Biochemical Pharmacology, Vol. 18, pp. 1157–1167. Pergamon Press. 1969. Printed in Great Britain

PROPERTIES OF SODIUM- AND POTASSIUM-ACTIVATED ADENOSINE TRIPHOSPHATASES OF RAT BRAIN—EFFECT OF CYCLOPROPANE AND OTHER AGENTS MODIFYING ENZYME ACTIVITY*

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(*Received* 26 *July* 1968; *accepted* 8 *November* 1968)

Abstract—Two ATP-hydrolyzing enzyme systems (ATPases), possibly lipoprotein in nature have been isolated from particulate fractions of rat cerebral cortex homogenates. They consist of at least two components, one Mg^{++} dependent and the other Mg^{++}, Na^+ and K^+ dependent. ATP hydrolysis is measured both by orthophosphate and hydrogen ion liberation and a lack of stoichiometry exists (H^+ ion release two times orthophosphate formation) over the first 90 sec of the reaction. H^+ ion and phosphate release are sensitive to strophanthin, but only in the presence of Na^+ and K^+. Sulfhydryl inhibitors including *p*-hydroxymercuribenzoate (POMB) and iodoacetate (IAA) inhibit both basic and Na^+-K^+-activated ATP hydrolysis, but the enzyme system is more resistant to *N*-ethylmaleimide (NEM), especially the initial "spurt" of H^+ ion release. Certain gases have an action on enzyme activity, both to decrease (cyclopropane) or increase it (air, nitrogen) at pressures above one atmosphere.

The significance of the lack of stoichiometry between H^+ ion and orthophosphate release and the action of inhibitory agents are discussed.

CONSIDERABLE evidence is available suggesting an interaction of sodium- and potassium-activated adenosine triphosphatases (Na^+-K^+-ATPases) and cation translocation at excitable membranes (see review by Skou[1]). Recent studies of the nature of such enzyme systems derived from brain microsomes include their further characterization[2–4] and attempts at solubilization.[3–5] Of particular pertinence to the present investigation have been demonstrations of the sensitivity of this enzyme system(s) to cardiac glycosides (multiple authors), certain sulfhydryl inhibitors[2, 6, 7] and also a number of anesthetic agents including barbiturates,[7] ethanol[8–10] and halothane.[11]

This paper describes the separation of lipoprotein fractions[12] from cortical grey matter of rat brain which are enriched in Na^+-K^+-ATPase activity. Such activity is measured both by phosphate release from ATP and by H^+ ion production. Evidence is presented suggesting that on addition of substrate to such systems, the initial release (0–90 sec) of H^+ ion is two to three times that of inorganic phosphate. The

* This investigation was supported by United States Public Health Service Grant GM 13835 (to A. T.).

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possible significance of this parameter of the enzyme activity is discussed. The sensitivity of the lipoprotein Na^+-K^+-ATPases to sulfhydryl reagents, cyclopropane and also air and nitrogen at elevated pressures is reported.

EXPERIMENTAL

Preparation of lipoprotein fractions. The preparation of fractions containing Na^+-K^+-ATPase activity was modified from that reported by Ahmed and Judah.[12] Cerebral cortex tissue was separated from the brains of Sprague–Dawley rats (male, weight 250–300 g) and cleared of white matter and capillaries. A 10% homogenate of the tissue was prepared in a medium containing 0·25 M mannitol, 5 mM EDTA (Tris salt) at a final pH 6·8, using a Dounce homogenizer (pestle A). The homogenate was centrifuged at 8000 *g* (av.) for 20 min in a refrigerated centrifuge. Following separation of the supernatant (S) and resuspension of the pellet (P) in the original medium, each was frozen and stored at −10° for 24 hr.

Lipoprotein fractions could be isolated from either the pellet or the supernatant by the following procedure. After thawing, the fraction was centrifuged at 100,000 *g* (av.) for 35 min in the Spinco model L ultracentrifuge and the supernatant discarded. The residue was resuspended in a medium containing 0·25 M mannitol, 1·0 mM EDTA (Tris salt), 30 mM histidine-HCl, 0·1% deoxycholate and 30 mM Tris-HCl at pH 6·9, using the Dounce homogenizer (pestle A) and centrifuged at 20,000 *g* (av.) for 50 min. The pellet was resuspended in 6 ml of 0·25 M mannitol containing 20 mM Tris-HCl (pH 6·9) to which was added 9 ml of a solution containing 2·1 M sucrose, 5 mM EDTA (Tris salt) and 20 mM Tris-HCl (pH 6·9). After gently homogenizing, the suspension was centrifuged at 62,000 *g* (av.) for 60 min in a swinging bucket rotor. The layer formed at the top of the sucrose was removed by spatula, suspended in 0·25 M mannitol and 20 mM Tris-HCl (pH 6·9) and stored frozen at −10°. This fraction was designated lipoprotein I (LPI) if derived from the original pellet (P) and lipoprotein II (LPII) if isolated from the supernatant (S).

