STUDIES ON HEART MUSCLE LIPASES
AND
STUDIES ON 3',5'-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

by

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ABSTRACT

PART I STUDIES ON HEART MUSCLE LIPASES

The study of the role of lipids in supplying the energy requirements of the heart has attracted widespread attention, particularly within the past decade. It is now known that the heart, under normal conditions, oxidizes lipids as its main source of energy.

Numerous investigators have studied the in vivo and in vitro uptake and utilization of exogenously supplied lipids in the form of triglycerides, free fatty acids and ketone bodies. However, very few have studied the utilization of endogenous lipids by the working heart.

We have examined the relative importance of both endogenous glycogen and triglycerides for supplying the caloric needs of the isolated beating rat heart, and found that under the perfusion conditions used, endogenous glycogen appears to supply the initial source of energy.

A lipase in rat cardiac tissue was also examined. The enzyme had a pH optimum near 6.8, and was strongly inhibited by 0.2 M NaF and by 2×10^{-4}M diisopropylfluorophosphate. Most of the activity was found in the nuclear fraction of tissue homogenates. The enzyme hydrolyzed both monoolein and monostearin, and possessed much less activity against tripalmitin. The enzyme also rapidly hydrolyzed the monostearin component of Ediol^{R} (a commercial coconut oil emulsion widely used in lipase studies), and the implications of these findings are discussed. It was concluded from these studies that a lipase other than lipoprotein lipase exists in rat myocardium.

PART II STUDIES ON CYCLIC 3', 5'-NUCLEOTIDE PHOSPHODIESTERASE

In recent years, the study of the role of cyclic 3',5'-adenosine monophosphate (cyclic 3',5'-AMP) in the regulation of several biological reactions and processes has received widespread attention. The presence of a physiological mechanism for terminating the action of cyclic 3',5'-AMP in biological systems would therefore be expected. Indeed, an enzyme, cyclic 3',5'-nucleotide phosphodiesterase has been shown to exist in most mammalian tissues which have been studied for its activity. The central nervous system, particularly the cerebral cortex, possesses a very high activity of this enzyme.

In this study, cyclic 3',5'-nucleotide phosphodiesterase was partially purified from rabbit brain and its properties were studied. The enzyme required Mg^{++} ions for activity and was inhibited by 2×10^{-4}M theophylline. Cyclic 3',5'-dAMP, cyclic 3',5'-GMP and cyclic 3',5'-dGMP were hydrolyzed by the brain diesterase at approximately one-half the rate at which cyclic 3',5'-AMP was hydrolyzed. Little activity against cyclic 3',5'-CMP, cyclic 3',5'-dCMP and cyclic 3',5'-TMP was detected, although cyclic 3',5'-UMP was hydrolyzed at approximately 13% of the rate at which cyclic 3',5'-AMP was hydrolyzed. The brain diesterase therefore possessed a high specificity for cyclic 3',5'-nucleotides with purine bases. Optimum enzyme activity was observed near pH 7.0, and the activity was stimulated about 1.5-fold by 0.06 M imidazole. The K_m value of the enzyme with cyclic 3',5'-AMP as substrate

was approximately 0.8×10^{-4}M. The properties of the partially purified phosphodiesterase from brain were thus very similar to the diesterases which have been purified from beef and dog hearts.

A study of the intracellular localization of the brain diesterase indicated that about 50% of the activity was located in the 105,000 x g supernate. The microsomal and mitochondrial fractions also contained considerable amounts of diesterase activity, but little activity was located in the nuclear fraction.

A survey of cyclic 3',5'-nucleotide phosphodiesterase activity in several available specimens of the plant kingdom indicated the absence of this enzyme activity in these organisms. However, appreciable levels of diesterase activity were detected in E. coli.

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FFA	Free fatty acid
DFP	Diisopropylfluorophosphate
EDTA	Ethylenediaminetetraacetic acid
5'-AMP	Adenosine 5'-phosphate
ATP	Adenosine 5'-triphosphate
UDPG	Uridine diphosphate glucose
Cyclic 3',5'-AMP	Adenosine 3',5'-phosphate
Cyclic 3',5'-UMP	Uridine 3',5'-phosphate
Cyclic 3',5'-dAMP	Deoxyadenosine 3',5'-phosphate
Cyclic 3',5'-dGMP	Deoxyguanosine 3',5'-phosphate
Cyclic 3',5'-GMP	Guanosine 3',5'-phosphate
Cyclic 3',5'-dCMP	Deoxycytidine 3',5'-phosphate
Cyclic 3',5'-TMP	Thymidine 3',5'-phosphate
Cyclic 2',3'-AMP	Adenosine 2',3'-phosphate
Pi	Inorganic phosphate

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PART I

STUDIES ON HEART MUSCLE LIPASES

INTRODUCTION

It is widely known that during starvation, the mammalian organism derives virtually all of its energy requirements from fat catabolism. Less widely realized, perhaps, is the fact that even under normal physiological conditions, mammals depend to a large extent on the oxidation of fats for energy. For example, Fredrickson and Gordon (20) measured the expired $C^{14}O_2$ after injection of C^{14}-labelled albumin-bound long-chain fatty acids into man and indicated that up to 50% of the energy could have been derived from fatty acids during the post-absorptive state. Various tissues and organs have been examined for their ability to oxidize lipids *in vivo* and *in vitro*. In 1930, Richardson *et al.*(65) found that the respiratory quotients of incubated, excised renal and muscular tissues were consistently intermediate between 0.7 and 1.0, and suggested that fats may have participated in the oxidative process. Artom (66), Volk *et al.*(68) and Geyer and associates (69) demonstrated that when carboxyl-labelled C^{14}-fatty acids were incubated with kidney, liver, spleen, heart, lung, brain, skeletal muscle and testis slices, all of these tissues were capable of oxidizing the fatty acids to $C^{14}O_2$. The *in vitro* uptake of fatty acids by the isolated diaphragm was measured by Wertheimer and Ben-Tor (70), and the oxidation of octanoic acid by this tissue was demonstrated by Hansen (71). According to Neptune *et al.*(90,91), isolated diaphragms possess the ability to utilize endogenous lipids. When rat diaphragms were incubated for 4 hours in a substrate-

free medium, there was a slight net decrease in tissue free fatty acids, triglycerides and especially in total phospholipids. Fritz and co-workers (72) measured the uptake and oxidation of acetate-1-C^{14}, octanoate-1-C^{14} and palmitate-1-C^{14} by the isolated diaphragm and skeletal muscle at rest and at work. Their data indicated that electrically stimulated skeletal muscles oxidized twice as much palmitate than resting muscles in the presence or absence of added glucose. Freidberg et al.(73) showed that after 5 minutes of moderate exercise, the plasma FFA concentration in man dropped from approximately 0.82 to 0.61 mM, then increased sharply to about 1.2 mM after cessation of activity. Similar observations were recorded by Issekutz and Miller (74) in dogs, who noted that the decrease in plasma FFA levels was accompanied by a 5-fold increase in oxygen uptake. Fritz (63) has suggested, therefore, that during moderate, sustained work, if the oxygen supply is adequate, skeletal muscles can oxidize a considerable amount of lipids for energy. Indeed, when Havel and his associates (21) infused palmitate-1-C^{14} intravenously into human subjects walking on a treadmill at 3-4 miles per hour, they found a rapid mobilization of fatty acids (presumably from adipose tissue), and an increased rate of oxidation of palmitate-1-C^{14}. The uptake and oxidation of labelled long-chain fatty acids by skeletal muscle in vivo was measured at rest and during electrical stimulation by Spitzer and Gold (40). Their data agreed with the concept that FFA are oxidized by skeletal muscle at rest, and that

FFA oxidation increases during muscular activity. Andres et al.(75) measured the differences in arteriovenous concentrations of O_2, CO_2, glucose and lactate in forearms of human subjects at rest, and concluded that under basal conditions the oxidation of glucose could account for only 7% of the oxygen uptake. Since the mean respiratory quotient of forearm muscle was 0.80, these investigators suggested that the major non-carbohydrate material which served as fuel under the conditions of their experiment was lipid. From the foregoing, it is clear that both diaphragm and skeletal muscles are capable of oxidizing fatty acids directly for energy. Recently, Masoro et al.(92) found that no decrease in endogenous triglycerides or in any of the muscle phospholipids had occurred in monkey skeletal muscles which had been stimulated for 5 hours in situ. They concluded, therefore, that skeletal muscles are capable of oxidizing only exogenously supplied lipids.

The utilization of fatty acids for energy by the brain has been studied both in vivo and in vitro. According to Gordon and Cherkes (77) and Quastel and Wheatley (78), the brain does not utilize lipids for energy. On the other hand, a number of investigators have indicated that the brain is capable of oxidizing lipids to a limited extent (68, 69, 79, 80). However, in view of a number of earlier observations on the R.Q. of the brain (81), it would appear that although the brain possesses the enzymes for oxidizing lipids, the amount of energy derived from this source is insignificant

compared to that contributed by the oxidation of glucose.

Other tissues which have been examined for their ability to oxidize lipids are adipose tissue (82) and liver (66, 68, 69). Isolated mitochondria from the latter tissue actively oxidize lipids, especially in the presence of carnitine (83, 84, 85).

It is well known that the heart functions largely aerobically and one might expect therefore that this organ would be particularly adapted for the oxidation of lipids. Perhaps two of the earliest investigators to suggest that the heart must utilize some substrate other than carbohydrates were Visscher and Mulder (18) in 1930. Using the isolated heart-lung preparation, these investigators discovered that even after 6 hours of work, the same amount of glycogen was found as in the normal, unworked hearts. Furthermore, they suggested that since all the carbohydrates in the heart-lung system could not account for the total energy requirements of the heart, some other non-carbohydrate substrate must have been utilized during the 6 hours of work. Eight years later, Visscher (19) presented quantitative evidence that the non-carbohydrate source may be fat. Again using the heart-lung preparation, he showed that during 3 hours of cardiac work, the total fat content of ventricular muscle decreased from 3.71 ± 0.76 to 3.18 ± 0.68 g per 100 wet weight of cardiac tissue.

In spite of Visscher's investigations (18, 19) and Cruikshank's review in the 1930's (94) on myocardial

metabolism, interest in cardiac lipid metabolism appears to have subsided for almost two decades, until Bing and his associates (22, 86, 87) in 1953 and 1954 reported on the *in vivo* uptake of fatty acids by the myocardium in human subjects. They found that at blood fatty acid levels of 1.105 ± 0.286 mEq/100 ml, the extraction of fatty acids was 0.016 ± 0.013 mEq/100 ml. Their datum is not highly impressive, but their reports on the *in vivo* uptake of fatty acids by the human heart appear to have attracted the interest of numerous investigators to the area of cardiac lipid metabolism. While measuring the transport rate of plasma FFA, Gordon (88) also noted the myocardial extraction of FFA *in vivo*. In accordance with earlier observations, the uptake of FFA by the myocardium *in vivo* was also demonstrated by Ballard *et al.* (89). Their data indicated that in fasting dogs, free fatty acids accounted for only 23% of the total fatty acids extracted by the heart, while the esterified fatty acids made up the other 77%. Similar conclusions were reached by Scott and coworkers (24) who measured the myocardial removal of FFA under normal and pathological conditions in dogs. It is perhaps pertinent to mention here that when Bragdon and Gordon (93) injected C^{14}-labelled chylomicrons into fasted rats and analyzed the various tissues for radioactivity, the tissues with the highest specific activity were the liver and heart, which accounted for about 50% and 25% respectively of the total activities found. More recently, Rothlin and Bing (23) showed that oleic acid was extracted by the heart to a greater

extent than any other long-chain fatty acids in arterial blood. Similar results have been obtained with the isolated perfused heart (98).

It must be emphasized that these in vivo studies, though important, nevertheless gave only an indication that lipids may have been utilized by the heart, since only their uptake by the myocardium was measured. The strongest evidence that lipids in the form of FFA and triglycerides are not only taken up but also oxidized by the heart for energy has come from more recent studies utilizing the isolated perfused heart. Evans and his associates (27) used a closed perfusion system to demonstrate that isolated rat hearts converted about 90% of the palmitate-1-C^{14} in the perfusion medium to $C^{14}O_2$ in 60 minutes. That short-chain fatty acids (C^{14}-labelled acetate, propionate, n-butyrate, n-octanoate) were also readily oxidized directly by isolated dog hearts to $C^{14}O_2$ was shown earilier by Cavert and Johnson (95). Opie et al.(26), Shipp (97) and Shipp and his associates (96) found that whereas palmitate-1-C^{14} was taken up and oxidized by hearts obtained from both fed and fasted rats, glucose-U-C^{14} was oxidized only by hearts obtained from fed rats. Furthermore, when both substrates were made available to the isolated hearts from either fed or fasted rats, palmitate-1-C^{14} was preferentially taken up and oxidized over glucose-U-C^{14}.

Studies with free fatty acids as substrates for the myocardium demonstrated unequivocally their importance as an energy source. However, the question as to whether circu-

lating triglycerides (which actually represent the majority of the total circulating fatty acids *in vivo*) are taken up and oxidized by the myocardium remained unanswered until recent years. The relative importance of esterified fatty acids in supplying the energy demands of the heart was suggested earlier by Ballard *et al.*(89), who found that 77% of the total fatty acids extracted by the heart *in vivo* was in the esterified form. The uptake and oxidation of triglycerides by the isolated perfused rat heart were studied by Gousios and co-workers (33). These investigators found that 30% of the total $d < 1.006$ lipoproteins (labelled with C^{14}-tripalmitin) in the perfusion medium was extracted by hearts from starved rabbits and that 10% of the extracted triglycerides was oxidized to $C^{14}O_2$. Hearts obtained from fed rabbits extracted 15% of the available C^{14}-tripalmitin and oxidized 8% of the extracted triglyceride to $C^{14}O_2$. Olivecrona (31) and Olivecrona and Belfrage (32) injected C^{14}-glycerol-H^3-palmitate-labelled chylomicrons intravenously into rats and examined the distribution of the labels in a number of tissues, including liver, heart and adipose tissues. They concluded from their studies that the heart (and adipose tissue) took up chylomicron triglycerides intact, and that extensive and rapid hydrolysis of this glyceride occurred probably near the plasma membrane. The uptake and oxidation of $d < 1.006$ lipoprotein triglycerides by the isolated rat heart was also demonstrated by Delcher and associates (34) who found that about 40%-90% of the total CO_2 was derived

from the exogenously supplied triglycerides, and thus concluded that lipoprotein triglyceride fatty acids were the primary source of fatty acids for the heart.

It should be mentioned briefly that other substrates have also been shown to be rapidly metabolized by the heart. Williamson and Krebs (29), Williamson (30) and Hall (99) demonstrated that acetoacetate, acetate and β-hydroxybutyrate were, as might be expected, oxidized rapidly by the perfused rat heart in preference to glucose.

In summary, the evidence presented by numerous investigators demonstrates clearly that a variety of mammalian tissues are capable of oxidizing lipids for energy. The heart, in particular, appears to depend primarily upon lipids as its source of energy. The importance of FFA in myocardial metabolism has been reviewed recently by Evans (28) and by Bing (100). The comparative aspect of muscle metabolism, with special emphasis on the importance of lipid metabolism in insects, birds and fishes has been reviewed by Drummond and Black (76).

The intracellular fate of FFA's which are transported to the myocardium (either complexed with serum albumin or as chylomicron triglycerides) has been examined, and the evidence indicates that not all of the FFA's are directly oxidized by the myocardium; a portion is re-esterified to triglycerides and phospholipids. For example, Shipp (97) found that when rat hearts were perfused with 0.5 mM palmitate-1-C^{14} for 30 minutes, 4.69 ± 0.16 μmoles of palmitate

were taken up per gram weight of tissue, 2.34 ± 0.24 μmoles were recovered as $C^{14}O_2$, and 1.68 ± 0.06 μmoles recovered as tissue lipids. In hearts perfused for 1 hour with 0.5 mM palmitate-1-C^{14}, 65.4 ± 2.3% of the C^{14} in tissue lipids was recovered as triglyceride, 14.1 ± 1.1% as phospholipids, 6.6 ± 0.5% as FFA, and 13.9 ± 1.7% as cholesterol. The synthesis of triglycerides in rat hearts *in vivo* from injected palmitate-1-C^{14} was shown earlier by Borgstrom and Olivecrona (101). According to Stein and Stein (103), the isolated perfused rat heart incorporated palmitate-1-C^{14} into tissue lipids, the triglycerides accounting for 70-75%, and the phospholipids, 25-30%, of the label incorporated. Qualitatively, similar observations have been recorded by Shipp *et al.* (35) and by Olson (102). Hence it is reasonable to assume that FFA's which are taken up in excess of the immediate energy requirements are re-esterified and stored in the form of phospholipids or triglycerides. Indeed, electron micrographs of cardiac muscle cells often show abundant amounts of lipid droplets often adhering to the mitochondria.

The utilization of these *endogenous* lipids for energy by the myocardium was investigated by Shipp and his associates (35, 36, 104) and also by Denton and Randle (37). Essentially, the observations made by Shipp *et al.* were that when glycogen-depleted rat hearts (whose intracellular lipids had been pre-labelled with C^{14} *in vivo*) were perfused in a closed system with substrate-free buffer, the production of $C^{14}O_2$ gave direct evidence that endogenous lipids were

oxidized. Furthermore, they stated that the net decrease in endogenous C^{14}-labelled phospholipid content alone could account for over 75% of the total metabolic CO_2 formed under these conditions. However, in direct contrast to these observations, Denton and Randle (37) showed that after 60 minutes of substrate-free perfusion, the triglyceride content fell from 18.7 ± 0.8 to 8.7 ± 0.7 μmoles per gram dry weight of tissue, and there was no change in endogenous phospholipid levels. These contradictory observations present much difficulty in assessing the exact role of endogenous lipids as potential fuel for the working heart muscle, and therefore this particular aspect of cardiac metabolism must still be considered open for further investigation.

The role of endogenous lipids in supplying the energy requirements of the heart may be uncertain, but as mentioned earlier, the importance of exogenously supplied chylomicron triglycerides in this respect cannot be over-emphasized. Complete agreement exists among researchers (31, 32, 33, 34) that chylomicron triglycerides are taken up intact by the myocardium and subsequently hydrolyzed rapidly to FFA's. These observations immediately suggest that lipolytic enzymes must exist in heart cells to hydrolyze triglycerides to FFA and glycerol. Indeed, an active enzyme, lipoprotein lipase, was characterized in cardiac tissue in 1955 by Korn (38, 39). This enzyme was also detected in liver, kidney, spleen, aorta, lung, skeletal muscle and adipose tissue, by Korn (38, 47), as well as in post-heparin plasma (42) and diaphragm (50).

Lipoprotein lipase has been extensively studied by numerous investigators, and since it has been the subject of a fairly recent review (41), only the salient features of this enzyme will be presented. Lipoprotein lipase has been reported purified from post-heparin plasma 1480-fold by Hollett and Meng (42) using isoelectric precipitation and ammonium sulfate fractionation procedures. The purified preparation was optimally active at pH 8.5 and its activity destroyed by heating for 5 minutes at 50°. The natural substrate for lipoprotein lipase appears to be chylomicrons, which are composed of about 90% triglycerides, some phospholipids, cholesterol esters and about 2% protein. Coconut oil emulsions and other artificial triglyceride preparations are attacked at only a slow rate by the enzyme, unless small quantities of serum are present (39). A unique property of lipoprotein lipase is its sudden appearance in the circulation after heparin administration, as first observed by Hahn (105). Indeed, the enzyme is eluted within minutes from adipose tissue and heart when these organs are perfused with buffer containing heparin and serum (43, 44, 45, 46). The pH optimum for this enzyme is near 8.5 and its activity is inhibited 100% by 0.2 to 1.0 M NaCl (38, 43, 45, 48) and 30-60% by protamine sulfate, 20 mg/ml (43, 48). The enzyme is very slightly (0-7%) inhibited by 0.2 M NaF (43, 48, 49). Hollenberg (50) and Alousi and Mallov (17) have noted a 2-3 fold increase in lipoprotein lipase activity in hearts obtained from 3-4 day fasted rats. A similar increase in enzyme activity was observed by Nikkila *et al.*

(51, 52) in the myocardium of rats which had been subjected to moderate exercise for 90 minutes. The mode of action of lipoprotein lipase has been investigated by Borgstrom and Carlson (53), Carlson and Wadstrom (54) and most recently, by Payza et al. (106). Carlson and Wadstrom's data (54) indicates clearly that chylomicron triglycerides are hydrolyzed rapidly to monoglycerides, but the hydrolysis of the latter glyceride occurred very slowly. This was illustrated by the rapid (150-fold) increase in monoglyceride content within the first 5 minutes of incubation, accompanied by decreases in triglyceride and diglyceride levels. Payza and co-workers (106) have similarly shown that monoglycerides accumulated when an artificial coconut emulsion (Ediol^{R}) was used as substrate. It is reasonable to conclude, therefore, that monoglycerides are not hydrolyzed to any extent by lipoprotein lipase, and that its action is specific for triglycerides, and may even extend to diglycerides. The physiological importance of lipoprotein lipase in cardiac energy metabolism cannot be underestimated.

In adipose tissue, a lipase possessing properties considerably different from that of lipoprotein lipase was shown to exist by Rizack (107). Using Ediol^{R} as substrate, he showed that the enzyme was inhibited 16% by 0.6 M NaCl, 7% by 8 x 10^{-4}M EDTA, 66% by 0.2 M NaF, but not inhibited by protamine sulfate, 300 µg/ml. The optimum activity was observed near pH 6.5 as compared with pH 8.5 for lipoprotein lipase. The most interesting feature of this lipase was

that it could be re-activated when incubated with epinephrine and tissue sediment, suggesting an important means of controlling free fatty acid release from adipose tissue. It was later reported by Rizack (108) that the enzyme could be activated *in vitro* by 2×10^{-5}M cyclic 3',5'-AMP. Much interest is currently being directed toward the possibility that this enzyme is under hormonal control and therefore regulates the output of free fatty acids from adipose tissue.

Recently, Bjorntorp and Furman (13) reported that a lipolytic activity, similar to that observed by Rizack (107) in adipose tissue, existed in rat hearts. Using Ediol^{R} as substrate and crude extracts as the enzyme source, these investigators indicated that the lipolytic activity was optimal near pH 6.8, slightly inhibited (8%) by 0.5 M NaCl but strongly inhibited (68-100%) by 0.2 M NaF. It was not inhibited by protamine sulfate, 400 µg/ml. Furthermore, they reported that when heart tissue from fasted rats was incubated in the presence of epinephrine, 1 µg/ml, the activity increased from 12.70 ± 1.30 to 13.60 ± 1.60 (µmoles FFA released/g tissue/hour). They concluded that in addition to lipoprotein lipase, another lipolytic component existed in rat cardiac tissue, whose function was perhaps analogous to that of the lipase found in rat adipose tissue by Rizack (107).

Evidence supporting the concept that lipids play a major role in supplying the energy demands of the mammalian heart has been presented. It may be concluded that the heart *in vivo* derives part of its energy from the direct oxidation of

albumin-bound free fatty acids which are taken up from the arterial circulation. However, the fuel for muscle metabolism is to a much greater extent derived from exogenously supplied chylomicron triglycerides and very low density lipoproteins. It follows, therefore, that cardiac lipases must play a vital role in providing a source of oxidizable fatty acids for the myocardium. The question as to whether endogenously stored cardiac lipids are readily mobilized and utilized for energy has not been unequivocally answered. The work to be described in this thesis was undertaken to further study the nature of lipolytic activities in heart muscle. Special attention has been directed toward lipolytic activity other than lipoprotein lipase activity. We have been particularly interested by the suggestion that a cardiac lipase may exist which is activated by epinephrine. An investigation to provide additional insight into the possible utilization of endogenous triglycerides by isolated perfused rat hearts is also reported.

EXPERIMENTAL PROCEDURE

Materials

Glycogen was obtained from Nutritional Biochemical Company. Diazyme^{R}, an amyloglucosidase preparation, was purchased from Miles Chemical Company. Glucostat^{R} reagent, which contains glucose oxidase and horseradish peroxidase, was purchased from Worthington Biochemical Corporation. Ediol^{R}, which was generously provided by Dr. Martin Rizack of the Rockefeller Institute, New York, contains coconut oil 50%, sucrose 12.5%, glyceryl monostearate 1.5%, and polyoxyethylene sorbitan monostearate 2.0%.

Bovine serum albumin (Fraction V) was purchased from Sigma Chemical Company and purified before use by the method of Goodman (1) as follows: 50 g of the crude albumin was dissolved in 200 ml of glass-distilled water by simply placing the albumin powder over the water and allowing it to dissolve overnight. The resultant dark amber solution was then lyophilized; the residue was powdered with mortar and pestle, covered with anhydrous 2,2,4-trimethylpentane containing 5% acetic acid, and finally placed in the cold room overnight. As much as possible of the acetic acid-trimethylpentane extraction solvent was then aspirated, and the albumin washed twice with anhydrous trimethylpentane. Agitation of the albumin suspension during the extraction process with organic solvents was kept to a minimum to reduce the extent of protein denaturation. After aspiration of the trimethylpentane, the

albumin was again covered with the anhydrous 5% acetic acid-trimethylpentane mixture, and stored in the cold room overnight. The removal of the acetic acid-trimethylpentane mixture and washing with anhydrous trimethylpentane was repeated. The organic solvent was removed under vacuum, and the powder obtained was taken up in a suitable volume of glass-distilled water. To remove the last trace of acetic acid, the albumin solution was dialyzed by continuous flow for 3 days against a total volume of 60 liters of demineralized water, followed by 20 liters of glass-distilled water. The solution was then lyophilized and the extracted albumin stored in the deepfreeze until required. Commercial albumin (Fraction V) contains about 0.60 eq FFA/mole. After extraction by the method of Goodman (1) just described, the content of FFA is reduced to about 0.14-0.18 eq/mole.

Monoolein (Calbiochem, "90% pure") was made free of trace triglyceride contaminant by adsorption on 80-200 mesh silicic acid, followed by elution with chloroform:methanol, 2:1. Tripalmitin was obtained from Eastman Organic chemicals and purified ($>$ 99%) by silicic acid chromatography. Tripalmitin-1-C^{14} (96% pure) was purchased from Nuclear-Chicago Corporation. Commercial monostearin was obtained from the Faculty of Pharmacy, U. B. C., and re-crystallized twice from hot ethanol before use. Silica gel GF (Merck) was secured from Canadian Laboratories.

Methods

I. Perfusion Studies

Normal fed and 3-day fasted female Wistar rats weighing between 275 and 325 grams were used. The animals were stunned by a blow on the head, their hearts removed immediately and attached to a cannula of a Langendorf perfusion apparatus. The apex of the hearts was secured to a Stratham Force Displacement Transducer, a 5-gram tension applied, and the rate and strength of contractions recorded on a Grass Model 5D polygraph. The flow rate was adjusted as required for maximal efficiency of the heart, usually between 5 and 8 mls per minute. The perfusion medium was carbogenated Krebs-Ringer bicarbonate solution at pH 7.4, 37°. When epinephrine was added to the perfusion fluid, it was injected with a Lambda Pump Driver at the rate of 0.2 to 1.0 µg per minute. When heparin was used, it was injected at the rate of 60 µg per minute.

At the end of the perfusion period, the hearts were removed from the apparatus and the auricles cut away and discarded. The ventricles were carefully blotted to remove excess water and divided in two in such a way as to provide approximately equal parts of the left and right ventricular tissues for subsequent glycogen and triglyceride analyses. Samples thus obtained were immersed in liquid nitrogen within one minute following termination of perfusion, then assayed on the following day.

Tissue Glycogen -- Tissue glycogen was assayed enzymatically

according to Johnson et al (2). A sample of ventricular tissue weighing between 150-250 mg was placed in a graduated 12-ml centrifuge tube containing 1.0 ml of 30% KOH. The tube was placed in boiling water for 20 minutes, the contents cooled, and 1.25 ml 95% ethanol was added to precipitate the glycogen. The contents of the tube were mixed thoroughly with a glass rod. The tube was chilled in ice for 15 minutes, then the contents heated to a boil in a water bath. The precipitate was collected by centrifugation for 15 minutes, using a bench top Model H International centrifuge. The supernatant fluid was decanted and the precipitate dissolved in 1.0 ml glass-distilled water. Then 1.25 ml 95% ethanol was added to re-precipitate the glycogen, and the tube chilled and centrifuged as before. The sediment was taken up in 2.0 ml of glass-distilled water, and usually an 0.2 ml aliquot was taken for glycogen determination.

The amyloglucosidase solution used for the glycogen assay was prepared by mixing 200 mg Diazyme^{R} with 100 ml 0.1 M potassium phosphate buffer, pH 6, and filtering. The enzyme solution was stored at 4^{o}, and discarded after one week. The glucose oxidase-horseradish peroxidase reaction mixture (Glucostat^{R} x 4) was prepared by first dissolving the contents of the smaller (chromagen) vial in 4.0 ml methanol. The contents of the larger (enzyme) vial were then dissolved in about 380 ml of buffered glycerol (4 volumes glycerol plus 6 volumes 0.04 M potassium phosphate buffer, pH 7), the chromagen solution added, and made up to 400 ml

with buffered glycerol. This preparation was stored in the deepfreeze in small individual quantities, and was stable to repeated freezing and thawing.

