Purification and Comparative Properties of Isoenzymes of Nicotinamide–Adenine Dinucleotide Phosphate–Isocitrate Dehydrogenase from Rat Heart and Liver

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1. Rat liver and heart major isoenzymes of NADP–isocitrate dehydrogenase have each been purified about 100-fold by a combination of ammonium sulphate fractionation and chromatography on ion-exchange cellulose and their properties compared. 2. The properties were similar in respect of pH, inhibition by Hg^{2+} and Michaelis constants for isocitrate and NADP. 3. Some of the properties of the isoenzymes were different. 4. The heart isoenzyme was activated about 210% by 0.8M-ammonium sulphate whereas the liver isoenzyme was unaffected. The heart isoenzyme showed greater sensitivity to inactivation by heat (30°C for 30min), whereas the liver isoenzyme was more sensitive to inactivation by *p*-chloromercuribenzoate and by Cu^{2+} . 5. The Michaelis constants with 3-acetylpyridine–adenine dinucleotide phosphate showed a twofold difference between liver and heart isoenzyme. 6. The differential sensitivity to heat and its mainly non-cytoplasmic location may be an explanation of the failure of plasma isocitrate dehydrogenase activity to increase after a myocardial infarction.

There are two types of NADP–isocitrate dehydrogenase [threo-D₅-isocitrate–NADP oxidoreductase (decarboxylating); EC 1.1.1.42] in mammalian tissues. These isoenzymes differ in intracellular location and in tissue distribution, and in the cytoplasm one predominates in liver, the other in heart (Bell & Baron, 1968). The object (Islam, 1970) of the present study was to purify these two isoenzymes from rat tissues and to study their properties, with special reference to the phenomenon of the failure of isocitrate dehydrogenase activity in plasma to increase in man after myocardial infarction, in contrast with the increase that occurs in hepatocellular disease (Wolfson *et al.*, 1958; Bell *et al.*, 1962).

Materials and Methods

Materials

Animals. Healthy male Wistar rats (150–200g) were used. They were killed by spinal dislocation and the tissues perfused with 0.15M-NaCl at 0°C. The hearts (total about 60g from 55 animals) and the livers (total about 100g from 10 animals) were removed, blotted, weighed and then homogenized at 4°C in 20% glycerol containing 1mM-EDTA, pH7.0, by using 10vol. of solution/g wet wt. of tissue, in a chilled Potter–Elvehjem homogenizer for four successive 20s intervals. The homogenates were im-

mediately centrifuged at 59000g for 1h at 2°C in the no. 21 rotor of the Spinco model L preparative ultracentrifuge, and the supernatant was decanted.

Blood was taken by cardiac puncture from freshly killed rats and spun at 4°C to obtain serum.

Glassware. All glassware was cleaned with chromic acid and thoroughly rinsed in water. The glassware used in the purification steps was given a final wash with a solution containing 2.0g of EDTA and 1.35g of NaHCO_3 /litre (pH7.0).

Chemicals. All chemicals used were of A.R. grade.

NADP (β) grade V, NHDP† (deamino-NADP) and APADP were obtained from Sigma Chemical Co., Kingston upon Thames KTC 7BH, U.K. Thio-NADP was from the Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. (Initially, substituted coenzymes were a gift from Dr. N. O. Kaplan.) Ammonium sulphate was the 'specially low in heavy metals, for enzyme work' grade supplied by British Drug Houses Ltd., Poole, Dorset, U.K. Saturated ammonium sulphate solution (pH6.8–7.0) was prepared in 0.005M-sodium phosphate buffer containing 1mM-EDTA. The pH was rechecked before use. Glycerol was the spectroscopic grade from BDH Ltd. DL-Isocitrate (trisodium salt) was obtained from the Boehringer Corp. D-Glucose 6-phosphate, glucose 6-phosphate dehydrogenase (135 units/mg

† Abbreviations: NHDP, nicotinamide–hypoxanthine dinucleotide phosphate; APADP, 3-acetylpyridine–adenine dinucleotide phosphate; thio-NADP, thionicotinamide–adenine dinucleotide phosphate.

