Enhancement of Brain Glutamate Dehydrogenase Activity and Glutamate Oxidation by Adenine Nucleotides

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SUMMARY

The rate of oxidation of glutamate by brain homogenates or mitochondria was enhanced approximately 4-fold by 5′-AMP, cyclic 3′,5′-AMP, or 5′-ADP. These nucleotides were equally effective in augmenting glutamate oxidation, while 3′-AMP, 2′-AMP, and 2′,3′-AMP were without effect. Cyclic 3′,5′-AMP did not increase the rate of glutamate oxidation in heart or testis homogenates but produced 13% and 89% stimulation by liver and kidney homogenates, respectively. Kinetic and gel filtration evidence indicates that glutamate dehydrogenases of brain, liver, and kidney mitochondria are not identical in their properties. The enhancement of activity of the brain enzyme by 5′-AMP or cyclic 3′,5′-AMP was dependent on the pH of the reaction mixture and the concentration of NAD. At pH 8.0, the apparent K_m for NAD was decreased from 2 mM in the absence of adenine nucleotides to 0.4 mM or 0.5 mM in the presence of 5′-AMP or cyclic 3′,5′-AMP, respectively. At pH 7.5, 5′-AMP evoked twice the stimulation produced by corresponding concentrations of cyclic 3′,5′-AMP. Kinetic evidence indicates that brain glutamate dehydrogenase contains at least two cooperative sites for NAD in the absence of adenine nucleotides, and only one site in the presence of these activators. Density gradient studies suggest that the molecular weight of the activated enzyme is approximately 250,000.

INTRODUCTION

A number of recent observations suggest that 5′-AMP, 5′-ADP, and cyclic 3′,5′-AMP may be involved in the regulation of energy metabolism (1). Frieden (2, 3) has demonstrated the ability of 5′-AMP and 5′-ADP to enhance liver glutamate dehydrogenase, and these nucleotides have been found to alter the catalytic activity of isocitrate dehydrogenase (4) and phosphofructokinase (5). Mansour (6) has shown that 5′-AMP and cyclic 3′,5′-AMP activate phosphofructokinase isolated from guinea pig heart and from a parasitic liver fluke. Similar effects of 5′-AMP have been reported for phosphofructokinases obtained from rabbit muscle and kidney and from yeast (7, 8).

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Recent studies by Sutherland and coworkers (9–13) and by others (14–18) have indicated that cyclic AMP, which is formed from ATP by the enzyme adenyl cyclase, is involved in the regulation of various metabolic pathways, including glycogenolysis in liver and heart, lipolysis in fat tissue, and steroidogenesis in adrenal cortex. Inasmuch as glutamate oxidation is of importance in energy metabolism in the brain, it seemed of interest to examine the actions of various adenine nucleotides, including cyclic 3′,5′-AMP, on glutamate metabolism in brain preparations.

MATERIALS AND METHODS

Adenosine 3′,5′-monophosphoric acid, adenosine 2′,3′-monophosphoric acid, adenosine 5′-monophosphate, adenosine 5′-diphosphate, adenosine 2′-monophosphate, and

adenosine 3′-monophosphate were purchased from Sigma Chemical Company. They were found to be 99% pure when assayed spectrophotometrically, and were determined to be homogeneous by a descending paper chromatographic technique with a solvent system consisting of 3 volumes of 1 M ammonium acetate (adjusted to pH 7.5 with ammonium hydroxide) and 7 volumes of 95% ethanol. Molar absorbance and R_F values were virtually identical with those previously reported by P-L Biochemicals.

Water used in the experiments was triple-distilled from a Pyrex glass apparatus. Other reagents obtained from commercial sources were purified when necessary.

Sprague-Dawley rats of either sex, weighing 250–300 g, were decapitated, after which their brains were removed within 15–20 sec and placed in 0.25 M sucrose at 0°. All preparative procedures were conducted at 0–4°. The brains were blotted on filter paper, weighed, and homogenized in sufficient 0.25 M sucrose to yield a 20% homogenate. Homogenization was performed by four or five passes of a Teflon pestle in a glass homogenizer; the tightly fitting pestle was driven at approximately 1200 rpm.

When mitochondria were to be isolated, the 20% homogenate was diluted with 1 volume of 0.25 M sucrose and centrifuged at $1000 \times g$ for 10 min to sediment unbroken cells, nuclei, and debris. The resulting supernatant fluid was centrifuged at $12{,}000 \times g$ for 10 min, and the ensuing pellet, containing the mitochondrial fraction, was washed by resuspending it in the original volume of 0.25 M sucrose, followed by centrifugation at $10{,}000 \times g$ for 10 min. This washing procedure was repeated twice.

