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ACTION OF METHANETHIOL ON MEMBRANE (Na^+, K^+)-ATPase OF RAT BRAIN

GREGORY QUARFOTH, KHALIL AHMED, DONALD FOSTER and LESLIE ZIEVE

Toxicology Research Laboratory, Minneapolis Veterans Hospital, Department of Laboratory Medicine and Pathology, and Department of Medicine, University of Minnesota, Minneapolis, Minnesota 55417, U.S.A.

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Abstract The action of methanethiol (CH_3SH) on rat brain (Na^+,K^+)-ATPase was examined. The results show that CH_3SH acts at several sites on the enzyme system. The effects are characterized by an inhibition of the ATPase activity, but a concurrent stimulation of the associated K^+-dependent phosphatase. The inhibitory effect of CH_3SH was of an apparently mixed type with respect to the activation of the ATPase by Na^+ or by ATP suggesting that CH_3SH may inhibit the formation of phosphoenzyme intermediate in the ATPase reaction, and the inhibition may not be fully reversed by increasing Na^+. Methanethiol inhibited the activation of the ATPase by K^+ in an apparently uncompetitive manner, whereas it produced a competitive stimulation of the K^+ activation of the K^+-dependent phosphatase activity by increasing the affinity of K^+ for the enzyme. There was no significant change in the apparent K_m for the substrate *p*-nitrophenyl phosphate for the phosphatase activity. These effects of CH_3SH may be relevant to its toxicity, and offer a possible molecular site of its action with implications for the encephalopathy of hepatic failure.

One of the features of hepatic failure is the abnormal production of methanethiol, ethanethiol, and dimethylsulfide. These agents have been detected in the urine [1] and breath [2] of patients in hepatic coma. In experimental animals, these agents are capable of producing reversible coma [3], and methanethiol appears to be the most potent of the three. However, to our knowledge, no biochemical studies on the action of methanethiol are reported in the literature. Therefore, the biochemical basis of its toxicity remains unclear. The rapid onset of coma on exposure of rats to methanethiol and its reversibility, suggest that the possible locus of action of CH_3SH is on a function of the nerve membrane. In a preliminary report [4] we showed that methanethiol did not inhibit mitochondrial oxidative phosphorylation suggesting that it did not interfere with cellular energetics. On the other hand, membrane-associated (Na^+,K^+)-ATPase, believed to be involved in the transport of alkali metal cations across cell membranes, was inhibited by various mercaptans, methanethiol being the most potent one [4]. In other work [5] we have demonstrated that methanethiol can stabilize erythrocyte membrane against hemolysis, a property in common with general anesthetic agents [6] which may explain the comatogenic action of methanethiol. However, unlike the usual effective anesthetic agents, it appears that methanethiol, at concentrations which afford protection against hemolysis, also demonstrates a strong inhibition of brain (Na^+,K^+)-ATPase; the close dosage relationship for the inhibition of these two membrane-associated phenomena may be a basis of methanethiol toxicity. The present work describes the action of methanethiol on the kinetics of the activation of (Na^+,K^+)-ATPase by its various ligands. A preliminary account of this work has been given [4, 5].

MATERIALS AND METHODS

Chemicals. Methanethiol was obtained from Eastman Organic Chemicals, Rochester, New York and rubber septums from Arthur H. Thomas Company, Philadelphia, Pennsylvania. The details concerning other reagents used have been published previously [7, 8].

