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On the Purification of Liver Alcohol Dehydrogenase

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A modified procedure is described for the preparation of crystalline alcohol dehydrogenase from horse liver, which gives a greater yield of enzyme of apparently higher specific activity than previous methods. The recrystallised product has always contained two components on electrophoresis. The major component has been isolated by chromatography, and its properties determined.

Liver alcohol dehydrogenase has in recent years been the subject of detailed kinetic study [1-4], and its purification and characterisation is therefore a matter of some importance. Following the work of Lutwak-Mann [5], in which partial purification of the enzyme of horse liver was effected by acetone precipitation, heating to remove unwanted protein, and precipitation with ammonium sulphate, Bonnichsen and Wassén [6] crystallised the enzyme after fractionation with ethanol and ammonium sulphate. In a later method [7], which gave a more active product [2], acetone precipitation was omitted, and treatment with ethanol-chloroform mixture was introduced to remove haemoglobin [8].

A further slight modification, which apparently gave a still more active enzyme, was described by Bonnichsen and Brink [9]: the essential steps were (1) heat treatment of the aqueous liver extract, (2) fractionation with ammonium sulphate, (3) ethanol-chloroform treatment, (4) fractionation and crystallisation with ethanol.

The properties of material prepared by this method and repeatedly recrystallised to a constant activity/E_{280} ratio were described recently [10,11]. The specific extinction coefficient at 280 mμ (1 mg/ml, 1 cm) agreed with previous values [7,9], but the specific activity was again increased, by 30 % compared with that reported by Bonnichsen and Brink and by 60 % compared with that of Bonnichsen's earlier preparation [2]. Nevertheless, the protein was not completely homogeneous on electrophoresis.

A further modification of the purification procedure will be described here which has consistently given a crystalline product of this same high

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activity in twice the yield of previous methods. The product has always contained a small proportion of a second active component on electrophoresis, which can be removed by chromatography. The properties of the electrophoretically homogeneous main component will be described in this and in the following paper [12].

PURIFICATION PROCEDURE

The principal differences from the previous methods [7,9] are that the ethanol-chloroform treatment is carried out first on the liver extract, and after heat treatment and ammonium sulphate fractionation, the crude product is chromatographed on carboxymethyl cellulose; the subsequent crystallisation procedure also differs slightly. The assay of the enzyme has been described previously [10].

(1) *Extraction.* Ground horse liver (5 kg) is extracted with twice the amount of water for 2 h at room temperature, or overnight in the cold, and centrifuged (2 300 r.p.m., 45 min, International Serum Centrifuge, capacity 12 l).

(2) *Removal of haemoglobin.* To the dark brown, slightly turbid extract (10 l) a mixture of ethanol (1 600 ml 95 % ethanol + 800 ml chloroform) is added very slowly with vigorous stirring. After 10 min, with occasional stirring, the denatured haemoglobin is centrifuged off (2 300 r.p.m., 30 min). The green-brown supernatant (10 l) is evaporated under reduced pressure; the minimum amount of octanol is added to reduce foaming, and the flask is immersed in a water bath at 55°. Most of the organic solvents should be removed. The dark green-brown solution (8—9 l), which may contain some precipitate of heat-denatured protein, is thoroughly dialysed at room temperature against phosphate buffer pH 7, $\mu = 0.02$ (2 × 20 l, 18 h).

(3) *Ammonium sulphate fractionation.* The enzyme is precipitated between 0.55 and 0.70 saturation at room temperature. The pH should be kept at 6.0—6.5 by the addition of ammonia. 3.5 kg ammonium sulphate per 10 l solution is added and, after 2 h the brown or green-brown precipitate is removed by centrifuging and discarded (<0.5 g enzyme). To the clear, yellow-green supernatant another 4.7 kg per 10 l is added; after 2 h, the crude enzyme is centrifuged down (2 300 r.p.m., 1 h) as a cream precipitate, and the clear yellow supernatant (<0.1 g enzyme) is discarded. (Difficulty in separating the precipitate indicates that organic solvents have not been completely removed.) The precipitate is washed into dialysis sacs with the minimum volume of phosphate buffer, $\mu = 0.1$, pH 7 (*ca.* 400 ml), and thoroughly dialysed against the same buffer (3 × 4 l) to remove ammonium sulphate, when it dissolves to give a clear yellow-brown solution.

