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BARKER, Kenneth Leroy, 1939—
CONTROL BY ESTRADIOL AND PROGESTERONE
OF THE ACTIVITIES OF CERTAIN ENZYMES IN
THE LACTATING MAMMARY GLAND AND THE
UTERUS OF RATS.

The Ohio State University, Ph.D., 1964
Chemistry, biological

University Microfilms, Inc., Ann Arbor, Michigan
CONTROL BY ESTRADIOL AND PROGESTERONE OF THE ACTIVITIES
OF CERTAIN ENZYMES IN THE LACTATING MAMMARY
GLAND AND THE UTERUS OF RATS

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Kenneth Leroy Barker, B.Sc., M.Sc.

* * * * * * *

The Ohio State University
1964

Approved by

Thomas M. Ludwick
Adviser
Department of Dairy Science
ACKNOWLEDGMENTS

I would like to express my most sincere appreciation to Dr. Thomas Ludwick, Professor of Dairy Science, whose guidance and encouragement was ever present in my graduate program. He has been a most perfect example of a person in the academic world with a strong sense of human values.

To Dr. John Gander, I wish to extend my thanks for his assistance in the initiation and development of the biochemical aspects of this problem.

I wish to thank Dr. Noland Van Demark and Dr. James Fleeger for the use of the equipment in their laboratory and for their technical assistance.

I extend my gratitude to the personnel of the Department of Dairy Science, especially those on the N. C.-2 Federal Breeding Project, whose support and friendship have made this study a pleasant and worth-while undertaking.

My most sincere thanks must go to my parents for their encouragement and assistance throughout all of my educational endeavors.
VITA

July 15, 1939  Born - Columbus, Ohio

1960. . . .  B.Sc., The Ohio State University, Columbus, Ohio

1961-1962 .  .  Research Assistant in Reproductive Physiology, Department of Dairy Science, The Ohio State University, Columbus, Ohio

1962. . . .  M.Sc., The Ohio State University, Columbus, Ohio

1962-1964 .  .  Research Assistant on The Ohio and Federal N. C.-2 Dairy Cattle Breeding Research Project, The Ohio State University, Columbus, Ohio

PUBLICATIONS


FIELDS OF STUDY

Major Field: Dairy Science


Studies in Chemistry. Professors Albert L. Henne, James Mortenson, William White, and Quentin VanWinkle

Studies in Biometrics. Professors Walter R. Harvey and William B. McIntosh
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INTRODUCTION

In recent years much of the research in the field of biology has been centered on the biochemical activities of cells and cell systems. Essentially all of the cell's activities are mediated through enzymes. Enzymes are the tools of the cell which are used for phenotypic manifestation of the cell's genotype. Theoretically, each somatic cell of an organism contains within its nucleus all of the information required to produce every enzyme found within the organism. Through the various stages of differentiation in higher organisms the various genotypes become expressed and organs and other specialized tissues are formed. Since all of the cells have the same genotype, it is apparent from the existence of these various tissue types that some form of control of gene action must exist which differs between cells and cell types.

Although the various organs have distinct morphological and physiological differences, they are not without a considerable amount of dependence upon the other organs within the entire system. In higher organisms there is a considerable degree of control imparted by an organ on the other organs in the system. One of the major control mechanisms between various tissues in higher organisms is mediated through hormones. Hormones are essentially metabolic
products of one group of specialized cells which can act on certain
target organs or cells to direct their activity in such a way that
their action can be integrated into the entire system to maintain a
symbiotic relationship.

Perhaps some of the most interesting control relationships in
mammals are those associated with the process of reproduction. The
very existence and maintenance of the species is dependent upon the
proper functioning of these control processes. In the female, for
example, the maternal gamete must be produced, made available to
fertilization, fertilized, and nurtured in the proper internal
environment for growth and development of the fetus. When the new
individual has reached a certain stage in its development it must be
born into its semi-independent environment. Throughout this stage it
is nurtured by its mother until it is able to survive in its com­
pletely independent environment. All of these events are dependent
upon various orderly changes in metabolic activity of the female.
To a very large degree these processes are under the controlling
influence of the hormones associated with the ovary.

It is the purpose of this dissertation to determine some of
the mechanisms of control of the metabolism of the uterus and the
mammary gland of the female by the ovarian hormones, estradiol and
progesterone. The primary emphasis will be placed on the effects of
these hormones on specific enzyme systems of these two organs.
REVIEW OF LITERATURE

Considerable interest has developed recently in the general area of metabolic control at the cellular level. In this review, the general topics of the mechanisms of control by hormones, the general endocrinology of the uterus and the mammary gland, and the endocrine control of the metabolism of these two organs will be considered.

Mechanisms of control by hormones

Essentially all of the cell's activities are mediated through enzymes. The control of the biochemical processes which are mediated through enzymes can occur in several ways. Enzymically catalyzed reactions are dependent upon enzyme concentration, the enzyme's affinity for its substrates, the velocity at which the enzyme can catalyze its reaction, the activation and inhibition properties of the enzyme and the concentration of substrates available for the reaction. As most of the research concerning control at the enzyme level has been done with lower forms of unicellular organisms, much of this portion of the review will at times seem to include topics other than those related to hormones.

Control of enzyme synthesis

There are two types of enzymes based upon the degree of homeostasis of their relative concentrations within the cell.
are "constitutive" enzymes, those which remain at constant levels in the cell, and there are "adaptive" enzymes, those which vary in concentration depending on the status of the cell (Vogel, 1957). It is through the adaptive enzymes that much of the cellular control activities are mediated.

**Biological synthesis of protein.** Protein synthesis is by necessity unlike the biological synthesis of other molecules such as polysaccharides, lipids, co-enzymes, etc., which are dependent upon enzymes for the direction of their synthesis. As enzymes are themselves protein, a paradoxical situation of perpetual motion would have to be postulated (Crick, 1958). Recent reviews, Berg (1961), Gros et al. (1961), Simpson (1962), and Spiegelman (1963) indicated that the whole phenomenon of protein synthesis is the bridge between genotype and the phenotypic expression of genotype at the cellular level.

The scheme of protein synthesis involves first the process of "transcription" of the genetic code contained in deoxyribonucleic acid (DNA) into ribonucleic acid (RNA). There appears to be three basic types of RNA (Gros et al., 1961; Spiegelman, 1963). There is (1) translatable or "messenger" RNA which can be translated into polypeptide sequences by the ribosomal system, (2) non-translatable or "ribosomal" RNA which is found generally in conjunction with ribonucleoprotein to form the ribosomes of the cell, and (3) translational or "soluble" RNA which acts as the amino acid acceptor and transfer agent.
Once formed, the messenger RNA passes through the cytoplasm of the cell and associates with the ribosome (Spiegelman, 1963; Gros et al., 1961). It is this RNA which is the template for the specific polypeptide being formed. The process of transporting the amino acids to the ribosome bound messenger RNA, as stated earlier, is a function of the soluble RNA. This transport process begins with the activation of the amino acid by a specific amino acid activating enzyme. This enzyme exhibits a high degree of specificity toward the amino acid which it activates. Each amino acid is bound to its specific activating enzyme and is then transferred to a specific molecule of soluble RNA. The amino acyl RNA complex is then transferred to the ribosome in a specific order depending on the complementary triplet coded nucleotide base sequences contained in the two RNA molecules. The amino acids, which are now arranged sequentially on the ribosome, undergo a reaction which is probably mediated through some sort of "zipper" enzyme, and the polypeptide is formed and released from the ribosome. Upon release of the polypeptide, the soluble and the messenger RNAs are released from the ribosome to either repeat the process or be degraded by ribonucleases (Berg, 1961; Simpson, 1962).

General model of the regulation of enzyme synthesis. By studying the effects of mutations in various regions of the chromosomes of certain microorganisms, it was observed that mutations can affect enzyme synthesis in two ways. They can alter the amount of enzyme synthesized or they can alter the structure of the enzyme to the extent of changing or eliminating its biological activity. By
chromosome mapping techniques, it is possible to locate the site of the mutation. From these studies it was noted that mutations in one region of a chromosome cause structural alterations. Other mutations occurring both adjacent to this site and on other chromosomes are capable of altering rates of enzyme formation. These latter mutations can either elevate the rate of enzyme synthesis to a constitutive level or completely eliminate enzyme formation (Pardee et al., 1959; Jacob and Monod, 1961a, 1961b; Monod et al., 1962).

Based on mutation effects and chromosome mapping techniques a model was set forth by Jacob and Monod (1961b). This model consists of a regulator gene, an operator gene, and a series of structural genes. The operator gene and the structural genes constitute a functional unit called the operon.

The assumptions upon which the Jacob and Monod model are based follow:

(1) The primary product of structural genes is messenger RNA.

(2) The synthesis of messenger RNA is sequentially oriented and can be initiated only at certain regions or "operator genes."

(3) The genetic material contains determinants, functionally distinct from structural genes (and operator genes) called "regulator genes." The regulator genes produce a repressor which is capable of blocking the activity of the operator gene.

(4) The repressor can react with small molecules (effectors) which can either inhibit or activate the action of the repressor on the operator gene.
The structural gene. The structural gene is considered to be that segment of the chromosome which is considered to be the template for messenger RNA. The structural gene has been identified primarily on the basis of the effects of mutations within its boundary. It has been shown, using the $\beta$-galactosidase system of *E. coli*, that mutations in the structural gene will alter the resulting protein. Heterozygotes with respect to the $\beta$-galactosidase gene produce a protein that is antigenically similar to $\beta$-galactosidase but with no affinity for $\beta$-galactosides. Both proteins are synthesized at the same rate. It is found that such mutations in the structural genes, alter only the quality, not the quantity of the synthesized protein (Perrin *et al.*, 1960). Riley *et al.* (1960) have demonstrated that the structural gene is responsive immediately to the positive action of the operator gene. In mutant strain crossing experiments they have demonstrated that enzyme synthesis per each newly formed zygote is constant from the time of penetration of the $z^+$ ($\beta$-galactosidase positive) structural gene into the receptor $z^-$ cell.

The kinetics of enzyme induction indicate that the product of the structural gene has to be a molecule with properties similar to those of messenger RNA. It has to be an unstable intermediate, such as a low molecular weight polyribonucleotide, due to the fact that enzyme synthesis per cell is not an additive function with time (Riley *et al.*, 1960). Gros *et al.* (1961), Spiegelman (1963), and Brenner *et al.* (1961) have identified an RNA fraction that has the needed properties of messenger RNA. Also, they have
shown that the nucleotide base ratios of this RNA corresponds closely to that of DNA.

The operator gene. The operator gene is that portion of the operon which is thought to be the site of the initiation of the transcription process (Jacob and Monod, 1961a, 1961b). By chromosome mapping techniques it is thought to be located directly adjacent to the structural gene(s). The operator gene and the structural gene(s) together comprise a functional unit called the operon.

Using mutant strains of *E. coli*, Jacob et al. (1960) have demonstrated that the operator gene segment directs only the activity of the cis, or adjacent, structural gene(s). The observation that the operator gene cannot initiate transcription on the trans structural gene(s) indicates that the mode of action is not via a diffusible intermediate. The authors hypothesize that the mode of action of the operator gene is dependent upon its direct contact with the structural gene chain. It is thought to act by initiating some sort of peristaltic wave of transcription.

There are two types of mutations which can occur in the operator gene. There are constitutive-operator mutants and operator-negative mutants. As will be discussed later, it is generally thought that a repressor substance produced by the regulator gene acts by inhibiting the action of the operator gene. The repressor binds with a high degree of specificity to the operator gene and is probably dependent upon the latter's nucleotide base sequence (Jacob and Monod, 1961b).
A constitutive-operator mutant is one in which a mutation within the operator gene makes it unsusceptible to the binding of the repressor. Under these conditions the operator tends to cause continuous initiation of structural gene activity and the resultant protein reaches a constitutive level. Organisms with a constitutive-operator mutation on only one chromosome tend to exhibit levels of the resultant protein which are less than half that obtained by maximal induction of a non-mutant strain (Jacob and Monod, 1961b). When these hybrid mutants are subjected to induction, the synthesis of the specific protein is equal to that of the fully induced non-mutant strain.

Operator-negative mutants appear to be unable to synthesize proteins corresponding to the cis, structural gene which follows the mutated operator (Buttin, 1961). This effect could be the result of either an irreversible binding of the operator or an inherent inability to initiate transcription.

The phenomenon of coinduction is observed in numerous systems. It involves the simultaneous induction of two or more enzymes at an identical rate. There are two mechanisms which could result in coinduction. One system is based on the common effects of one regulator gene on several operons. This system is characterized by the arginine repressed enzyme system (Maas, 1961). With this type of coinduction it is easy to visualize the synthesis of enzymes in ratios other than one to one, depending on the relative affinities of the various operator genes for the repressor.
The other system involves a series of structural genes that are controlled by one operator gene. The most commonly cited example of this type of coinduction is the lactose system of *E. coli* (Monod et al., 1951; Jacob et al., 1960). This system involves the coinduction of at least three enzymes involved in the metabolism of β-galactosides. Ames and Garry (1959) observed a similar phenomenon in the repressible histidine system of *S. typhimurium*. These systems are quantitatively coinduced or corepressed, i.e., all of the enzymes are synthesized in identical amounts. Evidence has been presented which indicates that transcription is initiated at the operator gene and passes successively through each structural gene to the end of the operon (Jacob and Monod, 1961b). These authors postulate that this type of coinduction is important in the synthesis of a series of polypeptides used to build polymeric proteins.

The regulator gene. It has been shown that mutations in certain regions of the chromosome other than the operon can and do alter the rate of synthesis of certain proteins (Pardee et al., 1959; Cohen and Jacob, 1959). As was discussed earlier, the regulator gene is thought to act by influencing the activity of the operator gene (Jacob and Monod, 1961a, 1961b; Monod et al., 1962). The nature of this effect is in a dynamic state of flux in current literature. Pardee et al. (1959) have shown that the regulator gene need not be on the same chromosome to impart its effect. In
heterozygote mutants carrying one gene for inducibility and one for
constitutivity, the involved protein synthesis was of an entirely
inducible nature. This indicated, not only that the gene for
inducibility was dominate, but that some diffusible intermediate
must be responsible for the inductive action of the regulator gene
on the trans structural gene. The current concept is, that under
"normal" conditions the diffusible intermediate, i.e., repressor,
binds reversibly with the operator gene and prevents the initiation
of transcription (Pardee et al., 1959; Jacob and Monod, 1961a,
1961b).

