Purification and Some Properties of Mouse Brain L-Glutamic Decarboxylase*

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ABSTRACT: L-Glutamic acid decarboxylase has been purified from mouse brain to the extent of approximately 150- to 200-fold by successive chromatographic procedures on calcium phosphate gel, DEAE-cellulose, and Sephadex. Throughout the purification steps the enzyme was stabilized with pyridoxal phosphate (pyridoxal-P) and aminoethylisothiuronium bromide. The pH optimum of the enzyme shifted toward the alkaline side during the purification, from approximately 6.4–6.5 in the original homogenate to 7.2–7.5 in the Sephadex fractions, and the K_m values were increased during purification.

The enzyme is inhibited by anions, the halide anions inhibiting in the order $I^- > Br^- > Cl^- > F^-$. A study of the inhibition by Cl^- ions showed it to be competitive with substrate. The extent of inhibition decreased progressively with increasing pH. Since the inhibition by Cl^- ions occurs within physiological ranges it is suggested that Cl^- ion fluxes during nerve activity may play a role in the control of the rate of production of γ-aminobutyric acid from L-glutamic acid by the decarboxylase.

γ-Aminobutyric acid (γ-ABA)[1] has been shown to have inhibitory physiological effects in experiments with a wide variety of vertebrate (Krnjevic, 1964) and invertebrate test systems, presumably because it can increase the stability of both pre- and postsynaptic neuronal membranes by increasing their permeability to K^+ and Cl^- ions (for review, see Roberts *et al.*, 1964). γ-ABA is found in large concentrations only in the central nervous system of vertebrate organisms and is formed to a large extent, if not entirely, from L-glutamic acid by an L-glutamic acid decarboxylase (GAD) which is found specifically in the central nervous system, largely in the gray matter (Roberts and Eidelberg, 1960; Roberts *et al.*, 1964). Elucidation of a number of the important biological properties of the γ-ABA system awaits the purification and detailed study of GAD. Previous attempts to purify the GAD from mouse brain, the richest source of this enzyme, repeatedly have met with failure because of the lability of the enzyme. The stabilization of the enzyme with pyridoxal phosphate (pyridoxal-P) and aminoethylisothiuronium bromide (AET) (M. Takacs and E. Roberts, 1965, personal communication) made possible the present degree of purification.

Materials and Methods

Materials. Brains of Swiss mice were employed as the source of enzyme. Large numbers of mice were given us by Riker Laboratories. Pyridoxal-P was donated to us by California Corp. for Biochemical Research, Los Angeles, Calif. L-[1-^{14}C]Glutamic acid prepared by synthesis and resolution also was obtained from the latter source. Calcium phosphate gel was prepared by the procedure of Tisèlius *et al.* (1956). Sephadex was purchased from Pharmacia Fine Chemicals, New York, and DEAE-cellulose, Lot No. 1236 was obtained from Brown Co., New Hampshire. Other analytical grade reagents were obtained from regular commercial sources.

Starting Material for Enzyme Purification. Earlier studies of brain GAD (Roberts and Frankel, 1951a,b; Roberts and Simonsen, 1963) were made using mouse brain acetone powders. Attempts at purification of the enzyme from the acetone powders were not successful because the various extraction procedures gave extracts possessing high concentrations of poorly separable proteins. Studies of the subcellular distribution of GAD by Salganicoff and De Robertis (1965) showed that this enzyme is relatively highly concentrated in nerve ending particles which sediment in the "crude mitochondrial" pellet from sucrose homogenates. This pellet was employed as a starting material for subsequent purification in the present study.

In a typical preparation approximately 1000 mice were killed by cervical dislocation, the whole brains minus the brain stem removed rapidly, and a 10% homogenate made in ice-cold 0.25 M sucrose in a motor-driven glass homogenizer with a Teflon pestle. A heavier fraction was removed by centrifugation for 15 min at 900*g* in a refrigerated centrifuge and the "crude

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[1] Abbreviations used in this work: γ-ABA, γ-aminobutyric acid; GAD, L-glutamic acid decarboxylase; AET aminoethylisothiuronium bromide; pyridoxal-P, pyridoxal phosphate.

TABLE I: Steps in the Purification of Mouse Brain Glutamic Decarboxylase.

