of oxidized enzyme–imino acid complex. As shown in Fig. 2 (C and D), the shoulder peak of absorption at 490 mμ could be still observed, after the oxygen in solution was exhausted as indicated by the measurement of oxygen consumption. Therefore, the possibility of the existence of the above complex seems to be negative. Thus the complex should be considered to be that of the enzyme and substrate.

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Betaine aldehyde dehydrogenase: assay and partial purification

Betaine aldehyde dehydrogenase (betaine-aldehyde:NAD oxidoreductase, EC 1.2.1.8) is the enzyme responsible for the oxidation of betaine aldehyde to betaine. The activity of this enzyme has been previously measured either manometrically[1] or spectrophotometrically[2]. The present communication describes a procedure for the partial purification and the necessary conditions for the fluorometric assay of the enzyme.

Assay. The buffer substrate was prepared just prior to each analysis and contained the following constituents at the indicated final concentrations: 100 mM Tris buffer (pH 8.1); 5 mM Cleland's reagent (Calbiochem.); 1 mM NAD^+; and 4 mM betaine aldehyde.

The betaine aldehyde was prepared from 2,2-diethoxy-ethyltrimethylammonium iodide (Aldrich, D8370) as described by ROTHSCHILD AND GUZMAN-BARRON[3]. The stock solution was approx. 400 mM and was stored at −20°.

1 μl of water or enzyme (equivalent to 0.4 μg protein) was added to a pointed tube of 2.5 mm internal diameter containing 10 μl of ice-cold incubation mixture. The

contents of the tubes were mixed without warming and were incubated at 38° for 15 min. At the end of the incubation period, the tubes were returned to an ice-water bath. Aliquots (8 μl) were removed and transferred to a fluorometer tube (Corning 9820) containing 1.0 ml of a solution which was 50 mM in K_2HPO_4 and 1 mM versene. The native fluorescence of the NADH produced was determined in a Farrand filter fluorometer against a freshly standardized solution of NADH[4]. Protein was measured by the procedure of LOWRY *et al.*[5].

During the purification procedure two blanks were routinely performed. In the complete incubation medium described, water was substituted for the tissue giving rise to a water blank. A second incubation medium was prepared in which the aldehyde was omitted giving rise to a tissue blank. In the purification procedure to be described, the fractions resulting from the Sephadex fractionation yield tissue blanks in which the fluorescence is no greater than water blanks. The assay as described is dependent upon the addition of NAD^+.

Enzyme purification. Rat liver (40 g) obtained from males of the Wistar strain, were homogenized in 50 mM Tris buffer (pH 7.6) containing 2 mM each of neutralized versene and cysteine. The preparation of the homogenate and all centrifugations and other manipulations were performed at 0–4°. The homogenate (800 ml) was centrifuged at 75 000 × *g* (average) for 45 min in a Spinco Model L preparative ultracentrifuge. The supernatant (Sn) was brought to 65% saturation with solid $(NH_4)_2SO_4$ (Mann, enzyme grade) and the pH adjusted (approx. 7) with 1 M NH_4OH. All further centrifugations were at 12000 × *g* for 20 min unless otherwise stated. The 65% saturated solution was centrifuged and the precipitate was extracted with 200 ml of an $(NH_4)_2SO_4$ solution (40% satn.) containing 2.5 mM each of neutralized versene and cysteine. After centrifugation to remove the insoluble protein, the resulting supernatant was brought to 70% saturation by the addition of solid $(NH_4)_2SO_4$ and again centrifuged. The resultant precipitate was taken up in less than 20 ml of 50 mM Tris buffer (pH 7.6) containing 2.5 mM each versene and Cleland's reagent (this medium is subsequently referred to as TVC). This preparation (P5) was stored at −20° for one month before the purification could be continued. Concentration of this solution was then accomplished by means of a DiaFlo Model 50 ultrafiltration cell (Aminco). The concentration by ultrafiltration also results in a removal of some $(NH_4)_2SO_4$. This lowers the viscosity of the solution and thus facilitates Sephadex chromatography. However, this step can be replaced by taking up the $(NH_4)_2SO_4$ precipitate in less than 10 ml TVC and dialyzing overnight. The resulting protein solution (7 ml) was placed directly on an ascending flow Bio-Gel P-60 column previously equilibrated with TVC. The column 100 cm × 2.5 cm had a void volume of approx. 120 ml. Elution was accomplished with TVC. The fractions (10 ml each) containing the enzyme (tubes UF 13–18) were combined (CUF) and Cleland's reagent was added so that the final concentration was 5 mM. This increase in concentration of Cleland's reagent increases the enzyme recovery during the next step. This solution was heated for 30 min at 50° then centrifuged at 12 000 × *g* for 30 min. The supernatant fraction (HCUF) was dialyzed overnight against 20 vol. TVC. The final preparation (DHCUF) contained 30% of the original activity and only 8% of the starting protein (Table I). The major loss of enzyme activity was either due to the $(NH_4)_2SO_4$ precipitation or due to the delay that occurred in the fractionation procedure. All other steps in the purification gave excellent recovery of enzyme. This preparation as described is free of NADH oxidase activity,