Assay of ATPase activity. The conventional system used for the assay of ATPase was as follows: ATP (Tris salt), 5 mM; KCl, 10 mM; NaCl, 110 mM; $MgSO_4$, 5 mM and Tris buffer (pH 7·2), 20 mM, in a final volume of 0·5 ml. When NaCl or KCl was omitted, equimolar amounts of choline chloride were substituted. Enzyme samples were added to the mixture incubated at 37° and the reaction terminated at 10 min by addition of perchloric acid (final concn, 5% w/v). A 0·1–0·2 ml-portion of the supernatant was taken for phosphate (iP) determination by the procedure of Martin and Doty,[13] scaled down to the range 1–10 μg of phosphate in a final volume of 3 ml.

The rate of ATP hydrolysis was also measured by the rate of liberation of H^+. The incubation mixture (see above), in a specially constructed reaction vessel, was equilibrated at 37° and stirred by means of a magnetic bar. The essential features of the apparatus for measuring H^+ release are shown in block diagram form in Fig. 1. The components used were as follows: electrode (Beckman combination, model 39183); operational amplifier (Keithley, model 300); digital voltmeter (Electro Instruments, model 620); digital recorder (Honeywell, model 9047). The use of such methods for ATP hydrolysis estimation is well documented.[14, 15]

Protein was determined by the biuret procedure of Gornall, Bardawill and David.[16]

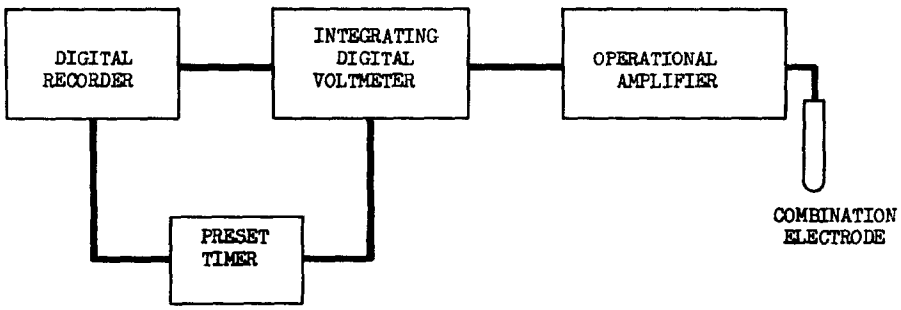


FIG. 1. Apparatus for measuring small changes in H^+ ion concentration. The components were: Beckman combination electrode 39183, Keithley operational amplifier 300, Electro Instruments voltmeter 620 and Honeywell digital recorder 9047. Rapid and accurately timed sampling was possible using a repeat timer, 0–6 sec (Industrial Timer Corp.). The electrode was placed in a glass-jacketed reaction vessel with a circular magnetic stirrer and hydrogen ion liberation measured in a total vol. of 1 ml of reaction medium.

Reagents and chemicals. Tris-ATP, Tris base and mannitol were supplied by Sigma Chemical Co. Strophanthin G was obtained from Mann Biochemicals, N.Y.

