Purification and some Properties of Cytoplasmic Aspartate Aminotransferase from Sheep Liver

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1. A procedure for the purification of the cytoplasmic isoenzyme of aspartate aminotransferase from sheep liver is described. 2. The purified isoenzyme shows a single component in the ultracentrifuge at pH 7.6 and forms a single protein band on agar-gel electrophoresis at pH 6.3 or 8.6, as well as when stained for protein or activity after polyacrylamide-gel or cellulose acetate electrophoresis at pH 8.8. 3. Immunoelectrophoresis on agar gel yields only one precipitin arc associated with the protein band, with rabbit antiserum to the purified isoenzyme. By immunodiffusion, cross-reaction was detected between the cytoplasmic isoenzymes from sheep liver and pig heart, but not between the cytoplasmic and mitochondrial sheep liver isoenzymes. 4. The $s_{20,w}$ of the enzyme is 5.69S and the molecular weight determined by sedimentation equilibrium is 88900; 19313 molecules of oxaloacetate were formed/min per molecule of enzyme at pH 7.4 and 25°C. 5. The amino acid composition of the isoenzyme is presented. It has about 790 residues per molecule. 6. The holoenzyme has a maximum of absorption at 362nm at pH 7.6 and 25°C. 7. A value of 2.1 was found for the coenzyme/enzyme molar ratio. 8. The purified enzyme revealed two bands of activity on polyacrylamide-gel electrophoresis at pH 7.4 and an extra, faster, band in some circumstances. These bands occurred even when dithiothreitol was present throughout the isolation procedure. 9. Three main bands were obtained by electrofocusing on polyacrylamide plates with pI values 5.75, 5.56 and 5.35. 10. Structural similarities with cytoplasmic isoenzymes from other organs are discussed.

Aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) has been studied in some mammalian tissues (Fleisher *et al.*, 1960; Marino *et al.*, 1966; Bertland & Kaplan, 1968; Magee & Phillips, 1971) and especially in pig heart (Jenkins *et al.*, 1959; Martínez-Carrión *et al.*, 1967; Wada & Morino, 1964; Doonan *et al.*, 1972b; Ovchinnikov *et al.*, 1973). Two isoenzymes have been reported, an anionic cytoplasmic isoenzyme and a cationic mitochondrial isoenzyme with different kinetic and structural properties (Boyd, 1966; Martínez-Carrión & Tiemeier, 1967). The complete amino acid sequence of the pig heart cytoplasmic isoenzyme has been published (Ovchinnikov *et al.*, 1973). The latest values reported for the molecular weight of the pig heart cytoplasmic isoenzyme (92000–93000) (Feliss & Martínez-Carrión, 1970; Polyanovsky, 1972) agree well with that estimated from the sequence data (Doonan *et al.*, 1972a).

Several subforms have been described for each aspartate aminotransferase isoenzyme from pig heart (Martínez-Carrión *et al.*, 1967) and rat brain (Magee & Phillips, 1971), among other organs. They have been isolated and have the same molecular weight and amino acid composition, showing also complete antigenic identity by immunodiffusion in agar gel (Martínez-Carrión *et al.*, 1967). So far, their precise

nature and origin is not clear (Ivanov & Karpeisky, 1969).

In spite of the important role of aspartate aminotransferase in the liver, very little has been published about the properties of the enzyme from this source. The canine liver isoenzymes (Fleisher, 1960) and the rat liver isoenzymes (Hook & Vestling, 1962; Harpring, 1965; Boyd, 1966) have been partially purified. Immunochemical and kinetic studies on the human liver isoenzymes (Nisselbaum & Bodansky, 1964) as well as for the ox liver isoenzymes (Wada & Morino, 1964) have been reported.

In this paper we describe the purification of sheep liver cytoplasmic isoenzyme to a high degree and report some of its structural properties. We also give evidence for the presence of subforms in preparations of this enzyme.

Materials and Methods

Materials

Reagents. NADH, 2-oxoglutaric acid, dithiothreitol and ovalbumin were purchased from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Malate dehydrogenase, pig heart cytoplasmic aspartate aminotransferase and cytochrome *c* were obtained from Boehringer (London) Corp., London

W5 2TZ, U.K. Sperm-whale myoglobin, acrylamide and TEMED (*NNN'*-tetramethylethylenediamine) were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Bisacrylamide and 6-benzamido-4-methoxy-*m*-toluidinediazonium chloride were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. Naphthalene Black, Coomassie Brilliant Blue and Fast Blue B were purchased from Gurr, London N.W.9, U.K. Sephrapure III and Gelman high-resolution buffer were obtained from Gelman Instrument Co., Ann Arbor, Mich., U.S.A. Ionagar 2 and Freund's complete adjuvant were obtained from Difco Laboratories, Detroit 1, Mich., U.S.A. Whatman DEAE-cellulose was obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K. Sephadex G-100 and G-200 and Blue Dextran were purchased from Pharmacia (Great Britain) Ltd., London W5 5SS, U.K. Polyvinylpyrrolidone was from May and Baker Ltd., Dagenham, U.K. Ampholine was obtained from LKB Instruments Ltd., London S.E.20, U.K. L-Aspartic acid was from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K. Bovine serum albumin was obtained from Calbiochem Ltd., London W1H 1AS, U.K. All other chemicals were A.R. grade from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

Buffers. 0.20M-Sodium-potassium phosphate buffer, pH 7.4, was made by mixing 0.20M- Na_2HPO_4 (40.5 ml) and 0.20M- KH_2PO_4 (9.5 ml) and adjusted to pH 7.4. 20mM-Sodium-potassium phosphate buffer, pH 7.4, was prepared by diluting the above-mentioned buffer ten times with glass-distilled water and adjusted to pH 7.4. 0.03M- Na_2HPO_4 - KH_2PO_4 buffer, pH 7.5, was 25 mM in Na_2HPO_4 , 5 mM in KH_2PO_4 and adjusted to pH 7.5. Gelman high-resolution buffer, pH 8.8, corresponds to a 0.05M-sodium Tris-barbital buffer. 0.15M-NaCl-17.6mM-sodium-potassium phosphate buffer, pH 7.6, corresponds to a solution 16.2 mM in Na_2HPO_4 and 1.4 mM in KH_2PO_4 and 0.15M-NaCl (all final concentrations). 50mM-Sodium-potassium phosphate buffer, pH 6.3, was 50 mM in KH_2PO_4 , adjusted to pH 6.3 with NaOH. 25mM-Sodium-potassium phosphate buffer, pH 6.3, was prepared by diluting twice the 50mM-sodium-potassium phosphate buffer, pH 6.3, with glass-distilled water. 0.05M-Sodium phosphate buffer, pH 7.4, was made by mixing 0.20M- Na_2HPO_4 (40.5 ml) and 0.20M- NaH_2PO_4 (9.5 ml), and was diluted fourfold and adjusted to pH 7.4. 0.01M-Sodium phosphate buffer, pH 7.4, was prepared by diluting fivefold the 0.05M-sodium phosphate buffer, pH 7.4, with glass-distilled water.