Fractions	Total Act. (%)	Total Protein (%)	Specific Act.[a]	Purification
Original homogenate	100	100	0.43	1
Lysate of "crude mitochondrial fraction"	25.5	3.6	3.2	7.1
Calcium phosphate gel filtration	4.8	0.19	10.6	25.2
DEAE-cellulose fraction	1.6	0.021	32.3	76.4
Sephadex fraction	0.35	0.0022	68.6	158.0

[a] Micrograms of γ-ABA formed per minute per milligram of protein under the standard conditions of assay.

mitochondrial" fraction was sedimented from the supernatant fluid by centrifugation for 15 min at 17,400*g* in the No. 30 rotor of the Spinco L-2 centrifuge. After removal of the supernatant fluid, the pellet was recentrifuged for 15 min at 55,000*g* to remove as much entrained sucrose as possible. The pellet was rehomogenized in an amount of nitrogen-gassed glass-distilled water which was one-tenth of the volume of the original suspending fluid. The suspension was stored on ice for 20 min to permit osmotic rupture of particles and then centrifuged for 60 min at 100,000*g*. The supernatant fluid was decanted and a sufficient quantity of 0.1 M potassium phosphate buffer, pH 7.2, was added to make the buffer concentration in the supernatant 1 × 10^{-3} M. Pyridoxal-P and AET were added so that the final concentrations of these substances were 10^{-4} and 10^{-3} M, respectively. In all procedures to be discussed subsequently, the solutions employed in equilibration of columns, in elution procedures, and during dialysis contained the above concentrations of pyridoxal-P and AET. The above supernatant of the water-lysed "crude mitochondrial" preparation was the starting material for further purification of the GAD. Such enzyme preparations were fairly stable at cold room temperatures. Excessive exposure to light was avoided throughout the subsequent procedures because the enzyme was found to be sensitive to light.

Assay Procedures. The method for the measurement of GAD activity, described in detail previously (Roberts and Simonsen, 1963), depends on the evolution of $^{14}CO_2$ from L-[1-^{14}C]glutamic acid under anaerobic conditions. Substrate was prepared by dissolving L-[1-^{14}C]glutamic acid in 0.1 M potassium phosphate buffer, pH 6.2, to which was added carrier glutamic acid at pH 6.5. The substrate solution also contained pyridoxal-P at 10^{-4} M final concentration. A 0.1-ml aliquot of the above substrate containing 20.8 μmoles of glutamate (0.074 μc) was placed in the incubation vessel and the vessel was gassed with prepurified nitrogen. The reaction was initiated by the injection of 1 ml of enzyme preparation at pH 6.5 containing 100 μmoles of potassium phosphate, 0.1 μmole of pyridoxal-P, and 1 μmole of AET, unless stated otherwise. The incubations usually were carried out for 30 min at 37° in a Dubnoff metabolic incubator. The reaction was terminated by the injection of 0.15 ml of 8 N H_2SO_4, the vessels were incubated for another 90 min to achieve quantitative absorption of $^{14}CO_2$ by Hyamine base, and the samples were counted, all as previously described (Roberts and Simonsen, 1963).

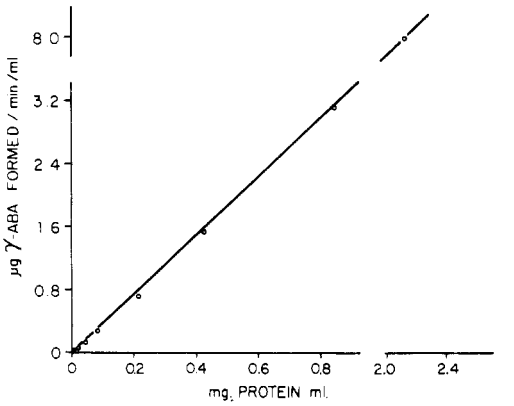

FIGURE 1: Proportionality of GAD activity to protein content of water lysate of "crude" mitochondrial fraction.

The enzyme activities usually were expressed as micrograms of γ-ABA formed per minute per milligram of protein (specific activity), or as micrograms of γ-ABA formed per minute per milliliter. Crude homogenates were assayed in the presence of 0.25 vol. % Triton X-100 to ensure maximal liberation of the enzyme (Van Kempen *et al.*, 1965). The assay was found to be linear at all protein concentrations in the various preparations used. Typical results obtained with the lysate of the "crude mitochondrial fraction" are shown in Figure 1.

