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THE EFFECT OF ENDOTOXIN ON HEPATIC ADENYL CYCLASE ACTIVITY

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
ADP	Adenosine diphosphate
Cyclic AMP	Cyclic 3',5'-Adenosine monophosphate
ATP^{C14}	Adenosine 5'-triphosphate-8-C^{14} disodium (29.2-34.7 Mc/mMole)
5'-AMP	5'-Adenosine monophosphate
BSA	Bovine serum albumin
3',5'-PDE	3',5'-nucleotide phosphodiesterase
Tris buffer	Tris (hydroxymethyl) amino methane HCl
Endotoxin	Lipopolysaccharide
Theophylline	Aminophylline
Pi	Inorganic phosphate
LD_{5C}	Dose which is lethal to 50% of the animals in 24 hours
rpm	Revolutions per minute
ml	Milliliter
mg	Milligram
ug	Microgram
ul	0.001 milliliter
uM	Micromolar
mM	Millimolar
^{o}C	Degrees centigrade
g	Gram
mu	Millimicron (1 mu = 10^{-7} cm)

LIST OF ABBREVIATIONS -Continued

M	Molar
N	Normal
v/v	Volume to volume
w/v	Weight to volume
kg	Kilogram
$ZnSO_4$	Zinc Sulfate
$Ba(OH)_2$	Barium hydroxide
cm	Centimeter
$MgSO_4$	Magnesium sulfate
HCl	Hydrochloric Acid
O.D.	Optical Density
Km	Substrate concentration at which reaction proceeds at half-maximal velocity
Vmax	Maximal Velocity
Mc	Millicurie
mMole	Millimole
uMole	Micromole
pMole	Picomole

THE EFFECT OF ENDOTOXIN ON HEPATIC ADENYL CYCLASE ACTIVITY

CHAPTER I

INTRODUCTION

More than 100 years ago endotoxins were detected in cell-free filtrates of autolyzed gram-negative bacteria (Billroth, 1865) which indicated that these substances might be released spontaneously by some cells. This material was thought to be an internal constituent of gram-negative bacteria, hence the designation "endotoxin." This concept was discarded in 1933 when Boivin, Mesrobeanu, and Mesrobeanu extracted a toxic lipopolysaccharide from intact bacteria which retained both their morphology and staining characteristics.

The structure visualized by electron microscopy and the physical properties determined by chemical analysis indicate that the cell walls of gram-negative bacteria are composed of a multilayered mosaic of lipoprotein and lipopolysaccharide (Murray, Steed, and Elson, 1965). The outer three layers form globules which communicate freely with the extracellular fluid. The detachment of these globules from intact cytoplasmic membranes is related to the appearance of lipopolysaccharide in the extracellular fluid (Work, Knox, and Vesk, 1966; Mergenhagen, Bladen, and Hsu, 1966).

The pathological manifestations of gram-negative bacterial

infections are caused by the endotoxin content of these microorganisms. All mammalian species succumb to endotoxin with evidence of circulatory failure. As early as 1897 the similarities between the severe hypotension accompanying gram-negative septicemia and the shock state following hemorrhage were noted by Boise. Waisbren (1951) confirmed the similarity of the cardiovascular response to endotoxins regardless of the bacterial species involved and considered this response to be a general characteristic of toxic gram-negative lipopolysaccharides.

There has been considerable interest in the shock syndrome produced by gram-negative bacteria, first described clinically in 1951 (Waisbren, 1951; Borden and Hall, 1951). The toxic manifestations of endotoxemia have proven remarkably resistant to treatment. A mortality rate of greater than 60 per cent is encountered in septic shock, and has not been improved significantly over the past 50 years (Spittel, Martin, and Nichols, 1956; McCabe and Jackson, 1962; Martin and McHenry, 1962). The lack of knowledge concerning the mechanism involved in endotoxin shock persists despite the vast number of scientific publications concerned with lipopolysaccharides of gram-negative bacteria.

Molecular Characteristics and Systemic Distribution of Endotoxin

Many structural aspects of gram-negative lipopolysaccharides are not known. Structural analysis and electron microscopic studies of endotoxin have revealed an inner core polysaccharide to which a lipid component is covalently attached. This linkage between carbohydrate and lipid appears to be unique, since it has not been found elsewhere in nature (Treffers, 1946; Nowotny, 1961). The polysaccharide portion consists of a heptose polymeric backbone with attachments of organic phos-

phorus, O-phosphorylethanolamine, and antigenically active polysaccharide side chains (Work, Knox, and Vesk, 1966).

The detailed structure of the lipid portion of endotoxin is unknown save for its primary structure. The major constituents of E. coli endotoxin lipid are glucosamine, fatty acids, organic phosphorus, and 3-deoxyoctulosonate (Heath et al., 1966). Efforts have been made to isolate the biologically active moiety by removing inert components of the complex, both by hydrolytic (Palmer and Gerlough, 1940) and nonhydrolytic techniques (Ribi et al., 1961). These procedures appear to cause crucial changes in the tertiary structure of the split products or irreversible deformations of the molecular frame of the parent complex (Treffers, 1946; Nowotny, 1961; Nowotny, 1962). For these reasons attempts to isolate the "toxic principle" of bacterial lipopolysaccharides have been uniformly unsuccessful. Experimental evidence suggests that the toxic properties reside in the specific steric configuration of the endotoxin molecule and not in some discrete component (Tripodi and Nowotny, 1966). Invariably degradation of the parent compound is accompanied by diminished activity.

Although the cells which are the primary target involved in endotoxin poisoning have not been identified, the marked leukopenia and platelet destruction which is known to follow injection of endotoxin indicates that the reticuloendothelial (RE) system has been severely damaged (Crafton and DiLuzio, 1969). The RE system was first implicated in the host response to endotoxin in 1947 by Beeson. Furthermore, labelled endotoxin injected intravenously is cleared rapidly from the circulation and is localized in the RE system, particularly in the liver (Braude, 1964; Herring et al., 1962). Apparently the response to endotoxin involves the

entire reticuloendothelial system including the endothelial lining of blood vessels (Rubenstein, Fine, and Coons, 1962) and the spleen (Braude, 1955) as well as platelets, granulocytes, and monocytes (Brunning, Woolfrey, and Schrader, 1964).

Description of the Endotoxin Shock Syndrome

The circulatory changes produced by endotoxin vary from one species to another both in the timing of the circulatory failure and the degree of response. The hemodynamic effects of endotoxin have been studied in greatest detail in the dog. Since there are more data concerned with the development of the shock state in the dog, this species will be discussed first for comparative purposes.

Most striking in the early circulatory response to endotoxin is the sharp rise in portal venous pressure (MacLean and Weil, 1956; MacLean et al., 1956; Weil et al., 1956) at which time the central venous pressure is known to fall (Ebert et al., 1955; Freedberg, Haimovici, and Blumgart, 1944). Even the isolated perfused liver responded with an increase in vascular resistance and a gain in weight (MacLean et al., 1956). The combination of increased weight and vascular resistance is indicative of hepatic venular constriction. Further, when the liver is removed, there is no immediate fall in arterial pressure or venous return. In the dog, the early drop in blood pressure after endotoxin is due to a drop in cardiac output which is related to a decrease in venous return from pooling of blood in the splanchnic region (MacLean et al., 1956; Freedberg, Haimovici, and Blumgart, 1944; Brobmann et al., 1970).

Typically, the initial hypotension is followed by a period when the arterial pressure approaches normal levels. The blood pressure then

falls once more and the animal dies in shock. This final prolonged hypotension is not caused by splanchnic pooling, nor could it represent irreversible shock induced by the brief initial period of hypotension (Weil et al., 1956). Terminally the cardiac output is greatly reduced (Ebert et al., 1955) as well as the oxygen consumption (Freedberg, Haimovici, and Blumgart, 1944; Maxwell et al., 1959).

The conscious dog is characterized by tachypnea, rigors, vomiting, and diarrhea. Ataxia, apathy, and stupor are also seen with terminal tonic or clonic convulsions; respiration stops before the heart beat (Franke, 1944; Penner and Bernheim, 1951; Swan and Jacobson, 1967).

In contrast, the cat becomes hyperneic after intravenous endotoxin and may die of acute pulmonary edema during the first five minutes. The portal pressure rises only slightly and there are no signs of congestion in the splanchnic region (Hinshaw et al., 1958). There is a striking rise in pulmonary vascular resistance accompanied by pulmonary venous constriction (Kuida et al., 1958).

Unlike the dog, hepato-splanchnic pooling after the injection of endotoxin was not observed in the rabbit (Hinshaw et al., 1958). However, an acute fall in blood pressure (Braude et al., 1955; Leese, Poel, and Berman, 1950) was observed. Following the initial drop in blood pressure, there was frequently a return towards normal only to gradually decline over several hours until death (Heiffer, Mundy, and Grimm, 1958). Intense constriction of the ear vessels with later dilation was reported by several investigators (Boquet et al., 1948; Pinkstone, 1935).

After a fatal dose of endotoxin, the guinea pig becomes restless during the first hour and the respiratory rate increases. By three hours

the animal is resting on its abdomen with the hind legs extended (Roberts, 1949). Microscopic observations have revealed intense mesenteric vascular constriction and circulation time is prolonged (Boquet et al., 1947).

Terminally in the rat, convulsions and respiratory failure occur (Ross, 1957). The blood pressure may fall early in the second hour after intravenous injection of endotoxin (Zweifach, Nagler, and Thomas, 1956) or may remain relatively stable for three or four hours until terminal collapse (Zweifach and Hershey, 1957; White, Ross, and Barajas, 1966).

The effects of endotoxin in man are seen when small doses of purified endotoxin have been given, when blood or intravenous solutions contaminated with gram-negative organisms have been used, or when patients have developed serious infections. In the more severe reactions cyanosis, shallow rapid respiration, and oliguria develop (Altschule and Freedberg, 1945). The liver blood flow has been found to rise (Bradley and Conan, 1947; Hamrick and Myers, 1955) and vascular resistance is reduced (Maxwell et al., 1953). The arteriovenous oxygen difference has been shown to decrease with little change in arterial oxygen saturation (Altschule, Freedberg, and McManus, 1945; Bradley et al., 1945; Moser et al., 1959).

Studies which were conducted as the shock-like state developed showed tachycardia and a falling arterial pressure in association with a low cardiac output, a drop in venous pressure, and a diminished venous return (Bradley et al., 1945). The fall in venous return which occurs in man cannot be ascribed to hepato-splanchnic pooling as occurs in the dog (Braude et al., 1953; Stevens et al., 1953).

In all mammalian species studied, the invasion of the blood stream by gram-negative bacteria or the injection of extracted bacteria is

followed by inevitable failure of the circulation (Delaunay and Lebrun, 1947). In principle shock can occur as a result of a decline in cardiac filling and (or) a decline in overall vascular resistance. The cardiac output has been shown to decline in those instances where it has been measured and can be related to a drop in venous return (MacLean and Weil, 1956; Weil et al., 1956; Udhoji et al., 1963; Bynum et al., 1971).

After the intravenous injection of endotoxin, there is no significant change in hematocrit or plasma volume (Favorite and Morgan, 1942; Gibson and Kopp, 1938). As would be predicted from these data, the infusion of dextran or blood to maintain arterial pressure does not improve the survival rate (Lillehei and MacLean, 1958). There is evidence that the peripheral vessels dilate during the terminal phase of endotoxin shock (Zweifach et al., 1956; Zweifach, 1958); there are also examples of vessels which dilate early in endotoxin shock (Frolich, Scott, and Dooley, 1962; Brobmann et al., 1970).

Thus the discrepancy between vascular capacity and blood volume is a function of both declining venous return and peripheral resistance which, in turn, lead to a fall in cardiac output and arterial pressure. The cause of these vascular changes has not been established, although a variety of mechanisms have been postulated including neural mediation (Weil et al., 1956), a direct action of endotoxin on vascular smooth muscle (Zweifach, 1964), and indirect effects through a liberated chemical mediator (Hinshaw, Jordan, and Vick, 1961; Jacobson, Mehlman, and Kalas, 1964; Vick, 1964).

The Effects of Endotoxin on Carbohydrate Metabolism

It has generally been believed that the primary defect which led

to circulatory collapse following systemic endotoxemia was a disturbance in the reactivity of vascular smooth muscle (Zweifach, Nagler, and Thomas, 1956; Zweifach and Thomas, 1957). While many toxic reactions to endotoxin can be monitored through alterations in cardiovascular function, changes in the circulation could be secondary to more fundamental general cellular responses to endotoxin. Recent studies have shown that endotoxemia is associated with a variety of biochemical and physiological abnormalities, many of which could account for the lethality and the cardiovascular effects.

Over the past decade evidence has accumulated which demonstrates the existence of a primary metabolic alteration at the cellular level under numerous conditions in a wide variety of cells. Endotoxin damages the cellular targets of the host animal by interfering with normal metabolism (Nowotny, 1969). Initial investigations involved experiments with various types of malignant tissue in the presence of endotoxin. Persistent stimulation of both aerobic and anearobic cellular glycolysis preceded cellular necrosis during treatment with endotoxin. The degree of stimulation of tumor glycolysis observed *in vitro* with a series of endotoxin preparations paralleled their potencies in eliciting toxic phenomena *in vivo*. The reverse was also true; glycolysis was unaffected in tumors insensitive to endotoxin (Woods *et al.*, 1961a; Woods *et al.*, 1961b). These striking results were confirmed in normal tissue also.

Endotoxin-induced stimulation of glycolysis has been demonstrated in such diverse cells as polymorphonuclear leukocytes (Cohn and Morse, 1960), brain (Giger, 1961), and bone marrow (Yunis and Harrington, 1960). Fukuda concluded that the severe depletion of liver glycogen which

developed during systemic endotoxemia contributed significantly to the lethal outcome, since animals which had been rendered tolerant to endotoxin did not show this type of glycogen depletion (Fukuda, 1963; Fukuda and Akiyama, 1963). Berry, Smythe, and Young (1959) reported that injection of endotoxin resulted in an 80 per cent reduction in total body carbohydrate. Further, pretreatment with dibenzylene (α adrenergic-blocking drug) neither protected against the lethal effect of endotoxin nor did it alter the related carbohydrate changes. These data, supported by previous investigations (Nickerson and Goodman, 1947; Boquet and Izard, 1951; Hamosh and Shapiro, 1960), suggested that the adrenal medulla did not play a major role in the endotoxin-induced alterations in carbohydrate metabolism. Kun (1948) showed that endotoxin completely inhibited the *in vivo* conversion of glucose to liver glycogen.

Another line of evidence related the pathological changes produced by endotoxin to the stimulation of glycolysis, formation of lactic acid, and reduction in pH (Weil and Miller, 1961). These authors suggested that critical alterations in cellular function resulted from the metabolic acidosis.

The mechanism by which glycogen depletion occurs is unknown. Berry and Smythe (1963) pointed out that the loss of tissue carbohydrate could not be explained as an indirect result of elevated metabolic rate accompanying hyperthermia, a conclusion supported by previous investigations (Previte and Berry, 1963). Moreover, similar effects could be produced by endotoxin *in vitro* in liver slices and homogenates (Hamosh and Shapiro, 1960), thus reinforcing Berry's contention that the action was a direct effect of endotoxin on a metabolic pathway.