The entire concept of "adaptive enzymes" appears to be
related to the control of either the regulator gene activity or the
action of the repressor on the operon (Jacob and Monod, 1961a). The
phenomenon of "repression" is thought to occur by the reversible
binding of the involved compound (effector) to the repressor. The
complex which is formed possesses the ability to suppress the
involved operon. The uncomplexed repressor in this case is unable
to impart this effect. The net effect in the case of "repression"
is that the synthesis of enzymes is controlled (inhibited) by the
end products or end product analogs of the involved enzyme system
(Jacob and Monod, 1961b).

The phenomenon of "induction" is also thought to involve the
reversible binding of an effector compound with the repressor. In-
duction is thought to differ from repression in that the effector-
repressor complex is incapable of binding to the operator gene.
In this case the free repressor is the active form which can inhibit transcription (Jacob and Monod, 1961b). Induction, unlike repression, is thought to be of importance in permitting an organism to synthesize enzyme systems required for unusual catabolic pathways. Repression, on the other hand, is considered to be a control mechanism for normal biosynthetic processes (Vogel, 1957, 1958; Gorini and Maas, 1958; Maas and Gorini, 1957; Gorini, 1960; Pardee, 1961).

As in the case of the operator genes, there are two basic types of mutations which can occur in the regulator gene: constitutive-regulator mutations and super-repressed-regulator mutations. Again, the structure of the synthesized protein is not altered. The constitutive-regulator mutation results in an uncontrolled synthesis of the involved proteins. The site of mutation is distinct from the structural gene. These observations are reported by Pardee et al. (1959) and by Jacob and Monod (1961b) for the inducible β-galactosidase system of *E. coli*. Repressible systems of the tryptophan pathway (Cohen and Jacob, 1959) and the arginine pathway (Gorini et al., 1961; Maas, 1961) of *E. coli*, are also known to have constitutive-regulator mutations. This type of mutant is recessive since a single mutation would still have a homologous regulator gene capable of synthesizing the repressor. The mode of action of this type of mutation is probably the alteration of the repressor to such an extent as to render it incapable of complexing with the operator gene. The superrepressed-regulator mutation is one which exhibits
complete dominance over inducibility. It results from a mutation which prevents the complexing of the repressor and the effector (Jacob and Monod, 1959, 1961a, 1961b; Monod et al., 1963).

The identity of the cytoplasmic repressor remains a mystery today. It must (1) be able to bind very specifically to the operator gene, (2) be capable of interacting with effector molecules of various chemical structures, and (3) be reversibly bound to the operator gene and rather easily degraded in the free state (Jacob and Monod, 1961a, 1961b; Jacob et al., 1962; Paigen, 1962). It has been suggested that the most logical product of the regulator gene is a polyribonucleotide. Paigen (1962) suggests that a "censor" RNA (regulator) complementary to the base sequence of the operator gene is the logical molecule for the regulator. His hypothesis is dependent upon the assumption that transcription of DNA occurs from only one strand, with the second strand being used as a means of displacing the products of transcription by the reforming of hydrogen bonds with its complement. The "censor" RNA would act by binding to the operators DNA strand to prevent this displacement. He suggests that the effectors modify this procedure by inactivation or activation of the "censor" RNA. Monod et al. (1963) suggest that it would be logical to expect a binding of the repressor, which he suspects to be a polyribonucleotide, and the effector molecule to an allosteric enzyme. This allosteric bound enzymes complex could then act on the operator gene to modify its activity. Prior to the
concept of messenger RNA being the product of the structural gene, Vogel (1957a, 1957b, 1958) suggested that repressors and inducers might act by suppressing or stimulating the release of the newly synthesized enzymes from their enzyme forming templates on the ribosome. The observation by Pardee and Prestidge (1959) that chloramphenicol and 5-methyl tryptophan are incapable of blocking the formation of the repressor adds support to the concept of induction and repression at the DNA level.

Several environmental factors are capable of altering the effects of inducers and repressors. Vogel (1960) has observed a "pace setting" phenomenon in derepressed systems. He contributes this to the decreased number of ribosomes brought about by the semi-starved state of the cell culture.

The evolution of adaptive enzyme systems. The genetic make-up of all life as we know it today is probably a reflection of the selection of mutations which have had a desirable effect on the ability of the host organism to compete in a changing environment. Pardee (1961) has indicated that the most rapid growing and the most adaptable organism will be in the most favorable position to maintain its integrity. Vogel (1957) suggests that adaptive enzyme synthesis permits the cell to make the most efficient use of its raw materials. If Paigen's (1962) hypothesis that the regulator gene is complementary to the operator gene is true, it is easy to visualize the formation of new control genes by such chromosomal aberrations as crossing over and fragmentation.
The fact that mutations capable of changing the gene control mechanism occur with regular frequency is born out by the observations of Cocito and Vogel (1958). They found that continuous repression of the repressible acetylmornithinase system of E. coli for 3 to 7 days resulted in an 85 to 90 per cent loss of repressibility. This was attributed to the selection of non-repressible mutants occurring during the repressed period. Their data suggested that continually repressed systems were more prone to mutation.

It is easy to visualize that hormones could act at the level of the cell nucleus to control the synthesis of adaptive enzymes. They could act as either repressor or inducer substances. Cell membrane permeability to other inducer or repressor substances could also be affected by hormones. Competition between hormones for the binding site of the products of the regulator gene may give rise to antagonisms between hormones. Synergistic effects between hormones could also be attributed to the hormones' combined action on this gene regulating substance. That the phenomenon of enzyme induction does occur in mammalian systems is born out in the observations that several of the transaminases and tryptophane pyrrolase synthesis is controlled by hormones of the adrenal cortex (Knox, 1958; Greengard, 1963).

Control through isozymes

With the advent of the newer techniques for separation of proteins, it has become obvious that many functionally similar
proteins in the living cell exist in different molecular forms (Blumberg, 1958). Markert and Moller (1959) have named these multiple molecular forms of enzymes, "isozymes." These proteins vary all the way from very subtle to very complex differences, depending on the enzyme, tissue, and organism from which they come. It is suspected that the existence of isozymes is of extreme importance to the organism. They offer a means of metabolic control through their differences in substrate specificity, sensitivity to inhibitors, response to altered physiological conditions and location within tissues and individual cells (Allen, 1961).

As most of the work on isozymes has been done with lactic dehydrogenase (LDH), it is deemed necessary to briefly describe this enzyme and its isozyme properties. Cahn et al. (1962) have shown that LDH from animal sources exists in five electrophoretically different forms when subjected to starch gel electrophorosis at pH 8.6. The relative amounts of each isozyme is dependent upon the tissue from which it came. Heart muscle from most species exhibits a preponderance of the isozyme which migrates farthest in the anodal direction of the starch gel. Skeletal muscle gives primarily cathodal migrating isozymes. The other three isozymes are equally spaced between the two extreme forms. Appella and Markert (1961) and Markert (1963) have shown that the LDH molecule is a tetramer composed of four monomeric subunits, each of which have a molecular weight of about 34,000±2,000. The monomers have
been demonstrated by subjecting the native LDH molecule to 5M guanidine hydrochloride or 12M urea. They have observed that there exists two different monomers, with different physical and electrophoretic properties. They have been named muscle type (M) and heart type (H) because the H monomer dominates the composition of heart muscle LDH and the M monomer dominates skeletal muscle LDH (Cahn et al., 1962). The three intermediate bands have been shown to be "hybrids" composed of both monomers. Allowing the superscript to indicate the number of each monomer in the tetramer, the cathodal most band has the monomer ratios of with the other isozymes being M^3H, M^2H^2, MH^3, and H^4 respectively as they approach the anode. Markert (1963) has demonstrated that the tetrameric association is a random process depending upon the concentrations of the individual monomers. Upon dissociation of equal amounts of M^4 and H^4 LDH isozymes by freezing in 1 M NaCl, he observed that upon recombination all five bands were generated in the binomially distributed ratios of 1:4:6:4:1.

The enzymic properties of the various isozymes are also different. Kaplan's laboratory has provided much work concerning the differential binding and velocity parameters of the LDH isozymes when analogs of diphosphopyridine nucleotides are employed as cofactors. H^4 type LDH exhibits marked substrate inhibition when higher concentrations of pyruvate are employed (Kaplan, 1961). Recently, Dawson et al. (1964) have reported Km values of 8.9X10^-5 M, 5.2X10^-4 M, and 3.2X10^-3 M for H^4, H^2M^2, and M^4 respectively. Turn-over numbers for
and were reported to be 45,500 and 93,400 under maximum velocity conditions.

While the tetramer is generally considered to be the active LDH species, it is reported by Millar (1962) that an active form with a molecular weight of 72,000 exists. As the molecular weight of the tetramer is on the order of 135,000, he suggests that the dimer can also exhibit some LDH activity.

Brand et al. (1962) have reported difference in thermostability between the LDH isozymes of beef and chicken. They report that the $H^+$-type dehydrogenase is somewhat more stable than the $M^+$-type. This stability relationship is also observed when the isozymes are subjected to urea inactivation. Markert and Appella (1961) have surveyed, in a rather complete manner, many other possible physical differences and have concluded that the major differences exist only in electrical properties and differential cofactor analog binding.

Based upon the differences in electrical potentials of the two extreme forms of LDH, Wieland and Pfleiderer (1961) determined their amino acid composition. Based on their results, the differences in composition of lysine, histidine and aspartate between the two monomers are sufficient to account for the difference in electrophoretic mobilities at pH 8.6 of the various isozymes.

Immunological techniques have also proven fruitful in the investigation of LDH isozymes. Kaplan and White (1963), Cahn et al. (1962), Markert and Appella (1963), Nisselbaum and Bodansky (1959),
and Nisselbaum et al. (1961) have successfully produced antisera to the various LDH isozymes. They observed that anti-H\(^h\) gave a positive precipitation test with isozymes H\(^h\), H\(^3\)M, H\(^2\)M\(^2\) and HM\(^3\) but not M\(^h\). Anti-M\(^h\) will react with all forms except H\(^h\). Anti-H\(^2\)M\(^2\) was found to precipitate all LDH isozymes. Markert and Appella (1963) suggest that three basic types of neutralizing antibodies are formed. These are HH, HM, and MM types. The monomers of LDH are shown to be antigenically inactive, as the reactivity to the above antibodies is dependent upon the tetrameric structure. The incomplete neutralization of LDH by certain antibodies is taken by these investigators as further evidence of an enzymically active dimer. They also suggest that species differences exist between similar electrophoretic forms.

The protein subunit phenomenon observed in LDH is not unique to that enzyme system. It has been demonstrated that glutamic dehydrogenase (GDH) can be broken down into subunits by certain physiological agents. Unlike LDH, GDH subunits possess catalytic activity. The subunits are actually molecules of alanine dehydrogenase (ADH). The aggregation of the ADH subunits to form GDH is controlled by many factors. Frieden (1959a, 1959b, 1959c, 1962) has studied the effects of various substrates and nucleotides on the state of aggregation of the subunits. Wolff (1957, 1962a, 1962b) has studied the effect of thyroid hormones on the state of aggregation of GDH. Tomkins et al. (1961) and
Yielding and Tomkins (1960, 1962) have observed the effects of several steroids on this process. The significance of these effects will be discussed later. Talal et al. (1964) have demonstrated, immunologically, that at least two different monomers exist. One of these is believed to have some GDH activity in addition to ADH activity.

The monomeric subunit phenomenon has also been observed in homoserine dehydrogenase (Sturani et al., 1963). Although most of the literature on the subunit phenomenon is on dehydrogenases, evidence indicates that certain kinase enzymes are also found in the various states of aggregation (Kenkare and Colowick, 1963). Perhaps in the future when the various subunits of other enzymes are studied, they, too, will be found to possess activity completely different from that of the aggregate of state as in the case of glutamic dehydrogenase.

Metabolic control by isozymes. As essentially all of the cells' activities are mediated through enzymes, the control of activity of these enzymes should permit control of the cell's activity. Enzyme catalyzed reactions are dependent upon enzyme concentration, substrate concentration, the enzyme's affinity for the substrate, the enzyme's affinity for inhibitors, and the velocity at which the enzyme can catalyze the reaction.

There are several ways in which enzyme concentrations can be altered. One mechanism is exemplified by the glutamic dehydrogenase enzyme. By regulating the state of aggregation of the
subunits, the enzyme quantity can be regulated. Frieden's work (1959a, 1959b, 1959c, 1962) indicates that to a certain extent this degree of aggregation is controlled by certain substrates of the reaction. He observed that NADH and NADPH while being substrates for the reaction are capable of causing an increase in the state of aggregation of the subunits. Certain other factors such as ATP, ADP, and GTP cause a disaggregation of subunits. Due to the lack of complete understanding of all of the interconversions of carbohydrate and amino acid metabolism the complete significance of the various nucleotide effects is uncertain. The fact that NADH and NADPH are products of catalytic processes and substrates for many biosynthetic processes gives a teleological reason for their involvement at this control site. Under states of high nutritional levels they can "tool-up" a pathway for their use in protein anabolism. Wolff (1957, 1962a, 1962b), Tomkins et al. (1961), and Yielding and Tomkins (1960, 1962) found that thyroid hormones and estrogens also promote the disaggregation of the GDH molecule. This is an example of how hormones can alter enzyme concentrations at their target organs. This mechanism may, in part, account for increased $O_2$ uptake by certain tissues brought about by thyroxine and estrogen. By blocking the GDH pathway they could force more complete oxidation of $\alpha$-ketoglutarate via the remainder of the citric acid cycle.

In general, substrate concentrations at the site of enzyme action can be altered in only a few ways. Compartmentalization of
the cell is one important way. Isozymes such as isocitric dehydro-
genase (IDH) and malic dehydrogenase (MDH) probably function in this
manner. These two enzymes exist in at least two forms (Markert and
Moller, 1959). One form of each is present in the soluble portion
of the cell and one form of each resides in the mitochondria. These
two types of enzymes are capable of utilizing similar substrates in
different cellular locations. Kadenbach et al. (1964) have shown
that thyroid hormones are capable of increasing the concentrations
of D-IDH at the expense of T-IDH. This affords a mechanism for
increased catabolic processes within the mitochondria. Substrate
concentrations for other enzymes may be altered indirectly by
isozymes whose products or substrates are common with the substrate
of that enzyme (Pardee, 1959).

