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Studies of the Nature of the Inhibitory Action of Inorganic Phosphate, Fluoride, and Detergents on 5′-Adenylic Acid Deaminase Activity and on the Activation by Adenosine Triphosphate*

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SUMMARY

Studies of the nature of the inhibitory action of inorganic phosphate, fluoride, and some detergents on 5′-adenylic acid deaminase activities of rat brain and liver show that inorganic phosphate, as well as ATP, is an effector of the deaminase.

Inorganic phosphate was found to be a competitive inhibitor in the absence of ATP, but in the presence of ATP, the nature of the inhibition was uncompetitive. ATP strongly prevented the inhibition by inorganic phosphate. It appears that inorganic phosphate competes with the substrate, AMP, and with the activator, ATP, simultaneously at the active site and the effector site, respectively.

Fluoride inhibited the deaminase noncompetitively in the absence of ATP, and uncompetitive effect was obtained in the presence of ATP. It is likely that fluoride competes with ATP in binding to the effector binding site, and in the absence of ATP fluoride occupies the effector binding site to modify the activity.

Deoxycholate and lauryl sulfate acted as activators or inhibitors, depending upon the enzyme concentrations and the inhibitor concentrations. The inhibition was also dependent upon the substrate concentrations and therefore showed a mixed type in nature. ATP could prevent the inhibitory effect, but higher deoxycholate concentrations reduced or diminished the ATP-activated activity. It appears that deoxycholate or lauryl sulfate can affect the ATP-binding site in modifying the ATP-activation as well as the deamination.

The physiological significance of the relationship between inorganic phosphate and ATP in regard to AMP metabolism and consequently to intermediary metabolism is discussed.

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In previous communications, we reported a method of purification along with some physicochemical and enzymatic properties of 5′-AMP deaminase from rabbit skeletal muscle (1), although catalytic properties of the enzyme had not been completely studied. The roles of the deaminase in muscle function and in cell metabolism are not yet clear, in spite of the wide distribution of deaminase among various organs of different species and microorganisms (2, 3). The deaminase may be the main enzyme in controlling the AMP concentration in cells. An alteration of the concentration of AMP or IMP through the action of the deaminase could conceivably result in changing the rates of nucleic acid biosynthesis and of other reactions. AMP has also been recognized as an activator or inhibitor of some enzymatic reactions. Phosphorylase *b* or *b′* is activated by 5′-AMP, and, furthermore, no other nucleotides have been able to replace 5′-AMP for the activation (4). More recently, it was found that 5′-AMP acts as a regulator of NAD-isocitric dehydrogenase (5), phosphofructokinase (6–8), fructose 1,6-diphosphatase (9), NADH oxidase (10), amidotransferase (11), and adenylosuccinate synthetase (12). Thus, it is obvious that 5′-AMP plays an important role in controlling the purine biosynthesis and interconversion, glycolysis, gluconeogenesis, and respiration in cells, besides acting as a substrate. Attempts have been made to study the deaminase in brain, liver, and other tissues, in order to understand the physiological significance of the deaminase in AMP metabolism.

In this paper, we present some findings concerning the nature of fluoride inhibition which we could not clearly elucidate previously (1). The results regarding the interaction between orthophosphate and ATP and the effect of some detergents on AMP deaminase activity are also presented.

EXPERIMENTAL PROCEDURE

Materials—Sprague-Dawley rats, which were maintained on commercial, complete rat diet (laboratory chow), were used as normal control animals. AMP, ADP, ATP, and IMP from Sigma; 2′-AMP, 3′-AMP, adenine, and adenosine from Schwarz BioResearch; deoxycholate from Mann; and other analytical reagents from the Fisher Scientific Company and Mallinckrodt Chemical Works were used.

