The Preparation and Properties of Crystalline Alcohol Dehydrogenase from Liver

By K. DALZIEL*
Department of Biochemistry, University of Sheffield, Sheffield 10

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Kinetic studies of alcohol dehydrogenase prepared from horse liver by different methods have given different results (Theorell, Nygaard & Bonnichsen, 1955; Dalziel, 1958; Theorell, 1958; K. Dalziel, unpublished work, 1957). The most active crystalline preparations have been obtained by a modification of earlier methods (Bonnichsen, 1950; Bonnichsen & Brink, 1955) in which the principal new feature was chromatography of the crude enzyme preparations on carboxymethylcellulose before crystallization (Dalziel, 1958). Such products were homogeneous in the ultracentrifuge (Ehrenberg & Dalziel, 1958), but electrophoresis indicated the presence of small proportions of a second active component, and it was suggested that this may be formed from the major component during agitation of the liver extract with ethanol and chloroform to denature haemoglobin (Tsuchihashi, 1923; Bonnichsen, 1950). It has now been found that this step can be omitted, and haemoglobin removed instead by modification of the ammonium sulphate fractionation and chromatography. Some properties of the crystalline product are described, and kinetic data are briefly considered with reference to earlier work.

EXPERIMENTAL

Reagent solutions and phosphate buffers, I 0·1, were made up in glass-distilled water.

Coenzymes and substrates. Ethanol and acetaldehyde were redistilled, and the latter was stored at $-15°$. Diphosphopyridine nucleotide (DPN) and the disodium salt of the reduced form ($DPNH_2$) were from Sigma Chemical Co. and were assayed by measurement of the change in light-absorption at 340 mμ accompanying complete enzymic reduction or oxidation with alcohol dehydrogenase and excess of substrate, taking the millimolar extinction coefficient of DPNH as 6·22 (Horecker & Kornberg, 1948). The several samples used assayed as 84–88 % DPN and 68–83 % $DPNH_2(Na_2)$ by weight.

Assay method. Enzyme concentrations were estimated as described by Dalziel (1957*a*) by measurement of the time taken for the formation of a given amount of $DPNH_2$ from alcohol and DPN at pH 10 and 23·5°. The measurements were made either spectrophotometrically or fluorimetrically. In the spectrophotometric method the enzyme concentration in mg./ml. is equal to 1·13 tv, where v is ml. of enzyme solution used and t is time (sec.) required for an increase of extinction at 340 mμ of 0·200. The specific extinction coefficient (1 mg./ml., 1 cm. path) of apparently pure enzyme, prepared by the previous method, from which the minor component had been removed by chromatography, was 0·42 at 280 mμ (Dalziel, 1958). The purity of fractions in the present preparations is calculated from the enzyme concentration, determined by assay, and the extinction at 280 mμ, E_{280}, by the empirical expression $0{\cdot}42 \times$ mg./ml.$/E_{280}$.

Initial-rate measurements. These were made with a recording fluorimeter, based on that of Theorell & Nygaard (1954). Initial-rate measurements were made essentially as described by Theorell *et al.* (1955), and maximum rates were estimated by double reciprocal plots (cf. Dalziel, 1957*b*).

A Perspex cylinder served as a fluorescent standard, and for enzyme assays the instrument was periodically calibrated with enzyme solutions assayed by the spectrophotometric method. For initial-rate measurements, calibration of the standard directly against $DPNH_2$ solutions of known concentration is not satisfactory because the coenzyme samples always contain a fluorescent compound that is not coenzymically active with liver (and yeast) alcohol dehydrogenase. After rapid oxidation of $DPNH_2$ by excess of acetaldehyde and alcohol dehydrogenase at pH 7, significant absorption at 340 mμ and also fluorescence emission remain, both amounting to about 7 % of the initial values. The method used was to compare the fluorescence of the standard, F_S, with the fluorescence changes, F_T, accompanying complete enzymic reduction of DPN at pH 10 and complete enzymic oxidation of $DPNH_2$ at pH 7, by using solutions of coenzymes that had been assayed spectrophotometrically as described above. The results of the two types of measurement agreed, but the plot of the relative fluorescence, F_R ($= F_T/F_S$), against concentration, c, is not linear because of light-absorption by the $DPNH_2$ (Fig. 1). The effect of this small curvature on the calculation of initial rates of $DPNH_2$ oxidation is greater than might be supposed at first sight, since the quantity required to convert the measured rate of change of fluorescence intensity, dF_R/dt, into rate of change of $DPNH_2$ concentration, dc/dt, is the reciprocal of the slope of the tangent to the curve of Fig. 1, dF_R/dc, at the appropriate initial $DPNH_2$ concentration. This quantity is best obtained by an alternative linear plot derived from an empirical equation to the curve of Fig. 1:

