Isolation and Characterization of a Hydroxyproline-Containing Protein from Soluble Extracts of the Leaves of Sandal (*Santalum album* L.)

By ULIYAR V. MANI and AMURTUR N. RADHAKRISHNAN

Wellcome Research Unit, Christian Medical College Hospital, Vellore-632004, Tamil Nadu, India

(Received 28 December 1973)

1. A hydroxyproline-containing protein was isolated from the soluble fraction of sandal leaves (*Santalum album* L.) and the purified protein was homogeneous by disc electrophoresis. 2. It is a glycoprotein containing 16% carbohydrate, the components of which were mainly arabinose, with only small amounts (about 5%) of galactose. The principal amino acids were glutamic acid, aspartic acid, glycine, alanine, arginine, lysine, proline and hydroxyproline, which together comprised 60% of the total. The number of acidic amino acids exceeds the number of basic amino acids. By Sephadex gel filtration, the approximate molecular weight was found to be about 63000. The ratio of residues of hydroxyproline to those of arabinose was 1:2. 3. The native protein is resistant to the action of several proteolytic enzymes. After partial hydrolysis with 0.1 M-HCl, the protein became susceptible to attack by Pronase but remained resistant to collagenase.

The cell wall of some plants contains a structural protein component designated 'extensin' which is characterized by its high hydroxyproline content (Lampert, 1965). The glycoprotein nature of this protein and the fact that hydroxyproline is covalently bonded to arabinose through an *O*-glycosidic linkage was established by Lampert (1967, 1969). The presence of hydroxyproline-rich material in the cytoplasm has been reported (Steward & Pollard, 1958; Olson, 1964; Lampert, 1965; Cleland, 1968). From kinetic studies, Cleland (1968) suggested that a portion of the hydroxyproline-containing protein of the cytoplasm may be the precursor of wall protein. More direct evidence for the role of the cytoplasmic fraction as a precursor of the wall protein has come from the work of Chrispeels (1970). Brisk & Chrispeels (1972) isolated hydroxyproline-containing protein fractions from membranous organelles of the cytoplasm and from the cell-wall material of carrots in an electrophoretically homogeneous form.

Previous studies from our laboratory (Kuttan & Radhakrishnan, 1970) showed that *trans*-4-hydroxy-L-proline in sandal leaves was associated mainly with two fractions, a 'wall' fraction and a 'soluble' fraction, roughly in equal proportions. Studies of incorporation of [^{14}C]proline into the cytoplasmic and wall fractions revealed that the label in the soluble fraction was not readily incorporated into the wall fraction. In the present study the procedure for the isolation of one of the hydroxyproline-containing proteins from the soluble fraction of sandal leaves in an electrophoretically homogeneous state is described. This protein is shown to be a glycoprotein with arabinose as its major carbohydrate moiety.

Materials and Methods

Chemicals

All chemicals used were of analytical-reagent or the best commercially available grade: L-proline and *trans*-hydroxy-L-proline (Calbiochem, San Diego, Calif., U.S.A.); 4-dimethylaminobenzaldehyde (Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.); Coomassie Brilliant Blue, galactose oxidase, collagenase (type III, from *Clostridium histolyticum*, protease-free), crystalline trypsin, crystalline α -chymotrypsin, Pronase (type VI, from *Streptomyces griseus*) (all from Sigma Chemical Co., St. Louis, Mo., U.S.A.); marker proteins, bovine serum albumin, myoglobin, cytochrome c, ovalbumin (Schwarz-Mann, Orangeburg, N.Y., U.S.A.); DEAE-Sephadex A-50, Blue Dextran 2000, Sephadex G-100 and G-50 (Pharmacia, Uppsala, Sweden). $(\text{NH}_4)_2\text{SO}_4$ was purchased from Sarabhai Chemicals, Baroda, India. Alumina C₁ was prepared by the procedure of Willstatter & Kraut (1923). Amidoschwartz 10B was from E. Merck, Darmstadt, Germany.