Methods—5′-AMP, adenosine, and adenine deaminase activities were measured either spectrophotometrically or colorimetrically. The spectrophotometric method introduced by Kalckar (13) was based on the decrease of optical density at 265 mμ or on its increase at 240 mμ. Experiments at high substrate concentrations were carried out in test tubes in which the reaction was stopped by the addition of perchloric acid. After proper dilution with water, the optical density was determined with a spectrophotometer at 265 and 240 mμ. The colorimetric method was based on the production of ammonia with the use of phenol-hypochlorite reagent (14). The basic assay mixture consisted of 0.1 M succinate (Na^+), pH 6.5, 1×10^{-2} M AMP (Na^+), enzyme solution, and additional compounds. Water or a NaCl solution was added to keep the final sodium concentration constant in the reaction mixture (1.0 ml in total), since Askari (15) reported that the deaminase in red cells and rabbit brain was activated by monovalent cations. ATP (Na^+) (1×10^{-3} M) or sodium deoxycholate (1×10^{-3} M or 2×10^{-3} M) or both were added to the assay mixture as the above mentioned additional compounds. The assay mixture, excluding the enzyme solution, was previously incubated in a 30° water bath for 5 min. After a certain period of incubation of 30° with enzyme, 0.2 ml of 1 N perchloric acid was added to stop the reaction. The supernatant fluid was collected after centrifugation at $2000 \times g$ for 10 min. A 0.1-ml aliquot was diluted with water to 10 ml and its optical density was measured at 265 and 240 mμ. A 0.1- or 0.2-ml aliquot of the supernatant fluid was used for the ammonia determination. Under the conditions described for the usual assay system in the presence and absence of ATP or deoxycholate or both, the reaction was first order with respect to AMP, as shown previously by using crystalline AMP deaminase preparation from rabbit skeletal muscle (1) (Fig. 1). Initial velocity was calculated on the basis of the first order velocity constant. Inorganic phosphate was determined by the method of Lowry and Lopez (16), and the protein concentration was determined by the biuret reagent as described elsewhere (17).

The gray matter of rat brain of liver was homogenized with 0.25 M sucrose by a Teflon and glass homogenizer. A 10% homogenate in 0.25 M sucrose was filtered through four layers of gauze to remove the coarse particles. The homogenate was centrifuged at $75{,}000 \times g$ for 60 min to separate the cytoplasmic and particulate fractions. The particulate fraction was washed once with a 0.25 M sucrose solution, and the washing was discarded.

The adenine, adenosine, and ATP deaminase activities were determined under various conditions to verify the AMP deaminase activity measurement. No adenine deaminase activity could be detected at various substrate concentrations in the presence of ATP or deoxycholate or both, and within the pH range between 5.5 and 9.5, although adenosine deaminase activity could be detected within this pH range. ATP or deoxycholate or both did not modify the production of ammonia from adenosine. The rate of ammonia liberation from adenosine at concentration 6×10^{-3} M was about $\frac{1}{100}$ of that from AMP. The production of ammonia from adenosine, therefore, was negligible during the assay of AMP deaminase activity. The addition of adenine or adenosine to the assay mixture for AMP deaminase did not affect the liberation of ammonia during the incubation. ATP alone or with deoxycholate did not cause significant ammonia production.