Methods

Enzyme assay. Aspartate aminotransferase activity was assayed at pH 7.5 and 25°C by the method of Karmen (1955) with reagent concentrations as used by

Boyd (1966). One unit of enzyme activity is defined as that amount of enzyme that catalyses the formation of 1 μmol of oxaloacetate/min at 25°C in 0.20M-sodium-potassium phosphate buffer, pH 7.4.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Agar-gel electrophoresis. High-voltage agar-gel electrophoresis on microscope slides was performed as described by Wieme (1959), except that the buffer was 0.03M- Na_2HPO_4 - KH_2PO_4 (pH 7.5 and 10.1) as recommended by Boyd (1966). Tests for aspartate aminotransferase isoenzyme were carried out by the method of Boyd (1966).

Cellulose acetate electrophoresis. This was carried out on Sephrapure strips (2.54 cm $\times$ 17.14 cm) at pH 8.8 by using Gelman high-resolution buffer. Voltage was maintained at 300 V for 1 h at 23°C. Protein was stained with a saturated solution of Naphthalene Black in 2.5% (v/v) acetic acid-25% (v/v) methanol.

Polyacrylamide-gel electrophoresis. Analytical electrophoresis in disc and in vertical slab gel was carried out at 4°C. Protein bands were localized by staining with 0.25% (w/v) Coomassie Brilliant Blue in methanol-acetic acid-water (4:1:4, by vol.). Excess of stain was removed electrophoretically by applying 10-15 mA/tube in 5% (v/v) acetic acid solution. Enzymic activity was located on polyacrylamide gels either (a) as described by Banks *et al.* (1968), by using Fast Blue B (the gels were stained overnight at room temperature and excess of reagent was removed by using a destaining solution that contained 7.5% v/v, acetic acid and 5% v/v, methanol in water) or (b) as described by Decker & Rau (1963) with 6-benzamido-4-methoxy-*m*-toluidinediazonium chloride as the staining reagent. The red colour developed at room temperature within 5-10 min and was stable for at least 24 h.

Polyacrylamide disc-gel electrophoresis was performed at pH 8.9 as described by Davis (1964) and at pH 7.4 as recommended by Williams & Reisfeld (1964). However, for both systems only one gel concentration was used throughout, corresponding to the small pore (7.5% w/v, acrylamide) as suggested by Scandurra & Cannella (1972). The samples were applied by layering at the top of the gel after addition of some glycerol or sucrose at a final concentration of 33% (w/v). The runs were carried out at 2 mA/tube for 15 min and then at 3 mA/tube for 2 h at 4°C. Each gel was sliced in two, and one half was tested for protein and the other for enzymic activity.

Vertical-slab gel electrophoresis was performed in an Ortec 4200 apparatus (Ortec Inc., Oak Ridge, Tenn., U.S.A.) by using a pulsed constant power supply (Ortec 4100). Electrophoresis was towards the anode at a current of 1 mA/sample, which was pulsed at 150 pulses/s until the tracking dye entered the running gel, and then at 3 mA/sample and 225 pulses/s.

The runs were done at pH7.4 by the method of Williams & Reisfeld (1964). No sample gel was used, the sample being applied instead in sucrose at a final concentration of 33% (w/v). The electrophoresis was allowed to proceed for 1 h in excess of the time that the tracking dye took to leave the running gel.

Isoelectric focusing. Isoelectric focusing on polyacrylamide plates (21.6cm × 16.5cm) in the range pH4–7 (obtained by mixing equal amounts of ampholyte pH4–6 and ampholyte pH5–7) was done by using the technique of Awdeh *et al.* (1968). Purified samples were dialysed extensively against 20mM-sodium-potassium phosphate buffer, pH7.4. Samples (80–200 µl) at a protein concentration from 0.5 to 2.5 mg/ml were applied to the surface of the gel on a small piece of filter paper. Haemoglobin samples were also applied to the plates as controls for each run. All the runs were carried out at 4°C for 24 h. The voltage was gradually increased during the first 3–4 h as the pH gradient was established so as to maintain a maximal constant current of 6 mA. The potential difference was finally maintained at 500 V for 20 h, the current dropping to about 2 mA during this period. After the run had ended, the gel was allowed to reach room temperature (Salaman & Williamson, 1971). The pH gradient was then determined by measuring the pH directly along the plate (at 1 cm intervals) with a flat glass-calomel combination electrode (Pye-Unicam, Cambridge, U.K.). A plot of pH versus distance along the gel was drawn. The gel was then stained with Coomassie Blue by the procedure of Awdeh *et al.* (1968) and the pH values of the bands were located with the aid of the plot.

Absorption spectra. These were recorded at 25°C with a Unicam SP.800 recording spectrophotometer, in 1 cm cells and against the appropriate blanks as reference.

Amino acid analysis. The enzyme was dialysed against glass-distilled water for 24 h. The salt-free protein was freeze-dried and then hydrolysed in an evacuated sealed glass ampoule with 6 M-HCl for 24, 48 and 72 h at 108°C. Samples were then analysed on a Technicon Microanalyzer (Technicon Instruments Co., Chertsey, Surrey, U.K.) by the procedure of Beale & Kent (1968). Cysteine was measured as cysteic acid after oxidation of the sample with performic acid followed by hydrolysis with 6 M-HCl for 48 h at 106°C (Hirs, 1967). Tryptophan was measured spectrophotometrically (Bencze & Schmid, 1957) in separate samples.

