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# PURIFICATION AND PROPERTIES OF TWO SUCCINATE SEMIALDEHYDE DEHYDROGENASES FROM HUMAN BRAIN

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## Summary

In human brain there are two major isoenzymes of succinate semialdehyde dehydrogenase (succinate-semialdehyde:$NAD^+$ oxidoreductase, EC 1.2.1.24). They are composed of two apparently identical subunits with a molecular weight of 69 000. The $K_m$ (limits) for their substrates $NAD^+$ and succinate semialdehyde are $1.6 \cdot 10^{-5}$ M and $3.7 \cdot 10^{-6}$ M, respectively, for one enzyme, and $1.85 \cdot 10^{-5}$ M and $2 \cdot 10^{-6}$ M, respectively, for the other. These values, and other kinetic data obtained from the two enzymes are not very different. However the enzymes differ in the following respects: their behaviour on ion exchange and 5′-AMP affinity columns, their isoelectric points, their tryptic fingerprints and in their amino acid compositions.

## Introduction

Succinate semialdehyde dehydrogenase (succinate-semialdehyde:$NAD^+$ oxidoreductase, EC 1.2.1.24) in the nervous system is of great interest since it catalyses the oxidation to succinate of succinic semialdehyde, a product of the degradation of 4-aminobutyrate, the major inhibitory transmitter. The other two enzymes of the 4-aminobutyrate shunt pathway, glutamate decarboxylase (EC 4.1.1.15) and 4-aminobutyrate:2-oxoglutarate transaminase (EC 2.6.1.19) have been more extensively studied [1—9]. Rat brain succinate semialdehyde dehydrogenase has recently been purified and characterized; no isoenzymes were detected [10]. However, initial work by Embree and Albers on the human enzyme [11] suggested the presence of isoenzymes. This is confirmed in the present work in which two isoenzymes were purified and their properties studied.

## Materials and Methods

### *Reagents*

Succinate semialdehyde was prepared by hydrolysis of γ-ethoxy-butyrol-

actone and assayed enzymatically with pure rat brain succinate semialdehyde dehydrogenase [19]. Other reagents were of analytical grade.

*Enzyme assays*

In order to detect the enzyme from column chromatography eluates, 50-$\mu$l samples were pipetted into tubes containing cold buffer consisting of 100 mM Tris · HCl (pH 8.0)/50 mM KCL/3 · $10^{-4}$ M $NAD^+$/0.1 mM EDTA/20 mM 2-mercaptoethanol. The reaction was started by rapid addition of succinate semialdehyde to a final concentration of 1 · $10^{-4}$ M and the tubes were incubated at 37°C for 5 min. After cooling, the fluorescence of the NADH produced was measured in a Zeiss fluorimeter at excitation 355 nm emission 470 nm.

For quantitative enzyme assays of samples from each step of the purification, the same incubation medium was used except that Triton X-100 was added to a final concentration of 1% to the incubation medium in which the activity of the initial homogenate was assayed. Triton increased the yield in this fraction but had no effect and was omitted from other samples. The incubation medium was maintained at 37°C in thermostatted cuvettes in the fluorimeter and the reaction was started by addition of 50 $\mu$l of suitably diluted enzyme. The rate of increase in fluorescence was recorded directly (initial rates) and the fluorimeter was calibrated by a solution of NADH previously assayed by its optical density at 340 nm.

*Protein determinations*

Lowry's method [12] was used for samples obtained before ion exchange chromatography. At later stages when the protein concentration was low, the fluorescamine method was used [13].

*Enzyme purification*

*Extraction.* 382 g of human brain which had been obtained about 12 h post-mortem and kept frozen at —70°C until use, were suspended in a total volume of 1.5 litres of cold medium containing 5 mM mercaptoethanol, 2 mM sodium EDTA pH 7.2 and 0.5 mM phenylmethylsulphonyl fluoride.

The suspension was homogenised for a total of 6 min in a commercial food blender at maximum speed allowing intervals to prevent significant heating. The homogenate was briefly centrifuged at low speed to remove the resultant foam. The homogenate was then centrifuged at 15 000 $\times g$ for 2 h. About 1 litre of clear supernatant was obtained.

