

CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE
AND ESTROGEN ACTION

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RONALD T. LAFRENIERE, B.Sc.

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CURRICULUM VITAE

NAME: Ronald Thomas J. Lafreniere

BORN: Sudbury, Ontario; September 8, 1944

SCHOLASTIC TRAINING:

B.Sc.....Chemistry.....Universite Laurentienne.....1965

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LIST OF ABBREVIATIONS

HK; Hexokinase

PFK; Phosphofructokinase

PK; Pyruvate kinase

G-6-PDH; Glucose 6-phosphate dehydrogenase

6-PGDH; 6-Phosphogluconate dehydrogenase

α -GPDH; α -Glycerophosphate dehydrogenase

NAD; Nicotinamide adenine dinucleotide

NADH; Nicotinamide adenine dinucleotide (reduced)

NADP; Nicotinamide adenine dinucleotide phosphate

NADPH; Nicotinamide adenine dinucleotide phosphate (reduced)

ADP; Adenosine 5'-diphosphate

ATP; Adenosine 5'-triphosphate

AMP; Adenosine 5'-monophosphate

2'-AMP; Adenosine 2'-monophosphate

3'-AMP; Adenosine 3'-monophosphate

2',3'-AMP; Adenosine 2',3'-monophosphate

Cyclic AMP; Adenosine 3',5'-monophosphate

Dibutyryl Cyclic AMP; N⁶-O^{2'} dibutyryl adenosine 3',5'-monophosphate

DNA; Deoxyribonucleic acid

RNA; Ribonucleic acid

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I. INTRODUCTION

The multiplicity of levels at which hormones act on biological systems makes them versatile tools in exploring the various regulatory mechanisms involved in the maintenance of dynamic homeostasis. Recent studies have demonstrated that estrogenic hormones exert an important regulatory influence on uterine biochemical differentiation and control metabolic processes in the uterus by acting as inducers of certain functionally-related, key, rate-limiting enzymes. We have, however, only little insight as to how do the estrogens produce their regulatory effects on cellular metabolism in this tissue. Evidence indicates that steroid hormones may act by a variety of different mechanisms. Karlson and Sekeris, in 1966, stated that "Physiological processes can be regulated in various ways, and hormones acting as regulatory messengers may possibly use not one, but several of the regulatory systems." (1).

The concept that the cyclic nucleotide, adenosine 3',5'-monophosphate (cyclic AMP), may function as an intracellular mediator in the mechanism of action of various hormones has attracted considerable attention. Although definitive evidence for a single triggering event in the action of estrogens on the uterus is still not available, the suggestion has been raised that estrogenic hormones might also act through the agency of cyclic AMP. In the present investigation, an attempt has been made to examine further the possible involvement of this cyclic nucleotide in estrogen-induced biochemical responses in the uterus.

It is known that carbohydrate metabolism plays an important role in the maintenance of normal uterine function. One of the characteristic effects produced by estrogens is the marked stimulation of uterine carbohydrate metabolism as evidenced by enhanced glycogen accumulation and increased activities of several important enzymes involved in the utilization of glucose. Recent work has shown that estrogenic hormones regulate glucose metabolism by exerting effects on receptor sites at the source of enzyme formation to switch on the biosynthesis of certain key glycolytic and hexose monophosphate shunt enzymes in the uterus. In order to examine the role played by cyclic AMP in the observed biochemical responses, a study was carried out to investigate the ability of exogenously administered cyclic AMP to mimic the estrogen-induced changes in uterine carbohydrate-metabolizing enzymes. In particular, this study has been directed towards the question of whether cyclic AMP is capable of mimicking the known effects of estrogens on uterine glycogen synthesis as well as on the activities of certain key glycolytic and hexose monophosphate shunt enzymes in uteri of both immature and ovariectomized rats.

II. LITERATURE REVIEW

A. ESTROGEN STIMULATION OF UTERINE FUNCTION

The uterus is an organ which is dramatically influenced by hormones. Among all the physiological effects of estrogen, the most striking is its action upon the uterus. In response to a single physiological dose of estradiol, the atrophic uterus of the ovariectomized rat is rapidly converted to an actively growing organ. As early as 1 hour after the administration of estradiol, there is a generalized hyperemia of the uterus with an accumulation of fluid throughout the tissue. The imbibition of water reaches a maximum after 4-6 hours of hormone treatment. There are also early increases in glycogen and phospholipid content of the uterus followed by an easily measurable increase in RNA and protein synthesis. Since many important uterine parameters increase after estrogen administration, an important problem has been to ascertain the initial event triggered by estrogens and the molecular mechanism by which this hormone stimulates macromolecular synthesis in the uterus.

(a) Increase in Tissue Mass

Estrogens have been shown to stimulate many reactions in the uterus. Estrogenic effects have been studied by measuring acute changes in the size of uterine structures, increases in the weight of the uterus or some of its components, and alterations in enzyme activities and in the rate of turnover of a cellular component. There are changes

in uterine cellular structures; the cells become swollen, the mitochondria vesiculated and the endoplasmic reticulum spread apart during the first 6 hours after estrogen administration. The nucleolar size increases (2) and there are changes in the epithelial basement lamina (3) as well as in the cell membrane (4). Administration of estrogens to ovariectomized rats is known to increase nuclear volume (5), mitotic rate (6), hyperemia (7), water accumulation (8), dry weight (9), glycolysis (10), the synthesis of uterine phospholipids (11), glycogen (12), cholesterol (13), RNA (14), protein (15) and DNA (16). The first 6 hours following estradiol treatment have been regarded as the induction phase; while there is a dramatic change in the water content of the tissue, little or no accumulation of protein, RNA, or DNA occurs during this period. The period from 6 to 24 hours is referred to as the RNA-accumulation phase, during which the protein accumulates in the wake of the rising RNA content of the uterus (2). While no increase in DNA occurs in the initial 24 hours of estrogenic response, a DNA-synthesis phase does occur sometime between 40 and 72 hours (17). Thus, the initial response is one of hypertrophy rather than hyperplasia - in fact, the hypertrophy appears to be a necessary step in preparation for hyperplasia (2).

(b) Amino Acid Accumulation

It is believed that at least nine of the amino acids found in protein (the essential amino acids: L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tyrosine and

L-valine) must be delivered to the uterine sites of protein synthesis from the extracellular fluid. Although the uterus can synthesize some of the non-essential amino acids found in protein (18,19), it is not known whether sufficient quantities are produced to meet the cellular demands. While the transport of valine- C^{14} into the uterus remained unaltered up to 12 hours following an intramuscular injection of 1.0 mg of estradiol (20), Halkerston et al (21) demonstrated that estradiol stimulated the accumulation of a non-utilizable amino acid, 2-aminoisobutyrate- $1-C^{14}$, in the rat uterus. Mueller and Herranen (19) found that oxidation of radioactive glucose- $1-C^{14}$, pyruvate- $2-C^{14}$ and acetate- $1-C^{14}$ to CO_2 in surviving segments of the rat uterus was not altered after 6 hours of estradiol treatment. However, Nicolette and Gorski (22) and MacLeod and Hollander (23) observed an increased production of CO_2 from glucose as early as 2 hours after estrogen treatment. The oxidation of $NaC^{14}COO^-$, glycine- $2-C^{14}$ and labelled formate remained unaffected up to 6 hours after hormone injection, although the incorporation of radioactivity from these substances into protein was enhanced (19). The incorporation of serine into purine bases and its oxidation to CO_2 were also stimulated by estradiol treatment (18). Furthermore, estradiol has been shown to increase the incorporation of many free extracellular amino acids into uterine protein (24).

It is apparent that estrogens influence the metabolism of some amino acids and amino acid precursors within the uterine cells. However, whether the stimulation of transport of these materials across

the cell membrane or within the cell to the loci of amino acid activation and subsequent peptide bonding is a principle mode of action of estrogenic hormones is yet to be established.

(c) RNA Biosynthesis

The rate of uterine RNA synthesis and its relationship to overall protein biosynthesis is of considerable importance in elucidating the mechanism of action of estrogens. As early as 1948, Jeener (16) observed an increase in the uterine RNA content 24 hours following administration of estradiol to mice without a concomitant rise in the concentration of DNA. This finding was soon confirmed in the rat (25) and since then, most investigators have used the rat for such studies. Grauer et al (25) demonstrated an even more rapid effect of estrogens by using the incorporation of a radioactive RNA precursor as a measure of RNA synthesis. The uptake of $P^{32}O_4$ into the uterus was stimulated within 3 hours after estradiol treatment and its incorporation into the acid insoluble uterine subcellular fraction was enhanced at 6 hours. Administration of estradiol also increased formate and glycine incorporation into nucleic acid moieties of surviving uterine segments within 6-8 hours (19).

Davis, Meyer and McShan (26) demonstrated an increased incorporation of radioactive phosphorus into uterine RNA 24 hours after administration of the hormone. An increase in total RNA of the incubated uterine segment, measured as acid-insoluble uridine and adenine, occurred 12 hours following a single injection of estradiol

(14). In addition, incorporation of radioactivity from $\text{NaHC}^{14}\text{O}_3$ and orotic acid 6-C^{14} into acid insoluble uridine increased 12-13 hours after estradiol treatment (14). Using the same in vitro system, it was shown that estradiol promotes the incorporation of adenine- 8-C^{14} into the adenine and guanine residues of RNA within 7 hours (2). Hamilton (27) confirmed these findings using a very small dose of the estrogen which was presumably closer to the physiological concentration. As little as 0.03 μg estradiol or 0.6 μg estrone injected intravenously into an ovariectomized rat was sufficient to stimulate incorporation of glycine- 2-C^{14} into uterine protein and incorporation of phosphate- P^{32} into nucleotides of the mixed nucleic acid fraction.

Mueller et al (28) demonstrated that puromycin not only blocked protein synthesis, but also prevented the usual acceleration of RNA synthesis induced by estrogens and inhibited the imbibition of water. With the use of actinomycin D, it was found that the synthesis of uterine RNA was decreased to very low levels in both the control and estrogen-treated animals (29). The consensus is that estradiol activates some aspect of RNA synthesis probably messenger-RNA, or even just one messenger-RNA for the synthesis of some rate-limiting enzyme, before any generalized increase occurs in the synthesis of total proteins. In this regard, several workers have presented evidence for the estrogenic stimulation of RNA-polymerase. Gorski (30) reported that the activity of uterine RNA-polymerase is increased one hour after estrogen injection. Both actinomycin D and puromycin were shown to block effectively the estradiol-induced increases in uterine RNA-

polymerase. Barker and Warren (31,32) demonstrated that estradiol-17 β , both in vivo and in vitro, was capable of stimulating the capacity of purified uterine chromatin to act as a template for DNA-dependent RNA-polymerase. It was postulated that estrogens first cause an increase in RNA polymerase and perhaps small amounts of other critical proteins, followed by increases in the synthesis of phospholipid, RNA and bulk protein.

(d) Amino Acid Activation

The utilization of ATP in activating eight of the L-amino acids naturally occurring in protein (leucine, tryptophan, cysteine, methionine, valine, tyrosine, isoleucine and glycine) was stimulated within 3 hours following the administration of estradiol. A nearly linear increase in the rate of amino acid dependent inorganic pyrophosphate-P³²-ATP exchange occurred up to 24 hours after estrogen treatment (33). No evidence of activation of other amino acids was obtained in the crude supernatant fractions used in this study. All of the amino acids that were shown to stimulate ATP-pyrophosphate-P³² exchange in uterine muscle did so in skeletal muscle (34) and all of the amino acids that did not stimulate this reaction in skeletal muscle behaved similarly in this respect in uterine muscle. It is of interest that of the amino acids demonstrated to stimulate ATP-pyrophosphate-P³² exchange, L-leucine and L-tryptophan displayed the highest level of this activity in both tissues. It is not understood whether the stimulation of uterine ATP-pyrophosphate-P³² exchange activities is the result of enzymatic activation, de novo

enzyme synthesis, or a combination of both, nor is it known whether this stimulation is a primary action of estrogens.

(e) Protein Biosynthesis

Studies involving the use of radioactive isotopic protein precursors for measuring newly formed proteins have demonstrated that an increase in protein synthesis indeed occurs in the uterus of the ovariectomized animal shortly after administration of a single dose of estradiol (15,24,35). Noteboom and Gorski (36) reported that there were two stages of uterine protein synthesis in response to estrogen treatment. Within 2-4 hours after estrogen, a wave of generalized protein synthesis occurred which seemed to involve proteins present in all subcellular fractions of the uterus. This was preceded by a stage during the first 2 hours in which estrogen had no significant effect on overall incorporation of amino acids into proteins. During these first 2 hours however, there was marked stimulation of RNA synthesis which was blocked by puromycin. These studies were extended (37,22) to show that cycloheximide, a protein synthesis inhibitor which differs markedly from puromycin in its chemical structure, also prevented the early increases in RNA and lipid synthesis as well as in glucose metabolism. Since actinomycin D also blocked the various aspects of estrogen response (22,29,38-40), it was suggested that estrogen action is dependent upon RNA synthesis at these early time periods. That the synthesis of nuclear RNA as well as the uptake of RNA precursor by the uterus is stimulated as early as 2 minutes after

injection of estradiol, indicated that one of the earliest effects of this hormone occurred at the transcriptional level of the uterine cell (41,42). The immediacy of these effects suggested that membrane activity and transcription of DNA are stimulated by estrogens to result in the rapid synthesis of specific molecules of RNA which presumably initiate and support the subsequent stimulation of protein synthesis (42).

The molecular action of estrogens on the uterus seems to involve a sequence of steps (43). First, estrogen interacts stereospecifically with a receptor in the target tissue i.e. uterus. The second step involves a change in the biological activity of this receptor protein as a result of its interaction with estrogen, a mechanism about which no definitive information is yet available. It is believed that this primary interaction is responsible for an increase in glucose metabolism, lipid and RNA synthesis as well as a number of other responses. The fact that these responses are all blocked by inhibitors of RNA and protein synthesis at a time when no overall protein synthesis is noted suggested that the synthesis of certain specific enzymes may be involved. The events of the third or amplification step of estrogen action arise as a consequence of the first two events and include many metabolic changes which contribute to the increase in early overall protein synthesis. Toft and Gorski (44) and Notedos and Gorski (45) have indeed isolated and characterized a macromolecular component from the uterus which has the

attributes of a specific receptor that binds to estrogens.