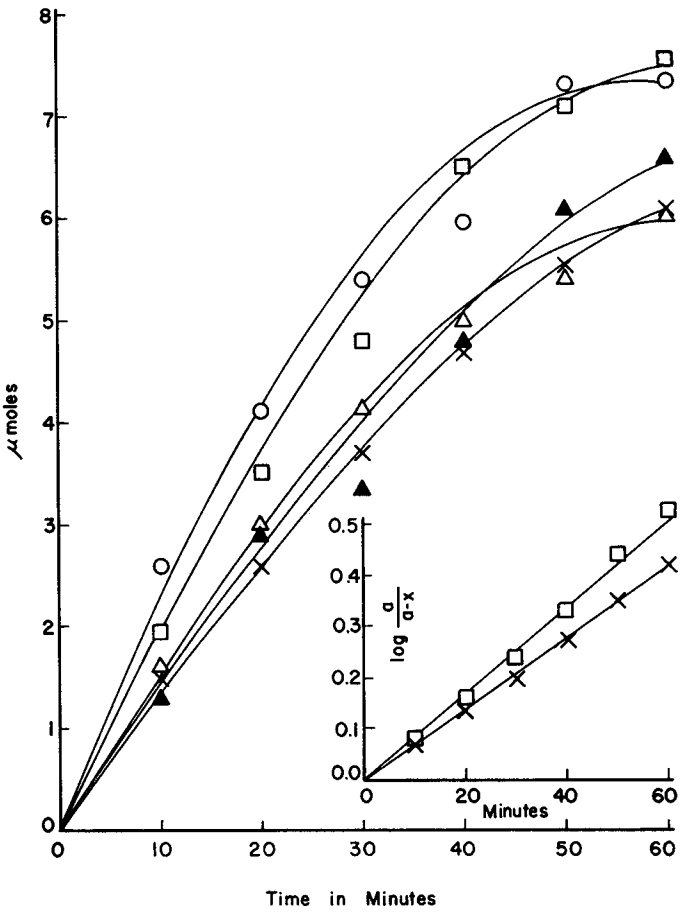

FIG. 1. Ammonia formation and changes in optical density at 265 and 240 mμ as a function of the incubation time. A rat brain particulate preparation and AMP (1×10^{-2} M) were incubated in 0.1 M succinate buffer, pH 6.5, at 30° with the addition of deoxycholate (1×10^{-3} M) with or without ATP (1×10^{-3} M). Determination based on ammonia formation in the presence of deoxycholate (×) or deoxycholate plus ATP (□). Determination based on changes in optical density at 265 mμ in the presence of deoxycholate (△) or deoxycholate plus ATP (○) and at 240 mμ in the presence of deoxycholate (▲).

RESULTS

Effect of Deoxycholate, Triton X-100, Lauryl Sulfate, and Tween 20 on Deaminase Activity of Rat Brain and Liver—Several detergents were used to disperse the subcellular particles in order to eliminate a possible masking phenomenon, since deaminase activity was found in the nuclear, mitochondrial, intermediary, and microsomal fractions. It was also desired to test the possibility that lipid might be involved in the deamination. Deoxycholate between 2×10^{-4} and 2×10^{-3} M was found to inhibit the deaminase in the cytoplasmic fraction from brain and liver (Figs. 2 and 3). It seemed that ATP tended to prevent the inhibitory action by the lower concentrations of deoxycholate. Higher concentrations of deoxycholate reduced or diminished the ATP

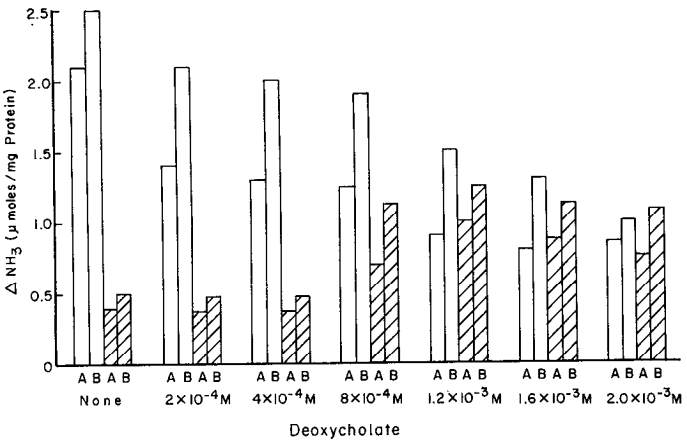

FIG. 2. Ammonia production in various deoxycholate concentrations. A cytoplasmic (*open bar*) or a particulate (*hatched bar*) fraction from rat brain was incubated in 0.1 M succinate buffer, pH 6.5, with AMP (1×10^{-2} M) in the presence (*B*) and absence (*A*) of ATP (1×10^{-3} M) at 30° for 30 min. The deamination is expressed in terms of micromoles per mg of protein per 30 min.

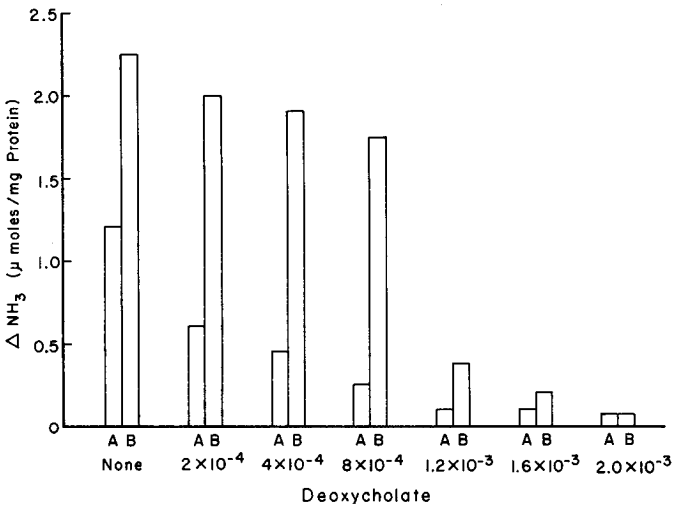

FIG. 3. Ammonia production in various deoxycholate concentrations. A cytoplasmic fraction from rat liver was incubated in 0.1 M succinate buffer, pH 6.5, with AMP (1×10^{-2} M) in the presence (*B*) and absence (*A*) of ATP (1×10^{-3} M) at 30° for 30 min. The deamination is expressed in terms of micromoles per mg of protein per 30 min.