Protein contents were determined by a modification (Miller, 1959) of the Lowry procedure (Lowry *et al.*, 1951). The blank values in the protein method were elevated by the presence of pyridoxal-P and AET. Whenever possible, samples were diluted fivefold

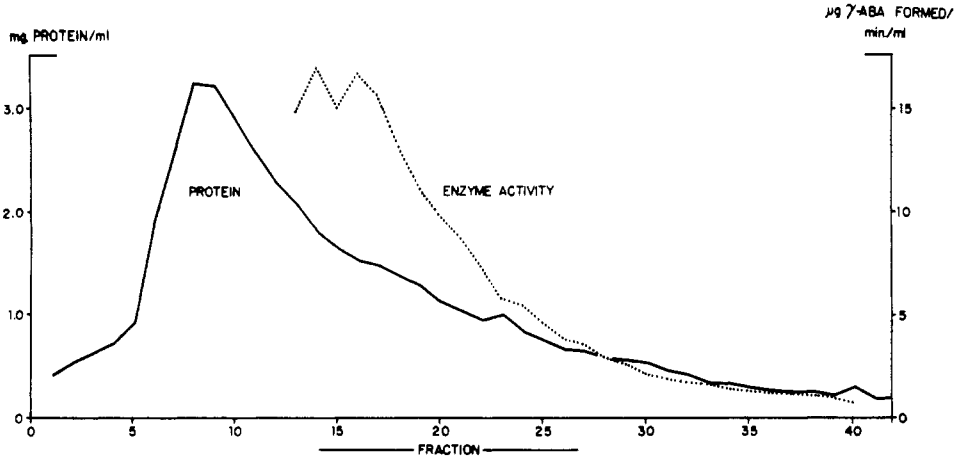

FIGURE 2: Calcium phosphate gel chromatography of GAD.

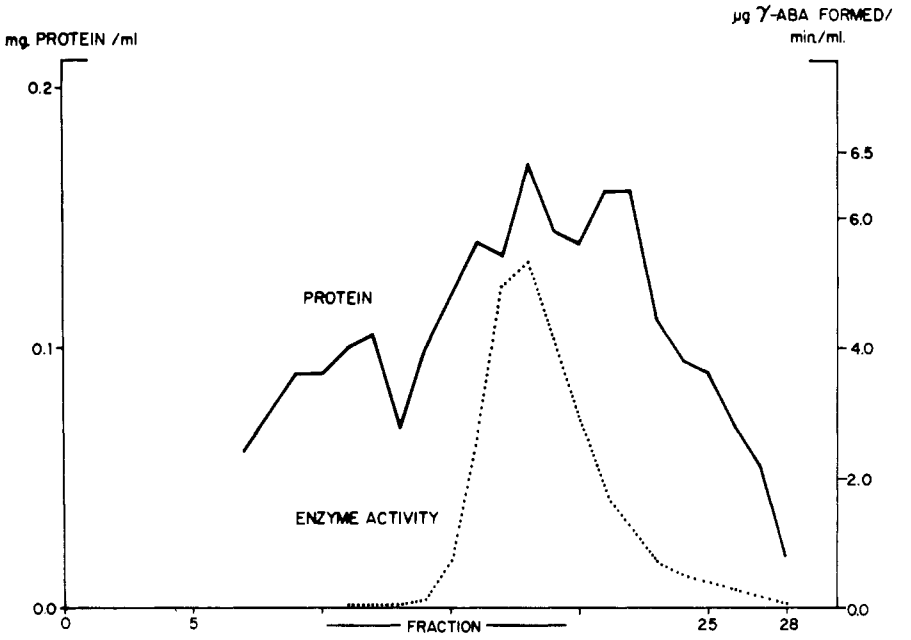

FIGURE 3: Chromatography of GAD on DEAE-cellulose.

or more prior to assay. Samples containing only small amounts of protein were dialyzed against distilled water.

Results and Discussion

Purification Steps. The results of a typical purification are shown in Table I. The aqueous extract prepared from the "crude mitochondrial" pellet contained approximately 27% of the GAD activity of the whole homogenate and only approximately 4% of the protein. This represented approximately a sevenfold increase in specific activity of the extract over the homogenate. Many attempts were made to fractionate this extract with ammonium or sodium sulfate with little success. Chromatography on calcium phosphate gel employing nonlinear ionic gradients for elution made it possible to obtain another threefold increase in specific activity with approximately a 20% recovery of the enzyme activity applied to the column (Table I, Figure 2). Prior to use the calcium phosphate gel was equilibrated with a solution identical with that in which the enzyme extract was contained. The gel (14 ml/extract from 100 mouse brains) was packed in a chromatographic column, 1 cm in diameter, to a height of 20 cm and the remainder of the column was filled with Hyflo Super Cel (Fisher). The enzyme extract was allowed to adsorb under gravity flow and the column was washed with one gel volume of solution of the same composition: 0.001 M potassium phosphate buffer,