Ultracentrifuge studies. These were carried out with a Beckman-Spinco model E ultracentrifuge equipped with Rayleigh interference optics. Samples were previously dialysed for 24 h against 0.15 M-NaCl–17.6 mM-sodium-potassium phosphate buffer, pH7.6. Sedimentation-velocity runs were performed by using schlieren optics in 4°-sector cells, with 12 mm aluminium centrepieces, at 23°C and at a rotor speed

of 60000 rev./min, giving 260000g (r_{av} 6.5 cm). Sedimentation-equilibrium experiments were conducted by using the short-column meniscus-depletion method of Yphantis (1964). In these runs, 4 mm solution columns in double-sector cells equipped with sapphire windows were used at constant temperature (23°C). The attainment of the equilibrium was determined experimentally by measuring fringe displacements at given radial positions on successive photographs. A water blank was run after each experiment to correct for window distortion.

Molecular-weight determination by gel filtration. The molecular weight of the enzyme was also determined by the gel-filtration procedure of Andrews (1965). A Sephadex G-200 column (1.4 cm × 90 cm) equilibrated with 0.15 M-NaCl–17.6 mM-sodium-potassium phosphate buffer, pH7.6, was used. The following proteins were used as standards: cytochrome *c* (mol.wt. 12400); sperm-whale myoglobin (mol.wt. 17800); ovalbumin (mol.wt. 45000); bovine serum albumin (mol.wt. 67000). Blue Dextran was used for the determination of the void volume. The enzyme samples ranged from 10 to 16 mg/2 ml of buffer.

Preparation of antisera. Cytoplasmic aspartate aminotransferase from sheep liver obtained after chromatography with Sephadex G-100, as well as cytoplasmic aspartate aminotransferase from pig heart (specific activity 180 units/mg), were individually dialysed against 0.15 M-NaCl. Rabbits weighing 3.2 kg were injected intramuscularly with either sheep liver enzyme (2.3 mg) or pig heart enzyme (3 mg) in Freund's complete adjuvant. The rabbits were bled by ear puncture 14, 29 and 47 days after injection. The sera were separated by centrifugation and stored at –20°C.

Immunochemical analysis. The antisera obtained as described above were used directly for immunochemical assays. Double diffusion on agar gel was performed as described by Ouchterlony (1967) at room temperature. Immunoelectrophoresis at pH8.6 was carried out in agar gel (1.5%, w/v, Ionagar 2 in 25 mM-sodium diethyl barbiturate, pH8.6, containing 0.1% NaN₃) with 50 mM-sodium diethyl barbiturate, pH8.6, in the reservoir (Feinstein, 1968). Immunoelectrophoresis at pH6.3 was carried out in agar gel (1.5%, w/v, Ionagar 2 in 25 mM-sodium-potassium phosphate buffer, pH6.3, containing 0.1% NaN₃) with 50 mM-sodium-potassium phosphate buffer, pH6.3, in the reservoir (Munn *et al.*, 1971). Duplicate plates were run for 3 h at 4.7 V/cm and at 4°C. In one of the plates immunodiffusion was carried out at room temperature, and the other was stained for protein. This second plate was first fixed by immersion for 30 min in ethanol-acetic acid-water (5:1:5, by vol.) and then covered with filter paper and allowed to dry at 37°C. It was then stained with a saturated solution of Naphthalene Black in the fixer mixture for 5–

10 min. The plate was finally destained in the fixer mixture.

Enzyme purification procedure

The centrifugations were performed in a MSE 18 high-speed refrigerated centrifuge. All steps were carried out at 0–4°C unless otherwise specified. Ammonium sulphate concentrations are expressed as percentage saturation at 0°C.

Stage 1. Clun Forest sheep were killed by a shot in the head and bled out. The livers were collected immediately, diced and rinsed in cold 0.25 M-sucrose. A 20% (w/v) homogenate was prepared in 0.25 M-sucrose with a Teflon-glass homogenizer (Wesley-Coe Glass Co., Cambridge, U.K.) by using ten up-and-down strokes. Differential centrifugation of the homogenate was performed. It was first centrifuged at 650g for 10 min. The pellet was washed once and the 650g supernatant and the wash were pooled and centrifuged at 12000g for 10 min.

Stage 2. Heat-treatment of the supernatant was carried out as described by Banks *et al.* (1968) for the pig heart cytoplasmic isoenzyme but 2-oxoglutarate (Jenkins *et al.*, 1959) and pyridoxal phosphate (Wada & Morino, 1964) were added as stabilizers, to final concentrations of 0.001 M and 0.01 mM respectively. The 12000g supernatant from stage 1 (in portions of 400 ml) was heated at 70°C for 15 min, cooled to 4°C and the precipitate was removed by centrifugation.

Stage 3. The supernatant from stage 2 was further purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation. Optimum precipitation occurred between 64 and 72% saturation, with an overall purification factor of 69 and a yield of 37%. When the range of 50–65% saturation was used, as recommended by Boyd (1966) for the rat liver isoenzyme, an overall purification factor of 25 and a yield of 30% were obtained. Protein precipitating overnight in the optimum range was collected and redissolved in 0.01 M-sodium phosphate buffer, pH 7.4, dialysed against the same buffer and concentrated by ultrafiltration by means of an Amicon Diaflo ultrafiltration cell (Amicon, High Wycombe, Bucks., U.K.) fitted with a PM-30 filter, to a volume below 10 ml.

Stage 4. The sample was applied to a column (2.5 cm × 22.5 cm) of DEAE-cellulose (Whatman DE 32) equilibrated with 0.01 M-sodium-potassium phosphate buffer, pH 7.4. The cytoplasmic isoenzyme was retarded under these conditions and its elution could be obtained by applying a linear concentration gradient (0.01–0.05 M) of sodium phosphate buffer, pH 7.4, at a constant flow rate of 40 ml/h. Any mitochondrial isoenzyme still present was eluted in the void volume at this stage. Cytoplasmic isoenzyme fractions with the highest specific activity (fractions 21–28 inclusive in Fig. 1) were pooled, dialysed against 0.15 M-NaCl–17.6 mM-sodium-potassium phosphate buffer, pH 7.6, and concentrated to a volume below 2 ml by ultrafiltration.

Fig. 1. DEAE-cellulose chromatography of the dialysed ammonium sulphate fraction

The sample (319 mg/5.9 ml) was loaded on a column (2.5 cm × 22.5 cm) and eluted with a linear gradient of 0.01–0.05 M-sodium-potassium phosphate buffer, pH 7.4, at 4°C, and at a flow rate of 40 ml/h. Fractions (3.4 ml) were collected. ●, E_{280} ; ▲, enzymic activity.

