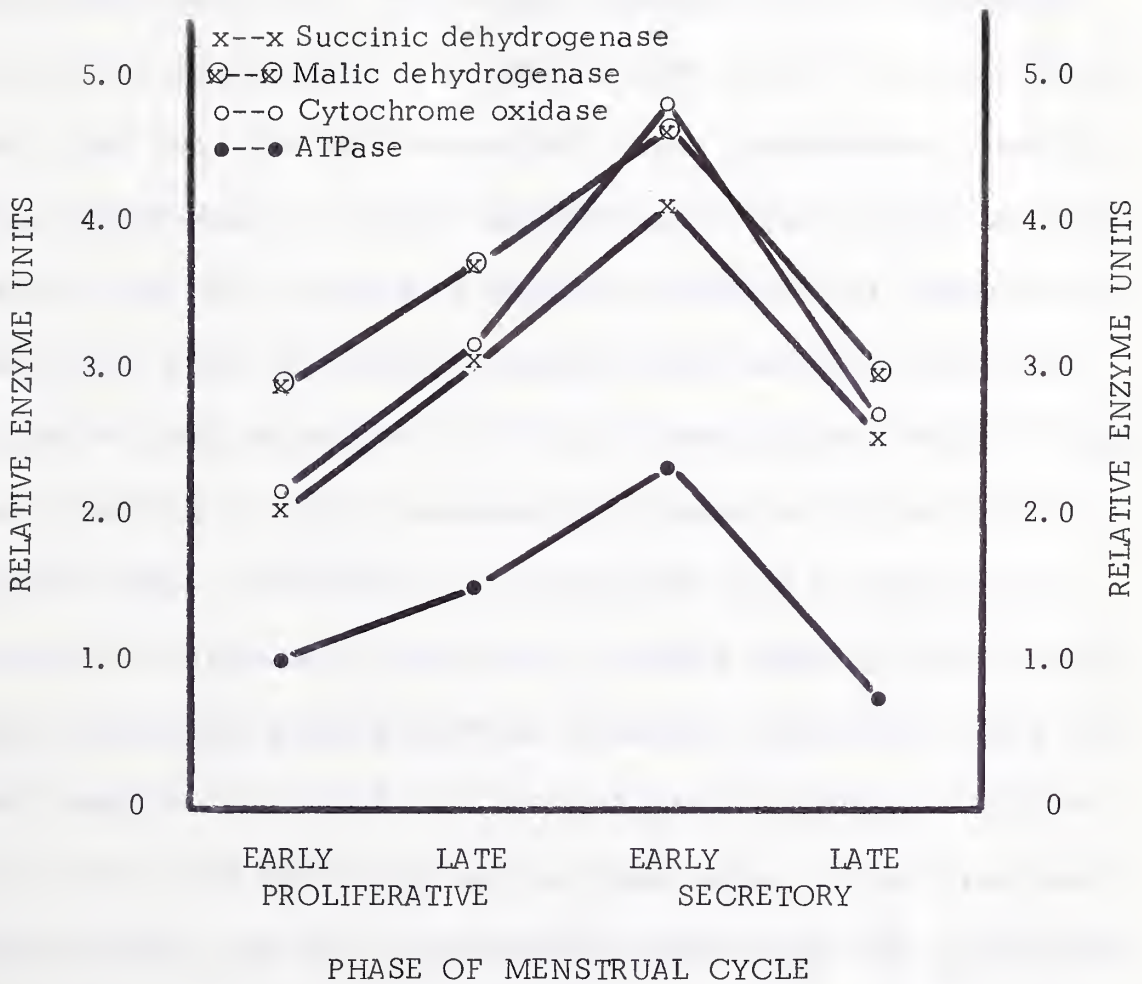


Fig. 2 Cyclic variations in enzyme activities during the menstrual cycle. (from Stuermer and Stein(51)).

three mechanisms is active to a greater or lesser degree, and identification of the primary mechanism cannot be made on the basis of this data alone.

Related data is found in the work of Saldarini and Yochim, who studied oxygen uptake in the rat uterus as influenced by estrogen and progestogen (48). Their studies examined oxygen consumption during pseudo-pregnancy in normal rat uteri, uteri from ovariectomized rats, and uteri from rats treated with either progesterone or estrone plus progesterone. The data demonstrates a rise in oxygen consumption in uteri from normal rats beginning on day one of pseudo-pregnancy and a fall in oxygen consumption after day five, during the phase of luteal regression. Uteri from ovariectomized animals showed an initial fall in oxygen consumption, followed by a rise to subnormal levels. Treatment with progesterone gave a pattern quite similar to that found in normal rats, whereas treatment with estrone and progesterone showed elevation of oxygen consumption above normal levels for the initial three days of pseudo-pregnancy, followed by a more rapid fall during the next three days. It was also found that although uteri from progesterone treated animals had near-normal levels of oxygen consumption during pregnancy, tissue growth did not occur. In uteri from animals treated with estrone in addition to progesterone, uterine weight followed a normal growth pattern. These

investigators felt their observations suggested that the high respiratory activity of early pseudo-pregnancy reflects progestational effect and that growth-stimulation during the same period is due to estrogenic influences.

An additional observation made in the same paper showed that, despite wide variations in uterine weight, water content varied in the narrow range of 80.3% to 83.9% during all phases of estrus and pseudo-pregnancy (48). This indicates that observed variations in tissue respiration and enzyme activity per gram wet weight of tissue were due to actual changes in metabolic activity and not due solely to fluctuations in water content. These findings have implications in examining the data from other sources on enzyme activity per unit of tissue weight where determinations of variation in water content are not available.

Hughes et al. have carried out a series of studies on the carbohydrate pathways in human endometrium in which they measured the activity of numerous enzymes as well as glycogen, water and lactate content during the menstrual cycle (12, 28, 29, 30). In one of their earlier studies, the parameters measured seemed to fall into two groups, those which peaked immediately before ovulation and those which peaked immediately after ovulation (28). The activities of glucose-6-phosphatase and alkaline phosphatase as well as glucose

content of human endometrium peaked 14 - 16 days after the preceding menstrual period. In contrast, lactic dehydrogenase, isocitric dehydrogenase and malic dehydrogenase activities, as well as glycogen and lactic acid content, peaked 17 - 19 days following the preceding period. The activity of glucose-6-phosphate dehydrogenase was found to be unchanged. Also of interest, water content was found to vary from 80 to 83% during the proliferative phase, and between 78 to 84% during the secretory phase of the cycle.

In another series of experiments Hughes et al. measured glycogen content, glycogen synthetase activity and glycogen phosphorylase activity in endometrium from normal and infertile patients (30). In this study glycogen content was found to vary from 150 to 1000 mg. % in normals with a rapid rise during the 12th - 15th days of the cycle and a peak during the 16th - 20th days of the cycle. In contrast, glycogen content in infertile patients varied between 150 and 300 mg. %, without a rapid rise in the 12th - 15th days. Glycogen synthetase activity in normals showed a pattern similar to glycogen content, with a rapid rise in the period between the 12th and 15th days, and a peak between the 16th and 20th days. Again, activity of this enzyme in the infertile group failed to show a significant rise during the period from the 12th - 15th days. Both glycogen content and glycogen synthetase activity showed rapid increases

during the pre-ovulatory period, at which time the endometrium is being stimulated by estrogens alone.

Further findings revealed that the activity of glycogen phosphorylase did not begin its rapid rise until the 16th - 20th day of the cycle, and did not peak until the 20th - 25th days of the cycle, during which time the endometrium was being stimulated by progesterone in addition to estrogen. It was concluded that estrogen initiates the process of glycogen synthesis, which was completed under the additional influence of progesterone, whereas progesterone seemed to have a stimulatory effect on glycogen phosphorylase necessary for glycogenolysis.

Barker, Warren and others studied glucose metabolism in the uteri of rats treated with estradiol (5, 6, 70). Using glucose labelled with C^{14} at the 1-carbon and 6-carbon positions, they found that carbon dioxide formation from both the C-1 and C-6 positions increased during the first two hours after treatment. In the period 6 - 24 hours after treatment, there was increased carbon dioxide production from the C-1 carbon; the C-1/ C-6 ratio increased linearly after estrogen treatment. Incorporation of C-1 and C-6 into lipid was found to be equal and to increase after estrogen treatment. Estradiol seemed to favor utilization of glucose via the hexose monophosphate shunt, and the rate of utilization was found to be

controlled by the amount of TPN available (6). Whether these results were due to rapid synthesis of new enzymatic systems, or to alteration in activity of pre-existing enzymes was not determined. However, no change was observed in total activity of glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase, key enzymes in the hexose monophosphate pathway.

Mueller, McCorquodale, and Herranen studied the effect of estradiol treatment of rats on the uterine incorporation of amino acids into protein (35, 38, 39). Within three hours after injection of estradiol, the activity of the amino acid activating enzymes in the 105,000 x g supernatant was found to be increased. By five hours after injection the incorporation of labelled glycine into protein was found to be increased over that found in uteri from untreated animals. Thus an activation of the protein synthetic machinery was shown to occur early, followed by an increase in actual protein synthesis. Again, it was not possible to determine whether the increased activity of amino acid activating enzymes represented de novo synthesis or simply activation of pre-existing enzymes.

Pincus and others studied the effect of steroids on the carbonic anhydrase activity in rabbit uteri, finding that enzyme activity increased markedly during pregnancy, beginning shortly after mating (42). This rise in carbonic anhydrase activity was found to be due to the effects

of progestins and was also found to be abolished by the administration of estrogenic compounds (37, 42). Nicholls and Board studied the activity of carbonic anhydrase in human endometrium and found it to be increased by increasing the total dose and by prolonging the duration of therapy with oral progestins (40). Knudsen and others reported that the progesterone induced elevation of carbonic anhydrase activity in rabbit uteri could be inhibited by administration of carbonic anhydrase inhibitors such as Diamox (32). However, the normal proliferative response to progesterone in the rabbit was not affected by this treatment. The precise significance of these elevations of carbonic anhydrase is not clear, for no clear role in the metabolic processes of the endometrium has been elucidated for this enzyme. It is possible to speculate that the increased activity of carbonic anhydrase facilitates removal of carbon dioxide produced by both endometrium and implanting ovum, although why a requirement exists for large increases in this enzyme awaits clarification in further studies.

Szego and Davis have published some very interesting experiments on the effect of intravenously administered estradiol on cyclic-AMP levels in rat uteri (56, 57). They found that within fifteen seconds after intravenous administration of low doses of estradiol, total uterine content of cyclic-AMP was elevated significantly over

levels found in control animals (56). That this elevation of cyclic-AMP might be due to liberation by estrogen of a biogenic amine was tested by treatment of animals with a beta-adrenergic blocking agent prior to injection of the estradiol. Under these conditions, the elevation of cyclic-AMP was found to be inhibited (57). Alpha-adrenergic blocking agents were found to have no effect. Thus estrogens were shown to elevate cyclic-AMP by means of interaction with the beta-adrenergic receptors in the rat uterus.

In a similar type of experiment Rosenfeld *et al.* studied the effect of progesterone on cyclic-AMP levels and adenyl cyclase activity in the chick oviduct (47). They reported that progesterone given *in vivo* caused a delayed and progressive activation of adenyl cyclase, noted at three hours after treatment and becoming maximal 24 hours after injection. Cyclic-AMP levels were found to increase as well. Beta-adrenergic blocking agents did not inhibit the *in vivo* adenyl cyclase response to progesterone, nor did epinephrine, a beta-adrenergic stimulator, cause the enzyme to be activated. However, progesterone added *in vitro* to homogenates of chick oviduct failed to produce activation of adenyl cyclase. Administration *in vivo* of a cyclic-AMP analog did not induce synthesis of avidin, known to be a specific response to progesterone (47). Thus progesterone and estradiol have been shown to activate adenyl cyclase in the repro-

ductive tract. That separate adenyl cyclase systems may be involved can be inferred from a difference in the time courses in the enzyme response to the two hormones and from a difference in susceptibility to blockade by beta-adrenergic blocking agents.