*Ammonium sulphate precipitation.* The supernatant was kept at 0°C and ammonium sulphate was slowly added with stirring to a final concentration of 80% saturation. The pH was maintained at 7.2 throughout by small additions of concentrated $NH_4OH$. The suspension was centrifuged for 1.5 h at 15 000 $\times g$ and the pellets were redissolved in about 150 ml of dialysis medium.

*Dialysis.* The solution was dialyzed for 2 days against three changes of 10 l medium consisting of 2 mM phosphate buffer pH 7.2, 5 mM mercaptoethanol and 0.1 mM EDTA. This procedure and all the following steps were performed in a cold room at about 5°C.

*Column chromatography.* All buffers contained 2 mM phosphate pH 7.2,

5 mM mercaptoethanol and 0.1 mM EDTA. If the eluates were to be stored before further use, the buffers also contained 10% glycerol. This prevents loss of activity in frozen samples. All columns were extensively rinsed with starting buffer before use.

*First ion exchange chromatographies.* It has previously been established that under our conditions part of the enzyme activity is retained by DEAE-cellulose and the part which is not retained is fixed by CM-cellulose under the same conditions. Thus two columns were connected in series. The first consisted of 2.5 × 15 cm of CM-cellulose (Whatman CM 52) and the second of 2.6 × 25 cm of DEAE-cellulose (Whatman DE 52). The dialyzate was thus adsorbed onto both columns simultaneously. After extensive rincing with starting buffer, the columns were separated from each other. The DEAE-cellulose column was eluted with a linear gradient of 1 litre of 0 to 100 mM KCl, and the CM-cellulose column eluted with a linear gradient of 500 ml of 0 to 100 mM KCl. The eluates were collected in 5-ml fractions.

*5'-AMP Sepahrose affinity chromatography.* The active fraction from the CM-cellulose column (hereafter called "CM" enzyme) was adsorbed directly onto a 1 × 10 cm column of Pharmacia 5'-AMP substituted Sepharose 4B. After rinsing, the column was eluted with a linear gradient of 200 ml, 0 to 2 mM AMP. The active fraction from the DEAE-cellulose column (hereafter called "DE" enzyme) was first dialyzed to remove KCl and then applied to a similar AMP Sepharose column. After rinsing it was eluted with 200 ml of a linear gradient of 0 to 0.5 mM AMP.

*Second ion exchange chromatographies.* The DE enzyme eluted from the affinity column was absorbed onto a second DEAE-cellulose column (1 × 10 cm) and eluted with a linear gradient of 100 ml 0 to 100 mM KCl. The CM enzyme was adsorbed onto a 1 × 10 cm hydroxyapatite column. After rinsing, a gradient of 200 ml of 0 to 2.5 mM KCl was applied. This was insufficient to elute all of the enzyme. Thus, elution was continued with about 200 ml of 2.8 M KCl. As the resultant enzyme was very dilute, it was concentrated using diaflo cell (Aminco) equipped with a PM 10 membrane. To lower the KCl concentration, the enzyme was rediluted with 2 mM phosphate buffer, pH 7.2, containing 20 mM mercaptoethanol, 0.1 mM EDTA and 10% glycerol and then the enzyme was reconcentrated with the same apparatus.

*Physical properties and structure*

*Molecular weights of native enzymes.* This was determined by gel filtration on a 2.5 × 75 cm column of Sephadex G200 by the method of Andrews [14]. The column was equilibrated with 2 mM phosphate buffer, pH 7.2, containing 100 mM KCl, 0.1 mM EDTA and 5 mM mercaptoethanol. About 5 μg of enzyme was placed on the column in a total volume of 4 ml containing the following molecular weight markers: γ-globulin, bovine serum albumin, ovalbumin and myoglobin. The column was eluted with the equilibration buffer. The markers were detected by their absorbance at 280 nm, and the succinate semialdehyde dehydrogenase was assayed enzymatically.

*Molecular weights of the subunits.* These were determined by SDS gel electrophoresis as described by Weber and Osborn [15]. The gels contained 1% SDS with or without 6 mM urea. 10 μg of enzyme were incubated for 15 min

at 37°C in the electrophoresis buffer containing 0.1% SDS and 1% mercaptoethanol. The following molecular weight markers were used: γ-globulin, bovine serum albumin, ovalbumin and chymotrypsinogen A. Bromophenol blue was used to mark the front. The mobilities were calculated by the original technique.

