Biochemical Pharmacology, Vol. 24, pp. 663–669. Pergamon Press, 1975. Printed in Great Britain.

REVERSIBLE INHIBITION OF MAMMALIAN BRAIN ACETYLCHOLINESTERASE AND SODIUM POTASSIUM-STIMULATED ADENOSINE TRIPHOSPHATASE BY CYCLOPROPANE*

USHA R. MAHESHWARI, SIN-LAM CHAN and ANTHONY J. TREVOR

Department of Pharmacology, University of California at San Francisco, San Francisco, Calif. 94143, U.S.A.

(*Received* 1 *June* 1974; *accepted* 13 *September* 1974)

Abstract—Membrane-bound and purified forms of acetylcholinesterase (AChE) derived from beef brain caudate nucleus tissue were inhibited reversibly by cyclopropane at low gas pressures (0·025 to 0·25 atm). Inhibition followed mixed kinetics which suggested interactions of the anesthetic gas with both active sites(s) and other sites on the enzyme molecule. At gas pressures of 1 atm and higher, cyclopropane inhibited membrane-bound and solubilized preparations of brain Na^+-K^+-ATPase without affecting Mg^{2+}-ATPase activity. This inhibition was reversible, followed uncompetitive kinetics and was not due to pressure *per se*. $AT^{32}P$-labeling experiments suggested that cyclopropane inhibited Na^+-K^+-ATPase at or before the phosphorylation step in the enzyme reaction cycle.

The enzymes acetylcholinesterase (AChE; EC 3.1.1.7) and Na^+-K^+-stimulated, Mg^{2+}-dependent adenosine triphosphatase (Na^+-K^+-ATPase; EC 3.6.1.3) both play important roles in the control of neuronal excitability. Modulation of the activities of these enzymes by drugs having actions on the central nervous system could contribute to the neuropharmacological effects of such agents. In this respect, AChE is sensitive to various anesthetic agents [1–3], atropine [4] and neuromuscular blocking agents [5]. Similarly, brain Na^+-K^+-ATPase activity can be inhibited by various centrally acting drugs including ethanol [6], halothane and ether [7].

The present work concerns an examination of the interactions of the inert gas anesthetic cyclopropane with mammalian brain AChE and Na^+-K^+-ATPase, since preliminary studies showed that the agent had inhibitory actions on these enzymes [2, 8]. The limited chemical reactivity of cyclopropane is of interest in terms of the possible physicochemical forces underlying interactions between small molecular weight ligands and macromolecules [9]. Since purified forms of brain AChE were available [10], studies were carried out on these and on membrane-bound forms of the enzyme to ascertain the possible contribution of preparative artifacts to any observed effects. Similar comparative studies were made on membrane-bound and solubilized forms of brain Na^+-K^+-ATPase. The concentration dependence, reversibility and kinetics of inhibition by cyclopropane are reported and discussed in terms of gas interactions with these enzyme macromolecules.

* This work was supported in part by Research Grant NS 10913 and Training Grant DE-00020-15 from the National Institutes of Health.

METHODS

Preparation of AChE and Na^+-K^+-ATPase

AChE from beef brain caudate nucleus tissue was solubilized and purified by the method of Chan *et al.* [10]. Three forms of the enzyme were separated by gel filtration (A, B and C), which on the basis of their molecular weights have been suggested to be hexamer, tetramer and dimer respectively. Synaptosomal AChE was prepared following the procedure of DeRobertis *et al.* [11]. The crude mitochondrial fraction was submitted to osmotic shock and centrifuged at 20,000 *g* for 30 min. The pellets were resuspended in 0·32M sucrose, layered on a sucrose density gradient and centrifuged at 50,000 *g* for 2 hr. Fractions 2 and 3 were combined to determine protein concentration and AChE activity. For Na^+-K^+-ATPase preparations, a 10% homogenate of beef brain cortical grey matter was prepared in a medium containing 0·32 M sucrose and 1 mM EDTA adjusted to pH 6·9 with Tris (disodium salt). Preparation of microsomal fractions of the homogenate and subsequent lubrol extraction were carried out by the method of Shirachi *et al.* [12]. The final protein concentration of the microsomal fraction and the lubrol extract was 15 to 20 mg/ml and 2·5 to 3 mg/ml respectively.