In moving to the effect of added hormones on tissue slices and homogenates incubated in vitro, it is worthwhile to pursue further the demonstrated interrelationship of cyclic-AMP and estrogen. Szego demonstrated in another series of experiments that rat uteri incubated in vitro in the presence of cyclic-AMP and labelled amino acids revealed a stimulation of amino acid incorporation into protein (55). Hechter and others published similar observations, finding that cyclic-AMP stimulated incorporation of amino acids into protein as well as synthesis of lipids, glycogen, and RNA (21, 22). This stimulatory effect of cyclic-AMP was found to be dependent upon RNA synthesis and could be abolished by addition of actinomycin D, an inhibitor of RNA synthesis (21). Gorski et al. found that the response of rat uteri to estradiol could be blocked by inhibition of RNA and protein synthesis (15). Thus cyclic-AMP and estradiol have been reported to have similar effects on synthesis of protein, RNA and lipids.

O'Malley and others examined the effect of progesterone and estrogens on the chick oviduct and found that estradiol increased

the synthesis of RNA in the nucleus, in association with increased activity of nuclear RNA polymerase (41). Progesterone is known to stimulate the synthesis by the oviduct of a specific protein avidin; this stimulatory effect was thought to be due to alteration in the synthesis of messenger-RNA in the nucleus (41). Thus both estradiol and progesterone were found to alter the expression of genes through changes in the messenger-RNA produced in the nucleus. In a related study, Dingman et al. found that treatment of chicks with estrogen and progesterone produced a large increase in nuclear transfer-RNA and a small increase in cytoplasmic transfer-RNA (13). It was hypothesized that the transfer-RNA could be a rate limiting factor in protein synthesis and that hormonal control of availability of specific transfer-RNA species could have a regulatory effect on cytoplasmic protein synthesis. Stumpf has reported that in target tissues, radioactive labelled estradiol was found to be concentrated in the nuclei of the cells (52). Target tissues studied included uterus, vagina and oviduct; in non-target tissues such as liver and adrenal, no nuclear concentration of radioactive label was found. These findings were suggestive, as were those of O'Malley, Dingman and others, of a genomic effect of the hormones in target tissues of the reproductive tract.

Further data on the effect of estrogens and progesterone on oxidative metabolism comes from the work of Wade and Jones, who studied the effect of steroids on oxygen consumption and inorganic phosphate uptake (69). In a reaction mixture containing ATP, cytochrome-C, glucose, hexokinase, α-ketoglutarate, rat liver mitochondria and steroid, it was found that progesterone 6.4×10^{-4} M both inhibited oxygen consumption and showed a net liberation of inorganic phosphate. By contrast, other steroids, including estradiol, testosterone, pregnanediol and 17 α-hydroxyprogesterone, inhibited oxygen uptake to a lesser degree, but showed a net uptake of inorganic phosphate. Thus progesterone was the only steroid tested which produced a P:O ratio of zero. In the presence of other substrates progesterone was found to be equally effective in inhibiting oxygen consumption and in producing a net liberation of phosphate. Progesterone was the only steroid producing a net hydrolysis of ATP instead of a net synthesis. This data suggested that progesterone was acting to inhibit oxygen consumption at a site within the electron transport chain.

Disappearance of DPNH in the mitochondrial preparation was found to be accelerated by progesterone as well as by the other steroids tested. The increase in rate of disappearance of DPNH was greatest in the presence of progesterone.

Progesterone alone was also found to inhibit the reduction of cytochrome C but not its oxidation. In fresh mitochondria, where oxidation and phosphorylation remain coupled, progesterone completely uncoupled these two processes. As a result, progesterone was compared with dinitro-phenol (DNP), a known uncoupler of oxidation and phosphorylation. Both compounds were found to uncouple oxidation and phosphorylation in a similar manner.

The fact that progesterone caused accelerated removal of hydrogen from DPNH and at the same time inhibited cytochrome C reduction suggested that a site of action of progesterone occurred in the electron transport chain between DPNH and cytochrome C.

In other experiments Wade and Jones compared the effect of progesterone and DNP on ATPase activity in rat liver mitochondria (68). Both DNP and progesterone were found to activate ATPase in the mitochondria. Other steroids tested, including estradiol and testosterone, had no effect on ATPase activity. This data led to the conclusion that progesterone and DNP have similar modes of action on ATPase activity and oxidation phosphorylation in mitochondria.

Other investigators have reported inhibition of oxygen consumption in response to various steroids. Guidry et al. reported that estradiol decreased oxygen consumption of tissue homogenates (16). Cochran and DuBois reported that the oxidation of α-ketoglutarate by

whole tissue homogenates was inhibited by testosterone, ethinyl estradiol, progesterone, and diethylstilbestrol; estradiol and estrone were found to be ineffective as inhibitors of α-ketoglutarate oxidation (8). This data seems to provide further evidence that steroids, and particularly progesterone, may effect cell processes through alteration of the oxidative processes with concomitant changes in concentrations and availability of such co-factors as DPN and ATP.

An extended series of investigations carried out by Hagerman, Villee and co-workers provides further information on the effect of steroids on oxidative metabolism. In the early 1950's Hagerman and Villee observed a cyclic variation in oxygen consumption by human endometrium, associated with cyclic changes in glycogen content, lactate production, pyruvate oxidation and carbon dioxide production from glucose (17). The variation in glycogen content, suggested possible mediation by progesterone. Subsequently these investigators found that human endometrial slices, when incubated in vitro in the presence of 4×10^{-6} M estradiol, showed increased oxygen consumption when compared to controls (18). Progesterone was found to have no effect. This effect of estradiol on oxygen consumption was found in human placenta to be due to increased activity of the Krebs cycle pathways, without effecting lactate production, indicating no effect on glycolytic pathways (60). The stimulatory effect of estradiol on

oxygen consumption was found to occur through increased activity of the citrate-isocitrate-α-ketogluterate interconversion steps. Estradiol was seen to have a possible effect on electron transport (60). The site of action of estradiol was then localized to a DPN linked isocitrate dehydrogenase found in the 57,000 x g supernatant of human placental homogenates (14, 61, 63, 67). Hagerman and Villee subsequently showed that estradiol was bound by the activated enzyme system, and the activity of various other hormones was examined (19). Active hormones included 17α-estradiol and estrone, while 17β-estradiol, estriol, progesterone, testosterone and stilbesterol were found not to effect the enzyme (64). It is of interest that a potent synthetic estrogen, stilbesterol, was found to be ineffective in reproducing the effects observed with 17β-estradiol.

Subsequently Talalay and Williams-Ashman reported that catalytic quantities of TPN greatly increased the response of placental proparation to estradiol (58). They found that the apparent stimulation by estradiol of a DPN dependent isocitrate dehydrogenase actually represented a coupling of a TPN-dependent isocitrate dehydrogenase with a soluble transhydrogenase system, giving the following reactions:

$$\text{ISOCITRATE} + \text{TPN} \longrightarrow \alpha\text{-KETOGLUTARATE} + CO_2 + \text{TPNH}$$

$$\text{TPNH} + \text{DPN} \longrightarrow \text{TPN} + \text{DPNH}$$

$$\text{SUM: ISOCITRATE} + \text{DPN} \longrightarrow \alpha\text{-KETOGLUTARATE} + CO_2 + \text{DPNH}$$

It was demonstrated that any TPNH generating enzyme, such as glucose-6-phosphate dehydrogenase, could be substituted for the isocitrate dehydrogenase. Thus the transhydrogenation reaction was shown to be the step sensitive to estradiol.

Talalay and Williams-Ashman observed that in mammalian tissues, TPNH is present in greater concentrations than TPN, while DPN is present in higher concentrations than DPNH. The ratio $\frac{[TPNH]\,[DPN]}{[TPN]\,[DPNH]}$ is thus quite high; this ratio also represents the equilibrium constant of the transhydrogenase reaction shown above. This was seen to have important implications regarding the activities of reactions dependent upon DPNH and TPNH and possible influence by estrogens on the DPNH and TPNH linked reactions.

A second paper by Talalay, Hurlock and Williams-Ashman presented evidence supporting the idea that the estradiol sensitive trans-

hydrogenase was a revisible 17β-hydroxysteroid dehydrogenase, with specificity for both TPN and DPN (59). The following reactions were thought to occur:

ESTRADIOL-17β + DPN ⟶ ESTRONE + DPNH

ESTRONE + TPNH ⟶ ESTRADIOL-17β + TPN

SUM: TPNH + DPN ⟶ DPNH + TPN

A similar steroid dehydrogenase had been reported previously by Langer and Engel (33, 34). Thus it was felt that the transhydrogenation and dehydrogenation functions were common to a single enzyme.

Villee and co-workers published observations which confirmed those of Talalay that transhydrogenation was the site of estrogen sensitivity (65, 66, 67). However, Hagerman and Villee then reported separation of the transhydrogenase activity from the dehydrogenase activity in placental preparations (20). This was followed by a report by Abe, Hagerman and Villee that an estrogen dependent transhydrogenase was present in human myometrium, in the absence of any estradiol dehydrogenase activity (1). However, this myometrial trans-

hydrogenase was sensitive to diethylstilbesterol, whereas the placental transhydrogenase was not stimulated by this synthetic estrogen (2, 58, 64). Jarabak et al. had reported extensive purification of the dehydrogenase activity from placenta and found no separation of dehydrogenase from transhydrogenase activity (31). Thus, the question of whether or not the estradiol sensitive dehydrogenase and transhydrogenase functions of human placenta were the same or different enzymes could not be resolved.

Hollander, Hollander and others studied the placental estrogen sensitive system and found results consistent with those of Talalay, supporting the idea that transhydrogenation was a function of the dehydrogenase activity (24). These investigators also examined the structural specificity of the estrogen-sensitive process and found that a hydroxyl or keto group at position 17 was essential for activity (23). It was also found that 17α-dihydroequilenin had a high affintiy for the enzyme, but was devoid of any stimulating effect.

In the above mentioned experiments on estradiol sensitive transhydrogenase function, enzyme activity was measured by the rate of appearance of DPNH produced via transhydrogenation from TPNH to DPN. However, none of the investigators determined the effect of steroids on the reaction in which DPNH was re-converted to DPN.

Yielding, Tomkins and others examined the effect of steroids on DPNH oxidation by particulate fractions from rat liver and found that steroids, especially progesterone, estradiol and diethylstilbesterol, inhibited the oxidation of DPNH (72). These results were thought to be independent of any transhydrogenase reaction. The mechanism of this inhibition seemed to be by interruption of electron flow from DPNH to cytochrome C. Further studies indicated that the locus of steroid inhibition of the DPNH-cytochrome C reductase was between flavoprotein and coenzyme Q or cytochrome b (73). Progesterone and estradiol were found to act at the same site.

Hollander and Stephens, on the other hand, reported that estrogens activated a DPNH oxidase in particulate fractions of rat uteri (25). An enzyme which converted DPNH to DPN was found to be present in increased activities in the uteri of ovariectomized rats treated with estrogen. The relationship of these findings to those of Yielding and Tomkins is not at all clear.

To summarize, ovarian steroids have been found to influence a great number of cell functions, including DNA, RNA, lipid and protein synthesis, oxygen consumption, concentration of various substances such as glycogen, lactate and cyclic-AMP, activities of mitochondrial and soluble enzymes, of carbohydrate metabolism, and hydrogen transfer from pyridine nucleotides.

Results to be presented in this paper will examine further the effect of steroids on the placental transhydrogenase system, on a soluble placental DPNH oxidase, and on activation of glycogen-phosphorylase and adenyl cyclase in rabbit endometrium.

