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SUCCINIC SEMIALDEHYDE DEHYDROGENASE FROM HUMAN BRAIN

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Abstract—Succinic semialdehyde dehydrogenase, the enzyme that catalyzes the oxidative step in the γ-aminobutyrate "shunt", has been purified from human brain and its properties are presented. Noteworthy are the low Michaelis constant, the occurrence of substrate inhibition, the specificity for NAD as the electron acceptor, and the arsenite sensitivity that is potentiated by thiols. Also of interest are the effects of anticonvulsant drugs, steroid hormones, and certain organic compounds upon the activity of the enzyme. A mechanism may be outlined whereby inhibition of succinic semialdehyde oxidation could result in an elevation of brain γ-aminobutyrate and, according to one theory, a consequent elevation of seizure threshold.

GAMMA-aminobutyrate (γ-AB) has been the focus of extensive biochemical and biophysical investigation since its discovery in the mammalian central nervous system.[1, 2] A great deal of information concerning its metabolism and pharmacologic effects is now available as a result of further investigation in a number of laboratories. On the basis of previously described enzyme-catalyzed reactions,[3–5] Roberts proposed a shunt, bypassing the conventional α-ketoglutarate oxidase system.[6] Through this alternate pathway α-ketoglutarate could be withdrawn from the Krebs tricarboxylic acid (TCA) cycle as glutamate by transamination with γ-AB; the carbon chain of γ-AB, then in the form of succinic semialdehyde (SSA), could be oxidized and re-enter the TCA cycle at the level of succinate. Isotope-labeling patterns in experiments employing ^{11}C-labeled γ-AB with intact animals and with a variety of brain preparations have been in complete accord with the actual operation of a unique alternative pathway in brain[7, 8] and the metabolites of the "γ-AB shunt", glutamate, γ-AB, and SSA, have been shown to support oxidative phosphorylation in cerebral tissue;[9] however, it remained for the oxidative step of γ-AB metabolism to be examined in detail in mammalian brain. A specific succinic semialdehyde dehydrogenase (SSADH) was identified in the brain of several species.[10] The purification and characterization of this enzyme, which catalyzes the oxidative step in the "γ-AB shunt", was subsequently undertaken in this laboratory.[11] We report our findings in the investigation of the oxidative metabolism of γ-AB as catalyzed by SSADH from human brain.

MATERIALS AND METHODS

Preparation of the enzyme. Human brain was obtained at autopsy from subjects dead for a relatively short period (less than 8 hr) and kept cool prior to autopsy. Cases

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selected were free from clinical and gross neuropathological disease. The material was frozen and stored at −20° until extraction and purification of SSADH was begun. The preparation of the enzyme was carried out at 5°. Cerebral cortex with small amounts of subjacent white matter was weighed and homogenized in 3 vol quartz-distilled water in a groundglass homogenizer. Enzyme activity recovered by homogenization in water was equal to that obtained when pyrophosphate buffer or NAD plus buffer is used or when mercaptoethanol is added to any extracting media.

After centrifuging for 30 min at 14,000 *g*, the supernatant fluid was decanted and the residue re-extracted with 2·5 vol quartz-distilled water. The tissue was extracted twice with water.

The combined supernatant fluid was fractionated with ammonium sulfate. Attempts to precipitate the enzyme with nucleic acid were unproductive. The enzyme activity was found to precipitate between 0·15 and 0·25 g/ml of ammonium sulfate. The enzyme yield was greater when the ammonium sulfate solution was not neutralized (pH 4·8–5·0) than when the pH was adjusted to 7·0. In order to remove endogenous substrates and obtain a more purified protein fraction, the preparation was reprecipitated several times between the same limits of ammonium sulfate concentration, with a density gradient column[12] to adjust the salt concentration. The enzyme was redissolved in 0·1 M sodium pyrophosphate buffer (pH 7·4) and stored at −20° until assayed. Enzyme activity was greater when the ammonium sulfate fraction was redissolved and stored in buffer rather than water.