$$F_R = Ac - Bc^2 \quad (1)$$

hence

$$dF_R/dc = A - 2Bc \quad (2)$$

and

$$F_R/c = A - Bc. \quad (3)$$

* Sorby Research Fellow of the Royal Society.

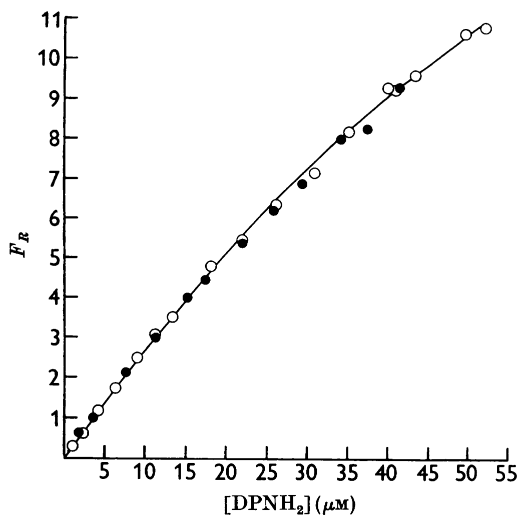

Fig. 1. Calibration curve for the fluorimeter. Relation between the fluorescence of $DPNH_2$ solutions divided by the fluorescence of the standard and the $DPNH_2$ concentration. The $DPNH_2$ fluorescence was measured as the change of fluorescence intensity accompanying complete enzymic oxidation of $DPNH_2$ (●) and complete enzymic reduction of DPN (○). The fitted line was calculated from equation (1) and the constants were obtained from Fig. 2.

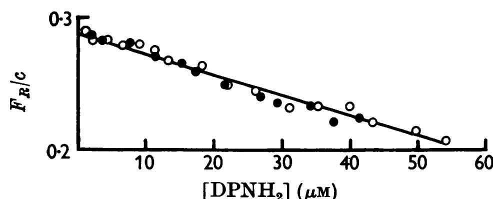

Fig. 2. Relation between the specific fluorescence of $DPNH_2$ divided by the fluorescence of the standard (F_R/c) and the $DPNH_2$ concentration, determined by complete enzymic oxidation of $DPNH_2$ (●) and complete enzymic reduction of DPN (○). The intercept and slope give values for the constants in equation (2) from which the calibration factor is calculated (see text).

In accordance with equation (3), the plot of F_R/c against c is linear (Fig. 2) and the constants $A = 0{\cdot}286$ and $B = 0{\cdot}0015$ are obtained as the intercept and slope. The required calibration factor, dc/dF_R, is then obtained from equation (2), and is in fact the $DPNH_2$ equivalent of the standard for any initial $DPNH_2$ concentration. The values are 3·5 μM when the initial $DPNH_2$ concentration is zero, i.e. for measurements of the initial rate of alcohol oxidation, and 4·4 μM for initial-rate measurements of aldehyde reduction with an initial $DPNH_2$ concentration of 20 μM.

