Seed proteins of cowpea, vigna unguiculata (L.) walp.

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SEED PROTEINS OF COWPEA, VIGNA UNGUICULATA (L.) Walp.

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To my parents

A B S T R A C T

A number of experiments were designed to extract, separate and characterise the major seed proteins of cowpea, Vigna unguiculata (L.) Walp. cv. Prima and to determine their nutritional status by carrying out sulphur-amino acid analyses. Attempts to isolate legumin by various methods were unsuccessful and legumin is either absent or it occurs in small amounts in this cultivar. Three protein subunits made up more than two-thirds of the protein extracted; these had apparent molecular weights 56,200, 53,000 and 48,500. Major subunits and other smaller molecular weight subunits of the total globulins were partially separated by urea-ion-exchange chromatography and characterised. The major subunits are glyco-polypeptides and low in sulphur-amino acids. However, some of the smaller molecular weight subunits contain relatively higher levels of these essential amino acids.

The time and rate at which various molecular components change during fruit development was also investigated. Seed fresh weight was found to be the best parameter to follow the course of fruit development in cowpea. There was a differential rate of synthesis between globulin subunits and the rate of synthesis of the individual major subunits also varied during seed development. The greatest rate of total globulin synthesis was during the drying-out stage just before maturity. Analyses of the nitrogenous components in the developing pod at different stages indicated that the pod acted as a nitrogen reservoir in the early stages of development.

Differences were found in the protein profiles of one cultivar of V. unguiculata and its wild relatives.

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INTRODUCTION

Man has domesticated legumes for over ten millenia and these plants are therefore one of his most ancient food crops. Legumes are important agriculturally mainly for the production of seeds which are rich in protein, in some cases up to 50% on a dry weight basis. These seeds are used for human consumption in both the tropic and temperate zones and also for animal feed in the temperate zones. In some agricultural areas the whole plant of some legume species is utilised as a fodder crop. Legumes also play an important role in improving soil fertility by replenishing nitrogen through nitrogen-fixation, a free source of nitrogen input for mixed rotation farms of subsistence agriculture.

WHO (1951, 1953) and FAO (1964, 1970) Reports indicate that protein malnutrition is a widespread and acute problem in many developing countries. The shortage of protein in a diet especially for children has serious repercussions on health (Waterlow and Alleyne, 1971; Scrimshaw and Gordon, 1968). For mainly socio-economic reasons a large section of the affected population's diet will not include animal proteins for many decades to come and cereals, the basic staple food in many countries, have a low protein/calorie ratio. So where a more or less vegetarian diet is consumed, legumes are a significant source of protein. Most cereals are also limiting nutritionally in the essential amino acid, lysine (Munck, 1972). Grain legumes could play a vital role in supplementing this deficient amino acid in cereal based diets. Thus one possible method to alleviate the protein malnutrition problem is to attempt to increase production of vegetable protein by increasing grain legume production (U.N., 1968). This method

is not only efficient in terms of conversion of energy but also one which is likely to be feasible in many countries. However, in their turn, the protein of many legumes contain limiting sulphur-amino acids, cysteine and methionine. To overcome this problem Boulter et al. (1973) have suggested manipulating the relative ratios of storage proteins in legumes with different sulphur-amino acid contents. A similar situation in maize is illustrated by the high lysine varieties such as Opaque 2. The ratio of the major proteins, zein (low in lysine) to glutelin (relatively high in lysine), in Opaque 2 is reversed when compared to normal maize lines (Mertz et al., 1964).

In order that the breeder may identify high protein and high sulphur-amino acid lines, some basic biochemical information is required on the proteins of legume grains.

Investigations on the classification, isolation, physico-chemical characterisation and chemical composition of major proteins of grain legumes has been going on since the late nineteenth century. A pioneer in the field of seed protein fractionation was Osborne, who first published a major classification of seed proteins according to the classical solubility scheme (Osborne, 1924):

1. Albumins soluble in pure water;
2. Globulins soluble in neutral salt solutions;
3. Prolamins soluble in 70-80% ethanol but insoluble in water or pure ethanol;
4. Glutelins soluble in dilute acids or alkalis but insoluble in water, neutral salt solution or ethanol.

Prolamins and glutelins predominate in cereals but in legume grains the greater part of the protein of the order of 80-90%, is contributed by the seed storage globulins. Osborne's classifica-

tion of major seed proteins based on solubility criteria is not totally exclusive and was questioned by Altschul et al. (1964) but is still used as it provides a working definition in seed protein separation.

Osborne and his co-workers (1898a, 1907) separated the globulins of pea into two components, which they classified as legumin and vicilin. The basis of the chemical classification was (i) legumin in solution did not coagulate when heated to 100° whereas vicilin coagulated upon heating to 95-100°, (ii) legumin was less soluble in dilute salt solutions than vicilin and (iii) legumin contained a higher sulphur content than vicilin.

The next major investigation of legume seed proteins followed on the development of the ultracentrifuge by Svedberg and Lysholm (1927). Daniellson (1949) examined the seed globulins from thirtyfour species in the family Leguminosae and found in all, except in some Acacia species and in Trifolium repens, at least two globulin components with sedimentation coefficient of approximately 7S and 11S. From coagulation experiments Daniellson connected legumin with the component that had a sedimentation constant of 12.64S and vicilin with the component that had a sedimentation constant of 8.10S at infinite dilution. The molecular weight of vicilin was found to be 186,000 and that of legumin 331,000. He also concluded that his results confirmed the hypothesis that closely related species have approximately the same relative concentrations of the two components.

Although the Leguminosae is the third largest family of the seed plants (over 600 genera) most workers after Daniellson concentrated on a few species. Major investigations have been

carried out on the following tribes and genera:-

Glycineae (*Glycine*), Lupineae (*Lupinus*) Stylosantheae (*Arachis*), Vicieae (*Pisum* and *Vicia*) and Phaseoleae (*Phaseolus* and *Vigna*).

These investigations have used various methods of extraction and characterisation of the proteins (chromatography, association-dissociation of seed proteins, immunochemical techniques, isoelectric focusing, N-Terminal analysis, chemical composition and electrophoresis) which often makes it difficult to compare the data from one laboratory with that of another.

Of the abovementioned genera the most intensive investigations have been carried out on an economically important legume grain crop, the soya bean, *Glycine max* (L.) Merr. Soya beans have a long history of use as a protein foodstuff in Asia. Today in the U.S.A., the largest producers of soya beans, it is a major source of edible oil and the meal provides protein for animal feed.

Proteins extracted from soya beans are heterogeneous in molecular size at pH 7.6 to 7.8, 0.5 ionic strength. The range in size is shown by ultracentrifugation, which separates four well-resolved fractions having approximate sedimentation constants of 2S, 7S, 11S and 15S with unfractionated soya bean protein extracts (Naismith, 1955; Wolf and Briggs, 1956) and also on examination of fractions after gel filtration with Sephadex G-200 (Hasegawa *et al.* 1963). Each of the four sedimentation peaks does not represent a single component (Wolf, 1969) and subsequent fractionation has yielded a number of different proteins from the 2S and 7S fractions. The 2S fraction (22% of total) contains several trypsin inhibitors, cytochrome c, allantoinase and two globulins with no known biological

activity. The 7S fraction (37% of total) contains at least four different proteins: beta amylase, hemagglutinin, lipoxygenase and 7S globulin. The 7S globulin corresponds to 50% of the total 7S fraction (Wolf and Smith, 1961). Another one-third of the total protein is made up of the 11S globulin (also called glycinin) which may account for the bulk of the 11S fraction. This fraction should be distinguished from the old term "glycinin" proposed by Osborne and Campbell (1898), which represents a heterogeneous mixture of proteins. The 15S fraction (11% of total) has not yet been isolated and characterised.

Among these four components rapid and reversible association and dissociation reactions under changes in pH, ionic environment and type of salt present have been reported (Rackis et al.,1957). The 7S globulin in the pH range 7.0 to 9.0, 0.5 ionic strength exists as a monomer but at 0.1 ionic strength, the 7S is entirely converted to a new species having a sedimentation coefficient of approximately 9S as a result of dimerization (Roberts and Briggs, 1965). Likewise the 11S globulin forms a disulphide polymer on lowering of ionic strength (Naismith, 1955; Briggs and Wolf, 1957). The ability to form disulphide-linked polymers thus contributing to insolubility (Briggs and Wolf, 1957; Nash and Wolf, 1967) is an added complexity for protein extraction and separation methods.

The 11S and 7S globulin fractions have been extensively investigated as the two fractions make up more than 50% of the total soya bean protein.

The 11S globulin or glycinin has a quaternary structure consisting of 12 subunits based on the following N-Terminals:

glycine (8), phenylalanine (2) and either leucine or isoleucine (2) (Catsimpoolas et al., 1967). But the distribution of the 12 N-Terminals is also reported as glycines (6), phenylalanines (2), leucines (2) and isoleucines (2) (Badley et al., 1975). The reasons given by Badley et al. (1975) for the difference in the analyses is lack of resolution for leucine and isoleucine by some methods and incomplete recovery of N-Terminal amino acids. The 12 subunits have a molecular weight of about 330,000 ± 20,000 and have different isoelectric points, either acidic or basic. Okubo et al. (1969) reported that the basic subunits contained only glycine as N-Terminal amino acid and indicated that the acidic subunit fraction contains no N-Terminal glycine.

Urea gel electrophoresis (Okubo et al., 1969; Kitamura and Shibasaki, 1975) and isoelectric focusing (Catsimpoolas et al., 1967) suggests that there are three different kinds of basic subunits.

Isoelectric focusing indicate three different kinds of acidic subunits (Catsimpoolas et al., 1971) but with urea gel electrophoresis four kinds of acidic subunits were separated and characterised with phenylalanine (2) and leucine or isoleucine (2) as N-Terminal amino acids (Kitamura and Shibasaki, 1975a). The amino acid compositions of the separated acidic subunit fractions were roughly similar and this together with the results of double gel immunodiffusion techniques indicate possible homologous primary structures of the acidic subunits (Kitamura and Shibasaki, 1975b).

The acidic and basic subunits have molecular weights of 37,200 and 22,300 respectively (Catsimpoolas et al., 1971). Kitamura's additional acidic subunit has a molecular weight of

45,000 (Kitamura and Shibasaki, 1975a).

The total sulphur-amino acid in the 11S component is 2.1g/total amino acids (Kitamura and Shibasaki, 1975a).

Several workers have observed that the 7S globulin fractions although homogeneous in an ultracentrifuge, contains several components when subjected to chromatography.

Hasegawa et al. (1963) showed that the water extractable soya bean proteins consisted of seven protein fractions on gel filtration with Sephadex G-200 and that four fractions had different sedimentation coefficients of approximately 7S. By the use of hydroxylapatite column chromatography to separate the globulins, Wolf and Sly (1965) suggested the presence of at least three components and Vaintraub (1964) found at least four chromatographic components with 7S sedimentation coefficient. Except for lysine the same N-Terminal amino acids as reported by Roberts and Briggs (1965) for 7S globulins were found in Vaintraub's 7S fractions.

Electrophoretic studies also show that the 7S components of soya bean proteins consist of several proteins. Thus Hill and Breidenbach (1974a) isolated the major 7S protein by sucrose density gradient sedimentation and demonstrated three major and three minor protein bands by polyacrylamide discontinuous gel electrophoresis.

In recent years Thanh et al.(1975a,b) fractionated purified 7S globulin into at least five components by DEAE-Sephadex A-50 chromatography. Their preliminary electrophoretic results indicate that the five 7S components are composed of four major subunits in different ratios.

Catsimpoolas and Ekenstam (1969) using two different

preparations (Koshiyama, 1965; Roberts and Briggs, 1965) characterised two 7S components by immunoelectrophoresis - these were called γ-conglycinin and β-conglycinin respectively.

The 7S proteins are disrupted by the usual reagent capable of dissociating proteins into their subunits. In 8M urea or 4M guanidine hydrochloride the total 7S protein fraction dissociates from MW 180,000 to components of MW 22,500 and 34,000 by SDS treatment. Experimental evidence suggests that the subunits of 7S globulin are not held together by disulphide bonds (Koshiyama, 1971; Wolf, 1956). Ten N-Terminal residues of the 7S globulin were found: aspartate, alanine, glycine, valine, tyrosine, serine (2), glutamate (2) and leucine (isoleucine) (Roberts and Briggs, 1965). Although Koshiyama (1968) reported the same eight amino acids, he found one N-Terminal glutamate and thus nine N-Terminal residues. When γ-conglycinin was examined by dissociation and electrophoresis, nine subunits were also obtained (Catsimpoolas, 1970).

There is evidence that soya bean contains a 7S protein which is a glycoprotein (Koshiyama, 1966). Furthermore a glycopeptide has been isolated and found to contain the same carbohydrate content as in the starting material (separated 7S protein) i.e. 39 mannose and 12 glucosamine residues per mole of glycopeptide or glycoprotein; the sugars were present in the 7S protein as a single polysaccharide unit (Koshiyama, 1969).

The total sulphur-amino acids in the 7S globulin is relatively low, 0.51 g / 100 g protein (Koshiyama, 1968).

Research into lupin seeds was pioneered by Ritthausen (1882), Osborne and Campbell (1897) and Osborne and Harris (1905) who showed that there were two types of globulins present. Later the physico - chemical studies of Daniellson (1949) and Joubert (1955a, 1955b, 1955c, 1956 and 1957a) indicated the presence of three major globulins with sedimentation coefficients approximately

2S, 7S and 11S.

Three major globulins, termed conglutins α, β and γ of the seeds of Lupinus angustifolius cv Uniwhite have been separated by Blagrove and Gillespie (1975) by preliminary ammonium sulphate fractionation. Conglutin γ differs from conglutin α and β and most other legume storage proteins in its relatively high contents of cystine and methionine, 3.93 (residues %).

The structure of the two peanut globulins, arachin and conarachin (Johns and Jones, 1916; 1917) is complex and conflicting results have been obtained with this species. N-Terminal analyses and denaturation studies have been carried out on arachin by Johnson and Shooter (1950) and Cater and Naismith (1958) and their results indicate a subunit structure for arachin. Tombs (1965) described four of the subunits, two with molecular weights of 35,000 (α, β) and two with molecular weights of 10,000 (γ, δ). Three forms of arachin, A, B and A1 were found with different ratios of these subunits by Tombs (1965) and Tombs and Lowe (1967) who suggested the following model: arachin A was represented by $\alpha 4\ \beta 4\ \gamma 2\ \delta 2$, B by $\beta 8 \gamma 2 \delta 2$ and A1 by $\alpha 8\ \gamma 2\ \delta 2$. There is disagreement however, to the above, when arachin was examined by Singh and Dieckert (1973) who support the following structure: nine subunits with molecular weights ranging from 15,000 to 65,000. Ion-exchange chromatography has been used to isolate the major component of the conarachin fraction, α-conarachin, and this protein was shown to undergo association from a 7.8S form to a 12.6S form (Dechary et al., 1961).

In the tribe Vicieae, two species have been investigated in some detail - Pisum sativum and Vicia faba.

As mentioned previously, the terms legumin and vicilin were first used in reference to the globulins from seeds of Pisum sativum. Legumin and vicilin of pea have different isoelectric points, pH 4.8 and 5.5 respectively. This property was used to separate both proteins by the isoelectric precipitation procedure of Danielsson (1949) (Vaintraub and Gofman, 1961). They found the N-Terminals for legumin to be glycine (8) and leucine (4) and for vicilin, aspartic acid (2) and serine indicating twelve and three different polypeptide chains respectively. Grant and Lawrence (1964) determined similar N-Terminals after separating the two globulins by chromatography on DEAE-cellulose. Reported total sulphur-amino acid compositions were low 0.4 (mole %) and similar in both their legumin and vicilin preparations. However, they obtained one subunit fraction from preparative gels which had 1.1 (mole %) sulphur-amino acids.

The first major work on the storage proteins, legumin (11S) and vicilin (7S) of Vicia faba was by Bailey and Boulter (1970; 1972). They reported that legumin contained three N-Terminal residues, leucine, glycine and threonine and that the three polypeptide chains have molecular weights of 56,000, 42,000 and 23,000. Further studies on legumin, prepared by zonal isoelectric precipitation indicated that it was composed of two types of subunits, acidic and basic with molecular weights of 36,000 and 22,000 respectively (Wright, 1973). Vicilin consists of at least three proteins (Wright, 1973) and contains four subunits and four N-Terminal amino-acids: leucine, threonine, serine and lysine (Bailey and Boulter, 1972). As with the 11S and 7S component of soya beans, the 11S of Vicia faba contains much more total sulphur-amino acids than the 7S fraction (Bailey and Boulter, 1970; 1972).

Three species (Phaseolus vulgaris, Phaseolus aureus and Vigna unguiculata) belonging to the tribe Phaseoleae will be mentioned. The major protein from Phaseolus vulgaris has been separated and characterised as Glycoprotein II (Pusztai and Watt, 1970; Racusen and Foote, 1971). Pusztai found the total sulphur-amino acid content was 0.97g/100g dry weight of protein. Another purified protein has been named Glycoprotein I; it contains 0.06g cystine and 1.16g methionine/100g protein (Pusztai, 1966). Recently Derbyshire and Boulter (1975) have reported a legumin-like protein for this plant. Hall and his co-workers (McLeester et al., 1973; Sun et al., 1974; Sun and Hall 1975) have separated and characterised two fractions G1 and G11 and claimed that these fractions exhibited characteristics of legumin and vicilin (as defined by Osborne and Campbell 1898) respectively. This correlation has been questioned by Wright and Boulter (1973).

Ericson and Chrispeels (1973) reported that the storage proteins in Phaseolus aureus are in close agreement with the properties of vicilin and legumin of Vicia faba as obtained by Millerd et al. (1971).

Work on Vigna unguiculata was first published by Osborne who reported that proteins of cowpea mainly consisted of three globulins (Osborne and Campbell, 1897). The predominant globulin he termed, vignin and although it resembled the legumin of pea, it was essentially different in composition and properties. The second globulin resembled phaseolin (not precipitated from saline solution by acetic acid) found in Phaseolus vulgaris and Vigna angularis. And the third globulin which was extremely soluble in very dilute salt solutions he described as legumelin.

After Osborne no published work appeared on cowpea until Joubert (1957) found the proteins from two cowpea varieties contained a major 7.3S component which was reversibly dissociated. Ammonium sulphate fractionation using 0 to 50% and 50 to 100% saturation of total protein extract followed by DEAE-cellulose chromatography of the two fractions revealed a mixture of legumin and vicilin in the 0 to 50% fraction and only vicilin in the 50 to 100% fraction (Platsynda and Klimenko, 1970).

The cowpea is one of twentyseven genera in the tribe Phaseoleae of the family Papilionaceae (Leguminosae) (Heywood, 1971). The species has been described by various Latin names but a recent taxonomic treatment of Vigna by Verdcourt (1970) classified cowpea as Vigna unguiculata (L.) Walp. subsp. unguiculata (= Vigna sinensis (L.) Savi ex. Hassk.). Three subspecies of Vigna unguiculata were recognised; the other two subspecies referred to catjang and asparagus-bean (yard-long-bean).

Cowpea is widely grown in the tropics and is a common legume in Italy and Spain: it is described by different names in different countries (see Appendix). By Vavilov's criteria (1951) Africa is probably the centre of origin of cowpea since, of 170 species listed for Vigna, 120 are found in Africa (Faris, 1963); also wild relatives of cowpeas have only been reported in West and Central Africa (Faris, 1963; FAO, 1973). Rachie and Roberts (1974) conclude from Sanskritic sources that the cowpea must have reached Egypt, Arabia and India very early. The early Greeks and Romans knew of it and it was introduced by the Spaniards into the West Indies in the 16th century, reaching the United States about 1700. Today cowpea is the most important pulse crop in Africa (Rachie, 1973) and in Nigeria it is a main source of dietary protein (Oyenuga,

1967). The agronomic reasons for selecting cowpea as one of the legume species for improvement have been listed by Rachie (1973). However, the yields of cowpea are very low and several lines of research are being pursued to increase seed production (Rachie and Roberts, 1974). The nutritive value of mature cowpea seed meals has been determined (Boulter et al., 1973; Dividich, 1973; Oke, 1975) and cowpeas have been found to be deficient in cysteine and methionine. Of the toxic constituents usually found in legumes, protease inhibitors exist in cowpeas (Ventura and Filho, 1966; Jaffe, 1973; Royer et al., 1974) and cyanogens (Jaffe, 1950; Montgomery, 1964) but no hemagglutinins have been reported (Liener, 1962).

Very little biochemical information is available on cowpea proteins and further work is needed in order to supply the plant breeder with additional tools in his task of attempting to improve cowpeas for human nutrition.

This thesis gives the results of experiments designed to separate and characterise the major seed proteins of cowpea and to determine their nutritional status by carrying out total sulphur-amino acid analysis in order to supply basic information which could be of use practically.

The time and rate at which the major seed proteins are produced during seed development was also investigated for the same reasons. The nitrogenous components in the developing pod at different stages was also determined.

The range of protein pattern variation between one cultivar of Vigna unguiculata and its wild relatives was ascertained as possible sources of different genetic material.

MATERIALS

I Biological materials

Seeds of Vigna unguiculata (L.) Walp. were obtained from the International Institute of Tropical Agriculture, Ibadan, Nigeria; mature seeds of var Prima were used for extraction and separation of major proteins, immature seeds of accession Tvu 201-1D were used for total sulphur determination and wild relatives investigated for protein patterns were accessions Tvnu 36, 37, 40, 41, 65, 73, 74, 75, 76 and 89.

The strains of Rhizobium used to induce nodulation in cowpea roots were supplied by Rothamstead Experimental Station, Harpenden, Herts.

II Chemicals and Reagents

With the exception of those products listed below, chemicals were obtained from British Drug House (BDH) Ltd., Poole, Dorset and were of analytical grade when necessary.

Sephadex G-50, medium, was obtained from Pharmacia Ltd., 75 Uxbridge Road, London W.5.

Whatman DE 52 Anion-exchange cellulose was obtained from Whatman Ltd., Springfield Mill, Maidstone, Kent.

Visking dialysis tubing was obtained from Gallenkamp Ltd., Stockton-on-Tees.

Bovine serum albumin; fraction V powder;

Ovalbumin, Grade V;

Pepsin;

Myoglobin;

Lysozyme;

Cytochrome c (horse heart);

L-leucine;

were obtained from Sigma (London) Chemical Company, Norbiton Station Yard, Kingston-upon-Thames, Surrey.

D-mannose and Glucosamine-HCl

were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Human Group O erythrocytes

were obtained from a local hospital.

METHODS

I GROWTH OF BIOLOGICAL MATERIALS

Developing Seed and Pod

Seeds of cowpea, V. unguiculata (cv Prima) were used in this investigation. Plants were grown in the greenhouse at a minimum temperature of 18°C and with supplementary lighting to give a 12 h daily light period during 1974 and 1975. Three seeds were sown in each 10 cm plastic pot containing John Innes No. 1 compost. The seeds germinated in 5 days and seedlings were later selected for uniformity in size and vigour and thinned to one seedling per pot.

Ten days after sowing the pots were inoculated with a suitable Rhizobium culture to induce nodulation. There was a lag of 7 to 10 days between inoculation and the establishment of a nitrogen fixation system. After about 8 weeks from sowing the first flowers appeared and every day during the flowering period (about four weeks) fresh and fully opened flowers were tagged. Outcrossing is very low and as flowers open only for a few hours, seed ages are given as days after anthesis or flowering.

Pods from tagged flowers were harvested daily at about the same time of the day. As soon as possible after harvest, seeds were removed from their pods. A pod usually contained 8 to 14 seeds of approximately equal size; sometimes two or three seeds were underdeveloped (smaller) and were discarded. Then the coefficient of variation of the fresh weights of seeds in any pod did not exceed 4%.

Pod length and fresh weight was recorded and the seeds were counted, weighed and the length measured along the long axis.

Both pod and seeds were stored at -20°C or freeze-dried if not used on the same day when harvested.

II PREPARATION OF COWPEA MEALS

Cowpea meals were prepared from mature dry seeds of V. unguiculata (cv Prima) by milling in a Janke and Kunkel K.G. water-cooled mill (type A10). The finely milled meal (mesh size greater than 40) was defatted by repeated extractions with hexane at 4° until the hexane was colourless. Defatted meal was then rinsed with petroleum ether and air dried.

When only small amounts of material was available of the seeds of wild relatives, meals of these varieties were prepared from seeds by milling with a Glen Creston Hammer Mill, type DFH 48, equipped with 0.7 mm screen.

Developing seeds were macerated in a pestle and mortar before extraction or analysis.

III DETERMINATION OF MOISTURE CONTENT AND DRY WEIGHT

Weighed duplicate samples were dried to constant weight at 105°, allowed to cool in a dessicator and then re-weighed to determine their water content. In some cases, seeds and pods were dried to constant weight by lyophilisation.

IV DETERMINATION OF TOTAL NITROGEN CONTENT

The nitrogen content of cowpea meals, protein fractions, seeds (mature and developing) and pods were determined by the micro-Kjeldahl method of Varley (1966) as modified by Evans and Boulter (1974). The determinations were carried out automatically using a Carlo Erba apparatus.

V DETERMINATION OF TOTAL SULPHUR CONTENT

Meals were digested with a mixture of perchloric and nitric acid and total sulphur was estimated using the method of Mottershead (1971) and Evans and Boulter (1974).

VI DETERMINATION OF PROTEIN IN SOLUTION

Protein was estimated by the method of Lowry *et al.* (1951). A calibration graph was prepared using solutions of bovine serum albumin as the standard protein.

VII E X T R A C T I O N

A) Nitrogen and Protein

(i) Sodium dodecyl sulphate extraction

10 mg of various cowpea meals were extracted and dissociated for electrophoresis as described in section XI (i) (b). Insoluble material was removed by centrifugation before the extract was made dense with glycerol.

(ii) Buffered salt extraction pH 8.0

Two different extractants were employed:-

(1) 0.4M NaCl, 0.066M phosphate buffer pH 8.0 (standard buffer);

or

(2) 0.02M borate buffer pH 8.0 (borate buffer).

Defatted meal (2 g) was blended in 50 ml extractant for three 30 sec periods, using a MSE overhead homogeniser at ambient temperature. The extract was filtered through two layers of muslin then clarified by centrifugation for 30 min at 20,000 x g. The residue was dispersed in 25 ml of the same extractant and stirred overnight at 4°. The re-extracted residue was filtered, centrifuged and then dried at 105° for 2 h.

(a) Efficiency of extraction

Nitrogen determinations were carried out on all extracts and residues. To determine protein extraction efficiency portions of the clear extract were dialysed against the same extractant buffer. After 24 h, nitrogen estimations were performed on the non-diffusible part.

Trichloroacetic acid was added to all clear extracts to a final concentration of 7.5% (w/v). The precipitated protein was collected by centrifugation and washed twice in 7.5% trichloroacetic acid at the centrifuge. The washed precipitates were dissolved in 0.1N NaOH for nitrogen determinations.

(b) Globulin preparation

The combined supernatant from the borate buffer extract was dialysed overnight against 0.033M sodium acetate buffer pH 5.0. The precipitate from this dialysis was collected by centrifugation at 20,000 x g for 30 min washed with distilled water and then freeze-dried - pellet was designated the globulin fraction (Pusztai and Watt, 1974).

Further dialysis of the supernatant against fresh acetate buffer pH 5.0 did not yield any more precipitate. The supernatant was then dialysed against distilled water, precipitate collected by centrifugation, freeze-dried and designated the albumin fraction.

(iii) NaOH soluble protein

Alkali - soluble protein was extracted from developing material (pods and macerated seeds) with three successive

extractions with N NaOH (1 : 5 meal-solvent ratio) using an overhead homogeniser.

(iv) Ascorbic acid extraction

This was performed essentially as described by Wright and Boulter (1973) and McLeester et al. (1973). All procedures were at 4°. Meal (5g fresh weight) was homogenised with 50 ml of 0.5M NaCl, 0.25M ascorbic acid and the homogenate filtered through muslin and the filtrate centrifuged at 23,000 x g for 30 min. The supernatant was decanted and further clarified by centrifugation at 23,000 x g for 30 min (2X). An equal volume of deionised water was added to the clear supernatant and the resulting precipitate (globulin fraction G1) was centrifuged down at 23,000 x g for 30 min and washed with deionised water. A small portion of supernatant was diluted with twice its volume of deionised water to check that there was no further precipitation. The remainder of the supernatant was dialysed against deionised water for 15 h, the water being changed at least three times. The precipitate formed was pelleted by centrifugation at 23,000 x g for 30 min and designated G2A. The supernatant was dialysed for a further 24 h and 48 h periods and the respective precipitates designated G2B and G2C.

B) Free Amino Acids

(i) Seeds

The freeze-dried seeds of each stage were ground in a pestle and mortar. 80% ethanol was added to the meal (1 : 10 meal-solvent ratio) before homogenising for 1 min at top speed in an MSE homogeniser. The residue was removed by centrifugation and re-extracted twice and supernatants were combined.

(ii) Pods

The freeze-dried pods from each stage were cut into small pieces with scissors and soaked in hot 80% ethanol (1 : 20 meal-solvent ratio). The slurry was homogenised and after filtering, the procedure repeated twice. All filtrates and washings were combined and mixed with an equal volume of petroleum spirit in a separating funnel to remove the chlorophyll.

An appropriate aliquot of the ethanol extract at each stage was used for analysis. The amount of soluble amino nitrogen was determined using the ninhydrin method described by Moore and Stein (1948, 1954). L-leucine was used as a standard.

VIII FRACTIONATION OF COWPEA SEED PROTEINS

(i) Isoelectric precipitation

The method used by Danielsson (1949) and Bailey and Boulter (1970) was employed. Meal was extracted with standard buffer and the extractions clarified by centrifugation. The supernatant was adjusted to 70% saturation with $(NH_4)_2SO_4$. The precipitate was serially dispersed in standard buffer made to the following concentrations of NaCl: 0.2M, 0.3M and 0.4M. The resulting solutions of protein were adjusted to pH 4.7 by the addition of 1N acetic acid.

(ii) Ammonium sulphate fractionation

Protein was extracted from 5 g defatted meal with 50 ml of standard buffer. An attempt was made to fractionate the proteins by precipitations or ' salting out ' at definite concentrations of ammonium sulphate between the limits 0 to 50%, 50 to 65%, 65 to 70% and 70 to 85% of saturation.

(iii) Zonal isoelectric precipitation

The method used was similar to that described by Shutov and Vaintraub (1965) and Wright (1973) and is outlined below:

(a) Protein sample preparation

5 g of defatted meal was extracted with phosphate buffer. The extract was passed through a Sephadex G-25 column (3.5 cm x 15.5 cm) to remove any small molecules. Ammonium sulphate was then added to the extract to 70% saturation and centrifuged, to pellet the protein.

(b) Buffers

0.2M NaCl, 0.05M citric acid adjusted to pH 4.7 with dilute NaOH (citrate buffer)

0.2M NaCl, 0.05M NaH_2PO_4 adjusted to pH 8.0 with dilute NaOH (phosphate buffer)

Both buffers contained 0.01M mercaptoethanol.

