Biochem. J. (1970) **120**, 607–612
Printed in Great Britain

Studies on Phytochrome

TWO PHOTOREVERSIBLE CHROMOPROTEINS FROM ETIOLATED OAT SEEDLINGS

BY T. S. WALKER* AND J. L. BAILEY†
Twyford Laboratories Ltd., Twyford Abbey Road, Park Royal, London N.W.10, U.K.

(*Received* 6 *July* 1970)

1. The photoreversible chromoprotein phytochrome was extracted from etiolated oat seedlings. The final purification step revealed that there were two photoreversible coloured components. 2. The amino acid composition, spectra and Svedberg coefficients of each component are reported.

The photoreversible plant chromoprotein phytochrome has been isolated and purified from etiolated oat seedlings (Siegelman & Firer, 1964; Siegelman & Hendricks, 1965; Mumford & Jenner, 1966; Briggs, Zollinger & Platz, 1968), etiolated rye seedlings (Correll, Steers, Edwards, Suriano & Shropshire, 1966) and the alga *Mesotinium* and the liverwort *Sphaerocarpus* (Taylor & Bonner, 1967). Although the chemical and physical properties of phytochrome have been described by these various workers only Briggs *et al.* (1968) presented evidence for the existence of phytochrome as two different-sized molecules. We now report our findings in this respect.

MATERIALS AND METHODS

Etiolated oat (*Avena sativa* var. Condor, grown in Kent) seedlings were grown and harvested under the same conditions as previously described (Walker & Bailey, 1968).

Extraction procedure. Etiolated seedlings (2.6kg) were macerated in a 4-litre Waring stainless-steel blender with 2.6 litres of 0.1M-potassium phosphate buffer, pH7.8. Unless otherwise stated all buffers were at pH7.8 and contained 0.05M-2-mercaptoethanol.

The macerated mixture was filtered on 24cm-diam. Buchner funnels with a 6mm pad of Solka floc as a filter aid. The filtrate was clarified by centrifugation and concentrated by stirring sufficient dry Sephadex G-50 (coarse-grade beads) into the extract to form a thick slurry. This was filtered on Buchner funnels and the Sephadex cake rinsed with 0.01M-potassium phosphate buffer. The concentration procedure was repeated twice. After concentration the mixture was centrifuged in the preparative Spinco model L ultracentrifuge in the no. 21 rotor at 15000rev./min for 30min. This gave 410ml of clarified concentrated mixture from 3.8 litres of dilute extract, with 95% recovery of photoreversible material as measured by the methods of Hendricks, Butler & Siegelman (1962) by using the relationship:

$$\Delta(\Delta E) = (E_{660} - E_{725})_{730\ \text{Irrad.}} - (E_{660} - E_{725})_{645\ \text{Irrad.}}$$

The photoactive concentrated mixture was loaded on to a column (60cm × 10cm) of Sephadex G-100 equilibrated with 0.01M-potassium phosphate buffer, eluted with the same buffer at 150–200ml/h and the eluate was collected in 30ml fractions. Small samples were monitored for photoreversibility.

The fractions showing highest photoreversibility were pooled and loaded on to a column (20cm × 7cm) of DEAE-cellulose equilibrated with 0.01M-potassium phosphate buffer and eluted under constant hydrostatic head with a gradient of 1 litre each of 0.01M- and 0.25M-potassium phosphate buffer at 200–250ml/h. The eluate was collected in 30ml fractions and E_{280} and the photoreversible response of the fractions were determined.

