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STIMULATION OF MITOCHONDRIAL MEMBRANE PHOSPHOLIPASE A_2 IN A HIBERNATOR, *SPERMOPHILUS COLUMBIANUS*

by

© DANIEL JOSEPH PEHOWICH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Stimulation of mitochondrial membrane phospholipase A_2 in a hibernator, *Spermophilus columbianus*", submitted by Daniel Joseph Pehowich in partial fulfilment of the requirements for the degree of Master of Science.

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DATE . June 27 / 1980 . . . . .

ABSTRACT

An increase in membrane fluidity to allow cellular functions to continue at low temperatures appears to be a prerequisite for hibernation. The phospholipid components of mitochondrial membranes are different between the active and hibernating states. The primary means by which phospholipids are synthesized or modified is through hydrolysis by the membrane-bound phospholipases and the subsequent reacylation of different fatty acid moieties.

The focus of this study was to explore the mechanism by which membrane lipids are altered in order to increase fluidity during hibernation. The release of free fatty acids from liver mitochondria was used to assess mitochondrial membrane phospholipase A_2 activity in order to determine if indeed the lipid nature and fluidity of the membrane can be altered by this enzyme.

Intact hepatocytes and liver mitochondria from a hibernator, the Columbian ground squirrel, *Spermophilus columbianus*, were used to observe the effectiveness of norepinephrine (NE), 3',5'-cyclic AMP (cAMP), prostaglandin E_1 (PGE_1) and A-23187, a Ca^{2+} ionophore, in stimulating endogenous phospholipase A_2 activity and the hydrolysis of phospholipids. The phospholipase A_2 activity was monitored by measuring the levels of free fatty acids (FFA) in mitochondria and their release from mitochondria using a ^{63}Ni isotopic assay.

Norepinephrine did not affect the levels of free fatty acids in mitochondria when incubated either directly with isolated mitochondria or with intact hepatocytes. Norepinephrine and cAMP also failed to significantly increase the release of FFA from isolated mitochondria.

The incubation of mitochondria with A-23187 resulted in a 57% increase in the mitochondrial FFA concentration after 10 minutes. Incubation with PGE_1 also stimulated the release of FFA from isolated mitochondria. However, A-23187 or PGE_1 did not stimulate FFA release if Ca^{2+} was absent from the medium or if mitochondrial Ca^{2+} uptake was blocked by ruthenium red. It was concluded that mitochondrial phospholipase A_2 activity can be stimulated by a cyclic flux of Ca^{2+} across the membrane with a consequent hydrolysis of membrane phospholipids.

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INTRODUCTION

Hibernation is a unique physiological state in that it allows a homeotherm to survive cold stress during winter released from the burden of the high energy demands of maintaining a high body temperature. The energy conservation during hibernation is considerable and has been estimated to be as high as 80 to 90 percent (Wang, 1978). Many species of hibernators can maintain their body temperature near 2-4°C for weeks at a time and yet still preserve the ability to rewarm to 37°C within a matter of hours. The dynamic changes in metabolism which occur as the hibernator arouses are even more astonishing when one considers that these changes are occurring as body temperature increases from near freezing to normothermia.

Obviously the ability to undergo such temperature perturbations must require some rather unique adaptations at the cellular level in order to sustain the integrity of life supporting functions. At the low temperatures experienced during hibernation, heart rates have been recorded as low as 2 beats per minute, both in the intact and isolated hearts (Lyman and Blinks, 1959). At the same time respiration is depressed to a single breath every few minutes (Lyman and Chatfield, 1955). In contrast, when a non-hibernator experiences a depression in body temperature, it becomes hypothermic and cannot rewarm spontaneously. In hypothermia, ventricular fibrillation and heart failure occur at a body temperature of 15-20°C (Covino and Hannon, 1959); respiratory failure becomes evident at about 19°C; and nerves fail to conduct impulses at 9°C (Popovic and Popovic, 1974).

It is thus clear that hibernators do possess adaptations which

prevent the impairment of cellular functions at extremely low physiological temperatures. What these adaptations are and how they operate has been a matter of speculation for some time, and as yet no clear picture or unifying theme has evolved (Willis, 1978). One common aspect of the comparisons between hibernation and hypothermia outlined above is that they are all membrane-associated functions. It would seem logical to focus attention on the structure and function of membranes in order to elucidate a key to survival at low temperatures.

Mitochondria play a specific and essential role in energy metabolism and are thought to be associated with a number of other functions. For example, mitochondria are the loci of the urea cycle, enzymes for the metabolism of phosphoglycerides and other lipids, and many other enzymes such as those involved in nitrogen metabolism (Cohn, 1969). The mitochondrion is characterized by a double membrane, one 50 to 100 Å inside the other. Certain enzymes, mainly those of the electron transport chain, are intimately associated with the inner membrane, while others, such as the oxidative enzymes of the Kreb's cycle, are located within the matrix or interior of the mitochondrion. All of these enzymes contribute to respiratory activity.

Membrane lipids, the backbone of membrane structure, appear to be an important factor in the normal operation of membrane-bound enzymes, such as those of cellular respiration. As well, membrane lipids are responsible in part for membrane permeability barriers (Kimelberg, 1976). Alteration or removal of certain lipids may bring about changes ranging from alterations in kinetics or stability to complete inactivation of some enzymes (Aloia and Rouser, 1975). Raison *et al* (1971) have demonstrated that the rate of oxygen uptake by liver mitochondria shows

pertubations from a strictly linear temperature function, i.e. when the respiratory activity is plotted against the reciprocal of absolute temperature (the Arrhenius plot), the slope of the line is not constant over the temperature range 5-37°C. Rather there is a break in the slope at or near 23°C, indicating an impairment or modification of the function of the membrane-bound enzymes. This is contrary to the observed behaviour of most soluble enzymes which display a straight-line relationship with a gradual reduction in activity as temperature is lowered (Raison, 1972; Lenaz *et al*, 1972). Further studies have shown that membrane-bound enzymes are influenced by the physical state of the membrane lipids. This is revealed by a coincidental phase transition in the lipids at or near 23°C (Raison *et al*, 1971). During such a phase transition it is thought that the oxidative enzymes may actually undergo a configurational change when the lipid components of the membrane change from a liquid-crystalline to a gel state. Singer (1972) has postulated a Liquid-Globular Protein Mosaic model which proposes hydrophobic interactions between the fatty acid chains and the hydrophobic regions of membrane proteins. Thus a physical change in the phospholipids would be reflected in any proteins interacting with them. What is manifested when a temperature-induced phase-transition occurs is an increase in the apparent energy of activation of the particular enzyme involved. An increase from 9.1 to 16.7 kcal/mole in activation energy for succinoxidase from liver mitochondria of active ground squirrels is characteristic of such a change (Raison and Lyons, 1971). On the other hand, Arrhenius plots of the mitochondrial respiratory activity of hibernating ground squirrels showed a single activation energy of 13.8 kcal/mole between 5-37°C, indicating no phase

transition has occurred within this temperature range (Raison and Lyons, 1971). Because temperature-induced detrimental effects are observed in non-hibernating animals near or below the transition temperature (23°C), it is logical to assume that the nature of the lipids plays an important role in maintaining the functional integrity of the membrane.

Another important aspect to consider is that membrane alterations seen during hibernation may not be completely related to a global change in membrane fluidity. That is, localized prevention of a phase-transition may be all that is necessary to ensure functional integrity. McElhaney (1974) has demonstrated that some organisms can survive and function with up to 50% of their membranes in the solid state. Rat liver mitochondria and microsomes have been shown to be sufficiently fluid to remain in a liquid-crystalline state at temperatures near 0°C yet the animal cannot survive below 15-20°C (Martonosi, 1974). Thus, although the functional significance of a global phase-transition is still debatable, the possibility of a phase shift in isolated regions appears to be likely.

The absence of a detectable phase-transition in the membranes of hibernators suggests that the membrane lipids are altered or protected in some manner during hibernation. Possible schemes to eliminate the barrier of a temperature-induced phase-transition include the enhancement of membrane unsaturated fatty acids (Hazel and Prosser, 1974) and the incorporation of a "cryoprotective" agent such as cholesterol (Steim *et al*, 1969). Alternative mechanisms include a reduction of hydrocarbon chain length (Khuller and Goldfine, 1974) and the use of phospholipids such as phosphatidylcholine which contain weakly bonding polar groups (Papahadjopoulos, 1977). All of these modifications serve

to decrease the temperature at which lipids shift from a liquid crystalline to a gel phase by increasing disorder or "fluidity" and thereby increase the temperature range of membrane bound enzyme functions.

Comparing rat liver mitochondria with those of trout, Richardson and Tappel (1962) observed that the poikilotherm did not exhibit a phase-transition at low (4°C) temperature, and that this correlated with a higher degree of unsaturation of membrane fatty acids. This has since been supported by data from Lyons and Raison (1970), Wilson and Fox (1971) and Kimelberg and Papahadjopoulos (1972).

More recent studies, however, have indicated that the degree of unsaturation may not determine membrane fluidity *per se*. Rather, it would seem that the nature of the lipids present in the membrane is a major factor in maintainence of fluidity at low temperature as well. Aloia *et al* (1974) have found that the molar percentages of glycerophosphatides decrease during hibernation, whereas the percentage of lysoglycerophosphatides increases (glycerophosphatides are a group of phospholipids characterized by glycerol, the alcohol with which the fatty acids and phosphoric acid are esterified; lysoglycerophosphatides are formed from the hydrolysis of an acyl group from a diacly glycerophosphatide). Arrhenius plots showing the activation energy of succinate oxidation, along with the evidence from electron spin resonance (ESR) studies, indicate that an 8 molar percent increase of the lyso compound of certain glycerophosphatides is sufficient to prevent a phase-transition from occurring. The ESR data demonstrates that the lyso derivatives can increase the disorder of the membrane phospholipids, and in this manner, maintain fluidity (phase-transitions

are thought to result in a disorder-order confirmational transition of the hydrocarbon chains of the lipids). Analysis of intact membranes (Schecter *et al*, 1974) and lipid water models (Gulik-Krzywicki, 1975) suggests that a small fraction of the hydrocarbon chains may be involved in the disorder-order transition under normal physiological conditions. This may induce a local segregation of the lipid molecules which in turn may alter the protein-lipid interactions and thereby exert a regulatory effect on certain membrane functions. It would seem, therefore, that the amount of disorder-order is very important to membrane function, both at and below normal body temperatures.

If the assumption that the nature of its lipid moieties determine the membrane's integrity is valid, then the factors which control lipid composition are the most probable means with which an animal can have some input into compensating for thermal stress. In other words, if the animal can control the physical and chemical properties of membrane lipids to a certain extent, it may be able to alleviate the problems created by temperature-induced changes in these lipids.

Lysoglycerophosphatides, thought by Aloia *et al* (1974) to enhance membrane fluidity, are produced by the hydrolysis of glycerophosphatides by the phospholipases. Of primary importance to membrane structure is the enzyme phospholipase A (EC 3.1.1.4). Phospholipase A was first thought to hydrolyze both acyl groups from phosphoglycerides, but more detailed study has revealed that there are actually two "A" enzymes: phospholipase A_1 (specific for the 1-acyl ester) and phospholipase A_2 (specific for the 2-acyl ester), both of which give rise to lysophosphoglycerides (Waite and vanDeenan, 1966). Phospholipase A_2 was found to be located mainly in mitochondria while A_1 is now thought

to be concentrated in lysosomes. Phospholipase A_2 has a wide distribution and it is generally believed that the production of small amounts of lysoglycerides by this enzyme is one of the chief ways by which fatty acid composition of tissues is regulated.