NaI-treated Na^+-K^+-ATPase was obtained by the method of Nakao *et al.* [13] with the following minor modifications. The precipitate was washed with 5 mM EDTA and was centrifuged at 50,000 *g* for 15 min. The above washing and centrifugation were repeated twice.

The final pellet was resuspended in 0·32 M sucrose to give a protein concentration of 1 to 1·2 mg/ml and kept frozen until use.

Analytical determinations

AChE assay. AChE activity was measured using the colorimetric procedure of Ellman *et al.* [14] at room temperature. To study the effect of cyclopropane on the enzyme activity, all reagents except the substrate were placed in an optical cuvette which was part of a modified Thunburg tube. A mercury manometer attachment was used to measure gas pressures up to 1 atm and control AChE activity was determined in the presence of air at the same pressures.

ATPase assay. The conventional system used for the assay of Na^+-K^+-ATPase activity contained 5 mM Tris-HCl, 10 mM KCl, 110 mM NaCl, 5 mM $MgSO_4$, 75 mM Tris buffer (pH 7·0) and the enzyme in a total volume of 0·5 ml. Mg^{2+} ATPase activity was measured by substituting choline chloride (equimolar amounts) for NaCl and KCl. Phosphate was determined by the method of Martin and Doty [15]. To study gas effects on enzyme activities, specially designed reaction tubes and an airtight pressure bomb with a pressure gauge were used. All the reagents were placed in the central well of the tubes, and the substrate was placed into the side arm. The tubes containing the reaction mixture were placed into the pressure chamber and the entire system was degased with a vacuum pump for 2 min. Gas was then introduced at the desired pressure and 10–15 min was allowed for equilibration before starting the reaction with ATP. The pressure bomb was tipped through 90° and the reaction was allowed to proceed for 10 min and then stopped by addition of perchloric acid (final concn. 5%, w/v). To study the reversal of cyclopropane effects, a gas manifold apparatus previously described by Kwan and Trevor [16] was used. The reagents were the same as mentioned in the conventional phosphate assay procedure except that the total volume in this case was 2·5 ml.

Labeling procedure

The NaI-treated enzyme was labeled by the addition of ATP (γ-^{32}P) 10^6 cpm by the procedure of Nagano *et al.* [17]. The final concentration of ATP was adjusted to 0·5 to 5 mM as desired and the reaction was carried out for time periods up to 15 sec at 0·2° using the gas manifold apparatus. Gas effects were observed in the presence of: (a) Na^+ and Mg^{2+} ions, and (b) Na^+, K^+ and Mg^{2+} ions. The centrifugations, washings and homogenizations of the labeled protein were carried out as described by Nagano *et al.* [17], and portions of the supernatant were saved after each washing. The final precipitate was suspended uniformly in 2 ml water and 6 ml aquasol. Radioactivity in the final pellet and in each wash was measured using a Packard Tricarb liquid scintillation spectrometer, model 3375. The efficiency of the system was determined using the internal standard method as 82 ± 3·1 per cent.

Reactions carried out under the above conditions in the presence of Na^+ and Mg^{2+} ions approached steady state kinetics (pseudo-steady state) in that the rate of phosphorylation was linear with time and substrate concentrations remained virtually unchanged. While the concentration of phosphorylated enzyme is assumed to be increasing during the reaction, this variation did not influence reaction linearity. In control experiments with Mg^{2+} alone, the rate of phosphorylation was 7 per cent of that in the presence of Mg^{2+} and Na^+.

Protein

Protein was determined by the procedure of Lowry *et al.* [18]. Bovine serum albumin was used as standard.