(c) Preparation of Sephadex G-50

The resin was swollen in several volumes of citrate buffer at room temperature and the fines removed.

(d) Elution of protein

The protein pellet obtained above was dissolved in 20 ml of the phosphate buffer and dialysed overnight against the same buffer. 10 ml of the protein solution was applied to the column. The column was washed with the phosphate buffer and the protein then eluted. Column eluant was monitored continuously at 280 nm using an LKB 8300 Uvicord II ultraviolet analyser in conjunction with a LKB Chart Recorder Type 6520-4. Fractions were collected using an LKB Minirac fraction collector.

Gel filtration

Fractions from peak IIA of the zonal isoelectric precipitation (marked on Fig. 8) were pooled and dialyses conducted against distilled water containing 0.01M 2-mercaptoethanol for 24 h at 4°. The content of the dialysis bags were freeze-dried and before chromatography resolubilised in 10 ml of standard buffer containing 0.01M mercaptoethanol.

The salt - soluble proteins were fractionated at room temperature on a 3.5 cm x 35 cm column packed with Sephadex G-200. Proteins were eluted from the column with the standard buffer. Flow rate was maintained at 28 ml/hour and fractions were collected every fifteen minutes.

Effluent fractions were monitored by Unicam SP800 ultraviolet spectrophotometer for absorption at 280 nm. Fraction peaks at 280 nm were pooled and dialysed for 72 h against distilled water.

(iv) Ion - exchange Chromatography on DEAE-cellulose (DE52) by Grant and Lawrence (1964) method

Cowpea proteins were extracted from defatted meals with standard buffer. Protein precipitated between 50 to 100% ammonium sulphate was dissolved in 0.1M NaCl, phosphate buffer ph 8.0 (0.02M Na_2HPO_4, 0.02M NaH_2PO_4) made 0.01M in mercaptoethanol and dialysed for 24 h against the same buffer. Chromatographic separations of these globulins was carried out in columns (1.1 cm x 30 cm) packed with DEAE-cellulose (DE52) equilibrated with the phosphate buffer. Elution of adsorbed material was effected by increasing sodium chloride concentration from 0.1M to 0.35M.

IX PREPARATION OF DISSOCIATED GLOBULINS AND SEPARATION OF SUBUNITS USING ION-EXCHANGE CHROMATOGRAPHY WITH UREA

Fractionation of the globulins of V. unguiculata obtained as insoluble material at pH 5.0 (see Method Section VII (ii) (b)) was carried out on a 1.25 cm x 25 cm column of DEAE-cellulose (Whatman DE52) equilibrated with 0.076M Tris-citrate buffer pH 8.7 made 8.0M in urea and 0.01M in mercaptoethanol (starting buffer) at room temperature. Approximately 90 mg of protein sample was dissolved for 1 h at room temperature in 1 ml of the starting buffer but made 10.0M in respect to urea. The solutions were centrifuged to remove any insoluble materials in the sample before being applied to the column. The column was then rinsed with starting buffer until no A_{280} absorbing material i.e. the unadsorbed fraction, was eluted. A linear 0 to 0.35M NaCl gradient in starting buffer was used to elute the adsorbed material on the ion-exchange cellulose. Elution gradients were determined by conductivity measurements on the effluent. When the eluate was 0.35M in respect to NaCl, elution was continued with 2.0M of NaCl in starting buffer to remove as much of the adsorbed material as possible.

The transmission of the column effluent was monitored automatically at 280 nm by a LKB 8300 Uvicord II. The absorbance of each fraction at 280 nm was also recorded by using a Unicam SP1800 ultraviolet spectrophotometer and cells of 1 cm light path. Fractions of the main chromatographic peaks were also scanned between 230 and 300 nm for the spectrophotometric determination of the absorption maxima (λ max) and to estimate whether there was any contamination with nucleic acids.

The pooled fractions were extensively dialysed for 72 h

against water and then freeze-dried. The lyophilised samples were weighed to determine approximate recoveries and then stored at -20°C; lyophilised fractions were subject to SDS electrophoresis.

X DEIONISATION OF UREA SOLUTIONS

All solutions containing urea were prepared from concentrated ' Analar ' urea solutions after passage through a column of mixed bed resin (' Amberlite ' monobed resin MB-1, analytical grade).

XI POLYACRYLAMIDE GEL ELECTROPHORESIS

(i) Gel systems

The following gel systems were employed:-

(a) non-dissociating pH 8.3 (Ornstein, 1964; Davis, 1964) 7.5% (w/v) acrylamide gels were used except that the spacer gel was omitted.

(b) dissociating (SDS) pH 7.0

The method of Schapiro et al. (1967) and Weber and Osborn (1969) was used to determine the subunit profiles of the samples being investigated.

Meal (10 mg) or protein (approx. 2 mg) was added to 1 ml of 0.2M phosphate buffer pH 7.0, 2.5% (w/v) in sodium dodecyl sulphate and 2% (v/v) 2-mercaptoethanol in a tube previously heated to 100°. After two min of incubation the tubes were cooled in running tap water for one min. This solution was divided into two tubes each with 0.5 ml and to one was added 0.5 ml of standard protein, myoglobin in phosphate buffer (1 mg/1 ml).

0.25 ml of 80% (v/v) glycerol and 6 drops of tracking dye solution (0.04% bromo-phenol blue) was added to each tube.

Preliminary experiments at three gel concentrations (15%, 10% and 7.5%) were tried and 10% (w/v) acrylamide was found to be the most generally useful. 20 µl and 80 µl of samples (± myoglobin were loaded on 10% acrylamide under reservoir buffer and electrophorised at room temperature using a current of 47 mA cm^{-2} until the tracking dye had migrated approximately 55 mm.

(ii) Staining

(a) protein

The gels were stained in 1% (w/v) amido black (Napthalene Black 12 B) in 7% (v/v) acetic acid or 0.1% (w/v) coomassie brilliant blue R250 in methanol : acetic acid : water (90 : 7 : 43 by vol.) and then destained by diffusion in their respective solvents for 2 to 3 days.

(b) glycoprotein

At least 200 µg of protein extracted with standard buffer was electrophorised for staining of sugar residues of glycoproteins. 100 µg of protein was loaded on other gels run at the same time and stained with coomassie blue to localise all the protein subunits.

The method used to detect glycoproteins was the PAS staining techniques of Zacharius et al.

(1969) and Glossman and Neville (1971) as adapted by Croy (1976)

(iii) Recording of electrophoretic patterns

Three methods have been used: 1) photographs, 11) densitometric traces obtained by scanning the gels in transmission at 570 nm (for amido black) and 580 nm (for PAS and coomassie blue) using a Gilford chromoscan and, 111) line drawings.

(iv) Molecular weight determination

Since electrophoretic mobility of proteins after reduction is proportional to log MW in SDS media, the molecular weights of unknown proteins were determined from a calibration curve using proteins of known molecular weights (see Fig.1). The following proteins (molecular weights in parentheses) were used as standards:

serum albumin (64,000), ovalbumin (43,000) γglobulin (25,000 and 55,000), pepsin (35,000) myoglobin (17,000), cytochrome c (12,400) and lysozyme 14,300).

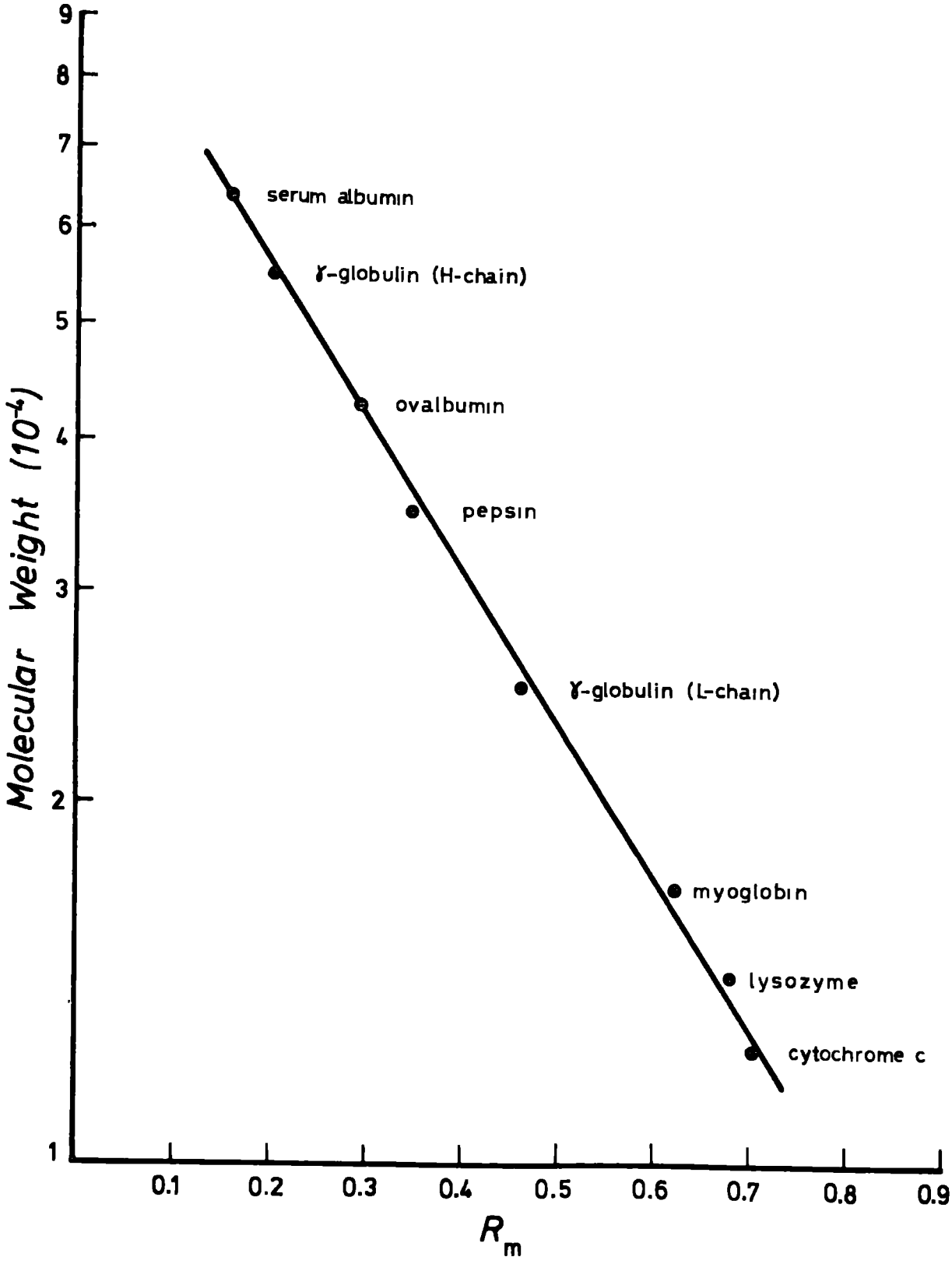
Quoted molecular weights standardised to that of myoglobin were obtained from gels in which a mixture of cowpea proteins of unknown molecular weights and standard protein (myoglobin) were electrophorised. Relative mobilities (Rm) of subunits have been calculated relative to the migration of the bromophenol blue band.

(v) Determination of subunit ratios

Subunit ratios were determined using a

F I G. 1

Molecular weight calibration graph for the dissociating (SDS) pH 7.0 polyacrylamide gel electrophoretic system.


Molecular Weight (10^{-4})
9
8
7
6
5
4
3
2
1
serum albumin
γ-globulin (H-chain)
ovalbumin
pepsin
γ-globulin (L-chain)
myoglobin
lysozyme
cytochrome c
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
R_m

quantitative measurement of the amount of dye bound to each subunit on SDS gels by integrating the densitometric traces of the gels.

X11 ANALYTICAL ULTRACENTRIFUGATION

This was performed using a Christ Omega II 70000 Ultracentrifuge. Centrifugations were carried out at 40,000 rev/min and 60°. Sedimentation coefficients and $s_{20,w}$ values were determined as described by Svedberg and Pedersen (1940).

X111 SEROLOGICAL METHODS

(i) Agglutination test

Protein samples dissolved in 0.9% (w/v) sodium chloride ph 7.0 were mixed with an equal volume of a 2% (v/v) suspension of washed group O erythrocytes in the same solution, at room temperature.

(ii) Production of antiserum

Rabbits were injected by a course of 12 to 14 intravenous injections, spread over 6 to 7 weeks, of seed proteins (5 to 15 mg of protein/injection) in standard buffer. The animals were killed and their blood collected 5 days after the last injection.

(iii) Immunoelectrophoresis

A micro method based on that of Grabar and Williams (1955) and Hirschfield (1960) was used. The conditions were as follows: A solution of a 0.75% agar gel in Tris-EDTA-Borate buffer pH 9.0 (Dudman and Millerd, 1975), was melted and 2 ml spread on a glass micro-slide (75 mm x 25 mm) with a pipette. Seed samples were applied in preformed wells 27.5 mm from one end of the slide

- this end, during electrophoresis, was the cathode end. Electrophoresis of the seed proteins was conducted in a Shandon apparatus at a voltage of approximately 200V at the power pack and continued until after the centre of the bromo-phenol blue spot had migrated 30 to 35 mm towards the anode. The polyvalent antiserum (0.2 ml) was placed into the troughs and allowed to diffuse for 3 days at 4°C. The interaction of antigen and antiserum produced precipitin arcs which were stained with 0.1% (w/v) coomassie blue and then destained with 7% (w/v) acetic acid.

XIV ANALYTICAL CHEMICAL METHODS

(i) Amino acid analysis

Samples in 6N HCl were hydrolysed for 22 h and sometimes 72 h *in vacuo* at 105° in sealed Pyrex tubes. The amino acid composition of hydrolysates were analysed on a Locarte automatic loading amino acid analyser.

(ii) Cysteine determination

Cysteine ($\frac{1}{2}$-Cystine) was determined after performic acid oxidation as cysteic acid by the method of Schram *et al.* (1954)

(iii) N-Terminal amino acid analysis

N-Terminal amino acids were determined by the dansyl method of Gros and Labouesse (1969).

(iv) Neutral sugars determination

Neutral sugars were estimated by the phenol-sulphuric acid method (Dubois *et al.*, 1956). Mannose was used as a standard.

(v) Hexosamine determination

Total hexosamines were determined using the Morgan-Elson reaction (Morgan and Elson, 1934) as recommended by Good and Bessman (1964) after prior hydrolysis with 4N HCl for 6 h (Spiro, 1966). Glucosamine-HCl was used as a standard.

RESULTS

I THE EXTRACTION AND CHARACTERISATION OF THE PROTEIN OF COWPEA MEALS

A) Preliminary analyses

Before considering the actual extraction and characterisation of the cowpea protein the moisture content of the defatted meals was determined to be 10.4 ± 0.04%. The total nitrogen content of such meals was 4.34 ± 0.07% after correcting for moisture.

B) Efficiency of extraction of nitrogenous materials

64% of the total nitrogen in the meal was extracted during a first extraction in standard buffer (see Table 1). An additional 7.5% nitrogen was solubilised in the second extract from the residue. The nitrogen content of the residue not solubilised was 21% of the original total nitrogen of the meal.

54% of the total meal nitrogen in the initial extract remained in the dialysis sac when the extract was dialysed against the extractant buffer overnight. Not all material precipitated with trichloroacetic acid dissolved in 0.1N NaOH which probably accounts for the lower protein N figures for material precipitated than for protein N of the non-diffusible part.

The total nitrogen and protein nitrogen contents of the fractions obtained by extraction with borate buffer were very similar to those prepared by use of standard buffer.

A 100 g of defatted cowpea meal contained 12.2 g of globulin and 0.15 g of albumin (both proteins weighed after lyophilisation). The globulin fraction contained 15.91 ± 0.2% nitrogen on a dry weight basis.

T A B L E 1

Extraction of protein and other nitrogenous materials from cowpea, Vigna unguiculata (cv Prima) meals

Dispersing agent	N extracted (g / 100 g of total meal N)	Protein N extracted (g / 100 g of total meal N) (a)	(b)	Protein N (g / 100 g of nitrogen extracted)	Residue N
0.4M NaCl, phosphate buffer pH 8.0					
1st extract	64.0	34.0	54.0	84.4	
2nd extract	7.5	4.2			21.0
0.02M borate buffer					
1st extract	64.0	36.0	56.0	87.5	
2nd extract	8.7	5.1			19.6

The amount of protein nitrogen extracted is estimated :-

(a) from materials precipitated with tricholoacetic acid and

(b) from the non-diffusible part of the extract.

C) Protein profile of meals

(i) Polyacrylamide gel electrophoresis

a) alkaline extraction

When the standard buffer extract was analysed by electrophoresis in the non-dissociating gel system a major band Rm 0.12 was obtained together with faster moving minor components with Rm's 0.2 and 0.4 (Fig. 2(a)).

b) SDS extraction

The major polypeptides after SDS electrophoresis of a SDS extract of defatted cowpea meal (Fig 2b) had apparent molecular weights: 85,000, 56,200, 53,000, 48,500, 40,000, 31,300 and 27,000. A minor subunit, apparent molecular weight 20,000 had the lowest staining intensity and was masked by the standard myoglobin used in the gel (see Fig. 3). The subunits with molecular weights 56,200, 53,000 and 48,500 together retained more than two-thirds of the total stain retained in the gel.

To make comparisons with electrophoretic profiles presented later, relative mobilities of the polypeptide chains are given in parentheses: 85,000 (Rm 0.06), 56,200 (Rm 0.2), 53,000 (Rm 0.23) 48,500 (Rm 0.25), 40,000 (Rm 0.32), 31,300 (Rm 0.4), 27,000 (Rm 0.45) and 18,500 (Rm 0.58). The relative mobilities of the same subunits differed on an average by $\pm$ 5% from one electrophoretic run to another.

c) Ascorbic acid extraction

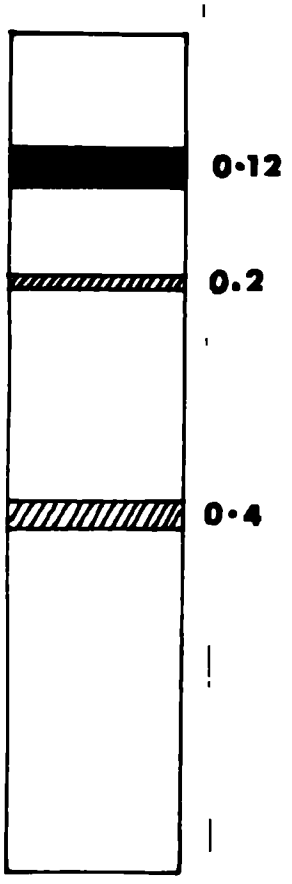
Meals extracted with ascorbic acid were diluted and dialysed according to the procedure given in the Methods (Section VII (A) (iv)). On polyacrylamide electrophoresis seven bands were observed on gels in the globulin fraction G1 (see Fig. 5). The

F I G. 2(a)

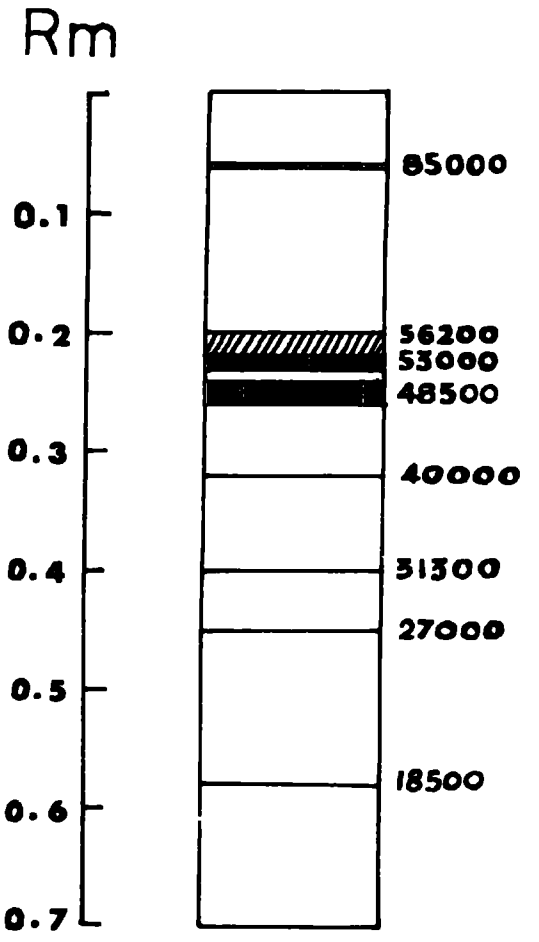
Disc electrophoretic pattern of standard buffer extract of cowpea meal. Mobilities were calculated relative to bromophenol blue.

F I G. 2(b)

SDS electrophoretic pattern of SDS extract of cowpea meal with molecular weights of subunits and relative mobility (Rm) scale.


0·12
0·2
0·4

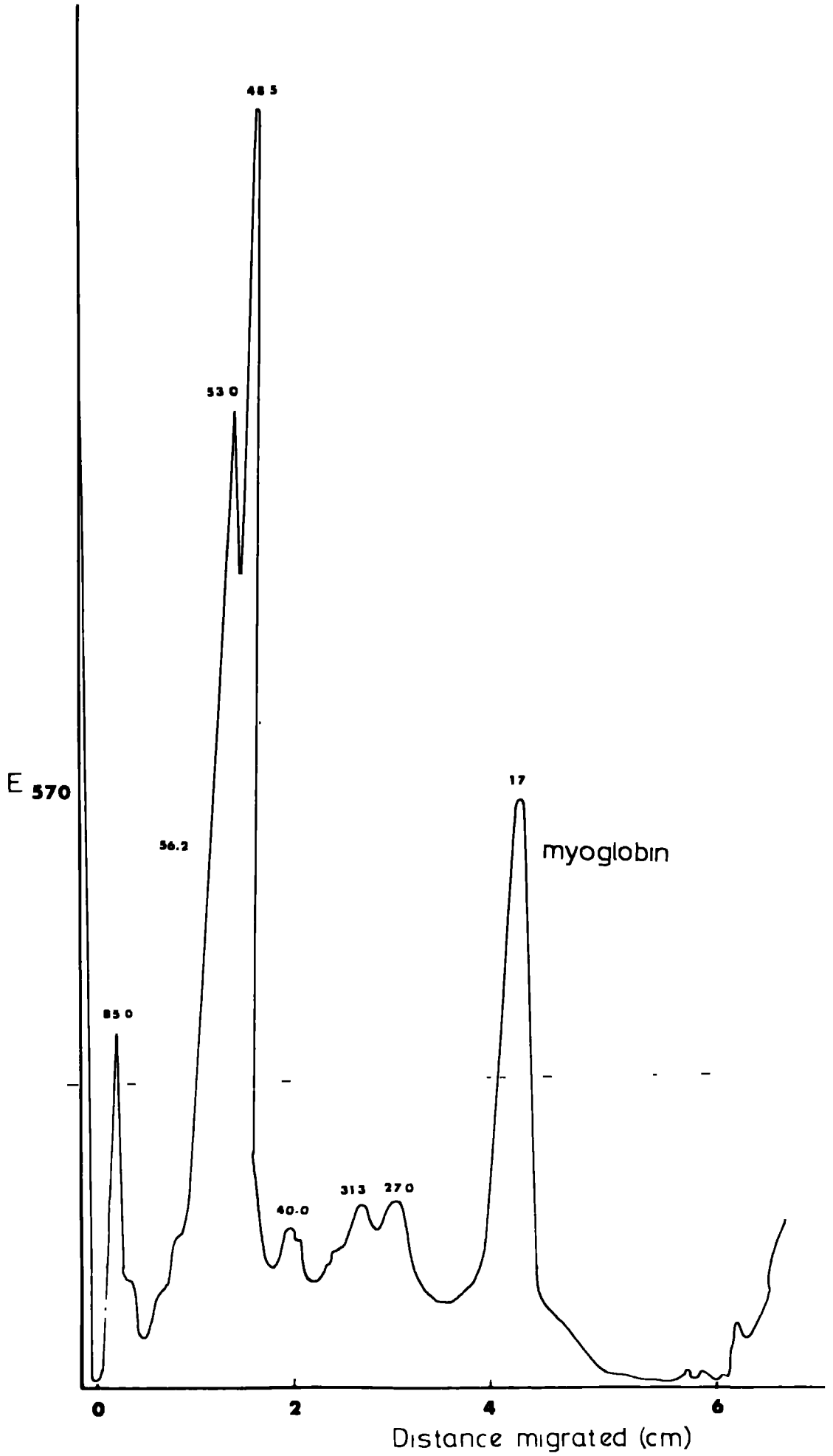
(a)


Rm
0.1
0.2
0.3
0.4
0.5
0.6
0.7
85000
56200
53000
48500
40000
31300
27000
18500

(b)

F I G. 3

SDS electrophoretic profile of SDS extract of cowpea meal. Numbers above peaks refer to the molecular weights (10^{-3}) of the corresponding polypeptides.


48 5
53 0
E 570
56.2
17
myoglobin
85 0
40.0
31 3
27 0
0
2
4
6
Distance migrated (cm)

F I G. 4

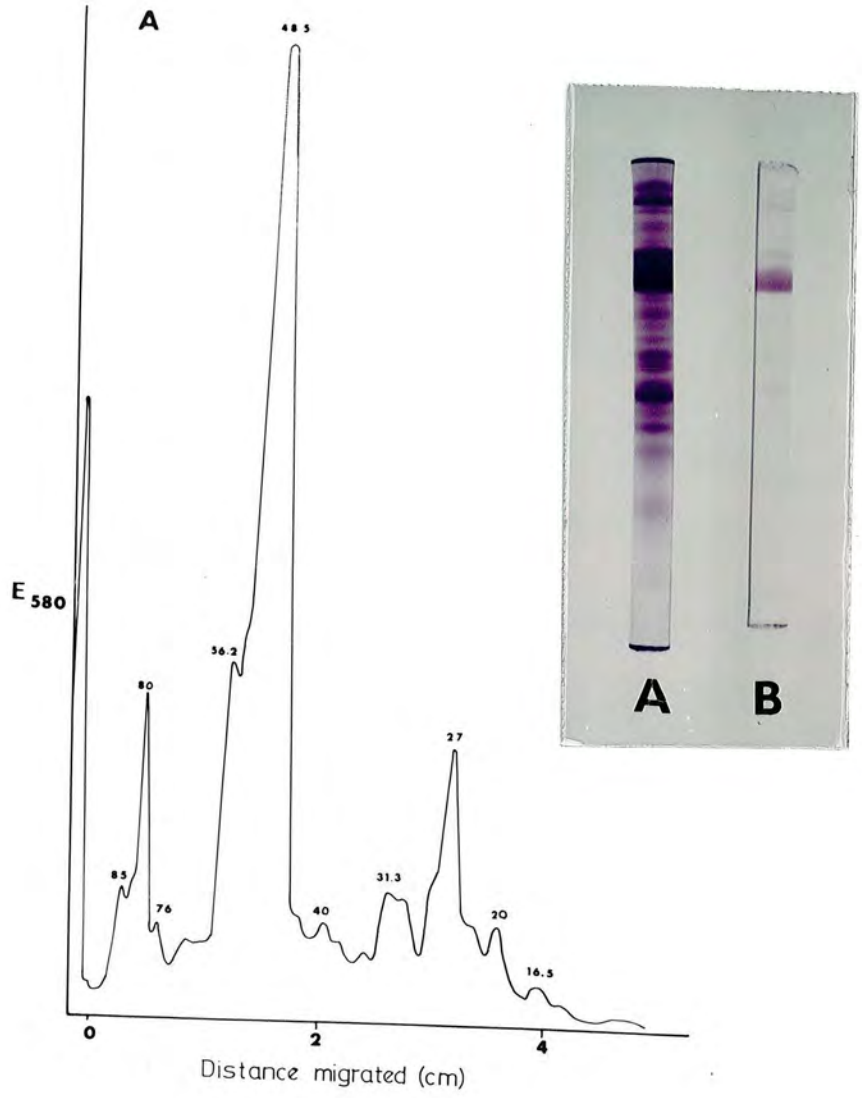
SDS electrophoretic profiles and photographs of gels of standard buffer extracts of cowpea meals with:

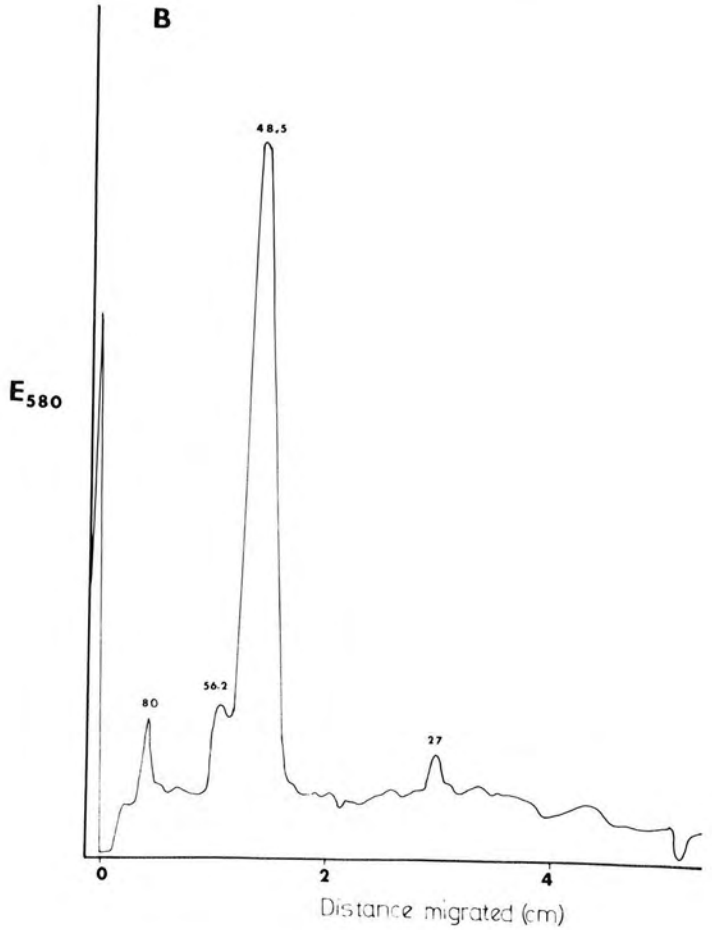
(A) coomassie blue

and

(B) PAS.

The numbers refer to the molecular weights (x 10^{-3}) of the associated peaks.


