The Sequences of the Coenzyme-binding Peptide in the Cytoplasmic and the Mitochondrial Aspartate Aminotransferases from Sheep Liver

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The sequences of the coenzyme-binding peptide of both cytoplasmic and mitochondrial aspartate aminotransferases from sheep liver were determined. The holoenzymes were treated with NaBH_4 and digested with chymotrypsin; peptides containing bound pyridoxal phosphate were then isolated. One phosphopyridoxyl peptide was obtained from sheep liver cytoplasmic aspartate aminotransferase. Its sequence was Ser- N^{ϵ} -(phosphopyridoxyl)-Lys-Asn-Phe. This sequence is identical with that reported for the homologous peptide from pig heart cytoplasmic aspartate aminotransferase. Two phosphopyridoxyl peptides with different R_f values were isolated from the sheep liver mitochondrial isoenzyme. They had the same N -terminal amino acid and similar amino acid composition. The mitochondrial phosphopyridoxyl peptide of highest yield and purity had the sequence Ala- N^{ϵ} -(phosphopyridoxyl)-Lys-Asx-Met-Gly-Leu-Tyr. The sequence of the first four amino acids is identical with that already reported for the phosphopyridoxyl tetrapeptide from the pig heart mitochondrial isoenzyme. The heptapeptide found for the sheep liver mitochondrial isoenzyme closely resembles the corresponding sequence taken from the primary structure of the pig heart cytoplasmic aspartate aminotransferase.

The purification of sheep liver cytoplasmic aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) and the study of some of its structural properties have been reported in a previous paper (Campos-Cavieres & Munn, 1973). This enzyme has physicochemical properties and molecular weight similar to those of the well-known pig heart cytoplasmic aspartate aminotransferase, although their amino acid compositions are different (Feliss & Martínez-Carrión, 1970; Bossa *et al.*, 1973; Ovchinnikov *et al.*, 1973). They also present common antigenic determinants (Campos-Cavieres & Munn, 1973), similar to the cytoplasmic aspartate aminotransferases from other mammalian tissues (Wada & Morino, 1964; Nisselbaum & Bodansky, 1966; Shrawder & Martínez-Carrión, 1973). Several studies have been made aimed at identifying groups essential for the enzymic activity of pig heart cytoplasmic aspartate aminotransferase (Fasella & Turano, 1970; Polyanovsky *et al.*, 1973; Birchmeier *et al.*, 1973; John *et al.*, 1973; Peterson & Martínez-Carrión, 1970). The coenzyme, pyridoxal phosphate, has been shown to bind to the ϵ -amino group of a lysine residue of the pig heart cytoplasmic isoenzyme (Turano *et al.*, 1961; Hughes *et al.*, 1962) and the sequence of the corresponding chymotryptic tetrapeptide has been determined (Polyanovsky & Keil, 1963; Morino & Watanabe, 1969). The complete amino acid sequence of this enzyme has been published (Ovchinnikov *et al.*, 1973; Doonan *et al.*, 1974).

The mitochondrial isoenzyme of aspartate aminotransferase has been described in several tissues (Fleischer *et al.*, 1960; Wada & Morino, 1964; Nisselbaum & Bodansky, 1964; Boyd, 1966; Bertland & Kaplan, 1968; Magee & Phillips, 1971; Scandurra & Cannella, 1972; Shrawder & Martínez-Carrión, 1973) and has also been isolated and purified from sheep liver (I. G. Pashev & E. A. Munn, unpublished work). The mitochondrial and cytoplasmic isoenzymes of aspartate aminotransferase from a given tissue present differences in their amino acid composition, and physicochemical and kinetic properties; in addition, they show no antigenic determinants in common (Shrawder & Martínez-Carrión, 1973; Wada & Morino, 1964; Nisselbaum & Bodansky, 1966; Magee & Phillips, 1971; Campos-Cavieres & Munn, 1973). However, a mitochondrial isoenzyme is antigenically related to its counterpart from other tissues (Wada & Morino, 1964; Shrawder & Martínez-Carrión, 1973).