Chemicals

Acetylthiocholine (ATC), Tris–ATP and trizma base were obtained from Sigma Chemical Co. (St. Louis, Mo.); 5,5-dithiobis-(2-nitrobenzoic acid) was from Aldrich Chemical Co., Inc. (Milwaukee, Wis.); affinose-202, was from Bio-Rad. Labs (Richmond, Calif.); Sephadex G-200 was from Pharmacia (Uppsala, Sweden); and edrophonium chloride and lubrol were gifts from Hoffmann-La Roche and ICI American, Inc., respectively; gases were obtained from Ohio Medical Products (Madison, Wis.); aquasol and

Table 1. Effect of cyclopropane on various brain AChE preparations*

Preparation	Specific activity (μmoles ATC/mg protein/hr) Low [S] Control	Low [S] Gas	Low [S] % Inhibition	High [S] Control	High [S] Gas	High [S] % Inhibition
Homogenate	11 ± 0·3	7 ± 0·2	37	27 ± 1·0	22·7 ± 0·9	15
Mitrochondrial fraction	7 ± 0·2	4 ± 0·1	42	18 ± 0·8	15·0 ± 0·5	16
Synaptosomal fraction	15 ± 0·5	10 ± 0·2	33	40 ± 1·2	34·6 ± 1·0	13

* Various AChE preparations were obtained as detailed in Methods. Enzyme activity was measured both at high and low substrate concentrations (1 and 0·1 mM ATC respectively). Cyclopropane was present at 0·1 atm. Values are the means of three to four experiments ± S.E.

Table 2. Effect of cyclopropane on various brain AChE preparations*

AChE preparations	Specific activity (m-moles ATC/mg protein/hr) Low [S] Control	Low [S] Gas	Low [S] % Inhibition	High [S] Control	High [S] Gas	High [S] % Inhibition
Pure Form A	216 ± 5	155 ± 3	28	580 ± 6	511 ± 8	12
Form B	185 ± 8	135 ± 4	27	535 ± 5	467 ± 4	12
Form C	220 ± 6	144 ± 4	34	610 ± 6	500 ± 6	18

* Various AChE forms were obtained as detailed in Methods. Enzyme activity was measured both at high and low substrate concentrations (1 and 0·1 mM ATC respectively). For form C the high and low substrate concentrations were 0·5 mM and 0·05 mM ATC. Cyclopropane was present at 0·1 atm. Values are the means of four to five experiments ± S.E.

$AT^{32}P$ were obtained from New England Nuclear. $AT^{32}P$ had a specific activity of 14·6 Ci/m-mole. Other reagents or chemicals used were of reagent grade.

RESULTS

Cyclopropane inhibition of particulate and purified preparations of brain AChE

The AChE activities present in crude particulate preparations of brain tissue and purified forms of the enzyme were sensitive to cyclopropane at low gas pressures. The inhibitory effects of 0·1 atm cyclopropane on two particulate fractions prepared from a homogenate of beef brain caudate nucleus tissue and on three highly purified forms of the enzyme (A, B and C) are shown in Tables 1 and 2. In all cases, the degree of cyclopropane inhibition was quantitatively similar and was more marked at low substrate concentrations. In control experiments, AChE activity was not influenced by changes in air pressure between 0·01 and 1·0 atm.

Influence of cyclopropane pressure on AChE inhibition

The partial pressure of gas used in these studies was quite low and hence the concentration of cyclopropane in solution was assumed to be proportional to its pressure in the gas phase in accordance with Henry's Law. In Fig. 1, the percentage inhibition of activity of various forms of AChE is plotted as a function of cyclopropane pressure (0·025 to 1 atm). At low partial pressure of the gas, inhibition increased linearly with increase in partial pressure and reached a maximum between 0·1 and 0·25 atm, suggesting gas saturation of the enzyme.

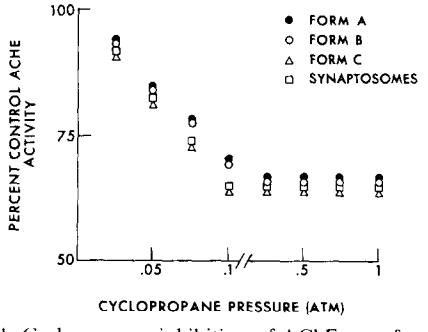

Fig. 1. Cyclopropane inhibition of AChE as a function of pressure. The three purified forms and synaptosomal AChE were prepared as described in Methods. Control AChE activities were 580 m-moles (A), 535 m-moles (B), 610 m-moles (C) and 40 μmoles (synaptosomal) ATC hydrolyzed/mg of protein/hr respectively. Each point represents the mean of four experiments.

