Phytochemistry, 1978, Vol 17, pp. 605–609 Pergamon Press Printed in England

CHICKPEA SEED PROTEINS: MODIFICATION DURING GERMINATION

K. GANESH KUMAR and L. V. VENKATARAMAN

Protein Technology Discipline, Central Food Technological Research Institute, Mysore 570013, India

(*Revised received* 10 *August* 1977)

Key Word Index—*Cicer arietinum*; Leguminosae; chickpea; seed proteins; germination; protein modification.

Abstract—Changes in the proteins of chickpea during a 12-day germination period are reported using techniques of gel filtration, DEAE-cellulose chromatography, polyacrylamide gel (PAG) electrophoresis and ultracentrifugation. In the ultracentrifuge, the total proteins of dormant seeds resolve into 3 components which have the sedimentation coefficients of 2.2 S, 6.9 S and 10.3 S respectively. On germination, the presence of fractions of lower sedimentation coefficient indicates possible degradation of these components; in the early stages, the degradation rate of the 7 S fraction is higher, while the 10 S fraction is broken down faster in the later stages. Gel filtration experiments indicate the possibility of degradation of high polymer into intermediary products. Increase in the relative mobility of protein components on PAG and elution constant on DEAE-cellulose chromatographs indicates an increase in the net negative charge of the protein fractions. The accumulation of subunits of the proteins is negligible during the germination period.

INTRODUCTION

The nitrogenous constituents of leguminous seeds are primarily in the form of storage or reserve proteins [1, 2]. These storage proteins are concentrated in subcellular organelles called protein bodies [3, 4]. During germination the protein contained in the protein bodies is degraded finally to amino acids [5] by the enhanced activity of the proteolytic enzymes. The amino acids are either translocated to the developing axis [6] or utilised in *de novo* synthesis of metabolic proteins in the cotyledons [7].

The characteristics of the storage proteins of legume seeds and their mobilization during germination have been extensively studied in peas (*Pisum sativum*), groundnut (*Arachis hypogea*), soybean (*Glycine max*) and mung bean (*Phaseolus aureus*) using the techniques of gel and immuno electrophoresis, ion exchange chromatography and ultracentrifugation [6, 8–11]. Degradation of the polymeric constituents like, protein and carbohydrates of chickpea with progressive stages of germination has been reported [12–15]. However, an extensive literature search has not revealed detailed work on the alterations of protein characteristics in cotyledons during germination of chickpea.

In this study, we present data on the characterization, of the proteins of chickpea. The techniques of gel filtration, sedimentation velocity, ion-exchange chromatography and gel electrophoresis are used to follow protein changes in seeds for up to 12 days from germination.

RESULTS

Growth of seedlings

Germination of seeds was uniform under the experimental conditions described. During germination, the growth kinetics of the axis (Fig 1) followed the pattern of a typical sigmoid curve. Until day 3 the axis showed little increase in dry wt. However, after this period a steep rise in dry wt was observed. With the onset of germination, dry wt of the cotyledons progressively decreased.

Changes in nitrogenous constituents

The total N and protein N (expressed per pair of cotyledons, on dry wt basis) of the cotyledons showed a progressive decrease (Fig. 2). Pronounced decrease of cotyledonary protein (*ca* 67 %) with concomitant increase in the amount of non-protein N was observed between day 6 and 9. The amide N values were not altered significantly along the germination period studied.

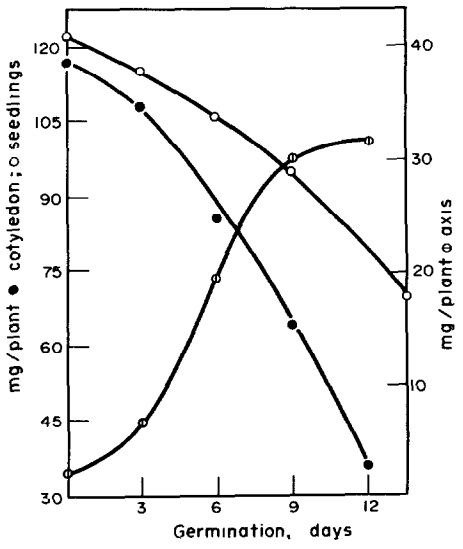

Fig. 1. Growth kinetics of chickpea germination.