Purification of enzyme. Ground fresh horse liver (2 kg.) is extracted with 4 l. of water for 2 hr. at room temperature or overnight at 2°. By $(NH_4)_2SO_4$ fractionation at pH 6·5 and 2°, 80% of the enzyme is precipitated between 0·5 and 0·67 saturation, and much haemoglobin is left in the final supernatant. To the extract, after centrifuging, $(NH_4)_2SO_4$ (300 g./l.) is added, and after 2 hr. the precipitate, containing catalase and ferritin, is removed in the Servall centrifuge (G.S.A. head, 8000 rev./min., 1 hr.). To the clear red supernatant solution, $(NH_4)_2SO_4$ (110 g./l.) is added and after 2 hr. the pale-red precipitate containing the enzyme is separated in the centrifuge as before.

The precipitate is taken up in the minimum volume of cold 0·05 M-sodium phosphate buffer, pH 7, to give a thick gel, and more buffer is added gradually with shaking in the cold until a heavy crystalline precipitate (haemoglobin, ? aldolase) is formed. The suspension (about 200 ml.) is centrifuged (SS1 head, 14 000 rev./min., 60 min.) and the precipitate discarded. Further dilution and centrifuging may be necessary to obtain a clear red solution.

The solution is heated at 52° for 15 min., cooled and centrifuged. The heavy precipitate of denatured protein is washed with buffer, and the combined supernatant solution and washings are dialysed against 0·05 M-phosphate buffer, pH 7 (3 × 2 l.), and then against 0·04 M-phosphate buffer, pH 6·0 (2 × 2 l.).

Chromatography is carried out at 2° on carboxymethylcellulose, prepared according to Peterson & Sober (1956) except that washing and drying with ethanol are omitted. The adsorbent was equilibrated with 0·04 M-phosphate buffer, pH 6·0, and packed by gravity in a column (3 cm. × 50 cm.), which gives a flow rate of 50 ml./hr. The dialysed enzyme solution (about 350 ml.) is run in, and the column washed with buffer until the runnings, initially yellow–brown, are colourless, and the specific extinction at 280 μ is <0·2. Haemoglobin and alcohol dehydrogenase are firmly adsorbed. Development and elution are effected with phosphate buffer, pH 6·6, I 0·1. As the haemoglobin front spreads down the column, a yellow band forms in front of it. The first yellow eluate, about 10 ml. containing enzyme of low purity, is discarded. In the subsequent orange and pale-red fractions, 75% of the enzyme, purity 0·4, is obtained in about 150 ml.

The enzyme is crystallized by the addition of 90% ethanol to give a final concentration of 15% (v/v) with chilling to −7°, and left at this temperature for 48 hr. The pale-yellow enzyme precipitate is separated in the centrifuge, washed twice with 15% ethanol in 0·05 M-phosphate buffer, pH 7, and dissolved in the minimum volume of this buffer to which NH_3 has been added to raise the pH to about 9·5. The enzyme is then twice recrystallized by dialysis against phosphate buffer containing 6% of ethanol, as described by Dalziel (1958).

Dry-weight determinations. These were made by heating solutions of the recrystallized enzyme at 110° and cooling in a vacuum over P_2O_5 to constant weight.

Thiol groups. These were estimated by spectrophotometric titration of the enzyme with p-chloromercuribenzoate (Boyer, 1954).

RESULTS

A typical schedule for the enzyme purification is given in Table 1, and the elution from carboxymethylcellulose is summarized in Table 2. Much haemoglobin is removed in the ammonium sulphate fractionation, and most of the remainder is retained by the carboxymethylcellulose. The large purification in the chromatography step is mainly

Table 1. *Purification of horse-liver alcohol dehydrogenase*

Step	Volume (ml.)	Enzyme (mg./ml.)	Enzyme Total (g.)	Yield (%)	Purity
Extract	3700	0·54	2·00	100	—
Ammonium sulphate	285	5·5	1·56	78	—
Heat, dialysis	380	3·5	1·33	66	0·04
Chromatography	182	5·5	1·00	50	0·40
Cryst., 15% ethanol	29	27·6	0·80	40	0·70
Recryst., 6% ethanol	16·5	37·5	0·62	31	0·98
Recryst., 6% ethanol	16·2	33·4	0·54	27	1·02

Table 2. *Elution of alcohol dehydrogenase from carboxymethylcellulose*

Volume (ml.)	Colour	Enzyme (mg./ml.)	Enzyme Total (mg.)	Purity
10	Yellow	4·0	40	0·20
21	Pink	15·0	315	0·42
100	Red	5·3	530	0·46
40	Red	2·5	100	0·35
20	Red	2·0	40	0·30

due to the removal of foreign protein, including the whole of the aldehyde dehydrogenase, in the washings at pH 6.

