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ISOLATION OF LEGUMIN-LIKE PROTEIN FROM *PHASEOLUS AUREUS* AND *PHASEOLUS VULGARIS*

E. DERBYSHIRE and D. BOULTER

Department of Botany, University of Durham, Durham City, England

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Key Word Index—*Phaseolus aureus*; *P. vulgaris*; Leguminosae; seed storage protein; seed globulins; protein bodies; legumin.

Abstract—An 11S seed globulin has been isolated from *Phaseolus aureus* and *P. vulgaris* by zonal isoelectric precipitation and the MWs of the constituent subunits determined. The protein of *P. vulgaris* occurs in the protein body fraction and its chemical composition, including the *N*-terminal amino acids and amino acid composition has been determined. The similarity between the 11S globulin of the two *Phaseolus* spp. and legumin from other legumes is discussed.

INTRODUCTION

Legumin, an 11S globulin first isolated as an impure fraction from seeds of *Pisum sativum* [1,2] has been purified from *Vicia faba* seeds where it represents approximately 75% of the storage protein [3–5]. Legumin-like globulins are also major seed storage proteins of other legume species for example *Vicia sativa* [6] and *Glycine max* [7–9] and they probably occur widely among flowering plants [10]. Globulins which sediment as 11S components have been identified in several species of *Phaseolus* and were equated with legumin by Danielsson solely on the basis of their sedimentation coefficients [2]. The only species of *Phaseolus* from which an 11S globulin has been isolated previously is *P. aureus* [11] and the subunit composition reported for this globulin differs from that of legumin-like globulins.

We have now isolated a legumin-like globulin from *Phaseolus aureus* and *P. vulgaris* and have characterized that of the latter species.

RESULTS

The crude globulin preparations from *Phaseolus vulgaris* and *P. aureus* were each separated into two protein fractions by zonal isoelectric precipitation [5,12]. One of these fractions consisted of protein which was soluble at pH 4·7 and eluted from the column in a volume of 50 ml immediately after the void volume (130 ml), while the other consisted of protein insoluble at pH 4·7 and which was substantially retarded (V_e approximately equal to 2·5 V_o) and eluted in a volume of 90 ml. The retarded fraction from each species accounted for approximately 15% of the protein recovered (250/300 mg) and was homogeneous in the ultracentrifuge (s_{20} approx. 11S) except for traces (approx. 5%) of a larger component (s_{20} approx. 16S). By comparison the unretarded protein was heterogeneous at pH 7 in the ultracentrifuge and for both species the Schlieren diagrams showed a large 7S peak which was not completely resolved from a small 2S-3S peak: a 6S shoulder was also present in the 7S peak of *P. vulgaris*. When the unretarded fraction of *P. vulgaris* was examined in the ultracentrifuge at pH 6·2 and I = 0·15 more than 50% of the 7S component observed at pH 7 was replaced by a peak which corresponded to 18S protein and under these conditions the sedimentation coefficient of the 6S component of *P. vulgaris* was 6·1S i.e. it had a calculated MW of approximately 130000. The unretarded protein of *P. aureus* did not associate to 18S at pH 6·2 and I = 0·15. Agglutinating activity was detected only in the unretarded fractions; agglutination by that of *P. vulgaris* was more rapid than by that of *P. aureus*.

The protein of the retarded fraction from each species migrated during disc electrophoresis as a major band with mobility 0·15 relative to that of the bromophenol blue marker band and two slower and one faster minor bands. The proteins were dissociated by treatment with sodium dodecyl sulphate (SDS) and 2-mercaptoethanol (2Me) into major subunits with apparent MWs 62000 and 20000; only trace amounts of stain were retained by these subunits when half gels were stained for glycoprotein by the PAS procedure.

