SEPARATION OF ALDEHYDE REDUCTASES AND ALCOHOL DEHYDROGENASE FROM BRAIN BY AFFINITY CHROMATOGRAPHY: METABOLISM OF SUCCINIC SEMIALDEHYDE AND ETHANOL

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Enzyme activities which reduced aldehydes such as succinic semialdehyde in rat brain cytosol were separated by affinity chromatography on NADP-Sepharose. The reduction of succinic semialdehyde which was previously thought to be catalyzed by alcohol dehydrogenase or lactate dehydrogenase was shown to be primarily catalyzed by enzymes both separable from and characteristically different from these enzyme activities. Brain alcohol dehydrogenase activity was shown to be <1mU/gm brain. Brain activity reducing succinic semialdehyde to γ-hydroxybutyrate, a known hypnotic, was also shown to differ from previously described aldehyde reductases.

Brain tissue has been demonstrated to contain multiple molecular forms of aldehyde reductase (E.C.1.1.1.2) (1,2,3) as well as alcohol dehydrogenase (E.C.1.1.1.1) (4) and questions have been posed as to which class of enzymes is responsible for the reductive metabolism of aldehydes in brain (5). Aldehydes, such as lactaldehyde, which have been used to characterize brain aldehyde reductase (3) are also substrates for alcohol dehydrogenase (6) and acetaldehyde a substrate of alcohol dehydrogenase may also serve as substrate for partially purified aldehyde reductase (7,1). Recently it has been reported (8) that brain alcohol dehydrogenase catalyzes the conversion of succinic semialdehyde to the known hypnotic γ-hydroxybutyrate (9). However, a previous report (10) attributed the reduction of succinic semialdehyde in brain to the action of lactate dehydrogenase (E.C.1.1.1.28).

We attempted a preparative procedure by which we could separate various pyridine nucleotide dependent oxido-reductases from brain and attribute the metabolism of particular aldehydes to the separated enzyme proteins. It was also of particular interest to obtain a preparation of

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alcohol dehydrogenase from brain wherein one could measure the oxidation of ethanol by direct spectrophotometric analysis. Previous studies of alcohol dehydrogenase activity in brain have utilized an assay coupled to the reduction of lactaldehyde to estimate enzyme activity (4,11). The conversion of ethanol to acetaldehyde by horse liver alcohol dehydrogenase, however, has been demonstrated to be increased nearly 100 fold by the inclusion of lactaldehyde in assay mixtures (12).

MATERIALS AND METHODS Adult male rats of the CFR strain were decapitated, brains were removed, washed, and a 20% homogenate was prepared in 10 mM sodium phosphate pH 8.0. The homogenate was centrifuged at 106,000xg for 90 min and the clear supernatent fluid was recovered. This fluid was fractionated using ammonium sulfate. Protein precipitating between 35 and 70% saturation with ammonium sulfate was recovered and desalted by gel filtration chromatography on Sephadex G-25. Column eluate which contained protein was pooled and concentrated using an Amicon filter apparatus. This concentrated solution will be referred to as the ammonium sulfate fraction. The ammonium sulfate fraction was further fractionated using affinity chromatography on NADP linked Sepharose. Fractions eluted from this column which metabolized aldehydes (see legend Fig. 1) were pooled, concentrated, and further characterized.

"Coupled" assays of alcohol dehydrogenase activity (12) were performed as described by Raskin and Sokoloff (4). Reaction mixtures contained L-lactaldehyde (8mM), NAD^+ (0.5 mM), ethanol (200 mM) and enzyme in 50 mM sodium phosphate pH 7.4. Mixtures were incubated at 37° for 30 min and the propanediol produced from lactaldehyde was quantified as described by Jones and Riddick (13).

"Direct" assays of alcohol dehydrogenase activity were performed by measuring spectrophotometrically the conversion of NAD^+ to NADH in reaction mixtures containing NAD^+ (5 mM), ethanol (16 mM) and enzyme in 100 mM sodium pyrophosphate pH 8.8.