(f) Estradiol Induction of Uterine Enzymes

Recent studies have shown that estrogenic hormones may regulate uterine glucose metabolism by exerting their effects on receptor sites at the source of enzyme formation to turn on or off the biosynthesis of the three important rate-limiting enzymes viz. hexokinase, phosphofructokinase and pyruvate kinase, involved in the process of glycolysis (46,47,48). The three enzymes decreased markedly on castration and returned to the normal range following estrogen treatment. Investigations with estriol, estrone, diethylstilbestrol and estradiol-17 β revealed that the enzymatic responses to these estrogens parallel their known physiological potencies with estradiol-17 β being the most potent inducer of these enzymes. While a single injection of estradiol-17 β (10.0 μ g/100 g) by the intramuscular route produced significant increases in enzyme activities at 4 hours, greater increases were observed as early as 2 hours after intraperitoneal or intravenous administration of this hormone. The increases in uterine enzyme activities were dose-dependent. The estradiol-induced enhancement of uterine glycolytic enzymes was blocked effectively by actinomycin, puromycin, cycloheximide, 5-fluorouracil and ethionine, indicating that these enzymatic responses involved stimulation of both RNA and protein synthesis.

The estrogen-stimulated changes in uterine glycolytic enzymes showed positive correlation with the enhanced production of lactate and α -glycerophosphate by uterine supernatants (49). The production of lactate

and α -glycerophosphate was enhanced markedly from both glucose and glucose 6-phosphate as substrates by uteri of estradiol-treated ovariectomized rats. The time-course of the estrogen-induced increases in the formation of these metabolites paralleled that for the estradiol stimulation of the three key, glycolytic enzymes. In addition, the estrogen-induced enhancement of lactate and α -glycerophosphate production was completely blocked by inhibitors of protein synthesis. Based on these observations, Singhal and Valadares (48) have suggested that estrogenic hormones induce simultaneously the de novo synthesis of all three of the key, rate-limiting glycolytic enzymes in order to amplify the process of glycolysis in uterine tissue.

Since the exogenous administration of estradiol induced several uterine glycolytic enzymes, Valadares et al (50) investigated the influence of fluctuations in the level of endogenous estrogens on these enzymes in the normal cycling rat. Uterine weight and the activities of hexokinase, phosphofructokinase, pyruvate kinase as well as those of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were minimal at diestrus and reached maximum values at estrus. When cycloheximide was administered during early proestrus, the expected increases in enzyme activities were significantly inhibited. These authors concluded that alterations in the level of uterine glycolytic and hexose monophosphate shunt enzymes parallel the changes in the amounts of endogenous estrogens which, in physiological concentrations elicit profound changes in the protein-synthesizing ability of the uterus.

More recently, Singhal et al (51) reported that an inverse relationship exists between the estrogen-stimulated biochemical responses in the uterus and the age of the animal. Administration of a single intramuscular injection of estradiol produced a significant uterotrophic response in rats up to 12 months of age with maximum increases in 1-month old animals. Likewise, the most pronounced increases in the activities of uterine phosphofructokinase and phosphohexose isomerase were induced by the hormone in 1-month old rats. In older animals, however, administration of the hormone resulted in considerably smaller increases in uterine enzymes and in 24-month old rats, estradiol failed to exert any appreciable effect. In contrast, the glycogen-synthesizing system remained relatively sensitive to the action of estradiol-17 β in uteri of 24-month old animals, although, maximum stimulation of uterine glycogen synthesis also was produced in animals of 1-2 months of age.

(g) Uterine Glycogen Synthesis

An increase in glycogen concentration in the uterus has been considered to be an important metabolic event observed after the administration of estrogens to ovariectomized rats (12). Cecil and Bitman (52) demonstrated that following estradiol treatment, uterine glycogen increased almost five-fold in 16 hours. These authors also showed that cycloheximide increased uterine glycogen by 60% in 4.5 hours and by 100% in 15-19 hours in both fed and fasted ovariectomized rats. Valadares et al (53) found that in addition to cycloheximide, actino-

mycin D and ethionine stimulated uterine glycogen synthesis in fed ovariectomized animals. It is of interest that administration of ethionine to estradiol-treated animals resulted in further enhancement of uterine glycogen and none of the inhibitors of protein synthesis examined, prevented the estradiol-induced stimulation of glycogen synthesis. These investigators have suggested that the stimulation of uterine glycogenesis either by inhibitors of RNA and protein synthesis or by estradiol itself does not require prior stimulation of protein synthesis.

In essence, the primary mechanism of action of estradiol, whether on some aspect of metabolism or by direct regulation of the genetic control of protein biosynthesis has not yet been elucidated. Only when the site or sites of action of estrogen have been pinpointed, the mechanism involved in the regulation of uterine metabolism will be clearly understood.

B. INVOLVEMENT OF CYCLIC AMP IN HORMONE ACTION

Hormones play a crucial role in maintaining dynamic homeostasis in a way that a certain balance between the various cells and tissues is achieved and that the organism survives in an environment which, were it not for hormones, may have been extremely unfavourable for the continued existence of that organism (54). Previous investigations have demonstrated that estrogens are capable of exerting a variety of biochemical and physiological effects on the uterus (46-48,55). One can raise a very important fundamental question, namely, how are these

effects of estrogens propagated in the target organ? Although different hormones may act by a variety of different mechanisms, increasing evidence indicates that many of them act by a "two-messenger system". According to this concept, the first messenger, the hormones themselves, travel from their cells of origin to the cells of their target tissues to stimulate therein the formation of a second messenger. It is this second messenger which then does the real work of the hormone and orders the affected cell to perform whatever function seems to be required at that particular time and place (54,56-58). The first such second messenger to have been identified and indeed the only one to have been identified to date (perhaps with the exception of cyclic guanosine monophosphate) is adenosine 3',5'-monophosphate (cyclic AMP) (54).

(a) Cyclic 3',5'-Adenosine Monophosphate

Adenosine 3',5'-phosphate was discovered in 1957 by two separate groups of investigators. Cook, Lipkin and Markham (59) were studying the hydrolysis of adenosine triphosphate in the presence of barium hydroxide and found several products besides the two major products (adenylic acid and inorganic pyrophosphate), one of these being the cyclic AMP (60,61). At about the same time, a heat stable factor, the accumulation of which was increased by epinephrine and glucagon (62-64) was isolated from hepatic tissue and crystallized by Sutherland and Rall (64,65). Both groups demonstrated that the compound had an adenine, ribose, phosphate ratio of 1:1:1 and contained no monoesterified

phosphate. Cyclic AMP is a mononucleotide of adenylic acid with the phosphate groups attached to carbons 3' and 5' of the ribose moiety. The ultraviolet spectrum and the molar extinction coefficient of cyclic 3',5'-AMP are essentially the same as those of adenosine 5'-monophosphate. The compound is very stable chemically and may be boiled at neutral or slightly acid or alkaline pH for one half hour or more with no appreciable loss of activity (64). Studies on the structure of cyclic AMP by means of X-ray diffraction revealed two forms with glycosidic bond angles different from that of 5'-AMP (66).

(b) Derivatives of Cyclic AMP

The synthesis of a series of acyl derivatives of cyclic AMP substituted at the 6-amino and 2'-O positions was described by Falbriard et al (67). All of these derivatives were found to be more active than the parent compound in stimulating the activation of phosphorylase in liver slices. These derivatives, particularly the dibutyryl analogue, have been used successfully in a number of recent investigations. In several cases, such as the parotid gland (68) and adipose tissue (69), dibutyryl cyclic AMP was found to be effective under conditions in which cyclic AMP proved to be virtually inactive. Although the exact reason for the greater potency of dibutyryl cyclic AMP is not known, it is generally believed that this analogue is more resistant to the action of phosphodiesterase, is more lipid soluble and penetrates the cell membrane much more readily than the parent compound.

(c) Adenyl Cyclase

The level of cyclic AMP at any given moment depends upon the activity of at least two enzymes. Adenyl cyclase catalyses the formation of cyclic AMP from ATP in a reaction which requires magnesium as a co-factor (70). In addition, cyclic AMP is inactivated by a specific phosphodiesterase which catalyses its hydrolysis to 5'-AMP (71). It has been shown that in liver, epinephrine and glucagon act by increasing adenyl cyclase without affecting the activity of phosphodiesterase (72). In the case of liver, as in most animal tissues that have been investigated, adenyl cyclase appears to be associated with the plasma membrane. (73,74). However, studies on the subcellular distribution of this enzyme do not exclude the possibility that a fraction of the enzyme may also be associated with intracellular organelles. For example, the adenyl cyclase activity of brain was found in all particulate fractions (75) and a nerve ending fraction exhibited half the specific activity of the crude mitochondrial starting material (76). It is important to realize that fractions of cell homogenates sedimenting as nuclei, mitochondria or microsomes contain fragments of plasma membrane, although it is not inconceivable that the enzyme does occur in other intracellular structures in heart (77), kidney (78), adrenal (79), myometrium and mammary glands (78).

In addition to epinephrine and glucagon, a variety of hormones stimulate the activity of adenyl cyclase and thus increase the intracellular level of cyclic AMP. Adenyl cyclase has been shown to

decrease markedly in failing myocardium (80) and stimulation of activity immediately following the addition of thyroid hormone to heart homogenates has been reported (81). De novo synthesis of adipose tissue adeny cyclase in hyperthyroid rats has been reported (82) as well as an increased activity during fasting (83). Renal cortex cyclase activity (84) almost doubled after water deprivation and endogenous cyclic AMP was elevated to a greater degree in the cortex than in the medulla (85). These alterations were attributed to the action of endogenous vasopressin which is released during water deprivation.

(d) Cyclic Nucleotide Phosphodiesterase

The other enzyme which influences the level of cyclic AMP is a relatively specific phosphodiesterase which catalyses the hydrolysis of cyclic AMP to 5'-AMP (71). This enzyme is more widely distributed in cells than is adeny cyclase, being partly particulate and partly soluble (71,76). Cheung (86) found that brain phosphodiesterase was inhibited by ATP and pyrophosphate and was also subject to inhibition by citrate. The inhibition of phosphodiesterase by these agents seemed to be of a mixed type in contrast to the competitive inhibition produced by methylxanthines (71,87). Of the methyl xanthines examined, theophylline was found to be the most potent inhibitor of phosphodiesterase (71). In addition to the methyl xanthines, several other drugs such as puromycin (88) and certain benzothiadiazines (89-91) have been shown to inhibit the activity of cyclic nucleotide phos-

phodiesterase. It is probable that a number of effects of these drugs are related to this action although it should be emphasized that these agents have numerous other actions which are not related to phosphodiesterase inhibition. An interesting example of this is the ability of theophylline to inhibit protein synthesis from the adrenal gland (92). Mandel and Kuehl (93) have shown that high concentrations of triiodothyronine are also capable of inhibiting phosphodiesterase activity in several tissues.

Imidazole has been found to stimulate the activity of phosphodiesterase from brain (94), liver (95), adipose tissue (96) and heart (71). Imidazole antagonised the serum calcium response to dibutyryl cyclic AMP in parathyroidectomized rats (97) and reduced the lipolytic response of isolated adipose tissue to cyclic AMP (98). That imidazole stimulates phosphodiesterase and thereby lowers the level of cyclic AMP is suggested (98), although the intracellular concentration of imidazole is very low in comparison to the concentration required to stimulate the enzyme. In addition to imidazole, other agents such as nicotinic acid (99) and insulin (100) have been reported to stimulate the activity of phosphodiesterase.

(e) Cyclic AMP and Hormone Action

The hypothesis that cyclic AMP might be an intracellular mediator of many hormonal effects has received considerable support during the last ten years. Once elevated, cyclic AMP mimics many of the actions ascribed to the hormone which stimulated its synthesis.

Hormones known to exert some of their effects by changing the level of cyclic AMP in cells of their target tissues include, in addition to epinephrine and glucagon, adrenocorticotrophic hormone (101-103), vasopressin (104), luteinizing hormone (105,106), thyroid stimulating hormone (103,107), serotonin (108,109), histamine (110) and prostaglandins (111,112).

Adenyl cyclase is activated by different hormones in different tissues and is believed to function both as a discriminator for environmental signals and as a signal generator (58). The reasons for the hormonal specificity of adenyl cyclase are unknown, but it would appear that the molecular configuration of at least one part of the adenyl cyclase system must differ from one tissue to another (72). By this mechanism, the initial intercellular signal, represented by the first messenger, is changed to a signal (the second messenger) which is capable of acting within the cell. This signal may undergo amplification in a variety of ways with the ultimate response being dependent upon the enzymatic profile of the type of cell involved. Although the nature of these effects depends on the target tissue, they are the same in each case as those specifically produced by the hormone (58). Metabolic responses which have been shown to be influenced by cyclic AMP include steroidogenesis (101-106, 113), glucose oxidation (107,114), gluconeogenesis (115,116), ketogenesis (117), lipolysis (118,119) and release of insulin (120). It has been known for many years that the hormonal activation of hepatic phosphorylase is mediated by cyclic AMP (121). The control of the activity of

glycogen synthesis also appears to be dependent upon the intracellular level of cyclic AMP (122). Likewise, phosphofructokinase has been shown by Mansour to be influenced by changes in the concentration of this cyclic nucleotide (108). Although cyclic AMP is known to activate a variety of enzymes, any definitive evidence to suggest that it exerts its regulatory influence by an allosteric mechanism is still lacking.

In general, three different approaches have been used for studying the possible involvement of cyclic AMP in hormone action (54,58). First the specificity of washed broken cell preparations of the adenylyl cyclase system of the tissue is compared with the known specificities of the hormone on the "physiological" or ultimate response of the tissue. The second approach involves the simultaneous measurement of the intracellular concentration of cyclic AMP in intact cell preparations and the physiological response produced by the hormone. While these two approaches can satisfactorily eliminate the involvement of cyclic AMP from the action of a given hormone, they cannot prove the role of cyclic AMP unequivocally, because it is conceivable that the changes in cyclic AMP levels and the ultimate response are not directly related but are only coincidental. Consequently, a third approach has been used in which the ability of exogenously administered cyclic AMP to reproduce the physiological and biochemical effects of the hormone is examined. This has been accomplished in several cases, e.g. the effects of glucagon and

epinephrine on liver, the steroidogenic effects of several hormones on their endocrine target organs, the permeability changes of the toad bladder produced by vasopressin, and other hormone actions have been mimicked by cyclic AMP at high concentrations (54,58). It should be pointed out that while none of these approaches alone can clearly implicate cyclic AMP in hormone action, positive findings in all cases would provide compelling evidence for the involvement of this cyclic nucleotide.