The most active fractions (fractions 27–39) were again combined and re-equilibrated with 0.01M-potassium phosphate buffer by passing the solution down a column (40cm × 7cm) of Sephadex G-50. The active solution was irradiated with far-red light (for details see Walker & Bailey, 1968) for 20min and then adsorbed on a column (5cm × 2.5cm) of DEAE-cellulose also equilibrated with 0.01M-potassium phosphate buffer. During loading and subsequent elution with 0.25M-potassium phosphate buffer the column was constantly irradiated with far-red light. The eluate was collected in 5ml fractions. The three or four fractions with highest activity were combined and loaded on to a column (125cm × 2.5cm) of Sephadex G-200. The column, previously equilibrated with 0.25M-potassium phosphate buffer, was arranged for upward flow displacement (Porath & Bennich, 1962) and was eluted at 15ml/h by use of a LKB peristaltic pump. The column eluate was collected in 5ml fractions and E_{280} and the photoreversible responses of the fractions were determined.

The most photoactive fractions (fractions 79–93) were again combined and re-equilibrated with 0.01M-potassium phosphate buffer by passage down a small column (30cm × 5cm) of Sephadex G-50. After irradiation with far-red light the solution was adsorbed on a small column (2cm × 3cm) of DEAE-cellulose previously equilibrated with 0.01M-potassium phosphate buffer and finally eluted

* Present address: The Lord Rank Research Centre, Lincoln Road, High Wycombe, Bucks., U.K.

† Present address: School of Biological Sciences, Australian National University, Canberra, A.C.T., Australia.

with 0.25M-potassium phosphate buffer. During this procedure the column was again irradiated with far-red light. The eluate was collected in 2ml fractions and the two or three fractions with the highest photoactivity were combined.

The photoactive solution was dialysed overnight against 5 litres of 0.05M-tris–acetate buffer, pH 8.2 at 25°C and pH 8.6 at 0°C, containing 0.05M-2-mercaptoethanol; after dialysis a small amount of sucrose was added to the sample and 1ml portions were loaded on to each of four polyacrylamide-gel columns (10cm × 1.8cm). Electrophoresis was carried out at 200V at 20mA/tube for 3.5h, the proteins migrating towards the anode. During electrophoresis a green zone followed by a blue zone was observed migrating through the gel columns.

After electrophoresis the gel columns were removed from their glass tubes, the gels stiffened by immersion in a salt–ice freezing mixture and the coloured zones cut from the columns. A lengthwise segment was cut from one column and stained with 0.1% Amido Black to locate the protein components. The spectra of the two coloured components were determined while they were still in the gel slices. The two photoreversible coloured components were recovered from the gel slices by the technique of Thornber, Smith & Bailey (1966).

The photoactive solutions were dialysed overnight against 5 litres of 00.5M-tris–acetate buffer and the sedimentation coefficients determined in a Spinco model E analytical ultracentrifuge. After the sedimentation coefficients had been determined the protein solutions were dialysed against 5 litres of glass-distilled water. The water was changed after 1, 2 and 5 days. The dialysed protein solutions were freeze-dried and the total sample was hydrolysed for amino acid analysis.

Sephadex. The various grades of Sephadex used for chromatography were swollen in the elution buffers and the columns were packed in the cold-room.

DEAE-cellulose. Whatman DE11 DEAE-cellulose (1 mequiv./g) was used in DEAE-cellulose chromatography and was prepared by the precycling method recommended by the manufacturers.

Polyacrylamide-gel electrophoresis. The gel columns were prepared in glass tubes (12.5cm × 1.8cm constant internal diam.) and electrophoresis was carried out in the apparatus described by Ornstein & Davis (1961) and modified to accept the larger tubes. The final gel concentration used was 7.5% (cross-linking 1.66%). The gels were equilibrated by electrophoresis for 30min at 200V at 20mA/tube before the samples were applied.

Photoreversibility measurements and spectra. Photoreversibility measurements and spectral determinations were carried out as described previously (Walker & Bailey, 1968).