Reduction of aldehydes was monitored spectrophotometrically in mixtures containing NADH or NADPH (0.16 mM), enzyme, and aldehyde substrate in 100 mM sodium phosphate pH 7.4. All incubation mixtures were one ml in total volume and spectrophotometric assays were performed at 25°.

L-lactaldehyde was prepared from D-threonine as described by Huff and Rudney (14). Prior to use the pH of the solutions was adjusted to pH 7.4 and lactaldehyde was dedimerized by heating at 80° for 10 min.

Succinic semialdehyde was prepared from hydroxybutyrolactone as described by Taberner et al (15). The presence of succinic semialdehyde was determined as described by Salvador and Albers (16). The quantity of this and other aldehyde substrates in solutions was assertained using horse liver alcohol dehydrogenase (1).

NADP-Sepharose was synthesized by the procedure of Larson and Mossbach (17). Protein was determined by the method of Lowry et al (18) with bovine serum albumin as the standard.

RESULTS Rat brain cytosol catalyzed the reduction of the aldehydes listed in Table 1 in the presence of either NADPH or NADH. Pyrazole, a known inhibitor of alcohol dehydrogenase (19), had little effect on this activity and acetaldehyde reduction in the presence of NADH could not be detected.

TABLE I

REDUCTION OF ALDEHYDES BY RAT BRAIN CYTOSOL

SUBSTRATE	COFACTOR	RELATIVE SPECIFIC ACTIVITY[1,2]
p-nitrobenzaldehyde (5×10^{-4}M)	NADPH	100
	NADH	12
L-lactaldehyde (2×10^{-3}M)	NADPH	59
	NADH	55
Succinic Semialdehyde (5×10^{-4}M)	NADPH	11
	NADH	5
Acetaldehyde (1×10^{-3}M)	NADH	n.d.[3]

Enzymatic activity was monitored spectrophotometrically as described in the text.

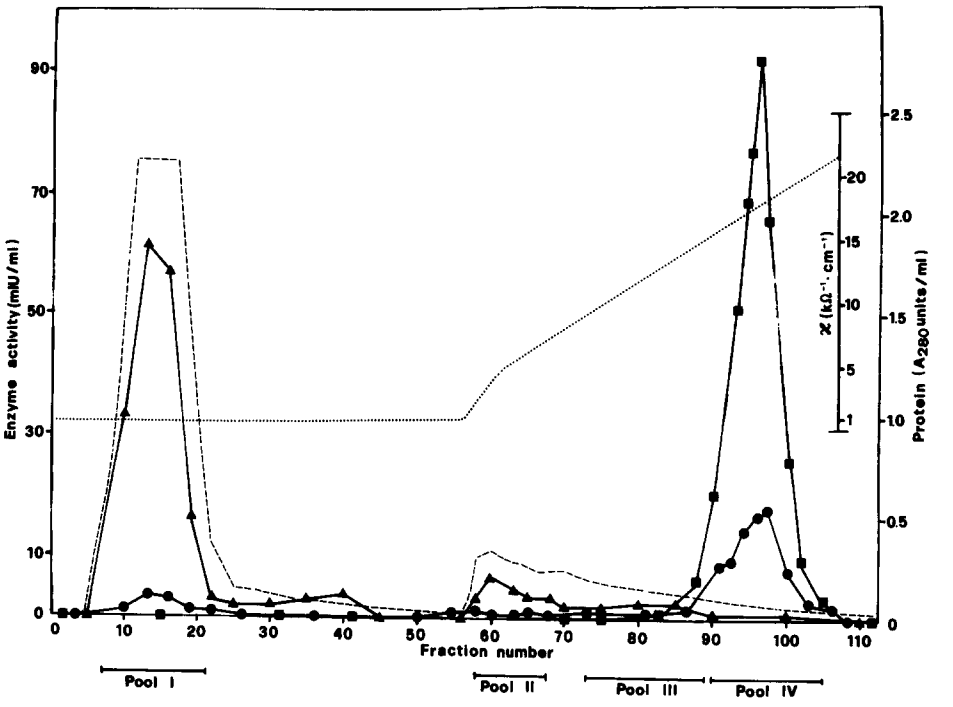
1. Relative specific activity was taken as the specific activity obtained with the various aldehydes and cofactors divided by the specific activity obtained with p-nitrobenzaldehyde and NADPH multiplied by 100. The specific activity obtained with p-nitrobenzaldehyde and NADPH was 10.3 mU/mg protein.

