The Preparation of Crystalline Human L-Lactate–Nicotinamide–Adenine Dinucleotide Oxidoreductase Isoenzyme 1 involving Preparative Polyacrylamide–Gel Electrophoresis

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A method is presented for the preparation of human heart lactate dehydrogenase (L-lactate–NAD⁺ oxidoreductase; EC 1.1.1.27) isoenzyme 1; this involves the use of polyacrylamide-gel electrophoresis as a preparative step. The yield was about 10% with a final specific activity of 220 units/mg of protein, one unit being defined as the amount of enzyme catalysing the oxidation of 1 μ mol of NADH/min at 25°C, in the presence of 0.33 mM-pyruvate. The crystalline preparation contained less than 2% of the other isoenzymes, was homogeneous in the ultracentrifuge and showed only a trace of protein contamination on polyacrylamide-gel electrophoresis. Some properties of the crystalline isoenzyme are reported; $E_{1\text{cm}}^{1\%} = 13.2$ at 280 nm, $s_{20,w}^0 = 7.43$ S, $pI = 4.6$, and the apparent K_m for pyruvate = 1.02×10^{-4} M. The human isoenzyme and the isoenzyme from pig heart differ with respect to amino acid composition, electrophoretic mobility and solubility. It is possible that these differences do not involve the active site, or sites, but are due to changes in amino acid residues elsewhere in the molecule. The importance of purified human LDH-1 isoenzyme with regard to enzyme radioimmunoassay is emphasized.

Methods for the preparation of crystalline lactate dehydrogenase (L-lactate–NAD⁺ oxidoreductase, EC 1.1.1.27) have been available for many years and the literature now contains procedures using a wide variety of source species and tissues (Straub, 1940; Kubowitz & Ott, 1943; Schwert *et al.*, 1962; Reeves & Fimognari, 1963; Pesce *et al.*, 1964) including human heart muscle (Nisselbaum & Bodansky, 1961; Pesce *et al.*, 1967). Only in the more recent of these papers have preparations of pure isoenzymes been claimed. We have, however, been unable to prepare a truly homogeneous sample of human heart isoenzyme LDH-1 by any of these methods. In our hands the final product always contained appreciable amounts of isoenzyme LDH-2. Investigations of the enzyme's structure and certain of its catalytic properties are now well advanced, by using lactate dehydrogenases prepared from several animal species and tissues (Adams *et al.*, 1972, 1973; Jeckel *et al.*, 1973; Kaloustian & Kaplan, 1969; Pesce *et al.*, 1967; Pfeleiderer *et al.*, 1970; Taylor *et al.*, 1973). To provide suitable human material for comparative work, we decided to prepare about 100 mg of isoenzyme LDH-1 in crystalline form and showing negligible contamination with the other isoenzymes. We have utilized the preparative polyacrylamide-gel-electrophoresis technique developed

in this laboratory (Hodson & Latner, 1971). It is also hoped that the LDH-1 isoenzyme that we have prepared will eventually be used to produce crystals large enough for X-ray crystallography. Moreover, the importance of producing a highly purified LDH-1 isoenzyme is highlighted by the increasing application of immunological techniques to enzyme assay.

Materials and Methods

Materials

2-Mercaptoethanol, Tris, MTT Tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and phenazine methosulphate were purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.; DEAE-cellulose (Whatman type DE11) was from H. Reeve Angel and Co. Ltd., London E.C.4, U.K.; seamless regenerated cellulose tubing (Visking) was from the Scientific Instrument Centre Ltd., London W.C.1, U.K.; Naphthalene Black 10B was from Searle Scientific Services, High Wycombe, Bucks., U.K.; *NN'*-methylenebisacrylamide was from Kodak Ltd., Kirkby, Liverpool, U.K.; and NAD⁺, NADH, sodium pyruvate and pig heart isoenzyme LDH-1 were from Boehringer Corp. (London) Ltd., London W.5, U.K. (NH₄)₂SO₄ specially low in heavy metals, and other reagents, generally of AnalaR grade, were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