Fig. 2. Elution profile in Sephadex G-200

Pooled fractions (21–28 inclusive) from DEAE-cellulose chromatography were dialysed, concentrated and loaded on a column (1.4 cm $\times$ 90 cm) of Sephadex G-200 equilibrated with 0.15 M-NaCl–17.6 mM-sodium–potassium phosphate buffer, pH 7.6. The sample was eluted with the same buffer at a flow rate of 10 ml/h and at 4°C. Fractions (2.6 ml) were collected. ●, E_{280} ; ▲, enzymic activity.

Stage 5 (Sephadex G-200 chromatography). The concentrated sample obtained from stage 4 was placed on a column (1.4 cm $\times$ 90 cm) equilibrated with 0.15 M-NaCl–17.6 mM-sodium–potassium phosphate buffer, pH 7.6. On elution with the same solution (flow rate 10 ml/h) two peaks were obtained, the enzyme being recovered in the first peak, as shown in Fig. 2. Enzyme fractions with the highest specific activity were collected, dialysed against 0.15 M-NaCl–17.6 mM-sodium–potassium phosphate buffer, pH 7.6, and concentrated by ultrafiltration to a volume of 1 ml.

Stage 6 (Sephadex G-100 chromatography). This additional purification step was applied only to samples to be used for ultracentrifuge analysis, to ensure that they were free of particle aggregates. The sample was applied to a column (1 cm $\times$ 104 cm) equilibrated with 0.15 M-NaCl–17.6 mM-sodium–potassium phosphate buffer, pH 7.6. The enzyme was eluted with the same buffer, the fractions with the highest specific activity being used immediately for the ultracentrifuge studies.

In one experiment, a preparation was made in the presence of dithiothreitol, a reagent known to keep the thiol group in the reduced form. Dithiothreitol (0.2 mM final concentration) was added to the homogenizing medium and the same concentration was maintained during all the purification stages.

Results

Purification of the cytoplasmic isoenzyme

The yield and purification obtained for a typical preparation are summarized in Table 1. The 12000g supernatant obtained in stage 1 was always contaminated with the mitochondrial isoenzyme, as shown by high-voltage agar-gel electrophoresis and enzyme assays at pH 6 and 7.4 (Boyd, 1966). Chromatography (stage 4) yielded two peaks of protein, the cytoplasmic isoenzyme being eluted in the second one (Fig. 1). Ultracentrifuge analysis of the concentrated material obtained from the pooled DEAE-cellulose fractions showed that it consisted of two components of about the same concentration with sedimentation coefficients of 3.30S and 5.45S. The first peak obtained by gel filtration on Sephadex G-200 corresponded to the 5.45S component and contained all the enzymic activity (Fig. 2). The second peak corresponded to the 3.3S component.

Electrophoretic behaviour

Purified sheep liver cytoplasmic aspartate aminotransferase (after chromatography in Sephadex G-200) migrates towards the anode as a single band on cellulose acetate electrophoresis at pH 8.8.

On polyacrylamide-gel electrophoresis at pH 8.8 this fraction also migrates as a single band (Fig. 3 and Table 2), whereas at pH 7.4 it shows an additional band of higher mobility (β band) when either fresh or aged (stored at -20°C during 1 month) samples are run. A third band of even higher mobility (γ band) was observed for an aged sample that had been repeatedly thawed and re-frozen. All three bands of the purified cytoplasmic aspartate aminotransferase always showed enzymic activity. The mobilities of the bands are approx. 8×10^{-10} (α band), 8.3×10^{-10} (β band) and $9.4 \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ (γ band) at pH 7.4. The same three-band pattern of enzymic activity was obtained for the supernatant from stage 2. The 12000g supernatant, however, shows only α and β activity bands, even after twofold concentration by ultrafiltration.

Since Arrio-Dupont *et al.* (1970) have reported that 0.1 mM-dithiothreitol prevents the appearance of multiple subforms of pig heart cytoplasmic isoenzyme, one-half of a sheep liver was processed as usual (preparation B) (Table 2), and the other half was rinsed, homogenized and treated always in the presence of 0.2 mM-dithiothreitol (preparation C). Treatment with dithiothreitol did not prevent the appearance of the bands nor change the electrophoretic pattern of the respective fractions. Moreover, although the samples of 12000g supernatants (stage 1) were subjected to electrophoresis immediately after they were obtained, both α and β bands of enzyme activity were observed in the treated and untreated preparations.

Table 1. *Purification of sheep liver cytoplasmic aspartate aminotransferase*

The enzyme was purified from 1 kg of sheep liver. One unit of enzyme activity is defined as that amount of enzyme that catalyses the formation of 1 μ mol of oxaloacetate/min at 25°C in 0.20 M-sodium-potassium phosphate buffer, pH7.4, under the conditions described by Boyd (1966). Experimental details are given in the text.

Stage	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)	Purification factor
1 12000g supernatant*	2900	33 500	69 600	0.48†	—	1
2 Heated supernatant (15 min, 70°C)	2520	28 600	6230	4.59	100	9.5
3 Ammonium sulphate residue (64–72 g/100 ml)	5.9	10 600	319	33.2	37	69.2
4 Pooled DEAE-cellulose eluate	2.0	3750	42.7	87.8	13	182.9
5 Pooled Sephadex G-200 eluate	2.5	3320	15.3	217	12	452

* The 12000g supernatant is always contaminated to some extent with the mitochondrial isoenzyme from sheep liver; for this reason the yields are calculated from the second step on the grounds that the mitochondrial isoenzyme is less stable to heat-treatment than the cytoplasmic one (Martínez-Carrión *et al.*, 1967).

† The specific activity of this supernatant was the highest of all preparations (average = 0.29 unit/mg of protein). However, this supernatant did not contain more than the usual amount of contaminating mitochondrial isoenzyme, as evidenced by high-voltage agar-gel electrophoresis and enzyme assays at pH6 and 7.4 (see Boyd, 1966).

Fig. 3. *Diagrams of the electrophoretic patterns of the purified enzyme in polyacrylamide discs*

Samples were loaded at O. (a) Fresh or aged sample, pH8.8; (b) fresh or aged sample, pH7.4; (c) aged sample repeatedly frozen and thawed, pH7.4 (for details see the Materials and Methods section).

A portion of purified enzyme from preparation C was incubated with 1 mM-dithiothreitol for 48 h at 4°C. Disc electrophoresis at pH7.4 after the incubation still yielded both α and β bands.