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of protein) and NADP-isocitrate dehydrogenase (10 units/mg of protein) were from Sigma. *N*-Methylphenazonium methosulphate was from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and 2,5-diphenyl-3-(4,5-demethylthiazol-2-yl)tetrazolium bromide from George T. Gurr Ltd., High Wycombe, Bucks., U.K. Ion-exchange celluloses (CM11, formerly graded as CM70; DE11, formerly graded as DE50) were purchased from Whatman, Reeve Angel Ltd., London EC4V 6AY, U.K. Sephadex G-25 (coarse grade: particle size 100–300 μ m) was from Pharmacia, Uppsala, Sweden. Hydrolysed starch for starch-gel electrophoresis was from Connaught Medical Research Laboratories, Toronto, Canada. The Visking tubing washing solution contained 2.0 g of EDTA, 1.37 g of NaHCO₃ and 0.5 ml of β -mercaptoethanol/litre.

Assay procedures

Isocitrate dehydrogenase was estimated by the colorimetric procedure of Bell & Baron (1960) when large batches were analysed in following the course of the purification, and by the spectrophotometric method of Wolfson & Williams-Ashman (1957) as modified by Bowers (1959) when small numbers of samples were used. These two methods gave identical results. The spectrophotometric method was more convenient for smaller batches of assays.

Isocitrate was assayed in the Michaelis constant experiments by the method of Siebert (1963), and NADP by the method of Klingenberg (1963). Protein was determined by the method of Lowry *et al.* (1951)

with an assayed (10 mg of protein nitrogen/ml) bovine albumin as a standard (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.).

Starch-gel electrophoresis

Electrophoresis was by the vertical starch-gel method of Smithies (1959) with a 10% starch gel. The starch gel was prepared in the citrate-phosphate buffer of Fine & Costello (1963), which consists of 7.0 ml of 0.2 M-citric acid plus 43.0 ml of 0.2 M-Na₂HPO₄ plus 950 ml of water. The bridge buffer solution consisted of 30 ml of 0.2 M-citric acid plus 240 ml of 0.2 M-Na₂HPO₄ and 1230 ml of water. Electrophoresis was done at 4°C for 18 h at 10 V/cm and 30 mA. The isoenzymes were located by using a staining solution consisting of 0.03 M-MnCl₂ (1.0 ml), 0.1 M-trisodium isocitrate in 0.1 M-tris buffer, pH 7.5 (2.0 ml), 0.1% 2,5-diphenyl-3-(4,5-demethylthiazol-2-yl)tetrazolium bromide (2.0 ml), 1% NADP⁺ (1.0 ml) and 0.1% *N*-methyl phenazonium methosulphate (1.0 ml). The gel was incubated at 37°C in the dark for 1–4 h.

Purification of heart isoenzyme (Table 1)

The supernatant after ultracentrifugation of the tissue homogenate was brought to 50% saturation with ammonium sulphate by slow addition and constant slow stirring over a period of 85 min and then was kept for 3 h with constant slow stirring at 4°C. The suspension was centrifuged. The 50% supernatant was brought to 75% saturation over a period

Table 1. *Purification of rat heart isocitrate dehydrogenase*

Protein concentration was measured by the method of Lowry *et al.* (1951) and isocitrate dehydrogenase activity by the colorimetric method of Bell & Baron (1960).

Fractions	Volume (ml)	Total protein (mg)	Total activity (i.u.)	Specific activity (i.u./mg of protein)	Total yield (%)	Times purified (total)
Supernatant	560	1400	224	0.16	100	1.0
40–60% precipitate desalted	20	380	138	0.36	62	2.2
CM-cellulose column fractions (14–16)	60	78	288	3.69	128	23.0
Column fractions concentrated with Carbowax	12	72	240	3.33	107	20.8
51% (NH ₄) ₂ SO ₄ supernatant	22	46	1155	25.0	515	—
51–69% (NH ₄) ₂ SO ₄ precipitate	7	38	220	5.73	98	36.8
51–69% (NH ₄) ₂ SO ₄ supernatant	32.5	7	17	2.43	2	—
51–69% precipitate desalted	10	35	220	5.73	98	39.4
DEAE-cellulose column fractions	20	20	160	8.00	71	50.0
First gel supernatant	20	19	160	8.42	71	52.8
Sixth gel supernatant	23	9	138	15.33	61	96.0
Concentrate	10.5	9	130	14.44	58	90.2
0–75% (NH ₄) ₂ SO ₄ precipitate pooling	10	8	126	15.75	56	98.4