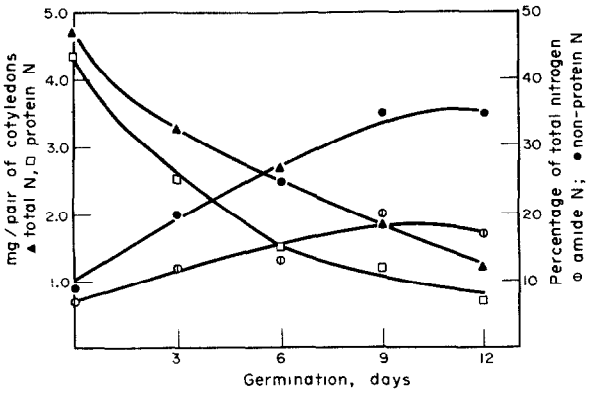

Fig. 2. Changes in the nitrogenous constituents of chickpea cotyledons during germination.

Gel filtration studies

On fractionation on Sepharose-6B gel column, the total cotyledonary proteins of ungerminated seed gave 4 fractions with Ve/Vo value 1.16 (I), 2.11 (II), 2.74 (III) and 3.52 (IV) respectively (Fig. 3). The first turbid fraction eluted after the void volume (Vo). Fraction II and III had no nucleic acid contamination, whereas fraction IV contained nucleic acids. None of the fractions was homogeneous on PAG electrophoresis. The major constituents of fractions II, III and IV were shown to be 10 S, 7 S and 2 S proteins respectively in the sedimentation velocity experiments.

The elution behaviour and relative percentage distribution of the protein fractions altered as a result of germination (Fig. 3a–e). In the elution pattern, no quantitative change in fraction I was observed, whereas the peak of fraction II gradually shifted towards that of fraction III, indicating its degradation. The profile of fraction III did not show any significant variation.

During the initial stages of germination (up to day 3), the relative proportion of fraction II increased whereas that of fractions III and IV decreased. This may be due to a greater degradation of these two fractions than that of fraction II at this stage of germination. This is also supported by the observed increase in Ve/Vo values of fractions III and IV, from 2.56 to 2.58 and from 3.52 to 3.78 respectively.

At day 6, maximum degradation of proteins occurred. The Ve/Vo value of fraction IV of this stage was the same as that of the ungerminated one (*ca* 3.52) and its relative amount was at its minimum (*ca* 10%). This newly formed fraction may represent the degradation product of the high MW protein fraction. Further its possible formation from fraction II between day 3 and 6 was indicated (a) by its (fraction IV) low relative abundance at day 6 (*ca* 10%) and the subsequent increase in the proportion of this fraction during successive stages of germination (viz. day 9, 24% and day 12, 42%) and (b) by the decrease in the proportion of fraction II (from 35 to 28%) with concomitant increase in Ve/Vo (from 2.41 to 2.53). The later stages of germination was characterized by (a) increase in the proportion of fraction IV and (b) absence of fraction II by day 12.

DEAE-cellulose chromatography

On DEAE-cellulose ion-exchange chromatography, proteins of ungerminated cotyledons were fractionated into 6 well defined components (Fig. 4). The 4 main fractions were eluted at NaCl concentrations of 0.09, 0.18, 0.28 and 0.32 M respectively. Among them the protein fractions with 0.28 M and 0.32 M elution constant could be identified with globulins. The proteins extracted from the germinated seeds behaved differently on DEAE-cellulose column in the following respect (Fig. 4a–d). With germination, the elution constant of the 4 major fractions increased. The percentage of the unadsorbed protein fraction increased as germination reached the day 12 period.