Assay of methanethiol. Methanethiol (CH_3SH) stock solutions were prepared in ethanol and stored at −20° in glass bottles fitted with a tight 13 × 18-mm rubber septum. These solutions were kept no longer than three months. The precise concentration of CH_3SH in these solutions was measured initially and every two or three weeks thereafter by g.l.c. using mercuric methylmercaptide as a standard according to the procedure of Doizaki and Zieve [9]. For this purpose, a 5-μl sample of the ethanolic stock solution of CH_3SH (brought to 0°) was rapidly injected into a 9-ml glass bottle fitted with a tight rubber septum (13-mm size) and containing 1 ml of double-distilled H_2O. The CH_3SH was then quantitatively released into the gas phase by heating the bottle to 70° for 25 sec with constant shaking. A 0.5-ml sample of the gaseous phase was removed (using a gas syringe), and injected into the gas chromatograph which had been previously standardized [9]. Each determination was made in several replicates which gave good reproducibility. It is noteworthy that the glass bottles used should have very tight rubber septums to avoid leakage of CH_3SH and, further, these stoppers should be discarded after each use. Routine weekly or biweekly assay of CH_3SH in stock solutions is essential, since control experiments showed a gradual decline in the concentration of CH_3SH in these solutions over a period of several months, due possibly to leakage or absorption in the rubber septum.

The partition of CH_3SH between the aqueous and

gaseous phases was determined for the actual reaction medium used for the assay of the (Na^+,K^+)-ATPase. For this purpose, 12-ml conical glass tubes which could be tightly stoppered with 14 × 18-mm rubber septums were used as the reaction vessels. These were filled with 2 ml of the standard (Na^+,K^+)-ATPase assay medium containing 35 μg of the enzyme protein. Experiments were performed to measure (a) the solubility of CH_3SH in this medium under the precise experimental conditions employed for the ATPase assay, and (b) to estimate the time required for the equilibration of CH_3SH between the liquid and gas phase in the reaction tube when samples were transferred from 0° to 37° (at which temperature the ATPase assays were performed). A known amount of CH_3SH solution was injected into these tubes; the precise amount of this CH_3SH was determined in parallel experiments by adding the same volumes to stoppered tubes containing 2 ml of H_2O, heating to 70° for 45 sec, and measuring the CH_3SH released in the gas phase as described above. For equilibration experiments (b, above) a known amount of CH_3SH was introduced into the tubes containing 2 ml of the ATPase assay medium. These tubes were then incubated on ice for 10 min followed by incubation for varying periods of time (0–12 min) at 37°, with constant shaking, at which times samples of the gas phase were analysed for CH_3SH. The results of this control experiment, shown in Fig. 1 (inset), demonstrate that the concentration of CH_3SH in the gas phase did not alter after the initial incubation period of 5 min. This indicated that the concentration of CH_3SH in the liquid phase (the ATPase assay medium) remained constant during the experiment. A CH_3SH solubility curve for the ATPase assay medium (a, above) was prepared by incubating several concentrations of CH_3SH for 10 min on ice followed by 12 min at 37° with constant shaking. A sample of gas phase was then assayed and the amount of CH_3SH in the liquid phase was estimated by the difference between this amount and the total amount of CH_3SH added (which had been estimated separately as outlined above). The concentration of CH_3SH in the complete (Na^+,K^+)-ATPase assay system was thus calculated and used to describe all the experimental work. The graph of the solubility of CH_3SH in the ATPase assay system is given in Fig. 1.

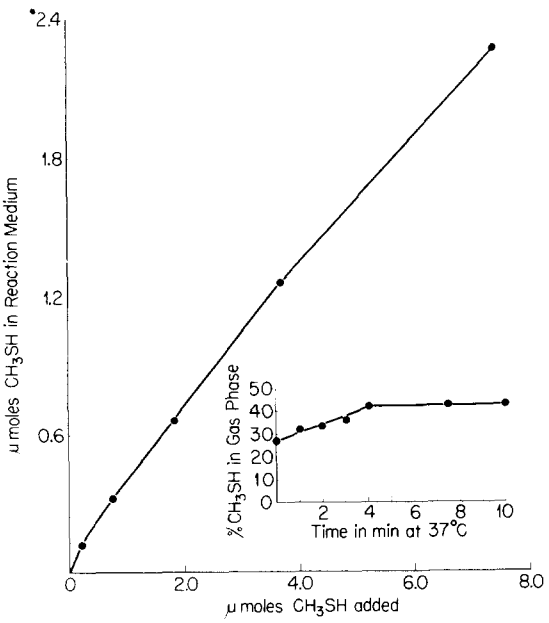

Fig. 1. Solubility of CH_3SH in the standard ATPase assay medium, and (inset) equilibration time necessary to achieve a constant CH_3SH concentration in solution. All the details are described in Methods.