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Methods

Lactate dehydrogenase assay. The method of Bergmeyer *et al.* (1963) was used. Where large numbers of assays were required, this method was adapted for use with a reaction-rate analyser (Ultralab system; LKB Instruments Ltd., South Croydon, Surrey, U.K.). One unit of enzyme activity is defined as the amount of enzyme catalysing the oxidation of $1\ \mu\text{mol}$ of NADH/min at 25°C , in the presence of $0.33\ \text{mM}$ pyruvate.

Protein assays. The method used was that of Lowry *et al.* (1951), except that the reagent solutions were stored separately and mixed immediately before use. Human serum albumin (Blood Products Laboratory, Lister Institute, Elstree, Herts., U.K.), was normally used as a standard. For the routine assay of column fractions, the E_{280} was determined. For preparations of lactate dehydrogenase approaching homogeneity, an $E_{1\text{cm}}^{1\%}$ of 13.2 at 280nm was used (see the Results section) to calculate the amount of protein present.

Electrophoresis procedures. Starch-gel electrophoresis was carried out by the method of Smithies (1959) with Tris-borate-EDTA buffers, pH 8.6 (Boyer *et al.*, 1963), and the isoenzymes were detected by a method essentially that of Latner & Skillen (1961). In certain cases, an enzyme preparation was diluted to give a series of samples with lactate dehydrogenase activities of, usually, 500, 50, 10 and 5 units/ml. Starch-gel electrophoresis was performed simultaneously on all the samples and the isoenzymes were detected as described above. Comparison of the intensity of the band due to isoenzyme LDH-2 in the most concentrated sample with the intensity of the isoenzyme LDH-1 bands in the more dilute samples enabled a sufficiently accurate assessment to be made of the proportion of isoenzyme LDH-2 present.

The method of Davis (1964) was used for disc electrophoresis in polyacrylamide gels. Proteins were stained with Naphthalene Black 10B and the lactate dehydrogenase isoenzymes were detected as described by Latner & Skillen (1961).

Preparative polyacrylamide-gel electrophoresis was carried out by the method of Hodson & Latner (1971). The gel was prepared from a solution containing 7.5% (w/v) acrylamide mixture [95% acrylamide–5% *NN'*-methylenebisacrylamide (w/w)], 0.05% (w/v) ammonium persulphate and 0.1% (v/v) *NNN'*-tetramethylethylenediamine in 0.05 M-Tris-acetic acid buffer, pH 8.6, containing 1 mM-lithium lactate and cast in a mould to provide one elution chamber and a sample slot 5 cm long, 1 cm deep and 0.5 cm wide. The same buffer containing lithium lactate was used in the electrode compartments and to fill and flush the elution chamber on the anode side.

Sedimentation studies. These were carried out at 20°C in a Spinco model E analytical ultracentrifuge

with schlieren optics. For the determination of the sedimentation coefficient, the two samples examined contained 8 and 5.53 mg of protein/ml of 0.01 M- KH_2PO_4 -NaOH buffer, pH 6.5, containing 0.1 M-KCl. Photographs were taken at 33 and 55 min after a speed of 60000 rev./min had been reached.

Determination of extinction coefficients. A sample of the crystalline preparation, suspended in saturated $(\text{NH}_4)_2\text{SO}_4$ solution, was redissolved by dilution with water and exhaustively dialysed against double-distilled water. The dialysis residue was centrifuged at $34000g$ for 30 min, the supernatant diluted to a known volume and its E_{280} determined. The material was dried at 110°C to constant weight, the flask being stoppered immediately on removal from the oven and before cooling and weighing, after equilibration in the balance case. The results were adjusted for the change in weight of a similar vessel containing the same volume of double-distilled water and also dried at 110°C to constant weight.