Oxygen uptake by homogenates or mitochondrial suspensions with glutamate as substrate was determined at 37° by conventional manometric techniques (19). Routinely, the buffer and enzyme preparation were added to the reaction vessel, and after a 10-min equilibration period the manometer stopcocks were closed. Substrate and NAD were then added from the side arm. Unless indicated, the final concentrations of reagents, in a total volume of 3.0 ml, were: 0.01 M phosphate buffer, pH 7.5 (prepared by adjusting a solution of KH_2PO_4 to pH 7.5 with 1 N NaOH); 0.01 M glutamate, pH 7.5; 1 mM NAD, pH 6.5; 1 mM theophylline, pH 7.5; and 0.5 ml of enzyme preparation representing 100 mg of brain tissue (approximately 15 mg of protein) in 0.25 M sucrose. Manometer readings were recorded at 7.5-min intervals for 30 min.

Glutamate dehydrogenase preparations were prepared by disrupting mitochondrial membranes by a 30-sec treatment with a Branson Sonifier at a setting of 3 amp, followed by centrifugation at $175{,}000 \times g$ for 1 hr. The resulting clear enzyme solution was dialyzed by passing the preparation through a 1×30 cm Sephadex G-25 column equilibrated with 0.05 M phosphate buffer, pH 7.4. Glutamate dehydrogenase activity was measured by observing the rate of NADH formation at 340 mμ with a Gilford model 2400 spectrophotometer and recorder. The authenticity of the NADH formed in these experiments was verified by the disappearance of 340 mμ absorption after addition of dilute HCl or excess alcohol dehydrogenase and acetaldehyde to the cuvettes.

The gel filtration columns were prepared with Sephadex G-200 which had been sieved through a 200-mesh screen. The particles retained by the sieve were allowed to swell for 72 hr in 0.05 M phosphate, pH 7.4, at room temperature. The gel was then poured into a 1×55 cm glass column at 4°, and the column was equilibrated by washing with buffer for 24 hr. The effluent was collected in 0.5-ml samples by means of a Buchler refrigerated fraction collector.

In the sedimentation velocity studies, 0.2 ml of enzyme solution was applied to linear sucrose density gradients (8–20%) and centrifuged at $175{,}000 \times g$ for 6 hr in a Spinco model LH centrifuge equipped with an SW-39L rotor.

Ammonia determinations were performed by Conway microdiffusion techniques (20). Protein was determined by the biuret method with three-times recrystallized bovine serum albumin as a standard.

RESULTS

Enhancement of oxygen uptake in rat brain homogenates by cyclic 3',5'-AMP or 5'-AMP. Table 1 shows that the rate of oxidation of glutamate by brain homogenates, as measured by the rate of oxygen uptake, was approximately 1.0 μl/30 min/mg of protein in the absence of nucleotide. When 0.33 mM cyclic 3',5'-AMP or 5'-AMP was added to the incubation mixture, the rate of oxygen uptake was increased about 380%. All rates were determined by subtracting the values obtained in the absence of substrate and were linear up to 30 min. Similar results were obtained when glutamine was employed as the substrate instead of glutamate.

TABLE 1

Effects of cyclic 3',5'-AMP and 5'-AMP on oxidation of various substrates by rat brain homogenates

Each Warburg flask contained, in a final volume of 3.0 ml, 10 mM phosphate buffer (pH 7.4), 1 mM theophylline, 1.0 mM NAD, 0.03 mM $MgCl_2$, and, where indicated, 0.33 mM cyclic 3',5'-AMP or 0.33 mM 5'-AMP. The final concentrations of substrates were 10 mM. Each flask also contained 0.5 ml of 20% rat brain homogenate (approximately 16 mg of protein). Details of the procedure are described in the text.

Substrate	Nucleotide	Q_{O_2}[a]
L-Glutamate	None	1.06
L-Glutamate	3',5'-AMP	4.81
L-Glutamate	5'-AMP	4.69
α-Ketoglutarate	None	3.06
α-Ketoglutarate	3',5'-AMP	5.38
α-Ketoglutarate	5'-AMP	4.94
Pyruvate[b]	None	1.88
Pyruvate	3',5'-AMP	4.69
Pyruvate	5'-AMP	4.38
Succinate	None	3.50
Succinate	3',5'-AMP	3.56
Succinate	5'-AMP	3.50
L-Malate	None	1.88
L-Malate	3',5'-AMP	1.81
L-Malate	5'-AMP	1.75
NADH	None	4.31
NADH	3',5'-AMP	3.75
NADH	5'-AMP	3.94

[a] Q_{O_2} values represent the average of three experiments and are expressed as microliters of oxygen uptake per milligram of protein in 30 min at 37°. Corrections were made for oxidation of endogenous substrates as described in the text.