Assay methods

The following determinations were carried out as indicated: hydroxyproline (Neuman & Logan, 1950), proline (Schweet, 1954), protein (Lowry *et al.*, 1951), total sugars (Dubois *et al.*, 1956), arabinose (Roe & Rice, 1948), amino sugars (Gatt & Berman, 1966), sialic acid (Warren, 1959), galactose by the galactose oxidase method (Worthington Enzyme Manual, 1972) and uronic acids by Dische's carbazole method (1947), modified by Bitter & Muir (1962).

Hydrolytic procedures for quantitative analysis

(a) *Hydroxyproline and proline.* The samples for the determination of hydroxyproline and proline were hydrolysed in sealed tubes with 6M-HCl at 140°C for 3h unless otherwise stated. The coloured samples were treated with activated charcoal to remove the interfering materials before chemical determination. Charcoal had no effect on the recovery of both *cis* and *trans* isomers of hydroxyproline (Kuttan & Radhakrishnan, 1970). Full recoveries were obtained even at 12mg of charcoal/20µg of hydroxyproline (U. V. Mani & A. N. Radhakrishnan, unpublished work).

(b) *Amino acids.* For amino acid analysis the protein was hydrolysed with 6M-HCl at 140°C for 3h in the presence of indol-3-ylpropionic acid (Gruen & Nicholls, 1972). The amino acid analysis was carried out by the method of Benson & Patterson (1965) in a two-column automatic amino acid analyser (locally assembled, but similar to Beckman model 120B) with Beckman custom research resins PA 28 and PA 35. Hydrolysis of the sandal protein at 110°C for 24h resulted in poor recoveries of a number of amino acids.

(c) *Individual sugars.* For sugar analysis the protein was hydrolysed at 140°C for 3h in sealed glass ampoules with 0.05M-H₂SO₄. The hydrolysate was passed successively through small columns (0.8cm×8cm, bed vol. 5ml) of Dowex 50 (×8; H⁺ form; 200–400 mesh) and Dowex 1 (acetate form; 200–400 mesh) and 5 bed volumes of water washings were collected, concentrated and made up to a known volume. Total sugars were determined in this fraction as given before. The neutral sugars were separated by paper chromatography as described by Mukerjee & Sri Ram (1964). After detection of the sugars in guide strips by spraying with alkaline AgNO₃ or aniline-phthalate, arabinose and galactose in the paper eluates were determined as given above. In the hydrolytic procedure used above there was no significant destruction of sugars and the overall recovery of the individual sugars, arabinose and galactose, in the subsequent steps was approx. 90%. These values were obtained by using as reference the value for total sugars (Dubois *et al.*, 1956), with glucose as standard.

(d) *Partial acid hydrolysis of the protein.* Mild acid hydrolysis of the purified sample was carried out for 2h at 90°C with 0.1M-HCl. The sample after neutralization with 0.1M-NaOH was subjected to gel filtration on a Sephadex G-50 column (1.2cm×90cm; bed vol. 135ml). About 90% of the sugars was removed by this procedure without a significant effect on the protein, but for a small hump in the inner volume. Over 90% of the original hydroxyproline was present in these two Sephadex fractions. Also, there was no detectable hydroxyproline in the retarded fractions containing most of the sugars.

(e) *Hydroxyproline glycosides from the glycoprotein.*

The glycoprotein was hydrolysed in sealed ampoules for 18h at 110°C with saturated Ba(OH)₂. The hydrolysate was carefully neutralized with 0.1M-H₂SO₄ and the insoluble salt was removed by centrifugation. The hydroxyproline glycosides in the clear supernatant were separated by ion-exchange chromatography as described by Lampert (1967) on a column (1.2cm×20cm; bed. vol. 25ml) of the sulphonic acid resin Dowex 50 (X2; H⁺ form). The hydroxyproline glycosides and other materials were eluted with a 0–0.2M-HCl gradient (Varigrad device, Technicon Chromatography Corp., Chauncy, N.Y., U.S.A.) and fractions (5ml) were collected mechanically. The fractions were analysed for sugars directly and for hydroxyproline after hydrolysis with 6M-HCl as described above.