Amino acid analyses. A sample of the protein was hydrolysed in 6 M-HCl at 110°C in a sealed, evacuated tube, for 20 h and approx. 40 h. To remove the HCl, the hydrolysate was dried by rotary evaporation, the residue redissolved in deionized water and the solution again evaporated. The wash with water and evaporation was repeated and the final residue taken up in 0.02 M-HCl (0.8 ml) and 62.5% (w/v) sucrose (0.2 ml). A sample (250 μl) representing between 200 and 300 μg of hydrolysed protein was applied to a column of Technicon C2 resin and analysed by the Technicon AutoAnalyser method.

Isoelectric focusing. A solution containing 0.615 mg of crystalline isoenzyme LDH-1 was dialysed for 16 h against 2 litres of 0.01 M-Tris-HCl buffer, pH 7.4, containing 1 mM-lithium lactate. The dialysis residue (about 1 ml) was then subjected to isoelectric focusing by using the equipment developed by LKB (electrofocusing column, type 8100-10) and the technique described by Haglund (1971), with minor modifications given by Latner *et al.* (1970). A potential of 300 V was applied for 46 h, the apparatus being cooled to 4°C . On completion of the focusing, fractions (1.5 ml) were collected with the aid of a peristaltic pump, assayed for lactate dehydrogenase activity and their pH measured.

Detection of haem pigments. Haem pigments were detected by measuring their absorbance at 412 nm.

Results

Preparation of isoenzyme LDH-1 from human heart muscle.

This incorporates part of the procedure of Reeves & Fimognari (1963). Unless otherwise stated, all steps were carried out at 0 – 4°C .

Fig. 1. Elution profile of lactate dehydrogenase from DEAE-cellulose

About 4.35×10^4 units of lactate dehydrogenase in water adjusted to pH 7.0 with $0.5\text{M-K}_2\text{HPO}_4$ were applied to a column (50 cm $\times$ 4.5 cm) of DEAE-cellulose prepared and eluted by using a combined NaCl and pH gradient as described by Reeves & Fimognari (1963). The volume of each fraction was 14 ml. Solutions were applied to the top of the column at the points indicated by the letters, as follows: A, $0.06\text{M-KH}_2\text{PO}_4$; B, $0.06\text{M-KH}_2\text{PO}_4$ containing 0.06M-KCl ; C, $0.09\text{M-KH}_2\text{PO}_4$ containing 0.09M-KCl ; D, $0.14\text{M-KH}_2\text{PO}_4$ containing 0.14M-KCl ; E, $0.20\text{M-KH}_2\text{PO}_4$ containing 0.20M-KCl . Numbers in square brackets refer to the isoenzyme composition of selected single fractions as determined by starch-gel electrophoresis (tr, trace). Fractions 350–380 were pooled and concentrated. ●, Lactate dehydrogenase activity (units/ml); ○, protein measured as E_{280} ; △, haem pigment measured as E_{412} ; —, pH of fractions.

Steps 1 and 2. Crude extract and DEAE-cellulose chromatography. These two steps were carried out exactly as described by Reeves & Fimognari (1963). The elution profiles for lactate dehydrogenase, protein and haem pigment obtained after DEAE-cellulose chromatography are shown in Fig. 1. The first major peak of enzyme activity consisted mainly of isoenzyme LDH-2, and the second, larger, peak contained mainly isoenzyme LDH-1. However, isoenzyme LDH-2 was detected throughout the isoenzyme LDH-1 peak and was still being eluted with the trailing end of the second peak. The fractions containing the major part of this second peak were pooled, concentrated to a small volume (about 6 ml) by ultrafiltration (Gregory, 1954) by using Visking tubing and dialysed for 8 h against 500 ml of $0.05\text{M-Tris-acetic acid buffer}$, pH 8.6, containing $1\text{mM-lithium lactate}$ and for 16 h against a further 500 ml of the same buffer. At this stage, starch-gel electrophoresis demonstrated that the isoenzyme LDH-2 comprised about 15% of the total lactate dehydrogenase activity.