The mechanism of activation of phospholipase A_2 is not perfectly clear, but it is well known that the physical properties of the substrate can markedly influence the activity and that the enzyme has an absolute and specific requirement for calcium (Wells, 1972).

The possibility that phospholipase A_2 can play a role in maintaining membrane fluidity was well demonstrated by Charnock *et al* (1973) when incubation of microsomal membrane preparations, obtained from renal cortex, with phospholipase A_2 (bee venom) resulted in straight line Arrhenius plots for oubain sensitive ($Na^+ + K^+$)-ATPase which had previously exhibited breaks in the plot. The question that remains, however, is how is phospholipase A_2 controlled (i.e. activated in sufficient quantities and at an appropriate time) to increase membrane concentrations of lysoglycerides in order to maintain fluidity?

One possible agent capable of stimulating hydrolysis of phospholipids is norepinephrine. Norepinephrine has been reported to increase the concentrations of unesterfied fatty acids in nerve endings and synaptic membranes, although results with mitochondria were ambiguous (Price and Rowe, 1972). As mentioned above, unsaturated fatty acids are thought to be a factor determining membrane fluidity; therefore any mechanism which increases non-esterified fatty acid levels may influence fluidity. Other work by Gullis and Rowe (1975) has shown that the stimulation of hydrolysis of phosphotidylcholine (a phosphoglyceride) by phospholipase A_2 in synaptic membranes and synaptosomes is possible

with norepinephrine and other putative transmitters. Hydrolysis of phospholipids can also be stimulated by cyclic nucleotides (cAMP, cGMP, cCMP and cUMP), the actions of the cyclic nucleotides being enhanced by Ca^{2+} (Gullis and Rowe, 1975). In other studies (Scarpa and Lindsay, 1972) it has been demonstrated that Ca^{2+} stimulates phospholipase A_2 activity in liver mitochondria. In plasma membranes prepared from rat heart ventricles, Vyvoda and Rowe (1976) observed a decrease in the net activity of phospholipase A_2 with 1.0 μM norepinephrine but an increase with 10 μM norepinephrine. They concluded that the membrane contained both phospholipase A_2 and a system for the acylation of endogenous phospholipids and that norepinephrine played a role in the net effect of these enzymes.

Increasing levels of Ca^{2+} have been shown to interact with negatively charged phospholipids (e.g. diphosphatidylglycerol) and raise the temperature of the lipid phase-transition (Papahadjopoulos, 1977). Viret and Leterrier (1976) observed a decrease in fluidity in synaptic vesicles with increasing Ca^{2+} with a consequent increase in the phase-transition temperature. These observations suggest that calcium could be involved in the control of membrane structure and function and thus may have a role in hibernation. Elevated Ca^{2+} levels have been observed during hibernation in heart muscle, where concentrations increase as much as 60 percent in hamsters (Ferren *et al*, 1971), serum (Ferren *et al*, 1971), skeletal muscle, cerebrospinal fluid (Bito and Roberts, 1974), and liver (Behrisch, 1978). The significance of these increases is not known but may be related to the maintenance of irritability and contractility in muscle at reduced body temperature (Ferren *et al*, 1971). Aloia and Pengelley (1979) suggest that

higher levels of Ca^{2+} may be compensated for by the reduction of negatively charged phospholipids observed during hibernation. The ordering effect produced by a combination of high Ca^{2+} and acidic phospholipids such as diphosphatidylglycerol would tend to increase rather than decrease the phase transition temperature and thus be detrimental to hibernation.

It would thus seem plausible that the activation of phospholipase A_2 in the mitochondrial membrane may provide a mechanism by which the nature of membrane lipids can be altered in preparation for hibernation. It would also appear that Ca^{2+} may play an intergral role in any scheme of lipid modification by virtue of its requirement by phospholipase A_2 and the recent evidence that levels of the ion increase during hibernation.

RATIONALE

Overall Objectives

The physiological phenomena peculiar to hibernation are both intriguing and at the same time perplexing. From information gathered to date we have gained an appreciation of the economics of hibernation, with respect to energy conservation, and we have evolved a basic framework in which to probe the aspects of physiology and biochemistry unique to hibernation. Many questions remain unanswered however, and this study is intended to provide some insight into one of the primary components of the ability to hibernate, i.e. the functional significance of membrane fluidity.

In particular this study was undertaken in an attempt to elucidate a possible mechanism for the alteration of membrane fluidity and to examine the implications of altered fluidity on a vital aspect of cellular function, mitochondrial respiration.

Specific Questions and Relevant Experiments

From the literature it is evident that the alteration of the lipid components of membranes proceeds via the hydrolysis of existing lipid species and subsequent substitution of different components. The primary enzyme involved in the synthesis of membrane phospholipids under such a scheme is phospholipase A. For example PLA_2 will cleave a fatty acid moiety from a phospholipid molecule allowing a different fatty acid to be reacylated at that position. This event can have the effect of altering the nature of the phospholipid. One could thus ask the question as to what stimulates the activity of mitochondrial PLA_2 in order to shift the make-up of membrane phospholipids in a direction bene-

ficial to survival during hibernation.

As norepinephrine has been demonstrated to be an effective modulator of PLA_2 activity in other tissues it was reasoned that NE may stimulate PLA_2 activity in mitochondria of ground squirrels as a means of altering membrane fluidity.

Experiment 1. Incubation of Intact Liver Cells with NE - the effects of NE on the activity of mitochondrial PLA_2 were examined using isolated hepatocytes from active ground squirrels. Intact cells were used to test our hypothesis as catecholamine receptors have been postulated to exist on the plasma membrane.

Cyclic AMP has been shown to mediate the effects of NE and therefore it was reasoned to be appropriate to observe the effects of cAMP on the stimulation of PLA_2.

Experiment 2. Incubation of Intact Liver Mitochondria with NE, Cyclic AMP and Phospholipase A_2 - intact mitochondria from active ground squirrels were incubated in the presence of cAMP to determine what effects, if any, this cyclic nucleotide or "second messenger" had on PLA_2 activity. Mitochondria were also incubated with NE to determine if it had any effect inside the cell, and as well, PLA_2 was added to observe the response of mitochondria to exogenous phospholipase.

As PLA_2 requires a specific cation, Ca^{2+}, for activation it would seem appropriate to determine if mitochondrial PLA_2 can be stimulated by altering the availability of the calcium ion.

Experiment 3. Incubation of Intact Liver Mitochondria with A-23187 - intact mitochondria were incubated in the presence or absence of Ca^{2+} to determine if the existence of the ion in the surrounding medium could stimulate PLA_2 activity. As well as A-23187, an ionophore

specific for Ca^{2+}, was added as it purportedly promotes a cyclic flux of Ca^{2+} across the mitochondrial membrane and thus presumably would expose the Ca^{2+} to the PLA_2 which is located in the outer membrane. A Ca^{2+} chelator (EGTA) and an inhibitor of Ca^{2+} uptake (ruthenium red) were used to verify any changes in PLA_2 activity due to Ca^{2+} cycling.

A-23187 is not a naturally occurring ionophore and therefore any activity it may induce may not reflect a true physiological response. Naturally occurring substances which have been shown to have ionophoretic properties are the prostaglandins, in particular PGE_1.

Experiment 4. Incubation of Intact Liver Mitochondria with PGE_1 - intact mitochondria were incubated in the presence of PGE_1, a prostaglandin shown to complex and transport Ca^{2+} across mitochondrial membranes. The mitochondria were pre-loaded with Ca^{2+} and PGE_1 added to initiate the cyclic flux of the ion. Preparations to which ruthenium red had been added were also used to observe what effect, if any, PGE_1 had on PLA_2 activity if Ca^{2+} was prevented from crossing the mitochondrial membrane.

If the Ca^{2+} ionophore A-23187 is effective in stimulating phospholipase A_2, the next logical step would be to determine if membrane fluidity is altered with an increase in lysophosphoglycerides and non-esterified fatty acids in the mitochondrial membrane. An indirect measurement of fluidity is through the observation of the activity of a membrane-bound enzyme, the activation energy of which is dependent upon the fluidity of the membrane.

Experiment 5. The Measurement of O_2 Consumption in Rat Liver Mitochondria after Incubation with A-23187 - intact mitochondria isolated from rat liver were incubated with A-23187 prior to the measurement of

succinoxidase activity. The temperature dependence of succinate oxidase was observed in rat liver mitochondria between 20 and 34°C, a range of optimal activity for components of oxidation-phosphorylation and above the temperature at which a phase transition may occur. Representative values of succinate oxidation (as measured by O_2 consumption) over this temperature range provide an activity/temperature relationship with which to determine the apparent energy of activation for the succinoxidase enzyme. If the hydrolysis of membrane phospholipids occurs through the activity of PLA_2 and if the resultant change in lipid pattern alters fluidity, then a change in the activation energy of succinate oxidase should be observed.

MATERIALS AND METHODS

General

Double glass-distilled deionized water was used to prepare all aqueous solutions. Isolation and reaction media were prepared with pre-boiled distilled water in order to eliminate CO_2. All glassware was washed in hot water with *Alconex* detergent, rinsed in distilled water and oven- dried overnight. Glassware used in FFA determinations was soaked overnight in chromic acid prior to washing.

Experimental Animals

Columbian ground squirrels, *Spermophilus columbianus*, were captured on the eastern slopes of the Rocky Mountains near Gorge Creek, Alberta. These animals were housed in the University of Alberta Biological Animal Services facilities prior to investigation. Animals under study were placed in environmental chambers and kept at 4°C under total darkness. A diet of Purina Rat Chow and vitacubes with occaisional fresh lettuce was provided.

A weight record of all animals was kept in order to follow the annual hibernating cycle (ground squirrels typically gain weight in preparation for hibernation). Hibernating animals were designated as those animals which were in the proper weight phase and which had experienced several bouts of hibernation. Active animals were kept under the same conditions as hibernators but showed no indication of hibernating. Ground squirrels were food deprived for several days in order to promote hibernation and were considered to be active and in a non-hibernating state if hibernation did not ensue.

Isolation of Intact Hepatocytes from Ground Squirrel Liver

Intact hepatocytes were isolated from 24 hour fasted rats and ground squirrels using a combination of procedures described by Seglen (1976) and Berry (1974).

A. General. The highest yields of intact, viable parenchymal cells would appear to be obtained by perfusing the isolated liver under physiological conditions (Seglen, 1976). In this study livers were first pre-perfused with a medium containing the Ca^{2+} chelator EGTA in order to initiate the washout of Ca^{2+}-dependent adhesion factor (Amsterdam and Jamieson, 1974) and then perfused with a medium containing collagenase and Ca^{2+}. After only 10 min of perfusion, a high yield of viable hepatocytes can be extracted from the perfusion apparatus.

B. Apparatus. It is essential that the liver and media perfusing it be maintained at a constant temperature throughout the procedure. To achieve this, all flasks containing the perfusion media were kept in a constant temperature water bath at 37°C. Recirculation of the perfusion was regulated with a peristaltic pump (Sage Instruments Inc.) fitted with silicone tubing and glass connectors. The liver was removed from the animal after cannulation and placed in a perfusion chamber, consisting of a glass dish 9.5 cm in diameter and 3 cm deep. The liver sat on a mesh covered with sterile gauze. The entire preparation was covered with a glass cover in order to maintain a constant temperature and humidity. Perfusate leaking from the preparation was collected through an outlet in the bottom of the chamber and returned to the perfusate reservoir. The temperature of the perfusion chamber was maintained at 37°C by a glass heating coil connected to a circula-

ting water bath. The perfusate reservoir was oxygenated with 95% O_2 and 5% CO_2 which had been passed through a gas scrubber.