A
48.5
80
56.2
85
76
40
31.3
27
20
16.5
E_{580}
0
2
4
Distance migrated (cm)
A
B


B
48.5
80
56.2
27
E_{580}
0
2
4
Distance migrated (cm)

F I G. 5

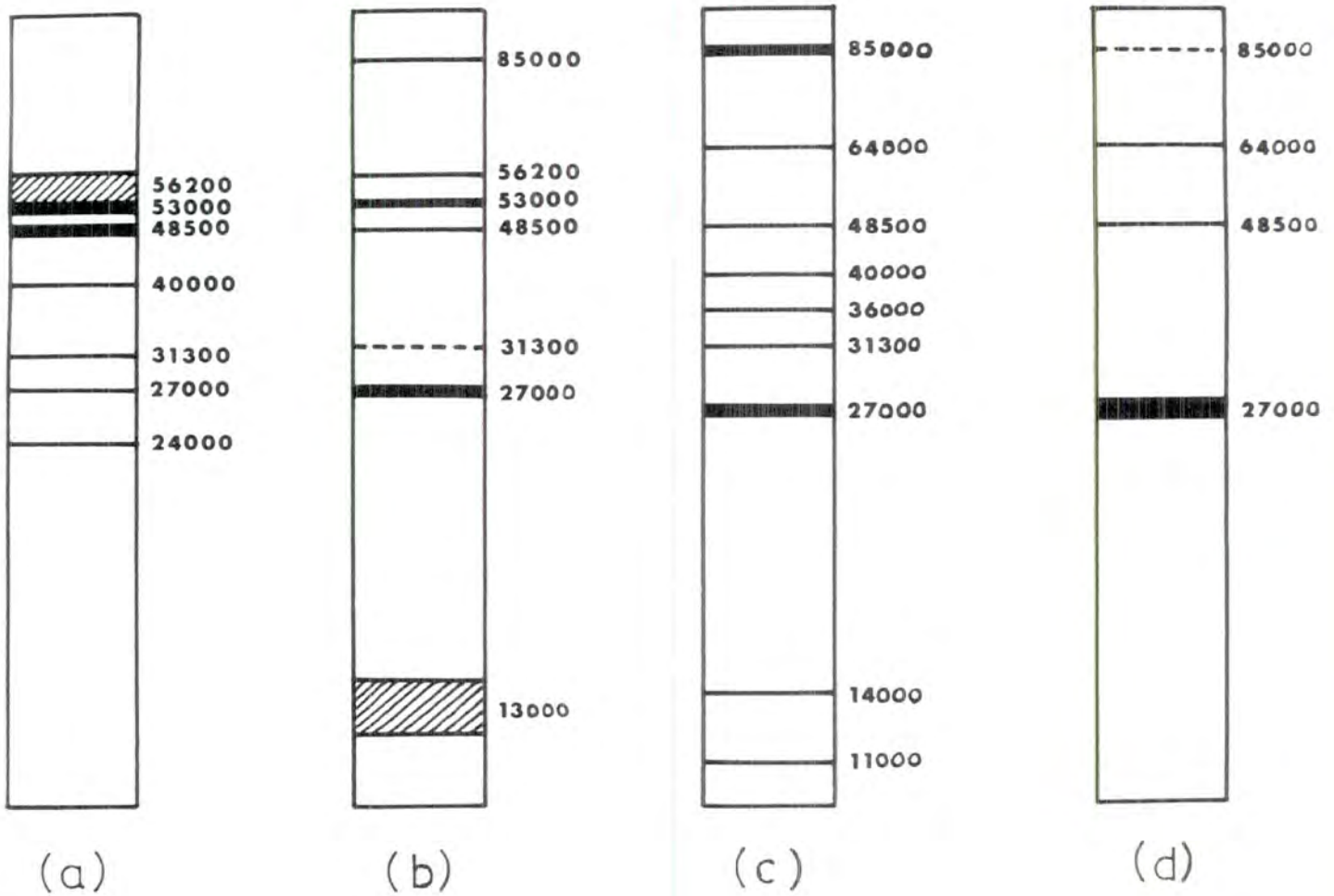
SDS electrophoretic patterns of ascorbic acid extraction and precipitates of globulin fraction:

(a) G 1 - by dilution 1:1

(b) G 2A - dialysis 15 h

(c) G 2B - dialysis 15-39 h

(d) G 2C - dialysis 39-63 h


56200
53000
48500
40000
31300
27000
24000
(a)
85000
56200
53000
48500
31300
27000
13000
(b)
85000
64000
48500
40000
36000
31300
27000
14000
11000
(c)
85000
64000
48500
27000
(d)

molecular weights of the major subunits were 53,000 and 48,500; and bands with MW 56,200, 40,000, 31,300, 27,000 and 24,000 were also prominent.

The precipitate from the first dialysis (fraction G2A) had two major bands - MW 53,000 and 27,000, bands with MW of 85,000, 56,200 and 48,500 were also prominent. Trace amounts of a band with MW 31300 were also observed. On further dialysis after 24 h the precipitate (G2B) had major bands of MW 85,000 and 27,000; other prominent bands in this fraction had MW 64,000, 48,500, 40,000, 36,000, 31,300, 14,000 and 11,000. After 39 h to 63 h dialysis, fraction G2C had only one major band MW 27,000 together with minor bands MW 85,000, 64,000 and 48,500.

When a mixture of fraction G2C and Human group O erythrocytes was incubated for 3 h at room temperature no agglutination of the erythrocytes was observed.

d) Standard buffer extraction and PAS staining

Four bands with molecular weights of 80,000, 56,200, 48,500 and 27,000 stained positively with PAS staining technique indicating that there are at least four glycoprotein subunits as these subunits corresponded with bands stained for protein only with coomassie blue (Fig. 4). It is possible that subunit MW 53,000 is also a glycoprotein but was not resolved as the gel was overloaded with respect to protein. Other subunits with molecular weights of 85,000, 76,000, 40,000, 31,300, 20,000 and 16,500 did not stain with Schiff's reagent.

(ii) Analytical ultracentrifugation

The total standard buffer (pH 8.0) extract of cowpea

meals was examined by ultracentrifugation analysis. The results of this showed that the major proteins of cowpeas were composed of only one component - the $s_{20,w}$ value was 7.2S (see Fig. 6).

II FRACTIONATED PROTEINS OF COWPEA

A) Isoelectric precipitation

When meals were extracted with NaCl, irrespective of salt concentration, the SDS pattern of the acid precipitate was similar to the standard buffer extract (see Fig. 4). After adding acid, protein precipitated around pH 5.5 and after centrifuging this solution no protein precipitated when the supernatant was further acidified to pH 4.7.

B) Ammonium sulphate precipitation

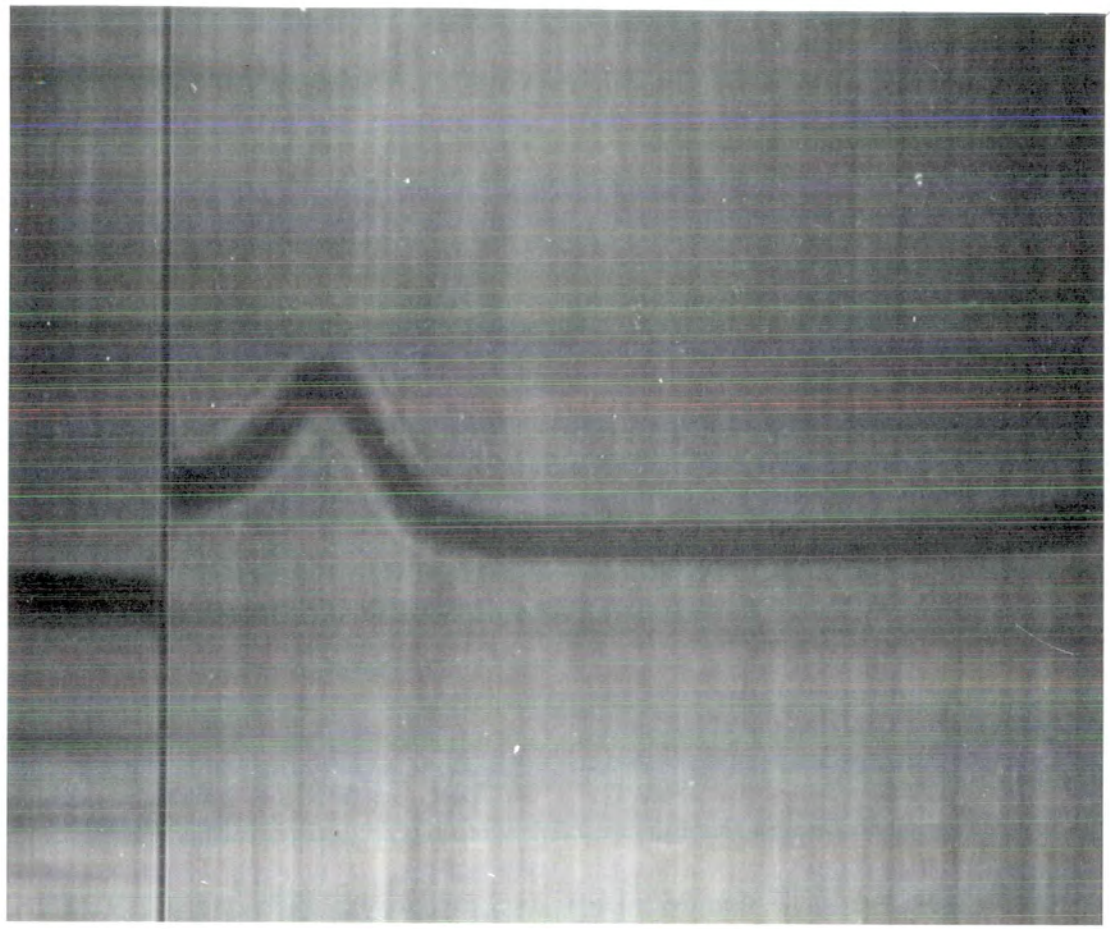
Analyses on SDS gels of all ammonium sulphate fractions (see Methods for details) showed that subunits MW 56,200, 53,000 and 48,500 are present in each case (Fig. 7 and Table 2). In the 0% to 50% range, the above subunits and subunits MW 71,000, 40,000 and 27,000 were prominent; subunits MW 85,000 and 64,000 were the major bands and subunits MW 31,300, 18,500 and 13,000 were minor components

Seven bands were observed in gels of the 50% to 65% range. The molecular weight of the major subunits were 53,000, 48,500 and 27,000; bands MW 85,000 and 56,200 were also prominent and bands MW 40,000 and 31,300 were minor components.

In the 65% to 70% range bands with MW 56,200, 53,000, 48,500 and 27,000 were the major components, bands MW 85,000 and 31,300 were prominent and traces of band MW 40,000 were present. The 70% to 85% range was similar to the 65% to 70% range except that band MW 40,000 was no longer present.

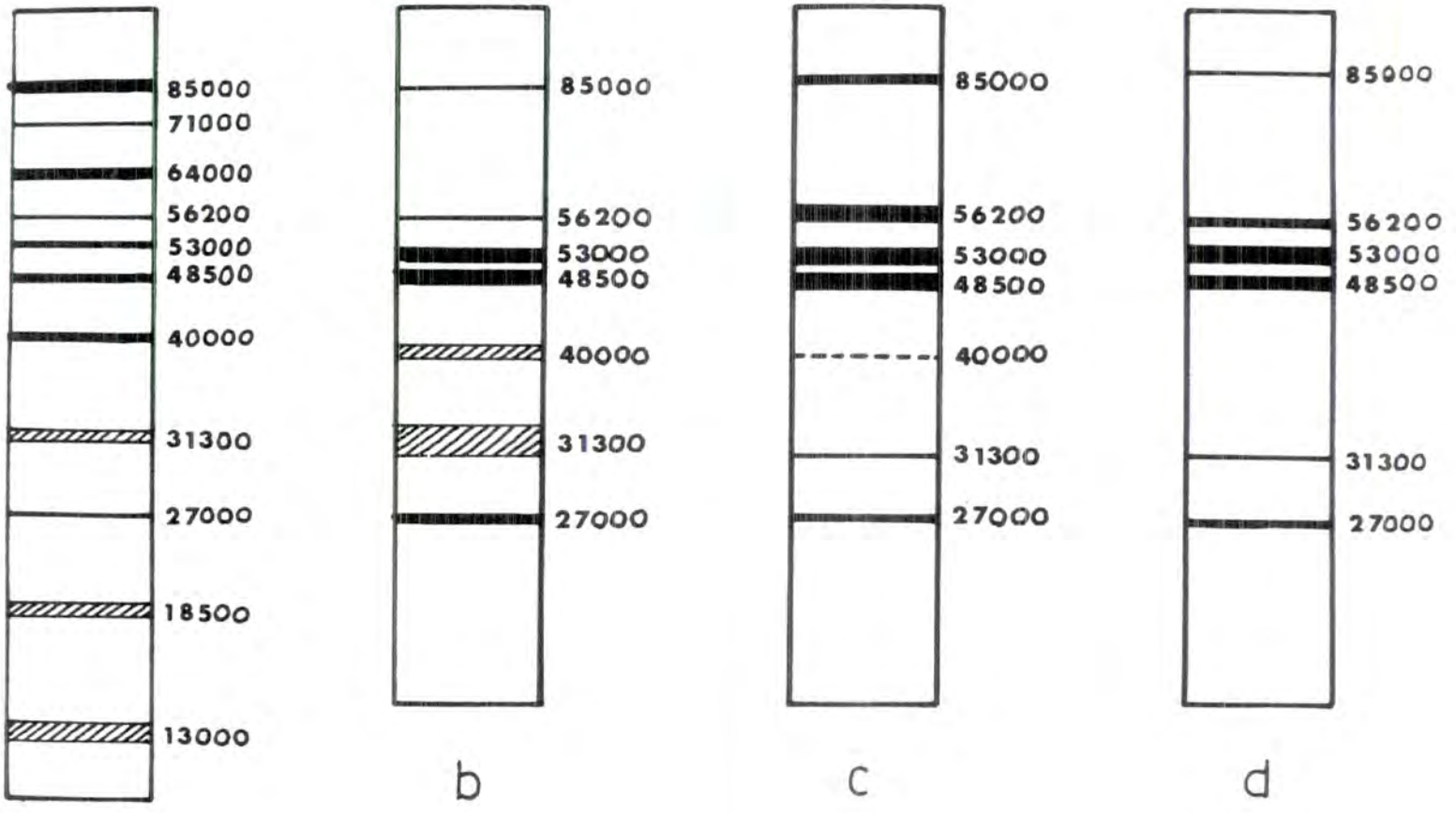
FIG. 6

Ultracentrifuge pattern of total extract of cowpea meal proteins in standard buffer. Photograph was taken 45 min after centrifuge had reached a speed of 40,000 rev / min. Bar angle 60°. Direction of sedimentation: left to right.



F I G. 7

SDS electrophoretic patterns of the ammonium sulphate fractions between the limits:
(a) 0 to 50% (b) 50 to 65% (c) 65 to 70% (d) 70 to 85% of saturation.


85000
71000
64000
56200
53000
48500
40000
31300
27000
18500
13000
a
85000
56200
53000
48500
40000
31300
27000
b
85000
56200
53000
48500
40000
31300
27000
c
85000
56200
53000
48500
31300
27000
d

T A B L E 2

Molecular weight values of dissociated ammonium sulphate precipitate fractions of protein extracts of cowpea meals

Fractions $(NH_4)_2SO_4$ % saturation	Molecular weight of subunits ($\times 10^3$)
0 to 50	<u>85.0</u> 71.0 <u>64.0</u> . 56.2 <u>53.0</u> 48.5 40.0 31.3 27.0 18.5 13.0
50 to 65	85.0 56.2 <u>53.0</u> <u>48.5</u> 40.0 31.3 27.0
65 to 70	85.0 56.2 <u>53.0</u> <u>48.5</u> 40.0 31.3 27.0
70 to 85	85.0 <u>56.2</u> <u>53.0</u> <u>48.5</u> 31.3 27.0

Molecular weight values underlined are major components

C) Zonal isoelectric precipitation

When the extracted proteins of cowpea meals were subjected to zonal isoelectric precipitation some of the protein was retarded as shown in the elution profile (Fig. 8).

The unretarded fraction was designated Peak I and the retarded fraction Peak II. This latter fraction was composed of two partially-resolved Peaks IIA and IIB with two minor peaks in between which contained only a small quantity of protein and were not further investigated.

Subunits with MW 56,200, 53,000 and 48,500 were present as major components in all three peaks but in different proportions in each case (Fig. 9).

In Peak I bands with MW 56,200, 53,000 and 48,500 were major and present in almost equal proportions; bands with MW of 40,000, 31,300, 27,000 and 23,000 were also prominent.

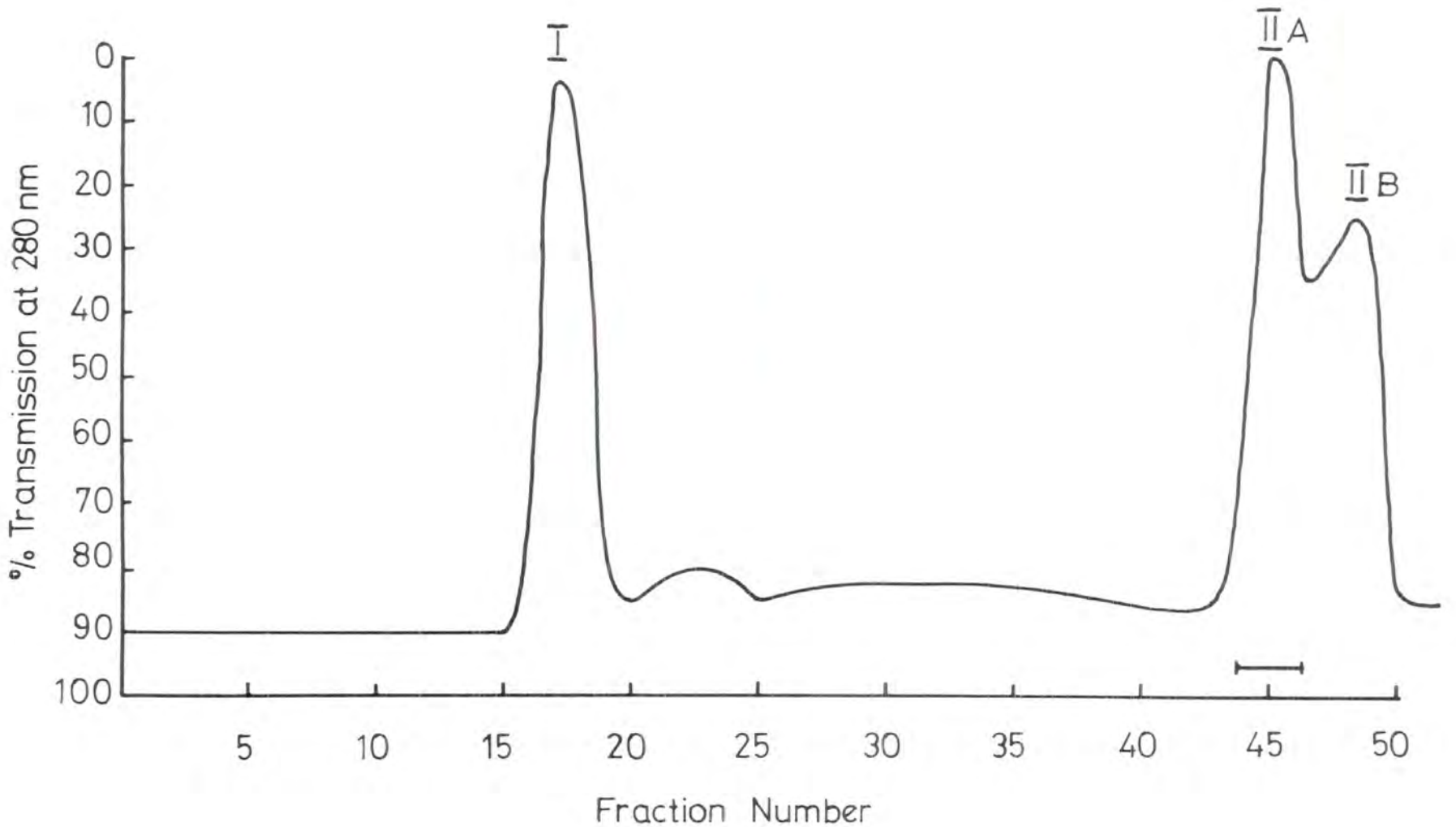
Peak IIA showed a definite enrichment of subunit with MW of 48,500 with 8 other subunits which were prominent - MW 90,000, 85,000, 64,000, 56,200, 40,000, 27,000, 18,500 and 16,500; traces of subunit MW 53,000, 31,300 and 23,000 were also observed. The major bands of Peak IIB have molecular weights of 56,200, 53,000 and 16,500 and included other prominent bands with molecular weights of 53,000, 42,000, 40,000, 36,000 and 31,300.

Gel filtration on Sephadex G-200

Peak IIA was then subjected to permeation chromatography when two peaks were obtained (Fig. 10) and the subunit patterns obtained when the two peaks were subjected to SDS gel electrophoresis are shown in Fig. 11.

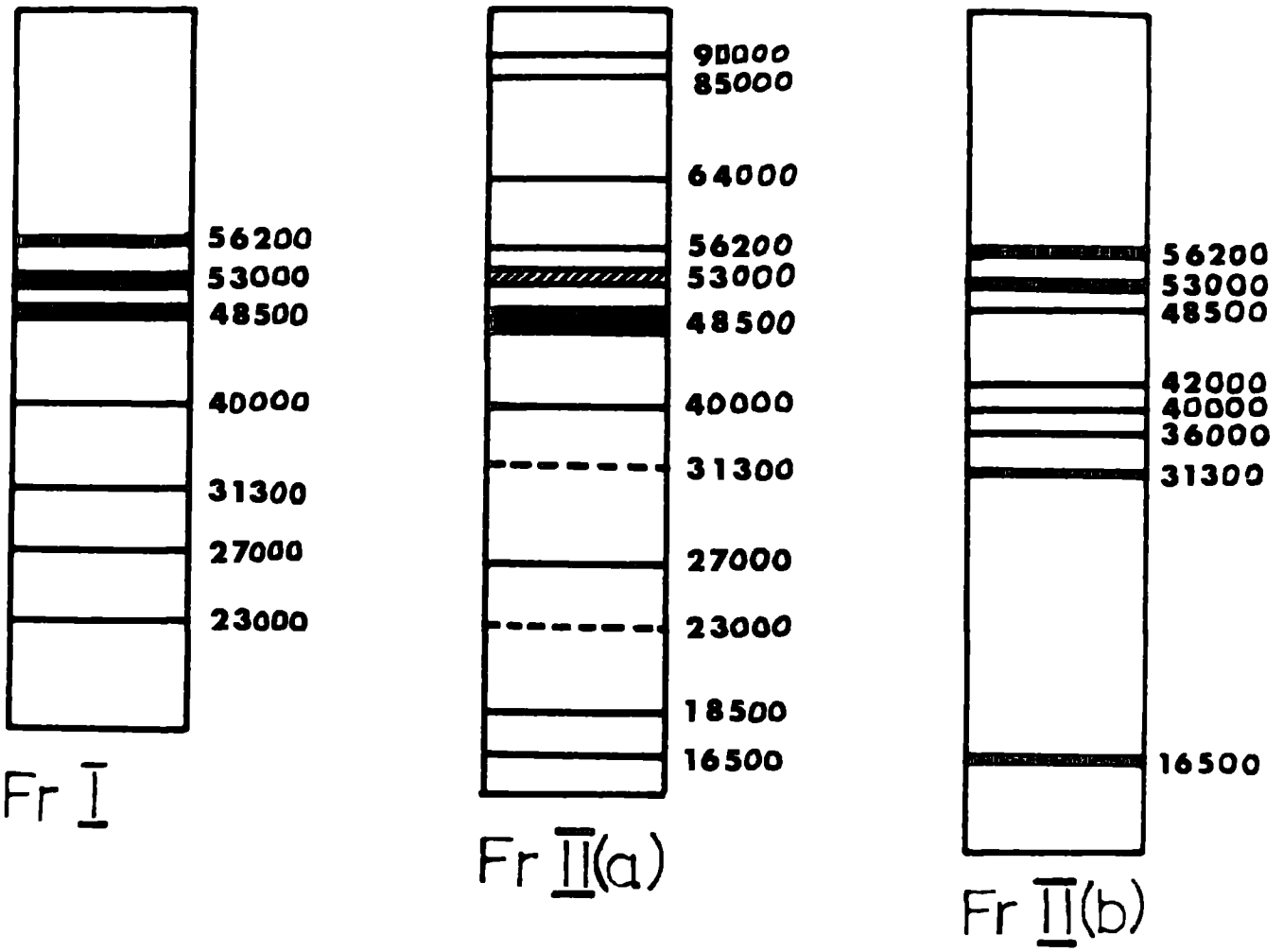
F I G. 8

Elution profile for zonal isoelectric precipitation of proteins of cowpea meals. Elution was performed on a 3.5 x 45 cm column at a rate of 10 ml/h. 7.5 ml fractions were collected.


I
II A
II B
0
10
20
30
40
50
60
70
80
90
100
% Transmission at 280 nm
5
10
15
20
25
30
35
40
45
50
Fraction Number

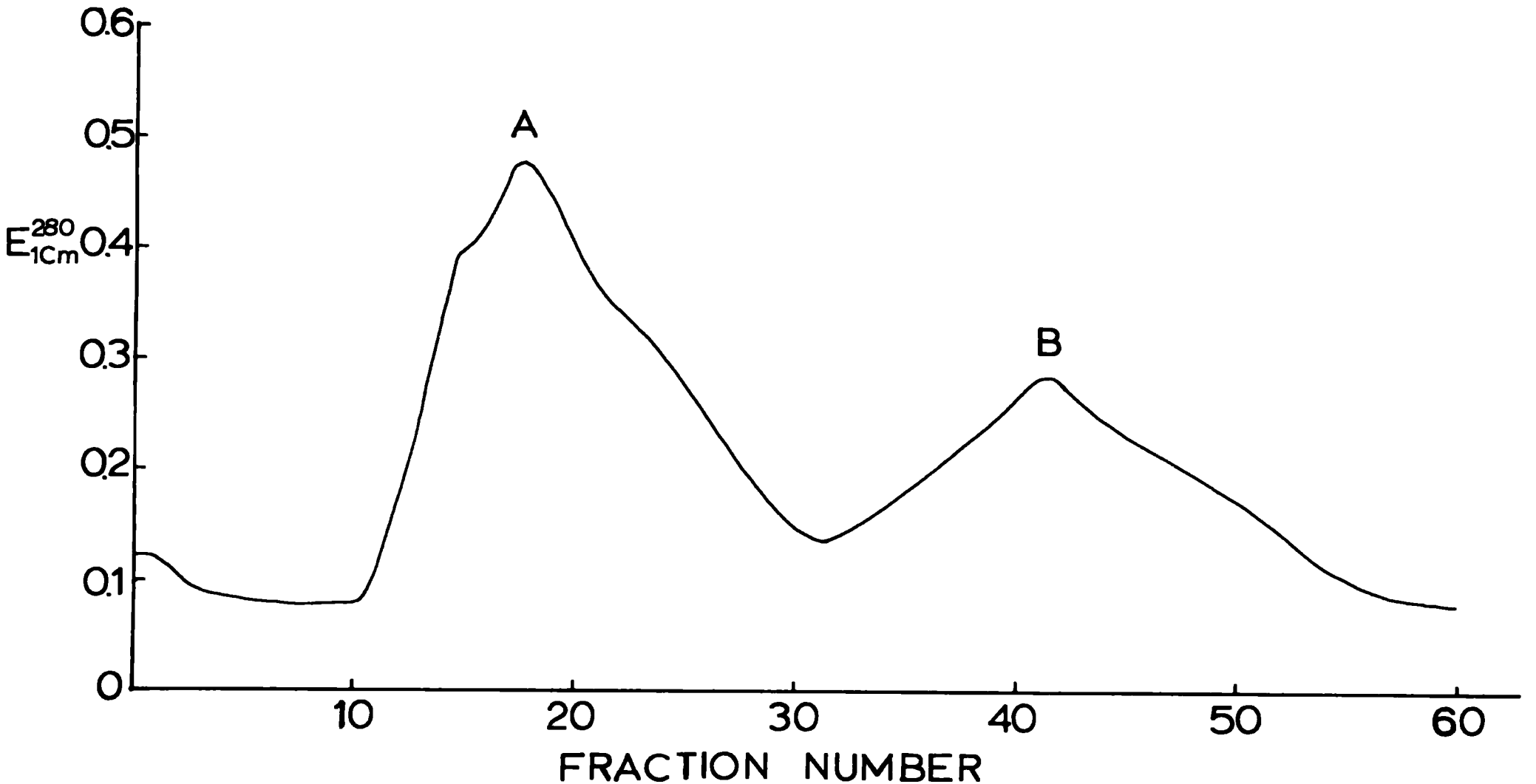
F I G. 9

SDS electrophoretic patterns and subunit molecular weights of the unretarded fraction (Fr. I) and the retarded fractions (Fr. IIa and Fr. IIb) obtained by zonal isoelectric precipitation of cowpea globulins.


56200
53000
48500
40000
31300
27000
23000
Fr I
90000
85000
64000
56200
53000
48500
40000
31300
27000
23000
18500
16500
Fr II(a)
56200
53000
48500
42000
40000
36000
31300
16500
Fr II(b)

F I G. 10

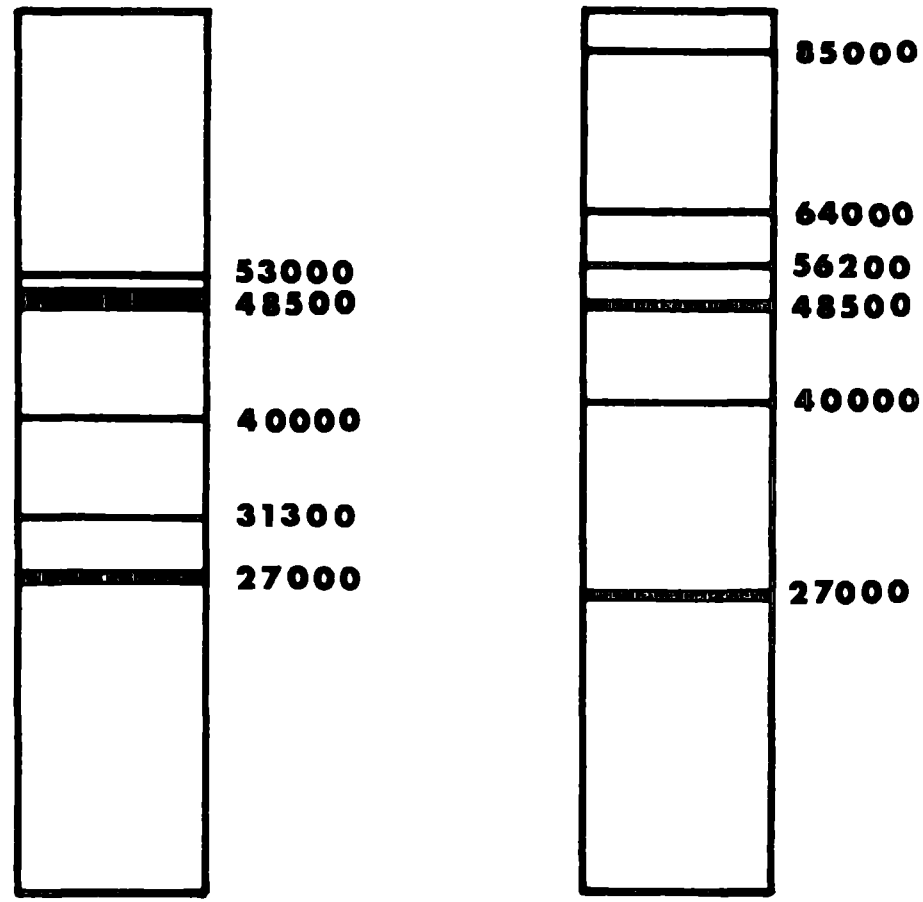
Elution profile for gel filtration on Sephadex G-200 of Peak IIA (from zonal isoelectric precipitation). Elution was performed on a 3.5 x 35 cm column at a rate of 28 ml/h. 7 ml fractions were collected.