Sedimentation coefficients. The two isolated components were equilibrated with 0.05M-tris–acetate buffer by overnight dialysis. They were then centrifuged simul-

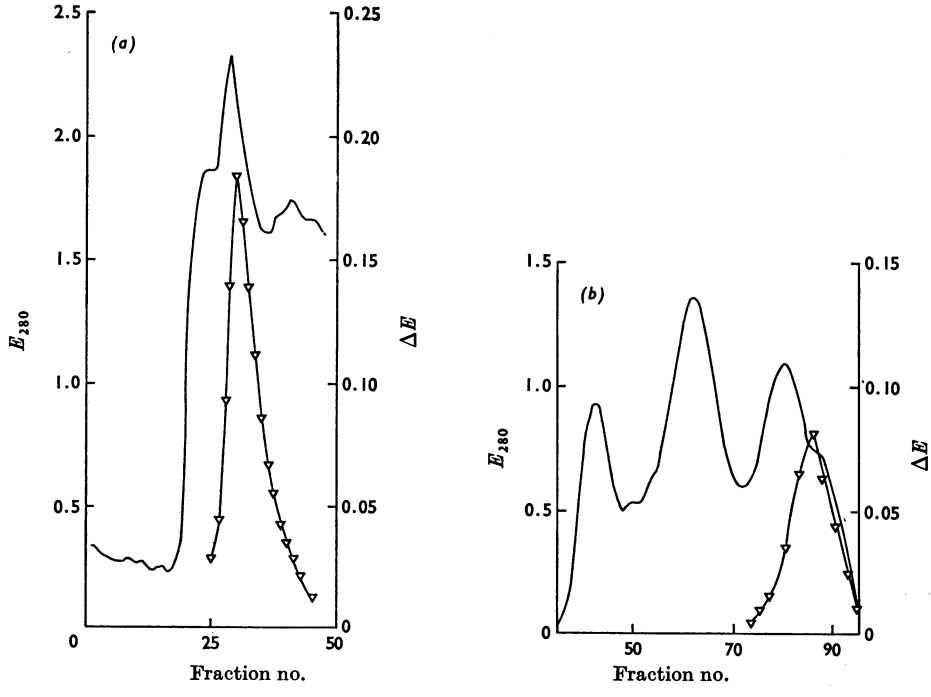

Fig. 1. Elution diagrams of DEAE-cellulose chromatography (*a*) and Sephadex G-200 chromatography (*b*) of photoactive solutions. E_{280} in the DEAE-cellulose eluate was measured in 10mm cuvettes and photoreversibility in 400mm cuvettes. For the Sephadex G-200 eluate the cuvettes used were 5mm and 10mm respectively. ——, E_{280}; ▽—▽, photoreversibility.

taneously in an analytical D rotor in the Spinco model E analytical ultracentrifuge. The blue component was placed in a cell with a 1°C positive-wedge window and the green component in the standard cell. The blue protein was subsequently remeasured in the artificial-boundary cell at 59780rev./min to obtain a more accurate value for the sedimentation coefficient.

Amino acid analysis. The freeze-dried proteins were each dissolved in 4ml of 6M-HCl and hydrolysed at 110°C for 24h in sealed acid-resistant glass ampoules. Before being sealed the ampoules were evacuated several times and flushed with pure N_2. After hydrolysis excess of acid was removed on a rotary evaporator and the samples were redissolved in 0.1M-sodium citrate buffer, pH2.2, and analysed in an EEL amino acid analyser.

RESULTS

The elution diagrams presented in Figs. 1(*a*) and 1(*b*) show that phytochrome migrated as a single peak both in ion-exchange and in gel-filtration chromatography. Even when partially purified phytochrome solution, concentrated after Sephadex G-200 chromatography, was rechromatographed on Sephadex G-200 by recycling chromatography the photoactive protein migrated as a single peak (T. S. Walker, unpublished work). There was at this stage in the purification procedure no indication that the photoactive material was heterogeneous. It was clear, however, that some loss of photoactivity accompanied by a change in the photoreversibility characteristics had occurred. Fig. 2 shows the spectra of the concentrated phytochrome solutions after (*a*) DEAE-cellulose chromatography and (*b*) Sephadex G-200 chromatography.