RESULTS

Activity and strophanthin sensitivity of cerebral lipoprotein ATPases. Enzyme preparations were separated from both a combined nuclear/mitochondrial fraction (8000 *g* (av.), 20 min) and a microsomal fraction (1,000,000 *g* (av.), 35 min) of a rat cerebral cortex homogenate. Their separation via flotation on sucrose suggests that they exist in the form of lipoproteins[12] and they were, therefore, designated lipoprotein I and II respectively. Both preparations contained basic ATPase activity in the presence of Mg^{++}, which could be stimulated by addition of Na^+ and K^+ to the reaction medium (Table 1). Cation concentrations for maximal stimulation of

TABLE 1. ACTIVITY OF CEREBRAL LIPOPROTEIN ATPASES*

Fraction	Concn of strophanthin-G	ATP split (μmoles/mg protein/hr) in presence of			Inhibition (%)
		Mg^{++} only	Mg^{++} plus Na^+ plus K^+	ΔP	
Lipoprotein I		16·4	45·4	29·0	
	2×10^{-5}M	16·1	26·4	10·3	65
Lipoprotein II		19·0	72·1	53·1	
	2×10^{-5}M	18·6	35·1	16·5	69

* Lipoprotein fractions were isolated from rat cerebral cortex tissue (see text). The reaction mixture (final vol. 0·5 ml) contained ATP, 5 mM; $MgSO_4$, 5 mM; Tris buffer (pH 7·3), 20 mM; and where indicated, NaCl, 110 mM and KCl, 10 mM. Strophanthin-G was added to the mixture prior to addition of enzyme and ATP. Incubation was carried out at 37° for 10 min and the reaction terminated by addition of 0·1 ml 25% perchloric acid. An aliquot of the supernatant was taken for inorganic phosphate determination. The ATPase values are expressed as μmoles ATP split/mg protein/hr and represent the means of five experiments.

ATPase activity were similar for both preparations (Na^+, 110 mM; K^+, 10 mM). The magnitude of this stimulatory effect was greater in the case of the lipoprotein of microsomal origin (approximately 300 per cent) than for lipoprotein I (200 per cent). In both preparations, the Na^+ and K^+ stimulated portion of the ATPase activity, but not the basic activity in the presence of Mg^{++} alone, was inhibited by strophanthin-G. In experiments not shown in Table 1, this inhibition was reversed by increasing K^+ concentration of the reaction medium.

H^+ ion liberation during ATP hydrolysis. In addition to estimation of orthophosphate release, the rate of hydrolysis of ATP was also measured by following the rate of H^+ ion liberation. A direct comparison of the two methods were made by sampling the incubation mixture at varying time intervals during ATP hydrolysis and estimating orthophosphate content. Figure 2 shows that after the initial 2–3 min, the rates of