The kinetic parameters of $K_m$ and $V_m$ may also be changed by
isozymes. The primary example of this phenomenon is LDH. As was
mentioned previously, the various isozymes have different kinetic
parameters. Dawson et al. (1964) reported $K_m$ value for pyruvate of
8.9$\times$10$^{-5}$ M and 3.2$\times$10$^{-3}$ M for $H^+$ and $M^+$ respectively. $K_m$ values
for lactate were reported by Vesell (1963) as 1.2$\times$10$^{-5}$ M and
2.6-50$\times$10$^{-5}$ M respectively. They have also reported a twofold
difference in turnover numbers between the two isozymes. In
addition to the variations in substrate affinities, it is generally
accepted that the $H^+$-type isozymes are greatly inhibited at high
substrate concentrations (Hunter and Markert, 1957; Markert and
In general, those tissues high in the H-type isozymes are capable of converting smaller amounts of pyruvate to lactate. High pyruvate concentrations tend to inhibit the LDH reaction and forces the cell to oxidize it through the citric acid cycle. Tissues high in the H-type isozymes are therefore highly subject to impairment due to conditions resulting in anoxia. Those tissues high in the M-type isozymes, on the other hand, are capable of handling high concentrations of pyruvate at a rapid rate. Tissues high in this form of the enzyme are capable of building up an oxygen debt under anaerobic conditions (Wilson et al., 1963).

The abilities of various tissues to handle pyruvate is also under hormonal control to a certain extent. Allen (1961), Dawson et al. (1964), and Goodfriend and Kaplan (1963, 1964) have demonstrated that in certain organs such as the uterus the relative amounts of the various LDH monomers is under control of various steroids. They found that in cells capable of protein synthesis, estradiol was capable of increasing the average percentage of the M monomer in the LDH isozymes. They hypothesize that estradiol selectively acts as an inducer of the biosynthesis of the M-type monomer. This is in accord with the protein synthesis scheme set forth by Jacob and Monod (1961). Goodfriend and Kaplan (1964) and Dawson et al. (1964) have reported that other steroids (testosterone and progesterone) also cause an elevation in total LDH activity in uterine
extracts without altering the ratio of the two monomers. They suggest this is evidence of two independent structural genes responsible for the synthesis of the two monomers. The control of the two structural genes must also be somewhat independent due to the differential action of estradiol and the latter two hormones. Allison et al. (1963, 1964) have observed that thyroid hormones cause a disappearance of the Mn isozyme of rabbit liver. Here again is a method whereby thyroxine can force substrate to be utilized by the citric acid cycle. It can limit the ability of the cell to form lactate from large amounts of pyruvate, through the differential kinetics of the various isozymes of LDH. Vesell et al. (1962) and Vesell and Philip (1963) have observed that differences in isozyme patterns of tissues are lost as the tissue is cultured in vitro. They suggest that this is evidence of control of isozymes by products of other organs and tissues in the animal's system.

Control of pre-existing enzymes

There are two basic kinetic parameters of an enzyme through which control can be imparted upon a biochemical reaction. One parameter influences the velocity at which the reaction can occur and the other influences the substrate concentration at which the reaction can occur most effectively. The two parameters are Vm (maximum velocity theoretically attained under optimum substrate concentration) and Km (Michaelis' constant which represents the substrate concentration giving half-maximum velocity). Km is often
designated as $K_s$, or substrate constant (Dixon and Webb, 1958). The velocity of an enzymically catalyzed reaction is related to these two parameters by the equation:

$$v = \frac{V_m S}{K_m + S}$$

where $v$ is the velocity of the reaction and $S$ is the substrate concentration. From this relationship it is obvious that substrate concentration affects the velocity of the reaction. As was discussed earlier the substrate concentration is probably influenced most by cell membrane permeability and the rates of adjacent enzymes in an enzyme sequence comprising a metabolic pathway (Pardee, 1959).

Metabolites and other compounds can affect an enzyme's activity by their action as activators and inhibitors. It is possible for these compounds to alter the apparent $V_m$ and $K_m$ parameters of the enzyme (Friedenwald, 1954). These compounds are observed to reversibly bind to the enzyme with a certain degree of specificity. By invoking various changes in tertiary and quaternary structure of the enzyme molecule, the activity of the enzyme can be greatly modified. Inhibitors can act by decreasing the apparent $V_m$ or increasing the apparent $K_m$ or by affecting both of these parameters. Activators act in an opposite direction to that of inhibitors. By decreasing the apparent $K_m$ the enzyme would take on the ability to more effectively compete with other enzymes for common substrates when the concentration of that substrate is relatively low. Increasing the apparent $V_m$ as is the case with certain kinds of
activators results in a proportional increase in rate of utilization of the substrate. It is possible for activators and inhibitors to affect both parameters simultaneously (Pardee, 1959). By acting as an activator or an inhibitor, hormones could control both the rate of the enzyme activity and its ability to compete with other enzymes for common substrates. This latter effect would allow a hormone to direct the activity of various branched metabolic pathways.

An example of a hormone acting as an inhibitor and an activator is the effect of estrogen on the glutamic dehydrogenase enzyme. As was discussed earlier estradiol in fairly high concentrations causes a disaggregation of the glutamic dehydrogenase molecule into subunits which possess alanine dehydrogenase activity. The hormone is therefore acting as an inhibitor of glutamic dehydrogenase and an activator of alanine dehydrogenase (Tomkins et al., 1961; Talal et al., 1964; and Yielding and Tomkins, 1960, 1962).

Control of cell membrane permeability

As has been discussed earlier, control of substrate levels at the site of the enzyme has the potential of being a very good control mechanism by which hormones may act. Changes in cell membrane permeability toward various substrates could effectively regulate intracellular substrate levels. In most instances it has been very difficult to demonstrate hormone action in cell-free \textit{in vitro} enzyme systems (Szego, 1956). It was therefore suggested that most hormones act at the cell membrane level and that the
difficulty in demonstrating hormone action was the inability to prepare active cellular systems for in vitro studies. Spaziani and Szego (1958, 1959) have demonstrated that a single injection of estradiol-17-β causes a release of endogenous histamine in the rat uterus. Cortisol as well as antihistamine injections at the time of estradiol injection prevented this release of histamine. Histamine is a well-known vasodilator and is thought to control the permeability of blood vessels. These investigators suggest that histamines play a role in the basic mechanism of estradiol action.

Noall and Allen (1961) observed that 30 minutes following estradiol administration there was an increase in penetration of amino acids into rabbit uterus. Estradiol administration was found necessary prior to sacrifice of the animals for subsequent in vitro amino acid incorporation experiments. Spaziani (1963) made similar observations using labeled hexoses and pentoses. Mueller et al. (1961) have demonstrated that puromycin, an inhibitor of protein synthesis, not only inhibited several characteristic biosynthetic processes which were normally induced by estradiol, but also inhibited water imbibition and cell membrane permeability. They suggest that early estradiol effects are therefore mediated through some protein, probably enzymic in nature.

Endocrinology of the uterus and mammary gland

Although numerous endocrine products control the functions of the uterus and the mammary gland the ones of primary importance
are produced by the pituitary and the ovary. Numerous distinct changes in the activities of these organs occur depending on the reproductive status of the individual. These changes are characteristic of various stages of the estrous cycle, pregnancy, and lactation.

The estrous cycle, pregnancy, and lactation

Three hormones produced by the anterior pituitary of the female are of primary importance in her reproductive processes (Turner, 1960). They are (1) follicle-stimulating hormone (FSH), (2) luteinizing hormone (LH), and (3) luteotrophin (LtH). These three hormones are all polypeptide in nature, although the FSH and LH possess some form of a carbohydrate moiety.

The primary function of FSH is the stimulation of growth and maturation of ovarian follicles. Although FSH promotes follicular growth, it is incapable of stimulating estrogen production and ovulation when administered as the only gonadotrophin to hypophysectomized rats (Velardo, 1960; Van Dyke et al., 1950).

LH in combination with FSH is required for the production of estrogen by the ovarian follicle. LH is often referred to as the ovulation inducing hormone as it is considered to be the impetus for ovulation in most species.

LtH or prolactin is a hormone with several diverse functions. It is responsible for the initiation and maintenance of functional
corpora lutea and is required for the development and secretory activities of the mammary gland.

Estrogens and progesterone are produced for the most part by the ovary. The three primary estrogens are estradiol, estrone, and estriol. Estrogens are thought to be produced by the theca interna layer of the developing ovarian follicle. The estrogens are characterized as steroids having eighteen carbon atoms. They have an aromatic "A" ring, lack a methyl group at C-10 and possess a phenolic hydroxyl group at C-3.

Progesterone is a product of the granulosa layer of the functional corpus luteum of both the diestrus phase of the estrous cycle and pregnancy. The placenta and adrenal cortex are also capable of synthesizing progesterone (Amoroso, 1955). The progesterone molecule is a steroid with twenty-one carbon atoms of which two comprise a side chain at C-17. Turner (1960) suggests that in general the estrogens stimulate primarily growth processes while progesterone encourages tissue differentiation.

Upon stimulation of the ovary by FSH there is a rapid proliferation of the theca cells around several primary oocytes. If LH is also present these cells begin to produce estrogens (Van Dyke et al., 1950). As further proliferation of this tissue occurs, the ovum continues to mature and greater quantities of estrogens are produced. The estrogen titer has been shown by these investigators to inhibit the release of FSH and stimulate
the release of LH. When the titer of LH is sufficiently great ovulation occurs. Velardo (1960) reported that both FSH and LH are required for ovulation in the hypophysectomized rat. Certain species require mating to stimulate ovulation (Donovan and Harris, 1955).
It is suggested that the elevation in LH levels requires a neurohumoral factor in both induced and spontaneously ovulating species. Hansel et al. (1958) have demonstrated by the use of atropine that the LH release is dependent upon some neural pathway. They tested the hypothesis that oxytocin release by the neurohypophysis might be the mechanism of LH release and found that oxytocin could not overcome the atropine block in the cow.

After the rupture of the mature follicle the site of ovulation becomes engorged with blood and lymph and develops into the corpus luteum. As was stated earlier the corpus luteum is the primary source of progesterone. Van Dyke, et al. (1950) have detected progesterone production prior to ovulation by the follicular cells. Everett (1951) suggested that the presence of small amounts of progesterone hastens the release of LH and subsequently ovulation. Robinson (1954) also implicates a need for progesterone in the induction of estrus. Larger amounts of progesterone, however, were found to inhibit LH release.

The involvement of progesterone is considered to be at the level of the neurohumoral factor required for ovulation. LH is required for the secretion of progesterone by the corpus luteum.
Everett (1956) has been able to maintain corpus luteum activity for extended periods with autographs of rat pituitary glands and attributes this to prolactin effects. Hammond (1956) suggests that the maintenance of the corpus luteum in the rabbit by this gonadotrophin is secondary to its stimulation of estrogen secretion by the corpus luteum. Exogenous estrogens were found to be capable of extending the diestrus phase by maintenance of progesterone producing ability. Upon the gradual withdrawal of luteal activity, FSH is again released and the cycle is perpetuated.

During pregnancy in many species, secondary sources of both steroids and gonadotrophins increase (Hagerman, 1964; Diczfalusy, 1964). The placenta serves as an excellent source of chorionic gonadotrophin during the early phases of pregnancy in women. After about 120 days of pregnancy estrogen and pregnandiol levels, as measured in urine, increase to great proportions. Hagerman (1964) presents an extensive list of enzymes found in the placenta. From this list it is evident that most of the enzymes required for steroid biogenesis are present in the placenta. Diczfalusy (1964) suggests that the fetal adrenal is very important in production of steroids during mid-pregnancy. He suggests that placental progesterone is utilized by the fetal adrenal to synthesize a multitude of other steroids.

The exact hormonal control of parturition is unknown. Estrogen promotes a rhythmic contraction of the uterus. Oxytocin
from the neurohypophysis is able to synergize with estrogen to greatly intensify the uterine contractions (Hays and Van Demark, 1953). One of the current theories of parturition is that the withdrawal of progesterone at parturition leaves the estradiol effects on uterine motility unopposed and the frequency and intensity of the uterine contractibility can increase (Csapo, 1959). Oxytocin levels are also found to be rather high at parturition.

Control of the uterus

Under the influence of estrogens the uterus makes several characteristic changes. In most species there is at first a marked increase in uterine weight due to increased water content. Later there is an increase in solids, indicating increases in growth and multiplication of the uterine cells. These changes are accompanied by an increase in permeability to water, electrolytes, amino acids, and sugars (Szego and Roberts, 1953). Glucose utilization, RNA synthesis, lipid synthesis, and protein synthesis are increased (Nicoldtte and Gorski, 1964). Progesterone and the adrenal cortex hormones prevent these rapid changes in proliferation and general metabolism.

During the follicular phase the endometrium makes very striking changes. The uterine glands become enlarged and are rather simple and straight with few branches. During the luteal phase, progesterone causes a rather conspicuous increase in the
thickness of the endometrium. The glands become further enlarged, branched, and convoluted (Malbandov, 1958).

Shortly after estrus or the follicular phase, there is considerable change in the fluids of the uterus. There is an increased quantity of fluids in mucoproteins. Blood is often found in these secretions as a result of the highly vascular nature of the tissue. As the estrous cycle progresses the amount of fluids at first decreases and then increases as progesterone levels increase (Olds and Van Demark, 1957).

The secretions of the female tract during early diestrus are probably the original source of nutrition of the fertilized ova when it reaches the uterus. Barker (1962) observed a stimulation of aerobic spermatozoan metabolism by fluids of the female reproductive tract of the cow produced during estrus. It was considered that this stimulation may play some role in the capacitation of spermatozoa.