(f) *Treatment with proteolytic enzymes and collagenase.* Proteolytic digestion of the protein with trypsin, α-chymotrypsin and Pronase was carried out by the method of Cogoli & Semenza (1973) with a protein/enzyme ratio of 10:1 (w/w). With Pronase, incubation with intact protein and partially hydrolysed protein was carried out for 24h and the products then subjected to gel filtration on a Sephadex G-50 column (90cm×1.2cm; bed vol. 135ml). Treatment with collagenase of the intact protein or of the partially hydrolysed protein was performed in 0.05M-Tris-HCl buffer containing 0.36mM-CaCl₂, pH7.5, with a protein/collagenase ratio of 20:1 (w/w) for 24h and the products were then subjected to gel filtration on a Sephadex G-50 column of the same dimensions as given above.

Results

Purification of a hydroxyproline-containing protein from the soluble extracts of sandal leaves

Step 1: crude extract. Freshly collected mature leaves from sandal were freed of the midribs and cut into small pieces. All operations hereafter were carried out between 0° and 5°C unless otherwise stated. A 30% homogenate of the cut leaves (400g) in 0.02M-Tris-HCl buffer, pH7.4, containing isotonic (1.15%) KCl was prepared in a Waring Blendor. The homogenate was centrifuged first at 1000g for 5min to remove the cell walls, nuclei and cell debris, and then at 20000g for 20min, and the sediment discarded. The clear brown supernatant fraction (1200ml) was concentrated by precipitation with solid (NH₄)₂SO₄ (756g; 95% satn.) and stirring was continued for 30min after the addition of (NH₄)₂SO₄. The mixture was then centrifuged at 10000g for 30min, the pellet dissolved in 0.02M-Tris-HCl buffer, pH7.4, and dialysed against the same buffer for 48h. The precipitation step and the dialysis also helped to remove all the free *cis*-4-hydroxy-

Table 1. Purification of hydroxyproline-containing protein from soluble fraction of sandal leaves

For the designation of the fractions see the Results section.

Fraction	Volume (ml)	Total protein (mg)	Hydroxyproline		Recovery (%)
			Total (μ mol)	(μ mol/mg of protein)	
1. Crude extract	200	8000	240	0.03	100
2. DEAE-Sephadex	40	196	59	0.30	25
3. Alumina C _γ gel	10	106	55	0.52	23

Fig. 1. DEAE-Sephadex A-50 column chromatography

The elution schedule was as follows: fractions 1–3, equilibrating buffer; fractions 4–6, 0.05M-KCl; fractions 7–9, 0.1M-KCl; fractions 10–12, 0.15M-KCl; fractions 13–15, 0.2M-KCl; fractions 16–18, 0.4M-KCl; equilibrating buffer, 0.02M-Tris-HCl, pH7.4, was present throughout in the KCl elutions. Fractions 19–21 were obtained with 0.5M-NaOH elution without buffer.

L-proline, of which there is a great deal in the extract, which otherwise would seriously interfere with determination of *trans*-4-hydroxy-L-proline. The dialysed concentrated extract is designated 'crude extract' in Table 1.

Step 2: DEAE-Sephadex A-50 column chromatography. The sample from the previous step (volume

