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THE EFFECT OF HYDROXYLAMINE AND *N*-METHYLHYDROXYLAMINE ON BEEF BRAIN MICROSOMAL ($Na^+ + K^+$)-ATPase

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SUMMARY

Although both hydroxylamine and *N*-methylhydroxylamine inhibited beef brain microsomal ($Na^+ + K^+$)-ATPase these reagents were found to be of only limited use in probing the mechanism of this enzyme which is thought to involve a protein-bound acyl phosphate group. Results obtained with hydroxylamine were difficult to interpret owing to the potassium-like effects of traces of ammonia. Experiments with *N*-methylhydroxylamine did not permit differentiation between a mechanism involving an acyl phosphate on the enzyme surface which was inaccessible to the reagent from one in which the acyl phosphate is not an intermediate. These findings were disappointing in view of the proven usefulness of hydroxylamine for the investigation of enzyme mechanisms involving a high energy acyl group.

INTRODUCTION

In 1957 SKOU[1] reported that crab nerve microsomes contained a Mg^{2+}-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) which was stimulated by the simultaneous presence of Na^+ and K^+, and inhibited by ouabain. The data which have accumulated since then (see reviews by SKOU[2] and JUDAH AND AHMED[3]) provide strong circumstantial evidence that ($Na^+ + K^+$)-ATPase is identical with or an integral part of the active cation transport mechanism in cell membranes. The elucidation of the mechanism of this enzyme should thus lead to a better understanding of the active cation transport process.

In studies of the ($Na^+ + K^+$)-ATPase mechanism, γ-labeled [^{32}P]ATP has proved to be particularly useful. Several laboratories[4–10], for example, have demonstrated a Na^+-dependent transfer of the terminal phosphate of [^{32}P]ATP to protein in microsomal ($Na^+ + K^+$)-ATPase preparations. The subsequent addition of K^+ to the medium then initiated a rapid loss of inorganic phosphate from the enzyme. Labeled phosphoprotein, isolated by trichloroacetic acid precipitation of the enzyme from reaction mixtures containing Na^+, Mg^{2+}, [^{32}P]ATP but not K^+, was found to lose P_i at high pH or when treated with hydroxylamine[8,9,11]. Since these are reactions

characteristic of acyl phosphates, the following two-step mechanism for the hydrolysis of ATP by ($Na^+ + K^+$)-ATPase was postulated:

$$E\text{-COOH} + \text{ATP} \overset{Na^+,Mg^{2+}}{\rightleftharpoons} E\text{-COOP} + \text{ADP}$$

$$E\text{-COOP} + H_2O \xrightarrow{K^+} E\text{-COOH} + P_i$$

This mechanism is attractive for two reasons: It is in accord with kinetic studies[12,13] in that it ascribes separate roles to Na^+ and K^+. It also provides for the transfer of chemical energy directly from ATP to the enzyme in the form of an acyl phosphate. The latter could then readily be used directly or indirectly in the cation transport process.

Experiments in our own[14] and other laboratories[15] have shown that hydroxylamine rapidly dephosphorylates beef brain microsomes labeled in the presence of Mg^{2+}, Na^+ and [^{32}P]ATP:

$$E\text{-COOP} + NH_2OH \rightarrow E\text{-CONHOH} + P_i$$

At 0.8 M, however, hydroxylamine had little effect on ($Na^+ + K^+$)-ATPase activity[14,15]. This result was somewhat surprising since the enzyme should have been rapidly inactivated by hydroxylamine if ATP were being hydrolyzed *via* an enzyme-bound acyl phosphate intermediate (see, however, ref. 2). However, BADER AND BROOM[16] have found it possible to demonstrate the inactivation of rabbit kidney ($Na^+ + K^+$)-ATPase by hydroxylamine when low concentrations of Ca^{2+} were included in the incubation medium.

In an attempt to resolve these anomalies we have employed a more highly purified microsomal preparation from beef brain[17] to re-examine the interaction of hydroxylamine with ($Na^+ + K^+$)-ATPase and have obtained additional data on the effects of *N*-methylhydroxylamine. Our results show that the unique properties of microsomal ($Na^+ + K^+$)-ATPase place severe limitations on the usefulness of hydroxylamine and *N*-methylhydroxylamine as a means of elucidating the mechanism of this enzyme.