The aim of the present study was to establish whether the observed lack of common antigenic loci between the two sheep liver isoenzymes implies radical differences in the amino acid sequence of the active site. Since the identification of the coenzyme-binding peptide provides direct information on the location of the active site of these enzymes, the sequences of the phosphopyridoxyl-binding peptide for both the cytoplasmic and mitochondrial aspartate aminotransferases from sheep liver have been determined and compared with

those already reported for both pig heart isoenzymes (Morino & Watanabe, 1969).

Materials and Methods

Reagents and enzymes

Cytoplasmic aspartate aminotransferase from sheep liver was prepared by the method described by Campos-Cavieres & Munn (1973). Mitochondrial aspartate aminotransferase from sheep liver was obtained by modifications of the procedure of Boyd (1966) developed by I. G. Pashev & E. A. Munn (unpublished work). Mitochondria isolated by differential centrifugation of homogenates of sheep liver in 0.25M-sucrose were extracted with butanol in the presence of 50mM-sodium succinate buffer, pH6.0, prepared as described by Dawson *et al.* (1969). The aqueous phase was dialysed against 10mM-potassium-sodium phosphate buffer, pH7.4, to remove butanol. The buffer was prepared as described by Campos-Cavieres & Munn (1973). Sodium succinate buffer, pH6.0, pyridoxal phosphate and 2-oxoglutarate were added to final concentrations of 0.15M, 0.01mM and 1mM respectively and the solution was then heated at 70°C for 15min. The proteins remaining in solution were precipitated with $(\text{NH}_4)_2\text{SO}_4$ between 50 and 90% saturation, redissolved in 5mM-Tris-HCl buffer, pH8.5, and dialysed against the same buffer prepared as described by Dawson *et al.* (1969). The isoenzyme was further purified by DEAE-cellulose chromatography in this buffer (Boyd, 1966). Fractions with the highest specific activity were pooled and concentrated by ultrafiltration and finally purified by gel filtration on Sephadex G-100 equilibrated with 0.15M-NaCl-17.6mM-sodium-potassium phosphate buffer prepared as described by Campos-Cavieres & Munn (1973). Both enzymes were pure as shown by ultracentrifuge analysis and polyacrylamide-gel electrophoresis. The pig heart cytoplasmic aspartate aminotransferase was a suspension of 10.7mg of protein/ml in 2.9M- $(\text{NH}_4)_2\text{SO}_4$ solution, specific activity 174units/mg, purchased from Whatman Biochemicals Ltd., Springfield Mill, Maidstone, Kent, U.K. One unit of enzyme activity is the amount of enzyme that catalyses the formation of 1 μ mol of oxaloacetate/min at 25°C in the assay conditions described by Campos-Cavieres & Munn (1973). Chymotrypsin was a crystallized salt-free preparation from Worthington Biochemical Corp., Freehold, N.J., U.S.A. *N*^ε-(Phosphopyridoxyl)lysine was prepared as described by Dempsey & Christensen (1962), but by using phosphopyridoxal instead of pyridoxal and protecting the α -amino group of DL-lysine by formation of the Cu^{2+} complex, as described by Neuberger & Sanger (1944). Iodoacetic acid was purified by extracting an

aqueous solution (2.3g/10ml) with 1ml of chloroform. Dithiothreitol, amino acid standard solution and ninhydrin were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Polyamide layer sheets (measuring 15cm $\times$ 15cm), dansyl chloride (1-dimethylaminonaphthalene-5-sulphonyl chloride), and trifluoroacetic acid were obtained from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K. NaBH_4 and 2-mercaptoethanol were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. HCl (microanalytical reagent), phenyl isothiocyanate and pyridine were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. The last two compounds were redistilled before use. All other chemicals were analytical grade.