200ml; 8000mg of protein) was loaded on a DEAE-Sephadex A-50 column (5cm×52cm; bed vol. 1000ml) equilibrated with 0.02M-Tris-HCl, pH7.4, and 1 litre fractions were collected. After initial washing with 3 bed volumes (3 litres) of equilibrating buffer, the hydroxyproline-containing proteins were eluted each time with 3 litres of eluent, 0.02M-Tris-HCl, pH7.4, but containing different concentrations of KCl (0.05–0.2M) as indicated in Fig. 1, and finally with 0.5M-NaOH. In addition to a major fraction in the breakthrough fraction and buffer washings, there were two major and three minor peaks of protein containing hydroxyproline in the elution profile (Fig. 1). The hydroxyproline protein in the breakthrough fraction and buffer washings (fractions 1–3, 3 litres) after concentration with $(\text{NH}_4)_2\text{SO}_4$ precipitation and on rechromatography on a second DEAE-Sephadex A-50 column was again not held on the column. This showed that the column was not overloaded and that the breakthrough fraction was a distinct fraction of hydroxyproline-containing material. Of the two major peaks from the 0.02M-Tris-HCl-KCl elution, the peak consisting of fractions 12–14 emerging earlier gave a 3-fold enrichment in the hydroxyproline content per mg of protein whereas the peak eluted with 0.5M-NaOH gave no enrichment. Fractions 12–14 chosen for further study were pooled (volume, 3litres) and concentrated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 85% saturation. The pellet thus obtained was dissolved in 0.02M-sodium phosphate buffer, pH6.5, and dialysed against the same buffer (3litres) for 18h with three buffer changes. The bag contents were then centrifuged (10000g, 30min; Sorvall model RC-2 centrifuge) to remove some insoluble material. The supernatant fraction (designated 'DEAE-Sephadex fraction' in Table 1) was used for further purification.

Step 3: negative adsorption on alumina C_γ gel. The fraction from the previous step (volume 40ml; 196mg of protein) was diluted with 0.02M-sodium phosphate buffer, pH6.5, to a concentration of 2.5mg of protein/ml and was then treated with 62ml of alumina C_γ gel (13mg of solids/ml; gel/protein ratio 4:1, w/w). After stirring for 30min, the gel was centrifuged and the pellet was washed with 20ml of the same buffer. The supernatant fraction and

washings were pooled (volume 100ml; protein 135mg) and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (85% satn.). After centrifugation, the pellet was dissolved in 0.2M-sodium phosphate buffer, pH 6.5, and dialysed against the same buffer for 18h with three changes of buffer. This fraction is designated 'Alumina C_γ gel' fraction in Table 1.

Table 1 gives a summary of the steps in the purification procedure. The protein was purified 17-fold, as measured by the increase of the content of hydroxyproline per mg of protein, with an overall recovery of 23%.

Characterization and properties of the purified protein

Homogeneity. The homogeneity of the purified protein was checked by disc electrophoresis (Davis, 1964). The protein moved as a single band both in this procedure (Plate 1a) and on treatment with sodium dodecyl sulphate (Weber & Osborn, 1969) in the presence and absence of β -mercaptoethanol. The behaviour in sodium dodecyl sulphate-polyacrylamide-gel electrophoresis also suggested that the protein was probably a single polypeptide chain (Plate 1b).

Molecular weight. An estimate of the molecular weight of the purified protein was obtained by the gel filtration method on a Sephadex G-100 column (2.2cm $\times$ 57cm; bed volume 200ml) as described by Andrews (1964). The void volume of the column as determined with Blue Dextran 2000 was 61ml. The molecular weight of the hydroxyproline-containing protein ($V_e/V_0 = 1.54$) was estimated to be approx. 63000 by comparison with pure marker proteins

for which V_e/V_0 values were as follows: cytochrome c, 2.46; myoglobin, 2.23; ovalbumin, 1.72; bovine serum albumin, 1.50.

Amino acids. The amino acid composition of the purified protein is given in Table 2(a).

Sugars. The total sugar analysis by the phenol- H_2SO_4 procedure showed the carbohydrate content in the protein to be 16%. Amino sugars, keto sugars, uronic acid and sialic acid were absent from this glycoprotein. The presence of arabinose and traces of galactose was established by paper chromatography and confirmed by co-chromatography with authentic sugars. A quantitative determination of arabinose and galactose is shown in Table 2(b); arabinose accounted for 95% of the sugar moiety, the remainder being galactose. The ratio of hydroxyproline to arabinose in the isolated protein was 1:2 (w/w).