Further characterization was then carried out on the protein recovered from the retarded fraction of both species. The protein was first precipitated by the addition of $(NH_4)_2SO_4$ to 70% saturation and then redissolved in a small volume of 0·01 M 2-Me. This method of recovery was used rather than precipitation by dialysis since protein precipitated by dialysis against deionized water did not redissolve in 0·4 M NaCl–0·01 2 Me–0·05 M NaH_2PO_4, pH7. The recovered protein of the retarded fractions sedimented in the ultracentrifuge with s_{20w} = 11·6S (*P. vulgaris*) and 11·4S (*P. aureus*) at pH 7·2 and I = 0·4 except for a minor component with

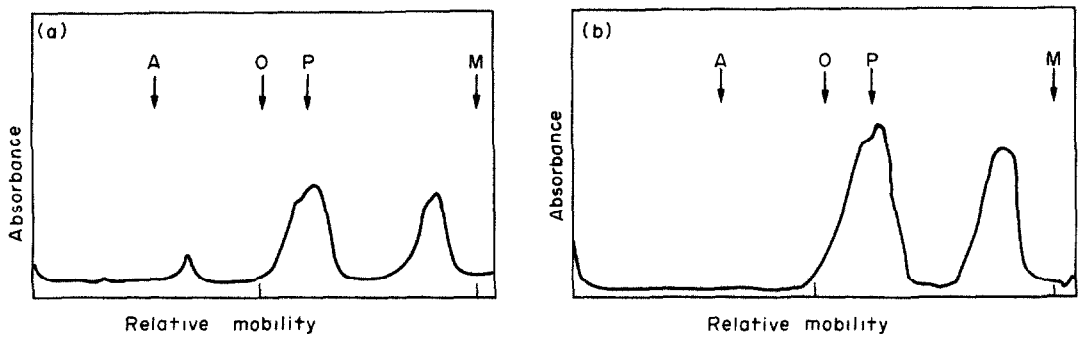

Fig. 1. SDS polyacrylamide gel electrophoresis of reduced dissociated 11S globulins from (a) *Phaseolus aureus*, and (b) *Phaseolus vulgaris*. Proteins (80 μg) were run on 10% gels and stained in Amido Black. The mobilities of serum albumin (A), ovalbumin (O) and pepsin (P) relative to that of myoglobin (M) are shown for comparison.

s_{20w} = 16S. The approximate MWs of the 11S components are 340000 and 330000 respectively. The gel patterns obtained by disc electrophoresis of the native proteins and continuous electrophoresis of the subunits of protein dissociated with SDS and 2 Me were the same as those obtained directly with the retarded fractions before the precipitation and recovery step. However, SDS-2Me treatment preceded by incubation of the proteins in 6 M urea (1 hr at 45°) dissociated them both to subunits with MWs 37000, 34000 and 20000 (Fig. 1).

The nitrogen and sulphur contents of the 11S globulin of *P. vulgaris* were 17·8 (w/w) and 0·44% (w/w) respectively, and 0·5% (w/w) carbohydrate was present (Table 1). Glycine, leucine, threonine and methionine were determined as *N*-terminal amino acids, and the amino acid composition of the protein is presented in Table 1.

Table 1. The chemical composition of 11S globulin of *Phaseolus vulgaris*

Amino acid composition (g/100 g protein)*	
Asp	8·6
Thr	3·9
Ser	5·0
Glu	12·8
Pro	3.8
Gly	3·6
Ala	3·8
Val	5·5
Met	1.4
Ile	4.5
Leu	7·7
Tyr	3·7
Phe	5·2
Lys	7·9
His	3.2
Arg	6.0
½ Cys	0·5 ± 0·05†
Trp	1·0 ± 0·1†
Kjeldahl N	17·8 ± ·2% (w/w)†
Total S	0·41 ± ·1% (w/w)†
Carbohydrate	0·5 ± ·07% (w/w)†
N-terminal amino acids	glycine, leucine, threonine, methionine

* Best values of means of duplicate samples hydrolysed for 20 and 70 hr, except values for Thr and Ser which were obtained by extrapolation to zero time, ½ Cys (which was determined as cysteic acid after 20 h hydrolysis only), and Trp which was determined colorimetrically [41]. † Means and errors of duplicate samples.

Approximately 10% of the protein of the fraction (G1A) which was precipitated from an ascorbate extract of *P. vulgaris* sedimented in the ultracentrifuge as an 11S component at pH 7·2 and I = 0·4, although it was not completely resolved from the major 7S peak of this fraction. Its probable identity with the legumin-like globulin referred to above is indicated by the presence of subunits with MWs of 62000 and 20000 as minor components when the G1A protein was dissociated by treatment with SDS and 2Me: other minor bands, MWs 55000 and 33000, were present in the gels and the MWs of major components were 50000, 47000 and 24000. The G1A fraction agglutinated red blood cells.