The uterine myometrium is capable of extensive contractile activity. Hays and Van Demark (1953) have indicated that in the ovariectomized cow both estrogen and progesterone are capable of stimulating uterine motility. Csapo (1959), however, suggests that in laboratory animals uterine motility is decreased by progesterone. Van Demark and Hays (1951) also observed a synergistic effect of oxytocin and estrogens on the stimulation of uterine motility. Epinephrine was a potent inhibitor of these contractions.
Control of the mammary gland

The mammary gland is controlled by a very complex series of endocrine interactions. In this section an attempt will be made to summarize the hormonal control of gross mammary gland activities during the growth and development phase and its lactational phases.

Growth and development. The growth and development of the mammary gland in most species occurs for the most part from puberty through gestation. The ovarian hormones control the growth of both the duct and alveolar or secretory systems. At puberty the ovaries begin to produce greater amounts of estrogens. At this time the duct system undergoes extensive development. Alveolar development occurs to a very limited degree during the period from puberty to the onset of pregnancy. Although large doses of estrogens are capable of stimulating some alveolar development it is thought that the relatively small amounts of progesterone produced during the luteal phases of the estrus cycle accounts for the limited degree of alveolar development during this period (Benson et al., 1957). Pregnancy results in a situation in which the mammary gland is under continual progesterone stimulation. During this phase, alveolar growth is extensive. In the earlier phases of pregnancy the levels of progesterone are relatively low when compared with the levels in later pregnancy when the placenta produces extensive amounts of this hormone. This latter source of progesterone probably accounts for the more extensive alveolar development as
the pregnancy approaches term. Lyons et al. (1958) demonstrated a synergism of estrogen and progesterone in stimulating mammary gland growth. Their results with guinea pigs indicated that the absolute amounts of the two hormones play a more important part in mammary tissue growth than does the ratio of the two hormones. Turner et al. (1956), however, presented data which strongly implicate the estrogen/progesterone ratio in the development of a "nearly normal" functional cow's udder. The growth and differentiation of the mammary gland is not strictly limited to the glands of the female. Jacobsohn (1961) sites several examples of induction of growth and differentiation in the mammary gland of the male by administration of ovarian hormones. Other steroids, namely the androgens and adrenalcorticoids, act synergistically, with estrogens and progesterone when administered at low levels, on mammary tissue growth and differentiation. Higher levels were inhibitory.

Lyons et al. (1958) summarized a series of experiments on mammary growth and development in hypophysectomized, oophorectomized, and adrenalectomized rats. Their data indicated that adrenalcorticoid hormones, estrogens, progesterone, prolactin, and somatotrophin are all required to develop a gland which in their estimation was normally developed when examined histologically.

Abraham et al. (1960a) were able to establish "nearly normal" lactation in hypophysectomized rats as measured by various biochemical criteria with hydrocortisone acetate and prolactin.
Lactation. Lactation is initiated in most mammalian species at or near parturition. Prolactin is perhaps the key hormone in the initiation of lactogenesis at parturition. Several investigations, however, indicated that prolactin alone is insufficient to initiate milk secretion in hypophysectomized animals which possessed suitably developed mammary glands. A source of corticotrophin or, more directly, corticoids from the adrenal cortex were required in these animals in addition to prolactin (Cowie, 1961). Although a controversy exists as to the exact mechanism of the initiation of lactation, it appears as though the withdrawal of the estrogen-progesterone complex at term allows for the action of prolactin to be manifested.

After the initiation of lactation, prolactin continues to be required to maintain milk production. In addition, some source of a corticoid with the properties of cortisol are required. This requirement was found to be satisfactorily provided in the ovariectomized and adrenalectomized rat by progesterone. Ovariectomy had no effect on milk secretion (Cowie, 1961). Somatotrophin appears to bolster the maintenance of lactation in some species. Its action appears likely to be indirect through increased supply of milk precursors. Abraham et al. (1960a) observed no difference in biochemical responses of the mammary gland in hypophysectomized, lactating rats maintained with prolactin and hydrocortisone acetate when somatotrophin was injected. Thyroid hormone, like somatotrophin
appears to function in a secondary manner in the maintenance of lactation in the rat (Lyons et al., 1958).

Another hormone of considerable importance to the lactational process is oxytocin. Peterson and Ludwick (1942) demonstrated the presence of the milk "let down" factor in the blood of freshly milked cows using a perfused udder technique. This factor was shown to be oxytocin.

**Endocrine control of metabolism of the uterus and mammary gland**

In the previous sections of this review it has been implied that control of cellular activities by hormones is dependent upon control of enzymically catalyzed reactions. An attempt was made to present the normal changes in hormone relationships that might have an effect either directly or indirectly on the uterus and mammary gland. The purpose of this section is to review the metabolic pathways in the two organs and the known effects of hormones on these pathways.

**Control of metabolism of the mammary gland**

The mammary gland is a rather unusual organ capable of considerable changes in metabolic activity. These changes occur in a very systematic manner depending on the physiological state of the gland. At the peak of lactation, there is considerable biosynthetic activity within the gland. This is evident from the
capabilities of bovine mammary tissue during its periods of lactation. About fifty pounds of mammary tissue can produce up to one hundred pounds of milk containing approximately three and a half pounds of butterfat, three and a half pounds of protein, and five pounds of lactose.

**General metabolism.** Considering the extensive biosynthetic activity of the mammary gland it is evident that its catabolic processes must also be quite active to provide the needed energy. As with most tissues the mammary gland utilizes glucose as its predominant source of energy. Mammary gland tissue slices are capable of considerable anaerobic glycolytic activity as measured by lactic acid production (Terner, 1952). This high rate of anaerobic glycolysis is subject to variations brought about by substrate concentration changes. Folley and French (1949a, 1949b) reported that both glucose and mannose could stimulate this pathway. It is of importance to note, however, that the Embden-Meyerhof pathway is incapable of accounting for all of the lactic acid formed under anaerobic conditions (Folley and French 1949a). Hansen and Carlson (1961) have recently summarized work that indicates either directly or indirectly that all of the enzymes and metabolic pathways of anaerobic glycolysis are present in the mammary gland.

Lactic acid accumulation is usually demonstrable only when mammary slices are incubated under anaerobic conditions with glucose (Terner, 1952). This can be accounted for by the findings of Moore and Nelson (1952) that the mammary gland possesses an active
citric acid cycle. They indicated that the rate limiting step of the citric acid cycle was the oxidation of isocitrate.

The pentose phosphate pathway has been shown by several investigations to be a well used pathway in the lactating mammary gland (Glock and McLean, 1953a, 1953b, 1954, 1958; Glock et al., 1956; McLean, 1958a; and Abraham et al., 1954). Abraham's group has estimated that approximately sixty per cent of the total glucose utilized by the rat mammary gland proceeds by way of this pathway. The workers in the laboratories of Glock and McLean have shown that most of the variation in the rate of utilization of glucose which occurs during lactation is associated with the pentose phosphate pathway. The Embden-Meyerhof pathway proceeds at a rather constant rate throughout lactation. The pentose phosphate pathway plays a vital role as a generator of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). NADPH is quite important in most cytoplasmic biosynthetic processes. NADP⁺-malate dehydrogenase and NADP⁺-isocitrate dehydrogenase are other enzymes found in the mammary gland capable of generating NADPH (Matthes, 1963).

Horecker and Hiatt (1956) summarized the biosynthetic processes requiring NADPH. This product is required for the reductive biosynthesis of fatty acids. The reductive carboxylation of pyruvate to malate requires NADPH. Reductive aminations of α-ketoacids to their respective amino acids also utilize this reduced coenzyme. Finally the process of steroid synthesis uses
NADPH for ring closures, demethylation in cholesterol synthesis, and the hydroxylation of steroids. McLean (1958b) has measured the levels of the nucleotides in mammary tissue. The reduced form of the NADP+ is greater than the oxidized form in the mammary gland. Nicotinamide adenine dinucleotide (NAD+) has an opposite relationship to that of NADP+. The total NAD+ nucleotides are about five times the quantity of the NADP+ nucleotides. According to Levy (1961, 1963) glucose-6-phosphate dehydrogenase is quite specific for NADP+ but under rather nonphysiological conditions will reduce NAD+. Hollander et al. (1958) have demonstrated transhydrogenase activity in mammary tissue. According to Talalay and Williams (1958, 1960) these transhydrogenases allow the balance of the NAD+ and NADP+ nucleotides to readily exchange their oxidation states depending on the metabolic needs and status of the cell.

While carbohydrate catabolism is quite vital to the mammary gland, it is also found to possess carbohydrate anabolic activities of a rather unique nature. Lactose, or milk sugar, is a product of this organ. Lactose is a molecule composed of a glucose and galactose moiety linked through a β-galactosidic bond. It is thought to be synthesized from two molecules of glucose-1-phosphate (Malpress, 1958). One molecule of glucose-1-phosphate is first bound to a molecule of uridine triphosphate (UTP) through UDP glucose pyrophosphorylase. UDP-glucose is then subjected to the action of UDP galactose-4-epimerase to form UDP-galactose.
UDP-galactose is then reacted with a second molecule of glucose-1-phosphate to form lactose-1-phosphate plus UDP through a galactosyl transferase (Gander et al., 1956, 1957). A phosphatase is then thought to cleave the phosphate from lactose-1-phosphate forming lactose.

One additional aspect of lactose synthesis is the incorporation of acetate into the molecule. Wood (1957a) has indicated that acetate and glycerol carbons go preferentially into carbons 4, 5, and 6 of the galactose molecule. He implies that the enzymes of the pentose phosphate pathway are probably important in the synthesis of this molecule. Wood et al. (1957a, 1957b) suggested that the two carbohydrate moieties of lactose may be derived from two alternate pathways. The synthesis of lactose from acetate may therefore reveal an alternate pathway of formation of UDP-galactose.

The synthesis of lipids in the mammary gland has been the subject of many research endeavors. There have been many studies concerning the relationship of carbohydrate and fat metabolism in lactating rat mammary gland (McLean, 1962, 1964; Abraham et al., 1957, 1959, 1960a, 1960b; Abraham and Chaikoff, 1959, 1959; Balmain et al., 1954; Balmain and Folley, 1951; Folley and Greenbaum, 1960; Chernick, et al., 1950; and Dils and Popjak, 1962). When fatty acid synthesis by a particulate from mammary gland extract begins from acetate as a substrate, it has been found that ATP, manganese, coenzyme A, glutathione, NADPH, biotin, and bicarbonate are required
When malonyl Co A is the initial substrate instead of acetate the synthesis of fatty acids occurs somewhat faster. The cofactor requirements of this system are ATP, coenzyme A, manganese, NADPH, biotin, and bicarbonate (Ganguly, 1960; Wakil, 1962; and Dils and Popjak, 1962). Dils and Popjak (1962) observed that malonate cannot be converted into malonyl Co A in the mammary gland.

The close association between lipid synthesis and carbohydrate metabolism is twofold. The obvious association is the formation of acetate from glucose and its use as a carbon source for fatty acids (Abraham and Chaikoff, 1959). Fatty acid synthesis has been shown to be closely correlated to the amounts and rate of synthesis of NADPH (Langdon, 1957; Tepperman and Tepperman, 1958; Abraham et al., 1959, 1960b; Seperstein and Fagan, 1957, 1958). The NADPH is required for the reduction of \( \alpha-\beta \) unsaturated acyl Co A derivatives. McLean (1960, 1962, 1964) has attempted to assess the cause and effect relationship between fatty acid synthesis and the activity of the pentose phosphate pathway. Using phenazine methosulphate, an artificial electron acceptor, she has observed that a deficiency of an NADP\(^+\) limits the activity of the pentose phosphate pathway. Indirectly she has come to similar conclusions using insulin and bicarbonate stimulation of fatty acid synthesis as a means of stimulating the pentose phosphate pathway.

Protein synthesis in the mammary gland is the primary source of proteins in the milk. Campbell and Work (1952) have shown that
little plasma protein finds its way into milk as such. The
synthesis of protein is dependent upon an abundant source of blood
amino acids. The fact that active protein synthesis occurs is also
implied by the characteristic increase in RNA associated with in­
creased milk production in the rat mammary gland. DNA has been
shown to remain relatively constant throughout lactation, indicating
that cellular proliferation activities are somewhat constant
(Greenbaum and Slater, 1957).

Aside from the complete complement of enzymes required for
the various pathways observed in mammary tissue a few miscellaneous
enzymes associated with other less completely detailed enzymic
processes have been observed. Arginase and alkaline phosphatase
have been observed in mammary tissue (Folley and Greenbaum, 1947).
Glutamate dehydrogenase and glutamate-aspartate transaminase were
found to be present by Greenbaum and Greenwood (1954). A few
other enzymes found to be present have been listed by Hansen and
Carlson (1961). These are β-glucuronidase, adenosine deaminase,
guanine deaminase, guanosine deaminase, acid phosphatase and
apyrase. Hollander et al. (1958) have demonstrated an estrogen-
dependent isocitrate transhydrogenase in human breast tissue.
Villee et al. (1960) and Abe et al. (1964) have observed other
estrogen sensitive transhydrogenase in mammary glands as well as
other estrogen target tissues.

The lactational patterns of the rat as indicated by milk
yields indicate a somewhat linear increase in milk yield from
parturition to twenty days of lactation. At this time the glands begin to involute and the volume drops off rapidly (Folley and Greenbaum, 1947). A number of enzymes known to play an important role in biosynthetic processes follow a pattern that is quite similar to that of the milk yield. Glock and McLean (1954, 1953a), McLean (1960), and Matthes et al. (1963) have followed the lactational cycle levels of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and observed that the pattern fits closely that of milk and fat yield. Folley and Greenbaum (1947) had similar results with arginase and alkaline phosphatase. McLean (1958b) and Dickens and Glock (1950) have observed a direct relationship between milk yield and the levels of NADPH and NADH in the rat mammary gland.

**Hormonal control of metabolism.** Perhaps the most effective hormone in the regulation of the metabolic activities of mammary tissue is insulin. Folley and McNaught (1958) have demonstrated both in vitro and in vivo stimulation of lipid synthesis. Folley and Greenbaum (1960) and McLean (1960) observed a stimulation of the pentose phosphate pathway by insulin which they attributed to increased lipid synthesis and its association to this pathway.

Adrenalectomy has been shown to inhibit the activity of both glycolysis and the pentose phosphate pathway as measured by labeled glucose metabolism (Greenbaum and Darby, 1964a) and the activity of the enzymes of this pathway (Willmer, 1960). Cowie and Folley
(1948) suggest secondary changes such as malnutrition may account for part of the effects of adrenalectomy.