In 1960 Hamosh and Shapiro confirmed the previous findings of *in vivo* and *in vitro* effects of endotoxin on carbohydrate metabolism in liver and diaphragm homogenates and slices of both normal and adrenalectomized animals. They concluded that one of the enzymes participating in the phosphorolytic breakdown of glycogen to glucose was stimulated by endotoxin. Since phosphorylase was known to be the rate-limiting enzyme in the reaction sequence (Sutherland and Cori, 1951), this enzyme was chosen for further study. Hamosh and Shapiro showed that the effect of endotoxin on carbohydrate metabolism was due to an increased reactivation of phosphorylase rather than to an increase in the synthesis of the enzyme or an inhibition of its inactivation. Maximal phosphorylase activity followed endotoxin administration in both normal and adrenalectomized animals.

These findings led to the conclusion that similar biochemical steps were involved in the host response to endotoxin and in initiation of glycolytic stimulation (Woods, Landy, and Shear, 1959; Woods *et al.*, 1961b). Based on this general concept, a theory was developed which relates the cytotoxicity of endotoxin *in vivo* to stimulation of glycolysis as follows: Certain cells are unable to tolerate a persistent increase in the rate of glycolysis and suffer various forms of injury and malfunction. This primary injury to sensitive cells by excess glycolysis secondarily damages even those cells able to survive endotoxin-induced glycolysis. Although this theory does not define the precise mechanism involved in eliciting glycolysis, the initial event in endotoxin poisoning at the cellular level of metabolism is considered to be excess glycolytic stimulation.

Tripodi and Nowotny (1966) pointed out that the mechanism involved in endotoxin lethality must be related ultimately to chemical and physical characteristics of the endotoxin complex. In assessing the structure-function relationships of endotoxin, the following points should be considered: 1) endotoxins have a molecular weight ranging from one to twenty million and great insolubility in aqueous solutions (Beer and Braude, 1966); these physico-chemical characteristics make it unlikely that endotoxin could penetrate intact cell surfaces; 2) since endotoxins possess a marked ability to complex with a variety of natural products (Rudbach and Johnson, 1966; Rudbach, Miller, and Ribi, 1967) and have been shown to bind to cell surfaces (Neter, 1956; Brunning, Woolfrey, and Schrader, 1964; Springer and Horton, 1964), it is likely that the reactive site for endotoxin is a component of cell membranes. For several years increasing attention has been focused on the cell membrane as evidence was provided that these were not inert structures but directly determined many of the cell's responses to its environment by governing the entrance of outside factors, by determining its interactions with other cells and by altering intracellular metabolic patterns (Kalckar, 1964a; Kalckar, 1964b; Sundararajan, Rapin, and Kalckar, 1962; Rapin and Mayer, 1966); 3) endotoxins are extremely potent poisons; a lethal dose in mammals is of the order of one mg/kg body weight. Any theory designed to explain the toxicity of bacterial lipopolysaccharides must account for the fact that a relatively small number of molecules are responsible for profound and widespread pathological alterations in the host.

Effects of Cyclic AMP on Carbohydrate Metabolism

Early experiments which led to the discovery of cyclic AMP were

designed to explain the production of glucose in the liver by epinephrine and glucagon (Rall, Sutherland, and Wosilait, 1956; Rall, Sutherland, and Berthet, 1957; Rall and Sutherland, 1962). Previously glycogen phosphorylase was found to be the rate-limiting enzyme in the conversion of glycogen to glucose (Sutherland and Cori, 1951). Phosphorylase has been shown to exist in two forms: phosphorylase b which is inactive and phosphorylase a which is active. Whether phosphorylase b or a predominates at any given time depends upon the relative rates of phosphorylase kinase and phosphorylase phosphatase reactions (Krebs et al., 1966). Of these two converting enzymes, the kinase is involved in actual regulatory control. Activation of phosphorylase kinase is accompanied by the conversion of phosphorylase b to a which, in turn, accelerates glycogenolysis.

The mechanism by which epinephrine and glucagon elicited glycogen breakdown to glucose in the liver was shown in crude liver homogenates fortified with purified inactive phosphorylase, ATP and magnesium ion. Conversion of the inactive phosphorylase b to the active phosphorylase a was observed. In a supernatant fraction of liver homogenate which contained the phosphorylase but was separated from the particulate matter of the cell, epinephrine and glucagon did not activate phosphorylase (Rall, Sutherland, and Berthet, 1957). Furthermore, addition of the particulate fraction of the liver homogenate in the absence of the hormones did not activate phosphorylase. Phosphorylase activation occurred only in the presence of both the hormone and the membrane fraction of the homogenate.

Thus, it was shown that the hormones did not directly affect the level of phosphorylase activity but did require the participation of an intermediate sequence of events which was furnished by the particulate

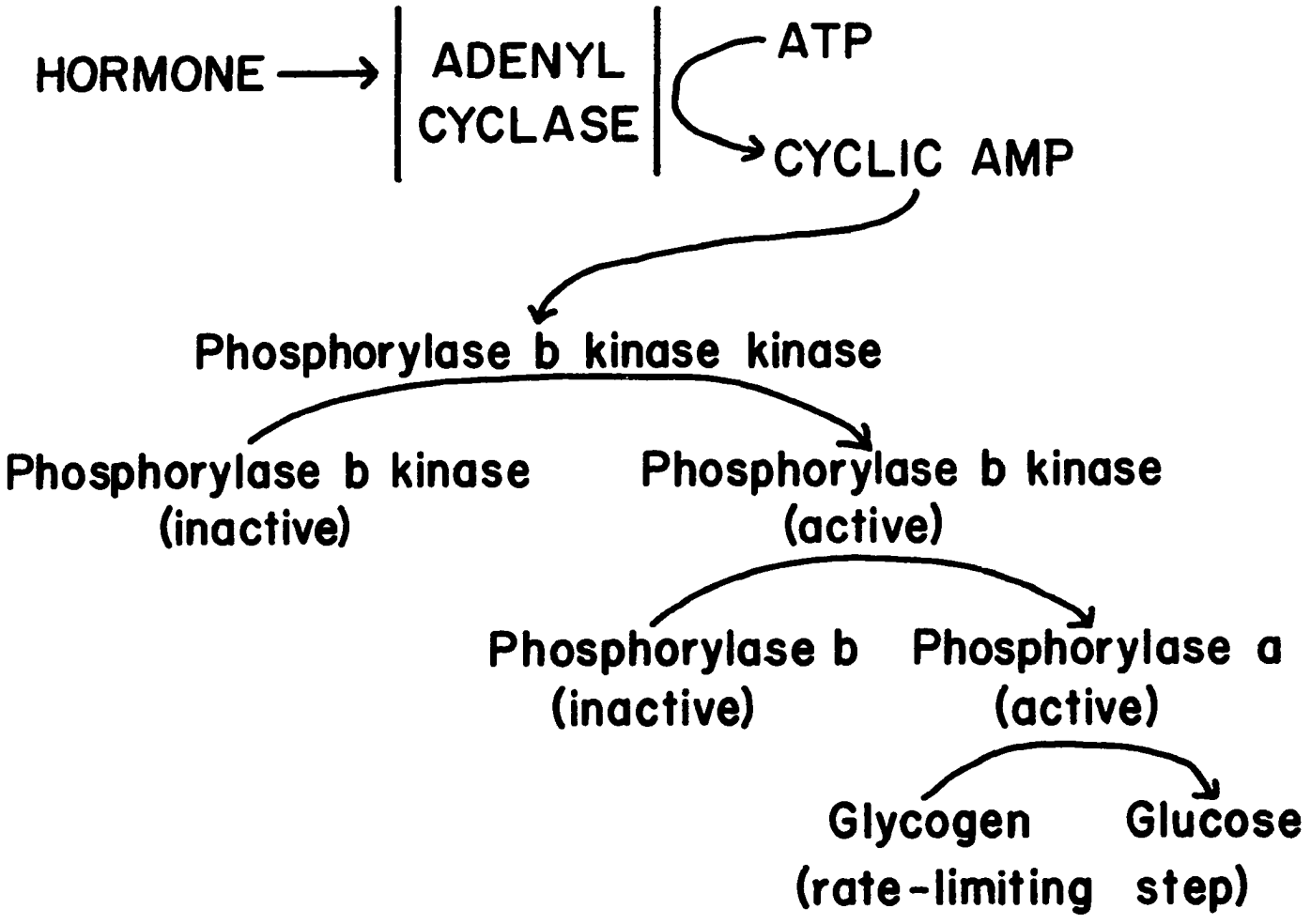
fraction of the homogenate. In subsequent experiments a heat stable factor was isolated and purified from hepatic tissue. It was named adenosine-3',5'-monophosphate, or more simply cyclic AMP. The accumulation of cyclic AMP was increased by epinephrine and glucagon and this preceded or occurred spontaneously with the activation of phosphorylase (Rall and Sutherland, 1958; Sutherland and Rall, 1958). The effect of cyclic AMP on hepatic phosphorylase has been shown to be so specific that this reaction is utilized in the assay of cyclic AMP levels in biological preparations (Rall, Sutherland, and Berthet, 1957; Rall and Sutherland, 1958). Krebs et al. (1966) demonstrated that the cyclic AMP increased the activity of phosphorylase b kinase rather than inhibited the phosphatase. The particulate fraction of the homogenate was found to contain the enzyme adenyl cyclase which specifically catalyzed the formation of cyclic AMP from ATP (Sutherland and Rall, 1957).

Later investigations revealed the complexity of the chain of reactions with the discovery and purification of a cyclic AMP-dependent phosphorylase b kinase kinase (Walsh, Perkins, and Krebs, 1968). This enzyme was identified as the receptor protein for cyclic AMP by gel filtration and kinetic studies. Using the former procedure, the cyclic AMP which was bound to phosphorylase b kinase kinase was quantitatively recovered (Hummel and Dryer, 1962). In the latter studies, cyclic AMP had no significant effect on the apparent Km for ATP but enhanced the maximal velocity eleven fold (Corbin and Krebs, 1969). These data provided convincing evidence that phosphorylase b kinase kinase was the direct receptor protein for cyclic AMP-induced activation of glycogenolysis.

To summarize, the interaction of hormones with membrane bound adenyl cyclase catalyzes the conversion of ATP to cyclic AMP. This initial sequence of reactions is followed by the binding of cyclic AMP directly to phosphorylase b kinase kinase; this enzyme accelerates the activity of phosphorylase b kinase. The catalytic conversion of phosphorylase b to a is the rate-limiting step in the conversion of glycogen to glucose, a reaction which is maximally stimulated by active phosphorylase b kinase. All these events have been carefully documented and constitute a generally well-accepted hypothesis (Walsh, Perkins, and Krebs, 1968; Posner, Stern, and Krebs, 1965) which is illustrated in Figure 1.

Since these initial studies of the effects of cyclic AMP on carbohydrate metabolism, it has been established that cyclic AMP accelerates carbohydrate utilization through three key pathways which involve specific rate-limiting enzymes; those involved in the acceleration of glycogenolysis, in the stimulation of gluconeogenesis, and in the inhibition of glycogen synthesis. In the perfused liver an increase in cyclic AMP concentration was the first event detected after exposure to glucagon or epinephrine, and preceded the increase in glycogenolysis by about forty seconds (Exton and Park, 1968). While glycogenolysis is accelerated through activation of phosphorylase as previously discussed, gluconeogenesis has also been shown to be under the direct influence of cyclic AMP. Utilizing lactate or amino acids as the substrate in perfused rat livers, Exton and Park (1969) demonstrated changes in intermediary metabolites in the presence of cyclic AMP which clearly pointed to the activation of phosphoenolpyruvate carboxykinase. This enzyme, known to occupy the rate-limiting step in the conversion of noncarbohydrate substrates to glucose,

Figure 1. Hormonal stimulation of glycogenolysis by cyclic AMP. Physiologically the hormone is carried from its cells of origin to its target cell. The receptor site is thought to be related to adenyl cyclase and to be in contact with the extracellular fluid. Hormonal binding activates the adenyl cyclase which increases the rate of formation of cyclic AMP within the cell.


HORMONE
ADENYL CYCLASE
ATP
CYCLIC AMP
Phosphorylase b kinase kinase
Phosphorylase b kinase (inactive)
Phosphorylase b kinase (active)
Phosphorylase b (inactive)
Phosphorylase a (active)
Glycogen (rate-limiting step)
Glucose

is also stimulated by hormones or cyclic AMP in a liver organ culture system (Wicks, 1969).

Finally, cyclic AMP has been shown to block glycogen formation. Glycogen synthetase has been shown to exist in two forms; conversion by phosphorylation of the I to the D form is equivalent to inactivation. This reaction is catalyzed by transferase I kinase, the activity of which is increased by cyclic AMP (Huijing and Larner, 1966; Bishop and Larner, 1969). The regulation of glycogen synthetase activity is thus similar to that of phosphorylase; both involve the participation of cyclic AMP with the respective kinase enzyme. In the case of glycogen synthetase, however, phosphorylation of transferase I kinase leads to a decrease in activity of the synthetase and a decrease in the formation of the α-1,4 linkages of glycogen.

From the experimental evidence which has been discussed, there can be little doubt that the adenyl cyclase-cyclic AMP system must be classified as a key regulator of carbohydrate metabolism. In the presence of cyclic AMP, glycolysis is maximally stimulated while non-carbohydrate substrates are shunted into the glycolytic pathway and glycogen synthesis is virtually stopped. The result is inevitable depletion of carbohydrate stores and the preferential utilization of glucose for cellular metabolism.

The Role of the Adenyl Cyclase-cyclic AMP System in Cell Function

The processes involved in the transfer of information within a cell and among different cells are essential for regulation and adaptation of the organism to a changing environment. If metabolic regulatory processes did not exist, conditions would be decidedly unfavorable for the continued existence of that organism. Hormones and chemical

transmitters are known to be essential for a physiological environment to be maintained.

Recently a common pathway through which hormonal agents can elicit their diversified biological responses has been postulated (Butcher, Baird, and Sutherland, 1968). This fundamental sequence of events may be visualized as a two-messenger system. According to this concept the first messengers, the peripheral hormones or chemical transmitters themselves, travel from their cells of origin to the cells of their target tissue to cause an alteration in the intracellular level of a second messenger. This second messenger is the transducer of the hormonal signal into the biological response (Sutherland et al., 1967).

As an intracellular mediator, cyclic AMP has been shown to participate in such diversified cellular responses as the initiation of permeability changes in kidney tubules (Orloff and Handler, 1967), the modification of protein synthesis in the liver and other tissues (Pryor and Berthet, 1960; Khairallah and Pitot, 1967), and the alteration of intracellular enzyme activity (Exton and Park, 1968). Investigators have speculated about the role of cyclic AMP in active transport processes (Pierce, et al., 1971).

The formation of cyclic AMP is catalyzed from its precursor ATP by the specific action of adenyl cyclase. The crucial role of this enzyme as a hormonal receptor is reflected in its ubiquitous distribution in biological systems. Adenyl cyclase has been identified in the membrane fraction of all nucleated mammalian cells as well as those of birds, reptiles and amphibians (Sutherland, Rall, and Menon, 1962; Davoren and Sutherland, 1963). Many invertebrates including insects, worms and

bacteria have been shown to possess a functional adenyl cyclase mechanism (Mansour and Menard, 1960; Makman and Sutherland, 1965). Obviously this enzyme was evolved early and has been maintained through subsequent evolution.