The incubation mixture for glycogen determination consisted of the following: 0.2 ml aliquot of the glycogen solution, 1.0 ml of Glucostat^{R} reagent, 1.0 ml of Diazyme^{R} solution, and 0.8 ml glass-distilled water. The mixture was incubated for 1 hour at 37^{o} and the reaction stopped by the addition of 0.5 ml 2 N HCl. The optical density was read at 400 mμ in a Beckman DU spectrophotometer, using a light path of 1.0 cm. The standard curve for glycogen in the range, 5-100 μg is illustrated in Fig. 1. Recovery experiments indicated 90-95% recovery of added glycogen.

Tissue Triglycerides -- Tissue triglycerides were extracted by the method of Folch et al (7). Ventricular tissues weighing between 150 and 250 mg were minced and homogenized for at least 7 minutes in a glass mortar (with a loose fitting Teflon motor-driven pestle) with 18 volumes of chloroform-methanol mixture (2:1). The flaky suspension was filtered through paper into a 12-ml centrifuge tube, using 2 volumes of the chloroform-methanol mixture as a final rinse. Four volumes of glass-distilled water was then added, the tube shaken by hand, and centrifuged. The upper phase was carefully removed with a pipette, and a 2.0 ml aliquot of the lower phase transferred to a screw-capped tube and evaporated to dryness under a gentle stream of nitrogen. The residual lipid was assayed for triglycerides by the original

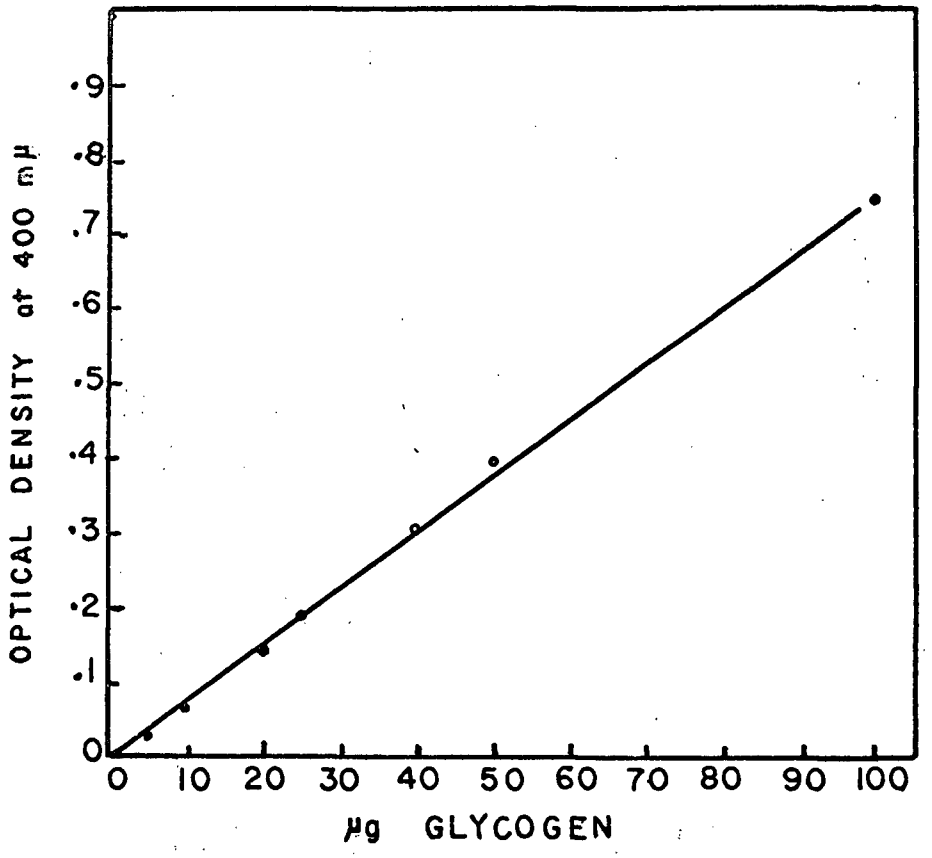


FIG. 1 Glycogen concentration versus optical density.

The assay of glycogen was performed with the coupled amyloglucosidase-glucose oxidase technique of Johnson et al. (2) as described in the text, except that the quantities of glycogen were varied.

method of Van Handel and Zilversmit (3) as modified by Jagannathan (4), except that silicic acid and diisopropyl ether were substituted for zeolite and chloroform, respectively. To the lipid residue was added 1.2 g activated silicic acid followed by 7.5 ml diisopropyl ether, and the contents shaken on a mechanical shaker for 30 minutes. After centrifugation at low speed, a 4.0 ml aliquot was transferred to another screw-capped tube and the organic solvent evaporated to dryness under a gentle stream of nitrogen. The rate of evaporation was increased by immersing the tube in a water bath at 60°. To this residue was added 1 drop of 2.5% KOH and 1.0 ml of aldehyde-free ethanol, and the triglycerides hydrolyzed to glycerol and fatty acid salts by heating for 30 minutes at 60°. Two drops of 6% acetic acid were then added, and the contents evaporated to dryness in an oven at 55° using a gentle stream of air to hasten the process. To the dry contents were added 10.0 ml petroleum ether (b.p. 35-60°) and 2.0 ml 0.7 N H_2SO_4. The tube was then capped tightly and inverted 25 times. The petroleum ether layer was removed by aspiration and discarded. To remove the last traces of the organic solvent, the tube was heated in an oven for 15 minutes at 60° under a gentle stream of air. Three drops of 25 mM sodium metaperiodate solution were then added, and the contents of the tube mixed thoroughly. After 10 minutes, 0.2 ml of freshly prepared sodium bisulfite (10% w/v) solution was added and the contents mixed thoroughly. Ten ml of chromotropic acid solution were finally added, the tubes

shaken and heated in a boiling water bath for 30 minutes. After cooling to room temperature, 1.0 ml of 5% thiourea solution was added, the contents mixed and the intensity of the colour was read at 570 mμ in Beckman DU spectrophotometer using a light path of 1.0 cm.

Aldehyde-free ethanol was prepared by heating 1000 mls ethanol under reflux for 60 minutes with 20 g zinc dust and 20 g KOH. The ethanol was then distilled, discarding the head and tail fractions. Chromotropic acid reagent was prepared in subdued light by first dissolving 2.24 g chromotropic acid in 200 mls H_2O, then adding this solution to 900 mls of sulfuric acid solution (300 mls H_2O plus 600 mls concentrated H_2SO_4). The reagent was stored in the dark and prepared fresh every two weeks.

The standard curve for tripalmitin is shown in Fig. 2. Recovery experiments carried through from the silicic acid extraction step indicated recoveries in the range 95-98%.

Both glycogen and triglyceride tissue levels are expressed as mg/g dry weight of ventricular tissue. This is based on the observation that the dry/wet weight ratios were 25.4% and 20.5% for non-perfused hearts, and hearts perfused over 5 minutes, respectively.

II. Cardiac Lipase Studies

Lipase Assay -- Standard lipase assays were performed in screw-capped tubes at 37° on a Dubnoff metabolic shaker. Incubation time was 30 minutes. The incubation mixture contained 60 mM potassium phosphate buffer, pH 6.8, 20 mg

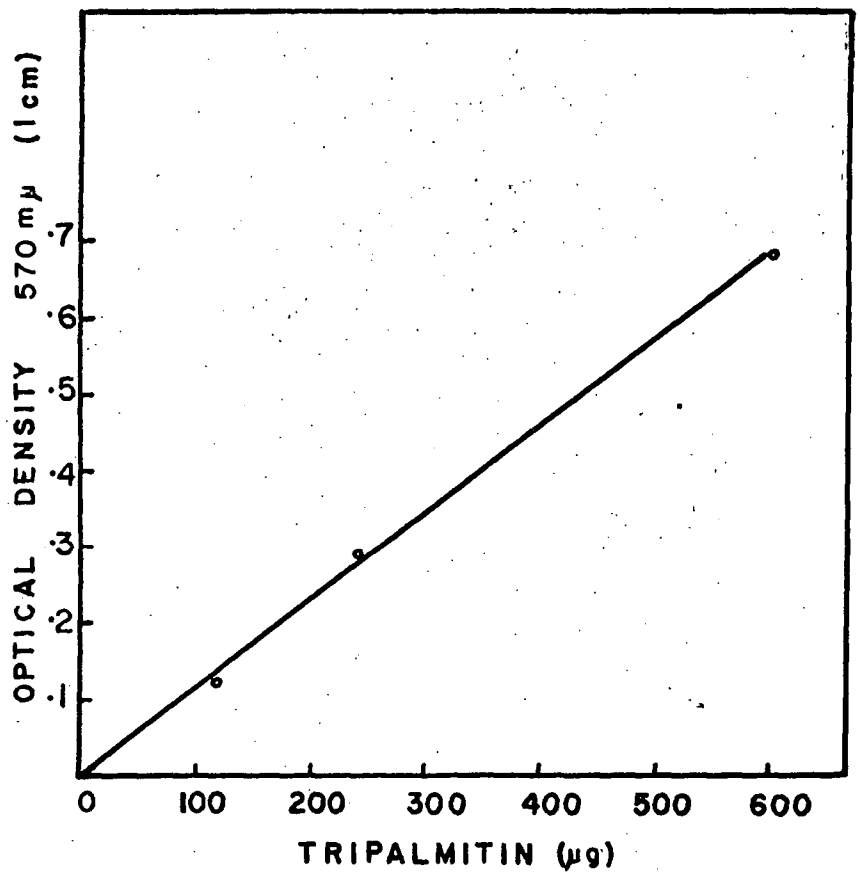


FIG. 2 Tripalmitin concentration versus optical density.

The assay was performed as described in the text except that the initial silicic acid extraction step was omitted. Recovery experiments indicated 95-98% recovery of added tripalmitin when carried through the silicic acid extraction step.

purified bovine serum albumin, enzyme preparation, the appropriate substrate, and glass-distilled water to make a final volume of 1.0 ml. When Ediol^{R} served as substrate in the standard assay, 0.1 ml of a 1:19 dilution was used. The amount of monoolein as substrate was either 5 or 15 µeqs per reaction mixture. Tripalmitin was used at a concentration of 2.5 mg per ml of reaction mixture. Lipolytic activity was measured by the method of Duncombe (5), as modified by Vaughan et al.(6) as follows: The reaction was stopped by the addition of 1.0 ml of a mixture containing 0.9 M triethanolamine, 0.1 N acetic acid, and 5% cupric nitrate.$3H_2O$. The purpose of this treatment is to convert the FFA formed to the chloroform-soluble copper soaps. Chloroform, 6.0 ml, was added and the tubes shaken on a mechanical shaker for 15 minutes. After brief centrifugation, the aqueous copper solution and the denatured protein were removed by suction. An aliquot (0.2-2.0 ml) of the chloroform layer was removed, made up to a final volume of 2.0 ml with chloroform, and 0.25 ml of freshly prepared 0.1% diethyldithiocarbamate (prepared in n-butanol) was added. The intensity of the colour was read in a Beckman DU spectrophotometer at 440 mµ using a light path of 1.0 cm. The standard curve for palmitic acid is shown in Fig. 3. In the author's opinion, this method is very much superior for long chain fatty acid determination to the microtitrimetric method of Dole (117) which has been used for many years.

The conditions for the assay utilizing tripalmitin-1-C^{14}

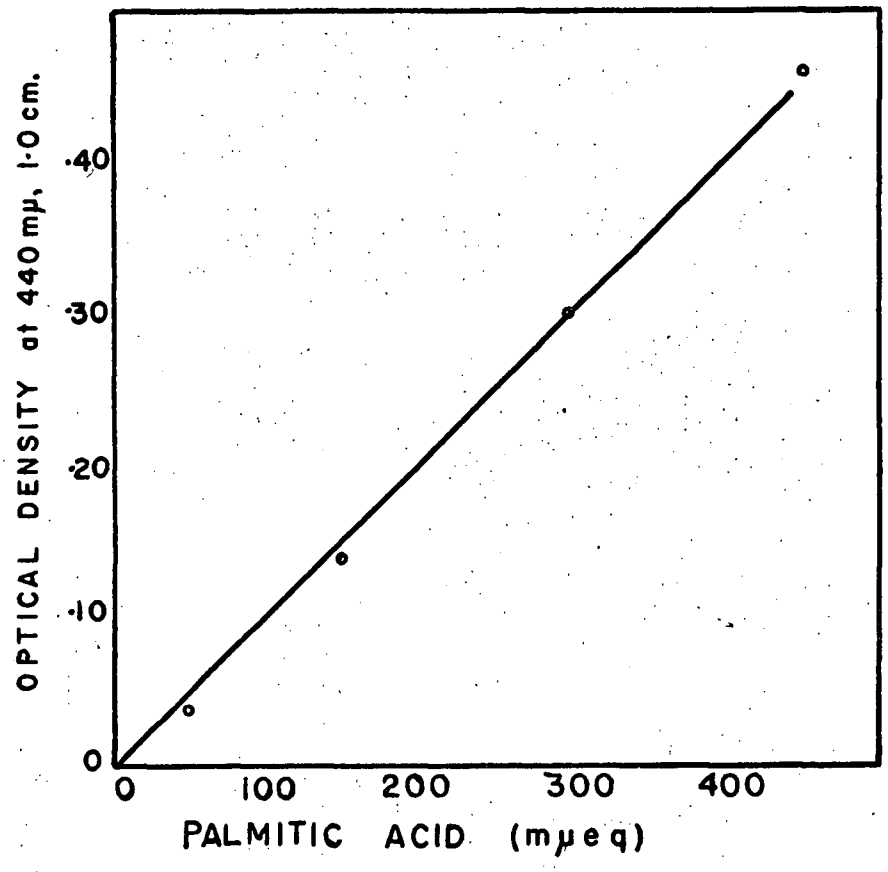


FIG. 3 Palmitic acid concentration versus optical density.

To 1.0 ml of mixture containing 20 mg albumin and 60 mM phosphate buffer, pH 6.8, was added 6.0 ml chloroform containing the indicated amounts of palmitic acid. Extraction and subsequent assay were performed as described in the text.

were essentially identical to the standard assay which contained Ediol^R as substrate, except that the volume of Ediol^R (1:19) used was 0.05 ml instead of 0.1 ml.

One mμ unit of enzyme activity is defined as that amount which produced 1.0 mμ equivalent FFA/60 min at 37°. Specific activity is defined as the number of mμeqs FFA produced/mg protein in 60 minutes at 37°.

Protein was determined by the biuret method (14) and optically by the method of Warburg and Christian (61).

Preparation of Substrates -- Monoolein suspension was prepared by heating 570 mg monoolein in 32 ml 0.25 M sucrose containing 5% acacia (pH 7) at 70°. The mixture was homogenized in a Servall Omnimixer at maximum velocity for 30 seconds at 70°, then slowly cooled with the omnimixer operating at a lower velocity. This procedure gave a satisfactory suspension which remained stable for a considerable length of time. Similarly, very stable suspensions of tripalmitin (25 mg/ml) and monostearin (50 μeq/ml) were prepared at pH 7.0. The substrate preparations were stored at room temperature.

Ediol^R labelled with tripalmitin-1-C^{14} was prepared by carefully evaporating a suitable aliquot of toluene containing about 5 μ curies activity, and mixing the residue with 2.5 ml of a 1:19 dilution of Ediol^R at 60° for at least 60 minutes on a mechanical shaker. Shaking was continued while the mixture was cooled to room temperature.

Measurement of Hydrolysis of Tripalmitin-1-C^{14}-- Hydrolysis of tripalmitin-1-C^{14} was followed by removing an aliquot of

the chloroform layer obtained during the standard FFA assay, and evaporating it to dryness in a centrifuge tube. The residue was quantitatively taken up in small volumes of chloroform and spotted on a thin layer chromatograph plate, using silica gel GF (Merck) as the adsorbent. The plates were developed with freshly distilled chloroform, then exposed to iodine vapour to allow detection of the FFA and glycerides. The triglyceride spots were consistently and clearly defined, moving just behind the solvent front. The lower glycerides and the fatty acids did not separate consistently nor completely to allow an individual quantitative analysis of these components. Therefore these products of triglyceride hydrolysis were scraped off together and counted as one component. Fifteen mls of Liquifluor (Nuclear-Chicago) containing 0.4% PPO (2,5-diphenyloxazole) and 0.05% POPOP (p-bis [2-(5-phenyloxazolyl)] -benzene) in toluene was added to the counting vials, the contents thoroughly swirled and the radioactivity counted in a Nuclear-Chicago scintillation counter. Correction for quenching was made for each vial by the channels ratio method.

Preparation of Enzyme Extract -- All ventricular tissues used for the preparation of extracts were obtained from fed, female Wistar rats weighing between 200 and 300 grams. Unless otherwise indicated, all hearts were perfused for 5 minutes with Ringer-Tyrode solution, pH 7.4, containing heparin, 20 µg per ml, before homogenization. This procedure effectively removes blood from the tissues and also elutes a considerable

amount of lipoprotein lipase from the heart. According to Robinson and Jennings (46), about 50% of the lipoprotein lipase activity of the heart is eluted in 60 minutes, and the rate of enzyme release is highest during the first few minutes.

Ventricular tissues pooled from 10 to 15 rat hearts were homogenized for three 1-minute periods at 0° in 5 volumes of 0.25 M sucrose containing 0.05 M Tris, pH 7, using a Servall Ommimixer. The homogenate was centrifuged at 105,000 x g for 60 minutes, and the sediment thus obtained was re-homogenized in 50% of the original volume of 0.25 M sucrose solution, pH 7, containing 0.1% Triton X-100. The homogenate was again centrifuged at 105,000 x g for 60 minutes at 0° and the supernatant fluids combined. Four mg sodium acetate per ml wereadded to the combined supernatant fractions and the pH was adjusted to 5.9 with the dropwise addition of 1.0 N acetic acid. Following equilibration in an ice bath for 20 minutes, the precipitate was collected by centrifuging for 60 minutes at 37,000 x g at 0° and discarded. The supernatant fluid was taken to pH 5.2 with 1.0 N acetic acid and equilibrated in an ice bath for 30 minutes before centrifuging at 37,000 x g for 60 minutes at 0°. The precipitate was taken up in 0.25 M sucrose, pH 7.

The activity of the pH 5.2-5.9 fraction was quite unstable to freezing and thawing, about 50% of the activity being lost after overnight storage at -20°. Therefore much of the work described later in the text was performed with freshly prepared pH 5.2-5.9 enzyme extracts.

RESULTS

I Perfusion Studies

It is common knowledge that the isolated mammalian heart continues to function for hours when perfused with "physiological" solutions (e.g. Tyrode's) which contain glucose as energy source. This extremely useful technique has been employed by numerous investigators to study the various biochemical aspects of cardiac metabolism, and has recently been used widely in studies on lipid metabolism by the heart. Our first studies were designed to determine whether hearts perfused without substrate were capable of utilizing endogenous triglycerides as energy source.

During the course of this work, Shipp et al. (35) reported that phospholipids were utilized to a much greater extent than were triglycerides by the isolated perfused rat heart perfused without substrate. This seemed rather unlikely, considering the generally accepted view that phospholipids play primarily a structural role in mammalian tissues. Furthermore, Denton and Randle (37) have reported recently that no decrease in phospholipid levels occurred during substrate-free perfusions, whereas tissue triglyceride contents decreased. In the present studies, phospholipid levels were not measured. Instead an attempt was made to compare the relative rates of utilization of endogenous triglycerides with that of glycogen under varying experimental conditions.

Fasting has been shown to increase the glycogen content

of rat hearts (8) and the triglyceride content of guinea pig hearts (118). Fasting also increases lipoprotein lipase activity in rat hearts (50). We have included studies on hearts from animals fasted for 3 days before sacrifice. The purpose of fasting the animals was two-fold: First, to provide a larger store of endogenous substrates so that the hearts could be perfused for longer periods of time, and second, to examine the possibility that increased cardiac lipase activity due to fasting might increase the rate of tissue triglyceride breakdown and utilization during perfusion.

The effects of epinephrine and heparin upon the utilization of endogenous substrates were also investigated. The glycogenolytic action of epinephrine is well known. However, the possibility that epinephrine might also cause an increase in lipolytic activity in cardiac tissue, as it does in adipose tissue seemed most attractive, and was used in the perfusion studies to test this possibility. The release of lipoprotein lipase activity from cardiac tissue slices and from isolated heart by heparin has been demonstrated (46). This suggested, then, that the perfusion of rat hearts with heparin might cause a reduction in tissue lipase content, resulting in a decreased rate of disappearance of endogenous triglycerides.

Initial ventricular levels of glycogen in the hearts of fed rats were 10.1 $\pm$ 1.3 mg/g dry weight, and 20.9 $\pm$ 0.8 mg/g dry weight in hearts from 3-day fasted rats ("CONTROL" panel, Fig. 4). The glycogen levels in hearts from fed rats decreased

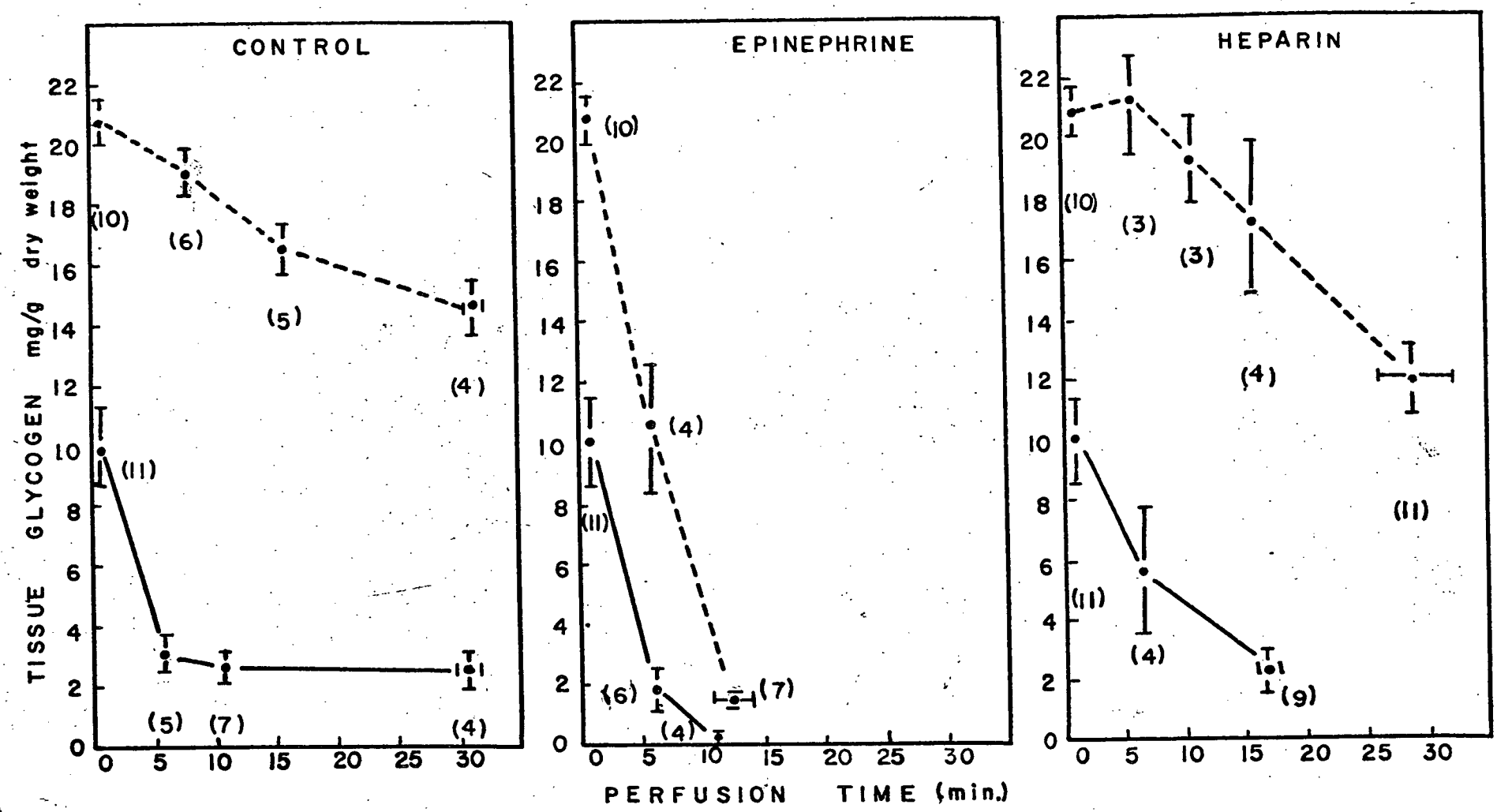


FIG. 4 Tissue glycogen contents in hearts from fed (——) and fasted (----) rats during substrate-free perfusion.

Control hearts were perfused for the indicated period of time with substrate-free Krebs-Ringer bicarbonate media, pH 7.4 at 37°. Epinephrine, 0.2-1.0 µg/min, and heparin, 60 µg/min, were injected into the perfusion system just above the cannula. Tissue glycogen contents are shown as the means ± standard error of the mean of the number of observations in parenthesis (vertical bars). The horizontal bars represent the mean time and range over which hearts were collected.

at a faster rate than those from fasted rats when perfused with substrate-free medium. In contrast to hearts from fasted rats, hearts from fed animals generally developed arrhythmia and decrease in contractile force within 10 minutes of perfusion. On the other hand, hearts from fasted rats maintained good rhythm and contractions for much longer periods of time, often longer than 25 minutes.

As might be expected, the addition of epinephrine to the perfusion medium caused a rapid depletion of tissue glycogen, which was especially noticeable in the hearts from fasted animals (see "EPINEPHRINE" panel, Fig. 4). Furthermore, epinephrine appeared to have caused a more complete depletion of tissue glycogen, as compared with the control series. In fact, the glycogen content after 10 minutes of perfusion in some of the epinephrine treated (fed) rat hearts was barely detectable. The effect of epinephrine on cardiac function was to increase both rate and force for the first 3-5 minutes, followed by a rapid decline in cardiac function. Within 8-12 minutes, most of the epinephrine treated hearts were virtually non-functional. Arrhythmias were very frequently encountered in the presence of epinephrine. It should perhaps be noted that when the perfusate from the epinephrine-treated fasted series was assayed for glucose, none could be detected.

Addition of heparin to the perfusion medium appeared to have little effect on the rates of glycogen depletion in both fed and fasted rat hearts ("HEPARIN" panel, Fig. 4).

Whereas fasting caused a significant increase in cardiac

glycogen content, there was no significant increase in tissue triglyceride level in hearts obtained from fasted rats. This observation is in contrast to the observations of Wittels (118) who found increased triglyceride levels in hearts of fasted guinea pigs. Perfusion of about 30 minutes duration (Fig. 5) appeared to cause some decrease in triglyceride levels in both fed and fasted rats. However, considering the relatively large variation in the tissue triglyceride values, and the small population of rats used, it is suggested that if any changes in triglyceride levels did occur, these changes were probably not very significant during the 27-30 minutes of perfusion. No decrease in triglyceride levels were noted in hearts perfused with epinephrine or with heparin. In fact, an apparent increase in triglyceride levels appeared. This apparent increase in tissue triglyceride levels in the epinephrine and heparin perfused series ("EPINEPHRINE" and "HEPARIN" panels, Fig. 5) is most difficult to interpret. It is inconceivable that triglycerides were synthesized under these perfusion conditions. It seems more likely that some factor may have been produced as a direct result of perfusion with epinephrine and heparin, giving rise to anomalously high values for triglycerides.

The apparent decrease in tissue triglyceride levels observed during perfusion of the control series must be substantiated with more data. Since more potential energy is contained per weight of triglycerides than is contained in glycogen, the decrease in triglycerides will probably not be

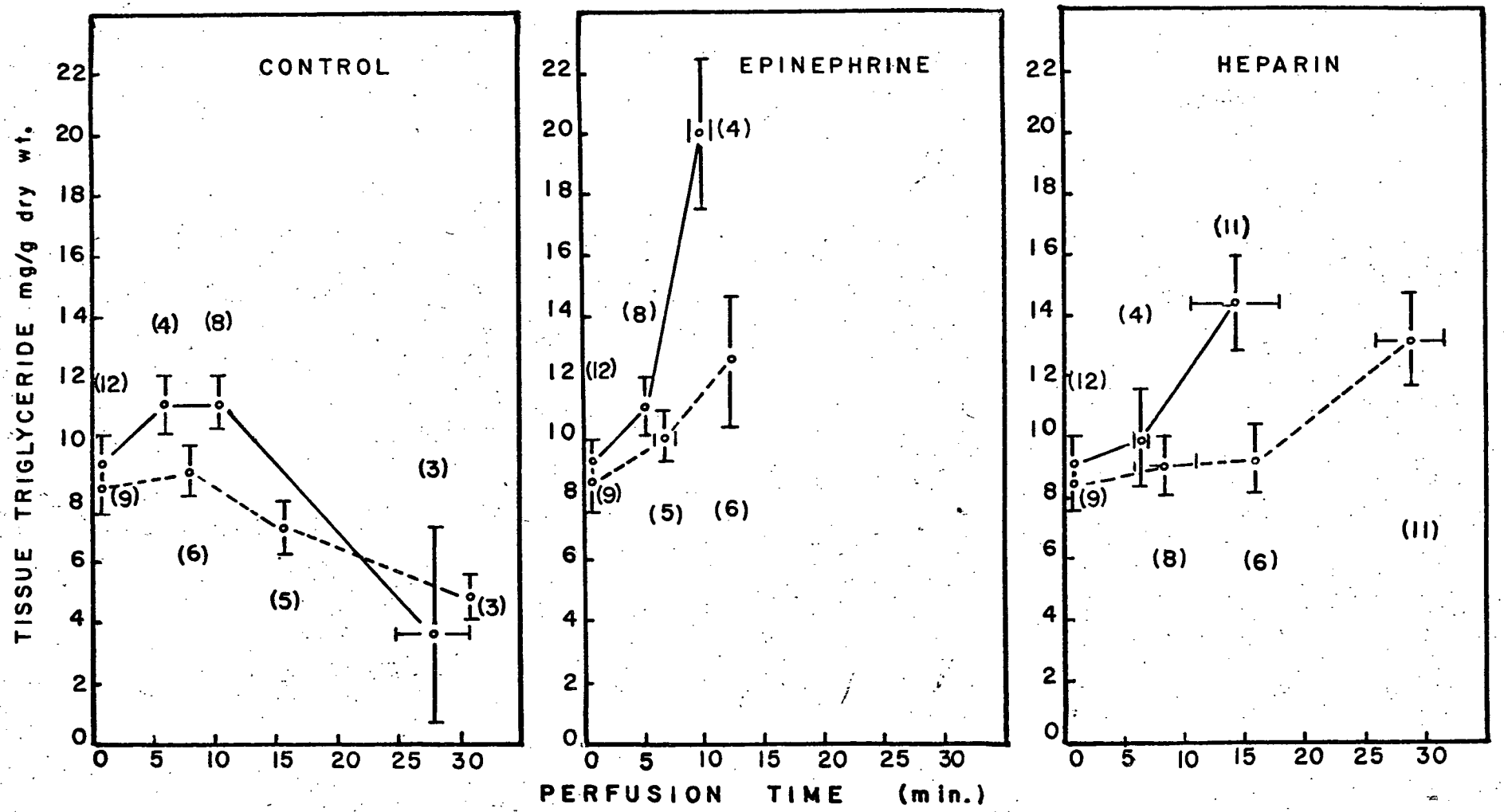


FIG. 5 Tissue triglyceride contents in hearts from fed (——) and fasted (----) rats during substrate-free perfusion.