Despite the fact that cyclic AMP may be a second messenger in the action of a variety of hormones, little work has been carried out on the possible involvement of this nucleotide in the action of steroid hormones. Hechter et al (123) have suggested that cyclic AMP may be involved in the action of estrogens on the uterus. Cyclic AMP was found to increase uterine RNA and protein synthesis in isolated uterine horns and led to a wide-spread stimulation of biosynthetic processes involving glycogen and all classes of lipids. The stimulatory effects of cyclic AMP were inhibited but not abolished by actinomycin and thus appeared to be dependent upon stimulation of new RNA synthesis (123). These investigators also presented evidence which eliminated the possibility that the cyclic nucleotide exerted the observed in vitro effects by serving as an energy source or RNA precursor in the system. Upon direct analysis of the uterus for endogenous cyclic AMP, Szego and Davis (124) found that the level of uterine cyclic AMP was markedly depleted following ovariectomy. It was further demonstrated that 15 seconds after an intravenous injection

of estradiol-17 β (1 μ g/100 g) to ovariectomized rats, uterine cyclic AMP was approximately doubled. More recently, Singhal et al (125) demonstrated that exogenous cyclic AMP produced testosterone-like induction of hexokinase, phosphofructokinase, pyruvate kinase and of glucose 6-phosphate dehydrogenase in the seminal vesicles of castrated and immature rats. The dibutyryl analog of cyclic AMP resulted in greater increases in vesicular enzymes than the parent compound and it was suggested that cyclic AMP may play the role of a second messenger in androgen action.

In the present investigation, an attempt has been made to examine the ability of exogenously administered cyclic AMP to produce stimulation of uterine glycogen and of several important carbohydrate-metabolizing enzymes known to be induced by estrogens (46-48,55). The data obtained lend support to the view that cyclic AMP may be involved in triggering the known metabolic effects of estrogens on the rat uterus.

III. MATERIALS AND METHODS

A. ANIMALS

Young female rats of the Wistar strain weighing either 45 to 50 g (immature) or 150 to 160 g (mature) maintained on Master Laboratory chow and water ad libitum were used throughout this study.

B. SURGICAL PROCEDURES

(a) Ovariectomy

Bilateral ovariectomies were performed in mature rats under light pentobarbital anesthesia (4 mg/100 g). The hair from the back was clipped and the area cleaned with zephiran. An incision about 1 cm long was made in the skin midway between the last rib and the knee about 1 cm lateral to the spinal muscles. A second incision was made through the muscle layer and into the peritoneal cavity. The ovary seen below the incision embedded in a mass of fat was withdrawn, separated from the fat and tied off with a silk ligature. It was then cut away and removed. The incision in the muscle was closed with one or more sutures and the skin closed with a wound slip. The ovary from the opposite side was similarly removed. The animals were returned to their respective cages and used only after a post-operative period of two weeks had elapsed.

(b) Adrenalectomy

About half of the ovariectomized animals also underwent

adrenalectomy according to the method described by Zarrow et al (126). A small cut was made through the muscle layer, taking care to avoid cutting the muscles around the vertebral column. The perirenal fat was grasped with forceps and carefully pulled through the opening. The fat below the gland was grasped with curved forceps and the adrenal was separated from the kidney with a second pair of forceps used to tear the fat beneath the gland. The excised gland was placed on a moist filter paper and carefully examined to make sure that the entire gland had been removed. The incision was closed with silk sutures and the animals were given a single intramuscular injection of deoxycorticosterone acetate (100 µg) suspended in oil. A period of two weeks was allowed for recovery.

C. SAMPLE PREPARATION

Rats were stunned, killed by decapitation and exsanguinated. The uteri were rapidly removed, dissected free of adhering fat and connective tissue, weighed on a Roller-Smith torsion balance (Federal Pacific Electric Co., Newark, N.J.) and placed immediately in beakers immersed in crushed ice. The pooled tissues were finely minced with scissors and 5% homogenates prepared in isotonic KCl, pH 7.4. Homogenization was effected with a chilled Potter-Elvehjem homogenizer (Fisher Scientific Co., Fairlawn, N.J.), fitted with a teflon plastic pestle turning at 700 r.p.m. for exactly 60 seconds. The homogenate was centrifuged at 100,000 X g at 5°C for 30 minutes in a refrigerated I.E.C. model B-60 centrifuge (International Equipment Co., Needham

Heights, Mass.). The supernatant fluids were decanted in glass vials which were kept immersed in crushed ice throughout the course of the experiment.

D. ENZYME DETERMINATIONS

Enzyme activities were assayed in the supernatant fluid under strictly linear kinetic conditions. All enzyme assays (excepting phosphofructokinase) were conducted at 340 m μ in a constant recording Unicam spectrophotometer model SP 800. (Unicam Instruments, Ltd., Cambridge, England), thermostated at 37°C. Changes in optical density were recorded for a period of at least 5 minutes and the final pH and temperature were checked at the termination of each assay. Enzyme activities were calculated as micromoles of substrate metabolized per hour per g of tissue times the weight of the organ as described by Singhal et al (47).

(a) Hexokinase

Hexokinase activity was measured according to the method of DiPietro and Weinhouse (127). The assay was based on the rate of formation of NADPH from NADP in a system coupled with glucose 6-phosphate dehydrogenase. The assay medium contained the following in a total volume of 2.5 ml at the designated final concentrations: glucose 6-phosphate dehydrogenase (1.43 μ g protein); glycyl-glycine buffer (pH 7.5), 50 mM; MgCl₂, 7.5 mM; ATP, 5 mM; NADP, 0.75 mM; cysteine, 2 mM and glucose, 0.5 mM. The reaction was initiated by

addition of the supernatant fluid corresponding to 10 mg wet weight of the tissue and changes in absorbance were recorded against a blank devoid of ATP and glucose.

(b) Phosphofructokinase

This enzyme was assayed according to a modification (128) of the procedure of Lea and Walker (129) based on determining the amount of triose phosphates (130) formed from fructose 1,6-diphosphate. The enzyme activity was determined at 37°C in a reaction mixture which contained the following components in a final volume of 1 ml: tris buffer (pH 7.4), 50 μ moles; $MgCl_2$, 2 μ moles; ATP, 2 μ moles; hydrazine, 55 μ moles; fructose 6-phosphate, 10 μ moles; aldolase, 1 μ g protein. The reaction was started by addition of the supernatant fluid equivalent to 20 mg wet weight of the tissue. The incubation was carried out for 0, 5, 10 and 15 minutes and at these time intervals, the reaction was stopped by the addition of 1 ml of ice-cold trichloroacetic acid (10%). The samples were filtered through Whatman No. 1 filter paper. 1.2 ml of 2N NaOH was added to the protein free supernatant and the amount of alkali-labile phosphate formed was measured according to the method of Fiske and Subbarow (131).

(c) Pyruvate Kinase

Pyruvate kinase activity was determined by measuring the disappearance of NADH in an assay system coupled with lactate dehydrogenase employing a modified procedure of Weber et al. (132).

The reaction mixture (final volume, 3.0 ml) contained the following components added in the given order: tris buffer (pH 7.4), 41.7 mM; $MgSO_4$, 6.25 mM; KCl, 25 mM; phosphoenolpyruvate, 5.3 mM; ADP, 1.3 mM; NADH, 0.22 mM; lactate dehydrogenase, 0.1 mg of protein. The reaction was initiated by adding an appropriate dilution of the supernatant fluid and the changes in optical density were recorded against a blank which contained no phosphoenolpyruvate.

(d) Glucose 6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase

The activities of these hexose monophosphate shunt enzymes were assayed according to the differential method of Glock and McLean (133) which is based on the reduction of NADP to NADPH. The assay medium contained the following in a total volume of 2.5 ml at the designated final concentrations: glycyl-glycine buffer (pH 7.5), 50 mM; $MgCl_2$, 7.5 mM; D-glucose 6-phosphate, 2 mM; 6-phosphogluconic acid, 2 mM. In the presence of 6-phosphogluconic acid and glucose 6-phosphate as substrates, the activity measured represented the sum of both enzymes. The activity of 6-phosphogluconate dehydrogenase was obtained using 6-phosphogluconic acid as the sole substrate and glucose 6-phosphate dehydrogenase activity was then calculated as the difference. The reaction was initiated by the addition of the supernatant fluid corresponding to 10 mg wet weight of the tissue and changes in absorbance were recorded against blanks devoid of the appropriate substrate.

(e) α -Glycerophosphate Dehydrogenase

The activity of α -glycerophosphate dehydrogenase was assayed by measuring the rate of disappearance of NADH in an assay system coupled with aldolase according to the procedure of Freedland (134). The following components were added to the reaction mixture (final volume, 3.0 ml) in the given order of addition: tris buffer (pH 7.4), 33.3 mM; NADH, 0.22 mM; aldolase, 50 μ g of protein; supernatant fluid corresponding to 25 mg wet weight of the tissue. The reaction was started by adding D-fructose 1,6-diphosphate (36 mM) and changes in optical density were recorded against a blank which contained no substrate.

(f) Isocitrate Dehydrogenase

Isocitrate dehydrogenase activity was measured according to the method of Ochoa (135) which is based on the formation of NADPH from NADP. The assay medium contained the following in a total volume of 3.0 ml: glycyl-glycine (pH 7.4), 25 mM; $MgCl_2$, 0.6 mM; NADP, 0.045 mM; DL-isocitrate, 0.6 mM. The reaction was started by adding the supernatant fluid equivalent to 2.5 mg wet weight of the tissue and changes in optical density were recorded against a blank containing no isocitrate.

E. GLYCOGEN ESTIMATION

The uteri were rapidly excised, weighed, and placed immediately in 1 ml of boiling 30% KOH. After the disintegration of the tissue in KOH, glycogen was purified by adding 0.05 ml of saturated Na_2SO_4

and 1.5 ml of 95% ethanol. The precipitate was digested in a water bath (70°C) for 10 minutes and the tubes were centrifuged at 4,000 r.p.m. for 30 minutes. The supernatant was discarded and the precipitate redissolved in water and made up to a known volume. A 2.0 ml aliquot was used to determine the glycogen content according to the anthrone method of Seifter et al (136). Uterine glycogen was calculated as µg per g of tissue and expressed as total glycogen per organ.

F. CHEMICALS

All reagents were of the purest grade available and were dissolved in glass distilled water unless stated otherwise. Glucose 6-phosphate dehydrogenase, NAD and NADH were obtained from Boehringer Chemical Co. (London). Cyclic AMP was purchased from Calbiochem (Los Angeles, Calif.). Glycogen was obtained from Nutritional Biochemicals (Cleveland, Ohio). Adenosine, ADP, ATP, AMP, 2',3'-AMP, 2'-AMP, 3'-AMP, 3',5'-Cyclic AMP, fructose 6-phosphate, D-fructose 1,6-diphosphate, phosphoenolpyruvate, 6-phosphogluconic acid, NADP, DL-isocitrate, aldolase, lactic dehydrogenase, estradiol-17β and theophylline were purchased from the Sigma Chemical Co. (St. Louis, Mo.). N⁶-O^{2'} dibutyryl cyclic AMP was kindly donated by Dr. Nelboek of Boehringer Mannheim, West Germany. Cycloheximide was a gift from Dr. Babcock (Upjohn Co., Kalamazoo) and actinomycin D was obtained from Merck, Sharp and Dohme (Rahway, N.J.).

Cyclic AMP was dissolved in 0.9% NaCl, brought to pH 7.4 by adding 2% Na₂CO₃ and injected intraperitoneally. Theophylline, dibutyryl cyclic AMP, actinomycin D and cycloheximide were also dissolved in physiological saline. Estradiol-17 β was dissolved in ethanolic-0.9% NaCl solution and injected intramuscularly. Control animals were injected with the appropriate vehicle solution.

G. STATISTICAL ANALYSIS

The results were subjected to statistical evaluation and significant differences between the means (calculated as p values) are shown. No statistical significance is indicated when the p value was >0.05 .

IV. RESULTS

A. EFFECTS OF CYCLIC AMP ON UTERINE METABOLISM IN IMMATURE RATS

The data on the effects of cyclic AMP, 5'-AMP and theophylline on uterine weights and glycogen content are presented in Table I. Whereas 5'-AMP failed to exert any appreciable effect on these parameters, administration of cyclic AMP increased organ weights to 157% and uterine glycogen to 259% of the control values. Greater increases were observed when theophylline was injected concurrently with cyclic AMP. Thus, treatment with cyclic AMP and theophylline enhanced uterine weights to 200% and glycogen content to 429% of the values observed in control rats. It is of interest that theophylline itself, increased the uterine weight and glycogen to 150% and 135%, respectively, when compared with the control values.

Table II demonstrates that both theophylline and cyclic AMP also stimulated the activities of uterine hexokinase, phosphofructokinase and pyruvate kinase, the three key glycolytic enzymes as well as glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, the two enzymes involved in the hexose monophosphate shunt pathway of carbohydrate metabolism. When cyclic AMP and theophylline were given together, greater increases were observed in all uterine enzymes investigated; hexokinase increased to 262%, phosphofructokinase to 339%, pyruvate kinase to 247%, glucose 6-phosphate dehydrogenase to 281% and 6-phosphogluconate dehydrogenase to 291% of the control values. Treatment with 5'-AMP, on the other hand, produced no

TABLE I

EFFECTS OF CYCLIC AMP, 5' AMP AND THEOPHYLLINE ON UTERINE WEIGHTS
AND GLYCOGEN CONTENT IN IMMATURE RATS

Means \pm S.E. represent at least 4 animals in each group. Cyclic AMP and 5' AMP were injected at a dose of 10 mg/rat intraperitoneally, in 2 equally divided doses at 8 hour intervals. Theophylline (10 mg/rat) was also given intraperitoneally in 2 equal doses concurrently with cyclic AMP. All animals were sacrificed 16 hours following initiation of the treatment. The data are also given in percentages (in parentheses) taking the values of control animals as 100%.

TREATMENT	UTERINE WEIGHT (mg)	GLYCOGEN (μ g)
CONTROL	28 \pm 1 (100)	29.4 \pm 1.9 (100)
5' AMP	31 \pm 1 (110)	33.1 \pm 2.3 (113)
CYCLIC AMP	44 \pm 2 (157)*	76.3 \pm 4.0 (259)*
THEOPHYLLINE	42 \pm 1 (150)*	39.7 \pm 0.1 (135)*
CYCLIC AMP + THEOPHYLLINE	56 \pm 1 (200)*	126.4 \pm 6.3 (429)*

*Statistically significant difference when compared with the values of control rats ($p = <0.05$).