Reversibility of cyclopropane inhibition of AChE

Cyclopropane inhibition of AChE was freely and completely reversible irrespective of the preparation or the degree of purity of the enzyme. Figure 2 shows the changes in reaction rate of a synaptosomal and purified form (C) of AChE when exposed to 0·1 atm of the gas and after removal of cyclopropane. The time required for either the addition or removal of gas was approximately 1 min, and changes in enzyme reaction rate were apparent immediately after resumption of recording.

Kinetics of AChE inhibition of cyclopropane

The Lineweaver–Burk plot of enzyme activity vs substrate concentration for the pure C form of AChE in the presence of 0·1 atm cyclopropane is shown in

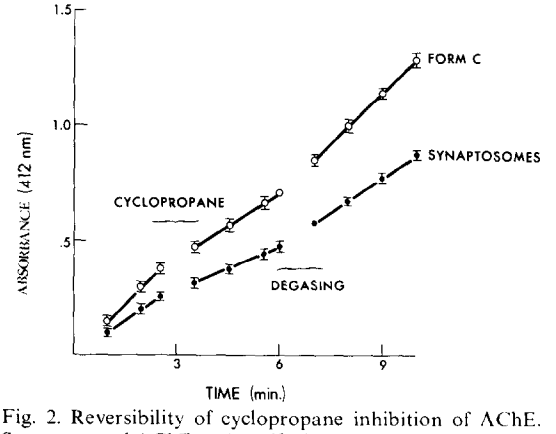

Fig. 2. Reversibility of cyclopropane inhibition of AChE. Synaptosomal AChE and purified form C were prepared as described in Methods. Cyclopropane (0·1 atm) was added and removed at the indicated times. Values are the means of four experiments ± S.E.

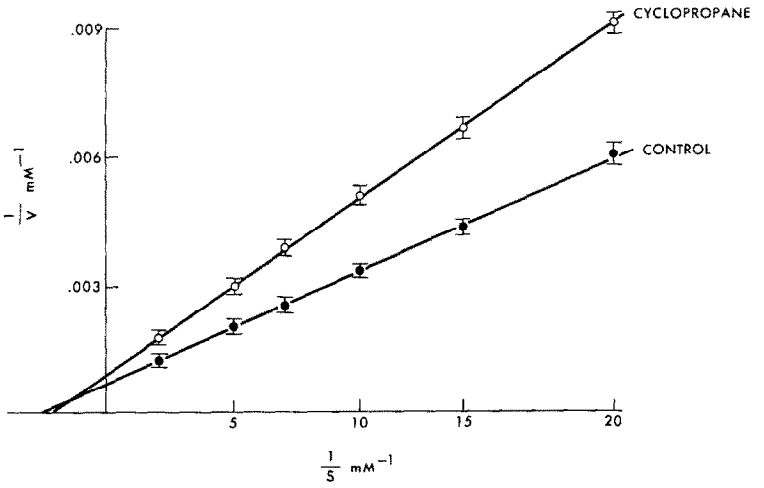

Fig. 3. Lineweaver–Burk plot of cyclopropane inhibition of AChE form C. Substrate concentrations ranged from 0·05 to 0·5 mM ATC, and cyclopropane was used at a pressure of 0·1 atm. Values are the means of four experiments ± S.E.

Table 3. Effect of cyclopropane on various Na^+-K^+-ATPase preparations*

Preparation	Specific activity (μmoles Pi/mg protein/hr) Mg^{2+}-ATPase		Na^+-K^+-ATPase		% Inhibition
	Control	Cyclopropane	Control	Cyclopropane	Na^+-K^+-ATPase
Homogenate	9·7 ± 0·5	9·1 ± 0·8	16 ± 0·5	10 ± 0·8	38
Microsomal fraction	8·3 ± 0·6	7·7 ± 0·6	26 ± 0·8	15 ± 0·5	42
NaI	6·8 ± 0·9	6·3 ± 0·8	43 ± 1·0	22 ± 1·0	49
Lubrol	5·8 ± 0·5	5·3 ± 0·4	58 ± 1·3	28 ± 1·0	51

* Various brain Na^+-K^+-ATPase preparations were obtained as detailed in Methods. Enzyme activity was measured at optimal substrate concentration (5 mM ATP) with and without Na^+ and K^+. Cyclopropane was present at 3·5 atm. Values are the means of four experiments ± S.E.