Assay of the enzyme. The assay system consisted of 0·1 M sodium pyrophosphate buffer at pH 9·2, 3×10^{-6} M 2-mercaptoethanol, 4×10^{-4} M NAD, enzyme, $2{\cdot}7 \times 10^{-5}$ M succinic semialdehyde. Total assay volume was 1·022 ml. Enzyme activity was followed by measuring the production of NADH fluorimetrically[13] in a Farrand model A fluorimeter, with 365 mμ Hg excitation and reading fluorescence at 480 mμ.

RESULTS

Properties of the enzyme

The enzyme was found to exhibit maximal activity between pH 9·2 and 9·3 (Fig. 1). The apparent Michaelis constant (K_m) for succinic semialdehyde is $5{\cdot}3 \times 10^{-6}$ M

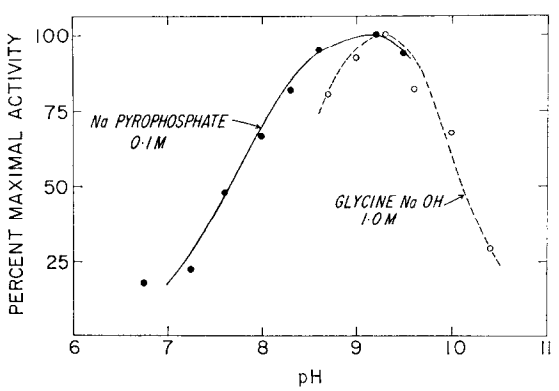

FIG. 1. Dependence of SSADH activity on pH. Maximal activity was found at 9·2 to 9·3. Fluorimetric assay as described in the text.

at pH 9·2 in the presence of 4×10^{-4} M NAD. At SSA concentrations above $2{\cdot}7 \times 10^{-5}$ M, the phenomenon of substrate inhibition is evident (Fig. 2). Inhibition of the type observed is not produced by the addition of high concentrations ($9{\cdot}7 \times 10^{-3}$ M) of the product, succinate. The enzyme exhibits a high degree of substrate specificity and does not reduce NAD at a significant rate in the presence of acetaldehyde, glyoxylic acid, triosephosphate, or 5-hydroxypentanal at the low concentrations at which SSA is utilized. Butyraldehyde is slowly oxidized: 40 per cent of the control rate at 10^{-1} and 55 per cent at 10^{-3} M.

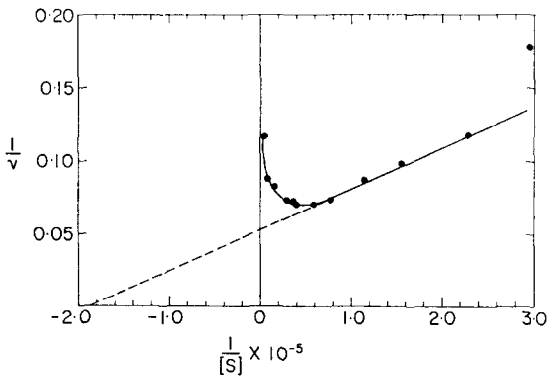

FIG. 2. Effect of substrate concentration on reaction velocity. K_m for SSA is 5·3 to 10^{-6} M. Substrate inhibition is evident at SSA concentrations above $2{\cdot}7 \times 10^{-5}$ M. Assay conditions as described in text.

Three aromatic aldehydes, phthaldehydic acid, *m*-, and *p*-hydroxybenzaldehydes, were studied because of their varying orientations of a negative substituent in relation to the aldehyde group. None of these compounds serves as a substrate for the enzyme; on the contrary, they are inhibitory. The *m*- and *p*-hydroxybenzaldehydes have a profound inhibitory effect, and phthaldehydic acid inhibits to a lesser degree. Dopamine (3,4-dihydroxyphenylethylamine) has no inhibitory effect.

The NAD concentration-reaction velocity kinetics under standard assay conditions produces a curve that suggests two species of the enzyme (Fig. 3). Nicotinamide-adenine dinucleotide phosphate (NADP) is inactive as an electron acceptor for the oxidation of SSA as catalyzed by SSADH from human brain; however, 3-acetyl pyridine- and deamino-analogs of NAD are reduced at essentially the same rate as NAD.

