Heterogeneity of Liver Alcohol Dehydrogenase on Starch-Gel Electrophoresis

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1. Purified horse-liver alcohol dehydrogenase is heterogeneous on starch-gel electrophoresis in several buffer systems. 2. The electrophoretic pattern is altered by the addition to the buffers of oxidized or reduced coenzymes, isobutyramide, metal ions or metal-chelating agents. 3. The effect of coenzymes on the pattern suggests that the major cause of the observed heterogeneity is not the existence of isoenzymes, but the presence in the enzyme preparations of coenzyme–enzyme complexes or complexes with other nucleotides similar to, but less reactive than, the coenzymes. 4. Metal ions and chelating agents influence the electrophoretic separation by partial denaturation and inactivation of the enzyme.

Considerable interest has been aroused in recent years by the observation that a number of purified enzyme preparations are heterogeneous when examined by various fractionation procedures; these findings have resulted in the formulation of the concept of isoenzymes, i.e. the association of a particular catalytic activity with more than one form of enzyme protein (see the review by Wieland & Pfleiderer, 1963). With lactate dehydrogenase, the origin of the observed heterogeneity is found in the formation of hybrid molecules containing different proportions of two types of sub-units (Appella & Markert, 1961; Cahn, Kaplan, Levine & Zwilling, 1962), but in many instances the nature of the multiple enzymic forms remains unexplained. In the present paper the separation of multiple zones of activity of purified liver alcohol dehydrogenase (EC 1.1.1.1) by starch-gel electrophoresis is described and an explanation of the nature of these zones is advanced.

MATERIALS AND METHODS

Horse-liver alcohol dehydrogenase was purified by the method of Bonnichsen & Brink (1955); some preparations were further purified by chromatography on CM-cellulose (Dalziel, 1958, 1960). The E_{280}/E_{260} ratio for the several preparations varied from 1·3 to 1·4, and the molarity of the enzyme solutions from 60 to 120 μM.

Horizontal starch-gel electrophoresis (Smithies, 1955) was carried out in trays 17 cm. × 10·5 cm. with a gel thickness of about 0·7 cm. The samples were applied on strips of Whatman 3MM filter paper inserted into slots cut in the centre of the gels. A voltage gradient of 6 v/cm. was applied, giving a current of 25–30 mA for 16 hr. at room temperature. The sliced gels were stained for protein with Amido Schwartz or Nigrosine and for alcohol-dehydrogenase activity by incubating at 37° in 30 ml. of 0·1 M-Na_2CO_3–$NaHCO_3$ buffer, pH 10, containing 0·015 ml. of ethanol, 6 mg. of NAD^+, 1 mg. of *N*-methylphenazonium methosulphate and 10 mg. of nitro-blue tetrazolium. The substrate-dependent reduction of NAD^+ is thus coupled to the reduction of nitro-blue tetrazolium to give an insoluble purple formazan at the zones of enzyme activity.

Several buffer systems (described in the Results section) were used to investigate the effects of changes in pH and anions. In most cases the electrode compartments were filled with 0·1 M-buffer, which was diluted to 0·01 M for preparation of the gel, but when borate buffer was used the concentrations were 0·3 M and 0·03 M respectively. A human blood serum sample was run on each gel so that the migration of the enzyme zones could be compared with that of known protein fractions.

The effect of adding certain substances to the buffers was studied. These substances, which were added in the concentrations noted in the Results section to both the electrode compartment and gel buffers, can be divided into three classes: substrates and coenzymes (acetaldehyde, ethanol, NAD^+ and NADH); metal ions and metal-chelating agents (EDTA and 1,10-phenanthroline); compounds expected to form complexes with liver alcohol dehydrogenase (isobutyramide, pyrazole and urea).

Before certain electrophoretic runs both types of enzyme preparation were treated in the following ways. Norit charcoal (20 mg.) was added to 1 ml. of enzyme solution (containing 6 mg. of enzyme) and allowed to stand for 8 hr. The charcoal was removed by centrifugation. Alternatively, further portions of enzyme solution (70 μM) were separately dialysed against NaCl (3 M), glycine, pH 10 (2·5 M), urea (4 M) and dodecyl sulphate (1%, w/v) for 12 hr. at 4°. Each

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solution was then dialysed for 12–24hr. at 4° against phosphate buffer, pH 7·1, before starch-gel electrophoresis.