Fig. 3. The plot suggests mixed kinetics of inhibition (competitive and noncompetitive), and this was also the case for other purified AChE forms (A and B) and for membrane-bound (synaptosomal) enzyme.

Cyclopropane inhibition of brain Na^+-K^+-ATPase preparations

Particulate and partially solubilized forms of brain Na^+-K^+-ATPase were less sensitive than AChE to the inhibitory action of cyclopropane. Table 3 shows the effect of cyclopropane at a pressure of 3·5 atm on various preparations containing Na^+-K^+-ATPase activity. The gas had no significant effect on basic Mg^{2+}-ATPase activity over the pressure range studied (0·25 to 4 atm). Table 4 shows that inhibition of Na^+-K^+-ATPase was dependent on cyclopropane pressure, a small but significant effect ($P < 0·05$) occurring at 1 atm. Cyclopropane inhibition was also reversible as

Table 4. Influence of cyclopropane pressure on Na^+-K^+-ATPase activity*

Cyclopropane pressure (atm)	Enzyme activity (μmoles Pi/mg protein/hr) Control	Cyclopropane	% Inhibition
1	58 ± 2	51 ± 1	12
2	55 ± 2	41 ± 2	26
3	60 ± 1	38 ± 1	36
4	58 ± 1	25 ± 0·5	60

* Lubrol-solubilized brain Na^+-K^+-ATPase was prepared as detailed in Methods. Enzyme activity was measured at optimal substrate concentration (5 mM ATP). Values are the means of three to five experiments ± S.E.

Table 5. Reversibility of cyclopropane inhibition of Na^+-K^+-ATPase*

Time (min)	Enzyme reaction rate (μmoles/mg protein/min)	% Change
2–4 Air-atmospheric pressure	0·36 ± 0·02	
8–10 Cyclopropane	0·24 ± 0·03	−35
14–16 Air	0·35 ± 0·02	−3

* Lubrol-solubilized brain Na^+-K^+-ATPase was prepared as detailed in Methods. The reaction was initiated by addition of 5 mM ATP and run at 25°. Cyclopropane (2·3 atm) was added after 4 min and removed after 10 min. Control reaction rate was linear for approximately 20 min. Values are means of four experiments ± S.E.

shown by the data in Table 5. The enzyme reaction was initiated by addition of substrate to a lubrol extract of a brain microsomal fraction. After sampling, the reaction mixture was degased and exposed to cyclopropane at 2·3 atm for 4 min and then resampled. After removal of the gas, the reaction was allowed to proceed for a further 4-min period.

Specificity of cyclopropane inhibition of brain Na^+-K^+-ATPase

It has been reported that various gases at high pressures can modify the reaction rates of a number of enzymes [19]. However, cyclopropane inhibition of brain Na^+-K^+-ATPase does not appear to be due to pressure *per se*. Figure 4 shows the effect of hydrostatic pressure, nitrogen, oxygen and cyclopropane each at a pressure of 4·3 atm on Na^+-K^+-ATPase activity of a lubrol extract of a brain microsomal fraction. Cyclopropane at this pressure had a marked inhibitory action, while the other conditions caused a small but consistent stimulation of Na^+-K^+-ATPase activity.

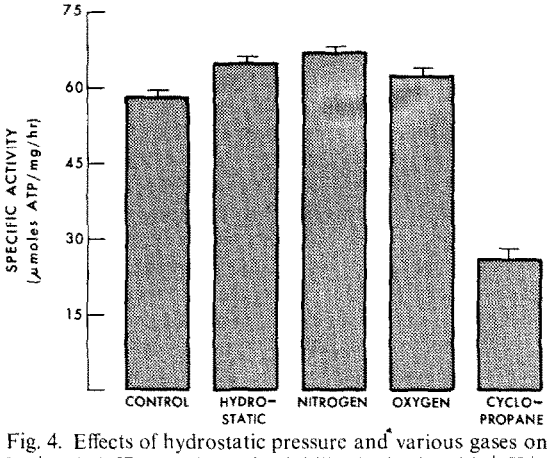

Fig. 4. Effects of hydrostatic pressure and various gases on Na^+-K^+-ATPase. Lubrol-solubilized brain Na^+-K^+-ATPase was prepared as detailed in Methods. Enzyme activity was measured with optimum substrate concentration (5 mM ATP), and controls were carried out in the presence of air at 1 atm. Gases and hydrostatic pressure were applied at 4·3 atm. Values are means of four experiments ± S.E.