Perfusion conditions as described under Fig. 4.

evident immediately under these relatively short perfusion conditions. Hence, in order to demonstrate conclusively that tissue triglycerides do decrease during substrate-free perfusions, it would likely be necessary to perfuse hearts for longer periods, perhaps up to 60 minutes. However, it was found extremely difficult to maintain hearts in good working order for periods much longer than 30 minutes under the perfusion conditions used in these experiments. These results unfortunately shed little light upon the relative importance of endogenous triglycerides to the isolated perfused heart. It is tentatively concluded that the immediate endogenous source of myocardial energy is derived from the breakdown of tissue glycogen when hearts are perfused with substrate-free media, at least during the initial phases of perfusion. Perhaps decreased levels of triglycerides become apparent after longer periods of time.

II Cardiac Lipases

Triglycerides in blood (in the form of chylomicrons and very low density lipoproteins) are taken up intact, i.e. without prior hydrolysis, by cardiac cells. When one considers that perhaps 90% of the circulating lipids which are extracted by the heart is in the form of triglycerides, it is obvious that the role of cardiac lipases must be extremely important for providing a constant source of free-fatty acids for oxidation by the myocardium.

In many tissues, the sequence of triglyceride hydrolysis to fatty acids proceeds enzymatically in the following manner:

triglycerides ⟶ diglycerides ⟶ monoglycerides ⟶ FFA, where the initial step is considered rate-limiting. In cardiac tissues, an enzyme system exists which hydrolyzes triglycerides to diglycerides. This triglyceride-hydrolyzing enzyme, lipoprotein lipase, probably hydrolyzes diglycerides to monoglycerides as well. Another lipase, similar to the epinephrine-sensitive lipase of adipose tissue (107), has been reported to exist in rat hearts (13). The existence of a lipase which is specific for diglycerides has not yet been shown in heart or in any other tissue, although there is some evidence to strongly indicate that lipoprotein lipase also attacks diglycerides. On the other hand, the presence of monoglyceride-splitting lipases has been demonstrated in a number of tissues, including adipose tissue (6, 16), liver (55), and the intestinal mucosa (56-59), but its presence has not been shown in cardiac tissue.

The general procedure for studying any enzyme is to determine first whether its activity exists in whole homogenates or in intact systems. The next step is to purify it as a discrete entity so that its properties may be better studied without the problem of contamination by other enzymes possessing similar properties. This general approach was followed in the present work for investigating the lipolytic activities in rat hearts.

Although numerous enzymes have been extensively purified, and many even crystallized, most lipases have resisted purification. In fact, no lipase has yet been crystallized.

Therefore, studies on most lipases up to the present time have usually been done on crude extracts of tissues, leading to much confusion in the literature with respect to some of the properties of lipases. Another major obstacle in the study of lipases is the technical difficulty of preparing suitable substrates for the enzymes. For example, there are no standard triglyceride substrates for triglyceride-hydrolysing lipases. The fatty acid moiety of a triglyceride substrate may be saturated, unsaturated, long, medium or short chain, and the physical nature of the substrate may be an oil-in-water emulsion or a crude glyceride suspension in a suitable aqueous buffer. Finally, all known methods of assaying lipolytic activity are considerably more laborious and technically cumbersome than most commonly used enzyme assays. All these reasons contribute to the fact that the study of lipases has lagged far behind the study of enzymes in other areas of the biological system.

In spite of the difficulties anticipated, an attempt was nevertheless made to study the lipases of cardiac tissue, with particular emphasis on lipases other than lipoprotein lipase.

A. EXISTENCE OF NaF-INHIBITED LIPASE

Rat hearts were perfused for 5 minutes with Krebs-Ringer bicarbonate solutions containing 20 µg/ml heparin. The ventricular tissues were pooled and homogenized in 10 volumes of 0.25 M sucrose, pH 7.0 at 0-4° with the aid of a Potter-Elvehjem Homogenizer. The crude homogenate was filtered

through cheesecloth and used directly for assay of lipolytic activity at pH 6.8, using Ediol^{R} (1:19) as substrate as described in the Experimental section. A rapid liberation of free fatty acids occurred, as may be seen in Fig. 6, and the enzyme activity was proportional to incubation time up to 30 minutes.

When similar experiments were performed in the presence and absence of 0.2 M NaF, again using Ediol^{R} as substrate, it was noted that a constant inhibition of about 40% occurred throughout the course of the reaction (Fig. 7). This experiment was performed at a pH suboptimal for lipoprotein lipase and without pre-activating the Ediol^{R} with serum. Under these conditions, the activity of lipoprotein lipase would be minimized. Since NaF is known <u>not</u> to be an inhibitor of lipoprotein lipase, the observed 40% inhibition in the crude system suggested that there indeed was an active lipase or lipases present in cardiac tissue in addition to lipoprotein lipase. Inhibition by NaF would indicate that the enzyme might be similar to the non-lipoprotein lipase of adipose tissue described by Rizack (107).

At this point, it must be emphasized that Ediol^{R} was used as substrate for measuring lipolytic activity in these experiments. The major advantage of this emulsion is that it is an extremely smooth and stable oil-in-water triglyceride preparation. However, a serious disadvantage of Ediol^{R} as substrate is that it contains monostearin as a stabilizing agent, and this monostearin could well serve as

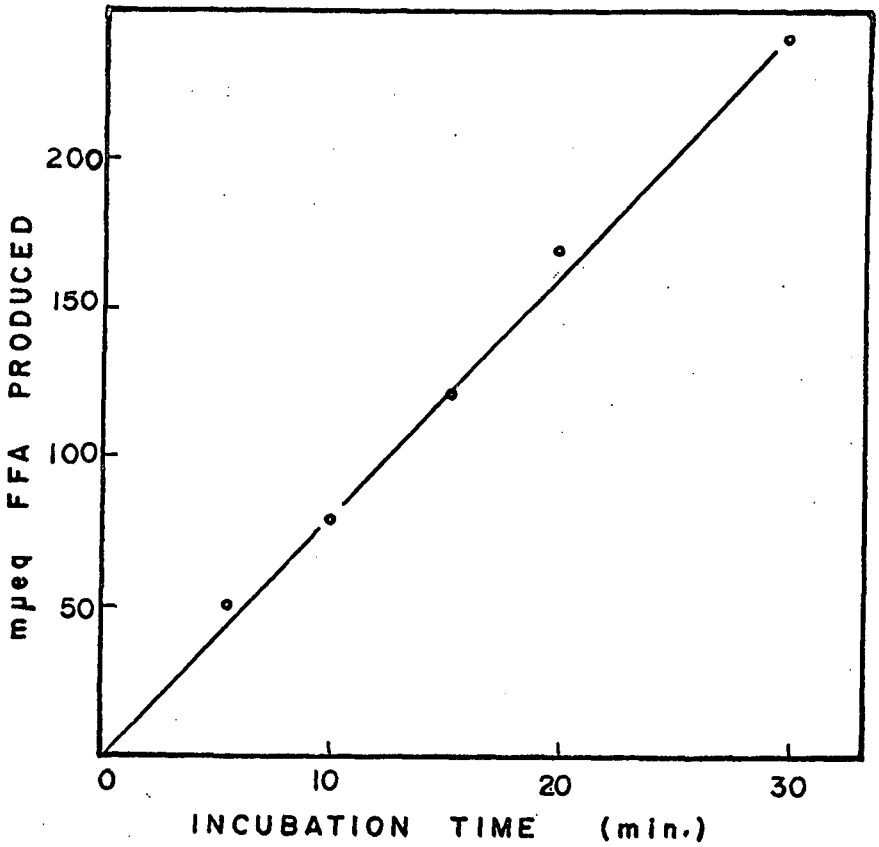


FIG. 6 Time course of lipolytic activity.

The reaction mixture contained 60 mM phosphate buffer, pH 6.8, 20 mg purified bovine serum albumin, 0.10 ml of 1:19 dilution of Ediol, 0.04 ml whole homogenate in a total volume of 1.0 ml. Incubation was at 37° in a Dubnoff Metabolic Shaker. Each reaction mixture contained 0.42 mg protein.

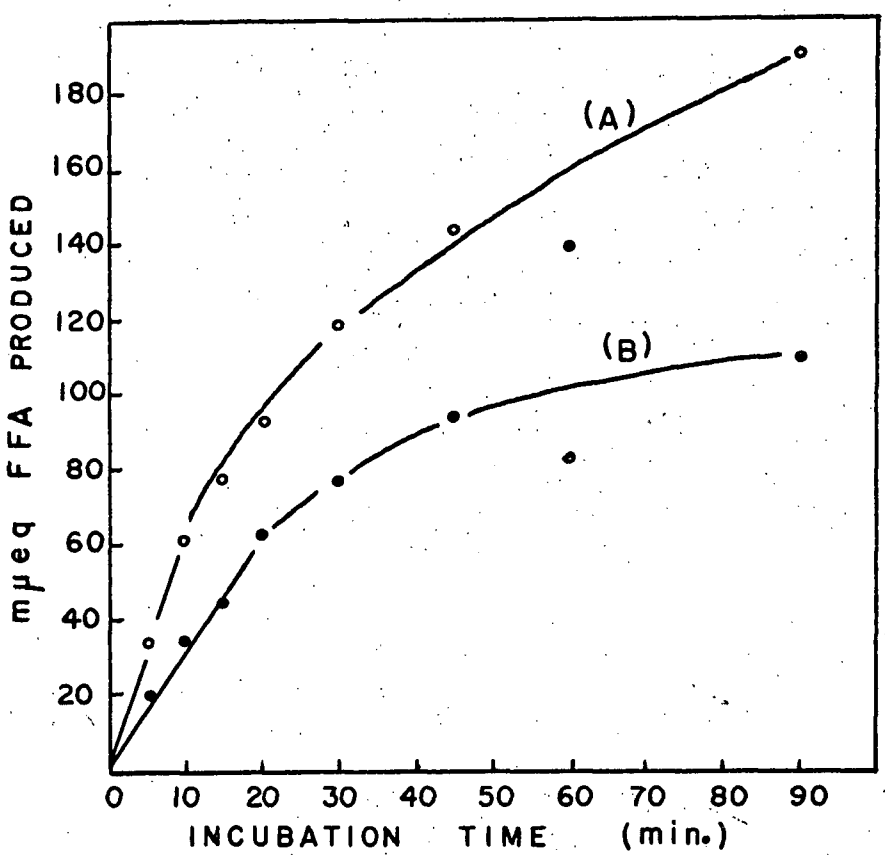


FIG. 7 Inhibition of lipolytic activity in crude heart homogenates by NaF.

The incubation mixture contained all components of the standard assay as described in the text, except that 0.15 ml of 1:19 dilution of Ediol was used as substrate. Curve "A" - control. Curve "B" - NaF, 0.2 M included in reaction mixture. Each reaction mixture contained 0.21 mg protein. The homogenate was stored overnight at -20° and thawed before use.

substrate for a lipolytic enzyme. Experiments were therefore performed in order to explore further the nature of the NaF-inhibited lipolytic activity in cardiac tissue by using substrates prepared from monoglycerides or triglycerides only.

When monostearin (suspended in 5% acacia solution) was used as substrate, it was rapidly hydrolyzed by the heart homogenate (Fig. 8, Curve A). Activity again was proportional to time. Of particular interest was the observation that in the presence of 0.2 M NaF, a constant 40% inhibition of lipolytic activity was obtained (Curve B). This degree of inhibition was almost identical to that observed when similar experiments using Ediol^{R} was performed (Fig. 7), thus strongly suggesting that the NaF-inhibited lipolytic activity measured with Ediol^{R} as substrate may have been due to the hydrolysis of its monostearin component. Furthermore, when tripalmitin alone was used as substrate, the lipolytic activity was so small as to make accurate and reliable lipolytic measurements exceedingly difficult (Curve C). NaF (0.2 M) did not appear to inhibit the hydrolysis of tripalmitin, although the low activities observed made the interpretation of the data difficult (Curve D). When, however, the incubation mixture was supplemented with rat serum, a rapid hydrolysis of tripalmitin occurred. The data in Table I shows a greater than 12-fold increase in lipolytic activity when the tripalmitin substrate was presented to the enzymes in the form of a lipoprotein complex. This experiment clearly indicated not only the presence of lipoprotein lipase activity in

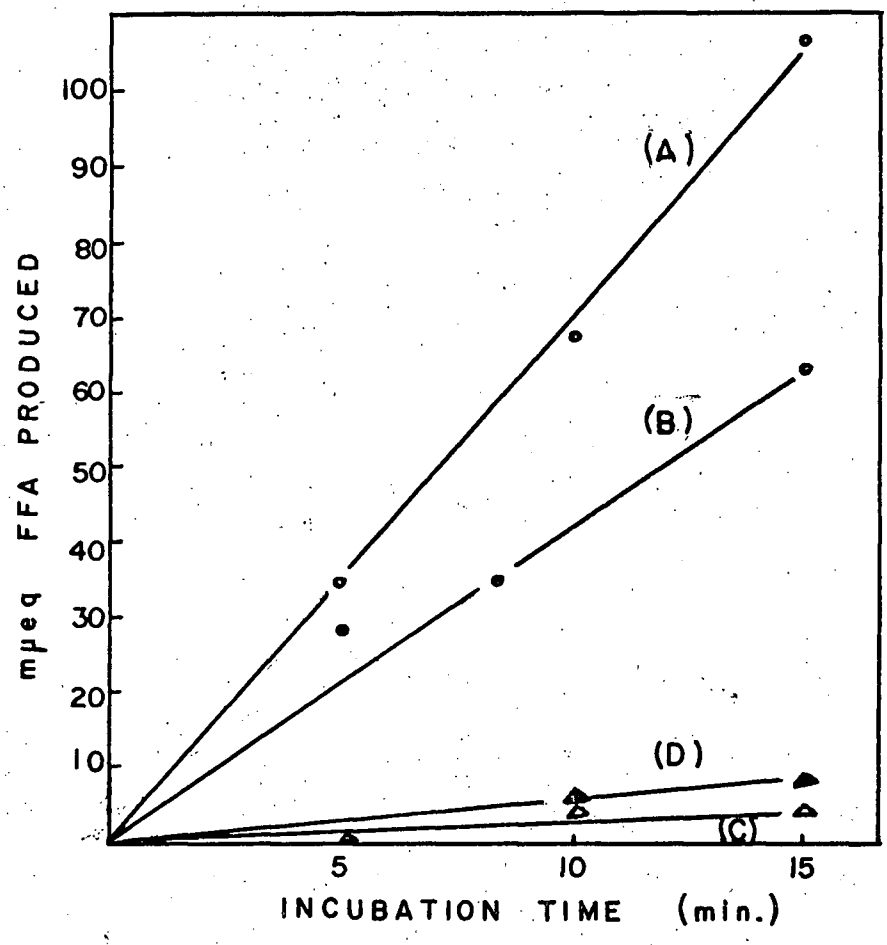


FIG. 8 Hydrolysis of monostearin and tripalmitin, and effect of NaF.

Line "A" represents the time course of lipolytic activity of the whole homogenate when the substrate was monostearin (5 ueq/ml) in the standard assay. Line "B" represents inhibition of monostearin hydrolysis by 0.2 M NaF. Line "C" represents the lipolytic activity of the homogenate (same volume used as in "A") when the standard incubation mixture contained 2.5 mg tripalmitin as substrate. Line "D" represents the hydrolysis of tripalmitin in the presence of 0.2 M NaF. The hydrolysis of tripalmitin was consistently so small that accurate lipolytic measurements were extremely difficult. Each reaction mixture contained approximately 0.42 mg protein.

TABLE I

Effect of rat serum on the hydrolysis of tripalmitin by partially purified extract of cardiac tissue.

The complete system contained 20 mg purified bovine serum albumin, 50 mM Tris buffer, pH 8.5, 2.5 mg tripalmitin which had been pre-incubated for 30 minutes at 37° with 0.1 ml rat serum, 0.1 ml of enzyme extract, and sufficient water to make a final volume of 1.0 ml.

Reaction mixture	Lipolytic Activity mμeq FFA/60 min at 37°
Complete system	99
Complete system without serum	8

cardiac extracts, but also demonstrated the marked dependence of this enzyme on serum-activated triglycerides. More important, the data provided additional evidence that an enzyme other than lipoprotein lipase exists in rat hearts. The enzyme appeared to hydrolyze monoglycerides with greater facility than triglycerides. The ability of the lipase to liberate free fatty acids from Ediol^{R} is likely due to its action on the monostearin component of this preparation. The enzyme already seemed similar to that reported by Bjorntorp and Furman (13) who used Ediol^{R} as substrate. The possibility existed that the activity these authors had measured was actually the hydrolysis of monoglyceride. Further studies were therefore necessary to clarify the source of fatty acids arising from Ediol^{R}. One could accomplish this by following the hydrolysis of either C^{14}-labelled monoglycerides or C^{14}-triglycerides when these glycerides were added to Ediol^{R} and subjected to lipase activity in heart extracts. Since labelled long-chain monoglycerides were not readily available, tripalmitin-1-C^{14} was used. The rate of hydrolysis of tripalmitin-1-C^{14} and the rate of total fatty acids released were measured simultaneously. It was assumed that fatty acids produced in the absence of tripalmitin-1-C^{14} hydrolysis must have been derived from the monoglyceride component of Ediol^{R}. Furthermore, it was thought that the effects of known lipase inhibitors could be more clearly observed in such a system. As may be seen in Fig. 9, the hydrolysis of tripalmitin-1-C^{14} in Ediol^{R} occurred at an appreciable rate in the presence of

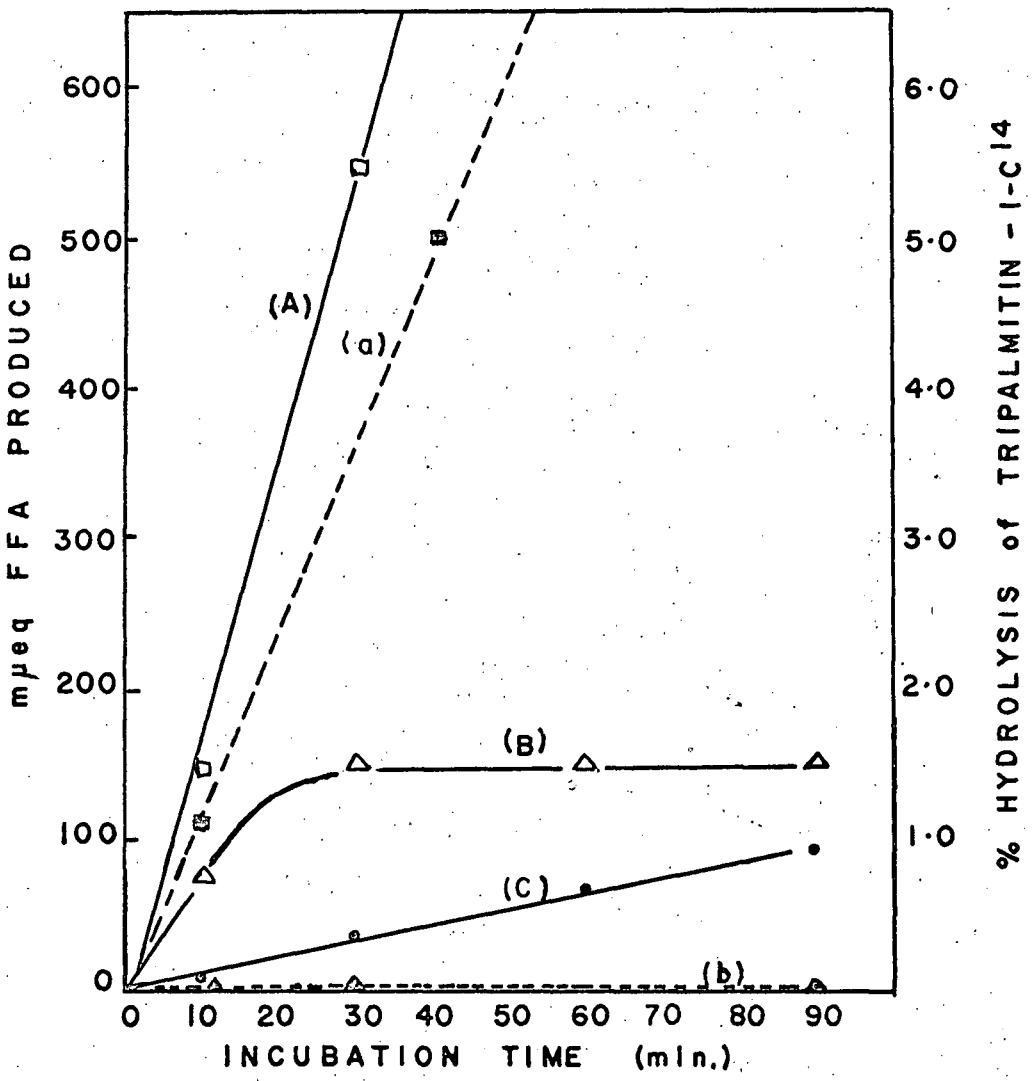


FIG. 9 Time course of Ediol hydrolysis in the presence of lipase inhibitors.

Lines "A", "B", and "C" represent the time course of total fatty acids produced during the experimental conditions as outlined below. Lines "a" and "b" represent the rate of tripalmitin-1-C^{14} hydrolysis during the same interval.

Lines "A" and "a" - The reaction mixture contained 120 mg bovine serum albumin, 50 mM Tris buffer, pH 8.5, 0.3 ml rat serum, 0.3 ml of 1:19 dilution Ediol containing tripalmitin-1-C^{14} (0.5 μ curie/ml), 0.6 ml 6000 x g rat heart supernate, and sufficient water to make 6.0 ml. Incubation was performed at 37° in a Dubnoff Metabolic Shaker and 1.0 ml aliquots removed at the indicated time intervals for the assay of total free fatty acids produced (line "A") and also tripalmitin-1-C^{14} hydrolyzed (line "a").

Lines "B" and "b" - Reaction conditions were essentially

the same as indicated above for "A" and "a", except that 50 mM Tris buffer pH 7.0 was used and the system also contained NaCl, 0.5 M.

Line "C" - Reaction conditions were as for "B" and "b", except that the system also contained NaF, 0.2 M.

The rate of tripalmitin-1-C^{14} hydrolysis (line "a") was proportional to incubation time for 90 minutes.

rat serum (line "a"). The total fatty acids produced during this period was also appreciable (line "A"). In the presence of 0.5 M NaCl (and absence of serum), the hydrolysis of tripalmitin-1-C^{14} was completely abolished (line "b"). This indicated that under these conditions (i.e., 0.5 M NaCl, no serum), lipoprotein lipase activity against triglycerides was completely inhibited. However, even under conditions where the hydrolysis of tripalmitin-1-C^{14} was completely inhibited, FFA release was still evident in the system (line "B"). Hence, this lipolytic activity was attributed to the hydrolysis of monostearin in Ediol^{R}. If this were the case, the addition of 0.2 M NaF to the reaction mixture should result in further inhibition of lipolytic activity, and as may be seen, this was indeed observed (line "C"). This experiment therefore gave additional support to the original observation that when Ediol^{R} was used as substrate in the "non-activated" state, a large proportion of free fatty acids liberated was derived from the hydrolysis of its monoglyceride component. It also supported the view that it is the hydrolysis of the monoglyceride component of Ediol^{R} which is inhibited by NaF.

It was noted that the rate of monoglyceride hydrolysis (line "B") did not change after about 25 minutes of incubation. This consistent observation in these experiments was due to the fact that under the conditions of the assay, the amount of monoglyceride was limiting the reaction. Ediol^{R} contains approximately 1.5% monostearin. This concentration of monostearin is equal to 40 µeq/ml. In those experiments utilizing

tripalmitin-1-C^{14}, each 1.0 ml of reaction mixture contained 0.05 ml of a 1:19 dilution of Ediol^{R}. Hence the total amount of monostearin available as substrate was about 100 mμeqs. Considering the noticeable and inevitable shrinkage in volume of Ediol^{R} during storage over a two year period, it is not unreasonable to assume that about 150 mμeqs of monostearin were perhaps available as substrate per reaction tube. This would explain the consistent plateauing of reaction rate observed at around 25-30 minutes incubation in those experiments which were designed specifically to show only monoglycerides being hydrolyzed.

The results of these experiments clearly demonstrated that the lipolytic activity in rat hearts possessing properties different from lipoprotein lipase was an active NaF-inhibited enzyme which rapidly hydrolyzed the monostearin component of Ediol^{R}. Since Ediol^{R} has been so widely used in the study of lipase, especially in adipose tissue, one wonders if results have not been occasionally misinterpreted.

B. PREPARATION OF PARTIALLY PURIFIED EXTRACT FROM HEART TISSUE

The presence of a NaF-inhibited monoglyceride-hydrolyzing lipolytic system in rat hearts having been established, it was considered essential that the enzyme be isolated so that its properties could be better understood. As anticipated, the satisfactory purification of this enzyme proved extremely difficult.

Some of the problems encountered during the attempt to

purify the enzyme will be noted here, but a detailed discussion will be reserved for the final Discussion section. First, about 75-85% of the enzyme activity was bound to the particulate fractions of the cells. Attempts to solubilize the enzyme using a number of standard techniques were in most instances unsuccessful. For example, preparations of acetone powders, repeated freezing and thawing, deoxycholate treatment, sonication at 9 and 20 kilocycles for various periods of time, sonication combined with deoxycholate treatment were tried in vain. The technique which gave reasonably satisfying results was the use of 0.1% Triton X-100, a non-ionic synthetic detergent. When a 105,000 x g pellet was re-homogenized with Triton X-100, a 2- to 3-fold increase in the amount of enzyme activity was noted in the subsequently obtained 105,000 x g supernatant fluid. This technique solubilized about 30-40% of the total enzyme activity in cardiac cells, and was therefore adopted as the basis for further attempts to purify the enzyme.

Purification of the enzyme from the 105,000 x g supernatant fluid was also extremely difficult. Again a number of classical techniques of enzyme fractionation were employed. Repeated attempts using high temperatures, ethanol precipitation methods, calcium phosphate gels, Sephadex G-200 columns and zinc-ethanol treatment failed to give a satisfactory purification. However, both ammonium sulfate fractionation and the isoelectric precipitation methods did provide a small measure of purification. The isoelectric precipitation tech-

nique was adopted as the second step in the proposed further purification of the enzyme. Treatment of the extract (obtained by isoelectric precipitation) with Sephadex G-200 and ammonium sulfate were tried but the enzyme resisted further purification. A major obstacle to purification was the instability of the lipolytic system in the isoelectric precipitate fraction, particularly to freezing and thawing. Since a considerable amount of time had already been expended in efforts to solubilize and purify the enzyme, it was decided to use the partially purified isoelectric precipitate fraction for the study of the enzyme. The acid precipitation method gave about a 3 to 4-fold purification over the combined supernatant fraction, and a yield of approximately 45%. The overall yield, however, was only 10% when based on the total activity of the whole homogenate, owing essentially to the extremely insoluble nature of the enzyme. It should be mentioned that the degree of purification obtained was about the same when either Ediol^{R} or monoolein was used as substrate for measuring lipolytic activity. The partial purification obtained is shown in Table II.

Lipolytic activity of the isoelectric precipitate was directly proportional to enzyme concentration (Fig. 10) over a range of protein which constituted a reliable assay.

C. PROPERTIES OF CARDIAC MONOGLYCERIDE-HYDROLYZING ENZYME

Using the partially purified isoelectric precipitate preparation (hereafter referred to as "partially purified extract") of rat heart, a number of experiments were performed

TABLE II

Partial purification of cardiac monoglyceride-splitting enzyme.

The activities in the first and second high speed supernate fractions are included to indicate the increase in solubilization obtained with Triton X-100. The standard lipase assay was employed with 15 µeqs monoolein as substrate. One mµ unit of enzyme activity is that amount which produced 1.0 mµeqs FFA/60 min/37°. Specific activity is defined as mµ units enzyme activity/mg protein/60 min/37°.