RESULTS

After electrophoresis in phosphate buffer at pH 7·1, four enzymically active zones of alcohol dehydrogenase were visible on the cathode side of the origin consisting of a group of three regularly spaced zones (zones 2, 3 and 4 in Fig. 1) with a fainter more-cathodal band also present (zone 1 in Fig. 1). Corresponding, though fainter, zones were visible by protein staining. Comparison with the migration of human serum protein showed that the main enzyme zones moved in the same direction and at the same rate as the γ-globulins, which have isoelectric points in the range pH 6·3–7·3, confirming the reported isoelectric point for liver alcohol dehydrogenase of pH 6·8 (Dalziel, 1958). The cathodal movement of the enzyme at this pH is thus presumably largely due to electroendosmosis. A similar pattern was observed after electrophoresis in tris–phosphate and tris–acetate buffers at pH 7·1, and also in phosphate buffers at pH 5·5 and pH 9·0, though in the former the zones were displaced towards the cathode and in the latter towards the anode. In tris–chloride buffer, pH 7·1, and borate buffer, pH 8·4, however, a faint extra zone was seen in the more cathodal region (zone 1a in Figs. 2*c* and 2*d*); in tris–chloride buffer the two most cathodal zones appeared somewhat more intense than in borate buffer. There were no marked differences between the patterns given by the two types of alcohol-dehydrogenase preparations, except that the faint most-cathodal zones appeared relatively slightly weaker in the chromatographically purified enzyme.

The addition of NAD^+ (0·5mM) to the borate buffer system, pH 8·4, altered the pattern of zones

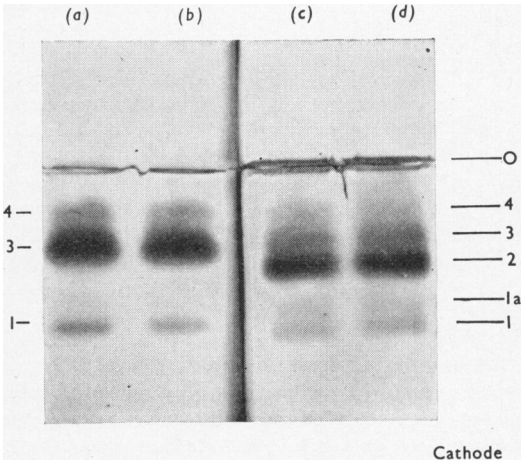

Fig. 2. Effect of adding NAD^+ (0·5mM) on the separation of liver alcohol dehydrogenase on starch gel in borate buffer, pH 8·4. The gel is stained to show enzyme activity. (*a*) and (*b*), With NAD^+ present; (*c*) and (*d*), controls without the addition of NAD^+. The numbers denote the active liver alcohol-dehydrogenase zones in controls. O, Origin.

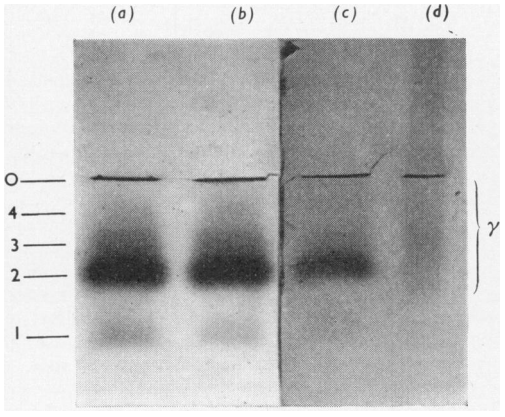

Fig. 1. Starch-gel electrophoresis of liver alcohol dehydrogenase in phosphate buffer, pH 7·1. (*a*) and (*b*), Enzyme activity stain; (*c*), protein stain; (*d*), serum protein. The numbers denote the active liver alcohol-dehydrogenase zones. O, Origin; γ, γ-globulins.

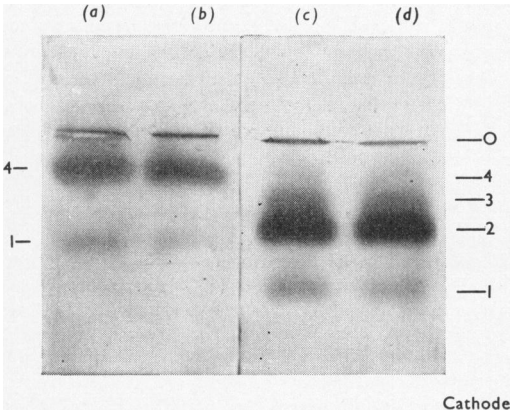

Fig. 3. Effect of adding NADH (50μM) on the separation of liver alcohol dehydrogenase on starch gel in phosphate buffer, pH 7·1. The gel is stained to show enzyme activity. (*a*) and (*b*), With NADH present; (*c*) and (*d*) controls without the addition of NADH. The numbers denote the active liver alcohol-dehydrogenase zones in controls. O, Origin.

as shown in Figs. 2(*a*) and 2(*b*), in which zone 3 is the predominant zone: this buffer was used because of the greater stability of the enzyme–NAD^+ complex at this pH. When NADH (50μM) was added to phosphate buffer, pH 7·1, the pattern shown in Figs. 3(*a*) and 3(*b*) was obtained, with zone 4 as the major component; some samples of NADH gave an extra zone between the main active band and the origin (zone 5 in Fig. 4*b*). The separation of the proteins of the reference serum sample was unaltered by these additions.