In mammalian organisms adenyl cyclase has been identified as the receptor for a large number of naturally occurring chemical transmitters. Adenyl cyclase appears to be the beta receptor of the sympathetic nervous system (Robison, Butcher, and Sutherland, 1967). According to the classification of Ahlquist (1948) and Furchgott (1959), beta receptor activation by adrenergic agents is responsible for the dilation of vascular smooth muscle, for the increased rate and force of cardiac contraction, and for the acceleration of glycogenolysis. In all these responses, cyclic AMP has been found to accumulate in the presence of the catecholamines (Murad, Rall, and Sutherland, 1960; Murad et al., 1962). Further, under those circumstances where the measurements have been made, the increase in cyclic AMP preceded or occurred simultaneously with the biological response (Murad et al., 1962; Robison, Butcher, and Sutherland, 1967). Another experimental approach has been the duplication of the hormonal response by cyclic AMP or its derivatives (Henion, Sutherland, and Posternak, 1967).

The role of adenyl cyclase-cyclic AMP is by no means limited to transmitters of the autonomic nervous system. Adenyl cyclase activity has been related to such potent biological compounds as histamine (Rall and Lakuchi, 1966), serotonin (Stone and Mansour, 1967), angiotensin (Kaplan, 1965), prostaglandins (Butcher and Baird, 1968), renin (Mickelakis, Caudle, and Liddle, 1969), and the majority of pituitary hormones (Gilman

and Rall, 1966; Grahame-Smith et al., 1967).

The mechanism responsible for the termination of the response to cyclic AMP has been thoroughly investigated. A magnesium-dependent cyclic 3',5'-nucleotide phosphodiesterase (3',5'-PDE) is the only enzyme known to inactivate cyclic AMP. This reaction has been shown to involve hydrolysis at the 3'-position of cyclic AMP to yield 5'-AMP (Sutherland and Rall, 1958). Like adenyl cyclase, 3',5'-PDE has been found in most tissues. However, the latter enzyme has a more complex distribution, being found in both soluble and particulate tissue fractions (Sutherland, Rall, and Menon, 1962). In those tissues where the activities of adenyl cyclase and 3',5'-PDE have been compared, the latter enzyme has been reported to be in much higher concentration. It would appear that intracellular levels of cyclic AMP may also be enhanced by sequestration or compartmentalization of cyclic AMP or of 3',5'-PDE (DeRobertis et al., 1967). The same cellular response is often much greater towards mediators which increase cyclic AMP by activating adenyl cyclase than to agents which increase cyclic AMP by inhibiting 3',5'-PDE (Murad, Rall, and Sutherland, 1962; Rall and West, 1963).

The standard free energy of hydrolysis of the 3' bond of cyclic AMP to 5'-AMP has been calculated by Greengard, Hayaishi, and Colowick (1969) to be -11,900 calories. By way of comparison, the standard free energy of hydrolysis of the γ-phosphate of ATP under the same conditions is about -8,900 calories (Alberty, 1969). Thus the standard free energy expended for the hydrolysis of cyclic AMP is about 3000 calories more than that for the hydrolysis of the terminal phosphate of ATP. These measurements, supported by other experiments (Greengard, Rudolph, and

Sturtevant, 1969), provided substantial support for the conclusion that the 3' bond of cyclic AMP is of high energy content. In contrast to other high energy compounds, almost all of which are unstable and rapidly hydrolyzed, cyclic AMP in aqueous solution is a quite stable compound. These characteristics are thought to be significant in the unique role of cyclic AMP in cellular metabolism although the physical relationship between cyclic AMP and its receptors remains obscure.

The regulatory properties of adenyl cyclase have been postulated to reside in subunits of the enzyme, not unlike the subunits identified by Gerhart and Schachman (1965) in the case of aspartate transcarbamylase. According to this scheme, the hormones or chemical transmitters would interact with a site on a regulatory subunit which faces the extracellular space. The conformational changes which result from this interaction are transmitted to the catalytic subunit on the inside of the cell membrane. Modification of the active site influences the effectiveness of the interaction of the enzyme-substrate complex which is reflected by a change in the reaction rate. Direct evidence for or against the existence of such subunits as components of adenyl cyclase must await purification of the enzyme. Regardless of the conformational design of this enzyme, the biological role of the adenyl cyclase-cyclic AMP system has been recognized as a fundamental reaction which forms the crucial link between extracellular mediators and the intracellular processes they regulate (Bowness, 1966; Schwartz and Hechter, 1966).

The Working Hypothesis

The indispensable role of adenyl cyclase in the regulation of cellular processes and the possible effect of endotoxin on this enzyme

were the points of primary interest for this dissertation. The experimental design of the research was developed to answer the following question: Does endotoxin activate adenyl cyclase?

The similarities of the responses of the cardiovascular system to endotoxin shock and beta receptor stimulation appeared to be significant, expecially in the light of recent identification of adenyl cyclase as the beta receptor. Previous work in a number of laboratories clearly indicated that endotoxins elicited a marked response at the level of carbohydrate metabolism. Woods et al. (1961b) developed a concept that the primary site of endotoxin-induced injury involved the same reactive site as that responsible for endotoxin stimulation of glycolysis. The thorough work of Hamosh and Shapiro (1960) on the activation of phosphorylase by endotoxin and the information that activation of hepatic phosphorylase by cyclic AMP is quite specific (Rall, Sutherland, and Berthet, 1957; Rall and Sutherland, 1958) strongly suggested a cause and effect relationship.

Therefore, it can be postulated that endotoxin was bound to a site on the membrane so as to activate the adenyl cyclase molecule. Adenyl cyclase activity was measured in the presence of endotoxin under a variety of experimental conditions to clarify the mechanism involved.

The following objectives were accomplished by this research: 1) Measurement of the response of hepatic adenyl cyclase to the injection of a lethal dose of endotoxin in vivo; 2) Measurement of adenyl cyclase activity in tissue homogenates incubated with endotoxin; and 3) Determination of a direct effect of endotoxin on adenyl cyclase.

The initial studies were pilot experiments which consisted of the administration of endotoxin to dogs, rats and guinea pigs and the

subsequent assay of adenyl cyclase activity in liver biopsies. The technique for the assay of adenyl cyclase was refined under the proposed experimental conditions. The guinea pig was chosen as the most suitable laboratory animal for this study, since hepatic congestion did not occur following endotoxin administration (splanchnic pooling is marked in the dog and rat in endotoxin shock).

After the technique and experimental design were established, the actual dissertation was begun. A lethality study was done to establish the LD_{50} dose for the endotoxin preparation which was used throughout this study.

The next phase of the study was designed to show the validity of the technique used to assay adenyl cyclase activity. This consisted of verification of sample purity with ascending paper chromatography, of tissue dilution studies to establish the relationship between enzyme concentration and levels of activity, and of elution patterns for the column chromatographic separation of cyclic AMP.

The liver was selected as the tissue for enzyme assays since this organ is known to be a major site of pathological changes in endotoxin shock and the role of adenyl cyclase in liver metabolism has been documented. Experiments were designed to measure the response of hepatic adenyl cyclase in endotoxin-shocked guinea pigs. A subsequent series of experiments involved the comparison of adenyl cyclase activity in an untreated liver homogenate with an identical homogenate incubated with endotoxin.

In order to further clarify the results of the previous experiments, two additional experimental designs were developed to determine

whether the *in vitro* response of adenyl cyclase was due to endotoxin or to the release of some intermediary compounds from the tissue. These experiments utilized both centrifugation and dialysis in a attempt to separate any intermediaries which might be released from the tissue by endotoxin and to record the activity of adenyl cyclase under these circumstances. Finally the effect of endotoxin on the activity of phosphodiesterase was determined to assess this additional route of regulation of cyclic AMP concentrations.

CHAPTER II

MATERIALS AND METHODS

Overall Experimental Design

The experiments reported here can be classified as *in vivo* or *in vitro*. The assay of enzyme activity in tissue taken from guinea pigs in shock or during control conditions is defined as *in vivo*; an assay performed on tissue from untreated animals which is then incubated either with endotoxin or with buffer is defined as *in vitro*.

The activation of adenyl cyclase in endotoxin-shocked animals would indicate that the enzyme is activated in the shock state. The assay of adenyl cyclase activity in tissue from untreated animals which is subsequently incubated with or without endotoxin would distinguish between the participation of blood-borne intermediaries and the direct effect of endotoxin on the tissue. If activation were found *in vitro*, in the absence of an intact blood supply, this would be evidence for a direct effect of endotoxin on the tissue.

Additional *in vitro* experiments were designed to detect the presence of an intermediary which might be released from the tissue itself by endotoxin. Two procedures were used which served to separate small molecular weight compounds from the endotoxin. First, a liver homogenate was dialysed against Tris buffer in the presence of endotoxin. The

dialysate was used to resuspend an untreated pellet of liver homogenate. This preparation was compared with appropriate controls. If this dialysate were to activate adenyl cyclase, this would indicate the presence of some small active molecules which were released from the tissue, since this compound could and endotoxin could not cross the dialysis membrane.

Second, an homogenate of liver was incubated with endotoxin and the mixture centrifuged. The supernatant fluid was discarded, the tissue pellet was resuspended in Tris buffer, and the activity of adenyl cyclase was measured. If this procedure resulted in a loss of adenyl cyclase activity, this would suggest that some active intermediary was discarded in the supernatant fluid; if the activity was similar to a tissue pellet resuspended in endotoxin rather than Tris buffer, this would be evidence for a direct effect of endotoxin on adenyl cyclase.

Finally 3',5'-PDE activity was measured in a crude liver homogenate in the presence of endotoxin to assess the directional changes in cyclic AMP concentration which might result from degradation of the nucleotide.

The combined results from these experiments should allow description of the relationship between endotoxin and cyclic AMP in the liver.

Materials

Animals

Albino male and female guinea pigs weighing from 300 to 600 g were obtained from Dr. Glenn S. Bulmer, Department of Microbiology and Immunology, University of Oklahoma Medical Center.

Reagent Chemicals and Materials

All the chemicals and solvents used were reagent grade and were used as obtained unless specified otherwise.

Chemicals were obtained from the following sources: Ion exchange resin (AG 50-X4), BioRad Laboratories, Richmond, Virginia. Potassium Monobasic Phosphate and Sodium Fluoride, J. T. Baker Chemical Company, Phillipsburg, New Jersey. Tris buffer and Theophylline, Sigma Chemical Company, St. Louis, Missouri. Bovine albumin, Travenol Laboratories, Inc., Los Angeles, California. Mg SO_4, Matheson Coleman and Bell, Los Angeles, California. Lipopolysaccharide B, E. Coli 0127;B8, Difco Laboratories, Detroit, Michigan. Cyclic AMP, free acid crystalline and ATP, P-L Biochemicals, Milwaukee, Wisconsin. (8-14 C) ATP disodium, specific activity 29.2-34.7 Mc/Mmol, Schwarz Bioresearch, Inc., Orangeburg, New York. Ammonium Acetate, Exanol, Aquasol, New England Nuclear, Boston, Massachusetts. L-Ascorbic Acid, Ammonium Molybdate, $ZnSO_4$, $Ba(OH)_2$ and HCl, Fisher Scientific Company, Fair Lawn, New Jersey. Ether, Mallenckrodt Chemical, St. Louis, Missouri. Dialysis Membrane, Union Carbide Corp., Chicago, Illinois. Pasteur capillary pipets, Owens-Illinois, Toledo, Ohio. Disposable glass scintillation vials, Isolab Incorp., Akron, Ohio. Parafilm, American Can Co., Neenah, Wisconsin. Whatman 3 MM chromatographic paper, H. Reeve Angel and Co., Inc., Clifton, New Jersey. Snake venom, Crotelus Atrox, Sigma Chemical Co., St. Louis, Missouri. Trichloro Acetic Acid, Sulfuric Acid, Matheson, Coleman and Bell, Norwood, Ohio.

Instruments

Substitution of the instrumentation used during this research

was unavoidable.

Spectrophotometers: Spectronic 600, Bausch and Lomb, Rochester, New York, and Model 139, Hitachi, Ltd., Tokyo, Japan.

Refrigerated centrifuges: International Model HR-1, International Equipment Co. and Sorvall RC-3, Ivan Sorvall, Inc., Norwalk, Conn.

Liquid Scintillation Spectrometers: Mark I and 720 Series, Nuclear-Chicago, Chicago, Illinois and Packard 3950, Packard Intsrument Co., Le Grange, Illinois.

Analytic balance: Mettler H20, W. H. Curtin and Co., Atlanta, Georgia.

Metabolic shaking incubators with constant temperature water bath: Dubnoff, Precision Scientific, Chicago, Illinois and Metabolyte, New Brunswick Scientific Co., Inc., New Brunswick, New Jersey.

Mixer: Vortex K-550-G, Scientific Industries, Inc., Springfield, Massachusetts.

pH meter: Corning 12, W. H. Curtin and Co., Atlanta, Georgia.

Magnetic stirrer: Magnestir, Lab-Line Instruments, Inc., Melrose Park, Illinois.

Multi-Purpose Rotator: Model 120, Scientific Industries, Inc., Springfield Massachusetts.

Methods

Management of Experimental Animals

Both male and female guinea pigs, weighing 300 to 600 g were used in these experiments. The animals, allowed free access to food and water, were housed, six per cage, in the animal quarters at the University of Oklahoma Medical Center. Experimental animals were matched for age

and weight.

Lethality Study

The LD_{50} dose of endotoxin was established using 10 guinea pigs ranging in weight from 332 g to 550 g. Endotoxin was suspended in saline (2 mg/ml) and injected intravenously in a dose of 1.5 mg/kg. Those animals which were alive 24 hours post-injection were counted as permanent survivors.

Preparation of Liver Homogenate for *In Vitro* Assay

One guinea pig was used for each experiment. At the time of assay the animal was lightly anesthetized with ether. A midline incision was made to expose the liver. A portion of the liver was quickly removed and gently homogenized in a glass homogenizer in Tris buffer (pH 7.4) at 0^{o} C. The homogenate was filtered into a test tube through a thin layer of glass wool and maintained at 0^{o} C until the homogenate was transferred to the incubation medium. The time which elapsed between removal of the liver biopsy and transfer of the homogenate to the incubation chamber was between 5 and 15 minutes.

Preparation of Liver Homogenate for *In Vivo* Assay

Two guinea pigs of comparable age and weight were used in each experiment. The experimental animal was injected intravenously with double the LD_{50} dose of endotoxin and was sacrificed 4 hours later. This time interval was chosen for the following reason: In 4 hours all treated animals exhibited symptoms of acute stress, inactivity and prostration. Control animals were injected intravenously with an equal volume of sterile saline.

At the time of the assay, both animals were lightly anesthetized with ether and a midline incision was made. A portion of liver was removed from each animal, immediately homogenized in a glass homogenizer in Tris buffer (pH 7.4) at 0° C, and was filtered through thin layers of glass wool into labelled test tubes which were maintained at 0° C until transferred to the incubation medium. The time which elapsed between removal of the liver tissue and transfer of the homogenates to the incubation chambers was between 10 and 15 minutes.