Kinetics of Na^+-K^+-ATPase inhibition by cyclopropane

Figure 5 shows Lineweaver–Burk plots of enzyme activity vs substrate concentration in the presence of cyclopropane at 2·0 and 3·5 atm pressure. In both cases, the parallelism of the experimental plots with that of control (in air at atmospheric pressure) is indicative of uncompetitive or exclusive Type C inhibition [20]. Data in Fig. 5 were obtained using a lubrol extract of a brain microsomal fraction, but identical inhibitory kinetics were observed for other preparations containing Na^+-K^+-ATPase activity.

Cyclopropane inhibition of $AT^{32}P$ phosphorylation of microsomal Na^+-K^+-ATPase

$AT^{32}P$ phosphorylation and dephosphorylation of a brain microsomal preparation containing Na^+-K^+-ATPase activity were measured at various ATP concentrations. Cyclopropane at a pressure of 2·5 atm caused a significant inhibition of phosphorylation (Table 6) which followed uncompetitive kinetics (Fig. 6) similar to the gas effect on the over-all Na^+-K^+-ATPase reaction.

Dephosphorylation of the phospho-enzyme form of the ATPase is stimulated by the presence of K^+ ions. Table 6 shows that the addition of K^+ (10 mM) to the

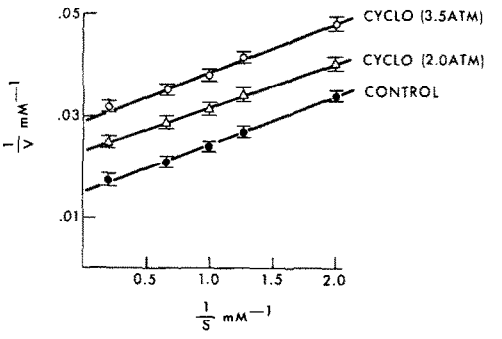

Fig. 5. Lineweaver–Burk plot of cyclopropane inhibition of Na^+-K^+-ATPase. Lubrol-solubilized brain Na^+-K^+-ATPase was prepared as detailed in Methods. Substrate concentrations ranged from 0·5 to 5 mM ATP. Values are the means of four experiments ± S.E.

Table 6. Effect of cyclopropane on $AT^{32}P$ labeling of brain ATPase*

Additions	Phosphorylated protein (pmoles ^{32}P/mg protein)	% Change due to K^+
$Na^+ + Mg^{2+}$	220 ± 6	
$Na^+ + Mg^{2+} + K^+$	140 ± 4	−37
$Na^+ + Mg^{2+}$ + cyclopropane	135 ± 3	
$Na^+ + Mg^{2+}$ + cyclopropane + K^+	88 ± 3	−35

* Brain microsomal preparations containing Na^+-K^+-ATPase activity were incubated with $AT^{32}P$ for a period of 15 sec as detailed in Methods. When present, cyclopropane (2·5 atm) was allowed to equilibrate with the reaction mixture for 10 min prior to addition of substrate. Values are the means of four to five experiments ± S.E.

reaction mixture caused a 37 per cent decrease in total radioactivity in the microsomal pellet, presumably due to accompanying dephosphorylation. Although cyclopropane inhibited phosphorylation of the enzyme, the gas did not appear to modify the effect of K^+. The percentage decrease in total radioactivity caused by K^+ was quite similar both in the presence of cyclopropane or in the absence of the gas. While the effect of cyclopropane on dephosphorylation was not measured directly, the above data suggest that inhibition of the enzyme occurs predominately at (or before) the phosphorylation step in the reaction sequence.