C. Enzymes. Collagenase (Type IV, Sigma Chemical) was dissolved in the perfusion medium at a concentration of 0.05%. Because this grade of collagenase does not dissolve completely, the suspension was centrifuged (18,000 rpm for 3 min) prior to use. Hyaluronidase (Type I, Sigma Chemical) was dissolved in the perfusate at a concentration of 0.1%.

D. Perfusion Medium. The liver was first perfused with a modifed Hanks buffer consisting of 8.0 g NaCl, 0.4 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.06 g $NaHPO_4 \cdot 2H_2O$, 0.06 g KH_2PO_4, 2.19 g $NaHCO_3$, 0.19 g EGTA and 20.0 g bovine serum albumin in one litre adjusted to pH 7.4. The collagenase buffer was composed of 6.9 g NaCl, 0.36 g KCl, 0.13 g KH_2PO_4, 0.295 g $MgSO_4 \cdot 7H_2O$, 0.374 g $CaCl_2 \cdot H_2O$, 2.0 g $NaHCO_3$ and 20.0 g bovine serum albumin in one litre adjusted to pH 7.4. After perfusion was complete, the disrupted cells were incubated in a shaking water bath in the same buffer minus collagenase and less Ca^{2+} (0.18 g). After purification, the isolated hepatocytes were suspended in a buffer consisting of 4.0 g NaCl, 0.4 g KCl, 0.18 g $CaCl_2 \cdot 2H_2O$, 0.13 g $MgCl_2 \cdot 6H_2O$, 0.15 g KH_2PO_4, 0.1 g Na_2SO_4, 7.2 g HEPES in one litre and adjusted to pH 7.6.

E. Procedure. Animals were fasted overnight before isolation of liver cells. Some authors state that the nutritional state of the animal is important for qualitative results (Berry, 1974); however, Seglen (1976) observed no difference in preparations from fasted or well fed animals. The animal was first anesthetized with sodium pentobarbital and restrained on a surgical board made of plexiglass through which a number

of holes had been drilled to facilitate drainage of blood and perfusate from the preparation. The abdomen was opened with a transverse incision and the skin retracted. The intestines were displaced to the left side of the abdominal cavity to provide access to the liver and its blood supply. A loose ligature was placed around the vena porta approximately 1.5 cm from its entrance to the liver and distal to its bifurcation. A 15 gauge stainless steel cannula connected to the silicone tubing was laid in position alongside the vena porta. A combined filter unit and bubble trap made of glass 0.5 cm x 5.0 cm and filled with cotton wool was placed in line between the cannula and the perfusate reservoir. The peristaltic pump was adjusted to a flow rate of 20 ml per minute with preperfusion buffer oxygenated and warmed to 37°C. The vena cava was severed to allow perfusate efflux. The vena porta was carefully cut about half way through with fine scissors and the cannula was inserted. The cannula was secured and the chest cavity quickly opened and the upper vena cava cut. The flow rate was increased to 50 ml per min and perfusion was allowed to proceed *in situ* while the preparation was examined to determine whether the cannulation and perfusion were successful. The liver had an overall tan coloring without any apparent swelling. The liver was then carefully excised from the carcass by cutting the vascular and biliary supply and the ligaments to the intestine and abdominal walls. Care was taken when transferring the liver to the perfusion chamber to avoid twisting of the lobes and possible damage to the cannulated vena porta. The liver was placed on a gauze covered mesh in the chamber and the cover placed on top. At this point the liver had been flushed with the preperfusion medium for approximately 5 min and the perfusion with the col-

lagenase buffer had just begun. Perfusion was continued with recirculating collagenase buffer at 50 ml per min for 7-10 min, or until the vena porta ruptured. By this time, the liver had swelled to nearly twice its original size and appeared soft and edematous. Perfusate was leaking profusely from its surface.

The liver was next placed on a 250 ml beaker of oxygenated wash buffer at 37°C where it was carefully disrupted and the connective tissue cut away. The hepatocyte suspension was incubated a further 8-10 min with constant shaking in order to separate the cells further and to fully complete digestion. After filtering through cheese cloth, the suspension was cooled on ice to 0-4°C before further purification. The hepatocytes were purified by filtering through a series of nylon filters from 250-61 µm mesh. Filtration was best achieved by fitting a piece of mesh over the end of a plexiglass cylinder, 5 cm in diameter and 8 cm long. The suspension was poured into the cylinder and by gently moving the cylinder up and down in a beaker of fresh medium, minimum damage to the hepatocytes occurred. After filtration with a 61 µm mesh the final suspension contained only single intact cells.

F. Determination of Viability. Hepatocytes were examined under the light microscope after isolation in order to assess their morphological integrity. Intact cells were identified as such by virtue of a rounded shape, refractility and the exclusion of Trypan Blue. Damaged cells were easily identified by their flattened, irregular shape and the presence of Trypan Blue within the cytoplasm. The preparations were refiltered through 61 µm mesh if necessary to attain an intact population of greater than 95 percent.

G. Isolation of Mitochondria from Hepatocytes. Intact mitochrondria

were isolated from hepatocytes as described by Gellerfors and Nelson (1979). Isolated hepatocytes become resistant to mechanical damage with the result that the force required to homogenize the cells leads to damaged and uncoupled mitochondria. Sonication of the hepatocytes yielded mitochondrial preparations that were tightly coupled with respiratory control ratios equivalent to those obtained from whole liver. A 10 to 60 sec sonication at low intensity was sufficient to totally disrupt the hepatocytes. An important precaution taken was to sonicate the cells in ice-cold isolation medium and to keep the sonicating vessel in ice during the procedure.

Isolation of Mitochondria

Animals were killed with a blow to the head. Livers were excised within 90 sec and plunged into ice-cold isolating medium. After weighing, the liver was minced with a razor blade and rinsed several times with fresh isolating medium to remove any residual blood. The tissue was then homogenized in a Potter-Elvejem type homogenizer with a teflon pestle. The pestle was turned on a lathe to a diameter which provided a clearance of 0.026" between it and the walls of the homogenizer. The isolation medium consists of 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4, 1.0 mM EGTA and 0.5 mg/ml BSA. The medium adjusted to pH 7.4 with Triethanolamine.

Rat liver mitochondria were isolated essentially as described by Bustamante *et al*, (1977). The liver was disrupted by one or two passes of the pestle and the brei was diluted to a volume of approximately 160 ml and divided amongst four 50 ml polyethylene centrifuge tubes. The tubes were then centrifuged at 600 g for 3 min, the supernatant decanted and the pellet resuspended with a single pass of the teflon

pestle. This step was repeated three times in order to obtain a maximum yield of mitochondria. The pooled supernatant was discarded and the sediment gently resuspended with a glass pipette. The mitochondrial preparation was then further purified by centrifugation at 20,000 g for 10 min, resuspending the pellets in fresh medium and centrifuging at 3000 g for three min. This low speed centrifugation removes any contamination which may have been carried from the nuclear fraction. A final spin at 20,000 g for 15 min yields a tightly packed pellet which was rinsed with fresh isolating medium and resuspended to a final concentration of approximately 50 mg protein per ml.

When ground squirrel mitochondria are isolated as described above, the resultant preparations are typically of low yield and quality. Assuming that ground squirrel liver mitochondria, and in particular that of hibernating ground squirrels, may have different sedimentation coefficients, the isolation procedure was modified. After homogenization as described for rat liver, the brei was first centrifuged at 20,000 g for 10 min. This permits the removal of most of the fat released during the homogenization process which can be detrimental to mitochondrial integrity. The sediment was then resuspended and centrifuged at 600 g for 5 min. The resultant supernatant was saved and the sediment resuspended and centrifuged again. This step was repeated three times for optimum recovery of mitochondria. The pooled supernatants were then spun at 15,000 g for 10 min to isolate the mitochondrial fraction. A low speed (600 g for 15 min) centrifugation was then used to remove any contamination from the nuclear fraction. A final spin at 20,000 g for 15 min results in a tightly packed mitochondrial pellet which was rinsed with fresh isolating medium and

resuspended to a final concentration near 50 mg protein per ml. Both the rat and ground squirrel mitochondria were kept on ice until use.

Assessment of Preparations

A number of criteria are used to determine the viability of the mitochondrial preparation. The homogeneity of the suspension obtained as described above was assessed with the use of an electron microscope. A sample was fixed in suspension with 5% glutaraldehyde in phosphate buffer at pH 7.4 and centrifuged to form a pellet. The pellet was then post fixed with 1% osmium tetroxide in phosphate buffer, dehydrated in a graded series of ethanols and propylene oxide and embedded in Epon 812. Sections 500-700 Å thick were cut by means of a Reichert ultramicrotome, stained with methanolic uranyl acetate and lead citrate, and viewed in a Siemens 102 electron microscope.

Another criterion used was the addition of exogenous NADH to the mitochondrial preparation while O_2 consumption was monitored. A well-prepared suspension of mitochondria should not oxidize NADH as this substrate does not pass through the mitochondrial membrane (Lehninger, 1949).

The final criterion which must be met is demonstration of a high respiratory control ratio, i.e. ADP:O, an acceptable ratio for liver mitochondria being 4 or more with succinate as substrate.

Measurement of Oxidative Activity

Apparatus

Respiration was measured polarographically with a Clark type PO_2 electrode fitted to an Instrumentation Laboratories 113 PO_2 Analyzer. The standard reaction medium consisted of 200 mM mannitol, 63 mM sucrose, 5 mM HEPES, 0.1 mM EGTA, 5 mM $MgCl_2$, 1.25 µM rotenone and

2 mg/ml bovine serum albumin. Succinate was used as substrate at a concentration of 5 mM. The reaction medium was adjusted to pH 7.4 at each measurement temperature with 2 M triethanolamine. The PO_2 electrode was housed in a plexiglass jacket which fitted snugly into a glass reaction chamber (Fig. 1). A water jacket surrounding the reaction chamber maintained the desired temperature ±0.1°C. To measure respiration, air-saturated medium was added to the reaction chamber and any air bubbles removed such that the final reaction mixture was essentially a closed system. Aliquots of mitochondria and other additions were made to the reaction chamber with a Hamilton syringe through a groove between the PO_2 electrode and the wall of the chamber. Continuous rates of O_2 consumption were recorded on a chart recorder (Linear Instruments Corp.) which provided a permanent record of oxidative activity.