FRACTION	TOTAL ACTIVITY	SPECIFIC ACTIVITY
(No. 1) 105,000 g supernate	220,000	956
(No. 2) " " obtained with Triton X-100	646,800	3480
COMBINED 105,000 supernates	766,800	1980
pH 5.2-5.9 isoelectric precipitate	333,333	7000

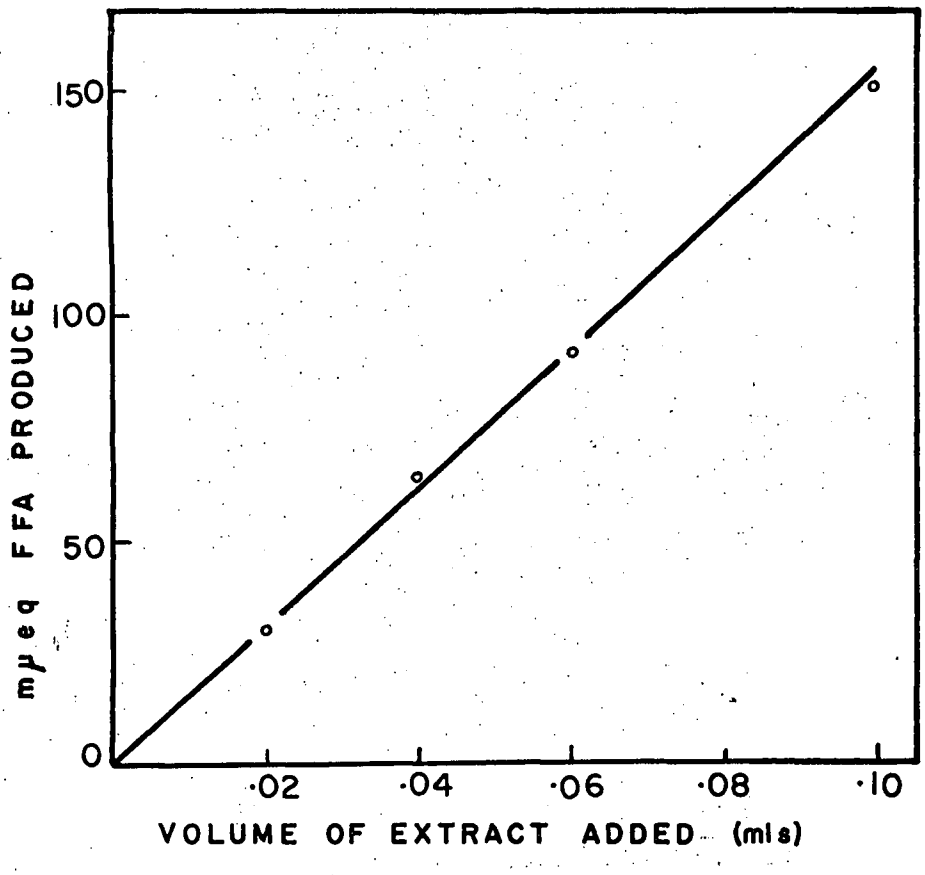


FIG. 10 FFA production as a function of enzyme concentration with monostearin as substrate.

The standard assay was employed, and the enzyme used was the partially purified isoelectric preparation.

to study some of the properties of this enzyme. It was hoped that information obtained from such studies would greatly assist in the assignment of a possible physiological role of the enzyme in cardiac tissues.

1. Albumin Requirement -- Under physiological conditions, long chain free fatty acids are normally transported in the blood as free fatty acid-albumin complexes. This binding of free fatty acids to albumin effectively solubilizes a considerable amount of otherwise insoluble FFA's which have been released into the circulation from adipose tissues. For the same reason, albumin has frequently been added to lipolytic reaction mixtures to function as a FFA-acceptor in the *in vitro* system. As may be seen in Fig. 11, the requirement for albumin in the assay mixtures was not absolute, but the presence of more than 12 mg albumin increased the activity about 2-fold. Routinely, 20 mg of purified bovine serum albumin was included in the assay system.

2. pH-Optimum -- When monoolein was used as substrate, the activity of the enzyme extended over a wide pH range (pH 6.0-9.0) with the maximum activity exhibited near pH 6.5-7.0 (Fig. 12). This observation is consistent with the report of Bjorntorp and Furman's (13) that a sodium fluoride-inhibited lipolytic activity having a pH optimum near pH 6.8 existed in rat hearts. The pH optimum of lipoprotein lipase is near 8.5. Since, in the experiment, no triglyceride or diglyceride substrate and no serum was present, and since the reaction was performed in the presence of 0.5 M NaCl, this enzyme can be

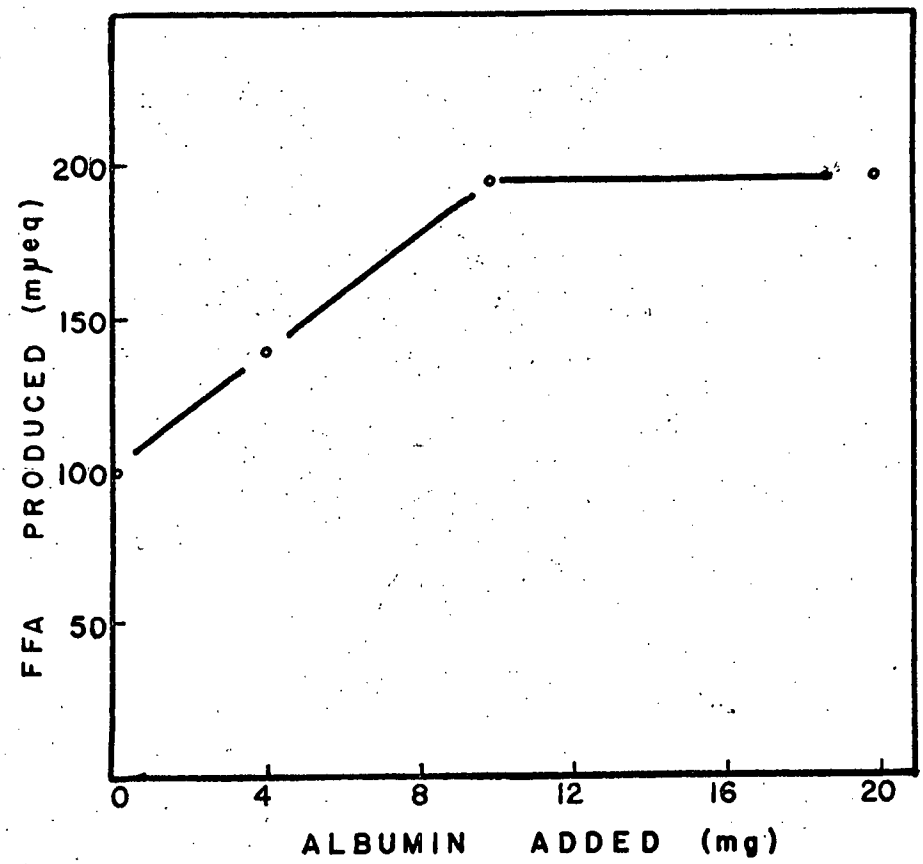


FIG. 11 Effect of concentration of albumin on lipolytic activity.

Incubation was carried out according to the standard assay, using 0.1 ml of 1:19 dilution of Ediol as substrate, except that the albumin concentration was varied as indicated. The enzyme used was the partially purified pH 5.2-5.9 preparation.

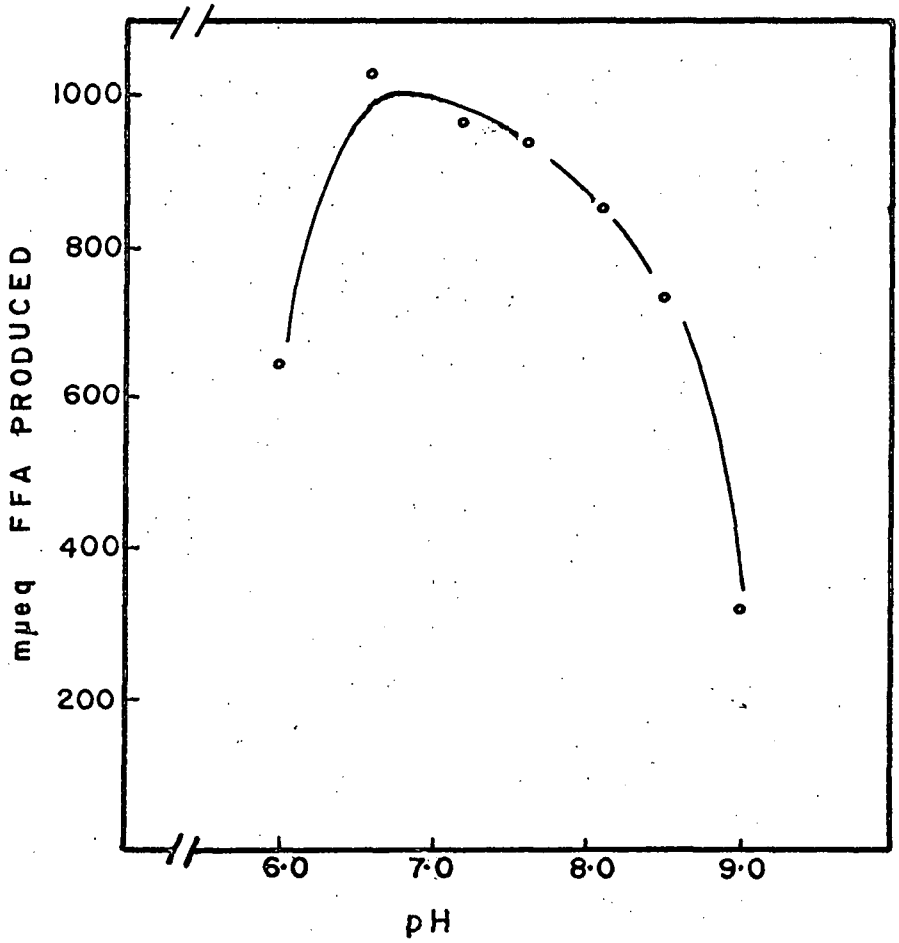


FIG. 12 Hydrolysis of monoolein by partial purified lipase as a function of pH.

The incubation mixture contained 15 μeq monoolein, 20 mg purified bovine serum albumin, 0.5 M NaCl, enzyme, 0.08 M Tris and 0.08 M phosphate buffers at the pH's indicated. The incubations were performed in a total volume of 1.0 ml for 60 minutes at 37° on a Dubnoff Metabolic Shaker.

readily distinguished from lipoprotein lipase.

3. Temperature Optimum -- The optimum temperature for the *in vitro* enzymatic hydrolysis of monostearin was near 45° (Fig. 13). This optimum is beyond the normal physiological range expected of any enzyme in biological systems. Therefore, the significance of this observation can only be extended to the enzymatic hydrolysis of monostearin *in vitro*, and is of academic interest only. However, it further emphasizes the similarity between this enzyme and the monoglyceride-splitting enzyme from adipose tissue because Vaughan *et al.*(6) has shown that this enzyme has a temperature optimum of 45°.

4. Effect of Physical State of Substrate -- One of the criteria used for distinguishing between true lipases and non-specific esterases is that the former does not normally attack esters in aqueous solution (109). In order to study the nature of the lipolytic activity in the partially purified extract, the enzyme was added to various concentrations of monoolein. A serial dilution of monoolein was made such that at low concentrations, the reaction mixture was optically clear, presumably because the monoolein was in true solution. At higher concentrations, the substrate was insoluble and in the form of a suspension. Careful visual examination of a series of reaction tubes prior to addition of enzyme indicated that the turbidity of the reaction mixture became apparent at monoolein concentrations in the region of 1.5 or 2.25 μeq/ml. When the lipolytic activity was measured in the usual manner,

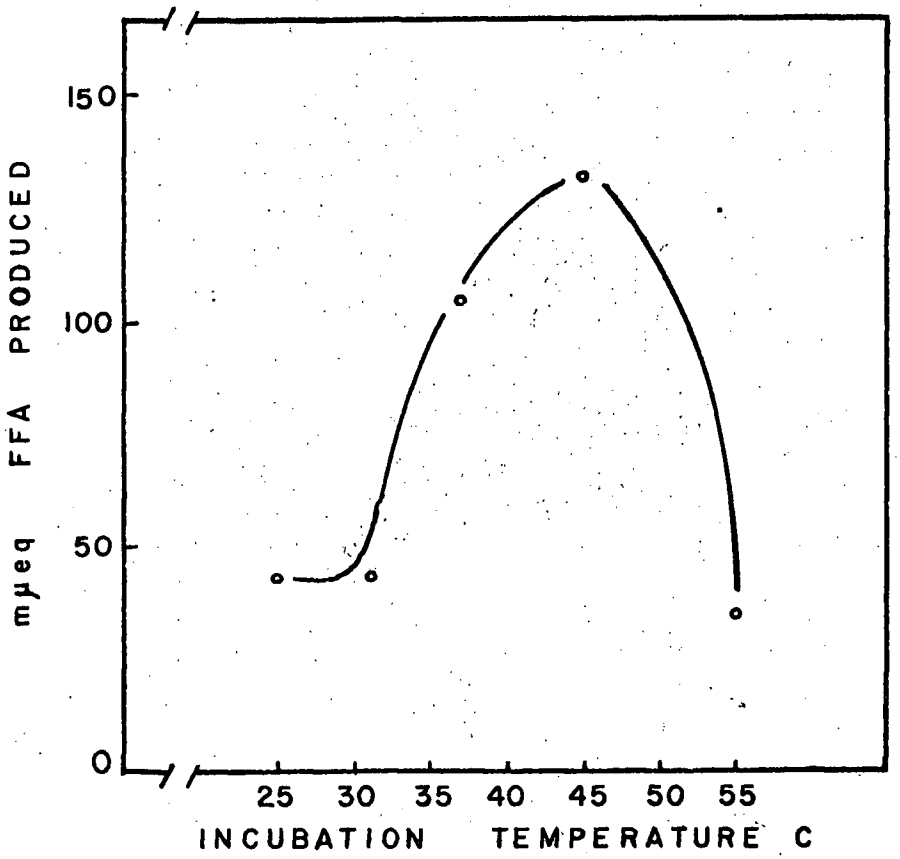


FIG. 13 Effect of temperature on cardiac monoglyceride-hydrolyzing lipase activity.

The standard assay was employed using monostearin as substrate, except that the incubations were performed at the temperatures indicated. Control tubes were also incubated, and the enzyme added after the reactions were stopped.

it was observed that a marked increase in activity occurred near 2.0-4.0 μeq/ml monoolein concentration (Fig. 14). It is readily conceded that the visual estimation of turbidity gave only a rough approximation of the actual physical state of the monoolein substrate in aqueous media. Nevertheless, the marked increase in lipolytic activity over a relatively narrow monoglyceride concentration range suggested strongly that the enzyme is a true lipase, and not a simple non-specific esterase.

5. Effect of Some Enzyme Inhibitors on Monoglyceride-hydrolyzing Lipase Activity -- Many enzyme inhibitors have been employed in order to identify the active sites and to study the mechanism of action of enzymes. It appears, however, that the use of inhibitors in the study of lipases has been largely restricted to distinguishing one lipase activity from another. Even when used for this purpose, many inconsistencies are noted in the literature with respect to the effect of inhibitors on mammalian tissue lipases, presumably due to the use of crude enzyme preparations and grossly impure substrates.

It was hoped that the use of some known lipase inhibitors in this study would yield additional information as to whether the cardiac monoglyceride-splitting lipase was similar to those which have been reported to exist in adipose tissue and in the intestinal mucosa. The results of these studies are presented in Table III, including the data obtained by other investigators for reference.

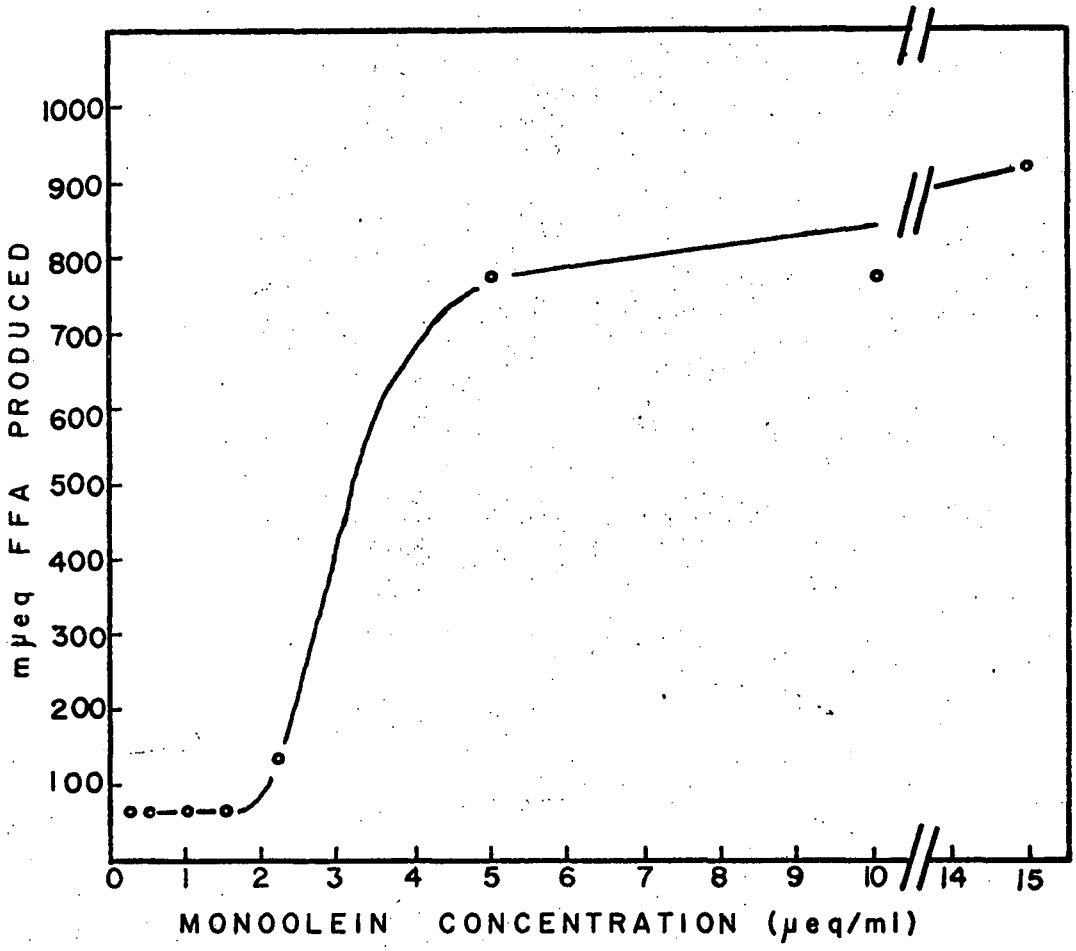


FIG. 14 Effect of monoolein concentration on the activity of the partially purified cardiac lipase.

The reaction mixture contained all components of the standard assay, except that the monoolein concentration was varied as indicated and 0.5 M NaCl was included.

TABLE III

Inhibition of Monoglyceride-splitting Lipase
by Various Compounds

The standard assay was used with 15 ueq monoolein as substrate, except that 0.5 M NaCl was included in all the reaction mixtures. The various inhibitors were pre-incubated for 15 minutes at 37° with the partially purified enzyme preparation prior to addition of substrate.

COMPOUND	THIS STUDY		Kupieki (16)		Vaughan et al. (6)		Strand et al. (15)		Pope et al. (59)	
	conc.	% inhib.	conc.	% inhib.	conc.	% inhib.	conc.	% inhib.	conc.	% inhib.
NaF	0.15M	75	0.20M	45	---	--	0.20M	84	1×10^{-3}M	100
Isopropanol	(0.02ml/ml)	0	---	--	(0.025ml/ml)	0	(0.025ml/ml)	0	---	--
DFP	2×10^{-4}M	70	1×10^{-5}M	39	5×10^{-4}M	97	---	--	1×10^{-6}M	100
EDTA	5×10^{-4}M	0	5×10^{-4}M	0	---	--	---	--	---	--
Protamine sulfate	400 µg/ml	0	300 µg/ml	0	---	--	---	--	---	--
N-Ethyl Maleimide	1×10^{-4}M	25	1×10^{-4}M	33	---	--	---	--	---	--
Iodoacetic acid	1×10^{-4}M	0	1×10^{-3}M	0	---	--	---	--	---	--
P-chloromercuri-benzoate	---	--	1×10^{-4}M	79	---	--	---	--	1×10^{-4}M	0
Substrate	Monoolein		Monoolein		Monostearin		Monoolein		Monoolein	
Monoglyceride-splitting lipase source	Partially purified from rat heart		Partially purified from rat adipose tissue		Crude extract adipose tissue		Crude extract adipose tissue		Highly purified from rabbit intestinal mucosa	

The most potent inhibitors of the monoglyceride hydrolyzing activity were NaF and DFP, and the effects observed were in good agreement with those seen for monoglyceride-hydrolyzing lipases of other tissues. The mechanism of inhibition by NaF is not known, but the relatively high concentrations normally required to show this inhibition restricts the usefulness of this compound, except perhaps to distinguish between monoglyceride-hydrolyzing activity and lipoprotein lipase activity. On the other hand, DFP inhibited the enzyme at a relatively low concentration. DFP is known to combine irreversibly with the -OH function of enzymes (e.g. esterases), thus blocking the active site of the enzyme. In this respect, the monoglyceride-hydrolyzing enzyme appears to resemble an esterase.

The complete absence of inhibition by protamine sulfate (400 µg/ml) was expected since any lipoprotein lipase activity which may have been present in the extract would have been effectively inhibited under the conditions of the assay.

The presence of EDTA (5×10^{-4}M) did not inhibit the activity, which likely indicates that no positively changed metal cations were required for activity.

The alkylating agents, N-ethylmaleimide and iodoacetate, used at the same concentrations would have been expected to give similar results. Therefore the inhibition observed with N-ethylmaleimide, but not with iodoacetate is difficult to reconcile, although it must be noted that Kupieki (16) obtained essentially similar results with these compounds. The effects

of these inhibitors on the monoglyceride-splitting lipase activity of heart tissue is in good agreement with those observed for the monoglyceride-hydrolyzing enzymes studied in other tissues, and further indicated the similarity of these activities.

6. Intracellular Localization -- It has been previously mentioned that the enzyme was bound to tissue particles. The intracellular distribution of the monoglyceride-splitting lipase was investigated in order to provide some evidence as to its possible physiological function in cardiac tissue. As shown in Table IV, about 85% of the activity was bound to the particulate components of the cell, about 50% of the total activity being located in the nuclear fraction. In contrast, when tripalmitin was used as substrate, the only cellular fraction to exhibit any lipolytic activity was the microsomal fraction (Table IV), although the activity was so low as to make accurate determinations difficult. In these experiments, 0.5 M NaCl was included in the assay system in order to inhibit lipoprotein lipase activity. The particle-bound nature of the monoglyceride-hydrolyzing lipase in heart tissue is similar to that reported recently by Pope et al. (59) for the purified monoglyceride lipase of intestinal mucosa, in which only about 10% of the total activity was located in the soluble fraction.

TABLE IV

Distribution of monoglyceride-splitting lipase and triglyceride lipase activities in various fractions of a rat heart homogenate.

Ventricular tissues were pooled from 6 rat hearts and homogenized in 12 volumes of 0.25 M sucrose containing 0.05 M Tris buffer, pH 7.0, for two 1-minute intervals using a Servall Omnimixer to obtain the whole homogenate. The nuclear fraction was obtained by centrifuging the homogenate at 250-300 x g for 15 minutes. The lightly packed sediment was washed with 15 mls 0.25 M sucrose pH 7.0, re-centrifuged and the supernatant fluids combined. The mitochondrial and microsomal fractions were obtained by centrifuging the combined supernatant fluids at 6000 x g x 15 minutes and 105,000 x g x 60 minutes respectively. All procedures were carried out at 0-4°. Standard assay conditions were employed, using either monoolein or tripalmitin as substrates, except that NaCl 0.5 M was also included in each reaction mixture. The assay for triglyceride-splitting activity was performed using 10 times more extract than used for the monoglyceride-splitting lipase activity, and incubation was for 120 mins at 37°. Enzyme activity is as defined in the text.

Fraction	Total Activity (mμ units)	%
MONOGLYCERIDE-SPLITTING LIPASE		
Whole Homogenate	536,500	(100%)
Nuclear Fraction	287,500	53.5
Mitochondrial Fraction	110,000	20.5
Microsomal Fraction	75,000	14.0
105,000 x g Supernate	80,600	15.0
TRIGLYCERIDE-SPLITTING LIPASE		
Whole Homogenate	2590	(100%)
Nuclear Fraction	0	0
Mitochondrial Fraction	0	0
Microsomal Fraction	2000	77.2
105,000 x g Supernate	0	0

DISCUSSION

The object of the perfusion studies was primarily to investigate the possibility that endogenous triglycerides were utilized by the heart, and to compare this utilization with that of glycogen when no exogenous substrates were available. In general, the changes observed in the glycogen levels of rat ventricular tissue during fasting and during substrate-free perfusions were not unexpected. The increase in fasting cardiac glycogen levels deserves further comment. Although Cruikshank (110) observed that pancreatectomy caused a shift in liver glycogen to cardiac glycogen, the first to observe this phenomenon in fasting was Evans (8) in 1934. Evans found that glycogen content increased in rat hearts from 341 ± 15 mg/100 g to 578 ± 14 mg/100 g wet weight after a 48-hour fast. Then Lackey and co-workers (111-113) found that cardiac glycogen increased in the alloxan diabetic state (111), that there was a direct relationship between blood ketone concentrations and cardiac glycogen levels (112, 113). Next, it was shown by Russell and Bloom (114) that growth hormone was necessary for glycogen to increase in heart during fasting. Lukens (115) suggested that the increase in cardiac glycogen was due to the increased amount of FFA reaching the heart, owing to the action of growth hormone on adipose tissue. This suggestion was later confirmed by Bowman (116). More recently, Newsholme and Randle (9) and Garland and co-workers (10) showed that ketone bodies,

FFA, and pyruvate effectively inhibit the glycolytic enzyme, phosphofructokinase in perfused rat hearts. Parmeggiani and Bowman (11) demonstrated further that the inhibition of phosphofructokinase was due to the increased tissue levels of citrate.

The increase in cardiac glycogen content during fasting and its immediate decline upon re-feeding was observed during the course of this work. As may be noted in Fig. 15, cardiac glycogen increased over 2.5-fold during seven days of fasting. The immediate depletion of glycogen after one day of re-feeding to the control level indicates a very rapid utilization and/or mobilization of glycogen to other tissues of the body, probably the liver.

The slower rate of glycogen disappearance from perfused (control) hearts from fasted rats as compared with those from the fed animals suggested that <u>in vivo</u>, the biological mechanisms for utilizing lipids was accelerated in fasting rat hearts. In fact, the triglyceride levels of the fasted group in the "control" series appeared to decrease during perfusion, suggesting perhaps, that endogenous triglycerides may have been utilized under these perfusion conditions. However, in view of the unexplainable increases in triglyceride levels found under different perfusion conditions, it is difficult to state categorically at this time that triglycerides were mobilized and oxidized by the working rat heart. At best, the data is interpreted as only suggestive that this might have been the case.

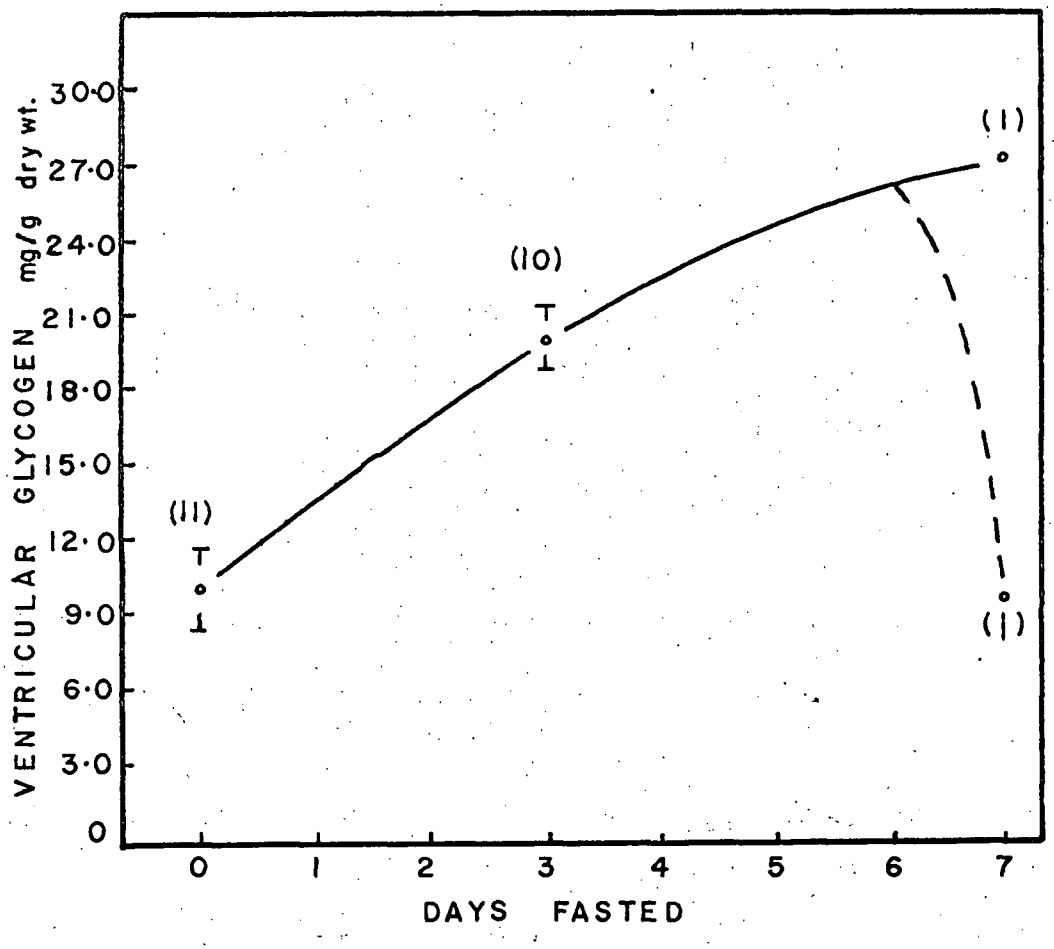


FIG. 15 Effect of Fasting on Myocardial Glycogen Level.