activation on the activity. In contrast to this, the deaminase activity in the particulate fraction was increased about 2-fold, but higher concentrations of deoxycholate tended to depress the activity. Triton X-100 (3×10^{-4}) depressed very slightly the ammonia formation when AMP alone was incubated with the cytoplasmic fraction, but no further inhibition was observed by increasing the Triton X-100 concentration up to 3×10^{-3} M. The formation of ammonia was not depressed when ATP was present in the reaction mixture, however. The activity in the particulate fraction was increased, and the ATP-activated activity became more prominent in the concentrations between 3×10^{-4} and 3×10^{-3} M. The effect of lauryl sulfate on deaminase activity in the cytoplasmic fraction was greater than that of deoxycholate and could also be prevented by the presence of ATP. The activities in the absence and presence of ATP were depressed by lauryl sulfate (1×10^{-4} M) to 20 and 70%, respectively. Tween 20 was also used, and the results obtained were quite similar to the effect of Triton X-100. These results indicate that nonionic detergents had no inhibitory effect on the cytoplasmic fraction, but dispersed the particulates and consequently elevated the activity in the concentrations used. Heparin (0.1 to 1.0 mg per ml) and caprylate (between 1.5 ×

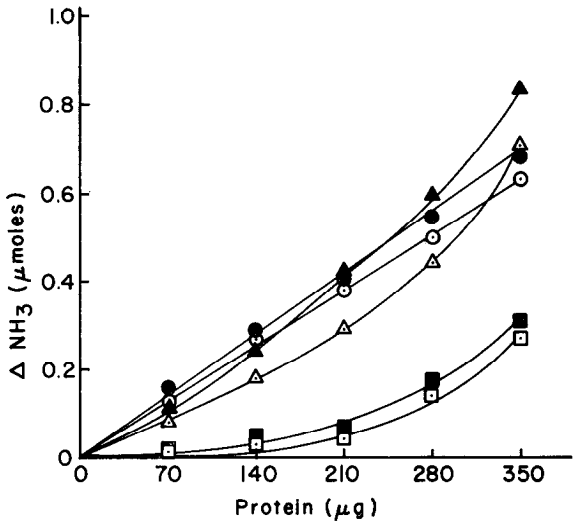

FIG. 4. Ammonia formation as a function of enzyme concentration in the presence and absence of deoxycholate or lauryl sulfate. A cytoplasmic fraction from rat brain was incubated in 0.1 M succinate buffer, pH 6.5, with the following additional compounds. AMP (1×10^{-2} M) (⊙); AMP and ATP (1×10^{-3} M) (●); AMP and lauryl sulfate (2×10^{-4} M) (⊡); AMP, ATP, and lauryl sulfate (■); AMP and deoxycholate (1×10^{-3} M) (△); and AMP, ATP, and deoxycholate (▲). The deamination is expressed n terms of micromoles per 30 min.

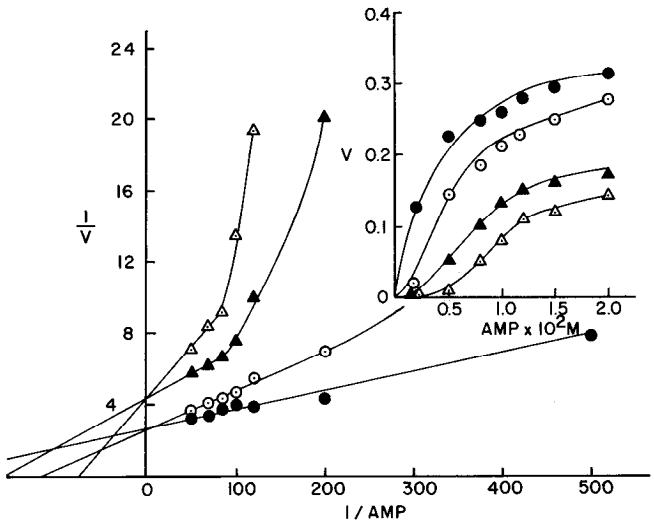

FIG. 5. Substrate concentration and rates of deamination in the presence and absence of deoxycholate. The reciprocal of the ammonia production is plotted against the reciprocal of AMP concentration. A rat brain particulate fraction was used as an enzyme source. The reactions were carried out in 0.1 M succinate buffer, pH 6.5, at 30° for 30 min. Initial velocity (*V*) was calculated on the basis of the first order velocity constant and expressed as micromoles per min per mg of protein in 1 ml of the reaction mixture. AMP concentration is in molar concentration. AMP alone (⊙); AMP and ATP (1×10^{-3} M) (●); AMP and deoxycholate (2×10^{-3} M) (△); AMP, ATP, and deoxycholate (▲).