(4) *Heat denaturation.* The dialysed solution is kept at 52° for 15 min, cooled to room temperature and centrifuged. The precipitate is washed with a small volume of buffer, and the combined supernatant and washings, containing 3—4 g enzyme, purity 5—10 %, are dialysed against phosphate buffer, pH 6.0, $\mu = 0.05$.

(5) *Chromatography.* Carboxymethylcellulose, prepared according to Peterson and Sober [13] (except that drying with ethanol is omitted), and equilibrated with phosphate buffer, pH 6.0, $\mu = 0.05$ is packed under 30 cm Hg pressure for a few hours in a column, 3 × 65 cm. The chromatography is carried out at +2°, under the same pressure, with a flow rate of about 60 ml/h. After the enzyme is put onto the column, it is washed with the same buffer until the runnings are colourless. The enzyme is completely retained, together with a yellow pigment. The runnings are first brown, then yellow, and finally colourless. The enzyme, together with the yellow pigment which may be used as an indicator, is rapidly eluted with phosphate buffer, pH 8, $\mu = 0.2$. Over 90 % of the enzyme should be recovered from the column, in a volume of 150 ml: purity 30—40 %.

(6) *Crystallisation.* (a) The enzyme solution is cooled to 0°, and cold 90 % ethanol is added gradually with continuous chilling to a final ethanol concentration of 30 % and a temperature of −12°. The mixture is left at this temperature overnight. The amorphous precipitate containing the whole of the enzyme is separated from the yellow supernatant

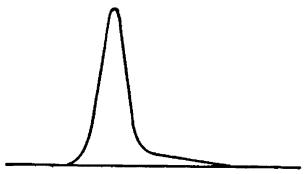

Fig. 1. Electrophoresis of recrystallised enzyme in the 10 ml Tiselius apparatus. $I = 15.5$ mA. Time = 16 h. Sodium phosphate buffer. pH 6.0. $\mu = 0.1$.

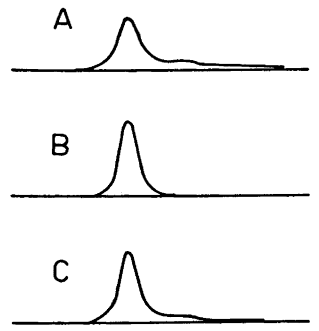

Fig. 2 Electrophoresis in the 10 ml Tiselius apparatus. $I = 15.5$ mA. Sodium phosphate buffer. pH 6.0. $\mu = 0.1$.
A. Recrystallised unresolved enzyme after 21 h.
B. Chromatographic fraction I after 22 h.
C. Mixture of chromatographic fraction I + II after 16 h.

by centrifuging at −12°, washed once with 30 % ethanol in phosphate buffer, and well drained. (b) The remaining steps are carried out at +2°. The precipitate is washed into a dialysis sac with the minimum volume (ca 20 ml per g enzyme) of phosphate buffer, $\mu = 0.1$, pH 7, to which ammonia has been added to raise the pH to 9 and is dialysed against the same buffer until the precipitate largely dissolves to give a pale yellow solution containing some turbidity and precipitate of denatured protein. The solution is then dialysed overnight against phosphate buffer, $\mu = 0.05$, pH 7, and then against the same buffer containing 6 % ethanol for 24 h to crystallise. The crystals are separated by centrifuging, washed once with a little 6 % ethanol dialysate, and dissolved as before in phosphate-ammonia, pH 9. Denatured protein is removed by centrifuging. The very pale yellow solution contains 80 % of the enzyme removed from the column, purity [10] 95 %; the remainder is lost in the 6 % ethanol supernatant and washings. (c) On recrystallisation with 6 % ethanol in the same manner, and removal of denatured protein, the pure [10], colourless crystals are obtained in a yield of 2 g per 5 kg liver.