of 180 min and left at 4°C for 18 h with constant slow stirring, and then it was centrifuged. The 50–75% precipitate was dissolved in 40% saturated ammonium sulphate solution (20 ml) containing 1 mM-EDTA and brought to 60% saturation; it was then left for 2 days before being centrifuged. The precipitate was dissolved in 15 ml of 5 mM-trisodium citrate buffer (containing 20% glycerol and 1 mM-EDTA, pH 6.2). The dissolved precipitate was desalted on a Sephadex G-25 column (3 cm × 38 cm) at 4°C, which was equilibrated with the 5 mM-trisodium citrate buffer, pH 6.2. The column was eluted with the same buffer at a rate of 15 ml/h. All fractions containing isocitrate dehydrogenase activity were checked with Nessler's reagent and barium chloride for complete absence of ammonium sulphate. Fractions containing the highest isocitrate dehydrogenase activity were combined. The heart NADP-isocitrate dehydrogenase isoenzymes were separated on a CM-cellulose column (2 cm × 37.7 cm) that was packed at room temperature and equilibrated with the 5 mM-trisodium citrate buffer, pH 6.2. The combined fractions (vol. 20 ml) from the previous purification step were added to the column and allowed to run in under gravity. Stepwise elution of the isoenzymes with increasing concentrations of trisodium citrate buffer was carried out, 20 ml fractions of eluate being collected.

The rate of elution was slow (15–20 ml/h) as a red layer, probably haemoglobin, remained at the top of the column up to the thirteenth column fraction. With the higher concentration of buffer (0.1 M) the red layer started moving and the rate of elution became faster (50–60 ml/h). Finally the red layer was eluted and after this the main active component in a yellow band appeared. The entire chromatography was done at 4°C and took 32 h. Both the protein and the enzyme activity were eluted into two fractions, the one being unretarded by the column and the second eluting in 0.1 M-trisodium citrate buffer. Starch-gel electrophoresis (Fig. 1) revealed that the first fraction (5 mM buffer) consisted of the heart 'fast isoenzyme', and the second fraction (0.1 M buffer) was the heart 'slow isoenzyme', which was the major isoenzyme present in heart tissue. The fractions with the highest concentration of slow isoenzyme (60 ml) were combined and concentrated with Carbowax M-20. Thin Visking tubing (8/32 in) previously soaked in Visking washing solution for several days was thoroughly washed with water and dried. It was then packed with finely ground Carbowax M-20 and coiled into a cylinder containing the combined slow isoenzyme fractions. This was left for 14 h at 4°C, and then the tubing was removed and thoroughly drained. This concentrated isoenzyme solution (12 ml) was brought to 51% saturation by the addition of saturated ammonium sulphate solution at 4°C, and left for 6 h with constant slow stirring and then centrifuged. The precipitate, which contained little activity, was dis-

Fig. 1. Starch-gel electrophoresis of separated column fractions of isoenzymes of isocitrate dehydrogenase

Heart isoenzymes were separated on a CM-cellulose column with increasing concentrations of trisodium citrate buffer, pH 6.2, and liver isoenzymes on a DEAE-cellulose column with increasing concentrations of sodium phosphate buffer, pH 7.0. Starch-gel electrophoresis was with the citrate-phosphate buffer, pH 7.0, of Fine & Costello (1963).