DISCUSSION

Inert gas anesthetics such as xenon and cyclopropane are capable of interacting with certain proteins including myoglobin and hemoglobin as shown by Schoenborn [21, 22] and because of the limited chemical reactivity of the gas molecules, such associations have been ascribed to Van der Vaal's forces [23]. The present study demonstrates that interactions can occur between cyclopropane and two membrane-bound enzymes from mammalian brain which result in modification of enzyme activities. Cyclopropane, at gas pressures equivalent to those used in the induction of anesthesia, caused a reversible inhibition of both membrane-bound and highly purified forms of brain AChE (Tables 1 and 2). No significant differences were observed between the forms of the enzyme in terms of sensitivity to cyclopropane or in the kinetics of inhibition (Figs. 1 and 3). Since purified forms of AChE contain no lipid constituents [24], changes in enzyme activity appear to be due to direct interaction of the gas with the enzyme protein. Unlike most inhibitors of AChE, cyclopropane is incapable of any molecular interactions involving covalent, ionic or hydrogen bonding. Hence AChE inhibition by cyclopropane is presumed to result from the summation of relatively weak interactions (of the Van der Vaal's type) between the gas molecule and certain atoms of the protein molecule. The kinetics of inhibition suggest the existence of at least two regions of the enzyme molecule with which cyclopropane can interact, one of which is in the vicinity of the active site(s). Similar inhibitory kinetics have been reported for the interactions of another anesthetic agent, diethylether, with purified forms of eel AChE [1] and the brain enzyme [2]. The inhibition of AChE by these anesthetics at low gas pressures offers a reasonable explanation for the parasympathomimetic effects observed when they are used clinically.

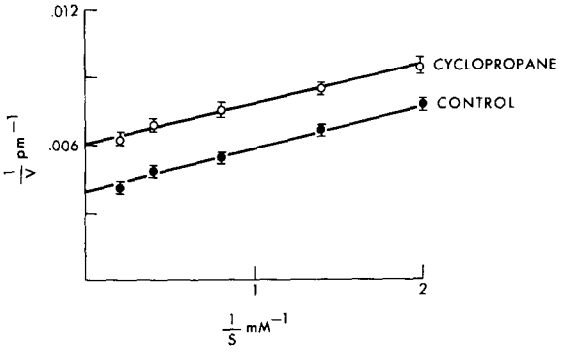

Fig. 6. Lineweaver–Burk plot of cyclopropane inhibition of $AT^{32}P$ phosphorylation of Na^+-K^+-ATPase. NaI-treated brain Na^+-K^+-ATPase was prepared and the phosphorylation reaction carried out as detailed in Methods. Substrate concentrations ranged from 0·5 to 5 mM ATP, and cyclopropane was present at a pressure of 2·5 atm. The 1/V axis represents reciprocal values for rates of phosphorylation expressed as pmoles ^{32}P/mg of protein(15 sec. Values are the means of four experiments ± S.E.

Inhibition of brain Na^+-K^+-ATPase by cyclopropane (Table 3) is considered to be more of theoretical than physiological importance, since higher gas pressures were required for significant effects on enzyme reaction rate. This enzyme system has been shown to be sensitive to various drugs having depressant effects. Cyclopropane inhibition of the "Ion Transport" ATPase has some specificity, since the gas has little effect on Mg^{2+}-ATPase activity (Table 3). In addition, control experiments show that inhibition is not the result of a pressure phenomenon *per se* (Fig. 4) and does not involve denaturation, since the effect is freely reversible (Table 5). The kinetics of inhibition are not unprecedented, since uncompetitive inhibition has been reported for the inhibition of glutamate dehydrogenase by sodium alkyl suflates [25] and for the inhibi-

tion of phenylethanolamine-*N*-methyltransferase by 5,6-dichloro-2-amino benzimidazole [26]. Mechanistically, uncompetitive inhibition is interpreted as involving either an interdependence between substrate and inhibitor in terms of binding or an interaction of the inhibitory molecule with an E–S complex rather than with free enzyme. The reaction cycle for Na^+-K^+-ATPase has been proposed by Albers [27], Siegel and Goodwin [28] and Tobin *et al.* [29] to involve a Mg^{2+}- and Na^+-dependent phosphorylation, transformation of "high energy" phospho-enzyme to a form of lower energy, followed by K^+-dependent dephosphorylation. Inhibition of phosphorylation by cyclopropane follows the same kinetics (Fig. 6) as that for the gas effect on the over-all Na^+-K^+-ATPase (Fig. 5). This suggests that cyclopropane acts in the reaction sequence at (or before) the phosphorylation step. In view of the limited chemical reactivity of the gas, it is possible that this action may provide a useful probe for further elucidation of the complex steps involved in the reaction cycle of Na^+-K^+-ATPase.