With both NADH (50μM) and isobutyramide (50mM) present in the phosphate buffer, the mobility towards the cathode of the main enzyme zone was still further decreased (Fig. 4*c*). In each case, protein staining confirmed the positions of the major active zones. When pyrazole (10μM or 1mM) was added together with NAD^+, the ternary complex appeared to be so tight that enzyme activity was inhibited and the effect on the pattern could not be determined. Incorporation of ethanol into borate buffer, pH 8·6 (3mM), or into phosphate buffer, pH 7·1 (30mM), did not alter the pattern of alcohol-dehydrogenase zones, nor did inclusion of acetaldehyde in phosphate buffer at pH 6·0 (1mM) or at pH 7·1 (30mM), except that in the last experiment the intensity of the zone closest to the origin was slightly decreased.

The addition of EDTA (5mM) or metal ions (Mg^{2+} and Fe^{3+}, each 30μM) produced similar effects on the electrophoretic pattern, reducing it to a single weak band. Protein staining showed a trail of material cathodal to the origin that did not correspond to regions of enzymic activity when compared with gels stained to show dehydrogenase action, indicating that these effects were due to denaturation and inactivation. Increasing the concentration of metal ions to 60μM each resulted in complete inactivation. 1,10-Phenanthroline (0·1 or 3·3mM) also produced enzyme inactivation but with less denaturation evident by protein staining.

Pretreatment of the enzyme solutions with sodium chloride or glycine or with Norit charcoal had no effect on the separation in phosphate buffer. After treatment with urea the faint most-cathodal alcohol-dehydrogenase zone was no longer visible. Treatment with dodecyl sulphate resulted in heavy protein staining in the sample slot, with some evidence of enzyme activity in this region, but no zones were visible on the gel.

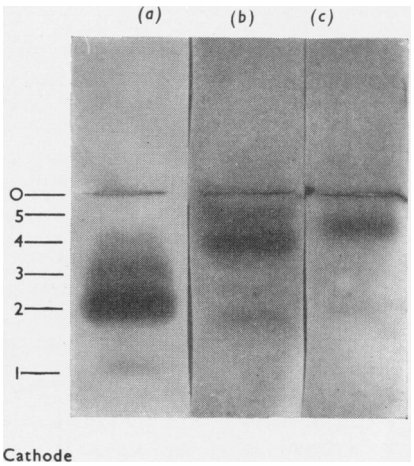

Fig. 4. Effect of adding NADH (50μM) and isobutyramide (50mM) on the separation of liver alcohol dehydrogenase in phosphate buffer, pH 7·1. The gel is stained to show enzyme activity. (*a*), Control; (*b*), with NADH added; (*c*), with NADH and isobutyramide added. The numbers denote the active liver alcohol-dehydrogenase zones in controls. O, Origin.

DISCUSSION

The most pronounced changes in the electrophoretic pattern were brought about by the addition of oxidized or reduced coenzymes to the buffers. In these experiments the major enzyme zones presumably represent the oxidized and reduced coenzyme–enzyme complexes respectively. Comparison with the controls (Figs. 2, 3 and 4) suggests that the three major components seen in pH 7·1 buffer are, from the origin to the cathode, enzyme–NADH (zone 4), enzyme–NAD^+ (zone 3) and free enzyme (zone 2), an order that is in agreement with the expected differences in the charge at this pH (the possible nature of zone 1 is discussed below). The enzyme protein is close to its isoelectric point and thus has little net charge: the single net negative charge of NAD^+ and the double net negative charge of NADH would consequently have a marked effect on the mobility of the complexes. The regular spacing of the three zones in the major group is also compatible with a series of substances differing by regular increments of charge.

The E_{280}/E_{260} ratios for the enzyme preparations could indicate that nucleotides are present in varying amounts. Treatments designed to dissociate nucleotides, e.g. Norit charcoal, sodium chloride, glycine and urea in high concentrations or dodecyl sulphate, had no effect on the main group of bands. Fritz & Jacobson (1963) have suggested attachment of NAD^+ in varying amounts as the reason for the appearance of 15 lactate-dehydrogenase bands on electrophoresis of mouse-muscle extracts in the presence of low concentrations of mercaptoethanol, and have shown that the number of bands is altered by changing the concentration of

mercaptoethanol. This reagent interfered with the staining reaction for alcohol dehydrogenase, but did not appear to affect the number of zones visible by protein staining.