*Cyanogen bromide cleavage.* About 20 μg of each enzyme were precipitated with 12% trichloroacetic acid and the pellets redissolved in 250 μl of 70% formic acid. 1 mg of cyanogen bromide was added and the solution was kept in a sealed tube at room temperature for 15 h. Then, the solution was lyophilised and redissolved in 0.3 ml of 0.2 M phosphate buffer pH 8 containing 1% SDS and 1% mercaptoethanol. The solution was incubated for 15 min at 60°C before electrophoresis.

The electrophoretic separation of the resultant peptides was carried out by a slight modification of the technique of Swank and Munkres [16]. The gels consisted of 12.5% acrylamide and 1.25% bisacrylamide buffered with 0.15% Tris/phosphoric acid pH 6.8 containing 0.1% SDS and 6 M urea.

*Tryptic fingerprinting.* About 100 μg of each enzyme were precipitated with 12% trichloroacetic acid. After centrifugation the precipitates were washed twice with 0.5 ml acetone, and then redissolved in 0.2 ml of 0.5 M phosphate buffer pH 7.2 to which was added in each case 2 μg of trypsin (8000 units/mg) previously treated with diphenylcarbamyl chloride to eliminate chymotrypsin activity. Incubation was carried out for 24 h at 37°C then the pH was adjusted to 8.8 with 1 M NaOH. Dansylation of the peptides produced was carried out according to the technique of Zanetta et al. [17]. Dansyl-OH was eliminated by passage through a column of Dowex 50WX8, $H^+$ and rinsing with 0.01 M acetic acid. The eluates containing the dansyl peptides were lyophilysed, redissolved in 200 μl acetone/1 M HCl (9 : 1, v/v) and subjected to two dimensional chromatography on silica gel thin layer plates.

1st dimension: methyl acetate/25% ammonia/isopropanol (9 : 4 : 6, v/v) for 2 h; 2nd dimension: isobutanol/acetic acid/water (15 : 4 : 2, v/v) for 4.5 h. The fluorescent spots were detected with an ultraviolet lamp and were compared for the two enzymes.

*Amino acid composition.* 50 μg of protein were precipitated with 12% trichloroacetic acid and after centrifugation, the precipitate was washed with 1 M HCl. After recentrifugation, the protein was hydrolysed in a sealed tube in 250 μl of 6 M HCl for 18 h at 110°C. The amino acid composition was determined using a Technicon amino acid analyser. Tryptophan which is destroyed by this method, was not determined.

*Isoelectric focussing.* This was performed on a plate of Ultrodex gel containing Ampholines in the range pH 4 to 10, 0.1 mM EDTA, 10 mM mercaptoethanol and 3 M urea according to the technique of Radola [18]. Urea was used to avoid artifacts due to protein aggregation. Higher concentrations could not be used, as all activity would be destroyed. 3 ml of the dialyzed ammonium sulphate precipitate fraction was applied to the gel. The anode consisted of 1 M phosphoric acid, and the cathode of 1 M sodium hydroxide. Migration took place for 16 h at a constant power of 6 W during which time the plate was maintained at 4°C by circulating cold water. The plate was then divided into 30 fractions with the aid of a grid. Each fraction was removed, diluted with

1 ml of $H_2O$ and the pH was measured. Then the pH of each fraction was brought to 7.2 with concentrated phosphate buffer before enzyme assay.

*Kinetic studies*

*Determination of Michaelis constants, and inhibition studies.* These were performed in 100 mM phosphate buffer, pH 7.2, containing 50 mM KCl/20 mM mercaptoethanol/0.1 mM EDTA. This pH is below the optimum of 8.6 but the enzyme is more stable under these conditions, particularly when low substrate concentrations were used, thus initial rates could be measured for a longer period. Reactions were started by addition of the enzyme diluted with a solution of bovine serum albumin 10 mg/ml, glycerol 10%, mercaptoethanol 20 mM and EDTA 0.1 mM. The incubation medium containing the substrates and inhibitors: AMP and 4-hydroxybenzaldehyde, was maintained at 37°C in the fluorimeter cuvettes. Reaction rates were recorded directly. The results were plotted by Lineweaver-Burk's method and the Michaelis constants were calculated by secondary plots of the intercepts versus the inverse of the substrate concentrations, and the inhibition constants by secondary plots of the slopes versus the inhibitor concentrations.