10^{-3} and 1×10^{-2} M) did no significantly affect the activities of either cytoplasmic or the particulate fraction. The degree of inhibition by deoxycholate or lauryl sulfate was dependent upon the enzyme concentrations used (Fig. 4), and also upon the substrate concentrations (Figs. 5 and 6). These compounds were found to inhibit more strongly at low enzyme concentrations. This can be interpreted as an indication of strong binding of inhibitor by enzyme, or as a modification of the enzyme molecule by the inhibitors. Deoxycholate shifted the hyperbolic curves to the sigmoidal curves (Fig. 5). On the basis of the results, it appears that deoxycholate or lauryl sulfate affects the ATP-binding site in modifying the ATP-activation, as well as the active site, for there is a relationship between AMP concentrations and degree of the inhibition; ATP can prevent the inhibitory effect, and higher deoxycholate concentrations reduce or diminish the ATP-activated activity (Table I).

Effect of P_i on Deaminase Activity—It is likely that this enzyme is one of the allosteric enzymes as reported recently by Cunningham and Lowenstein (18) with the use of a deaminase preparation from calf brain. There was no linear relationship between rate of deamination and AMP concentration at lower substrate levels. When ATP was added to the reaction mixture at different concentrations, the sigmoidal curve gradually shifted to hyperbolic curve (Fig. 5). When a rat brain particulate preparation was incubated in 0.1 M succinate buffer, pH 6.5, with 2×10^{-3} M P_i

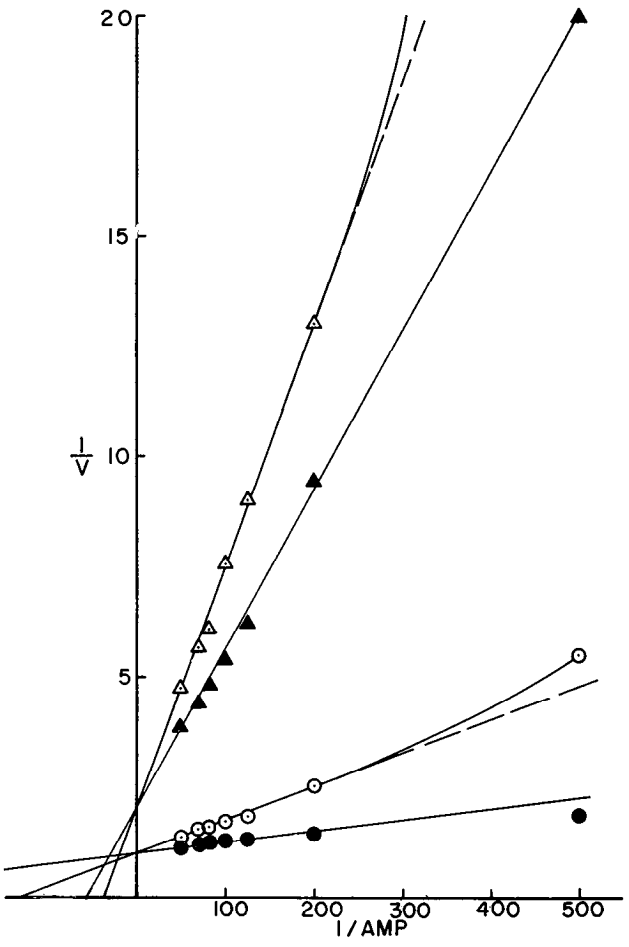

FIG. 6. Substrate concentration and rates of the deamination in the presence and absence of ATP or deoxycholate or both. The reciprocal of the ammonia production is plotted against the reciprocal of AMP concentration. A rat brain cytoplasmic fraction was used. The reactions were carried out in 0.1 M succinate buffer, pH 6.5, at 30° for 30 min. Initial velocity (V) was calculated on the basis of the first order velocity constant and expressed as micromoles per min per mg of protein in 1 ml of the reaction mixture. AMP concentration is in molar concentration. AMP alone (⊙); AMP and ATP (1×10^{-3} M) (●); AMP and deoxycholate (1×10^{-3} M) (△); AMP, ATP, and deoxycholate (▲).