TABLE I

SUMMARY OF PURIFICATION PROCEDURE FOR BETAINE ALDEHYDE DEHYDROGENASE

For explanation of fraction symbols see text.

Fraction	*Protein (mg)*	*Specific activity (μmoles/g protein per h)*	*Total activity (μmoles/h)*
Sn	3 320	5 200	16 700
P_5	1 040	5 480	5 650
UF 13	4	41 000	168
UF 14	25	36 600	910
UF 15	88	11 900	1 040
UF 16	135	8 500	1 150
UF 17	111	7 800	860
UF 18	65	3 500	226
CUF	429	13 100	5 600
HCUF	250	20 500	5 100
DHCUF	244	19 500	4 730

does not contribute to the fluorescence blank, is free of choline dehydrogenase (EC 1.1.99.1) activity and is stable for at least one month at −70° with repeated freeze-thaw. If stored at −20° there is only 10% of the activity after 3 months, while storage at −70° preserves at least 30% of the original activity up to 6 months.

The pH optimum for betaine aldehyde dehydrogenase is 8.6 to 9.0. Using Tris

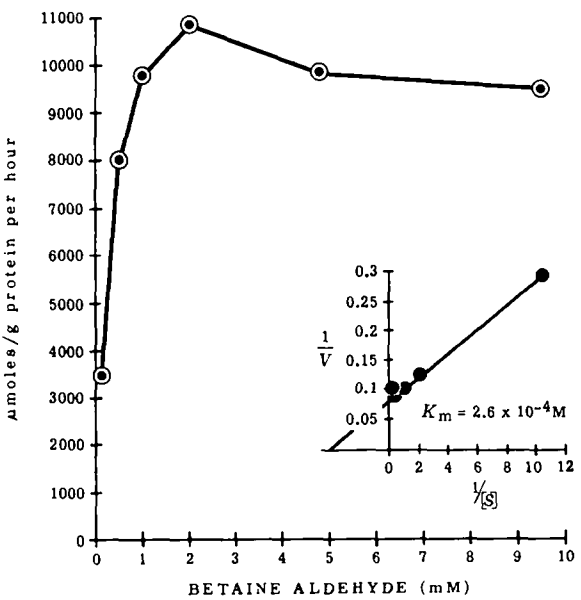

Fig. 1. Effect of substrate concentration on betaine aldehyde dehydrogenase activity. Except for the variation in concentration of betaine aldehyde all conditions are as described.

buffer at pH 8.1 about 90% of optimum activity is obtained. Phosphate buffers at this same pH are inhibitory.

Betaine aldehyde in a concentration of 1 mM gave maximum activity. Increasing the concentration to 10 mM caused a slight decrease in the activity of the enzyme (Fig. 1).

The apparent K_m, obtained by a Lineweaver–Burk plot, is $2.6 \cdot 10^{-4}$ M, (Fig. 1) which is in excellent agreement with that obtained by ROTHSCHILD AND GUZMAN-BARRON[3].

The preparation described was tested as to the "specificity" of the aldehyde dehydrogenase. All conditions were as described above, except that different aldehydes were substituted for the betaine aldehyde in the same final concentration. The rate of oxidation of acetylaldehyde and propionaldehyde was less than 10% of that for betaine aldehyde. The low activity toward acetaldehyde suggests that non-specific aldehyde dehydrogenases have been removed to a considerable extent.

The primary purpose of this study was to isolate a partially purified dehydrogenase that could be used in the development of an assay system for choline dehydrogenase. The partial purification of choline dehydrogenase is now in progress and it is expected that these two enzymes may be of considerable use in developing a sensitive fluorometric method for endogenous choline and acetylcholine in tissue extracts.

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