*Determination of pH optima.* For pH values up to 9, pyrophosphate buffers were used. Above 9, glycine/NaOH buffers were used. All buffers contained 50 mM KCl, 20 mM mercaptoethanol and 0.1 mM EDTA. The reaction rates for each enzyme were determined in the same experiment in the same buffers.

*NAD specificity.* For both enzymes, NAD was substituted by an equimolar concentration of NADP, and the relative reaction rates recorded.

## Results

*Purification*

Table I shows a typical purification as described under Materials and Methods. One unit is defined as the enzyme activity which catalyses the formation of 1 $\mu$mol of NADH per min at 37°C. Specific activity is in units per mg of protein.

The CM enzyme obtained from this preparation had a specific activity of 16 and appeared pure by polyacrylamide gel electrophoresis. In this preparation, the DE enzyme with a specific activity of 7.5 contained about 20% of an impurity judged by the same criterion. In a previous preparation, the DE enzyme had a specific activity of 16.3 and appeared to be pure. Only these enzymes which give a single band on SDS polyacrylamide gels were used for structural studies.

The CM enzyme was quantitatively retained by the AMP Sepharose column and required a gradient of 0 to 2 mM AMP for elution, whereas the DE enzyme is never more than 50% retained by this column but can be eluted with a 0 to 0.5 mM gradient of AMP.

*Molecular weights of native enzyme and subunits.* The molecular weights of both enzymes were both found to be 145 000 ± 5000 by Sephadex G-200 chromatography, and those of the subunits to be 69 000 ± 5000 by SDS gel electrophoresis.

*Cyanogen bromide cleavage.* There are 8 common bands for the two

TABLE I

PURIFICATION OF TWO SUNNICATE SEMIALDEHYDE DEHYDROGENASES FROM 382 g OF HUMAN BRAIN

| Fraction | Volume (ml) | Units (ml) | Total units | Protein (mg/ml) | Units (mg/protein) | Yield (%) | Purification (-fold) |
|---|---|---|---|---|---|---|---|
| Homogenate | 1500 | 0.097 | 146 | 34 | 0.003 | 100 | 1 |
| Supernatant | 1010 | 0.18 | 185 | 12.5 | 0.015 | 127 | 5.1 |
| $0 \rightarrow 80\%$ $(NH_4)_2SO_4$ | 220 | 0.53 | 115 | 17.8 | 0.03 | 79 | 10 |
| DE Cellulose | 248 | 0.22 | 55 | 1.6 | 0.14 | 38 | 48 |
| AMP-Sepharose | 65 | 0.26 | 17 | 0.06 | 4.3 | 11.5 | 1516 |
| 2nd DE Cellulose | 27 | 0.41 | 11 | 0.05 | 6.5 | 7.5 | 2281 DE enzyme |
| CM Cellulose | 112 | 0.39 | 44 | 0.64 | 0.61 | 30 | 215 |
| AMP-Sepharose | 128 | 0.085 | 10.8 | 0.01 | 8.5 | 7.4 | 2980 |
| Hydroxyapatite | 11 | 0.26 | 2.9 | 0.017 | 16 | 1.9 | 5600 CM enzyme |

enzymes (Fig. 1). There is one extra band for the CM enzyme which is not in common with those of the DE enzyme. This result indicates that the methionine distribution is quite similar in the two enzymes.

*Tryptic fingerprinting.* See Fig. 2 for a comparison of the peptide maps produced by tryptic digestion. There are 57 spots for the CM enzyme and 77 for the DE enzyme. Many of the major spots do not coincide for the two enzymes.

*Amino acid composition.* See Table II for a comparison of the amino acid composition of the two enzymes. There are notable differences in the relative percentages of glutamate + glutamine, glycine, proline and isoleucine.