Preparation of (Na^+,K^+)-ATPase. Sprague Dawley rats, weighing about 250 g, were used as the source of brain tissue. The preparation of (Na^+,K^+)-ATPase was based on the procedure of Ahmed and Judah [10], except that in the various preparative media mannitol was replaced by sucrose. The final enzyme preparation after an additional wash in a medium consisting of 0.25 M sucrose 10 mM imidazole HCl and 1 mM EDTA, pH 7.4 (24°) was suspended in the same medium, and was stored frozen in small aliquots. It was stable over several months. The specific activity of the (Na^+,K^+)-stimulated ATPase on the average was 125 μmoles of Pi released per mg of protein per hour. The basic Mg^{2+}-stimulated component was generally between 5 and 10% of the total Mg^{2+} + Na^+ + K^+-dependent ATPase. All other details concerning this preparation have been published previously [7, 8, 10, 11].

Assay of enzyme activities. The standard reaction medium for the determination of Mg^{2+} + Na^+ + K^+-stimulated ATPase activity consisted of 3 mM $MgCl_2$, 3 mM ATP (Tris salt), 110 mM NaCl, 10 mM KCl, and 30 mM Tris HCl buffer, pH 7.4 at 37°, and 5–50 μg of brain microsomal membrane protein, in a final volume of 2 ml. The basis Mg^{2+}-stimulated component of the ATPase was determined by omitting K^+ from the reaction.

K^+-stimulated *p*-nitrophenyl phosphatase (K^+-*p*-NPPase) was assayed in the presence of 3 mM $MgCl_2$, 10 mM KCl, 3 mM *p*-nitrophenyl phosphate (Tris salt), and 50 mM Tris HCl buffer, pH 7.4 at 37°, and 40–50 μg of enzyme protein, in a final volume of 2 ml. The basic Mg^{2+}-stimulated component, which was about 5–10% of the total phosphatase activity, was estimated by omitting K^+ from the reaction. To insure that both the ATPase and *p*-NPPase assays were performed during the linear phase of the reactions, the total amounts of substrates hydrolysed were kept below 10% of the amounts present at the beginning of the reaction [7].

The above assays were performed in glass tubes fitted with tight rubber septums (14-mm size) described above. It is important to use tightly-capped tubes to achieve a good reproducibility of experiments. These tubes containing all of the reagents, were placed on ice, and following the addition of the enzyme, were capped with the rubber septums. The solutions of CH_3SH were introduced into the tubes by means of Hamilton syringes which had been cooled on a slab of dry ice prior to use. To the control experimental tubes, appropriate volumes of ethanol were added by the use of the same techniques. The tubes were then allowed to stay on ice for a period of 10 min. They were then transferred to a 37° bath, and were incubated for an appropriate period of time (usually 9–30 min). The reaction was terminated by the addition of 1 ml of ice-cold 15% (w/v) trichloro-

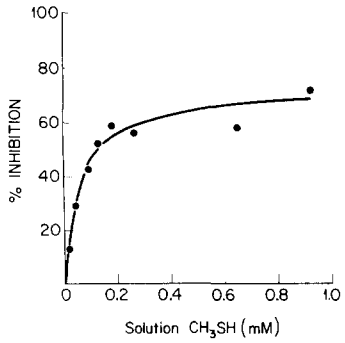

Fig. 2. Inhibition of (Na^+,K^+)-ATPase by CH_3SH. 1-10 μl of stock ethanolic CH_3SH solutions were used in order to vary the concentration of CH_3SH as shown. Suitable ethanol controls were included throughout. 36 μg of the enzyme protein was present; all other details are the same as given under Methods.

acetic acid. All other details of these assays, and the chemical methods used for Pi analysis, estimation of protein etc., have been detailed previously [7, 10, 12]. Suitable blanks were included in all experiments. Any variations from these procedures have been described under the appropriate legends to tables or figures.