The sex hormones have been studied in relation to the intensity of milk production by Folley and Kon (1938). They have observed that estradiol in relatively large doses inhibited lactation as does testosterone. Progesterone and androsterone had no effect. Folley and Young (1939) and Sykes et al. (1942) reported that prolactin stimulated milk fat production in the cow. Gaull and Villee (1959) have demonstrated that estradiol stimulates metabolic activity of anterior pituitary and suggests that this stimulation may control anterior pituitary hormone release. The actions of estradiol on the mammary gland could therefore act indirectly via the pituitary.

Control of metabolism of the uterus

The uterus is one of the primary target organs of estrogen action. It is observed to possess the enzymes for the glycolytic and pentose phosphate pathways of glucose metabolism, citric acid cycle, pathways for lipid synthesis, and the pathways of glycogen synthesis. As nearly all of the studies of uterine metabolism have been in relation to estrogen effects this section will cover both general metabolism and effects of estrogen on metabolism.

Injection of estradiol or estrone into either ovariectomized or immature rats is followed by an increase in anaerobic and aerobic
glycolysis in the surviving uterine tissue (Carroll, 1942; Hagerman and Villee, 1953; Sweeney, 1944; Kerly, 1940; and Roberts and Szego, 1953). Roberts and Szego (1953) and Kerly (1940) have considered the response as a function of time and observe that four hours after estrogen injection there is an increased glycolytic rate which is not accompanied by an increased respiration rate. Twenty hours after injection, however, there is a substantial increase in both glycolysis and respiratory activity.

Nicolette and Gorski (1964) have followed the metabolism of uniformly labeled glucose-$C^{14}$ in the rat uterus two hours after estrogen administration. Within this interval of time glucose was found to be incorporated into protein, RNA, lipid, and CO$_2$. Actinomycin D, an inhibitor of RNA synthesis, blocked these responses, thus indicating the dependence of these estrogen responses on protein synthesis.

Increased uterine lipid synthesis was also noticed by MacLeod and Hollander (1961) and Aizawa and Mueller (1961). The latter group reported that the in vivo uterine synthesis of ethanolamine, choline, inositol phospholipids and cholesterol is stimulated by a single intravenous injection of $17\beta$-estradiol into ovariectomized female rats. They also observed that estradiol exerted a stimulatory in vitro effect on phospholipid synthesis as measured by orthophosphate-$P^{32}$ incorporation.

Increased glycogen content has been noted in rat, rabbit, and human uterine tissue when estrogenic hormones are administered
Progesterone was found to reduce glycogen levels even when estradiol was administered simultaneously. Brody and Westman (1958) compared effects of these two hormones on the endometrium and myometrium and suggest that the progesterone-estrogen antagonism effects are observed primarily in the endometrium.

Many investigators have suggested that the primary responses of the uterus to estrogen injections are mediated through estrogens influence on protein synthesis. Estrogens stimulate the incorporation of labeled amino acids into protein (Mueller, 1953; Herranen and Mueller, 1956; and Hamilton, 1963). Gorski and Axman (1964) demonstrated that cycloheximide, an inhibitor of protein synthesis, inhibits most of the uterine responses due to estrogen. Ui and Mueller (1963a, 1963b) and Wilson (1963) obtained similar results using actinomycin-D, another inhibitor of protein synthesis which acts by preventing RNA synthesis.

Estrogen is known to stimulate several processes involved in RNA synthesis. After estrogen stimulation Gorski and Nicolette (1963) noted that the incorporation of labeled orthophosphate into RNA was increased. Jervell et al. (1958) have noticed that estradiol stimulates RNA synthesis and has no effect on DNA synthesis in the rat uterus. Jolley et al. (1963) using sea urchin embryos observed a similar stimulation of RNA synthesis but an inhibition of DNA synthesis and embryonic cleavage when estrogen was included in the
incubation medium. The rate of biosynthesis of the purine and pyrimidine bases is also increased by estradiol. Jervell et al. (1958) observed an increased rate of incorporation of $^{14}O_2$ into adenine, guanine, uridine, and other acid soluble nucleotides in surviving uterine segments. Using labeled orthophosphate and estrogen stimulation, Gorski and Mueller (1963) observed the greatest increase in incorporation of the label into uridine triphosphate as compared to other acid soluble nucleotides in the uterus. Herranen and Mueller (1956) and Mueller and Herranen (1956) observed a 5-6 fold increase in incorporation of a serine-$^3C_1^4$ label into purine bases. This carbon of serine is a source of one carbon unit used by the tetrahydrofolic acid system of one carbon metabolism. Two such one carbon units are incorporated into each purine molecule. Noteboom and Gorski (1963) observed an increase in RNA polymerase, an enzyme required for transcription of the DNA message into RNA following estradiol injection. They suggest that estrogens' action on the uterus is due to the early synthesis of some specific proteins such as RNA polymerase.

Estrogens also have an influence on the end site of protein synthesis. Greenman and Kenney (1963, 1964) have observed an increased number of uterine ribosomes following estradiol injection. The efficiency of the ribosomes, i.e., protein synthesized per milligram of ribosomal protein, was not changed by estradiol administration. Wilson (1962), however, observed an
increased efficiency of the ribosomes isolated from chick oviducts when estrogen was administered. Using labeled leucine Kalman and Opsall (1961) observed an increased specific activity of ribosomal nucleoprotein in the chick oviduct following estrogen administration. Using labeled orthophosphate Gorski and Nicolette (1963) also observed an increased specific activity in ribosomal RNA. Lio and Williams (1962) observed results quite similar in nature to those of estrogen in the effects of testosterone on the prostate gland of the rat. Their conclusion was that testosterone stimulated the synthesis of messenger RNA and not the efficiency of the ribosomes in this organ.

The site of estrogen accumulation has been estimated histologically using autoradiographic techniques and $^{14}$C and $^3$H labeled estradiol. Ulberg and Bengtsson (1963) noticed an accumulation of this compound or its metabolites in the adrenal cortex, follicular granulosa cells, and the nucleus of the secretory cells of the uterine glands. DePaepe (1960) also noticed an accumulation of the label in the peri-glomerular space of the kidney. These observations may give support to the suggestion of Hammond (1956) that estrogen is the primary stimulus for progesterone synthesis by the granulosa cells of the corpus luteum and the suggestions of McKerns (1963b) that estrogens regulate the synthesis of adrenal steroids.

There are a number of other enzymes that increase in uterine tissue under the influence of estrogens. Alkaline
phosphatase is known to increase under the influence of estrogens (Boutselo et al., 1963; Arzac and Blanchard, 1948). Estrogen promotes an increase in uterine $\beta$-glucuronidase content (Boutselo et al., 1963; and Knox et al., 1956). Brody (1958) observed an increased level of phosphorylase activity in the human myometrium under the influence of estrogens. Esterase activity is also increased in uterine tissue following estrogen administration (Knox et al., 1956). Dawson et al. (1964) and Goodfriend and Kaplan (1964) observed an increased rate of lactic dehydrogenase activity in uterine tissue following estradiol administration.

As was described in an earlier section of this review, these investigators observed that the "muscle type" lactic dehydrogenase was stimulated to a greater extent than the "heart type" form of the enzyme.

Other hormones are known to affect the synthesis of protein in the rat uterus. Lostroh (1963) has been able to maintain protein synthesis of mouse uteri in vitro with an organ culture technique. He observed that insulin and aldosterone are both capable of stimulating total protein synthesis under these conditions. Szego and Roberts (1948) observed that progesterone and deoxycorticosterone inhibited the early estrogen response of water inhibition into the uterus. They suggest that these hormones exhibit a "damping effect" on the estrogen response.
Estrogens and progesterone are known to have an affinity for certain proteins (DeMoor et al., 1963; Hansjuergen, 1963). With their affinity for proteins it is conceivable that steroids could also affect preformed enzymes by direct changes in kinetic parameters. McKerns (1960, 1963) suggests that both natural and synthetic estrogen are competitive inhibitors of glucose-6-phosphate dehydrogenase from adrenal tissue and anterior pituitary tissue. Marks and Banks (1960) were unable to demonstrate this competitive inhibition on the same enzyme from similar sources. Dehydroisoandrosterone and pregnenolone are known to be non-competitive inhibitors of mammalian glucose-6-phosphate dehydrogenase (Marks and Banks, 1960; Tsutsui et al., 1962).
MATERIALS AND METHODS

The procedures employed in this study were designed to evaluate the effects of 17β-estradiol and progesterone on the levels of several enzymes of the general pathways of glucose utilization in uterine and lactating mammary gland tissues of the rat. The enzymes to be evaluated were selected because of their position at sites capable of controlling branched metabolic pathways. The enzymes which were selected were dehydrogenases. The assays for such enzymes involve following the rate of formation or utilization of the reduced form of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) which are cofactors in the reactions these enzymes catalyze. The assay for these reduced cofactors is dependent upon their specific absorption of light at the wavelength of 340 millimicrons.

Following a preliminary study of several enzymes, investigations of greater detail were performed on the effects of estradiol and progesterone on the levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADPH-malic dehydrogenase in the lactating mammary gland and the levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and lactic dehydrogenase in uterine tissues of ovariectomized rats.
The effects of ovariectomy on the levels of the above enzymes in rat mammary glands during various stages of lactation have not been reported in the literature. It was therefore considered necessary to establish the ovariectomy effects prior to further experimentation on the mammary gland enzymes. The effects of 17β-estradiol and progesterone on the levels of these enzymes in lactating mammary glands were then studied. The effects of 17β-estradiol and progesterone on the levels of the enzymes in the uterus of ovariectomized rats were also evaluated. Actinomycin D, an inhibitor of protein synthesis was then used as a tool to determine the nature of the response of the enzymes studied in uterine tissue to estradiol administration.

The data were analyzed by comparing the weighted means of specific enzyme activities and total enzyme activities and tissue activities of similarly treated rats and tissues both graphically and statistically. The significance of weighted mean differences was tested using the t-test as described by Ostle (1954). The mean values for enzyme activities within similarly treated animals and tissues were weighted according to the amount of protein recovered per organ.

Source of rats

The albino rats used in this study were raised in a local colony. The lactating mammary glands were from animals undergoing
their first lactation. The animals used in the mammary gland experi-
ments were bred at 3 to 4 months of age at a body weight of 180 to
220 grams. Bilateral ovariectomy was performed by the dorsal
approach on ether anesthetized rats two days following parturition.
The litter size was maintained at 8-10 pups. The animals were
maintained on a diet of Purina Laboratory Chow fed ad libitum.
Enzyme assays were performed on control and ovariectomized animals
at 5, 10, 15, and 20 days post partum to evaluate the effects of
ovariectomy on lactational changes in enzyme levels. The effects
of estradiol and progesterone on the levels of the mammary gland
enzymes in ovariectomized lactating rats were determined for
animals sacrificed on their tenth day of lactation.

The uterine tissue used in the series of experiments to
determine the effects of estradiol and progesterone on uterine
enzymes was from albino rats 3 to 3 1/2 months of age, weighing from
180 to 200 grams. Ether anesthetized animals were bilaterally
ovariectomized by the dorsal approach. These animals were
sacrificed five days after ovariectomy.

**Hormonal treatments**

To determine the effects of estradiol and progesterone on
the enzyme levels in lactating mammary glands the hormones were
administered to groups of four ovariectomized lactating rats.
Estradiol was injected by the intraperitoneal route at the level of
5 micrograms in 0.25 milliliters of safflower oil daily for five days.
just prior to enzyme assay. Progesterone was injected in a similar manner at the rate of one milligram daily for the same period of time. The control group received a sham dose of 0.25 milliliters of the safflower oil daily for the same period of time.

The effects of estradiol and progesterone on the enzymes in uterine tissue were evaluated in ovariectomized rats. Because of the small amount of tissue available in the control and progesterone treated rats, tissues from two similarly treated rats were pooled before preparing the tissue for assay. As the numbers involved in each pooled sample varied at times, the means of specific activities and total activities were weighted by the amount of protein recovered after the tissues were processed. Five micrograms of estradiol and one milligram of progesterone in 0.25 milliliters of safflower oil were injected daily by the intraperitoneal route. Both hormones were administered together to one group to determine the combined effects. A control group received the oil carrier only. The hormone treatment was begun three days prior to sacrifice.

The effects of actinomycin D on estrogen's effects on the enzymes of the uterus were evaluated in two groups of ovariectomized rats. Actinomycin D at the rate of 70 micrograms per 100 grams of body weight was injected intraperitoneally with the daily injections both of estradiol in oil and a sham dose of oil. The injections were begun two days prior to sacrifice. This dosage of actinomycin D is in accord with that used by Greengard and Acs (1962) and Goodfriend and Kaplan (1964) for similar purposes in the rat.
**Preparation of tissues**

The mammary gland extracts used in this investigation were prepared as follows. Both abdominal mammary glands were removed through a ventral midline incision. The skin was pulled from the abdominal muscles by rolling the thumb at the point of connection and pulling the skin. The gland, which adheres to the skin, was then grasped at the caudal most point of the gland and pulled in its entirety from the skin. The two glands were placed immediately into about ten volumes ice-cold 0.25 M. sucrose-0.05 M. tris(hydroxymethyl)aminomethane (tris) buffer of pH 7.5. The glands were kneaded in the ice-cold sucrose-tris buffer to remove as much of the endogenous milk as possible. After blotting the two glands dry on filter paper they were weighed in tared weighing pans. The glands were then homogenized in one volume (w/v) of fresh ice-cold sucrose-tris buffer. Homogenization was done in an iced eight-ounce mortar and pestle using washed sea sand as an abrasive. All homogenizing was done in a cold room at 3°C. The homogenate was centrifuged at 35,000 xg for 45 minutes in a Servall refrigerated centrifuge set at 3°C. After decanting the supernatant into a precooled container the pellet was rehomogenized in the iced mortar and pestle with a second volume of ice-cold sucrose-tris buffer. This homogenate was centrifuged as above. The second supernatant was pooled with the first and the volume total was recorded. After mixing the two fractions thoroughly, about two milliliters were applied to a previously washed Sephadex-G-25 column (0.8 centimeters in diameter by 20.0 centimeters in height) which had been equilibrated with the
sucrose-tris buffer to remove endogenous substrates. The material was eluted from the column with the ice-cold sucrose-tris buffer. About 3 to \(3\frac{1}{2}\) milliliters of eluate, after the protein front, was collected for subsequent assays. This desalting procedure was carried out in the cold room at \(3^\circ\) C.