The recrystallized enzyme behaved similarly to the product of the earlier method on electrophoresis in the Tiselius apparatus (Dalziel, 1958, Fig. 1). In phosphate buffer, I 0·1, there was evidence of a few per cent of a second component moving in advance of the main component at pH 7·6 and pH 7·9, and behind it at pH 6·0, although it did not separate as a distinct separate peak even after 20 hr.

The extinction at 280 mμ of a solution of the recrystallized protein containing 1 mg./ml. in 1 cm. depth was 0·42 ± 0·01, an average of measurements on four preparations.

To 3 ml. samples of enzyme solution in phosphate buffer, pH 7, containing 0·5–9·6 μmoles of enzyme/l., 0·1 ml. of *p*-chloromercuribenzoate solution was added to give a final concentration of 62 μmoles/l. The extinction of the mixture at 250 mμ was measured after 3 and 60 min., and the increase due to mercaptide formation calculated. The results indicated 24 thiol groups reacting in 3 min. and 28 reacting in 60 min. (Fig. 3). Reaction was in fact practically complete in about 15 min. The increase of extinction with excess of enzyme between 3 and 60 min. is probably due to slight turbidity, which becomes marked after 2 or 3 hr. with high enzyme concentrations, whereas the solutions of low concentration remain clear.

The activity of the enzyme after treatment with various amounts of *p*-chloromercuribenzoate at room temperature and pH 7 for 15 min. was tested by measurements of the initial rate of ethanol oxid-

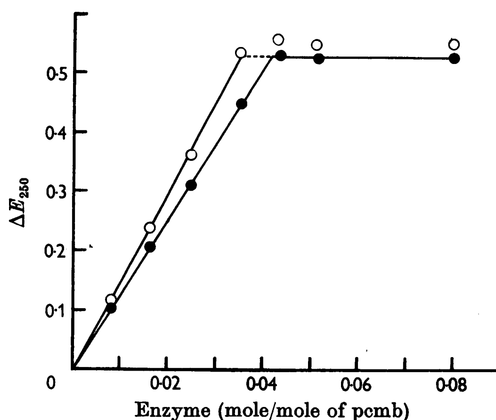

Fig. 3. Spectrophotometric titration of liver alcohol dehydrogenase with *p*-chloromercuribenzoate (pcmb). The extinction change at 250 mμ was measured after 3 min. (●) and 60 min. (○).

ation at pH 10 with high ethanol (8 mM) and DPN (0·42 mM) concentrations. The enzyme solutions contained 10 μmoles/l. and 5 μl. volumes were used in 3 ml. of reaction mixture for the rate measurements. Combination of 34, 67 and 100% of the titratable thiol groups caused 64, 93 and 100% inhibition respectively.

The enzyme is reasonably stable in 0·05 M-phosphate buffer solution, pH 7, provided the solutions are not too dilute. At concentrations of 10 μM or more there is no loss of activity after several days at 2°, whereas 1 μM-solutions lose 5% of activity in 24 hr. At room temperature, the more dilute solution loses 5% of activity in 4 hr. At pH 6 the enzyme is a little more unstable, and loses 10% of activity in 4 hr. at room temperature in 1 μM-solution. Similar results were obtained for crystalline enzyme prepared according to Bonnichsen & Brink (1955).