0.6
0.5
E^{280}_{1Cm} 0.4
0.3
0.2
0.1
0
A
B
10
20
30
40
50
60
FRACTION NUMBER

F I G. 11

SDS electrophoretic pattern of fractions obtained by gel filtration on Sephadex G-200:

(a). Peak A (b) Peak B


53000
48500
40000
31300
27000
(a)
85000
64000
56200
48500
40000
27000
(b)

Both peaks contained subunit MW 48,500 as major and also subunit with MW 40,000 and 27,000 as prominent components. Both peaks had additional but different prominent bands: Peak A had bands MW 53,000 and 31,500 and Peak B had bands with MW 85,000, 64,000 and 56,200.

D) Ion-exchange chromatography

The extracted proteins of cowpea were resolved into three fractions by ion-exchange chromatography on DEAE-cellulose (Fig. 12). One fraction was not absorbed and was eluted with the starting buffer and the other two fractions were adsorbed into the ion exchanger and were eluted only after the NaCl concentration of the buffer was increased to 0.21M NaCl and 0.25M NaCl respectively; separation was not complete.

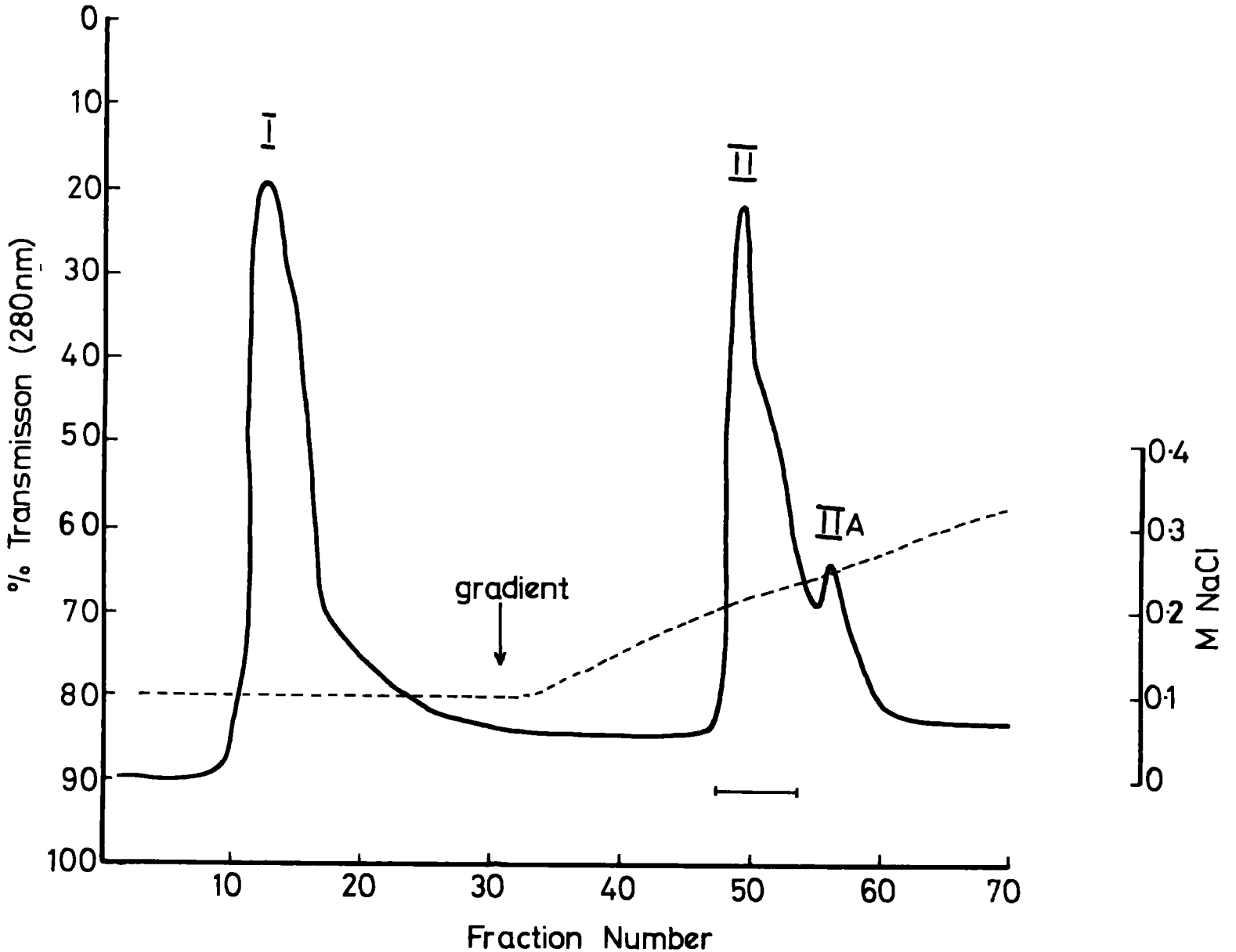
SDS gel electrophoresis of samples taken from the peaks are shown in Fig. 13. The unadsorbed fraction Peak I was composed of five bands. The molecular weights of the major subunits were 56,200, 53,000 and 48,500 in almost equal proportions and bands with MW 36,000 and 27,000 were almost prominent.

Peak II had subunits MW 53,000 and 48,500 as major and subunits MW 46,000, 40,000 and 31,300 as minor components. The other partly resolved fraction (Peak IIA) was composed of subunits MW 53,000 and 40,000 as major, subunit MW 20,000 as a minor component and traces of subunit MW 46,000. When analysed in the non-dissociating gel system, Peak IIA gave two bands with Rm 0.36 and 0.75.

To improve resolution, several variations were attempted starting sodium chloride concentration of the eluting buffer with

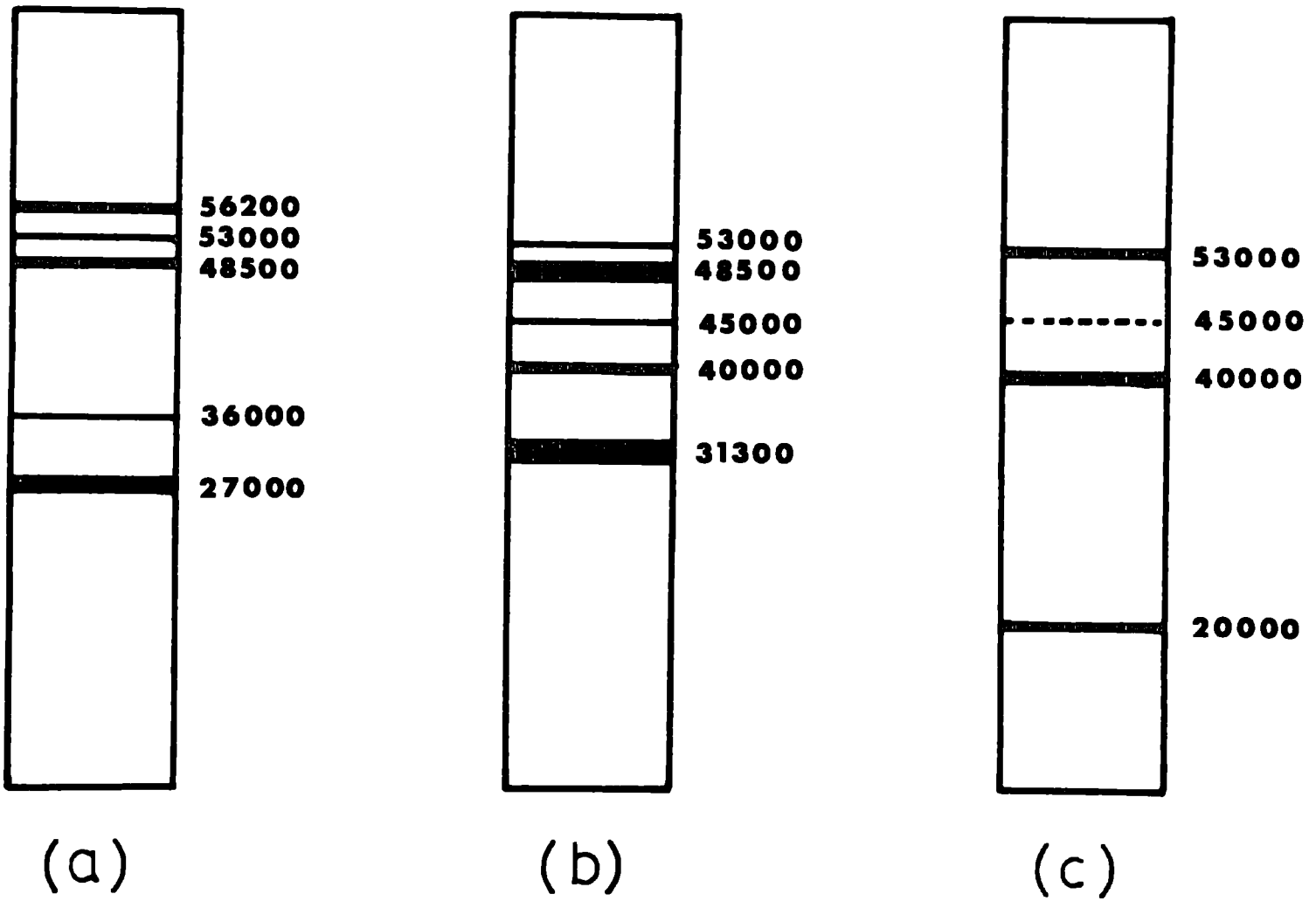
F I G. 12

Chromatogram of cowpea globulins on DEAE-cellulose. The column (1.1 x 30 cm) was equilibrated with 0.02M phosphate buffer (pH 8.0) made 0.1M in NaCl and 0.01M in mercapto-ethanol. Elution was at a rate of 5.2 ml/h and 4 ml fractions were collected.


0
10
20
30
40
50
60
70
80
90
100
% Transmisson (280nm)
I
II
IIA
gradient
10
20
30
40
50
60
70
Fraction Number
0·4
0·3
0·2
0·1
0
M NaCl

F I G. 13

SDS electrophoretic patterns of (a) Peak I (b) Peak II and (c) Peak IIa obtained by ion exchange chromatography of proteins of cowpea.


56200
53000
48500
36000
27000
(a)
53000
48500
45000
40000
31300
(b)
53000
45000
40000
20000
(c)

0.07M and 0.15M NaCl, longer column (1 cm x 90 cm) and less steeper sodium chloride elution gradient. None of the above changes made a significant difference to the separation of the major proteins.

III CHARACTERISATION OF GLOBULIN SUBUNITS PARTIALLY SEPARATED BY ION-EXCHANGE CHROMATOGRAPHY WITH UREA

A) Chromatography of an extract of total globulins

(i) Chromatography

Globulin preparations subjected to DEAE - cellulose chromatography exhibit an elution pattern typical to that shown in Fig. 14.

(ii) Ultraviolet absorption spectrum scan of separated fractions

The ultraviolet absorption spectrum scan between 230 nm and 300 nm of tubes 7, 10, 23, 43 and 67 from the main peaks is shown in Fig. 15 and in Table 3 are some of the main characteristics.

TABLE 3 Ultraviolet absorption characteristics of cowpea globulin fractions separated on a DEAE-cellulose in 8.0M urea column

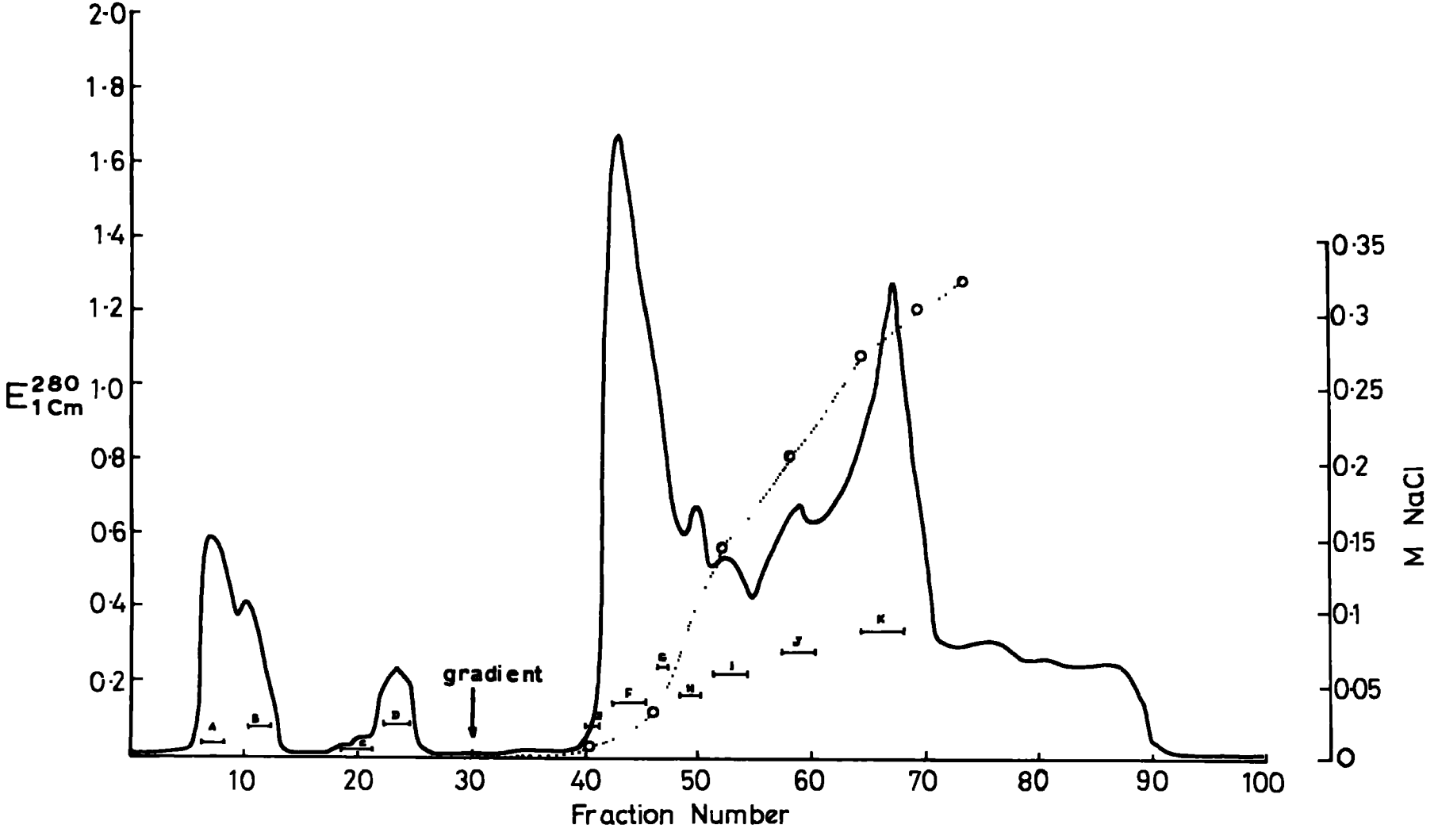
Fraction No.	K max_{nm}	280 : 260 nm ratio
7	280	9.6
10	273	1.3
23	240	2.4
43	280	2.3
67	275	1.7

(iii) Electrophoretic analyses of column fractions

The fractions off the column were pooled as indicated in Fig. 14 and extensively dialysed against distilled water. The protein that precipitated out was lyophilised and a part electro-

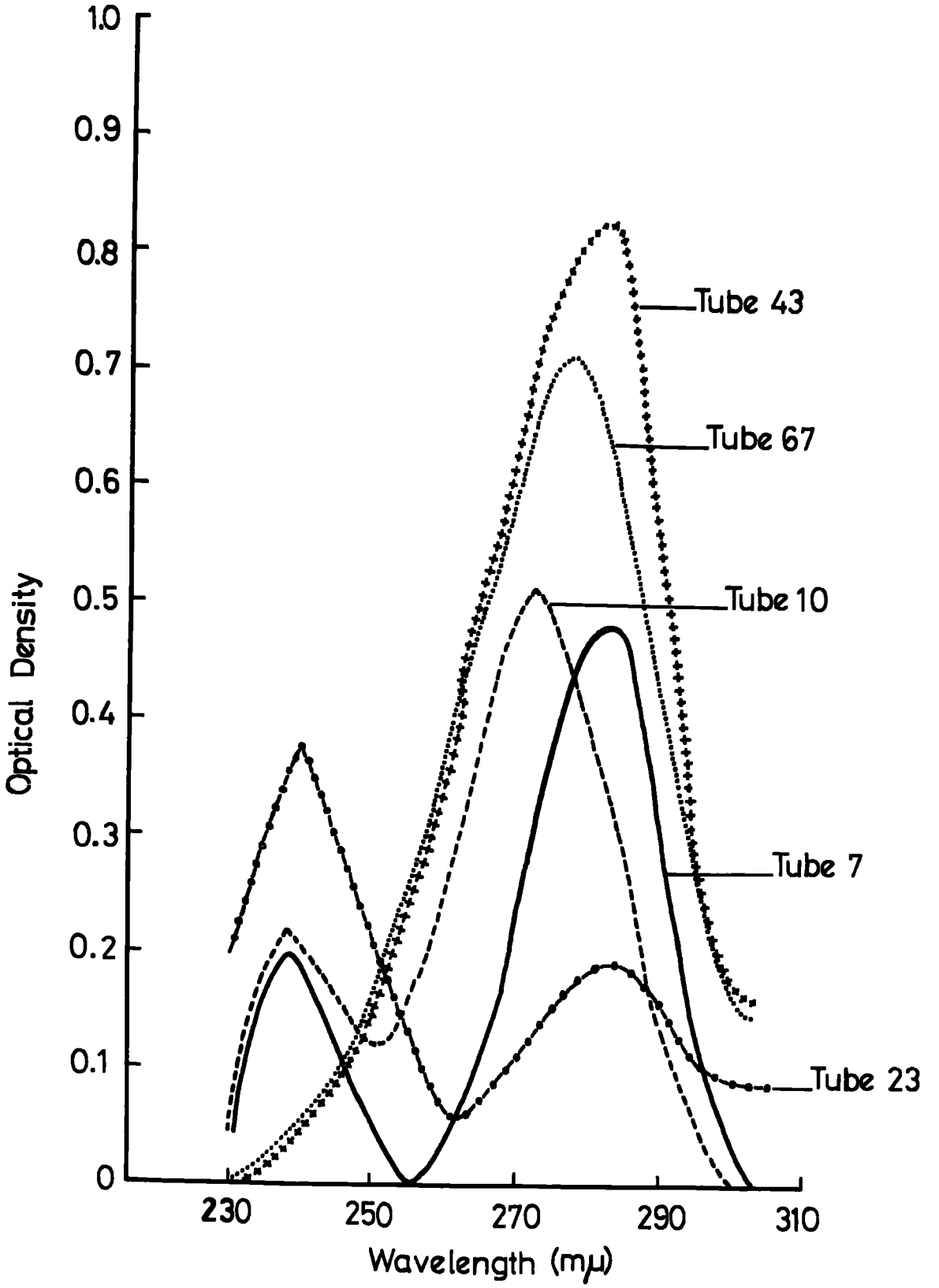
F I G. 14

Elution profile for ion exchange chromatography of globulins of *Vigna unguiculata* on DEAE-cellulose in 8.0M urea. About 90 mg of protein was applied on a column (1.25 x 25 cm) which was equilibrated with 0.076M Tris-citrate buffer pH 8.7 made 8.0M in urea and 0.01M in mercaptoethanol. Fractions 4.0 ml. Flow rate: 5.2 ml/h.


2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
$E^{280}_{1\,Cm}$
gradient
A
B
C
D
E
F
G
H
I
J
K
10
20
30
40
50
60
70
80
90
100
Fraction Number
0·35
0·3
0·25
0·2
0·15
0·1
0·05
0
M NaCl

F I G. 15

Ultraviolet absorption spectra of cowpea globulin fractions off a urea-DEAE-cellulose column. All spectra were measured in the starting buffer but in the case of those fractions eluted with the salt gradient, the spectra were measured also with the NaCl contained in the fraction.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Optical Density
230
250
270
290
310
Wavelength (mμ)
Tube 43
Tube 67
Tube 10
Tube 7
Tube 23

phorised. The SDS electrophoretic patterns obtained are shown in Fig. 16. The subunits with MW 56,000, 53,000 and 48,500 were adsorbed whereas some of the smaller molecular weight subunits were chromatographically separated by use of the starting buffer. When the adsorbed protein was eluted with a salt gradient, subunit MW 56,000 was eluted with subunit MW 48,500 at a salt concentration of less than 0.05M NaCl. But most of subunit MW 48,500 with a small amount of subunit MW 53,000 was eluted with 0.25M NaCl. The fraction which showed the highest enrichment of subunit MW 53,000 was desorbed at about 0.5M NaCl. Thus stepwise elution was used at 0.05M, 0.25M and 0.5M NaCl for further preparative separations of subunits MW 56,000, 53,000 and 48,500.

(iv) Cysteic acid content

The results of the analyses for cysteic acid content of the pooled unadsorbed fractions is given in Table 4.

(v) Recovery

Between 45% to 50% by weight of the total globulin loaded was recovered off the DEAE-cellulose column from pooled fractions of peaks that were dialysed and then lyophilised. The total unadsorbed fractions which did not contain the subunits MW 56,000, 53,000 and 48,500 made up 20% to 25% by weight of the recovered protein.

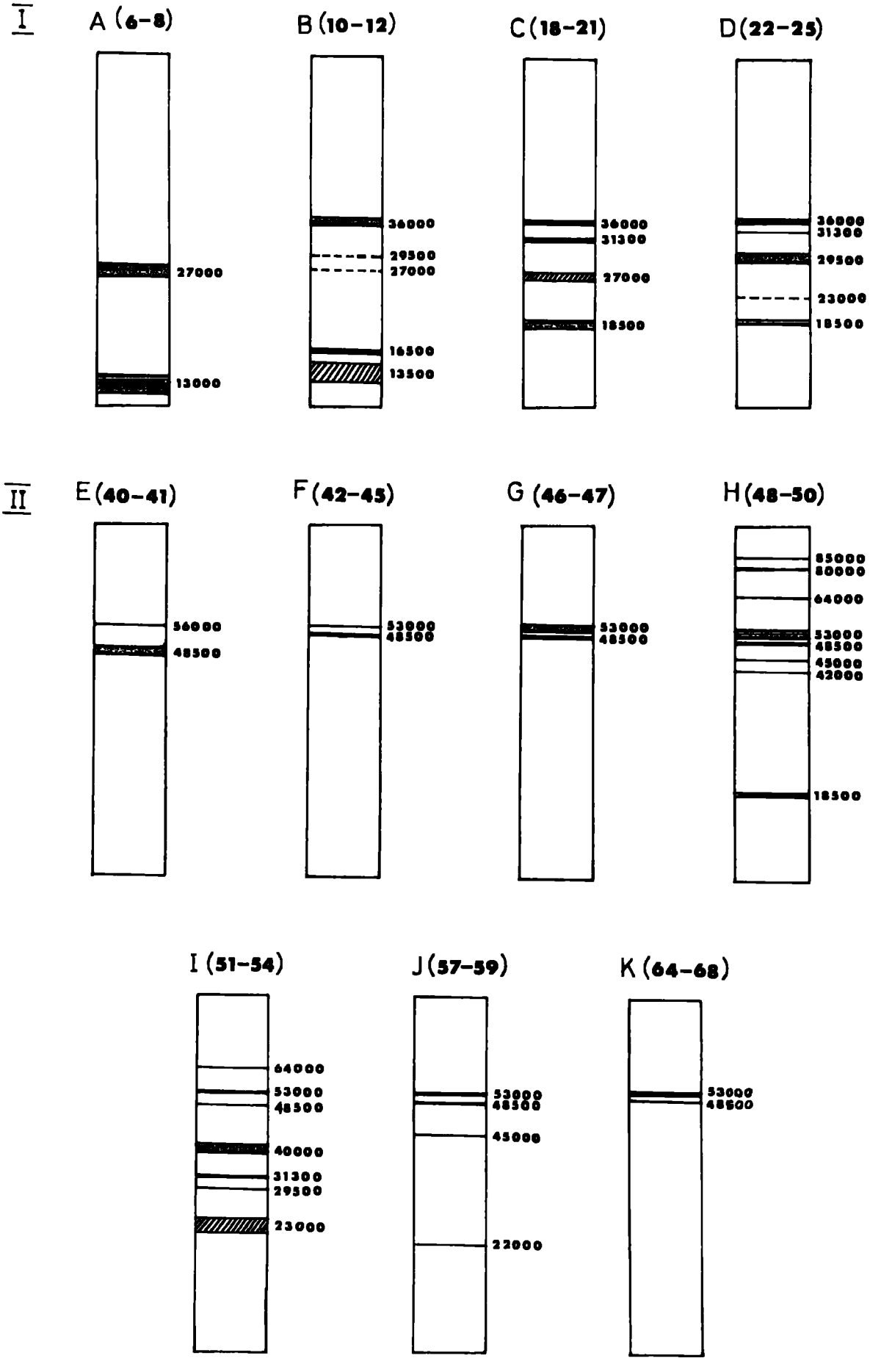
B) Rechromatography of two major subunit fractions

(i) Chromatography

Lyophilised proteins from fractions 40 - 41 and 46 - 47 (Fig. 16) enriched with major subunits MW 53,000 and 48,500 respectively were rechromatographed separately under the same

F I G. 16

SDS electrophoretic patterns of fractions obtained by ion chromatography with urea on Whatman DE 52 DEAE-cellulose column: (I) unadsorbed fractions (II) adsorbed fractions. Letters and figures above each pattern correspond with fractions of the elution profile (see Fig. 14).


I
A (6-8)
27000
13000
B (10-12)
36000
29500
27000
16500
13500
C (18-21)
36000
31300
27000
18500
D (22-25)
36000
31300
29500
23000
18500
II
E (40-41)
56000
48500
F (42-45)
53000
48500
G (46-47)
53000
48500
H (48-50)
85000
80000
64000
53000
48500
45000
42000
18500
I (51-54)
64000
53000
48500
40000
31300
29500
23000
J (57-59)
53000
48500
45000
22000
K (64-68)
53000
48500

T A B L E 4

Cysteic acid content and Rm values of unadsorbed fractions of globulin on chromatography on urea-DEAE-cellulose column

Fraction numbers	Rm values	Cysteic acid content (g / 100 g protein)
6 to 8	0.46 0.70	0.71
10 to 12	0.35 0.42 0.45 0.62 0.69	0.54
18 to 21	0.35 0.39 0.45 0.57	0.70
22 to 25	0.35 0.38 0.43 0.5 0.56	0.30

Rm values of major components are underlined

conditions as those described before. The effectiveness of this rechromatography were assessed by gel electrophoresis. At 0.225M NaCl concentration, subunit MW 48,500 was eluted and at 0.5M NaCl subunit MW 53,000 was eluted about 80% enriched. The electrophoretic profiles of the two protein fractions are illustrated in Fig. 17 and Fig. 18.

(ii) Moisture content of subunits

The moisture content of subunit MW 48,500 and enriched subunit MW 53,000 were found to be 8.62% and 9.96% respectively.

(iii) Nitrogen content of subunits

The total nitrogen content of subunit MW 48,500 and enriched subunit MW 53,000, after correcting for the water content, were 18.61% and 18.38% respectively.

(iv) Amino acid composition of subunits

The amino acid analyses of 22 h and 72 h hydrolysates of subunit MW 48,500 and enriched subunit MW 53,000 are given in Tables 5 and 6. Sample weights were corrected for moisture content.

(v) Carbohydrate composition of subunits

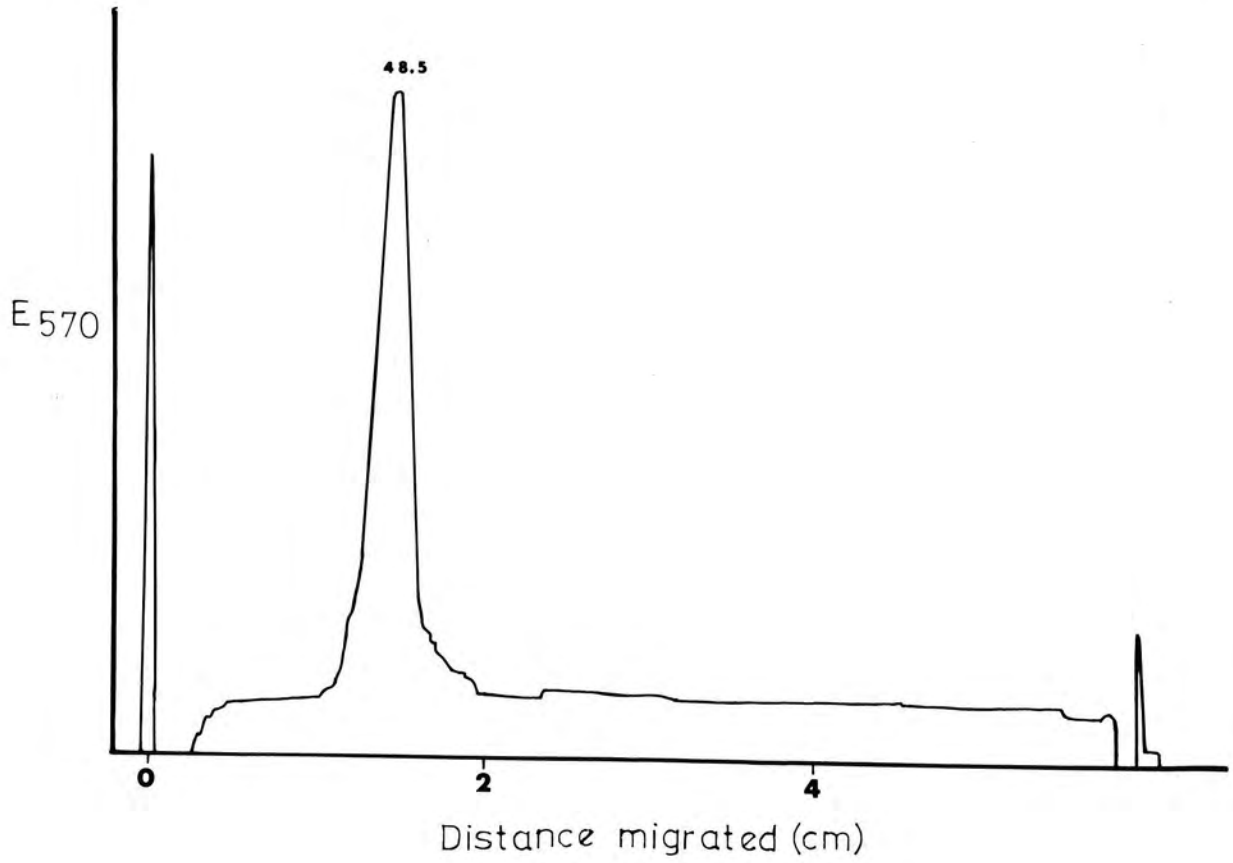
The neutral sugar content with mannose used as a standard of subunit MW 48,500 and enriched subunit MW 53,000 were found to be 1.9% and 2.2% respectively. When 2 mg samples of both fractions were examined no hexosamine component could be demonstrated.