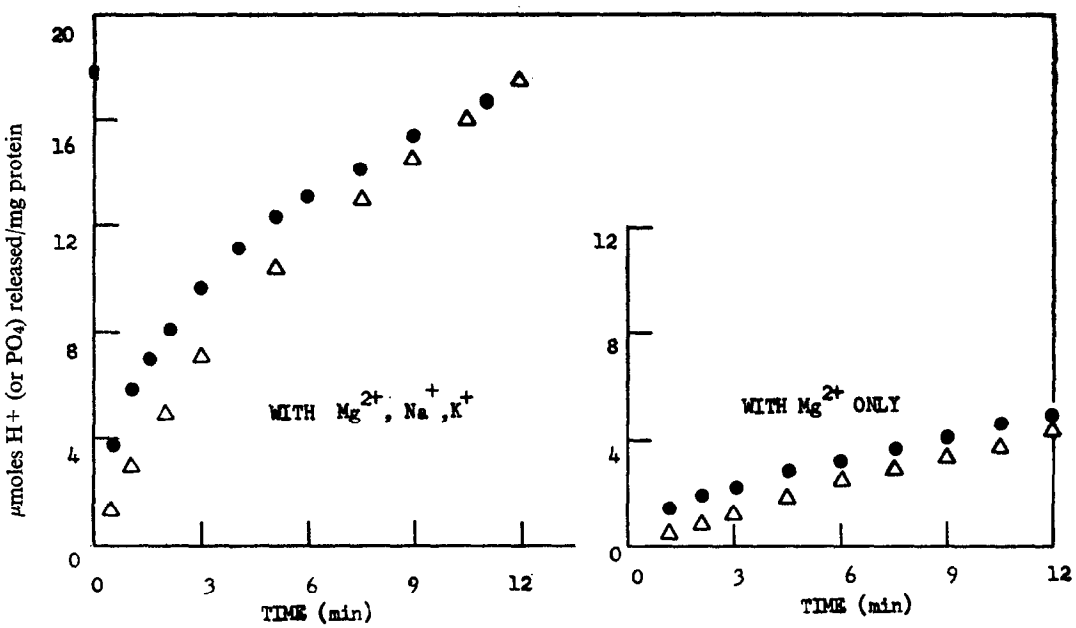
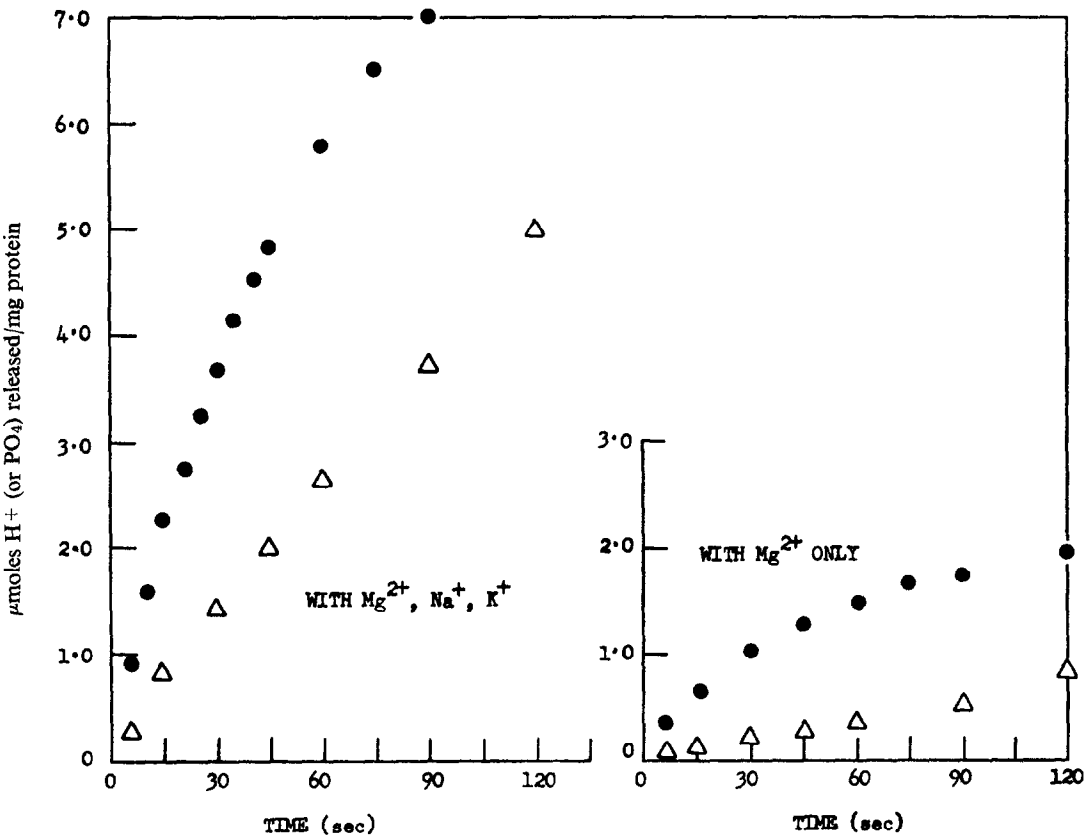


FIG. 2. Hydrogen ion and phosphate release during ATP hydrolysis. Lipoprotein fractions were isolated from rat cerebral cortex (see text). The reaction mixture contained ATP, 5 m: $MgSO_4$, 5 mM; Tris buffer (pH 7·0) 20 mM; and where indicated, NaCl, 110 mM and KCl, 10 mM. Incubation was at 37° with enzyme concentrations between 50–150 μg of protein. Hydrogen ion (●) and orthophosphate (△) concentration were measured as indicated in the text. Each point represents the mean value derived from five experiments.

H^+ ion and orthophosphate release were similar at pH 7·2, suggesting that the reaction measured under these conditions was:

$$(MgATP^{2-} + H_2O \rightarrow MgADP^- + HPO_4^{2-} + H^+).$$

A closer examination of the initial reaction following ATP addition to the system indicated that the initial rate of H^+ ion liberation was faster than that of orthophosphate (Fig. 3), both in the presence of Mg^{++} alone and when enzyme activity was stimulated by addition of Na^+ and K^+.