Materials and Methods

Materials

Biochemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, and P-L Biochemicals, Inc., Milwaukee, Wisconsin. Steroid hormones were obtained from Mann Research Labs, New York, New York. Radiochemical supplier was Schwarz-Mann, Orangeburg, New York. Equipment and sorbents for thin-layer chromatography were obtained from Brinkmann, Inc., Westbury, New York.

Methods

I. Transhydrogenase

Preparation of enzyme

The method used was a modification of the one described by Talalay and Williams-Ashman (58). Human term placentas were obtained within fifteen minutes of delivery, fibrous tissue was removed and sections were washed in two changes of ice cold physiologic saline. After measurement of wet weight, the tissue was homogenized in 4-5 volumes of ice cold 0.25 M sucrose pH 7, using an ice jacketed Waring Blendor. Centrifugation was carried out at 2000 x g

for 15 minutes at 2^{o} C. in a Sorvall RC2-B refrigerated centrifuge. The supernatant was then centrifuged at 90,000 x g for one hour at 2^{o} C. in a Beckman model C ultracentrifuge. The supernatant was quick frozen in a dry ice-acetone bath and lyophilized overnight. The lyophilized preparation was stored at -20^{o} C., where it was stable for approximately one week. Prior to use, lyophilized enzyme preparation was reconstituted in 10-15 volumes of Tris buffer 0.01 M pH 7.4, producing an initial optical density at 340 mu of 0.4 - 0.8 optical density units.

Assay of transhydrogenase activity:

Reconstituted lyophilized enzyme was centrifuged at 5,000 x g x 15 minutes to clear it of precipitated particles. Each cuvette contained 300 umoles 0.01 M Tris pH 7.4, 1.4 umoles DPN, 10 umoles glucose-6-phosphate, one unit glucose-6-phosphate dehydrogenase, 50 umoles $MgCl_2$, 1.0 ml. reconstituted enzyme preparation and sufficient distilled water to make a final volume of 3.0 ml. Hormones were added dissolved in 10 ul. of dioxane to give the appropriate final concentration. Estradiol 5×10^{-6} M, corresponded to 4 ug. per cuvette; progesterone at the same concentration corresponded to 4.62 ug. per cuvette. Control cuvettes contained 10 ul. of dioxane without hormone.

Reactants plus enzyme were placed in the cuvette and optical density at 340 mu was recorded for 10 - 20 minutes until a stable baseline rate was obtained. At that point 0.02 umoles of TPN were added in 0.1 ml. of distilled water to initiate the transhydrogenation reaction. Optical densities were then followed for a further period of 45 - 60 minutes, during which time all reactions appeared to be linear.

Assay of DPNH oxidase activity

Enzyme was prepared and reconstituted as above. Cuvettes contained 300 umoles 0.01 M Tris pH 7.4, 50 umoles $MgCl_2$, 1.0 ml. enzyme, 0.5 umoles DPNH, and distilled water to a final volume of 3.0 ml. Hormones were added in 10 ul. of dioxane. Reactions were followed for one hour at 340 mu, and were found to be non-linear.

II. A. Phosphorylase activity after ovulation in rabbits:

Post-ovulatory New Zealand white rabbits were anesthetized with 35 mg./ kg. of pentabarbital. The reproductive tract was exposed and the uterine vessels on either side clamped. Uteri were excised, the endometrium isolated immediately and placed in ice cold 0.1 M NaF pH 7.0. Care was taken to isolate the tissue in ice cold NaF within 2 - 3 minutes after excision. Tissue was then blotted dry and its wet weight determined. Homogenization of 100 - 250 mg. of tissue per ml. of 0.1 M NaF pH 7.0 was carried out in a hand-powered ground glass Pyrex homogenizer of 2 ml. capacity. Total volume of homogenizing fluid was 1.0 or 2.0 ml. depending on amount of tissue obtained. After homogenization, tissue was kept in ice where it was stable for up to two hours until assays were performed. All assays were begun within sixty minutes after homogenization.

The phosphorylase assay was Hug's modification of Sutherland's assay (26, 27, 53). Each homogenate was assayed at least in duplicate, both in the presence and absence of adenosine monophosphate (AMP). At zero time 0.1 ml. of homogenate was pipetted into 0.4 ml. of substrate which had been incubated at 37° C. for two minutes previously. Substrate contained 0.032 M glucose-1-phosphate, 2%

glycogen, plus and minus 0.002 M AMP at pH 6.0. At 15 and 30 minutes 0.2 ml. of reaction mixture was removed and pipetted into 1.0 ml. of 5% TCA. Zero time samples were prepared by pipetting 0.04 ml. of homogenate directly into 1.0 ml. of 5% TCA plus 0.16 ml. of substrate plus and minus AMP. At the end of the reaction, the tubes were spun at 1,000 x g for five minutes to sediment the protein precipitate, and then 0.8 ml. was removed and placed in a 15 ml. test tube for assay of phosphate content.

Volume was made up to 5.0 ml., 1.0 ml. of 5 N H_2SO_4 was then added and the tubes were mixed. Following this, 1.0 ml. of 2.5% ammonium molybdate, 0.5 ml. of phosphate reducer, and distilled water to a final volume of 10.0 ml. were added. Phosphate reducer contained 3.6 gm. Na_2SO_4, 36 gm. $NaHSO_3$, and 0.6 gm. aminonaphtholsulfonic acid in a volume of 300 ml. The tubes were mixed and allowed to stand for 20 minutes, after which time they were read in a Klett colorimeter with a 660 mu red filter. Two phosphate standards were prepared containing 1 uM phosphate per tube.

Enzyme activity was calculated on the basis of the optical density change in 30 minutes and is expressed as umoles inorganic phosphate liberated / minute / gm. wet weight of tissue.

B. Activation of phosphorylase by intravenous steroids:

Rabbits were anesthetized as above and after exposure of both uterine horns, the vessels on one side were clamped and that horn excised. Endometrium was immediately isolated and homogenized as above. After removal of one uterine horn, the animal was injected with a suspension of steroid in 0.9% saline with benzyl alcohol as a preservative. Steroid suspensions contained 10 mg./ ml.; dosages were 10 mg. / kg. body weight.

Five minutes after intravenous injection of the steroid suspension, the vessels of the remaining uterine horn were clamped and the horn removed. Tissue was then isolated and homogenized as before. Both the pre- and post-injection samples were then assayed for phosphorylase activity and the two compared.

III. A. Adenyl cyclase assay

Two animals were anesthetized as before and the endometrium pooled and homogenized in 4 volumes of ice cold 0.25 M sucrose, 0.1 M PO_4 pH 7.5, using a hand-driven Pyrex ground glass homogenizer with 2 ml. capacity. The homogenate was split into 0.2 ml. aliquots to which 1 ul. of dioxane with or without hormone was added, to give a final concentration of steroid of 10^{-5} M in the homogenate.

The assay system was that described by Cohen and Bitensky, and Bitensky et al. (4, 9). At zero time 10 ul. of homogenate with or without hormone was added to 10 ul. of reaction mixture which had been incubated at 30° C. The final reaction mixture contained $MgSO_4$ 3.5×10^{-3} M, glycylglycine 4×10^{-2} M, EDTA 1×10^{-4} M, aminophylline 6.6×10^{-3} M, phosphoenolpyruvate 2×10^{-2} M, pyruvate kinase 2 ug., ATP-8-C^{14} (specific activity 40 - 50 mC/ mmole) 6.6×10^{-4} M, and phosphate buffer 0.045 M pH 7.5. After a six minute incubation at 30° C., tubes were placed in a boiling water bath for three minutes. Following this, 20 ul. of distilled water containing 2×10^{-2} M cyclic-AMP was added to each tube. Ten ul. of each reaction tube was spotted in duplicate on thin-layer chromatography plates prepared and run as follows.

B. Separation of cyclic-AMP from other nucleotides:

Separation was done using Bitensky's modification of the technique of Randerath (3, 45, 46). Thin layers were prepared from a mixture of 18 gm. PEI cellulose, 12 gm. MN300 cellulose, 1.5 gm. soluble starch and 150 ml. distilled water which was poured on 8" x 20" glass plates, with a thickness of 0.5 mm. After baking dry, 10 ul. samples of reaction mixture were spotted, 6 to 8 per plate, using a hot air blower to dry the samples as they were spotted. Plates were developed in a descending direction, using a filter paper wick, for nine hours with solvent A (30% 1 M ammonium acetate in water, and 70% methanol). After development with solvent A, plates were air dried, rewicked and run for 10 hours in solvent B (25% glacial acetic acid, 25% distilled water and 50% 1-butanol). Afterwards, plates were again air dried, the cyclic-AMP marker was located with short-wave ultra-violet light and the spots were shaved off. Samples were placed in glass vials and counted in a Packard Tri-Carb model 3375 Scintillation counter using toluene based scintillation fluid.

Recovery of cyclic-AMP calculated from chromatography of H^3 labelled cyclic-AMP was greater than 90%.

Results and Discussion

Placental transhydrogenase:

Initially it was necessary to determine that the placental extracts prepared as described earlier actually possessed the capacity to transfer hydrogen from TPNH to DPN; also it was necessary to determine whether or not this hydrogen transfer activity could be stimulated by 17β-estradiol. As seen in Figure 3, the placental extract exhibited a low level of transhydrogenase activity prior to the addition of TPN. After TPN was added, the rate of change in optical density increased markedly in the cuvette containing estradiol 5×10^{-6} M, but remained essentially constant in both the control cuvette lacking hormone and in the cuvette containing progesterone 5×10^{-6} M. Thus the following points were established: (1) the extract possessed transhydrogenation activity; (2) this activity could be stimulated by estradiol; and (3) progesterone, in the same concentration as that of estradiol, lacked any stimulatory activity when compared to the control.

The effect of varied concentrations of hormone was then examined. Talalay and Williams-Ashman had determined that estradiol 5×10^{-6} M gave maximal stimulatory effect (58). Comparing Figures 3 and 4, in which the same placental preparation was used, it is seen that