TABLE II

EFFECTS OF CYCLIC AMP, 5' AMP AND THEOPHYLLINE ON UTERINE GLYCOLYTIC AND HEXOSE MONOPHOSPHATE SHUNT ENZYMES IN IMMATURE RATS

Each value is the mean \pm S.E. of 3 determinations of enzyme activity in uteri pooled from 5-7 rats. Cyclic AMP and 5' AMP were injected at a dose of 10 mg/rat intraperitoneally in 2 equally divided doses at 8 hour intervals. Theophylline (10 mg/rat) was also given intraperitoneally in 2 equal doses concurrently with cyclic AMP. All animals were sacrificed 16 hours following initiation of the treatment. The data are also given in percentages (in parentheses) taking the values of control animals as 100%.

TREATMENT	HK	PFK	PK	G6-PDH	6-PGDH
CONTROL	3.4 \pm 0.3 (100)	3.3 \pm 0.1 (100)	72.2 \pm 5.1 (100)	3.7 \pm 0.2 (100)	1.1 \pm 0.1 (100)
5' AMP	3.1 \pm 0.1 (91)	3.6 \pm 0.1 (109)	76.6 \pm 4.2 (105)	3.4 \pm 0.1 (92)	1.2 \pm 0.1 (109)
CYCLIC AMP	7.7 \pm 0.4 (226)*	6.6 \pm 0.1 (200)*	113.8 \pm 7.6 (158)*	8.7 \pm 0.2 (285)*	2.4 \pm 0.1 (218)*
THEOPHYLLINE	6.2 \pm 0.5 (182)*	6.2 \pm 0.2 (176)*	111.5 \pm 4.8 (155)*	6.8 \pm 0.5 (183)*	2.1 \pm 0.3 (191)*
CYCLIC AMP + THEOPHYLLINE	8.9 \pm 0.8 (262)*	11.2 \pm 0.4 (339)*	178.0 \pm 6.3 (247)*	10.4 \pm 0.5 (281)*	3.2 \pm 0.1 (291)*

*Statistically significant difference when compared with the values of control rats ($p = <0.05$).

The following abbreviations are used: HK, hexokinase; PFK, phospho-fructokinase; PK, pyruvate kinase; G6-PDH, glucose 6-phosphate dehydrogenase; 6-PGDH, 6-phosphogluconate dehydrogenase.

significant change in any of these enzymes.

B. TIME-COURSE OF CYCLIC AMP-INDUCED CHANGES

In order to follow the time-course of cyclic AMP-stimulated changes in uterine metabolism, sequential changes were studied for a period of 24 hours in immature rats following treatment with cyclic AMP and theophylline. Fig. 1 demonstrates that uterine weights increased respectively, to 113%, 141%, 155%, 189%, 200% and 185% of the control values at 1, 2, 4, 8, 16 and 24 hours following the administration of cyclic AMP and theophylline. More pronounced increases were observed in uterine glycogen which rose to 122%, 159%, 199%, 339%, 558% and 341% of the control values, after 1, 2, 4, 8, 16 and 24 hours. The results presented in Fig. 2 illustrate the time-course of cyclic AMP and theophylline-induced increases in the activities of glycolytic and hexose monophosphate shunt enzymes in uteri of immature rats. All enzymes increased significantly following the administration of cyclic AMP and theophylline as early as 1 hour when hexokinase was 159%, phosphofructokinase 161%, pyruvate kinase 157%, glucose 6-phosphate dehydrogenase 160% and 6-phosphogluconate dehydrogenase was 173% of the control values. Uterine enzymes increased progressively at 2, 4 and 8 hours and attained maximal values 16 hours after the beginning of cyclic AMP treatment. Uterine hexokinase was elevated to 262%, phosphofructokinase to 339%, pyruvate kinase to 247%, glucose 6-phosphate dehydrogenase to 281% and 6-phosphogluconate dehydrogenase

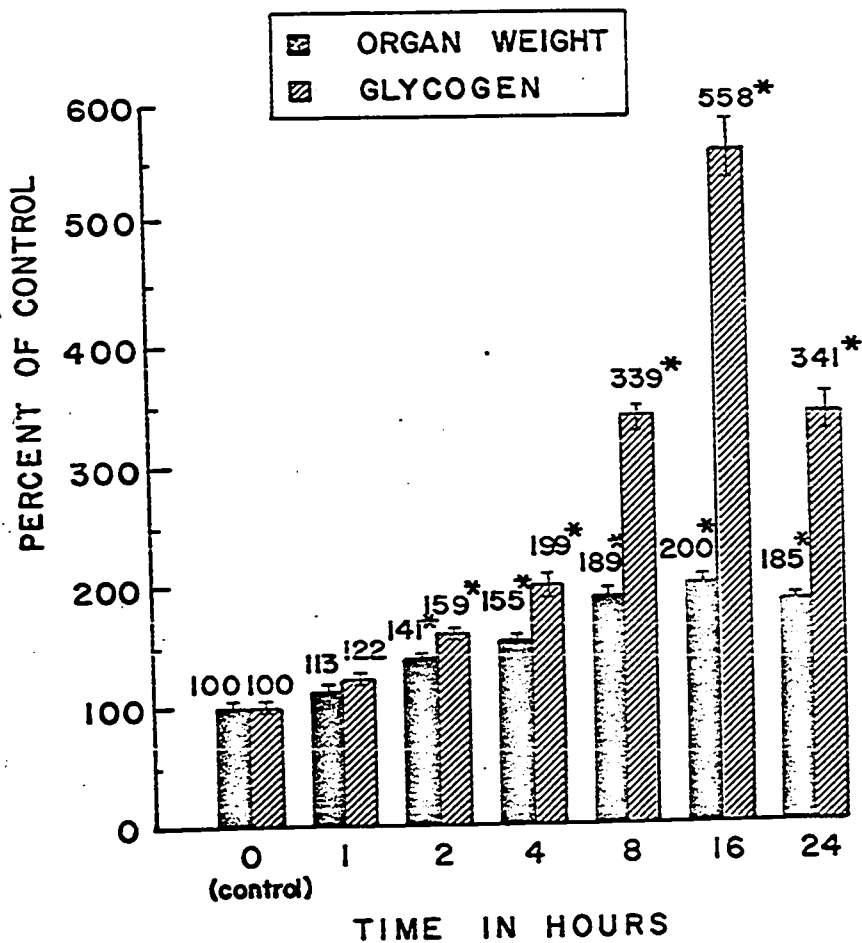


Fig. 1. Time-course of cyclic AMP and theophylline-induced increases in uterine weight and glycogen concentration. Each bar represents the mean \pm S.E. of 4 animals. Immature rats were injected simultaneously with cyclic AMP (10 mg/rat) and theophylline (10 mg/rat) by the intraperitoneal route, in 2 equally divided doses at predetermined intervals and then sacrificed after 1, 2, 4, 8, 16 and 24 hours. Data are given in percentages taking the values of control animals as 100%. *Statistically significant difference when compared to the values of control rats ($p = <0.05$).

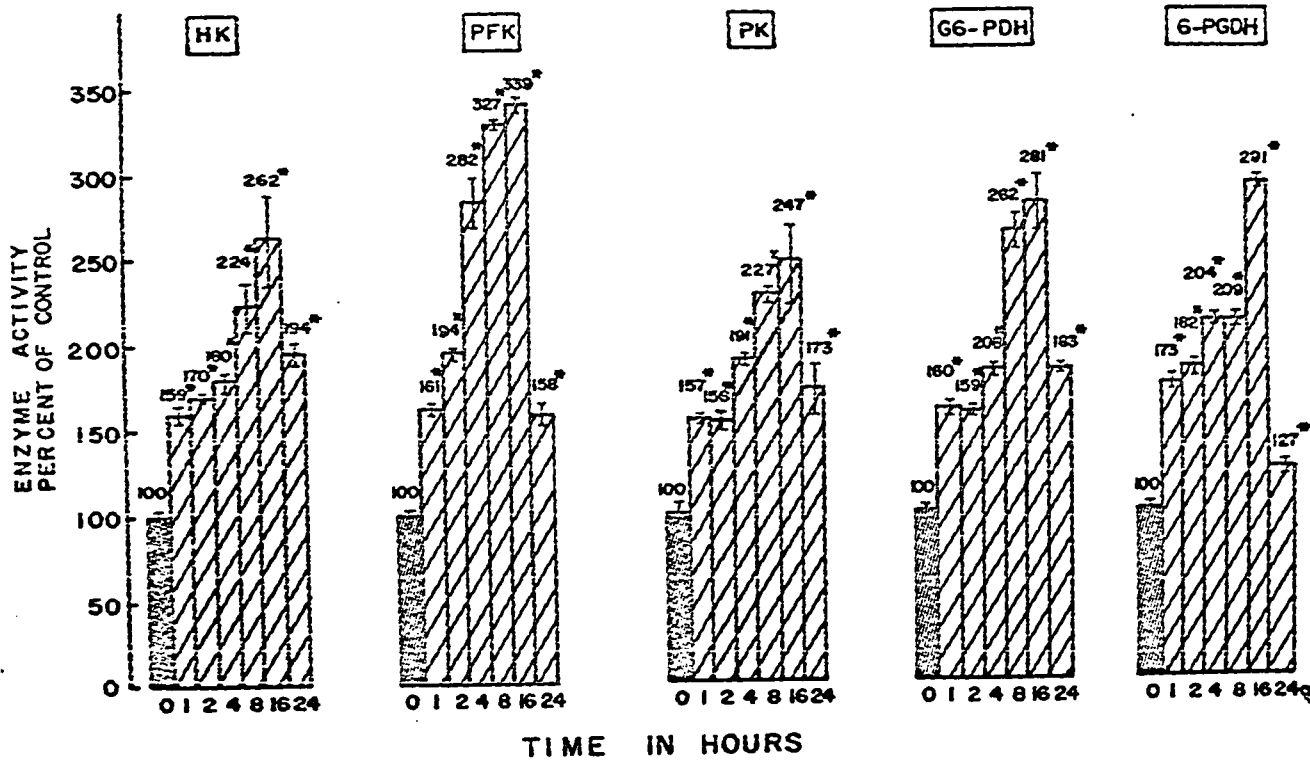


Fig. 2. Time-course of cyclic AMP and theophylline-induced stimulation of certain uterine enzymes. Each bar represents the mean \pm S.E. of 3 values each obtained by pooling uteri from 5-7 rats. Immature rats were injected with cyclic AMP (10 mg/rat) and theophylline (10 mg/rat) intraperitoneally, in two equally divided doses and sacrificed after 1, 2, 4, 8, 16 and 24 hours. The data are given in percentages taking the control values as 100%. The following abbreviations are used: HK, hexokinase; PFK, phosphofructokinase; PK, pyruvate kinase; G6-PDH, glucose 6-phosphate dehydrogenase; 6-PGDH, 6-phosphogluconate dehydrogenase. *Statistically significant difference when compared to the values of control animals ($p = <0.05$).

to 291% of controls 16 hours following the administration of cyclic AMP and theophylline.

C. INFLUENCE OF VARYING DOSES OF CYCLIC AMP

The data presented in Fig. 3 and Table III show that the observed cyclic AMP-induced changes in uterine enzymes as well as in the glycogen content were related to the dose of the cyclic nucleotide. When cyclic AMP was injected at a dose of 1 mg/rat, uterine weights increased to 154%, phosphofructokinase to 154%, pyruvate kinase to 183%, glucose 6-phosphate dehydrogenase to 217% and 6-phosphogluconate dehydrogenase to 191% of the control values. Increasing the dose to 2.5, 5.0, 10.0 and 15.0 mg/rat produced further enhancement of uterine enzymes; maximum increases were observed with the 15 mg dose when hexokinase increased to 290%, phosphofructokinase to 363%, pyruvate kinase to 306%, glucose 6-phosphate dehydrogenase to 354% and 6-phosphogluconate dehydrogenase to 395% of the values noted for control rats. Table III demonstrates the response of uterine glycogen to varying amounts of cyclic AMP. This parameter was more sensitive to the action of cyclic AMP than were the enzymes and as little as 0.2 mg of cyclic AMP stimulated uterine glycogen to 169% of the control values. The glycogen content was increased further with increasing dosages of cyclic AMP and maximal values were observed with the 10.0 mg dose (558%).

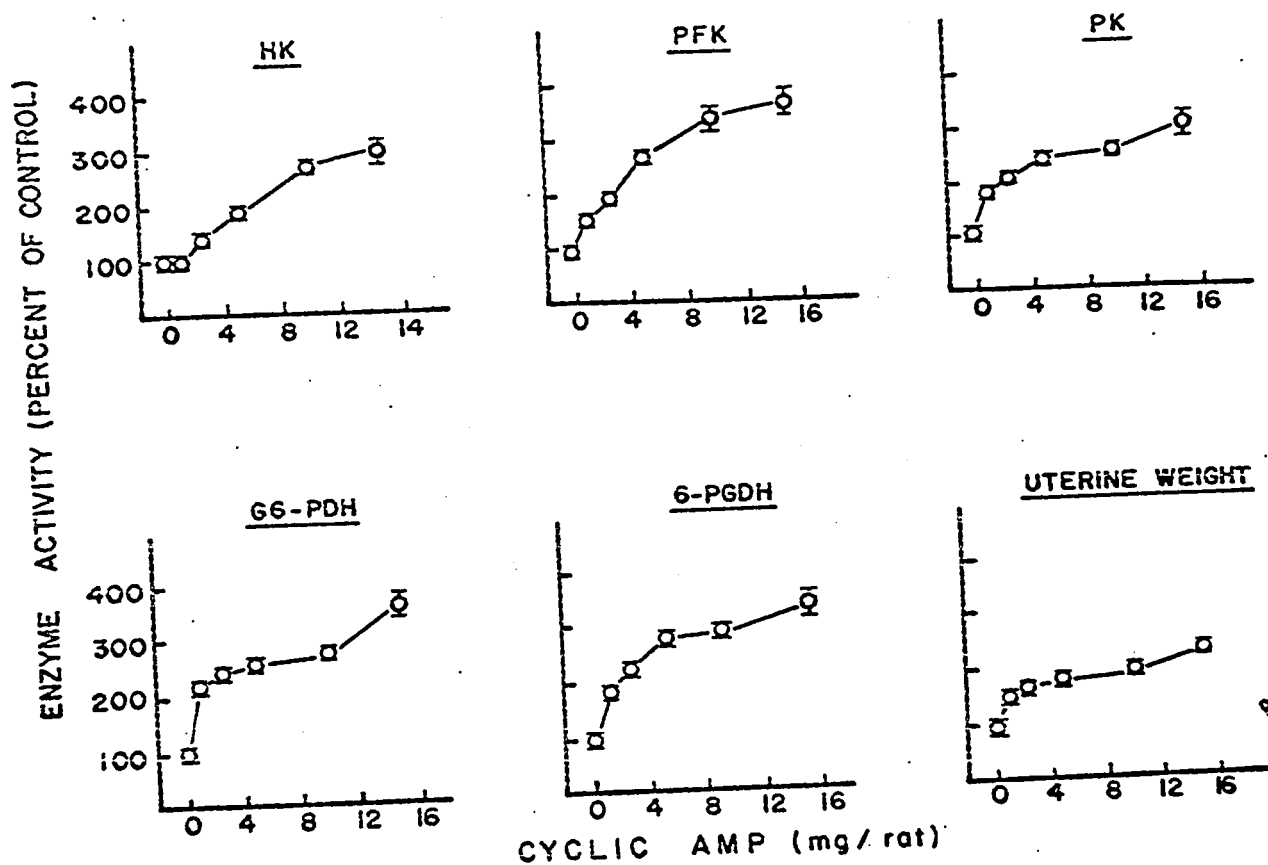


Fig. 3. Effect of varying doses of cyclic AMP on uterine glycolytic and hexosemonophosphate shunt enzymes. Each point represents the mean \pm S.E. of 3 values each obtained by pooling uteri from 5-7 immature rats. Varying doses of cyclic AMP (1-15 mg/rat) were administered along with theophylline (10 mg/rat) in 2 equally divided doses at 8 hour intervals. All animals were sacrificed 16 hours after the first injection of cyclic AMP and theophylline. Data are also given in percentages taking the values of control rats as 100%.

