# Sheep Liver Serine–Threonine Dehydratase. I. Purification and Evidence for Covalently Linked α-Ketobutyrate as Its Cofactor†

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ABSTRACT: L-Serine–threonine dehydratase (EC 4.2.1.16) from sheep liver has been obtained as a highly purified preparation as shown by ultracentrifuge studies and analytical disc gel electrophoresis. The dehydratase has a molecular weight of 98,000 ± 10,000 and is composed of two nonidentical subunits with molecular weights of 41,000 and 47,000. The 41,000 subunit is covalently linked to the carbonyl reagent-sensitive coenzyme which has been identified as α-ketobutyric acid.

In this communication we report the purification scheme for obtaining highly purified sheep liver serine–threonine dehydratase, its molecular weight, subunit properties, and coenzyme. In past communications (Davis and Metzler, 1962; Nishimura and Greenberg, 1961; McLemore and Metzler, 1968) indirect evidence has been presented to suggest that pyridoxal phosphate is required for the dehydration of serine and threonine by this dehydratase. This communication, however, for the first time reports the spectral properties of sheep liver dehydratase which fails to show an absorption maximum in the 400–414-nm range; a range in which all pyridoxal phosphate containing serine–threonine dehydratases have been observed to absorb (Davis and Metzler, 1972). Thus this dehydratase resembles serine–threonine dehydratase and urocanase from *Pseudomonas putida* (Cohn and Phillips, 1974; George and Phillips, 1970), histidine decarboxylase (Riley and Snell, 1968), adenosylmethionine decarboxylase (Wickner, et al., 1970), and proline reductase (Hodgins and Abeles, 1967), in having a non-pyridoxal phosphate–carbonyl cofactor.

## Experimental Procedure

*Materials.* Sheep livers were obtained fresh from Wilson Sinclair of Cedar Rapids, Iowa. Enzyme grade ammonium sulfate was obtained from Mann, Orangeburg, N.Y. L-Threonine, DEAE-Sephadex, G-200 Sephadex, bovine serum albumin, aldolase, and ovalbumin were obtained from Sigma Chemical Co., St. Louis, Mo. Bio-Gel A 1.5m (100–200 mesh) and A 0.5m (100–200 mesh) were obtained from Bio-Rad of Richmond, Calif. All other chemicals were from common commercial sources.

*Assay.* Enzyme assays have been previously described by Davis (1965). A unit of enzyme is that which will produce 1 μmol of keto acid/hr at pH 7.2 in phosphate buffer. Specific activity is defined as units per milligram of protein. Protein was measured by the method of Lowry (1951) and by the absorbance at 280 nm. The Lowry method and the absorbance at 280 nm were consistent with each other after the Agarose gel purification step assuming a 1-mg/ml protein solution to have an absorbance of 1.0 at 280 nm. Spectra were recorded on a Cary 15 spectrophotometer. Ultracentrifugation studies were done on a Spinco Model E ultracentrifuge, and radioactivity was determined on a Beckman LS-150 scintillation counter.

*Gel Electrophoresis.* Disc gels were run by the procedure of Davis (1964) with the modification of staining with Coomassie Blue. Sodium dodecyl sulfate gels were run by the procedure of Laemmli (1970). The activity stain developed by Feldberg and Datta (1970) was used to locate the band showing threonine dehydratase activity.

## Results

In attempting to purify sheep liver serine–threonine dehydratase, one is plagued by the variable enzyme level from one sheep liver to another. In order to procure enzyme the following scheme was followed, which allowed for the sampling of up to 24 livers over a short period of time. Eight to 24 livers was obtained directly off the processing line and carried to the laboratory on wet ice. Each liver was tagged and a 10-g sample removed and homogenized for 30 sec in a Waring Blendor with 20 ml of buffer.[1]

The homogenate was centrifuged and the supernatant assayed by the 2,4-dinitrophenylhydrazine method after a 5-min incubation. An absorbance reading of 0.5–1.0 indicated a liver having approximately $10^4$ units of activity. Only livers of this activity and above were normally purified. Once active livers were identified, highly purified enzyme could be obtained by the following procedure.

Step 1. Extraction of Enzyme. Active livers were washed with cold distilled water, ground in a meat grinder, and homogenized in a Waring Blendor for 30 sec with twice their volume of buffer.

Step 2. Chloroform Treatment. The homogenate was cooled to 0° and 5 ml of chloroform, precooled to −20°, was added per 100 ml of liver homogenate. The mixture was homogenized in a blender for 20 sec and then quickly transferred to chilled centrifuge cans and centrifuged at 16,000*g* for 15 min in a Sorvall GSA rotor.