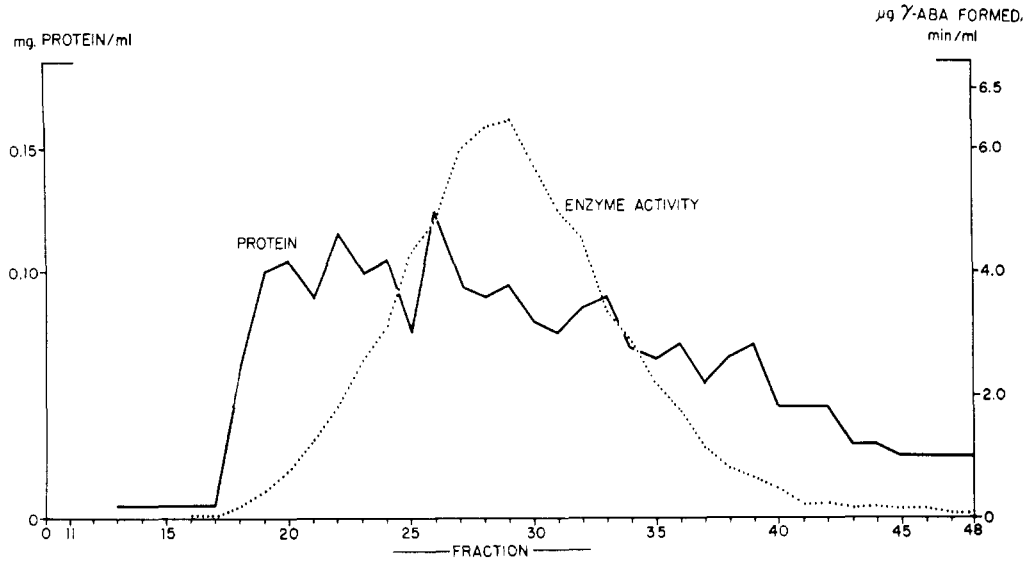

FIGURE 4: Chromatography on a mixed-bed Sephadex column.

pH 7.2, 1×10^{-4} M pyridoxal-P, and 1×10^{-3} M AET. After allowing the column to drain, a nonlinear gradient of phosphate buffer, pH 7.2, was allowed to run through. The mixing chamber, containing 100 ml of 0.01 M buffer, was connected to a separatory funnel placed above it which contained 300 ml of 0.1 M buffer, and the outflow was connected to a closed chromatographic column. Fractions of 5 ml each were collected and protein contents and enzyme activities were estimated. To utilize the calcium phosphate procedure successfully it was necessary to employ freshly prepared enzyme extract. In a typical experiment (Table I, Figure 2) the five fractions with the highest specific activity (fractions 13–17) were pooled and dialyzed overnight at 4° against a 50-fold volume of 0.005 M Tris–phosphate buffer, pH 7.2.

A 1-cm column was packed to a height of 10 cm with DEAE-cellulose, previously equilibrated with buffer having the same composition as that employed for dialysis using a chromatography pump and the same flow rate as used in the subsequent elution procedure. The sample was applied with a chromatography pump, and the column was washed with the dialysis buffer. Elution of the column was accomplished with a gradient of increasing ionic strength and decreasing pH in the manner described by Moore and McGregor (1965). In some of the more recent experiments use has been made of a multichambered gradient device manufactured by Buchler Instrument Co., constructed according to Peterson and Sober (1959). The solutions employed were identical with those previously described by Moore and McGregor (1965) with the exception that all of our solutions contained pyridoxal-P and AET. Prior to the determination of activity all samples were dialyzed extensively against 0.1 M potassium phosphate buffer. The two highest fractions (Figure 3, Table I) had 9.7 mg of protein

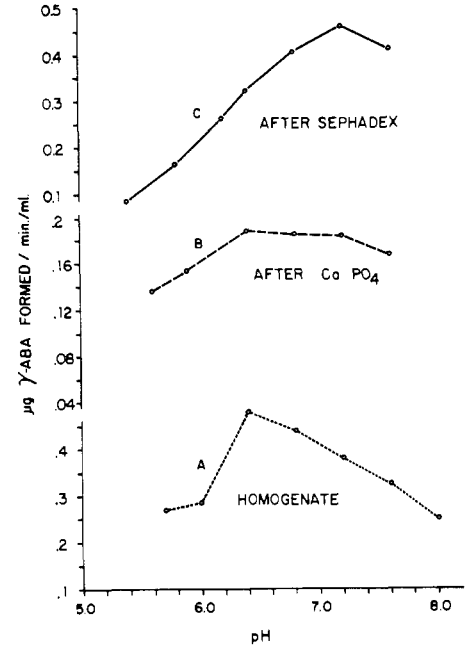

FIGURE 5: pH–activity curves of GAD at various stages of purification.

with specific activity 75-fold greater than that of the original homogenate.