carded and the ammonium sulphate saturation of the supernatant was increased to 69%. The suspension was left at 4°C for 18 h with constant stirring and then centrifuged. The 51–69% precipitate was taken up in 5 mM-sodium phosphate buffer (7 ml) containing 20% (v/v) glycerol and 1 mM-EDTA, pH 7.0, and desalted on a Sephadex G-25 column. A DEAE-cellulose column was equilibrated with 5 mM-sodium phosphate buffer containing 20% glycerol and 1 mM-EDTA, pH 7.0, at 4°C. The desalted solution of the 51–69% precipitate was added to the column and allowed to run in under gravity. Stepwise elution was done at 4°C, and 20 ml fractions were collected with stepwise elution and increasing concentrations of phosphate buffer. The slow-running isocitrate dehydrogenase isoenzyme was eluted in the 5 mM-sodium phosphate buffer, and the most active fractions (20 ml) were combined and treated with calcium phosphate gel (Tiselius *et al.*, 1956). The isoenzyme solution was treated with 1.0 ml of gel/mg of protein and the mixture was stirred occasionally over the next 15 min. The resultant supernatant solution was treated with 2.0 mg of gel/mg of remaining protein. This was followed by four or more treatments with gel, the supernatant solution from each preceding step being treated with 5.0 mg of gel/mg of remaining protein: the progress of the fractionation was followed by enzyme assay at each step. This calcium phosphate gel treatment removed inactive protein until a point was reached when an excessive amount of isoenzyme

Table 2. *Purification of rat liver isocitrate dehydrogenase*

Protein concentration was measured by the method of Lowry *et al.* (1951) and isocitrate dehydrogenase activity by the colorimetric method of Bell & Baron (1960).

Fractions	Volume (ml)	Total protein (mg)	Total activity (i.u.)	Specific activity (i.u./mg of protein)	Total yield (%)	Times purified (total)
Supernatant	1050	10500	1890	0.18	100	1.0
Final gel supernatant	1720	4816	1806	0.37	95	2.0
50% (NH ₄) ₂ SO ₄ supernatant	400	3196	1700	0.56	89	3.0
50–75% (NH ₄) ₂ SO ₄ precipitate	50	1800	1350	0.75	71	4.2
40–60% (NH ₄) ₂ SO ₄ precipitate desalted	30	1140	1230	1.08	65	6.0
DEAE-cellulose column fractions (32–34)	60	84	750	8.92	39	40.6
Column fractions concentrated with Carbowax	10.4	78	520	6.66	27	37.0
51–69% precipitate desalted	10	38	494	13.00	26	72.2
CM-cellulose column fractions (2, 3)	20	32	520	16.25	27	90.2
First gel supernatant	20.5	32	530	16.56	28	92.0
Sixth gel supernatant	26.5	26	482	18.54	25	102.8
0–75% (NH ₄) ₂ SO ₄ precipitate pooling	10	26	480	18.46	25	102.5

with high specific activity was removed by the gel. This activity was recovered if necessary as follows.

The gel residue was treated with 10% saturated ammonium sulphate solution with constant slow magnetic stirring for 20 min. The suspension was then centrifuged at $4000 \pm 100g$ for 30 min. The residue, which contained very little activity, was discarded and the supernatant was retained. The last gel supernatant was concentrated to a protein concentration of approx. 1 mg/ml with Carbowax CM-20. The concentrate (10.5 ml) was brought to 75% saturation with ammonium sulphate and left for 18 h with constant slow stirring at 4°C, and then centrifuged. The precipitate was dissolved in 10 ml of 0.1 M-tris-HCl buffer containing 20% glycerol and 1 mM-EDTA, pH 7.5.

Purification of liver isoenzyme (Table 2)

The liver supernatant (1050 ml) prepared in the same way as the heart supernatant was slightly turbid, owing to lipids that could not be removed by centrifugation, and it proved advantageous to have a calcium phosphate gel treatment as the first stage in purification of the liver isoenzyme. The liver supernatant was treated with 1.0 mg of gel/mg of protein, followed by 2.0 mg of gel/mg of protein, and by 5.0 mg of gel/mg of remaining protein. This preliminary treatment with calcium phosphate gel was followed by ammonium sulphate fractionation as for the heart isoenzyme up to the stage where the 60% precipitate was obtained. This precipitate was taken up in 5 mM-sodium phosphate buffer (25 ml) containing