Expression of results. The (Na^+,K^+)-ATPase activity (ΔPi) refers to μmoles of Pi formed in the presence of Mg^{2+} + Na^+ + K^+ minus that in the presence of Mg^{2+} + Na^+. K^+-stimulated *p*-nitrophenyl phosphatase is calculated as ΔPi, micromoles of Pi formed in the presence of Mg^{2+} + K^+ minus that in the presence of Mg^{2+}. All the values were calculated from the initial rates of the enzyme activities which were established from time course experiments in the absence and presence of CH_3SH.

RESULTS

Inhibition of (Na^+,K^+)-ATPase by CH_3SH. The result given in Fig. 2 illustrates the effect of increasing concentrations of CH_3SH on (Na^+,K^+)-ATPase activity. When the concentration of CH_3SH is gradually increased, there is a rapid increase in the inhibitory effect up to a concentration of 0.1 mM which caused about 50% inhibition of the ATPase activity. However, as the concentration of CH_3SH is increased further, the increase in the inhibitory effect is very gradual so that at 1 mM CH_3SH the enzyme was inhibited by some 72%. Owing to technical difficulties of achieving concentrations of CH_3SH greater than 1 mM in the reaction, the effect of higher concentrations of CH_3SH could not be measured. However, from these data it appears that CH_3SH may not be able completely to inhibit the (Na^+,K^+)-ATPase. This conclusion was also confirmed by an analysis of the above data according to the method of Dixon [13], i.e. a plot of $1/v$ vs concentration of CH_3SH, where a concave downward curve was obtained (Figure not given). The basic Mg^{2+}-stimulated portion of the ATPase was either unaffected by CH_3SH or sometimes was slightly increased (about 10%). The inhibition of the ATPase was not time-dependent and did not increase over a time course of 30 min. The inhibition produced by CH_3SH was freely reversed when the inhibitor was removed by dilution of the assay medium so as to reduce the concentration of CH_3SH, or by washing the enzyme [7, 12] after an exposure to the inhibitor. Further, the addition of 2 mM dithiothreitol or 2 mM 2-mercaptoethanol to the reaction medium did not alter the inhibitory effect of CH_3SH.

Effect on the activation of (Na^+,K^+)-ATPase by Na^+. The effect of CH_3SH on the activation of (Na^+,K^+)-ATPase by Na^+ was investigated in the presence of 1.5 mM K^+ and 0.19 mM CH_3SH while varying Na^+ from 0.5 to 3.3 mM (Fig. 3) or in the presence of 30 mM K^+ and 0.66 mM CH_3SH while varying Na^+ from 10 to 60 mM. In the absence of CH_3SH, the data in the former case could be described by linear plots as $(1/V)^{1.2}$ vs $1/Na^+$ [11], or as $(1/V)^{1.3}$ vs $1/Na^+$ [14], whereas in the latter case (i.e. 30 mM K^+ and Na^+ from 10 to 60 mM), the best fit in the double reciprocal plot was obtained by plotting $(1/V)^{1.3}$ vs $1/Na^+$ (Figure not given). This is in accord with the observations of Lindenmayer *et al.* [14]. The data in the presence of CH_3SH could be treated in the same fashion in either of the two cases. It is clear from Fig. 3 that CH_3SH inhibited the Na^+ activation of the ATPase by reducing the apparent V_{max} but increasing the apparent K_m for Na^+. These results suggest that the CH_3SH inhibition of (Na^+,K^+)-ATPase is of a mixed type (competitive plus non-competitive) with respect to Na^+ [13].