TABLE III

EFFECTS OF VARYING AMOUNTS OF CYCLIC AMP ON UTERINE GLYCOGEN CONTENT
IN IMMATURE RATS

Means \pm S.E. represent 4 animals in each group. Cyclic AMP (0.2-10.0 mg/rat) in varying doses was administered with theophylline (10 mg/rat) in 2 equally divided doses at 8 hour intervals. All rats were sacrificed 16 hours after the first injection. Data are also given in percentages taking the values of control rats as 100%.

DOSE OF CYCLIC AMP (mg/rat)	GLYCOGEN (μ g)	PERCENT OF CONTROL
0.0 (Control)	29.4 \pm 1.9	100
0.2	49.2 \pm 2.8	169*
1.0	76.4 \pm 4.7	262*
2.5	100.3 \pm 10.9	345*
5.0	116.6 \pm 2.5	403*
10.0	161.8 \pm 8.7	558*

*Statistically significant difference when compared with the values of control rats ($p = <0.05$).

D. EFFECTS OF ADENOSINE AND SEVERAL ADENINE NUCLEOTIDES

In order to examine whether the observed effects were specific to cyclic AMP, the influence of adenosine and several adenine nucleotides was investigated on uterine metabolism in immature rats. The results presented in Tables IV and V show that treatment with adenosine, 2',3'-cyclic AMP, 2'-AMP, 3'-AMP and 5'-ATP resulted in no significant effect on either the uterine weights and glycogen content or any of the enzymes investigated. Administration of 5'-ADP, although producing no change in glycolytic enzymes and glucose 6-phosphate dehydrogenase, increased slightly (127%) the activity of 6-phosphogluconate dehydrogenase. The results suggest that a degree of specificity exists in the action of cyclic AMP and demonstrate that of the compounds tested, only cyclic AMP was capable of triggering the observed metabolic responses in the uterus.

E. THEOPHYLLINE-INDUCED POTENTIATION OF ESTRADIOL ACTION

The methylxanthines such as theophylline would be expected to promote the accumulation of cyclic AMP by inhibiting the enzyme, phosphodiesterase. If cyclic AMP were to act as an intracellular mediator in the known effects of estrogens on the uterus, treatment with theophylline might be expected to enhance the influence of small doses of the hormone on uterine metabolism. The data presented in Table VI demonstrate the ability of theophylline to potentiate the action of a submaximal dose of estradiol-17 β on uterine glycogen

TABLE IV

EFFECT OF ADENOSINE AND SEVERAL ADENINE NUCLEOTIDES ON UTERINE WEIGHTS AND GLYCOGEN CONTENT IN IMMATURE RATS

Means \pm S.E. represent 3-4 rats in each group. Adenosine and various adenine nucleotides were injected at a dose of 10 mg/rat intraperitoneally, in 2 equally divided doses at 8 hour intervals and the animals were killed after 16 hours. Data are also given in percentages (in parentheses) taking the values of control rats as 100%.

TREATMENT	UTERINE WEIGHT (mg)	GLYCOGEN (μ g)
CONTROL	31 \pm 3 (100)	38.1 \pm 2.7 (100)
ADENOSINE	29 \pm 1 (93)	39.4 \pm 2.6 (103)
2',3' CYCLIC AMP	29 \pm 1 (94)	39.7 \pm 3.8 (104)
2' AMP	27 \pm 3 (87)	31.6 \pm 2.0 (83)
3' AMP	30 \pm 2 (97)	41.7 \pm 4.2 (109)
5' ADP	29 \pm 2 (93)	40.0 \pm 2.3 (105)
5' ATP	28 \pm 3 (90)	40.2 \pm 2.9 (105)

The following abbreviations have been used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

TABLE V
EFFECTS OF ADENOSINE AND SEVERAL ADENINE NUCLEOTIDES ON UTERINE GLYCOLYTIC AND HEXOSE MONOPHOSPHATE SHUNT ENZYMES IN IMMATURE RATS

Each value represents the mean \pm S.E. of 3 determinations of enzyme activity in uteri pooled from 5-7 immature rats. Adenosine and various adenine nucleotides were injected at a dose of 10 mg/rat intraperitoneally, in 2 equally divided doses at 8 hour intervals and the rats were killed after 16 hours. Data are also given in percentages (in parentheses) taking the values of control animals as 100%.

TREATMENT	HK	PFK	PK	G6-PDH	6-PGDH
CONTROL	3.4 \pm 0.3 (100)	3.3 \pm 0.1 (100)	72.2 \pm 5.1 (100)	3.5 \pm 0.3 (100)	1.1 \pm 0.1 (100)
ADENOSINE	3.6 \pm 0.2 (106)	3.5 \pm 0.2 (106)	82.6 \pm 5.0 (116)	3.7 \pm 0.1 (106)	1.3 \pm 0.2 (119)
2',3' CYCLIC AMP	3.8 \pm 0.1 (112)	3.4 \pm 0.1 (103)	81.2 \pm 15.3 (112)	4.0 \pm 0.4 (114)	1.3 \pm 0.1 (119)
2' AMP	3.7 \pm 0.2 (109)	3.6 \pm 0.5 (109)	85.6 \pm 12.3 (118)	3.8 \pm 0.1 (109)	1.4 \pm 0.3 (127)
3' AMP	3.4 \pm 0.1 (100)	3.7 \pm 0.1 (112)	89.4 \pm 7.0 (124)	3.6 \pm 0.3 (103)	1.2 \pm 0.1 (109)
5' ADP	3.7 \pm 0.1 (109)	3.9 \pm 0.4 (118)	89.0 \pm 4.5 (122)	3.7 \pm 0.1 (106)	1.4 \pm 0.0 (127)*
5' ATP	3.6 \pm 0.0 (106)	3.6 \pm 0.1 (109)	87.2 \pm 2.7 (121)	4.0 \pm 0.7 (114)	1.2 \pm 0.1 (109)

*Statistically significant difference when compared with the values of control rats ($p = <0.05$).

TABLE VI

THEOPHYLLINE-INDUCED POTENTIATION OF ESTRADIOL ACTION ON UTERINE
GLYCOGEN IN IMMATURE RATS

Means \pm S.E. represent 4 animals in each group. Groups of immature rats were injected with estradiol-17 β (0.1 μ g or 5.0 μ g/rat) intramuscularly, and killed after 16 hours. Theophylline (10 mg/rat) was injected by the intraperitoneal route in 2 equally divided doses at 8 hour intervals. Data are also given in percentages (in parentheses) taking the values of control animals as 100%.

TREATMENT	GLYCOGEN (μ g)	PERCENT OF CONTROL
CONTROL	34.6 \pm 4.5	100
THEOPHYLLINE	55.2 \pm 4.5	159*
ESTRADIOL-17 β (0.10 μ g)	55.9 \pm 5.6	160*
ESTRADIOL-17 β (5.0 μ g)	96.8 \pm 4.2	277*
ESTRADIOL-17 β (0.10 μ g) + THEOPHYLLINE	140.8 \pm 5.8	389*

*Statistically significant difference when compared with the values of control rats ($p = <0.05$).

in immature rats. Treatment with theophylline (10 mg) and estradiol (0.1 μ g) increased the level of uterine glycogen to 159% and 160%, respectively. However, when the same dose of estradiol (0.1 μ g) was injected concurrently with theophylline, the increase observed in uterine glycogen (389%) was of a much greater magnitude and was even higher than that produced by the 5.0 μ g dose of estradiol.

The potentiating effect of theophylline on estrogen-induced changes in uterine glycolytic and hexose monophosphate shunt enzymes is illustrated in Figs. 4 and 5. Administration of theophylline increased uterine weights to 150%, hexokinase to 182%, phosphofructokinase to 176%, pyruvate kinase to 155%, glucose 6-phosphate dehydrogenase to 183% and 6-phosphogluconate dehydrogenase to 191% of the control values. Likewise, estradiol (0.1 μ g) produced small, yet statistically significant increases in the activities of all enzymes investigated. However, when the same dose of estradiol was injected concurrently with theophylline, the increases produced in uterine glycolytic and hexose monophosphate shunt enzymes were much greater and were comparable to those observed with the high dose of estradiol (5.0 μ g). The data indicate that when a submaximal dose of estradiol (0.1 μ g) is administered along with theophylline, the increases produced in various uterine enzymes as well as in the glycogen content are of a magnitude similar to that elicited with the 5.0 μ g dose of the hormone.

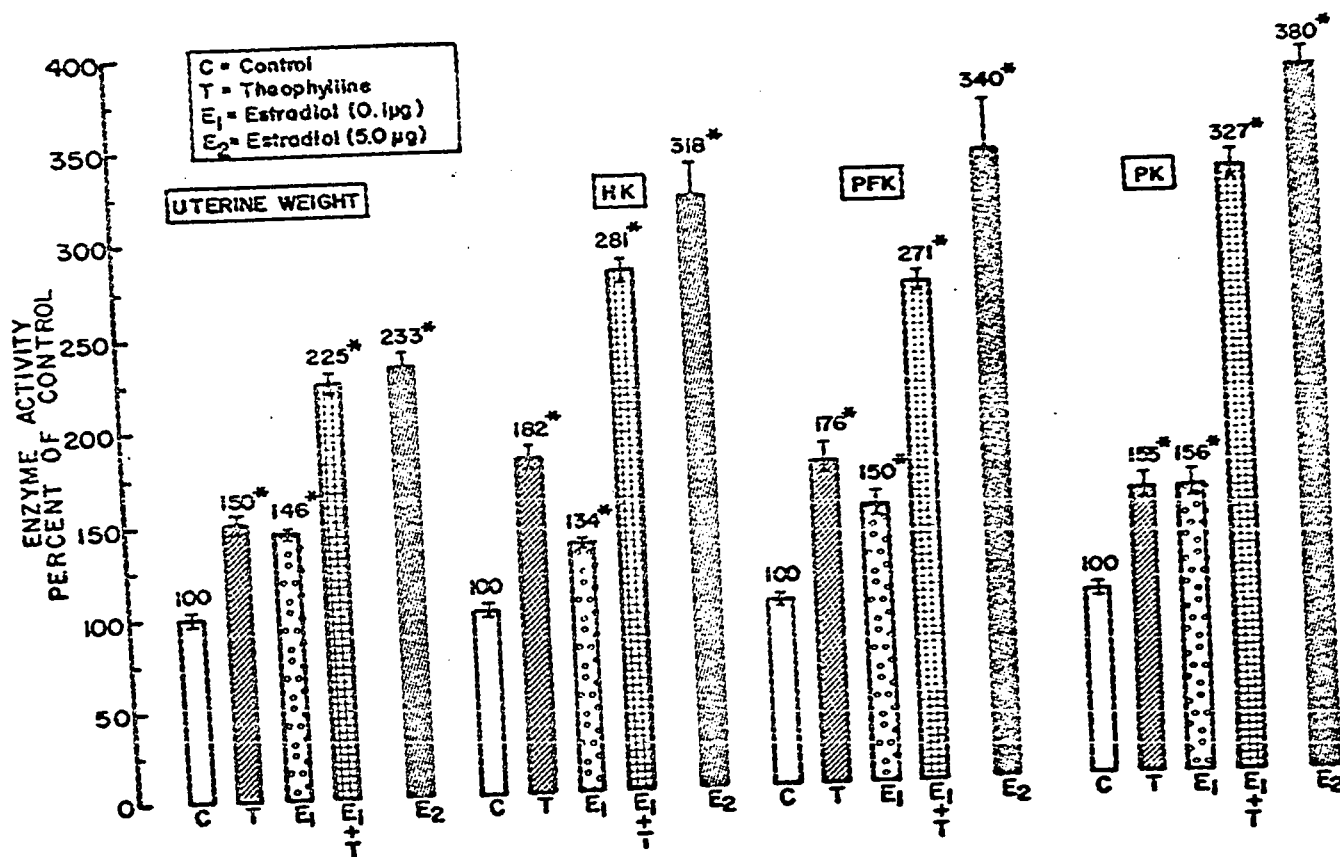


Fig. 4. Theophylline-induced potentiation of estradiol action on uterine glycolytic enzymes. Each bar represents the mean \pm S.E. of 3 values each obtained by pooling uteri from 5-7 rats. Immature rats were injected with estradiol-17 β (0.1 μ g or 5.0 μ g/rat) intramuscularly, and killed after 16 hours. Theophylline (10 mg/rat) was administered by the intraperitoneal route in 2 equally divided doses at 8 hour intervals. Data are given in percentages taking the values of control animals as 100%. *Statistically significant difference when compared to the values of control rats ($p < 0.05$).