Rats were fasted for the duration indicated. A rat was re-fed ad lib. on the sixth day and the ventricular glycogen content determined after 24 hours re-feeding (dotted line). Tissue glycogen contents are shown as the means ± standard error of the mean. The number of animals used is shown in parenthesis.

The increased rate of depletion of glycogen from epinephrine-perfused hearts was expected. It was calculated that within about 10 minutes, between 1.8 to 3.0 mg of glycogen actually disappeared from a heart of a fasted rat under the influence of epinephrine. Since it was difficult to believe that all the glucose derived from glycogenolysis under these conditions was being converted to lactate, or even oxidized by the Krebs cycle, the perfusate was analysed for glucose, but none could be detected by the glucose-oxidase method.

The mechanism of action of epinephrine on glycogenolysis is perhaps the best understood of all hormones investigated. Briefly, epinephrine promotes the formation of cyclic 3',5'-AMP from ATP by stimulation of an enzyme, adenyl cyclase. Cyclic 3',5'-AMP then brings about the conversion of inactive phosphorylase b to active phosphorylase a, mediated by phosphorylase b kinase. Phosphorylase a stimulates glycogenolysis, yielding glucose-1-phosphate. This scheme of glycogenolysis has been reviewed by Sutherland (12).

The data for the tissue triglyceride series is most difficult to interpret. If the increases in triglyceride content of epinephrine and heparin perfused hearts were real, this would be a most interesting observation, but the idea that triglycerides were actually synthesized during substrate-free perfusions is most unacceptable to the author. It is more likely that some product of glycogenolysis was extracted into the organic layer during the extraction of tissue lipids. Chromotropic acid would then later combine with the compound

to give falsely high values for triglycerides.

The experiments with epinephrine and heparin therefore yielded no useful information regarding the breakdown of triglyceride by the heart. On the other hand, triglycerides may have been hydrolyzed and metabolized in the "CONTROL" series. It would appear that in order to demonstrate clearly whether triglycerides are indeed utilized by the heart, perfusion conditions must be altered somehow so that hearts will function normally for 60 minutes or more, even under substrate-free medium. Further studies using longer periods of perfusion (without epinephrine or heparin) and using a larger population of rats would most likely be necessary before the disappearance of triglycerides in rat hearts can be conclusively demonstrated.

Evidence has been presented which clearly establishes that a lipolytic activity other than lipoprotein lipase exists in rat myocardium. The properties of the enzyme differ greatly from those of lipoprotein lipase, so that the two enzymes can be distinguished even in crude preparations. In many respects, the properties of the enzyme are similar to monoglyceride-hydrolyzing lipases reported in adipose tissue (6, 15, 16) and intestinal mucosa (56, 58, 59), and particularly to the Ediol^{R}-hydrolyzing enzyme system in rat hearts (13).

The pH optimum of the monoglyceride-hydrolyzing enzyme of cardiac tissue agrees with the optimum pH of 6.8 reported by Bjorntorp and Furman (13) for an unspecified cardiac enzyme which actively hydrolyzed Ediol^{R}. The monoglyceride-hydrolyzing activity in adipose tissue described by Strand et al.

(15) also has a pH optimum within this range (pH 7.0). On the other hand, Kupieki (16) and Vaughan and co-workers (6) have reported pH optimums of 7.5 and 8.0, respectively, for the same monoglyceride-splitting enzyme in adipose tissue. These inconsistencies may have arisen from the fact that different buffers and monoglyceride substrates were used in these studies. The pH optimum of the cardiac enzyme is significantly lower than that of the intestinal mucosa lipase which is reported to have an optimum pH of 7.8 (56) and 8.5-9.0 (59). The monoglyceride-splitting enzyme of rat and hog liver has a pH optimum of 8.2 (55).

The optimal temperature (45°) for the *in vitro* lipolytic activity of the heart lipase enzyme is consistent with that found by Vaughan and associates (6) for the enzyme in adipose tissue. No other temperature optima have been reported for further comparison.

The relative activity of monoglyceride-hydrolyzing systems against di- and triglycerides have been described for the intestinal mucosa. Senior and Isselbacher (56) observed the comparative rates of tri-, di-, and monoglyceride hydrolysis to be 0.3, 1.6, and 91.9 respectively. Pope and associates (59) found the relative rates of hydrolysis of tri-, di-, and monoolein to be 0.0, 0.0, and 1.0 respectively. McPherson *et al.* (58) observed that monoolein was hydrolyzed 16 times as fast as triolein. In adipose tissue, the comparative rates were 1, 43, 73, and 100 for triolein, 1,2-diolein, 1,3-diolein, and monoolein respectively as

reported by Strand and co-workers (15). Again in adipose tissue, Kupieki's data (16) indicates the relative rates are 0.19, 0.17, and 1.00 for tri-, di-, and monostearin. The accumulated evidence indicates that whereas the intestinal mucosa enzyme is highly specific for monoglycerides, the adipose tissue enzyme is relatively less specific in this respect. In the present study, no experiments were designed specifically to investigate the activity of the cardiac enzyme with respect to its activity against di- and triglycerides. However, from the data collected from occasional experiments in which both tripalmitin and monostearin (or monoolein) were used as substrates, it may be readily seen that the activity against monoolein and monostearin were significantly higher than against tripalmitin (Table V). Comparison of tripalmitin and monostearin hydrolysis in one experiment (Fig. 8) showed that the rate of monostearin hydrolysis was as much as 20-fold greater than that of tripalmitin hydrolysis. No definite statement can be made at present regarding the absolute specificity of the cardiac enzyme. There is no doubt that the enzyme hydrolyzes monoglycerides at an appreciably faster rate than it does triglycerides, but much more data is required to indicate its actual degree of substrate specificity. For example, the hydrolysis rates of mono-, di-, and triolein as compared with the rates of mono-, di-, and tripalmitin would be of interest. If it is established that the enzyme is highly specific for monoglycerides only, then its action on the 1- and 2-isomers of monoglycerides,

TABLE V

Relative rates of Hydrolysis of Monoolein, Monostearin and Tripalmitin

	SUBSTRATE	ACTIVITY mμeq FFA/60 min	RELATIVE ACTIVITY (Tripalmitin-1.0)
EXPT. A	Monoolein	34.0	12.1
	Tripalmitin	2.8	1.0
EXPT. B	Monostearin	42.0	3.8
	Tripalmitin	11.0	1.0
EXPT. C	Monostearin	208.0	5.2
	Tripalmitin	38.0	1.0

In Experiment "A", the microsomal fraction of heart tissue was used as the enzyme source. Standard assay conditions were used except that the reaction mixture containing monoolein and triolein were incubated in 0.05 M Tris buffer, pH 7.0 and 8.5 respectively.

In Experiment "B" and "C", the enzyme preparations were the isoelectric precipitate fraction and whole homogenate (1:10) respectively. Standard assay conditions were employed.

and its activity against medium and short chain monoglycerides must be investigated. Further studies of this nature, of course, must await a more extensive purification of the enzyme than has been obtained in this study.

The 75% inhibition of the cardiac lipolytic enzyme by 0.2 M NaF compares favourably with the similar type of lipolytic activity reported in adipose tissue (15, 16) and intestinal mucosa (59) where inhibitions of 84%, 45%, and 100% respectively have been reported. Since NaF does not inhibit lipoprotein lipase, the potent inhibitory action of 0.2 M NaF must indicate the presence of another lipolytic system in cardiac and other tissues. The inhibition by relatively low concentrations of DFP appears to be a common feature of monoglyceride-splitting enzymes. Thus, when discussing the possibility that the 300-fold purified monoglyceride-splitting enzyme in the intestinal mucosa may be an esterase (rather than a lipase), Pope and associates (59) have agreed that their enzyme was, in fact, a lipase, owing to the observation that "an increasing solubility of the substrate is associated with a decreasing rate of hydrolysis". It was likewise indicated in this study that the increased activity of the cardiac enzyme was associated with the increased availability of insoluble substrates.

The physiological role of lipoprotein lipase in heart is not completely understood at the present time. Its major role may be to hydrolyze exogenously supplied triglycerides to diglycerides, monoglycerides and FFA. Since the enzyme is

rapidly eluted from heart and adipose tissue *in vitro* by heparin, its location on or near the plasma membrane has been postulated. Alousi and Mallov (17) found the following distribution of lipoprotein lipase in cardiac cells: nuclear fraction, 1.33 ± 0.14; mitochondrial fraction, 0.83 ± 0.25; microsomal fraction, 1.16 ± 0.14; soluble fraction, 0.99 ± 0.13. The total enzyme activity was 4.14 ± 0.34. The high concentrations of the enzyme activity in the nuclear and microsomal fractions give support to the concept that lipoprotein lipase is essentially a membrane-located enzyme.

The intracellular distribution of the cardiac monoglyceride-hydrolyzing lipase (Table IV) also indicated that the enzyme is membrane-bound. Therefore, one might reasonably speculate that the primary physiological role of the monoglyceride-hydrolyzing lipase in cardiac tissue might be that of completing the final step in the hydrolysis of triglycerides. A similar co-ordinated lipolytic system is also thought to exist in the hydrolysis of triglycerides in the intestinal tract *in vivo*. It is known that pancreatic lipase hydrolyzes triglycerides to monoglycerides, and that its action essentially stops at this stage of the hydrolytic process in the intestinal lumen. However, after monoglycerides are absorbed into the intestinal mucosal cells, they are either further hydrolyzed to glycerol and FFA, or are re-esterified to higher glycerides. It is highly attractive to speculate that perhaps in the case of cardiac cells as well, the triglycerides in chylomicrons and very low density lipoproteins are first hydro-

lyzed to monoglycerides at the outer surface of the cells (including the lumen of the endoplasmic reticulum), then absorbed, and completely hydrolyzed by the monoglyceride-splitting lipase, or re-esterified to higher glycerides. However, this idea is not supported by isotopic studies which indicated that triglycerides are taken up intact by the heart. Therefore one is left with the alternative idea that the actions of both lipoprotein lipase and the monoglyceride-splitting lipase occur intracellularly, but on or near the inner aspect of the plasma membrane.

Some of the problems encountered during attempts to purify the monoglyceride-splitting lipase activity from cardiac tissue have already been briefly mentioned. However, the difficulties involved in attempting to solubilize and purify the enzyme cannot be over-emphasized. The enzyme was tightly bound to the particulate materials of the cell and was extremely resistant to the usual solubilizing techniques. It is noted that Pope and co-workers (59) used 0.3% sodium deoxycholate to rupture the microsomal particles and thus solubilized the enzyme prior to purification and study of the monoglyceride-hydrolyzing enzyme in the intestinal mucosa. In our hands, 0.1% deoxycholate treatment resulted in some solubilization, but this technique also resulted in low recoveries of activity. Although butanol extraction was considered as a possible technique, the reasonably satisfactory solubilization obtained with Triton X-100 obviated the necessity for its use.

The degree of purification of the monoglyceride-hydrolyzing enzyme in heart tissue is admittedly very small.

Nevertheless, it should be born in mind that with the possible exception of plasma lipoprotein lipase, pancreatic lipase and the most recently reported monoglyceride-hydrolyzing lipase of intestinal mucosa, extensive purification of lipase in biological systems have been overwhelmingly unsuccessful. Kupieki (16) for example, succeeded in obtaining a modest 4.8-fold purification of a monoglyceride-hydrolyzing lipase from adipose tissue. Nevertheless, when one considers the importance of lipid metabolism in mammalian systems, it is essential that greater efforts be directed in the future toward purifying and studying the lipase in these organisms.

The indiscriminate use of Ediol^{R} as substrate for measuring lipolytic activity has been criticized in this thesis. With the aid of tripalmitin-1-C^{14} incorporated into Ediol^{R}, the present study has shown that even when the hydrolysis of triglycerides in Ediol^{R} was completely abolished, significant amounts of free fatty acids were still being produced during the initial 20-25 minutes of the reaction. Since the addition of 0.2 M NaF caused a further consistent and significant (70-75%) decrease of this lipolytic activity, the source of these FFA's must have been derived from the monostearin component of Ediol^{R}. From these observations, incidentally, it was suggested that the unspecified Ediol^{R}-hydrolyzing activity in extracts of rat hearts which was reported by Bjorntorp and Furman (13) was probably entirely due to the hydrolysis of the monoglyceride component. Other workers have recently looked upon the use of Ediol^{R} in lipase studies

with some suspicion. Very recently, Kupieki (16) reported that the lipolytic activity of an adipose tissue extract prepared as described by Rizack (107) liberated as much free fatty acids from monostearin as from Ediol^R (i.e., 10.80 versus 9.84 μeq/hr/100 mg tissue from monostearin and Ediol^R, respectively). Furthermore, Kupieki (16) found that the pH optima were virtually indistinguishable when either of these substrates were used. He concluded that ". . . it appears (that) when the hydrolysis of Ediol^R is used to follow lipolytic activity, the results can be misleading; the added monostearin which serves as an emulsifier in Ediol^R can account for a large part of the FFA released by this substrate". The first hint, however, that the monoglyceride component of Ediol^R may be rapidly hydrolyzed was offered by Vaughan et al. (6) who observed that glycerol production was not proportional to adipose tissue homogenate concentration when Ediol^R was used as substrate. When the amount of glycerol derivable from the stated amount of monostearin present in Ediol^R was subtracted from the amounts of glycerol produced, the "corrected" glycerol production was linear with respect to homogenate concentration and a straight line was obtained through the origin. The results of the present study therefore not only support the suspicions of Kupieki (16) and Vaughan et al. (6) but provided direct evidence that the monostearin component in Ediol^R is, in fact, rapidly hydrolyzed by cardiac enzymes.

It is safe to conclude from these studies that a lipase

other than lipoprotein lipase exists in rat myocardium. Some of its properties have been described. Much further study will be required to clarify its true physiological function in overall cardiac energy metabolism. The knowledge will come only with success in purifying the enzyme. Whether it will be subject to regulation through epinephrine or some other hormone can only be a matter of speculation at the present time.

PART II

STUDIES ON 3',5'-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

INTRODUCTION

In recent years, the molecular regulation of cellular activity has been one of the most intensively studied areas in biochemistry. It is not our purpose here to review the mechanisms by which the living cell regulates its metabolic activity. We wish only to point out that many small molecules, bearing no necessary structural relation to substrate or product can profoundly influence the action of an enzyme in either a positive or negative direction. The physiological implications of such allosteric effects are boundless. One such compound which has attracted much attention as an effector or mediator of important enzymatic reactions is adenosine 3',5'-cyclic phosphate (cyclic 3',5'-AMP).

The discovery of cyclic 3',5'-AMP in mammalian tissues in 1958 originated from studies on liver phosphorylase. In 1951, Sutherland and Cori (119) noted that when liver slices were incubated with epinephrine and glucagon, the glycogen content decreased (glycogenolysis) and phosphorylase activity increased. Studies on liver phosphorylase, the enzyme which degrades glycogen to glucose-1-phosphate, revealed that it existed in an active and inactive form. Epinephrine was shown to mediate glycogenolysis by shifting the balance of liver phosphorylase in favour of the active enzyme. The enzyme, phosphorylase phosphatase, was isolated which catalyzed the inactivation of highly active liver phosphorylase (120, 121). At this time, Rall and Sutherland (122) also reported on

another enzyme which catalyzed the conversion of inactive liver phosphorylase to the active form. This enzyme, which was given the name phosphorylase kinase, required Mg^{++} ions and ATP for activity. From their studies, these investigators concluded that epinephrine stimulated glycogenolysis not by acting on phosphorylase, but by acting in some way on the latter enzyme, phosphorylase kinase. Rall and his associates (123) then made the important observation that when particulate fractions of dog liver homogenates obtained by low speed centrifugation were incubated with epinephrine (or glucagon) together with ATP and Mg^{++} ions, a heat-stable factor was produced which stimulated the activation of inactive liver phosphorylase. It became clear that the action of epinephrine was indirect, in that it stimulated the synthesis of a heat-stable factor in cell particles which in turn acted on phosphorylase kinase, with the result that phosphorylase was activated, leading to increased glycogenolysis. This factor was soon isolated from dog liver and crystallized by Sutherland and Rall (124, 125) in 1958, and was finally characterized (126) as adenosine 3',5'-cyclic phosphate (Fig. 16).

An enzyme system in liver which catalyzed the formation of cyclic 3',5'-AMP was first reported by Rall and Sutherland (127) in 1958. In the presence of Mg^{++} ions, ATP, epinephrine and glucagon, 1200 x g particles of liver (and also of heart, skeletal muscle and brain) formed significant amounts of cyclic 3',5'-AMP. The name "adenyl cyclase" was adopted by

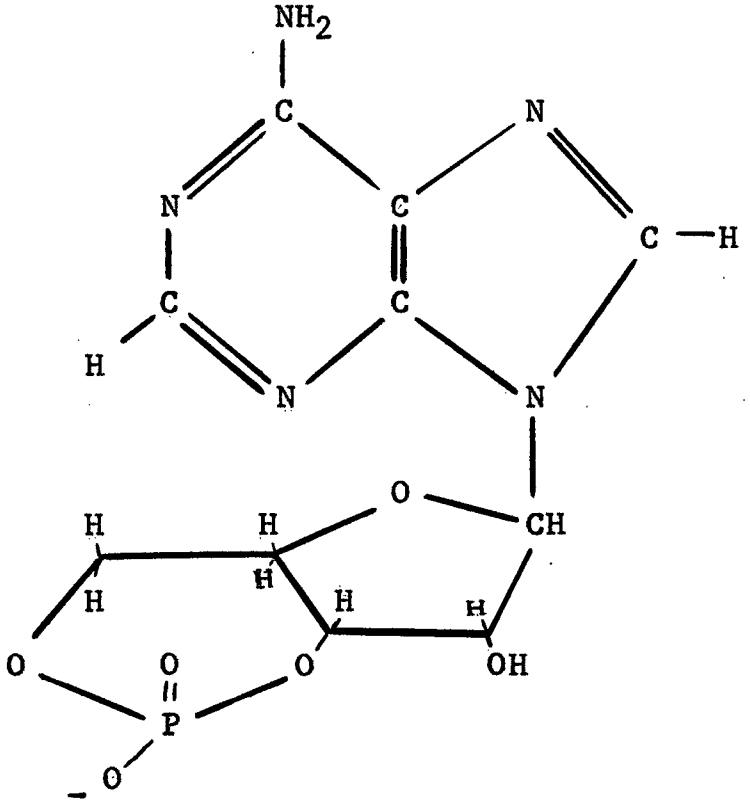


FIG. 16 Structural formula of adenosine-3',5'-phosphate (cyclic 3',5'-AMP).

Sutherland et al.(128) for the enzyme. Adenyl cyclase was detected in all animal tissues studied (128), except in dog red blood cells. The tissues possessing the highest specific activity were cerebral cortex and cerebellum of beef, calf, sheep and pig. Equally high activities were found in liver flukes (Fasciola hepatica) and in earthworms (Lumbricus terrestris). Intermediate levels of cyclic 3',5'-AMP-forming activity were found in testis, uterus, intestinal mucosa of dog, the liver of cat and rat, skeletal muscle of rabbit and blood cells of pigeons. Low in adenyl cyclase activity were rat epididymal fat pads, fly larva, and minnow. The relative activities (on protein basis) in dog tissues were: brain cortex (11.0), spleen (2.0), skeletal muscle (2.0), heart ventricle (2.0), lung (1.5), kidney cortex (1.0), liver (0.5), aorta (1.0), intestinal muscle (1.0), femoral artery (1.5) and adipose tissue (1.0). Studies on the intracellular localization of this enzyme indicated that it might be derived from plasma membranes or from nuclei. Later studies by Davoren and Sutherland (129) with pigeon erythrocytes demonstrated that no adenyl cyclase activity was associated with the nuclei. It appeared, therefore, that adenyl cyclase was located on the plasma membrane of cells. The preparation of the enzyme in a purified form was seriously hampered by its association with particulate materials of the "nuclear" fraction, by its instability, and by its close association with Triton X-100 after solubilization (128). The purification of adenyl cyclase was 2- to 3-fold from brain and about 15-fold from liver.

It was previously mentioned that epinephrine stimulated the synthesis of cyclic 3',5'-AMP, and it is now known that the immediate site of action of epinephrine is adenyl cyclase. Murad and associates (130) studied the relative potencies of several catecholamines on the adenyl cyclase system of dog myocardial and liver particles. In the myocardial system, the relative potencies were as follows: L-isopropylnorepinephrine (7.8), L-epinephrine (1.0), L-norepinephrine (1.0) and D-epinephrine (0.12). In the liver system, the relative potencies were: L-isopropylnorepinephrine (4.0), L-epinephrine (1.0), L-norepinephrine (1.0). Dichloroisopropylnorepinephrine (DCI), an adrenergic blocking agent, blocked the stimulating effect of catecholamines. The effect of epinephrine on the particulate preparations of adenyl cyclase from brain were also invetigated by Klainer et al (131). These workers observed that in the presence of epinephrine, a 2-fold increase in cyclic 3',5'-AMP formation by particulate preparations from the cerebellum was obtained. Cyclase preparations from the cerebral cortex, pons, medulla, and spinal cord were also stimulated by epinephrine.

It has become clear that cyclic 3',5'-AMP plays an important role in mediating the metabolic effects of catecholamines. The participation of cyclic 3',5'-AMP in the glycogenolytic response of the liver to epinephrine is shown in Fig. 17. Essentially, a similar series of enzymatic reactions occur in skeletal muscle (132) and in cardiac muscle (133). The metabolic role of cyclic 3',5'-AMP in glycogenolysis has thus

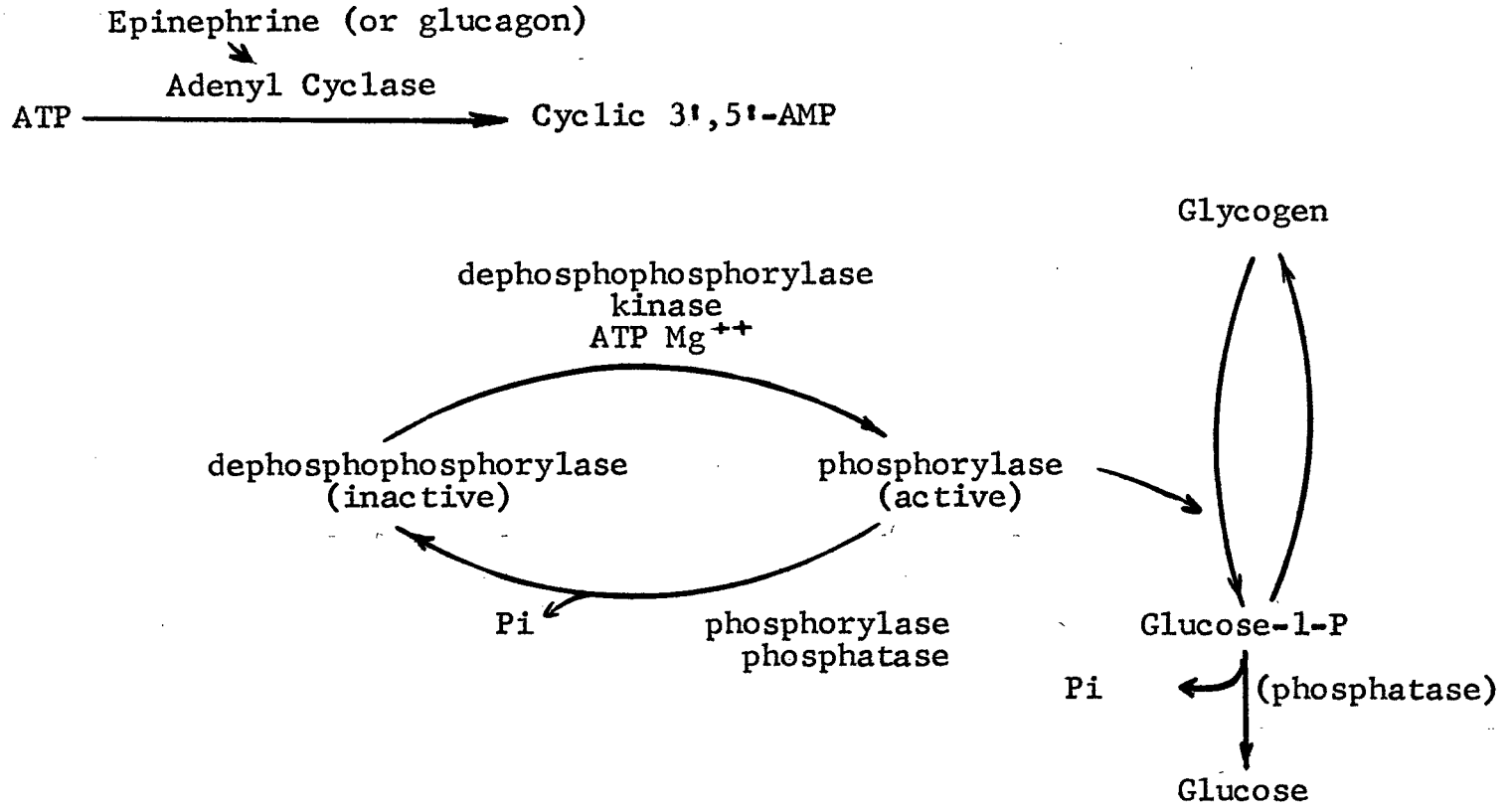


FIG. 17 Mediation of cyclic 3',5'-AMP in the glycogenolytic response of liver to epinephrine (or glucagon).

been firmly established.

Investigations by several workers have indicated that the role of cyclic 3',5'-AMP in biological systems extends far beyond its participation in the process of glycogenolysis. The activity of a number of enzyme systems (other than the phosphorylase system in liver, heart and skeletal muscle) have been shown to be influenced by cyclic 3',5'-AMP. Haynes and Berthet (134) found that the addition of adrenocortical hormone (ACTH) to adrenal tissue slices caused a rapid and specific activation of phosphorylase in these tissues. Furthermore, Haynes (135) showed that when ACTH was added to slices of beef adrenal cortex, cyclic 3',5'-AMP accumulated in these tissues. When cyclic 3',5'-AMP itself was added, phosphorylase activity increased. Thus, he concluded that the action of ACTH on the adrenal cortex was mediated by cyclic 3',5'-AMP.

Another enzyme which is affected by cyclic 3',5'-AMP is glycogen synthetase (UDPG-α-glucan Transglucosylase). Belocopitow (136) observed a decrease in glycogen synthetase activity in rat diaphragms after these tissues were incubated with epinephrine. In order to investigate further the mechanism of action of epinephrine on glycogen synthetase, he incubated a 4000 x g supernatant of rat skeletal muscle homogenates with cyclic 3',5'-AMP and ATP. When cyclic 3',5'-AMP was omitted from the system, an increase in synthetase activity was observed. When both ATP and cyclic 3',5'-AMP were omitted, a further increase in the enzyme activity was observed. These observations led Belocopitow (136) to suggest that cyclic

3',5'-AMP may have been inhibiting the synthetase system. Recently, Rosell-Perez and Larner (137) showed that the action of cyclic 3',5'-AMP on the inhibition of glycogen synthetase activity was closely associated with enhancing the conversion of the "I" (Independent, active) form of the enzyme to the "D" (Dependent, inactive) form. It has become evident, therefore, that the inactivation of glycogen synthetase coupled with the simultaneous activation of phosphorylase by cyclic 3',5'-AMP would constitute an important role for the cyclic nucleotide in the regulation of glycogen breakdown and synthesis. In 1960, Mansour and his co-workers (138) made an interesting observation relating to the phosphorylase system in the liver fluke, Fasciola hepatica. These workers demonstrated that the activation of phosphorylase in this organism was obtained not with epinephrine, but with 5-hydroxytryptamine (serotonin). Furthermore, their studies showed that serotonin caused a rapid increase in cyclic 3',5'-AMP levels, and also that activation of phosphorylase was mediated by the cyclic nucleotide. Mansour (139) also demonstrated that glycolysis in homogenates of liver flukes was regulated by the activity of phosphofructokinase (PFK), and that stimulation of glycolysis by serotonin resulted in a marked increase in PFK activity. Mansour and Mansour (140) reported that cyclic 3',5'-AMP could activate liver fluke PFK which had been inhibited by ATP, and that the cyclic nucleotide could also activate an inactive preparation of PFK. Similar effects of cyclic 3',5'-AMP in PFK activity have been demonstrated with the enzyme isolated from mammalian cardiac tissue (141, 142) and skeletal

muscle (143).