After the final purification step of polyacrylamide-gel electrophoresis Amido Black staining of the gels indicated the presence of five distinct protein components. A green component was coincident with the fastest running of these, with a blue component running 3mm behind it (Fig. 3). The coloured components were cut from the gel columns and the spectra measured. Photoreversion of the samples in the gel slices was carried out in the spectrophotometer cuvettes, the cuvettes being immersed in ice. Spectra of the chromoproteins were obtained after irradiation with red light and subsequently after irradiation with far-red light. Difference spectra were plotted by hand as insufficient material was available from any one extraction to enable difference spectra to be obtained directly. Both components were photoactive, showing no appreciable loss of photoreversibility after three photoreversion cycles.

The spectral properties of the two coloured components was significantly different (Fig. 4). Both components showed some deviation from published spectra for electrophoretically pure phytochrome (Mumford & Jenner, 1966) in that absorption in the red end of the spectrum was much less than that in the blue. The difference spectrum of the blue protein was very similar to that for native phytochrome, but that of the green protein showed total loss of photoactivity in the blue–near-ultraviolet region of the spectrum and additionally showed a considerable sharpening of the peak absorbing in the Soret-band region at 380nm. In both components the wavelength of the peak

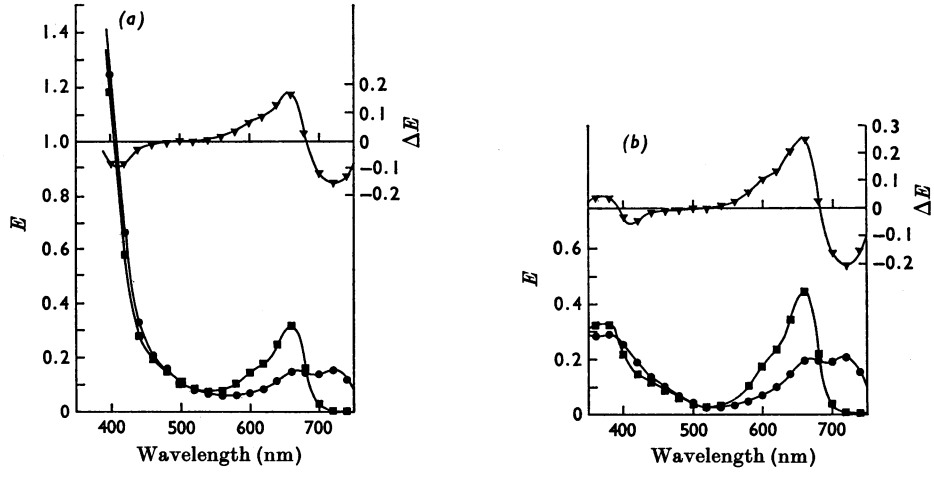

Fig. 2. Spectra of the concentrated photoactive solution before (*a*) and after (*b*) Sephadex G-200 chromatography. The spectra were measured in 10mm cuvettes and difference spectra obtained in the spectrophotometer with the 645nm-irradiated sample in the reference beam. ■, P_r spectrum; ●, P_{fr} spectrum; ▼, difference spectrum.

absorption of the P_r form had moved from 660nm to 656nm.

The amino acid analyses presented in Table 1 are the mean of two analyses on 24h hydrolyses of proteins from two separate extracts. A surprising feature of the analyses was the apparent lack of tyrosine in the proteins, even though the normal precautions were taken to prevent oxidation during hydrolysis. The trace amounts of tyrosine present in some of the chromatograms were too small to be calculated.