An 11S molecular component was detected also when the protein of the protein body fraction of *P. vulgaris* was examined in the ultracentrifuge at a protein concentration of 10 mg/ml at pH 7·2 and I = 0·4; it was a minor component only and was not completely resolved from the major 7S peak, which had a 6S shoulder. Subunits with MW of 62000 and 20000 were minor components of protein body protein dissociated without the use of urea, and subunit with MW 50000, 47000, 34000, 31000 and 24000 were the major components of this fraction.

DISCUSSION

Zonal isoelectric precipitation has been employed previously to isolate pure legumin from protein precipitated by ammonium sulphate precipitation from initial extracts of *Vicia faba* [5] and *V. sativa* [12,13]. However we have found it necessary, when isolating the 11S globulins from *Phaseolus* spp. by this procedure to first remove small molecules from the initial extracts by molecular sieving and also to inhibit oxidative processes. The 11S globulins isolated from *Phaseolus aureus* and *P. vulgaris* are insoluble at pH 4·7 at I = 0·3 and they each have sedimentation coefficients, MWs and subunit sizes which are similar to those of legumin of *Vicia faba* [5] and *V. sativa* [13,14]. The 11S globulin of *P. vulgaris* and legumin of *V. faba* are similar in amino acid composition, *N*-terminal amino acids and in their low content of carbohydrate.

The *N*-terminal amino acids of legumin of members of the Vicieae are glycine, leucine and threonine [3,5,13,15,16] and those of *Glycine max* are glycine, leucine and phenylalanine [8]: unlike the situation in *Phaseolus vulgaris* therefore, *N*-terminal methionine

has not been reported in these legumins. Several explanations for the apparent finding of *N*-terminal methionine can be proposed. It may be there as a true *N*-terminal of the protein or it could be a contaminant if the protein were not completely purified. A further possibility arises since the synthesis of polypeptide chains in the cytoplasm of eukaryotes is initiated by *N*-terminal methionyl residues which are subsequently cleaved from the completed chains [17,18]. Thus the *N*-terminal methionyl residue detected may be the initiating methionyl residue.

The presence of contaminating material could explain the low levels of carbohydrate found in several 11S globulin preparations [10]; however it could also indicate that legumin contains a glycoprotein subunit. The carbohydrate content of subunits isolated from 11S globulins of legumes have not been reported but a 2·7S subunit of the 12S globulin of *Brassica napus* contains 4–5% carbohydrate [19].

The three banded pattern of subunits with MWs 37000, 34000 and 20000 found in SDS gels of dissociated 11S globulin from *Phaseolus aureus* and *P. vulgaris* is also found in dissociated legumin from *Vicia faba* [5] and *V. sativa* [14]. The subunit with MW 62000 seen in some of the *Phaseolus* preparations would appear to be an association product and a component of similar MW is also seen in dissociated legumin preparations from *V. faba* and *V. sativa* under special conditions. In dissociated 11S globulin from *Glycine max* only two sizes of subunits, with MWs 37000 and 22000 have been separated so far [9]. Dissociated 11S globulin from *Lupinus angustifolius* has subunits with MWs 63000, 53000, 40000 and 20000 which differ somewhat from the above [20]. However the complete dissociation and separation of the 11S globulin subunits are very dependent on the conditions employed and a critical comparison can be made between subunit compositions of various globulins only when the latter have been dissociated under identical conditions and their subunits examined by a variety of techniques.

The high nitrogen and dicarboxylic acid/amide contents of the 11S globulin of *Phaseolus vulgaris* relative to those of a standard protein [21], its occurrence in the protein body fraction and its similarity to legumin suggest strongly that this protein is, like other legumins, a storage protein. Whereas the 11S globulins of *Vicia faba* and *Glycine max* contribute substantially to the nutritional value of these beans, the major globulin of *Phaseolus vulgaris* is Glycoprotein II [22,23] and legumin represents a much smaller proportion of the protein of these beans. However, the composition of the storage proteins is different in different cultivars of the same species [24–27] and other cultivars of *Phaseolus aureus* and *P. vulgaris* may have higher legumin contents and be of greater nutritional value than those used in the present investigation. Procedures based on those employed here may play a part in screening programmes for the selection of lines with improved nutritional status.