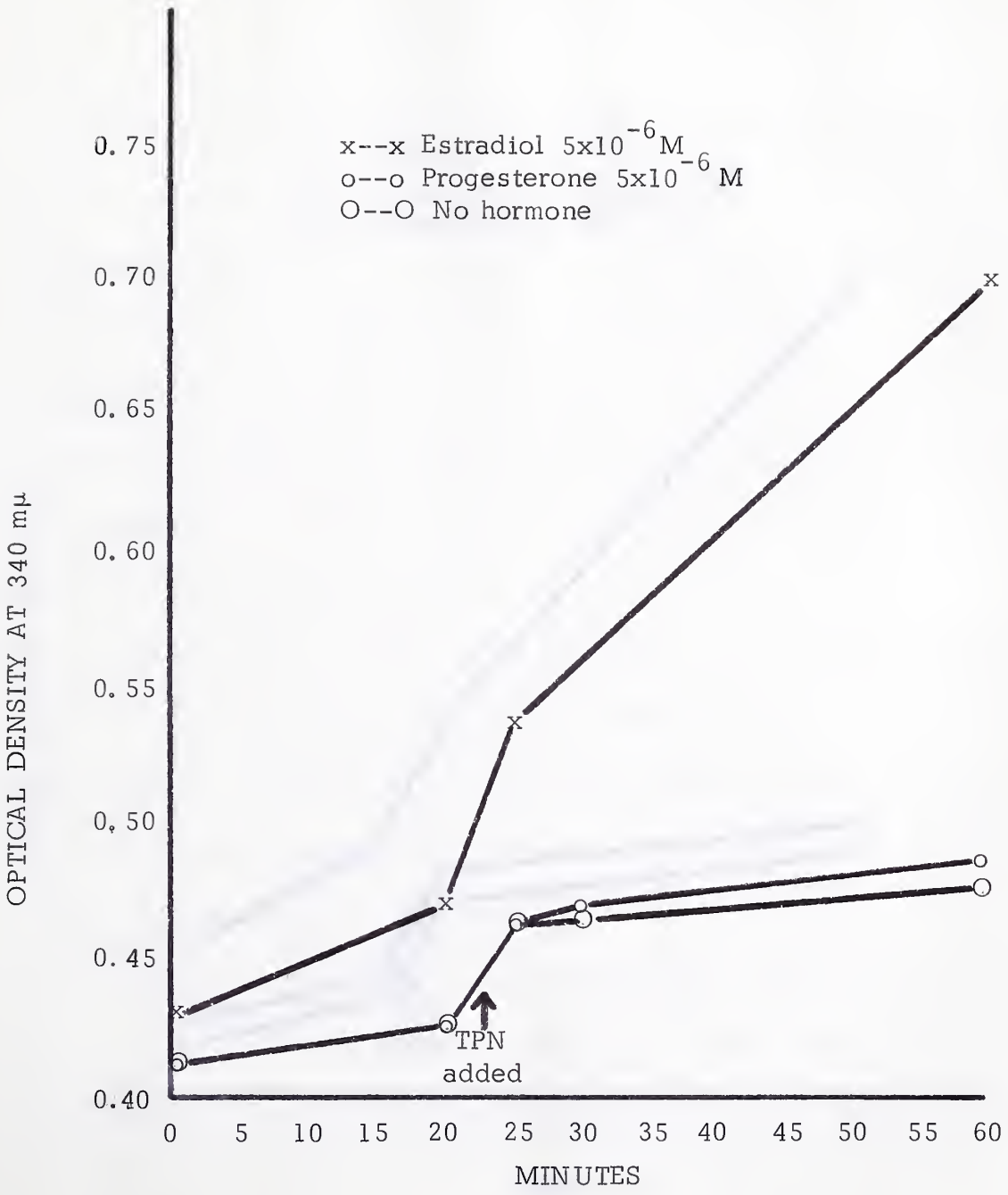


Fig. 3 Effect of estradiol and progesterone on placental trans-hydrogenase activity.

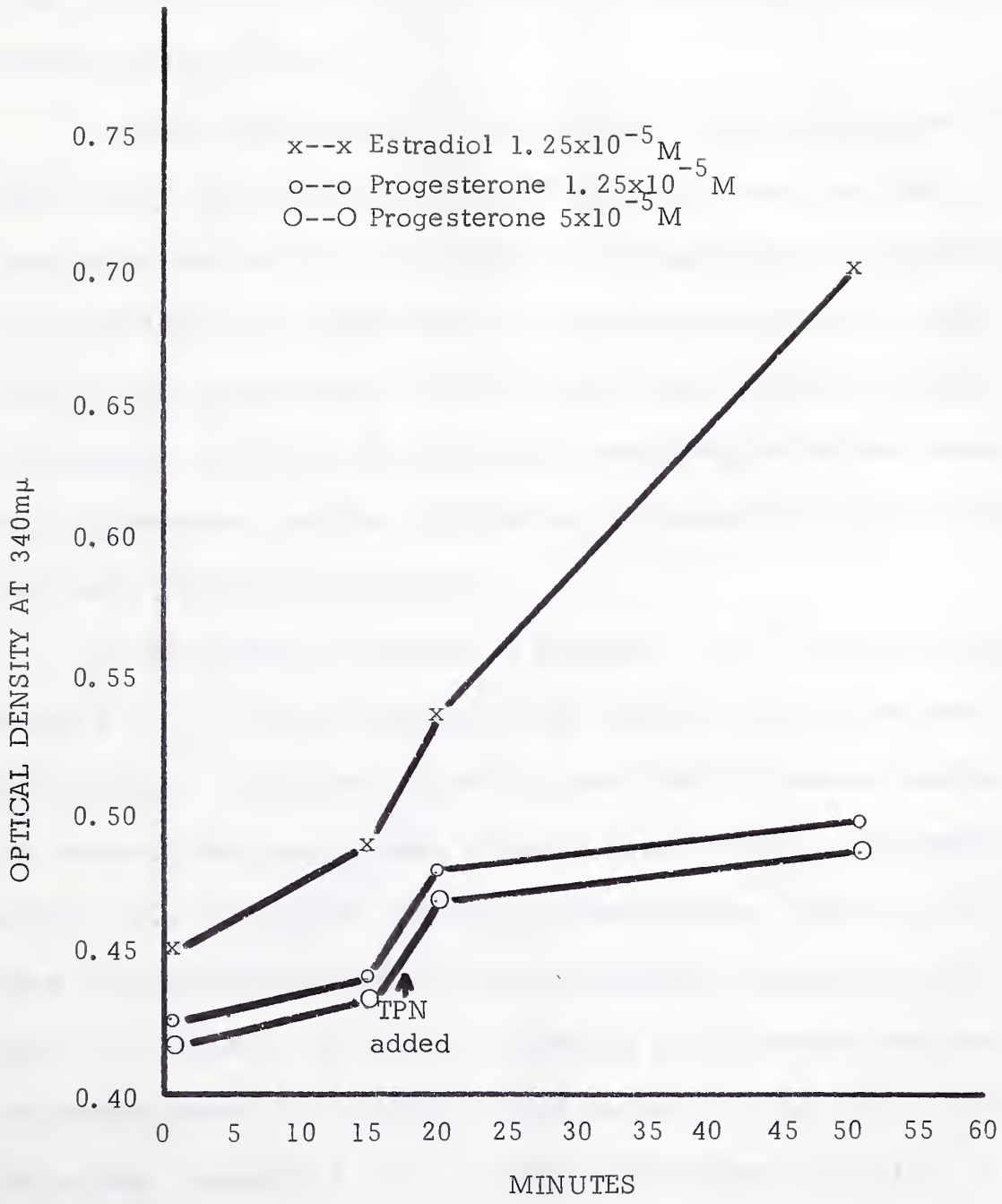


Fig. 4 Effect of varied concentrations of hormones on transhydrogenation.

increasing the concentration of estradiol to 1.25×10^{-5} M produces no additional stimulatory effect. Also, progesterone in concentrations of 1.25×10^{-5} M has no stimulatory activity when compared to controls lacking hormone.

The point that remained to be clarified, and one which had not been touched upon by any previous investigators, was the effect on transhydrogenase activity of estradiol and progesterone in combination. The possibility of an antagonistic or synergistic effect was of great interest since progesterone acts only upon tissue previously primed by estrogen. Solely in the case of an ovariectomized animal treated with progesterone does this hormone act on tissue which has not been previously influenced by estrogen.

For this reason, the effect of estradiol 5×10^{-6} M plus progesterone 5×10^{-5} M was compared to the effects of these hormones acting singly. In Figure 5 it can be seen that the cuvette containing estradiol plus progesterone possessed approximately 10% greater activity than the cuvette containing estradiol alone. Again, the cuvette containing progesterone alone demonstrated essentially no activity. In Figure 6, the effect of estradiol in combination with varied concentrations of progesterone was compared to the effect of estradiol alone. Estradiol 5×10^{-6} M plus progesterone 1.25×10^{-5} M possessed approximately 20% greater activity than estradiol alone

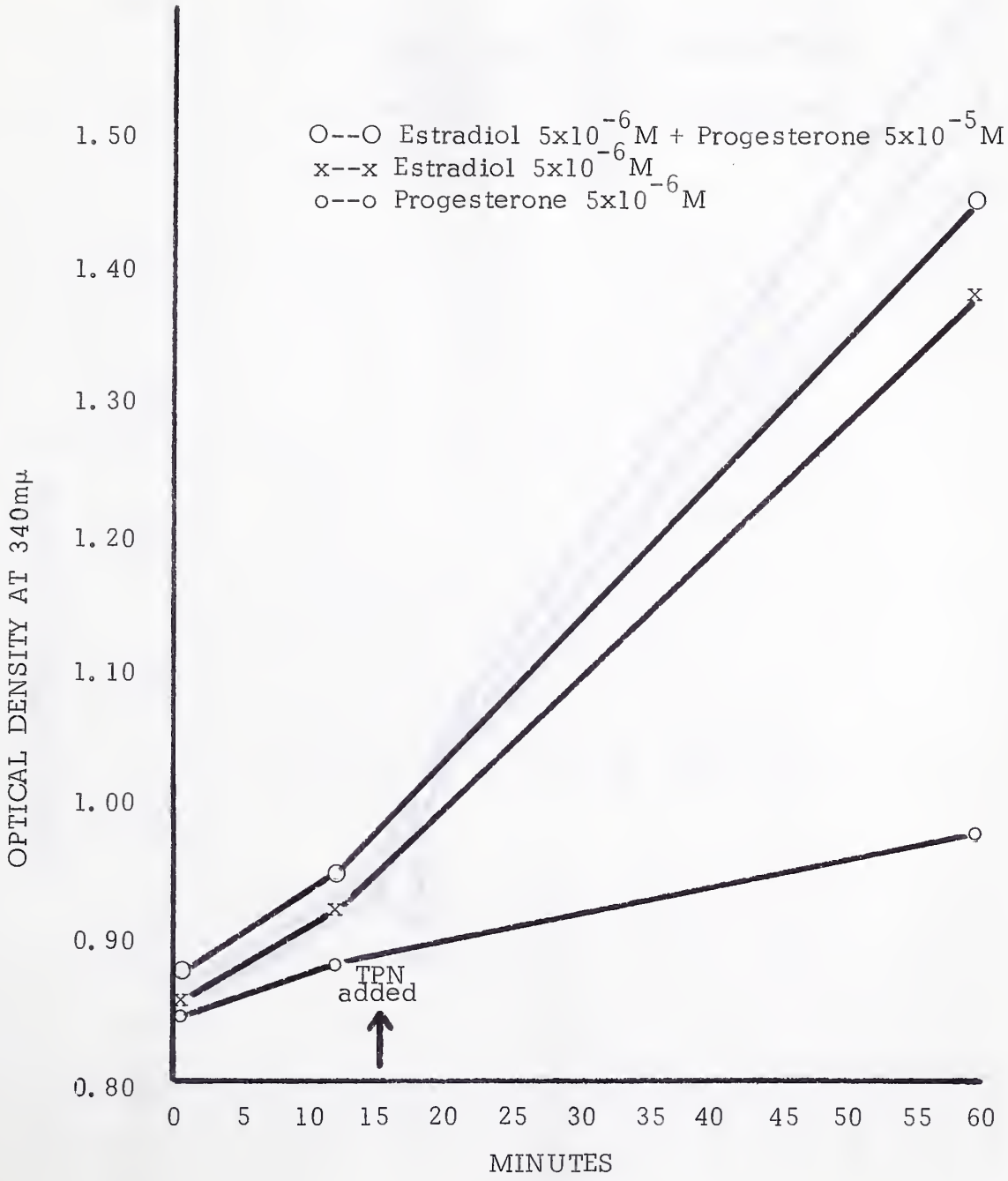


Fig. 5 Potentiation of estradiol effect by progesterone.