Isoelectric focusing in polyacrylamide gel

The technique of Awdeh *et al.* (1968) for isoelectric focusing in thin layers of polyacrylamide gel was used to examine the microheterogeneity of purified samples of the cytoplasmic isoenzyme from sheep liver. Polyacrylamide gel containing Ampholine carrier ampholytes in the range pH4–7 was used. Three samples of the purified sheep liver cytoplasmic isoenzyme and two samples of haemoglobin were loaded side by side on the surface of each gel. Plate 1A (a) shows the pattern obtained for the enzyme. Three main bands can be seen with pI values of 5.75 ± 0.04 ($n = 5$), 5.56 ± 0.02 ($n = 7$) and 5.35 ± 0.03 ($n = 6$). The differences in mean pI values between the two plates were 0.18 (pI5.75), 0.08 (pI5.56) and 0.00 (pI5.35). The splitting of each band that is apparent probably reflects the distribution of the glutamine/glutamic acid residues, since the components of each band are less than 0.06 pH unit apart (Awdeh *et al.*, 1968).

Absorption spectrum

The cytoplasmic aspartate aminotransferase holoenzyme from sheep liver has an absorption maximum at 362 nm in 0.15 M-NaCl–17.6 mM-sodium-potassium phosphate buffer, pH7.6, at 25°C. Similar results have been reported for the cytoplasmic aspartate aminotransferase from ox liver (Wada & Morino, 1964), pig heart (Martínez-Carrión *et al.*, 1967), chicken heart (Bertland & Kaplan, 1968) and rat brain (Magee & Phillips, 1971). According to Martínez-Carrión *et al.* (1967), the

Table 2. *Electrophoretic behaviour of the cytoplasmic isoenzyme of aspartate aminotransferase in polyacrylamide-gel discs and slab vertical polyacrylamide gels, with regard to the presence of subforms*

All the samples listed below were run on both types of polyacrylamide-gel electrophoresis. The system of Williams & Reisfeld (1964) was used at pH 7.4 and that of Davis (1964) at pH 8.8. The gels were sliced and one half was stained for enzymic activity and the other for protein. With the purified enzyme fractions (rows 3–6, 10 and 14) the protein bands found corresponded to enzyme-activity bands. The nomenclature of the bands is that of Martínez-Carrión *et al.* (1967). Preparations B and C were done in parallel and come from the same sheep liver; one-half of the organ was treated as usual (preparation B) and 0.2 mM-dithiothreitol (final concentration) was added throughout to the other half (preparation C). Purified enzyme refers to the enzyme fraction obtained after Sephadex G-200 chromatography. Fresh samples are those that were run immediately after the corresponding fraction was obtained. Aged samples are those from fractions stored at -20°C for a period of 1 month (for details see the Materials and Methods section).

	Sample	pH	Enzyme-activity bands
Preparation A	1 12000g supernatant, aged	7.4	α, β
	2 Heated supernatant, aged	7.4	α, β
	3 Purified enzyme, fresh	8.8	α
	4 Purified enzyme, aged	8.8	α
	5 Purified enzyme, aged	7.4	α, β
	6 Purified enzyme, aged, repeatedly thawed and refrozen	7.4	α, β, γ
Preparation B	7 12000g supernatant, fresh	7.4	α, β
	8 Heated supernatant, fresh	7.4	α, β, γ
	9 Pellet 64–72%-satd. $(\text{NH}_4)_2\text{SO}_4$, fresh	7.4	α, β
	10 Purified enzyme, fresh	7.4	α, β
Preparation C	11 12000g supernatant + dithiothreitol, fresh	7.4	α, β
	12 Heated supernatant + dithiothreitol, fresh	7.4	α, β, γ
	13 Pellet 64–72%-satd. $(\text{NH}_4)_2\text{SO}_4$ + dithiothreitol, fresh	7.4	α, β
	14 Purified enzyme + dithiothreitol, fresh	7.4	α, β

Fig. 4. *Sedimentation-velocity pattern for the purified enzyme*

The protein concentration was 8.6 mg/ml in 0.15 M-NaCl–17.6 mM-sodium–potassium phosphate buffer, pH 7.6; temperature = 21°C ; bar angle = 50° . The picture was taken 64 min after 260000g (r_{av} , 6.5 cm) was reached. Sedimentation was from left to right.

material absorbing at near 360 nm at this pH would be the α and/or β subforms of the enzyme, but not the γ subform.

The specific extinction coefficient ($E_{1\text{cm}}^{1\%}$) for the holoenzyme of sheep liver cytoplasmic aspartate aminotransferase at 362 nm is $14.1 \text{ ml} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ in 0.15 M-NaCl–17.6 mM-sodium–potassium phosphate

buffer, pH 7.6, at 25°C . The E_{280}/E_{362} ratio is 8.8 under the same conditions.

Ultracentrifuge studies

Sedimentation velocity. Velocity experiments showed a single component in each of the protein samples after Sephadex G-200 and G-100 chromatography (Fig. 4). The dependence of the sedimentation coefficient on protein concentration was determined at protein concentrations ranging from 0.21 to 0.86% (w/v) in 0.15 M-NaCl–17.6 mM-sodium–potassium phosphate buffer, pH 7.6, I 0.2, at 20 – 23°C . There was a negative slope of -0.489 in the plot of $s_{20,w}$ against concentration; the value of 5.69 S was determined for $s_{20,w}^0$ by extrapolation to zero concentration.

Sedimentation equilibrium. The high-speed technique of Yphantis (1964) was applied to 0.021–0.087% (w/v) solutions of the enzyme in 0.15 M-NaCl–17.6 mM-sodium–potassium phosphate buffer, pH 7.6 and I 0.2. Initially, the rotor speed was 16000 rev./min, giving 18500g (r_{av} , 6.5 cm) and the final photograph was taken 30 h after this speed was reached. The speed was then increased to 18000 rev./min to give 23500g and maintained for 13 h at 23°C . The value

Table 3. *Amino acid composition of cytoplasmic aspartate aminotransferase from sheep liver and pig heart*

The amino acid composition for the sheep liver cytoplasmic isoenzyme was derived from the average of 24–48 and 72h hydrolysates, with the exception of threonine, serine and tryptophan (see footnotes). Individual analyses were corrected to 100% recovery of DL-norleucine. For further details see the Results section. Data reported for the pig heart cytoplasmic isoenzyme (Doonan *et al.*, 1972b) are reproduced for comparison.