*Isoelectric focusing.* There are two peaks of activity which center at pH 5.6 and 7.2 (Fig. 3).

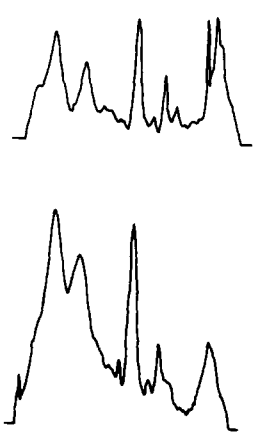

Fig. 1. Densitometry of peptide bands on SDS gels after cyanogen bromide cleavage. Upper: CM enzyme; lower: DE enzymes.

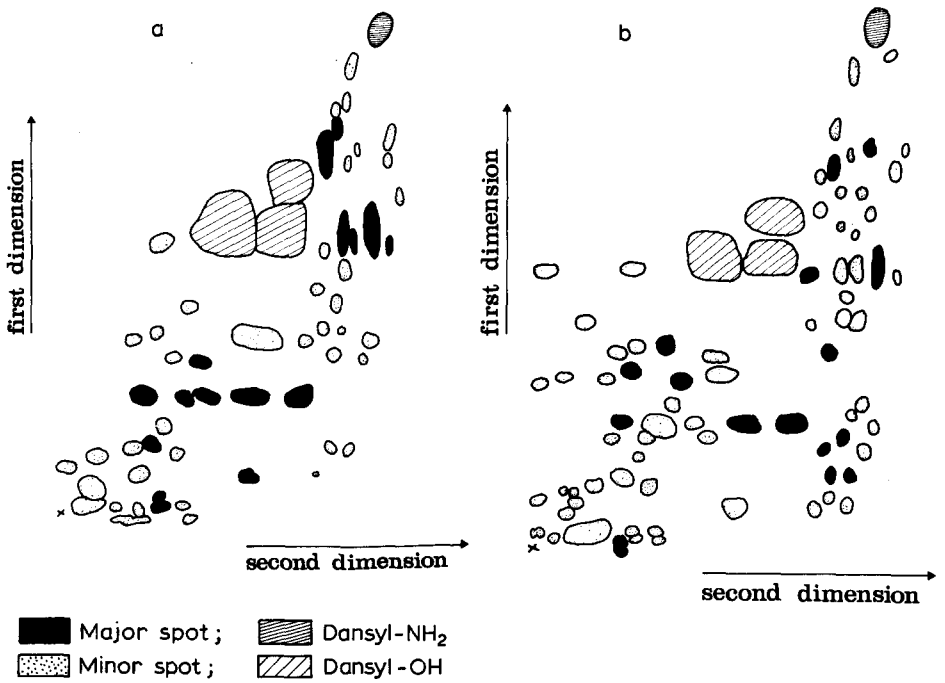


Fig. 2. Tryptic fingerprints. (a) CM enzyme; (b) DE enzyme.

*Kinetic properties*

The $K_m$ values (limit) found for the substrates NAD and succinate semialdehyde for the DE enzyme are $1.61 \cdot 10^{-5}$ M, respectively. For the CM enzyme the

TABLE II

AMINO ACID COMPOSITION

| Amino acid | Residues per mol | | Percentage of total | |
|---|---|---|---|---|
| | CM enzyme | DE enzyme | CM enzyme | DE enzyme |
| Glutamate + glutamine | 81 | 28 | 6.8 | 2.4 |
| Aspartate + asparagine | 79 | 75 | 6.6 | 6.4 |
| Threonine | 171 | 227 | 14 | 19 |
| Serine | 95 | 103 | 8.0 | 8.8 |
| Glycine | 116 | 58 | 9.8 | 4.9 |
| Half-cystine | 3 | 4 | 0.25 | 0.34 |
| Methionine | 2 | 2 | 0.17 | 0.17 |
| Valine | 92 | 141 | 7.7 | 12 |
| Proline | 38 | 2 | 3.2 | 0.17 |
| Arginine | 56 | 43 | 4.7 | 3.7 |
| Histidine | 51 | 47 | 4.3 | 4.0 |
| Lysine | 74 | 75 | 6.2 | 6.4 |
| Phenylalanine | 48 | 47 | 4.0 | 4.0 |
| Tyrosine | 2 | 6 | 0.17 | 0.51 |
| Leucine | 109 | 94 | 9.2 | 8.0 |
| Isoleucine | 65 | 113 | 5.5 | 9.6 |
| Alanine | 109 | 98 | 9.2 | 8.4 |
| Tryptophan | — | — | — | — |
| Total | 1189 | 1173 | | |