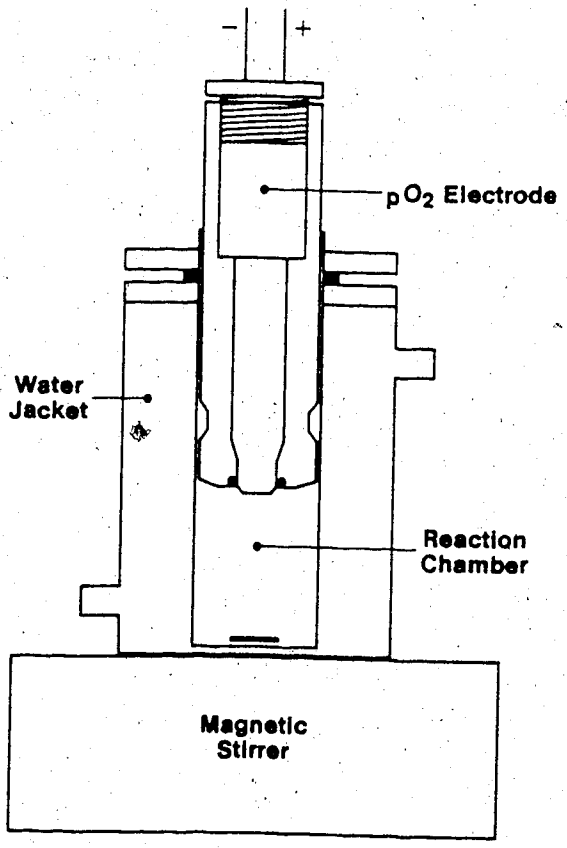
Determination of Oxygen Solubility

Oxygen solubility of the reaction medium was determined using xanthine oxidase as described by Billiar *et al* (1970). The stoichiometry of the xanthine oxidase reaction allows for the calibration of O_2 solubility of the medium, i.e.

$$\text{Xanthine} + H_2O + O_2 \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{Uric acid} + H_2O_2$$

Therefore, each mole of xanthine oxidized requires one mole of oxygen. In this manner, if the recorder was calibrated to 100 percent O_2 content with air saturated medium, the number of moles of xanthine needed to deplete all of the oxygen (i.e. a zero reading on the recording) is equal to the moles of oxygen dissolved in the medium. The xanthine concentration was determined by measuring the absorption at 270 nm and a molar absorbancy index of 0.10×10^4 (Kalckar, 1947). This method

Figure 1. Oxygen consumption apparatus. A Clark-type micro-electrode is placed inside a plexiglass jacket which fits snugly into a glass reaction chamber. Mitochondria and other additions are introduced to the reaction with a Hamilton syringe. A groove along the length of the electrode jacket permits additions to the medium without allowing the entry of air bubbles. The reaction chamber is surrounded by a water jacket which can regulate the temperature at which respiration is measured to ±0.1°C.


−
+
pO_2 Electrode
Water Jacket
Reaction Chamber
Magnetic Stirrer

appeared to be simpler than that of Estabrook (1967) who used electron transport particles isolated from mitochondrial membranes. As well, the use of xanthine oxidase eliminated the need to sacrifice an animal for the calibration procedure.

Protein Determination

The protein content of mitochondrial suspensions used for oxygen consumption and FFA analysis was determined by the procedure of Lowry *et al* (1951) as modified by Markwell *et al* (1978). A 200 µl aliquot of each suspension was solubilized in 3 ml of an alkaline copper reagent. The reagent consists of 2.0% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate and 1% sodium dodecyl sulphate to which was added 4% $CuSO_4$ at a ratio of 100:1. Contrary to observations of Markwell *et al* (1978) the stock alkaline reagent was not stable for more than a couple of days. The precipitate that formed on standing could be prevented if the SDS was stored separately and added immediately before solubilization of the samples.

After 60 min of incubation at room temperature, a 0.3 ml of phenol reagent (Folin-Ciocalteau) diluted 1:1 with distilled water was added to the samples and the samples mixed thoroughly. After a further 45 minute incubation absorbance was read at 600 nm on a Pye Unicam Ultraspectrophotometer. A standard curve was obtained using BSA standards and a reagent blank was prepared with each set of samples. Because this method is quite sensitive (10-200 µg/ml), the mitochondrial samples were usually diluted accordingly before measurement.

Fatty Acid Determination

Extraction

Fatty acids were extracted by a modification of the procedure of

Dole (1956). The extraction mixture consisted of isopropanol/heptane/ 1N H_2SO_4 in a ratio of 40:10:1. An aliquot of mitochondrial suspension was pipetted into a glass-stoppered test tube containing 3 ml of ice cold extraction mixture. The contents were then mixed for 60 sec on a *Vortex* mixer. The resulting mixture stood in ice for 10 min before 2.8 ml distilled water and 0.6 ml heptane were added and the mixture shaken again for 60 sec (Ko and Royer, 1967). After light centrifugation, 0.5 ml of the upper phase was transferred to a 1.5 ml microcentrifuge tube containing 0.5 ml chloroform and 10 mg silicic acid. The silicic acid was activated beforehand by warming at 120°C overnight and then stored in a tightly sealed reagent bottle. The fatty acid-silicic acid suspension was shaken for 60 sec on a *Vortex* mixer and then centrifuged for 10 min. This silicic acid treatment has been shown to remove large amounts of interfering substances (mostly non-polar phospholipids). An aliquot of the supernatant was used directly in the ^{63}Ni assay.

^{63}Ni Assay

A radiochemical assay for long chain fatty acids based on the solubilization of a Ni-fatty acid complex in chloroform-heptane was used for the quantitative determination of membrane free fatty acids. As described by Ho (1970), a 200 µl aliquot FFA in chloroform-heptane was added to a microcentrifuge tube containing 500 µl chloroform-heptane (3:1; v/v) and 50 µl of a nickel nitrate solution containing ^{63}Ni (New England Nuclear). The tube was shaken on a *Vortex* mixer for 60 sec and then centrifuged 600 x g. A 50 µl aliquot of the upper organic phase was then pipetted to a liquid scintillation vial containing 17 ml Bray's solution. Activity was counted on a Nuclear Chicago Mark IV Liquid Scintillation Counter. Duplicate determinations were made for

each mitochondrial sample. Counts were corrected for a reagent blank and the fatty acid content derived from palmitic acid standards. The FFA content can also be determined using the specific activity of ^{63}Ni using the formula

$$\text{nmole FFA} = \frac{\text{(sample blank cpm) x (organic phase volume) x 2}}{\text{(aliquot counted) x (specific activity of } ^{63}Ni\text{, cpm/mole)}}$$

where the organic phase is 700 µl, the molar ratio of FFA/Ni is 2, and the aliquot counted is 50 µl. The specific activity of the ^{63}Ni solution was found to be 7.1×10^3 cpm/nmole with a counting efficiency of 32%.

Using palmitic acid standards ranging from 1 to 40 nmoles per assay it was confirmed that, using the procedure described above, the molar ratio of FFA bound to ^{63}Ni is 2:1 (slope of the line was 2.01 ± 0.12 for 14 determinations with a correlation coefficient of 0.995) (Fig. 2).

The recovery efficiency was determined using ^{3}H-Palmitate (New England Nuclear). The sodium salt of the labelled palmitate was prepared by drying an aliquot of ^{3}H-Palmitate *in vacuo* and dissolving in 0.04 N NaOH in methanol. The methanol was evaporated in air and the ^{3}H-Palmitate dissolved in bovine serum albumin. An aliquot of known activity was added to mitochondrial suspension and incubated for 10 min. The amount of ^{3}H-Palmitate recovered after incorporation into the mitochondrial membranes was 95.6 ± 1.2% for 6 determinations.

It has been noted by Raison (personal communication) that phospholipase activity can be stimulated during the extraction of lipids from membranes which can lead to spurious interpretations when postulating seasonal changes in fatty acid patterns in mitochondrial membranes.

However, by routinely having carried out the extraction proceedure on ice, there was no significant increase in fatty acids found with time. When aliquots of mitochondria containing small amounts of FFA were assayed (less than 2 nmoles/mg) the variability between replicates was high. This was overcome by the addition of an internal palmitate standard to increase the total counts measured. The unknown was derived after subtracting the counts for the added palmitate.

Data Analysis

The difference between the means of experimental and control values were tested for significance using an unpaired Student's *t*-distribution (Sokal and Rohlf, 1969). The level of significance was set at $p<0.05$.

The relationship between respiration rate and temperature was determined with an Arrhenius analysis of the data. Log_{10} transformations of respiratory rates (V_{max}) were plotted against the reciprocal of absolute temperature (1/T) to obtain the Arrhenius equation such that:

$$E_a = \frac{2.303\ T_1 \cdot T_2\ (\log K_1 - K_2)\ R}{T_1 - T_2} \quad \text{(eqn. 1)}$$

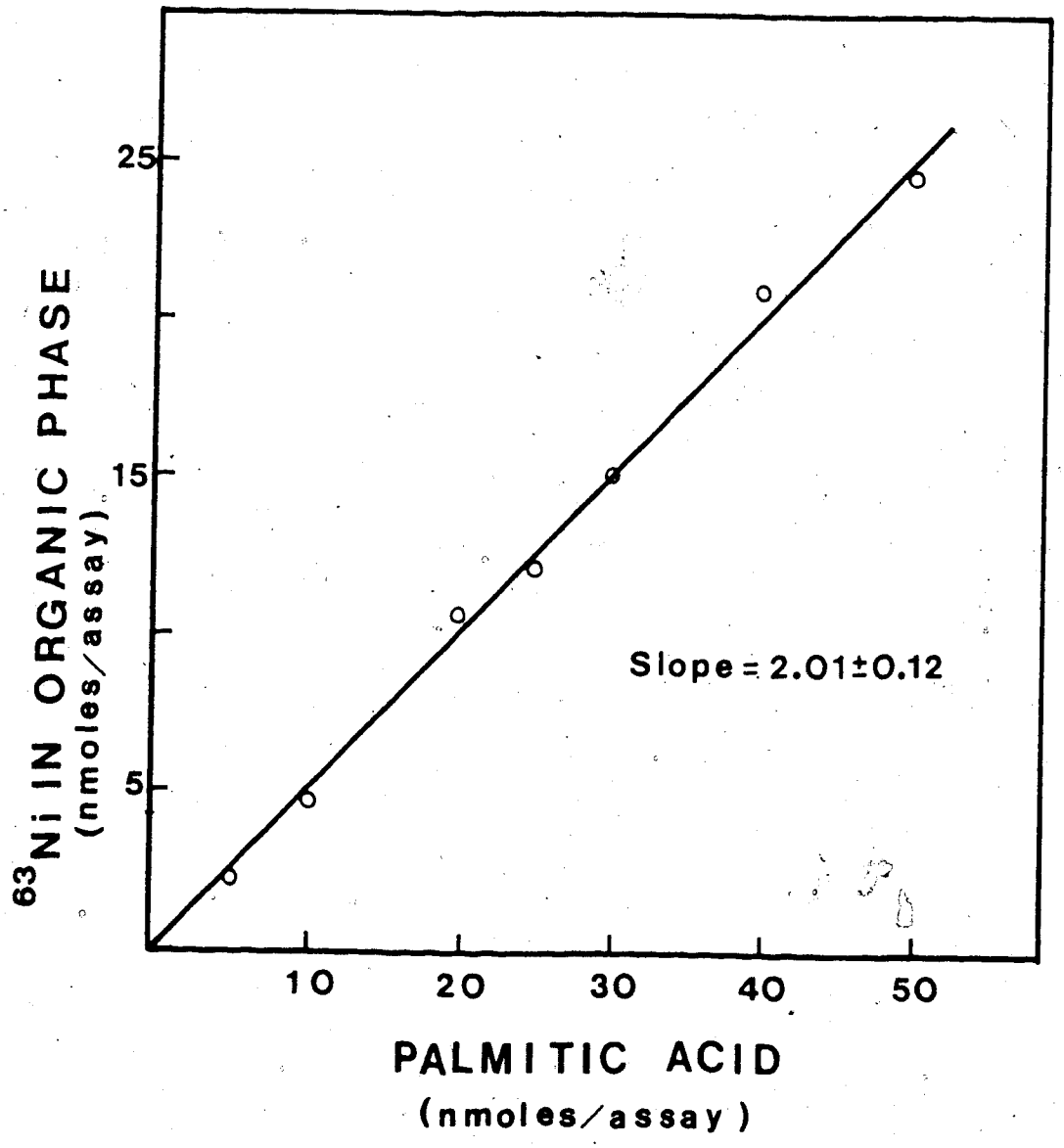
where E_a = apparent energy of activation, R is the gas constant (1.987 cals/mole^{-1} deg^{-1}) and K_1 and K_2 are the maximum velocities at absolute temperatures T_1 and T_2. When comparing the relationship of activity to [illegible] at a single temperature (e.g. 37°C), the means of the rates of [illegible]mption/mg protein/min are compared using the paired Student's *t*-distribution.