	Sheep liver cytoplasmic isoenzyme (mol of amino acid/mol of protein of mol.wt. 89000)		Pig heart cytoplasmic isoenzyme reported nearest integer (mol of amino acid/mol of protein of mol.wt. 92000)
	Calc. mol	Nearest integer	
Asx	78.1	78	88
Thr	43.7*	44	52
Ser	53.9*	54	54
Glx	70.8	71	82
Pro	53.6	54	46
Gly	62.6	63	54
Ala	57.5	58	62
Cys	11.3†	11	12
Val	49.4‡	49	58
Met	20.4	20	10
Ile	34.9‡	35	36
Leu	71.6	72	76
Tyr	24.4*	24	24
Phe	38.1	38	42
Lys	39.7	40	40
His	21.8	22	16
Arg	36.8	37	50
Trp		18§	22

* Extrapolated to zero time.

† As cysteic acid after performic acid oxidation.

‡ Extrapolation was not necessary since liberation was constant after 24h hydrolysis.

§ Determined separately.

of 0.732 was used for the partial specific volume of the enzyme, calculated from the amino acid composition (Cohn & Edsall, 1943).

All plots of sedimentation-equilibrium data were linear, thus indicating homogeneity within the experimental error.

The molecular weight obtained by this method for the cytoplasmic aspartate aminotransferase from sheep liver was 88900 ± 570 (mean $\pm$ S.E.M., $n = 9$).

Molecular weight and activity

Molecular activity. On the basis of a molecular weight of 89000 and a maximal specific activity of 217 units/mg, a molecular activity of 19313 molecules of oxaloacetate formed/min per molecule of enzyme, at 25°C and pH 7.4, was calculated.

Molecular weight by gel filtration. By using Sephadex G-200 equilibrated with 0.15M-NaCl–17.6mM-sodium–potassium phosphate buffer, pH 7.6, 4°C, a value of 86000 ± 1270 (mean $\pm$ S.E.M., $n = 4$) was

obtained for the approximate molecular weight of sheep liver cytoplasmic aspartate aminotransferase. A similar value (90000) has been reported for the pig heart cytoplasmic enzyme (Churchich & Farrelly, 1969) by the same method.

Amino acid composition. The mean values obtained for amino acid analysis are presented in Table 3. The values corresponding to different hydrolysis times as well as those for different cysteine and tryptophan determinations (see the Materials and Methods section for details) all agree within the experimental error (below 5%). Each analysis of the hydrolysates was corrected for 100% DL-norleucine recovery.

On the basis of a molecular weight of 89000 (see under 'Ultracentrifuge studies') the total number of amino acid residues of the sheep liver cytoplasmic aspartate aminotransferase was 787. This enzyme has a relatively higher content of proline, histidine and especially methionine and a lower content of arginine than the cytoplasmic isoenzyme from other sources.

Stability. At stage 1 of the purification, only 2% of

EXPLANATION OF PLATE 1A

Isoelectric focusing of sheep liver cytoplasmic isoenzyme of aspartate aminotransferase after Sephadex G-200 chromatography

The technique of Awdeh *et al.* (1968) was used (see the Materials and Methods section) with an Ampholine range of pH4–7. pI values shown are the means of five to seven determinations. (a) Sample from tube 35 after Sephadex G-200 chromatography; protein concentration 0.5 mg/ml, volume 200 μ l (loaded at cathode end). (b) Sample from tube 34 after Sephadex G-200 chromatography; protein concentration 0.83 mg/ml, volume 120 μ l (loaded at cathode end).

EXPLANATION OF PLATE 1B

Immunochemical reactions of sheep liver cytoplasmic isoenzyme: double diffusion in agar gel

Rabbit anti-(sheep liver cytoplasmic aspartate aminotransferase) antiserum was placed in the centre well, except in (f) in which the centre well contained anti-(pig heart cytoplasmic aspartate aminotransferase isoenzyme) antiserum. (c) Sheep liver cytoplasmic isoenzyme in the outer wells. (d) Sheep liver mitochondrial isoenzyme was placed in the outer wells (1), (3) and (5) and sheep liver cytoplasmic isoenzyme in the others. (e) Pig heart cytoplasmic isoenzyme was placed in the outer wells (2), (4) and (6) and sheep liver cytoplasmic isoenzyme in the alternate wells. (f) Pig heart cytoplasmic isoenzyme was placed in the outer wells (2), (4) and (6) and sheep liver cytoplasmic isoenzyme was in the others.

EXPLANATION OF PLATE 2

Immunoelectrophoresis of purified sheep liver cytoplasmic isoenzyme of aspartate aminotransferase

Electrophoretic and precipitation patterns from duplicate plates are shown. Rabbit antiserum to sheep liver cytoplasmic isoenzyme was placed in the slots. (a) pH 8.6; (b) pH 6.3 (for experimental details see the text).

the enzyme activity is lost per month on storage at -20°C , but the purified fraction loses up to 30–40% of its activity per month when stored at -20°C in dilute solutions (up to 2mg/ml).

Immunological reactivity

Double diffusion on agar gel of sheep liver cytoplasmic aspartate aminotransferase against rabbit anti-(sheep liver cytoplasmic enzyme) gives only one precipitation band (Plate 1B, c).

A similar result was obtained when the sheep liver cytoplasmic enzyme was tested by immunoelectrophoresis at pH 8.6 against the same antiserum. Plate 2(a) shows the precipitation pattern and the position of the protein after the electrophoretic migration of the duplicate. To increase the mobility of the enzyme, a sample was run at pH 6.3 towards the cathode (Plate 2b). Although the isoelectric point of the enzyme is under 6 (see below), it is displaced further in this case owing to endosmosis in the agar gel. Only one precipitation line was obtained again on immunoelectrophoresis corresponding to the position of the spot on a second agar plate run in parallel and stained for protein.

No cross-reaction was detected between the mitochondrial and the cytoplasmic aspartate aminotransferase isoenzymes from sheep liver when both were tested by double diffusion on agar gel against sheep liver cytoplasmic aspartate aminotransferase (Plate 1B, d).

Cross-reaction with spur formation between the cytoplasmic isoenzyme from sheep liver and the cytoplasmic pig heart enzyme was found by double diffusion on agar gel (Plate 1B, e and f), by using either antiserum to sheep liver cytoplasmic isoenzyme or the antiserum to pig heart cytoplasmic isoenzyme.