Step 3. Polyacrylamide-gel electrophoresis. The

dialysis residue was centrifuged for 30 min at $60000g$ and the supernatant taken for preparative polyacrylamide-gel electrophoresis (Hodson & Latner, 1971). The material was divided into three batches each containing about 100 mg of protein and these were subjected to electrophoresis separately. Fractions were collected over a period of 25 h, while a potential of 40 V was applied across the electrodes, giving a current of 25 mA. The distribution of protein and enzyme activity in the fractions is shown in Fig. 2. Where the proportion of isoenzyme LDH-2 did not exceed 2% of the total enzyme activity, the fractions were pooled and concentrated by ultrafiltration.

Step 4. Crystallization. The concentrated material from the three electrophoresis batches (about 10 ml) was dialysed for 48 h against 2 litres of 35% satd. $(\text{NH}_4)_2\text{SO}_4$ (Green & Hughes, 1955) containing $1\text{mM-lithium lactate}$ and $1\text{mM-mercaptoethanol}$, the dialysis residue centrifuged at $34000g$ for 30 min and the insoluble material discarded. The supernatant was transferred to a small Visking sac previously prepared by boiling in 1mM-EDTA and washing in

Fig. 2. Separation of a lactate dehydrogenase preparation by polyacrylamide-gel electrophoresis

About 6×10^3 units of lactate dehydrogenase in 0.05M-Tris-acetic acid buffer, pH8.6, containing 1mM-lithium lactate were added to the sample slot of a 7.5% polyacrylamide gel and subjected to a potential of 40V measured across the electrodes of the electrophoresis apparatus (Hodson & Latner, 1971). The isoenzyme LDH-2 in fractions 29–40 was found to be less than 2% of the total activity and these fractions were pooled and concentrated. ●, Lactate dehydrogenase activity; ○, protein measured as E_{280} . No absorbance at 412nm was found in these fractions.

Table 1. Purification of isoenzyme LDH-1 from human heart muscle

Details of the purification steps are given in the Results section.

Step	Volume (ml)	Total activity (kunits)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification
1. Crude extract	1415	43.5	11900	3.7	100	1
2. DEAE-cellulose chromatography	425	18.0	330	55	41	15
3. Polyacrylamide-gel electrophoresis	11	8.8	59	150	20	41
4. Crystallization	4.0	5.8	36	160	13	43
5. Recrystallization	3.8	4.15	19	220	9.5	60

water. Crystallization of the enzyme was carried out by dialysis against an $(\text{NH}_4)_2\text{SO}_4$ solution (initially 35%-satd.) containing 1 mM-lithium lactate, while the $(\text{NH}_4)_2\text{SO}_4$ concentration was increased slowly over several days. With 4.4mg of protein/ml in the Visking sac, crystallization took place when the $(\text{NH}_4)_2\text{SO}_4$ concentration reached 51% saturation.

Step 5. Recrystallization. The crystalline suspension was diluted with water until the crystals redissolved, and the whole of Step 4 was repeated. The final preparation was stored at -10°C as a suspension in saturated $(\text{NH}_4)_2\text{SO}_4$ solution containing 1 mM-lithium lactate.

Purification

The purification and recovery of isoenzyme LDH-1

at each step of the preparative procedure is given in Table 1, the overall yield being almost 10%, with a final specific activity of 220units/mg of protein. An alternative method of preparation, based in part on that of Pesce *et al.* (1967) and incorporating preparative polyacrylamide-gel electrophoresis, was also carried out. Although the yield was low, it gave a final product with a specific activity of 215 units/mg of protein and whose properties were indistinguishable from those of the product of the purifications described above.

Purity of the preparations

The ultracentrifuge schlieren patterns obtained for our crystalline preparation showed a single symmetri-

cal peak and gave a sedimentation coefficient ($s_{20,w}^0$) of 7.43S.