[illegible]iscontinuities in Arrhenius plots were determined using the regression coefficient (*b*) and the unexplained sum of squares (the deviations from the regression line). Starting at the lowest temperature, the regression coefficient was determined for successive V_{max} values with increasing temperature. Each consecutive V_{max} value was compared with the regression coefficient of the combined points preceding it (e.g. the slope *b* was determined between V_{max} at 5 and 7°C, which was compared with *b* between 7 and 9°C. The slope *b* be-

tween 9 and 11°C was then compared with the combined regression through values at 5, 7 and 9°C). When a point was found to be significantly different from the regression line preceding it ($p<0.01$) it was taken to represent a break in the Arrhenius plot and it, and all subsequent points, were excluded from the regression line. The same procedure was then taken starting at the highest temperature, calculating the slope b with decreasing temperature. The point at which the two straight lines intersected was taken as the temperature at which a discontinuity or break in the Arrhenius plot occurred. If there was no significant difference between points or slopes over the entire temperature range of 5-37°C, it was concluded that there was no break and only a single regression coefficient was applicable.

Activation energies (E_a) were compared by testing for the difference between the regression coefficients from which they were derived (eqn. 1). Significance was determined using critical values of the F-distribution (Sokal and Rohlf, 1969).

Figure 2. Molar ratio of ^{63}Ni to FFA in radiochemical assay. The palmitic acid standard curve for the ^{63}Ni assay for free fatty acids indicates that there is a 2:1 molar ratio of fatty acid to ^{63}Ni. The fatty acid content of samples extracted by the Dole procedure can thus be calculated directly from the specific activity of ^{63}Ni, which was determined to be 7.1×10^3 cpm/nmole. The points are the means of 12 determinations for each palmitate concentration.


^{63}Ni IN ORGANIC PHASE
(nmoles/assay)
25
15
5
Slope = 2.01±0.12
10
20
30
40
50
PALMITIC ACID
(nmoles/assay)

EXPERIMENTAL PROCEDURES

Incubation of Intact Hepatocytes

Intact hepatocytes were isolated from active ground squirrel liver as described. Aliquots of cells were suspended in a medium consisting of 10 mM Tris-HCl, 1.0 μM $CaCl_2$, 0.5 mM theophylline, 0.2 mM ATP and 0.4 mM $MgCl_2$. The pH was adjusted to 7.4 with HEPES. One ml of the cell suspension was placed in a 25 ml erlenmeyer flask and placed in a shaking water bath. The suspension was bubbled with 95% O_2-5% CO_2 during incubation. NE (L-Noradrenaline Bitartrate) was added in a final concentration of 50 μM. Typically a series of flasks were incubated at the same time with duplicate samples being taken at different time intervals. When the incubation period was for a set duration (e.g. 30 min) six replicates were used.

At the end of incubation, 2 volumes of ice-cold isolating medium were added and the samples placed on ice. All of the samples were centrifuged for 2 min at 50 x g and then washed again in cold medium. Mitochondria were isolated as described.

Incubation of Intact Mitochondria

Mitochondria were isolated from ground squirrel liver as described. The mitochondria were suspended in a medium of 250 mM sucrose, 10 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 0.5 mM theophyline, 0.4 mM $mgCl_2$ nad 0.2 mM ATP. One ml aliquots of mitochondria were placed in glass stoppered tubes in a 37°C water bath and the tubes flushed with 95% O_2-5% CO_2. Incubation was started after the addition NE, cAMP, or phospholipase A_2 (*Crotalus adamanteus*). At the end of incubation the reaction was stopped with the addition of 5 ml of Dole's extraction solvent and the

tubes placed on ice. Non-esterified fatty acids were extracted and measured as described.

Incubation of Intact Mitochondria With PGE_1 and A-23187

Mitochondria were isolated from ground squirrel liver as described. Aliquots of mitochondria suspended in 250 mM sucrose, 10 mM Tris HCl pH 7.5 and 1 mM $CaCl_2$ were incubated in glass stoppered test tubes at 37°C. PGE_1 and A-23187 (in ethanol) were added at a final concentration of 50 µM and 5 nmoles/mg protein respectively. The $CaCl_2$ concentration in the medium was equivalent to 200 nmoles Ca^{2+}/mg protein. The final mitochondrial concentration was 10 mg protein/ml.

After incubation the reaction was stopped with the addition of 5 ml Dole's extract and FFA isolated and measured as described.

Measurement of O_2 Consumption in Intact Rat Liver Mitochondria after Incubation with A-23187

Rat liver mitochondria were isolated as described. Aliquots of mitochondria were suspended in 200 mM mannitol, 63 mM sucrose, 5 mM succinate, 2.0 mM HEPES pH 7.4 and 1.25 µM rotenone. After equilibration for 2 min at 37°C, 150-200 nmoles/mg protein of Ca^{2+} ($CaCl_2$) was added, followed by 5 nmoles/mg protein of A-23187. After one min 2 mg/ml of BSA was added (it is thought the BSA binds A-23187 before it is taken up by the mitochondria). After 15 min of incubation the mitochondria were washed in ice-cold isolating medium, resuspended and O_2 consumption measured as described.

Measurement of Respiratory Rates

State IV and ADP-Stimulated Respiration

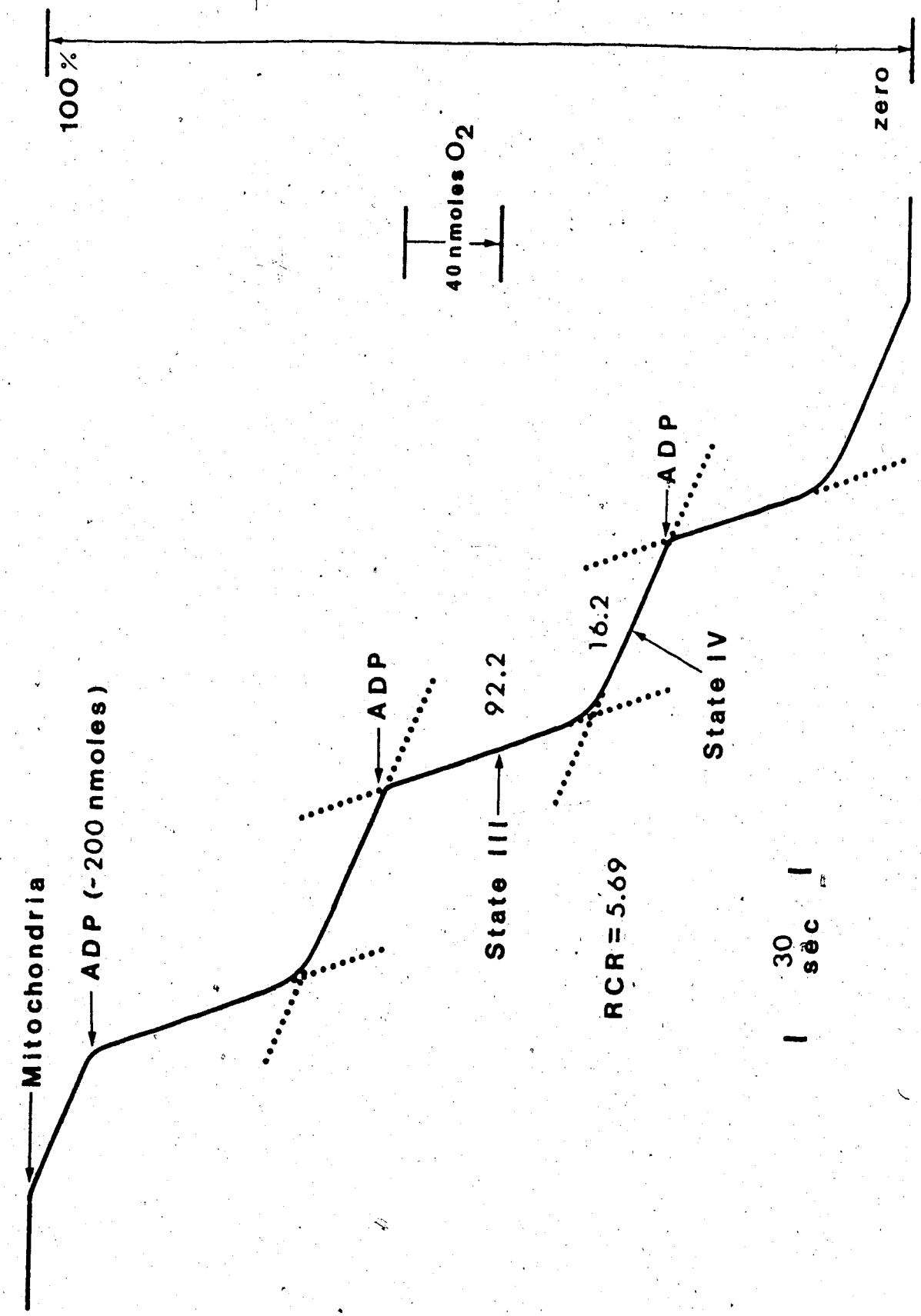
Air-saturated reaction medium was equilibrated at the temperature at which oxygen consumption was to be measured. The reaction chamber

was filled with medium and the PO_2 electrode inserted, with care taken to expel all air bubbles. The reaction medium consisted of 200 mM mannitol, 63 mM sucrose, 5 mM HEPES, pH 7.4, 0.1 mM EGTA, 5 mM $MgCl_2$, 1.25 µM rotenone, 5 mM sodium succinate, 2 mM Tris-phosphate and 2 mg/ml BSA. After calibration of the recorder, 100 µl of freshly isolated mitochondria was added to the reaction medium and the recording of O_2 consumption commenced. After 60 sec 150-200 nmoles of ADP in 2-5 µl was added. This resulted in a stimulation of O_2 consumption which after a short linear phase returned to the initial or State IV rate. Four or five additions of ADP usually was sufficient to exhaust the O_2 content of the medium with each addition generating a State III-State IV cycle. Triplicate measurements of O_2 consumption were made at each temperature. A schematic representation of State IV and ADP-stimulated respiration is shown in Fig. 3.

A-23187

As classified by Reed (1979), A-23187 belongs to the polyether-carboxylic acid family of antibiotic ionophores and is characterized by a pyrrole moiety. It is unique in that it is the only polyether ionophore specific for divalent cations. The ionophore forms an electrically neutral 2:1 antibiotic-alkali metal complex with a nitrogen atom in its structure participating in cation liganding. There is a general consensus that divalent cation transport by A-23187 is thus electroneutral, i.e. there are no net charge movements accompanying net cation transport. The lack of electrical effects indicates that the transmembrane transport of Ca^{2+} by the ionophore is via a mobile carrier mechanism rather than a gated pore (Pfeiffer *et al*, 1978), since the latter point is still the focus of some debate (Hutson, 1977).