The addition of the substrates acetaldehyde and ethanol was expected respectively to oxidize any reduced coenzyme and reduce any oxidized coenzyme in the enzyme solution, but, apart from some slight decrease in the amount of the presumed enzyme–NADH band in the presence of acetaldehyde, these additions were without effect. This result may indicate that the bound coenzyme is less than usually reactive, perhaps as a result of modification or degradation, or that the complexes contain nucleotides closely similar to NAD^+ and NADH.

The predominant active zone seen when isobutyramide and NADH are present together may represent a ternary enzyme–coenzyme–inhibitor complex. It has approximately the same mobility as a second band near the origin observed when certain NADH samples were added to the buffers (Fig. 4*b*), and which may thus represent a ternary complex due to a non-nucleotide inhibitor present in the NADH becoming visible at high coenzyme concentrations. It is possible that the additional zone seen at high NADH concentrations consists of a binary complex of the enzyme with a nucleotide other than NADH; postulation of such a complex requires, however, that the net charge should be different from that of the enzyme–NADH complex. A zone in this region was also detectable in some enzyme preparations without the addition of NADH, particularly in borate buffer. When isobutyramide and NADH were added at lower concentrations (7mM and 7μM respectively) no alteration in the pattern was produced. Since a ternary complex of enzyme, NADH and isobutyramide might be expected to be very tight, this result may indicate that the effect of isobutyramide on mobility is due rather to a configuration change brought about by high concentrations. The addition of isobutyramide to the buffers without the addition of NADH did not alter the pattern of zones; there is thus no evidence of formation of binary enzyme–isobutyramide complexes.

Watts & Donninger (1962) have shown that the number of zones seen on starch-gel electrophoresis of yeast alcohol dehydrogenase is decreased by freeing the system (buffers, filter-paper wicks etc.) from metal ions by treatment with EDTA or 1,10-phenanthroline. The decrease in the number of enzyme zones after the addition of chelating agents or metal ions seems to be the result of denaturation, and thus the explanation of the heterogeneity of liver alcohol dehydrogenase does not appear to lie in the formation of complexes with metal ions.

The position and intensity of the minor components (zones 1 and 1a in Figs. 2 and 3) of liver alcohol dehydrogenase travelling faster towards the cathode than the main group of zones are influenced both by the nature of the buffer anions and the presence of coenzymes. Zone 1a is abolished in the presence of NAD^+ (Fig. 2) whereas zone 1 is unaffected. With NADH present zone 1 also is retarded (Fig. 3). These components may therefore represent binary enzyme–buffer ion complexes or, more probably, ternary enzyme–coenzyme–ion complexes. It is difficult to see, however, why the formation of complexes with anions should give zones migrating more rapidly towards the cathode, unless some configuration change is involved. Disappearance of the most cathodal zone from the phosphate-buffer pattern after pretreatment of the enzyme solution with urea may be a result of dissociation of complexes of this type that may be formed during extraction and purification of the enzyme. A similar effect on the pattern was seen on adding urea (1M) to the phosphate buffer system.

The evidence presented for the existence of the enzyme–coenzyme complexes is indirect; specific methods of dissociating tightly bound nucleotides from the enzyme would be required to provide direct proof. Attempts to measure the E_{280}/E_{260} ratio of zones recovered from the gels have been unsuccessful because of the poor recoveries achieved. The heterogeneity of liver alcohol dehydrogenase on starch-gel electrophoresis can thus be explained without postulation of distinct multiple molecular forms, or isoenzymes, of this enzyme. If the suggestion that the main group of zones represents free enzyme and enzyme–coenzyme complexes is correct, starch-gel electrophoresis at a pH close to the isolectric point of the enzyme offers a good means of separating them because of the low diffusion, and correspondingly compact zones, obtained in this medium even when the net charge on the molecules is small. Electrophoresis at pH 10·6 (sodium carbonate–bicarbonate buffer), i.e. at a pH removed from the isoelectric point of the enzyme, showed only one anodal alcohol-dehydrogenase band, due perhaps to dissociation of complexes, particularly those containing NADH, but also probably to the fact that the charges on the coenzymes would have relatively less effect on the mobility of the complexes at this pH, compared with the greater net charge of the enzyme protein.

The possibility of separating enzyme–nucleotide complexes on starch gel is relevant to the investigation of the heterogeneity of dehydrogenases from a particular source, particularly as differences in properties such as heat stability or solubility can result from the conformational changes induced by coenzyme binding (McKinley-McKee & Donninger, 1963), and variations in these properties have been used as criteria for characterization of isoenzymes.

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