Effect on the activation of (Na^+,K^+)-ATPase by K^+. The activation of (Na^+,K^+)-ATPase by K^+ in the absence or presence of CH_3SH was studied by gradually increasing the K^+ concentration while keeping Na^+ at a constant level. For this purpose two concentrations of Na^+, i.e. 30 mM and 110 mM, were chosen and K^+ was varied from 0.2 to 0.6 mM (in the case of 30 mM Na^+, 0.19 mM CH_3SH) or from 0.8 to 10 mM (in the case of 110 mM Na^+, 0.63 mM CH_3SH). It is shown in Fig. 4 (data illustrated only from the latter case) that when the results were plotted according to Lineweaver and Burk [15], the presence of CH_3SH resulted in a decrease of both the apparent V_{max} and K_m in a manner which would be expected if the inhibition were of the uncompetitive type. The result was the same in the presence of 30 mM Na^+, with K^+ varying from 0.2 to 0.6 mM (not shown).

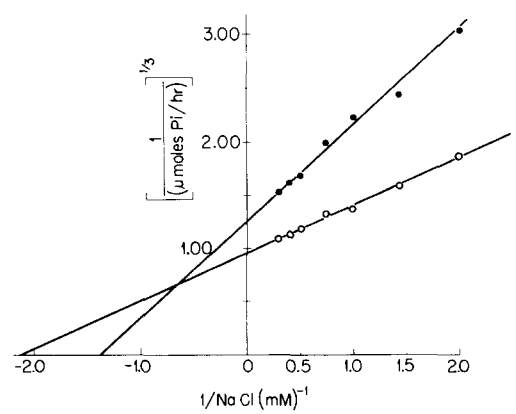

Fig. 3. The effect of CH_3SH on the activation of (Na^+,K^+)-ATPase by Na^+. The assay conditions were the same as for Fig. 2, except that Na^+ was varied as shown while keeping K^+ at 1.5 mM. ○, controls; ●, 0.19 mM CH_3SH.

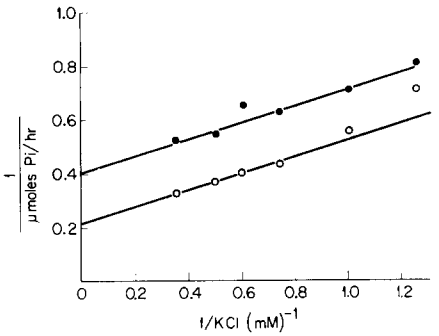

Fig. 4. The effect of CH_3SH on the activation of the (Na^+,K^+)-ATPase by K^+. 47 μg of enzyme was present in the assay; the conditions were the same as for Fig. 2 except that K^+ was varied as shown while keeping Na^+ at 110 mM. ○, controls; ●, 0.63 mM CH_3SH.

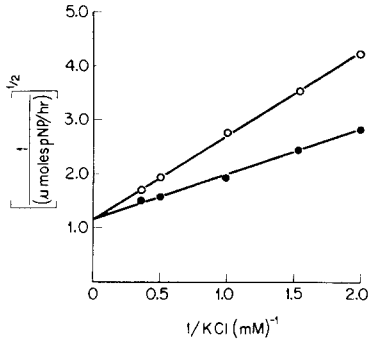

Fig. 6. The effect of CH_3SH on the activation of *p*-nitrophenyl phosphatase by K^+. All the details are the same as under Methods. K^+ was varied from 0.60 mM to 3.00 mM and the amount of enzyme present was 43 μg. ○, controls; ●, 0.54 mM CH_3SH.

Effect on the activation of (Na^+,K^+)-ATPase by ATP. The result given in Fig. 5 shows that when the concentration of ATP is varied while keeping all other conditons constant, the inhibition of the (Na^+,K^+)-ATPase by CH_3SH appeared to be of a 'mixed' type as characterized by a decrease in apparent V_{max} but increase in apparent K_m. Thus the effect of CH_3SH on substrate utilization by the ATPase is similar to its effect on the activation of the enzyme by Na^+. The result also suggests that whereas at low concentrations of ATP the inhibition by CH_3SH appears to be of a non-competitive type, it tends to approach a competitive relationship at higher levels of ATP (Fig. 5).