The unretarded fractions will not be discussed in detail here but the presence in *Phaseolus aureus* of high concentrations of 7S protein which does not associate to an 18S molecular species suggests, contrary to the suggestion of Ericson and Chrispeels [11], that this protein is not identical to the 7S Glycoprotein II of *P. vulgaris* [22]. Agglutinins with sedimentation coefficients of approximately 6S have also been isolated from *P. vulgaris* [28,29] and it is probable that these were the proteins of the unretarded fraction which sedimented as 6.1S species.

The Gl fraction of ascorbate extracts prepared from *Phaseolus vulgaris* by Sun *et al.* [30,31] is regarded by them as Glycoprotein II and an 11S protein was not detected, although they had reported previously that the sedimentation coefficient of Gl (32) was 11S. The subunits identified in dissociated Gl protein were also major components of a dissociated Gl fraction prepared by isoelectric precipitation [31] and they equate Glycoprotein II procedurally with legumin [31]. By contrast the GlA fraction which we isolated was heterogeneous and contained 11S protein, 7S protein (Glycoprotein II), and possibly a globulin agglutinin. The 11S globulins which have been isolated from various species as (relatively) pure proteins are very similar to legumin of *Vicia faba* when characterized by modern biochemical techniques [10]. They have similar sedimentation coefficients, MWs, subunit sizes, amino acid composition, N-terminal amino acids, low contents of carbohydrate and low solubilities at pH 4·7 at $I = 0·3$, and we regard them all as legumin by analogy with other well characterized proteins, for example the haemoglobins, although only when their amino acid sequences have been determined will it be clear whether they are functionally similar proteins or whether they are a group of proteins related by a common ancestry.

EXPERIMENTAL

Isolation of 11S globulins. Seed meals of *Phaseolus aureus* (green gram) and *P. vulgaris* cv. "Streamline", were dispersed with 10 g insoluble polyvinyl-pyrrolidone in 0·4 M NaCl–0·01 M 2 Me-0·1 M NaH_2PO_4 pH 7 (10 g meal/100 ml), and were blended for 3 min in a high-speed blender which was cooled in ice, and then stirred for 15 min. Each extract was filtered through 2 layers of muslin and clarified by centrifugation, then 60 ml clarified extract, containing approximately 1 g protein was applied to a column of Sephadex G-25 (6·6 cm × 20 cm), which had been equilibrated with 0·2 M NaCl-0·035 M NaH_2PO_4 pH 7–0·01 M 2 Me. The column was developed with the latter buffer. The chromatographic fractions which contained protein excluded from the Sephadex G-25 were bulked and adjusted to 70% saturation with respect to $(NH_4)_2SO_4$ and the protein which precipitated was quickly collected by centrifugation and redispersed in 10 ml 0·2 M NaCl–0·05 M NaH_2PO_4, pH 8–0·01 M 2 Mc (pH 8 buffer soln). Ammonium sulphate was removed from the protein soln by passing it through a column (3·4 × 20 cm) of Sephadex G-25, which had been equilibrated with the pH 8 buffer solution. After five-fold concentration of the protein solution by use of an Amicon ultrafiltration cell 8 ml, containing approximately 300 mg protein, was applied to a column (3·4 × 35 cm) of Sephadex G-50 which had been equilibrated with 0·2 M NaCl–0·05 M citric acid pH 4·7–0·01 M 2 Me and the column was then developed with the pH 8 buffer solution (zonal iso-electric precipitation (12)) at a flow rate of 25 ml/hr. Buffer solns which were employed had been deaerated and flushed with N_2 before the addition of 2 Me and were stored under N. The Sephadex used for chromatography had been deaerated before equilibration. Column eluates were monitored continuously at 280 nm by use of an LKB Uvicord II analyser.

Ascorbate extract. An ascorbate extract of meal of *Phaseolus vulgaris* was prepared by the method of McLeester *et al.* [32] and clarified by centrifugation. The clarified extract was diluted with two volumes of dionized water at 4° and the protein which precipitated was collected by centrifugation and designated the GlA fraction.

Protein bodies. These were isolated by centrifugation through glycerol, a method suggested by M. J. Chrispeels.