The above fractions were concentrated to 3.5 ml by ultrafiltration and employed in subsequent Sephadex chromatography. As a result of preliminary experiments it was determined that an optimal arrangement was to use a large double-bed column (Sephadex, 3 × 47 cm). The lower two-thirds was filled with Sephadex G-100 and the upper third with Sephadex

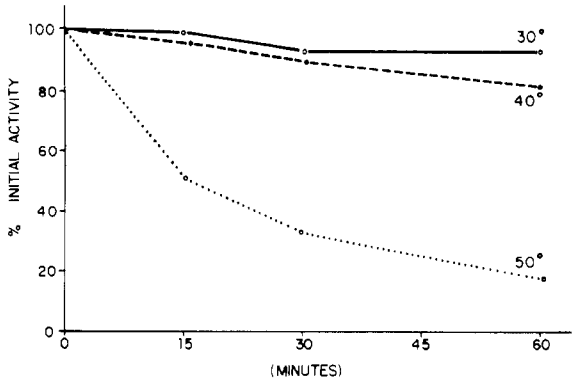

FIGURE 6: Thermal stability of GAD at three temperatures. Enzyme used was one which has been carried through the calcium phosphate gel chromatography (25-fold purified).

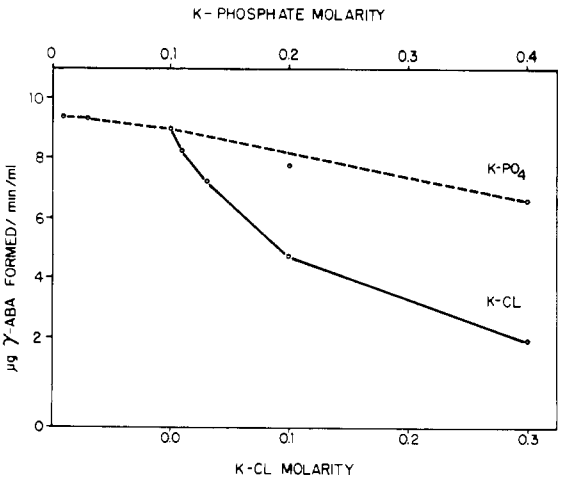

FIGURE 7: The influence on GAD activity of the addition of KCl or potassium phosphate to 0.1 M potassium phosphate buffer.

G-200. The gels previously had been equilibrated with 0.1 M potassium buffer, pH 6.5. After application of the sample to the column, elution was carried out with the above buffer at a flow rate of 20 ml/hr. The pooled fractions 27–30 (Figure 4, Table I) yielded 1 mg of protein with a specific activity of 159 relative to that of the starting material, but less than 1% of the original activity was recovered. Work is in progress to develop procedures which will improve the yield. The amounts of purified material were insufficient to allow physical studies of the protein. However, the characteristic K_D values (Gelotte, 1964) observed on separate chromatography on Sephadex G-100, G-200, and G-75 suggest that the molecular weight of GAD is in the range of 75,000–100,000.

Optimal pH for Enzyme Activity and K_m Values. The pH optimum of the crude GAD has been reported to be approximately 6.4 (Roberts and Simonsen, 1963). A similar value has been found in the present study for the original homogenate (curve A, Figure 5). The preparation obtained after calcium phosphate gel chromatography (curve B, Figure 5) showed essentially constant activity between pH 6.4 and 7.2, while the most highly purified preparation was maximally active at pH 7.2. The sequence of shifts in pH optimum has been observed during the course of a number of similar preparations and the optimum pH of a number of purified preparations ranged from 7.2 to 7.5. Interestingly, the K_m values for the crude enzyme (3×10^{-3} M) at pH 6.4 were lower than those observed for the calcium phosphate eluate ($3.6–4.0 \times 10^{-3}$ M), or for the preparation obtained on chromatography on Sephadex (7.9×10^{-3} M, measured at pH 7.2; $5.2–7.2 \times 10^{-3}$ M, measured at pH 6.4). This suggests that the purification steps employed may have produced configurational changes in the enzyme protein which resulted in a change in pH optimum and in a decreased affinity of the enzyme for the substrate. Since all enzyme measurements during the enzyme purification procedures were made at pH 6.4, it is likely that the purification factor reported in Table I for the most highly purified preparation, possessing a pH optimum of 7.2, is higher than the degree of purification stated.