Effect on the activation of p-nitrophenyl phosphatase by K^+. K^+-stimulated *p*-nitrophenyl phosphatase (K^+-*p*-NPPase) activity associated with the (Na^+,K^+)-ATPase, originally described by us [10, 16], is generally recognized as a model for the K^+-dependent phosphatase step in the overall ATPase reaction [17–20]. It was, therefore, of interest to examine the effects of CH_3SH on this activity. The result given in Fig. 6 shows that CH_3SH stimulates K^+-*p*-NPPase activity and that this stimulation appears to be of a competitive nature with respect to the activation of the enzyme by K^+ as characterized by an increased affinity of K^+ for the phosphatase activity. It is of interest that the Lineweaver and Burk plot [15] shown in Fig. 6 was made linear by plotting $1/K^+$ vs $(1/V)^{1.2}$ which may possibly be interpreted to indicate two K^+ activation sites for the phosphatase activity. This observation appears to be in agreement with the postulation of two K^+ sites for (Na^+,K^+)-ATPase by Lindenmayer *et al.* [14]. However, the same data could also be expressed by the Hill plot, which indicated a clear change in $K_{0.5}$ for K^+ in the presence of CH_3SH (not shown).

Effect on the utilization of p-nitrophenyl phosphate. When the effect of CH_3SH on K^+-*p*-NPPase was measured in the presence of 2 mM K^+, while varying the concentration of *p*-nitrophenyl phosphate, the stimulation appeared to be essentially of a non-competitive nature with respect to the substrate. This is suggested by an increase in the apparent V_{max}, but no significant change in the apparent K_m for *p*-NPP (Fig. 7). Thus, it appears that the stimulation of K^+-*p*-NPPase by CH_3SH is primarily a consequence of increased affinity for K^+ in the presence of CH_3SH.

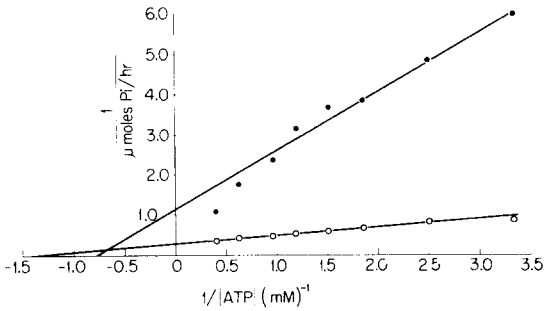

Fig. 5. Lineweaver Burk plot of the inhibition by CH_3SH of ATP activation of the (Na^+,K^+)-ATPase. ATP concentrations were varied from 0.30 mM to 2.50 mM; all other details were the same as given in Fig. 2. ○, controls; ●, 0.17 mM CH_3SH.

DISCUSSION

It is now generally agreed that membrane (Na^+,K^+)-ATPase represents the enzymic basis of Na^+ and K^+ transport across cell membrane [21].

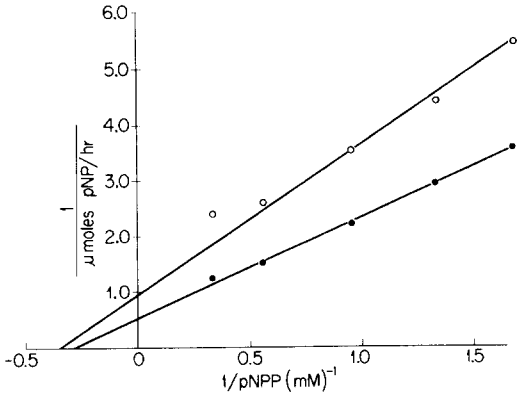

Fig. 7. Lineweaver Burk plot of the effect of CH_3SH on the substrate activation of the K^+-*p*-NPPase. The assay conditions were the same as detailed under Methods except that *p*-NPP was varied from 0.6 mM to 3.0 mM, while keeping K^+ at 2 mM. 43 μg of enzyme protein was present in the assay. ○, controls; ●, 0.53 mM CH_3SH.

The operation of this enzyme system, though not fully defined, proceeds through a series of steps resulting from the interaction of the various ligands with the enzyme. It is generally believed that the partial reactions of the enzyme involve a phosphorylation step yielding a phosphoenzyme (E_1-P) in the presence of Na^+ and Mg^{2+}, and that the E_1-P form of the phosphoenzyme undergoes a conformational change to a second form, E_2-P, which has a high affinity for K^+ and undergoes hydrolysis in its presence [22–25].