SSADH exhibits a striking sensitivity to arsenite, which is enhanced by mercaptoethanol; this is a property common among aldehyde dehydrogenases.[14] Enzyme activity is inhibited 50 per cent by $1{\cdot}5 \times 10^{-3}$ M arsenite in the absence of exogenous thiols. In the presence of 3×10^{-6} M 2-mercaptoethanol, 50 per cent inhibition of activity is obtained with only 1×10^{-5} M arsenite (Fig. 4).

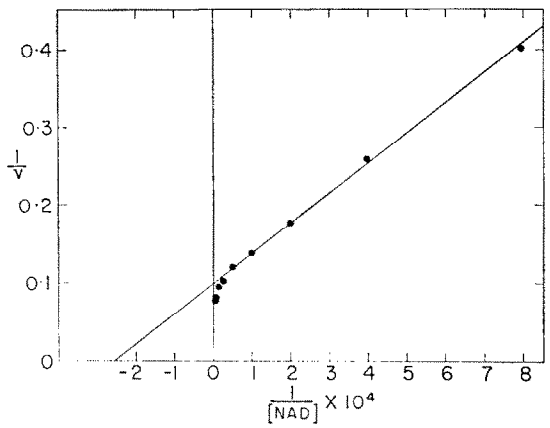

FIG. 3. Effect of NAD concentration on reaction velocity. Change of slope noted at higher concentrations of NAD is suggestive of two species of the enzyme. Standard assay conditions.

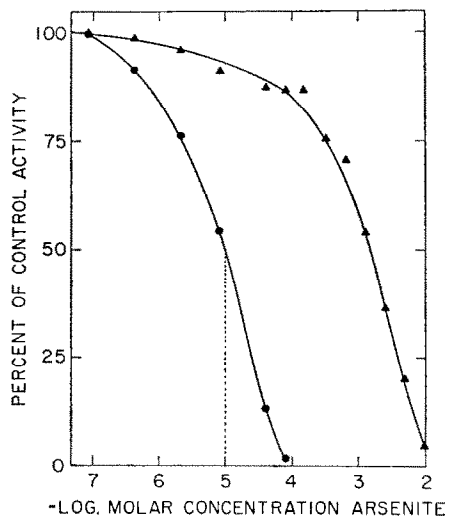

FIG. 4. Arsenite inhibition of SSADH and potentiation of this effect by mercaptoethanol. Without mercaptoethanol, ▲; with 3×10^{-6} M 2-mercaptoethanol, ●; otherwise standard assay conditions.

Borate at a concentration of $1{\cdot}3 \times 10^{-3}$ M inhibits SSADH activity approximately 50 per cent. This inhibition is not influenced by the presence of mercaptoethanol. The mechanism of borate inhibition is that of competitive inhibition for the coenzyme, NAD (Fig. 5). The dissociation constant for the borate-NAD complex (K_i) is $1{\cdot}6 \times 10^{-4}$.

Effects of drugs (*Table* 1)

Diphenylhydantoin was found to have an inhibitory effect upon SSADH activity. A concentration of $1{\cdot}8 \times 10^{-3}$ M Dilantin reduces enzyme activity approximately

50 per cent (Fig. 6). The kinetics of Dilantin inhibition is predominantly noncompetitive, as shown in Fig. 7. Several other compounds were evaluated for their possible influence upon SSADH activity. The glutarimide derivatives, Doriden and Elipten, were tested because of their sedative and anticonvulsive properties. Celontin, Milontin, and Zarontin, derivatives of succinimide with anticonvulsive action, were also tested.

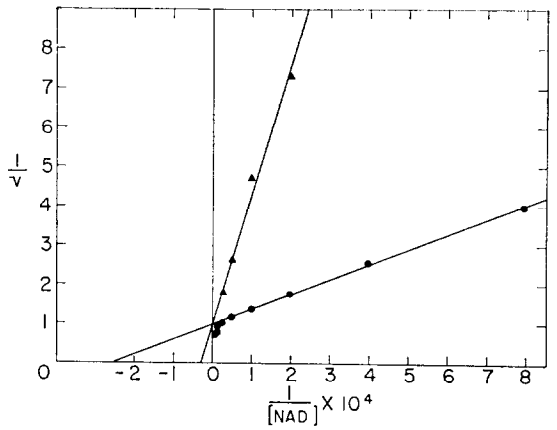

FIG. 5. Borate inhibition of SSADH activity by the mechanism of competitive inhibition for NAD. Borate $1{\cdot}3 \times 10^{-3}$ M, ▲; otherwise standard assay conditions.