Homogenate Dialysis Procedure

The liver homogenate was prepared as described for the in vitro assay. Following filtration of the homogenate through a thin layer of glass wool, 2.0 ml of the homogenate was placed in dialysis membrane tubing and dialyzed against 6.0 ml of Tris buffer (pH 7.4) for 1 hour at 5° C with constant gentle agitation.

Four sections of tubing were prepared. Two contained only the liver homogenate, one contained a 2.0 ml suspension of endotoxin (3 mg/ml) and one contained 2.0 ml of homogenate plus 5-7 ug endotoxin per mg dry weight of tissue.

During the same one hour period, four 3.0 ml fractions of liver homogenate were centrifuged at 15,000 rpm at 5° C. The supernatant fluid was discarded and 2.5 ml of the dialysate from each membrane was added to each. The tissue pellets were resuspended in the dialysate by thorough mixing on a Vortex. At this point, the assay of adenyl cyclase activity was performed.

The control incubate consisted of an untreated tissue pellet resuspended in dialysate from untreated liver tissue. A second untreated

tissue pellet was resuspended in dialysate from the endotoxin suspension to indicate if some small molecule from the endotoxin preparation could be responsible for the adenyl cyclase activity determined in the assay. A third untreated tissue pellet was resuspended in dialysate from untreated tissue but was subsequently added to incubation medium containing endotoxin to assure that endotoxin activated the adenyl cyclase in this preparation. The fourth untreated pellet was resuspended in dialysate from liver tissue to which endotoxin had been added prior to the start of the dialysis. This provided some evidence as to whether endotoxin was causing the release of a compound of small molecular weight from the tissue which was responsible for the activation of adenyl cyclase rather than being attributable to endotoxin directly.

Homogenate Centrifugation Procedure

The liver homogenate was prepared as outlined for the in vitro assay. Following filtration of the homogenate through a thin layer of glass wool, four 3.0 ml of liver homogenate were centrifuged at 15,000 rpm for one hour at 5° C. One of the fractions served as an untreated control. To one of the fractions, endotoxin (6-8 ug/mg dry weight) was added prior to centrifugation. The supernatant fluids were decanted and the tissue pellets were resuspended in Tris buffer (pH 7.4). The third and fourth preparations were not treated until the beginning of the assay procedure; then endotoxin (6-8 ug/mg dry weight) and fluoride (10 mM) were added.

The reasoning behind this experimental design was as follows: if endotoxin was causing the release of some small molecular weight compound which was responsible for activation of adenyl cyclase, then

incubating endotoxin with the homogenate prior to centrifugation should cause the smaller molecular weight compounds to appear in the supernatant fluid. Discarding the supernatant fluid and resuspending the tissue pellet in Tris buffer should diminish the activity from that of tissue treated with endotoxin after centrifugation. These four simultaneous assays of adenyl cyclase activity were performed to establish predictable relationships of adenyl cyclase activity.

Tissue Dry Weight Determination

The expression of adenyl cyclase activity included the units of tissue weight. This figure was derived from dry weight determinations of the homogenates used in the assay. Duplicate samples were prepared and the average of the two used in the calculations. One hundred ul of each tissue homogenate was placed in 2 tared glass tubes and these tubes were placed in a dessicator under vacuum for 36 hours. These tubes were then weighed on an analytic balance and the dry weight of the tissue was obtained by subtraction from the tare weight.

Preparation of Chromatographic Ion Exchange Resin

A cation exchange resin of the sulfonic acid type (purified and sized Dowex 50) was used in both the chromatographic purification of ATP^{C14} and in column chromatographic separation of cyclic AMP. This resin was washed in 1 N HCl and then rinsed with deionized distilled water to constant pH. A 50% slurry (v/v) of the resin was prepared and stored in the refrigerator until used.

Purification of ATP^{C14}

ATP^{C14} (29.2-34.7 Mc/mMole) was suspended in Tris buffer (pH 7.4).

This solution was eluted in 1.0 ml fractions on columns of resin (0.5 by 3.5 cm) with deionized distilled water. Five ul of each ml were transferred to scintillation vials containing 15 ml Aquasol. Counts per minute were determined for each ml. In most cases, only the first 2 ml which were collected were retained while trace radioactive contaminents in subsequent fractions were discarded. To prevent bacterial overgrowth, the purified ATP^{C14} fractions were poured into test tubes, covered with Parafilm, and frozen at 0° C until used in the assay procedure.

Elution Pattern for Cyclic AMP and ATP

The elution pattern for chromatographic separation of cyclic AMP was described in detail by Krishna, Weiss, and Brodie (1968). The ion exchange resin (50% v/v) was pipeted into columns 0.5 by 3.5 cm. These columns were prepared from Pasteur pipets plugged loosely with glass wool. A new elution pattern was determined whenever a new quantity of resin was used. To a stock solution containing 2 mM $MgSO_4$, 1% BSA, 30 mM theophylline, and 50 mM Tris, a known quantity of ATP^{C14} (29.2-34.7 Mc/mMole) and unlabelled cyclic AMP (10^{-3} M) was added. Either 500 or 750 ul of this solution were pipeted on the column. The column was eluted with deionized distilled water and 1.0 ml fractions collected. The radioactivity was determined in a liquid scintillation spectrometer and the corresponding ultraviolet absorption at 260 mu was determined spectrophotometrically for each of these fractions. The fractions which contained ATP were identified by the presence of radioactivity in the eluate; the O.D. readings identified the fractions which contained cyclic AMP.

All fractions were precipitated with 25 ul $Ba(OH)_2$(0.25 M) and 25 ul $ZnSO_4$ (0.25 M) and were centrifuged at 3000 rpm for 10 minutes.

The supernatant fluid was decanted and the precipitation and centrifugation step repeated. Following centrifugation, the radioactivity and O.D. for each fraction was determined.

Paper Chromatography

To further substantiate the technique for recovery of cyclic AMP, paper chromatography was used. Representative aliquots of cyclic AMP fractions from various experiments, as well as stock solutions of both ATP and cyclic AMP for comparison, were spotted on Whatman 3 MM filter paper. The ascending technique was used with ammonium acetate-ethanol (70% v/v ammonium acetate (1 M) and 30% v/v ethanol (95%)) as the solvent. Twenty-four hours later the chromatograms were dried and observed under ultraviolet light.

Assay of Adenyl Cyclase Activity

The assay procedure used in these studies was modified from a technique developed by Krishna et al. (1968). The rate of formation of cyclic AMP from its precursor ATP by the catalytic action of hepatic adenyl cyclase was measured.

The incubation medium for the assay of adenyl cyclase activity in each liver homogenate contained: Tris buffer, pH 7.4 (5×10^{-2} M); BSA (1% w/v); $MgSO_4$ (2×10^{-3} M); theophylline (3×10^{-2} M); ATP (1.6×10^{-3} M); ATP^{C14} (10-20 uCi); cyclic AMP (10^{-3} M) in a total volume of 5.0 ml.

A radioactive tracer (ATP^{C14}) is mixed with the unlabelled ATP substrate for the measurement of the rate of formation of cyclic AMP as follows: $ATP^{C14} \xrightarrow[\text{cyclase}]{\text{adenyl}}$ Cyclic AMP^{C14}.

Theophylline was added to the incubation medium to inhibit the hydrolysis of cyclic AMP to 5'-AMP by phosphodiesterase. $MgSO_4$ is a cofactor for adenyl cyclase. Unlabelled cyclic AMP was added to the incubation medium for two reasons: 1) to limit the enzymatic hydrolysis of newly formed radioactive cyclic AMP by dilution of the isotope; and, 2) to correct for the recovery of the purified fraction of cyclic AMP as well as for any loss due to enzymatic degradation during the incubation. In the experiments in which fluoride ion was added, sodium fluoride (10^{-2} M) was used. Fluoride ion is known to be a potent non-specific activator of adenyl cyclase (Sutherland, Rall, and Menon, 1962; Weiss, 1968).

Fifty ul of the incubation medium which contained a known concentration of the substrate ATP was transferred to a vial which contained 2 ml of distilled deionized water and 15 ml Aquasol. The radioactivity was measured in a liquid scintillation spectrometer. From this, the radioactivity (cpm) per uMole of ATP was calculated for each experiment. The determination of the efficiency of the spectrometer showed that it was not significantly different from one sample to another within the same experiment.

The reaction was initiated by the addition of 2.0 ml of liver homogenate (with or without the agent to be tested) to each incubation flask to make a total volume of 7.0 ml. At zero time, 10 minutes, and 15 or 20 minutes, 1.5 ml aliquots were removed from each incubation flask and were pipeted into 2 glass test tubes in equal volumes (0.75 ml duplicate samples). These tubes were immediately placed in a boiling water bath for 3 minutes to terminate the reaction. The tubes were removed from the boiling water bath and placed on ice.

The tubes were centrifuged at 10,000 rpm for 10 minutes and the supernatant fluid was decanted into dry glass test tubes. At this stage of the assay, the supernatant fluid was frozen overnight or the procedure was continued.

The slurry of resin was pipeted into columns (0.5 by 3.5 cm) and washed with deionized distilled water. Dry, graduated centrifuge tubes were placed under the columns to collect the eluate. Five hundred ul of supernatant fluid from each sample was chromatographed on each column. The columns were eluted with 2.0 ml of deionized distilled water; this fraction contained most of the ATP and ADP.

A duplicate set of centrifuge tubes replaced those used for the first collection. The columns were eluted with 3.5 ml of deionized distilled water. This fraction contained most of the cyclic AMP. To the cyclic AMP fractions, 200 ul each of $ZnSO_4$ (0.25 M) and $Ba(OH)_2$ (0.25 M) solutions were added, stirred with a Vortex for 15 seconds, and were centrifuged at 3000 rpm for 10 minutes. The concentrations of $Ba(OH)_2$ and $ZnSO_4$ were such that they neutralized each other to phenolphthalein when mixed in equal volumes. Therefore, this procedure did not alter the pH of the eluate. The supernatant fluid was transferred to a duplicate set of tubes by decantation; the precipitation and centrifugation steps were repeated.

The addition of $ZnSO_4$ and $Ba(OH)_2$ solutions quantitatively precipitated trace contaminants of ATP, ADP, 5'-AMP, 2'AMP, theophylline, inorganic phosphate, adenine and other nucleotides whereas more than 99% of the cyclic AMP remained in solution (Krishna, Weiss, and Brodie, 1968).

A 2.0 ml aliquot of each cyclic AMP fraction was added to 15 ml

of Aquasol and the radioactivity was measured in a liquid scintillation spectrometer. Incubations with heat-denatured enzyme were run as blanks and are shown in the figures as zero-time values which were subtracted from background radiation. In most cases these values were negligible.

The ATP fractions were not precipitated with $Ba(OH)_2$ and $ZnSO_4$. The radioactivity and the absorption at 260 mu were measured in the unprecipitated ATP fractions as was done for the cyclic AMP fractions. This allowed for the concentration of the substrate to be monitored throughout the reaction.

Rates of formation of cyclic AMP for each sample were derived from the following:

1) The absorbance at 260 mu of an aliquot of each cyclic AMP fraction. These readings were compared with the absorbance for a standard solution which contained a concentration of cyclic AMP equivalent to 100% recovery.

2) The radioactivity was counted for each sample and for 50 ul of incubation medium. From these data, the recovery of cyclic AMP in each sample was calculated and that information was used to correct each sample to 100% recovery.

3) The dry weight determinations for each ml of homogenate allowed for the calculation of the pMoles of cyclic AMP per mg of tissue. The aliquots of the samples which were removed at specific time periods and the reaction immediately terminated allowed for the calculation of pMoles of cyclic AMP formed per mg per unit time by the following formula:

$$\frac{Vol_T}{Vol_C} \times \frac{cpm^C}{Rec.} \times \frac{cpm}{50\ ul} \times \frac{50\ ul}{uMole\ ATP} \times \frac{1}{500\ ul} \times F \times \frac{7\ ml\ Inc.}{2\ ml\ H.} \times \frac{1\ ml\ H.}{mg\ dry\ weight\ H.} \times 10^6 = pMoles\ Cyclic\ AMP/mg\ dry\ weight$$

Vol_T = Final volume of eluate (cyclic AMP fraction = 4.0 ml)

Vol_C = Volume of eluate used to measure radioactivity (2.0 ml)

cpm^C = cpm of Vol_C

Rec. = Per cent recovery expressed as decimal fraction

50 ul = Amount of incubation medium which contained known amount of ATP

500 ul = Aliquot of supernatant fluid pipeted on each column

$F = \frac{1000 \text{ ul}}{1 \text{ ml}}$

Inc. = Total volume of incubation medium (5 ml) and homogenate (2 ml)

H. = Volume of homogenate

Dry Weight = Dry weight of homogenate

10^6 = pMoles/uMole

Assay of 3',5'-Nucleotide Phosphodiesterase Activity

This assay procedure was modified from the technique reported by Butcher and Sutherland (1962). The reaction sequence to be measured involved the hydrolysis of cyclic AMP to 5'-AMP by the 3',5'-nucleotide phosphodiesterase (3',5'-PDE) activity of the tissue. The addition of snake venom containing an excess of a potent 5' nucleotidase hydrolyzed the 5'-AMP formed by 3',5'-PDE to adenosine and inorganic phosphate.

The reaction mixture contained in a total volume of 1 ml the following: 300 ul of buffer or endotoxin; 100 ul of snake venom (1 mg/ml); 400 ul of cyclic AMP (2.5×10^{-3} M); and 200 ul of tissue homogenate. All reagents were prepared in Tris buffer (5×10^{-2} M) with $MgSO_4$ (3×10^{-3} M), pH 8.3.

Each sample had its own control which consisted of an identical

reaction mixture to which Tris buffer was added instead of the substrate cyclic AMP. The reaction mixtures were incubated at 37° C for 5, 10 and 20 minutes. The reaction was terminated by the addition of 200 ul of 55% TCA to each incubation flask.

The incubation mixture was centrifuged at 3000 rpm for 10 minutes, and the supernatant fluid was decanted.

Inorganic phosphate reagent consisted of 0.1% ammonium molybdate and 3% ascorbic acid in 0.2 N H_2SO_4. To 0.5 ml of supernatant fluid, 1.5 ml of inorganic phosphate reagent was added and thoroughly mixed with a Vortex. This solution was incubated immediately in a water bath at 37° C for 20 minutes. The absorption at 700 mu was read on a spectrophotometer. These readings were compared with a standard inorganic phosphate curve consisting of readings derived from known concentrations of KH_2PO_4 in 2.5% TCA. A standard curve was established with each experiment.

One unit of enzyme was defined as that amount which caused the production of 1 uMole of inorganic phosphate per minute at 37° C. Specific activity was expressed in units of enzyme per mg of protein. Protein was measured by the method of Lowry et al. (1951).

Statistical Presentation of Experimental Data

Standard deviations (S.D.) were derived from the following equation:

$$S.D. = \sqrt{\frac{x^2}{(N - 1)}}$$

In this equation x equals the deviation from the arithmetic mean and N is equal to the total number of experimental subjects. Standard errors (S.E.) were computed by dividing the S.D. by the square root of N.