From the foregoing results it is clear that CH_3SH is a fairly potent inhibitor of the (Na^+,K^+)-ATPase, and that its action is apparent at several sites on the enzyme system. These effects do not appear to be a generalized disruption of the membrane structure, since they are freely reversed, and further, the addition of 2-mercaptoethanol or dithiothreitol in the reaction did not prevent the inhibitory action of CH_3SH. The effect of CH_3SH on the (Na^+,K^+)-ATPase is characterized by an inhibition of the ATPase activity, but a concurrent stimulation of the associated K^+-dependent phosphatase. In this regard, the action of CH_3SH on these activities appears to resemble that of dimethylsulfoxide and deuterated water [7, 26–28]. However, it appears to differ from alcohols which tend to inhibit the K^+-dependent phosphatase, while stimulating the Na^+-dependent phosphoenzyme formation in the ATPase [29, 30]. With respect to the activation of the ATPase by Na^+ or ATP, the inhibitory effect of CH_3SH was of an apparently mixed type suggesting that CH_3SH would inhibit the formation of Na^+-dependent phosphoenzyme in the ATPase, but that the inhibition would not be fully overcome by increasing the concentrations of Na^+ or ATP. Preliminary studies have shown that CH_3SH indeed inhibits the phosphoenzyme formation in the (Na^+,K^+)-ATPase [4], which is only partially reversed by increasing the concentration of Na^+ in the reaction.* The second action of CH_3SH appears to be on the K^+-dependent steps in the ATPase. This is characterized by an increase in the affinity of K^+ for both the (Na^+,K^+)-ATPase and the associated K^+-phosphatase. This suggests that CH_3SH further interacts with the phosphoenzyme (E_2-P) to stabilize it in a conformation which facilitates the action of K^+ on it. Another possibility which may account for these observations on the action of CH_3SH on the (Na^+,K^+)-ATPase may reside in the possible stimulation of $E_1 \rightarrow E_2$ shift in the enzyme conformation by CH_3SH [8, 28]. Thus, despite our ignorance of the molecular mechanism of the interaction of CH_3SH with the membrane structure, it is possible to interpret its action on the (Na^+,K^+)-ATPase in the framework of the existing schemes of the operation of the enzyme system.

Our interest in the membrane actions of CH_3SH stems from the observation that this agent may be involved in the pathogenesis of hepatic coma [3]. However, it is not our intention to say that the onset of coma is mediated by CH_3SH by virtue of its action on the (Na^+,K^+)-ATPase. Other studies in our laboratory have shown that, in common with a variety of anesthetic agents [6], CH_3SH can stabilize the erythrocyte membrane against hypotonic hemolysis [5]. The concentrations at which CH_3SH produces a 50% protection against hemolysis of erythrocytes are of the same order as those which produce a 50% inhibition of the brain (Na^+,K^+)-ATPase shown above [32]. Thus, it may be speculated that the suggested *in vivo* toxicity of CH_3SH (e.g. in hepatic coma) is due to the narrow range of concentration at which this agent affects the diverse activities related to membrane function. It is of interest that the other agents implicated in a synergistic action with CH_3SH in the onset of experimental hepatic coma are NH_4^+ and fatty acids [3, 33].

The fatty acids inhibit both the membrane (Na^+,K^+)-ATPase, and mitochondrial oxidative phosphorylation [12, 31] at concentrations which are somewhat higher than the concentrations which stabilize erythrocyte membrane as expected of an anesthetic agent [6]. The mode by which NH_4^+ exerts a synergistic effect with CH_3SH in the production of coma [3] is not clear. It is conceivable that one of the sites of its action may be the (Na^+,K^+)-ATPase reaction, presumably because of the ability of NH_4^+ to substitute for K^+ in this system.

*D. Foster and K. Ahmed, unpublished data.

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