20% glycerol and 1 mM-EDTA, pH 7.6, and desalted on a Sephadex G-25 column. Chromatographic separation of the isoenzymes was done on a DEAE-cellulose column (2 cm × 37 cm). The column was packed at room temperature and equilibrated with 5 mM-sodium phosphate buffer containing 20% glycerol and 1 mM-EDTA, pH 7.6, at 4°C. The desalted enzyme extract was applied to the top of the column and allowed to run in under gravity. Stepwise elution with increasing concentration of phosphate buffer was carried out and 20 ml fractions were collected. The slow isoenzyme was eluted in the 5 mM-sodium phosphate buffer whereas the fast enzyme, which is the major liver fraction, was eluted by 0.02 M-sodium phosphate buffer. This separation was confirmed by starch-gel electrophoresis (Fig. 1). The most active fractions (60 ml) were combined and concentrated to a protein concentration of 7–10 mg/ml by the use of Carbowax M-20. Ammonium sulphate fractionation was then done to obtain a 51–69% precipitate as for the heart tissue. This precipitate was dissolved in 5 ml of 5 mM-sodium citrate buffer containing 20% glycerol and 1 mM-EDTA, pH 6.2, followed by desalting on a Sephadex G-25 column. The desalted precipitate was chromatographed on a CM-cellulose column, stepwise elution being done with increasing concentrations of sodium citrate buffer, pH 6.2, the most active fractions from the column being combined.

Treatment with calcium phosphate gel was carried out as for the heart isoenzyme. The last gel supernatant (26.5 ml) was brought to 75% ammonium sulphate saturation and then it was left for 18 h before being centrifuged as described above. The

0–75% precipitate was taken up in 10ml of 0.1M-tris-HCl buffer containing 20% glycerol and 1mM-EDTA, pH7.5.

Problems of purification

Stability. The basic problem during purification was maintenance of stability of the enzyme, especially with dilute solutions in low-ionic-strength buffers. The addition of glycerol (20–30%) to solutions containing isocitrate dehydrogenase was found to have a considerable stabilizing effect, as used by Colman (1968), and it was therefore included in solutions during purification. Lotspeich & Peters (1951) report that even traces of heavy-metal cations can inactivate this enzyme, and the use of glass-distilled water and inclusion of EDTA in purifying solutions proved to be essential.

Extraction. The extraction of this enzyme from pig heart by previous investigators (Adler *et al.*, 1939; Moyle & Dixon, 1956; Siebert *et al.*, 1957) was done with acetone. It was found that acetone caused variable loss of enzyme activity, and an alternative method of extraction by homogenizing in 20% glycerol containing 1mM-EDTA, pH7.0, was tried and found satisfactory.

Ammonium sulphate treatment. Solid ammonium sulphate in fractionation was avoided, because of the report by Moyle (1956) that solid ammonium sulphate inactivated the enzyme and the confirmation of this in the present work. Saturated ammonium sulphate solution was therefore used. Ammonium sulphate was found to cause an activation of the heart isoenzyme but not of the liver isoenzyme (Tables 1 and 2). For the first wide-range ammonium sulphate step it was found that fractionation between 50 and 75% ammonium sulphate saturation and a precipitating time of 18h at the 75% concentration and 3h at the 50% saturation gave the best purification with the least loss of enzyme activity.

Separation of the isoenzymes. The best separation of the liver isoenzymes of isocitrate dehydrogenase was obtained on a DEAE-cellulose column with sodium phosphate buffer, pH7.6. The recovery of liver isoenzymes from the DEAE-cellulose column was about 100%. Use of a CM-cellulose column and sodium citrate buffer, pH6.2, was the most suitable procedure for the heart. The recovery of the heart isoenzyme from the CM-cellulose column was always greater than 100%.

Treatment of concentrated column fractions with calcium phosphate gel. The best purification was achieved by adding sufficient calcium phosphate gel to absorb inactive protein, with the active enzyme left unabsorbed. The number of gel steps needed and the purification obtained was variable and it was thus necessary to carry out this fractionation in steps, analysing the enzyme activity at each stage.