Reduction of the aldimine bond of pyridoxal phosphate with the ϵ -amino group of lysine

These and all consecutive operations were performed with the minimum possible exposure to light. About 20mg of each of the three aspartate aminotransferase isoenzymes used was dissolved in 0.1M-sodium acetate buffer, pH5.35, at 4°C to give a final concentration of 10mg of protein/ml. Protein was determined by the method of Lowry *et al.* (1951). NaBH_4 (1mg/ml final concn.) plus one drop of octanol were added. Mixing was carried out at 4°C in a centrifuge tube, which was then immediately spun at low speed, for 15min in a bench centrifuge. The shift of the absorption maximum from 430 to 330nm was followed with the Pye-Unicam SP.800 spectrophotometer, to assess the completion of the reduction. Excess of reagents was removed by dialysis against glass-distilled water (adjusted to pH7 with 2M-Tris-HCl at 4°C). The sample was then dried in a desiccator. The molar extinction coefficient at 325nm ($1.015 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) reported for *N*^ε-(phosphopyridoxyl)-lysine, was used to calculate the amount of reduced enzyme (Anai *et al.*, 1973). Only 1% of the original enzymic activity remained after the above treatment was completed.

Enzyme alkylation and chymotryptic digestion

In a typical preparation, 10mg of dried enzyme obtained as described above, was dissolved in 1ml of 0.10M-Tris-HCl-6M-guanidine-2mM-EDTA, pH8.1. Dithiothreitol solution (45 μ l at 10mg/ml) was added and the tube was flushed with N_2 . The reduction was allowed to proceed for 1h at 37°C. Then 90 μ l of 1.25M-iodoacetic acid was added and the tube was flushed again with N_2 ; the mixture was kept at 37°C for 1.5h and the reaction stopped by the addition of two drops of 2-mercaptoethanol. The alkylated protein was dialysed exhaustively at 4°C

against 1% (w/v) NH_4HCO_3 , pH 8.0. Chymotrypsin solution was then added in a small volume (e.g., 30 μl into 3 ml) to the dialysed preparation. The ratio chymotrypsin/aspartate aminotransferase was 1:50 (w/w). The digestion was carried out for 20 h at 37°C, in the dark and with occasional stirring. The reaction was stopped by freeze-drying the clear solution obtained. The NH_4HCO_3 present in the mixture was eliminated by drying the samples under vacuum, dissolving in glass-distilled water and repeating the procedure as many times as necessary.

High-voltage paper electrophoresis

This was performed on Whatman 3MM paper with an apparatus similar to that described by Michl (1951). The buffer systems and coolants both at pH 6.5 and 3.5 were as described by Ambler (1963). In parallel with the samples, a mixture of standards with the following composition was run: lysine, arginine, histidine, alanyl-glycine, glycine, valine, ϵ -Dnp-lysine, glutamic acid, aspartic acid, taurine, cysteic acid and Xylene Cyanol FF (plus a spot of Red Pentel, for runs at pH 6.5). N^{ϵ} -(Phosphopyridoxyl)lysine (2 mg/ml) was used as a standard, in separate spots (10 μl) at both sides of the sample.

Paper chromatography

Descending chromatography was carried out on Whatman 3MM paper with butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) as the solvent. A mixture of standards with the same composition as used for high-voltage electrophoresis at pH 6.5, as well as N^{ϵ} -(phosphopyridoxyl)lysine, were used in parallel. The paper was equilibrated in the tank for 4 h, before the run. The chromatography was ended when the red marker reached the lower end of the paper.

Identification and isolation of phosphopyridoxyl peptides

N^{ϵ} -(Phosphopyridoxyl)lysine standard and phosphopyridoxyl peptides were detected by their characteristic intense blue fluorescence under a u.v. lamp.

(a) *Mitochondrial aspartate aminotransferase from sheep liver.* The reduced and alkylated enzyme was digested with chymotrypsin as described above. The dried, salt-free digest was dissolved in glass-distilled water and loaded on Whatman 3MM paper (1.5 mg of digest/cm). High-voltage electrophoresis at pH 6.5 was performed at 53 V/cm, toward the anode and for 140 min. After the run was completed, the strips showing intense blue fluorescence were cut out, sewn on to a new sheet of Whatman 3MM paper and subjected to descending chromatography for 19 h.

(b) *Cytoplasmic aspartate aminotransferase from pig heart or sheep liver.* The reduced and alkylated enzyme was digested with chymotrypsin as described above. The chymotryptic digest was dissolved in glass-distilled water and applied to the Whatman 3MM paper (1.5 mg of digest/cm). It was then subjected to high-voltage electrophoresis at pH 3.5 at 53 V/cm for 80 min. The band containing the phosphopyridoxyl peptide was cut out and sewn on to a new sheet of 3MM paper, and subjected to descending chromatography for 19 h. The blue fluorescent strip was then cut out from the chromatogram and further purified by high-voltage electrophoresis at pH 6.5 on 3MM paper for 140 min.