The uterine tissue extracts were prepared in a manner similar to the extracts from mammary tissue. Following a midline ventral opening of the abdominal cavity, the uterus was removed and placed immediately into ice-cold sucrose-tris buffer. The uterus was placed on a piece of filter paper and the fat and connective tissue was dissected away. The uterus was then weighed in tared weighing pans. The uteri from two similarly treated animals were pooled and homogenized in a mortar and pestle with two milliliters of ice-cold sucrose-tris buffer, using washed sea sand as an abrasive. The homogenate was centrifuged at 35,000 xg at \(3^\circ\) C. for 45 minutes. One to one and a half milliliters of the supernatant were applied to the Sephadex G-25 columns that were prepared as previously described. As before, the material was eluted from the column with cold sucrose-tris buffer. After the protein front was reached the next 3 to \(3\frac{1}{2}\) milliliters of eluate was collected for assay. Aliquots of the supernatants both before and after desalting were saved for protein assay in both the uterine and mammary gland tissue. Enzyme dilutions were made just prior to assay in ice-cold sucrose-tris buffer.
Assay of enzyme activity

Enzyme activities were measured by following the rate of reduction of NADP⁺ or the rate of oxidation of NADH, spectrophotometrically at a wavelength of 340 millimicrons in the presence of the specific substrate. The measurements were made in a Beckman DB spectrophotometer equipped with a Sargent SRL recorder with logarithmic gears. The chamber of the spectrophotometer was maintained at 25°C by circulating water from a refrigerated Warburg water bath through the chamber’s water-jacket. All reactions were carried out in standard Beckman cells with a one centimeter light path.

The change in optical density due to change in the oxidation state of the coenzyme was recorded as a function of time. The activity of the aliquot of enzyme in the reaction mixture was measured under initial velocity conditions. A unit of enzyme activity was considered to be that amount which caused an initial change in optical density of 1,000 per minute under the standardized conditions of the assay.

Each assay was carried out in triplicate. All components of the reaction except the enzyme and the specific substrate were mixed in a common flask. This reaction mixture and the aliquot of the enzyme preparation to be assayed were placed in the cell, mixed and incubated in a 25°C water bath prior to assay. Three cells were made up for each enzyme preparation and all were placed in water bath at the same time. The first cell was removed and
placed into the spectrophotometer after ten minutes of preincubation. The spectrophotometer was adjusted to zero optical density and checked for changes due to endogenous substrates. The reaction was initiated by adding the specific substrate to the cell and mixing with a plastic spatula. Recordings of optical density were begun immediately. The triplicate samples were assayed in succession. These general assay procedures were similar for all enzymes except lactic dehydrogenase. As this enzyme was assayed in the reverse direction, i.e., NADH was oxidized to NAD$^+$, the initial reading was adjusted to 0.83 optical density units before adding the substrate. This enzyme was followed by recording the decrease in optical density as a function of time. The amount of enzyme was adjusted to obtain changes in optical density from 0.005 to 0.05 units per minute.

Glucose-6-phosphate dehydrogenase activity was assayed by the procedures of Kornberg and Horecker (1955) with slight modification. The reaction mixture for each assay contained the following reagents.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (ml)</th>
<th>Micromoles per assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer 0.1M, pH 7.5</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>MgCl$_2$ 0.1M</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td>NADP 0.002M, pH 7.5</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>D-glucose-6-phosphate 0.02M</td>
<td>0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Enzyme in sucrose-tris buffer pH 7.5</td>
<td>to 3.0 milliliters</td>
<td>-</td>
</tr>
</tbody>
</table>

The procedure used for 6-phosphogluconate dehydrogenase was also that described by Kornberg and Horecker (1955). The amounts of the reagents used for this assay were as follows:
NADPH-malic dehydrogenase was assayed with a method similar to that described by Ochoa (1955). The reagents and amounts of these reagents employed in each assay were as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (ml)</th>
<th>Micromoles per assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer 0.1M, pH 7.5</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Mg Cl₂ 0.1M</td>
<td>.2</td>
<td>20</td>
</tr>
<tr>
<td>NADPH 0.002M, pH 7.5</td>
<td>.2</td>
<td>0.4</td>
</tr>
<tr>
<td>6-phospho-D-gluconate 0.02M</td>
<td>.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Enzyme in sucrose-tris buffer pH 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>3.0 milliliters</td>
</tr>
</tbody>
</table>

The lactic dehydrogenase assay was that described by Kornberg (1955). The assay was conducted with the following reagents:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (ml)</th>
<th>Micromoles per assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium phosphate buffer 0.1M, pH 7.5</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>NADH 0.002M, pH 7.5</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Sodium pyruvate 0.01M</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Enzyme in sucrose-tris buffer pH 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>3.0 milliliters</td>
</tr>
</tbody>
</table>

NADPH-isocitric dehydrogenase activity was measured in the preliminary studies by the following modification of Ochoa's (1955) method. The procedure was stated earlier in this section and the reagents used were as follows:
Reagent Volume Micromoles (ml) per assay

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (ml)</th>
<th>Micromoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer 0.1M, pH 7.5</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Mn Cl2 0.05M</td>
<td>.05</td>
<td>2.5</td>
</tr>
<tr>
<td>NADP+ 0.002M pH 7.5</td>
<td>.2</td>
<td>0.4</td>
</tr>
<tr>
<td>D-isocitrate .02M</td>
<td>.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Enzyme in sucrose-tris buffer pH 7.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>to 3.0 milliliters</td>
<td>-</td>
</tr>
</tbody>
</table>

To calculate specific enzyme activity it was essential to estimate the protein contained within the extracts used for assay. Protein assays were performed by the Folin method of Lowry et al. (1951). Optical density measurements were made on a Bosch and Lomb Spectronic 20 spectrophotometer. Crystalline bovine serum albumin was used for the standard protein solution. The protein content of the extracts was determined by comparing the spectrophotometer readings to those obtained with the standard protein solution. Duplicate observations were obtained for each extract. The protein content was taken as the mean of the duplicates. Protein standards were determined with each protein assay series to account for day to day variation in protein assay reagents.

Data collection and recording

The data for each rat in the mammary gland and uterus experiments were collected and recorded in a notebook on specially prepared mimeographed data sheets. Records were made of littering dates, surgery dates, and assay dates. An injection record was made for each treated animal. The weight of the tissue and the amounts of buffer added in the homogenization process were recorded.
The volumes of the extract prior to and following the Sephadex G-25
desalting process were recorded. The dilutions and amounts of the
enzyme activities of each of the triplicate observations on each
enzyme. The protein content of each extract and of each diluted
extract was determined and recorded.

Calculations

The effects of estradiol and progesterone on the enzymes in
mammary glands and uterine tissue were evaluated on three bases:
(1) specific activity (units of activity per milligram of protein),
(2) total activity (units of activity per total mammary gland or
units of activity per uterus), and (3) tissue activity (units of
activity per gram of tissue). The weighted means and standard
error of the deviations from the weighted means of the activities
for each enzyme within the tissues from similarly treated animals
were calculated and used for comparison purposes.

The calculations were as follows:

\[
\text{Specific activity} = \frac{\text{change in optical density}}{\text{milligram of protein per assay}}
\]

\[
\text{Total activity} = \text{specific activity} \times \text{total protein recovered}
\]

\[
\text{Tissue activity} = \frac{\text{total activity}}{\text{grams of tissue}}
\]

The change in optical density per minute was the mean of the
triplicate assays. Protein per assay was based on the protein
content of the desalted Sephadex G-25 eluate which was used for each
assay. The total protein recovered was based on the volume and protein content of the supernatant fraction prior to desalting. The assumption was made that there was no separation or removal of specific proteins from the supernatant in the desalting procedures. Sephadex G-25 has an approximate exclusion limit of 5,000; for example, it excludes all molecules of a molecular weight greater than 5,000 grams per mole. Since approximately one void volume of eluate was collected after the protein front, the protein composition was probably not changed. The tissue weight was taken as being the wet weight after blotting on filter paper.

The weighted mean of specific activity was the sum of the total enzyme activities per organ divided by the sum of the total protein per organ. The weighted mean of tissue activity was the sum of the total enzyme activities per organ divided by the organ weight. The weighted mean of total activity per organ was the sum of the total activities divided by the number of organs. This last value was different from the unweighted means only for the uterine tissue experiments of unequal numbers of animals per pooled sample.

The standard error of the deviations from the weighted mean (SEM) was calculated by the following formula:

\[ \text{SEM} = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} \]

where \( \bar{x} \) is the weighted mean, \( x \) is the individual variate.
Analysis

The data were analyzed both statistically and graphically. Statistical significance of differences between weighted means were tested by a one tailed t-test for unpaired variates as described by Ostle (1954). The formula for testing the differences between the two means was as follows:

$$ t_{\alpha} = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{\sum(x_1 - \bar{X}_1)^2}{n_1} + \frac{\sum(x_2 - \bar{X}_2)^2}{n_2}} \left( \frac{1}{n_1} + \frac{1}{n_2} \right)} $$

where \( \bar{X}_1 \) and \( \bar{X}_2 \) are the weighted means of the two treatments, \( X_1 \) and \( X_2 \) are the individual variates and \( n_1 \) and \( n_2 \) are the numbers of variates.

The null hypothesis that the population mean of the first group equals that of the second group was rejected if the calculated value of \( t_{\alpha} \) was less than \( -t_{\alpha}(n_1 + n_2 - 2) \) or greater than \( t_{\alpha}(n_1 + n_2 - 2) \). Three levels of probability (\( \alpha \)) were considered in the degree of significance of mean differences. Probability levels of \( P < .01 \), \( P < .05 \), and \( P < .10 \) were the levels of the test statistic considered before accepting the null hypothesis.

Graphical analysis was based on comparison of histograms of enzyme activity and enzyme activity expressed as per cent of the control's enzyme activity. This was used to detect trends that might exist in the data which were not readily tested in a statistical manner.
Special materials

The source of several rather specialized reagents used in this study is presented in the following list of special materials.

1. **17β-Estradiol, N.F., c grade**
   California Corporation for Biochemical Research
   Los Angeles, California

2. **Progesterone, U.S.P., c grade**
   California Corporation for Biochemical Research
   Los Angeles, California

3. **Nicotinamide Adenine Dinucleotide Phosphate (Monosodium Salt)**
   Sigma Chemical Company
   St. Louis, Missouri

4. **β-Dihydrronicotinamide Adenine Dinucleotide (Disodium Salt)**
   Sigma Chemical Company
   St. Louis, Missouri

5. **Glucose-6-Phosphate (Barium Salt)**
   Sigma Chemical Company
   St. Louis, Missouri

6. **6-Phosphogluconate (Barium Salt)**
   California Corporation for Biochemical Research
   Los Angeles, California

7. **Malic Acid**
   Sigma Chemical Company
   St. Louis, Missouri

8. **Pyruvate (Sodium Salt) Type II**
   Sigma Chemical Company
   St. Louis, Missouri

9. **Isocitrate (Barium Salt)**
   Nutritional Biochemicals Corporation
   Cleveland, Ohio

10. **Sephadex G-25 (Bead Form)**
    Pharmacia
    Uppsala, Sweden

11. **Actinomycin D**
    Donated by Dr. Laurient Michaud
    Merck, Sharp, and Dohm, Inc.
    Rahway, New Jersey
RESULTS AND DISCUSSION

Numerous biochemical changes associated with the stage of lactation have been observed in lactating mammary gland tissue. In general the levels of certain enzymes, cofactors, milk production, fat synthesis, and other compounds associated with biosynthetic processes increase until the onset of involution of the mammary gland. The height of metabolic activity is reached at about 18 to 19 days into lactation in the lactating rat. Glock and McLean (1954) suggested that enzyme activities and subsequent metabolic activities in the mammary gland and other tissues were correlated with the levels of female sex hormones.

Another target organ of the female sex hormones is the uterus. The uterus is observed to respond to estradiol stimulation within two to four hours after injection. This response is in the form of increased rate of CO₂ production, lipid synthesis, glycolysis, RNA synthesis, and protein synthesis (Nicolette and Gorski, 1964). In addition to these responses, oxygen uptake is increased twenty hours after estrogen injections.

In view of these observations, the objectives set forth in the materials and methods section were pursued and the following results were obtained.
The effects of ovariectomy on the activities of certain enzymes in lactating rat mammary gland tissue

The effects of ovariectomy on the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADPH-malic dehydrogenase in lactating rat mammary gland tissue are presented in Tables 1, 2, and 3 and Figures 1, 2, and 3. These data indicate that ovariectomy prevents the characteristic peak in activity of all three enzymes observed in the control animals on the fifteenth day of lactation. The specific activity, total activity, and tissue activity of glucose-6-phosphate dehydrogenase was significantly lower (P < .05) in the mammary gland tissue from ovariectomized rats at this stage of lactation. The differences observed in total activity and tissue activity of 6-phosphogluconate dehydrogenase between the ovariectomized and control groups approached significance (P < .10). While statistical significance was not indicated for all of the observed differences for enzyme activities throughout lactation between ovariectomized and control groups, the trends observed in Figures 1, 2, and 3 warrant consideration. In all cases the activity of the three enzymes in the glands from control rats was greatest on the fifteenth day of lactation. The activities of these enzymes decreased sometime after the fifteenth day and reached a level at the twentieth day that was about the same as that observed on the tenth day of lactation.
Ovariectomy prevents the peak in enzyme activity from occurring at the fifteenth day of lactation. The activities of all three enzymes in the ovariectomized animals continued to increase in a somewhat linear manner throughout the interval of lactation studied.

These trends indicate that ovariectomy has a modifying effect on the levels of these enzymes in the lactating rat mammary gland. The lack of complete dependence of the enzyme activity in the lactating mammary gland on the ovarian hormones is indicated by the fact that the levels of these enzymes continue to increase although at a slower rate following ovariectomy. Assuming the decrease in the activity of these enzymes is an indication of decreased mammary gland activity, these data also indicate that the ovarian hormones may bring about changes in the mammary gland which initiate involution of the secretory tissue.