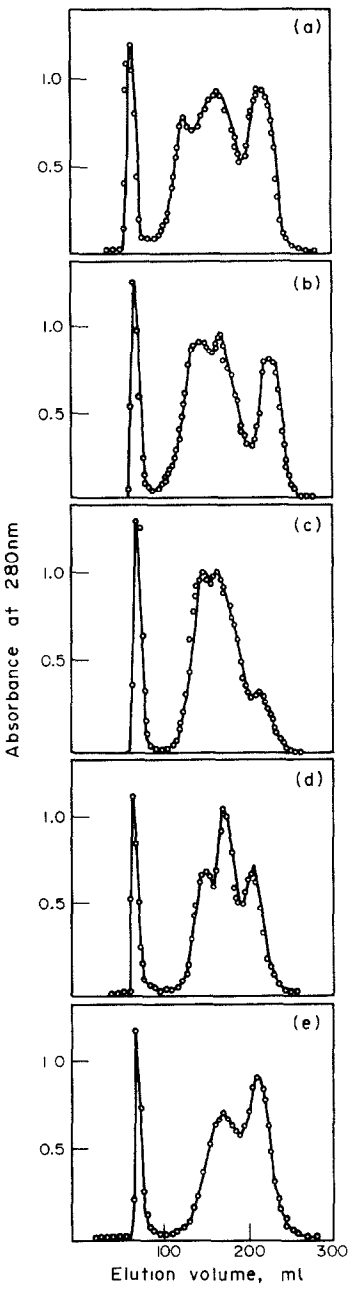

Fig. 3. Gel filtration patterns of chickpea cotyledonary proteins. (a) ungerminated; (b–e) 3,6,9 and 12 day germinated respectively.

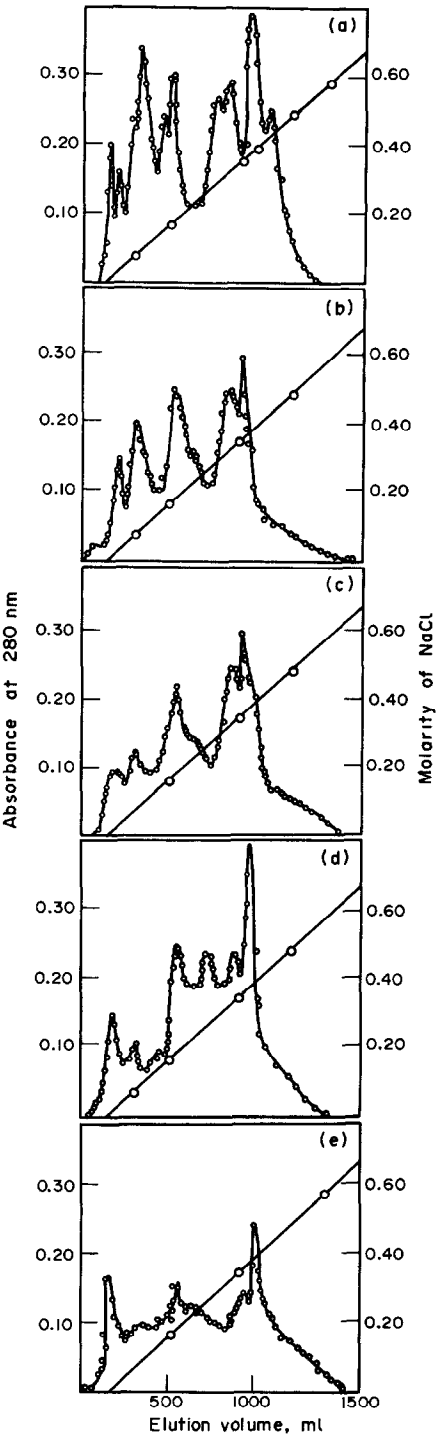

Fig. 4. DEAE-cellulose ion-exchange chromatographic patterns of chickpea cotyledonary proteins. (a) ungerminated; (b–e) 3, 6, 9 and 12 day germinated respectively.