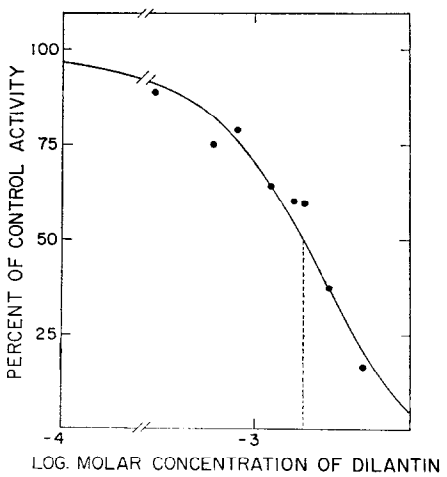

FIG. 6. Enzyme activity as a function of diphenylhydantoin concentration. Dilantin $1{\cdot}8 \times 10^{-3}$ M produces approximately 50 per cent inhibition of SSADH activity under standard assay conditions.

None of these drugs possesses the ability to inhibit enzyme activity to a greater extent than Dilantin. Of the succinimide and glutarimide derivatives studied, Milontin (N-methyl,α-phenylsuccinimide) and Zarontin (α-ethyl,α-methylsuccinimide) show the greatest degree of enzyme inhibition. SSADH activity in the presence of 5×10^{-3} M Milontin and $9{\cdot}7 \times 10^{-3}$ M Zarontin is approximately 79 per cent and 78 per cent respectively, of control activity under standard assay conditions.

Menthol and menthone were tested because of their reported inhibitory action on liver and kidney aldehyde dehydrogenase activity.[15] Menthone in 10^{-2} M concentration reduced SSADH activity to approximately 75 per cent of control activity. Menthol had no significant effect upon the activity of this enzyme.

The effect of several steroid hormones upon SSADH activity was studied. Dehydroepiandrosterone concentrations of 2·8 and $1{\cdot}4 \times 10^{-4}$ M reduce enzyme activity to approximately 62 per cent and 79 per cent, respectively, of control activity under

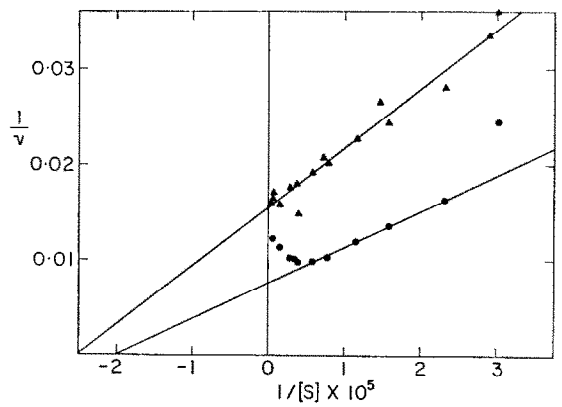

FIG. 7. Velocity of the reaction as a function of SSA concentration under standard assay conditions ●; and with $1{\cdot}8 \times 10^{-3}$ M diphenylhydantoin, ▲. The kinetics shows the mechanism of diphenylhydantoin inhibition to be predominantly noncompetitive.

standard assay conditions. In the absence of mercaptoethanol, $1{\cdot}4 \times 10^{-4}$ M dehydroepiandrosterone reduces SSADH activity to approximately 71 per cent of control activity in which mercaptoethanol was also omitted. Preincubation of the enzyme with SSA, NAD, or mercaptoethanol in the presence of dehydroepiandrosterone has no influence upon the steroid inhibition of SSA oxidation.

No inhibition of enzyme activity was detected in the presence of 5×10^{-3} M EDTA or 0·1 M sodium pyrophosphate. Less fluorescence of reduced NAD was observed in the presence of 8-hydroxyquinoline, but this was interpreted as due to quenching of NADH fluorescence because of the overlapping absorption spectra of these two compounds at the wavelength of excitation.