Number of molecules of pyridoxal phosphate bound per molecule of enzyme

The spectral shift of pyridoxal phosphate when released from the aspartate aminotransferase by alkali-treatment has been used to assess the number of coenzymes bound per molecule of enzyme (Martínez-Carrión *et al.*, 1965). A sample of purified sheep liver cytoplasmic enzyme that had been extensively dialysed against 20mM-sodium acetate buffer, pH 5.4, and containing 0.50mg of protein/ml, was treated with 0.1M-NaOH until pH 13 was reached (Martínez-Carrión *et al.*, 1965). The absorption spectra before and after the NaOH treatment were taken. The E_{390} obtained after the treatment with 0.1M-NaOH was 0.038 at 25°C . Since for pyridoxal phosphate ϵ_{390} in 0.1M-NaOH is $6.55 \times 10^{-3} \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Dawson *et al.*, 1969) and we have determined a molecular weight of 89900 for the sheep liver cytoplasmic isoenzyme of aspartate aminotransferase, a value of 2.1

may be calculated for the coenzyme/enzyme molar ratio.

Discussion

The sheep liver cytoplasmic isoenzyme of aspartate aminotransferase that we have prepared has a high degree of purity as shown both by its behaviour in the ultracentrifuge and by immunoelectrophoresis. A specific activity of 217 units/mg at 25°C (pH 7.4) has been obtained. Other liver cytoplasmic isoenzymes of aspartate aminotransferase that have been purified so far are those from ox liver (specific activity 187 units/mg) by Wada & Morino (1964) and from rat liver (specific activity 41.6 units/mg) by Boyd (1966). The value attained for the sheep liver isoenzyme resembles that reported for a highly purified preparation of rat brain cytoplasmic isoenzyme (239 units/mg) by Magee & Phillips (1971).

The value of 89000 that we have calculated for the molecular weight of the sheep liver isoenzyme from its behaviour in the ultracentrifuge is similar to the latest values reported for the corresponding pig heart isoenzyme (92000–93000) (Feliss & Martínez-Carrión, 1970; Polyanovsky *et al.*, 1972; Doonan *et al.*, 1972a). The value given for the molecular weight of the pig heart cytoplasmic isoenzyme shows good agreement with the estimate based on the total sequence data reported for the same isoenzyme by Ovchinnikov *et al.* (1973). The amino acid composition determined by us for the sheep liver cytoplasmic isoenzyme gives 787 amino acid residues per molecule (mol.wt. 89000), 37 residues less than in the pig heart cytoplasmic isoenzyme. The amino acid compositions are compared in Table 3.

The suggestion that the sheep liver and the pig heart cytoplasmic isoenzymes of aspartate aminotransferase possess structural similarities is strongly supported by the cross-reaction obtained by immunodiffusion on agar plates when a rabbit antiserum against purified sheep liver cytoplasmic isoenzyme was used. This result is confirmed by immunoelectrophoresis experiments that demonstrate a cross-reaction with a spur formation with the commercial pig heart cytoplasmic isoenzyme. There is then at least one antigenic determinant that is common to both the sheep liver and the pig heart cytoplasmic isoenzymes, as has also been observed for the cytoplasmic isoenzymes of aspartate aminotransferase from different organs in various mammals (Wada & Morino, 1964; Nisselbaum & Bodansky, 1966).

It is tempting to make similar comparisons of the cytoplasmic and mitochondrial isoenzymes from sheep liver, since a molecular weight of 86000 has been obtained for the mitochondrial isoenzyme of this organ in our laboratory as well as a number of 772 amino acid residues (tryptophan was not determined)

per enzyme molecule. Similar conclusions have been reported by Martínez-Carrión & Tiemeier (1967) for the pig heart isoenzymes, and Magee & Phillips (1971) have determined very similar molecular weights for the two isoenzymes from rat brain. Nevertheless we have found that the immunodiffusion in agar gel fails to show any cross-reaction between the cytoplasmic and the mitochondrial isoenzymes from sheep liver. The experiment, however, does not preclude the possibility that both isoenzymes from sheep liver may be structurally related in some way at non-antigenic sites.

The number of prosthetic groups per molecule of the sheep liver cytoplasmic isoenzyme puts an upper limit of 2 on the number of active centres per enzyme molecule. This number of prosthetic groups is common to all aspartate aminotransferases so far studied (Martínez-Carrión *et al.*, 1967; Martínez-Carrión & Tiemeier, 1967; Bertland & Kaplan, 1968; Scandurra & Cannella, 1972), and since the pig heart cytoplasmic isoenzyme appears to contain two identical subunits (Ovchinnikov *et al.*, 1973; Doonan *et al.*, 1972b), it seems justified to assume that the actual number of active centres in the sheep liver isoenzyme is 2. A catalytic centre activity of 9656min^{-1} may thus be calculated for the sheep liver cytoplasmic isoenzyme. From the data of Magee & Phillips (1971) a catalytic centre activity of 9440min^{-1} may be calculated for the rat brain cytoplasmic isoenzyme (mol.wt. 79000). It is unfortunate that no specific-activity data are available in the latest pig heart cytoplasmic isoenzyme preparations.

The appearance of multiple subforms on polyacrylamide-gel electrophoresis is a common feature of the cytoplasmic aspartate aminotransferases. The presence of up to three bands of protein or enzyme activity that we have found by using this technique with the cytoplasmic isoenzyme of sheep liver agrees with the pattern of three main protein bands that we have obtained by isoelectric focusing. We equate the bands with the subforms described by others and therefore propose pI values of 5.75 for the α subform, 5.56 for the β subform and 5.35 for the γ subform. Although the precise nature of the subform transition is obscure, both the α and the β subforms of the sheep liver cytoplasmic isoenzyme should either be present *in vivo* or be formed very quickly after the killing of the animal, since cytoplasmic fractions prepared under very mild conditions, and run immediately they were obtained, already showed the α and β subforms in the electrophoretic pattern. This finding confirms the results of Martínez-Carrión *et al.* (1967) with the pig heart cytoplasmic isoenzyme subforms. Arrio-Dupont *et al.* (1970) have reported that the appearance of subforms can be avoided with pig heart cytoplasmic isoenzyme by an early addition of 0.1 mM-dithiothreitol. However, we have been unable to inhibit the appearance of the α - β pattern by including 0.2 mM-dithio-

threitol in all media starting from the rinsing of the diced organ and subsequent homogenization, thus showing that at least for the sheep liver cytoplasmic isoenzyme, the α - β transition is not due to oxidation of thiol groups during the isolation and purification of the enzyme.