TABLE I

Rate of deamination and substrate concentration in presence and absence of ATP, deoxycholate, inorganic phosphate, and fluoride

See the legends of Figs. 5, 6, and 9 for detailed experimental conditions.

Enzyme source, and substrate and activator or inhibitor	Apparent K_m or K_p	Apparent V_{max} or V_p	Type of inhibition	K_i
	$\times 10^{-3}$ M	μmoles/min/mg protein		$\times 10^{-4}$ M
Cytoplasmic				
AMP	7.8	1.0		
AMP, ATP (1×10^{-3} M)	2.3	1.0		
AMP, deoxycholate (1×10^{-3} M)	28	0.5	Mixed type	
AMP, ATP, deoxycholate	16	0.48	Mixed type	
AMP, P_i (2×10^{-3} M)	34	1.0	Competitive	6
AMP, ATP, P_i	1.4	0.5	Uncompetitive	
AMP, fluoride (1×10^{-3} M)	7.8	0.44	Noncompetitive	8
AMP, ATP, fluoride	1.1	0.34	Uncompetitive	
Particulate				
AMP	8.3	0.38		
AMP, ATP	3.2	0.38		
AMP, deoxycholate (2×10^{-3} M)	14.7	0.24	Mixed type	
AMP, ATP, deoxycholate	6.3	0.24	Mixed type	

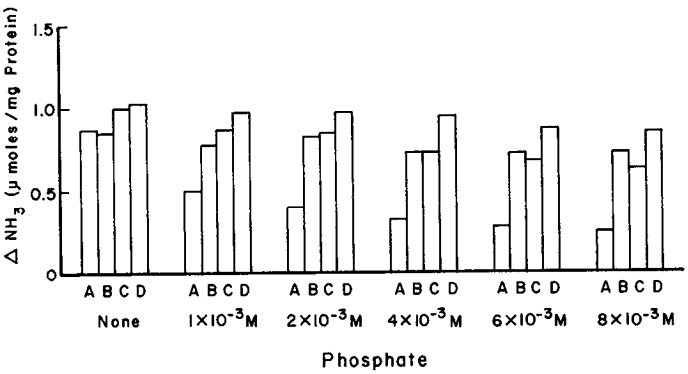

FIG. 7. Ammonia formation in various P_i concentrations. A rat brain particulate preparation was used as an enzyme source and the reactions were carried out in 0.1 M succinate buffer, pH 6.5, at 30° for 30 min. AMP (A); AMP and deoxycholate (B); AMP and ATP (1×10^{-3} M) (C); AMP, ATP, and deoxycholate (D). No ammonia formation was detected in various phosphate concentrations when ATP alone was added to the reaction mixture.

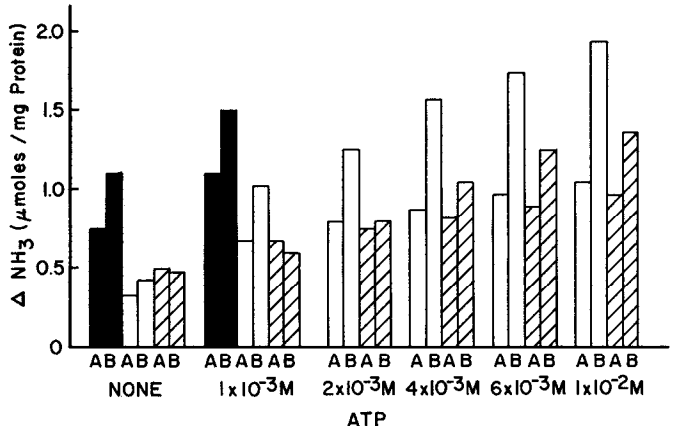

FIG. 8. The inhibitory effect of P_i or fluoride in various ATP concentrations. A rat brain particulate preparation was used as an enzyme source. The reactions were carried out in 0.1 M succinate, pH 6.5, at 30° for 30 min with various amounts of ATP in the absence of inhibitors (*solid bar*), in the presence of P_i (1×10^{-2} M) (*open bar*), or fluoride (1×10^{-3} M) (*hatched bar*). AMP alone (1×10^{-2} M) (*A*), AMP and deoxycholate (1×10^{-3} M) (*B*).