Electrophoresis and isolation of the main component

The recrystallised product has never been completely homogeneous on electrophoresis. The main component, which is 90 % or more of the total protein in the best preparations, has an isoelectric point of 6.8, phosphate, $\mu = 0.1$. The minor component, which moves behind the main peak at pH 6.0 (Fig. 1) and in advance of it at pH 7.4, was separated in the analytical Tiselius apparatus and found to be active. Because of the small proportion present, and the small difference between the mobilities, it has not been separated in quantity or as a distinct separate peak.

The main component can be obtained in an electrophoretically homogeneous form by chromatography on carboxymethyl cellulose. After the enzyme has been put on the column at pH 6.0, it is developed with phosphate buffer, $\mu = 0.05$, pH 6.8, until about 30 % of the enzyme has been eluted. In accordance with the electrophoretic properties and the chromatographic

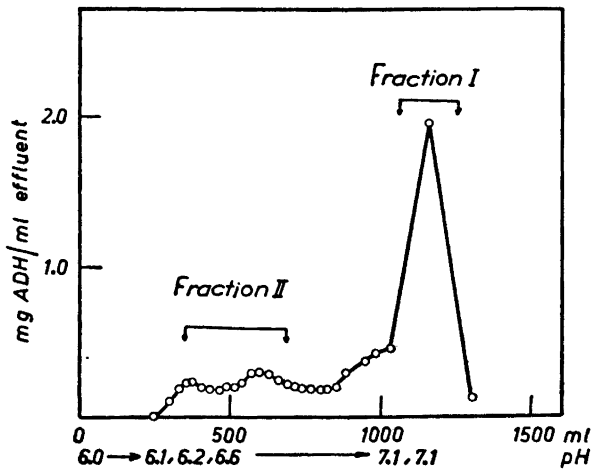

Fig. 3. Elution diagram of recrystallised enzyme on carboxymethylcellulose.

experiments described below, this fraction contains the minor constituent. The remainder of the enzyme is eluted with phosphate buffer, $\mu = 2.0$, pH 8.0, and is electrophoretically homogeneous.

Properties of the main component

The specific extinction coefficient (1 mg/ml, 1 cm) at 280 mμ was 0.42. In the spectrophotometric assay [10] the concentration of the enzyme solution is $\frac{1.13}{t_{0.2} \times v}$ mg/ml, where v is the volume in ml of the enzyme solution used and $t_{0.2}$ is the time in seconds required for the extinction at 340 mμ (1 cm layer) to increase by 0.200. The value of $\frac{1}{E_{280} \times t_{0.2} \times v}$ might be used as a "purity index". Determinations of the molecular weight, sedimentation constant and diffusion constant, and spectrophotometric titration with reduced diphosphopyridine nucleotide, are described in the following paper [12].

Further experiments on inhomogeneous preparations

In preparations in which dialysis after ethanol-chloroform treatment and evaporation was omitted, separation by centrifugation of the crude enzyme precipitated with 0.7 saturated ammonium sulphate was more difficult. The final recrystallised product was apparently pure, as judged by the purity index, but electrophoresis indicated the presence of 20—30 % of the minor component.

The electrophoretic behaviour of such a preparation is illustrated in Fig. 2 and chromatography of the same preparation in Fig. 3. The chromatography was carried out on carboxymethyl cellulose at +2°. 690 mg of recrystallised enzyme, purity index 215, in a concentration of 12 mg/ml was put on the column in phosphate buffer, $\mu = 0.05$, pH 6.0, and eluted first with phosphate buffer, $\mu = 0.05$, pH 6.6, and finally with phosphate buffer, $\mu = 0.1$, pH 7.1. The effluent was collected in 20—30 ml volumes and assayed by activity and E_{280} measurements. There was no significant variation in the purity index during the elution, but the elution diagram indicates the presence of a minor active component, estimated as 15 % of the total, although the separation from the main component is not clear cut. A similar result was obtained with another preparation.