[b] When pyruvate was used as a substrate, a final concentration of 0.5 mM L-malate was used as a "sparker" for the citric acid cycle.

Since brain contains the enzyme cyclic 3',5'-phosphodiesterase (21), which catalyzes the conversion of cyclic 3',5'-AMP to 5'-AMP, all of the results presented in this paper were obtained with 1 mM theophylline, which has previously been reported to be an inhibitor of cyclic 3',5'-phosphodiesterase (21). However, since 1 mM theophylline does not completely inhibit brain phosphodiesterase activity (22), the possibility that the enhanced oxygen uptake produced by cyclic 3',5'-AMP might have been due to conversion of this nucleotide to 5'-AMP was investigated. 8-^{14}C-Cyclic 3',5'-AMP (Schwarz BioResearch) in a final concentration of 0.33 mM was incubated at 37° for various times with 0.5 ml of 20% brain homogenate in a complete reaction mixture in the presence and absence of 1 mM theophylline. As shown in Table 2, after 45 min approximately 35% of the cyclic nucleotide was converted to 5'-AMP in the absence of theophylline, as compared with 18% in its presence. Inasmuch as 18% of the added cyclic 3',5'-AMP was hydrolyzed, the enhancement of oxygen uptake by the cyclic nucleotide might have been due in part to 5'-AMP or 5'-ADP. However, if cyclic 3',5'-AMP were ineffective in increasing glutamate oxidation, it is unlikely that linear rates of oxygen uptake would have been observed; probably an increase in the rate of the reaction with time would have been noted. In addition, glutamate oxidation by brain mitochondria was enhanced by cyclic 3',5'-AMP as well as by 5'-AMP.

As shown in Table 1, the rates of oxidation of succinate, malate, and NADH were not appreciably altered by the presence of cyclic 3',5'-AMP or 5'-AMP in the reaction mixture. However, these nucleotides enhanced α-ketoglutarate and pyruvate oxidation approximately 75% and 150%, respectively.

Enhancement of oxygen uptake in rat brain homogenates by various concentrations of nucleotides. In view of the reports

TABLE 2

Effect of theophylline on conversion of cyclic 3',5'-AMP to 5'-AMP by brain homogenates

To a complete reaction mixture as described in Table 1, 8-^{14}C-cyclic 3',5'-AMP (specific activity, 1.0 μCi/μmole) at a final concentration of 0.33 mM was added. The reaction mixture was incubated for the times shown at 37° in the presence or absence of 1 mM theophylline. The reaction was stopped by placing the tubes in boiling water for 10 min, and following centrifugation, aliquots were applied along with unlabeled nucleotides to polyethylinimine-impregnated cellulose thin layer plates. After the plates were developed with 0.1 M formate buffer, pH 3.3, spots observed with an ultraviolet lamp were removed from the plates, placed in 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid, and counted. Each determination was performed in duplicate, and values represent the averages of two experiments.

Time	Theophylline	5'-AMP[a]	Cyclic 3',5'-AMP
min	*mM*	*cpm*	*cpm*
15	0	410	4520
15	1.0	146	4788
30	0	1026	4198
30	1.0	427	4825
45	0	1778	3185
45	1.0	1008	4558

[a] Cyclic 3',5'-AMP (Schwarz) contained approximately 3% 5'-AMP upon separation by the above procedure (representing about 200 cpm in these experiments), and the values in this column were appropriately corrected.

by Breckenridge (23) and Aurbach and Houston (24) that the level of cyclic 3',5'-AMP in mouse brain was approximately 1 μmole/kg, and the finding by Kakiuchi and Rall (14) that norepinephrine produced a rapid increase in the concentration of cyclic 3',5'-AMP from 0.001 mM to approximately 0.02 mM in rabbit cerebral cortex slices, studies were performed to determine whether cyclic 3',5'-AMP at concentrations of the order of 1 μmole/kg of tissue might increase the rate of glutamate oxidation. The effects of cyclic 3',5'-AMP on rat brain homogenates which were aged at 0–4° for 24 hr as well as on fresh brain homogenates were investigated. The homogenates were aged to allow the cyclic phosphodiesterase to reduce the endogenous cyclic 3',5'-AMP level as much as possible. As shown in Fig. 1, cyclic 3',5'-AMP enhanced oxygen uptake by aged brain homogenates approximately 40% at a final concentration of 0.033 mM, and about 350% at a final concentration of 0.33 mM. Concentrations of cyclic 3',5'-AMP greater than 0.33 mM increased oxygen uptake to the same extent as 0.33 mM, indicating that maximal enhancement occurs at relatively high concentrations of the nucleotide. When studies were performed with 5'-AMP at various concentrations, the results were similar to those obtained with cyclic 3',5'-AMP. When 5'-AMP or cyclic 3',5'-AMP was added to the same reaction mixture, stimulation of glutamate oxidation was additive at total nucleotide concentrations less than 0.33 mM. However, in the presence of 0.33 mM 5'-AMP or 0.33 mM cyclic 3',5'-AMP, addition of cyclic 3',5'-AMP or 5'-AMP, respectively, produced no further enhancement of glutamate oxidation.