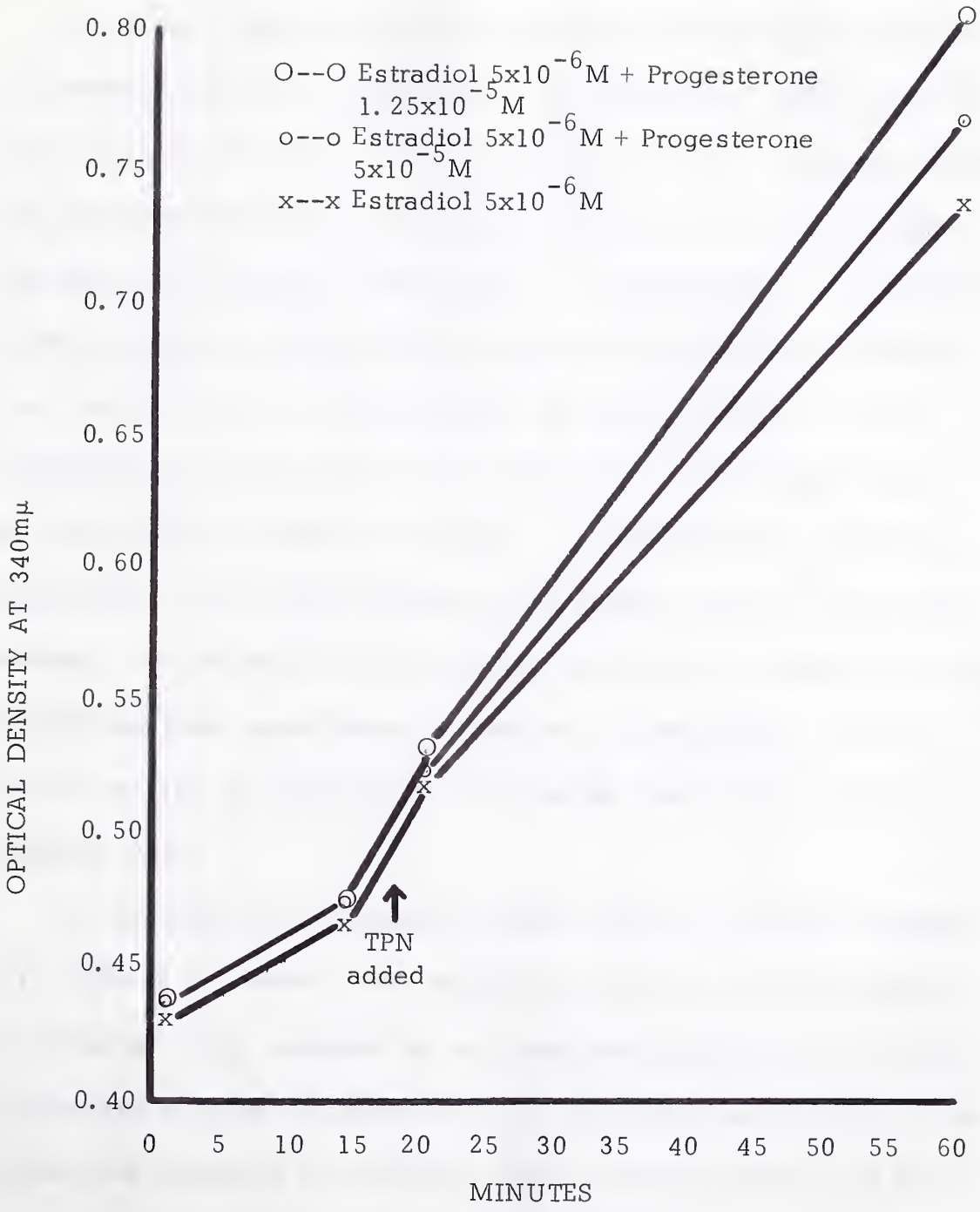
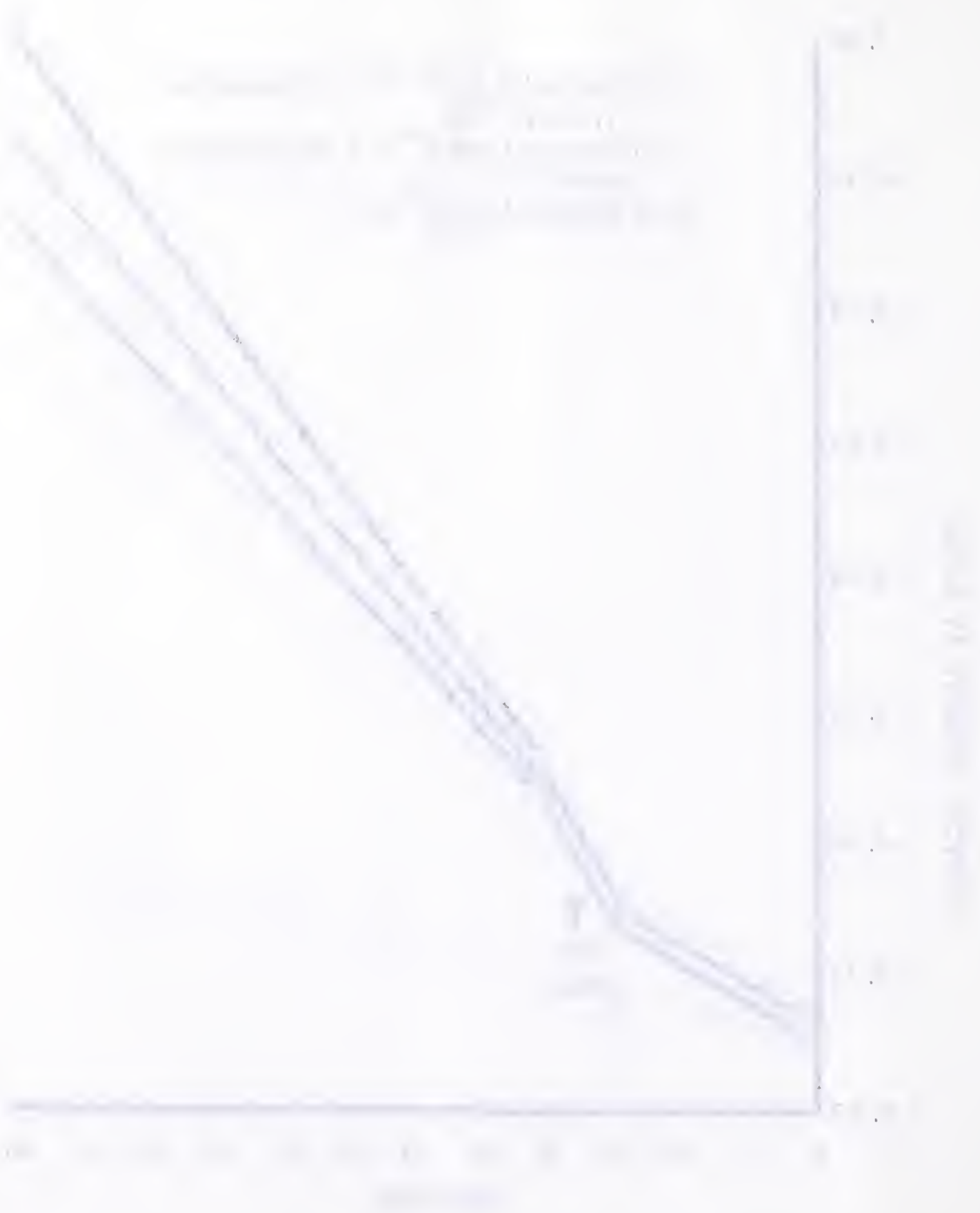


Fig. 6 Potentiation of estradiol effect by varied concentrations of progesterone.



and 10% greater activity than estradiol 5×10^{-6} M in combination with the same concentration of progesterone.

It is not clear what might be the cause of this potentiation of the estradiol effect by progesterone. If progesterone were bound by the enzyme at the same site that estradiol is bound, one would expect that progesterone would be least partially block the effect of estradiol when both hormones were present. If progesterone is not bound by the enzyme, no effect would be seen. An alternative explanation, which cannot be validated with the data available, is that progesterone may be bound by the enzyme at a site different from the site at which estradiol is bound. In the absence of estradiol, progesterone, even though bound by the enzyme, has no stimulatory activity. In the presence of estradiol, bound by the enzyme at a second binding site, progesterone possesses the capability of activating the enzyme to levels 10 to 20% greater than those produced by estradiol alone.

In the absence of separate binding sites for the two hormones, it is difficult to arrive at any explanation for the observed effects. As Hollander et al. reported on the structural specificity of the transhydrogenase enzyme, hormones such as dihydroequilenin could act as competitive inhibitors of estradiol; these hormones were bound by the enzyme at the same site as estradiol, thus displacing estradiol and

inhibiting its activity (23). Such a role for progesterone would explain inhibition of the estradiol effect, not the potentiation actually observed. Thus it seems reasonable to hypothesize the presence of a binding site for progesterone, separate from the estradiol site, that allows the progesterone to potentiate but not interfere with the estradiol effect. Furthermore, for progesterone to have any effect, estradiol must be simultaneously bound by the enzyme, as evidenced by the absence of any effect of progesterone alone.

Placental DPNH oxidase:

The reaction being followed in studying transhydrogenase activity actually involves measurement of optical density change due to the appearance of DPNH; the optical density change observed is the sum of DPNH produced by transhydrogenation from TPNH to DPN, and DPNH oxidized to DPN by the DPNH oxidases present in the soluble fraction of placenta. None of the investigators who studied the placental transhydrogenase actually examined the effect of steroids on the DPNH oxidases present in the preparation used.

The only available data is that of Yielding and Tomkins on the effect of steroids on the DPNH oxidase found in the microsomal fraction of the liver (72). Their results showed that both progesterone and estradiol produced significant inhibition of DPNH oxidation. A comparison of the effectiveness of inhibition of DPNH oxidation by these two hormones revealed that progesterone was the more effective inhibitor.

The findings of Yielding and Tomkins raise the possibility that the potentiation by progesterone of the estradiol effect on transhydrogenation might, in fact, represent inhibition of the DPNH oxidase by progesterone. Thus estradiol could be stimulating the production

of DPNH while progesterone could be inhibiting its degradation. The differences in activity of estradiol plus progesterone compared to estradiol alone might represent two different effects combining to give the same net result, namely the presence of a higher concentration of DPNH.

The effects of estradiol and progesterone on DPNH oxidation were examined, using the same soluble preparation of placenta as was used to study transhydrogenation. The results, shown in Figure 7, demonstrate that, contrary to the findings of Yielding and Tomkins, estradiol is a more effective inhibitor of DPNH oxidation than is progesterone. However, progesterone does possess the capacity to inhibit DPNH oxidation when compared to the control lacking hormone. Thus it is possible that the previous observation that estradiol plus progesterone possessed greater transhydrogenase activity than estradiol alone may be due to the capacity of progesterone to inhibit the oxidation of DPNH to DPN.