The phosphopyridoxyl peptides were always eluted by using constant-boiling HCl (containing 1 mg of phenol/ml) for samples for amino acid analysis, and with 1% acetic acid for sequence determination. The yield was expressed as nmol of pyridoxyl peptide obtained (as assessed by the amino acid composition) from the initial nmol of N^{ϵ} -(phosphopyridoxyl)lysine present in the reduced enzyme.

The strip containing the standards of each run was cut and stained by dipping the paper in cadmium-ninhydrin reagent [85 ml of 1% (w/v) ninhydrin in acetone plus 15 ml of a solution of 6 g of cadmium acetate in 30% (v/v) acetic acid] (Heilmann *et al.*, 1957).

The electrophoretic mobilities of the peptides at pH 6.5 were measured relative to aspartic acid (Offord, 1966), and at pH 3.5, relative to the mobility of alanyl-glycine in the same run, taking as mobility zero the mid-point of the taurine spot (Milstein & Deverson, 1971).

Amino acid analysis

Peptide samples were hydrolysed with constant-boiling HCl (containing 1 mg of phenol/ml) in evacuated sealed tubes for 19 h at 105°C. Quantitative amino acid analyses were carried out with an automatic Locarte amino acid analyser, by using a single column (0.45 cm $\times$ 23.5 cm) and a stepwise buffer system (0.1 M-citric acid-0.2 M-NaOH, adjusted to pH 3.25 with conc. HCl; 0.1 M-citric acid-0.2 M-NaOH, adjusted to pH 4.25 with conc. HCl; and 0.33 M-citric acid-1.0 M-NaOH, adjusted to pH 6.65 with conc. HCl) as described by Spackman *et al.* (1958).

The N^{ϵ} -(pyridoxyl)lysine was found in the amino acid chromatogram as a separate peak between NH_3 and arginine. Under the conditions used, this peak appeared after 230 min, NH_3 coming out at 210 min and arginine at 275 min. The amount of N^{ϵ} -(pyridoxyl)lysine was calculated by using the value of the constant for leucine (Morino & Watanabe, 1969).

Edman degradation and N-terminal amino acid analysis

The procedure described by Gray (1967) as sequential degradation plus dansylation was used. The Dns-amino acids were identified by t.l.c. on polyamide sheets (7.5 cm × 7.5 cm) as described by Gray (1972). Every sample was run together with Dns-amino acid standards prepared as described by Boulton & Bush (1964).

Results

As only small amounts (20–25 mg) of purified sheep liver isoenzymes were obtained, appropriate procedures were devised for the purification of the coenzyme-binding peptide from pig heart cytoplasmic aspartate aminotransferase, since this enzyme is commercially available and the sequence of its phosphopyridoxyl peptide has been established (Morino & Watanabe, 1969). The pig heart cytoplasmic holoenzyme was treated with NaBH₄, alkylated and then digested with chymotrypsin as described in the Materials and Methods section. During the purification, the chymotryptic phosphopyridoxyl peptide was followed by its characteristic blue fluorescence [compared with that of N^ε-(phosphopyridoxyl)lysine standard]. A three-step purification procedure was chosen: high-voltage electrophoresis on paper at pH 3.5, descending chromatography and then high-voltage electrophoresis on paper at pH 6.5 (see the Materials and Methods section). A purified peptide was then obtained (Table 1), which had the same amino acid composition as that

reported by Morino & Watanabe (1969). The amino acid sequence determined by the dansyl-Edman method is shown in Fig. 1, and is identical with that reported by Morino & Watanabe (1969). Two-step purification procedures, although yielding impure peptides for the cytoplasmic isoenzyme, were adequate for the mitochondrial isoenzyme (where high-voltage electrophoresis at pH 6.5 and chromatography was used).