in the presence and absence of deoxycholate for 10, 20, or 30 min, there was no difference in P_i and ammonia concentrations and there were no changes in optical density at 265 and 240 mμ, compared with those at zero time. Ammonia liberation, in the presence of the substrate, AMP, with or without ATP present, was inhibited by P_i (Fig. 7). Deoxycholate added to the reaction mixture apparently prevented the P_i inhibition to some extent either in the presence or absence of ATP. The inhibitory action of P_i was also reversed by higher concentrations of ATP in the absence of deoxycholate (Fig. 8). When the assay was conducted in the presence of deoxycholate, ATP not only reversed the inhibitory action of P_i but also activated the ammonia liberation further. P_i was found to be a competitive inhibitor in the absence of ATP, while in the presence of ATP it was an uncompetitive inhibitor (Fig. 9, Table I). Therefore, it seems clear that P_i competes simultaneously with the substrate, AMP, and with the activator, ATP, at different binding sites.

Effect of Fluoride on Deaminase Activity—On the basis of the previous experimental results with the use of rabbit skeletal muscle deaminase preparation, fluoride appeared to inhibit the deaminase by a mechanism which differed from that proposed for its effects on phosphate-transferring enzymes. The inhibition was dependent on pH, was not competitive to AMP, was not influenced by the presence of 2′-AMP, and was more pronounced at low enzyme concentrations (1, 19). Fluoride was found to be a strong inhibitor of the deaminase of rat brain (Fig. 10) as well as the enzyme of rabbit skeletal muscle. Fluoride apparently eliminated the potentiating effect of deoxycholate even in the presence of a lower concentration of ATP. Higher concentrations of ATP reversed the inhibitory effect of fluoride and brought the potentiating effect of deoxycholate back to the previous state (Fig. 8). Fluoride inhibited the deaminase noncompetitively in the absence of ATP and uncompetitively in the presence of ATP (Fig. 9, Table I). No inhibitory effect of CF_3COOH at various concentrations and at different pH values was found. The degree of the inhibitory action by fluoride was not enhanced by prior incubating of fluoride with an enzyme preparation for 15 min. The inhibition could also be reversed by dialyzing off fluoride. It is likely that fluoride competes with ATP in binding to the

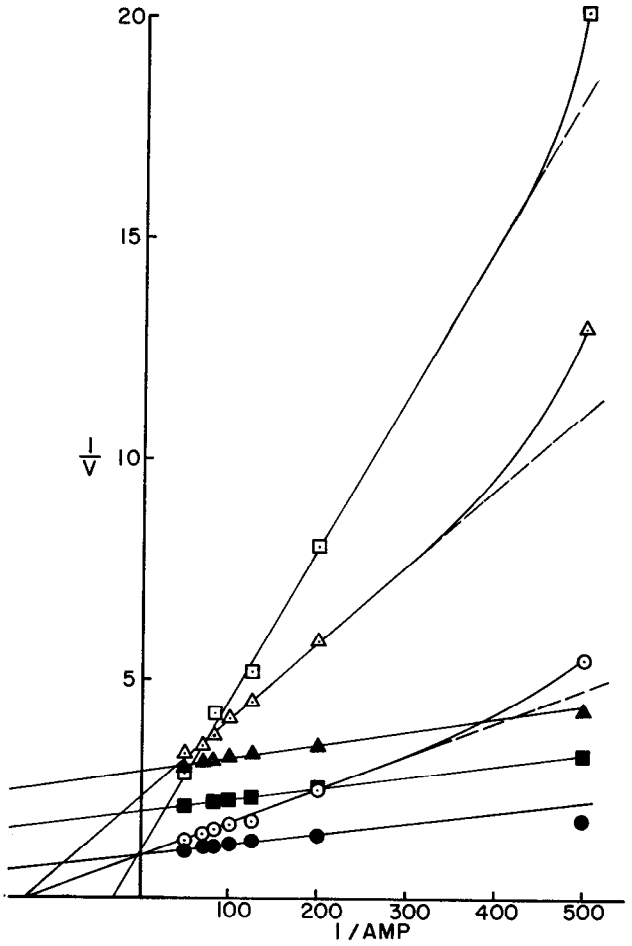

FIG. 9. Substrate concentration and rates of deamination in the presence and absence of ATP, deoxycholate, P_i, and fluoride. The reciprocal of the ammonia production is plotted against the reciprocal of AMP concentration. A rat brain cytoplasmic fraction was used as an enzyme source. The reactions were carried out in 0.1 M succinate buffer, pH 6.5, at 30° for 30 min. Initial velocity (V), was calculated on the basis of the first order velocity constant and expressed as micromoles per min per mg of protein in 1 ml of the reaction mixture. AMP concentration is in molar concentration. AMP alone (⊙); AMP and ATP (1×10^{-3} M) (●); AMP and P_i (2×10^{-3} M) (⊡); AMP, ATP, and P_i (■); AMP and fluoride (1×10^{-3} M) (△); AMP, ATP, and fluoride (▲).