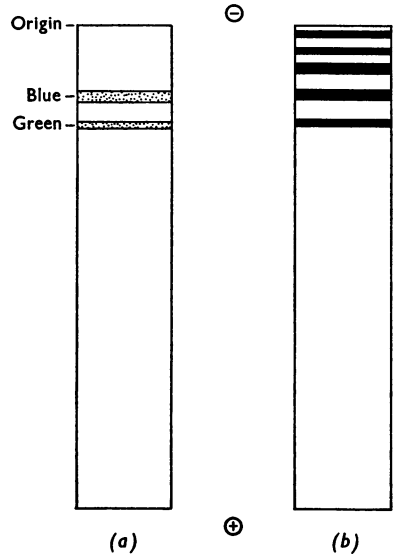

Fig. 3. Diagrams of the unstained preparative electrophoretic gel showing the position of the two coloured components (*a*) and diagram of the Amido Black-stained segment showing the position of the stained protein bands (*b*). For experimental details see the text.

The differences obtained for the numbers of amino acid residues in the two proteins are sufficiently marked in the cases of glutamic acid, glycine, alanine and isoleucine to be regarded as real differences. The difference of one residue between the analyses for both serine and histidine may be less significant.

Minimum molecular weights calculated from the analyses gave values of 10706 for the blue protein and 10142 for the green protein. The apparent specific volume for each of the proteins, calculated from the data of Schachman (1957), was for the blue protein $\bar{v} = 0.750$ and for the green protein $\bar{v} = 0.748$.

Ultracentrifugation of the two proteins showed that the green protein sedimented as two separate components. The corrected sedimentation coefficients for the two components were $s_{20,w}$ 1.86 and 6.61S. The sedimentation coefficient of the blue protein calculated from results obtained from a run in the artificial-boundary cell was $s_{20,w}$ 2.12S. Fig. 5 shows tracings from plates taken during these runs.

Molecular weights calculated from the sedimentation coefficients by the Atassi & Gandhi (1965) formulae were 19400 for the blue protein and 15900 and 119700 for the green protein. Molecular weights calculated by use of the Halsall (1967) formula were 26000 for the blue protein and 21400

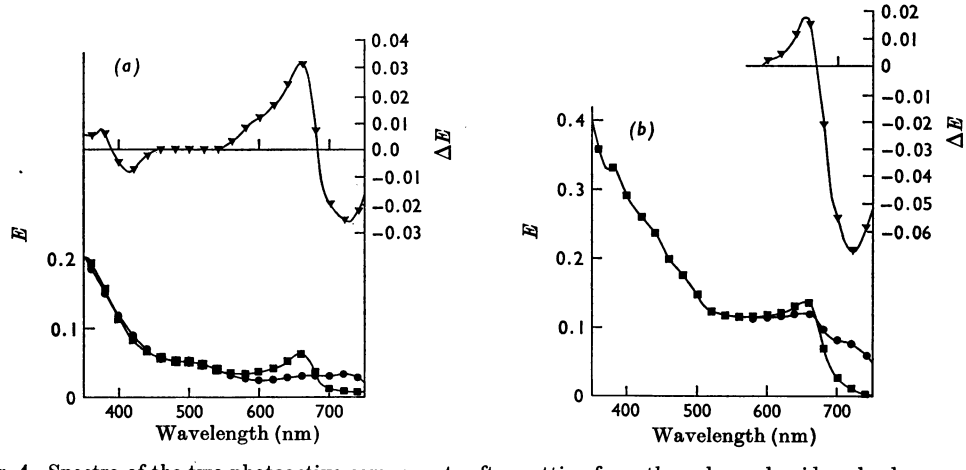

Fig. 4. Spectra of the two photoactive components after cutting from the polyacrylamide-gel columns. The spectra were measured, while the components were still in the gel slices, in 10mm cuvettes with a 10mm block of clear gel in the reference cuvette. (*a*) Spectra of the blue component; (*b*) spectra of the green component. ■, P_r spectrum; ●, P_{fr} spectrum; ▼, difference spectrum.

Table 1. *Amino acid composition of the green and the blue photoactive proteins*

The analyses were all corrected to a value of 1 μmol of leucine as this gave the most constant percentage recovery in all four analyses. The mean of the two analyses is presented with the upper and lower limits.