The observation that cyclic 3',5'-AMP mediated the glycogenolytic response of the liver to epinephrine led investigators to study the possibility that cyclic 3',5'-AMP might also mediate the lipolytic response of adipose tissue to epinephrine. Indeed, Rizack (144) showed that an epinephrine-sensitive lipase in cell-free extracts of adipose tissue could be activated by the addition of cyclic 3',5'-AMP in the presence of ATP and Mg^{++} ions. Butcher and his co-workers (145) found that when a derivative of cyclic 3',5'-AMP, N^6-2'-O-dibutyryl cyclic AMP was incubated with intact isolated fat cells, lipolysis was stimulated some 10-fold. Hence, another role for cyclic 3',5'-AMP, that of increasing the mobilization of free fatty acids from fat depots, has been established. The enzyme, tryptophan pyrrolase, which opens the indole ring of tryptophan to yield formylkynurenine, appears to exist in an active and an inactive form. Although studies on the effects of cyclic 3',5'-AMP on this enzyme system have not been extensive, there are indications that this enzyme can be converted from the inactive to the active form by cyclic 3',5'-AMP (146, 147), although a recent report (148) indicates that 5'-AMP, guanine, guanosine and 5'-GMP were also highly effective.

There are several other biological processes in which cyclic 3',5'-AMP has been implicated. For example, the positive inotropic response of the heart to epinephrine and other catecholamines has been widely studied and the indications

are that this process may also be mediated by cyclic 3',5'-AMP (149). Recent studies had shown that the inotropic response and the activation of cardiac phosphorylase were apparently unrelated (150, 151). Then Robison and his associates (149) observed that a single dose of epinephrine caused a 4-fold increase in cyclic 3',5'-AMP levels in cardiac tissue within 3 seconds after injection, while the contractile force increased about 1.4-fold at 20 seconds. These observations therefore favour the hypothesis that cyclic 3',5'-AMP may mediate the inotropic response of the heart to catecholamines.

It was mentioned earlier that the activation of adrenocortical phosphorylase by ACTH was shown to be mediated by cyclic 3',5'-AMP. Since ACTH action on the adrenal cortex stimulates the synthesis of corticosteroids, one might expect that cyclic 3',5'-AMP itself might mimic the action of ACTH. Indeed, Haynes et al. (152) found that when they added cyclic 3',5'-AMP to fragments of incubating rat adrenals, the total corticoid production was stimulated almost 5-fold. Recent studies have indicated that the stimulation of steroid production by the adrenal cortex was not solely due to the activation of phosphorylase. Roberts and co-workers (153, 154, 155) found that cyclic 3',5'-AMP selectively stimulated C-11β hydroxylase activity in rat adrenal homogenates fortified with glucose-6-phosphate and NADP, resulting in increased formation of corticosterone from exogenous 11-deoxycorticosterone or progesterone. They also showed that the conversion of progesterone to 11β-hydroxyprogesterone was increased.

These workers concluded that the action of 3',5'-AMP on the stimulation of steroidogenesis was independent of phosphorylase activation, NADPH generation and the presence of endogenous corticosteroid precursors. Roberts et al. (156, 157) showed that cyclic 3',5'-AMP also stimulated the hydroxylation of 11-deoxycorticosterone to 18-hydroxy-11-deoxycorticosterone, as well as the conversion of exogenous cholesterol to pregnenolone by isolated rat adrenal mitochondria. Karaboyas and Koritz (158) recently observed that cyclic 3',5'-AMP stimulated the incorporation of acetate into corticosterone, and the conversion of cholesterol to corticosterone. A report by Darrington and Kilpatrick (159) indicates that cyclic 3',5'-AMP stimulated the synthesis of two progestational steroids, 4-pregnen-20α-ol-3-one and progesterone by ovarian tissues of rabbits.

Pryor and Berthet (160) reported that the incorporation of leucine into protein of rat liver slices was inhibited when these tissues were incubated with cyclic 3',5'-AMP or with glucagon. Exton and Park (161, 162) have indicated that the effect of these hormones on gluconeogenesis appears to be mediated by cyclic 3',5'-AMP. Furthermore, these authors have suggested that the direct site of cyclic 3',5'-AMP action may be the activation of phosphopyruvate carboxylase and the hexose phosphate phosphatases. Strong evidence also exists that cyclic 3',5'-AMP may mediate the secretion of enzymes from rat parotid glands. Bdolah and Schramm (163) have indicated that when rat parotid slices are incubated with dibutyryl

cyclic AMP, the release of amylase from the glands is stimulated almost to the same extent as that observed with epinephrine. Orloff and Handler (164) reported that the addition of cyclic 3',5'-AMP to media in which toad bladders were immersed, caused a significant increase in the permeability of the membrane to water. Since vasopressin (antidiuretic hormone) is also known to elicit the same response, these authors suggested that the antidiuretic action of vasopressin might also be mediated by cyclic 3',5'-AMP. Recent studies on the action of vasopressin on the toad bladder by Strauch and Langdon (165) and by Handler and associates (166) have indicated that the action of this hormone may be a direct stimulation of adenyl cyclase. The cellular mode of action of vasopressin has been recently reviewed by Orloff and Handler (167). The decreased incorporation of acetate into fatty acids and cholesterol of liver slices, and the increased production of ketone bodies by epinephrine, glucagon and by cyclic 3',5'-AMP has been reported by Berthet (168). Cyclic 3',5'-AMP has also been implicated in sugar transport in the thyroid gland (169), and in the stimulation of hydrochloric acid secretion by gastric mucosa (170, 171). The widespread interest in cyclic 3',5'-AMP has led to the appearance of several review articles, the most recent of which are those on the metabolic effects of catecholamines by the following authors: Sutherland and Robison (172), Butcher (173), Krebs et al. (132), Mansour (174), and Exton and Park (162).

The participation of cyclic 3',5'-AMP in glycogenolysis,

steroidogenesis, ketogenesis, lipolysis, antidiuresis and possibly other physiological processes demonstrates clearly the exceedingly diversified and important role played by this cyclic nucleotide in regulating various biological processes. It would follow that an equally important physiological mechanism is necessary for the termination of the action of cyclic 3',5'-AMP in biological systems. Indeed, an enzyme which hydrolyzes cyclic 3',5'-AMP to 5'-AMP is present in most tissues. The existence of such an enzyme activity was first suggested by Sutherland and Rall (124) in 1958 while these authors were studying the properties of cyclic 3',5'-AMP formed by tissue particles. The enzyme has been subsequently purified from beef heart by Butcher and Sutherland (175) and recently from dog heart by Nair (176). Earlier, Drummond and Perrott-Yee (177) had studied the distribution of the diesterase in various mammalian tissues, and had found that nervous tissues, particularly the brain, possessed by far the highest activity. The kidney, heart, spleen and liver of rabbit contained only 10 - 25% of the activity of the brain. Studies made on the properties of the diesterase from brain revealed its absolute requirement for magnesium ions, and that the product of hydrolysis was exclusively 5'-AMP.

Considering the function of cyclic 3',5'-AMP in so many diverse biological processes, it follows that the diesterase must play an important role in regulating the action of the cyclic nucleotide in various tissues. The enzyme is more active in brain than in any other tissue. Adenyl cyclase is

also more active in brain than elsewhere in nature. The precise physiological function of cyclic 3',5'-AMP in nerve tissue is a problem of paramount importance. The work described in this part of the thesis constitutes a further study of the properties and partial purification of cyclic 3',5'-nucleotide phosphodiesterase from mammalian brain. Some studies on the distribution of the enzyme throughout the plant and animal kingdom are also reported.

EXPERIMENTAL PROCEDURE

Materials

Cyclic 3',5'-AMP and calf intestinal adenosine deaminase were purchased from Sigma Chemical Company. Cyclic 3',5'-GMP and cyclic 3',5'-UMP were prepared by Smith, Drummond and Khorana (178). Cyclic 3',5'-dAMP, cyclic 3',5'-dGMP, cyclic 3',5'-dCMP and cyclic 3',5'-TMP were prepared by Drummond, Gilgan, Reiner and Smith (179). Cyclic 2',3'-AMP was prepared by the method of Smith, Moffatt and Khorana (180). The cyclic nucleotides 3',5'-dAMP, 3',5'-GMP and 3',5'-dGMP were chromatographically pure. Crotalus adamanteus venom was purchased from Ross Allen's Reptile Institute, Silver Springs, Florida and from Sigma Chemical Company.

Methods

Standard Assays - Routinely, cyclic 3',5'-nucleotide phosphodiesterase activity was assayed according to Butcher and Sutherland (175) by measuring the release of inorganic phosphate when Crotalus adamanteus venom was included in the assay system. The snake venom contains a potent 5'-nucleotidase which hydrolyzes 5'-nucleotides to give the corresponding nucleoside and inorganic phosphate. The reaction mixture contained 0.54 μmoles cyclic 3',5'-AMP, 0.90 μmoles $MgSO_4$,

0.1 to 0.4 mg snake venom, 88 µmoles Tris buffer, pH 7.5, with an appropriate dilution of the phosphodiesterase sample being assayed, in a total volume of 0.9 ml. Incubations were performed at 30° for 30 minutes, and the reaction terminated by the addition of 0.1 ml cold 55% trichloroacetic acid. The reaction tubes were centrifuged for 10 minutes at 10,000 x g to sediment the denatured proteins, and 0.5 ml aliquots of the supernatant fluid taken for inorganic phosphate analysis based on the method of Fiske and SubbaRow (181) as modified by Butcher and Sutherland (175). Thus, to 0.5 ml aliquots of the supernate were added 0.1 ml 2.5% ammonium molybdate solution in 5 N H_2SO_4, 0.35 ml glass-distilled water and 0.05 ml reducing agent. Colour was allowed to develop for 15 minutes before reading at 720 mµ in a Beckman Model DU spectrophotometer, using a light path of 1.0 cm. The standard curve for inorganic phosphate as measured by this method is shown in Fig. 18. One unit of enzyme activity is defined as that amount which caused the liberation of 1 µmole of inorganic phosphate in 30 minutes at 30°. The specific activity of the enzyme is defined as the umoles of inorganic phosphate released per mg of enzyme protein in 30 minutes at 30°.

For kinetic experiments, the assay was based on the conversion of cyclic 3',5'-AMP to inosine in the presence of brain extract, snake venom and intestinal adenosine deaminase. (One unit of deaminase activity is defined as the number of µmoles of adenosine deaminated per minute at 30° in 0.1 M citrate buffer at pH 6.5 at an adenosine concentration of

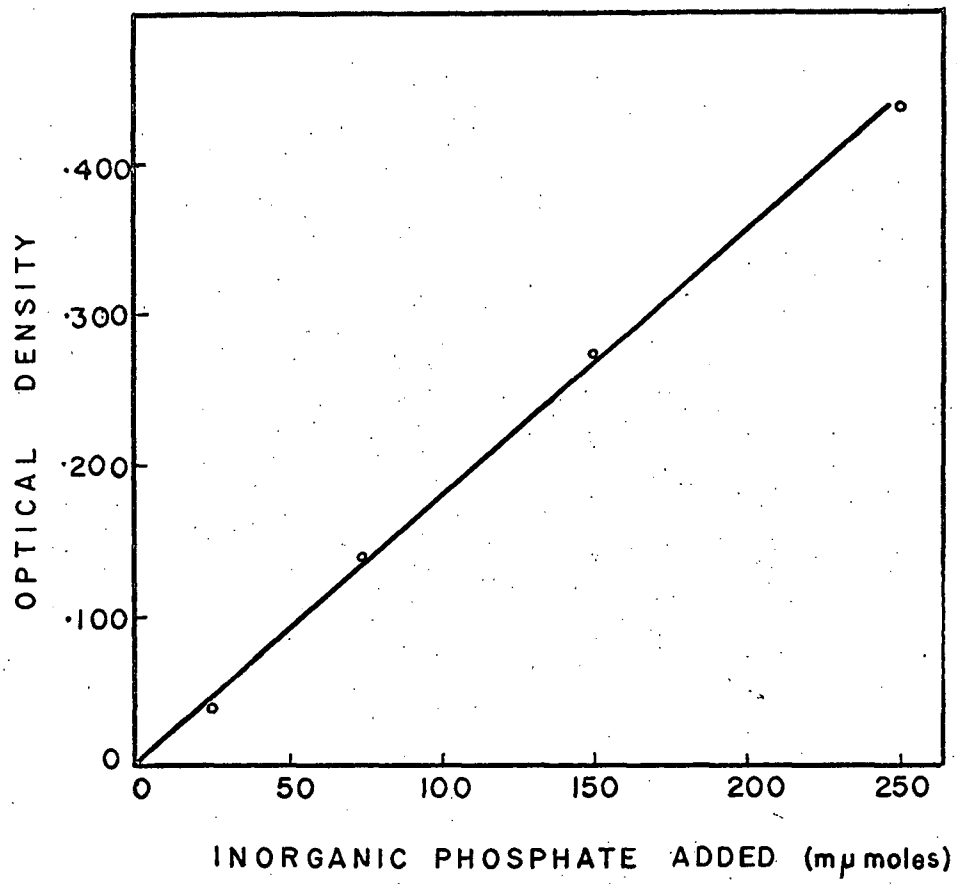


FIG. 18 Inorganic phosphate concentration curve.

To a series of tubes containing 0.2 ml 0.4 M Tris, pH 7.5, 0.05 ml 18 mM $MgSO_4$, 0.10 ml 55% trichloroacetic acid, were added the indicated amounts of inorganic phosphate and sufficient glass-distilled water to make a final volume of 1.0 ml. An 0.5 ml aliquot was taken from each tube and assayed for inorganic phosphate as described in the text.

0.45×10^{-4} M.) The concentrations of cyclic 3',5'-AMP were between 13 to 52 µM. The reaction mixture also contained 1.0 mM $MgSO_4$ and 88 mM Tris buffer at pH 7.5 in a final volume of 1.5 ml. After the addition of cyclic 3',5'-nucleotide phosphodiesterase, the decrease in absorbancy was followed at 265 mµ at 1-minute intervals using a Beckman Model DU spectrophotometer and a light path of 0.5 cm (Fig. 19).

For certain experiments where the identification of the reaction products by paper chromatography was considered advantageous, the assay system consisted of 0.25 µmoles cyclic 3',5'-AMP, 0.8 mM $MgSO_4$, 75-150 mM Tris buffer, pH 7.5, and enzyme in a total volume of 0.2 ml. In this system, no snake venom was used. The reaction was stopped after 15 minutes incubation at 30° by the addition of 0.02 ml glacial acetic acid. The tubes were centrifuged at 10,000 x g for 15 minutes and 0.02 ml aliquots of the supernate spotted on Whatman No. 1 filter paper. The chromatograms were developed by descending technique with isopropanol-ammonium hydroxide-0.1 M boric acid (7:1:2). This solvent system effectively separates adenine, adenosine, and the adenosine nucleotides.

Stock solutions of snake venom (8 mg/ml) used in the diesterase assay were prepared by dissolving the lyophilized powder in 0.02 M Tris, pH 7.5. Centrifugation was occasionally required to sediment insoluble particles. The snake venom activity was completely stable to repeated freezing and thawing over a period of several months. Protein was measured by the biuret method (182) and by the optical method of War-

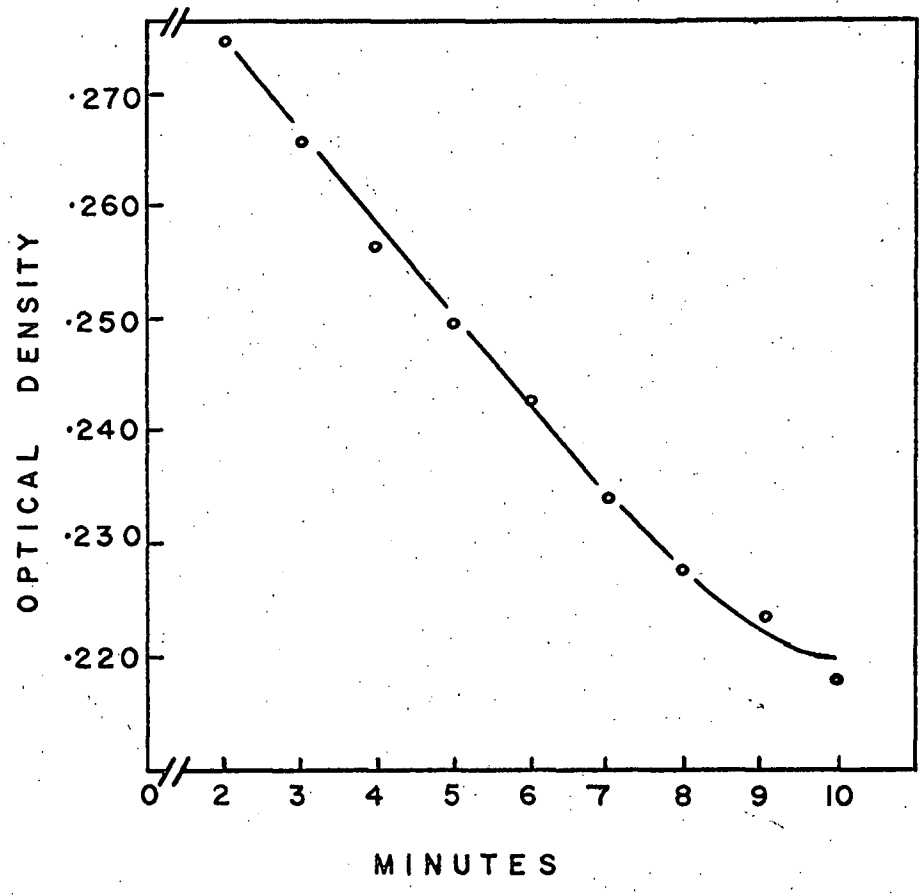


FIG. 19 Spectrophotometric Assay of Phosphodiesterase.

The incubation mixture contained 0.03 mM cyclic 3',5'-AMP, 1.0 mM $MgSO_4$, 88 mM Tris, pH 7.5, 0.15 units of intestinal adenosine deaminase, 0.32 mg snake venom, and 27 µg rabbit brain phosphodiesterase in a final volume of 1.5 ml. The reaction was started by the addition of the diesterase and $-\Delta_{265}$ was recorded at 1 minute intervals.

burg and Christian (183).

Enzyme Purification - Mature rabbits were stunned by a blow behind the head and the neck vessels severed immediately. The brains were removed, placed in ice and usually frozen before use. Only the cerebral lobes were used for the preparation of the extract. The tissue was homogenized in 10 volumes of 0.25 M unbuffered sucrose for 5 minutes at 0-4° using a glass mortar fitted with a motor-driven teflon pestle. All subsequent procedures, unless otherwise noted, were performed at 0-4°. The homogenate was centrifuged at 105,000 x g x 30 minutes, and the supernatant fluid thus obtained was set aside. The 105,000 x g sediment was re-homogenized with 5 original volumes of 0.25 M sucrose containing 0.1% deoxycholate. The homogenate was centrifuged for 30 minutes at 105,000 x g, and the supernatant fluids combined for the following ammonium sulfate fractionation step. It was found later that the centrifugation could be more conveniently performed at 37,000 x g x 60 minutes, giving essentially the same degree of purification.

Step 1 - Ammonium Sulfate Fractionation - The supernatant fluid was adjusted to 0.3 saturation by the addition of solid enzyme-grade ammonium sulfate with constant stirring over a 15-minute period. The pH was maintained between 6.9 and 7.1 by the dropwise addition of 1.0 N KOH. After at least 15 minutes equilibration, the precipitate was collected by centrifuging for 20 minutes at 37,000 x g. The precipitate was taken up to approximately 15% of the original combined super-

natant fluid volume using 1 mM imidazole, pH 7.5 containing 1 mM $MgSO_4$. The milky extract was dialyzed against 300 volumes of the same buffer (pH 7.5) for 3 hours in the cold room.

Step 2 - Repeated Freezing and Thawing - After dialyzing, the extract was centrifuged at 37,000 x g x 15 minutes to remove the heavy flocculent material, and the slightly cloudy supernatant fluid was stored at -20°. Upon thawing the extract, more flocculent material always appeared, which was readily removed by centrifugation. The supernate was re-frozen, thawed and centrifuged once more before taking the preparation to the next purification step, or was stored at -20°. About 5% of the enzyme activity was lost with the sediment, but no attempt was made to recover this activity. The repeated freezing and thawing of the first ammonium sulfate fraction usually gave a 6- to 10-fold purification of the enzyme, and an overall yield of about 15 to 20%.

Step 3 - Heat Denaturation and Acid Precipitation - After the repeated freezing and thawing procedure, 0.11 volumes of 0.5 M imidazole, pH 7.5, and 0.02 volumes of 0.5 M glycine, pH 10, was added to the clear supernate. The pH was taken to 10 by the addition of 1 N KOH, and the temperature of the solution brought quickly to 45°. After maintaining this temperature for about 15-16 minutes, the solution was immediately chilled by immersion in an ice-bath. The solution was then slowly taken to pH 5.8 with 0.3 N acetic acid and stirred for at least 15 minutes before centrifuging at 37,000 x g x 45 min-

utes. The precipitate was discarded, and the pH of the supernate brought back to 7.5 with 0.5 N KOH. The solution thus obtained was dialyzed against 300 volumes of 1 mM imidazole, pH 7.5 containing 1 mM $MgSO_4$ pH 7.5, with constant stirring for at least 6 hours.

Although the first ammonium sulfate step and the freezing and thawing gave reasonably consistent degrees of purification, the alkaline-heat, acid-precipitation step gave results which varied from one preparation to another. Unless otherwise indicated, this preparation was used for studying the properties of the brain phosphodiesterase. The overall yield at this step was about 5 to 10%, and the purification obtained ranged from 8- to 16-fold. The enzyme became exceedingly unstable with increasing purification, probably owing to the dilution of the enzyme during and after the alkaline-heat step. Concentration of the 6-hour dialysate obtained from the alkaline-heat step was accomplished by immersion of the dialysis bag into 1 litre of a 60% solution of sucrose. This technique resulted in a 90% decrease in volume of the dialysate within a few hours. The concentrated enzyme was stored at -20° for one week with no appreciable loss of activity.

Further purification of the enzyme could be obtained by taking a second (0.3 to 0.6 saturation) ammonium sulfate fraction after the alkaline-heat step. However, despite the 15 to 25-fold purification obtained by this method, the final yield of enzyme activity was low; hence the use of this step as a routine procedure was impractical. Nevertheless, this

preparation was occasionally used in some of the experiments where the use of a more highly purified preparation was indicated.

RESULTS

1. Preliminary - Before purification of the cyclic 3',5'-nucleotide phosphodiesterase from brain was attempted, the enzymatic components of the diesterase assay system were examined. The snake venom used in the diesterase assay contains a potent 5'-nucleotidase which hydrolyzes 5'-AMP to adenosine and phosphate. It was therefore necessary to determine the minimum quantity of snake venom required to hydrolyze all of the 5'-AMP produced by the diesterase under standard assay conditions. As may be seen in Fig. 20, 0.36 μmoles of 5'-AMP was almost completely hydrolyzed by 30 μg snake venom at 30° in 10 minutes. When the standard assay system was subsequently developed which contained 0.54 μmoles cyclic 3',5'-AMP, an excess (100-400 μg) of snake venom was routinely used in the incubation mixture in order to eliminate any possibility of the 5'-nucleotidase limiting the overall reaction rate. When 100 μg of snake venom was used, no cyclic 3',5'-nucleotide phosphodiesterase could be detected in the venom preparation. However, in some later experiments, larger amounts of snake venom were used in the assay system. Analysis for the presence of cyclic 3',5'-AMP hydrolyzing activity at these higher concentrations of venom indicated that a trace of diesterase activity was present, as shown in Table VI. Although the presence of diesterase activity in the snake venom had insignificant effect on the results of most experiments where brain diesterase activity being measured was high, data from those few experiments where the activity was low

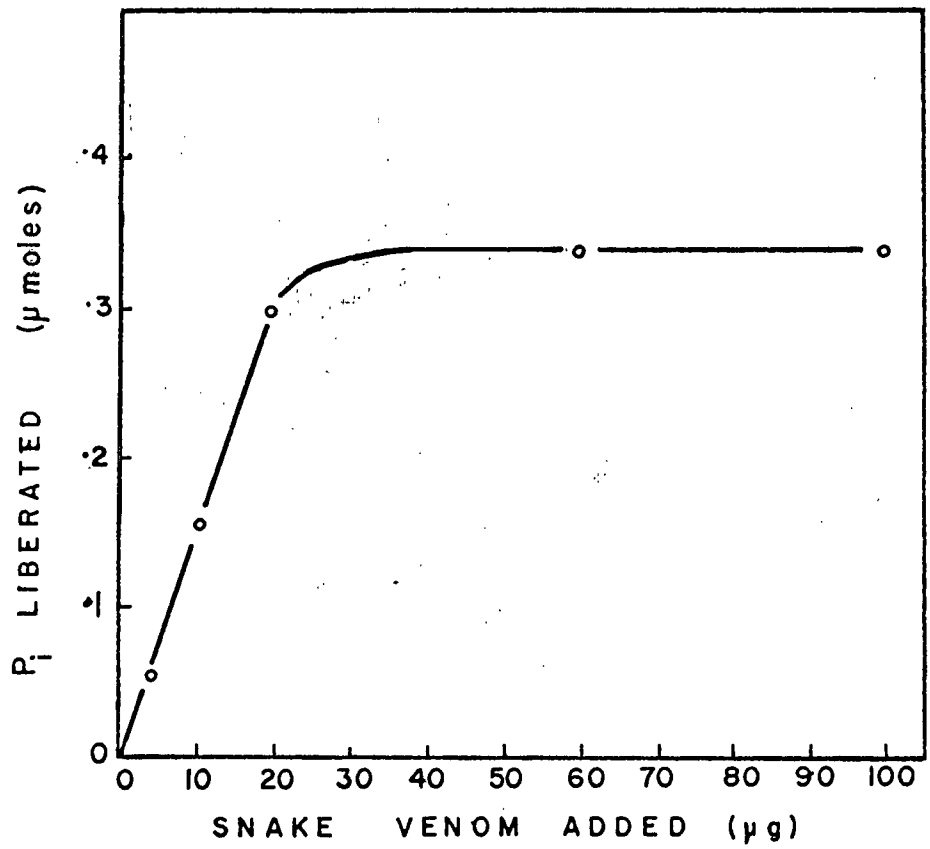


FIG. 20 Hydrolysis of 5'-AMP by snake venom (Crotalus adamanteus).

The incubation mixture contained 0.36 μmoles 5'-AMP, 20 mM $MgSO_4$, 40 mM Tris buffer, pH 7.5, in a final volume of 1.0 ml. The indicated amounts of snake venom were added to initiate the reaction. The reaction was terminated by the addition of 0.1 ml 55% trichloroacetic acid after 10 minutes incubation at 30°. Phosphate was analyzed as described in the text.

TABLE VI

Relative Activities of 5'-Nucleotidase and Cyclic 3',5'-Nucleotide Phosphodiesterase in Snake Venom.

The incubation mixture contained either 0.72 µmoles cyclic 3',5'-AMP or 5'-AMP, 18 mM $MgSO_4$, 0.04 M Tris, pH 7.5, the indicated amounts of Crotalus adamanteus venom (Sigma) and sufficient water to make 0.9 ml. The reaction was stopped by the addition of 0.1 ml 55% trichloroacetic acid after 30 minutes incubation at 30°. Inorganic phosphate was assayed according to the standard procedure as described in the text.

Amount of Snake Venom added (µg)	µmoles Pi released from 5'-AMP	& from cyclic 3',5'-AMP
0.43	0.01	0
0.86	0.03	0
2.15	0.09	0
4.3	0.24	0
8.6	0.45	0
21.5	0.75	.002
43.0	0.75	.004
129.0	0.77	---
215.0	0,74	.010

was corrected for the inherent cyclic 3',5'-nucleotide diesterase in the venom. Experiments indicated that 400 µg snake venom was capable of hydrolyzing 0.02 µmoles cyclic 3',5'-AMP in 30 minutes at 30°, and therefore this correction factor was used.

Preliminary experiments designed to test the validity of the coupled enzyme assay indicated that the rate of cyclic 3',5'-AMP hydrolysis was directly proportional to the amount of diesterase used (Fig. 21). The enzyme preparation used for these experiments was an extract of an acetone powder which had been prepared 2 years previously and stored at -20°. The experiments therefore indicated, in addition, that the brain diesterase was quite stable to storage when prepared in acetone powder form.

2. Partial Purification of Brain Phosphodiesterase - Drummond and Perrott-Yee (177) reported that a partial purification of rabbit brain diesterase was readily obtained by taking a 20,000 x g supernatant fraction of the whole homogenate to 0.4 saturation with ammonium sulfate. The enzyme activity was recovered from the precipitate. The present study also found most of the activity associated with the 0.4 saturated ammonium sulfate fraction, although the highest specific activity was recovered in the 0.40-0.45 saturated fraction. It was observed, however, that when 0.1% sodium deoxycholate was included in the 0.25 M sucrose solution used for re-homogenizing the initial 105,000 x g sediment, the diesterase was precipitated at lower ammonium sulfate concentrations.