DISCUSSION

Many properties of succinic semialdehyde dehydrogenase of human brain and rhesus monkey brain are closely similar. With minor exceptions the procedures for recovering the enzyme activity are the same. SSADH is not precipitated from human brain by nucleic acid as it is from monkey brain. Maximal SSADH activity is recovered by ammonium sulfate fractionation of either brain homogenate with essentially the same salt concentration: 0·15 to 0·25 g/ml for human brain and 0·17 to 0·27 g/ml for rhesus monkey brain.

The pH optima are 9·2 to 9·3 for human SSADH and 8·7 to 9·1 for the monkey enzyme. The apparent Michaelis constant for succinic semialdehyde is of the same order of magnitude, $5{\cdot}3 \times 10^{-6}$ M and $2{\cdot}7 \times 10^{-6}$ M, respectively, for human and monkey enzymes. The behavior of the enzymes with respect to cofactors and cofactor analogs is likewise similar: NADP will not serve as an electron acceptor for either human or monkey brain SSADH. This is in contrast to bacterial succinic semialdehyde dehydrogenase where NADP is several times more active than NAD as a substrate.[16] Deamino-NAD and 3-acetyl pyridine-NAD are both reduced at the same rate as NAD. The concentration–velocity kinetics for NAD with either enzyme produces a curve suggesting two species of the enzyme in both human and monkey. Slight activation by mercaptoethanol may be seen with SSADH from either human or monkey brain. There is preservation of enzyme activity by mercaptoethanol and by NAD when the enzymes are subjected to adverse conditions. Both enzymes show the arsenite sensitivity characteristic of aldehyde dehydrogenases and the enhancement of this sensitivity by thiols. The mechanism of sulfhydryl involvement in arsenite inhibition has been previously commented upon in some detail.[11, 17]

The three benzaldehyde derivatives with negative substituents variously oriented with respect to the aldehyde group, phthalaldehydic acid, *m*-, and *p*-hydroxybenzaldehydes, inhibit the reduction of NAD catalyzed by SSADH from either human or rhesus monkey brain. The interaction of SSADH with such substituted aldehydes is of possible relevance to the configuration and charge of the enzyme-substrate binding sites. The structural similarity of the hydroxybenzaldehydes to the aldehyde intermediates arising from the oxidation of phenolic amines by amine oxidase raises the possibility that these metabolites may influence the activity of SSADH *in vivo*.

In the process of selecting a buffer, in evaluating buffer systems, borate was found to have an inhibitory effect on the activity of SSADH from human brain as well as on that from monkey brain and bacteria. Since it was found for the human enzyme that the mechanism of borate inhibition is one of competition for NAD, it seems likely that the pyridine nucleotide coenzymes with their ribosyl groups form complexes with boric acid, which have an impaired ability to serve as electron acceptors.[18]

A reciprocal relationship has been proposed between the two parallel metabolic routes available in brain for the oxidation of α-ketoglutarate to succinate,[9] and several factors have been proposed as having regulatory roles in γ-AB metabolism. Jakoby points out that concentrations of α-ketoglutarate and oxidized NAD in brain may be rate limiting.[19] McKhann and Tower suggest that the availability of oxidized NAD alone may be important in the balance of activities between the two alternative metabolic pathways from α-ketoglutarate to succinate.[20] The apparent K_m of $5{\cdot}3 \times 10^{-6}$ M for SSA is noteworthy, since it indicates the enzyme's ability to operate effectively at low concentrations of SSA. This may account for the failure of SSA to accumulate as an endogenous metabolite in brain, despite the rapid metabolism *in vivo* of γ-aminobutyric acid (GABA). The equilibrium constant for GABA-glutamic transaminase (0·1),[21] and the prevailing ratio of glutamate to γ-AB in brain (3 : 1) would predict brain SSA concentration to be one-third of α-ketoglutarate whereas, in fact, it has not been detected as an endogenous metabolite.

