Phytochemistry, Vol. 25, No. 3, pp. 595–600, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00

PURIFICATION OF A STORAGE PROTEIN OF *PSOPHOCARPUS TETRAGONOLOBUS*

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(*Revised received* 17 *July* 1985)

Key Word Index—*Psophocarpus tetragonolobus*; Leguminosae; winged bean; seed storage protein; psophocarpin B_1; tuber storage protein; radio-immunoassay; germination; protein crystals.

Abstract—Psophocarpin B_1, one of the major seed storage proteins of *Psophocarpus tetragonolobus* (winged bean), has been purified to homogeneity and crystallized. It is an acidic protein with a pI of 5.5 and M_r of 20000, as determined by gel filtration in the absence of denaturating agents. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), indicates the absence of a quaternary structure of the protein. Using specific antibody raised in rabbits, a radio-immunoassay (RIA) method has been developed for its quantitation. It shows that 12% of the total soluble protein in the tuber of this plant is psophocarpin B_1 whereas it represents 9% of the seed protein. In the pods, it is only a minor component (1% of the total soluble protein) and it is not detectable in stems and leaves. Psophocarpin B_1 undergoes a time-dependent degradation in germinating seeds.

INTRODUCTION

The winged bean, a crop of the tropical regions, contains as much protein and oil in its seed as the soybean; and it has been hailed as 'the possible soybean for the tropics' [1]. Other parts of this plant such as the tuber, shoot, leaf, flower and pod are also edible and in this respect it may be more useful than the soybean. In spite of this high potential, it is cultivated as a subsistence crop in only a few tropical countries of the world, whereas the soybean, the world's premier protein crop, is produced on a large commercial scale. This disparity may be attributed to several factors, such as genetic improvement of the crop, the development of scientific agronomic practices and a thorough knowledge of the storage proteins, which are lacking in the case of the winged bean.

The characterization of the storage proteins of the winged bean is limited to the studies of Gillespie and Blagrove [2]. They obtained three fractions of these proteins and named them as psophocarpins A, B and C, respectively and carried out preliminary physico-chemical characterizations of these fractions. However, these fractions were not homogeneous as judged by SDS-PAGE [2]. Recently, in the course of his studies on the lectins of the winged bean, Kortt [3] has reported the resolution of the psophocarpin B fraction of Gillespie and Blagrove into various protein fractions by gel filtration. Lack of convenient methods which yield homogeneous preparations of storage proteins of this seed has greatly hampered further studies of these proteins. This prompted us to develop methods for the purification of some of these storage proteins, suitable for raising specific antibodies which may be used as a tool in our studies on the metabolism and molecular biology of the storage proteins of the winged bean. Here, we present the purification, crystallization, characterization and preliminary metabolic studies of one of the major proteins of the psophocarpin B fraction, which we have named as psophocarpin B_1, retaining the system of nomenclature introduced by Gillespie and Blagrove [2].

RESULTS

Purification of psophocarpin B_1 from winged bean seeds

All operations were carried out at 0–4°.

Preparation of crude extract

Soaked winged bean cotyledons (20 g), free from seed coat and embryonic axes, were homogenized in a blendor with 250 ml of 100 mM Tris–HCl, pH 8, containing 5 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM EDTA and 0.02% sodium azide, for a total period of 10 min, a few drops of octanol being added to prevent foaming. The homogenate was centrifuged at 16000 *g* for 1 hr at 4° and the turbid supernatant fluid was filtered through glass wool.

Acetone fractionation

To 225 ml of the crude extract, 75 ml of ice-cold acetone was added and stirred for 30 min and then centrifuged at 2500 *g* for 10 min. Another 450 ml of acetone was added to the supernatant solution, stirred for 30 min and centrifuged as above. The pellet was redissolved in a minimum volume of 100 mM potassium phosphate, pH 7.1 and the solution was centrifuged at 160000 *g* and 4° for 75 min. The clarified supernatant constituted the 25–70% acetone fraction.

Gel filtration on Sephadex G-50

The 25–70% acetone fraction (21.5 ml) was loaded on a Sephadex G-50 column (3 × 115 cm), equilibrated with

100 mM potassium phosphate buffer, pH 7.1 and proteins were eluted with the same buffer at a flow rate of 20 ml/hr. 10 ml fractions were collected. The eluted protein was monitored by measuring the absorbance at 280 nm and the elution profile is presented in Fig. 1. It shows two major protein peaks. The fractions (No. 35–42, Fig. 1) which showed considerable enrichment in psophocarpin B_1 on analysis by SDS-PAGE, were pooled and dialysed extensively against 25 mM potassium phosphate buffer, pH 7.1.