Proteolytic digestion. The native protein is resistant to the action of trypsin, chymotrypsin and collagenase as seen by the lack of ninhydrin-positive breakdown products by the method of Cogoli & Semenza (1973) but Pronase treatment gave evidence for a very limited cleavage of peptide bonds. Since some of the peptide products may be ninhydrin-negative, a more detailed study was made by following the progress of digestion by the absorbance at 215nm of the fractions obtained by Sephadex gel filtration. The native protein emerged as a single peak with an elution volume corresponding to the void volume (50ml) by gel filtration on a Sephadex G-50 column (1.2cm $\times$ 90cm; bed volume 135ml; equilibrating and elution buffer, 0.02M-sodium phosphate, pH 6.5). By gel filtration on a Sephadex G-100 column (1.0cm $\times$ 52cm; bed volume 40ml) the native protein again gave a single peak in the early inner volume. For further studies gel filtration on Sephadex G-50 columns was preferred in view of the ease of detection of hydrolytic fragments which would be expected to appear in the inner volume. The elution profile of the native protein and the protein after partial hydrolysis with 0.1M-HCl, is given in Fig. 2. The protein subjected to partial hydrolysis remained substantially unaffected but for a small hump showing 215nm absorbance in the inner volume. The major protein peak coinciding with the native peak and the minor hump were also observed with the 0.1M-HCl-hydrolysed protein by Sephadex G-100 gel filtration. Gel-filtration studies on the effect of Pronase showed very limited proteolysis of the native protein but almost complete digestion of the 0.1M-HCl-hydrolysed protein resulting in a number of peaks appearing in the inner volume (Fig. 3). The peptides of peaks 1, 2 and 3 together contained over 95% of the original hydroxyproline. Peak 4 gave no hydroxyproline on hydrolysis. Also, no free hydroxyproline was detected in the Pronase digests in the fractions appearing in the position of free hydroxyproline. These results suggest that the

Table 2. Chemical composition of hydroxyproline-containing protein from sandal

For details of assay procedure see the Materials and Methods section.

(a) Amino acid composition

Amino acid	Content (mol %)	Amino acid	Content (mol %)
Asp	10.6	Met	0.73
Thr	4.8	Ile	3.3
Ser	5.9	Leu	6.1
Pro	6.3	Tyr	3.7
Glu	12.1	Phe	3.5
Gly	13.4	Lys	4.3
Ala	8.6	His	1.3
Cys	0.9	Arg	5.8
Val	4.1	Hyp	5.9

(b) Sugar composition

Protein (mg)	Total sugar (mg)	Proline (μmol)	Hydroxy-proline (μmol)	Arabinose (μmol)	Galactose (μmol)
11.7	2.3	6.4	6.0	13.4	0.4

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of hydroxyproline-containing protein from sandal

(a) Disc gel electrophoresis of the sandal protein. A large amount of protein ($60\mu\text{g}$) was applied to detect any possible contaminant protein. The band was stained with 0.5% Amidoschwartz 10B in 7% acetic acid. (b) Sodium dodecyl sulphate-polyacrylamide-gel disc electrophoresis of the sandal protein in the presence (left) and in the absence (right) of β -mercaptoethanol. $40\mu\text{g}$ of protein was used. The staining reagent was Coomassie Brilliant Blue (0.25% in 50% methanol containing 9% acetic acid).

Fig. 2. Gel filtration of hydroxyproline-containing protein on Sephadex G-50 after partial acid hydrolysis

For conditions see the Materials and Methods section.
●, Intact protein; ○, partially hydrolysed protein.

Fig. 4. Separation of hydroxyproline-glycosides

An alkaline hydrolysate of hydroxyproline-containing protein (13.5mg) was fractionated on a column of Dowex-50 (X2) (H^+ form); for details see the Materials and Methods section.