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On a milligram-protein basis, the release of H^+ ions during the first 60 sec of stimulated ATPase activity was approximately 6 μmoles compared to the formation of 2·7 μmoles of orthophosphate. Between 90–120 sec after ATP addition, the rates of H^+ ion and orthophosphate release become equal. Since it was possible that the initial lack of stoichiometry was merely an artifact of the H^+ ion measuring procedure, a number of control experiments were carried out including; (1) addition of small quantities of standard acid and alkali to determine rate of mixing: pH stabilized within 3–4 sec; (2) addition of ATP to the system without enzyme; (3) addition of enzyme to the system without substrate; and (4) addition of substrate to medium with inactivated enzyme. There was no release of H^+ ions observable under the conditions of experiments 2–4. Some effort was made to estimate accurately the extent to which marginal differences between the pH of ATP solutions and media influenced the initial H^+ ion release, with the conclusion that the reaction system reaches equilibration within 5 sec.

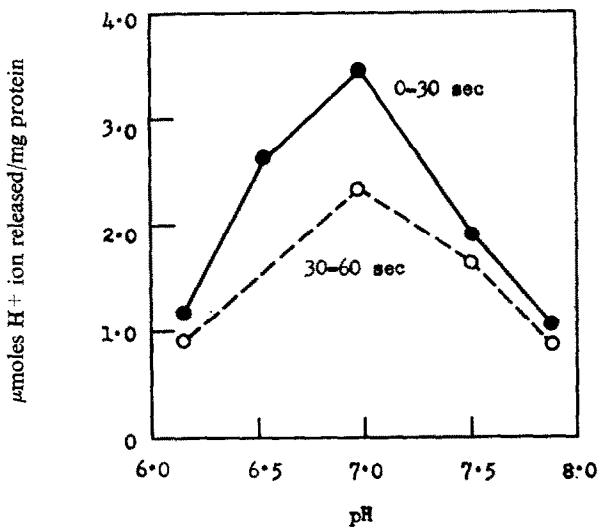

FIG. 4. pH Effect on hydrogen ion release during ATP hydrolysis. The reaction mixture contained $MgSO_4$, 5 mM; NaCl, 110 mM; KCl,10 mM; Tris buffer20m M; and lipoprotein enzyme (50–150 μg protein); incubated at 37°. Hydrogen ion release was measured during the 0–30 sec (●) and 30–60 sec (○) period following addition of ATP, 5mM. Each point represents the mean of four experiments.

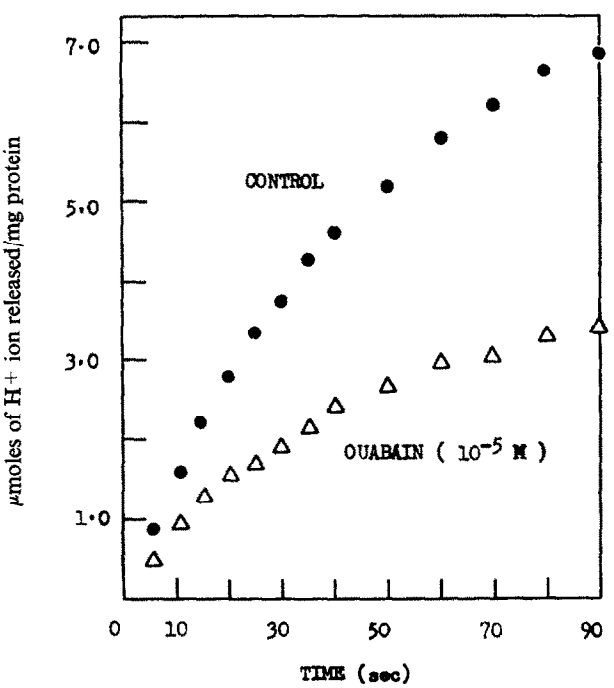

FIG. 5. Sensitivity to ouabain of hydrogen ion release during ATP hydrolysis. The reaction mixture contained $MgSO_4$, 5 mM; NaCl, 110 mM; KCl, 10 mM; Tris buffer, 20 mM; and lipoprotein enzyme, 50–150 μg protein, incubated at 37°. Hydrogen ion release was measured in the absence (●) and presence (△) of ouabain (10^{-5} M) following addition of ATP, 5 mM. Each point represents the mean of four experiments.