Seed meal (500 mg) of *Phaseolus vulgaris* was suspended in 5 ml 80% glycerol in 0·05 M NaH_2PO_4 pH 8 and centrifuged at 3000 *g* for 20 min and then at 23000 *g* for 20 min. The supernatant was layered over 10 ml 90% glycerol in the same buffer and centrifuged at 23000 *g* for 20 min. The pellet was resuspended in 5 ml 80% glycerol in buffer, large aggregates of material were removed by low speed centrifugation and the protein bodies were pelleted at 23000 *g*.

Analytical centrifugation. This was carried out at 40000 rpm and 20° in an Omega II 70000 ultracentrifuge. Proteins were centrifuged at concentrations of 5 mg/ml and 1 mg/ml and sedimentation coefficients, s_{20} and s_{20w} values were determined as described by Svedberg and Pedersen [33]. The Schlieren peaks obtained at the lower protein concentration were too small for accurate measurement and values quoted in the text were obtained at concentrations of 5 mg/ml, except where stated otherwise. Approximate MW's were calculated by the method of Halsall [34].

Dissociation of protein. Equal volumes of protein soln (2 mg/ml) and 2·5% SDS–0·1 M 2 Me–0·05 M Pi buffer pH 7 were mixed and heated for 3 min in a boiling water bath and then cooled in running tap water. Myoglobin was employed as a marker protein and after heating in SDS–2Me–Pi buffer pH 7, it was added to a portion of each dissociated protein sample to a final concentration of 0·7 mg/ml.

Polyacrylamide-gel electrophoresis. Undissociated proteins were examined in 7·5% gels by the procedure of Ornstein and Davis [35] but without the use of spacer gel. Dissociated proteins, 20 μl and 80 μl vols, were loaded under buffer onto 10% gels of 60 mm length in a continuous 0·1 M Pi, pH 7–10 mM sodium thioglycolate system (11,31): 15 mA/gel was maintained for 3 hr. Gels were stained for protein in 1% (w/v) Amido Black in 7% HOAc and destained by diffusion in 7% HOAc. Certains gels, on which the subunits of 11S globulin had been separated, were split longitudinally and one half of each gel was stained for protein while the other was stained for glycoprotein by the periodic acid–Schiffs base procedure [37]. The electrophoretic mobilities of the subunits relative to that of the marker protein were determined after staining and the apparent MW's of the subunits were estimated by reference to a calibration curve prepared using cytochrome *c*, myoglogin, pepsin, ovalbumin and serum albumin.

Agglutinin activity. Protein fractions were adjusted to pH 7 and a protein concentration of 1 mg/ml and then mixed with an equal vol of a 2% suspension of human group O red blood cells in 1% NaCl at RT.

Chemical composition. Total notrogen was determined by an automated micro-Kjeldahl method [38], and total sulphur was estimated by wet digestion with perchloric-nitric acid mixture, and subsequent turbidimetric analysis of $BaSO_4$ [39]. Carbohydrate was determined by the method of Dubois *et al.* [40]. The amino acid composition of acid hydrolysed (20 and 70 hr) protein was analysed on a Locarte automatic loading amino acid analyser. Tryptophan was determined colorimetrically [41] and cysteine plus cystine were determined by performic acid oxidation [42] and subsequent analysis of cysteic acid on the Locarte analyser. *N*-Terminal amino acids were determined by the procedure of Gros and Labouesse [43]; after hydrolysis each sample was loaded on one side of a polyamide sheet, a mixture of dansylated amino acids was loaded onto the reverse side and the *N*-terminal amino acids of the sample were identified after chromatographic separation by reference to the pattern obtained from the amino acid mixture.