DEAE-cellulose chromatography

The dialysed solution was loaded on a DEAE-cellulose column (2 × 45 cm), equilibrated with 25 mM potassium phosphate buffer, pH 7.1. The column was washed with 30 ml of the same buffer and the bound proteins were eluted with 500 ml of a linear gradient of 25–300 mM of the same buffer at a flow rate of 25 ml/hr. Fractions (5 ml) were collected and analysed as before. Three major protein peaks were observed (Fig. 2) and SDS-PAGE analysis revealed that seven fractions (No. 56–62, Fig. 2) eluted around 190 mM buffer concentration, contained purified psophocarpin B_1, free from other contaminating proteins. These fractions were pooled and concentrated by precipitation with solid ammonium sulphate.

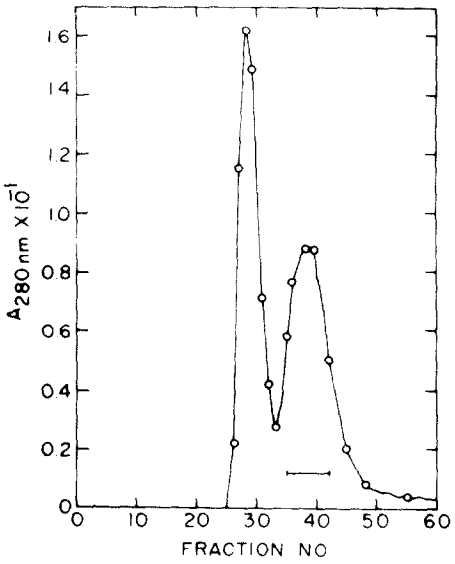

Fig. 1. Purification of psophocarpin B_1. Elution profile of proteins from the Sephadex G-50 column was obtained by measurement of $A_{280\,nm}$. Fractions enriched in psophocarpin B_1 are indicated by the horizontal bar.

Since there was no assay system specific for psophocarpin B_1, in the initial studies, the course of purification was followed by SDS-PAGE analysis of the fractions. The removal of contaminating proteins at different steps of purification is shown in Fig. 3. In later studies, after the standardization of RIA, the purification was followed by this method and a summary of a typical purification of psophocarpin B_1 is presented in Table 1. An overall recovery of about 20% was achieved.

Homogeneity, M_r and pI of psophocarpin B_1

The psophocarpin B_1 preparation obtained after the DEAE-cellulose chromatography step was essentially homogeneous as revealed by a single protein band on SDS-PAGE (Fig. 3, lane 4) as well as by a single protein band in alkaline PAGE in the absence of denaturating agents (data not shown).

The M_r of the native psophocarpin B_1 was estimated to be about 19 500 by gel filtration on Sephadex G-75, calibrated with several proteins of known M_r (data not shown) and that of the denatured protein by SDS-PAGE was around 20 200 (data not shown). Isoelectric focussing studies revealed a single protein band with a pI of 5.5 (pI calibration curve not shown).

Crystallization of psophocarpin B_1

The purified protein in the pooled fractions from the DEAE-cellulose chromatography step (Fig. 2) was precipitated by the addition of solid ammonium sulphate (60% saturation) and the precipitate was collected by centrifugation. It was dissolved in 100 mM Tris–HCl, pH 8 containing 0.4 M sodium chloride to give a protein concentration of about 20 mg/ml. Solid ammonium sul-

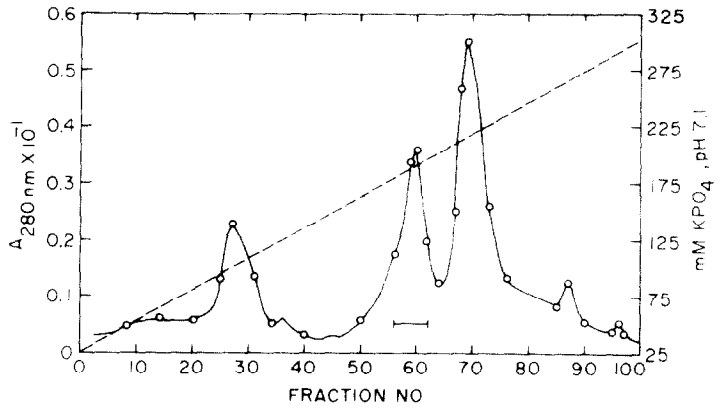

Fig. 2. Purification of psophocarpin B_1. Elution profile of protein from the DEAE-cellulose column, as measured by $A_{280\,nm}$, is represented by the solid line. The broken line represents the concentration of phosphate buffer.