METHODS

Beef brain microsomal ($Na^+ + K^+$)-ATPase was prepared from sucrose homogenates of gray matter as described by SCHONER *et al.*[17]. After assay of a representative sample with [^{32}P]ATP at 37° according to the procedure of CHIGNELL AND TITUS[18], activities of 4.5 μmoles of phosphate per h per mg of protein in the presence of $MgCl_2$ (5 mM) alone and an additional 65.5 μmoles/h per mg with 120 mM NaCl *plus* 30 mM KCl were found.

[^{32}P]ATP of specific activity from 6 to 8 mC/μmole was prepared by a modification[6,19] of the method of PFLEIDERER[20]. [^{32}P]ATP to be used in the ATPase assays was diluted with carrier ATP to give approx. 15 000 counts/min per μmole.

The incorporation of [^{32}P]phosphate into the microsomes was measured in reaction mixtures (200 μl final volume) containing 0.5 mM [^{32}P]ATP ($3 \cdot 10^6$ counts/min), 1.25 mM $MgCl_2$, 0.1 M Tris–HCl (pH 7.4) and, where indicated, 120 mM NaCl. After the addition of microsomal protein (approx. 0.2 mg) a steady-state level of

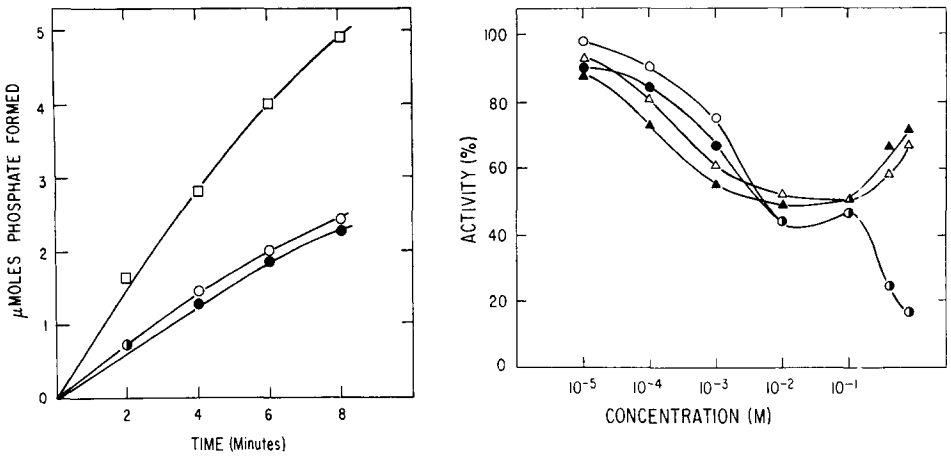


Fig. 1. The effect of hydroxylamine and *N*-methylhydroxylamine on the $(Na^+ + K^+$ - ATPase activity of beef brain microsomes .Final concentrations were ATP, 2 mM; NaCl, 120 mM; KCl, 20 mM; $MgCl_2$, 5 mM; Tris–HCl (pH 7.4), 100 mM. ●, hydroxylamine (100 mM); ○, .*N*-methylhydroxylamine (100 mM); □, control. Results are expressed as the number of μmoles of P_i released per mg of microsomal protein.

Fig. 2. Inhibition of beef brain microsomal $(Na^+ + K^+)$-ATPase by hydroxylamine and *N*-methylhydroxylamine as a function of inhibitor concentration. Incubation conditions are as in Fig. 1 except that the buffer was 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid. ○, hydroxylamine; ●, hydroxylamine, 0.06 mM $CaCl_2$; △, *N*-methylhydroxylamine; ▲, *N*-methylhydroxylamine, 0.06 mM $CaCl_2$.