Properties of the major liver and heart isoenzymes

The highly purified major heart (slow) and major liver (fast) isoenzymes were used for these experiments. All inhibitor and activator solutions were prepared in 0.1M-tris-HCl buffer unless otherwise specified, the final pH being adjusted to 7.5 before use. The spectrophotometric method of assay was used.

Effect of ammonium sulphate, ammonium chloride and sodium sulphate (Fig. 2). Initial experiments showed that the degree of activation depended both on the time of contact of the salt with the enzyme and on its concentration. No activation was observed if the salt was added to the enzyme solution and assay carried out immediately. A period of 30min contact before enzyme assay gave maximum effect. Solutions of the salts were added to the purified isoenzymes to give a final concentration between 0.13M and 1.30M and the solutions were kept at 4°C for 30min before assay of isocitrate dehydrogenase. Both ammonium sulphate and sodium sulphate, but not ammonium

Fig. 2. Effect of (a) sodium sulphate, (b) ammonium sulphate and (c) ammonium chloride on purified isocitrate dehydrogenase isoenzymes from rat heart (slow) and liver (fast)

Neutral solutions of the salts were added to the purified isoenzymes to give a final concentration between 0.13 and 1.3M, and the solutions were kept at 4°C for 30min before assay for isocitrate dehydrogenase activity: ●, heart isoenzyme; ○, liver isoenzyme.

Fig. 3. Effect of temperature on the stability of purified rat liver (fast) and rat heart (slow) isoenzymes of isocitrate dehydrogenase

The isoenzyme solution was preincubated for 5–60 min at the given temperature. It was cooled quickly to 25°C and then the reaction ingredients were added and the isocitrate dehydrogenase activity was measured: o, liver isoenzyme; ●, heart isoenzyme. (a) 25°C; (b) 30°C; (c) 35°C; (d) 40°C; (e) 45°C; (f) 50°C; (g) 55°C.

chloride, activated the heart isoenzyme. The activation with ammonium sulphate reached about twofold. In the main preparation, under different conditions, five- to six-fold activation was obtained (Table 1) with the heart preparation by using ammonium sulphate. The liver isoenzyme did not show a significant increase in activity with any of the salts at the concentration used.

Heat stability (Fig. 3). Appropriately dilute solutions of purified heart and liver isoenzyme were preincubated for 5–60 min at fixed temperatures in thermostatically controlled water baths. The samples were then cooled quickly to 25°C, the reaction ingredients were added and the enzyme activity was estimated. The heart isoenzyme was markedly less stable to heat than the liver isoenzyme. The largest difference in heat stability was seen at 30°C. Thus, with a preincubation time of 30 min, the liver isoenzyme retained 57% of its activity whereas the heart isoenzyme retained only 16%.

Effect of temperature on initial rate of reaction. The reaction rate increased linearly with temperature up to 28°C; further increase in temperature resulted in a

falling off of rate due to deactivation. Thus at 30°C the heart isoenzyme showed only 88% of its maximum activity, and the liver isoenzyme had just started to show deactivation. The isocitrate dehydrogenase measured in serum was not showing any inactivation at 30°C and presumably serum components protect the enzyme. The temperature coefficient in the range 18–28°C was found to be 2.58 for the heart isoenzyme, 2.42 for the liver isoenzyme and 2.56 for isocitrate dehydrogenase measured in serum.

Effect of pH on enzyme activity (Fig. 4). For these experiments tris-maleate buffer (Gomori, 1948) was used rather than tris-HCl so that a wider pH range could be covered. It was shown in initial experiments that use of tris-maleate buffer did not affect the enzyme activity measured. The initial reaction velocities were measured at values from pH 4.7 to 8.5, the pH in the cuvette being taken immediately on conclusion of the enzyme reaction. The optimum pH for both the heart and liver isoenzymes was 7.4 ± 0.2 and both isoenzymes showed similar pH-activity curves.