Sheep liver cytoplasmic aspartate aminotransferase

The phosphopyridoxyl peptide from sheep liver cytoplasmic aspartate aminotransferase was obtained and purified by the same procedure as described above for the pig heart cytoplasmic isoenzyme. Only one band with the characteristic blue fluorescence was found throughout. The amino acid composition of the purified peptide from sheep liver cytoplasmic isoenzyme is essentially the same as that found for the homologous peptide from the pig heart cytoplasmic isoenzyme, as shown in Table 1. The lysine found must have arisen from partial decomposition of phosphopyridoxyl-lysine during acid hydrolysis (Morino & Watanabe, 1969). As expected, phosphopyridoxyl-lysine did not appear after acid hydrolysis, since the phosphate group is released by this treatment. However, there were reasons to believe that N^ε-(phosphopyridoxyl)lysine was present as such in the coenzyme-binding peptide, as follows. (a) The presence of N^ε-(pyridoxyl)lysine and lysine in the amino acid chromatogram of the peptide (see Table 1). The same

Table 1. Amino acid composition of the purified chymotryptic phosphopyridoxyl-peptides from sheep liver cytoplasmic and mitochondrial aspartate aminotransferases

The amino acid composition obtained for the homologous peptide from pig heart cytoplasmic aspartate aminotransferase, is given for comparison. In 'purification procedure' HVE stands for high-voltage paper electrophoresis and BAWP refers to descending paper chromatography in butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.). (For details see the Materials and Methods section.)

Peptide from	No. of residues/molecule of peptide		
	Pig heart cytoplasmic isoenzyme	Sheep liver cytoplasmic isoenzyme	Sheep liver mitochondrial isoenzyme
Purification procedure	HVE pH 3.5, BAWP and HVE pH 6.5	HVE pH 3.5, BAWP and HVE pH 6.5	HVE pH 6.5 and BAWP
Amino acid			
Asp	1.0	1.1	1.1
Ser	0.9	0.9	—
Gly	—	—	1.1
Ala	—	—	0.9
Met	—	—	0.2
Leu	—	—	0.9
Tyr	—	—	0.5
Phe	1.1	0.9	—
Lys	0.1	0.3	0.3
N ^ε -Pxy-Lys	1.0	0.7	0.3

Table 2. R_F values and relative electrophoretic mobilities of N^{ϵ} -(phosphopyridoxyl)lysine and chymotryptic phosphopyridoxyl-peptides obtained from both sheep liver and pig heart cytoplasmic aspartate aminotransferases

N^{ϵ} -(Phosphopyridoxyl)lysine (N^{ϵ} -P-Pxy-lysine) was obtained as described in the Materials and Methods section. Electrophoretic mobilities at pH 3.5 were expressed relative to the mobility of alanylglycine in the same run, taking the midpoint of the taurine spot as zero mobility (Milstein & Deverson, 1971). Electrophoretic mobilities at pH 6.5 were measured relative to aspartic acid (Offord, 1966).

	N^{ϵ} -P-Pxy-lysine peptide		
	N^{ϵ} -P-Pxy-lysine	Sheep liver	Pig heart
Mobility at pH 3.5	0.66	0.67	0.67
Mobility at pH 6.5	0.36	0.27	0.28
R_F on chromatography	0.16	0.34	0.34

products were obtained when a standard of N^{ϵ} -(phosphopyridoxyl)lysine was acid-hydrolysed and analysed in the amino acid analyser. (b) In the coenzyme-binding peptide, as well as in the N^{ϵ} -(phosphopyridoxyl)lysine standard, the charge at pH 6.5 is given by the phosphate. Therefore, by applying the equation that relates mobility to molecular weight (Offord, 1966) to both compounds, peptide and standard, the mobility of the coenzyme-binding peptide can be calculated. The value thus obtained is very close to the experimental one at the same pH (Table 2, column 2).

Fig. 1 shows that the sequence determined for the coenzyme-binding peptide from the sheep liver cytoplasmic aspartate aminotransferase is identical with that of the homologous peptide from the pig heart cytoplasmic isoenzyme. The mobility of this peptide at pH 6.5 (Table 2) indicates that the aspartic acid which is formed on acid hydrolysis was originally present as asparagine (Offord, 1966). Further, the relative mobilities and R_F values of this peptide from both cytoplasmic isoenzymes are identical, and Morino & Watanabe (1969) had established the presence of asparagine in the pig heart cytoplasmic coenzyme-binding peptide, by digestion with leucine aminopeptidase.