Amino acid	Amount (μmol) (mean of two 24 h analyses)		No. of residues corrected to 10 leucine residues		Nearest whole no. of residues	
	Green protein	Blue protein	Green protein	Blue protein	Green protein	Blue protein
Asp	1.048±0.010	1.042±0.008	10.4	10.4	10	10
Thr	0.266±0.012	0.253±0.008	2.7	2.5	3	3
Ser	0.140±0.007	0.178±0.006	1.4	1.8	1	2
Glu	0.878±0.015	1.083±0.052	8.8	10.8	9	11
Pro	0.601±0.038	0.626±0.035	6.0	6.3	6	6
Gly	0.956±0.044	1.250	9.6	12.5	10	13
Ala	1.080±0.050	1.20	10.8	12.0	11	12
Val	0.915±0.033	0.908±0.038	9.2	9.1	9	9
Met	0.121±0.011	0.095	1.2	0.95	1	1
Ile	0.606±0.018	0.704±0.011	6.1	7.0	6	7
Leu	1.000	1.000	10.0	10.0	10	10
Tyr			Trace amount only			
Phe	0.448±0.007	0.395±0.013	4.4	4.0	4	4
Lys	0.758±0.008	0.803±0.003	7.6	8.0	8	8
His	0.261±0.013	0.197±0.003	2.6	2.0	3	2
Arg	0.441±0.017	0.370±0.014	4.4	3.7	4	4

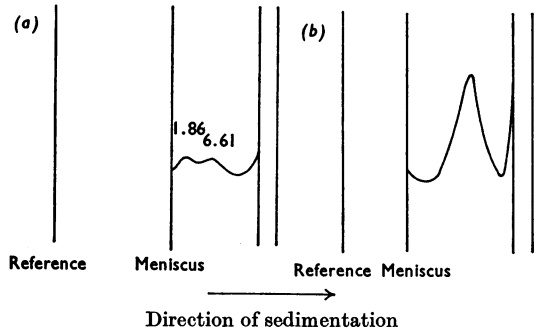

Fig. 5. Tracings of the schlieren patterns of the green protein (*a*) and the blue protein (*b*) in the artificial-boundary cell, both taken 32 min after reaching 59 780 rev./min in the ultracentrifuge.

and 143 200 for the green. From this it was concluded that the basic unit was twice the minimum determined from the amino acid analyses and that the polymer formed in the solution of green protein was a hexamer of the basic unit.

DISCUSSION

Studies on the decay of photoactive phytochrome in plant tissue (Butler & Lane, 1965), on the effect of various light regimes on the development of dodder seedlings (Lane & Kasperbauer, 1965) and on biochemical changes both in *Sinapsis alba* seedlings (Wagner & Mohr, 1966) and in turnip seedlings (Vince & Grill, 1966) have led these workers to suggest the existence of two types of phytochrome molecule in etiolated plant tissue. Spruit (1967) showed abnormal phytochrome spectra in etiolated leaves of *Pisum sativum* that differed from that of phytochrome spectra obtained from the stems of the same plants. Hillman (1968), however, was unable to repeat these results, even though using the same variety of pea (Krombek). Briggs *et al.* (1968) have since demonstrated the existence of two molecular species of phytochrome in oat seedlings with approximate molecular weights of 80 000 and 180 000. Purves & Briggs (1968) have shown the presence in various plant tissues of two and in one case three kinetically distinguishable populations of phytochrome.

Our results show that up to the Sephadex G-200 purification step phytochrome migrated as a single peak, and it was only on gel electrophoresis that two photoactive components became separable.

In spite of elaborate precautions to keep the phytochrome in the P_r form both by frequent irradiation with far-red light and by carrying out all the purification procedures in green light the spectral characteristics of the preparation gradually altered.