Maximum rates (molecules of substrate/sec./active centre) at 23·5° with enzyme purified by the present method were, at pH 7·15 and pH 6·0 respectively, 2·75 sec.$^{-1}$ and 1·6 sec.$^{-1}$ for ethanol oxidation and 100 sec.$^{-1}$ and 90 sec.$^{-1}$ for acetalde-

hyde reduction. These constants are based on an equivalent weight of 42 000 for the enzyme, which binds two molecules of coenzyme/molecule and has a molecular weight of 84 000 (Theorell & Bonnichsen, 1951; Ehrenberg & Dalziel, 1958). The data were obtained from secondary plots (Fig. 4) of the intercepts of primary plots according to Lineweaver & Burk (1934) of initial-rate measurements with simultaneously varied coenzyme and substrate concentrations, which give as intercepts ϕ_0 and ϕ'_0, the reciprocals of the maximum rates with infinitely large coenzyme and substrate concentrations (cf. Dalziel, 1957*b*).

The rates of ethanol oxidation were quite reproducible, but significantly different rates of aldehyde reduction were obtained with different samples of $DPNH_2$, especially at pH 6·0. The stated values are the largest and were obtained with the purest sample of $DPNH_2$. Other samples gave maximum rates up to 40 % smaller at pH 6·0. At pH 10, however, and also with yeast alcohol dehydrogenase at pH 6, all the $DPNH_2$ samples gave the same rates.

DISCUSSION

The omission of ethanol–chloroform treatment and subsequent distillation of the preparation under reduced pressure, introduced by Bonnichsen (1950), results in a shorter, milder and more convenient isolation procedure for liver alcohol dehydrogenase with the same high yield as the previous method (Dalziel, 1958). Carboxymethylcellulose has a large binding capacity for haemoglobin in slightly acid solutions (Peterson & Sober, 1956), and may be useful in other preparations also as a mild alternative to the Tsuchihashi denaturation procedure for the removal of haemoglobin. The fact that aldehyde dehydrogenase is not adsorbed by carboxymethylcellulose is proving