Sheep liver mitochondrial aspartate aminotransferase

The chymotryptic digest of the reduced and alkylated sheep liver mitochondrial isoenzyme yielded three phosphopyridoxyl peptides on high-voltage electrophoresis at pH 6.5, which were designated peptides m1, m2 and m3 according to their increasing relative mobilities (0.15, 0.29 and 0.46). The two peptides obtained in higher yields, m1 and m3, were further purified by descending chromatography. Peptide m1 yielded two phosphopyridoxyl peptides,

Fig. 1. Sequence of the chymotryptic phosphopyridoxyl peptides from sheep liver aspartate aminotransferase isoenzymes

Sequences of the phosphopyridoxyl peptides of both sheep liver isoenzymes, as determined in the present study. For comparison the sequences of the homologous peptides from pig heart cytoplasmic aspartate aminotransferase (Morino & Watanabe, 1969; also this paper) and from the pig heart mitochondrial isoenzyme (Morino & Watanabe, 1969) are also included. Pyridoxal phosphate binds the N^{ϵ} -amino group of the lysine in all these peptides. The sign $\rightarrow$ underneath a residue denotes that it has been identified by the dansyl-Edman procedure.

Sheep liver	Cytoplasmic	Ser-Lys-Asn-Phe $\rightarrow \rightarrow \rightarrow \rightarrow$
	Mitochondrial	Ala-Lys-Asx-Met-Gly-Leu-Tyr $\rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow$
Pig heart	Cytoplasmic	Ser-Lys-Asn-Phe $\rightarrow \rightarrow \rightarrow \rightarrow$
	Mitochondrial	Ala-Lys-Asn-Met $\rightarrow \rightarrow \rightarrow \rightarrow$

m1_a (R_F = 0.31) and m1_b (R_F = 0.51), whereas peptide m3 gave only one (R_F = 0.31). The yield of these peptides, calculated as described in the Materials and Methods section, was 13.3% for m1_a, 12.8% for m1_b and 6.4% for m3. These three peptides showed essentially the same amino acid composition, except for the methionine content, which was 0.4 in m1_b and 0.2 in the other two. The amino acid composition of peptide m1_a from sheep liver mitochondrial aspartate aminotransferase is shown in Table 1. This amino acid composition showed very low values for methionine and N^{ϵ} -(pyridoxyl)lysine. The low value for methionine is probably due to oxidation, since small amounts (less than 0.2 residue/molecule) of methionine sulfoxide and methionine sulphone were present. The amount of N^{ϵ} -(pyridoxyl)lysine is lower in this peptide than in the two cytoplasmic ones; it may be that the oxidized methionine reacts with N^{ϵ} -(phosphopyridoxyl)lysine during acid hydrolysis. Tyrosine, which is also low, is known to be usually partially destroyed by acid hydrolysis. It may also be that peptide m1_a is a mixture of two peptides, one of which is one residue (Tyr) longer than the other (see the sequence in Fig. 1). This is likely, considering the specificity of chymotrypsin (Kasper, 1970).

The N -terminal residue of the peptides m1_a and m1_b, determined by the dansyl procedure, was alanine. Of the two peptides obtained in greatest yield, m1_a and m1_b, the former was the purest. Its sequence was determined by the dansyl-Edman procedure (Fig. 1); the presence of tyrosine and methionine was confirmed. Methionine was the fourth residue, and this was confirmed by repeating the degradation. This residue appeared mainly as Dns-MetSO₂ (Harris, 1967).