The results of the above control experiments suggested that the rapid release of H^+ ions was indeed a characteristic of the initial hydrolysis rate of ATP by the lipoprotein enzyme systems. Further support for this suggestion was gained by examination of the pH dependency of H^+ ion release (Fig. 4). Two curves are shown, the upper representing H^+ ion release during the first 30 sec after addition of ATP and the lower, H^+ ion liberation between 30–60 sec. Both demonstrate pH sensitivity with a range and optimal value (pH 6·95) that closely parallels those observed for orthophosphate release. In these experiments involving H^+ ion release, a total change of less than 0·1 pH unit occurred and Fig. 4 demonstrates that such a change would not influence the reaction rate to any great extent.

H^+ ion liberation during ATP hydrolysis in the presence of Na^+ and K^+ was found to be sensitive to strophanthin at concentrations as low as 10^{-6}M. Figure 5 shows the inhibition of H^+ ion release during the first 90 sec of the reaction in the presence of 10^{-5} M-strophanthin (lower trace).

Sensitivity of lipoprotein ATPases to sulfhydryl inhibitors. The activity of the ATPases preparations in the presence of sulfhydryl inhibitors was measured both by orthophosphate release and H^+ liberation. The results shown in Table 2 represent

TABLE 2. SENSITIVITY OF LIPOPROTEIN ATPASE (LPI) TO SULFHYDRYL INHIBITORS*

	Initial rate of H^+ ion release (0–90 sec) (μmoles/mg protein/hr)		Rate of H^+ ion release (0–10 min) (μmoles/mg protein/hr)		Rate of orthophosphate release (0–10 min) (μmoles/mg protein/hr)	
	Basic	Na^+-K^+-stimulated	Basic	Na^+-K^+-stimulated	Basic	Na^+-K^+-stimulated
None	62	225	15·9	51·8	16·6	48·5
POMB (2×10^{-5})	—	168	—	43·0	—	26·6
POMB (10^{-4})	—	105	—	16·6	9·8	11·5
POMB (10^{-3})	34	45	9·2	12·2	7·9	8·2
IAA (5×10^{-3})	—	168	—	36·6	11·6	34·2
NEM (5×10^{-3})	—	195	—	38·0	8·2	29·0
NEM (5×10^{-3} 20′ preincubation)	50	175	8·5	29·5	7·8	24·4

* Lipoprotein fractions were isolated from rat cerebral cortex (see text). Rate of ATP hydrolysis, was measured by release of H^+ ions and orthophosphate in a reaction medium containing ATP, 5 mM; $MgSO_4$, 5 mM; Tris buffer (pH 7·2), 20 mM; and where indicated, NaCl, 110 mM and KCl 10 mM. Incubation was carried out at 37° for 10 min in the presence of the following sulfhydryl inhibitors at the concentrations shown: *p*-hydroxy mercuribenzoic acid (POMB); *N*-ethylmaleimide (NEM) and iodoacetic acid (IAA).

the sensitivity of ATPase activity of lipoprotein I to such agents, but both enzyme preparations demonstrated similar degrees of sensitivity. *p*-Hydroxymercuribenzoate (POMB) inhibited both the Mg^{++}, and the Mg^{++}, Na^+-K^+ component of the enzyme system. As measured by orthophosphate release, half-maximal inhibition of ATP hydrolysis in the presence of Mg^{++} alone was produced at a concentration of 5×10^{-4} POMB, whereas stimulated activity demonstrated greater sensitivity (half-maximal inhibition at 2×10^{-5} M). The initial rate of hydrogen ion release was also affected by POMB in a concentration-dependent manner. Iodacetic acid also inhibited both basic and Na^+-K^+-stimulated ATPase activities but higher concentrations of the agent were required for demonstrable effects (10^{-3} M). The initial release of H^+ ions was less sensitive to *N*-ethylmaleimide (NEM), even following a period of preincubation

(20 min/room temp.) of the enzyme with the agent, than orthophosphate release. This observation is of interest because it represents the single experimental situation in which we were able to distinguish H^+ ion release from orthophosphate release in terms of drug effects.