Effect of pH on stability (Fig. 4). Appropriately

Fig. 4. Effect of pH on the stability and activity of purified isocitrate dehydrogenase isoenzymes from rat heart and liver

For the stability experiments (a: ○, liver; ●, heart) the purified isoenzymes were kept at pH values between 4.7 and 8.5 (tris-maleate buffer) for 1 h at 4°C before enzyme assay at pH 7.4. For the activity experiments (b: ○, liver; ●, heart) the enzyme activity was measured at pH values between 4.7 and 8.5 with tris-maleate buffer.

dilute solutions of the isoenzymes were kept at pH values between 4.7 and 8.6, and left for 1 h at 4°C before measurement of enzyme activity at pH 7.4. Both the heart and liver isoenzymes were stable over a range of pH 5.5–7.8 with a sharp fall in stability beyond this range. The liver isoenzyme was slightly more stable than the heart isoenzyme; thus at pH 8.0 the liver isoenzyme retained about 80% of the total activity whereas the heart isoenzyme retained only 50%.

Effect of inhibitors on enzyme activity (Fig. 5). The effect of Hg^{2+} , Cu^{2+} and *p*-chloromercuribenzoate on purified heart and liver isoenzymes was investigated. Solutions of HgCl_2 and CuCl_2 were prepared in 0.1M-tris-HCl buffer and adjusted to final pH 7.5. *p*-Chloromercuribenzoate solution was prepared in 0.1M-glycylglycine buffer because of its insolubility in tris-HCl buffer. Inhibitor solutions (0.1 ml) of various concentrations were added to the reaction mixture in the cuvette just before enzyme assay and the initial reaction velocity was measured. Both the isoenzymes were inhibited to about the same

Fig. 5. Effect of inhibitors on purified isocitrate dehydrogenase isoenzymes from rat heart and liver

Neutral solutions of the inhibitors were added to the enzyme reaction mixture in the cuvette just before measurement of isocitrate dehydrogenase activity. Results with the isoenzyme from rat heart (●) and the isoenzyme from rat liver (○) are shown. (a) Cu^{2+} ; (b) *p*-chloromercuribenzoate; (c) Hg^{2+} .

extent by Hg^{2+} , showing complete inhibition at a final concentration of 32.2 μM . Cu^{2+} was a less effective inhibitor than Hg^{2+} but some difference was shown between the isoenzymes with respect to this ion. Thus, at a final Cu^{2+} concentration of 3.22 mM, heart isoenzyme showed only about 35% inhibition whereas liver isoenzyme showed 51% inhibition. Both isoenzymes showed complete inhibition at a final concentration of 32.2 mM. A more marked difference between the two isoenzymes was seen with respect to inhibition by *p*-chloromercuribenzoate. Thus, at a final inhibitor concentration of 0.322 μM , the heart isoenzyme retained 92% of its activity whereas the liver isoenzyme retained only 65% of its activity: it cannot be excluded that the

Table 3. *Effect of metal-ion activators on purified isocitrate dehydrogenase isoenzymes from rat heart (slow) and liver (fast)*

The metal ions (all salts in the chloride form) were made up in 0.15M-NaCl and adjusted to pH7.5 before use.

Metal ion	Concn. of metal ion (mM)	% of activity with Mn ²⁺	
		Heart isoenzyme	Liver isoenzyme
Mn ²⁺	0.161	100	100
	1.61	100	100
Mg ²⁺	0.161	45	42
	1.61	44	40
Co ²⁺	0.161	50	66
	1.61	50	66
Ni ²⁺	0.161	9	17
	1.61	9	14
Zn ²⁺	0.161	5	7
	1.61	5	6
None	0	0	0

Table 4. *Michaelis constants for rat liver (fast) and heart (slow) isocitrate dehydrogenase isoenzymes*

The initial reaction rates for purified isocitrate dehydrogenase isoenzymes were measured by using the spectrophotometric assay of Wolfson & Williams-Ashman (1957) as modified by Bowers (1959).