The measurement of DPNH produced is a net measurement dependent upon the differences in rate of reduction of DPN and the rate of oxidation of DPNH. Estradiol seems clearly to accelerate the rate of reduction of DPN, while progesterone alone has no apparent effect. Progesterone does have an appreciable effect on the rate of oxidation of DPNH. Estradiol and progesterone in combination may produce

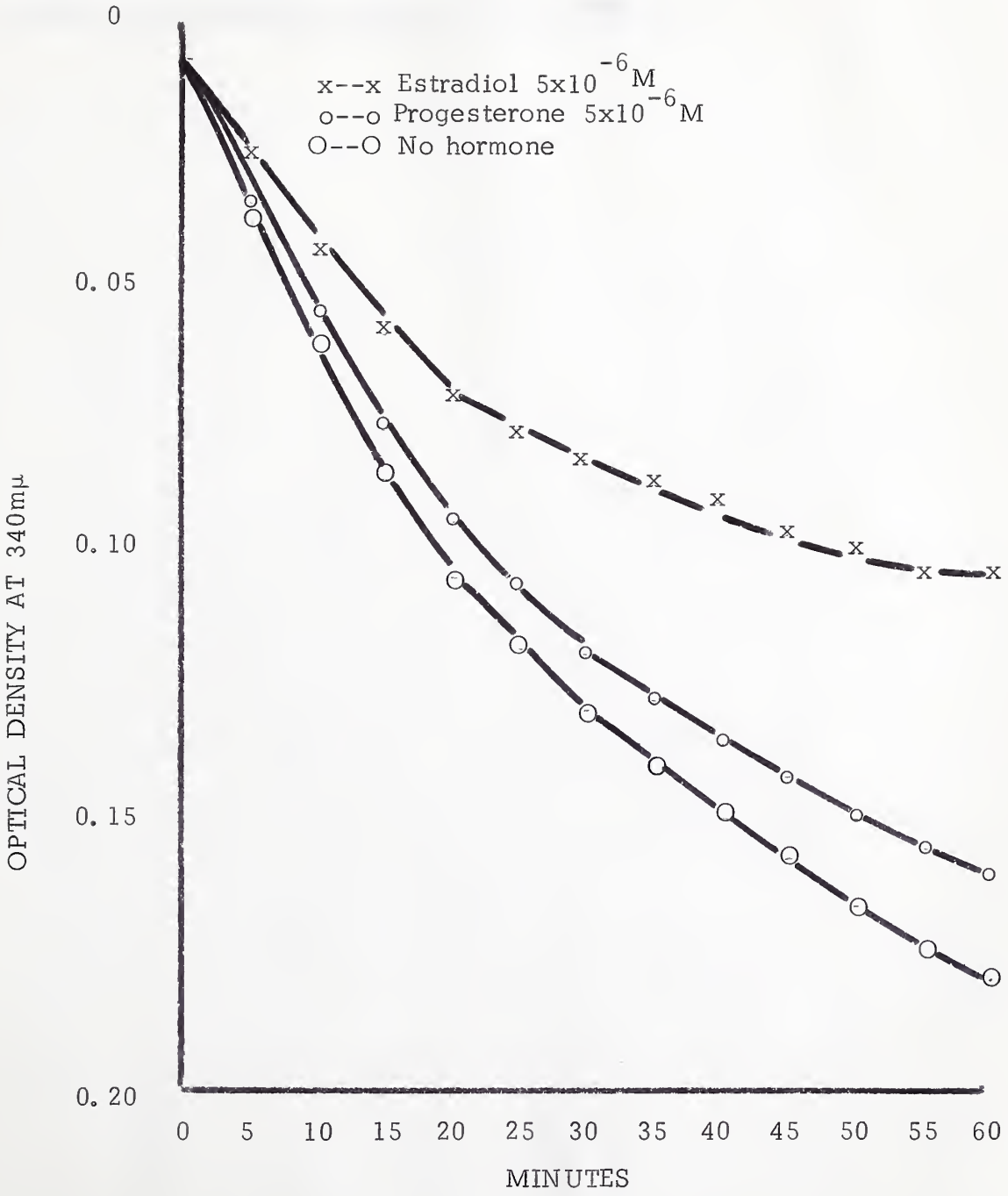


Fig. 7 Effect of estradiol and progesterone on oxidation of DPNH by placental preparations.

the observed effect of increased net production of DPNH if estradiol stimulates the forward reaction of DPN → DPNH, and progesterone inhibits the backward reaction of DPNH → DPN.

Phosphorylase activity in the endometrium of pregnant rabbits

In view of the results of Hughes et al. on the cyclic variations of phosphorylase activity in human endometrium and the conclutions of these investigators that phosphorylase was stimulated by the post-ovulatory presence of progesterone (30), it was decided to study the activity of phosphorylase in the endometrium of the post-ovulatory rabbit.

The rabbit provides an ideal opportunity to study the effects of progesterone on endometrial enzymes because rabbit endometrium is in a steady-state stimulation by estrogen until the time of ovulation (36). The rabbit is one of the few animals which ovulates in response to mating, ovulation occurring 10 to 12 hours after mating, depending on the species studied (36). Immediately after ovulation, the corpus luteum begins producting progesterone, and progestational effects such as an increase in carbonic anhydrase activity can be seen (42). That post-ovulatory changes are mainly due to progestational hormonal influence is seen by the fact that estrogens will abolish the observed increase in carbonic anhydrase activity after ovulation (37).

Therefore, it was felt that post-ovulatory changes in phosphorylase would be due to the effects of progesterone, either as a stimu-

lator of synthesis of new enzyme, or as an activator of pre-existing enzyme. The results shown in Table I and Figure 8 reveal a rise in endometrial phosphorylase activity by 24 hours after mating. Failure to demonstrate a rise at 12 hours after mating may indicate that the species of rabbit used does not ovulate until the period 12 to 24 hours after mating. Thus, within a few hours after ovulation, endometrial phosphorylase activity increases significantly over control values. Such a rapid rise in activity indicates either rapid induction and synthesis of new enzyme or activation of already present enzyme.

Additional information obtained from Figure 8 shows that endometrial phosphorylase is activated by adenosine monophosphate (AMP). This indicates that the endometrial phosphorylase is of the muscle type, that which is activated by AMP. This finding is in agreement with the data of Hughes et al. who obtained similar results with human endometrium (30). In contrast to endometrial phosphorylase is that found in rabbit liver. Experiments performed with rabbit liver revealed enzyme activities 30 to 50 times greater than those found in endometrium. In addition, no stimulation of liver phosphorylase by AMP was found.

The activity of endometrial phosphorylase is seen to peak at 24 hours and then to decline to levels still above those of the con-

TABLE I

PHOSPHORYLASE ACTIVITY
($\mu M\ PO_4$/min./gm.)

HOURS AFTER MATING	+ AMP ANIMAL #1	+ AMP ANIMAL #2	+ AMP AVE.	- AMP ANIMAL #1	- AMP ANIMAL #2	- AMP AVE.
12	2.20	2.82	2.51	1.71	1.58	1.65
24	5.45	5.62	5.53	4.34	3.52	3.93
48	3.07	3.13	3.09	2.72	1.97	2.35
72	3.56	2.98	3.29	2.81	2.95	2.88
96	3.16	3.66	3.41	2.96	3.34	3.15
120	3.44	4.63	3.98	3.18	4.30	3.74

Phosphorylase activity in the endometrium of the post-ovulatory rabbit. See Fig. 8..

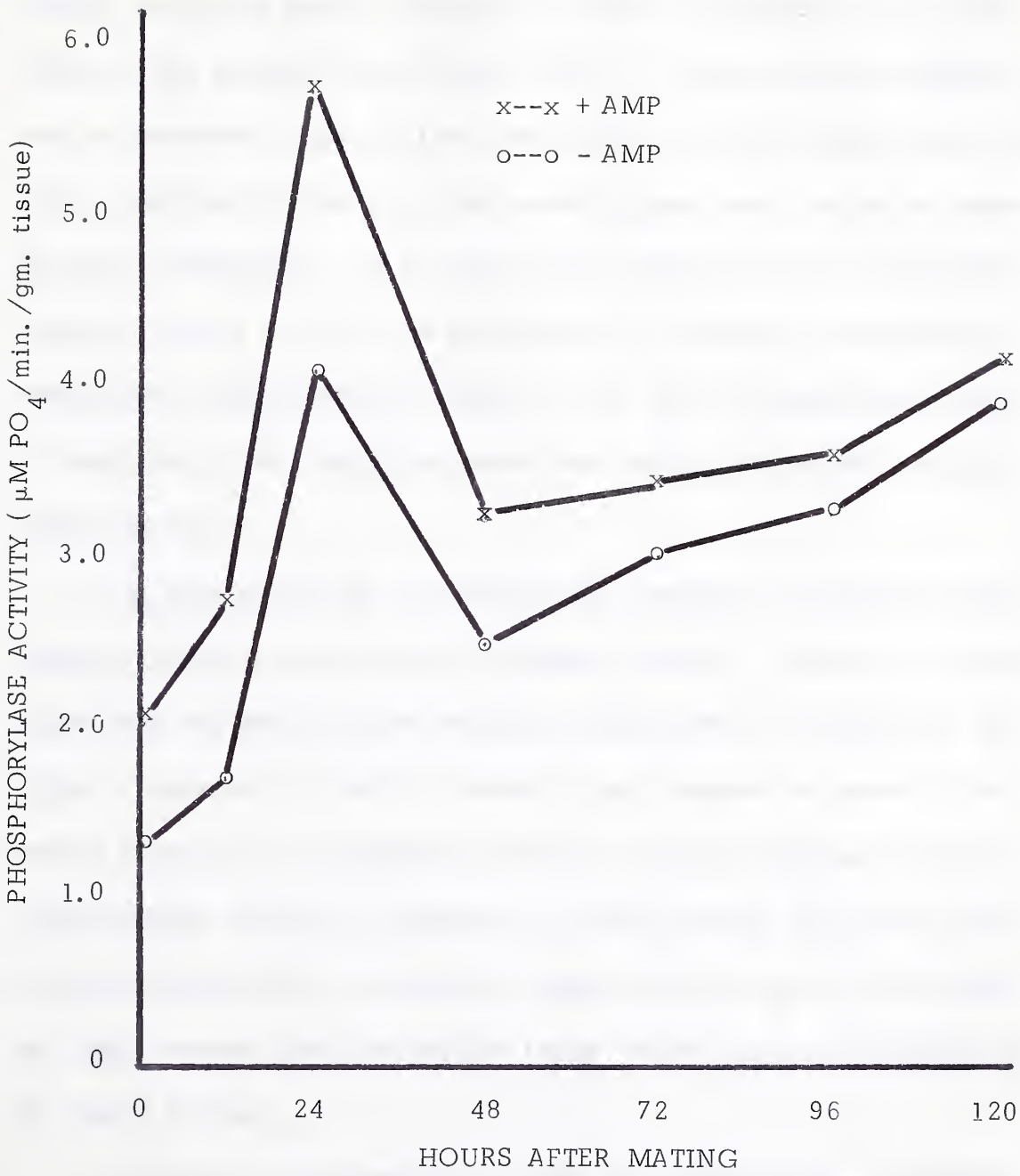


Fig. 8 Phosphorylase activity in the endometrium of the post-ovulatory rabbit.

trols. Enzyme activity was measured up until the fifth day after mating; after this period, during the sixth and seventh days, implantation of the fertilized ovum occurs (36). It was felt that differences in endometrium due to the local effects of implantation would make values obtained on the sixth and seventh days after mating of questionable significance. If a micro-assay system could be developed, requiring only 5 - 10 mg. of endometrium, it would be possible to examine the phosphorylase activity at the site of implantation and to determine if the implanting ovum has direct local effects on enzyme activity.

The role served by the changes in glycogen and glycogen metabolizing enzymes has not been precisely defined. However, it seems likely that the pre-ovulatory buildup of glycogen is important in providing a reservoir of readily available carbohydrate to serve as an energy source for the implanting ovum. The post-ovulatory rise in phosphorylase results in liberation of large amounts of glucose into the endometrial cell. Presumably, either glucose or its metabolites are then secreted into the uterine lumen where they are available as an energy source.

In humans, according to the data of Hughes et al., glycogen reaches a peak concentration of 1000 mg. % from a low of 150 mg. % (30). If this difference of 850 mg. % were broken down into glucose

and secreted into the uterine lumen, a substantial glucose concentration would be produced in the luminal fluid. Contrasted to this is the glucose concentration in human blood, which normally ranges from 60 to 100 mg. %. Degradation of glycogen and secretion into the uterine lumen would produce higher concentrations of glucose within the lumen and facilitate entry of glucose into the ovum by diffusion.

The ovum, prior to implantation, exists in the avascular environment of the uterine lumen where it must depend upon simple diffusion to provide necessary metabolites and to remove waste products. Metabolites must enter from the uterine fluid, and waste products must diffuse outwards into this fluid. A high external glucose concentration would accelerate diffusion into the ovum by producing a greater concentration gradient between the extracellular and intracellular fluid.