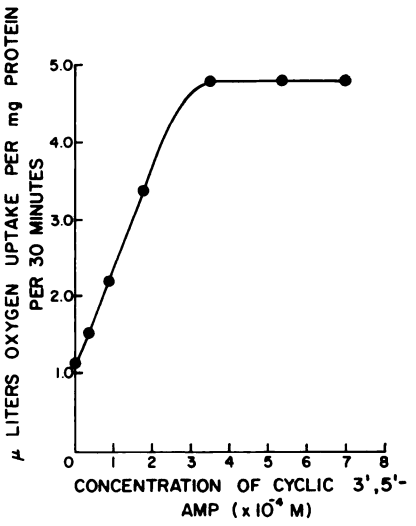

FIG. 1. *Rate of oxygen uptake by aged brain homogenates as a function of cyclic 3',5'-AMP concentration*

Conditions were those described in Table 1. Four rat brains were pooled and homogenized. Oxygen uptake was determined after 24 hr at 4°, following homogenization. Values represent an average of three experiments and are expressed as microliters of oxygen uptake per milligram of protein in 30 min at 37°.

Results similar to those shown in Fig. 1

were obtained when 0.01 M Tris buffer, pH 7.5, was used in place of 0.01 M phosphate buffer. However, enhancement of oxygen uptake in brain homogenates by cyclic 3',5'-AMP was markedly reduced when phosphate buffer concentrations greater than 0.05 M were employed.

Specificity of action of nucleotides. As shown in Table 3, 5'-AMP and 5'-ADP as well as cyclic 3',5'-AMP enhanced the rate of oxygen uptake by brain homogenates with glutamate as substrate. However, 3'-AMP, cyclic 2',3'-AMP, and 2'-AMP did not alter the rate of oxygen uptake. Similar results were obtained when brain mitochondria were used in place of homogenates.

TABLE 3

Effect of various nucleotides on oxygen uptake by rat brain homogenates with glutamate as substrate

Experimental conditions were the same as described in Table 1 and the text. Corrections were made for endogenous oxygen uptake as in Table 1. Sufficient nucleotide was added to give the final concentrations indicated. Q_{O_2} values represent microliters of oxygen uptake per milligram of protein per 30 min at 37° and represent the average of three experiments.

Nucleotide	Concentration	Q_{O_2}	Increase
	mM		%
None	0	0.90	
3',5'-AMP	0.33	4.00	344
3',5'-AMP	0.17	3.03	237
5'-AMP	0.33	3.85	328
5'-AMP	0.17	2.94	227
5'-ADP	0.33	3.77	319
3'-AMP	0.33	1.12	
3'-AMP	0.17	0.94	
2'-AMP	0.33	0.89	
2'-AMP	0.17	0.95	
2',3'-AMP	0.33	0.94	
2',3'-AMP	0.17	1.02	

Experiments were conducted to determine whether the effects of cyclic 3',5'-AMP on glutamate metabolism were specific for brain tissue. The data presented in Table 4 indicate that cyclic 3',5'-AMP markedly enhances glutamate oxidation by brain tissue, but has considerably less effect on oxygen uptake by other tissues. Oxygen uptake by brain homogenates was enhanced about 320% by 0.33 mM cyclic 3',5'-AMP, whereas this concentration of nucleotide did not significantly alter oxygen uptake by heart or testis homogenates in the presence of glutamate as substrate. Oxygen uptake by kidney and liver homogenates was enhanced 89 and 13%, respectively.

TABLE 4

Effect of cyclic 3',5'-AMP on oxygen uptake by various rat tissue homogenates with glutamate as substrate

Experimental conditions were the same as described in Table 1 and the text. Each flask contained 0.5 ml of a 20% homogenate in 0.25 M sucrose. Q_{O_2} values represent microliters of oxygen uptake per milligram of protein per 30 min at 37°. Corrections were made for endogenous oxygen uptake as described in the text.