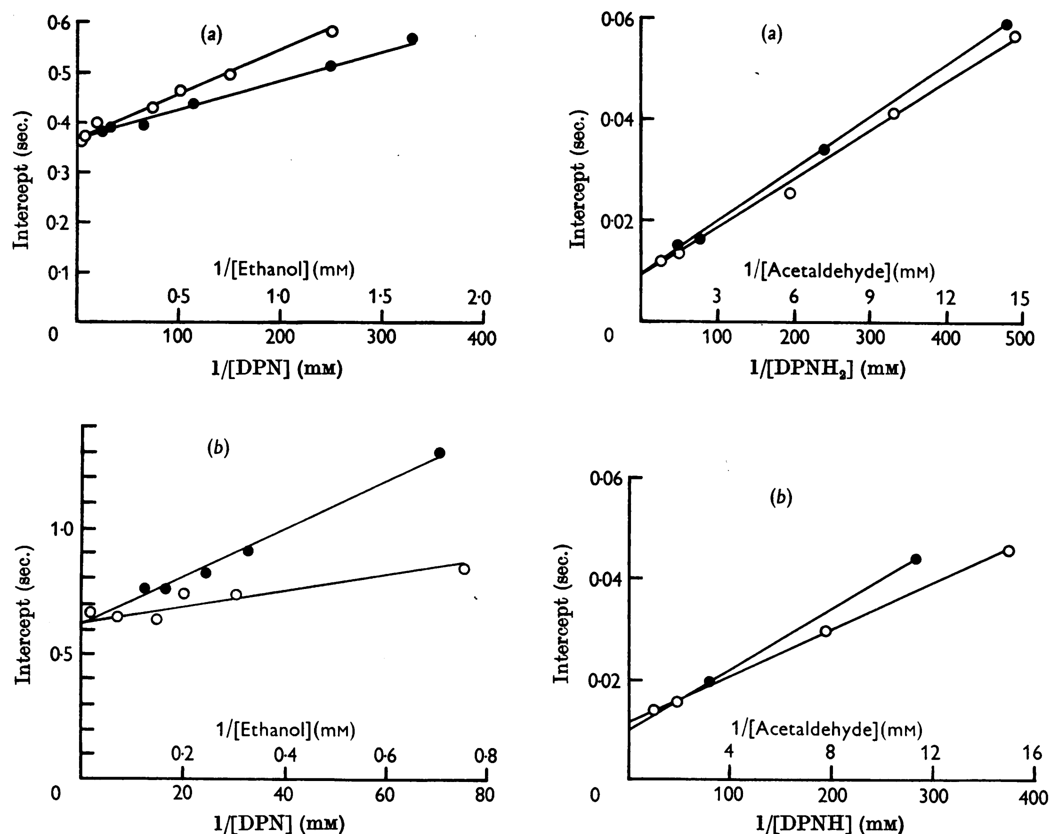

Fig. 4. Relations between reciprocal substrate concentrations and the intercepts of Lineweaver–Burk plots of the reciprocals of initial rates with unit enzyme concentration against reciprocal coenzyme concentration (●), and between reciprocal coenzyme concentrations and the intercepts of Lineweaver–Burk plots of reciprocal rates against reciprocal substrate concentration (○). Initial-rate measurements were made at 23·5° and (*a*) pH 7·15, (*b*) pH 6·0.

useful in the purification of this enzyme, since haemoglobin and alcohol dehydrogenase, which interferes with the spectrophotometric assay of aldehyde dehydrogenase (Racker, 1955), can be completely removed from crude liver extract by bulk adsorption.

The recrystallized enzyme appears to be identical with that obtained by the earlier procedure (Dalziel, 1958). The specific activities in ethanol oxidation at pH 10 (cf. Table 1) and pH 7·15 (Table 3) and the specific extinction at 280 mμ are the same, as is the electrophoretic behaviour at pH values above and below the isoelectric point. Thus the suggestion that the minor component is formed during the Tsuchihashi treatment is not substantiated. Dalziel (1958) found that kinetic data obtained with the recrystallized enzyme and with electrophoretically homogeneous material obtained by a second chromatography on carboxymethylcellulose were identical.

The presence in the molecule of 28 thiol groups reactive towards *p*-chloromercuribenzoate also agrees with earlier results (H. Theorell & J. S. M. McKee, personal communication) for both the Dalziel (1958) and Bonnichsen (1950) preparations. Inhibition by the mercurial also agrees quantitatively with results given by Witter (1960).

There was no significant difference in stability at pH 7 and 6 between the present product and a sample prepared according to Bonnichsen & Brink (1955). The observed activity losses were certainly no greater than those indicated by Theorell & Winer (1959) for a Bonnichsen & Brink preparation, and no explanation can be offered for the instability of enzyme prepared by the method of Dalziel (1958) at pH 6 reported by these workers.

Maximum-rate data reported for the various enzyme preparations are compared in Table 3. The two latest sets of data agree within experimental error. The maximum rate of ethanol oxidation at pH 7·15 is in each case an average of values from experiments with different enzyme and coenzyme samples which agreed to within ± 5 %. The data for pH 6·0 are from single experiments, and the error of initial-rate measurements for ethanol oxidation in the earlier work was relatively large because of interference from the reverse reaction.

The values given by Theorell *et al.* (1955) are all much smaller than the later ones. For ethanol oxidation a similar pH effect is indicated, and it might be assumed that the discrepancies simply represent a low specific activity of the enzyme preparation. The fact that Bonnichsen & Brink (1955) reported a higher specific activity for their preparation than that given by Bonnichsen (1950) supports this view. But the data for aldehyde reduction show relatively much bigger discrepancies. The assumption of a linear calibration curve for the fluorimeter up to 20 μM-DPNH$_2$ (Theorell *et al.* 1955) would give maximum rates perhaps 10 % too low (cf. Fig. 1), but the greater part of the discrepancies remain to be explained, especially at pH 6·0. Whereas the maximum rate at pH 6·0 is only half that at pH 7·15 according to Theorell *et al.*, the later data show no significant pH effect.