The purified preparations were shown to be composed of needle-shaped crystals of a mean length of about 25 μ m and a maximum length of about 45 μ m.

Disc electrophoresis in polyacrylamide gels of each sample of the purified isoenzyme gave a single densely staining protein band which corresponded exactly to the band obtained after location of the lactate dehydrogenase activity. Two extremely faint slower-moving protein bands were also seen. When the gels were stained for lactate dehydrogenase activity, two faint slower-moving bands were just visible after gross overloading of the gel.

Location of the lactate dehydrogenase activity after starch-gel electrophoresis showed two stained bands, one very dense band in the position expected for isoenzyme LDH-1 and a minor one in the position expected for isoenzyme LDH-2. The minor component was shown to be less than 2% of the total enzyme present by means of starch-gel electrophoresis of several dilutions of the preparation.

Specific extinction coefficient

The crystalline preparation gave an $E_{1\text{cm}}^{1\%}$ of 13.2

at 280 nm, based on the dry weight of the enzyme after heating at 110°C. Attempts to obtain an extinction coefficient for pig heart isoenzyme LDH-1 were unsuccessful, since the protein precipitated during the removal of $(\text{NH}_4)_2\text{SO}_4$ by dialysis. The human enzyme remained in solution during dialysis, provided that the suspension in $(\text{NH}_4)_2\text{SO}_4$ solution was redissolved by dilution beforehand.

Amino acid analysis

Table 2 shows the mean results of eight amino acid analyses of the crystalline preparation of human isoenzyme LDH-1 after hydrolysis for 20h. No significant difference was found between the results obtained after hydrolysis for 20h and for 40h, except for serine, where hydrolytic degradation decreased the estimated serine content from 7.3 mol/100 mol after 20h hydrolysis to 6.7 mol/100 mol after 40h hydrolysis. Table 2 also shows the mean result of two amino acid analyses of the purified pig heart isoenzyme LDH-1 after hydrolysis for 20h; in this case, hydrolytic degradation decreased the estimated serine content from 7.5 mol/100 mol after 20h hydrolysis to 6.8 mol/100 mol after 44h hydrolysis.

Michaelis constant for pyruvate

The reaction rates of the enzyme in 0.1 M-sodium phosphate buffer, pH 7.4, were obtained in the presence of 0.133 mM-NADH and pyruvate concentrations in the range 0.04–0.29 mM. A double-reciprocal plot (Lineweaver & Burk, 1934) gave an apparent K_m for pyruvate of 1.02×10^{-4} M.

Isoelectric point

Human isoenzyme LDH-1 was shown to have a pI of 4.6; only 2.8% of the initial enzyme activity was recovered.

Variation of lactate dehydrogenase activity with pH

The lactate dehydrogenase activity of the crystalline human preparation was measured in Na_2HPO_4 – KH_2PO_4 buffer ($I = 0.2$ mol/l) between pH 6.0 and 8.0 in the presence of 0.133 mM-NADH and 0.33 mM-pyruvate. Under these conditions at 25°C little variation in enzyme activity was found, other than a slight decrease in activity as the pH was raised (about 10% for one pH unit). An identical series of measurements using crystalline isoenzyme LDH-1 from pig heart also showed no distinct pH optimum; there was a 10% fall in activity between pH 6 and 7 and a further 30% fall between pH 7 and 8.