Tissue	Cyclic 3',5'-AMP	Q_{O_2}	Increase
	mM		%
Brain	0	1.01	
Brain	0.33	4.25	321
Kidney	0	2.92	
Kidney	0.33	5.52	89
Liver	0	3.95	
Liver	0.33	4.45	13
Heart	0	0.91	
Heart	0.33	0.95	0
Testes	0	0.68	
Testes	0.33	0.74	0

Effects of nucleotides on partially purified rat brain glutamate dehydrogenase. In the course of these studies it was observed that the rate of ammonia liberation as well as the rate of oxygen uptake by brain homogenates or mitochondria was enhanced by 5'-AMP or cyclic 3',5'-AMP, indicating that these nucleotides increased glutamate dehydrogenase activity. With glutamate as substrate, 5'-AMP (0.33 mM) or 3',5'-AMP (0.33 mM) markedly increased the rate of NAD reduction by a partially purified glutamate dehydrogenase obtained from sonically treated brain mitochondria, as shown by Fig. 2. The amount of activation produced by 5'-AMP was much greater than that produced by the cyclic nucleotide. However, at concentrations below 0.02 mM, the nucleotides were ineffective in enhancing the rate of NAD reduction. The rates of

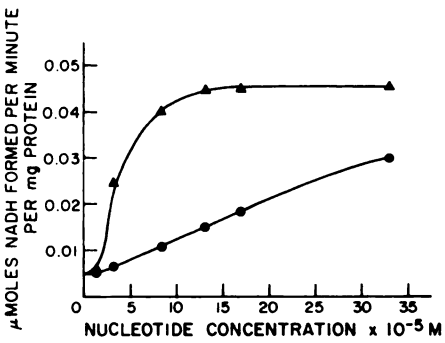

FIG. 2. *Effects of various concentrations of cyclic 3',5'-AMP and 5'-AMP on rat brain glutamate dehydrogenase*

Assays were carried out in a total volume of 3 ml with a final concentration of 0.01 M glycine buffer, pH 8.0; 0.01 M glutamate; 0.4 mM NAD; cyclic 3',5'-AMP (●——●) or 5'-AMP (▲——▲) as indicated; and approximately 1.0 mg of protein at 25°. The reaction was initiated by the addition of NAD.

NAD reduction in the presence and absence of nucleotide were linear for at least 1 min. Maximum activation by 5'-AMP or cyclic 3',5'-AMP was obtained in the time required to mix the enzyme and substrate in the reaction mixture. These results indicate that conversion of cyclic 3',5'-AMP to 5'-AMP was probably not essential for activation of brain glutamate dehydrogenase.

The ability of cyclic 3',5'-AMP or 5'-AMP to increase the activity of brain glutamate dehydrogenase is pH-dependent (Table 5). At pH 6.5 the nucleotides, in the concentrations indicated, were without effect, but as the pH of the reaction mixture was raised to 8.5, the enhancement of NAD reduction by cyclic 3',5'-AMP or 5'-AMP was increased to 8-fold and 18-fold, respectively. At pH 7.4, 0.33 mM cyclic 3',5'-AMP produced a 200% increase in brain glutamate dehydrogenase activity, whereas under similar conditions the activity of the enzymes obtained from rat liver and kidney mitochondria was increased by only 60% and 40%, respectively. That the nucleotide did not alter other enzymes concerned with glutamate metabolism was indicated by an absence of effect on the catalytic activity of brain glutamate-oxalacetate aminotransferase or glutamate decarboxylase.

TABLE 5

Activation of rat brain glutamate dehydrogenase by cyclic 3',5'-AMP and 5'-AMP at various pH values

The preparation of glutamate dehydrogenase from brain mitochondria is described in the text. Assays were performed by observing the rate of NADH formation spectrophotometrically at 340 mμ, and the rate is expressed as micromoles formed per minute per milligram of protein at 25°. The reaction was carried out in a total volume of 3.0 ml with a final concentration of 0.01 M phosphate buffer, pH 6.5 or 7.5, or with a final concentration of 0.01 M glycine buffer, pH 8.0 or pH 8.5; 0.01 M glutamate; 0.33 mM cyclic 3',5'-AMP or 0.33 mM 5'-AMP; 1.0 mM NAD; and approximately 1.0 mg of protein. The reaction was initiated by the addition of NAD.

pH	Nucleotide	NADH formed	Increase
		μmole/min/mg	%
8.5	None	0.0024	
8.5	3',5'-AMP	0.0217	800
8.5	5'-AMP	0.0460	1800
8.0	None	0.0072	
8.0	3',5'-AMP	0.0360	400
8.0	5'-AMP	0.0604	730
7.5	None	0.0144	
7.5	3',5'-AMP	0.0432	200
7.5	5'-AMP	0.0724	400
6.5	None	0.0072	
6.5	3',5'-AMP	0.0072	0
6.5	5'-AMP	0.0087	20