It has been suggested that the major discrepancies are due to an inhibitor in the enzyme prepared by Bonnichsen's method, which is removed in the chromatography step and is most active in acid solution (Theorell, 1958; Theorell & Winer, 1959). Alternatively an inhibitor may have been present in the coenzyme preparations used earlier (Dalziel, 1958). The latter explanation is supported by the present findings that significantly different rates of aldehyde reduction are obtained with different DPNH$_2$ samples in neutral and acid solutions, but not in alkaline solution. The inhibitor has not yet been identified, but Dalziel (1961) found that adenosine diphosphate ribose exhibits qualitatively similar inhibitory properties. Because of these findings, kinetic constants other than maximum rates, which can be calculated from the data of Fig. 4, have not been considered. Coenzyme preparations free from inhibitors must be obtained before reliable conclusions about mechanism can be drawn from kinetic constants for the forward and reverse reaction and overall equilibrium constants (Alberty, 1953; Dalziel, 1957*b*).

Table 3. *Maximum rates for liver alcohol dehydrogenase at* 23·5°
(*molecules of substrate/sec./active centre*)

		Maximum rate (sec.$^{-1}$)			
		Ethanol oxidation		Acetaldehyde reduction	
Reference	Enzyme preparation	pH 6·0	pH 7·15	pH 6·0	pH 7·15
Theorell, Nygaard & Bonnichsen (1955)*	Bonnichsen (1950)	0·75	1·8	20	43
Dalziel (1958;* unpublished work, 1957)	Dalziel (1958)	2·1	2·9	80	106
This paper	This paper	1·6	2·75	90	100

* Original data, based on equivalent weight 36 500, corrected for later value of 44 000.

SUMMARY

1. A method for the preparation of crystalline alcohol dehydrogenase from horse liver is described, which is simpler and milder than previous methods.

2. The product appears to be identical with that of the previous method, as judged by specific extinction at 280 mμ, specific activity and thiol group content, and is no more unstable than other preparations.

3. Maximum-rate data are reported and discussed. Discrepancies with the results of previous workers are attributed partly to the low specific activity of earlier preparations of the enzyme, and partly to the presence of an inhibitor in preparations of reduced diphosphopyridine nucleotide.

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Formation of Chromenols on Alumina from Quinones having an Isoprenoid Side Chain

BY F. W. HEMMING, R. A. MORTON AND J. F. PENNOCK*
Department of Biochemistry, The University of Liverpool

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The extraction of ubichromenol from human-kidney unsaponifiable lipids and the elucidation of its structure have been described (Laidman, Morton, Paterson & Pennock, 1959, 1960). Ubichromenol from this tissue was shown to be a cyclic isomeride of ubiquinone (50). Links (1960) has suggested that ubichromenol is not a natural product but is an artifact of isolation formed by the action of alumina on ubiquinone. Links was able to make ubichromenol by adsorption of ubiquinone on alumina and elution with acetone–hydrochloric acid after 24 hr. The process has been repeated by Green, Edwin, Diplock & McHale (1960) and by Shunk *et al.* (1960). The last-named were able to show that the ubichromenol could be eluted with methanol–ether, that is, without using an acidified eluent. We have also repeated Links's procedure on different ubiquinones and have also shown a conversion on acid-washed Brockmann Grade 2 alumina with the formation of a much cleaner product. Solanachromene (Rowland, 1958) has been produced by adsorption of koflerquinone (Kofler *et al.* 1959) on acid-washed alumina weakened with water. Vitamin K_1 has been subjected to a similar process.

* Cancer Research Fellow, University of Liverpool.

EXPERIMENTAL

Materials. Ubiquinone samples and koflerquinone were kindly donated by Hoffmann-La Roche and Co. Ltd., Basle (through Drs O. Isler and U. Gloor). Vitamin K_1 was purchased from Roche Products Ltd., Welwyn Garden City, Herts. Alumina (Grade O) was purchased from P. Spence and Sons Ltd., Widnes, Lancs, magnesium oxide