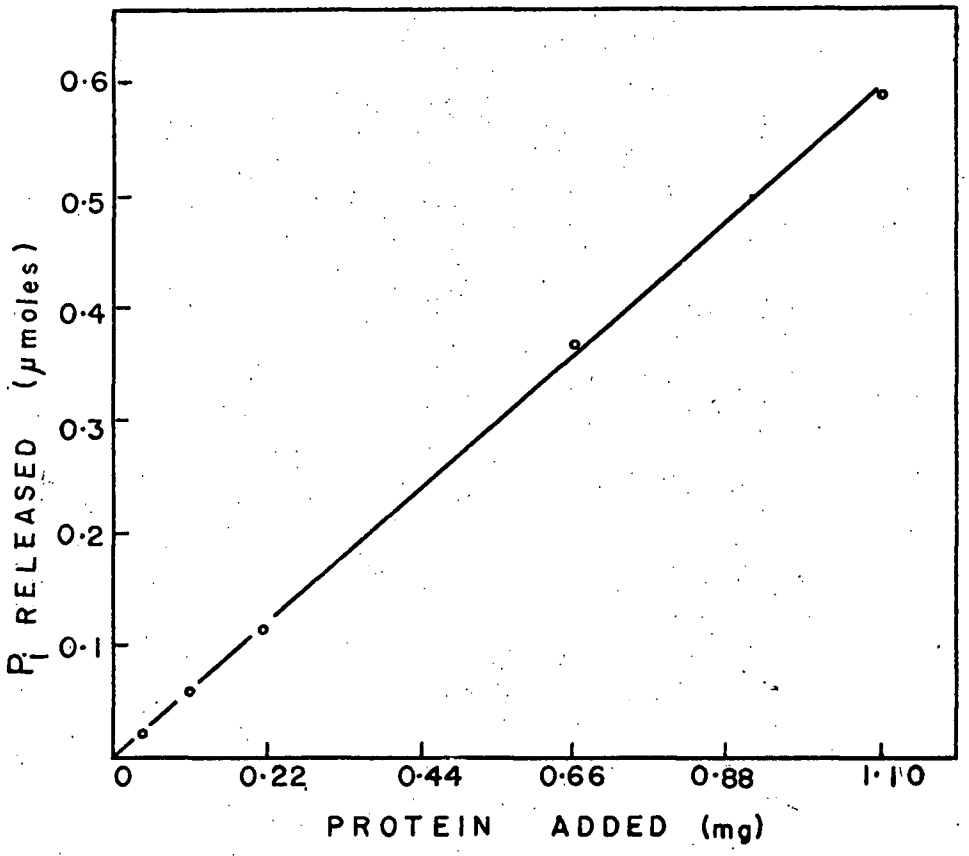


FIG. 21 Phosphodiesterase Activity as a Linear Function of Protein Concentration.

The reaction mixture contained 0.36 μmoles cyclic 3',5'-AMP, 20 mM $MgSO_4$, 40 mM Tris, pH 7.5, 60 μg snake venom, the indicated amounts of protein and sufficient water to make a final volume of 0.9 ml. The reaction was stopped by the addition of 0.1 ml trichloroacetic acid after 30 minutes incubation at 30°. The diesterase preparation used was an acetone powder extract of beef brain. Pi was assayed as described in the text.

Furthermore, repeated freezing and thawing of the dialyzed extract obtained from the 0.3 saturated ammonium sulfate fraction consistently resulted in a 6-10-fold purification. The alkaline-heat treatment followed by the acid precipitation frequently gave an additional 2-fold purification. An example of the purification of the diesterase from rabbit brain is shown in Table VII. The specific activity of the final preparation from brain was about half of that reported by Butcher and Sutherland (175) who purified the enzyme from beef heart. While this work was in progress, Nair (176) also reported purifying the diesterase from dog heart. The specific activity of the purified enzyme from dog heart was around 27.5, which is in the same range as that obtained for the beef heart enzyme. Attempts to purify the brain enzyme by the use of Sephadex G-200 columns, or by adsorption on calcium phosphate gels under various conditions were unsuccessful. Several attempts to purify the enzyme on DEAE-cellulose columns were also unsuccessful, owing to the instability of the enzyme in dilute solutions.

3. Properties of the Partially Purified Phosphodiesterase -

(a) The initial studies on brain diesterase by Drummond and Perrott-Yee (177) indicated that the rabbit brain diesterase had an absolute requirement for Mg^{++} ions and was completely inhibited by EDTA (1.0 mM). These observations were fully confirmed in the present study.

(b) The effect of 0.06 M imidazole on brain diesterase activity was investigated. As may be seen in Fig. 22, imi-

TABLE VII

Partial Purification and Yield of Cyclic 3',5'-nucleotide Phosphodiesterase from Rabbit Brain.

The cerebral cortex from a rabbit brain was fractionated as described in the text. Activities are also defined in the text.

Fraction	Total Activity	Specific Activity	% Yield	Purification
Homogenate	728	0.9	(100)	1.0
Combined Supernate (37,000 x g)	660	2.3	90	2.4
0.3 Ammonium sulfate	159	4.3	22	4.6
Frozen-Thawed twice	156	9.0	21	9.2
Alkaline-Heat and Acid precipitation	20	14.0	2.7	14.8

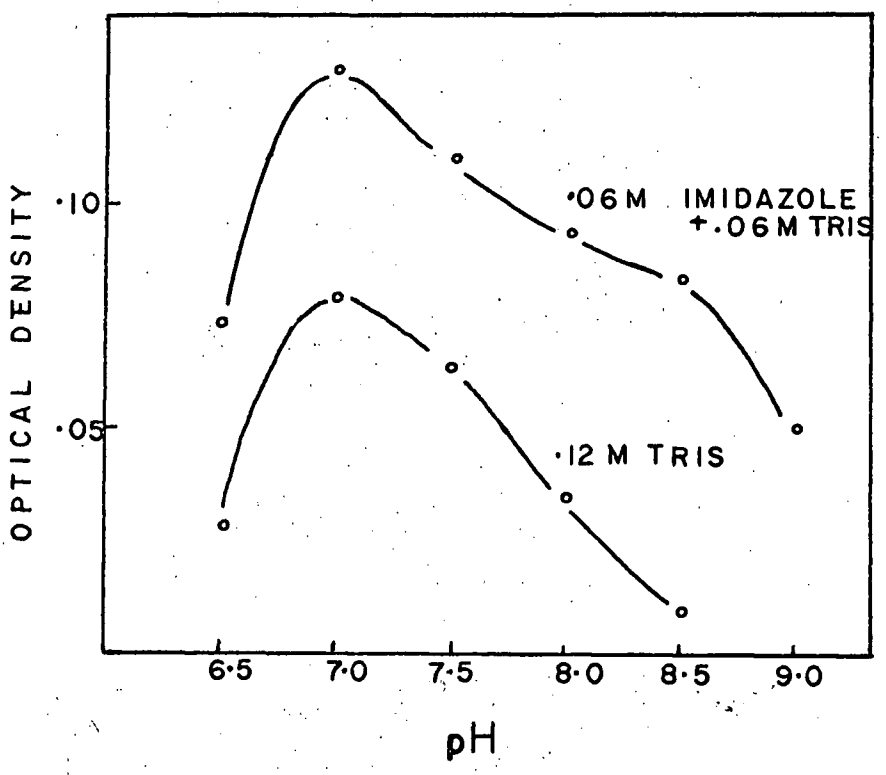


FIG. 22 pH Curve of Brain Cyclic 3',5'-Nucleotide Phosphodiesterase and Effect of Imidazole.

The reaction mixture contained 0.8 mM cyclic 3',5'-AMP, 1.3 mM $MgSO_4$, 4 µg enzyme protein of specific activity 19 units, and 0.06 M Tris plus imidazole or 0.12 M Tris. After a 20-minute incubation at 30°, the reaction was terminated by heating the tubes in boiling water for 1 minute. The pH of the reaction mixtures were adjusted to neutrality, snake venom (400 µg) was added, and the tubes incubated for 10 minutes at 30°. The reaction was stopped by the addition of 0.1 ml cold trichloroacetic acid. Inorganic phosphate was assayed as described in the text.

dazole caused a significant increase in diesterase activity over the entire pH range examined. Peak enzyme activity was near pH 7.0, whether imidazole was present or not. Stimulation of the beef heart enzyme by imidazole has also been reported by Butcher and Sutherland (175), although their data indicated little stimulation at pH 8.5.

(c) Inhibition by Theophylline in vitro - The methyl xanthines, particularly theophylline, are known to inhibit cyclic 3',5'-nucleotide phosphodiesterase. The effect of 2×10^{-4}M theophylline on the partially purified brain diesterase activity was investigated, and these results are shown in Fig. 23. The inhibition of brain diesterase by theophylline appears to be competitive in nature. The K_m of the enzyme was about 0.8×10^{-4}M with cyclic 3',5'-AMP as substrate. The K_m value obtained indicated a marked similarity to those values reported for the beef heart enzyme. Nair (176), however, has reported K_m values near 4.9×10^{-4}M for the dog heart diesterase.

(d) Cyclic 3',5'-dAMP/cyclic 3',5'-AMP activity ratios in successive fractions obtained during purification - It was reported (177) that the brain diesterase hydrolyzed cyclic 3',5'-dAMP at about 50% of the rate at which cyclic 3',5'-AMP was hydrolyzed. The present studies showed that the cyclic 3',5'-dAMP/cyclic 3',5'-AMP activity ratios were about 0.45. In order to determine whether cyclic 3',5'-dAMP and cyclic 3',5'-AMP might be hydrolyzed by the same or different enzyme, the cyclic 3',5'-dAMP/cyclic 3',5'-AMP activity ratios were

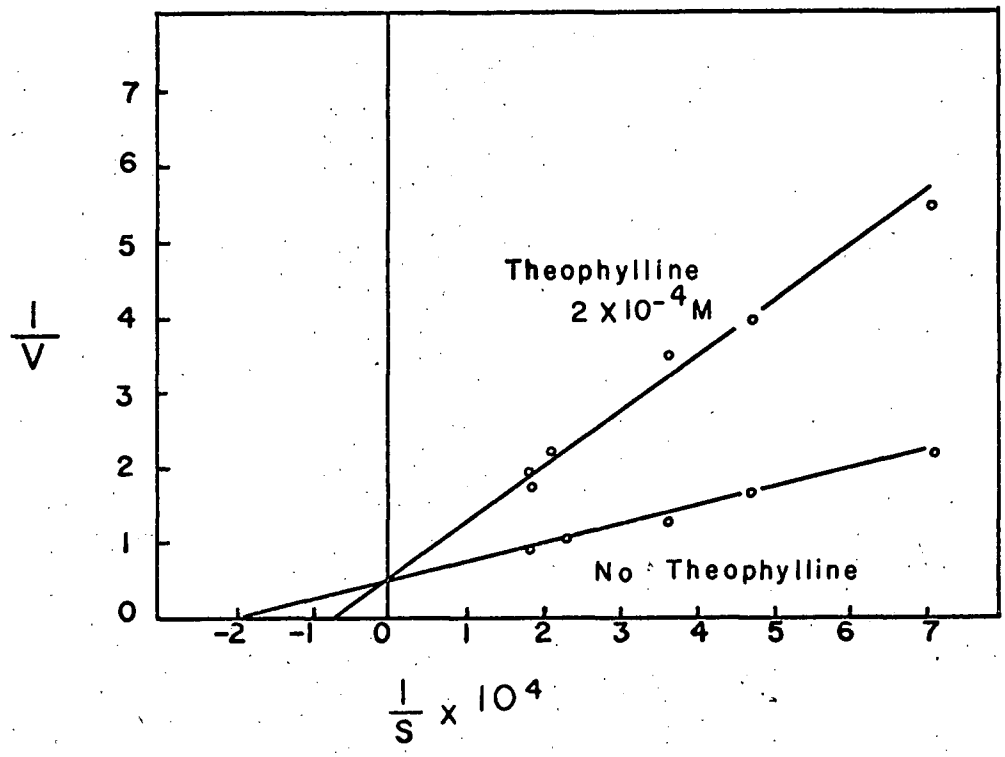


FIG. 23. Nature of Brain Cyclic 3',5'-Nucleotide Phosphodiesterase Inhibition by Theophylline.

The partially purified brain diesterase preparation (specific activity, 9.1) used in these experiments was stored at -20° in a concentrated sucrose solution. After thawing and making the appropriate dilution, the enzyme preparation was incubated in the presence of 2 x 10^{-4}M theophylline as described in the text for the spectrophotometric assay.

determined in the various fractions obtained during purification. These results are shown in Table VIII. Although there was a significant difference between the ratios obtained for the whole homogenate and the combined supernatant fractions, the succeeding fractions showed insignificant differences. It was also observed that when equimolar concentrations of cyclic 3',5'-dAMP were included with cyclic 3',5'-AMP in the standard phosphodiesterase assays, a 15-19% inhibition of diesterase activity was consistently obtained. These observations strongly indicate that both cyclic 3',5'-AMP and cyclic 3',5'-dAMP are hydrolyzed by the same enzyme. However, further investigations are necessary in order to show more conclusively whether these compounds are indeed hydrolyzed by the same diesterase.

(e) Hydrolysis Rates of other Purine and Pyrimidine cyclic 3',5'-nucleotides - The rate of hydrolysis of other cyclic 3',5'-nucleotides were investigated in order to further determine the specificity of the brain phosphodiesterase. The relative hydrolysis rates of all the cyclic 3',5'-nucleotides which were investigated are listed in Table IX. Included for comparison are the rates obtained for the brain and heart enzyme extracts by other investigators. The data clearly demonstrates the high specificity of the enzyme for purine cyclic 3',5'-nucleotides. Cyclic 3',5'-UMP was the only pyrimidine cyclic nucleotide which was hydrolyzed appreciably by the brain diesterase preparation, although the activity was only about 13% of cyclic 3',5'-AMP hydrolysis rates.

TABLE VIII

Cyclic 3',5'-dAMP/cyclic 3',5'-AMP activity ratios.

The cyclic 3',5'-nucleotide phosphodiesterase was partially purified as described in the text. The specific activity of the final preparation was 15.0. Assays were performed by the standard method as described in the text, except that cyclic 3',5'-dAMP was used at a concentration of 0.50 μmoles/ml.

Fraction	$\frac{\text{cyclic 3',5'-dAMP}}{\text{cyclic 3',5'-AMP}}$ Activity Ratio
Whole Homogenate	.76
Combined 105,000 x g supernatant	.53
0.3 Ammonium sulfate	---
Frozen-Thawed x2	.52
Alkaline-heat, Acid treatment	.45

TABLE IX

Relative Hydrolysis Rates of Purine and Pyrimidine Cyclic 3',5'-Nucleotides.

Diesterase activities were assayed by the standard procedure, except that the other cyclic 3',5'-nucleotides as indicated were substituted for cyclic 3',5'-AMP in the assay in equimolar concentrations.

COMPOUND	THIS STUDY (Rabbit brain)	DRUMMOND (177) (Rabbit brain)	BUTCHER & SUTHERLAND (175) (Beef heart)	NAIR (176) (Dog heart)	DRUMMOND et al (189) (Beef brain)
3',5'-AMP	1.00	1.00	1.00	1.00	1.00
3',5'-dAMP	0.45			1.30	0.50
3',5'-GMP	0.50	0.33		0.33	
3',5'-dGMP	0.48				0.44
3',5'-CMP	0.0	0.0		0	
3',5'-dCMP	<0.04				0.10
3',5'-UMP	0.13	0.11	0.17	0.12-0.15	
3',5'-TMP	<0.07				0.10

Hardman and Sutherland (184) have recently reported purifying a cyclic 3',5'-nucleotide phosphodiesterase from beef heart that hydrolyzed cyclic 3',5'-UMP at a much faster rate than it hydrolyzed cyclic 3',5'-AMP. Their findings suggest the possibility that the appreciable hydrolysis of cyclic 3',5'-UMP by the partially purified fraction of rabbit brain may be due to the presence of a separate enzyme for hydrolyzing cyclic 3',5'-UMP.

The hydrolysis of cyclic 3',5'-AMP, cyclic 3',5'-GMP and their deoxy analogues by the brain diesterase preparation were followed by paper chromatography (Figs. 24-27). Only one hydrolysis product could be detected for each of the cyclic nucleotides. It should be mentioned here that the isopropanol-ammonium hydroxide-0.1 M boric acid (7:1:2) solvent system effectively separates 3'- from 5'-nucleotides. Although no reference standards are shown for these compounds on these chromatograms, it was noted that the product of cyclic 3',5'-AMP hydrolysis was indistinguishable from authentic 5'-AMP. Furthermore, earlier studies by Drummond and Perrott-Yee (177) have shown that the hydrolysis products of cyclic 3',5'-GMP and cyclic 3',5'-AMP were their corresponding 5'-nucleotides. Therefore, it is reasonable to suggest that the products of cyclic 3',5'-deoxy-nucleotides were also their corresponding 5'-deoxynucleotides.

(f) Further studies on Specificity of Brain Diesterase - The presence of 5'-nucleotidase activity in the partially purified preparation of brain diesterase was investigated.

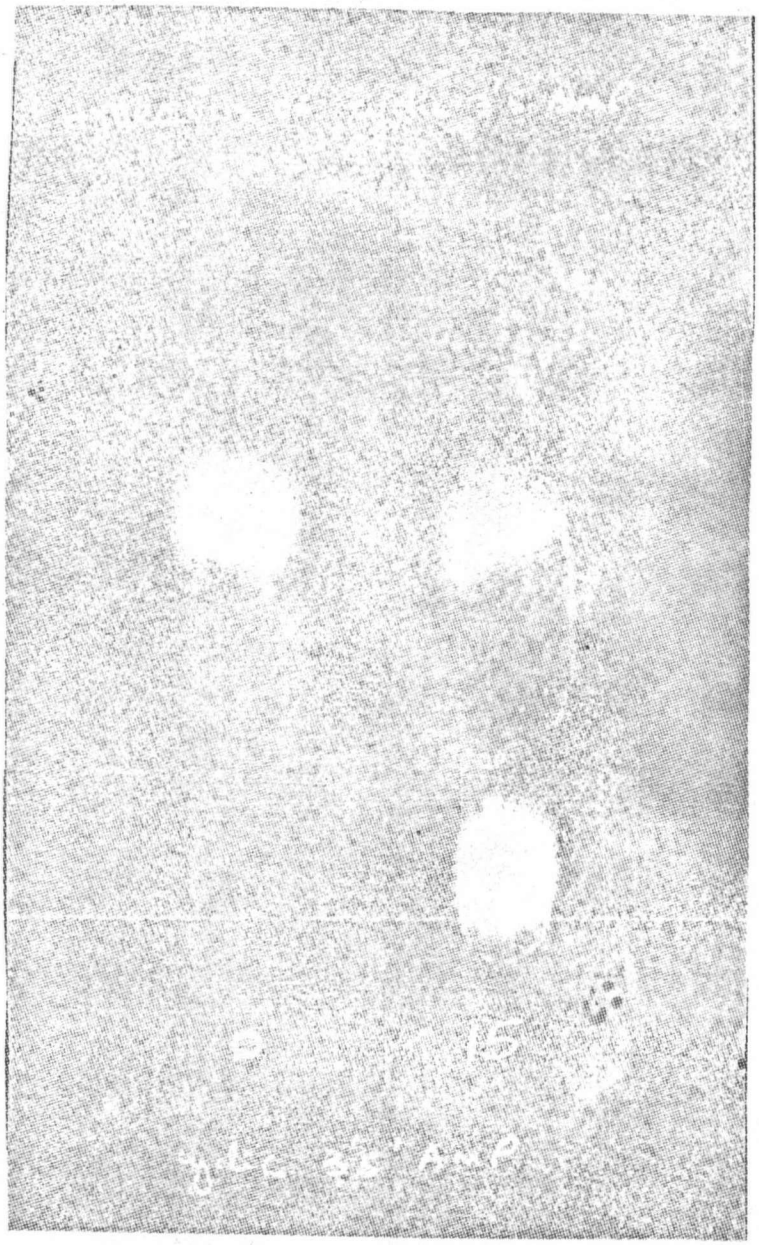


FIG. 24 Hydrolysis of Cyclic 3',5'-AMP by Partially Purified Brain Diesterase.

The reaction mixture contained 0.25 μmoles cyclic 3',5'-AMP, 0.9 mM $MgSO_4$, 150 mM Tris, pH 7.5, 45 μg partially purified enzyme protein, in a total volume of 0.11 ml. Incubation at 30° was stopped at 15 minutes by the addition of 0.02 ml glacial acetic acid. An aliquot (0.02 ml) was spotted on Whatman No. 1 filter paper and the chromatogram developed with isopropanol-ammonium hydroxide-0.1 M boric acid (7:1:2).

The "0" and "15" indicate duration of incubation (min). The solvent front extended 16 cm from the origin. 5'-nucleotides always remain near the origin under the conditions used.

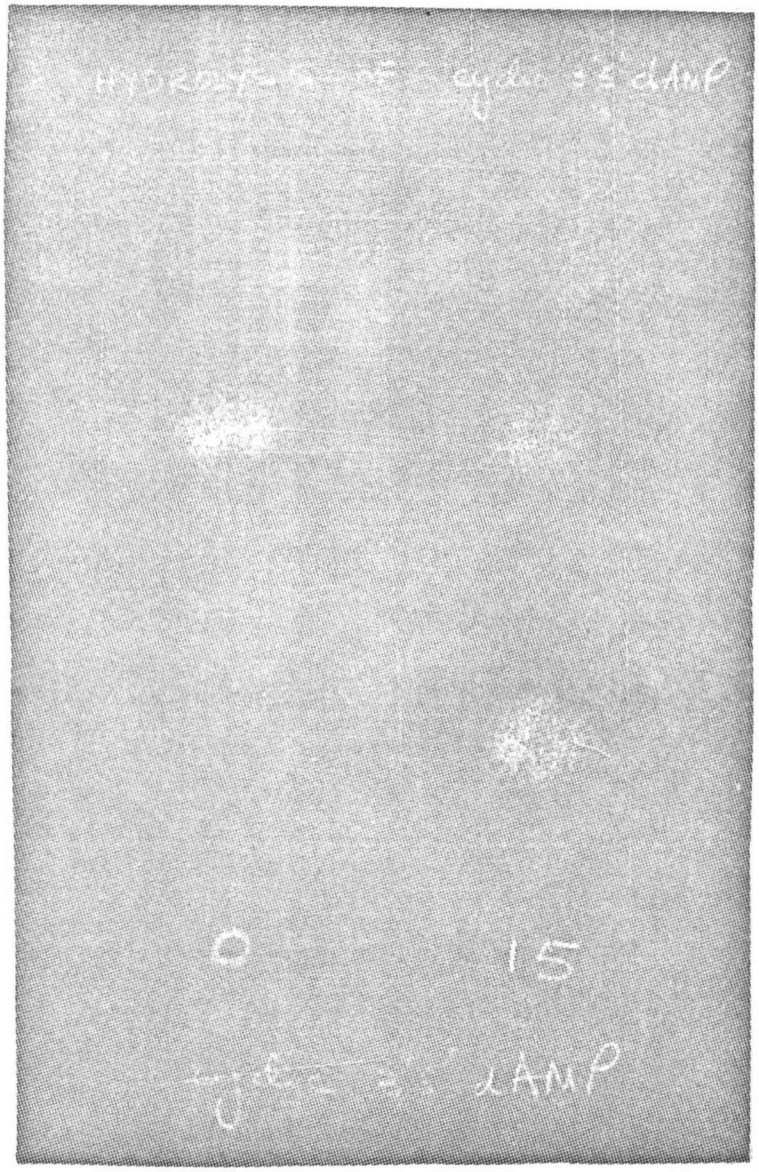


FIG. 25 Hydrolysis of Cyclic 3',5'-dAMP.

The enzyme incubation conditions and chromatographic procedures were as described under Fig. 24, except that the substrate was 3',5'-dAMP. The solvent front extended 16 cm from the origin.

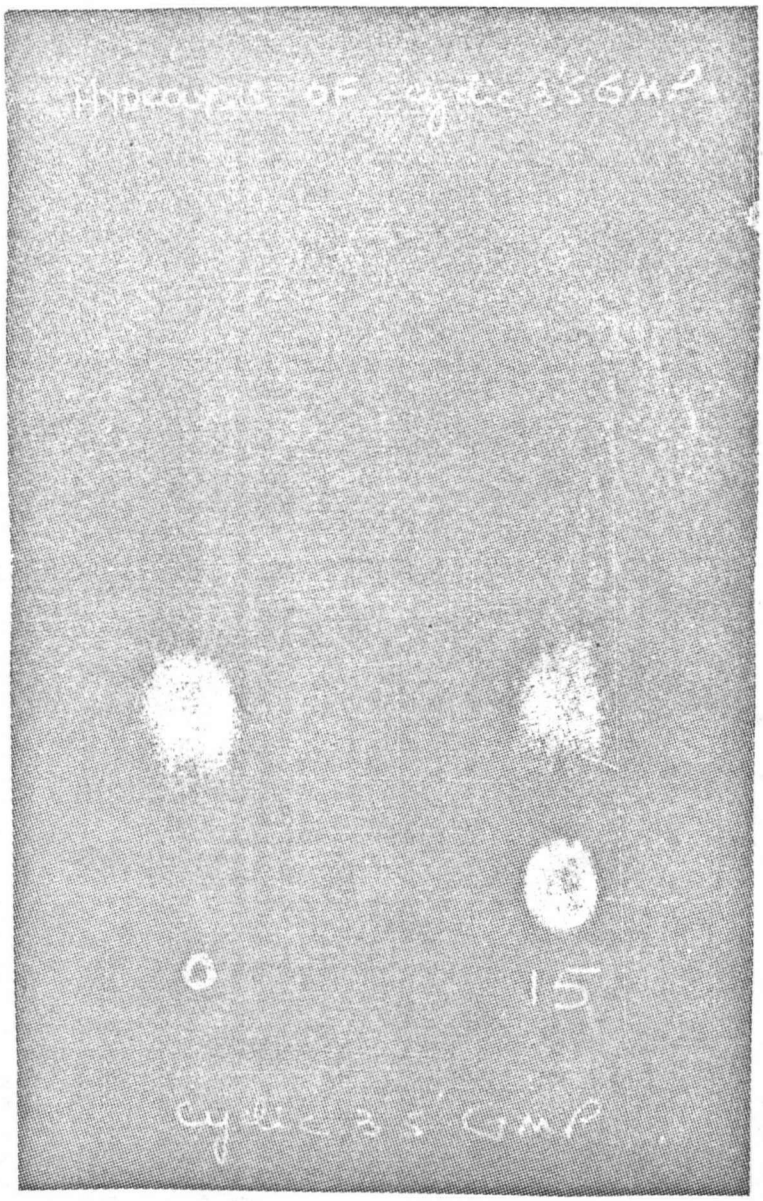


FIG. 26. Hydrolysis of Cyclic 3',5'-GMP.

The enzyme incubation conditions and chromatographic procedures were as described under Fig. 24, except that the substrate was cyclic 3',5'-GMP. The solvent front was 16 cm from the origin.

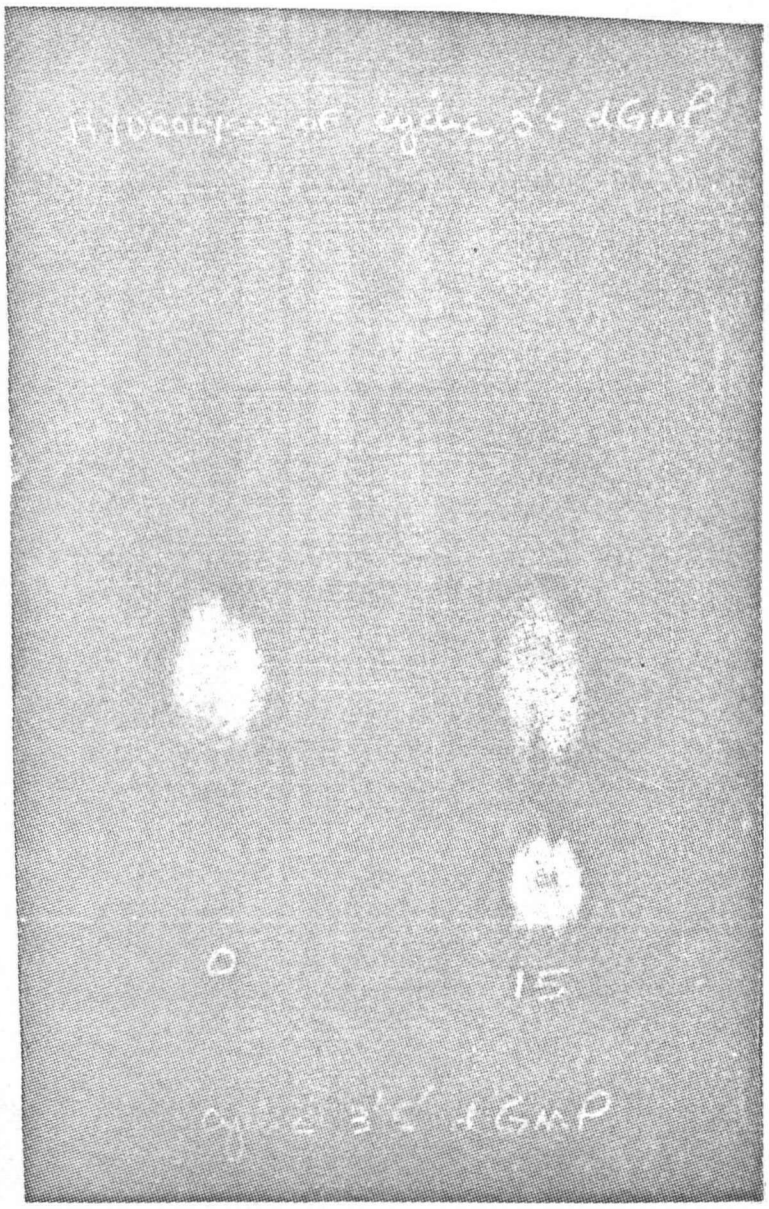


FIG. 27 Hydrolysis of Cyclic 3',5'-dGMP.

The enzyme incubation conditions and chromatographic procedures were as described under Fig. 24, except that the substrate was cyclic 3',5'-dGMP. The solvent front was 14.5 cm from the origin.