The absorption spectra of both proteins isolated after the final purification step of gel electrophoresis

showed that considerable denaturation had taken place although the blue protein had more closely resembled 'native' phytochrome than did the green protein.

This differential denaturation may be the consequence of either an initial small difference in the amino acid composition between two phytochrome species or the loss by one species during the final electrophoretic purification step of a small loosely bound peptide. The differences between the properties of the two proteins could be explained in either way.

The amino acid analyses of the two proteins do not bear a close resemblance to that published by Mumford & Jenner (1966), the lysine:arginine and leucine:isolecuine:valine proportions in particular being noticeably different.

The sedimentation coefficient for phytochrome has been given as 4.5S (Siegelman & Firer, 1964) and 9.5S (Correll *et al.* 1966). The 9.5S particle broke down to give a 1.8S particle when treated with surface-active agents. It was not reported whether the subunit was photoactive. Our results clearly indicated that the blue protein sedimented as one boundary only whereas the green protein sedimented as two. In view of the preceding purification steps it would appear that both proteins were initially of the same size but that during electrophoresis the green protein became altered so that when it was later extracted from the gel slices a hexamer formed.

Our results underline the differences in the properties of phytochrome observed by other workers in this field. Because of the obvious changes in the properties of phytochrome during our extraction procedure it would be unwise to conclude that our results offer incontrovertible proof of the existence of two phytochrome species.

We thank Mr M. Hatton, who carried out the amino acid analyses.

REFERENCES

Atassi, M. Z. & Gandhi, S. K. (1965). *Naturwissenschaften*, **52**, 259.

Briggs, W. R., Zollinger, W. D. & Platz, B. B. (1968). *Pl. Physiol., Lancaster*, **43**, 1239.

Butler, W. L. & Lane, H. C. (1965). *Pl. Physiol., Lancaster*, **40**, 13.

Correll, D. L., Steers, E., jun., Edwards, J. L., Suriano, K. R. & Shropshire, W., jun. (1966). *Pl. Physiol., Lancaster*, **41**, xvi.

Halsall, H. B. (1967). *Nature, Lond.*, **215**, 880.

Hendricks, S. B., Butler, W. L. & Siegelman, H. W. (1962). *J. phys. Chem., Ithaca*, **66**, 2550.

Hillman, W. S. (1968). *Biochim. biophys. Acta*, **162**, 464.

Lane, H. C. & Kasperbauer, M. J. (1965). *Pl. Physiol., Lancaster*, **40**, 109.

Mumford, F. E., & Jenner, E. L. (1966). *Biochemistry, Easton*, **5**, 3657.

Ornstein, L. & Davis, B. J. (1961). *Disc Electrophoresis* (Preprint). Rochester: Distillation Products Industries.

Porath, J. & Bennich, H. (1962). *Archs Biochem. Biophys.* Suppl. no. 1, p. 152.

Purves, W. K. & Briggs, W. R. (1968). *Pl. Physiol., Lancaster*, **43**, 1259.

Schachman, M. K. (1957). In *Methods in Enzymology*, vol. 4, p. 32. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Siegelman, H. W. & Firer, E. M. (1964). *Biochemistry, Easton*, **3**, 418.

Siegelman, H. W. & Hendricks, S. B. (1965). *Fedn Proc. Fedn. Am. Socs exp. Biol.* **24**, 863.

Spruit, C. J. P. (1967). *Biochim. biophys. Acta*, **143**, 260.

Taylor, A. O. & Bonner, B. A. (1967). *Pl. Physiol., Lancaster*, **42**, 762.

Thornber, J. P., Smith, C. A. & Bailey, J. L. (1966). *Biochem. J.* **100**, 14P

Vince, D. & Grill, R. (1966). *Photochem. & Photobiol.* **5**, 407.

Wagner, E. & Mohr, H. (1966). *Photochem. & Photobiol.* **5**, 397.

Walker, T. S. & Bailey, J. L. (1968). *Biochem. J.* **107**, 603.