Step 3. Heat Treatment. The supernatant from step 2 was taken to 70° in a boiling water bath and held at this temperature for 10 min. The sample was cooled to room

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[1] Buffer refers to 0.1 *M* pH 7.2 sodium phosphate and unless otherwise specified all operations were carried out at room temperature.

Table I: Purification of Serine–Threonine Dehydratase from Sheep Liver.

| Step | Total Units $\times 10^{-4}$ | Total Protein (mg) $\times 10^{-3}$ | Specific Activity | % Yield |
|---|---|---|---|---|
| Crude | 1.6 | 150 | 0.1 | |
| Chloroform | 21 | 38.0 | 5.5 | (100) |
| 1st heat | 20 | 2.60 | 77 | 95 |
| 1st precipitation | 17 | 0.760 | 223 | 81 |
| 2nd heat | 16 | 0.660 | 242 | 76 |
| 2nd precipitation | 14 | 0.340 | 411 | 66 |
| 3rd heat | 11 | 0.220 | 500 | 52 |
| Agarose column | 8.0 | 0.080 | 1000 | 38 |
| DEAE column | 2.2 | 0.012 | 1833 | 10 |

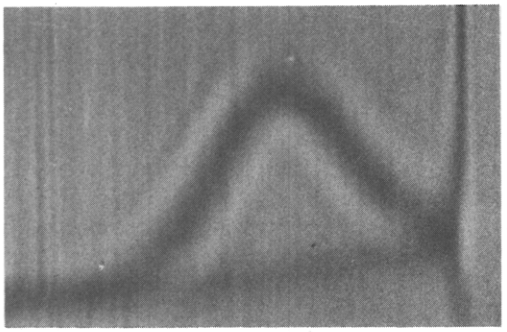

FIGURE 1: The schlieren pattern of serine-threonine dehydratase (7 mg/ml) after 32 min of centrifugation at 44,770 rpm.

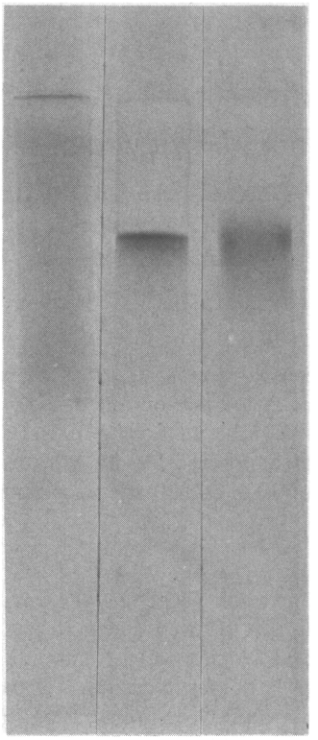

FIGURE 2: Disc gel of serine-threonine dehydratase. Gel A was run without mercaptoethanol. Gels B and C were run with 20 $\mu$l of a mixture of 10 $\mu$l of glycerol, 15 $\mu$l mercaptoethanol, and 50 $\mu$g of enzyme in a total volume of 100 $\mu$l. Gels A and B were stained for protein and gel C was stained for enzymatic activity.

temperature in an ice bath and the denatured protein removed by centrifugation at 16,000*g* for 10 min in a Sorvall GSA rotor.

Step 4. Supernatant from step 3 was made 40% saturated with ammonium sulfate at 25° by the addition of 243 g of salt/l. of solution. The solution was allowed to stand at room temperature for 10 min and the precipitate was removed by centrifugation. The dehydratase at this point appears to be inactive, however, after it is precipitated at 55% saturation with ammonium sulfate and resuspended in buffer the activity returns.

Steps 3 and 4 were repeated as many as four times. The enzyme usually precipitates in the presence of 40% ammonium sulfate during the third and fourth fractionations.

Step 5. After the last ammonium sulfate precipitation the enzyme was dissolved in a minimal amount of buffer and applied to a (2.0 cm × 100 cm) column of Bio-Gel A 0.5 (100–200 mesh). The enzyme was eluted with 0.1 *M* pH 7.2 phosphate buffer.

Step 6. The most active fractions from the Bio-Gel column were pooled and precipitated with 60% ammonium sulfate. The enzyme was dissolved in 0.1 *M* phosphate buffer and then equilibrated with 0.005 *M* buffer at pH 7.2 by dialysis or passage through G-25 Sephadex.