Sensitivity of lipoprotein ATPases to anesthetic gases. The action of certain gases including air, nitrogen, nitrous oxide, xenon and cyclopropane on the lipoprotein enzyme systems was examined at varying gas pressures, using a specially constructed stainless-steel pressure chamber. ATP hydrolysis was measured by orthophosphate release only, since the pressure chamber did not permit continual H^+ ion electrode recordings of enzyme activity. Nitrous oxide and the inert gas anesthetic xenon at pressures up to 50 psi had no effect on either preparation of the ATPase system in

TABLE 3. CYCLOPROPANE AND LIPOPROTEIN ATPASES OF RAT CEREBRAL CORTEX*

Pressure (psi)	Change in ATPase activity expressed as % of control value in air at 14 psi			
	Lipoprotein I		Lipoprotein II	
	With Mg^{++} only	With Mg^{++}, Na^+ and K^+	With Mg^{++} only	With Mg^{++}, Na^+ and K^+
Atmospheric	100	79	100	74
20	104	72	100	69
40	77	53	88	59
60	64	45	84	42
80	56	31	76	28

* Lipoprotein fractions were isolated from rat cerebral cortex tissue (see text). ATP hydrolysis was measured by orthophosphate release following exposure of the enzyme preparations to cyclopropane gas at varying pressures in a reaction mixture containing ATP, 5 mM; $MgSO_4$, 5 mM, Tris buffer (pH 7·2), 20 mM; and where indicated, NaCl, 110 mM and KCl, 10 mM. Incubation was carried out at 25°. The figures represent the means of five experiments.

TABLE 4. GAS PRESSURE AND LIPOPROTEIN ATPASES OF RAT CEREBRAL CORTEX*

Gas/pressure (psi)	Change in ATPase activity expressed as % of control value in air at 14 psi			
	Lipoprotein I		Lipoprotein II	
	With Mg^{++} only	With Mg^{++}, Na^+ and K^+	With Mg^{++} only	With Mg^{++}, Na^+ and K^+
Compressed air				
20	103	105	100	105
40	115	110	106	114
80	135	127	122	126
Nitrogen				
20	104	100	100	100
40	116	114	107	110
80	125	132	117	124

* Lipoprotein fractions were isolated from rat cerebral cortex tissue (see text). ATP hydrolysis was measured by orthophosphate release following exposure of the enzyme preparations to air and nitrogen at varying pressures in a reaction mixture containing ATP, 5 mM; $MgSO_4$, 5 mM, Tris buffer (pH 7·2), 20 mM; and where indicated, NaCl, 110 mM and KCl, 10 mM. Incubation was carried out at 25° for 10 min. The effect of the gases are expressed as a per cent of control ATPase activity in air at 25°. The figures represent means of five experiments.

terms of basic or Na^+-K^+-stimulated activity. Cyclopropane was found to inhibit the Mg^{++}, Na^+ and K^+ component of the enzymes to a greater extent than the Mg^{++}-ATPase activity, the pressures required to obtain half-maximal inhibition of the stimulated-ATPase activity being 44 psi. Basic Mg^{++}-ATPase activity was inhibited half-maximally at 80 psi for lipoprotein I, while this activity of the lipoprotein of microsomal origin was even less sensitive to the gas. In order to determine the effect of gas pressure per se, the activity of the enzyme preparation was measured in the presence of air and nitrogen at pressures up to 80 psi (Table 4). ATP hydrolysis both in the presence of Mg^{++} alone and with Na^+ and K^+ was stimulated between 20–30 per cent by air or nitrogen at this pressure. The reversibility of cyclopropane action on the enzyme systems was tested by exposing them to various pressures of the gas in the reaction medium, but in the absence of ATP. After 10 min (the usual reaction time), the gas was removed, substrate added and ATPase activity determined. This was found to be equal to the original activity of the preparations indicating that cyclopropane does not inhibit the ATPase enzyme activities by an irreversible denaturation process.

DISCUSSION

The present report concerns the isolation from rat cerebral cortex of enzyme complexes exhibiting Mg^{++}-dependent ATPase activity which is markedly stimulated by Na^+ and K^+. Such preparations can be isolated from crude mitochondrial or microsomal fractions and their high lipid content together with their property of flotation on sucrose suggests a lipoprotein nature.[12] Rate of ATP hydrolysis is measured both by orthophosphate release and H^+ ion liberation[14, 15] and careful examination of initial rates of hydrolysis indicates a lack of stoichiometry in that H^+ ion release occurs at a rate two times that of orthophosphate. Evidence is presented which argues against this initial rapid H^+ ion liberation resulting artifactually from the experimental method.