The following generalizations are possible regarding the selective degradation rate of various protein fractions during germination. In the initial stages, the protein fractions with elution constant 0.09 M and 0.15 M underwent greater degradation than in other fractions. The regular quantitative decrease in the amount of 0.09 M fraction suggested its non-accumulation and also its non-formation from other protein fractions. A constant stability in the proportion of 0.18 M eluted fraction indicated either a non-significant storage role until later stages of germination and/or its being in a state of equilibrium, its degradation to other components combined with its formation from higher polymers.

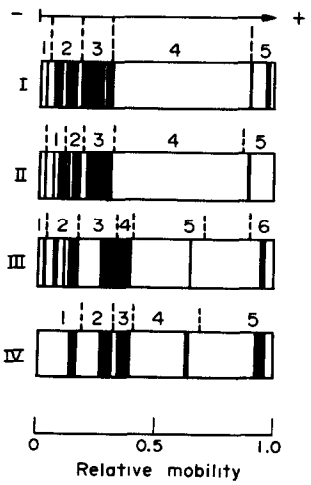

Fig. 5. Microdensitometric scanning of PAG patterns of chickpea cotyledonary proteins. (I—ungerminated; II to IV—3,6 and 9 day germinated respectively.)

PAG electrophoresis

(a) *Without dissociating agents.* The total proteins from the ungerminated seed gave 7 bands on PAG electrophoresis, with two fast moving and 5 slow moving components (Fig. 5). The protein pattern from the successive stages of germination differed from the ungerminated one. Increase in relative mobility of the fractions with respect (to the tracking dye), appearance of dissociation products and decrease in the relative abundance of the major band (with R_f 0.3) were some of the major attributes of the 'germination modified proteins' (Fig. 5).

(b) *With SDS.* Chickpea cotyledon proteins were dissociated in the presence of SDS to their respective subunits (Fig. 6). About 10 subunits could be detected on the gel. The 4 major subunits had MW 78000, 49000, 32000 and 21000 respectively. With proteins from the germinated cotyledons the SDS–PAG electrophoresis showed changes in these 4 subunits. As germination progressed, the relative abundance of the subunit with MW 78000 decreased and eventually disappeared at

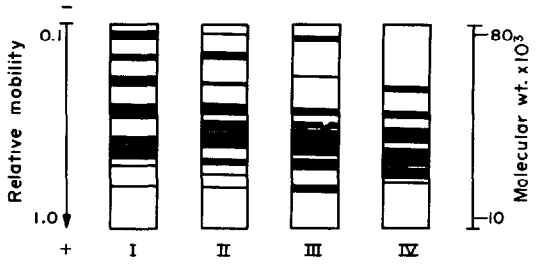

Fig. 6. Microdensitometric scanning of SDS–PAG patterns of chickpea cotyledonary proteins. (I—ungerminated; II to IV—3, 6 and 9 day germinated respectively.)

day 9. Degradation of the subunits into further smaller ones could be seen by the decrease in their relative abundance and appearance of newer subunits. At day 9, only two new subunits having MW of 24000 and 18000 were prominent.

Sedimentation velocity experiments

On ultracentrifugation the ungerminated chickpea protein gave 3 peaks with S values of 2.2, 6.9 and 10.3 respectively, with proportions of *ca* 1:4:5. With germination fractions with decreased S values appeared. At day 3, 3 peaks with S values of 1.2, 6.3 and 10 were observed in the proportion of 1:6:13 respectively. At day 9, the appearance of a new fraction with 3.9S was detected, while the other 2 major components of this stage were 5.65 (37%) and 8.85 (58%).