The sample was applied to a DEAE-Sephadex A-50 column (1 × 15 cm). The DEAE-Sephadex was washed by the method used by Peterson and Sober (1959) and equilibrated with 0.005 *M* buffer. The column was eluted stepwise with 0.05 *M* phosphate buffer and then 0.1 *M* buffer (pH 7.2). A small amount of dehydratase activity was eluted with the 0.05 *M* buffer and enzyme of up to 1800 units/mg of protein was eluted by the 0.1 *M* buffer.

The protocol of a typical preparation is shown in Table I. The overall yield is 10%. However, in the protocol shown, a tenfold increase in activity was obtained during the chloroform treatment and all subsequent yields are calculated from this step.

*Ultracentrifuge Studies.* Homogeneity of the purified serine–threonine dehydratase was checked by ultracentrifugation. The dehydratase was dialyzed against buffer for 24 hr and centrifuged in a double sector cell at 44,770 rpm at 20°. The bar angle was 50° and photographs were taken at 8-min intervals. Figure 1 shows the schlieren pattern after 32 min. The enzyme sedimented as a single peak with an $s_{20,w}$ value of 6.1 which is not extrapolated to infinite dilution.

*Properties of the Purified Enzyme on Disc Gels.* Dehydratase in the presence of mercaptoethanol showed a single band on disc gel (Figure 2). In the absence of mercaptoethanol, the enzyme smears as also shown in Figure 2.

*Estimation of Molecular Weight by Chromatography.* The molecular weight of serine–threonine dehydratase was determined by chromatographing the enzyme and marker proteins on columns of Bio-Gel A 1.5, 100–200 mesh (1 cm × 50 cm) and Sephadex G-200. The columns were calibrated with aldolase (mol wt 158,000), bovine serum albumin (mol wt 65,000 by column), and chymotrypsinogen A (mol wt 25,000). The molecular weight of the dehydratase was found to be 98,000 ± 10,000.

*Molecular Weight by Sodium Dodecyl Sulfate Gel Electrophoresis.* Enzyme from the DEAE column (40 $\mu$g) was preincubated with 25 $\mu$l of sodium dodecyl sulfate (20%), 12 $\mu$l of $\beta$-mercaptoethanol, and 25 $\mu$l of 1.0 *M* Tris buffer (pH 6.8). The samples were incubated at 100° for 2 min; 100 $\mu$l of the incubation mixture was electrophoresed on 10-cm 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate prepared according to Laemmli (1970).

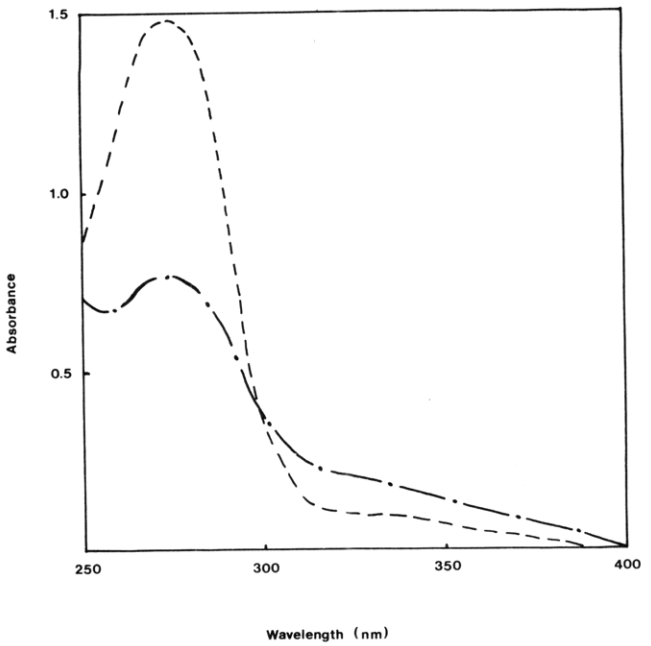


FIGURE 3: Spectrum of serine–threonine dehydratase: (- - -) native dehydratase; (· – · –) phenylhydrazone of native dehydratase). Protein concentrations of the native dehydratase and phenylhydrazine treated dehydratase were 1.5 and 0.7 mg/ml, respectively.