The results of molecular sieve studies are presented in Fig. 3 and show that the enzyme from brain mitochondria is eluted from a Sephadex G-200 column at 33% of the gel bed volume, while the rat liver and bovine liver (Sigma) enzymes are eluted at 42% of the gel bed volume. With protein concentrations (approximately 3.0 mg/ml) similar to those for the rat brain and rat liver enzymes, the rat kidney enzyme is eluted at 37% of the gel bed volume. The value for the bovine liver enzyme is similar to that obtained by Rogers, Hellerman, and Thompson (25) for bovine liver glutamate dehydrogenase. Since catalase which has a molecular weight of 2.5×10^5, is eluted at 42% of the gel bed volume, it appears that under the conditions used in this study the brain enzyme either has a molecular weight greater than 2.5×10^5 or is more highly

TABLE 6

Comparison of effects of adenine nucleotides on the K'_m for NAD and V_{max} for rat brain, liver, and kidney glutamate dehydrogenases

Enzyme source	K'_m[a]			V_{max}		
	Nucleotide absent	3',5'-AMP present	5'-AMP present	Nucleotide absent	3',5'-AMP present	5'-AMP present
	mM	mM	mM	μmole NADH formed/min/mg		
Brain	2.0	0.5	0.3	0.116	0.106	0.120
Liver	2.0	1.0		0.164	0.169	
Kidney	2.8	2.1		0.048	0.048	

[a] Represents the apparent Michaelis constant for NAD (concentration of NAD giving one-half maximal velocity).

associated than the other enzymes. In addition, as shown in Table 6, slight differences were observed for the apparent Michaelis-Menten (K'_m) constants for NAD with the brain, liver, and kidney enzymes in the presence or absence of nucleotide. Such findings, coupled with those portrayed in Table 4, indicate that the properties of glutamate dehydrogenases from different tissues may not be identical.

As shown in Fig. 4, when the activity, at pH 8.0, of brain glutamate dehydrogenase is plotted as a function of NAD concentration, a sigmoidal curve is obtained. This type of curve is indicative of a homotropic cooperative effect (26), and is in keeping with data published for other enzyme systems (27, 28). In the presence of 0.33 mM cyclic 3',5'-AMP or 5'-AMP, a hyperbolic curve is obtained, indicating that at low concentrations of NAD the adenine nucleotides markedly enhance the enzyme activity. When the data are plotted in a double-reciprocal form, a nonlinear relationship is obtained in the absence of adenine nucleotides, while in their presence the plot is linear. The apparent K'_m for NAD in the absence of nucleotide was found to be about 2.0 mM, but in the presence of cyclic 3',5'-

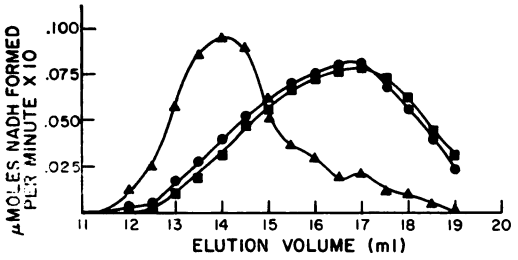

FIG. 3. *Elution pattern for glutamate dehydrogenase from rat brain (▲——▲), rat liver (■——■), and bovine liver (●——●) from a Sephadex G-200 column at 4°*

Blue dextran was eluted from the column in 12.5 ml, representing 29% of the gel bed volume, and catalase was eluted in 17 ml or 42% of the gel bed volume. One-milliliter samples (brain enzyme contained 3.3 mg of protein per milliliter, rat liver enzyme contained 3.0 mg of protein per milliliter, and bovine liver enzyme contained 10 μg of protein per milliliter) were placed on the column and were eluted with 0.05 M phosphate, pH 7.4. Enzyme activity was determined as described for Fig. 2 and in the text.

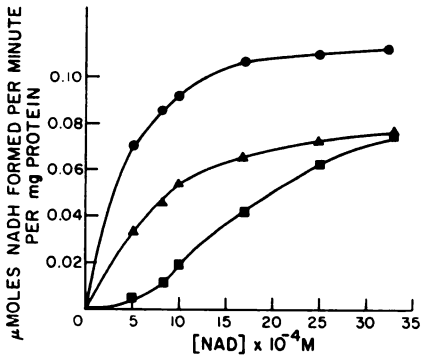

FIG. 4. *Plot of velocity vs. NAD concentration for rat brain glutamate dehydrogenase in the absence and presence of cyclic 3',5'-AMP or 5'-AMP*

Enzyme activities were determined as described for Fig. 2, at pH 8.0, 25°, and varying concentrations of NAD as indicated. The final concentration of cyclic 3',5'-AMP (▲——▲) or 5'-AMP (●——●) was 0.33 mM. ■——■, no adenine nucleotide present.