The reduction in S values of the 3 protein fractions along the germination period indicated possible degradation of the individual components. Qualitatively the degradation process on germination could be represented schematically as follows:

1. $10.3\ S \xrightarrow[3D]{} 10.0\ S \xrightarrow[6D]{} 9.3\ S \xrightarrow[9D]{} 8.8\ S$

2. $6.9\ S \xrightarrow[3D]{} 6.3\ S \xrightarrow[6D]{} 6.1\ S \xrightarrow[9D]{} 5.6\ S$

3. $2.2\ S \xrightarrow[3D]{} 1.2\ S \xrightarrow[6D]{} 1.1\ S \xrightarrow[9D]{} 1.0\ S$

Any critical evaluation of the above proposed scheme of 7 S fraction must take into account, the possible contribution from the degradation of 10 S fraction apart from the modification of the original 7 S fraction during germination.

DISCUSSION

The decrease in the cotyledon dry matter with progressive germination may be attributed to transport of degradation product of organic reserves to the growing axis and to respiratory losses [6, 10]. Degradation of reserve proteins by proteolytic enzymes during germination has been reported in chickpea seeds [13]. This may account for the decrease in cotyledon proteins with a concomitant increase in non-protein N.

The major cotyledon proteins in ungerminated chickpea seeds are salt soluble globulins which account for 80–90% of the total proteins. The globulins comprised 10 S and 7 S fractions (3:2) which were shown to be distinct entities. Existence of reversible association–dissociation system has been reported in ungerminated *Cicer arietinum*, *Vicia faba* and *Pisum sativum* [16, 17]. However, the changes in the proportion of peaks II and III during germination in our gel filtration experiments are not likely to be due to any reversible association–dissociation system. This was evident from our finding no difference in the gel filtration patterns with high and low ionic strength buffers (data not presented).

The net charge of the proteins was altered during germination. Increase in the relative mobility of the individual components on PAG electrophoresis and the elution constants on DEAE-cellulose chromatography indicated the possibility of an increase in the net negative charge of the protein fractions. Similar observations were reported in vicilin of *Pisum sativum* [6], phaseolin of *Phaseolus vulgaris* [18], arachin of *Arachis hypogea* [19] and in 7 S fraction of *Glycine max* [9]. These changes have been attributed to the progressive deamidation of of protein fractions during germination [19, 20].

Electrophoresis of total proteins showed changes as a result of germination. It is difficult to interpret these changes for the following reasons. Different protein components may have similar electrophoretic mobilities. It is also possible that both the monomeric and dimeric forms of the protein may both be present [21]. When SDS was incorporated in PAG, progressive decrease in the amount of subunits having MW range from 50000 to 70000 was observed with the formation of new subunits of 30000–40000. It is possible that the dissociation of reserve proteins into subunits preceeds final metabolism [20].

The sedimentation coefficient of the protein fractions decreased during germination indicating degradation of these components. The amount of the 2 S fraction was not altered significantly. This suggests that accumulation of subunits of reserve proteins during a 9 day germination period was negligible. A similar trend has been reproted in germinating vetch and soybean seeds [10].

Gel filtration and sedimentation velocity experiments suggested a higher degradation rate of 7 S fraction in the early stages of germination, while that of 10 S fraction during the later stages. In *Pisum sativum*, the degradative rates of legumin and vicilin were not different [22], while in *Glycine max* different rates of breakdown of the reserve proteins have been reported [9]. The higher degradation rate may be due to the complexity of protein structure that determines proteolytic susceptibility [23]. Schimke *et al.* [24] have reported that polypeptides of large MW tend to be degraded more rapidly than smaller ones in a wide variety of eukaryotic cells. Other conformational features like helical content and number of disulphide bonds may also play a crucial role in determining the degradation rate of proteins.

More detailed study on fractionation and characterization of some of the major protein components of chickpea and the conformational changes during germination are in progress and will be reported later.