Fig. 3. Pronase digestion of partially hydrolysed protein

Gel filtration was carried out on Sephadex G-50 after treatment with Pronase. For conditions see the Materials and Methods section. ●, Native protein after Pronase digestion; ○, partially hydrolysed protein after Pronase digestion.

native protein which is resistant to enzymic proteolysis, became susceptible to Pronase digestion after partial hydrolysis with 0.1M-HCl which removed the bulk of the sugars and which probably caused limited splitting of some peptide bonds.

Bacterial collagenase apparently had no action on the native protein or the protein after partial acid hydrolysis, since after collagenase treatment there were no additional peaks in the elution profile.

Hydroxyproline glycosides by alkaline hydrolysis of the protein

Alkaline hydrolysis of the protein gave hydroxyproline-containing glycosides which were separated on a Dowex-50 (X2) resin column (Fig. 4). Three major peaks were obtained. Peak 1, emerging first, consists of sugars and bound hydroxyproline but has

not been further characterized for the presence of free and bound sugars. Both peak 2 and peak 3 gave sugars and hydroxyproline only on acid hydrolysis. The ratio of hydroxyproline to sugars was 1:3 and 1:2 (w/w) respectively. Although direct analysis of individual fractions in the region of fractions 13–53 and 74–98 gave no significant amounts of sugar or hydroxyproline, pooled and concentrated fractions (in batches of five fractions) gave both sugars and hydroxyproline after acid hydrolysis. This result needs to be checked with larger amounts of protein. These results are reminiscent of those of Miller *et al.* (1972) with *Chlamydomonas*. Miller *et al.* (1972) reported the presence of free hydroxyproline (both *trans* and *cis* isomers) in alkali hydrolysates, the two peaks emerging towards the tail end of the Dowex 50 (X2) fractionation. However, with the sandal protein there was no evidence for the presence of free hydroxyproline in the alkali hydrolysates.

Discussion

There are several reports of hydroxyproline-containing proteins in plants, and of their synthesis, secretion and transport to the cell wall. The presence of hydroxyproline-containing proteins in the cytoplasm and in the cell-wall fractions of sandal leaves has been shown earlier (Kuttan & Radhakrishnan, 1970). So far, no report is available on the isolation of hydroxyproline-containing proteins from the soluble fraction in a pure form. Brisk & Chrispeels (1972) isolated a hydroxyproline-containing protein from the cytoplasmic membranous organelles of carrots. The hydroxyproline-containing protein isolated from sandal has a molecular weight of 63000, and consists of 84% protein and 16% carbohydrates, whereas the glycoprotein isolated by Brisk & Chrispeels (1972) contained 60% protein and 40% carbohydrates. There are significant differences in the amino acid composition of these two proteins and the one isolated by Lamport (1965) from the cell-wall material. The amino acid composition of wall proteins from different sources isolated by Lamport (1965) shows variations in hydroxyproline content, and the polar amino acid residues (especially hydroxy amino acids) are predominant, but glycine, tyrosine and cysteine are in average amounts and hydroxylysine is absent. The cell-wall protein isolated by Brisk & Chrispeels (1972) contained hydroxyproline (10.3mol%), proline (2.8mol%), serine (18.8mol%) and glycine (12.0mol%). The amino acid composition of our protein shows that the predominant amino acid was glycine (13.4mol%), whereas serine (5.9mol%) was low. Hydroxyproline (5.9mol%) and proline (6.3mol%) are present in about equal amounts, but the sum (12.2mol%) is similar to that of Brisk & Chrispeels' (1972) protein (13.1mol%). In both proteins the sum of the imino

acids is roughly equal to the content of glycine. The molar ratio of hydroxyproline to proline is 1:1 in our protein, but it is 4:1 in the protein isolated by Brisk & Chrispeels (1972), and this probably suggests that the sandal protein may be under-hydroxylated. The acidic amino acids (plus any glutamine and asparagine that there might have been before hydrolysis) exceed the sum of basic amino acids, whereas the protein isolated by Brisk & Chrispeels (1972) has more basic amino acids and has pI 9.1. Cell-wall proteins of a variety of plant species show a wide variation in this respect (Lamport, 1965). Both methionine and cysteine were present in the sandal protein, though the amounts were low. Another feature of the sandal protein is the absence of tryptophan.