It has been observed by several investigators (Folley and McNaught, 1958; Folley and Greenbaum, 1960; Abraham and Chaikoff, 1959; McLean, 1958a, 1958b, 1960, 1962, 1964) that the levels of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are closely correlated with the production of milk, fat synthesis levels of reduced nicotinamide adenine nucleotides and activity of the pentose phosphate pathway as measured by the production of $\text{C}^{14}\text{O}_2$ from 1-$\text{C}^{14}$-glucose. McLean (1962) has suggested that control of the pentose phosphate pathway is associated with the rate of oxidation of NADPH. She suggests that factors controlling fat
synthesis are primarily responsible for changes in the rate of oxidation of NADPH. Using various inhibitors of lipid synthesis she has been able to reduce the rate of C\textsubscript{14}O\textsubscript{2} production from 1-C\textsubscript{14}-glucose in mammary tissue slices. This loss of pentose phosphate pathway activity was restored by the addition of phenazine methosulfate, an artificial electron acceptor to the assay reaction media.

Using collodion to block the teats of the mammary gland on one side of a lactating rat, McLean (1964) has induced involution at mid-lactation in the blocked gland. Following experimental involution she observed that the levels of 6-phosphogluconate dehydrogenase, the rate limiting enzyme of the pentose phosphate pathway, doesn't decrease until nine hours after the decrease in fat synthesis. She suggests this is further evidence of control of the pentose phosphate pathway by fat synthesis.

Under in vivo conditions, the normal process of involution may still be controlled by the hormonal activities of the ovary. Shortly after parturition the rat exhibits ovarian follicle development. Following ovulation at this time, corpora lutea are developed and maintained throughout lactation. At about 20 to 21 days the ovary again begins to cycle. This time coincides with glandular involution and weaning of the suckling litter. As the pups continue to nurse their mothers, it is probable that involution precedes weaning and that the process of involution probably is dependent upon some hormonal change in the female. As was previously mentioned, the
data from this study tend to involve the functional ovary in the process of involution. Its effects, however, may not be directly on the mammary gland but may be mediated through changes in pituitary function. Perhaps the ovarian hormones could interfere with milk "let-down" and result in a condition similar to the collodion treatment used by McLean. They may also interfere with fat synthesis and in that way initiate involution directly at the mammary gland level. The possibility also exists that under in vivo conditions they could act directly on the pentose phosphate pathway to control the levels of the enzymes in this pathway in such a way as to limit mammary gland metabolic activity. Greenbaum and Darby (1964b) using the tranquilizing drug, reserpine, were able to demonstrate a reduction in fat synthesis in the mammary gland without affecting the activity of the pentose phosphate pathway. These results give support to the theory that fat synthesis does not completely control the pentose phosphate pathway. The effects of the major ovarian hormones, estradiol and progesterone, on the levels of these enzymes is considered in the next section.

The effects of estradiol and progesterone on the activities of certain enzymes in lactating rat mammary gland tissue

The effects of five daily injections of estradiol or progesterone on the activities of the enzymes studied in lactating mammary tissue are presented in Table 5. The total activities of
the three enzymes expressed as percentage of the sham injected control are presented in Figure 4. Tests of statistical significance indicated that the mean difference of total activity of glucose-6-phosphate dehydrogenase between the control and estradiol treated group approaches statistical significance ($P < .10$). The results expressed in Figure 4 tend to indicate that both estradiol and progesterone are capable of stimulating the total activities of the three enzymes studied. Estradiol had a more pronounced effect on the total activity of glucose-6-phosphate dehydrogenase than did progesterone. The activities of the other two enzymes were increased similarly by the two hormones.

These data tend to further indicate that the effects of the ovarian hormones at the tenth day of lactation are to modify the activity of the mammary gland, rather than to exhibit complete control. As was considered by Abraham and Chaikoff (1959) and McLean (1964), the large amount of variation in the enzymic activities of mammary glands in even very closely matched control groups is an indication of a multitude of unaccountable influencing factors. In such complex systems of control the alteration of one small aspect of the system may be compensated for by the other components of the system. Perhaps any dependence the mammary gland may have on the ovarian steroids could be overcome by adjustments in adrenal gland activity. Willmer (1960) has demonstrated that adrenalectomy results in a complete reduction in mammary gland activity. Possibly
adrenalectomy effects are also indirect through the general upset of nutritional and electrolyte balances in the system. Cortisone was found by this investigator to restore mammary gland activity and likewise the levels of enzymes of the pentose phosphate pathway. The effects of estradiol and progesterone on the mammary gland may be due to their actions or the actions of their metabolic products in a manner similar to the adrenal hormones.

Considering the apparent effects of other factors on the metabolic activities of the mammary gland, further studies on the mechanism of action of the ovarian hormones were conducted on uterine tissue. As this organ is under a greater degree of control by the ovarian hormones it was expected that the effects of these hormones would be more specific in nature.

The effects of estradiol and progesterone on the activities of certain enzymes in the uterus

The effects of injections of estradiol, progesterone, and a combination of estradiol and progesterone on the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and lactic dehydrogenase in the rat uterus are presented in Table 6 and Figures 5, 6, and 7. The data are presented in terms of specific activity, total activity, and tissue activity. Although these three criteria of enzyme activity were presented for the mammary gland studies, it was noted that the relationships between the three
criteria were similar. This is probably the result of the rather constant nature of the amounts of mammary gland tissue during lactation (Folley and Greenbaum, 1947). Unlike the mammary gland, the uterus changes markedly in size and water content under the effects of the treatments employed in this study. Specific activity measurements are considered to be a measure of the relative amounts of the total uterine protein comprised by the enzyme studied. Total activity is an indication of the gross effects of the treatment on enzyme activity in the uterus as a whole and accounts for the effects of change in size of the uterus. The differences between tissue activity and specific activity relationships are considered to be a function of water content of the uterine tissue.

The results of this investigation indicate that the activities as measured by all three criteria of the enzymes studied are increased as a result of estradiol administration. The levels of statistical significance are indicated in Table 6. It is noted in Table 8 that under the conditions of estradiol administration, uterine weight increased by about 85 per cent. The total activity of glucose-6-phosphate dehydrogenase increases by about 350 per cent. The changes in the other enzymes are similar but of a smaller magnitude. The specific activity changes indicate that of the total protein in the uterus, glucose-6-phosphate dehydrogenase is about doubled in relation to the other proteins under the influence of estradiol. The other enzymes again followed a similar but smaller change in specific activity.
Progesterone administered alone had no significant effect on the activities of any of the enzymes studied. When administered in conjunction with estradiol, progesterone inhibited the increase in total enzyme activity of pentose phosphate pathway enzymes. The inhibition of estradiol's effect on total activity of glucose-6-phosphate dehydrogenase by progesterone approached statistical significance (P < .10). These data suggest that progesterone may compete with estradiol at whatever site estradiol acts in changing the levels of the enzymes of the pentose phosphate pathway. As noted in Table 6, this inhibitory action of progesterone did not appear to occur toward estradiol's stimulation of lactic dehydrogenase activity, thus indicating that the site of action of estradiol's effects on this enzyme is different from that of the other two enzymes. These latter results are in accord with the observations of Dawson et al. (1964) which indicated an increased lactic dehydrogenase activity by progesterone as well as estradiol injections. Their data also suggest that the two sites of action are different.

The dependence of estradiol's effects on the activities of certain enzymes on active protein synthesis

Actinomycin D, an inhibitor of protein synthesis, has been used by several investigators to determine the site of action of certain hormones. Sekeris and Karlson (1964) suggest a scheme whereby several antibiotics could be used to pinpoint the level at
which hormone action could occur. Actinomycin D is thought to interfere with the synthesis of messenger RNA by the nuclear DNA. Garren and Howell (1963) and Greengard and Acs (1962) demonstrated that cortisone action on the synthesis of several enzymes was inhibited by actinomycin D injections. Talwar and Segal (1963) were able to inhibit the action of estrogens on the vaginal mucosa by local application of actinomycin D. Using this drug an attempt was made in this study to determine the nature of estrogen's stimulatory effect on the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and lactic dehydrogenase in the uterus. The data in Table 7 and Figure 8 indicate that the stimulation of enzyme synthesis by estradiol in the rat uterus is dependent upon messenger RNA synthesis. Figure 8 indicates that estradiol tends to allow some increase in enzyme activities in uterine tissue above that in the controls which received actinomycin D. Although these differences are probably insignificant they may indicate that estradiol effects begin prior to the inhibition effects of actinomycin D on protein synthesis when the two compounds are injected together. These data also suggest that the increased specific activities of these enzymes are due to active enzyme synthesis rather than a decreased rate of enzyme degradation. Noteboom and Gorski (1963) suggest that the synthesis of specific proteins is stimulated in early phases of the estrogen response. These data may indicate that enzymes in the pentose phosphate pathway
may be among the early proteins to be synthesized in the estrogen response.

The possible mechanism of control by estrogens and progesterone

The observation that the pentose phosphate pathway enzymes are increased in estrogen sensitive target organs provides a strong area for the possible control of metabolic processes in these organs by estrogens. It must be indicated, however, that this study does not indicate the cause and effect relationship of these enzymes to the other aspects of the estrogen response. As mentioned earlier it is observed that the early estrogen responses in the rat uterus are increased CO$_2$ production, increased lipid synthesis, increased rate of glycolysis, increased RNA synthesis, and increased protein synthesis. Later responses are observed to involve in addition to these, an increased rate of oxygen uptake.

The pentose phosphate pathway is one of the most active sources of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in most mammalian tissues. The NADP$^+$-isocitric dehydrogenase and NADP$^+$-malic dehydrogenase systems are also good sources of NADPH in mammary tissues. The pentose phosphate pathway is also characterized by the production of CO$_2$ from the number one carbon of glucose. Ribose-5-phosphate is an intermediate of this pathway and is thought to be the primary source of this compound in mammalian systems (Marks and Feigelson, 1957; and Tabohnick et al., 1958).
Nicolette and Gorski (1964) followed the utilization of glucose-U-C\(^{14}\) in the rat uterus and observed an increase in the production of labeled CO\(_2\). Although they did not determine that the CO\(_2\) was specifically from the number one position, it is quite possible that the CO\(_2\) could be generated in the early stages of the response by the oxidative decarboxylation of 6-phosphogluconate.

The addition of each two carbon unit to the fatty acid molecule required NADPH. Control of the pentose phosphate pathway could regulate the synthesis of fatty acids by regulating the levels of coenzymes. As was mentioned by McLean (1964) the cause and effect relationship of fatty acid synthesis and the activity of the pentose phosphate pathway is not completely clarified, although in the mammary gland the two are closely associated. It is possible that the observation of Aizawa and Mueller (1961), that estradiol stimulates, and progesterone inhibits the *in vitro* synthesis of phospholipids, may indicate that control of activity occurs at the level of lipid synthesis.

Ribonucleic acid synthesis and subsequent protein synthesis could be controlled by the pentose phosphate pathway. Under conditions of rapid growth there would be a need for increased amounts of ribose-5-phosphate used in the formation of RNA. The activity of the pentose phosphate pathway could be able to control protein synthesis by limiting ribose-5-phosphate pool sizes. The later increase in oxygen uptake could be the result of the synthesis of
estrogen sensitive transhydrogenases (Villee et al., 1960). Through the transhydrogenase reaction the NADPH could be reoxidized by NAD\(^+\) and the NADH could be completely oxidized by the NADH-cytochrome reductase system. Through this system the cells energy supply could be increased by the ATP formed by the electron transport system.

Nearly all results concerning the mechanism of estrogen action on its target tissues seems to point to the need of active protein synthesis. Mueller et al. (1961) indicate that certain proteins required for early stimulation of RNA synthesis are synthesized prior to the increased synthesis of RNA. It was suggested that certain inactive forms of these enzymes may be activated as the initial phase of the estrogen response. As in other systems it is possible that the removal of the newly synthesized protein from the ribosome is facilitated by compounds such as substrates, cofactors, or even inhibitors, which by their binding to the protein may change the affinity of the newly synthesized protein for the ribosome (Greengard and Acs, 1962). Estradiol and progesterone are known to bind to proteins (DeMoor et al., 1963; Hansjuergen, 1963). More specifically certain steroids have been shown by some investigators to be non-competitive inhibitors of glucose-6-phosphate dehydrogenase, possibly implicating these steroids in early elevation of this enzyme in tissues in which the levels of these hormones are quite high (Marks and Banks, 1960; and McKerans and Bell, 1960).
It is concluded in this study that increased glucose-6-
phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities
are part of the estrogen response in two target organs, the mammary
gland and the uterus. Since the time of assay of these enzymes was
during the latter period of the estrogen response, the relationship
of the elevation of the activity of these enzymes to the remainder
of the estrogen response is not presently known. It is possible that
control of these enzymes by estrogens is the cause of further estrogen
responses. The levels of these enzymes in the estrogen target
tissues could also be merely a reflection of rapid cellular bio-
synthetic activity. Increases in NADP⁺-malic dehydrogenase and
lactic dehydrogenase in mammary and uterine tissues respectively,
are also shown to be part of the later estrogen responses in these
tissues.
TABLE 1.—The effects of ovariectomy two days after parturition on the specific activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP⁺-malic dehydrogenase in rat mammary gland tissue during different stages of lactation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Specific Activity by Day of Lactation (Change in Optical Density per Minute per mg of Protein Expressed as a Weighted Mean±S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>Control</td>
<td>.647 ± .084</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>Ovariectomized</td>
<td>.590 ± .038</td>
</tr>
<tr>
<td>6-Phosphogluconate</td>
<td>Control</td>
<td>.073 ± .008</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>Ovariectomized</td>
<td>.072 ± .016</td>
</tr>
<tr>
<td>NADP⁺-malic Dehydrogenase</td>
<td>Control</td>
<td>.228 ± .050</td>
</tr>
<tr>
<td></td>
<td>Ovariectomized</td>
<td>.234 ± .042</td>
</tr>
</tbody>
</table>

aValue significantly different from control at P < .05.
TABLE 2.—The effects of ovariectomy two days after parturition on the total activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP⁺-malic dehydrogenase in rat mammary gland tissue during different stages of lactation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Total Activity by Day of Lactation (Change in Optical Density per Minute per Mammary Gland Expressed as a Weighted Mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>Control</td>
<td>125.24 ± 38.34</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>Ovariectomized</td>
<td>130.17 ± 8.45</td>
</tr>
<tr>
<td>6-phosphogluconate</td>
<td>Control</td>
<td>14.21 ± 3.87</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>Ovariectomized</td>
<td>15.90 ± 2.17</td>
</tr>
<tr>
<td>NADP⁺-malic Dehydrogenase</td>
<td>Control</td>
<td>44.18 ± 17.25</td>
</tr>
<tr>
<td></td>
<td>Ovariectomized</td>
<td>51.55 ± 13.59</td>
</tr>
</tbody>
</table>

*Value significantly different from control at P < .05.

bValue significantly different from control at P < .10.
TABLE 3.—The effects of ovariectomy two days after parturition on the tissue activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP⁺-malic dehydrogenase in rat mammary gland tissue during different stages of lactation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Tissue Activity by Day of Lactation (Change in Optical Density per Minute per Gram of Tissue Expressed as a Weighted Mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>Glucose-6-phosphate Dehydrogenase</td>
<td>Control</td>
<td>22.72 ± 5.57</td>
</tr>
<tr>
<td></td>
<td>Ovariectomized</td>
<td>21.67 ± 1.76</td>
</tr>
<tr>
<td>6-phosphogluconate Dehydrogenase</td>
<td>Control</td>
<td>2.58 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>Ovariectomized</td>
<td>2.65 ± 0.60</td>
</tr>
<tr>
<td>NADP⁺-malic Dehydrogenase</td>
<td>Control</td>
<td>8.02 ± 2.51</td>
</tr>
<tr>
<td></td>
<td>Ovariectomized</td>
<td>8.58 ± 1.40</td>
</tr>
</tbody>
</table>

<sup>a</sup>Value significantly different from control at P < .05.