There are a number of possible explanations for such a phenomenon occurring during ATP hydrolysis by these enzyme complexes. Wheeler and Whittam[14] have suggested that the formation of a phosphorylated intermediate during enzymic hydrolysis of ATP may manifest itself by an initial high rate of H^+ ion release. Evidence for the participation of intermediates in this reaction has been reported for systems derived from cerebral tissue,[17, 18] but the precise nature of their involvement in ATPase activity remains obscure.[19, 20] An alternative possibility is an associated proton release accompanying a rearrangement of cations on enzymic binding sites following interaction with ATP, in a manner discussed by Abood.[21] Similarly, it could reflect the activity of a (K^+) ATPase enzyme system involved in K^+–H^+ exchange as suggested by Fujita *et al.*[2] If proton release is associated with such mechanisms it appears to be of limited duration, at least during ATP hydrolysis by the enzyme system *in vitro*. This raises the possibility that phosphorylated intermediate formation and mechanisms involving cation rearrangement or K^+-H^+ exchange are also involved only during the initial stages of the reaction. Further suggestions would at best be speculative but a closer examination of this phenomenon, in relation to the possibility of conformational changes during enzyme activity as suggested by Robinson[22] and by Albers *et al.*,[23] would seem to be in order.

Although the two lipoprotein preparations are derived from different particulate fractions, the close similarity in properties suggests a common cellular origin, since the heterogeneous nature of brain subcellular fractions is well recognized. The preparations have properties, including strophanthin sensitivity, in common with preparations isolated by others.[7, 12, 24] Differences exist, however, with regard to sulfhydryl inhibitor susceptibility. Both the basic Mg^{++}-ATPase activity and the Na^+ and K^+ stimulated component were sensitive to POMB, with the latter showing greater sensitivity.[2, 7] The sensitivity of Na^+-K^+-activated ATP hydrolysing enzymes to NEM was initially reported by Skou.[25] The lipoprotein ATPases are comparatively insensitive to NEM, half-maximal inhibition occurring at 10 mM (or 5 mM NEM following 20 min preincubation at room temperature). Fujita *et al.*[2] found complete inhibition of brain-Na^+-K^+-AT-Pase at 1 mM NEM and, in contrast to our results, a complete insensitivity of basic Mg^+-ATPase activity. The initial rate of H^+ ion release during ATP hydrolysis was also singularly resistant to NEM, being inhibited only 13 per cent by a concentration of 5 mM. This observation may have some functional significance but presently remains obscure and will require further characterization studies before it can be revealed.

The inhibitory action of the "inert" gas anesthetic cyclopropane on the lipoprotein ATPases and the greater sensitivity of the Na^+ and K^+ activated component is of some interest in view of the reported action of other anesthetics on similar systems.[7, 11] Since the inert gases are incapable of forming ionic, covalent or hydrogen bonds under physiological conditions, considerations of the mechanism by which cyclopropane inhibits these enzyme systems must be restricted to the physical level of molecular interaction. The existence of such interactions involving London forces has been reported in recent studies on inert gas anesthetics.[26] That such physical interactions can result in functional sequelae is suggested by the observation of Gottlieb *et al.*[27] that helium group gases do inhibit active sodium transport systems in a pressure-dependent manner. The reversible inhibition of ATPase-enzyme preparations by cyclopropane could have significance regarding the anesthetic properties of the gas. Such considerations may be premature in view of the relatively high gas pressure required to inhibit the enzyme system, although this requirement could be related in part to the source of the ATPase preparations. Species differences in sensitivity to the inert gas anesthetics have been documented,[28] greater concentrations of these agents being required to induce anesthesia in lower mammals (including rodents) than in man. Nitrous oxide and xenon had no appreciable action on the ATPase preparations at pressures up to 50 psi. Both of these gases are less potent anesthetic agents than cyclopropane and it is possible that their interaction with enzyme systems is apparent only at greatly elevated pressures. The significance of ATPase activation by increased pressures of nitrogen and air remains obscure. Nitrogen and other inert gases at high pressures will inhibit certain oxygenases perhaps by competition with molecular oxygen.[29] The effects on "transport ATPases" can hardly be explained in terms of gas displacement but must be related to the physical presence of the gases perhaps at an intramolecular site,[26] the identification of which awaits further characterization of the enzyme system. Why such interaction results in enzyme activation *in vitro* is not known, but the phenomenon may well have implications of special pertinence to the contemporary physiological problems of deep submergence.

Acknowledgements—We wish to thank Dr. R. M. Featherstone for valuable discussion and Mr. Charles Pudwill for excellent technical assistance.

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