(vi) N-Terminal amino analysis

Only subunit MW 48,500 was examined by the dansylation procedure and one N-Terminal amino acid was identified, either leucine or isoleucine.

F I G. 17

SDS electrophoretic profile and photograph of gel of 48,500 molecular weight subunit obtained by urea-ion-exchange chromatography of globulins.


48.5
E570
0
2
4
Distance migrated (cm)

F I G. 18

SDS electrophoretic profile and photograph of gel of the enriched fraction of subunit MW 53,000 obtained by urea-ion-exchange chromatography of cowpea globulins.

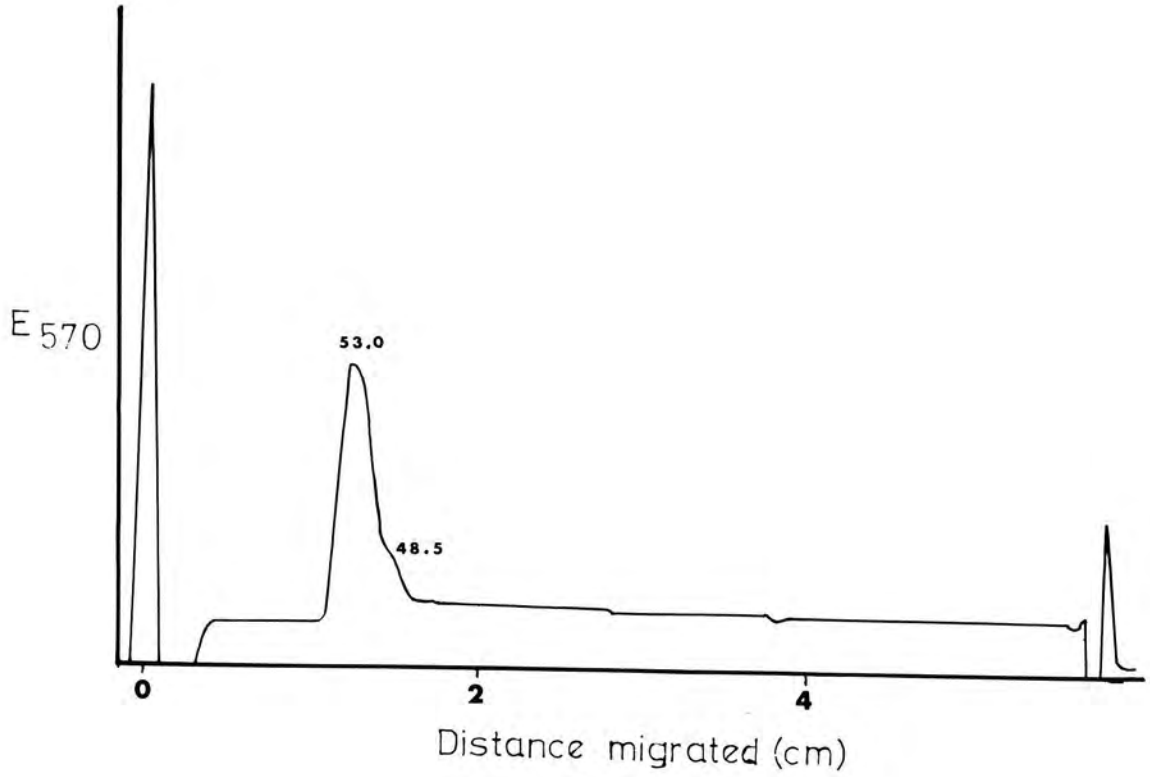

E 570
53.0
48.5
0
2
4
Distance migrated (cm)

TABLE 5

Amino acid analyses of

subunit MW 48,500

eluted from urea-DEAE-cellulose column

Amino acid	Recovery g amino acid residue / 100 g protein	
	22 h hydrolysis	72 h hydrolysis
Aspartic acid	11.92 ± 0.13	11.96 ± 0.26
Threonine	2.54 ± 0.01	2.31 ± 0.05
Serine	4.78 ± 0.07	4.13 ± 0.01
Glutamic acid	13.22 ± 0.08	13.17 ± 0.42
Proline	3.76 ± 0.60	3.73 ± 0.16
Glycine	2.21 ± 0.12	2.23 ± 0.07
Alanine	2.99 ± 0.13	2.91 ± 0.02
Cysteine[a]	0.23 ± 0.05	N.D.
Valine	4.30 ± 0.07	4.94 ± 0.01
Methionine	0.81 ± 0.21	0.73 ± 0.26
Isoleucine	4.15 ± 0.02	4.68 ± 0.01
Leucine	9.42 ± 0.19	9.29 ± 0.06
Tyrosine	3.12 ± 0.07	3.08 ± 0.07
Phenylalanine	7.05 ± 0.42	6.99 ± 0.03
Histidine	2.67 ± 0.14	2.64 ± 0.02
Lysine	5.56 ± 0.10	5.62 ± 0.05
Arginine	9.42 ± 0.42	7.55 ± 0.61
	88.15	85.96

a determined as cysteic acid after performic acid oxidation

N.D. not determined

The figures quoted refer to the mean values and errors from duplicate hydrolyses

TABLE 6

Amino acid analyses of enriched fraction of subunit MW 53,000 eluted from urea-DEAE-cellulose column

Amino acid	Recovery g amino acid residue / 100 g protein	
	22 h hydrolysis	72 h hydrolysis
Aspartic acid	8.42 ± 0.09	8.20 ± 0.23
Threonine	1.63 ± 0.02	1.57 ± 0.17
Serine	3.32 ± 0.04	2.96 ± 0.06
Glutamic acid	10.74 ± 0.05	10.29 ± 0.31
Proline	3.08 ± 0.04	2.97 ± 0.01
Glycine	1.97 ± 0.02	1.88 ± 0.06
Alanine	1.93 ± 0.01	1.86 ± 0.12
Cysteine[a]	0.37 ± 0.03	N.D.
Valine	2.52 ± 0.06	2.59 ± 0.06
Methionine	0.72 ± 0.01	0.60 ± 0.02
Isoleucine	2.13 ± 0.05	2.22 ± 0.11
Leucine	4.54 ± 0.01	4.31 ± 0.14
Tyrosine	1.68 ± 0.01	1.64 ± 0.06
Phenylalanine	2.53 ± 0.02	2.38 ± 0.14
Histidine	2.72 ± 0.03	3.26 ± 0.05
Lysine	4.25 ± 0.06	6.51 ± 0.08
Arginine	5.53 ± 0.02	5.10 ± 0.30
	58.08	58.34

[a] determined as cysteic acid after performic acid oxidation

N.D. not determined

The figures quoted refer to the mean values and errors from duplicate hydrolyses.

IV DEVELOPMENT FROM ANTHESIS TO FRUIT MATURITY

A) Parameter used to monitor the molecular changes during seed development

Cowpea seeds are very small especially in the early stages of development. When the seed has completed about 25% of its development on a seed age basis (seven days after flowering) fresh weight is in the range of only 7 to 10 mg (Fig. 19). Approximately 84% of the seed is moisture at this stage and so the pooling of seeds of several harvests was necessary to obtain sufficient dry weight material for analyses. This required a reliable parameter by which seeds that have reached a similar stage of physiological development i. e. nitrogen accumulation, could be identified and pooled for analysis.

A critical parameter to define the various stages of development had to be selected because of the rapid growth of cowpea seed. The period of development from flowering to maturity of cotyledons was less than thirty days (cf Vicia faba about 80 days, soya bean about 60 days). Thus an inherent factor increased the margin of error of obtaining overlapping stages when developing seeds were pooled in the case of cowpea.

The number of days after synthesis or flowering has often been used in studies of development of legume seeds (Bils and Howell, 1963; Smith, 1973; Hill and Breidenbach, 1974; Gritton et al., 1975 etc). The use of this parameter is based on the assumption that growth is only directly related to time and constant on different parts of the plant and throughout the fruiting period.

However, in our trials under the growing conditions used,

F I G. 19

Increase in mean seed size and seed fresh weight with time. Each point on the graph represents an average value for 30 seeds and from one harvest for all stages of development.

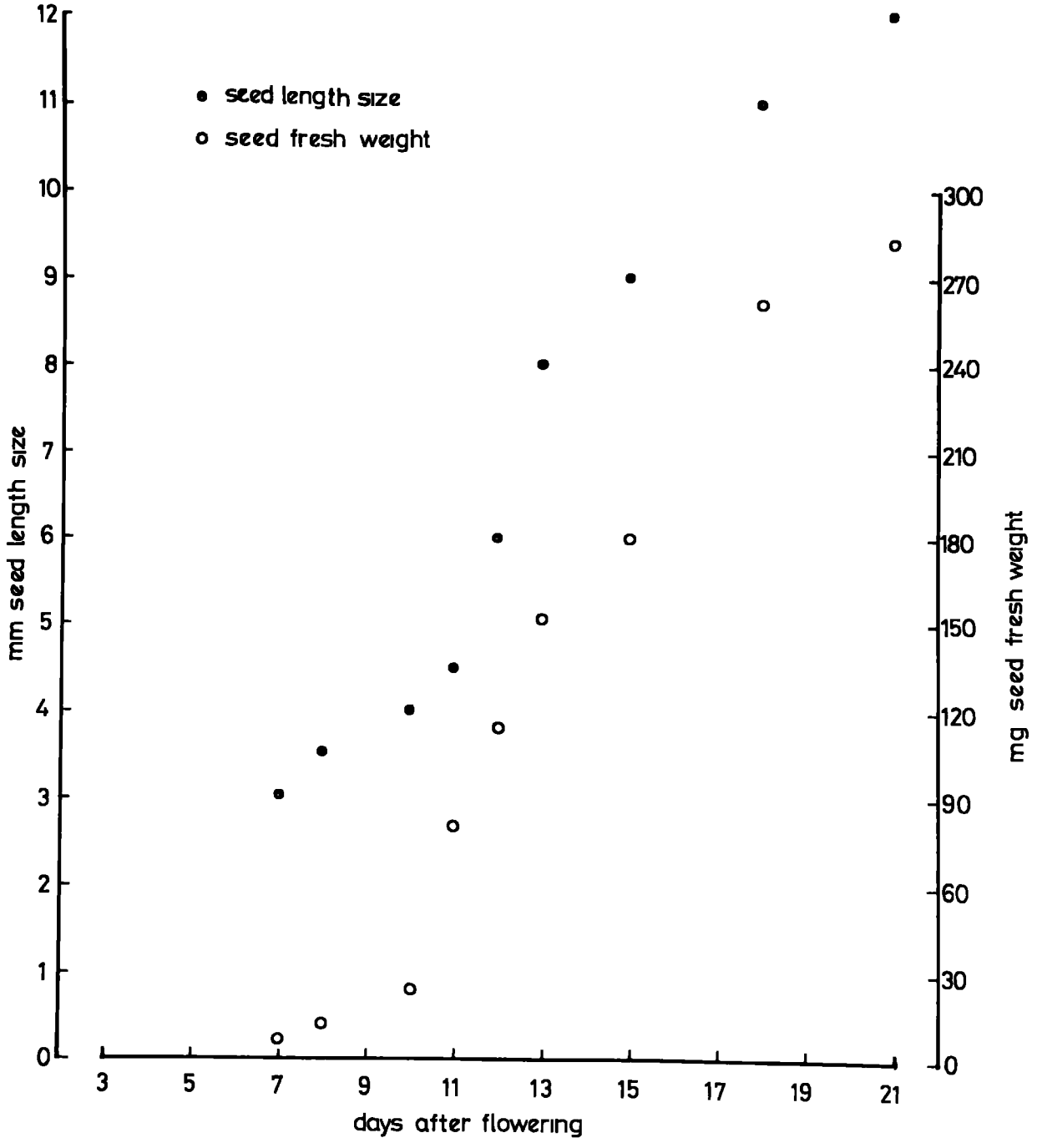

seed length size
seed fresh weight
mm seed length size
mg seed fresh weight
days after flowering
0
1
2
3
4
5
6
7
8
9
10
11
12
0
30
60
90
120
150
180
210
240
270
300
3
5
7
9
11
13
15
17
19
21

Fig. 20 shows that seeds of the same age can differ considerably in fresh weight and total nitrogen. For example at Day 14 there is a wide range of fresh weight and nitrogen content values of seeds which overlaps with the values obtained for some seeds during the developing period - 11 to 20 days.

Polyacrylamide gel electrophoresis has been used by several workers to determine the time at which different seed storage proteins are synthesised in the seed development period (Flinn and Pate, 1968; Wright and Boulter, 1972; Hall et al., 1972; Cherry, 1974; Hill and Breidenbach, 1974).

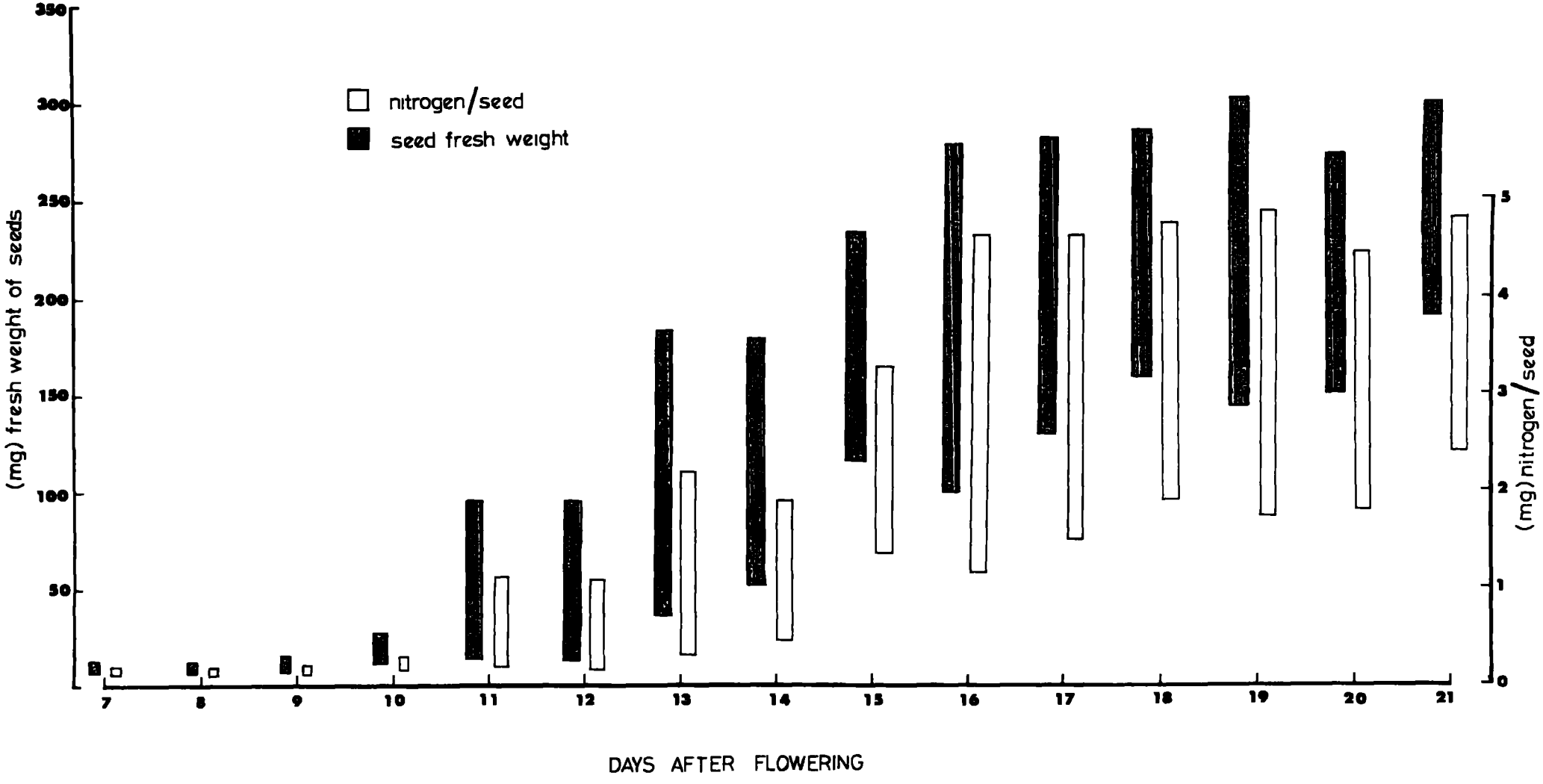
In preliminary experiments, SDS electrophoresis was used to determine the most reliable parameter to monitor the sequence of nitrogen accumulation and protein synthesis. Four parameters were considered: seed fresh weight, length of seed, length of pod and days after anthesis. More consistent densitometric scans of gel patterns (both in intensity of staining and number of bands) were found with seeds of approximately equal fresh weight and equal length of seeds from different harvests than with seeds of similar ages or length of pod.

Subsequently fresh weight was used as a basis to determine the course of nitrogen and sulphur accumulation and protein synthesis during cotyledon development. However, a plot of fresh weight increase and seed size against days after flowering from one harvest is given for comparison with material grown under other conditions (Fig. 19). The increase in fresh weight and seed size started slowly (only $\frac{1}{3}$ its maximum size by Day 10) and then increased rapidly until about Day 18 after which there was a further increase to maturity but not as rapid as the phase before.

F I G. 20

Distribution of fresh weight and nitrogen content per seed against days after flowering.

The range for each day after flowering represents the average fresh weights of seeds and nitrogen content per seed in a pod from at least 10 pods harvested at different times throughout the fruiting period. Vertical bars indicate the 95% confidence limits.


nitrogen/seed
seed fresh weight
(mg) fresh weight of seeds
(mg) nitrogen/seed
350
300
250
200
150
100
50
0
1
2
3
4
5
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
DAYS AFTER FLOWERING

B) Molecular changes during fruit development

(i) Seed

Globulins from developing seeds were prepared as described in Methods section VII (A) (ii) (b). The data presented in Figs. 21, 22 and 23 indicate the following developmental phases from anthesis to seed maturity in cowpea.

P h a s e 1 0 to 59 mg seed fresh weight
(about 0 to 11 days after flowering)

The seed developed slowly in this phase and accumulated only 6.5% of the quantity of dry matter found in mature seeds. The major constituent was water which attained a maximum value of 87% of fresh weight.

By comparison with later stages nitrogen accumulation was slow and rate of "globulin" synthesis was low both in dry weight accumulation and % nitrogen content.

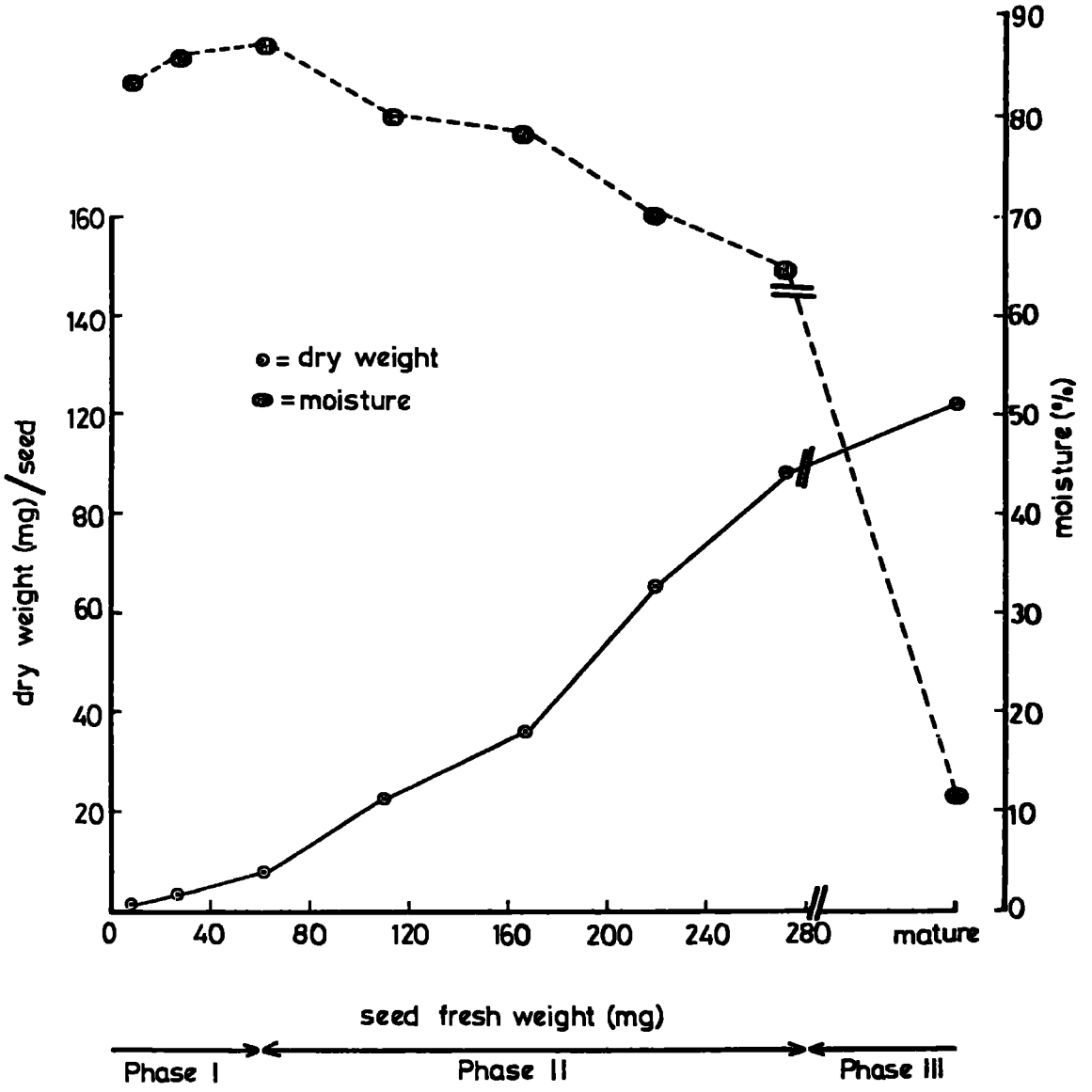
P h a s e II 60 to 289 mg seed fresh weight
(about 12 to 21 days after flowering)

At the beginning of this phase the seed showed deposition of protein in small cytoplasmic vesicles and larger vacuoles (Plate 1). This coincided with the beginning of the rapid increase of total "globulin" fraction (pH 5.0 insoluble fraction) and % N in "globulin". But between approximately 120 to 160 mg seed fresh weight there was a relatively slower rate of increase and then dry weight, total nitrogen and "globulin" fraction increased rapidly at a steady rate.

About 80% of the final content of dry matter and total

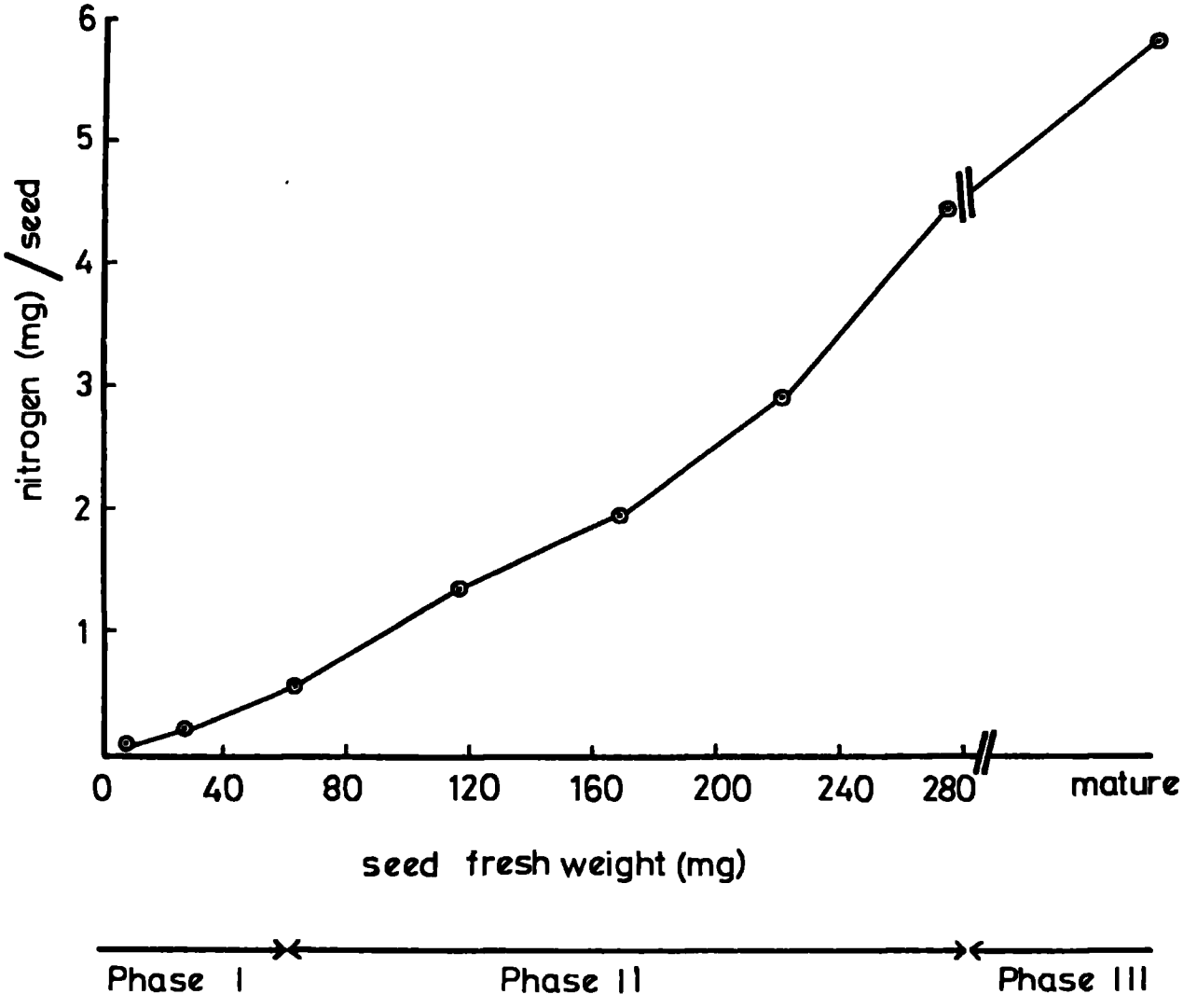
F I G. 21

Changes in dry weight and moisture content with seed fresh weight during development.


o = dry weight
● = moisture
dry weight (mg)/seed
moisture (%)
160
140
120
80
60
40
20
90
80
70
60
50
40
30
20
10
0
0
40
80
120
160
200
240
280
mature
seed fresh weight (mg)
Phase I
Phase II
Phase III

F I G. 22

Increase in nitrogen content with seed fresh weight during development.


nitrogen (mg) /seed
6
5
4
3
2
1
0
40
80
120
160
200
240
280
mature
seed fresh weight (mg)
Phase I
Phase II
Phase III

F I G. 23

Increase in seed globulin content and nitrogen content in globulin with secd fresh weight during development.
80 to 90% of total nitrogen was extracted from macerated seeds at each stagc of development considered.