CHAPTER III

EXPERIMENTAL RESULTS

Survival Studies

The LD_{50} of endotoxin was established for 10 young guinea pigs weighing from 332 g to 550 g. Endotoxin suspended in saline (2 mg ml^{-1}) was injected intravenously in a dose of 1.5 mg kg^{-1}. Under these conditions 50 per cent of the animals were alive 24 hours post-injection and were defined as permanent survivors.

Elution Pattern for Cyclic AMP and ATP

A mixture of purified radioactive ATP^{C14} and unlabelled cyclic AMP was fractionated with an ion-exchange column. The solution was pipeted into the column and the column was eluted with water. The effluent was collected in eight 1.0 ml fractions. An aliquot of each fraction was read in a spectrophotometer for its optical density at 260 mu and in a liquid scintillation spectrometer for its level of radioactivity (cpm). The ATP fractions were detected by the presence of radioactivity since ATP^{C14} was the only isotope present in the solution.

The resulting chromatographic pattern is shown in Figure 2. The level of radioactivity in each fraction is represented by the dotted line. ATP is not retained by the resin and consequently ATP^{C14} is found primarily in the first 2 to 3 ml of the effluent. The optical density of each 1.0 ml

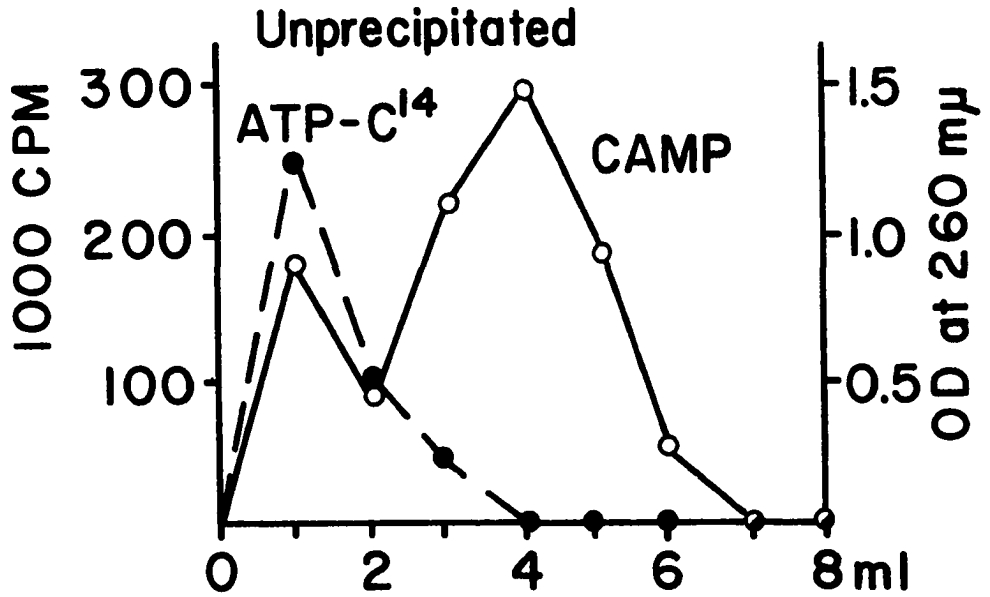


Figure 2. Chromatographic elution pattern for cyclic AMP and ATP.

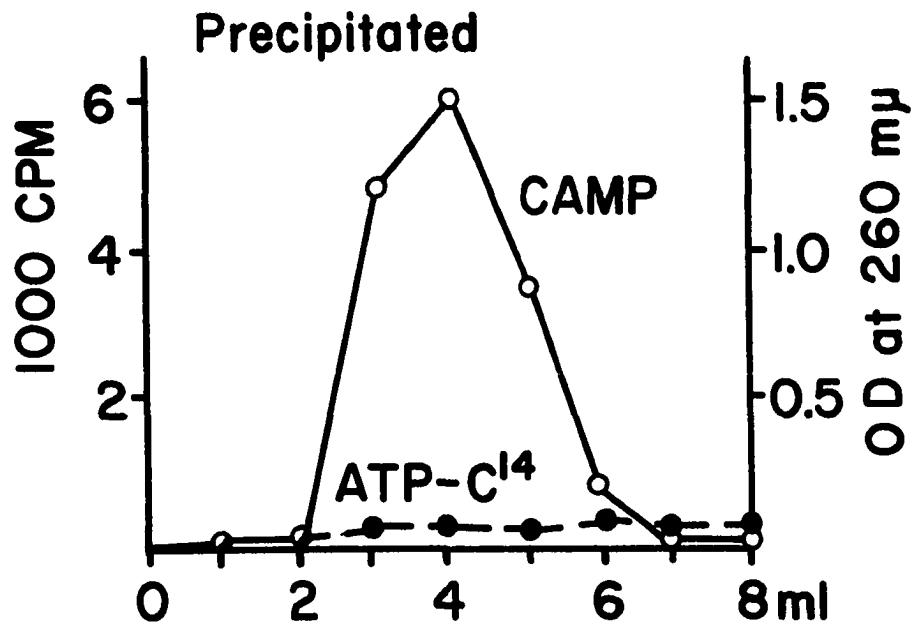


Figure 3. Chromatographic elution pattern for cyclic AMP and ATP after precipitation for the effluent with $Ba(OH)_2$ and $ZnSO_4$.

fraction revealed 2 peaks (solid line). The first peak corresponded to the highest concentration in the effluent of ATP^{C14}. The second peak represented the fractions of effluent which contained cyclic AMP. Thus, cyclic AMP was chromatographically separated from the ATP in the solution.

Figure 3 shows the level of radioactivity and the optical density of the same fractions that are illustrated in Figure 2 after precipitation of each with $Ba(OH)_2$ and $ZnSO_4$. The precipitation of the fractions by this procedure purified the cyclic AMP fraction by the removal of trace contaminants as evidenced by the loss of radioactivity from the effluent. The loss of the first peak in the optical density pattern indicated that this represented the absorption due to ATP in the effluent. The mean of the optical densities of the second peak for milliliter number 3 through 6 indicated that this fraction contained approximately 83 per cent of the cyclic AMP originally pipeted on the resin.

Comparison of Experimental Cyclic AMP Fractions with Known Standards of Cyclic AMP and ATP

Since the assay procedure for adenyl cyclase activity is a measure of the rate of formation of cyclic AMP, it is essential that the fractions which contained cyclic AMP be free of contaminants. Representative aliquots of cyclic AMP fractions from various experiments were spotted on Whatman 3 MM filter paper to be compared with stock solutions of cyclic AMP and ATP. Ascending chromatographic separation in a solvent of ammonium acetate and ethanol showed that cyclic AMP fractions which were collected from actual experiments migrated with the stock solution of cyclic AMP and were separated from the ATP (Figure 4). This procedure was repeated at various stages of the research with consistent results. Impurities

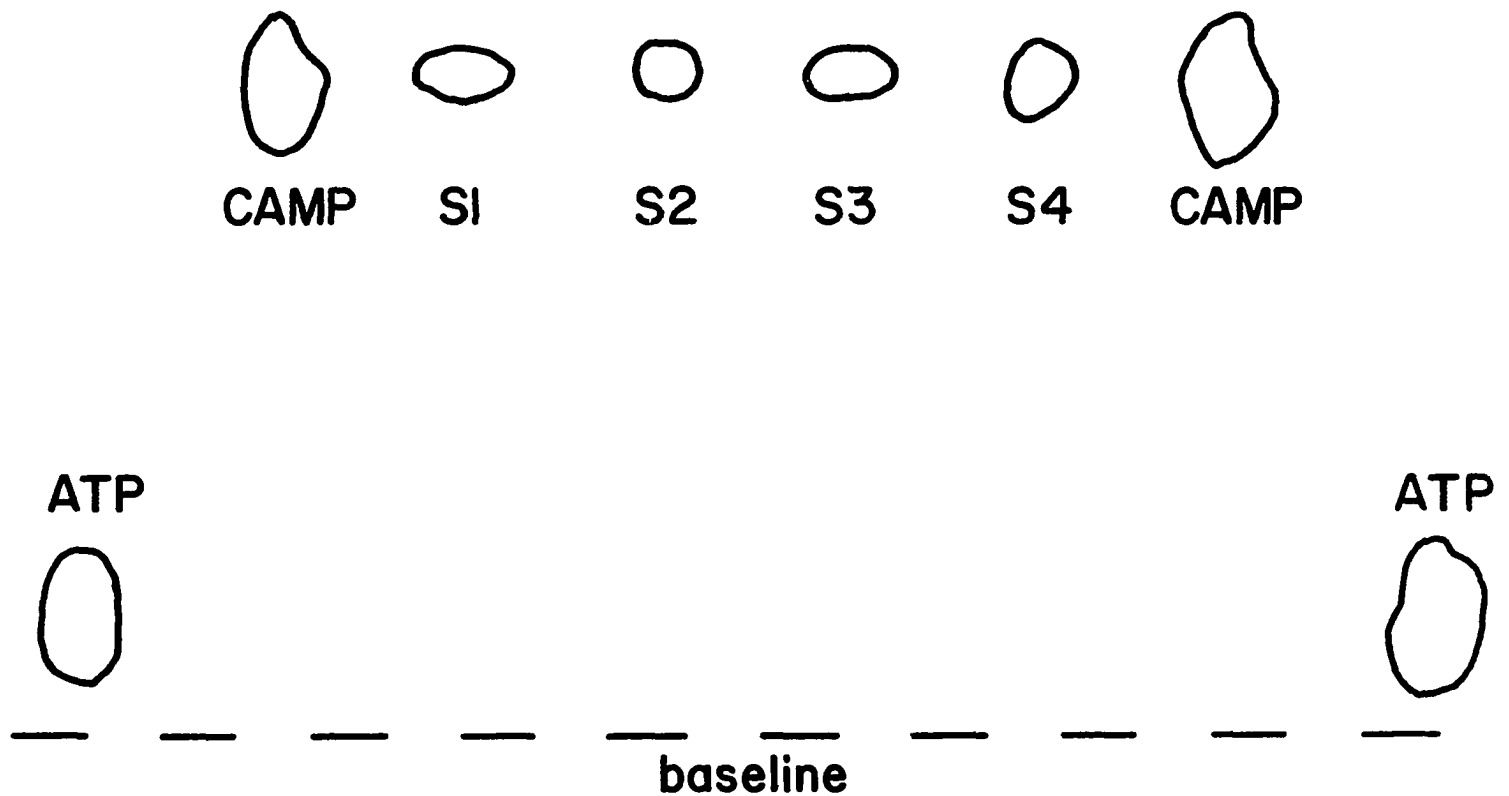


Figure 4. Comparison of experimental fractions of cyclic AMP with stock solutions of cyclic AMP and ATP by paper chromatographic studies. S_1, S_2, S_3, and S_4 - four different fractions of cyclic AMP which were collected from the same experiment.

were never detected in the cyclic AMP fractions derived from an experimental assay procedure. However, in one case an impurity was detected in the stock solution of cyclic AMP; this impurity appeared as a light spot located on the filter paper between those for ATP and cyclic AMP.

Paper chromatograms which were prepared from various experiments indicated that the cyclic AMP fractions which were derived under experimental conditions represented cyclic AMP only and were free from ATP and other metabolic impurities.

Tissue Dilution Studies

Periodically the assay system was checked to verify that the activity of adenyl cyclase in the crude homogenate was a function of tissue concentration. A portion of a homogenate which was prepared for a particular assay was diluted 1:1 with Tris buffer (pH 7.4). The assay was done simultaneously using both the diluted and undiluted homogenate. Cost considerations prevented this procedure from being followed for each experiment.

A 1:1 dilution of the homogenate caused adenyl cyclase activity to be reduced to approximately one half the activity of the undiluted homogenate. As illustrated in Figure 5, these results indicated that a proportional drop in activity occurred over the range of tissue concentration which was used in these studies. Similar results were obtained in all experiments in which this tissue dilution study was performed. Furthermore, this proportional reduction in activity occurred under all experimental circumstances: in the untreated control homogenate, in the homogenate incubated with endotoxin, and in the homogenate treated with sodium fluoride.

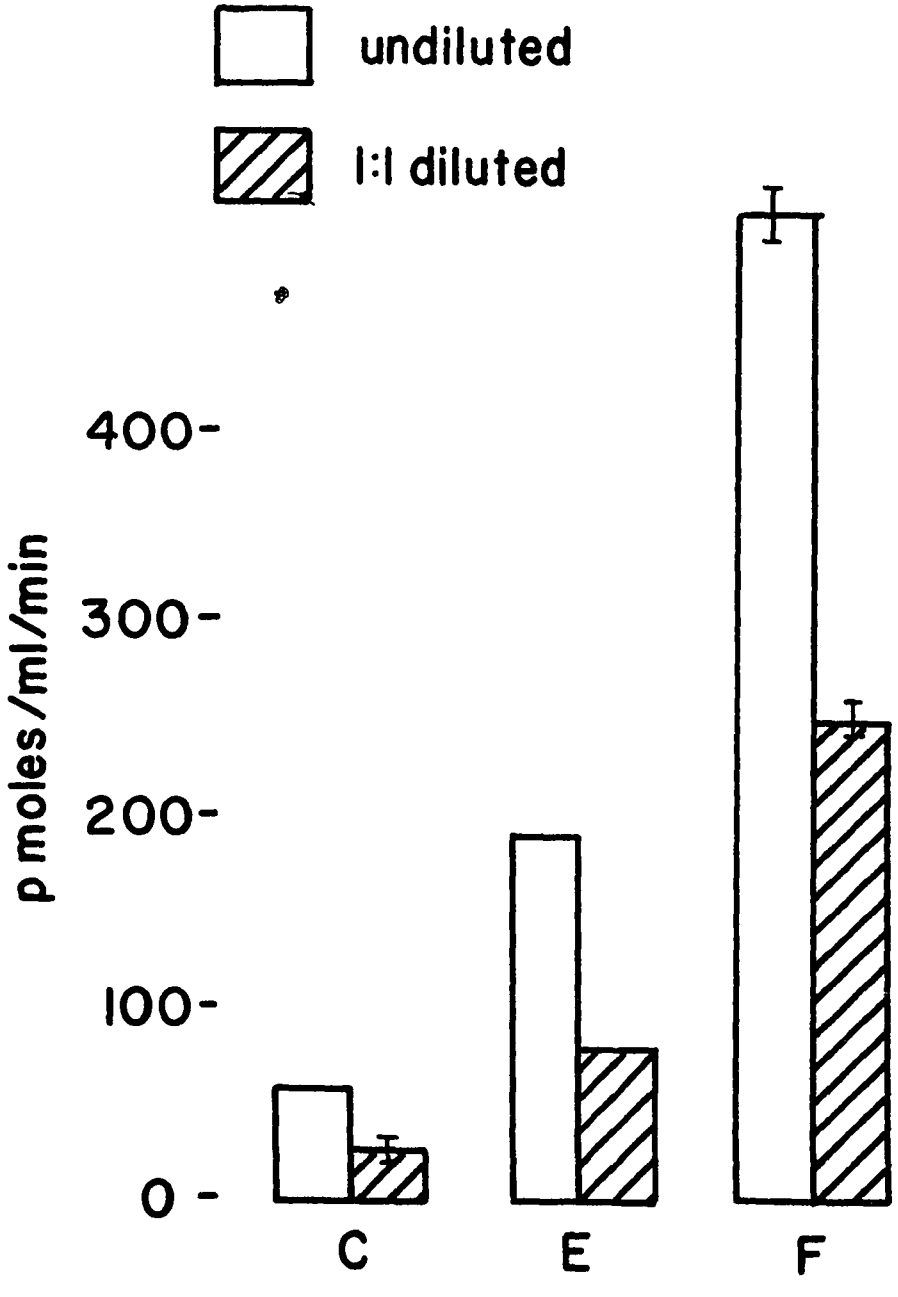


Figure 5. Adenyl cyclase activity as a function of tissue concentration. C - Control homogenate. E - Homogenate incubated with endotoxin (7 ug endotoxin mg^{-1} dry weight undiluted homogenate). F - Homogenate incubated with fluoride ion (10^{-2} M).