As shown in Fig. 28, no 5'-nucleotidase activity was present in the preparation.

The presence of a cyclic 2',3'-nucleotide phosphodiesterase activity was reported by Drummond and Perrott-Yee (177) in their ammonium sulfate preparation from rabbit brain. The partially purified preparation obtained as described in the present investigation also contained a considerable amount of cyclic 2',3'-nucleotide phosphodiesterase activity. However, when the fraction obtained by the alkaline-heat, acid precipitation method was further fractionated with ammonium sulfate (0.3-0.6 saturation), the cyclic 2',3'-nucleotide phosphodiesterase activity was completely eliminated (Fig. 29). Furthermore, the 0.3-0.6 saturated ammonium sulfate fraction yielded up to a 2-fold increase in purification of the 3',5'-nucleotide phosphodiesterase activity having specific activities in the range 15-25. However, the final ammonium sulfate step gave extremely low yield of enzyme and was not sufficiently reproducible to merit consideration as a routine technique for purifying the brain diesterase.

4. Cellular Distribution of Cyclic 3',5'-nucleotide Phosphodiesterase - Fractionation of rabbit brain into several cellular components was performed as described by De Robertis et al (185). The results (Table X) indicated that about 50% of the diesterase activity was located in the 105,000 x g supernatant fraction. The microsomal and mitochondrial fractions contained considerable amounts of diesterase activity, but little activity was located in the nuclear fraction.

TABLE X

Cellular Distribution of Cyclic 3',5'-Nucleotide Phosphodiesterase in Rabbit Brain.

The cerebral cortex from a rabbit was homogenized for 5 minutes in 8 volumes of 0.33 M sucrose (unbuffered) with the aid of a glass homogenizer fitted with a teflon pestle and centrifuged for 10 minutes at 900 x g. The sediment was washed twice with 0.33 M sucrose, and after re-centrifugation at 900 x g, the supernates were combined with the original 900 x g supernate. The mitochondrial fraction was obtained by centrifuging the combined supernates for 20 minutes at 11,500 x g. The sediment was again washed twice with 0.33 M sucrose. The microsomal fraction was obtained by centrifuging the combined 11,500 x g supernates for 30 minutes at 105,000 x g. The microsomal fraction thus obtained was washed once only. All procedures were carried out at 0-4°. Enzyme activity is defined in the text. The standard assay was used to determine diesterase activity.

Fraction	Total Activity (units)	% Total Activity
Whole homogenate	780	(100%)
Nuclear	22	2.8
Mitochondrial	63	8.1
Microsomal	103	13.2
105,000 x g supernate	394	50.5
Recovery	582	74.9

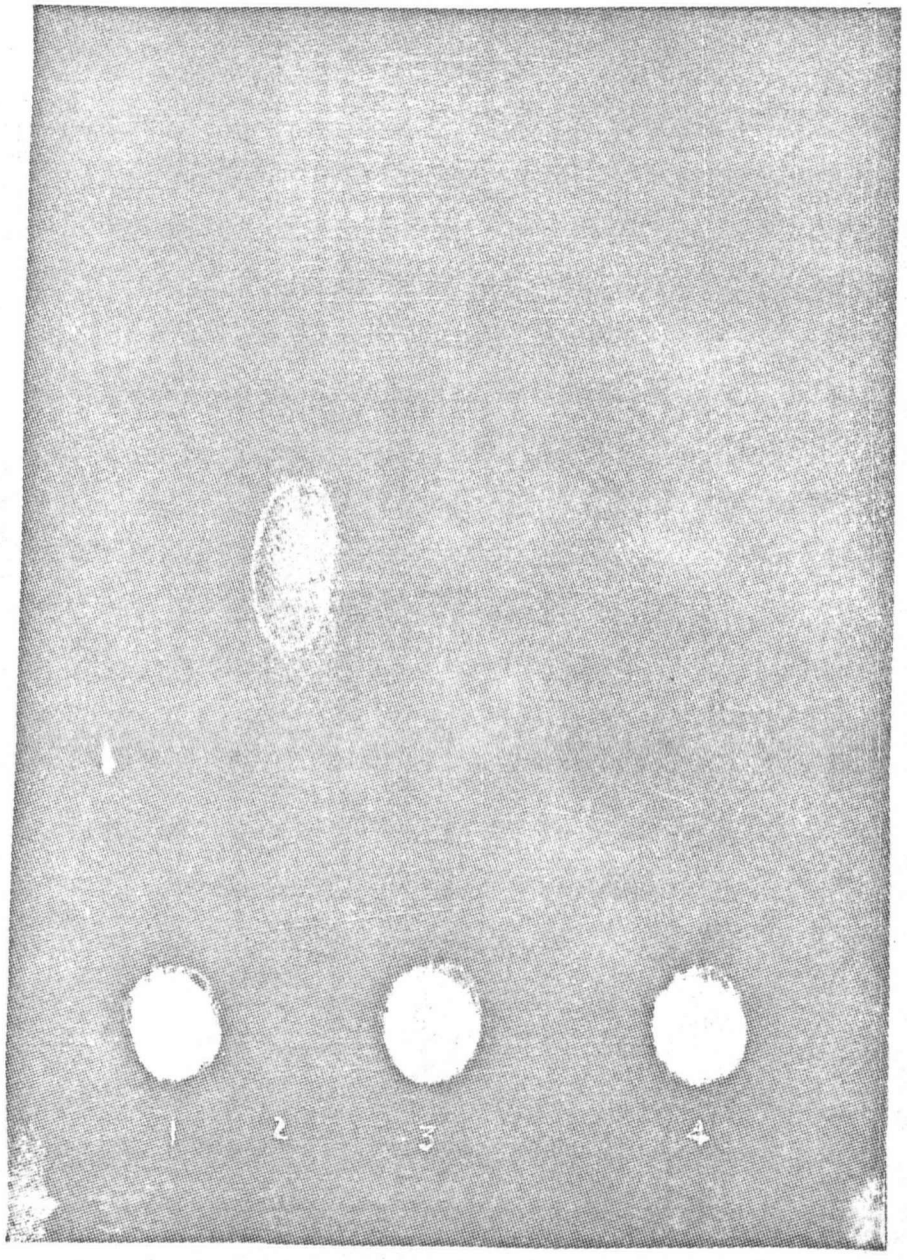


FIG. 28. Absence of 5'-Nucleotidase Activity in Partially Purified Brain Diesterase Fraction.

The reaction mixture contained 1.8 μmoles 5'-AMP, 0.9 mM $MgSO_4$, 80 mM Tris, pH 7.5, partially purified brain diesterase (specific activity, 14) and sufficient water to make 0.2 ml. Incubation was for 30 minutes at 30°, and the entire contents of the reaction mixture were spotted on Whatman No. 1 filter paper and developed as described in the text.

From the left, spot 1 - reference 5'-AMP; spot 2 - reference adenosine; spot 3 and 4, substrate 5'-AMP incubated in the absence and presence of 13.5 μg enzyme protein respectively.

The solvent front was 17 cm from the origin.

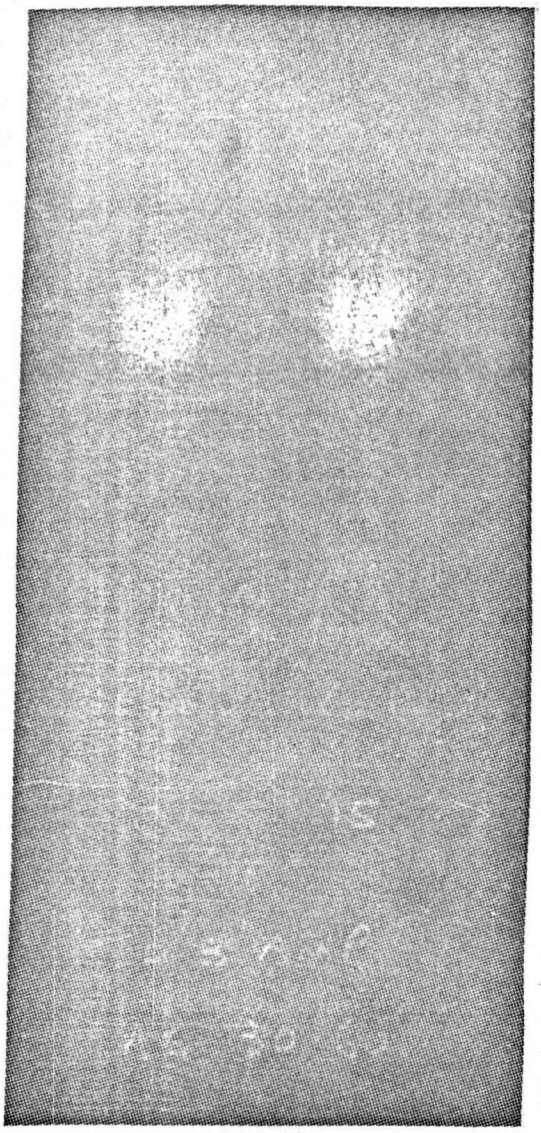


FIG. 29 Absence of Cyclic 2',3'-AMP Hydrolytic Activity in the Final (Ammonium sulfate 0.3-0.6 saturation) Preparation of Brain Diesterase.

The reaction mixture contained 0.24 μmoles cyclic 2',3'-AMP, 0.9 mM $MgSO_4$, 80 mM Tris, pH 7.5, 6 μg brain diesterase preparation, in a total volume of 0.2 ml. Incubation was for 15 minutes at 30°. Aliquots of 0.02 ml were taken, spotted on paper and developed as described in the text. Specific activity of the diesterase preparation was 27. The solvent front was 14.5 cm from the origin.

While this work was in progress, Cheung and Salganicoff (186) reported that 40% of the total activity was located in the mitochondrial fraction of rat brain. On the other hand, Drummond and Perrott-Yee (177) reported that the activity was localized entirely in the 100,000 x g supernatant fraction. It is therefore difficult to reconcile the differences observed between the results of the present study and those of the other authors. De Robertis et al.(185) have indicated that the fitting of the teflon plunger had to be specially determined in order to produce a minimal breakage of nerve terminals. It is therefore likely that the differences in the results reported on the cellular distribution of brain diesterase arise essentially from the extent to which cellular components are disrupted during homogenization.

The present study indicated little diesterase activity in the nuclear fraction (which also contains fragments of plasma membrane). These findings are in good agreement with those of Cheung and Salganicoff (186) who found only 7% of the activity in this fraction. Since the adenyl cyclase system which synthesizes cyclic 3',5'-AMP is located on the plasma membrane, it would appear that the adenyl cyclase system and the phosphodiesterase activities are spatially separated in the cell. It seems that such a spatial arrangement may perhaps be biologically important, since cyclic 3',5'-AMP must be given time to act before its destruction by the diesterase.

5. Distribution of Cyclic 3',5'-Nucleotide Phosphodiesterase in Various Areas of the Central Nervous System and in Lower

Organisms - As described earlier, cyclic 3',5'-AMP is involved in the regulation of several important biological processes. The enzyme, cyclic 3',5'-nucleotide phosphodiesterase, which terminates the action of cyclic 3',5'-AMP has been shown to exist in most of the higher organisms which have been examined for its activity. Because the diesterase must play a role in the regulation of intracellular cyclic 3',5'-AMP levels, the distribution of the enzyme was investigated in several tissues; namely, the human brain, the nervous system of the dog, in marine organisms and in the plant kingdom. As may be observed in Table XI, the diesterase activity was highest in the cerebral cortex of the human brain. The survey of the distribution of diesterase activity in various areas of the dog nervous system (Table XII) also indicated that the enzyme activity was highest in the cerebral cortex. The high content of diesterase activity found in the cerebral cortex is consistent with that observed by other investigators. The distribution of the enzyme in several available marine organisms which were examined is shown in Table XIII. The survey of diesterase activity in various areas of the human brain, dog nervous system and in marine organisms were performed by Mr. Lorne K. Massey, a medical student working in this laboratory.

Several available plants were also investigated for diesterase activity. As may be seen in Table XIV, no diesterase activity was detected in any of the plant specimens which were examined. Several techniques were used to ensure complete disintegration of yeast cells, but no activity was detected.

TABLE XI

Distribution of cyclic 3',5'-Nucleotide Phosphodiesterase Activity in Human Brain.

Specific activity is as described in the text.

Area of Brain	Specific Activity
Cerebral cortex - grey	1.6
- white	1.4
Cerebellar cortex	1.0
Pons	0.4
Corpus Callosum	1.0
Thalamus	1.0
Caudate nucleus	1.0
Vermis	0.8
Hypothalamus	0.4

TABLE XII

Cyclic 3',5'-Nucleotide Phosphodiesterase Activities in Various Areas of Dog Nervous System.

Specific activity is as described in the text.

Nervous Tissue	Specific Activity
Cerebral cortex	3.2
Cerebellum	0.4
Basal ganglion and internal capsule	2.0
Medulla	1.0
Pons	0.3
Spinal Cord - cervical	---
- upper thoracic	0.4
- lower thoracic	0.6
- lumbar	0.2
Midbrain	0.5
Hypothalumus	2.1
Caudate nucleus	2.9
Stellate ganglion	0.4
Thoracic sympathetic ganglion	0.05
Phrenic nerve	0.3
Sciatic nerve	0.2
Thoracic sympathetic axons	0.5
Cervical sympathetic axons	0.1
Cervical superior ganglion	0.1
Nodose ganglion	0.2
Vagus nerve	0.5
Thalamus	3.1

TABLE XIII

Distribution of Cyclic 3',5'-nucleotide Phosphodiesterase Activity in Several Marine Organisms.

Specific activity is as defined in the text.

Organism	Genus	Tissue	Specific Activity
Steelhead Trout	Salmo	brain	0.21
"		skeletal muscle	0.07
Salmon	Oncorhynkus	heart	0.34
Sea anemone	Metridium	muscle	0.54
Tubeworm	Nereis	(whole organism)	0.48
Sea urchin	Stronglyo-centrotus	gonads	0.26
" "	"	intestine	0.85
Oyster	Crosostrea	adductor muscle	0.0
Clam	Mytilus	adductor muscle	0.0
Snail	Thais	(whole organism)	0.45
Hermit Crab	Pagarus	(whole organism)	0.0
Sea cucumber	Stichopus	longitudinal muscle	0.25
Crab	Cancer	gill	0.0
"	"	liver	0.0
"	"	pancreas	0.0
"	"	heart	0.0
"	"	aorta	0.0

TABLE XIV

Distribution of Phosphodiesterase Activity in Plants and Micro-organisms.

Plant tissues were washed with 0.15 M KCl, frozen in liquid nitrogen, ground to a powder in a chilled mortar, and further homogenized in suitable volumes of 0.5 M Tris, pH 7.5. Whole homogenates were used for assaying diesterase activities, unless indicated otherwise. The assays were performed as described in the text for the chromatographic method of detecting diesterase activity.

All negative results were re-examined by using larger volumes of tissue homogenates or extracts and increasing the incubation time to 2 hours. B. ferrooxidans and E. coli cells were disrupted by sonication at 9 kc/sec for 30 and 10 minutes, respectively.

Specific activity as described in the text.

Organism	Specific Activity
Plants	
Higher plant leaf - (Genus Tradescantia)	0
Moss (Liverwort) - (Genus Lunularia)	0
Fungus (mycelium) - Coprinus macrorhizus	0*
Yeast - Saccharomyces cerevisiae	0*
Algae (Red) - Gymnogongrus norvegicus	0*
Algae (Green) - Spongomorpha coalita	0*
Algae (Blue) - Phaeostrophion irregulare	0*
Micro-organisms	
Bacteria - Bacillus ferrooxidans	0*
Bacteria - Escherichia coli	0.27*

* indicates 37,000 x g supernate and sediment were examined for activity.

On the other hand, Cheung (187) has reported recently that extremely low diesterase activity was present in the yeast, Saccharomyces carlsbergensis. Two available microorganisms were also examined for diesterase activity. It was noted that E. coli possessed an appreciable level of phosphodiesterase activity. In this organism, the entire activity was located in the 37,000 x g supernate. After the presence of diesterase activity in E. coli was demonstrated in the present study, Brana and Chytil (188) reported similar observations. These authors also noted that the diesterase activity was located in the supernatant fraction obtained after centrifugation of sonicated E. coli cells at 20,000 x g.

DISCUSSION

The elucidation of the roles of cyclic 3',5'-AMP is currently under intense study in many laboratories. Experiments with the adenyl cyclase system has led Sutherland (190) to propose a general picture of a two-messenger system for the expression of hormonal control in biological systems. He suggests that a hormone (first messenger) interacts with specific effector cells at the plasma membrane. This interaction results in the formation of a second messenger within the cell to modify intracellular enzyme activity. As a specific example, Sutherland cites the stimulation of adenyl cyclase by epinephrine (first messenger) which results in the increased biosynthesis of cyclic 3',5'-AMP (second messenger) and consequently, the several physiological effects of epinephrine which are observed. The scheme proposed by Sutherland (190) is illustrated in Fig. 30. In such a system, the activity of the phosphodiesterase must be equally important as that of adenyl cyclase in maintaining the required intracellular levels of cyclic 3',5'-AMP. As yet, no hormonal mechanism for controlling the activity of the phosphodiesterase has been detected.

The role of cyclic 3',5'-nucleotide phosphodiesterase in the brain is being actively investigated. Cheung and Salganicoff (186) have reported that the diesterase activity was located mainly in the cholinergic nerve endings and in the soluble synaptic neuroplasm. They suggested that the diesterase in brain was probably more closely associated with

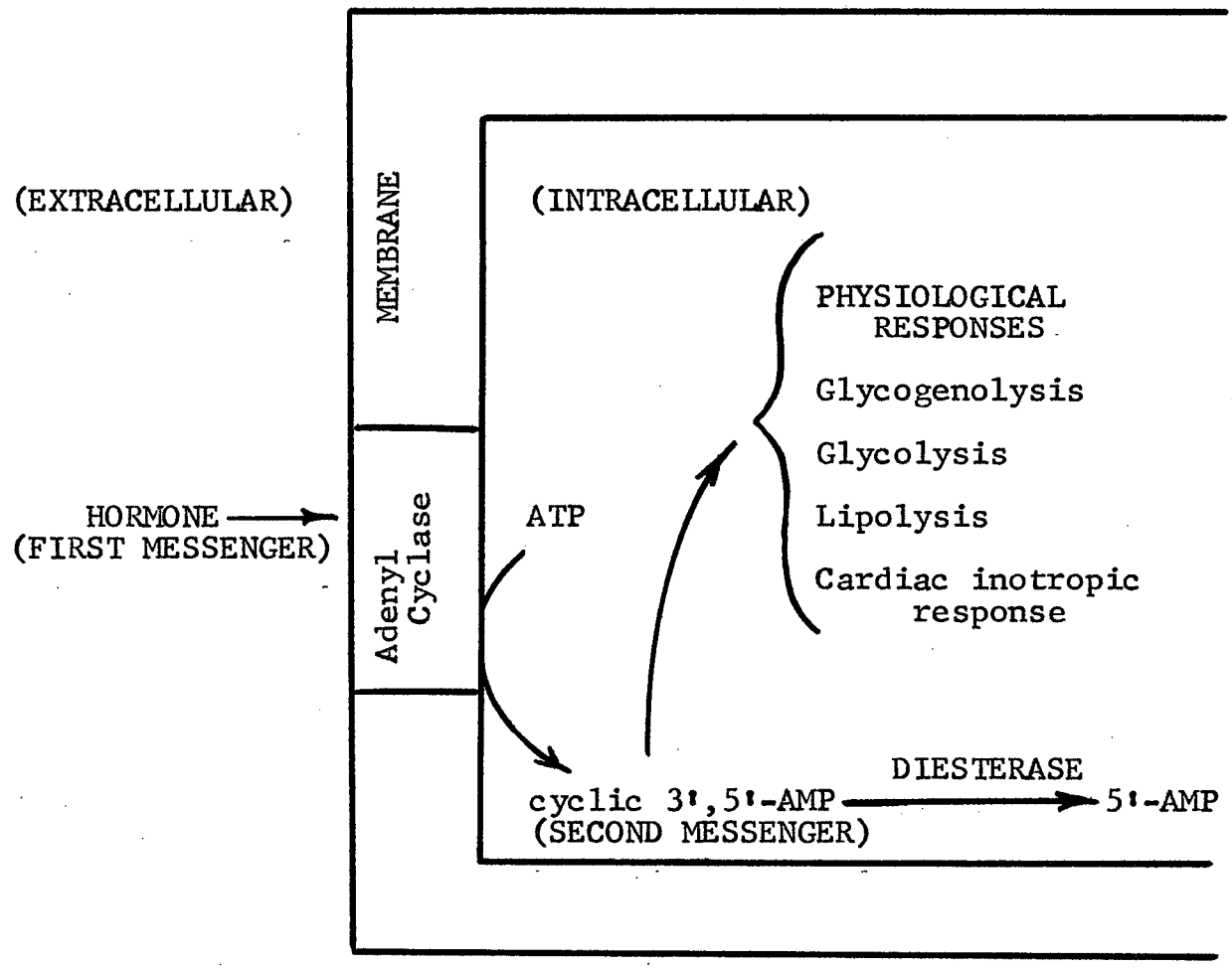


FIG. 30 The Two-Messenger Concept for the Expression of Hormonal Control as Modified from Sutherland(190).

the regulation of glucose metabolism than in synaptic transmission.

Studies of several properties of the partially purified cyclic 3',5'-nucleotide phosphodiesterase from rabbit brain revealed that the properties of the brain enzyme are very similar to the diesterase which has been purified from cardiac tissue. As reported earlier by Drummond and Perrott-Yee (177), brain diesterase catalyzed the conversion of cyclic 3',5'-AMP specifically to 5'-AMP. No other product was formed. It was also confirmed that the enzyme requires Mg^{++} ions for activity and was completely inactive in the presence of 1 mM EDTA. Earlier studies on brain and heart diesterases suggested that the activity of the enzyme was more selective for the cyclic 3',5'-nucleotide which contain purine bases than those with pyrimidine bases. Indeed, the present studies have shown that the enzyme has virtually no activity against pyrimidine cyclic 3',5'-nucleotides, with the exception of cyclic 3',5'-UMP. However, Hardman and Sutherland (184) recently reported that a phosphodiesterase was present in heart that hydrolyzed cyclic 3',5'-UMP at a much faster rate than cyclic 3',5'-AMP and other available cyclic 3',5'-nucleotides. Their observation therefore suggests that the slight but significant activity against cyclic 3',5'-UMP found in the partially purified brain preparation may have been due to another diesterase which was specific for cyclic 3',5'-UMP. It was noted that the hydrolysis of cyclic 3',5'-AMP was consistently inhibited by the presence of cyclic 3',5'-dAMP. Preliminary experiments

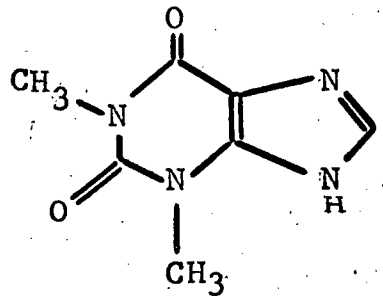
also indicated the inhibition of cyclic 3',5'-AMP hydrolysis by cyclic 3',5'-GMP and cyclic 3',5'-dGMP. The data strongly indicates that cyclic 3',5'-nucleotides possessing purine bases are hydrolyzed by the same enzyme.

ATP (0.125 mM and 1.25 mM) did not appear to inhibit the brain diesterase. This is in contrast to a recent report by Cheung (191) who indicated that brain diesterase was inhibited by ATP and pyrophosphate, and have attributed to ATP a regulatory role on the enzyme.

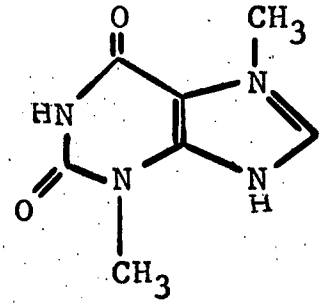
The specificity of brain diesterase for the cyclic 3',5'-diester linkage was unequivocally demonstrated when the final ammonium sulfate fractionation (0.3-0.6 saturation) was performed. This final step effectively removed the cyclic 2',3'-nucleotide phosphodiesterase activity by precipitating the enzyme into the 0-0.3 saturated ammonium sulfate fraction, leaving a highly purified preparation of cyclic 3',5'-nucleotide phosphodiesterase in the higher ammonium sulfate fraction. The present study also demonstrated that the brain diesterase is stimulated by imidazole. The quantitative response to imidazole is in agreement with that observed for heart diesterase. The K_m value of brain diesterase for cyclic 3',5'-AMP was about 0.8×10^{-4}M, which is similar to that observed for beef heart diesterase, but lower than that observed by Nair (176) for dog heart diesterase. The <u>in vitro</u> inhibition of brain diesterase by theophylline observed in the present investigation is also a common feature possessed by the brain and heart enzymes. The nature of theophylline inhibition

appears to be competitive in both instances. As one might expect, caffeine and theobromine also inhibits diesterase activity, although it has been reported (175) that these compounds were only about 16% as potent as theophylline in this respect. The ability of methyl xanthines to inhibit diesterase activity is most likely due to the similarity in the structures of the methyl xanthines to cyclic 3',5'-AMP, as indicated in Fig. 31. One might even speculate that the lower potency of caffeine and theobromine may be due to the presence of N^7-methyl groups on these compounds, which may interfere with their binding to the diesterase.

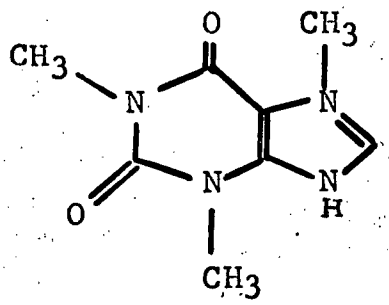
Theophylline has been known for many years to produce a number of pharmacological effects. The compound stimulates the central nervous system, particularly the cerebral cortex, although to a lesser extent than caffeine. However, theophylline is the most potent of all the methyl xanthines in its diuretic action on the kidney, its stimulation of cardiac and skeletal muscle, and its effect on the relaxation of smooth muscle. These pharmacological responses to theophylline administration is likely due to the inhibition of phosphodiesterase *in vivo*, which in turn would result in increased levels of cyclic 3',5'-AMP. For example, it is known that when skeletal muscle is exposed to methyl xanthines (e.g., caffeine), large quantities of lactic acids are produced. These observations indicate that glycogenolysis and glycolysis were stimulated owing to increased cyclic 3',5'-AMP levels in the tissues. Similarly, theophylline has been



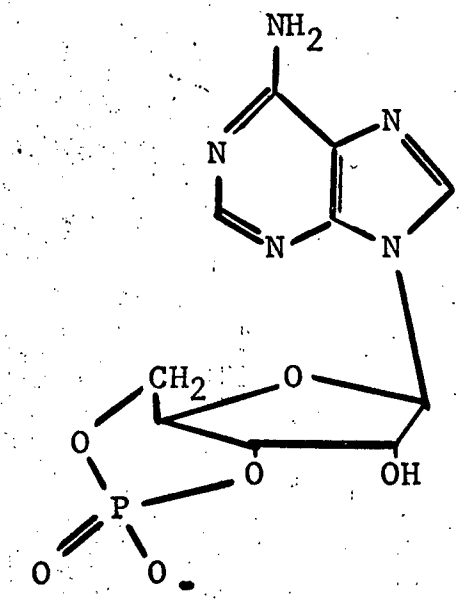
Theophylline
(1,3-Dimethyl xanthine)



Theobromine
(3,7-Dimethyl xanthine)



Caffeine
(1,3,7-Trimethyl xanthine)



Cyclic 3',5'-AMP

FIG. 31 Structural Formulae of Methyl Xanthines

reported to potentiate the cardiac inotropic response to norepinephrine (192), increase lipolytic activity in adipose tissue (193), stimulate steroidogenesis (159), increase amylase secretion from rat parotid gland (163) and increase the permeability of toad bladders to water (166). It would be of particular interest to investigate the possibility that the observed pharmacological effects of caffeine and theophylline upon the central nervous system might arise from the in vivo inhibition of brain phosphodiesterase, resulting in higher levels of cyclic 3',5'-AMP in the brain.

The method described in this study for the partial purification of cyclic 3',5'-nucleotide phosphodiesterase from brain is considerably more rapid and technically easier than that described for the purification of the enzyme from beef and dog hearts. However, this advantage is offset by the fact that the enzyme preparation generally has a specific activity equal to about one-half that reported for the heart preparations.

The use of purified diesterase preparations has aided in the measurement of cyclic 3',5'-AMP in biological materials. For example, Butcher and Sutherland (175) have used the diesterase as a biological tool in order to destroy cyclic 3',5'-AMP in extracts of urine, thus obtaining "tissue blanks" in the assay for the cyclic nucleotide in urine.

The universal distribution of cyclic 3',5'-nucleotide phosphodiesterase in higher organisms of the animal kingdom contrasts sharply with the absence of diesterase activity in

any of the plant specimens examined in this study. Sutherland (194) reported that no adenyl cyclase activity could be detected in plants. Therefore, these observations suggest that the biological importance of cyclic 3',5'-AMP may be confined largely to those organisms within the animal kingdom. The detection of diesterase activity in marine organisms indicates that cyclic 3',5'-AMP is also widespread in lower animal organisms. The biological importance of cyclic 3',5'-AMP in these organisms is of no less interest than its role in higher organisms.

The biological role played by cyclic 3',5'-AMP in the physiology of the nervous system is presently attracting widespread attention. Elucidation of its precise role in this tissue must await further investigation.

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