As another consequence of an avascular environment, the ovum must exist in conditions of low oxygen tension since there is no available capillary network to provide a continuous supply of oxygen. Thus the ovum depends primarily on anaerobic metabolic processes to provide energy prior to implantation and establishment of a blood supply. Comparison of the energy yield from one mole of glucose under aerobic and anaerobic conditions reveals that aerobic metabolism produces 38 moles of high-energy phosphate bonds per

mole of glucose, whereas anaerobic metabolism produces only 2 moles of high-energy phosphate bonds per mole of glucose. Under anaerobic conditions, the cell would therefore require almost twenty times as much glucose to produce the necessary amounts of high-energy phosphate bonds as it would require under aerobic conditions. A large amount of the energy produced by this twenty times greater quantity of glucose would be liberated as heat energy.

Under anaerobic conditions the cell relies on the glycolytic pathways producing lactate as an end product. By producing lactate, the DPNH generated by an earlier step in the glycolytic pathway is converted back to DPN, thus eliminating the need for oxidation of DPNH by oxygen in the mitochondria.

As a result of the inhibition of oxidative phosphorylation by progesterone demonstrated by Wade and Jones (69), the endometrium as well as the ovum is dependent upon anaerobic metabolism for energy supply. Since DPNH oxidation is inhibited by progesterone and estradiol as demonstrated in this paper and by Yielding and Tomkins (72), DPNH must accumulate within the endometrial cells. This would result in acceleration of reactions requiring DPNH and inhibition of reactions requiring DPN. Lack of available DPN would serve to inhibit Kreb's cycle metabolism, many reactions in this cycle requiring DPN as a hydrogen acceptor. However, for reasons mentioned

above, the glycolytic pathways would not be inhibited by lack of DPN since the pyruvate-lactate conversion serves to regenerate the DPN needed in an earlier step.

Uncoupling of oxidation and phosphorylation, a property of progesterone, combined with inhibition of DPNH oxidase leading to depletion of DPN, would result in almost complete dependence upon the anaerobic glycolytic pathways for energy production. Such a dependence upon glycolytic pathways would provide high levels of glycolytic pathway intermediates, which may themselves be utilized by the ovum.

Effects of intravenous steroids on rabbit endometrial phosphorylase

To examine further the effects of progesterone and estradiol on endometrial phosphorylase, and to provide further information on the mechanism by which progesterone causes a post-ovulatory rise in phosphorylase, normal rabbits were treated with intravenous steroids. Hug et al. have demonstrated in humans that intravenous glucagon will activate hepatic phosphorylase within 3-1/2 minutes after injection when compared to levels in a pre-injection biopsy specimen of the patient's liver (26). With this in mind, one uterine horn was removed and the endometrium homogenized, intravenous injection of an aqueous suspension of steroid was made, and the other uterine horn was removed 5 minutes after injection. The activity of phosphorylase in the pre-injection control was compared to that in the post-injection sample.

The data, shown in Table II, indicate that both progesterone and estradiol activate phosphorylase over control levels while animals receiving only a control injection of saline vehicle showed no difference between the two samples. These results were substantiated in further experiments. Whether the magnitude of activation produced by the two hormones has any significance is not clear. In any event,

TABLE II

	PRE-INJECTION CONTROL		POST-INJECTION 5 min.		PERCENT CHANGE	
	+AMP	-AMP	+AMP	-AMP	+AMP	-AMP
PROGESTERONE 10mg./kg.*	1.20	0.90	1.80	1.16	50	29
ESTRADIOL 10mg./kg.*	1.89	1.03	2.34	1.49	24	44
CONTROL saline 1ml./kg.	2.74	1.78	2.88	1.77	5	0

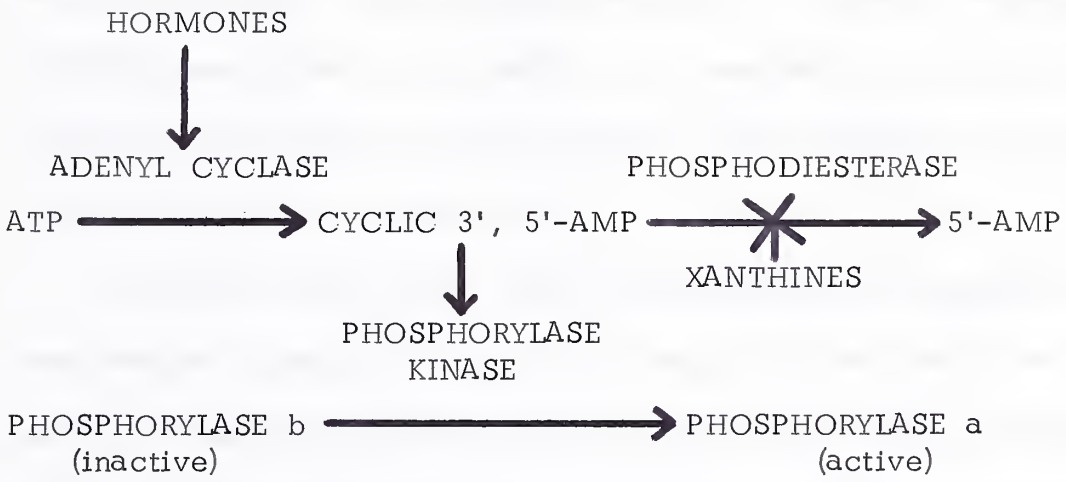
* All hormones injected as suspensions 10 mg./ml. in 0.9% saline with benzyl alcohol as preservative.

Effect of intravenous injection of progesterone and estradiol on phosphorylase activity of rabbit endometrium.

both hormones have a definite activating effect; these effects may be mediated through separate activating systems.

Effect of progesterone on endometrial adenyl cyclase

Hormones are known to activate phosphorylase through their action on the adenyl cyclase system (43, 46, 54). This chain of activation, shown below,



is sensitive to hormones at the step where ATP is converted to cyclic-AMP. Many tissues have been shown to contain adenyl cyclase activity; however, the adenyl cyclase found in a given tissue is sensitive to only certain hormones (7, 54). For instance, adenyl cyclase from adrenal is sensitive to ACTH but not to glucagon, adenyl cyclase from liver is sensitive to epinephrine and glucagon, whereas skeletal muscle adenyl cyclase is sensitive to epinephrine but not to glucagon (54). Thus there must exist variations in the enzyme or en-

zymes which convert ATP to cyclic-AMP that cause these proteins to be activated by only certain of the hormones.

In liver, which possesses adenyl cyclase activity sensitive to both epinephrine and glucagon, Bitensky et al. have provided evidence that separate adenyl cyclase systems are activated by these two hormones (4). Thus, in endometrium, the observed activation of phosphorylase by both progesterone and estradiol may be due to the presence of two adenyl cyclase systems, one activated by progesterone and the other by estradiol.

Szego and Davis have shown that injections of estradiol into rats produce immediate increases in adenyl cyclase activity as measured by a rise in total uterine cyclic-AMP levels (56). Rosenfeld et al. have shown that progesterone injections into chicks produce a delayed increase in levels of adenyl cyclase activity found in the oviduct (47). Szego and Davis further reported that beta-adrenergic blocking agents were capable of blocking the rise in uterine cyclic-AMP in response to estradiol injection (57). Rosenfeld et al. reported that the response of chick oviduct adenyl cyclase to progesterone was not effected by beta-adrenergic blockade (47).

Rosenfeld et al. were unable, however, to demonstrate in vitro activation of chick oviduct adenyl cyclase by progesterone (47). Prior to the publication of their data, it was decided to investigate the

effect of progesterone *in vitro* on rabbit endometrial adenyl cyclase.

It should be noted that the results of Szego and Davis, and Rosenfeld *et al.* were obtained using, respectively, intact rat uteri, and whole chick oviducts. Both rat uteri and chick oviduct are composed of large amounts of smooth muscle tissue, which has significant adenyl cyclase activity of its own (54). Thus the effects of hormones on adenyl cyclase in these tissues may have been due in some degree to effects on the smooth muscle. Of more interest in terms of effects on the implanting ovum would be to determine the manner in which hormones influenced adenyl cyclase present in endometrium.

For this reason, endometrial adenyl cyclase was assayed by the methods mentioned previously, in the presence and absence of progesterone 5×10^{-6} M. Four samples of homogenate with and without hormone were incubated and each chromatographed in duplicate. No difference in the activity of adenyl cyclase could be demonstrated between the samples containing progesterone and those lacking the hormone.

These results are consistent with those of Rosenfeld *et al.* but are somewhat contradictory to the observed effect of progesterone on endometrial phosphorylase. It is possible that the conditions of the assay used, which was the same as that used for liver, were

not appropriate for the endometrial enzyme system. A system that shows such variable sensitivity to hormones may also require variable reaction conditions for assay. At the present time, further work is being carried out to determine if suitable modification in the assay will reveal a stimulatory effect of progesterone on the endometrial adenyl cyclase system.

Summary

Data has been presented on various *in vivo* and *in vitro* effects of ovarian steroids. It has been shown that progesterone is ineffective in stimulating the soluble transhydrogenase system from human placenta but is capable of producing 10 to 20% greater activity in combination with estradiol than estradiol alone produces. It was further demonstrated that estradiol and progesterone inhibit soluble DPNH oxidase activity in human placental preparations, with estradiol producing a greater degree of inhibition.

The effects of steroids on rabbit endometrial phosphorylase were examined, with findings of an increase in phosphorylase after ovulation, presumably in response to progesterone. Intravenous injections of both progesterone and estradiol were found to produce increases in endometrial phosphorylase within five minutes when compared to pre-injection controls. These findings are indicative of possible activation of pre-existing phosphorylase through stimulation of adenyl cyclase. The contributions of enzyme activation and enzyme synthesis to the post-ovulatory rise in phosphorylase activity cannot be evaluated directly, but the acute effect of intravenous steroids certainly seems to indicate activation of pre-existing enzyme as a significant component.

Finally, the *in vitro* effect of progesterone on rabbit endometrial adenyl cyclase was examined. Progesterone was found to have no stimulatory effect in the assay system used. In view of the acute effect of intravenous progesterone on phosphorylase activity, the possibility exists that modifications in the assay conditions may reveal stimulation of adenyl cyclase by progesterone.

Ovarian steroids have been shown to effect diverse elements of the metabolic process, including the intracellular availability of the enzyme co-factors DPN, DPNH, TPN, and TPNH, and the activity of enzymes leading to the production of substrates which can serve as energy sources.

Bibliography

1. Abe, T., Hagerman, D.D., and Villee, C.A. Estrogen-dependent pyridine nucleotide transhydrogenase of human myometrium. J. Biol. Chem. 239: 414 (1964).

2. Adams, J.A., Jarabak, J., and Talalay, P. The steroid specificity of the 17β-hydroxysteroid dehydrogenase of human placenta. J. Biol. Chem. 237: 3069 (1962).

3. Bitensky, M. Personal communication. (1970)

4. Bitensky, M.W., Russell, V., and Robertson, W. Evidence for separate epinephrine and glucagon responsive adenyl cyclase systems in rat liver. Biochem. Biophys. Res. Commun. 31: 706 (1968).

5. Barker, K.L., and Warren, J.C. Estrogen control of carbohydrate metabolism in the rat uterus: Pathways of glucose metabolism. Endocrinology 78: 1205 (1965).

6. Barker, K.L., Nielson, M.H., and Warren, J.C. Estrogen control of uterine carbohydrate metabolism: Factors affecting glucose utilization. Endocrinology 79: 1069 (1966).

7. Catt, K.J. Hormones in General. Lancet, April 11, 1970, p. 763 ff.

8. Cochran, K.W., and DuBois, K.P. The influence of certain steroids on the oxidation of α-keto acids. Endocrinology 55: 10 (1954).

9. Cohen, K.L., and Bitensky, M.W. Inhibitory effects of alloxan on mammalian adenyl cyclase. J. Pharm. and Expt. Thera. 169: 80 (1969).