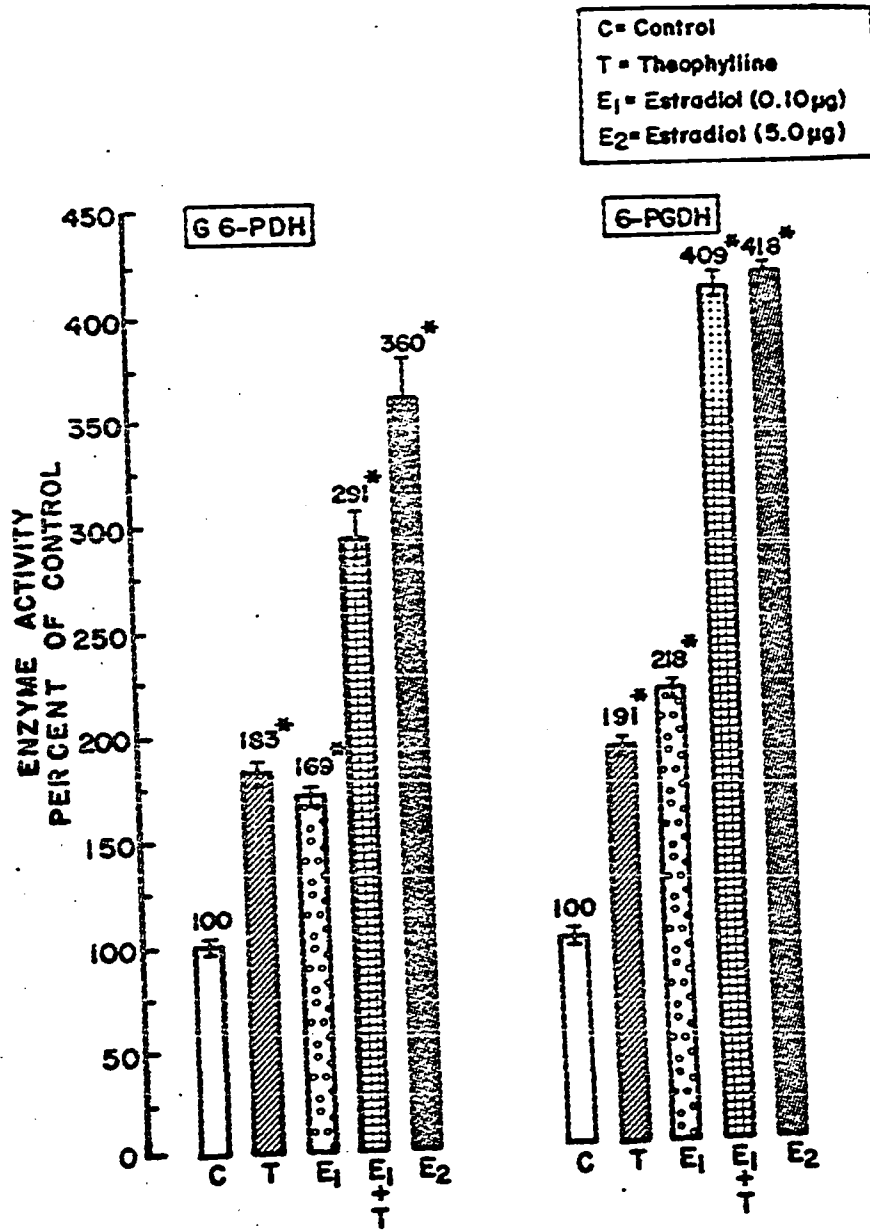


Fig. 5. Theophylline-induced potentiation of estradiol action on the two hexose monophosphate shunt dehydrogenases. Each bar represents the mean \pm S.E. of 3 values each obtained by pooling uteri from 5-7 immature rats. Experimental conditions were the same as described in Fig. 4. Data are given in percentages taking the values of control animals as 100%. *Statistically significant difference when compared with the control values ($p = <0.05$).

F. INFLUENCE OF CYCLIC AMP AND ESTRADIOL ON ISOCITRATE DEHYDROGENASE AND α -GLYCEROPHOSPHATE DEHYDROGENASE

The action of cyclic AMP on the activities of two other enzymes, isocitrate dehydrogenase and α -glycerophosphate dehydrogenase, was studied in order to examine whether the response produced for these enzymes was similar to that observed with estradiol. The results presented in Table VII demonstrate that estradiol-17 β in doses up to 5.0 μ g/rat induced no significant change in the activity of either of these enzymes. Also, neither theophylline nor cyclic AMP produced any appreciable increase in uterine isocitrate dehydrogenase or α -glycerophosphate dehydrogenase. When theophylline was given concurrently with cyclic AMP, both enzymes increased to approximately 133% of the control values although, the increase was statistically significant only in the case of α -glycerophosphate dehydrogenase. Simultaneous treatment with theophylline and estradiol also did not produce any measurable effect on either of the two enzymes. The results show that two of the uterine enzymes which do not respond to estradiol also fail to elicit any response to cyclic AMP and theophylline.

G. EFFECTS OF CYCLIC AMP IN OVARIECTOMIZED RATS

Since estradiol is known to stimulate uterine glycolytic and hexose monophosphate shunt enzymes in ovariectomized rats, it was of interest to investigate whether cyclic AMP also could produce a similar stimulation of these enzymes in uteri of ovariectomized animals. Table VIII shows that cyclic AMP and theophylline increased uterine

TABLE VII

INFLUENCE OF ESTRADIOL-17 β AND CYCLIC AMP ON UTERINE ISOCITRATE DEHYDROGENASE AND α -GLYCEROPHOSPHATE DEHYDROGENASE

Each value is the mean \pm S.E. of 3 enzyme determinations in uteri pooled from 5-7 rats. Groups of animals were injected either with estradiol-17 β (0.1-5.0 μ g/rat) or cyclic AMP (10 mg/rat in 2 doses) and sacrificed after 16 hours. Theophylline (10 mg/rat) was administered in 2 equally divided doses at 8 hour intervals concurrently with cyclic AMP or estradiol. Data are also given in percentages (in parentheses) taking the control values as 100%.

TREATMENT	ISOCITRATE DEHYDROGENASE	α -GLYCEROPHOSPHATE DEHYDROGENASE
CONTROL	10.4 \pm 0.7 (100)	3.0 \pm 0.3 (100)
ESTRADIOL-17 β (0.10 μ g)	9.8 \pm 0.3 (94)	2.4 \pm 0.3 (80)
ESTRADIOL-17 β (1.0 μ g)	11.0 \pm 0.4 (105)	2.5 \pm 0.2 (83)
ESTRADIOL-17 β (5.0 μ g)	13.0 \pm 0.6 (124)	2.6 \pm 0.4 (87)
THEOPHYLLINE	11.3 \pm 0.6 (108)	3.0 \pm 0.4 (100)
CYCLIC AMP	10.2 \pm 0.2 (99)	3.1 \pm 0.1 (103)
CYCLIC AMP + THEOPHYLLINE	13.7 \pm 1.0 (131)	4.0 \pm 0.1 (133)*
THEOPHYLLINE + ESTRADIOL-17 β (0.1 μ g)	11.7 \pm 0.3 (112)	2.5 \pm 0.5 (83)

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

TABLE VIII
 STIMULATION BY CYCLIC AMP AND THEOPHYLLINE OF UTERINE GLYCOLYTIC AND HEXOSE MONOPHOSPHATE SHUNT ENZYMES
 IN OVARECTOMIZED RATS

Each value is the mean \pm S.E. of 3 determinations of enzyme activity in uteri pooled from 3 rats. Cyclic AMP, 5' AMP and dibutyryl cyclic AMP were injected at a dose of 10 mg/rat intraperitoneally, in 2 equally divided doses at 8 hour intervals. Theophylline (10 mg/rat) was also given intraperitoneally in 2 equal doses concurrently with the cyclic nucleotide. All rats were sacrificed 16 hours following initiation of the treatment. Data are also given in percentages (in parentheses) taking the values of control rats as 100%.

TREATMENT	UTERINE WEIGHT (mg)	HK	PFK	PK	G6-PDH	6-PGDH
CONTROL	75 \pm 1 (100)	5.2 \pm 0.1 (100)	6.7 \pm 0.4 (100)	178.4 \pm 14.0 (100)	8.8 \pm 0.5 (100)	4.8 \pm 0.2 (100)
5' AMP	78 \pm 2 (104)	5.3 \pm 0.1 (102)	6.8 \pm 0.2 (101)	200.4 \pm 6.0 (112)	8.9 \pm 0.2 (101)	4.5 \pm 0.2 (94)
CYCLIC AMP	113 \pm 3 (151)*	7.1 \pm 0.2 (137)*	13.0 \pm 0.4 (190)*	340.8 \pm 7.8 (191)*	13.0 \pm 0.1 (148)*	7.5 \pm 0.3 (156)*
THEOPHYLLINE	109 \pm 2 (145)*	7.3 \pm 0.2 (140)*	12.3 \pm 1.0 (184)*	318.4 \pm 7.8 (180)*	12.6 \pm 0.2 (143)*	6.7 \pm 0.6 (138)*
CYCLIC AMP + THEOPHYLLINE	135 \pm 3 (180)*	9.3 \pm 1.5 (180)*	17.0 \pm 0.4 (254)*	342.4 \pm 28.0 (192)*	14.8 \pm 1.3 (171)*	10.4 \pm 0.2 (217)*
DIBUTYRYL CYCLIC AMP + THEOPHYLLINE	150 \pm 3 (200)*	12.5 \pm 0.1 (244)*	23.4 \pm 0.2 (349)*	470.3 \pm 25.4 (263)*	18.9 \pm 1.5 (215)*	12.9 \pm 0.7 (269)*

*Statistically significant difference when compared with the values of control rats ($p = <0.05$).

weight and hexokinase to 180%, phosphofructokinase to 254%, pyruvate kinase to 192%, glucose 6-phosphate dehydrogenase to 171% and 6-phosphogluconate dehydrogenase to 217% of the values observed in control rats. Whereas 5'-AMP exerted no significant effect, treatment with cyclic AMP or theophylline alone produced stimulation of uterine enzymes which was similar to that observed in immature animals. Administration of the N⁶-O^{2'} dibutyryl analogue of cyclic AMP along with theophylline resulted in much greater increases in uterine enzymes than those produced by cyclic AMP and theophylline and elevated hexokinase to 244%, phosphofructokinase to 349%, pyruvate kinase to 263%, glucose 6-phosphate dehydrogenase to 215% and 6-phosphogluconate dehydrogenase to 269% of the control values. It is of interest that whereas the combination of dibutyryl cyclic AMP and theophylline was well tolerated in mature ovariectomized rats, it produced seizure activity and death in all the immature animals.

H. EFFECTS OF INHIBITORS OF PROTEIN SYNTHESIS

Previous studies have shown that many of the biochemical and physiological responses induced by estrogens in the uterus of the ovariectomized rat are blocked by compounds which inhibit RNA and protein synthesis. The data presented in Fig. 6 show the effects of actinomycin D and cycloheximide on the cyclic AMP-induced stimulation of uterine glycolytic enzymes. When cyclic AMP and theophylline-treated ovariectomized rats were given either actinomycin D or cyclo-

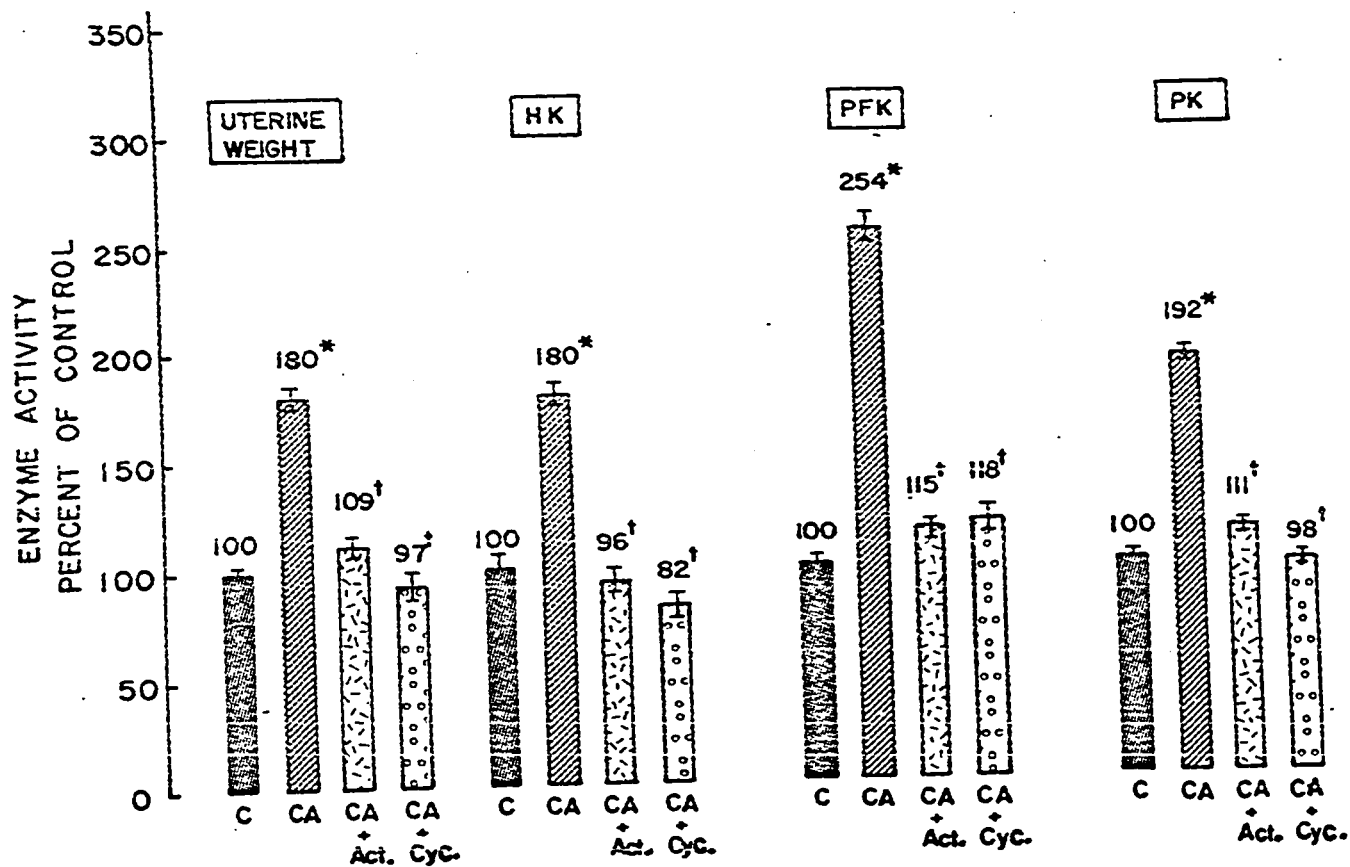


Fig. 6. Influence of actinomycin and cycloheximide on the cyclic AMP-induced increases in uterine glycolytic enzymes. Bars represent the mean \pm S.E. of 3 values each obtained by pooling uteri from 3 rats. Animals were injected with cyclic AMP (10 mg/rat) and theophylline (10 mg/rat) in 2 equally divided doses at 8 hour intervals and sacrificed after 16 hours. Actinomycin D (25 μ g/100 g) and cycloheximide (70 μ g/100 g) were given intraperitoneally, in two divided doses at 8 hour intervals 30 minutes prior to the injection of cyclic AMP. Data are given in percentages taking the values of control rats as 100%. The following abbreviations are used: C, control; CA, cyclic AMP and theophylline; Act, actinomycin D; Cyc, cycloheximide. *Statistically significant difference when compared with the values of control rats ($p = <0.05$). †Statistically significant difference when compared with the values of rats receiving cyclic AMP and theophylline ($p = <0.05$).

heximide, the increases observed in the activities of uterine hexokinase, phosphofructokinase and pyruvate kinase were completely inhibited and the values remained close to those noted for the control animals. Likewise, administration of these inhibitors completely blocked the cyclic AMP and theophylline-induced stimulation of uterine glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Fig. 7). The results indicate that new RNA and protein synthesis may be involved in the observed cyclic AMP-induced stimulation of uterine glycolytic and hexose monophosphate shunt enzymes.