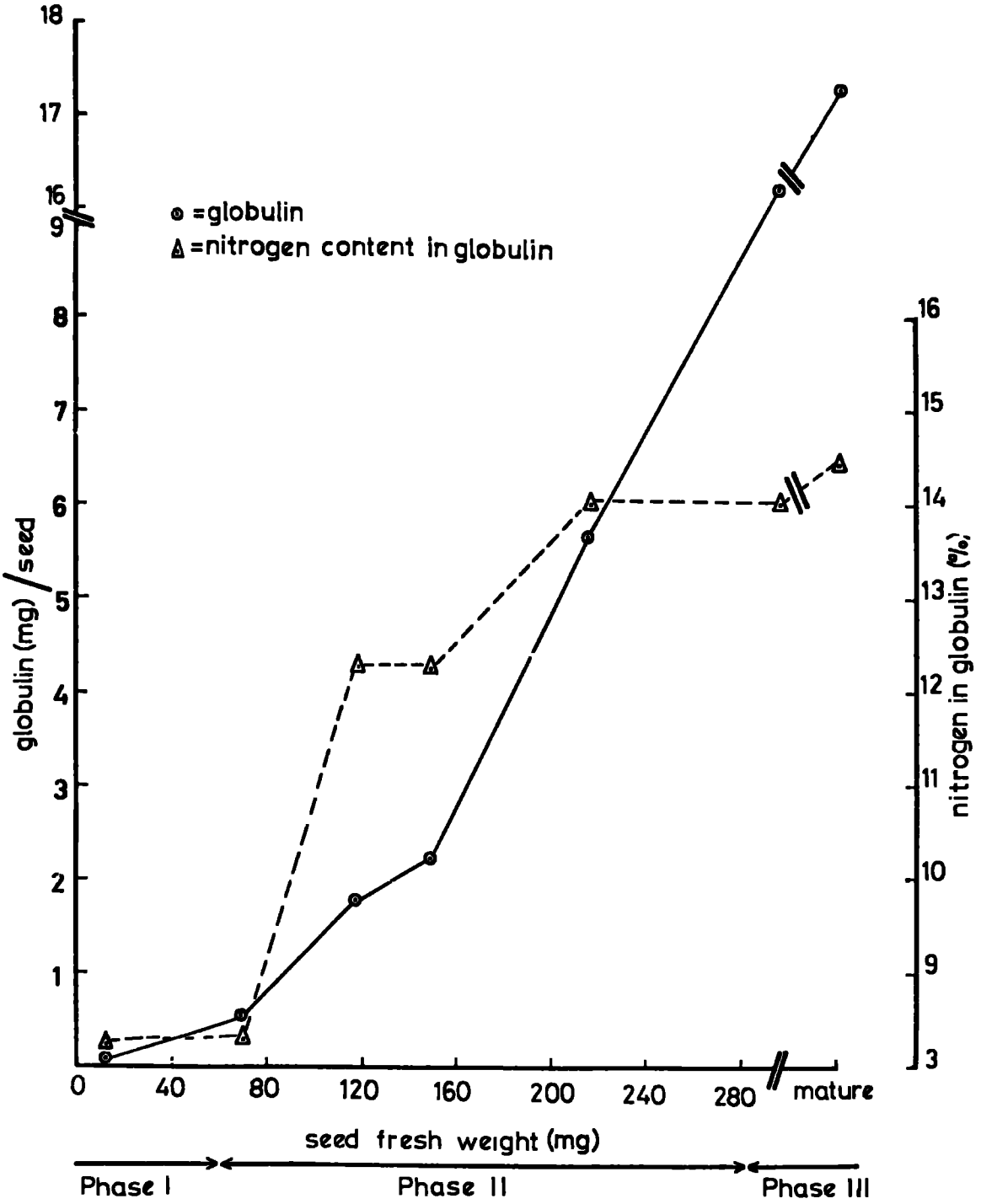
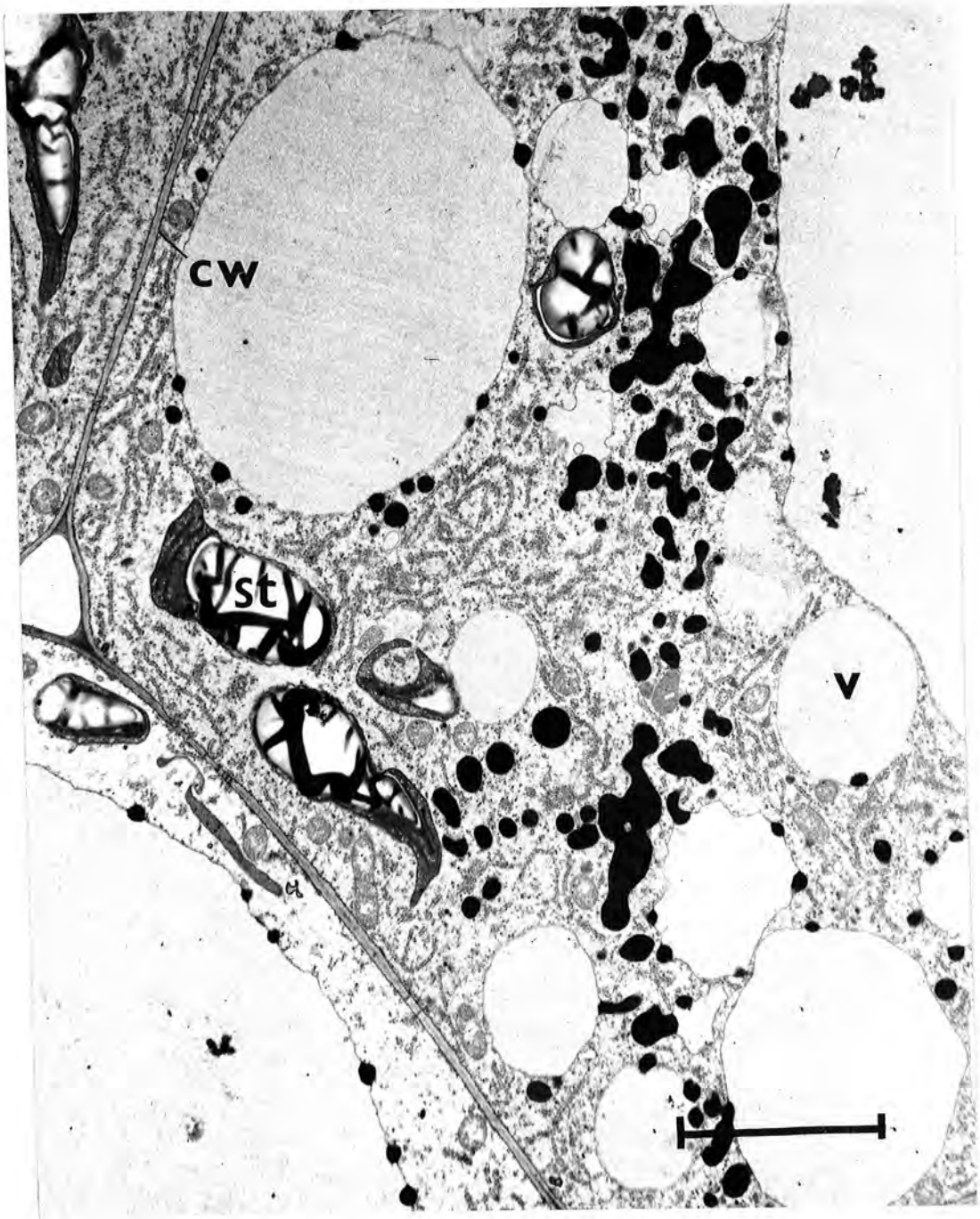

⊙ =globulin
△ =nitrogen content in globulin
globulin (mg) /seed
nitrogen in globulin (%)
seed fresh weight (mg)
mature
Phase I
Phase II
Phase III

PLATE 1

Part of cotyledonary cell of 60 mg seed showing deposition of protein in small cytoplasmic vesicles and larger vacuoles : bar represents 4μm

Abbreviations

cw = cell wall
st = starch grain
V = vacuole


CW
st
V

nitrogen was incorporated in the seed by the end of this phase. The major component of the rapid increase in dry weight initially was probably the accumulation of starch grains (Plate 2).

Water was lost, but at a slow rate - a decrease of 85% to 64% moisture by the end of this stage.

P h a s e III 290 to 130 mg seed fresh weight (drying out stage)

(about 22 to 26 days after flowering)

In the final stage to maturity both dry matter and nitrogen increased but at a slower rate than that of the end of the previous phase. One of the principal components of the increased dry matter was the globulin, almost double the weight had accumulated when compared with the total globulin content at the end of the previous phase. A mature seed showed a much higher concentration of protein bodies (Plate 3) when compared to the previous phase (Plate 2). The rapid synthesis of globulin was accompanied by a rapid dehydration of the seed - a decrease of 64% at the end of the previous phase to 10.4% moisture content at maturity.

The changes in globulin content extracted from developing cowpea seeds (the globulins obtained to produce the results given in Fig. 23 were used) were investigated by:

(a) Electrophoresis of dissociated globulins

No protein bands were visible on the gels loaded with "globulin" of seed fresh weight 8 mg and 62 mg. The components of globulins which were detected at various stages are shown in Table 7. A major band with Rm 0.2 which was visible in the gels but which was not resolved by densitometric scanning is included

P L A T E 2

Part of cotyledonary cell of 160 mg seed showing deposition of starch grains and protein bodies: bar represents 4μm

Abbreviations

cw = cell wall
pb = protein body
st = starch grain

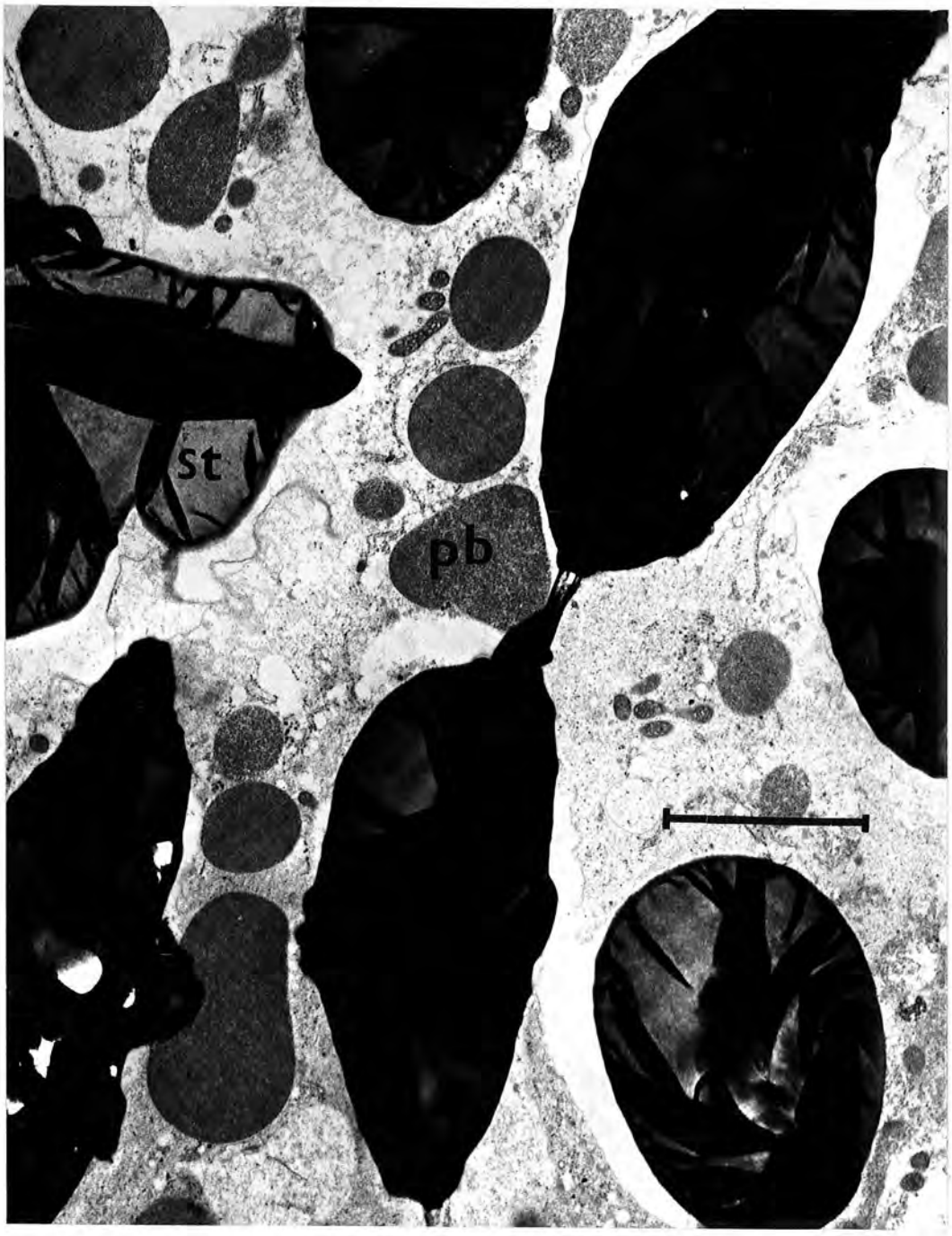

st
pb

PLATE 3

Part of cotledonary cell of mature seed showing protein bodies, starch grains and lipid reserve: bar represents 1.0μm

Abbreviations

cw = cell wall
l = lipid reserve
pb = protein body
st = starch grain

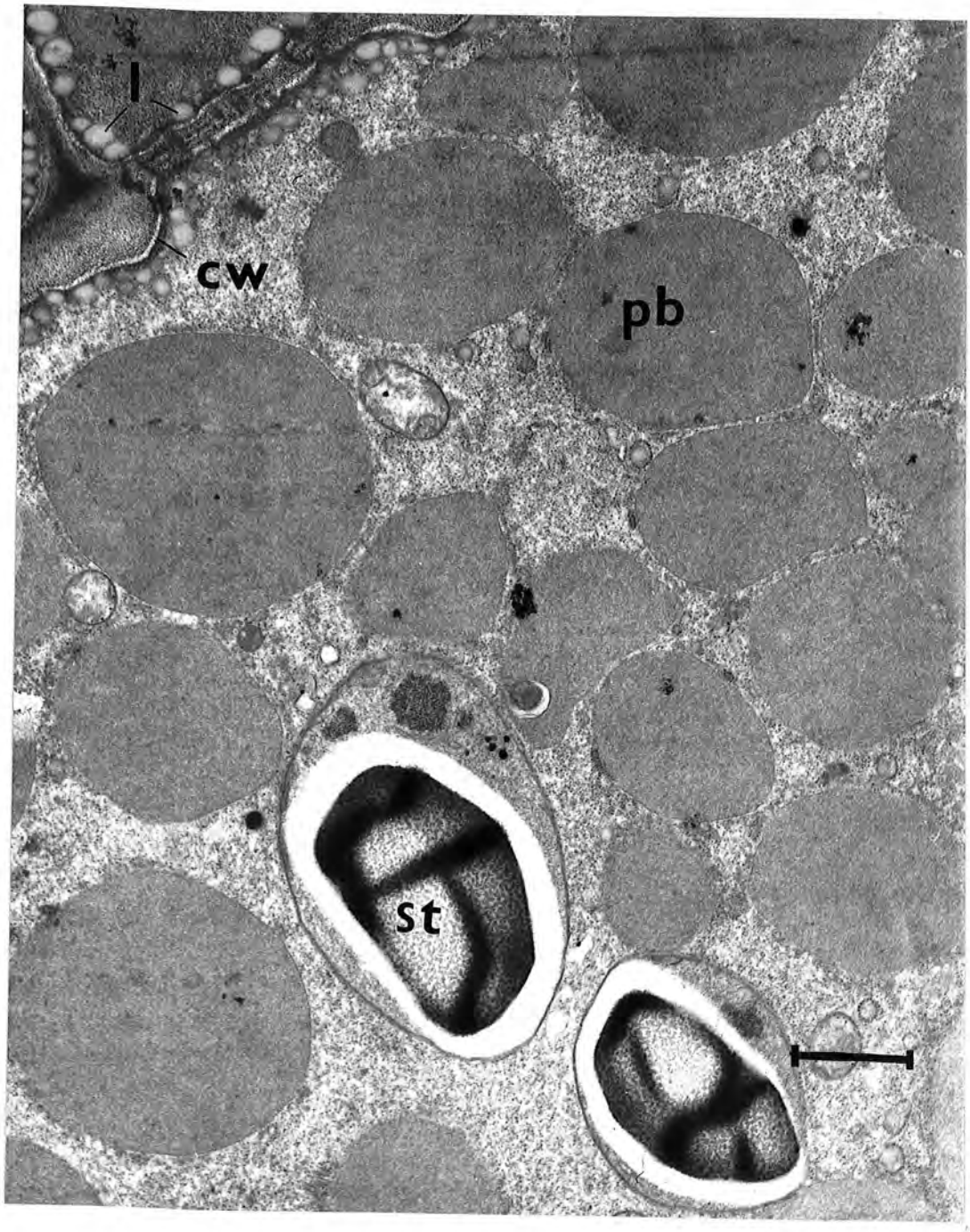

l
cw
pb
st

TABLE 7

Rm values of globulin samples of developing cowpea seeds in 10% SDS - acrylamide gels

Stage of development (seed fresh weight) mg	Rm values
8	no bands detected
62	no bands detected
110	0.22 0.25 0.41 0.45 0.58 0.73
164	0.2 0.21 0.24 0.40 0.45 0.50 0.58 0.73
220	0.2 0.22 0.24 0.32 0.41 0.46 0.53 0.58 0.73
270	0.2 0.23 0.25 0.31 0.41 0.45 0.51 0.58 0.71
mature	0.2 0.23 0.25 0.32 0.38 0.41 0.46 0.51 0.58 0.70

Rm values of major components are underlined

in the table. Changes in the synthesis of the subunits of globulins of developing cowpea seeds are illustrated by electrophoretic profiles in Figs. 24 a to e. At the 110 mg seed fresh weight the following subunits with Rm's 0.22, 0.25, 0.41, 0.45, 0.58 and 0.73 were observed. Globulins from seeds of later stages resolved on SDS gels showed additional bands and varying ratios of polypeptide chains to those given above for the 110 mg seed. The additional bands which were resolved at the later stages were: 164 mg (Rm's 0.2 and 0.5), 220 mg (Rm 0.32) and mature seeds (Rm 0.38). The relative staining intensities of the two major bands with Rm 0.23 and 0.25 changed during development and band Rm 0.25 increased progressively relative to band Rm 0.23. When the seed fresh weight was 110 mg, 164 mg and 220 mg the staining intensity of band Rm 0.23 was greater than that of band Rm 0.25 (Figs. 24 a, b, and c). However, their intensities were approximately equal at the stage of seed fresh weight 270 mg (Fig. 24 d) and on the seed attaining maturity band Rm 0.25 was the major component (Fig. 24 e).

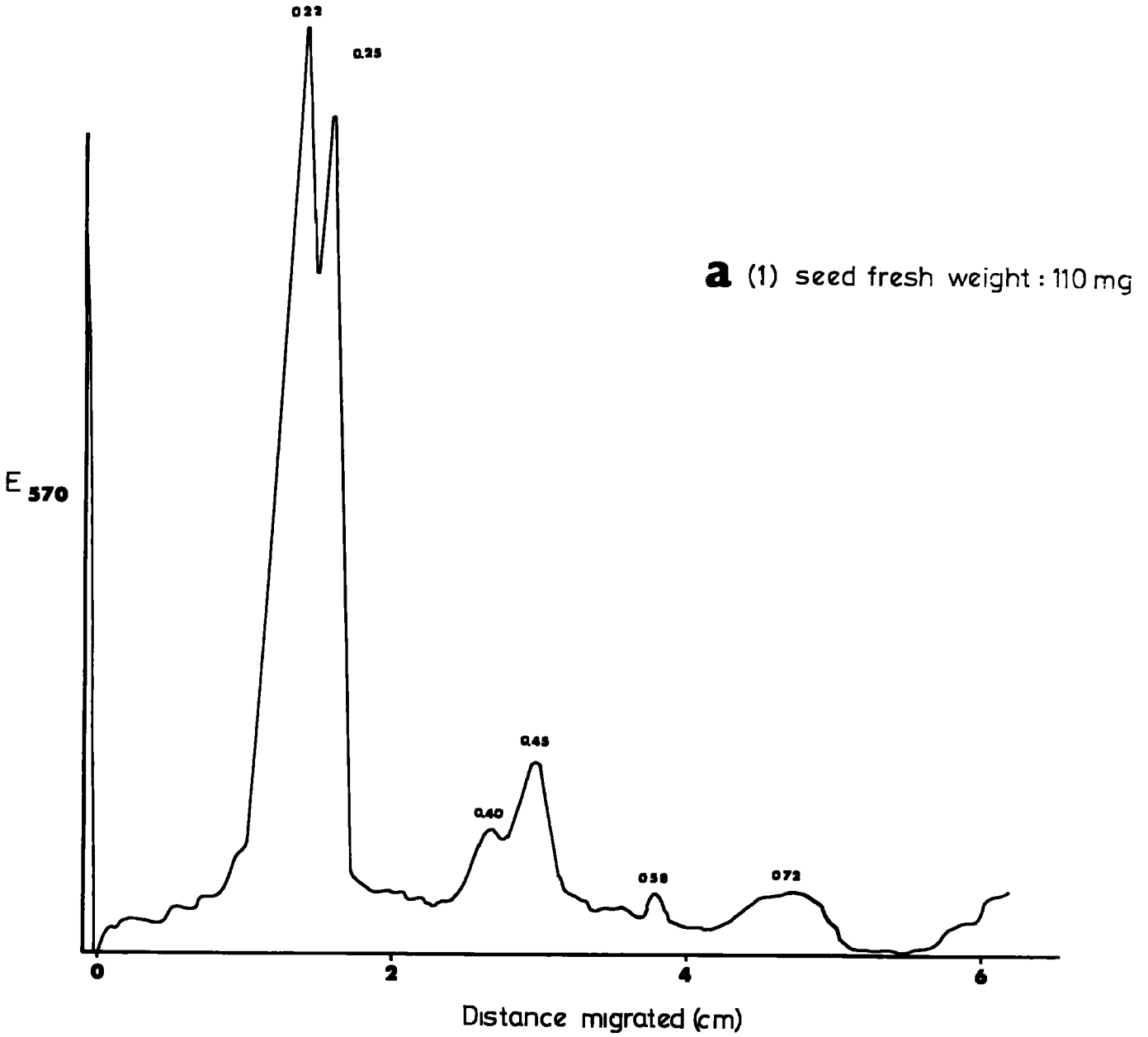
The electrophoretic pattern of the dissociated globulin of the mature seed (Fig. 24 e) is similar to the electrophoretic pattern of the SDS extract of cowpea meal (Fig. 3).

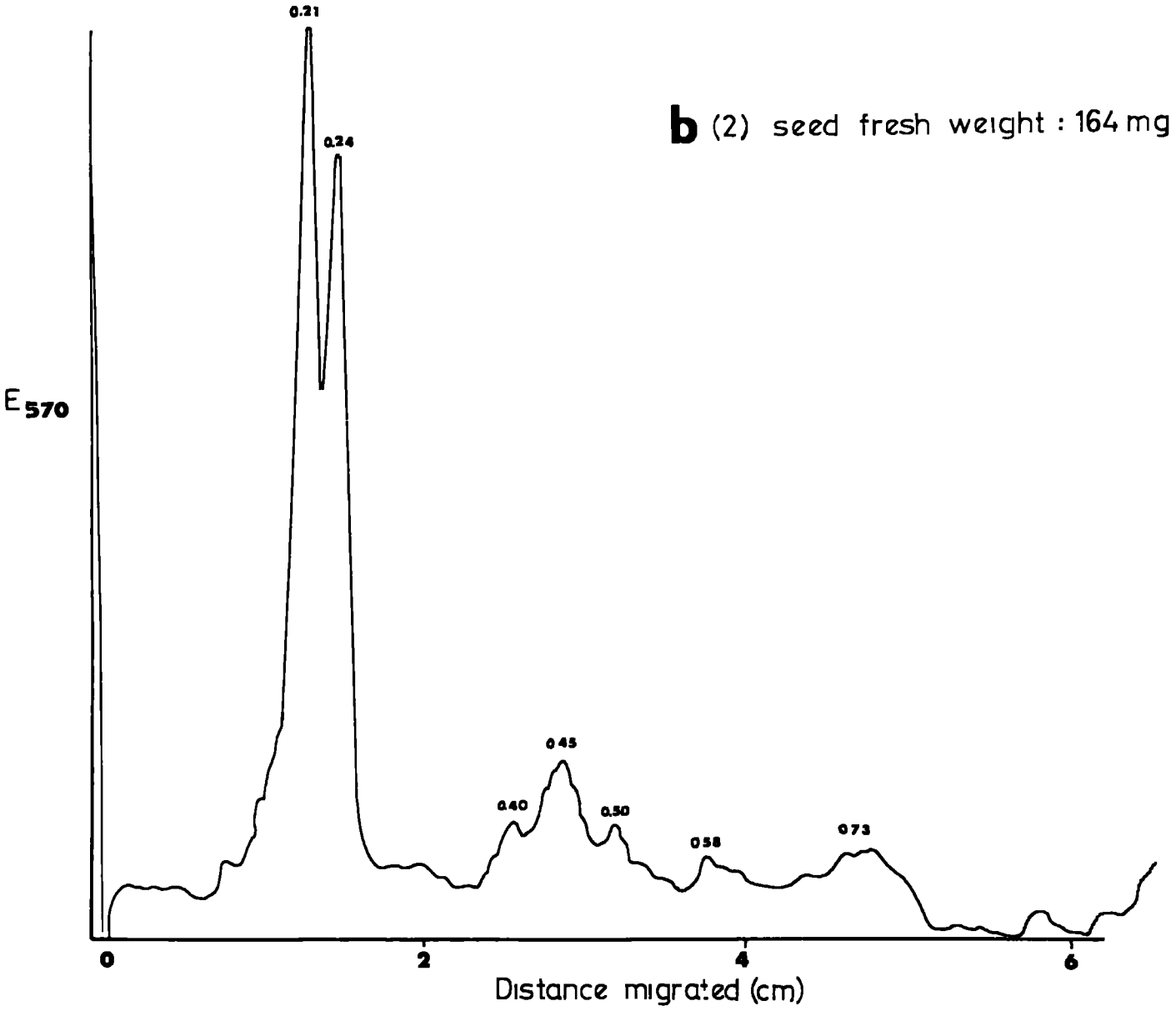
(b) Immunoelectrophoresis

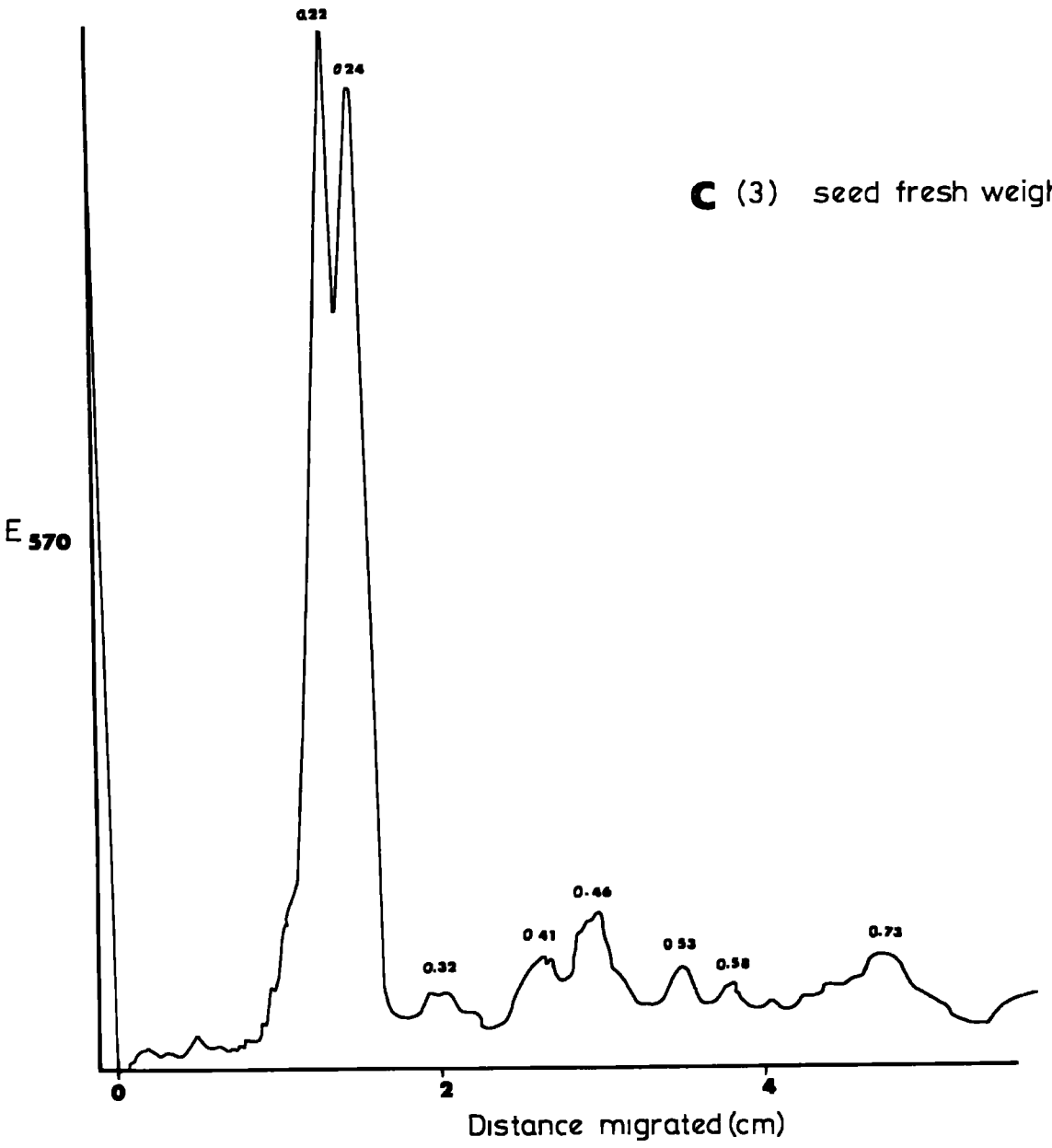
The antiserum employed was obtained from rabbits into which an unfractionated extract of mature seeds had been injected. Five precipitin arcs, designated a, b, c, d and e for convenience, were detected when globulin of developing cowpea seeds were immuno-electrophorised (Fig. 25 and Table 8). No arcs were visible on the slides loaded with "globulin" of seed fresh weight 8 mg and 62 mg. With immature seeds, which had developed further, precipitin arcs