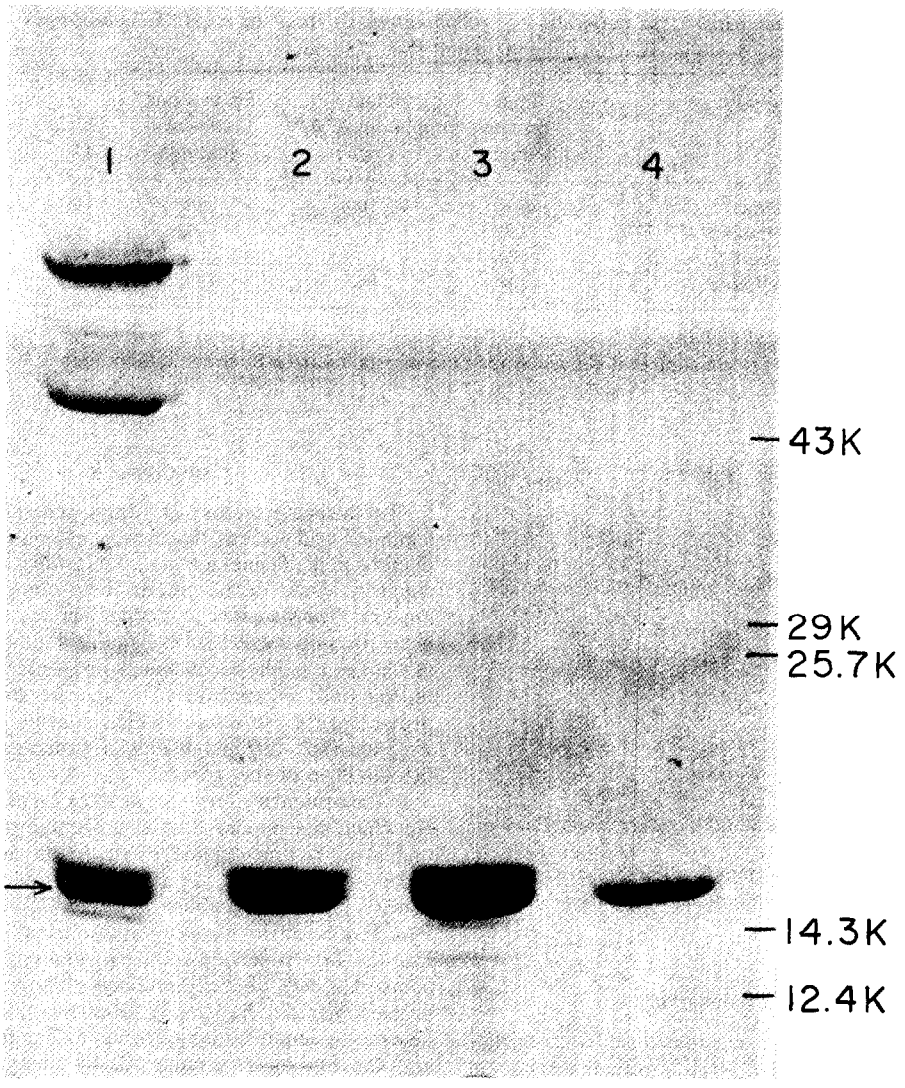

Fig. 3. Protein analysis by SDS-PAGE during the purification of psophocarpin B_1. At different stages of purification, protein was analysed by SDS-PAGE to assess the enrichment of psophocarpin B_1. Lane 1, crude extract (112 μg protein); lane 2, acetone fraction (66 μg); lane 3, gel filtration (75 μg); and lane 4, ion-exchange chromatography (15 μg). Numbers on the right margin represent various standard M_r markers. The arrow on the left indicates the protein band of psophocarpin B_1.

phate was gradually added to this solution till slight turbidity appeared. The solution was centrifuged and to the clear supernatant fluid a few tiny crystals of ammonium sulphate were added and left at 4° with slow stirring. After several days, microscopic needle-shaped crystals appeared (Fig. 4). The crystals could be stained readily with methylene blue and further identified as psophocarpin B_1 by SDS-PAGE and RIA.