AMP or 5′-AMP the apparent values were approximately 0.5 mM and 0.4 mM, respectively. Frieden (2) reported a value of 2.0 mM for the activation constant for NAD with bovine liver glutamate dehydrogenase and an apparent K'_m of 0.09 mM for NAD in the presence of 5′-ADP.

Figure 5 shows curves obtained when kinetic data are plotted according to the Hill equation (26),

$$\log \frac{v}{V_{max} - v} = n \log S + \log K$$

where V_{max}, v, S, n, and K are maximal velocity, initial reaction velocity, NAD concentration, a complex function of an interaction coefficient and the number of binding sites, and a complex equilibrium constant, respectively. The value of $n = 2.05$ for the slope of the Hill plot indicates that at least 2 molecules of NAD bind at different sites to exhibit a homotropic cooperative effect. The linear double-reciprocal plots obtained in the presence of nucleotide activator and a value of $n = 1.0$ for the slope of the Hill plot in the presence of nucleotide indicate that cyclic 3′,5′-AMP or 5′-AMP prevents interaction between NAD sites. As shown in Table 5, the nucleotides were ineffective in activating the enzyme at pH 6.5, and at this pH the double-reciprocal plots of velocity vs. NAD concentration were linear in the absence of nucleotide. In addition, at pH 6.5, the slope of the Hill plot was found to be 1.1 in the absence of activator. These results suggest an absence of interaction between binding sites for NAD at pH 6.5.

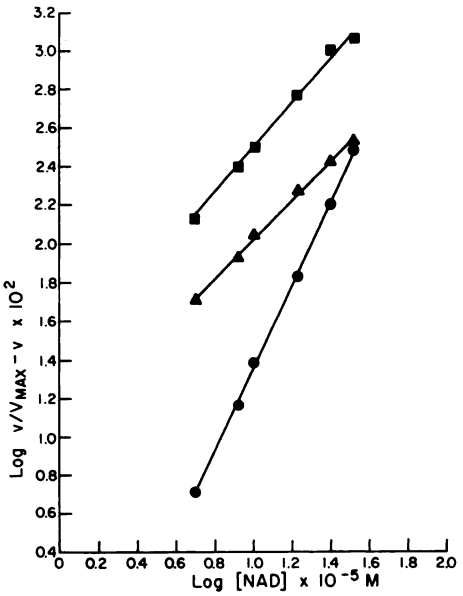

FIG. 5. *The data of Fig. 4 plotted according to the empirical Hill equation*

●——●, no adenine nucleotide present; ▲——▲, in the presence of cyclic 3′,5′-AMP; ■——■, in the presence of 5′-AMP.

In order to examine the possibility that association-dissociation mechanisms may be involved in activation of brain glutamate dehydrogenase by cyclic 3′,5′-AMP, the enzyme (0.3 mg) was placed on linear sucrose density gradients at pH 8.2 in the absence and presence of the cyclic nucleotide and was centrifuged at 175,000 × *g* for 6 hr. The results (Fig. 6) suggest that, at pH 8.2, the enzyme sedimented at a rate similar to that of catalase (mol wt 2.5×10^5) in both the presence and absence of the nucleotide. These observations are in

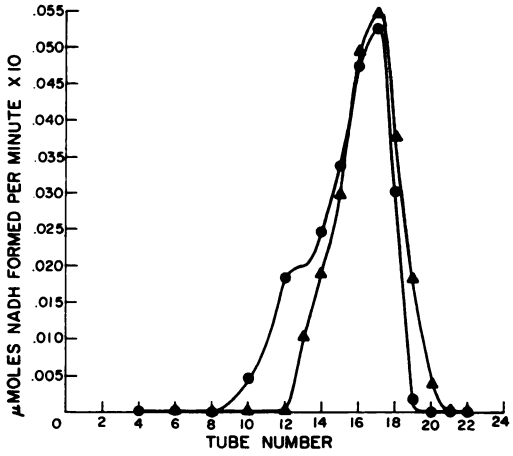

FIG. 6. *Elution patterns for brain glutamate dehydrogenase from sucrose density gradients*

The linear sucrose gradients were prepared as described in the text. Enzyme (0.2 ml containing 0.3 mg of protein) was applied to each gradient: ●——●, pH 8.2, in the absence of activator; ▲——▲ pH 8.2, in the presence of 0.33 mM cyclic 3′,5′-AMP. The enzyme activity of the contents of each fraction collected was determined at pH 7.4 with 0.33 mM 5′-AMP in the reaction mixture as described for Fig. 2.

keeping with those of Frieden (29) for the bovine liver enzyme and indicate that activation may not involve association of the 2.5×10^5 mol wt monomers. The elution pattern of relatively concentrated brain glutamate dehydrogenase (3.0 mg/ml) from a Sephadex column (Fig. 3) indicated that the enzyme was aggregated into units larger than 2.5×10^5 mol wt, but when 0.3 mg of enzyme protein was subjected to sedimentation velocity studies (Fig. 6), a molecular weight of approximately 2.5×10^5 was obtained.