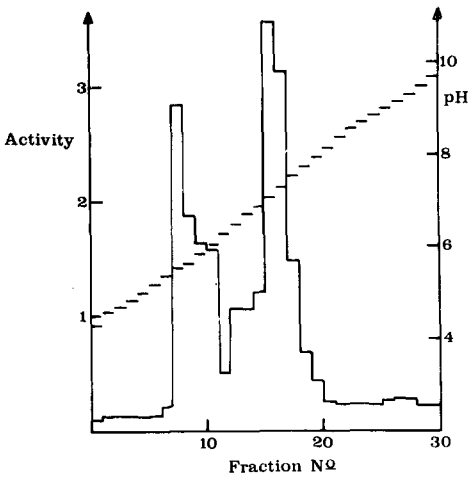


Fig. 3. Isoelectric focusing of dialysed ammonium sulphate precipitate fraction.

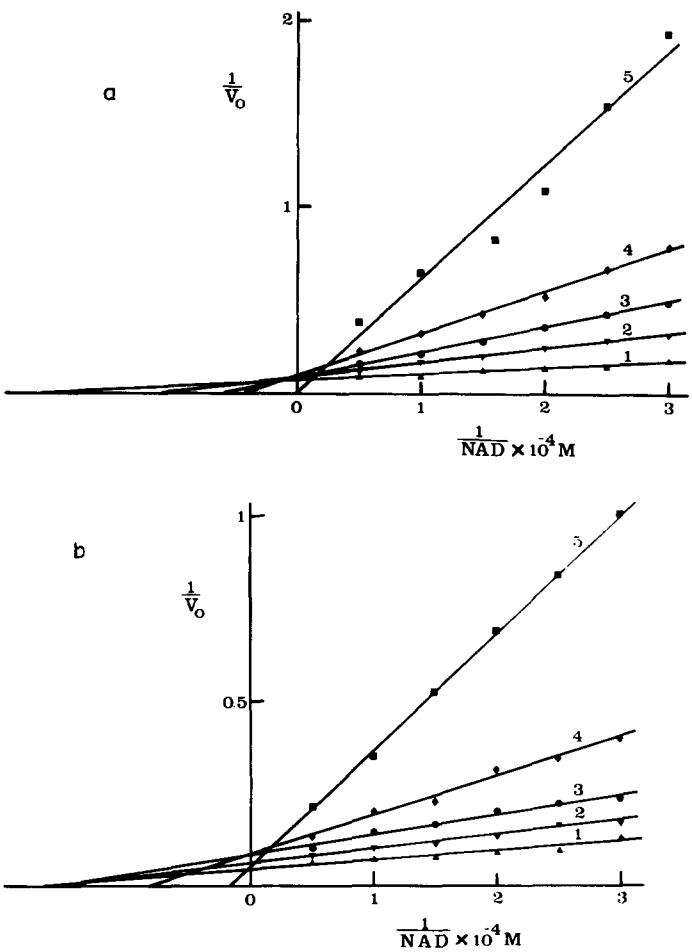


Fig. 4. Double-reciprocal plot of inhibition by AMP at variable NAD concentrations. (a) CM enzyme; (b) DE enzyme. (1), no inhibitor; 2, 0.5 mM AMP; 3, 1 mM AMP; 4, 2 mM AMP; 5, 5 mM AMP.

respective values are $1.85 \cdot 10^{-5}$ M and $2 \cdot 10^{-6}$ M. The slight differences in these values found for the two enzymes are not thought to be significant when the experimental errors are taken into account.