Figure 3. Evaluation of oxygen consumption and respiratory control ratio in a typical mitochondrial respiration experiment. The medium consisted of 200 mM mannitol, 63 mM sucrose, 5 mM HEPES, pH 7.4, 0.1 mM EGTA, 5 mM $MgCl_2$, 1.25 µM rotenone, 2 mM Tris-phosphate, 2mg/ml BSA and 5 mM sodium succinate. Respiration is initiated with the addition of mitochondria to the reaction medium and a series of State III/State IV cycles are induced with the addition of ADP. The ratio of State III to State IV respiration is the respiratory control ratio (RCR). The numbers next to each linear portion of the respiration trace represent the rate of O_2 consumption in nmoles/mg protein/min.


100 %
zero
40 nmoles O_2
Mitochondria
ADP (~200 nmoles)
ADP
92.2
State III
16.2
ADP
State IV
RCR = 5.69
30 sec

RESULTS AND DISCUSSION

Incubation of Intact Liver Cells with NE (Experiment 1).

Preliminary results indicated that there was a distinct elevation of FFA in mitochondria isolated from intact liver cells after incubation of the cells with noradrenaline bitartrate. Subsequent experiments did not confirm these findings and it was concluded that in the earlier experiments the hepatocyte preparations were not sufficiently viable to test the hypothesis. Improvements in the overall success of the isolation procedure led to consistently good preparations and only when viability was seen to be greater than 95 percent were the cells used.

When hepatocytes from active ground squirrels were incubated in Krebs-Henseleit buffer containing 2 mM ATP, 10 mM glucose and 1 mM theophyline at 37°C, the presence of 100 µM l-noradrenaline bitartrate did not significantly increase the amount of FFA recovered from the mitochondria. In fact, the amount of FFA extracted from the mitochondria decreased from 23.6 ± 4.8 nmoles/ mg protein to 20.6 ± 5.2 nmoles/mg protein after 60 min incubation (n=4). Untreated hepatocytes showed a similar decrease from initial values (22.7 ± 5.0 nmoles/mg protein (n=4)). Incubation with 10 µM NE produced similar results in that 15.54 ± 3.8 nmoles/mg protein of FFA were recovered after incubation for 60 min as compared to a value at time zero of 17.4 ± 5.2 nmoles/mg protein.

Thus, under the conditions employed, it would appear that NE does not initiate any significant changes in mitochondrial FFA levels and that the mitochondria are capable of regulating FFA concentrations in an *in vitro* system. These assumptions cannot be considered conclusive,

however, in view of the existence of a reacylation pathway which can re-esterify fatty acids as soon as they are released through hydrolysis. The reacylating system would have to be controlled to attach any significance to the observations.

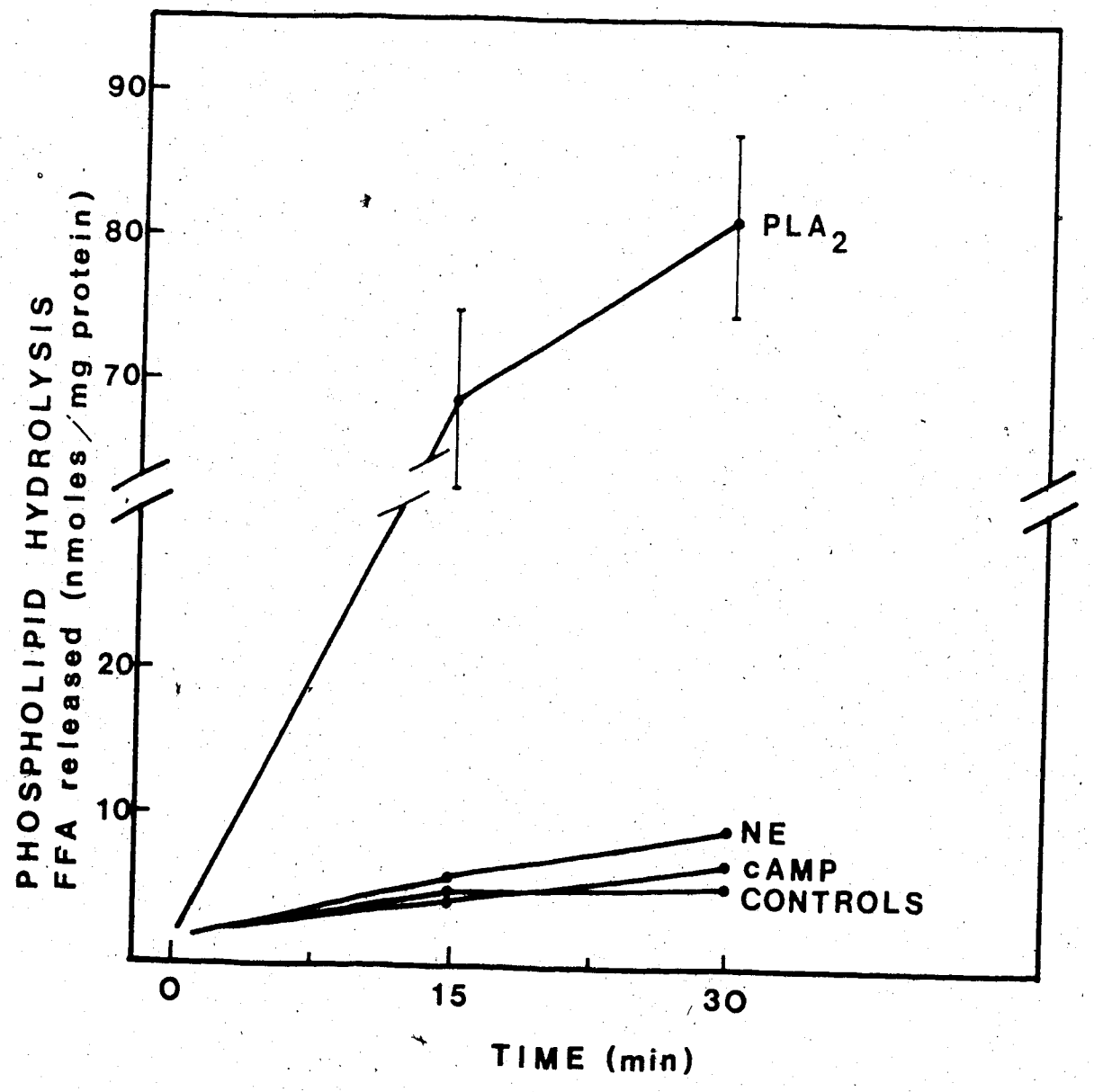
Incubation of Intact Liver Mitochondria with NE, Cyclic AMP and Phospholipase A_2 (Experiment 2).

When freshly isolated liver mitochondria from an active ground squirrel were incubated with phospholipase A_2 there was a rapid increase in the rate of hydrolysis of membrane phospholipids. At 37°C phospholipase A_2 from *Crotalus adamanteus* (50 µl of a 0.02% solution) catalyzed the release of 68.0 ± 8.8 nmoles of FFA per mg of protein after 15 min (n=4) (Fig. 4). A 30 min incubation under the same conditions resulted in the release of 81.4 ± 10.6 nmoles FFA per mg of protein (n=4). No such increase in FFA was observed if 2 mM EGTA was present in the reaction medium.

The incubation of ground squirrel liver mitochondria in the presence of 50 µM cyclic AMP or L-noradrenaline bitartrate (Fig. 4) did not significantly increase the release of FFA above control values. After 30 min the amount of FFA extracted from aliquots of mitochondria were 10.4 ± 4.2, 9.2 ± 3.8 and 7.6 ± 4.0 nmoles/mg protein for NE cAMP and controls respectively. As the SEM bars overlapped they were not shown in Fig. 4.

These results indicate that phospholipase A_2 is a potent hydrolytic agent and capable of liberating fatty acids from the mitochondrial membrane. The dramatic increase in FFA detected can also be considered as a confirmation of the validity of the FFA extraction and measurement techniques. The data shows that NE and cAMP do not stimulate the

Figure 4. Stimulation of phospholipase A_2 in mitochondria with NE and cAMP. Mitochondria were incubated at 37°C in a medium consisting of 250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 5 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.2 mM ATP and 0.5 mM theophylline. Aliquots of mitochondria were incubated after the addition of either 50 µM NE, 50 µM cAMP or 50 µl of a 0.02% solution of *Crotalus adamanteus* phospholipase A_2. Fatty acids were extracted and complexed with ^{63}Ni before counting in a scintillation counter. All points were corrected for background (zero-time incubation). (n=4).


PHOSPHOLIPID HYDROLYSIS
FFA released (nmoles/mg protein)
90
80
70
20
10
0
15
30
TIME (min)
PLA2
NE
cAMP
CONTROLS

activity of endogenous phospholipase A_2 activity above control values under the conditions used. It is also important to note that although Ca^{2+} was present in all cases, it did not appear to activate endogenous phospholipase A_2 activity but was required for the action of added phospholipase A_2 from *Crotalus adamanteus*.

Incubation of Intact Mitochondria with A-23187 (Experiment 3).

When freshly isolated mitochondria from active ground squirrels were incubated with the divalent cation ionophore A-23187, a significant increase in the release of FFA was observed. Mitochondria were preloaded with 100 nmoles of Ca^{2+}/mg protein and incubated with 5 nmoles of A-23187/mg protein for 10 min at 37°C. The total amount of FFA in the preparation increased from 13.30 ± 3.2 to 20.91 ± 2.8 nmoles/mg protein (n=3, triplicate determinations). Control levels of FFA were 14.27 ± 2.6 nmoles/mg protein and were not significantly different from initial values. The presence of 1.0 mM EGTA or 2 nmoles of ruthenium red/mg protein prevented an increase in FFA levels above initial values. If Ca^{2+} was not added prior to the addition of ionophore there was only a slight increase in FFA levels to 14.6 ± 3.1 nmoles/mg protein which was not significantly different from initial levels.

This data points to the ability of A-23187 to stimulate phospholipase A_2 activity in liver mitochondria of ground squirrels. The absence of any A-23187-induced stimulation of phospholipase activity when Ca^{2+} is absent or chelated suggests that Ca^{2+} is required for phospholipase activity. Because there was also no significant increase in phospholipase activity in the controls, which were also preloaded with Ca^{2+}, it would appear as though it is the cyclic flux of Ca^{2+} across the mitochondrial membrane brought about by the ionophore which

is responsible for the stimulation of phospholipase A_2.