Heat Stability. The enzyme is stable if kept at 30 and 40° for 1 hr prior to measurement of activity (Figure 6). However, a rapid loss of activity was noted at 50°. Preliminary experiments have shown the heat stability of the enzyme to be greatly increased when it is adsorbed on calcium phosphate gel. There was no loss of activity when enzyme adsorbed on gel was heated to 60° for 15 min. It is anticipated that this knowledge may be useful in attempting to improve the degree of purification at the calcium phosphate gel step of the procedure.

Inhibition by Anions. During the course of analysis of fractions obtained from DEAE-cellulose chromatography it was observed that the GAD was inhibited by the high concentrations of NaCl employed. The inhibition was readily reversible upon dialysis. The results of the experiment in Figure 7 show that KCl added to 0.1 M potassium phosphate is much more inhibitory than equimolar amounts of potassium phosphate. The above experiment and similar ones with NaCl showed that the inhibition largely could be attributable to the Cl^- ion. Subsequent experiments with enzyme carried through the Sephadex purification step gave the following percentage inhibitions with the sodium salts of various anions at pH 6.4 and 7.2, respectively: iodide, 94.8 and 84.7; nitrate, 79.6 and 68.4; sulfate, 77.1 and 61.5; bromide, 75.7 and 68.8; chloride, 67 and 58.6; acetate, 37.5 and 31.7; and fluoride 5.2 and 18.6. The inhibition by all of the above anions was shown to be reversible by dialysis. Measurement of the inhibition as a function of substrate concentration in the absence of Cl^- ions and in the presence of two concentrations of NaCl showed the inhibition to be competitive in nature (Figure 8).

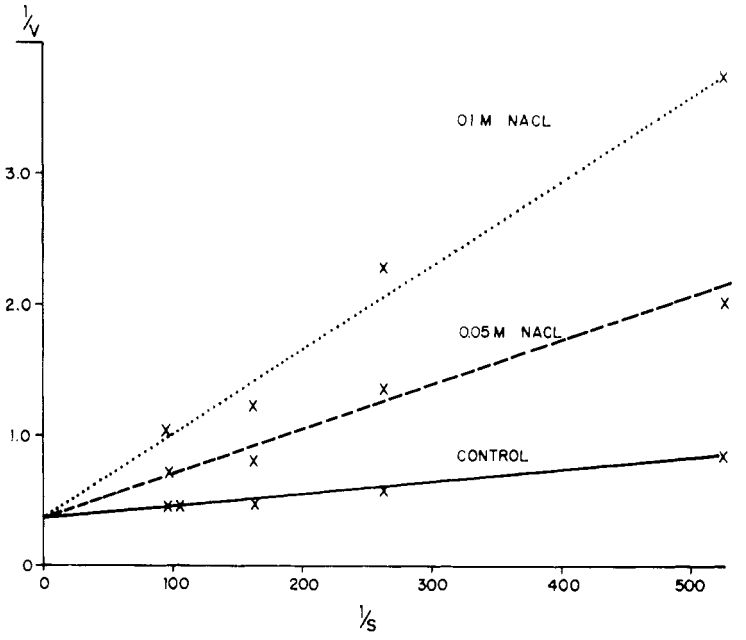

FIGURE 8: Linweaver–Burk plot in the absence and presence of two concentrations of NaCl.