Two fractions of the eluted enzyme, as indicated in Fig. 3, were concentrated and crystallised with alcohol, and dialysed. From fraction I, the main component, 340 mg enzyme, purity index 219, were obtained. The specific extinction at 280 mμ was 0.42, and the preparation was homogeneous on electrophoresis (Fig. 2 B). From fraction II, 40 mg, purity index 226, was obtained. The specific extinction was 0.47. Fractions I and II were mixed in the proportion 80 % I and 20 % II. The electrophoretic behaviour of the mixture (Fig. 2 C) is similar to that of the unresolved enzyme, and confirms the reality and identity of the minor active component observed in electrophoresis and chromatography.

DISCUSSION

In the purification procedures of Bonnichsen [7] and Bonnichsen and Brink [9], ethanol-chloroform treatment after ammonium sulphate fractionation does not remove all the haemoglobin, and the enzyme is crystallised from a deep red solution. By applying the ethanol-chloroform treatment directly to the liver extract, the haemoglobin is completely removed; on general grounds also there is something to be said for introducing this rather drastic treatment at as early a stage as possible. Chromatography prior to crystallisation gives a 6-fold increase fo purity, and is probably mainly responsible for the increased yield; any catalase or haemoglobin which may have escaped earlier steps is also removed here. Finally, a more rational and reproducible crystallisation procedure can be applied to the relatively pure, haemoglobin-free product.

The enzyme readily crystallises together with inactive material, and this seems to have been responsible for the low activity of the orginal crystalline product [6] compared with Bonnichsen's material. The reasons for the subsequent successive gains in activity reported by Bonnischsen and Brink [6], and by Dalziel [10] for a repeatedly recrystallised product of their procedure, are not se clear. These reports are based upon the spectrophotometric assay method of Theorell and Bonnichsen [2,10], in which the rate of reduction of the coenzyme by ethanol at pH 9.6 is measured. A similar gain in activity is indicated by fluorimetric mearurements of the maximum rate of this reaction at pH 7.15:2.5 sec^{-1} for Dalziel's preparation compared with 1.6 sec^{-1} for Bonnichsen's preparation [3], It is possible, of course, that inhibitors were present in the reagents used in the earlier work, perhaps in the less pure coenzyme

then available. There has been no further significant gain in activity by the present method of preparation. It may be mentioned, however, that the purity index of enzyme freshly eluted from carboxymethylcellulose during chromatography of the recrystallised product was usually about 10 % greater than that given above for the pure enzyme, but always decreased to the latter value after recrystallisation and dialysis.

The separation of a recrystallised enzyme into more than one active component by electrophoresis and chromatography recalls similar observations on other enzymes and proteins, especially the lactic acid dehydrogenase of heart muscle [14]. In the present case, the fact that the proportion of the minor component is increased in preparations in which alcohol, chloroform or octyl alcohol may not have been completely removed after the Tsuchihashi treatment suggests that it may be formed during the purification procedure. This question, and the properties of the minor component, require further investigation. It has been shown that the minor component has about the same specific activity as the major one, and in one determination a fraction containing a high proportion of the minor component gave a specific extinction at 280 mμ of 0.47, compared with 0.42 for the major component. This presumably accounts for the specific extinction of 0.45 previously reported [3,10] for the recrystallised enzyme.

The molecular weight of the homogeneous main component is 84 000; [12,15] the sedimentation constant is greater, and the diffusion constant smaller, than the values reported previously [2], which gave a molecular weight of 67 500 [2,11,15]. The difference in the diffusion constant is consistent with inhomogeneity of the earlier preparations. Nevertheless, the earlier finding that 2 molecules of DPNH are bound per molecule of enzyme has been reproduced with the homogeneous material [12].

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