Specificity of rabbit antisera

Immuno-electrophoretic studies with the rabbit antiserum raised against the purified psophocarpin B_1 gave a single precipitin band for the latter as well as for a crude winged bean seed extract. Similar single precipitin bands were obtained in double-diffusion studies on Ouchterlony plates (data not shown). These results attest to the specificity of the antiserum and its suitability for RIA.

Distribution of psophocarpin B_1

The total soluble protein and the distribution of psophocarpin B_1 were studied in various tissues of the plant by protein estimation and RIA. The results presented in Table 2, show that the seed contains the largest amount of protein, 9% of which is psophocarpin B_1 and the tuber has only about 10% soluble protein as compared to that of the seed. However, 12% of the soluble protein in the tuber is psophocarpin B_1. The protein content in the pod, the stem and the leaf is rather small and among them, only the pod has a small amount of psophocarpin B_1 (1% of the total soluble protein).

Table 1. Summary of the purification of psophocarpin B_1 from the seeds of *Psophocarpus tetragonolobus*

Steps	Total protein (mg)	Total psophocarpin B_1* (mg)	Sp. protein† content (mg/mg)	Yield (%)
1. Crude extract	4410	410	0.09	100
2. Acetone fraction (25–70%)	2039	372	0.18	91
3. Gel filtration	556	170	0.31	41
4. DEAE-cellulose	84	84	1.00	20

*As assayed by RIA.
†Specific protein content is defined as the ratio of psophocarpin B_1 to the total protein.

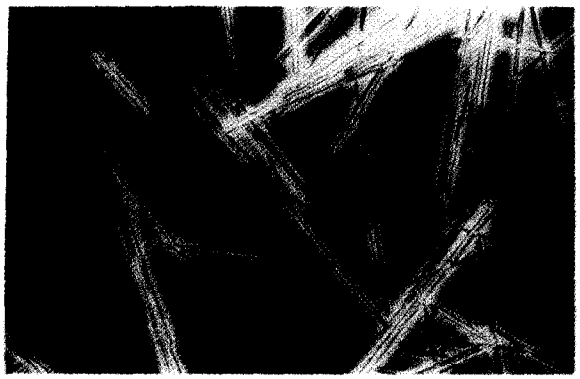

Fig. 4. Crystals of psophocarpin B_1. Photomicrograph was taken using a Carl Zeiss Photomicroscope III (× 430).

Psophocarpin B_1 and seed germination

Winged bean seeds were germinated under controlled conditions and the psophocarpin B_1 contents were quantitated by RIA in the cotyledon, the root and the shoot. Results presented in Table 3 show that there is a gradual disappearance of this protein in the cotyledon resulting in about 76% loss in 21 days of germination. In the growing root, small amounts of this protein were detected in only 10 and 15 day samples whereas in the shoot it was detectable only in the 10 day sample.

DISCUSSION

The storage proteins of legumes, generally classified as legumins and vicilins, have been extensively studied in *Glycine max*, *Pisum sativum*, *Vicia faba* and *Phaseolus vulgaris*. Many details about these complex molecules including the structure and organization of the encoding genes are now available [4–10]. Our studies dealing with the structure, physicochemical properties, tissue distribution and metabolism of a specific storage protein, provide some information which is lacking in the case of *Psophocarpus tetragonolobus*, a potentially important protein crop of the tropics.