I. INVOLVEMENT OF ADRENAL GLANDS

In order to examine the involvement of adrenal glands in the observed stimulation of uterine enzymes, the influence of cyclic AMP was investigated in adrenalectomized-ovariectomized rats. The data presented in Fig. 8 demonstrate that when cyclic AMP and theophylline were given concurrently to adrenalectomized-ovariectomized animals, uterine weights as well as the activities of hexokinase, phosphofructokinase and pyruvate kinase were elevated to the same extent as that observed in immature or ovariectomized rats. The administration of the dibutyryl analogue of cyclic AMP in conjunction with theophylline, produced greater increases in glycolytic enzymes than did the parent compound. The ability of the dibutyryl analogue to produce greater enzyme increases than cyclic AMP in adrenalectomized-ovariectomized rats was similar to that found in ovariectomized animals with the

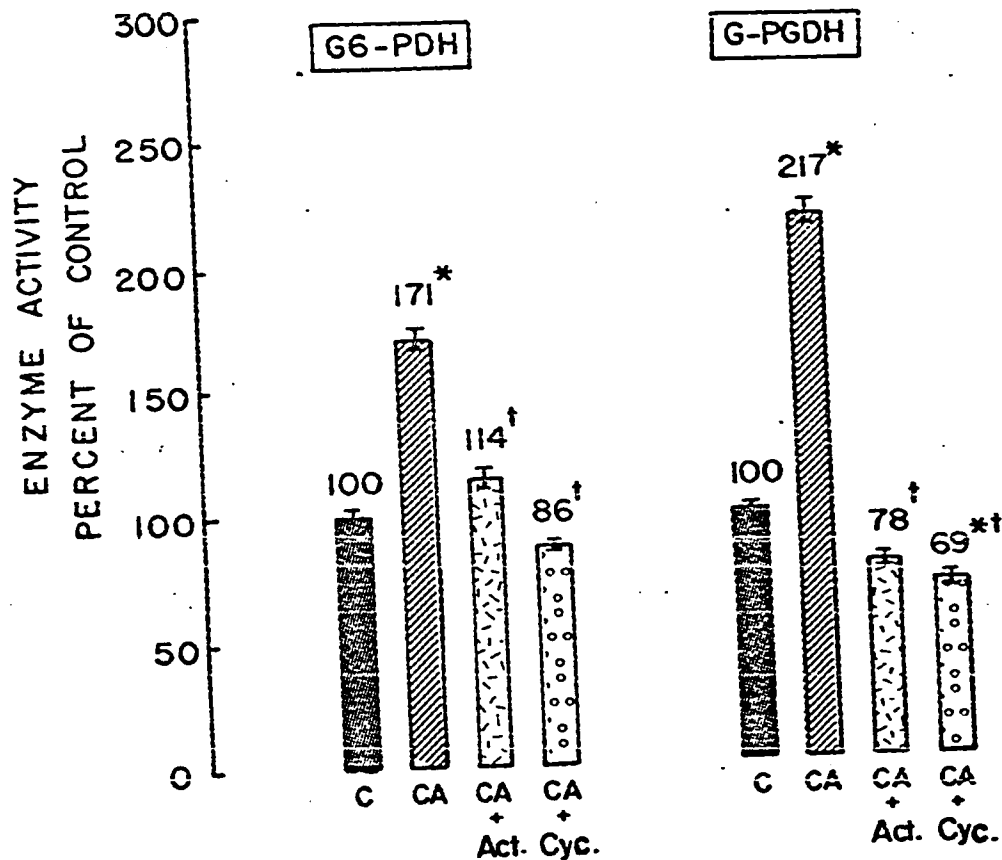


Fig. 7. Effect of actinomycin D and cycloheximide on cyclic AMP-induced increases in the two hexose monophosphate shunt dehydrogenases. Each bar represents the mean \pm S.E. of 3 values each obtained by pooling uteri from 3 ovariectomized rats. Experimental conditions were the same as described in Fig. 6. Data are given in percentages taking the values of control rats as 100%. *Statistically significant difference when compared with the values of control animals ($p = <0.05$). †Statistically significant difference when compared with the values of rats receiving cyclic AMP and theophylline ($p = <0.05$).

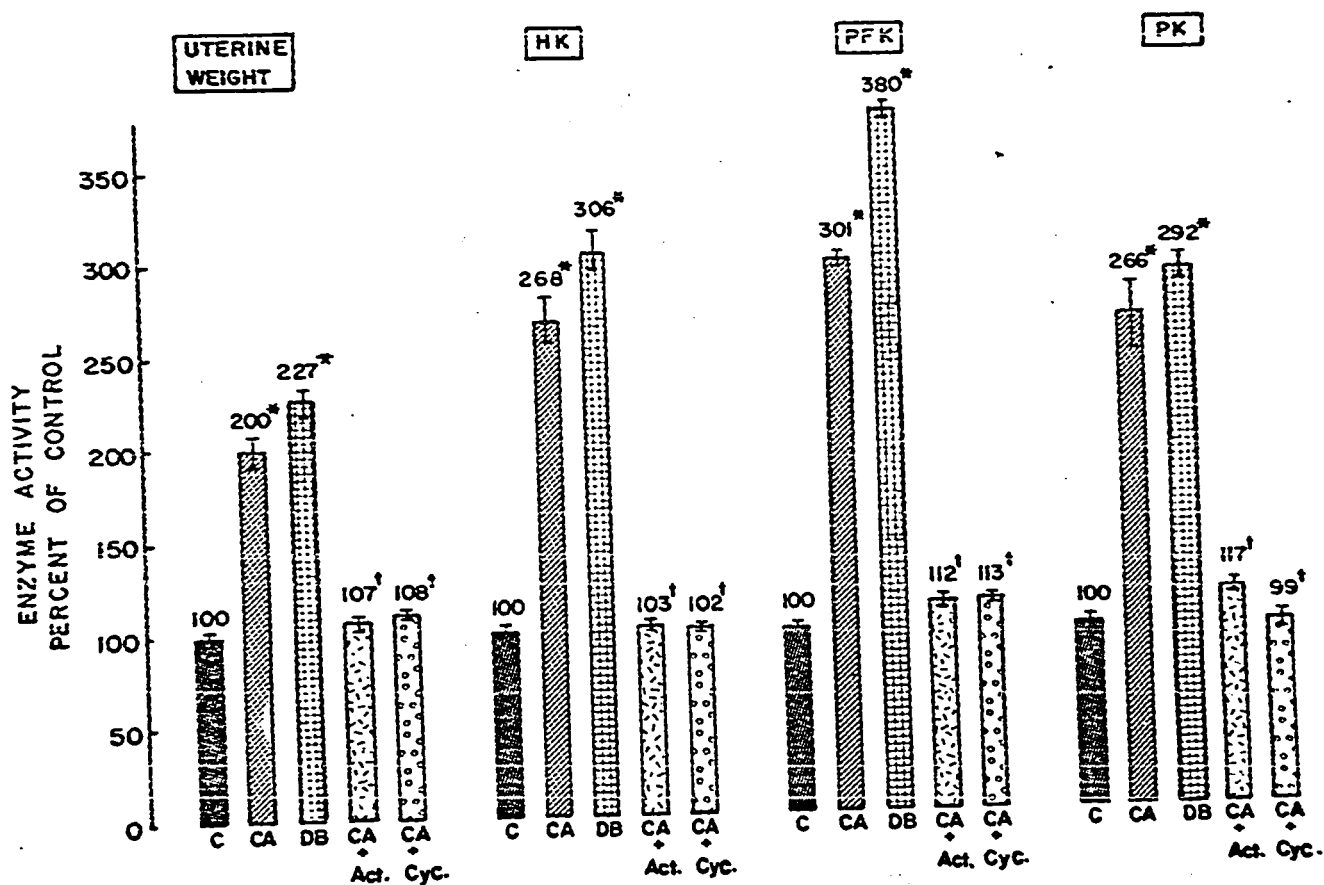


Fig. 8. Effect of cyclic AMP on uterine glycolytic enzymes in adrenal-ectomized-ovariectomized rats. Bars represent the mean \pm S.E. of 3 values each obtained by pooling uteri from 3 animals. Rats were injected with cyclic AMP (10 mg/rat) or dibutyryl cyclic AMP (10 mg/rat) in 2 equally divided doses at 8 hour intervals, and sacrificed after 16 hours. Theophylline (10 mg/rat) also was administered by the intraperitoneal route in 2 equally divided doses concurrently with the cyclic nucleotide. Actinomycin D (25 μ g/100 g) and cycloheximide (70 μ g/100 g) were given intraperitoneally, in two divided doses at 8 hour intervals 30 minutes before the injection of cyclic AMP. The data are given in percentages taking the control values as 100%. The following abbreviations are used: C, control; CA, cyclic AMP and theophylline; DB, dibutyryl cyclic AMP and theophylline; Act, actinomycin; Cyc, cycloheximide. *Statistically significant difference when compared with the values of control rats ($p = <0.05$). †Statistically significant difference when compared with the values of rats receiving cyclic AMP and theophylline ($p = <0.05$).

intact adrenals. Fig. 8 also demonstrates that when cyclic AMP and theophylline-treated adrenalectomized-ovariectomized rats were given either actinomycin D or cycloheximide, the observed enzyme increases were completely inhibited and the values remained near those of the control animals.

Table IX shows the influence of cyclic AMP and theophylline on the two hexose monophosphate shunt dehydrogenases in uteri of ovariectomized-adrenalectomized rats. Administration of cyclic AMP and theophylline elevated glucose 6-phosphate dehydrogenase to 387% and 6-phosphogluconate dehydrogenase to 256% of the control values. As expected, the dibutyryl analogue of cyclic AMP produced greater increases in the activity of both of these enzymes. Treatment with actinomycin D or cycloheximide resulted in complete inhibition of the cyclic AMP-induced increases in the activity of the two hexose monophosphate shunt dehydrogenases. Data suggest that neither the cyclic AMP-induced stimulation of uterine enzymes nor the inhibition of the enzymatic responses by actinomycin D and cycloheximide is dependent upon adrenal function.

TABLE IX

INFLUENCE OF CYCLIC AMP AND THEOPHYLLINE ON THE TWO HEXOSE MONOPHOSPHATE SHUNT DEHYDROGENASES IN UTERI OF ADRENALECTOMIZED-OVARIECTOMIZED RATS

Each value is the mean \pm S.E. based on 3 determinations of enzyme activity in uteri pooled from 3 rats. Cyclic AMP and dibutyryl cyclic AMP (each at a dose of 10 mg/rat) were injected intraperitoneally, in 2 equally divided doses at 8 hour intervals. Theophylline (10 mg/rat) was also administered by the intraperitoneal route in 2 equal doses concurrently with the cyclic nucleotide. Actinomycin D (25 μ g/100 g) or cycloheximide (70 μ g/100 g) were also given intraperitoneally twice at 8 hour intervals 30 minutes prior to the injection of cyclic AMP. All rats were sacrificed 16 hours following initiation of the treatment. Data are also given in percentages (in parentheses) taking the control values as 100%.

TREATMENT	GLUCOSE 6-PHOSPHATE DEHYDROGENASE	6-PHOSPHOGLUCONATE DEHYDROGENASE
CONTROL	9.2 \pm 0.2 (100)	4.1 \pm 0.2 (100)
CYCLIC AMP + THEOPHYLLINE	35.6 \pm 3.5 (387)*	10.5 \pm 1.0 (256)*
DIBUTYRYL CYCLIC AMP + THEOPHYLLINE	48.4 \pm 2.3 (526)*	12.2 \pm 1.0 (297)*
CYCLIC AMP + THEOPHYLLINE + ACTINOMYCIN	14.1 \pm 1.0 (153)*†	4.2 \pm 0.2 (103)†
CYCLIC AMP + THEOPHYLLINE + CYCLOHEXIMIDE	9.5 \pm 1.1 (103)†	3.8 \pm 0.1 (93)†

*Statistically significant difference when compared with the control values ($p = <0.05$).

†Statistically significant difference when compared with the values of cyclic AMP and theophylline-treated rats ($p = <0.05$).

V. DISCUSSION

A. ESTROGEN-LIKE EFFECTS OF CYCLIC AMP ON UTERINE METABOLISM

Hormones are generally thought to be the chemical messengers produced by special glands or cell groups and carried via the circulation to their target organs. There is, however, little agreement on how the hormones produce their diverse metabolic effects in responding tissues or cells. Indeed, the multiplicity of hormonal effects and the wide variations in the chemical structure of hormones make it quite conceivable that these biological substances act by a variety of different mechanisms.

Available evidence indicates that the action of certain hormones may be mediated by effects on the permeability of cell membranes. By this mechanism, hormones may control the availability of substrates for essential reactions and thus exert a selective control on cellular metabolism. Many of the actions of insulin on carbohydrate distribution and utilization may be accounted for by the effects of this hormone on permeability of the outer cell membrane (137). The estradiol-induced stimulation of the uptake of uridine, glucose, water and ions might also be due to its effects on the uterine cell membrane (7).