Electrophoretic mobilities of isoenzyme LDH-1 from human heart and pig heart

When the crystalline preparations of human heart LDH-1 and pig heart LDH-1 isoenzymes were subjected to starch-gel electrophoresis at pH 8.6, the pig heart enzyme showed a distinctly higher

Table 2. Amino acid composition of isoenzyme LDH-1

A sample of the purified protein was hydrolysed in 6M-HCl at 110°C in a sealed, evacuated tube for 20h. The HCl was removed by repeated evaporation and washing with deionized water, the final residue being taken up in 0.02M-HCl (0.8 ml) and 62.5% (w/v) sucrose (0.2 ml). A portion (250 μ l) representing between 200 μ g and 300 μ g of hydrolysed protein was applied to a column of Technicon C2 resin and analysed by the Technicon AutoAnalyser method. The values are expressed as residues/molecule, assuming mol.wt. 140 000 (Pesce *et al.*, 1967), the human data being the mean of eight analyses and the pig data the mean of two analyses. Tryptophan was not measured and its contribution has been ignored.

Amino acid	LDH-1 isoenzyme	
	Human heart	Pig heart
Lysine	114	96
Histidine	32	24
Arginine	33	33
Aspartic acid	142	143
Threonine	51	59
Serine	94	98
Glutamic acid	131	137
Proline	46	54
Glycine	99	104
Alanine	92	87
Valine	139	150
Half-cystine	11	15
Methionine	32	32
Isoleucine	82	84
Leucine	143	137
Tyrosine	29	31
Phenylalanine	20	20

mobility, so that a mixture of the two enzymes was resolved into two LDH-1 isoenzyme bands. The mobilities of both enzymes remained the same, whether subjected to electrophoresis separately or as a mixture and the mobility of the purified human heart isoenzyme LDH-1 was the same as that of LDH-1 in a crude preparation of human lactate dehydrogenase isoenzymes separated by electrophoresis at the same time.

Discussion

The specific activity of 220 units/mg of protein obtained for our crystalline preparation is lower than the values given by Pesce *et al.* (1967) for human heart isoenzyme LDH-1 and Reeves & Fimognari (1963) for pig heart isoenzyme LDH-1. Nisselbaum & Bodansky (1961) obtained a much higher specific-activity value, but after making allowances for their higher assay temperature, different substrate concentrations and definition of enzyme units, a value close to ours would probably be appropriate. A commercial pig heart isoenzyme LDH-1 preparation showed a specific activity of 275 units/mg of protein, by using our enzyme assay and an $E_{1\text{cm}}^{1\%}$ of 13.9 (Holbrook & Stinson, 1970); by using an $E_{1\text{cm}}^{1\%}$ of 11.6 (Pfleiderer & Jeckel, 1957), the same preparation gave a specific activity of 230 units/mg of protein. These values are considerably lower than the value of 360 units/mg of protein specified by the manufacturers of this preparation and the value of 344 units/mg of protein obtained by Reeves & Fimognari (1963). However, there is considerable variation in the assay methods used. The commercial pig heart preparation was assayed by the manufacturers at pH 7.0 with 0.2 mM-NADH, whereas Reeves & Fimognari (1963) used pH 7.4 and 0.167 mM-NADH, the pyruvate concentration being 0.76 mM in both cases. We assayed at pH 7.5 in the presence of 0.133 mM-NADH and 0.33 mM-pyruvate (Bergmeyer *et al.*, 1963); this pyruvate concentration gave the maximum rate of reaction under the conditions of our assay, as did 0.76 mM-pyruvate, although there was considerable substrate inhibition at higher pyruvate concentrations. It seems clear that for a given preparation our assay produces a lower rate of reaction and therefore a lower specific activity than assays used elsewhere. However, it may be that the human enzyme is intrinsically less active than that from the pig and in fact quite large variations in the specific activities of purified lactate dehydrogenase have been reported. Pesce *et al.* (1967) give values ranging from 800 to 1250 units/mg of protein for their H_4 (LDH-1) enzymes and from 974 to 1850 units/mg of protein for their M_4 (LDH-5) preparations.

The specific activity of the crystalline preparation given in Table 1 was almost identical with that of material prepared by an alternative method based on

that of Pesce *et al.* (1967) with the addition of preparative polyacrylamide-gel electrophoresis before crystallization. There was a similar low contamination with isoenzyme LDH-2, and needle-shaped crystals were readily obtained in both cases. The final products were indistinguishable from each other and we believe that the production of apparently identical material by two different methods adds to evidence for its purity.