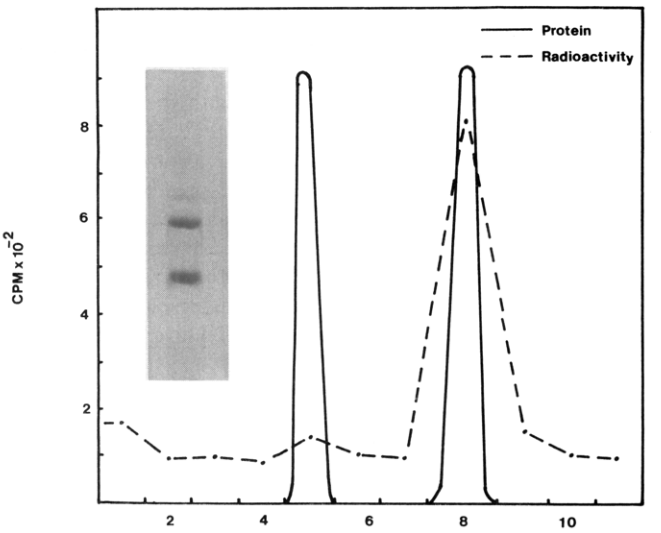


FIGURE 4: Incorporation of tritium into the 41,000 subunit of serine–threonine dehydratase. The subunits were separated by sodium dodecyl sulfate gel electrophoresis after $NaB^3H_4$ reduction.

Electrophoresis was carried out at 6 V/cm for 6 hr with Bromophenol Blue as a tracking dye.

Purified serine–threonine dehydratase was found to contain two subunits of molecular weights 47,000 and 41,000, when compared with standard proteins of known molecular weight in the same system.

*Absorption Spectra.* The spectrum of the purified dehydratase (1.5 mg/ml) in 0.1 *M* phosphate buffer (pH 7.2) was recorded over the range of 250–400 nm against a buffer blank. The spectrum of the active dehydratase in 0.1 *M* buffer lacks an absorption maximum associated with pyridoxal phosphate containing dehydratases (Figure 3). The spectrum of the phenylhydrazine-treated enzyme lacks an absorption band in the 300–350-nm range associated with phenylhydrazine-treated pyruvate-containing enzymes (Riley and Snell, 1968; Wickner et al., 1970). One does observe a decrease in the magnitude of the trough at 250 nm and an overall increase in absorbance in the 300–350-nm range which is indicative of the phenylhydrazine-treated $\alpha$-ketobutyrate-containing enzyme of George and Phillips (1970).

*Radioactive Labeling of Active Subunit.* Enzymatic activity of the dehydratase is destroyed when reduced by $NaBH_4$. Therefore, reduction with $NaB^3H_4$ can be used to locate the subunit containing the carbonyl cofactor. Dehydratase was reduced with $NaB^3H_4$, desalted on Sephadex G-25, and lyophilized and the subunits were separated by sodium dodecyl sulfate gel electrophoresis.

The gel was scanned, sectioned, dissolved in 30% hydrogen peroxide, and counted in a scintillation counter. Radioactivity was incorporated only in the 41,000 molecular weight subunit (Figure 4), indicating it is the subunit which contains the coenzyme.

*Coenzyme Identification.* Serine–threonine dehydratase was reduced with $NaB^3H_4$, dialyzed against two 3-l. changes of water, and lyophilized. The reduced enzyme was hydrolyzed in 6 *N* HCl in a sealed glass tube for 30 hr. The hydrolysate was applied to a Dowex 50 column which was equilibrated and eluted with 6 *N* HCl; 76% of the counts were recovered. The elutant was evaporated to dryness under vacuum and redissolved in distilled water. $\alpha$-Hydroxybutyric acid with tritium in the $\alpha$ position was prepared by reduction of $\alpha$-ketobutyrate with $NaB^3H_4$. The mobilities of the unknown and $\alpha$-hydroxybutyric acid were determined by cutting the chromatogram into 1-cm$^2$ sections for detection of radioactivity by scintillation counting. In basic solvent systems the known $\alpha$-hydroxybutyric acid was also detected with Bromophenol Blue (George and Phillips, 1970). The unknown cochromatographed with $\alpha$-hydroxybutyric acid in the following ascending systems: *n*-propyl alcohol, concentrated $NH_4OH$ (3:2), $R_f$ 0.78; 0.5 *M* ammonium acetate, acetone (1:3), $R_f$ 0.40; chloroform, ethanol, formic acid (50:50:1), $R_f$ 0.92. The unknown and $\alpha$-hydroxybutyric acid were shown to have identical mobilities by high voltage electrophoresis. In 1.0% $(NH_4)_2CO_3$ (pH 8.9) they migrated 16.0 cm in 2 hr at 1000 V. In pyridine–acetic acid–water (100:3:900) (pH 6.5) they migrated 6.0 cm in 2 hr at 1000 V. An average of 70% of the unknown radioactivity migrated with the standard.