Hepatic Adenyl Cyclase Activity During Endotoxin Shock

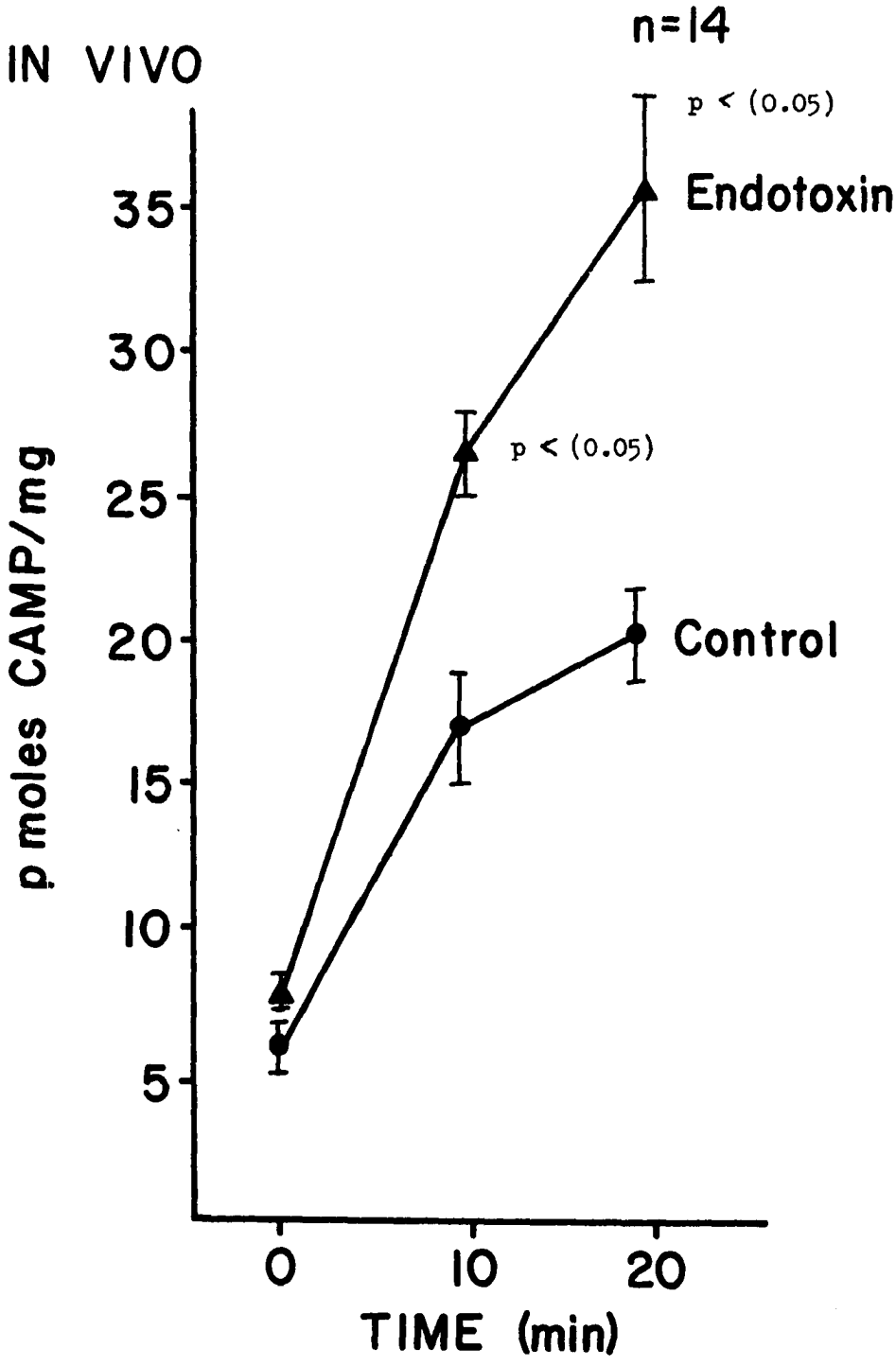
Seven guinea pigs were injected with 3.0 mg endotoxin kg^{-1} body weight. Seven guinea pigs which served as control animals were injected with an equivalent volume of sterile saline. Four hours post-injection, all animals which were administered endotoxin displayed symptoms associated with the shock state. At this time both control and treated animals were lightly anesthetized, midline incisions were made, and liver biopsies were taken from each animal. The tissue was prepared as outlined for the in vivo assay procedure and adenyl cyclase was measured in each homogenate under identical assay conditions.

In these 14 experiments, the activity of adenyl cyclase was always greater in the animals injected with endotoxin than in the corresponding saline-injected control guinea pigs. As shown in Figure 6, hepatic adenyl cyclase activity increased linearly with time in tissue from both saline-injected and endotoxin-injected guinea pigs. The data are presented as mean values ± S.E. The activity of adenyl cyclase in 7 guinea pigs in endotoxin shock was significantly greater than control ($p < 0.05$) at 10 and 20 minutes. The activation of adenyl cyclase as a result of the administration of endotoxin was a consistent finding in all 14 experiments. These results indicated that hepatic adenyl cyclase was consistently activated during the endotoxin shock syndrome.

Incubation of Hepatic Adenyl Cyclase with Endotoxin and Fluoride

The results from the experiments in which in vivo adenyl cyclase activation was measured in vitro during endotoxemia indicated that adenyl cyclase was activated during the shock state. Whether this enzyme was

Figure 6. Hepatic adenyl cyclase activity during endotoxin shock. Mean values, plus or minus standard errors, are shown. Each point represents the mean of 2 observations in each of 7 animals. Control animals (●) were injected with sterile saline. Treated animals (▲) were injected with 3 mg endotoxin kg^{-1} body weight.

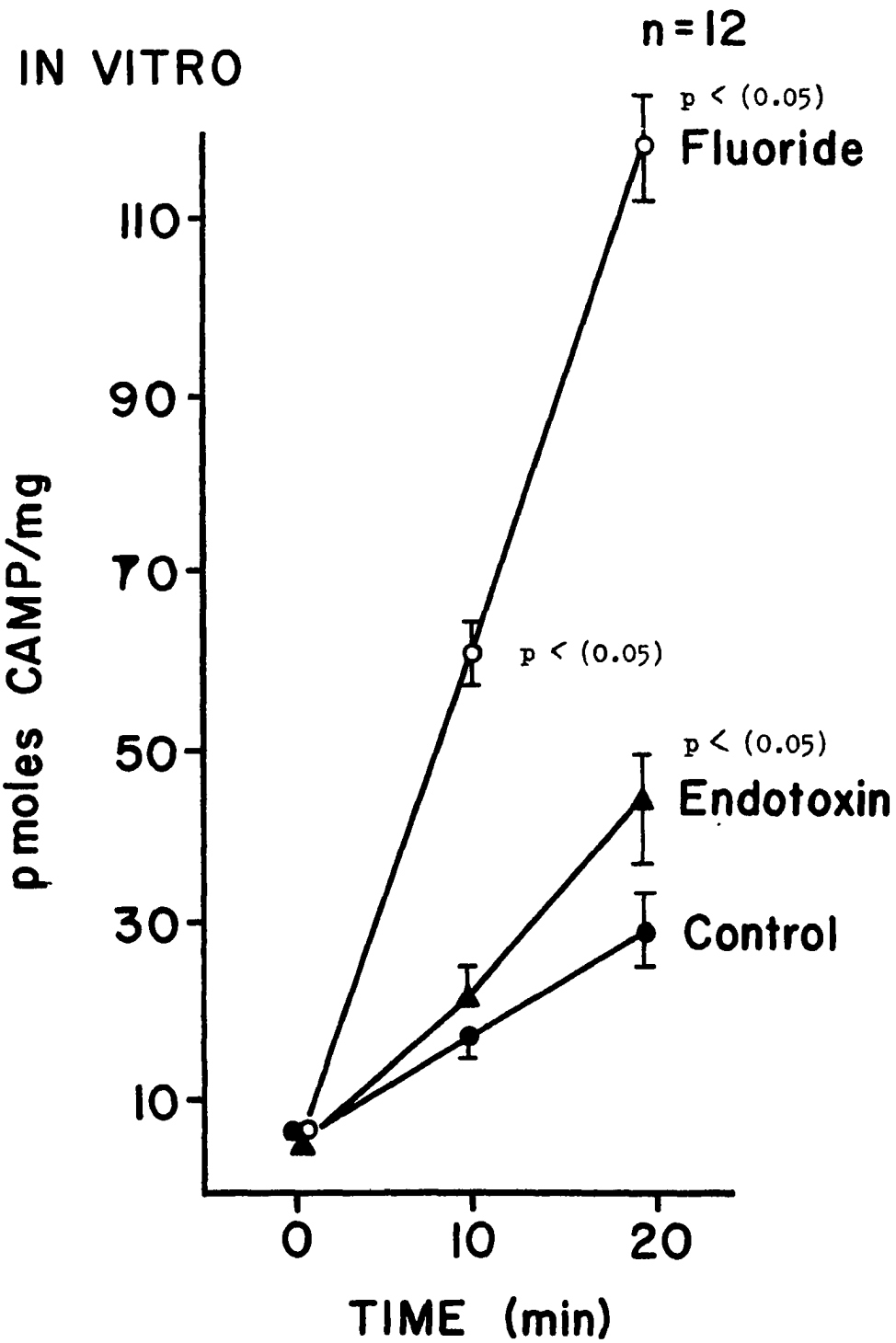

IN VIVO
n=14
p < (0.05)
Endotoxin
p < (0.05)
Control
p moles CAMP/mg
35
30
25
20
15
10
5
0
10
20
TIME (min)

activated as a result of direct binding with the endotoxin complex or was activated indirectly through an intermediary mechanism is unclear from in vivo experiments.

To distinguish between these two possibilities, adenyl cyclase was measured in vitro in liver homogenates which were prepared from 6 untreated guinea pigs. Each homogenate was divided into 3 equal portions. To the control homogenate only buffer was added. The second homogenate was incubated with endotoxin (5-7 ug mg^{-1} dry weight). The third homogenate was incubated with sodium fluoride (10^{-2}M). Fluoride ion, known to be a potent non-specific activator of adenyl cyclase (Sutherland, Rall, and Menon, 1962; Weiss, 1968), was used to demonstrate that the enzyme system could be more fully activated under these experimental conditions.

The results from 12 experiments are given in Figure 7. Each point is expressed as the mean value ± S.E. The activity of adenyl cyclase from the homogenates incubated with endotoxin were significantly greater than controls ($p < 0.05$) at 20 minutes; the activity of homogenates incubated with fluoride ion were significantly greater than those incubated with endotoxin ($p < 0.05$). These results show that adenyl cyclase is activated when the tissue is incubated in vitro with endotoxin. The intensity of stimulation appears to be less than that achieved with fluoride but is approximately twice that of the inactive enzyme. This activation cannot be an indirect manifestation of neural mechanisms, blood-borne intermediaries, or cardiovascular alterations since stimulation occurred during an in vitro incubation of hepatic homogenates with endotoxin. These results strongly suggest that adenyl cyclase is stimulated directly by endotoxin. However, the possibility has not been eliminated that endotoxin may cause

Figure 7. Incubation in vitro of hepatic adenyl cyclase with endotoxin (5-7 ug mg^{-1} dry weight) and with sodium fluoride (10^{-2} M). Mean values, plus or minus standard errors, are shown. Each point represents the mean of 2 observations in each of 7 animals. Control homogenates (●) were incubated with buffer. Endotoxin-stimulated homogenates (▲) were incubated with 5-7 ug mg^{-1} dry weight. Fluoride-stimulated homogenates (○) were incubated with 10^{-2} M sodium fluoride.


IN VITRO
n=12
p < (0.05)
Fluoride
p < (0.05)
p < (0.05)
Endotoxin
Control
pmoles CAMP/mg
110
90
70
50
30
10
0
10
20
TIME (min)

the local release of active metabolites from the tissue during the incubation period.

Centrifugation Experiments

If endotoxin caused the release of some active compound in hepatic tissue which was actually responsible for stimulating adenyl cyclase, then activation of the enzyme would still be an indirect rather than a direct effect of endotoxin. A possible sequence of events for such an indirect mechanism is illustrated in Figure 8.