Our yield was close to that obtained by Nisselbaum & Bodansky (1961), although less than that of Pesce *et al.* (1967). The recovery of isoenzyme LDH-1 that we achieved from the DEAE-cellulose column (41%), although much lower than the 69% recovery of isoenzyme LDH-1 obtained from pig heart by Reeves & Fimognari (1963), is part of a total recovery of 88% of the enzyme activity from the column. This indicates a lower percentage of isoenzyme LDH-1 in the total lactate dehydrogenase of human heart muscle compared with pig heart and is in agreement with values of 80% isoenzyme LDH-1 for pig heart (Reeves & Fimognari, 1963) and 60% isoenzyme LDH-1 for human heart (Wieland & Pfeleiderer, 1962). Consequently, our effective recovery of isoenzyme LDH-1 would be somewhat higher than the values given in Table 1.

The purity of our preparations was better than 98% with respect to isoenzyme LDH-1, with less than 2% isoenzyme LDH-2 and possibly very slight contamination with one or two other proteins. This was a more homogeneous preparation than one of those obtained previously (Nisselbaum & Bodansky, 1961) and, judging from the ultracentrifuge data, was more homogeneous than the preparation from pig heart (Reeves & Fimognari, 1963). Pesce *et al.* (1967) state that only pure isoenzyme LDH-1 was isolated. However, few data are given which relate specifically to the human heart enzyme and, in this laboratory, we were unable to obtain a preparation approaching homogeneity by their method. It is noteworthy that in a later paper from the same laboratory, dogfish muscle isoenzyme LDH-5, originally said to be homogeneous, was prepared by their usual method (Pesce *et al.*, 1967) and then further purified by using Sephadex G-100 to remove trace amounts of a contaminant of higher molecular weight than lactate dehydrogenase (Allison *et al.*, 1969).

Our crystals were produced in the presence of lithium lactate. It has been our experience that this procedure probably stabilizes isoenzyme LDH-1, although the substrate is not bound to the active site in the absence of coenzyme (Takenaka & Schwert, 1956). We were unable to devise conditions capable of producing either large crystals or crystals of a shape more amenable to crystallographic studies, as has been done with other lactate dehydrogenase isoenzymes (Adams *et al.*, 1972, 1973). Moreover, attempts elsewhere to produce big crystals from our

material were also unsuccessful (M. J. Adams, personal communication). By the use of a preparative method employing affinity chromatography (O'Carra & Barry, 1972) followed by ion-exchange chromatography, other workers (Eventoff *et al.*, 1974) have obtained isoenzyme LDH-1 from pig hearts in good yield. The purity was greater than 99%, which corresponds closely to that of our own material. Crystals suitable for X-ray diffraction analysis have, however, been grown from this pig isoenzyme preparation.

Values for the $E_{1\text{cm}}^{1\%}$ at 280nm, $s_{20,w}^0$ and K_m for pyruvate of lactate dehydrogenase isoenzymes have been obtained elsewhere (Pesce *et al.*, 1964, 1967; Fondy *et al.*, 1964; Kaloustian *et al.*, 1969; Nisselbaum & Bodansky, 1961). Our values for $s_{20,w}^0$ and K_m for pyruvate agree closely with the reported values for human heart-muscle isoenzyme LDH-1, which fall well within the range of values reported for this isoenzyme from various species. Our value of 13.2 for $E_{1\text{cm}}^{1\%}$ at 280nm is much lower than that of Nisselbaum & Bodansky (1961), although still close to the values reported for isoenzyme LDH-1 from other species. However, although Gibson *et al.* (1953) used the dry weight of rat liver lactate dehydrogenase to obtain a specific extinction coefficient, the dry weight has not been determined with regard to other reported isolations of lactate dehydrogenase isoenzymes. As we have found with pig heart isoenzyme LDH-1, Neilands (1952) mentions that ox heart lactate dehydrogenase is denatured in salt-free solutions and, consequently, protein concentrations have generally been measured by a Kjeldahl method (Neilands, 1952; Hakala *et al.*, 1956; Pesce *et al.*, 1964), the biuret method (Pesce *et al.*, 1967) or the Lowry method (Kaloustian *et al.*, 1969). We obtained an $E_{1\text{cm}}^{1\%}$ of 16.96 for human isoenzyme LDH-1 by using the Lowry method for protein determination (Lowry *et al.*, 1951). This is close to the previously reported value (Nisselbaum & Bodansky, 1961) and suggests that data obtained in this way are likely to be significantly different from data obtained by using dry-weight determinations, as we have found.