In our preparations an additional γ subform appears after the heating step or by aging of the samples, a fact that has also been observed for the pig heart (Banks *et al.*, 1968) and the rat brain isoenzymes (Magee & Phillips, 1971). It may well also represent in our case a transformation product of the α or β subforms (Banks *et al.*, 1968; Martínez-Carrión *et al.*, 1967). Since we have obtained consistently only one component in the ultracentrifuge and a single precipitation line in the immunoelectrophoresis experiments with the purified enzyme, it is most likely that the nature of the subform transition for the sheep liver enzyme is similar to that of the pig heart enzyme. In this last case isolated subforms have yielded identical molecular weights and amino acid compositions (Martínez-Carrión *et al.*, 1967). A current hypothesis is that the cytoplasmic isoenzyme subforms of aspartate aminotransferase are conformers with a different environment of charged groups, leading to a different net surface charge, since their presence is masked at pH 8.6 but is apparent at pH values near 7.4 (Banks *et al.*, 1968).

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References

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Arrio-Dupont, M., Cournil, I. & Duie, P. (1970) *FEBS Lett.* **11**, 144–146
- Awdeh, Z. L., Williamson, A. R. & Askonas, B. A. (1968) *Nature (London)* **219**, 66–67
- Banks, B. E. C., Doonan, S., Lawrence, A. J. & Vernon, C. A. (1968) *Eur. J. Biochem.* **5**, 528–539
- Beale, D. & Kent, C. M. (1968) *Proc. Technicon Amino Acid Colloq.* 6th p. 133.
- Bencze, W. L. & Schmid, K. (1957) *Anal. Chem.* **29**, 1193–1196
- Bertland, L. H. & Kaplan, N. O. (1968) *Biochemistry* **7**, 134–142
- Boyd, J. W. (1966) *Biochim. Biophys. Acta* **113**, 302–311
- Churchich, J. E. & Farrelly, J. G. (1969) *J. Biol. Chem.* **244**, 72–76
- Cohn, E. J. & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, pp. 370–381, Reinhold Publishing Corp., New York
- Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd edn., pp. 206–207, Clarendon Press, Oxford

- Decker, L. E. & Rau, E. M. (1963) *Proc. Soc. Exp. Biol. Med.* **112**, 144–149
- Doonan, S., Bossa, F., Barre, D., Carloni, M., Doonan, H. J., Hanford, R., Riva, F. & Walker, J. M. (1972a) *Abstr. Commun. FEBS Meet. 8th* 362
- Doonan, S., Doonan, H. J., Riva, F., Vernon, C. A., Walker, J. M., Bossa, F., Barra, D., Carloni, M. & Fasella, P. (1972b) *Biochem. J.* **130**, 443–452
- Feinstein, A. (1968) in *Chromatographic and Electrophoretic Techniques* (Smith, I., ed.), vol. 2, pp. 194–208, Pitman Press, Bath
- Feliss, N. & Martínez-Carrión, M. (1970) *Biochem. Biophys. Res. Commun.* **40**, 932–940
- Fleisher, G. A. (1960) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **19**, 6
- Fleisher, G. A., Potter, C. S. & Wakim, K. G. (1960) *Proc. Soc. Exp. Biol. Med.* **103**, 229–231
- Harpring, L. L. (1965) Ph.D. Thesis, University of Illinois, Urbana, Ill.
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197–198
- Hook, R. H. & Vestling, C. S. (1962) *Biochim. Biophys. Acta* **65**, 358–359
- Ivanov, V. I. & Karpeisky, M. YA. (1969) *Advan. Enzymol. Relat. Areas Mol. Biol.* **32**, 21–53
- Jenkins, T., Yphantis, D. & Sizer, I. (1959) *J. Biol. Chem.* **234**, 51–57
- Karmen, A. (1955) *J. Clin. Invest.* **34**, 131–133
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Magee, S. C. & Phillips, A. T. (1971) *Biochemistry* **10**, 3397–3405
- Marino, G., Greco, A. M., Scardi, V. & Zito, R. (1966) *Biochem. J.* **99**, 589–594
- Martínez-Carrión, M. & Tiemeier, D. (1967) *Biochemistry* **6**, 1715–1722
- Martínez-Carrión, M., Riva, F., Turano, C. & Fasella, P. (1965) *Biochem. Biophys. Res. Commun.* **20**, 206–211
- Martínez-Carrión, M., Turano, C., Chiancone, E., Bossa, F., Giartosio, A., Riva, F. & Fasella, P. (1967) *J. Biol. Chem.* **242**, 2397–2409
- Munn, E. A., Feinstein, A. & Greville, G. D. (1971) *Biochem. J.* **124**, 367–374
- Nisselbaum, J. S. & Bodansky, O. (1964) *J. Biol. Chem.* **239**, 4232–4236
- Nisselbaum, J. S. & Bodansky, O. (1966) *J. Biol. Chem.* **241**, 2661–2664
- Ouchterlony, O. (1967) *Handb. Exp. Immunol.* pp. 655–706
- Ovchinnikov, Yu. A., Egarov, C. A., Aldanova, N. A., Feigina, M. Yu., Lipkin, V. M., Abdulaev, N. G., Grishin, E. V., Kiselev, A. P., Modyanov, N. N., Braunstein, A. E., Polynovsky, O. L. & Nosikov, V. V. (1973) *FEBS Lett.* **29**, 31–34
- Polyanovsky, O. L. (1972) *Abstr. Commun. FEBS Meet. 8th*, 404
- Polyanovsky, O. L., Demidkina, T. V. & Egarov, C. A. (1972) *FEBS Lett.* **23**, 262–264
- Salaman, M. R. & Williamson, A. R. (1971) *Biochem. J.* **122**, 93–99
- Scandurra, R. & Cannella, C. (1972) *Eur. J. Biochem.* **26**, 196–206
- Wada, H. & Morino, Y. (1964) *Vitam. Horm. (New York)* **22**, 411–444
- Wieme, R. J. (1959) *Clin. Chim. Acta* **4**, 317–321
- Williams, D. E. & Reisfeld, R. A. (1964) *Ann. N.Y. Acad. Sci.* **121**, 373–381
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317