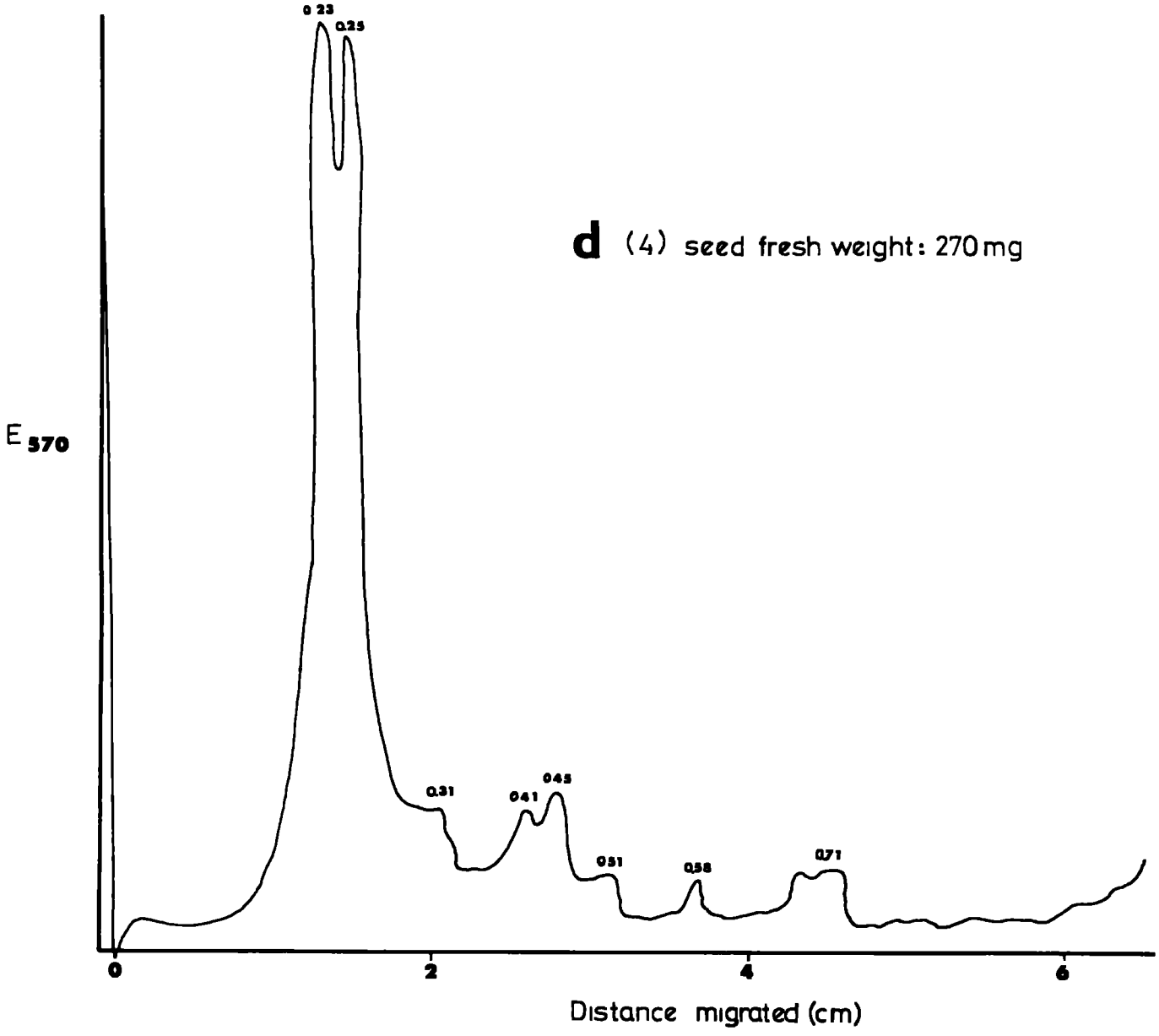
F I G. 24 (a to e)
=1 to 5

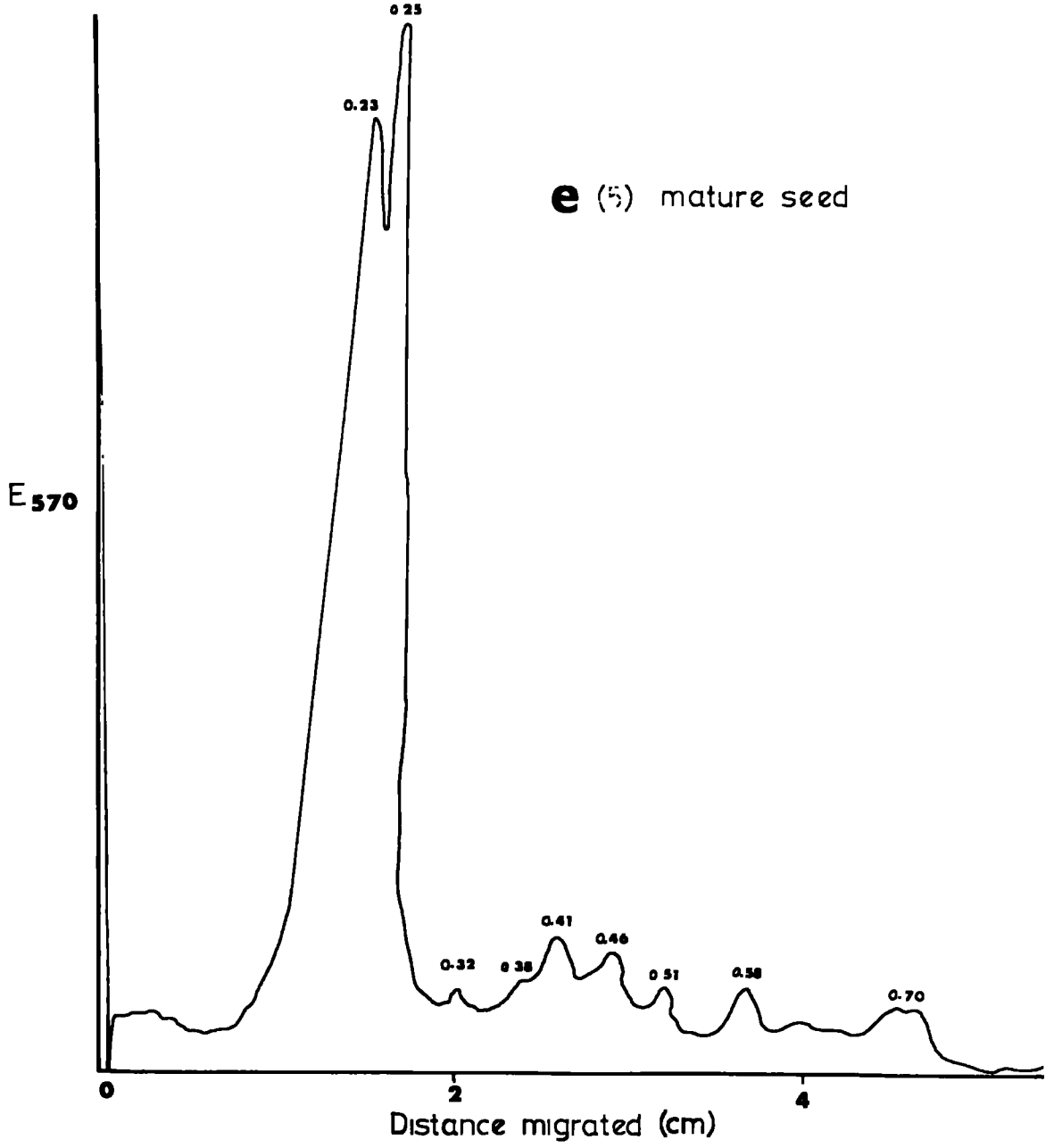
SDS electrophoretic profiles of globulins extracted from seeds at various stages of development. Seed fresh weight: (1) 110 mg (2) 164 mg (3) 220 mg (4) 270 mg (5) mature. The numbers refer to the molecular weights (x 10^{-3}) of the associated peaks.


a (1) seed fresh weight : 110 mg
E 570
0.22
0.25
0.40
0.45
0.58
0.72
0
2
4
6
Distance migrated (cm)


b (2) seed fresh weight : 164 mg
E570
0.21
0.24
0.40
0.45
0.50
0.58
0.73
0
2
4
6
Distance migrated (cm)


C (3) seed fresh weight : 220 mg
E 570
0.22
0.24
0.32
0.41
0.46
0.53
0.58
0.73
0
2
4
Distance migrated (cm)

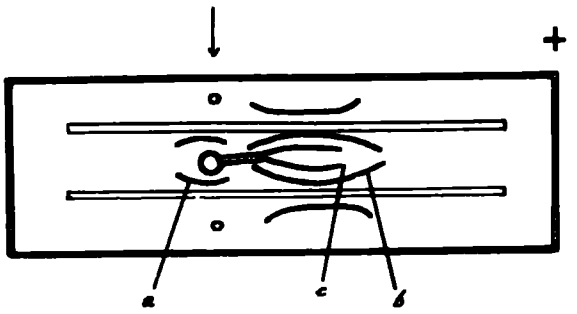

d (4) seed fresh weight: 270 mg
E 570
0.23
0.25
0.31
0.41
0.45
0.51
0.58
0.71
0
2
4
6
Distance migrated (cm)


e (5) mature seed
E570
0.25
0.23
0.32
0.38
0.41
0.46
0.51
0.58
0.70
0
2
4
Distance migrated (cm)

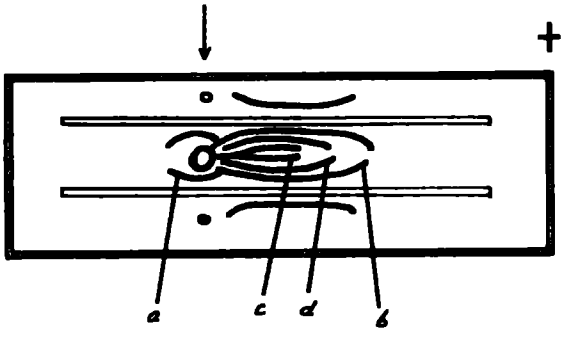
F I G. 25

Immunoelectrophoretic patterns of globulin antigens of cowpea seeds at various stages of development. The precipitin lines were obtained by using an antiserum produced against an unfractionated extract (pH 8.0) of mature seeds of cowpea.

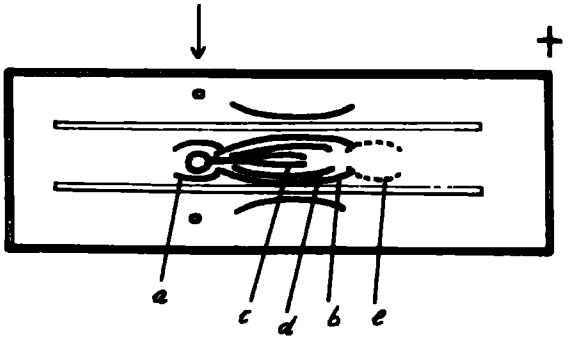
(I) SEED FRESH WEIGHT : 110 mg and 164 mg

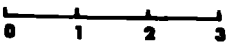


(II) SEED FRESH WEIGHT : 220 mg and 270 mg



(III) MATURE SEED





scale (cm)

were formed after immunoelectrophoresis. At the 110 mg seed fresh weight stage arcs a, b and c were observed. Later during development additional precipitin arcs were formed: 220 mg (arc d) and mature seed (arc e). Antigen component forming arc e is present in very small quantities as the arc it formed was faint.

TABLE 8

Precipitin lines detected after immunoelectrophoresis of globulins at various stages of seed development

Stage of development (seed fresh weight) mg	Precipitin lines
8	none
62	none
110	a, b, c
164	a, b, c
220	a, b, c, d
270	a, b, c, d
mature	a, b, c, d, e

Total sulphur content

The material used for determining total sulphur content was grown in Nigeria and was not the cultivar Prima used in all other experiments. Table 9 shows the sulphur content in cowpea at various stages of maturity.

TABLE 9

Total sulphur content in cowpea
V. unguiculata (accession Tvu 201-1D)

Days after flowering	Sulphur (mg) / seed
7	0.017
10	0.051
10+	0.126
14	0.216
18	0.255
21	0.288
22+	0.306

The accumulation of sulphur followed a similar pattern to changes in dry weight as shown in Fig. 21. Up to about Day 10 sulphur content was low, then increased rapidly to Day 21.

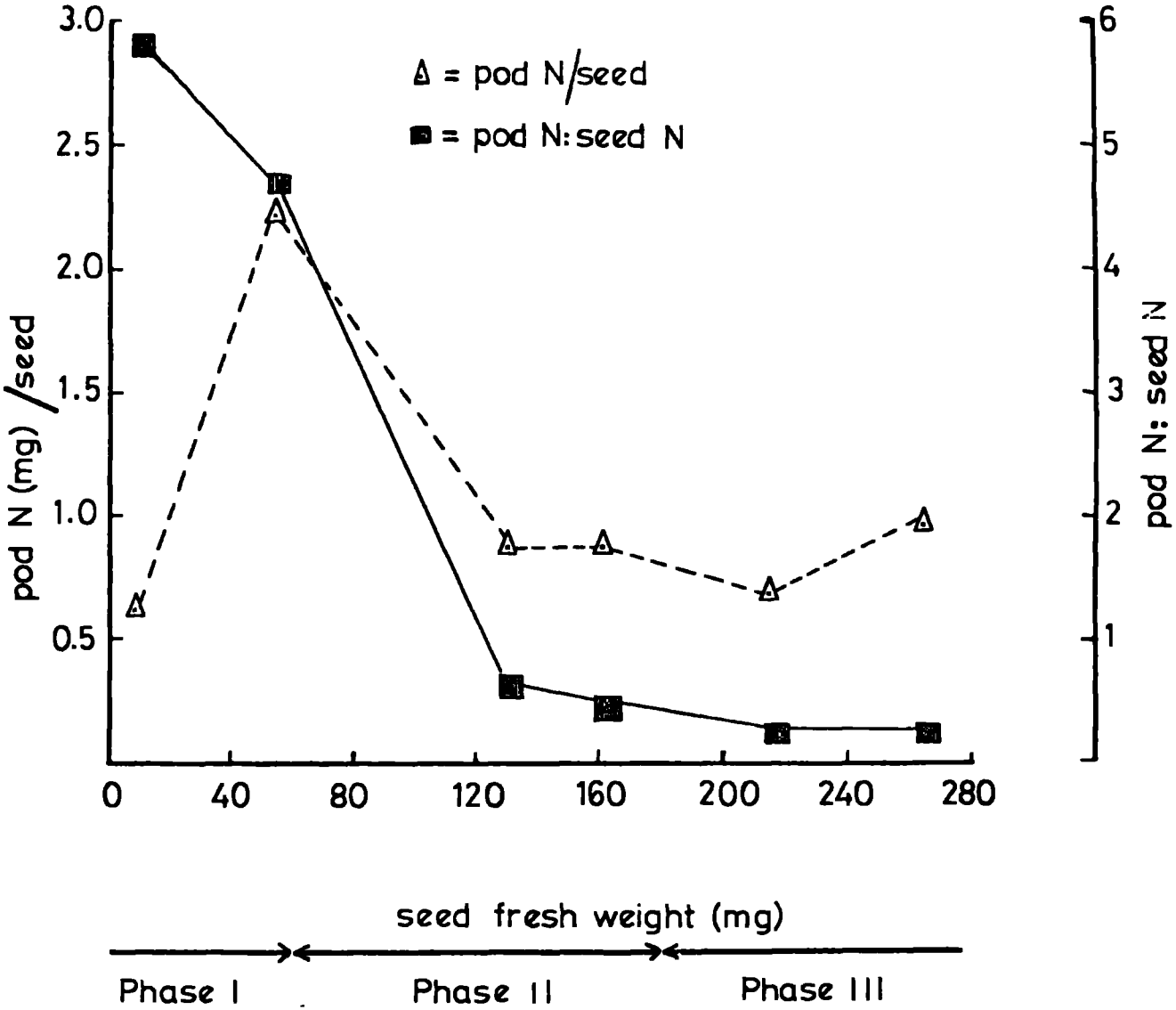
(ii) pod-seed system

Seed development at different stages could not be monitored using length or weight of a pod as the number of seeds in a pod was not constant. Thus seed fresh weight was used as before and data for content of pods has been presented as the total nitrogenous component divided by the number of seeds in the pod considered. The data in this section for the "pod" does not mean the whole fruit but only the carpel wall.

From the graphs showing the changes in nitrogenous components in the pod-seed system (Figs. 26, 27 and 28) the following phases were delimited:

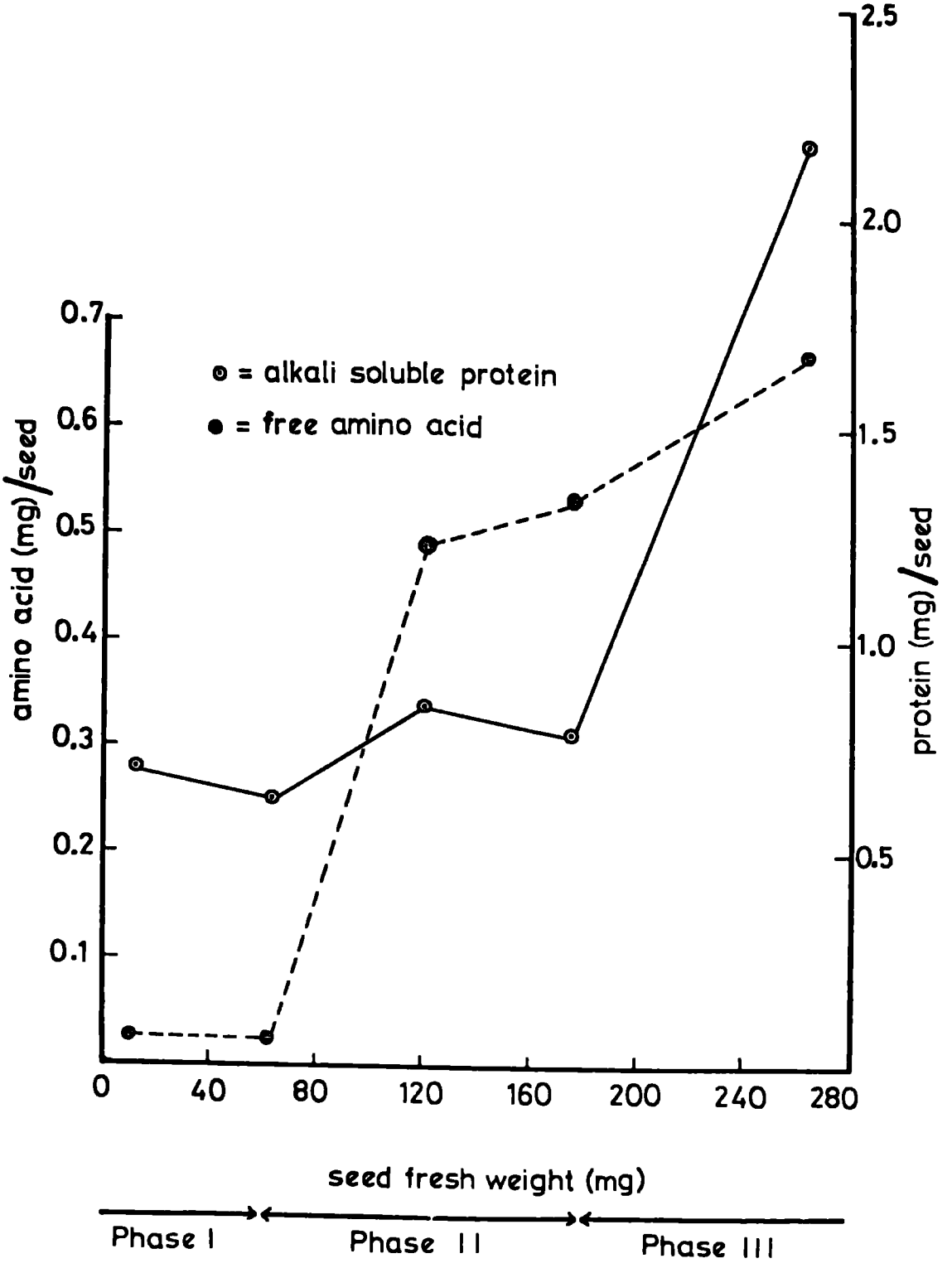
FIG. 26

Nitrogen content in pod and seed during development.


Δ = pod N/seed
■ = pod N:seed N
pod N (mg) /seed
pod N: seed N
3.0
2.5
2.0
1.5
1.0
0.5
6
5
4
3
2
1
0
40
80
120
160
200
240
280
seed fresh weight (mg)
Phase I
Phase II
Phase III

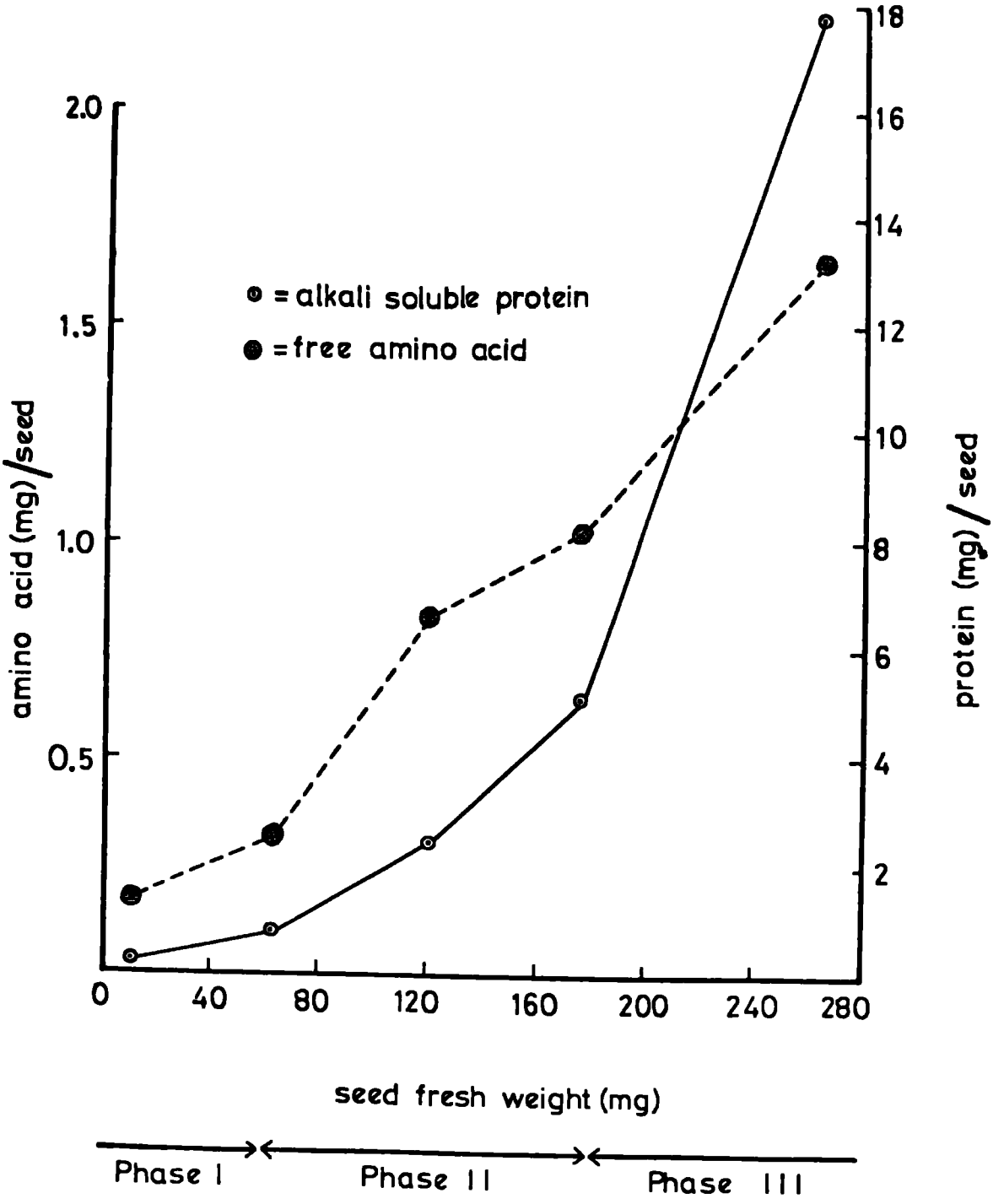
F I G. 27

Free amino acid and alkali-soluble protein content in pods during development.


⊙ = alkali soluble protein
● = free amino acid
amino acid (mg)/seed
protein (mg)/seed
0.7
0.6
0.5
0.4
0.3
0.2
0.1
2.5
2.0
1.5
1.0
0.5
0
40
80
120
160
200
240
280
seed fresh weight (mg)
Phase I
Phase II
Phase III

F I G. 28

Free amino acid and alkali-soluble protein content in individual seeds during development.


2.0
1.5
1.0
0.5
amino acid (mg) / seed
= alkali soluble protein
= free amino acid
18
16
14
12
10
8
6
4
2
protein (mg) / seed
0
40
80
120
160
200
240
280
seed fresh weight (mg)
Phase I
Phase II
Phase III

P h a s e I (0 to 59 mg seed fresh weight)

The pod N : seed N ratio fell throughout this period but remained well above unity.

The nitrogen content in the pod reached a maximum during this phase. However, the free amino acids and alkali-soluble protein in the pod were at their lowest levels. Thus more than 90% of the nitrogen was present as inorganic nitrogen in the pod.

The level of free amino acids in the seed was also low and the accumulation of alkali-soluble protein was at its lowest during this stage of pod-seed development.

P h a s e II (60 to 180 mg seed fresh weight)

The pod N : seed N ratio fell sharply, initially to a figure less than unity, and then subsequently more gradually.

The nitrogen level in the pod dropped rapidly to less than half the maximum and at the same time there was a very large increase in free amino acid. The alkali-soluble protein increased initially and then decreased slowly.

In the seed the free amino acid pool increased rapidly and the synthesis of alkali-soluble protein was higher than at the previous phase.

P h a s e III (181 to 280 mg seed fresh weight)

The pod N : seed N ratio continued to fall gradually and well below unity.

The level of nitrogen in the pod increased slowly towards the end of this phase. The free amino acid continued to increase

but it was the alkali-soluble protein component that increased substantially in the pod during this period in development.

The free amino acid continued to increase in the seed and the alkali-soluble protein accumulated more rapidly than during the previous phase. 3 times more protein accumulated than the total protein before this stage.

Amino acid composition

The amino acid compositions of developing pod and seed were determined separately at two different stages:

(i) seed fresh weight 70 mg (corresponding to the beginning of Phase II);

(ii) seed fresh weight 240 mg (corresponding to Phase III).

The results are presented in Tables 10 and 11.

V PROTEIN PATTERNS IN V. UNGUICULATA AND ITS WILD RELATIVES

The relative mobilities (Rm) values of the subunits of proteins present in the cultivated variety, Prima and the 10 wild relatives investigated are summarised in Table 12.

The electrophoretic band patterns of the cultivated variety, Prima had more similarities than differences in subunit mobilities when compared with the wild relatives.

Of the more intensely stained subunits only one differed in all the wild relatives (Rm 0.17 - 0.2) from the cultivated variety (Rm 0.16). With this exception four (Tvnu 36, 40, 41 and 89) of the wild relatives (Group A) had similar electrophoretic patterns to Prima. The remaining six wild relatives (Group B)

TABLE 10

The total amino acid compositions of pod and seeds at two different stages:

(i) seed fresh weight 70 mg

(ii) seed fresh weight 240 mg

All results are expressed as g amino acid residues recovered / 100 g of freeze-dried sample.

Amino acid	Pod		Seeds	
	(i)	(ii)	(i)	(ii)
Aspartic acid	1.26	0.96	2.06	3.20
Threonine	0.50	0.31	1.13	0.84
Serine	0.50	0.34	1.04	1.03
Glutamic acid	1.13	0.84	2.16	2.96
Proline	0.44	0.29	0.96	0.87
Glycine	0.43	0.28	0.86	0.79
Alanine	0.53	0.33	1.07	0.91
$\frac{1}{2}$Cystine	0.17	0.12	0.37	0.31
Valine	0.61	0.37	1.27	1.18
Methionine	0.15	0.09	0.43	0.30
Isoleucine	0.50	0.31	1.27	1.03
Leucine	0.85	0.55	1.56	1.72
Tyrosine	0.35	0.21	0.96	0.69
Phenylalanine	0.56	0.33	1.20	1.27
Histidine	0.29	0.16	1.31	0.91
Lysine	0.69	0.48	1.46	1.53
Arginine	0.59	0.33	1.82	1.77
	9.55	6.30	20.93	21.31

T A B L E 11

The total amino acid compositions of pod and seeds at two different stages:

(i) seed fresh weight 70 mg

(ii) seed fresh weight 240 mg

All results are expressed as mg amino acid residues recovered.

Amino acid	Pod		Seeds	
	(i)	(ii)	(i)	(ii)
Aspartic acid	4.81	6.79	3.13	16.64
Threonine	1.91	2.19	1.72	4.37
Serine	1.91	2.40	1.58	5.36
Glutamic acid	4.32	5.94	3.28	15.39
Proline	1.68	2.05	1.46	4.52
Glycine	1.64	1.98	1.31	4.11
Alanine	2.02	2.33	1.63	4.73
$\frac{1}{2}$Cystine	0.65	0.85	0.56	1.61
Valine	2.33	2.62	1.93	6.14
Methionine	0.57	0.64	0.65	1.56
Isoleucine	1.91	2.19	1.93	5.36
Leucine	3.25	3.89	2.37	8.94
Tyrosine	1.34	1.48	1.46	3.59
Phenylalanine	2.14	2.33	1.82	6.60
Histidine	1.11	1.13	1.99	4.73
Lysine	2.64	3.39	2.22	7.96
Arginine	2.25	2.33	2.77	9.20
	36.48	44.53	31.81	110.81

T A B L E 1 2

Rm and MW values of subunits of major proteins of *V. unguiculata* on SDS gels

var Prima		Wild relatives		
Rm	MW	Rm		MW
0.09	85,000	0.08 - 0.1	(10)	83,000 - 87,500
0.16	64,000	0.17 - 0.2	(10)	55,000 - 62,000
*0.21 - 0.25	56,200 - 48,500	*0.21 - 0.26	(10)	48,000 - 56,200
		0.31	(6)	45,000
		0.33	(6)	42,000
0.35	40,000	0.35	(4)	40,000
0.47	31,300	0.42 - 0.46	(10)	32,000 - 36,000
		0.48	(4)	30,500
		0.50	(2)	29,500
0.55	27,000	0.52 - 0.55	(10)	27,000 - 28,500
0.61	18,500	0.58 - 0.63	(10)	17,500 - 19,500

( ) number of accessions showing a band in the range stated

* resolved into 2 or 3 components within range stated

differed in having two subunits with Rm of 0.31 and 0.33 where Prima and Group A wild relatives have one subunit with a Rm of 0.35. From densitometric tracings, the amount of staining in the regions of Rm 0.31 and Rm 0.33 was almost twice the amount found in gels with band of Rm 0.35. Thus subunits Rm 0.33 and Rm 0.35 are within experimental error probably homologues; the former possibly being slowed down by the presence of subunit with Rm 0.31. Therefore one band (Rm 0.31) is present in Group B of the wild relatives and not found in Prima and Group A relatives. Also four of the six wild relatives in Group B had one or two additional subunits with Rm values of 0.48 (Tvnu 65, 73, 74 and 75) and 0.50 (Tvnu 65 and 75).

D I S C U S S I O N

In order to investigate the major proteins in the cowpea, an essential prerequisite was the examination of the efficiency of extraction of nitrogenous material from cowpea meals. Several factors affect the efficiency of solubilisation of the nitrogenous constituents of legume meals e.g. ionic strength and pH of the solvent, particle size of the meal, meal-solvent ratio, extraction times, temperature of extraction, number of successive extractions, mode of stirring and influence of lipids. With so many factors to consider, the extraction procedure was designed after consulting published works which had similar objectives with closely related and other species.

Above pH 7.0 the ionic strength of the extractant had little effect on the dispersion of the nitrogenous components of the seeds of Phaseolus vulgaris as almost 100% of the nitrogen was extracted between pH 7.0 and 9.0 at low or moderate ionic strength (Pusztai, 1965). Djang et al. (1953) reported that the yield of solubilised nitrogen from a defatted sample of Phaseolus aureus was greater than with lipid-containing meals provided the temperature was below 45°. Defatted meals were also used when extracting protein for subsequent fractionation in the experiments reported on here since Tombs (1967) has reported that without prior defatting, lipid emulsions were formed which made some separations impossible. Two successive extractions with 0.4M sodium chloride were used since with the results of Djang (1953) and Mohammad et al. (1973) further extractions did not recover any more nitrogen.

The degree of extraction obtained (71 to 73% of the total nitrogen) with 40-mesh cowpea meals was comparable with that obtained by Ainouz (1970) who also worked with cowpea but not to 88% nitrogen extraction efficiency obtained with investigations on Phaseolus vulgaris (Evans and Kerr, 1963; Pusztai, 1965; Ishino and Ortega, 1975). The efficiency of extraction could possibly be improved and thus the amount of nitrogen not dispersed in the seed reduced by the use of smaller particle size of meal. This was achieved with rice meals when solubility was compared between 40-mesh and 100-mesh samples (Cagampang et al., 1966).

The major proportion of the nitrogen in the seed is in the form of protein as indicated by the high percentage of total meal nitrogen that remained in the sac after dialysis. Pusztai (1965) obtained a similar result with Phaseolus vulgaris.

Landry et al. (1972) extracted the bulk of nitrogen (94%) of defatted barley seed with dilute aqueous solutions of SDS with added reducing agents. Since SDS is efficient in extracting nitrogen from seed meals, an SDS extract of a cowpea meal was electrophorised and the major proteins characterised. The meals yielded three subunits, which made up more than two-thirds of the protein extracted and whose apparent molecular weights are 56,200, 53,000 and 48,500. These subunits are hereinafter referred to as the major subunits. The other subunits obtained were with apparent molecular weights 85,000, 42,000, 31,300, 27,000 and 18,500. From non-dissociating gel electrophoresis it appears that there are at least three proteins present in extracts when meals are extracted with a standard buffer solution.

Ultracentrifugation of cowpea extracts gave a single

peak of approximate sedimentation coefficient 7S. Joubert (1957) also found the proteins from the cowpea varieties he examined to contain a major 7.3S component and which could be reversibly dissociated. The 7S component was equated by Daniellson (1949) with the globulin, vicilin.

The methods that have been utilised in attempts to separate the major cowpea seed proteins were solubility, size and possibly shape and charge; the object to fractionate the major proteins in cowpea were two-fold:

(a) to separate the major subunits with " apparent " molecular weights and approximate relative mobilities of 56,200 (Rm 0.2), 53,000 (Rm 0.23) and 48,500 (Rm 0.25). These molecular weights are apparent since it is possible when using the SDS method that the amino acid composition of a subunit affects to some extent the position after electrophoresis on gels. It is possible that these subunits have the same molecular weights and very different amino acid compositions.