In the present studies, the purification of psophocarpin B_1 which can be classified as a storage protein since it undergoes a time-dependent depletion during seedling growth (Table 3) [11, 12] has been carried out. Its homogeneity has been established by alkaline PAGE, SDS-PAGE, isoelectric focussing and immunological studies. For the first time one of the proteins from this plant source has been obtained in a crystalline form. Its M_r has been estimated to be around 20 000 by gel filtration in the absence of denaturating agents as well as by SDS-PAGE and the finding of similar M_r by these two methods indicates the absence of quaternary structure of the protein. While the earlier work of Gillespie and Blagrove [2, 13] provides some valuable information about the nature of the proteins of the winged bean in general, the present studies are directed towards the understanding of a single protein in a greater detail; and this required us to devise a procedure which yields a pure protein as judged

Table 2. Tissue distribution of psophocarpin B_1

Tissue	Soluble protein (mg/g wet tissue)	Psophocarpin B_1 mg/g wet tissue	Psophocarpin B_1 mg/mg total soluble protein	Psophocarpin B_1 Percent of total soluble protein
Seed*	220.5	20.5	0.09	9
Tuber	22.9	2.64	0.12	12
Pod	4.6	0.06	0.01	1
Stem	4	Negligible	—	—
Leaf	10	ND	—	—

*Values are taken from Table 1.
ND, Not detectable. Tissue homogenates were prepared as described in the text (Results section) and psophocarpin B_1 was assayed by RIA.

Table 3. Levels of psophocarpin B_1 at different stages of germination

Time of germination (days)	Psophocarpin B_1 (mg/g wet tissue)		
	Cotyledon	Root	Shoot
0	15	*	*
5	13	*	*
10	10	0.8	0.6
15	6	0.7	ND
21	3.6	ND	ND

*Not done.

ND, Not detectable. Seeds were germinated as described in the text and homogenates were prepared as usual. Psophocarpin B_1 was assayed by RIA.

by various accepted criteria of protein homogeneity.

In spite of the relative abundance of seed storage proteins, the purification of a specific storage protein is handicapped by the lack of a quick assay method as well as by the problem of heterogeneity which often makes these proteins complex and difficult to obtain as a single species. Earlier workers fractionated these proteins on the basis of differences in solubility and the monitoring was usually done by electrophoretic or sedimentation methods. Derbyshire *et al.* [12] have already emphasized the limitations of such conventional methods of storage protein fractionation. In these studies, we have adopted SDS-PAGE as the assay method which is rather slow and laborious. Nevertheless, this approach has enabled us to obtain a pure protein, suitable for the preparation of specific antibody and the latter, in turn, has led to the development of an RIA method, the sensitivity of which has been adjusted in the range of 0.1–9 μg of psophocarpin B_1, by minimizing the specific activity of ^{125}I-protein. If desired, the sensitivity can be increased to the pg level.

The finding of substantial amounts of psophocarpin B_1 in the tuber of this plant may be quite significant. To the best of our knowledge, this is the only report of the same storage protein occurring in large quantities in two specialized storage organs, i.e. the seed and the tuber and it raises intriguing questions regarding regulation of gene expression in these tissues. The storage protein genes are considered to be unique since their expression is known to occur only in the seeds at a specified stage of their development [14]. In view of the general interest in the mechanisms of regulation of gene expression, there exists an opportunity to investigate whether similar mechanisms are operative in the regulation of the same gene in two storage organs which develop in very different environments.

EXPERIMENTAL

Materials. SDS, PMSF and standard protein M_r markers were purchased from Sigma and DEAE-cellulose (DE-52), from Whatman. Sephadex G-50 (medium), Sephadex G-75 (superfine) and Pharmalyte were obtained from Pharmacia, and Iodogen from Pierce. Freund's complete and incomplete adjuvants were purchased from Difco. Carrier-free Na^{125}I was supplied by Bhabha Atomic Research Center, Trombay, India. All other reagents used were of analytical grade.

Winged bean seeds were obtained locally and for germination studies, the seeds were sterilized with a soln of $HgCl_2$ and washed thoroughly with sterile H_2O and allowed to germinate on moist filter paper at 30°. The cracking of the seed coat was counted as day zero and germination was continued on moist sterilized sand.

Polyacrylamide gel electrophoresis. Slab SDS-PAGE was carried out in 12% acrylamide as described in ref. [15]. Alkaline-PAGE was done in tubes using 7.5% acrylamide by the same procedure in the absence of SDS. Protein was stained by Coomassie blue.

Immunological studies. Rabbits were immunized by two intradermal injections of the homogeneous protein, emulsified in complete Freund's adjuvant followed by another dose of booster in incomplete Freund's adjuvant, given at 10 day intervals. A total of about 1.5 mg protein was injected per rabbit. After 7 days, the rabbits were bled through the ear vein; and bleeding was continued at weekly intervals. Sera were tested by immunodiffusion [16] as well as by immunoelectrophoresis in Tris–glycine buffer (pH 8.3) on 1.5% agarose plates [17].