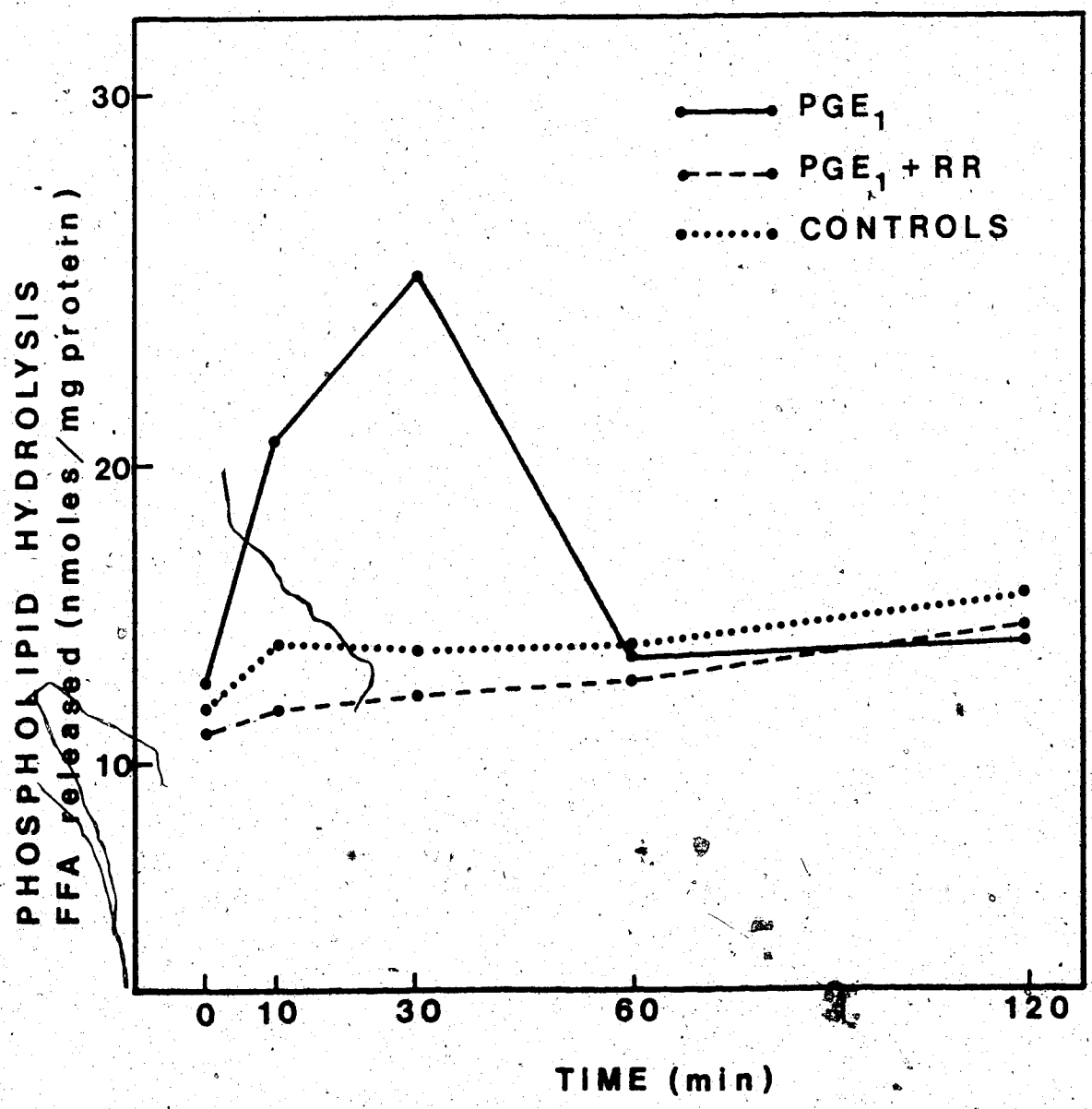
Incubation of Intact Liver Mitochondria with PGE_1 (Experiment 4).

When freshly isolated mitochondria from active ground squirrels were incubated in the presence of 100 µM prostaglandin E_1, there was a marked increase in phospholipase activity as determined by an increase in FFA. As can be seen in Fig. 5, the amount of FFA extracted increased from 12.75 ± 2.6 to 21.0 ± 3.7 nmoles FFA/mg protein, after 10 min incubation at 37°C. Untreated mitochondria FFA concentrations increased from 12.2 ± 4.1 to 14.0 ± 3.9 nmoles/mg protein, these changes not being significantly different. After 30 min. FFA levels in PGE_1-treated mitochondria were 26.0 ± 5.2 nmoles/mg protein and controls had decreased slightly to 13.9 ± 3.3 nmoles/mg protein. After 60 min, however, the total amount of FFA recovered from PGE_1 treated mitochondria decreased dramatically to 13.5 ± 5.7 nmoles/mg protein which was the same as controls (13.4 ± 3.8 nmoles/mg protein). After 120 min, the FFA levels in treated and untreated mitochondria increased slightly (14.3 ± 2.[illegible] and 14.8 ± 3.0 nmoles/mg protein).

The addition of 8 nmoles of ruthenium red/mg protein prior to the addition of PGE_1 resulted in no significant increase in FFA release (Fig. 5). Because the prostaglandins are derivatives of fatty acid precursors, it was thought that the increased FFA acids observed may have been due to ^{63}Ni binding to PGE_1 in the assay procedure. However, concentrations of PGE_1 from 10 to 200 µM added to blanks prior to Dole extraction and the FFA assay did not show any deviation from the blanks.

These observations indicate that PGE_1 is capable of stimulating phospholipase activity, under the conditions used, and that Ca^{2+} is required for the mediation of its effect. The decrease in FFA after

Figure 5. Stimulation of phospholipase A_2 in mitochondria with PGE_1. Mitochondria were incubated at 37°C in a medium consisting of 250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 5mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.2 mM ATP and 0.5 mM theophylline. PGE_1 was added to the incubation at a final concentration of 50 μM. Aliquots of mitochondria were also incubated with PGE_1 and ruthenium red, an inhibitor of Ca^{2+} uptake. (n=4).


PGE1
PGE1 + RR
CONTROLS
PHOSPHOLIPID HYDROLYSIS
FFA released (nmoles/mg protein)
30
20
10
0
10
30
60
120
TIME (min)

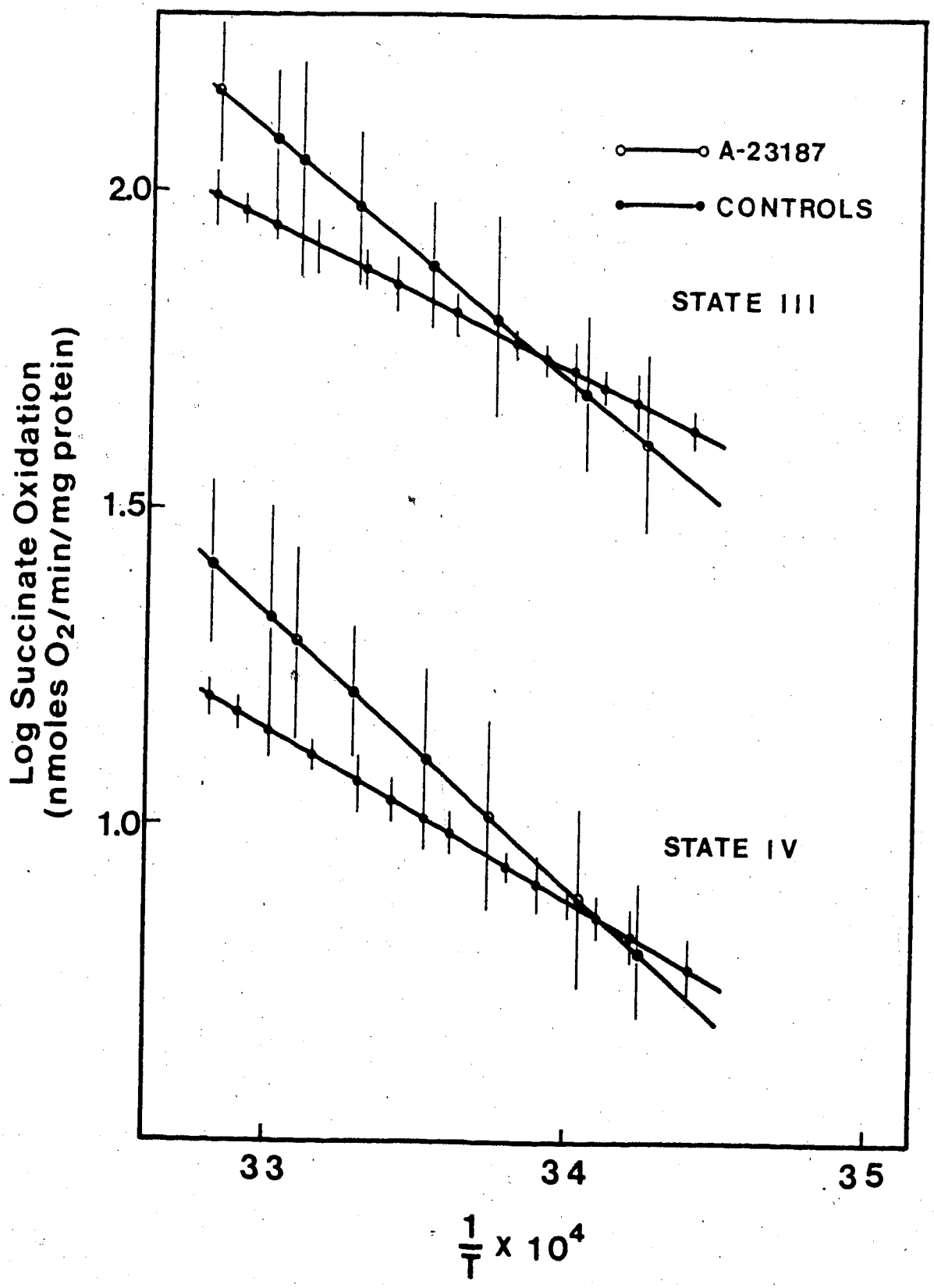
~30 minutes would seem to be evidence of the existence of a reacylating mechanism in the mitochondria. However, the possibility of oxidation of the FFA released cannot be ruled out.

Measurement of O_2 Consumption in Rat Liver Mitochondria after Incubation with A-23187 (Experiment 5).

The activation energy for succinate oxdiase was determined over the temperature range of 21-34°C in intact mitochondria from rat liver. When the E_a's for individual animals were determined they were not significantly different from the average Arrhenius E_a for pooled data ($n=6$; $p<.01$). The E_a for ADP-stimulated succinoxidase activity was 9.4 ± 0.6 kcal/mole; State IV was 10.3 ± 0.9 kcal/mole (Fig. 6).

After 15 min incubation with A-23187 the FFA content increased 58% from initial levels (20.2 ± 3.2 kcal/mole; n=4). When succinoxidase activity was subsequently measured the results were anomolous. Although the E_a's of individual preparations were significantly greater than controls, they were all significantly different from each other. As can be seen in Fig. 6, the average E_a after treatment was 16.6 ± 6.2 kcal/mole (the individual E_a's were 12.7, 14.2, 18.0 and 21.7 kcal/mole). These results indicate that A-23187, which has been shown in a previous experiment to stimulate PLA_2, can alter the E_a for succinate oxidase in rat liver mitochondria. The wide range in value obtained can be attributed to mitochondrial damage in that it has since been determined that the mitochondria become uncoupled and display characteristics of damaged mitochondria if more than 100 nmoles/mg protein of Ca^{2+} is accumulated under the conditions employed in this experiment. The addition of BSA to the incubation may have contributed to the decrease in membrane integrity in that it has been recently observed

Figure 6. Arrhenius plot of succinate oxidation in rat liver mitochondria after incubation with A-23187. Rat liver mitochondria were incubated at 25°C in 200 mM mannitol, 63 mM sucrose, 5 mM HEPES, pH 7.4, 5 mM succinate and 2 mg/ml BSA. The mitochondria were loaded with 150 nmoles/mg Ca^{2+} prior to the addition of 5 nmole/mg A-23187. After 15 min incubation, the mitochondria were washed and respiration measured as described in MATERIALS AND METHODS. The number of observations on which the activation energies are based are 54 for controls (n=6) and 36 for A-23187 treated (n=4). The range of rates measured at each temperature is shown.