Low speed centrifugation was used to separate soluble small molecular weight compounds which might be released from the tissue during incubation with endotoxin and which might be responsible for activation of adenyl cyclase. If tissue which was incubated with endotoxin was centrifuged at low speed, small molecular weight compounds would appear in the supernatant fluid. The high molecular weight cellular components and endotoxin would be found in the tissue pellet. By discarding the supernatant fluid and resuspending the tissue pellet in buffer, the activity of the adenyl cyclase in this preparation should differentiate between an indirect and a direct effect of endotoxin. If endotoxin acted indirectly through a mechanism similar to the one outlined in Figure 8, any active small molecular weight intermediary compound would be found in the supernatant fluid. Since the supernatant fluid was discarded, the activity of the enzyme should closely resemble that of an untreated control tissue and should be greatly reduced when compared with the activity of a homogenate incubated after centrifugation with endotoxin. However, if the stimulation of adenyl cyclase was due directly to endotoxin, centrifugation should have little or no effect on the level of activity when

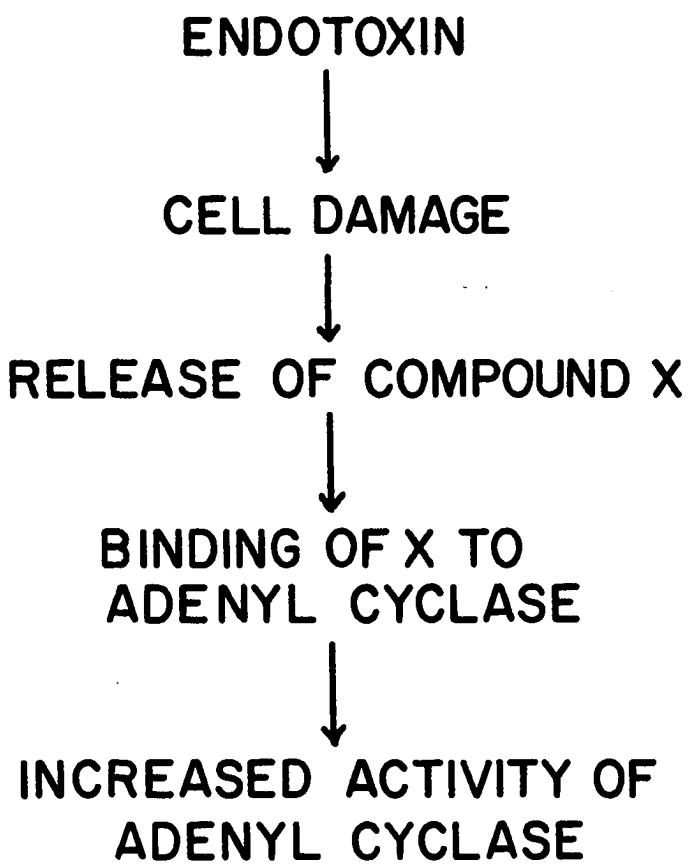
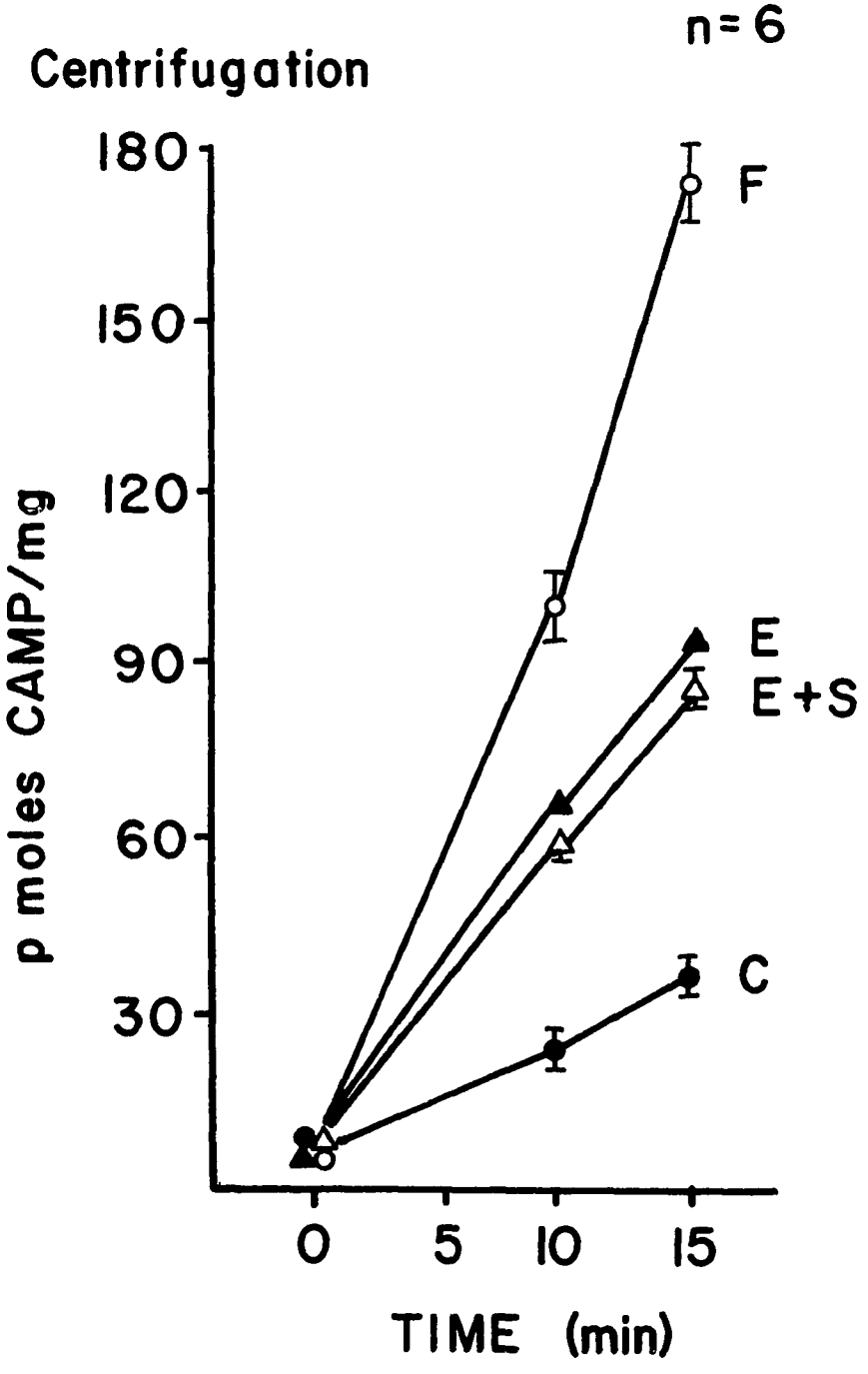


Figure 8. Possible mechanism for indirect activation of adenyl cyclase by endotoxin. X represents possible intermediaries such as polypeptides, catecholamines, serotonin, or lysosomal enzymes.

compared with a homogenate incubated with endotoxin after centrifugation.

The experimental design was as follows: For each assay a portion of liver was removed and was prepared as outlined for the homogenate centrifugation procedure. The results from these experiments are shown in Figure 9. One tissue suspension served as a control (C). The second was assayed in an incubation medium which contained endotoxin (6-8 ug mg^{-1} dry weight) (E). The third was assayed in an incubation medium to which sodium fluoride (10 mM) was added (F). The enzyme activity of these preparations was compared with the suspension of the tissue pellet which contained endotoxin prior to centrifugation (E + S). Each point represents the mean values ± S.E. for 6 experiments. In some cases the S.E. was too small to be represented on the graph. The activity of the enzyme remained approximately the same whether endotoxin was added to the tissue before or after the centrifugation procedure. These data show that an intermediary compound in the supernatant fluid was not responsible for activation of adenyl cyclase; stimulation of the enzyme occurred after the supernatant fluid was discarded. In addition the stimulation was equivalent to the level of activity achieved when endotoxin was added after centrifugation. These results support the concept of a direct interaction of endotoxin with adenyl cyclase. The enzymic activity which was measured in the preparation of a resuspended tissue pellet was greater than that detected in a crude homogenate (Figure 7). The increased sensitivity of the assay system under these conditions is attributable to the loss of soluble phosphodiesterase when the supernatant fluid was discarded. In addition, this procedure concentrates adenyl cyclase. A greater proportion of the resuspended tissue pellet than of a crude homogenate was composed of adenyl

Figure 9. The effect of centrifugation on activation of adenyl cyclase by endotoxin. Mean values, plus or minus standard errors, are presented. Each point represents the mean of 2 observations in each of 3 animals. C - Untreated tissue pellet resuspended in buffer. E - Untreated tissue pellet resuspended in buffer to which endotoxin (6-8 ug mg^{-1} dry weight) was added. E + S - Tissue pellet which was incubated with endotoxin (6-8 ug mg^{-1} dry weight) prior to centrifugation and resuspended in buffer. F - Untreated tissue pellet resuspended in buffer to which fluoride ion (10^{-2} M) was added.


n=6
Centrifugation
180
150
120
90
60
30
p moles CAMP/mg
F
E
E+S
C
0
5
10
15
TIME (min)

cyclase, since the soluble components of the tissue were discarded in the supernatant fluid.

Dialysis Experiments

Tissue dialysis was also used as a procedure to separate possible active compounds which were not a part of the endotoxin complex from the liver homogenate-endotoxin mixture. The rationale behind this experimental design was as follows: If polypeptides or other small molecular weight compounds were released by a mechanism similar to the one outlined in Figure 8, these molecules would diffuse across the dialysis membrane and appear as components of the dialysis fluid. Also if the endotoxin extract contained some small molecule as a contaminant which was responsible for activation of adenyl cyclase, dialysis should separate these small molecules from the high molecular weight endotoxin complex. Due to its large molecular weight (1 to 20 million), endotoxin would not penetrate the dialysis membrane and consequently only small molecules could appear in the dialysis fluid.

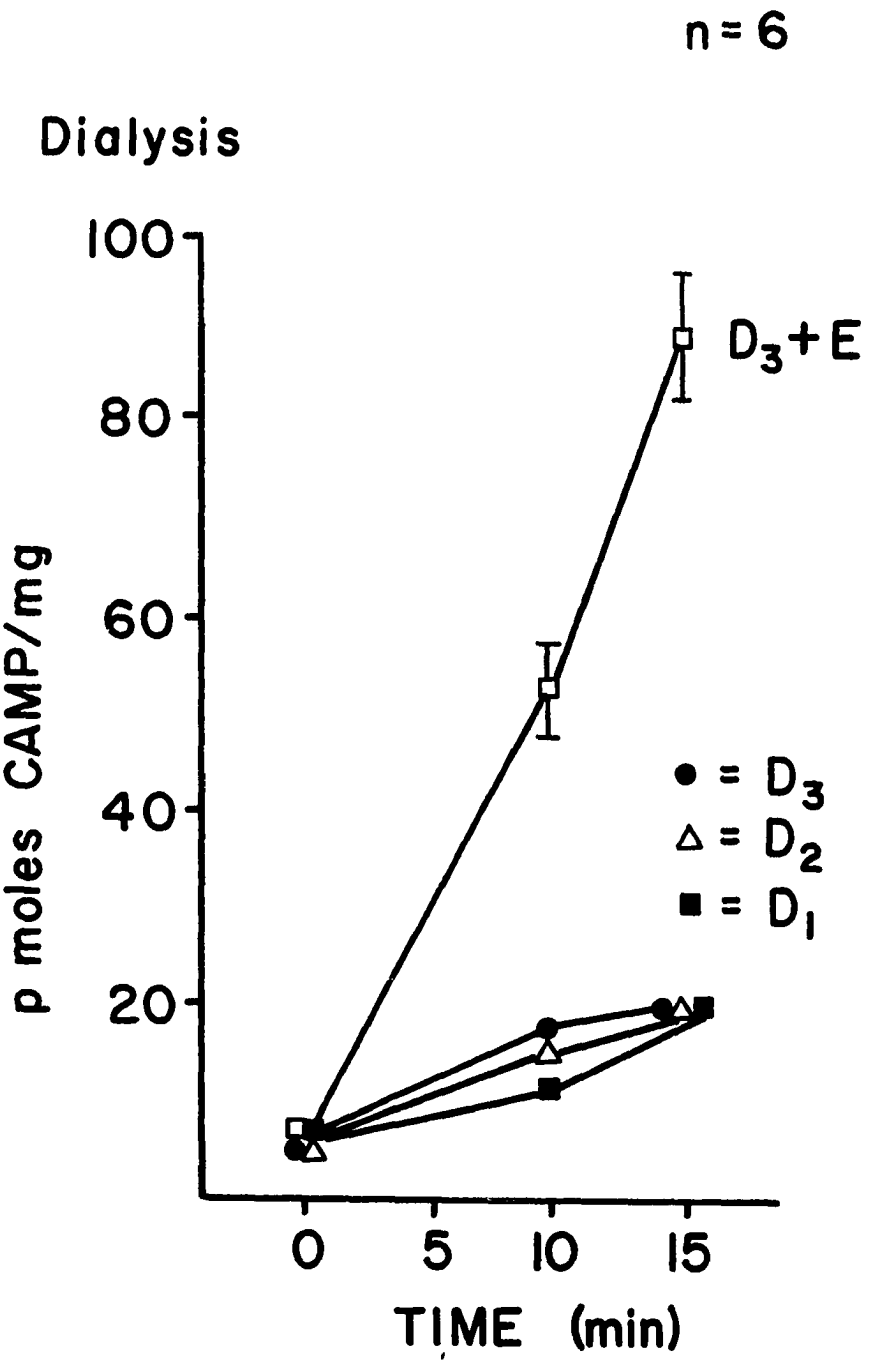
Three different suspensions were dialyzed. The tissue was prepared as outlined for the homogenate dialysis procedure. A suspension of liver homogenate which contained endotoxin, a suspension which contained only endotoxin, and two suspensions which contained untreated liver homogenate were dialyzed against Tris buffer (pH 7.4) for 1 hour at 5° C.

From the same homogenate, four identical fractions were pipeted into glass centrifuge tubes and tissue pellets were prepared by centrifugation. The supernatant fluids were discarded and the 4 tissue pellets were resuspended in 2.5 ml of dialysate from each of the preparations described previously.

Adenyl cyclase activities were measured in 4 identical tissue pellets prepared in the following manner: 1) control values were obtained from the activity of a tissue pellet resuspended in dialysis fluid from an untreated homogenate; 2) the presence of an active contaminant in the endotoxin extract was tested by resuspending a tissue pellet in a dialysate which was derived from a suspension of endotoxin; 3) the presence of an active intermediary compound was investigated by resuspending a tissue pellet in dialysate from a liver homogenate which contained endotoxin; 4) to furnish evidence that endotoxin was capable of activating adenyl cyclase in this preparation in the same manner as in the crude homogenate, a dialysate from an untreated homogenate was used to resuspend a tissue pellet; endotoxin was added to this suspension prior to the assay.

The results from six such experiments are shown in Figure 10. Each point represents the mean values ± S.E. In some cases the S.E. was too small to be illustrated on the graph. Under these experimental conditions, no difference in activity could be detected between the inactive control preparation and this preparation mixed with the endotoxin dialysis fluid. This suggests that the activation of adenyl cyclase is not due to some small molecular weight contaminant of the endotoxin extract. Similarly, no difference in activity could be detected between the inactive control preparation and this preparation mixed with the homogenate-endotoxin dialysis fluid. Stimulation of adenyl cyclase occurred only when endotoxin was directly added after dialysis. These experimental findings suggest that activation of adenyl cyclase required the direct participation of the endotoxin complex and liver tissue.