The inhibition patterns produced by AMP at variable NAD concentrations are not however identical for the two enzymes (Fig. 4). For the CM enzyme, the inhibition is principally competitive in nature. A secondary plot of slopes versus inhibitor concentration is linear and gives a $K_i$ value of 0.16 mM. For the DE enzyme, the inhibition is non-competitive at low AMP concentrations. At higher concentrations of AMP, the inhibition seems rather competitive. A secondary plot of slopes versus inhibitor concentration is nonlinear. However, using just the first three lines of the Lineweaver-Burk plot (AMP = 0.05 and 1 mM) an apparent $K_i$ of 0.85 mM is obtained.

Similarly, the inhibition patterns produced by 4-hydroxybenzaldehyde at

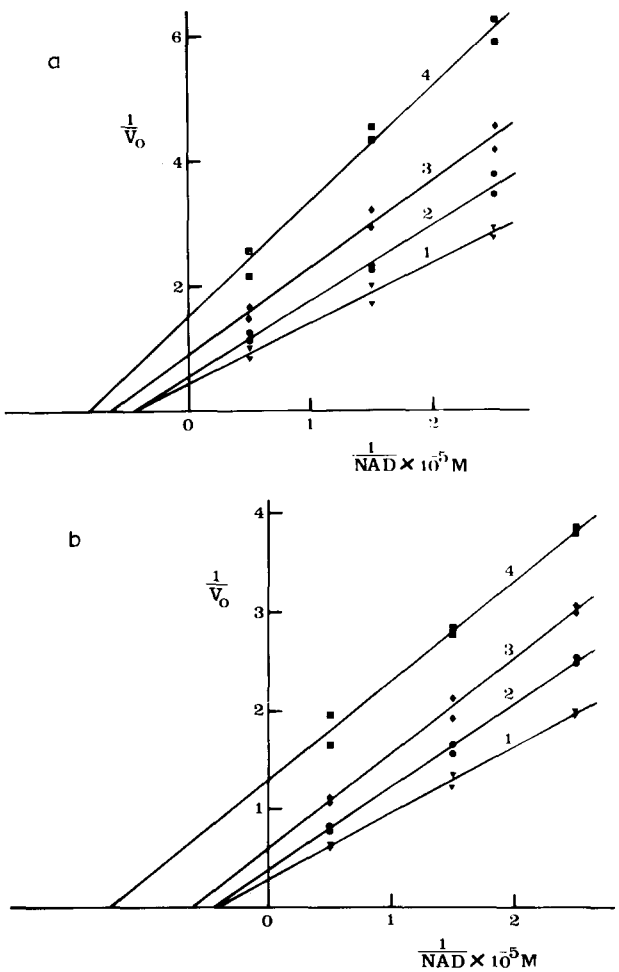


Fig. 5. Double-reciprocal plot of inhibition by 4-hydroxybenzaldehyde at variable NAD concentrations. (a) CM enzyme; (b) DE enzyme. 1, no inhibitor; 2, 0.05 mM 4-hydroxybenzaldehyde; 3, 0.1 mM 4-hydroxybenzaldehyde; 4, 0.2 mM 4-hydroxybenzaldehyde.

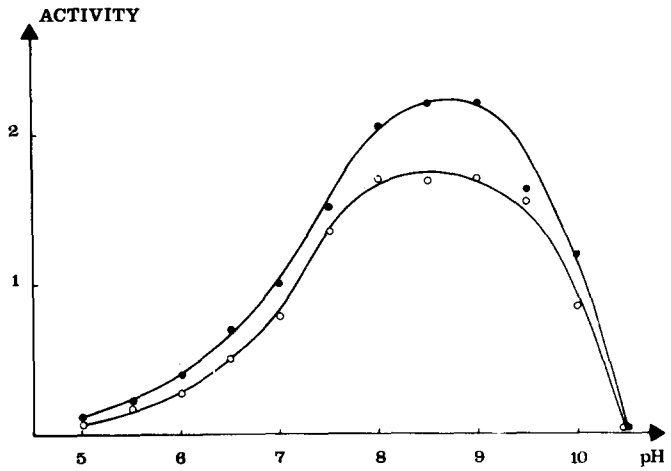


Fig. 6. pH activity profiles. ○, CM enzyme; ●, DE enzyme.

variable NAD concentrations are not identical for the two enzymes (see Fig. 5). In neither case is there a pure inhibition type. However, for the CM enzyme, a secondary plot of slopes versus inhibitor concentration is linear, whereas for the DE enzyme, it is non-linear.