## Discussion

In an earlier publication serine–threonine dehydratase from sheep liver was obtained with a specific activity of 776 units/mg of protein (Nishimura and Greenberg, 1961). The present communication describes a procedure for obtaining preparations of the enzyme with specific activities up to 1800 units/mg of protein. The molecular weight of serine–threonine dehydratase is 98,000 ± 10,000 by column chromatography and is composed of two subunits of molecular weights 41,000 and 47,000 as determined by sodium dodecyl sulfate gel electrophoresis. Reduction of the dehydratase with tritiated borohydride results in inactivation of the dehydratase. Tritium is incorporated only in the 41,000 subunit. This result indicates that the 41,000 molecular weight subunit contains the carbonyl coenzyme.

Because of the variability of enzyme levels in individual livers, it is necessary to assay large numbers of them to procure the dehydratase in significant amounts. Only about 10% of over 400 livers assayed over a 3-year period were found to contain $10^4$ units of dehydratase activity per liver

and only four were observed to contain above $10^5$ units/liver. About 85% of the livers assayed did not contain any measurable dehydratase activity.

The variability of dehydratase levels in sheep liver is well known (Nishimura and Greenberg, 1960; Davis and Metzler, 1962) and nonreproducible activation has been observed during this investigation. The presence of the enzyme in the serine-inactivated form has been suggested to account for this variability (Moss and Dian, 1965). However, another possible explanation for the variability may be the presence of an inactive protein precursor similar to the pyruvate containing histidine decarboxylase of Recsei and Snell (1973).

The enzyme, like most other dehydratases (Davis and Metzler, 1972), is inactivated when acting on serine as a substrate. We have recently observed a similar inactivation with L-$\beta$-chloroalanine as a substrate (unpublished results). This serine inhibition can be relieved by pyridoxal phosphate (Nishimura and Greenberg, 1961) or pH alteration (McLemore and Metzler, 1968). The effect of pyridoxal phosphate on the serine-altered enzyme has, in the past, led to the conclusion that this enzyme required as a carbonyl cofactor pyridoxal phosphate.

Inactivation by serine has been suggested to occur by cofactor resolution (Nishimura and Greenberg, 1961), by oxazolidine formation (McLemore and Metzler, 1968), and by alkylation of an essential group by aminoacrylate (Phillips, 1968). The L-$\beta$-chloroalanine observation would rule out oxazolidine formation as the mode of inhibition and the pH activation would tend to rule out cofactor resolution. Therefore, it would appear that the serine inhibition occurs by alkylation.

Alkylation by aminoacrylate could result in formation of an enzyme $\beta$-substituted serine. In the presence of pyridoxal phosphate this complex could undergo a nonenzymic $\beta$ elimination, thus explaining the reversal of the serine inhibitation by high levels of pyridoxal phosphate.

As a result of obtaining highly purified dehydratase, we can also for the first time investigate the spectral properties of the enzyme relative to cofactor requirement. The activity of the enzyme is independent of added pyridoxal phosphate (Sayre and Greenberg, 1956) and dialysis against buffer does not resolve a cofactor (Nishimura and Greenberg, 1961). Highly purified enzyme does not absorb light in the region where all pyridoxal phosphate requiring dehydratases absorb. These observations plus the reported inhibition of the enzyme by carbonyl reagents (Nishimura and Greenberg, 1961; Davis and Metzler, 1962) show that the dehydratase from sheep liver requires a carbonyl cofactor which is not pyridoxal phosphate.

Sheep liver serine–threonine dehydratase does not form a phenylhydrazone which absorbs like that of the adenosylmethionine decarboxylase isolated by Wickner et al., (1970) or the histidine decarboxylase isolated by Riley and Snell (1968) indicating the cofactor is not pyruvic acid. However, the phenylhydrazine-treated enzyme does resemble the phenylhydrazone of urocanase from *P. putida* (George and Phillips, 1970), suggesting that the coenzyme is $\alpha$-ketobutyrate. $\alpha$-Ketobutyric acid has been confirmed as the cofactor of sheep liver serine–threonine dehydratase by reduction of the holoenzyme and isolation of the reduced cofactor. The reduced cofactor cochromatographed with authentic $\alpha$-hydroxybutyric acid in three ascending and two high voltage systems, thus establishing sheep liver serine–threonine dehydratase as a non-pyridoxal phosphate containing dehydratase.

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