Radio-immunoassay. Psophocarpin B_1 was iodinated with ^{125}I using iodogen [18] to give 3×10^4 cpm/μg protein and the RIA method was standardized according to ref. [19]. Limiting amounts of decomplemented rabbit antiserum were incubated with predetermined amounts of labelled protein having constant count, in a total vol. of 350 μl for 8 hr at 25° and then it was further incubated for 12 hr at the same temp. with varying quantities of the unlabelled protein to compete with the radioactive protein. The resultant antigen–antibody complexes were precipitated with goat anti-rabbit IgG and the radioactivity was determined in an automatic gamma counter. On the basis of the linearity of the standard curve, the range of the assay was fixed between 0.1 and 9.0 μg of psophocarpin B_1.

Determination of M_r. For the native protein, M_r was determined by gel filtration on a Sephadex G-75 column (2 × 140 cm) equilibrated with 50 mM NaPi buffer, pH 7.2 containing 200 mM NaCl. Catalase and dinitrophenyl-lysine were used as markers for the void and internal vols of the column, respectively. M_r of the denatured protein was carried out by SDS-PAGE as described earlier. In both the cases, protein M_r markers were employed and linear standard curves were constructed.

Determination of pI. Isoelectric focussing was carried out on 5% polyacrylamide gel with Pharmalyte (pH 3–9) as the carrier ampholyte for 45 min at 510 V and 45 min at 1500 V. Protein was stained by Coomassie blue. The pI of several standard proteins was found to be linear with the distance of their migration.

Protein determination. Protein was assayed according to ref. [20] using bovine serum albumin as the standard.

Acknowledgements—This work was supported in part by the Department of Science and Technology (Government of India) and AR is a Junior Research Fellow under the Council of Scientific and Industrial Research, India. We thank Mr. Prasun Dutta of the Dept. of Zoology, Calcutta University, Calcutta, for the photomicrograph of the protein crystals.

REFERENCES

1. Anonymous (1981) in *The Winged Bean: A High-Protein Crop For the Tropics*, 2nd edn. National Academy Press, Washington, DC.
2. Gillespie, J. M. and Blagrove, R. J. (1978) *Aust. J. Plant Physiol.* **5**, 357.
3. Kortt, A. A. (1985) *Arch. Biochem. Biophys.* **236**, 544.
4. Chrispeels, M. J. (1984) *Phil. Trans. R. Soc. Lond.* **304**, 309.

5. Boulter, D. (1984) *Phil. Trans. R. Soc. Lond.* **304**, 323.
6. Sun, M., Slightom, J. L. and Hall, T. C. (1981) *Nature (London)* **289**, 37.
7. Nielsen, N. C. (1984) *Phil. Trans. R. Soc. London* **304**, 287.
8. Gatehouse, J. A., Croy, R. R. D. and Boulter, D. (1984) *CRC Crit. Rev. Plant Sci.* **1**, 287.
9. Bailey, C. J. and Boulter, D. (1970) *Eur. J. Biochem.* **17**, 460.
10. Bailey, C. J. and Boulter, D. (1972) *Phytochemistry* **11**, 59.
11. Larkins, B. A. (1981) in *Biochemistry of Plants* (Marcus, A., ed.) Vol. 6, p. 449. Academic Press, New York.
12. Derbyshire, J. W., Wright, D. J. and Boulter, D. (1976) *Phytochemistry* **15**, 3.
13. Blagrove, R. J. and Gillespie, J. M. (1978) *Aust. J. Plant Physiol.* **5**, 371.
14. Chlan, C. A. and Dure III, L. (1983) *Molec. Cell. Biochem.* **55**, 5.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680.
16. Ouchterlony, O. (1948) *Acta Pathol. Microbiol. Scand.* **25**, 186.
17. Grabar, P. and Williams, C. A. (1953) *Biochim. Biophys. Acta* **10**, 193.
18. Markwell, M. A. K. and Fox, F. C. (1978) *Biochemistry* **17**, 4807.
19. Hunter, W. M. (1978) in *Handbook of Experimental Immunology* (Weir, D. M., ed.) Chap. 14, 3rd edn. Blackwell Scientific Publications.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.