(b) to isolate legumin (if it occurs) as in other legumes this globulin has been shown to contain relatively higher levels of sulphur-amino acids.

From work on other legume species mentioned in the introduction, the 7S globulin is heterogeneous. The detection of heterogeneity has practical limits which are linked directly to the powers of separation and detection associated with the method. Thus non-dissociation disc electrophoresis alone is not sufficient for the complete characterisation of seed proteins in mixtures because of the proximity and overlapping of bands and formation of polymers (Catsimpoolas and Ekenstam, 1969). Diffuse bands on polyacrylamide gels were observed when 7S fractions of two other

species were electrophorised by Bailey and Boulter (1972) and Ericson and Chrispeels (1973). Discontinuous non-dissociating polyacrylamide gel electrophoresis also failed to detect multiple forms of Dolichos biflorus lectin (Carter and Etzler, 1975). For these reasons therefore dissociating SDS gel electrophoresis has been used to monitor the various fractions in this investigation.

To avoid disulphide interchange reactions (Wolf and Briggs, 1959) many of the solutions used in the isolation of the proteins and their separation contained mercaptoethanol as a reducing agent.

Ascorbic acid extraction has been used in an attempt to separate the major globulins in Phaseolus vulgaris by McLeester et al. (1973). They obtained a major globulin fraction termed G1 (comprising three subunits with molecular weights of 53,000, 47,000 and 43,000) free from traces of cross-contamination and their procedure permits a large amount of material to be handled (Sun and Hall, 1975). For these reasons their method was used on cowpea meals but it failed to isolate the major subunits free from the smaller molecular weight subunits in the G1 fraction. This method is also not suitable for seed protein extractions as below the isoelectric points of storage proteins, pH 3.0 to 5.0, phytic acid in meals reduced the solubility of seed proteins (Fontaine et al., 1946). Phytic acid is

present in cowpeas (Sundararajan, 1938) and the formation of protein-phytate complex interferes in protein fractionation of fresh extracts of seed (Smith and Rackis, 1957; Mikola *et al.*, 1962; Saio *et al.*, 1967).

However, only this method did give an enriched fraction of subunit with molecular weight 27,000. This subunit, a glycoprotein, did not agglutinate red blood cells and thus was not comparable with the glycoprotein lectins of subunit molecular weight 30,000 isolated from *Phaseolus vulgaris* by Pusztai and Watt (1974). Further investigation on the structure of the cowpea subunit MW 27,000 may explain the lack of agglutinating ability.

Two definitions of globulin have been used in this thesis. The first is salt solubility *sensu* Osborne (1924). The other definition is an operational one by Pusztai and Watt (1974) and is given in the Methods section. Pusztai and Watt (1974) prepared globulins under defined conditions of pH and ionic strength both of which parameters have an effect on the extraction and functional groups of proteins. Thus separation procedures based on Pusztai's definition are likely to give similar results with chemically similar starting material even if prepared at different times.

The major globulins, legumin and vicilin, of *Vicia faba* and *Pisum sativum* have different isoelectric points and Danielsson (1949) found that the major globulins in *Pisum sativum* could

be separated by a simple but effective method - isoelectric precipitation. This method was also used successfully to separate legumin at its isoelectric point pH 4.7 in different species of the genus Vicia by Bailey and Boulter (1970) and Vaintraub et al. (1962). However, with cowpeas all of the major proteins precipitated around pH 5.5, thus making the use of differences in isoelectric points of proteins unsuitable for separating the major proteins of cowpeas.

Ammonium sulphate fractionation has been used to separate globulins of some legume species (Jones and Horn, 1930; Jones and Csonka, 1932; Daniellson 1949; Blagrove and Gillespie, 1975). With cowpeas the 0 to 50% saturation fraction consisted of most of the subunits found in cowpea but with an enrichment of subunits MW 85,000 and 64,000. Platsynda and Klimenko (1970) also used this method with cowpea and identified this fraction as a mixture of legumin and vicilin by the use of paper electrophoresis. In the 50 to 85% saturation range of fractions there was a progressive enrichment of the major subunits with increasing ammonium sulphate saturation. Similarly, Platsynda and Klimenko (1970) found that the 50 to 100% fraction consisted of electrophoretically similar components which they described as vicilins. Thus "salting out", did not separate the major proteins but could be used in the initial stages of separation to obtain an enriched fraction of the major subunits.

A modification of the manual isoelectric precipitation method combining the technique of gel filtration (Porath, 1962) and described as zonal isoelectric precipitation has been used to separate seed proteins of Vicia (Shutov and Vaintraub, 1965; Scholz et al.,1974). With cowpeas, zonal isoelectric precipitation gave an enrichment of one of the major subunits, MW 53,000 in the first retarded fraction; the other two major subunits MW 56,200 and 48,500 were enriched in the second retarded fraction. But both retarded fractions also contained three of the same smaller molecular weight subunits indicating cross-contamination. The unretarded fraction contained most of the subunits that were also found in the retarded fractions.

In the retarded fractions, the molecular weights of the major subunits (56,200, 53,000 and 48,500) plus the number of smaller molecular weight subunits do not correspond with preparations of legumin by zonal isoelectric precipitations on work with Vicia faba (Wright and Boulter, 1974) and Phaseolus vulgaris (Derbyshire and Boulter, 1976; Barker, 1975). The legumin fraction of Wright and Boulter (1974) and Derbyshire and Boulter (1976) sedimented as a 11S in the ultracentrifuge and when dissociated with SDS and mercaptoethanol yielded two subunits, which had molecular weights in the region of 37,000 and 20,000; Barker's (1975) preparation yielded two subunits with molecular weights 60,000 and 20,000.

The first retarded fraction was subjected to permeation chromatography to try and purify the major subunit with molecular weight 48,500 by molecular size difference but it was unsuccessful.

Another method, ion exchange chromatography, which has

been used to separate the seed proteins exploits the differences in charge of the functional groups of proteins. Grant and Lawrence (1964) investigated pea seed globulins in sodium chloride extractions by DEAE-cellulose chromatography and confirmed the presence of two components, legumin and vicilin. Following their method the major subunits of cowpea were not separated except in the fraction eluted at 0.25M NaCl where only one of the major subunits as well as two smaller molecular weight subunits were found; on non-dissociating polyacrylamide gels that fraction was seen to consist of two protein bands.

The failure to detect legumin similar in physico-chemical character to that found in other legumes by any of the methods indicates that either this protein was not present or present in insufficient quantities to be detected. This conclusion is in agreement with immunochemical results of Dudman and Millerd (1975). They used an antiserum specific for the legumin and vicilin of Vicia faba to examine extracts of seeds of the Leguminosae Subfamily Faboideae for the presence of related storage proteins. Although proteins related to legumin were found to be widely distributed in the subfamily, no reaction for legumin was obtained with cowpea extracts. Similar findings have also been reported by Kloz (1971) who has worked extensively on the serology of the Leguminosae. He reported that Vigna sinensis differed serologically from Pisum, Vicia, Lens and Glycine.

Osborne (1897) obtained vignin, his major component of cowpea proteins by dilution of a salt extract. This protein preparation was heterogeneous but it probably contained a high proportion of the major subunits MW 56,200, 53,000 and 48,500.

All the methods attempted to separate the major subunits utilising their potential differences in solubility, size and charge to friction ratio failed due to the highly aggregated nature of the protein mixture. Thus it was necessary to dissociate the proteins before attempting separation. Denaturation then, once a process avoided with proteins, is a useful technique through which unusual separations have been achieved (Masaki and Soejima, 1972; Carter and Etzler, 1975a) and through which the basic chemistry of proteins can be probed (Tombs, 1967).

One problem with the use of urea is the presence of cyanate ions (Marier and Rose, 1964) which can lead to carbamylation of amino groups (Cole, 1961; Manson, 1962; Cejka et al., 1968). Even after deionising urea solutions new cyanate formations occur at pH values greater than 4 (Gerding et al., 1971). However the amino acid analysis of the separated fractions showed no detectable homocitrulline which would have appeared as a result of lysine reacting with cyanate in urea (Stark et al., 1960).

In this work ion exchange chromatography on DEAE-cellulose in 8.0M urea was the most successful method in terms of resolution; but in terms of yield, the amount of protein recovered was low.

The ultraviolet absorption of the first unadsorbed fraction and of the eluted fraction which contained only two of the major subunits corresponded to that of a pure protein solution (max. at 280 nm). The other fractions showed varying degrees of contamination with nucleic acid. Seed protein fractions contaminated with nucleic acid have also been reported by Obara and Kimura (1967); Sawai and Morita (1968) and Mohammad et al. (1973).

The major subunits were completely adsorbed on the DEAE-cellulose column in urea and the major proportion of the smaller molecular weight subunits were not adsorbed. The cysteic acid content of two of the unadsorbed fractions was as high as that published by Boulter and Derbyshire (1971) for legumin of Pisum sativum and Vicia faba. Thus there is potential for nutritional improvement, but the smaller molecular weight subunits should be further characterised.

The two major subunits with apparent MW 53,000 and 48,500 contain sugar residues as shown by PAS staining and phenol-sulphuric acid assay. The amino acid composition of subunit MW 48,500 and the enriched fraction of MW 53,000 are typical of globulins as they contain a high proportion of aspartic and glutamic acid residues and low quantities of the sulphur-amino acids. Leucine or isoleucine, the N-Terminals of subunit MW 48,500 is also found as an N-Terminal amino acid in both the 7S and 11S fractions of soya bean (Catsimpoolas et al., 1967; Roberts and Briggs, 1965) and Vicia faba (Bailey and Boulter, 1970; 1972). A subunit with Rm 0.25 on 10% SDS acrylamide gels also present in both the legumin and vicilin fractions during seed development of Vicia faba was reported by Wright and Boulter (1972). Subunit MW 48,500 in cowpea, too, had a Rm of 0.25 on 10% SDS acrylamide gels and one of the two N-Termini common to both storage proteins in Vicia faba. This suggests that there is a subunit common to vicilin and legumin present in Vicia faba and cowpea, but in the case of Vicia faba it is only a minor component.

Other species in the same tribe as cowpea, Phaseolus vulgaris and Phaseolus aureus contain major proteins with similar

subunit molecular weights, sugar content and N-Termini as the major subunits in cowpea. In Phaseolus vulgaris Barker et al. (1976) reported pH 4.7 soluble fraction (equated with Glycoprotein II of Pusztai and Watt, 1970) which contained two subunits, MW 50,000 and 47,000 and one of the N-Terminals found was leucine. With Glycoprotein II Pusztai and Watt (1970) reported the following carbohydrate content: 0.99% glucosamine and 4.46% of neutral sugar residues. This figure for neutral sugar residues is more than double the amount found in the major subunits MW 48,500 and 53,000 of cowpea. However in Phaseolus aureus Ericson and Chrispeels (1973) isolated a major protein fraction which contained among other minor components a high proportion of a subunit with MW 50,000. This protein fraction also contained 1.8% neutral sugars and 0.2% hexosamine. The similarity in size and carbohydrate content of the major subunit of cowpea and Phaseolus aureus confirms the conclusion by Kioz (1971) that cowpea is relatively closer to the Asiatic species of Phaseolus (P. aureus, P. Mungo) than to the American endemics (P. vulgaris and P. lunatus). In fact P. aureus has been reclassified from morphological evidence into the genus Vigna by Tourneur (1958) and Verdcourt (1970). Additional support for this revision has come from recent work on the electrophoretic mobilities of the major seed proteins of these species (Derbyshire et al., 1976).

The major vicilin-type proteins of which there are probably several have sedimentation coefficients of about 7S, isoelectric points around pH 5.5, are low in sulphur-amino acids and are glycoproteins (Derbyshire et al., 1976). Using these broad criteria the major subunits of cowpea with molecular weights 56,200, 53,000 and 48,500 could be said to be vicilin-type proteins.

Further work needs to be done on the separation and characterisation of the major subunits of cowpea. Since the major proteins are glycoproteins in two genera (Vigna and Phaseolus) which are important sources of food, the nutritional status of glycoproteins should also be investigated.

The study of developing legume pods and seeds at different stages can contribute to an understanding of the genetic and biochemical controls associated with the qualitative and quantitative regulation of globulin protein synthesis. This understanding may be useful in manipulating the process to improve protein quality and quantity in the field. Many studies have dealt with the accumulation of major protein components during the seed development in legumes including among others, Glycine max (Bils and Howell, 1963; Kondo et al., 1954), Pisum sativum (Beevers and Poulson, 1972; Daniellson, 1952), Vicia faba (Klimenko and Berezovikov, 1963), Phaseolus vulgaris (Loewenberg, 1955) and cowpea (Klimenko and Vysokos, 1972).

To monitor the accumulation of nitrogenous compounds a reliable gauge had to be selected as seed development is subject to variations from climatic factors and position of pod on the plant (Robertson et al., 1962). A number of papers published on seed development have used a chronological scale i.e. days from flowering or pollination; other workers have used seed colour (Racusen and Foote, 1971), seed length (Millerd et al., 1971; Hall et al., 1972), milky ripeness (Klimenko and Vysokos, 1972) and seed fresh weight (Raacke, 1957). In this work after experimental investigations on the basis that nitrogen and protein accumulation is a measure of the degree of physiological development, the conclusion was that seed fresh weight was the most reliable

parameter. A lack of relationship between seed age and development was also noted by Hall *et al.* (1972).

Dry weight would possibly be a better parameter as it removes one more variable i.e. moisture, but is impractical for the collection of material and drying may denature the proteins.

The changes in development from anthesis to fruit maturity in the seeds alone will be considered first.

As the globulins were not separated into homogeneous proteins by a relatively simple method the change in the globulin fraction as a whole was investigated. Most of the nitrogen in the developing seed was extracted, when preparing globulins from the various stages examined.

In the first phase (0 to 59 mg seed fresh weight) there was little development in terms of dry matter production and nitrogen and globulin accumulation, and when the globulin fraction was subjected to electrophoresis and staining it did not produce any pattern on SDS gels or immunoelectrophoretic slides. Thus for this phase the "globulin" fraction did not contain much protein. The first phase appears to correspond to the ' cell division and expansion ' stage reported by Opik (1968) for *Phaseolus vulgaris* and Briarty *et al.* (1969) for *Vicia faba*.

The major deposition of reserve materials (starch and protein) occurred in the second phase (60 to 289 mg seed fresh weight) and is similar in the pattern of synthesis to other species (Boulter, 1965; Draper, 1976).

This rapid synthesis of globulins takes place at about seed fresh weight 110 mg, which is approximately the half-way stage

from flowering to maturity. The 'lag' in dry weight, nitrogen and globulin accumulation for a short period in this phase has been observed by other authors (Burrows, 1967; Flinn and Pate, 1968).

No explanation has been put forward except the observation that the lag phase coincides with the disappearance of the endosperm and with the embryo completely filling the embryo sac (Carr and Skeene, 1961). It has been speculated that the role of the endosperm is not only to transmit nutrient substances from maternal tissues to the embryo but also to convert these substances chemically into suitable forms for the embryo (Paramonova, 1975; Modileyskii, 1953). If this speculation can be verified then it is possible that the lag phase is the transition period during a change of immediate source of nutrients for the developing seed.

During the ' drying out ' (Phase III) the synthesis of globulin differs from other species of legumes. Whereas the synthesis of proteins falls off during this period in soyabean (Hill and Breidenbach, 1974), Pisum sativum (Daniellson, 1952; Flinn and Pate, 1968) and Vicia faba (Klimenko and Berezovikov, 1963; Wright and Boulter, 1972) in cowpea, the amount of globulin synthesised is the highest ever for a comparable period. The rapid accumulation of protein right up to maturation has led to the suggestion that a longer seed development time might result in higher levels of protein. However insufficient genetic trials have been carried out with cowpea varieties to test the variation (if any) of seed development time.

The rapid increase in the amount of globulin coincided with an increase in the concentration of protein bodies on electron micrograph plates. Thus the globulins appear to be stored in

protein bodies as in other species.

The changes in the production of the three major subunits during the seed development period were not the same. The subunit with molecular weight 56,200 appeared on gels later than the subunits with molecular weights of 53,000 and 48,500 during the development period. The latter two smaller molecular weight subunits were synthesised at the same time but there was a reversal in their staining ratios when the globulin subunit pattern of an early stage (110 mg seed fresh weight) was compared with that of the mature stage. Thus the rate of synthesis of the two major subunits appeared to change so as to reverse the relative amounts of the two subunits accumulated by the time half the total globulin had been synthesised.

There is evidence that seed protein subunits are under separate genetic control from the data on Vicia faba reported by Wright and Boulter (1972). Further evidence is provided in Phaseolus vulgaris by Romero et al. (1975) that one major subunit is under genetic control and by Sun et al. (1975) that all three major subunits are translated from separate cistrons. However, the control mechanisms on the different and varying rates of synthesis of each of the major proteins in legumes have not been elucidated as yet.

Additional smaller molecular weight subunits appear on SDS gels as the seed develops towards maturity. The appearance of various subunits on SDS gels at different stages of development does not correspond exactly with the appearance of new precipitin lines on immunoelectrophoretic slides. This is probably due to the differential sensitivity of the two methods used to detect proteins.

But as SDS electrophoretic patterns showed different proteins are synthesised in the globulin fraction as development proceeds so did the immunoelectrophoretic patterns since increasing number of precipitin lines appeared. However, it is possible that the onset of all globulin synthesis occurs simultaneously but in the case of some proteins, initially the amounts synthesised are below the limits of sensitivity of SDS electrophoresis and immunoelectrophoretic methods. Later the rate of synthesis of these proteins could increase to alter their ratio in the total globulin fraction. For example, Millerd et al. (1971) using a sensitive immunological method have reported on the seed development of Vicia faba the presence of minute amounts of legumin at a very early stage (cotyledon length 5 mm) followed by a rapid increase in the rate of legumin accumulation from cotyledon length 10 mm. Even when a similar immunochemical method was employed, different sensitivities have been obtained. In the case of peanuts, Neucere et al. (1975) detected α-arachin in embryos 0.6 mm to 1 mm long but Dieckert and Dieckert (1972) were unable to detect arachin in peanut embryos less than 8 mm long. Neucere et al. (1975) suggest that different results were obtained because of differences in titre of the immune sera used.

The situation with regard to the synthesis of the major proteins of the cowpea is analogous to that of the change in the ratio during development of the major proteins, legumin and vicilin in Vicia faba and Pisum sativum. Although legumin is present in larger quantities than vicilin in the mature seed, more vicilin is synthesised than legumin during the early stages of seed development as indicated by several methods, namely sedimentation

characteristics in the ultracentrifuge (Daniellson, 1952), serological methods (Graham and Gunning, 1970) and isoelectric precipitation (Wright and Boulter, 1972). Similarly, with three genera of the tribe Phaseolae, Klimenko and Vysokos (1972) reported that the biosynthesis of vicilins predominated in the early stages of maturation and towards the end of maturation a rise in legumin occurred.

The first protein bodies in the cowpea appear when the seed fresh weight is about 60 mg (Harris and Boulter, 1976) which is approximately just before the half-way stage from flowering to maturity. During the early stages the ratio of the subunits present is different from the ratio that is present in the mature seeds when many more protein bodies are observed. The possibility exists that since the rate of synthesis of individual proteins alters during development and the number of protein bodies increase, different protein bodies contain different ratios of globulin protein component. To support this possibility there is some evidence from studies on protein bodies of other species. Some protein bodies in immature cotyledons of Vicia faba contain neither of the two globulins, a very few only vicilin and most grains contain both globulins (Graham and Gunning, 1970). Vogel and Wood (1971) using histochemical techniques found cystine- and cysteine-rich protein bodies in only two regions of a mature bean cotyledon. Recently, Barker et al.(1976) reported that the major storage proteins of mature seeds of Phaseolus vulgaris occurred in the protein bodies and their relative amounts were different in the outer and central parts of the cotyledon.

The accumulation of sulphur is correlated with dry weight and thus the major portion of the sulphur content in a developing

seed is probably a component of the globulin protein. If there are any high sulphur-amino acid globulin proteins they do not appear to be synthesised in significant quantities in the early stages of a maturing cotyledon.

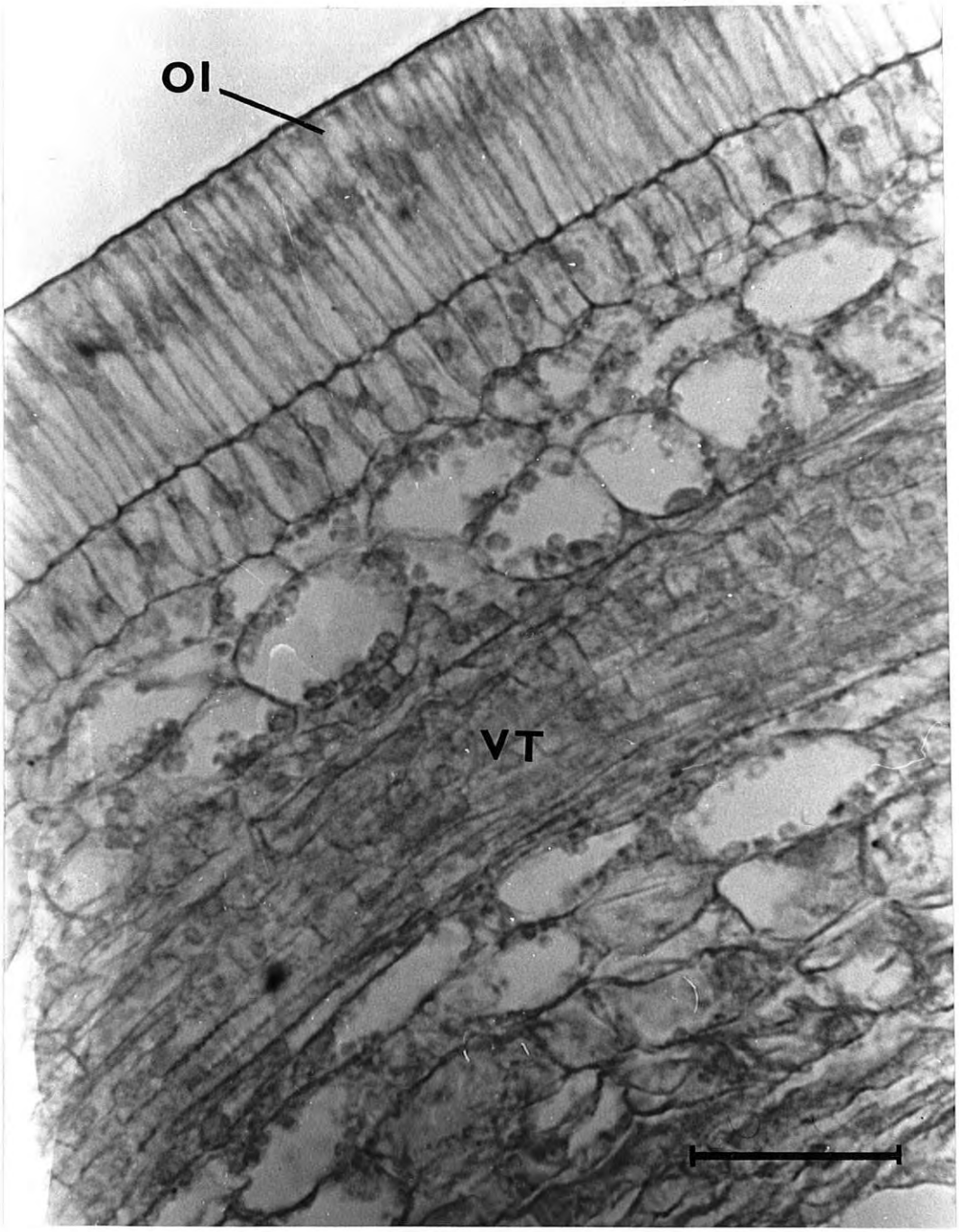
The relationship between the pod and the seed was also investigated. During phase I the ratio of pod nitrogen : seed nitrogen was at the highest i.e. 6 : 1, later during the drying out phase the ratio was reversed to 1 : 4. The pod therefore was acting as a reservoir of nitrogen in the early stages of development. The rapid accumulation of non-protein and non-amino acid nitrogen in the pod coincided with the period of minimum requirement of nitrogen by the seed i.e. the state of lowest accumulation of protein by the seed. At this early stage the amount of nitrogen in the form of protein and amino acids is in the order of 10% - although the exact form of the nitrogen compounds accounting for the rest was not investigated. Raacke (1957) with peas found the major proportion of the nitrogen of the pod consisted of amides and amino acids. At a comparable stage nitrate and nitrate reductase activity has been shown to be at its peak in leaves of the soyabean (Thibodeau and Jaworski, 1975) and pods of Phaseolus vulgaris (Schleiser and Muentz, 1974). Electron micrograph pictures show that during the early stages of development pod wall and seed wall are in close contact (N. Harris, personal communication). The outer wall of the seed at the point of contact consists of cells capable of conducting material (Plate 4). Thus nitrogenous compounds from the pod can diffuse to the seed through the testa or be translocated via the vascular tissue. Nitrate reductase activity was shown by Schleiser and Muentz (1974) in the testa of developing seeds of Phaseolus vulgaris and Vicia faba whereas very little activity was

PLATE 4

Part of seed coat of 60 mg seed showing outer integument and vascular tissue: bar represents 4μm

Abbreviations

OI = outer integument (palisade layer)
VT = vascular tissue


OI
VT

found in the cotyledons of both species. In these cases then nitrogen stored in the pod in the form of nitrates could be reduced when translocated to the testa by the enzyme situated there.

The amount of nitrogen incorporated in the seed in Phases II and III was greater than the increments of theoretically available nitrogen in the pod. So the pod may no longer act as a temporary reservoir of nitrogen in these phases. During a similar stage of development Thibodeau and Jaworski (1975) report that nitrate reductase activity in the leaves had dropped to 25% of maximum activity and the maximum levels of nitrogen fixation were reached suggesting that nitrate reductase is not so important at this phase.

Results from published papers and those reported above therefore suggest that much of the nitrogen destined for the developing seed was initially stored in an inorganic form in the pod. With the onset of synthesis of proteins in the seed the inorganic nitrogen stored in the pod was utilised. However, when the seed was rapidly synthesising proteins, reduced nitrogen from the nodules was probably the main source of nitrogen. During this stage nitrogen was not stored in the pod but directly translocated to the seed. The above scheme fits in with the suggestion of Flinn and Pate (1968) that mobilisation of nitrogen is a gradual process which gathers momentum as fruiting proceeds.

During the latter stages of seed development both nodules and seeds drain available photosynthate and this may lead to the breakdown of foliar proteins (Thibodeau and Jaworski, 1975). Similarly the greater solubility in alkali of the pod proteins may also represent a mobilisation of protein at this stage (Phase III).

The pod is homologous to the leaf (Muntz, 1973 a, b) and the breakdown of pod proteins may have rendered them soluble in alkali.

Several workers (Asif and Greig, 1972; Trevino and Murray, 1975 and Decau et al., 1975) have found a correlation between high protein content of legume seeds and high levels of nitrate in the pod. Thus further work needs to be done on the balance sheet of the various forms of nitrogen in the developing pod. The results could offer a potential to obtain higher protein yields by selecting for high nitrate reductase activity. It is this enzyme that is limiting often in nitrogen reduction (Beevers and Hageman, 1969).

Immature pods and seeds were examined at two stages to determine their nutritional status. One stage was before the pod developed a fibrous constitution, sometimes described as the splitting or snapping pod. The second stage examined was the period of maximum seed fresh weight just before the pod and seed lost their moisture. On a percentage dry weight basis the pod splitting stage (70 mg seed fresh weight) the seeds and the pod had a higher total amino acid and sulphur-amino acid content than the seeds and pods of the later stage (240 mg seed fresh weight). However, if absolute dry weights of individual pods and seeds were considered both total amino acid and sulphur-amino acid content are much higher at the later stage. So if most of the amino acid residues recovered in the analyses were available for human nutrition, later stages in pods and seed development have a higher nutritional value. This finding may not have a practical significance if the fibre content of the pod after the splitting stage is too great.

The domestication of legumes for human consumption involved among other changes an increase in size and weight of the seeds.

However the transition from the wild relatives to advanced cultivars has had the effect of narrowing the genetic base.

The variation in protein content and quality among the wild relatives of cowpea therefore should be further investigated as a basis for potential nutritional improvement; there should be no breeding incompatibility problems between wild relatives and cultivars as Rawal (1975) has evidence of natural hybridization among wild and cultivated cowpeas.

Variation in seed protein content of wild and cultivated species within a genus has been shown with Vicia (Ladizinsky, 1975) and with rice (Terrell and Wiser, 1975) but not within wild and cultivated forms of a species. In the latter case the differences are probably quantitative rather than qualitative.

In cowpeas, for example, some subunits are found only on SDS gels of meals of wild relatives and not the cultivar, Prima, yet these same subunits are found in semi-purified fractions of the cultivar Prima. Whether this is reflected in a higher sulphur content of the seed meals of wild relatives has still to be determined. Variation in amino acid composition has been reported in meals of cultivars of cowpea (Bliss, 1973; Boulter et al., 1973) and Phaseolus vulgaris (Hackler and Dickson, 1973) and so there is potential for selection of varieties for nutritional improvement. As the subunits of the cowpea are under separate genetic control the identification of a high sulphur-amino acid protein in a screening method would involve dissociation of the proteins.

Further investigation is required of both wild and cultivated forms of cowpea to select seeds with the most desirable amino acid profile.

APPENDIX

Latin name	Common name	Country	Reference
Vigna sinensis (L.) (Savi)	bean cowpea blackeyed bean Southern pea Indian pea cornfield pea	Nigeria	Ebong (1968)
V. unguiculata (L.) Walp. V. sinensis	macacar bean serido beans	Brazil	Lago et al. (1971) Prisco et al. (1975)
V. sinensis	chiang tou	China	Adolph and Chang (1935)
Dolichos sesquipedalis V. catiang V. sinensis	cowpea	India	Bhagvat (1935)
V. sinensis	Pois iconnu Pois iconnu a luzerne	Haiti	King (1964)
V. cylindrica V. sinensis V. sesquipedalis	Frijol precioso Frijol carita Habichuela	Cuba	Navia et al. (1955)
V. unguiculata (L.) Walp.	Haricot dolique Dolique de Chine Pois du Bresil Haricot a oeil noir Niebe	France	Busson et al. (1959-60)
V. unguiculata (L.) Walp. subsp. unguiculata	cowpea catjang asparagus - bean	U. S. A.	Gunn (1973)
V. unguiculata (L.) Walp.	lubia coupe		Rachie and Roberts (1974)

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A B B R E V I A T I O N S

The abbreviations used in this thesis are as recommended in 'Policy of the Journal and Instructions to Authors'. Biochem J. 145, 1 - 20 (1975), with the following additions:

2-mercaptoethanol	2-Me
N, N' - methylbisacrylamide	bisacrylamide or bis
N,N,N',N' - tetramethylethylenediamine ..	TEMED
Periodate-acid-Schiff	PAS
Sodium dodecyl sulphate	SDS

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