We obtained a value of 4.6 for the pI of human heart isoenzyme LDH-1. This is in close agreement with a pI of 4.9 for the most anodic lactate dehydrogenase isoenzyme obtained from human heart muscle by Chamoles & Karcher (1970). The most anodic lactate dehydrogenase component obtained from ox heart had a pI of 4.5 (Markert & Apella, 1961), as did isoenzyme LDH-1 from dog heart (M. J. Gallimore, unpublished work). The main component of human skeletal-muscle lactate dehydrogenase, LDH-5, has a pI of 9.4 (Weller *et al.*, 1973).

The pH required for the optimum activity of ox heart lactate dehydrogenase, assayed by using the pyruvate-to-lactate reaction, has been given as pH 7.8 (Meister, 1950) and as between pH 6.8 and 8.0

(Winer & Schwert, 1958). These authors believed that the optimum pH was dependent on the pyruvate concentration and all obtained no optimum above pH 6.0 when pyruvate concentrations were kept below values at which inhibition became important. Meister (1950) used 1.67mM-pyruvate to obtain his optimum of pH 7.8, and, in the presence of 8.18 μ M-pyruvate, showed a rapid fall in enzyme activity as the pH was increased from pH 5.5. Our data indicate that both the human and pig heart LDH-1 isoenzymes show similar, but not identical, properties and have no optimum above pH 6.0 when 0.33mM-pyruvate is used in the assay mixture. Substrate inhibition did not take place under the conditions that we used until the pyruvate concentration exceeded 2mM.

Our amino acid analysis for the human isoenzyme is generally in agreement with that of Pesce *et al.* (1967) and our analysis of the pig heart material is also similar to that reported previously (Wieland & Pfeleiderer, 1962). A comparison of our analyses of the material from the two sources shows that isoenzyme LDH-1 from pig heart, although somewhat similar to the human isoenzyme, has a lower content of lysine and possibly of histidine. This decrease in basic amino acid content could account for the higher mobility of the pig heart material compared with the human isoenzyme during starch-gel electrophoresis.

In comparison with the pig heart isoenzyme, our human isoenzyme LDH-1 preparation shows a higher content of certain basic amino acids, travels more slowly during starch-gel electrophoresis and has different solubility properties. These differences would indicate that isoenzyme LDH-1 from human heart is not identical with that from pig heart. It is notable, however, that both isoenzymes possess a number of similar properties, e.g. sedimentation coefficient and $E_{1\text{cm}}^{1\%}$ (Holbrook & Stinson, 1970; Pfeleiderer & Jeckel, 1957) and variations in activity with changing pH. The latter might suggest that the differences between the two molecules do not reside at the active site, or sites, but in the remainder of the molecule.

Now that radioimmunoassay techniques are being applied to the measurement of enzymes, it is obviously of great importance to obtain a human isoenzyme of high purity. Material prepared by our methods, as described in the present paper, should prove very suitable as an antigen for the measurement of human isoenzyme LDH-1 and would then make available a number of immunological procedures related to this isoenzyme, which could be applied to humans.

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