[^{32}P]intermediate was obtained for at least 30 sec (Fig. 3). Additions were made at 10 sec and the reaction stopped by the addition of 5 ml trichloracetic acid (10%) containing ATP and H_3PO_4 (1 mM each). The precipitates were filtered on Millipore filters (0.8-μ pore diameter) and washed with 5 ml of 5% trichloracetic acid containing carrier ATP and H_3PO_4 as above. After washing with 5 ml of water the filters were dried and placed in 10 ml of phosphor solution for counting. The latter was prepared by mixing 3 parts of ethanol with 7 parts of a toluene solution containing 0.4% 2,5-bis[5′-*tert.*-butyl benzoxazolyl (2′)]-thiophene. Preliminary experiments had shown that the Millipore filters retained more than 95% of the microsomal protein even when hydroxylamine or *N*-methylhydroxylamine was present. Aliquots of each filtrate were counted before and after charcoal absorption for estimation of P_i produced by the hydrolysis of [^{32}P]ATP[21].

Protein was determined by the method of LOWRY *et al.*[22] using a bovine serum albumin standard.

Hydroxylamine free base was prepared by dissolving the hydrochloride salt in anhydrous methanol and adding an equivalent amount of sodium methoxide. After the precipitated NaCl had been filtered off, the filtrate was evaporated and the residue distilled under reduced pressure to give free hydroxylamine as a colorless oil (b.p., 55° at 25 mm). *N*-Methylhydroxylamine was prepared in a similar manner (b.p., 38° at 23 mm). Aqueous solutions of hydroxylamine and *N*-methylhydroxylamine were prepared just before use and immediately adjusted to pH 7.4 by the addition of 6 M HCl.

RESULTS AND DISCUSSION

Hydroxylamine and its *N*-methyl analog inhibited the ($Na^+ + K^+$)-ATPase activity of the deoxycholate-treated microsomes (Fig. 1). This is in contrast to previously reported results which were obtained with microsomes prepared by the NaI method of NAKAO *et al.*[23]. As can be seen from Fig. 2 inhibition is not a simple function of concentration. At 0.01 M both hydroxylamine and *N*-methylhydroxylamine produced approx. 50% inhibition of enzyme activity which was not then affected by a 10-fold increase in inhibitor concentration (Fig. 2). When the concentration of hydroxylamine was increased above 0.1 M, further inhibition of ($Na^+ + K^+$)-ATPase activity was observed (Fig. 2). *N*-Methylhydroxylamine, however, was less effective when the concentration was raised above 0.1 M (Fig. 2). The reason for this phenomenon is not at present clear. In contrast to the report of BADER AND BROOM[16], the inclusion of a low concentration (0.06 mM) of Ca^{2+} in the incubation medium did little to modify the effect of hydroxylamine or *N*-methylhydroxylamine.

Since both hydroxylamine and *N*-methylhydroxylamine inhibited the ($Na^+ + K^+$)-ATPase activity of the deoxycholate-treated microsomes it became important to determine whether the same reagents could release inorganic phosphate from the labeled enzyme (*E*-COOP). As can be seen from Fig. 3 the Na^+ stimulated incorporation of [^{32}P]phosphate from γ-labeled [^{32}P]ATP into beef brain microsomes reaches a steady state in less than 10 sec which persists for a further 30 sec. The accompanying Na^+-stimulated increase in the rate of [^{32}P]phosphate production (Fig. 4) may be the result of a Na^+-stimulated dephosphorylation of the enzyme or perhaps the intermediate is inherently unstable. The addition of hydroxylamine (0.8 M) to microsomes incubated for 10 sec in the presence of Mg^{2+}, Na^+ and [^{32}P]ATP results in a fairly rapid reduction in the level of labeling such that the magnesium control level is reached in