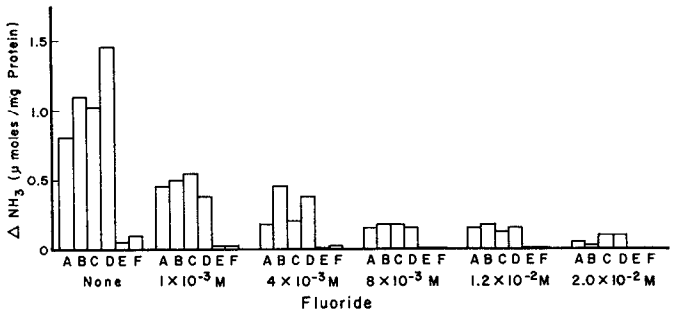

FIG. 10. Ammonia formation in various fluoride concentrations. A rat brain particulate preparation was used as an enzyme source and the reactions were conducted in 0.1 M succinate buffer, pH 6.5, in various fluoride concentrations with the following compounds. AMP (1×10^{-2} M) (*A*); AMP and deoxycholate (1×10^{-3} M) (*B*); AMP and ATP (1×10^{-3} M) (*C*); AMP, ATP, and deoxycholate (*D*); ATP (*E*); ATP and deoxycholate (*F*).

effector binding site, and in the absence of ATP fluoride occupies the effector binding site to modify the activity.

DISCUSSION

The physiological role of AMP deaminase in cells has been discussed ever since this enzyme was discovered. However, it seems that AMP deaminase may not directly participate in muscular contraction and relaxation processes for the following reasons: (*a*) Cain, Kushmerick, and Davies (20) reported that there were no significant net changes in either the adenine or hypoxanthine nucleotides during single contraction; (*b*) Kitagawa and Tonomura (21) detected no significant AMP deaminase activity in a purified contractile protein, myosin B, from adductors either by measuring ammonia liberation or by IMP formation; and (*c*) several types of muscle have been reported to be lacking deaminase (22). Further studies are necessary for a definite conclusion as to the direct participation of the deaminase in muscular contraction and relaxation processes.

It is likely as another possible physiological role that AMP deaminase regulates the concentration of cellular AMP, which can accelerate glycogenolysis and glycolysis by activating phosphorylase *b* and phosphofructokinase, and by depressing fructose diphosphatase. When ATP, a strong potentiator of AMP deaminase because of lowering the K_m value, decreases in concentration because of its utilization for muscular work and the like, and when P_i, an inhibitor of the deaminase, increases in concentration, AMP deaminase may essentially not function because of its higher K_m value, low ATP (activator), and high P_i (inhibitor) concentration. Thus, the concentration of AMP rises, and thus glycogenolysis and glycolysis, proceed to generate ATP. High ATP and low P_i concentrations, as a result of elevated oxidative phosphorylation will retard glycolysis because of the low AMP concentration. This phenomenon could be a part of the Pasteur effect if the results regarding the effect of nucleotides on enzyme activities, which have been obtained separately with the use of individual enzyme, can be applied to the over-all glycolytic or gluconeogenetic processes. Hydrocortisone or other hormones has been shown to modify glycolytic and gluconeogenetic processes by changing some of glycolytic or gluconeogenetic enzyme activities. It is possible that part of this hormonal or metabolic regulation on glycolysis and gluconeogenesis might have resulted from a change of AMP concentration, partly because of an alteration of AMP deaminase activity.

It is of interest to study further the potentiating effect of ATP on deaminase activity, which was originally discovered in brain by Muntz (23), and later in muscle by Lyubimova and Matlina (24), since the purified deaminase preparation from rabbit skeletal muscle was not potentiated by, nor did it require, ATP (1). We feel that there might be three types of AMP deaminase in cells, namely ATP-independent (ATP is not an essential factor), ATP-dependent (ATP is an essential factor), and ATP-activated (ATP is an activator). As for another possibility, there may be an ATP receptor in an intact original AMP deaminase molecule. This receptor might be dissociated or uncoupled during purification processes, at lower pH, or by heat treatment as described previously (1).

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