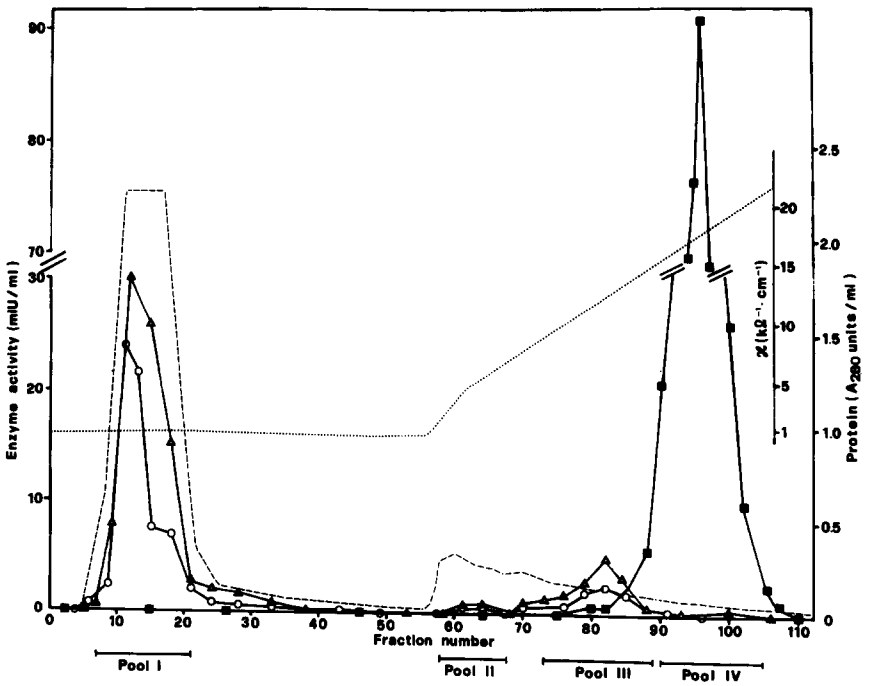
2. Incubation of reaction mixtures in the presence of pyrazole (1mM) produced <5% inhibition of enzyme activity.

3. n.d. = not detectable under our assay conditions.

Alcohol dehydrogenase activity in brain cytosol could, however, be demonstrated by means of the assay coupling the oxidation of ethanol to reduction of lactaldehyde. The rate of lactaldehyde reduction which was dependent on the presence of ethanol was found to be 25.8 $\pm$ 13.8 mU/gm brain (n=5). The reduction of lactaldehyde by brain cytosol and NAD^+ in the absence of ethanol was 13.6 $\pm$ 4.4 mU/gm brain. These values are similar to those previously reported by Raskin and Sokoloff (4) and ourselves (20). Pyrazole (1 mM) inhibited this alcohol dehydrogenase activity 98%.

Fractionation with ammonium sulfate was performed so as to maximize





the recovery of alcohol dehydrogenase activity measured by the coupled assay. Approximately 40% of the activity was recovered with a purification of two

fold by this procedure. However, as in cytosol, the reduction of aldehydes with this fraction was not inhibited by pyrazole.