Hormones also exert their characteristic effects by altering the activities of certain enzymes which control vital metabolic processes in the cell. Hormones may affect the rate of enzyme synthesis and degradation by acting on the protein-synthesizing machinery of the cell. Increased enzyme activity as a result of this mechanism thus

represents enzyme synthesis de novo and is referred to as "enzyme induction" (1). Studies during recent years have made it increasingly evident that hormones are capable of stimulating quantitative changes in the amounts of specific proteins (or enzymes) and that this mechanism represents an important means by which metabolic processes are regulated in a variety of animal tissues.

While evidence for any single triggering event involved with the regulatory influence of estrogens on uterine biochemical differentiation is still lacking, recent work by Szego and Davis (124) has implicated cyclic AMP in the mechanism of action of estrogenic hormones. A single intravenous injection of estradiol-17 β to ovariectomized rats was shown to double the concentration of uterine cyclic AMP within 15 seconds of its administration. The elevation in the level of cyclic AMP was also produced by diethylstilbesterol but not by estradiol-17 α , the compound which exerts no estrogenic effects on the uterus (124). The response was organ specific since estradiol-17 β failed to produce any change in cyclic AMP concentration of the diaphragm (124), and mesometrial fat pad (138), two tissues which do not respond to estrogens. The elevation of uterine cyclic AMP after estradiol treatment was shown to be blocked by two β -adrenergic blocking agents, propranolol and dichloroisoproterenol (138). Estrogens have been shown to alter the concentration of epinephrine in the uterus (139) and it has been found that epinephrine also can elevate the level of cyclic AMP in this tissue (69). More recently, Rosenfeld and O'Malley (140) demonstrated

that the administration of diethylstilbesterol acutely elevates the activity of adenyl cyclase in uteri of ovariectomized animals.

The results presented in this study demonstrate that cyclic AMP, when given concurrently with theophylline, is capable of producing estrogen-like increases in glycogen content as well as the activities of several carbohydrate-metabolizing enzymes in uteri of immature and ovariectomized rats. The observation that theophylline itself increased uterine glycogen and the three key glycolytic and two hexose monophosphate shunt enzymes raises the possibility that the endogenous cyclic AMP might act as a stimulator of uterine metabolism (59,72,125,141). Following the administration of cyclic AMP, uterine enzymes increased significantly as early as 1 hour and maximum increases were observed 16 hours after treatment with the cyclic nucleotide. The observed time-course of cyclic AMP-induced stimulation of uterine hexokinase, phosphofructokinase, pyruvate kinase and the two hexose monophosphate shunt enzymes is similar to that reported previously for estrogenic stimulation of these carbohydrate-metabolizing enzymes in this tissue (46-48). The increases in uterine weight as well as in enzyme activities were related to the dose of cyclic AMP and 1 mg of the cyclic nucleotide produced significant increases in the activities of most enzymes investigated. Uterine glycogen was more sensitive to the action of cyclic AMP than were the enzymes since as little as 0.2 mg of cyclic AMP resulted in measurable increases in glycogen content. It is of interest that these time-course and dose-response studies for the influence of cyclic AMP on the uterus are comparable

to those reported by Singhal et al for the andromimetic action of this nucleotide on the prostatic and vesicular enzymes in orchidectomized rats (125,156).

B. SPECIFICITY STUDIES WITH CYCLIC AMP

Studies by Hechter et al (123) demonstrated that the effects produced by cyclic AMP on processes such as the synthesis of uterine RNA, protein, phospholipid and glycogen were not specific to this nucleotide. These investigators noted that in addition to cyclic AMP, other substances such as glucose, adenosine and guanosine monophosphate produced estrogen-like effects on these biochemical responses in the uterus. They postulated that a set of specific nucleotides, not the cyclic AMP exclusively, generated by estrogen action may provide intracellular regulatory signals in the uterine anabolic response (123). Results of the present study show that adenosine, 5'-AMP, 2',3'-AMP, 2'-AMP, 3'-AMP, 5'-ADP and 5'-ATP produced no significant change in either the uterine weight, glycogen content or any of the enzymes investigated. The data suggest that a degree of specificity exists for the action of cyclic AMP on uterine glycolytic and hexose monophosphate shunt enzymes and that of the compounds tested, only cyclic AMP is capable of triggering the observed enzymic responses in the uterus. Recently, Sharma and Talwar (142) also demonstrated that the effects of cyclic AMP on the incorporation of labelled uridine and leucine into RNA and protein were specific to this nucleotide. Other compounds

such as adenosine monophosphate and guanosine monophosphate did not produce any stimulation of these parameters, at least in the first 15 minutes of incubation (142).

The response of the two uterine enzymes, isocitrate dehydrogenase and α -glycerophosphate dehydrogenase to cyclic AMP and theophylline was similar to that observed for estradiol. Neither the administration of estradiol, nor cyclic AMP nor theophylline produced any significant change in the activities of these two enzymes. Concurrent treatment of estradiol or cyclic AMP-injected rats with theophylline also failed to produce any measurable effect on the activity of uterine isocitrate dehydrogenase and α -glycerophosphate dehydrogenase. These results indicate a specificity in response since enzymes, which do not increase following the administration of estradiol, also do not respond to cyclic AMP and theophylline.

C. THEOPHYLLINE-INDUCED POTENTIATION OF ESTROGEN ACTION

Sutherland et al (58) emphasized the variety of approaches that must be used in order to firmly establish the role of cyclic AMP as an intracellular mediator in the action of a given hormone. If the cyclic nucleotide is involved in estrogen action, not only the effects of the hormone should be reproduced by exogenous cyclic AMP, the phosphodiesterase inhibitors would be expected to potentiate the metabolic effects produced by a submaximal dose of estradiol. The results obtained in the present study demonstrate that theophylline is

capable of potentiating the action of a small dose of the hormone on uterine glycogen as well as the activities of various carbohydrate-metabolizing enzymes investigated. When a submaximal dose of estradiol (0.1 μ g) was administered along with theophylline, the increases produced in uterine glycolytic and hexose monophosphate shunt enzymes and in the glycogen content were of a magnitude similar to that produced by the 5.0 μ g dose of the hormone. These results are in analogy to previous studies reporting the ability of methyl xanthines to potentiate lipolysis in fat cells caused by catecholamines, glucagon and ACTH (92,143-145), the effects of vasopressin on the toad bladder (146) and the cardiac inotropic responses to norepinephrine (147). Recently, theophylline has also been shown to potentiate the effects of isoproterenol on DNA synthesis in parotid glands (148).

D. EFFECTS OF ACTINOMYCIN D AND CYCLOHEXIMIDE

Administration of cyclic AMP and theophylline to ovariectomized rats produced effects on uterine hexokinase, phosphofructokinase and pyruvate kinase as well as glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which were similar to those produced in immature animals. N⁶-O^{2'} dibutyryl analog of cyclic AMP along with theophylline resulted in much greater increases in uterine enzymes than did the parent compound. Although this combination was well tolerated in mature ovariectomized animals, it produced seizure activity and death in all of the immature rats. The greater potency of the dibutyryl analog

of cyclic AMP in stimulating uterine biochemical responses can be attributed to its greater resistance to the action of phosphodiesterase, more lipid solubility and the fact that it penetrates cells more readily than the parent compound (72,125).

Pretreatment of ovariectomized rats with actinomycin D and cycloheximide, two inhibitors of RNA and protein synthesis, completely abolished the cyclic AMP and theophylline-induced increases in the activities of all uterine enzymes studied. Since these inhibitors prevent the estradiol-induced stimulation of uterine glycolytic and hexose monophosphate shunt enzymes (46-48), the results indicate that like estrogens, the cyclic AMP-stimulated increases in the activities of these uterine enzymes might also be the result of new RNA and protein synthesis. Furthermore, since actinomycin D is known to block DNA-directed synthesis of nuclear RNA (149), the cyclic AMP-induced enhancement of the uterine enzymes may involve participation of messenger-RNA synthesis at the gene locus. Hechter *et al.* have shown that the stimulation of uterine RNA and protein synthesis produced by cyclic AMP *in vitro* was inhibited, but not abolished by actinomycin D and that these effects of the nucleotide depend, at least in part, upon new RNA synthesis at the gene locus (123). More recently, Sharma and Talwar (142) also demonstrated that actinomycin D, in very low concentrations, can inhibit almost completely the synthesis of RNA in the uterus stimulated by cyclic AMP.

E. EFFECTS OF CYCLIC AMP IN ADRENALECTOMIZED-OVARIECTOMIZED RATS

Lippe and Szego (150,151) have questioned the validity of conclusions drawn from experiments involving the use of compounds which inhibit RNA and protein synthesis. They suggested that actinomycin D stimulates adrenal function and that the increased secretion of adrenal steroids counteracts the estrogen-induced responses by a mechanism unrelated to the direct action of these inhibitors on RNA and protein synthesis. 11-oxy,17-hydroxycorticosteroids produced specific inhibition of the estrogen-induced uterine responses such as hyperemia (7,152), water and inorganic phosphate accumulation (7), accentuated carbohydrate uptake and utilization (153), as well as the selective incorporation of amino acids into protein (7). More recently, Szego and Davis (154) demonstrated that the elevation of uterine cyclic AMP following administration of estradiol was also blocked by cortisol. The present study shows that when cyclic AMP and theophylline were administered to adrenalectomized-ovariectomized animals, uterine weight and enzyme activities increased to the same extent as that noted in ovariectomized rats. When cyclic AMP treated adrenalectomized-ovariectomized animals were given either actinomycin D or cycloheximide, the observed enzyme responses were completely blocked as they were in ovariectomized rats in the presence of intact adrenals. The data support the contention that both the cyclic AMP-induced stimulation of uterine enzymes as well as the prevention of the enzymic responses by inhibitors of RNA and protein synthesis are independent of adrenal function. Singhal

and Valadares (155) also have demonstrated that neither the estradiol-induced increases in uterine phosphofructokinase and phosphohexose isomerase nor the inhibition of these effects by actinomycin D was dependent upon the presence of adrenal glands. More recently, Singhal et al (156) drew a similar conclusion from experiments in which they examined the ability of these inhibitors to prevent the cyclic AMP-induced stimulation of several prostatic enzymes in adrenalectomized-orchidectomized animals.

Of the hormones that are currently believed to act through the agency of cyclic AMP, almost all are proteins (besides the biogenic amines) and even to date, definite evidence for the role of this cyclic nucleotide in the mechanism of action of steroid hormones is lacking. The results presented in this dissertation demonstrate that cyclic AMP may be involved in triggering the known effects of estrogens on uterine metabolism. The ability of theophylline to potentiate the action of a submaximal dose of estradiol on uterine glycogen, the three key glycolytic and two hexose monophosphate shunt enzymes (157) provides additional support for the role of cyclic AMP in estrogen action. Recently, Triner et al (158) have suggested that this nucleotide may be involved in the regulation of uterine motility and contractility. Since exogenous cyclic AMP is also known to produce testosterone-like induction of several carbohydrate-metabolizing enzymes in the rat prostate and seminal vesicles, evidence indicates that cyclic AMP may play the role of an intracellular mediator in the mechanism of

action of both the androgenic and estrogenic steroids in the cells of their target tissues (125).

VI. SUMMARY

The discovery that cyclic 3',5'-adenosine monophosphate (cyclic AMP) is involved in the mechanism of glycogenolytic action of catecholamines has encouraged the belief that many hormones may act via the agency of this cyclic nucleotide. Although evidence for any single triggering event in the regulatory influence of estrogenic hormones on uterine biochemical differentiation is still lacking, recent studies indicate that estrogens might also act through the cyclic AMP-adenyl cyclase system. The aim of the present study was to examine the ability of exogenously administered cyclic AMP to produce estradiol-like stimulation of several carbohydrate-metabolizing enzymes and glycogen synthesis in the rat uterus.

In immature rats, the administration of cyclic AMP concurrently with theophylline, markedly stimulated uterine glycogen content and the activities of hexokinase, phosphofructokinase, pyruvate kinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Although these glycolytic and hexose monophosphate shunt enzymes and uterine glycogen were elevated significantly as early as 1 hour, maximum increases were observed 16 hours following the administration of cyclic AMP. The increases in uterine weight and enzyme activities were related to the dose of the cyclic nucleotide and even 1 mg of cyclic AMP produced significant increases in most enzymes investigated. Uterine glycogen was more sensitive to the action of cyclic AMP since as little

0.2 mg dose of this nucleotide resulted in measurable increases in glycogen content. Theophylline, an inhibitor of phosphodiesterase that inactivates cyclic AMP potentiated the effects of a submaximal dose of estradiol-17 β on uterine glycogen and the activities of glycolytic and hexose monophosphate shunt enzymes.

In contrast to the estrogen-like effects of cyclic AMP, adenosine as well as several adenine nucleotides (5'-AMP, 2',3'-AMP, 2'-AMP, 3'-AMP, 5'-ADP and 5'-ATP) failed to produce any appreciable effect on uterine metabolism. Two uterine enzymes, isocitrate dehydrogenase and α -glycerophosphate dehydrogenase, which did not increase following the administration of estradiol-17 β , also failed to respond to cyclic AMP and theophylline. The results suggest that a degree of specificity exists for the action of cyclic AMP on uterine glycolytic and hexose monophosphate shunt enzymes and that of the compounds tested, only cyclic AMP is capable of inducing the observed enzymatic responses in the uterus.

In ovariectomized rats, the administration of cyclic AMP and theophylline also produced estrogen-like stimulation of these carbohydrate-metabolizing uterine enzymes. The N⁶-O^{2'} dibutyryl analog of cyclic AMP elicited more pronounced increases than did the parent compound. In addition, cyclic AMP was capable of stimulating uterine enzymes in adrenalectomized-ovariectomized animals, suggesting that the action of the cyclic nucleotide is independent of adrenal function. Actinomycin D and cycloheximide, inhibitors of RNA and protein synthesis, blocked the ability of cyclic AMP to enhance the activities of uterine

glycolytic and hexose monophosphate shunt enzymes in ovariectomized as well as adrenalectomized-ovariectomized rats. These results raise the possibility that like estrogens, the cyclic AMP-induced increases in uterine enzyme activities may also be the result of enzyme synthesis de novo.

The present study has examined the ability of exogenously administered cyclic AMP to mimic some of the known effects of estrogenic hormones on the uterus of the immature and ovariectomized rat. Since cyclic AMP treatment resulted in marked stimulation of uterine weight, glycogen synthesis and of the activities of several important carbohydrate-metabolizing enzymes, the data lend support to the view that cyclic AMP may be involved in triggering the known metabolic effects of estrogens on the uterus.

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