10. Cori, C.F., and Cori, G.T. The activity and crystallization of phosphorylase b. J. Biol. Chem. 158: 341 (1945).

11. Cori, G.T., and Illingworth, B. The effect of epinephrine and other glycogenolytic agents on the phosphorylase a content of muscle. Biochem. Biophys. Acta 21: 105 (1956).

12. Csermely, T., Demers, L.M., and Hughes, E.C. Organ culture of human endometrium: Effects of progesterone. Obstet. Gynec. 34: 252 (1969).

13. Dingman, C.W., Aronow, A., Bunting, S.L., Peacock, A.C., and O'Malley, B.W. Changes in chick oviduct RNA following hormonal stimulation. Biochem. 8: 489 (1969).

14. Gordon, E.E., and Villee, C.A. Spectrophotometric studies of the stimulation of human placental preparations by estradiol. J. Biol. Chem. 216: 215 (1955).

15. Gorski, J., Noteboom, W.D., and Nicolette, J.A. Estrogen control of the synthesis of RNA and protein in the uterus. J. Cell. Comp. Physiol. 66: supl 1, 91 (1965).

16. Guidry, M.A., Segaloff, A., and Altschul, A.A. Effect of α-Estradiol, some of its esters and estrone on the respiration of tissue homogenates. Endocrinology 50: 29 (1952).

17. Hagerman, D.D., and Villee, C.A. Effect of the menstrual cycle on the metabolism of human endometrium. Endocrinology 53: 667 (1953).

18. Hagerman, D.D., and Villee, C.A. Effects of estradiol on the metabolism of human endometrium in vitro. J. Biol. Chem. 203: 425 (1953).

19. Hagerman, D.D., and Villee, C.A. Estrogen-sensitive isocitric dehydrogenase. J. Biol. Chem. 229: 589 (1957).

20. Hagerman, D.D., and Villee, C.A. Separation of human placental trans-hydrogenase from estradiol-17β dehydrogenase. J. Biol. Chem 234: 2031 (1959).

21. Hechter, O.K., Yoshinaga, K., Cohn, C., Dodd, P., and Halkerston, I.D.K. In vitro stimulatory effects of nucleotides and nucleosides on biosynthetic processes in castrated rat uterus. Federation Proc. 24: 384 (1965).

22. Hechter, O.K., Yosinaga, K., Halkerston, I.D.K., and Birchall, K. Estrogen-like anabolic effects of cyclic 3', 5' adenosine monophosphate and other nucleotides in isolated rat uterus. Arch. Biochem. Biophys. 122: 449 (1967).

23. Hollander, V.P., Nolan, H., and Hollander, N. The structural specificity of the estrogen sensitive enzyme system in placental homogenates. J. Biol. Chem. 233: 580 (1958).

24. Hollander, V.P., Hollander, N., and Brown, J.D. Studies on the estrogen-sensitive enzyme system from placenta. J. Biol. Chem. 234: 1678 (1959).

25. Hollander, V.P., and Stephens, M.L. Studies on phenol-activated oxidation of reduced nucleotides of rat uterus. J. Biol. Chem. 234: 1901 (1959).

26. Hug, G., Schubert, W.K., and Schwachman, H. Imbalance of liver phosphorylase and accumulation of glycogen in a girl with progressive disease of the brain. J. Ped. 67: 741 (1965).

27. Hug, G., Schubert, W.K., and Chuck, G. Deficient activity of dephosphophosphorylase kinase and accumulation of glycogen in the liver. J. Clin. Invest. 48: 704 (1969).

28. Hughes, E.C., Jacobs, R.D., Rubulis, A., and Husney, R.M. Carbohydrate pathways of the endometrium: Effects on ovular growth. Am. J. Obstet. Gynec. 85: 594 (1963).

29. Hughes, E.C., Jacobs, R.D., and Rubulis, A. Effect of treatment for sterility and abortion upon the carbohydrate pathways of the endometrium. Am. J. Obstet. Gynec. 89: 59 (1964).

30. Hughes, E.C., Demers, L.M., Csermely, T., and Jones, D.B. Organ culture of human endometrium: Effect of ovarian steroids. Am. J. Obstet. Gynec. 105: 707 (1969).

31. Jarabak, J., Adams, J.A., Williams-Ashman, H.G., and Talalay, P. Purification of a 17-β hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. J. Biol. Chem. 237: 345 (1962).

32. Knudsen, K.A., Jones, R.C., and Edgren, R.A. Effect of a carbonic anhydrase inhibitor (Diamox) on the progesterone stimulated rabbit uterus. Endocrinology 85: 1204 (1969).

33. Langer, L.J., and Engel, L.L. Human placental estradiol-17β dehydrogenase. Federation Proc. 15: 296 (1956).

34. Langer, L.J., and Engel, L.L. Human placental estradiol-17β dehydrogenase. J. Biol. Chem. 233: 583 (1958).

35. McCorquodale, D.J., and Mueller, G.C. Effect of estradiol on the level of amino acid-activating enzymes in the rat uterus. J. Biol. Chem. 232: 31 (1958).

36. Marshall's Physiology of Reproduction. Editor A.S. Parkes. Longmans, Green and Co., Ltd., London (1956), p. 257 ff.

37. Miyake, T., and Pincus, G. Anti-progestational activity of estrogens in rabbit endometrium. Proc. Soc. Expt. Biol. Med. 99: 478 (1958).

38. Mueller, G.C., and Herranen, A. Metabolism of one-carbon fragments by surviving uteri from estradiol-treated rats. J. Biol. Chem. 219: 585 (1956).

39. Mueller, G.C. Some biochemical studies on the mechanism of activity of estrogens. Cancer 10: 716 (1957).

40. Nicholls, R.A., and Board, J.A. Carbonic anhydrase concentration in endometrium after oral progestins. Am. J. Obstet. Gynec. 99: 829 (1967).

41. O'Malley, B.W., McGuire, W.L., Kohler, P.O., and Korenman, S.G. Studies on the mechanism of steroid hormone regulation of synthesis of specific proteins. Recent Progr. Hormone Res. 25: 105 (1969).

42. Pincus, G., and Bialy, G. Carbonic anhydrase in steroid-responsive tissues. Recent Progr. Hormone Res. 21: 201 (1963).

43. Rall, R.W., Sutherland, E.W., and Berthet, J. The relationship of epinephrine and glucagon to liver phosphorylase. J. Biol. Chem. 224: 463 (1957).

44. Randerath, K., and Randerath, E. Ion-exchange chromatography of nucleotides on poly (ethyleneimine)-cellulose thin layers. J. Chromatog. 16: 111 (1964).

45. Randerath, K. Separation of constituent nucleotides of nucleic acids on ion-exchange thin layers. Pro Experimentis 20: 406 (1965).

46. Robison, G.A., Butcher, R.W., and Sutherland, E.W. On the relation of hormone receptors to adenyl cyclase; in Fundamental Concepts in Drug-Receptor Interactions. Academic Press, New York (1970), p. 59 ff.

47. Rosenfeld, M.G., Kissel, J.H., and O'Malley, B.W. Progesterone effects on adenyl cyclase activity and cyclic adenosine 3', 5'-monophosphate levels in the chick oviduct. Cytobios 5: 33 (1970).

48. Saldarini, R.J., and Yochim, J.M. Metabolism of the uterus of the rat during early pseudo-pregnancy and its regulation by estrogen and progesterone. Endocrinology 80: 453 (1967).

49. Spiegel, A.M., and Bitensky, M.W. Effects of chemical and enzymatic modifications of glucagon on its activation of hepatic adenyl cyclase. Endocrinology 85: 638 (1969).

50. Stein, R.J., and Stuermer, V.M. Cytodynamic properties of human endometrium, III. Variations in the nucleoprotein content of the endometrium during the menstrual cycle. Am. J. Obstet. Gynec. 61: 414 (1951).

51. Stuermer, V.M., and Stein, R.J. Cytodynamic properties of the human endometrium, V. Metabolism and the enzymatic activity of the human endometrium during the menstrual cycle. Am. J. Obstet. Gynec. 63: 359 (1952).

52. Stumpf, W.E. Nucleic concentration of ^{3}H-estradiol in target tissues. Dry mount autoradiography of vagina, oviduct, ovary, testes, mammary tumor, liver and adrenal. Endocrinology 85: 31 (1969).

53. Sutherland, E.W. Polysaccharide phosphorylase, liver; in Methods in Enzymology. S.P. Colowick and N. Kaplan, editors. Academic Press, Inc., New York (1955), p. 215 ff.

54. Sutherland, E.W., and Rall, T.W. The relation of adenosine-3', 5'-phosphate and phosphorylase to the actions of catecholamines and other hormones. Pharmacol. Rev. 12: 265 (1960).

55. Szego, C.M. Role of histamine in the mediation of hormone action. Fed. Proc. 24: 1343 (1965).

56. Szego, C.M., and Davis, J.S. Adenosine 3', 5'-monophosphate in rat uterus: acute elevation by estrogen. Proc. Natl. Acad. Sci. U.S. 58: 1711 (1967).

57. Szego, C.M., and Davis, J.S. Inhibition of estrogen-induced elevation of cyclic 3', 5'-adenosine monophosphate in rat uterus: I. By β-adrenergic receptor blocking drugs. Mol. Pharmacol. 5: 470 (1969).

58. Talalay, P., and Williams-Ashman, H.G. Activation of hydrogen transfer by steroids. Proc. Natl. Acad. Sci. U.S. 44: 15 (1958).

59. Talalay, P., Hurlock, B., and Williams-Ashman, H.G. On a co-enzymatic function of estradiol 17-β. Proc. Natl. Acad. Sci. U.S. 44: 862 (1958).

60. Villee, C.A., and Hagerman, D.D. Effect of estradiol on the metabolism of human placenta in vitro. J. Biol. Chem. 205: 873 (1953).

61. Villee, C.A. An estradiol-induced stimulation of citrate utilization by placenta. J. Biol. Chem. 215: 171 (1955).

62. Villee, C.A., and Gordon, C.C. Further studies on the action of estradiol in vitro. J. Biol. Chem. 216: 203 (1955).

63. Villee, C.A. Role of estrogens in regulating the metabolism of the placenta and endometrium. Fertility and Sterility 8: 156 (1957).

64. Villee, C.A., and Hagerman, D.D. Compounds with antiestrogenic activity in vitro. Endocrinology 60: 552 (1957).

65. Villee, C.A., and Hagerman, D.D. On the identity of the estrogen sensitive enzyme of human placenta. J. Biol. Chem. 233: 42 (1958).

66. Villee, C.A., Loring, J.M., and Sarner, A.M. The isocitric dehydrogenases of the placenta. Federation Proc. 17: 328 (1958).

67. Villee, C.A. Estrogens and uterine enzymes. Ann. NY Acad. Sci. 75: (2) 524 (1959).

68. Wade, R., and Jones, H.W. Effect of progesterone on mitochondrial ATPase. J. Biol. Chem. 220: 547 (1956).

69. Wade, R., and Jones, H.W. Effect of progesterone on oxidative phosphorylation. J. Biol. Chem. 220: 553 (1956).

70. Warren, J.C., Cheatum, S.G., Greenwald, G.S., and Barker, K.L. Cyclic variation of uterine metabolic activity in the golden hamster. Endocrinology 80: 714 (1967).

71. Watson, J.D. Molecular Biology of the Gene. W.A. Benjamin, Inc., New York, 1965.

72. Yielding, K.L., and Tomkins, G.M. Inhibition of the enzymic oxidation of DPNH by steroid hormones. Proc. Natl. Acad. Sci. U.S. 45: 1730 (1959).

73. Yielding, K.L., Tomkins, G.M., Munday, J.S., and Cowley, I.J. The effect of steroids on electron transport. J. Biol. Chem. 235: 3413 (1960).

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

_____

_____

NAME AND ADDRESS DATE