The order of inhibition by the various anions is very similar to the order often observed in studies of anion binding to proteins and anion exchangers (Ling, 1962). It was particularly instructive to plot the values for relative free energy of adsorption of several anions on Dowex 1 and 2, assuming the ΔF_{rel} for the acetate ion to be 0, against the percentage inhibition of GAD by these anions at 0.05 M concentrations (Figure 9). At both pH 6.4 and 7.2 there was a linear relationship between the ΔF_{rel} of adsorption on Dowex 1 and 2, both strongly basic resins, and the degree of inhibition of GAD. The degree of inhibition was less for each anion at pH 7.2 than at pH 6.4. These results suggest that the anionic inhibition may be attributable to the interaction with positively charged groups of the enzyme. The results of an experiment in which inhibition by 0.05 M Cl^- was determined at different pH values are consistent with such an interpretation (Figure 10), since the degree of inhibition decreased with increasing pH over a range in which the enzyme activity was increasing. The results (see curve C, Figure 10) also suggest that more than one type of cationic group may be involved in the interaction of GAD with Cl^- ions or other anions. The competitive nature of the inhibition (Figure 8) suggests that at least one of the positively charged groups with which the inhibitory anions can combine is at the active site of the enzyme. It previously has been suggested that sites of attachment of the carboxyl groups of glutamic acid to GAD may be an ϵ-amino group of lysine residue in the apoenzyme and the positively charged pyridine ring nitrogen of the coenzyme (Roberts and Simonsen, 1963). As in the case of GAD, fumarase is inhibited in decreasing order by iodide, bromide, and chloride, respectively (Massey, 1953). However, in the latter instance the inhibition was noncompetitive. Similarly, the monoamine oxidase from brain is inhibited in a noncompetitive manner by anions, the inhibition being less at higher levels of pH (Van Woert and Cotzias, 1966).

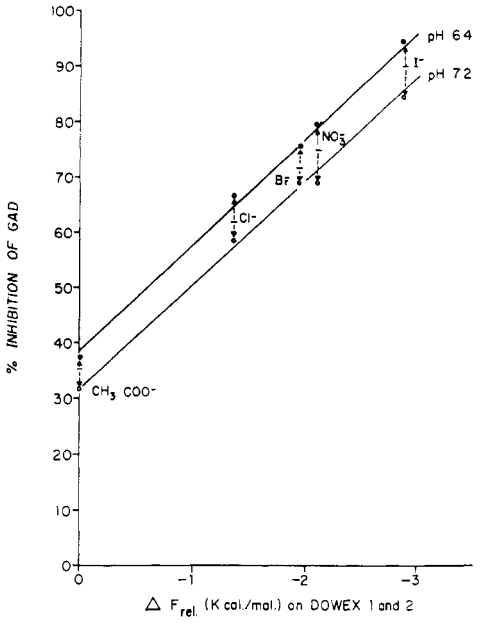

FIGURE 9: Linear relationship between inhibition of GAD by several anions at pH 6.4 and 7.2 and ΔF_{rel} of adsorption of these anions on Dowex 1 and 2 (glutamate concentration, 2×10^{-3} M).

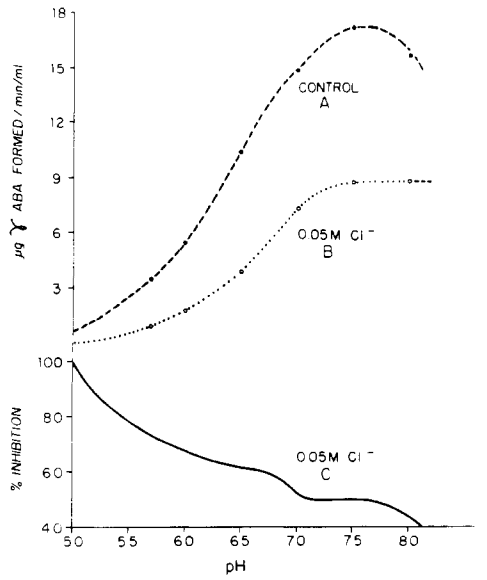

FIGURE 10: Activity of GAD (Sephadex prepared) in the presence and absence of Cl^- as a function of pH (glutamate concentration used, 2×10^{-3} M).

and McGregor (1965). The chromatographic behavior of GAD on DEAE-cellulose and preliminary experiments with starch gel electrophoresis showed that GAD is an acidic protein and suggests that it might be identical with that described by Moore and McGregor (1965). Experiments with agar gel double-diffusion tests employing GAD preparations at various stages of our purification procedure and antibody prepared against the protein of Moore and McGregor gave negative results[2] in all instances except in the case of the lysate of the "crude mitochondrial" pellet.