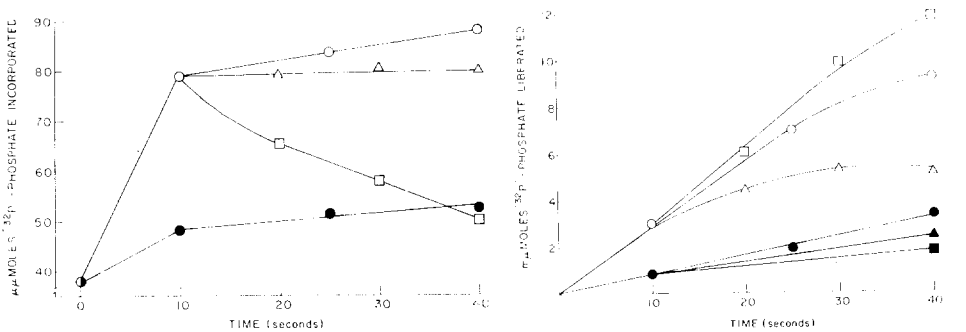


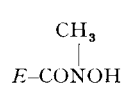
Fig. 3. The effect of hydroxylamine and *N*-methylhydroxylamine on the incorporation of [^{32}P]-phosphate from [^{32}P]ATP into beef brain microsomes. Final concentrations were [^{32}P]ATP, 0.5 mM; $MgCl_2$, 1.25 mM; Tris–HCl (pH 7.4), 100 mM. All tubes, with the exception of those designated ●, also contained NaCl, 120 mM. Beef brain microsomes (0.19 mg) were added at zero time and at 10 sec additions were made to give the following final concentration: ○, water; □, hydroxylamine, 800 mM; △, *N*-methylhydroxylamine, 800 mM. Since neither hydroxylamine nor *N*-methylhydroxylamine affected the labeling in the absence of NaCl these points were omitted for clarity.

Fig. 4. Effect of hydroxylamine and *N*-methylhydroxylamine on the release of [^{32}P]phosphate from [^{32}P]ATP by beef brain microsomes. These results were obtained from the experiment described in Fig. 3 using the technique described under METHODS.

about 30 sec (Fig. 3). During this time, however, the rate of P_i production increases (Fig. 4). This suggests that hydroxylamine is not dephosphorylating the enzyme by reacting chemically with an acyl phosphate at the active site since this would result in inactivation and a decrease in the rate of P_i production. Instead, hydroxylamine appears to be acting like K^+ which stimulates the rate of P_i release by promoting the enzymatic breakdown of the intermediate (see INTRODUCTION). While it is possible that hydroxylamine itself is responsible for this effect, it is more likely to be ammonia, one of the possible decomposition products. The NH_4^+ has been found to cause rapid dephosphorylation of microsomes labeled in the presence of Mg^{2+}, Na^+ and [^{32}P]ATP with a concomitant increase in the rate of P_i production[10].

N-Methylhydroxylamine, on the other hand, produces only a 25% depression in the level of labeled enzyme in 30 sec (Fig. 3), while at the same time the rate of inorganic phosphate production is markedly reduced (Fig. 4). This reduction in the level of labeling is in good agreement with the 26% which would be predicted if it is assumed that the rate of reaction of *N*-methylhydroxylamine with the enzyme-bound intermediate is the same as that with the trichloroacetic acid-precipitated phosphoprotein. Pseudo first-order rate constants for the displacement of phosphate by 0.8 M *N*-methylhydroxylamine at pH 7.4 and 37° have been calculated from unpublished data in this laboratory to be 0.56 min^{-1} for the denatured phosphoprotein and 0.88 min^{-1} for the model substrate, acetyl phosphate.

While contamination of hydroxylamine by ammonia makes it difficult to interpret results obtained with this reagent, it would appear from Figs. 3 and 4 that *N*-methylhydroxylamine is reacting with an acyl phosphate on the enzyme surface. If the reacting acyl phosphate is involved in the hydrolysis of ATP and if the resultant enzyme hydroxamate



is stable, then $(Na^+ + K^+)$-ATPase activity should be irreversibly inhibited by *N*-methylhydroxylamine. One of the features of irreversible inhibition is that the rate of the enzymatic reaction should decrease with time, eventually reaching zero A closer examination of Fig. 1 will show that this is not the case for *N*-methylhydroxylamine since the $(Na^+ + K^+)$-ATPase activity of the microsomes remains at about the 50% level during the entire 8-min incubation. This observation suggests that the inhibition of microsomal $(Na^+ + K^+)$-ATPase is the result of a "reversible" type of interaction. This was confirmed in a further experiment in which microsomes were preincubated with Mg^{2+}, Na^+, ATP and *N*-methylhydroxylamine (0.8 M) (*cf.* Figs. 3 and 4), isolated on a Millipore filter (the enzyme was inactivated by repeated washing and centrifugation) and then incubated with [^{32}P]ATP. Table I shows that exposure to 0.8 M *N*-methylhydroxylamine reduced the Na^+-dependent incorporation of [^{32}P]phosphate by only 15%.