REFERENCES

1. J. T. Roberts, B. S. Braswell and R. J. Kitz, *Abstr. of Scientific Papers—ASA Annual Meeting*, 165. Atlanta, Ga. American Society of Anesthesiologists. (1970).
2. U. R. Maheshwari, S. L. Chan and A. J. Trevor, *Proc. west. Pharmac. Soc.* **16,** 175 (1973).
3. U. R. Maheshwari, S. L. Chan and A. J. Trevor, *Pharmacologist* **15,** 183 (1973).
4. G. Kato, J. Yung and M. Ihnat, *Biochem. biophys. Res. Commun.* **40,** 15 (1970).
5. B. D. Roufogalis and E. E. Quist, *Molec. Pharmac.* **8,** 41 (1972).
6. Y. Israel, H. Kalant, A. E. Leblanc, J. C. Bernstein and I. Salazar, *J. Pharmac. exp. Ther.* **174,** 330 (1970).
7. I. Veda and W. Meiani, *Biochem. Pharmac.* **16,** 1370 (1967).
8. A. J. Trevor and J. T. Cummins, *Biochem. Pharmac.* **18,** 1157 (1969).
9. R. M. Featherstone and C. A. Muehlbacher, *Pharmac. Rev.* **15,** 98 (1963).
10. S. L. Chan, D. Y. Shirachi, H. N. Bhargava, E. Gardner and A. J. Trevor, *J. Neurochem.* **19,** 2747 (1972).
11. E. DeRobertis, M. Alberici, G. Rodriquez and J. M. Azcurro, *Life Sci.* **5,** 577 (1966).
12. D. Y. Shirachi, A. A. Allard and A. J. Trevor, *Biochem. Pharmac.* **19,** 2893 (1970).
13. T. Nakao, Y. Tashima, K. Nagano and S. Nakao, *Biochem. biophys. Res. Commun.* **19,** 755 (1965).
14. G. L. Ellman, K. D. Courtney, V. Andres and R. M. Featherstone, *Biochem. Pharmac.* **7,** 88 (1961).
15. J. H. Martin and D. M. Doty, *Analyt. Chem.* **21,** 965 (1949).
16. E. Kwan and A. J. Trevor, *Molec. Pharmac.* **5,** 236 (1969).
17. K. Nagano, I. Kanazawa and M. Nakao, *Biochem. biophys. Res. Commun.* **19,** 759 (1965).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 263 (1951).
19. H. R. Schreiner, *Fedn Proc.* **27,** 872 (1968).
20. J. M. Reiner, in *Behavior of Enzyme Systems*, p. 186. Von Nostrand-Reinhold Co., New York (1966).
21. B. P. Schoenborn, *Nature, Lond.* **208,** 700 (1965).
22. B. P. Schoenborn, *Nature, Lond.* **214,** 1120 (1967).
23. R. M. Featherstone, C. A. Muehlbacher, F. L. Debon and J. A. Forsaith, *Anesthesiology* **22,** 977 (1961).
24. W. Leuzinger and A. L. Baker, *Proc. natn. Acad. Sci.* **57,** 446 (1967).
25. S. R. Kenneth and C. Y. Stanley, *J. biol. Chem.* **247,** 3671 (1972).
26. R. G. Pendleton, I. B. Snow, C. Kaiser, J. Wong and H. Green, *Biochem. Pharmac.* **21,** 2967 (1971).
27. R. W. Albers, *A. Rev. Biochem.* **36,** 727 (1967).
28. G. J. Siegel and B. G. Goodwin, *J. biol. Chem.* **247,** 3630 (1972).
29. T. Tobin, S. I. Baskin, T. Akara and J. M. Brody, *Molec. Pharmac.* **8,** 256 (1971).