Substrate	Michaelis constants (μ M)	
	Heart isoenzyme	Liver isoenzyme
D-Isocitrate	5.0	3.0
NADP ⁺	20	25
APADP ⁺	83	200

Michaelis constants for the substrate and cofactors (Table 4)

The concentrations of isocitrate and of NADP were assayed as described above. The reactions were monitored at the appropriate absorption maxima of the reduced forms of the coenzymes, namely 340nm for NADP, 363nm for APADP, 338nm for NHDP and 398nm for thio-NADP.

The Michaelis constants were calculated by the method of Lineweaver & Burk (1934). The Michaelis constants for isocitrate and NADP were similar for both isoenzymes but a twofold difference between liver and heart isoenzymes was shown with the coenzyme analogue APADP. No reaction with either isoenzyme was observed when thio-NADP and NHDP were used.

purification had left different protective contaminating proteins for the two isoenzymes.

Effect of metal-ion activators on enzyme activity (Table 3). The metal ions used were Mn²⁺, Mg²⁺, Co²⁺, Ni²⁺ and Zn²⁺ (each metal as the chloride). The solutions were made in 0.15M-NaCl and adjusted to pH7.5 before use. In the absence of added metal ion neither of the isoenzymes showed any activity and maximum activation of both isoenzymes was obtained by the addition of manganese salt. With the heart isoenzyme and a metal-ion concentration of 0.161mM, Co²⁺ produced 50%, Mg²⁺ 45%, Ni²⁺ 9% and Zn²⁺ 4% of the activity of that with an equivalent amount of Mn²⁺. With the liver isoenzyme and the same metal-ion concentration, Co²⁺ produced 66%, Mg²⁺ 42%, Ni²⁺ 17% and Zn²⁺ 7% of the activity of that with an equivalent amount of Mn²⁺.

Discussion

NADP-isocitrate dehydrogenase exists as two isoenzymes, the 'fast' form being predominant in liver and the 'slow' form in heart (Tsao, 1960; Baron & Bell, 1962; Campbell & Moss, 1962). The present purification included separation of the isoenzymes on cellulose ion-exchange columns so that the 'slow' isoenzyme of heart (the predominant isoenzyme of this tissue) could be compared with the 'fast' liver isoenzyme, which is the predominant liver form. A marked difference between the two isoenzymes was in their stability to temperature, the heart isoenzyme being less stable. Campbell & Moss (1962) found with unpurified heart and liver total isocitrate dehydrogenase, a greater instability to heat of the heart fraction. Activation of the heart isoenzyme by SO₄²⁻ has not previously been reported and it may be that

addition of SO_4^{2-} to sera before estimation of isocitrate dehydrogenase after myocardial infarction may demonstrate that increase of isocitrate dehydrogenase does occur in myocardial infarction. The difference in stability of liver and heart enzymes to heat, and the fact that heart isocitrate dehydrogenase is mainly non-cytoplasmic (Bell & Baron, 1968) could together account for the fact that the activity of this enzyme rises in serum after liver cell destruction but not after myocardial infarction. Moyle (1956) and Siebert *et al.* (1957) noted the preservative quality of ammonium sulphate on isocitrate dehydrogenase but did not report activation. It was suggested by Moyle (1956) that SO_4^{2-} prevents the dissociation of some complex or binding in the enzyme molecule necessary for the preservation of enzymic activity and ultimately for the maintenance of the native state of the protein. Both isoenzymes were inhibited to a similar extent by Hg^{2+} whereas differences were seen with Cu^{2+} and *p*-chloromercuribenzoate. The strong inhibition produced by metal ions and *p*-chloromercuribenzoate shows the thiol nature of both heart and liver isocitrate dehydrogenase and is in agreement with reports by Lotspeich & Peters (1951) and Higashi *et al.* (1965).

Most reports on purification of NADP-isocitrate dehydrogenase have been concerned with the total isocitrate dehydrogenase content of the tissue and the purification achieved was less than in the present paper (Moyle & Dixon, 1956; Siebert *et al.*, 1957; Rose, 1960). After the completion of this work Illingworth & Tipton (1970) described a purification of NADP-isocitrate dehydrogenase from pig liver cytoplasm, the purification being five times that obtained in the present paper.

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