Chromatography of the ammonium sulfate fraction on NADP-Sepharose resulted in the separation of several peaks of enzyme activity capable of reducing aldehydes (Fig. 1). When various fractions reducing aldehydes were pooled (Fig. 1) and concentrated, alcohol dehydrogenase activity measured by the coupled assay was primarily localized in Pool II. The overall purification of alcohol dehydrogenase found in Pool II was 18 fold with a recovery of 12%. The specific activity of alcohol dehydrogenase in Pool II was 25.8 mU/mg protein as measured by the coupled assay as compared to less than 2 mU/mg in the other pools. On the other hand, using the direct assay for alcohol dehydrogenase activity in Pool II, a specific activity of 0.37 mU/mg protein was found. In the ammonium sulfate fraction the specific activity using the coupled assay ranged from 0.54 to 2.4 mU/mg protein and using the direct assay from 0.01 to 0.04 mU/mg protein. The ratios of specific activities measured in identical preparations were found to be 56 ± 6. Based on these figures, we calculated that the capacity of rat brain to oxidize ethanol without coupling enzyme activity to reduction of lactaldehyde was approximately 0.45 mU/gm brain.

Figure 1

CHROMATOGRAPHY OF ALDEHYDE REDUCING ENZYMES
ON NADP-SEPHAROSE

Columns were developed using 100 ml of 5 mM sodium phosphate pH 8.3 followed by a linear gradient of potassium chloride in the same buffer. Conductivity of collected fractions (····) is expressed as $K\Omega^{-1}.cm^{-1}$. Fractions (2.5 ml each) were assayed for enzyme activity as described in the text. Upper frame: (▲-▲-▲)enzyme activity with L-lactaldehyde and NADH as cosubstrates; (●-●-●) activity with L-lactaldehyde and NADPH as cosubstrates; (■-■-■)activity with p-nitrobenzaldehyde and NADPH as cosubstrates. Lower frame: (Δ-Δ-Δ) activity with succinic semialdehyde and NADPH as cosubstrates; (○-○-○) activity with succinic semialdehyde and NADH as cosubstrates; (□-□-□) activity with p-nitrobenzaldehyde and NADPH as cosubstrates. Protein in both frames denoted by (- - -). Fractions were pooled as denoted below the abscissa, concentrated when necessary and used for inhibitor studies summarized in Table 2.

Table 2 EFFECT OF INHIBITORS ON ENZYME ACTIVITIES ELUTED FROM NADP-SEPHAROSE

SUBSTRATE	COFACTOR	POOL I ACTIV	POOL I % INHIBITION			POOL II ACTIV	POOL II % INHIBITION			POOL III ACTIV	POOL III % INHIBITION		
			Pheno	Pyrazole	Oxalate		Pheno	Pyrazole	Oxalate		Pheno	Pyrazole	Oxalate
L-lactaldehyde	NADH	479	3	2	63	24	0	1	57	n.d.[1]	-	-	-
p-nitrobenzaldehyde	NADH	10	17	0	0	n.d.	-	-	-	n.d.	-	-	-
succinic semialdehyde	NADH	38	14	14	0	5	26	79	10	28	4	21	3
L-lactaldehyde	NADPH	7	35	0	20	5	90	0	0	274	86	2	0
p-nitrobenzaldehyde	NADPH	n.d.	-	-	-	n.d.	-	-	-	510	85	4	0
succinic semialdehyde	NADPH	64	31	0	0	3	12	0	8	35	35	4	0
ethanol (direct assay)	NAD^+	n.d.	-	-	-	0.4	-	99	-	n.d.	-	-	-
ethanol (coupled assay)	NAD^+	15	-	-	-	23	-	94	-	0.8	-	-	-

Fractions elluted from NADP-Sepharose were pooled as shown in figure 1 and reduction of aldehydes with either NADH or NADPH as cofactors was assayed as described under methods. Ethanol oxidation using either the "coupled" or "direct" assays was also assayed as described in the methods section. Activity is expressed as total mU recovered in each pool.

1. n.d.= not detectable under our assay conditions.