Our experiments therefore show that neither hydroxylamine nor its *N*-methyl analog are of use in probing the mechanism of microsomal $(Na^+ + K^+)$-ATPase. The unique cation requirements of this enzyme make it impossible to interpret data obtained with hydroxylamine because of possible contamination by ammonia. The interaction of *N*-methylhydroxylamine with microsomal $(Na^+ + K^+)$-ATPase is

TABLE I

THE EFFECT OF PRETREATMENT WITH *N*-METHYLHYDROXYLAMINE ON THE Na^+-DEPENDENT INCORPORATION OF [^{32}P]PHOSPHATE FROM [^{32}P]ATP BY BEEF BRAIN MICROSOMES

Microsomes were preincubated with $MgCl_2$ (5 mM), NaCl (120 mM), ATP (2 mM) and *N*-methylhydroxylamine (0.8 M) for 8 min at 37°. Aliquots, containing 0.2 mg of microsomal protein, were transferred to a Millipore filter (0.8-μ pore diameter), then washed with 5 ml of Tris–HCl buffer (50 mM, pH 7.4) and the filter aspirated dry. The microsomes retained on the filter were then exposed at room temperature (25°) to 0.3 ml of a solution containing, in final concentration, [^{32}P]ATP, 0.5 mM; $MgCl_2$, 1.25 mM; NaCl, 120 mM; Tris–HCl (pH 7.4) 100 mM. After 20 sec 10 ml of 10% trichloroacetic acid (containing ATP and H_3PO_4, each 1 mM) was added, aspirated through and the filter washed with 10 ml of 5% trichloroacetic acid containing carrier ATP and H_3PO_4 as above. After a further wash with water (10 ml) the filters were removed, dried and counted in a liquid scintillation spectrometer. Na^+-dependent incorporation was obtained by subtracting the labeling obtained in the absence of Na^+.

Treatment	*Na^+-dependent incorporation of [^{32}P]phosphate from [^{32}P]ATP (μmoles [^{32}P]-phosphate per mg protein)*
Mg^{2+}, Na^+, ATP	97.6
Mg^{2+}, Na^+, ATP, CH_3NHOH	83.0

complex (Fig. 2). Certainly the results presented in Fig. 3 suggest that *N*-methylhydroxylamine does react with an enzyme-bound acyl phosphate. However, Table I clearly shows that microsomes pretreated with *N*-methylhydroxylamine are still capable of incorporating [^{32}P]phosphate from [^{32}P]ATP. This could only mean that the interaction between *N*-methylhydroxylamine and the enzyme is "reversible". One possible explanation for such a phenomenon is that the enzyme itself is capable of removing the hydroxamate group blocking the active site.

$$E\text{–}\overset{\displaystyle CH_3}{\overset{|}{C}}ONOH + H_2O \rightarrow E\text{–}COOH + CH_3NHOH$$

However, such a mechanism would predict that, in the presence of Na^+, *N*-methylhydroxylamine should cause an increase in the rate of ATP hydrolysis (since *N*-methylhydroxylamine would be acting like K^+) whereas a decrease was observed (Fig. 4). In addition the brain microsomes could not hydrolyze the model substrates acetyl hydroxamate[14] and γ-glutamyl hydroxamate. Hydroxamates are also very stable at neutral pH so it is unlikely that spontaneous hydrolysis would occur. Although these results suggest that the acyl phosphate is not an intermediate, another interpretation is possible. If the acyl phosphate intermediate is protected from attack by *N*-methylhydroxylamine it could still be possible for this reagent to interact elsewhere on the enzyme surface and inhibit the Na^+-dependent phosphorylation step. This would produce a decrease both in the steady-state level of the intermediate (Fig 3) and in the rate of P_i release (Fig. 4). Such an interaction could be non-specific and reversible. Experiments with *N*-methylhydroxylamine do not permit differentiation between these two alternatives.

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