EXPERIMENTAL

Seeds and germination. Seeds of chickpea (*Cicer arietinum*) were obtained locally. They were surface sterilized with $HgCl_2$ (0.1% w/v) and thoroughly washed with H_2O. Germination was carried out in autoclaved moist vermiculite and grown at room temp. (28 ± 2°) in darkness. Only H_2O was provided during the germination period. Cotyledons of 3, 6, 9 and 12 day germinated seeds were taken up for protein studies. The cotyledons were freeze dried, defatted and powdered to 100 mesh size. For growth measurements, 3 replicates of 20 seedlings were harvested, separated into cotyledons and axis, weighed and dry wt determined.

Estimation of nitrogenous constituents. Powdered cotyledons were extracted with 10% (w/v) NaCl, buffered with 0.01 M borate buffer (pH 7.8) for 2 hr (at a solute to solvent ratio of 1:10). The slurry was centrifuged at 5000 rpm for 20 min. The clear supernatant was used for the estimation of total protein, non-protein and amide N. The total N and protein N (TCA-precipitable) were estimated by the micro Kjeldahl method. The non-protein N was estimated by the method of ref. [25] and amide N was estimated by the method of ref. [19].

Preparation and concentration of proteins. Powdered cotyledons obtained from dormant and germinated seeds were extracted in 0.01 M borate buffer of pH 7.8 for 2 hr. The extract was centrifuged and the clear supernatant was dialysed for 48 hr at 4° against 0.02 M Tris–glycine buffer of pH 8.3 for gel electrophoresis, against 0.03 M Pi buffer of pH 7.8 for gel filtration, against the extracting buffer for ion-exchange chromatography and against 0.02 M Tris–glycine buffer of pH 8.3, containing M NaCl for sedimentation velocity experiments. The concn of protein in the dialysate was determined by Lowry's method [26], using BSA as standard.

Gel filtration. Carried out on Sepharose-6B-100 gel in a 2 × 85 cm column. The flow rate was 25 to 30 ml/hr and 2.5 ml fractions were collected.

DEAE-cellulose ion exchange chromatography. DEAE-cellulose was adjusted to the initial pH of borate buffer (pH 7.8) and packed into a 2 × 13 cm column. 100 mg proteins were loaded on the column. A linear gradient of 0.1 M NaCl in borate buffer was established and the eluted protein fractions monitored at 280 nm. The concn of NaCl was determined by titration with standard 0.01 N $AgNO_3$ using K_2CrO_4 as indicator [27].

Polyacrylamide gel (PAG) electrophoresis. (a) *Without dissociating agent.* PAG columns (10%) were prepared and electrophoresis was conducted as in ref. [28]. (b) *SDS–PAG electrophoresis.* Protein samples were dialysed against 0.01 M Pi buffer of pH 7. Samples containing 5 mg/ml protein were incubated with 1% SDS and 1% 3-mercaptoethanol at 98° for 5 min. The SDS-PAG electrophoresis was conducted as in ref. [29].

Staining and quantitation of the protein fractions. Separated protein components on PAG were detected [1] by staining the gel column for 4 hr with 0.5% amido black followed by diffusion in 7.5% HOAc medium or by staining in coomassie brilliant blue for 12 hr followed by diffusion in a MeOH–HOAc–H_2O system. The protein–dye complex on the gel was scanned at 620 nm using the density scale of 1:1.3 in a chromoscanner. The area under each peak was measured and the relative percentages were calculated.

MW standards. Pepsin, α-chymotrypsin, ribonuclease and egg albumin were used as reference proteins in SDS–PAG electrophoresis.

Sedimentation velocity experiments. Expts were performed in a Spinco model E-analytical ultracentrifuge equipped with a rotor temp. indicator unit and phase plate Schlieren optics. A standard 12 mm duraluminium cell centrepiece was used. The expts were carried out at 28° with 1% protein soln using speeds of 59780 rpm. The enlarged tracings of the photographs were used for the calculation of the relative percentage of each fraction. Correction for the radial dilution was not made. The $S_{20,w}$ value of each peak was calculated by the standard procedure [30].