*pH activity profile.* There is no significant difference in these profiles. The pH optima for both enzymes are about 8.6 (Fig. 6).

*NAD specificity.* When NADP is substituted for NAD, 20% of the control activity is obtained with both enzymes.

## Discussion

The fact that two isoenzymes of succinate semialdehyde dehydrogenase in approximately equal quantities have been found in human brain is of interest and its significance with respect to the regulation of 4-aminobutyrate catabolism should be considered.

The possibility that the obtention of two isoenzymes is an artifact due to protein degradation cannot be ruled out. Unlike rat brain, it is impossible to obtain human brain immediately post-mortem. However, all precautions to prevent protein degradation during extraction were taken, i.e. the inclusion of the inhibitors of proteases, phenylmethylsulphonyl fluoride and a high concentration of EDTA. Moreover, two isoenzymes of succinate semialdehyde dehydrogenase from fresh mouse brain have recently been prepared in this laboratory (unpublished results).

In different preparations the specific activities of the initial homogenates varied considerably as did the final degrees of purification of the two isoenzymes despite using the same purification procedures. As is seen in Table I, the activity yield in the supernatant is greater than 100%. This demonstrates the difficulty in measuring this enzyme activity in homogenates with precision. Preliminary work using a gradient of polyacrylamide gel to measure the molecular weights of the enzyme indicated a value about twice that found by Sepha-

dex gel filtration, suggesting that the enzymes have a tendency to aggregate.

The only significant differences detected in the kinetic properties of the two enzymes is in the inhibition patterns produced by AMP and 4-hydroxybenzaldehyde. The different types of inhibition produced by low concentrations of AMP could explain their different behaviour on AMP affinity columns. The AMP concentration of such columns is calculated to be in the millimolar range.

In order to demonstrate that the differences between the two enzymes are not due to aggregation with other proteins, the isoelectric focussing was carried out in the presence of 3 M urea. Higher concentrations could not be used as all the enzyme activity was destroyed. It is possible that the two enzymes have a different cellular location, e.g. neurones and glia, but cellular fractionation requires fresh material and thus cannot be performed with human brain. As analogous results have been observed with mouse brain (unpublished results) this technique may be applied here to advantage.

## References


1 Wu, J.Y. and Roberts, E. (1974) J. Neurochem. 23, 759—767
2 Wu, J.Y. (1976) in GABA in Nervous System Function, (Roberts, E., ed.), pp. 7—55, Raven Press, New York
3 Blinderman, J.M., Maitre, M., Ossola, L. and Mandel, P. (1977) C.R. Acad, Sci. Paris, série D 285, 1079—1082
4 Schousboe, A., Wu, J.Y. and Roberts, E. (1973) Biochemistry 17, 2868—2873
5 Maitre, M., Ciesielski, L., Cash, C. and Mandel, P. (1975) Eur. J. Biochem. 57, 157—169
6 Cash, C., Maitre, M., Ciesielski, L. and Mandel, P. (1974) FEBS Lett. 47, 199—203
7 Bloch-Tardy, M., Rolland, B. and Gonnard, P. (1974) Biochimie 56, 823—837
8 John, R.A. and Fowler, L.J. (1976) Biochem. J. 155, 645—651
9 Maitre, M., Ciesielski, L., Cash, C. and Mandel, P. (1978) Biochim. Biophys. Acta 522, 385—299
10 Cash, C., Ciesielski, L., Maitre, M. and Mandel, P. (1977) Biochimie 59, 257—268
11 Embree, L.J. and Albers, R.W. (1963) Biochem. Pharmacol. 13, 1209—1217
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W. and Weigele, M. (1972) Science 178, 871—872
14 Andrews, P. (1964) Biochem. J. 91, 222—223
15 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
16 Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462—477
17 Zanetta, J.P., Vincendon, G., Mandel, P. and Gombos, G. (1970) J. Chromatogr. 51, 441—458
18 Radola, B.J. (1969) Biochim. Biophys. Acta 194, 335—338
19 Wermuth, C.G. (1964) Bull. Soc. Chim. 1, 471—472