DISCUSSION

In these studies, some degree of specificity was noted in the ability of 5′-AMP or 3′,5′-AMP to enhance the rate of oxidation of glutamate, inasmuch as 2′-AMP, 3′-AMP, and cyclic 2′,3′-AMP were ineffective in augmenting oxygen uptake. The stimulation of α-ketoglutarate oxidation by brain homogenates may be explained by the rapid conversion of this substrate to glutamate by transamination. The ability of cyclic 3′,5′-AMP to enhance glutamate oxidation by homogenates was found to be somewhat specific for brain tissue, since this nucleotide did not increase glutamate oxidation by heart or testis homogenates and evoked only slight stimulation of oxygen uptake when liver or kidney preparations were studied. With the partially purified enzymes from liver, kidney, and brain, differences were observed in the degree of activation by nucleotides, in the elution pattern from Sephadex G-200, and in the apparent K'_m for NAD in the presence of adenine nucleotides. These findings lend credence to the concept that the glutamate dehydrogenases from liver, kidney, and brain do not have identical properties.

The data presented in Fig. 4 and Table 6 show that cyclic 3′,5′-AMP and 5′-AMP decreased the K'_m for NAD from 2.0 mM to 0.5 mM and 0.4 mM, respectively. However, these nucleotides did not appreciably alter the maximal velocity (V_{max}) of the reaction. A number of investigators have found brain NAD levels to be approximately 0.2 mM (30). At that concentration of NAD the enzyme was found to possess little activity in the absence of activator and was fully active in the presence of activator. It has been reported that NAD levels in the liver are considerably higher than in the brain, i.e., 1.0 mM (30), and preliminary evidence indicates that at that NAD concentration the liver enzyme possesses considerable activity. Although the concentrations of other activators and inhibitors in these tissues must be considered, these results suggest that regulation of glutamate dehydrogenase by adenine nucleotides may be more important in brain than it is in liver.

It has been suggested that inhibition of bovine liver glutamate dehydrogenase activity by diethylstilbestrol (31), thyroxine (32), or NADH (2) might be related to the dissociation of the enzymatically active molecule (mol wt 10^6) into inactive units (mol wt 2.5×10^5). However, this dissociation may not be related to inhibition, since Rogers, Hellerman, and Thompson (25, 33) reported a molecular weight of 2.5×10^5 when the enzyme was markedly activated by methylmercuric bromide. In addition, Frieden and Colman (34) have suggested that activation of bovine liver glutamate dehydrogenase may involve conformational changes in the monomeric form of the enzyme. The results presented in this paper for rat brain glutamate dehydrogenase show that in the concentration range used for enzymatic assay, the enzyme is dissociated into units of about 2.5×10^5 mol wt in both the absence and presence of 5′-AMP or cyclic 3′,5′-AMP.

Mandel and Harth (35) demonstrated that the level of 5′-AMP in rat brain may vary between 0.025 and 1 mM, depending on the procedure used to kill the animals, and suggested that the normal level of 5′-AMP in brain is approximately 0.025 mM. It is of interest that the results reported herein show that 0.025 mM 5′-AMP appreciably enhanced glutamate dehydrogenase activity; however, considerably higher concentrations (Fig. 2) were required for maximal activation. Cyclic 3′,5′-AMP enhanced the rate of oxidation of glutamate by brain preparations only slightly at concentrations as low as 0.033 mM. This concentration is relatively close to the level of cyclic 3′,5′-

AMP reported for rabbit cortex slices following a brief incubation with norepinephrine and theophylline, i.e., 0.2 mM (14), but is considerably higher than the reported normal level of 0.001 mM (14, 23, 24). At concentrations of 5′-AMP below 0.1 mM, addition of 0.025–0.1 mM cyclic 3′,5′-AMP produced an additive effect on glutamate oxidation and glutamate dehydrogenase activity. From these results it would appear that at comparable concentrations cyclic 3′,5′-AMP is not as effective as 5′-AMP in enhancing brain glutamate dehydrogenase, but the effects of the cyclic nucleotide may be additive with 5′-AMP. To what extent these nucleotides are involved in the regulation of brain glutamate metabolism remains to be elucidated.

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