By pulse-chase experiments with [¹⁴C]proline Brisk & Chrispeels (1972) showed a precursor-product relationship between the membranous organelle fraction and the wall fraction with a carrot disc system. However, in sandal the cytoplasmic hydroxyproline fraction was not readily incorporated into the wall fraction (Kuttan & Radhakrishnan, 1970). Heterogeneity of the hydroxyproline-containing fraction has been observed both in the wall fraction (Kuttan & Radhakrishnan, 1970) and in the present study in the cytoplasmic fraction. It is at present not established which of these cytoplasmic fractions is incorporated into the wall fraction, which is also heterogeneous. Further studies are required to find out whether the protein associated with these seemingly different fractions is the same and whether the heterogeneity is due to differences in carbohydrate content or mode of linkage to the organelles. It is also important to note that glycoprotein lectins from potato tubers have been shown to contain hydroxyproline (Allen & Neuberger, 1973). The relationship between hydroxyproline-containing lectins and hydroxyproline-containing fractions associated with cell walls need to be established. It is tempting to speculate that the affinity of lectins for sugars may offer a mechanism for the initial attachment of hydroxyproline-containing fractions to the sugars of cell-wall material.

We are grateful to Professor S. J. Baker, Head of our Unit, for his keen interest. The project was supported by the Council of Scientific and Industrial Research, New Delhi, India, and by U.S. Public Law 480 funds.

References

- Andrews, P. (1964) *Biochem. J.* **91**, 222–223
- Allen, A. K. & Neuberger, A. (1973) *Biochem. J.* **135**, 307–327
- Benson, J. V., Jr. & Patterson, J. A. (1965) *Anal. Biochem.* **13**, 265–280
- Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334

- Brisk, M. & Chrispeels, M. J. (1972) *Biochim. Biophys. Acta* **257**, 421–432
- Chrispeels, M. J. (1970) *Biochem. Biophys. Res. Commun.* **39**, 732–737
- Cleland, R. (1968) *Plant Physiol.* **43**, 865–870
- Cogoli, A. & Semenza, G. (1973) *Eur. J. Biochem.* **33**, 40–48
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dische, Z. (1947) *J. Biol. Chem.* **167**, 189–198
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. I. (1956) *Anal. Chem.* **28**, 350–356
- Gatt, R. & Berman, E. R. (1966) *Anal. Biochem.* **15**, 167–171
- Gruen, L. C. & Nicholls, P. W. (1972) *Anal. Biochem.* **47**, 348–355
- Kuttan, R. & Radhakrishnan, A. N. (1970) *Biochem. J.* **119**, 651–657
- Lamport, D. T. A. (1965) *Advan. Bot. Res.* **2**, 151–218
- Lamport, D. T. A. (1967) *Nature (London)* **216**, 1322–1324
- Lamport, D. T. A. (1969) *Biochemistry* **8**, 1155–1163
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Miller, D. H., Lamport, D. T. A. & Miller, M. (1972) *Science* **176**, 918–920
- Mukerjee, H. & Sri Ram, J. (1964) *Anal. Biochem.* **8**, 393–394
- Neuman, R. E. & Logan, M. A. (1950) *J. Biol. Chem.* **186**, 549–556
- Olson, A. C. (1964) *Plant Physiol.* **39**, 543–550
- Roe, J. H. & Rice, E. W. (1948) *J. Biol. Chem.* **173**, 507–512
- Schweet, R. S. (1954) *J. Biol. Chem.* **208**, 603–613
- Steward, F. C. & Pollard, J. K. (1958) *Nature (London)* **182**, 828–832
- Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975
- Weber, R. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Willstatter, R. & Kraut, H. (1923) *Ber. Deut. Chem. Ges.* **56**, 1117–1121
- Worthington Enzyme Manual (1972) pp. 21–22, Worthington Biochemical Corporation, Freehold, N.J., U.S.A.