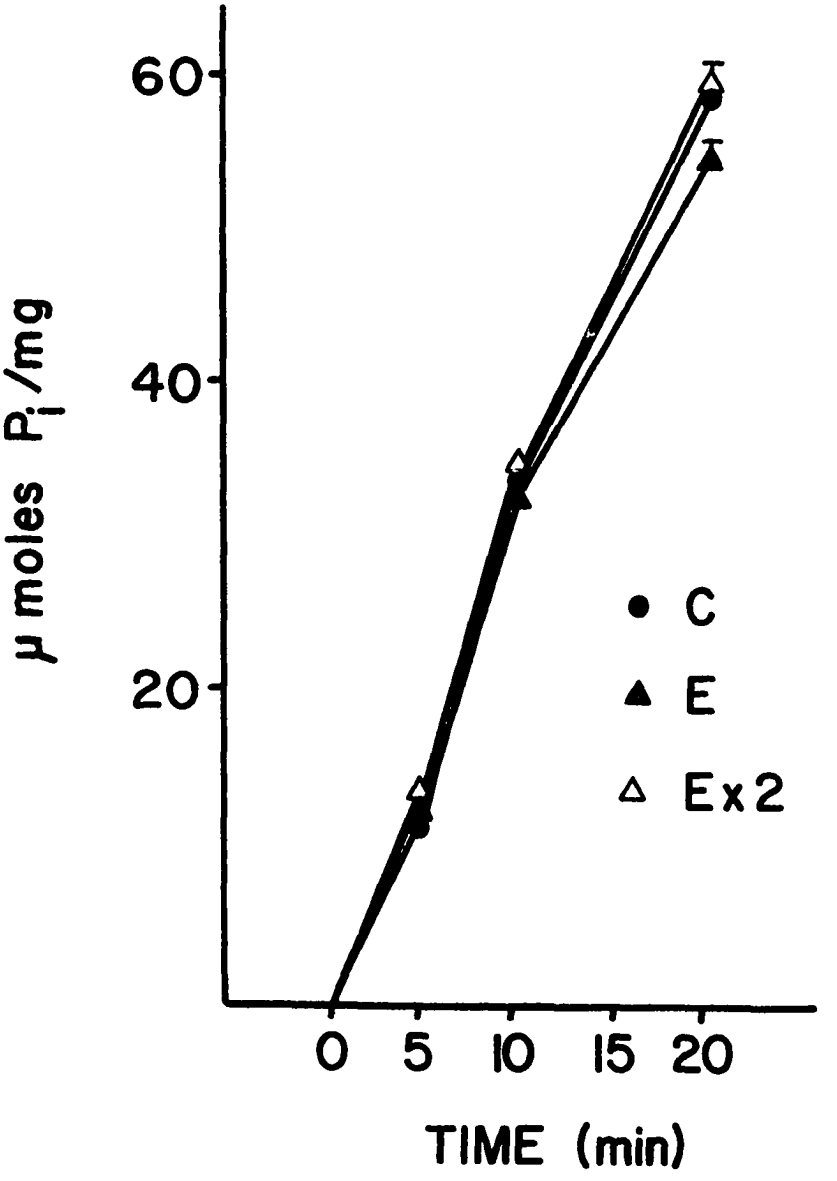
Figure 10. The effect of dialysis on the stimulation of adenyl cyclase by endotoxin. Mean values, plus or minus standard errors, are shown. Each point represents the mean of 2 observations in each of 3 animals. D_1 - Tissue pellet resuspended in dialysis fluid from a homogenate which was incubated with endotoxin (6-8 ug mg^{-1} dry weight). D_2 - Tissue pellet resuspended in dialysis fluid from a suspension of endotoxin (2 mg ml^{-1}). D_3 - Tissue pellet resuspended in dialysis fluid from untreated liver homogenate (control). D_3 + E - Tissue pellet resuspended in dialysis fluid from untreated homogenate to which endotoxin (6-8 ug mg^{-1} dry weight) was subsequently added.


n = 6
Dialysis
100
80
60
40
20
p moles CAMP/mg
D3+E
● = D3
△ = D2
■ = D1
0
5
10
15
TIME (min)

Incubation of Hepatic 3',5'-Nucleotide Phosphodiesterase with Endotoxin

In order to determine if endotoxin influences cyclic PDE activity and to assess the directional changes in cyclic AMP concentration under these conditions, cyclic PDE activity was measured in the presence of endotoxin. Two concentrations of endotoxin were used in each experiment. One was equivalent to the concentration of endotoxin which was used in the assay of adenyl cyclase; the other concentration was approximately twice that which was used in the assay of adenyl cyclase. Both were compared with an identical homogenate to which only buffer was added. The results from 9 such experiments are given in Figure 11. Each point represents the mean values ±S.E. In some cases the S.E. was too small to be represented on the graph. No difference in activity was evident between the homogenates incubated with endotoxin and the homogenates incubated with buffer. These results indicate that endotoxin neither activated nor inhibited cyclic PDE.

Figure 11. The effect of endotoxin on cyclic 3',5'-nucleotide phosphodiesterase activity. Mean values, plus or minus standard errors, are presented. Each point represents the mean of one observation in each of 3 experiments. C - Incubation medium contained buffer. E - Incubation medium contained endotoxin (7-8 ug mg^{-1} protein). E x 2 - Incubation medium contained (15-18 ug mg^{-1} protein). Phosphodiesterase activity expressed as uMoles Pi formed mg^{-1} protein.


60
40
20
μ moles P_i/mg
0
5
10
15
20
TIME (min)
C
E
Ex2

CHAPTER IV

DISCUSSION

There have been no previous reports which were concerned specifically with the effect of endotoxin on the adenyl cyclase-cyclic AMP system. Whether a direct cause and effect relationship exists between stimulation of adenyl cyclase and the toxicity of endotoxin has not been tested. Rather there is a body of indirect evidence which implicates adenyl cyclase as a receptor for the endotoxin complex.

Alterations in carbohydrate matabolism appear to be a consistent manifestation of endotoxin shock under a wide variety of experimental circumstances. Glycolytic stimulation has been shown to be proportional to the severity of the host response to endotoxin (Woods et al., 1961a; Woods et al., 1961b) and to result in severe depletion of carbohydrate reserves (Berry, Smythe, and Young, 1959). This response to the administration of endotoxin has been directly related to maximal activation of glycogen phosphorylase (Hamosh and Shapiro, 1960) and to marked inhibition of glycogen synthesis (Kun, 1948). The key position of cyclic AMP as a regulatory molecule in both of these reactions has been clearly demonstrated (Rall, Sutherland, and Berthet, 1957; Walsh, Perkins, and Krebs, 1968; Huijing and Larner, 1966). The results of the present investigation have demonstrated that adenyl cyclase is activated by endotoxin while

cyclic PDE is unaffected under similar experimental conditions. Therefore it is reasonable to assume that the intracellular concentration of cyclic AMP has increased following activation of adenyl cyclase by endotoxin.

The cardiovascular manifestations of endotoxin shock have been described by numerous investigators. The controversy as to whether this response is the cause or the effect of endotoxin has not been resolved. However it is clear that circulatory failure is an inevitable consequence of the administration of endotoxin (Waisbren, 1951). The identification of adenyl cyclase as the beta receptor of the sympathetic nervous system (Robison, Butcher, and Sutherland, 1967) classifies this enzyme receptor in a position of regulatory control of circulatory hemeostasis. The presence of adenyl cyclase in all tissues and the demonstration of adenyl cyclase activation both in vivo and in vitro by endotoxin suggests that this mechanism may be of some consequence in the vascular responses to endotoxin in view of the similarities between the circulatory response and the beta receptor stimulation.

The physico-chemical characteristics of endotoxin and adenyl cyclase are compatible with the concept of a molecular interaction at the cell membrane. The potency of endotoxin could be explained if the host response was a result of the binding of the toxic lipopolysaccharide to an enzyme receptor site. The location of adenyl cyclase on the cell membrane (Sutherland, Rall, and Menon, 1962) makes it a vulnerable target for a large molecular weight insoluble toxin. Evidence which supports the crucial role of adenyl cyclase in the regulation of intracellular metabolic pathways emphasizes the importance of this enzyme in the cellular response to its environment.

The adenyl cyclase-cyclic AMP system has been recognized as the crucial link between certain endogenous hormones or chemical transmitters and the intracellular processes they regulate. In the terminology of the two-messenger hypothesis, the experimental results of this dissertation support the concept that endotoxin is acting as a "false messenger" or an abnormal signal generator through the same reaction sequence that has been postulated as a common pathway for the various hormones and neurohumoral agents (Butcher, Baird, and Sutherland, 1968).

First the intravenous administration of endotoxin is followed by the activation of hepatic adenyl cyclase in vivo. This response appears to be consistently associated with the endotoxin shock syndrome. The most likely explanation for stimulation of adenyl cyclase appears to be a direct binding of endotoxin with adenyl cyclase. Activation of adenyl cyclase in vitro occurred simply by incubating endotoxin with a crude liver homogenate from a normal guinea pig. Under these latter circumstances activation could not be due to interposed neural mechanisms, vascular alterations, or circulating chemical transmitters.

Finally experimental procedures which were designed to identify active intermediary compounds gave negative results. If endotoxin caused the local release from the tissue of metabolites which activated adenyl cyclase, their presence was not detected by dialysis or by low speed centrifugation of the tissue incubated with endotoxin; both techniques should have separated soluble small molecular weight compounds from the endotoxin-tissue homogenate.

From these data, it was concluded that the most likely explanation for the experimental findings is as follows: Endotoxin activates adenyl

cyclase by binding directly to a receptor site on or associated with a regulatory portion of the adenyl cyclase molecule.

Although speculative, the following sequence of events (Figure 12) relates the conclusions derived from these studies to the hypothesis of Woods and coworkers (1961). Consistent with the results reported here, the binding of endotoxin to adenyl cyclase may be the initial reaction responsible for the endotoxin-induced glycolysis reported by numerous other investigators (Kun, 1948; Fukuda, 1963; Hamosh and Shapiro, 1960). Woods et al. (1961b) proposed that glycolysis, depletion of carbohydrate reserves, and hypoglycemia damaged "glycolytic-sensitive cells." Pathological alterations in cell function led to secondary damage to all cells and if severe enough, death occurred.

In conclusion, critical evaluation of the experimental data is appropriate. This research has measured adenyl cyclase activity in only one tissue of the guinea pig. Clearly before endotoxin-induced stimulation of adenyl cyclase can be regarded as a wide-spread cellular response, this relationship must be established in other species as well as in other tissues. Further, no direct experimental evidence was given for a direct cause and effect relationship between adenyl cyclase activation and endotoxin toxicity.

In all cases, adenyl cyclase activation was studied in broken cell preparations. Certainly this response might be at great variance with what would be observed in the intact cell. Tissue homogenization destroys many physical relationships which exist in the intact cell membrane. As a component of the cell membrane, adenyl cyclase is also subjected to some disruption of its organized structure. This makes absolute quantitation

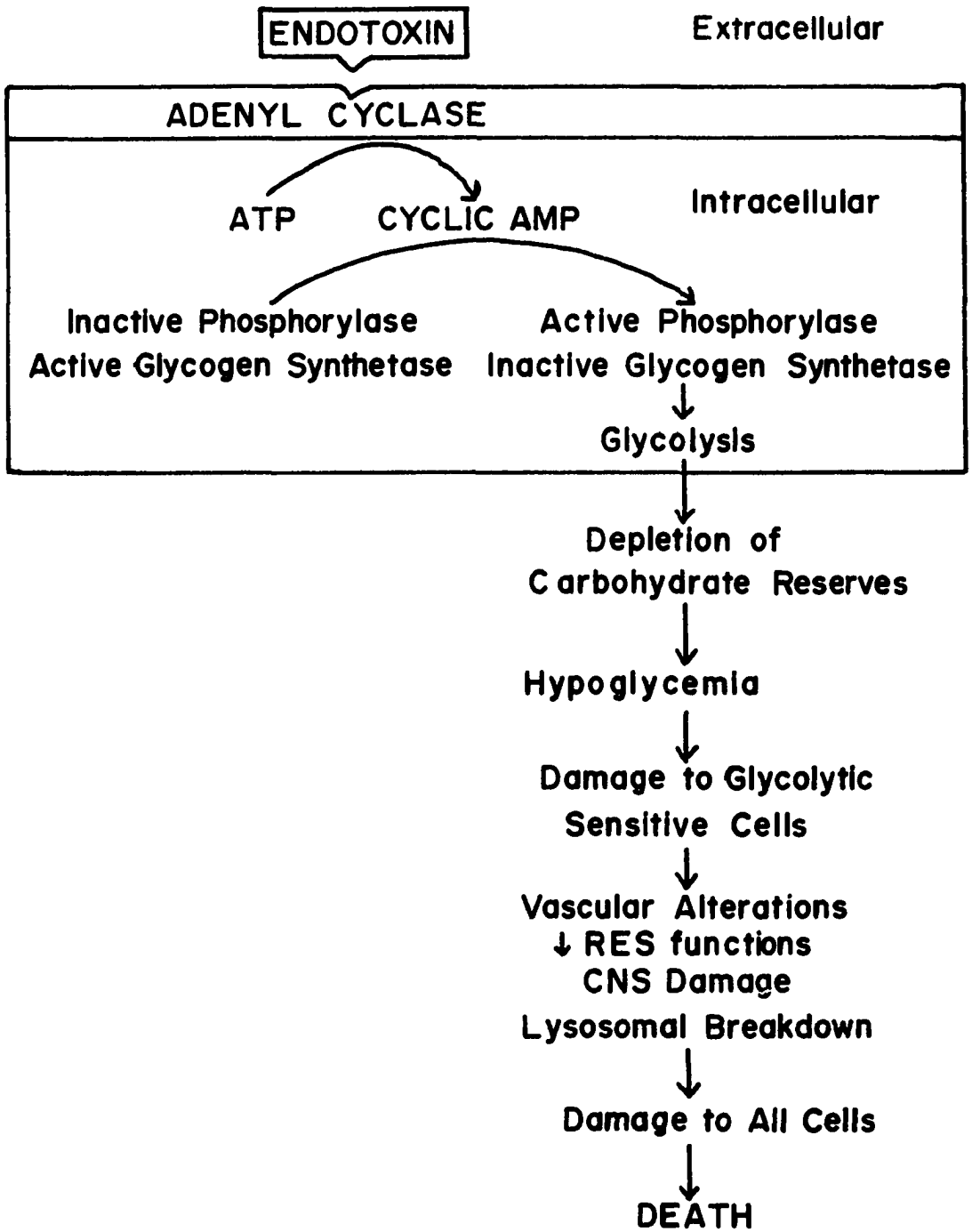


Figure 12. Possible mechanism for glycolytic stimulation by endotoxin.

of enzymatic activation impossible. Earlier investigations indicate that the intracellular accumulation of cyclic AMP in intact cells is often far in excess of that which can be associated with the assay of adenyl cyclase activation in broken cell preparations (Sutherland and Rall, 1958; Rall and Sutherland, 1958).

Finally, while this research strongly suggests a direct role for endotoxin in the activation of adenyl cyclase, the possibility of an intermediary mechanism has not been entirely excluded. For example, it is possible endotoxin caused the local release of an active intermediary which was bound so firmly to the adenyl cyclase receptor site that neither centrifugation nor dialysis was effective in isolating the intermediary compound.

CHAPTER V

SUMMARY

Evidence has been presented to support the hypothesis that endotoxin activates hepatic adenyl cyclase in the guinea pig. Stimulation of hepatic adenyl cyclase occured following the intravenous administration of double an LD_{50} dose of endotoxin into conscious guinea pigs. Activation of this enzyme was a consistent finding during endotoxin shock.

Endotoxin-induced activation of adenyl cyclase appeared to be a direct effect of the lipopolysaccharide on the enzyme. When compared with appropriate control preparations: 1) incubation of endotoxin in vitro with a crude liver homogenate from a normal guinea pig resulted in a 2 to 3 fold increase in adenyl cyclase activity; 2) dialysis of a liver homogenate which was incubated with endotoxin did not yield intermediaries in the dialysate which could themselves activate adenyl cyclase; and 3) centrifugation of a liver homogenate incubated with endotoxin and discarding the supernate did not alter adenyl cyclase activity of the homogenate. These studies demonstrate that incubation of endotoxin with a crude liver homogenate activated adenyl cyclase, and the activation appears to be a direct action of endotoxin.

In addition, a similar preparation showed that endotoxin neither inhibited nor stimulated cyclic phosphodiesterase activity. Therefore,

it is reasonable to assume that the net result of these two enzymatic reactions under the influence of endotoxin is an increased rate of formation and an increased concentration of cyclic AMP in the liver. These effects appear to be directly related to the activation of adenyl cyclase by endotoxin.

These findings are consistent with the concept that adenyl cyclase is the cellular receptor site for bacterial endotoxin.

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