- Birchmeier, W., Wilson, K. J. & Christen, P. (1973) *J. Biol. Chem.* **248**, 1751-1759
- Bossa, F., Barra, D., Carloni, M., Fasella, P., Riva, F., Doonan, S., Doonan, H. J., Hanford, R., Vernon, C. A. & Walker, J. M. (1973) *Biochem. J.* **133**, 805-819
- Boulton, A. A. & Bush, I. E. (1964) *Biochem. J.* **92**, 11P-12P
- Boyd, J. W. (1966) *Biochim. Biophys. Acta* **113**, 302-311
- Campos-Cavieres, M. & Munn, E. A. (1973) *Biochem. J.* **135**, 683-693
- Dayhoff, M. O. & Eck, R. V. (1969) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., ed.), vol. 4, pp. 85-87, National Biomedical Research Foundation, Washington, D.C.
- Dayhoff, M. O., Barker, W. C. & Hardman, J. K. (1972) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., ed.), vol. 5, pp. 53-66, National Biomedical Research Foundation, Washington, D.C.
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd edn., pp. 475-508, Clarendon Press, Oxford
- Dempsey, W. B. & Christensen, H. N. (1962) *J. Biol. Chem.* **237**, 1113-1120
- Doonan, S., Doonan, H. J., Hanford, R., Vernon, C. A., Walker, J. M., Bossa, F., Barra, D., Carloni, M., Fasella, P., Riva, F. & Walton, P. L. (1974) *FEBS Lett.* **38**, 229-233.
- Fasella, P. & Turano, C. (1970) *Vitam. Horm. (New York)* **28**, 157-194
- Feliss, N. & Martínez-Carrión, M. (1970) *Biochem. Biophys. Res. Commun.* **40**, 932-940
- Fleischer, G. A., Potter, C. S., Wakim, K. G., Pankow, M. & Osborne, D. (1960) *Proc. Soc. Exp. Biol. Med.* **103**, 229-231
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 469-475
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121-138
- Harris, J. I. (1967) *Methods Enzymol.* **11**, 390-398
- Heilmann, J., Barrolier, J. & Watzke, E. (1957) *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 219-220
- Hughes, R. C., Jenkins, W. T. & Fischer, E. H. (1962) *Proc. Nat. Acad. Sci. U.S.A.* **48**, 1615-1618
- John, R., Bossa, F., Barra, D. & Fasella, P. (1973) *Biochem. Soc. Trans.* **1**, 862-864
- Kasper, C. B. (1970) in *Protein Sequence Determination* (Needleman, S. B., ed.), pp. 137-184, Chapman and Hall, London
- 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
- Michl, H. (1951) *Monatsh. Chem.* **82**, 489-493
- Milstein, C. P. & Deverson, E. V. (1971) *Biochem. J.* **123**, 945-958
- Morino, Y. & Watanabe, T. (1969) *Biochemistry* **8**, 3412-3417
- Neuberger, A. & Sanger, F. (1944) *Biochem. J.* **37**, 515-518
- Nisselbaum, J. S. & Bodansky, O. (1964) *J. Biol. Chem.* **239**, 4232-4236
- Nisselbaum, J. S. & Bodansky, O. (1966) *J. Biol. Chem.* **241**, 2661-2664
- Offord, R. E. (1966) *Nature (London)* **211**, 591-593
- Ovchinnikov, Yu. A., Egorov, 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., Polyanovsky, O. L. & Nosikov, V. V. (1973) *FEBS Lett.* **29**, 31-34
- Peterson, D. L. & Martínez-Carrión, M. (1970) *J. Biol. Chem.* **245**, 806-813
- Polyanovsky, O. L. & Keil, B. A. (1963) *Biokhimiya* **28**, 306-311
- Polyanovsky, O. L., Timofeev, V. P., Shaduri, M. I., Misharin, A. Yu. & Volkenstein, M. V. (1973) *Biochim. Biophys. Acta* **327**, 57-65
- Scandurra, R. & Cannella, C. (1972) *Eur. J. Biochem.* **26**, 196-206
- Shrawder, E. J. & Martínez-Carrión, M. (1973) *J. Biol. Chem.* **248**, 2140-2146
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190-1206
- Torchinsky, Yu. M., Kochkina, V. M. & Sajgó, M. (1974) *Acta Biochim. Biophys.* **9**, 213-216
- Turano, C., Fasella, P., Vecchini, P. & Giartosio, A. (1961) *Atti Accad. Naz. Lincei Cl. Sci. Fis. Mat. Natur. Rend.* **30**, 532-537
- Wada, H. & Morino, Y. (1964) *Vitam. Horm. (New York)* **22**, 411-444