Comment

The brain GAD differs from the bacterial enzyme (Shukuya and Schwert, 1960), not only in regard to pH-optimum and K_m values but also in that the brain enzyme is inhibited by Cl^- and other anions while the bacterial enzyme is activated by anions. The order of diminishing effectiveness of activation by halides of the bacterial GAD was chloride, bromide, and iodide, which was the order of decreasing potency of inhibition of the brain enzyme. The explanation for the effects of anions awaits further characterization. However, the inhibition of brain GAD by Cl^- ions may already be envisioned to have some physiological importance. It has been shown that glutamic acid has a potent excitatory effect on neurons in both invertebrate and vertebrate preparations (Krnjevic, 1964; Crawford and Curtis, 1964; Takeuchi and Takeuchi, 1964). The possibility is open that glutamic acid may be an excitatory transmitter and that γ-ABA, which is made from glutamic acid by GAD, may be an inhibitory transmitter (Krnjevic, 1964; Takeuchi and Takeuchi, 1964). The variation of Cl^- content at nerve endings may regulate the GAD activity and thus determine the proportions of glutamic acid and γ-ABA which might be released on stimulation. Under specific conditions of glutamate and Cl^- concentrations it is even possible to suggest that the Cl^- concentration in a given nerve ending may determine whether it releases predominantly glutamate or γ-ABA when it is stimulated, and thus whether or not it has an excitatory or inhibitory function. In a preliminary study of Cl^- concentration on the degree of inhibition of a partially purified preparation of GAD in the presence of 2×10^{-3} M substrate, approximately 70 and 20% inhibition was observed at 0.1 and 0.02 M Cl^-, respectively, reasonable values for extracellular and intracellular concentrations of Cl^- ion.

Acknowledgments

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[2] The antibody was kindly supplied to us by Professor Lawrence Levine of the Biochemistry Department of Brandeis University, Waltham, Mass.

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Direct and ^{18}O-Exchange Measurements Relevant to Possible Activated or Phosphorylated States of Myosin*

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ABSTRACT: A careful search has been made for a covalently linked phosphoryl group in myosin which has cleaved one $AT^{32}P$ per mole in a "burst" reaction. No evidence for a phosphoryl myosin was obtained upon extraction of protein with phenol near neutral pH, or by measurement of ^{18}O incorporation into inorganic phosphate (P_i) formed when trichloroacetic acid–$H^{18}OH$ was used to stop adenosine triphosphate (ATP) hydrolysis. Further, with EDTA or phenol as stopping agents in the presence of $H^{18}OH$, lack of appreciable ^{18}O incorporation into P_i does not support possible presence of a labile acyl phosphate. When the "burst "reaction is carried out in $H^{18}OH$, the initial P_i formed shows extensive exchange with water oxygen. In continued hydrolysis, water oxygen appears exclusively in the P_i and not in the adenosine diphosphate (ADP) formed or in the unhydrolyzed ATP. Presence of 2,4-dinitrophenol slightly accelerates the oxygen-exchange reaction, but the *p*-mercuribenzoate inhibits the exchange. This *p*-mercuribenzoate inhibition appears distinct from the activation of adenosine triphosphatase (ATPase) by the mercurial.

Among the myriad studies on the ATPase[1] action of myosin, various experimental findings have raised the possibility of the formation of a phosphorylated or an activated form of myosin during ATP cleavage. These include the occurrence of an initial rapid phase or "burst" of ATP hydrolysis (Weber and Hasselbach, 1954). This has recently been studied in considerable detail by Tonomura and associates under conditions where the "burst" of P_i liberated is approximately 1 mole/mole of myosin (Tonomura and Kanazawa, 1965; Imamura *et al.*, 1965). The suggestion has been made that this reflects a transient phosphorylation of myosin (Tonomura and Kanazawa, 1965). Other pertinent findings are the extra oxygen incorporation from HOH into inorganic phosphate (P_i) that accompanies ATP hydrolysis (Levy and Koshland, 1959; Levy *et al.*, 1960), and the induced exchange between medium P_i and HOH (Dempsey *et al.*, 1963). Some plausible explanations for these exchanges involve reversible phosphorylation of an active site by P_i.

Although a considerable search, likely mostly unpublished, has been made for a phosphorylated myosin, no convincing evidence for a covalent phosphoryl intermediate has been reported. One purpose of the present experiments was to apply more sensitive methodology to the possible direct demonstration of a phosphorylated myosin, with particular attention to preparations just after occurrence of an approximately stoichiometric "burst" of P_i release. Correlated with these studies, the incorporation of water oxygen into P_i during or at the time of stopping the ATPase action was measured as a possible means of revealing formation of any very labile phosphorylated intermediate. Other experiments with ^{18}O are reported which indicate lack of formation of activated ADP or ATP during ATP hydrolysis, and suggest that myosin SH groups may be of particular importance in the extra oxygen exchange accompanying ATP cleavage.

Experimental Section

Myosin Preparation. Myosin was prepared from

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[1] ATPase, adenosine triphosphatase; ATP and ADP, adenosine tri- and diphosphates.