That this alcohol dehydrogenase activity from brain is not of primary importance in the metabolism of succinic semialdehyde is illustrated by Fig. 1 and Table 2. Although a small amount of pyrazole sensitive succinic semialdehyde reducing activity was noted in Pool II the major portion of enzyme activity metabolizing succinic semialdehyde was recovered in Pool I and III. Succinic semialdehyde reductase activity found in these pools was not inhibited by pyrazole or sodium oxalate, a known inhibitor of lactate dehydrogenase (21). Sodium oxalate (5 mM) was found to inhibit pyruvate reduction by brain cytosol ~95% in the present studies. Phenobarbital, an inhibitor of aldehyde reductase (22), inhibited the reduction of both lactaldehyde and p-nitrobenzaldehyde with Pool III 80-85%. However, reduction of succinic semialdehyde was much less affected by phenobarbital. These inhibition patterns and the elution profile of enzyme activity metabolizing succinic semialdehyde in the presence of NADH and NADPH indicate that this activity is different and separable from the bulk of alcohol dehydrogenase and the previously described aldehyde reductase activity (1,2,27). The major portion of the previously described aldehyde reductase activity was recovered in Pool IV. This activity was quite sensitive to inhibition by phenobarbital (>90% inhibition with 1 mM sodium phenobarbital) but was unaffected by pyrazole or sodium oxalate, and was nearly exclusively dependent on NADPH as a cofactor.

DISCUSSION The possible presence of alcohol dehydrogenase in brain led to speculation that oxidation of ethanol in this tissue would produce changes in redox couples (i.e. lactate/pyruvate) as in the liver. Contradictory reports have appeared on this matter (23,24). Rawat et al (23) found significant redox changes in brain after administration of ethanol and attributed these changes to oxidation of ethanol by brain tissue. On the other hand, Veloso et al (24) attributed the small changes they found in brain redox couples after ethanol to changes in pCO_2 caused by ethanol induced anesthesia. However, the activity of alcohol dehydrogenase was not measured

directly in these studies. Our partial purification of rat brain alcohol dehydrogenase provided a means of separating this enzyme from other dehydrogenases and reductases in brain and allowed us to make a direct estimate of alcohol dehydrogenase activity. Our results (0.45 mU/gm rat brain) would support the contention of Veloso, Passonneau and Veech (24) who predicted that enzyme activity oxidizing ethanol in brain was < 1 mU/gm. This low activity of alcohol dehydrogenase would add little to any change in intermediary metabolites in brain after ethanol administration. The use of the "coupled" assay in our studies produced a 50 fold greater value of alcohol dehydrogenase activity compared to estimates obtained by "direct" spectrophotometric measurements. Previous measurements of horse liver alcohol dehydrogenase activity by the assay coupling the reduction of lactaldehyde to the oxidation of ethanol resulted in approximately a 100 fold increase in the production of acetaldehyde over assays containing no lactaldehyde(12). Since rat alcohol dehydrogenase has been shown to be quite similar to the horse enzyme (25,26) we should expect that the rate limiting (NADH off) step would be bypassed in the coupled assay.

The contention that brain alcohol dehydrogenase activity is the major enzyme responsible for conversion of succinic semialdehyde to γ-hydroxybutyrate (8) in brain was not supported by our studies. Although the alcohol dehydrogenase activity in Pool II metabolized succinic semialdehyde, the major portions of enzymatic activity catalyzing the reduction of succinic semialdehyde had chromatographic properties quite different from alcohol dehydrogenase (Fig. 1). Greater enzymatic activity was found with NADPH as a cofactor (Table 1) and the sensitivities to inhibitors differed considerably from previously described dehydrogenases and reductases (Table 2). In addition, little contamination of our preparation would be expected from aldehyde reductase form 4.2 (1) since this enzyme has been shown to be localized primarily in mitochondria (Ris, Deitrich, von Wartburg: in preparation).

Fishbein and Bessman (10) proposed that brain lactate dehydrogenase was responsible for conversion of succinic semialdehyde to γ-hydroxybutyrate. However, sodium oxalate a known inhibitor of lactate dehydrogenase had little effect on metabolism of succinic semialdehyde (Table 2). Sodium oxalate did, however, inhibit the metabolism of lactaldehyde in the presence of NADH.

Thus, we ascribe the major portion of enzymatic activity reducing succinic semialdehyde to γ-hydroxybutyrate to previously undescribed enzymes which can be separated by means of affinity chromatography. Since these enzymes would be responsible for the production of a known sommifacent in brain further characterization of their properties is in progress in our laboratories.

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