The succinimide derivatives that clinically alter convulsive disorders—Celontin, Milontin, and Zarontin—attracted our attention because of their basic four-carbon, dicarbonyl structure similar to SSA. Our interest likewise extended to the glutarimide

derivatives, Doriden and Elipten, because of their basic structure of a five-carbon chain with terminal carbonyl groups and their anticonvulsant properties. The possibility that these anticonvulsant compounds, by virtue of a structural similarity to SSA, could alter the activity of SSADH was investigated. Inhibition of enzyme activity is indeed observed in the presence of the succinimide and glutarimide derivatives (Table 1).

TABLE 1. EFFECT OF VARIOUS CENTRAL NERVOUS SYSTEM DEPRESSANTS, STEROID HORMONES, AND ORGANIC COMPOUNDS UPON THE ACTIVITY OF SSADH FROM HUMAN BRAIN

	Concentration, M	Control activity, %
Drugs		
Diphenylhydantoin	$3{\cdot}6 \times 10^{-3}$	17
(Dilantin)	$1{\cdot}8 \times 10^{-3}$	55
	$8{\cdot}1 \times 10^{-4}$	79
N-Methyl, α-phenylsuccinimide	$2{\cdot}4 \times 10^{-3}$	88
(Milontin)	$4{\cdot}8 \times 10^{-3}$	79
α-Ethyl, α-methylsuccinimide	$9{\cdot}7 \times 10^{-3}$	78
(Zarontin)		
N-Methyl, α,α-methylphenylsuccinimide	$4{\cdot}8 \times 10^{-3}$	86
(Celontin)		
α-Ethyl, α-phenylglutarimide	$4{\cdot}8 \times 10^{-3}$	85
(Doriden)		
α-Ethyl, α-*p*-aminophenylglutarimide	$4{\cdot}8 \times 10^{-3}$	89
(Elipten)		
Steroids		
Dehydroepiandrosterone	$2{\cdot}8 \times 10^{-4}$	62
	$1{\cdot}4 \times 10^{-4}$	79
Estradiol	$4{\cdot}9 \times 10^{-4}$	107
Estrone	$2{\cdot}4 \times 10^{-4}$	97
Progesterone	$1{\cdot}2 \times 10^{-3}$	86
	$1{\cdot}2 \times 10^{-5}$	95
Pregnenolone	$1{\cdot}2 \times 10^{-3}$	96
Testosterone	$2{\cdot}4 \times 10^{-4}$	100
Aromatic carbonyl, hydroxyl, and carboxyl compounds		
m-Hydroxybenzaldehyde	1×10^{-3}	0
	2×10^{-4}	14
p-Hydroxybenzaldehyde	2×10^{-4}	10
Phthaldehydic acid	1×10^{-3}	77
Menthone	$9{\cdot}7 \times 10^{-3}$	75
Menthol	$9{\cdot}7 \times 10^{-3}$	93

Among anticonvulsant compounds with less obvious structural similarity to SSA, diphenylhydantoin was the most potent inhibitor of SSADH activity in the group of drugs tested. Enzyme activity is reduced approximately 50 per cent in the presence of $1{\cdot}8 \times 10^{-3}$ M diphenylhydantoin by a mechanism which is predominantly non-competitive inhibition (Fig. 7). It has been reported that rats receiving diphenylhydantoin showed decreased brain excitability as measured by electroshock seizure threshold and increased concentration of γ-AB in brain.[22] γ-AB-glutamate transaminase, which catalyzes the first step in the degradative metabolism of γ-AB, has been shown to be unaffected by diphenylhydantoin concentrations of 10^{-3} M.[23] Hence it would seem that the increased γ-AB is not a result of impaired transamination.

However, an increase in SSA concentration due to inhibition of SSA oxidation would be accompanied by an increase in γ-AB. The diphenylhydantoin concentration of $1{\cdot}8 \times 10^{-3}$ M is equal to 500 μg/ml, a level which, if present in serum, would certainly be extremely toxic; but lower concentrations of diphenylhydantoin producing lesser degrees of SSADH inhibition might still bring about a sufficient increase in brain γ-AB concentration or decrease in the turnover of γ-AB to produce clinically observable effects.

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