<sup>b</sup>Value significantly different from control at P < .10.
TABLE 4.—The effects of ovariectomy two days after parturition on the mean wet weights of rat mammary glands by stage of lactation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Wet Weights and Numbers of Animals&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td></td>
<td>Grams</td>
</tr>
<tr>
<td>Control</td>
<td>5.511</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>6.007</td>
</tr>
</tbody>
</table>

<sup>a</sup>The mammary gland weights and numbers reported in this table correspond with the data presented on the enzyme levels in the rat mammary gland by stage of lactation following ovariectomy two days after parturition.
TABLE 5.—The effects of estradiol and progesterone on the specific activities, total activities, and tissue activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP⁺-malic dehydrogenase in the mammary gland tissue from rats which were ovariectomized two days after parturition and in their tenth day of lactation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Specific Activity (Units per mg Protein)</th>
<th>Total Activity (Units per Total Gland)</th>
<th>Tissue Activity (Units per gm of Tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate Dehydrogenase</td>
<td>Control</td>
<td>.743 ± .174</td>
<td>148.36 ± 58.88</td>
<td>30.26 ± 7.14</td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>.946 ± .153</td>
<td>251.36 ± 68.92</td>
<td>44.51 ± 15.02</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>.775 ± .037</td>
<td>197.14 ± 17.77</td>
<td>32.96 ± 1.95</td>
</tr>
<tr>
<td>6-phosphogluconate Dehydrogenase</td>
<td>Control</td>
<td>.106 ± .014</td>
<td>21.14 ± 9.68</td>
<td>4.33 ± 1.01</td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>.112 ± .012</td>
<td>29.76 ± 6.83</td>
<td>5.27 ± 1.39</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>.125 ± .015</td>
<td>31.82 ± 5.27</td>
<td>5.32 ± .73</td>
</tr>
</tbody>
</table>
TABLE 5—Continued

Enzyme Activities (Expressed as Weighted Mean ± S.E.M.)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Specific Activity (Units per mg Protein)</th>
<th>Total Activity (Units per Total Gland)</th>
<th>Tissue Activity (Units per gm of Tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP⁺- malic Dehydrogenase</td>
<td>Control</td>
<td>.244 ± .049</td>
<td>48.59 ± 22.55</td>
<td>9.91 ± 2.87</td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>.251 ± .056</td>
<td>66.75 ± 22.99</td>
<td>11.82 ± 4.58</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>.255 ± .024</td>
<td>64.74 ± 7.89</td>
<td>10.82 ± 1.12</td>
</tr>
</tbody>
</table>

a One unit is that amount of enzyme which causes an optical density change of 1.000 per minute under the conditions of the assay.

b Value significantly different from control at P < .10.
TABLE 6.—The effects of estradiol and progesterone on the specific activities, total activities, and tissue activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and lactic dehydrogenase in the uterus of ovariectomized rats

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Specific Activity (Units per mg Protein)</th>
<th>Total Activity (Units per Total Gland)</th>
<th>Tissue Activity (Units per gm of Tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate</td>
<td>Control</td>
<td>.082 ± .011</td>
<td>.392 ± .068</td>
<td>1.977 ± .139</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>Estradiol</td>
<td>.178 ± .032b</td>
<td>1.767 ± .830c</td>
<td>4.836 ± 1.181b</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>.082 ± .010</td>
<td>.357 ± .123</td>
<td>1.611 ± .109</td>
</tr>
<tr>
<td></td>
<td>Estradiol plus</td>
<td>.115 ± .039</td>
<td>.897 ± .495d</td>
<td>2.912 ± 1.184</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-phosphogluconate</td>
<td>Control</td>
<td>.024 ± .009</td>
<td>.113 ± .037</td>
<td>.572 ± .182</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>Estradiol</td>
<td>.034 ± .010</td>
<td>.338 ± .016c</td>
<td>.924 ± .235c</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>.027 ± .013</td>
<td>.117 ± .075</td>
<td>.530 ± .254</td>
</tr>
<tr>
<td></td>
<td>Estradiol plus</td>
<td>.030 ± .010</td>
<td>.234 ± .075c</td>
<td>.759 ± .254</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 6.—Continued

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Specific Activity (Units per mg Protein)</th>
<th>Total Activity (Units per Total Gland)</th>
<th>Tissue Activity (Units per gm of Tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic Dehydrogenase</td>
<td>Control</td>
<td>$1.83 \pm 0.15$</td>
<td>$8.09 \pm 1.42$</td>
<td>$44.33 \pm 1.31$</td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>$2.51 \pm 0.35^{c}$</td>
<td>$23.07 \pm 3.53$</td>
<td>$67.19 \pm 16.43^{c}$</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>$1.61 \pm 0.02$</td>
<td>$6.64 \pm 2.33$</td>
<td>$30.39 \pm 4.61$</td>
</tr>
<tr>
<td></td>
<td>Estradiol plus</td>
<td>$2.42 \pm 0.56$</td>
<td>$18.28 \pm 2.07^{c}$</td>
<td>$60.61 \pm 0.45^{b}$</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) One unit is that amount of enzyme which causes an optical density change of 1.000 per minute under the conditions of the assay.

\(^b\) Value significantly different from control at $P < .01$.

\(^c\) Value significantly different from control at $P < .05$.

\(^d\) Value significantly different from control at $P < .10$. 
### TABLE 7—The effects of actinomycin D on the specific activities, total activities, and tissue activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and lactic dehydrogenase in the uterus of ovariectomized rats following estradiol injections

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Specific Activity (Units per mg Protein)</th>
<th>Total Activity (Units per Total Gland)</th>
<th>Tissue Activity (Units per gm of Tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate Dehydrogenase</td>
<td>Control plus Actinomycin D</td>
<td>.072 ± .013</td>
<td>.263 ± .109</td>
<td>1.492 ± .134</td>
</tr>
<tr>
<td></td>
<td>Estradiol plus Actinomycin D</td>
<td>.073 ± .015</td>
<td>.401 ± .098</td>
<td>1.353 ± .326</td>
</tr>
<tr>
<td>6-phosphogluconate Dehydrogenase</td>
<td>Control plus Actinomycin D</td>
<td>.019 ± .002</td>
<td>.074 ± .023</td>
<td>.392 ± .054</td>
</tr>
<tr>
<td></td>
<td>Estradiol plus Actinomycin D</td>
<td>.027 ± .006</td>
<td>.148 ± .041</td>
<td>.498 ± .137</td>
</tr>
<tr>
<td>Lactic Dehydrogenase</td>
<td>Control plus Actinomycin D</td>
<td>2.21 ± .47</td>
<td>8.75 ± 1.15</td>
<td>46.12 ± 7.36</td>
</tr>
<tr>
<td></td>
<td>Estradiol plus Actinomycin D</td>
<td>2.20 ± .31</td>
<td>12.20 ± 2.18</td>
<td>41.15 ± 7.23</td>
</tr>
</tbody>
</table>

*A One unit is that amount of enzyme which causes an optical density change of 1.000 per minute under the conditions of the assay.*
TABLE 8.—The effects of various treatments employed in this investigation on the wet weight of the uteri from ovariectomized rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight per Uterus</th>
<th>Number of Rats</th>
<th>Number of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>.1982</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Estradiol</td>
<td>.3655</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Progesterone</td>
<td>.2214</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Estradiol plus Progesterone</td>
<td>.3061</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Control plus Actinomycin D</td>
<td>.1898</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Estradiol plus Actinomycin D</td>
<td>.2964</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

*aThe numbers of samples are different from the numbers of rats due to the pooled sample techniques used in this phase of the investigation.*
Fig. 1.—The effects of ovariectomy two days after parturition on the levels of glucose-6-phosphate dehydrogenase in rat mammary gland tissue during different stages of lactation. Comparisons of ovariectomized rats (black) with control rats (white) are based on three criteria: (a) specific activity, (b) total activity, and (c) tissue activity.
Fig. 2.—The effects of ovariectomy two days after parturition on the levels of 6-phosphogluconate dehydrogenase in rat mammary gland tissue during different stages of lactation. Comparisons of ovariectomized rats (black) with control rats (white) are based on three criteria: (a) specific activity, (b) total activity, and (c) tissue activity.
Fig. 3.—The effects of ovariectomy two days after parturition on the levels of NADP⁺-malic dehydrogenase in rat mammary gland tissue during different stages of lactation. Comparisons of ovariectomized rats (black) with control rats (white) are based on three criteria: (a) specific activity, (b) total activity, and (c) tissue activity.
Fig. 4.—The effects of estradiol and progesterone on the total activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP⁺-malic dehydrogenase in the mammary gland tissue from rats which were ovariectomized two days after parturition and in their tenth day of lactation. Estradiol(E), progesterone(P), and the oil carrier(C) were injected daily from the fifth to the tenth day of lactation.
Fig. 5.—The effects of estradiol and progesterone on the specific activity, total activity, and tissue activity of glucose-6-phosphate dehydrogenase in the uterus of ovariectomized rats. Estradiol (E), progesterone (P), estradiol plus progesterone (EP), and the oil carrier (C) were injected daily from the second to the fifth day after ovariectomy.
Fig. 6.—The effects of estradiol and progesterone on the specific activity, total activity, and tissue activity of 6-phosphogluconate dehydrogenase in the uterus of ovariectomized rats. Estradiol (E), progesterone (P), estradiol plus progesterone (EP), and the oil carrier (C) were injected from the second to the fifth day after ovariectomy.
Fig. 7.—The effects of estradiol and progesterone on the specific activity, total activity, and tissue activity of lactic dehydrogenase in the uterus of ovariectomized rats. Estradiol(E), progesterone(P), estradiol plus progesterone(EP), and the oil carrier(C) were injected daily from the second to the fifth day after ovariectomy.
Fig. 8.—The effects of actinomycin D on the total activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and lactic dehydrogenase in the uterus of ovariectomized rats following estradiol injections. Estradiol(E), estradiol plus actinomycin D(EA), the oil carrier(C), and the oil carrier plus actinomycin D(CA) were injected daily from the second to the fifth day after ovariectomy.
SUMMARY

The evaluations of responses of the abdominal mammary glands of 34 lactating female rats were used to determine the effects of ovariectomy, at the second day of lactation, on the levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic dehydrogenase in this organ during lactation. It was concluded that ovariectomy caused the levels of these enzymes to be significantly lowered in the mammary gland on the fifteenth day of lactation. The ovariectomized rats did not exhibit the marked decrease in activity of these enzymes on the twentieth day of lactation, which was characteristic of the control group. This possibly implicates ovarian activity in the process of mammary involution.

Five daily estradiol injections were found to elevate the levels of all three enzymes in the lactating rat mammary glands removed on the tenth day of lactation. Progesterone had a similar effect on 6-phosphogluconate dehydrogenase, and NADP⁺-malic dehydrogenase but not glucose-6-phosphate dehydrogenase. It is concluded that control of the levels of these enzymes in the mammary gland of rats is not solely a function of the ovary. Ovarian hormones are thought to exhibit only a modifying effect on the levels of these enzymes in the mammary gland.

The levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and lactic dehydrogenase in the uterus are
more completely controlled by estradiol. The activities of all three enzymes increase in the uteri of ovariectomized rats upon estradiol injection. Progesterone injections did not affect the levels of these enzymes in the uterus. When administered at the same time, progesterone is capable of limiting the estrogen stimulated elevation of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the uterus. This indicates that the site of action of estradiol stimulation is different for lactic dehydrogenase than that of the other two enzymes. Using actinomycin D, an inhibitor of RNA synthesis and subsequent protein synthesis, it was shown that the elevated levels of the enzymes but not lactic dehydrogenase in the uterus were the result of increased synthesis of these enzymes rather than a decreased rate of degradation or the activation of inactive forms of the enzyme. The control of the pentose phosphate pathway in estrogen target tissues by estradiol may be the basis of the estrogen response. The fact that progesterone inhibits estradiol's stimulation of the synthesis of some of the enzymes of this pathway may be the very basis of the inhibitory effect of progesterone on some of the estrogen responses.
LITERATURE CITED


WOOD, H. G., SIU, P., and SCHAMBYE, P. 1957b. Lactose synthesis III. The distribution of C\textsuperscript{14} in lactose of milk after intra-arterial injection of acetate-\textsuperscript{1-}C\textsuperscript{14}. Arch. Biochem. Biophys., 69:390.