A-23187
CONTROLS
STATE III
STATE IV
2.0
1.5
1.0
33
34
35
Log Succinate Oxidation
(nmoles O2/min/mg protein)
1/T x 10^4

that lysophospholipids, the product of phospholipase A_2 activity, induce prolonged lysis of the mitochondrial membrane if the fatty acid moieties cleaved during their production are removed from the membrane (Jain, *et al*, 1980). BSA binds FFA to a great extent and may actually extract them from the membrane.

GENERAL DISCUSSION

Fluidity is a term used to describe the concept that membrane lipids and proteins are in a continual lateral motion and that the overall structure of the membrane is dynamic rather than static (Singer and Nicholson, 1972). From the Singer Fluid Mosaic Model of the membrane (1972) it can also be construed that fluidity refers to the molecular ordering of membrane lipids in relation to physiological function (McMurchie and Raison, 1979). The concept of a fluid membrane is a departure from our basic view of biological structures, yet the plasticity of structure in the membrane must be of fundamental importance (Singer, 1975). The need to increase fluidity during hibernation and the detrimental effects of temperature observed when fluidity is not compensated for, point to an inherent requirement for fluidity and molecular mobility.

The primary objective of the study was to examine some processes whose mechanisms are critically dependent on the fluidity of membranes and to propose a scheme by which a hibernating species can alter its membrane structure in preparation for hibernation.

The use of intact hepatocytes would appear to be an excellent means with which to study both cellular and sub-cellular processes. For example, the examination of a mitochondria-associated event, such as that attempted in this study, could be considered to more closely approximate *in vivo* conditions than if the mitochondria were obtained from disrupted cells.

When isolated hepatocytes from active ground squirrels were incubated with NE there was no detectable increase in the stimulation of phospholipase activity as determined by monitoring fatty acid levels.

These results certainly cannot be considered as equivocal, however, in view of our inability to control all aspects of cellular function. Chen *et al* (1978) were able to stimulate gluconeogenesis, a Ca^{2+} requiring function, in isolated hepatocytes from rat liver. Similar conditions to this study were employed and it was suggested that the 8 μM concentration of NE which stimulated gluconeogenesis did so via the release of an exchangeable pool of Ca^{2+} within the cell, presumably from the mitochondria. Similarly, the activation of protein kinase and glycogen phosphorylase in isolated rat liver cells has been achieved with the use of cyclic AMP, which is thought to be mediating the effect of catecholamines (Birnbaum and Fain, 1977). When Al-Shaikhaly and Baum (1977) attempted to stimulate phospholipase activity through the release of mitochondrial Ca^{2+} they observed that the effects of mercurials and thyroxine were initially masked by the activity of a reacylating system. From these observations we can only conclude that a proposed role for NE in the activation of mitochondrial PLA_2 remains tenable.

When mitochondria from active ground squirrels were incubated in the presence of cyclic AMP there was virtually no difference in FFA released when compared to untreated controls. Again we cannot accept this evidence as conclusive. Phospholipase activity has been demonstrated to be stimulated by cyclic 3', 5'-AMP in fat cell homogenates and isolated fat cells (De Cingolani *et al*, 1971). The conditions under which PLA_2 activity was increased were quite different from methods employed in this study; e.g. the observed increases in phospholipid hydrolysis occurred at pH 8.0 as compared to a pH of 7.4 in this case. It is thought the effect of cyclic nucleotides is through

the regulation of mitochondrial influx and efflux of Ca^{2+} (Mela, 1979) as both cAMP and Ca^{2+} are intricately involved in the control of various cellular responses. For example, the production of prostaglandins is known to involve cAMP, Ca^{2+} and phospholipase A_2, but the exact nature of their interactions is not clear (Laychock *et al*, 1977). Perhaps in the isolated mitochondrial preparation employed in this study, co-factors vital to any cAMP-evoked response were absent. There was sufficient Ca^{2+} in our medium to stimulate PLA_2 activity as was aptly demonstrated by the hydrolysis induced by phospholipase from *Crotalus adamanteus*. The information derived in other portions of this study would certainly warrant further investigation of cAMP as a modulator of mitochondrial Ca^{2+} flux and consequential activation of PLA_2.

The use of A-23187 to initiate a cyclic flux of Ca^{2+} across the mitochondrial membrane is a process to which cAMP may be analogous. The Ca^{2+} ionophore was seen to stimulate PLA_2 activity, as significant increases in FFA extracted from the mitochondrial preparations were observed. Parallel results were obtained by Pickett and his co-workers (1977) in which case they induced the release of arachidonic acid from platelets with 10 μM A-23187. Although the initial release of arachidonic acid from the membrane was quite high, the levels gradually decreased as the fatty acid was reaccumulated by the phospholipids, presumably via esterification in the Lands pathway. Pickett *et al* (1977) suggested that their data showed that the Ca^{2+} sensitive site of PLA_2 activity was not on the platelets surface. The same suggestion could also be proposed for the observations in this study. A-23187 is known to transport Ca^{2+} inward across the mitochondrial membrane (Gomez-Puyou and Gomez-Lojero, 1977) and this, coupled with the observa-

tion that Ca^{2+} outside the mitochondrion alone did not activate PLA_2, would make the idea of an activation site within the membrane a plausible conclusion. The lack of any response when ruthenium red was present in the medium is further evidence that Ca^{2+} cycling is essential for PLA_2 activation. We are not alone in making this assumption in that Parce *et al* (1978) have suggested that in coupled mitochondria, phospholipase A_2 is silent because of the inaccessibility of Ca^{2+} due to the organization of the mitochondrial membrane and the location of the enzyme within the membrane. Parce *et al* (1978) also state that PLA_2 may appear to be inactive under some conditions of measurement because it is usually compensated for by the reacylation of the lyso-compounds. Some important considerations to be made from the work of Parce *et al* (1978) and Siliprandi *et al* (1979), who obtained similar results when they induced Ca^{2+} movements with inorganic phosphate and diamide, are that in both of these cases the mitochondria were not coupled. In fact in both instances the mitochondria were irreversibly damaged and had lost the ability to generate enough energy to retain accumulated Ca^{2+}. In our case, the mitochondrial integrity was not decreased by the actions of A-23187 as demonstrated in a subsequent experiment when an abrupt return to a tightly coupled State IV rate of respiration was observed on the addition of ruthenium red.

Prostaglandin E_1 stimulated increases in FFA in much the same way as A-23187. Significant increases in FFA released to the medium were observed after only 10 min of incubation. It has been shown that PGE_1 acts as a mitochondrial Ca^{2+} ionophore and can transport ions across the membrane with no apparent decay in the integrity of the membrane (Malmstrom and Carafoli, 1975). As with A-23187, the addition of

ruthenium red to the preparation prevented any stimulation of phospholipase activity and leads to the conclusion that cycling of Ca^{2+} across the mitochondrial membrane is necessary for Ca^{2+} to be made accessible to phospholipase A_2. While it is interesting to note that there was little difference in the ability of A-23187 and PGE_1 to stimulate fatty acid release (i.e. in the context that the former is not a naturally occurring substance) it should be realized that the concentrations of PGE_1 used were considerably higher than those determined as physiological levels. Concentrations of PG in adrenal gland are typically less than 0.5 ng/ 2.5×10^5 cells (Laychock *et al*, 1977) as compared to a final concentration of 50 μM (50 nmole/mg) in the mitochondrial suspensions used in this study. Malstrom and Carafoli (1975) have shown that rat liver mitochondria can bind PGE_1 to a certain extent (maximum of about 3 nmoles/mg), however, these concentrations are not high enough to initiate Ca^{2+} cycling. Liver slices from active and hibernating woodchucks were shown to accumulate significant amounts of PGE_1 at 7 and 37°C (Salvador *et al*, 1978). The authors concluded that there was no adaptive significance to the uptake of PG as there was no difference in the amount accumulated at 7°C in active or hibernating animals. It appears that the functional efficiency of membranes was not taken into consideration, however, when this assumption was made. The membrane fluidity could be expected to be different at 7°C in the active and hibernating animals such that if the accumulcation of PG is via an active transport mechanism (presumably fluidity-dependent), similar amounts of PG taken up might reflect a net difference in transport efficiency.

The decrease of FFA with time observed after an initial high rate

of phospholipid hydrolysis by PGE_1 (Fig.5) can be explained either by the degradation of the PG or, most likely, by the cessation of Ca^{2+} cycling as a consequence of anaerobic conditions developing in the medium. Ca^{2+} cycling will only endure as long as energy is available. If however, the reacylation system is also inhibited when the mitochondria become de-energized (Siliprandi *et al*, 1979) we would not expect to see the FFA disappear. This observation will have to remain unresolved at this time.

A unifying theme that has emerged from this study is that Ca^{2+} plays an important role in maintaining the functional integrity of mitochondria during hibernation. It has been observed that the cyclic flux of Ca^{2+} across the membrane can stimulate membrane-bound phospholipase A_2 activity, an enzyme known to be essential in the altering of membrane lipids. Reports published subsequent to the results obtained in this study show conclusively that phospholipase A_2 is stimulated by the cyclic flux of Ca^{2+} and that because of the positional distribution of the phospholipid moieties, the products formed would be largely polyunsaturated fatty acids and lysophospholipids (Pfeiffer *et al*, 1979). The data also confirms that the inherent balance between Phospholipase A_2 and an intramitochondrial acyltransferase would result in a futile cycle of FFA release and reacylation which would not be detected by free fatty acid determinations and thus lead to an underestimation of phospholipase activity (Pfeiffer *et al*, 1979). This may have been the case in our early experiments with NE and cAMP. It is now apparent from the work of Pfeiffer and his co-workers (1979) that N-ethylmaleimide can effectively inhibit the ability of mitochondria to reacylate phospholipids and thus be a valuable tool for further investigations in this area.

While we have demonstrated the stimulation of phospholipase A_2 activity with A-23187, naturally occurring ionophores would have to initiate an analogous situation in the intact animal. There is data in this study to suggest that the prostaglandins, in particular PGE_1, may play such a role, however other substances may function in a similar manner. Roman *et al* (1979) have suggested that unsaturated fatty acids can initiate the cyclic flux of Ca^{2+} by forming a Ca^{2+}/fatty acid complex and acting as an ionophore. Such a scheme would have to be under tight control, however, to prevent a cascade effect as more and more fatty acids are released. Other agents which have been demonstrated to trigger Ca^{2+}-dependent phospholipase activity include mercurials and thyroxine (Al-Shaikhaly and Baum, 1977) and phospholipids (Tyson *et al*, 1976). Tyson *et al* (1976) found cardiolipin and phosphatidic acid to be highly efficient ionophores in a Pressman cell but have not demonstrated similar capabilities in an *in vivo* situation. Perhaps the most likely candidate for a physiological ionophore is thyroxine. Fiskum *et al* (1980) found that the treatment of thyroidectomized rats with T_3 lead to a 250% increase in Ca^{2+} efflux and concluded that the regulation of mitochondrial Ca^{2+} efflux by metabolites and hormones may be an important homeostatic mechanism in the control of Ca^{2+}- sensitive activities.

From the observations in this study and relevant literature it can be postulated that hibernators initiate the alteration of the lipid nature of cellular and organelle membranes through some as yet undetermined physiological signal. If phospholipase A_2 is in fact involved in the maintenance of membrane fluidity, its activity is regulated through the cyclic flux of Ca^{2+} across the membrane and this Ca^{2+} cycling itself would have to continue unhindered during all phases of hibernation.

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