Acknowledgements—The authors gratefully acknowledge the facilities extended by Dr. M. S. Narasinga Rao, Project Coordinator, Protein Technology Disicipline and Dr. B. L. Amla, Director, CFTRI, Mysore. K G thanks the Council of Scientific and Industrial Research, India for providing a research fellowship.

REFERENCES

1. Altschul, A. M., Yatsu, L. Y., Ory, R. L. and Engleman, E M. (1966) *Ann. Rev. Plant Physiol.* **17**, 113.
2. Koller, D., Mayer, A. M., Poljakoff-Mayber, A. and Klein, S. (1962) *Ann. Rev. Plant Physiol.* **13**, 437.
3. Tombs, M. P. (1967) *Plant Physiol.* **42**, 797.
4. Ashton, F. M. (1976) *Ann. Rev. Plant Physiol.* **27**, 95.
5. Boulter, D. and Barber, J. T. (1963) *New Phytologist* **62**, 301.
6. Beevers, L. and Guernsey, F. S. (1966) *Plant Physiol.* **41**, 1455.
7. Young, J. L., Huang, R. C., Vanecko, S., Marks, J. D. and Varner, J. E. (1960) *Plant Physiol.* **35**, 288.
8. Daussant, J. (1975) *The Chemistry and Biochemistry of Plant Proteins* (Harborne, J. B. and Van Sumere, C. F. eds) pp. 31–69. Academic Press, London.
9. Catsimpoolas, N., Campbell, T. G. and Meyer, E. L. (1968) *Plant Physiol.* **43**, 799.
10. Shutov, A. D. and Vaintraub, I. A. (1972) *Rast. Belki* 38.
11. Ericson, M. C. and Chrispeels, M. J. (1973) *Plant Physiol.* **52**, 98.
12. Radionova, N. A. (1957) *Byull. Gl. Botan. Sada* **28**, 56.
13. Azhar, S., Srivastava, A. K. and Krishnamurthi, C. R. (1972) *Phytochemistry* **11**, 3173.
14. Ganesh Kumar, K. and Venkataraman, L. V. (1976) *J. Food Sci. Tech.* **12**, 292.
15. Ganesh Kumar, K. and Ventakaraman, L. V. (1976) *Nutr. Rep. Internatl.* **13**, 115.
16. Jackson, P., Miltons, J. M. and Boulter, D. (1967) *New Phytologist* **66**, 47.
17. Boulter, D. and Derbyshire, E. (1971) *Chemotaxonomy of the Leguminosae* (Harborne, J. B., Boulter, D. and Turner, B. L. eds) pp. 285–308. Academic Press, London.
18. Pusztai, A. and Duncan, I. (1971) *Planta* **96**, 317.
19. Daussant, J., Neucere, N. J. and Yatsu, L. Y. (1969) *Plant Physiol.* **44**, 471.
20. Catsimpoolas, N., Ekenstam, C., Rogers, D. A. and Meyer, E. W. (1968) *Biochim. Biophys. Acta* **168**, 122
21. Derbyshire, E., Wright, D. J. and Boulter, D. (1976) *Phytochemistry* **15**, 3.
22. Danielsson, C. E, (1951) *Acta Chem. Scand.* **5**, 541.
23. Goldberg, A. L. and St. John, A. C. (1976) *Ann. Rev. Biochem.* **45**, 747.
24. Dice, J. F., Dehlinger, P. J. and Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 4200.
25. Bhatty, R. S. and Finlayson, A. J. (1973) *Cereal Chem.* **50**, 329.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
27. Rieman, W., Newes, J. D. and Naiman, B. (1951) *Quantitative Analysis* pp. 70–72. McGraw-Hill, New York.
28. Davis, B. J. (1964) *Ann. NY Acad. Sci.* **121**, 404.
29. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406.
30. Schachman, H. K. (1959) *Ultracentrifugation in Biochemistry* Academic Press, New York.