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REFERENCES

1. Osborne, T. B., (1924) *The Vegetable Proteins*, 2nd edn., Longmans, Green, London.
2. Danielsson, C. E. (1949) *Biochem. J.* **44,** 387.
3. Bailey, C. J. and Boulter, D. (1970) *European J. Biochem.* **17,** 460.
4. Wright, D. J. and Boulter, D. (1972) *Planta (Berlin)* **105,** 60.
5. Wright, D. J. and Boulter, D. (1974) *Biochem. J.* **141,** 143.
6. Vaintraub, I. A. and Shutov, A. D. (1966) *Biokhimiya* **31,** 726.
7. Wolf, W. J. (1972) *Food Technol.* **26,** 44.
8. Catsimpoolas, N., Rogers, D. A., Circle, S. J. and Meyer, E. W. (1967) *Cereal Chem.* **44,** 631.
9. Catsimpoolas, N., Kenney, J. A., Meyer, E. W. and Szuhaj, B. F. (1971) *J. Sci. Fd. Agric.* **22,** 448.
10. Derbyshire, E., Wright, D. J. and Boulter, D. (1976) *Phytochemistry* **15,** 3.
11. Ericson, M. C. and Chrispeels, M. J. (1973) *Plant Physiol.* **52,** 98.
12. Shutov, A. D. and Vaintraub, I. A. (1965) *Ukr. Biokhim. Zh.* **37,** 177.
13. Vaintraub, I. A. and Nguyen Thanh Thien (1968) *Dokl. Akad. Nauk SSSR* **180,** 1239.
14. Vaintraub, I. A. and Nguyen Thanh Thien (1971) *Mol. Biol.* **5,** 59.
15. Grant, D. R. and Lawrence, J. M. (1964) *Arch.. Biochem. Biophys.* **108,** 552.
16. Jackson, P., Boulter, D. and Thurman, D. A. (1969) *New Phytol.* **68,** 25.
17. Boulter, D., Ellis, R. J. and Yarwood, A. (1972) *Biol. Rev.* **47,** 113.
18. Zalik, S. and Jones, B. L. (1973) *Ann. Rev. Plant Physiol.* **24,** 47.
19. Goding, L. A., Bhatty, R. S. and Finlayson, A. J. (1970) *Can. J. Biochem.* **48,** 1096.
20. Blagrove, R. J. and Gillespie, J. M. (1975) *Aust. J. Plant Physiol.* **2,** 13.
21. Smith, M. H. (1966) *J. Theoret. Biol.* **13,** 261.
22. Pusztai, A. and Watt, W. A. (1970) *Biochim. Biophys. Acta* **207,** 413.
23. Racusen, D. and Foote, M. (1971) *Can. J. Botany* **49,** 2107.
24. Tombs, M. P. (1964) *Nature, London* **200,** 1321.
25. Adriaanse, A., Klop, W. and Robbens, J. E. (1969)) *J. Sci. Fd Agric.* **20,** 647.
26. Gillespie, J. M. and Blagrove, R. J. (1975) *Aust. J. Plant Physiol.* **2,** 29.
27. Davis, D. R. (1973) *Nature (New Biol.)* **245,** 30.
28. Pusztai, A. and Watt, W. A. (1974) *Biochim. Biophys. Acta* **365.** 57.
29. Barker, R. D. J., Derbyshire, E. and Boulter, D. (1976) *Phytochemistry* **15.** (in press).
30. Sun, S. M., McLeester, R. C., Bliss, F. B. and Hall, T. C. (1974) *J. Biol. Chem.* **249,** 2118.
31. Sun, S. M. and Hall, T. C. (1975) *J. Agric. Food Chem.* **23,** 184.
32. McLeester, R. C., Hall, T. C., Sun, S. M. and Bliss, F. A. (1973) *Phytochemistry* **12,** 85.
33. Svedberg, T. and Pedersen, K. O. (1940) *The Ultracentrifuge*, Oxford University Press, London.
34. Halsall, H. B. (1967) *Nature, London* **215,** 880.
35. Ornstein, L. and Davis, B. J. (1961) *Disc Electrophoresis*, Preprint by Distillation Products Inductries (Eastman Kodak Co.), Rochester, New York.
36. Weber, K., Pringle, J. R. and Osborn, M. (1972) *Methods Enzymol.* **26c,** 3.
37. Zacharius, R. M., Zell, T. E., Morrison, J. H. and Woodlock, J. J. (1969) *Anal. Biochem.* **30,** 148.
38. Varley, J. A. (1966) *Analyst* **91,** 119.
39. Mottershead, B. E. (1971) *Laboratory Practice* **20,** 483.
40. Dubois, M., Gillies, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28,** 350.
41. Bencze, W. L. and Schmidt, K. (1957) *Anal. Chem.* **29,** 1193.
42. Moore, S. (1963) *J. Biol. Chem.* **238,** 235.
43. Gros, C. and Labouesse, B. (1969) *European J. Biochem.* **7,** 463.