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PROTEINS OF DEVELOPING, MATURE AND GERMINATING SEEDS OF *PHASEOLUS VULGARIS*

A thesis submitted in accordance with the
requirements of the University of Durham
for the degree of Doctor of Philosophy

by

R.D.J. BARKER, B.A. (Cantab.)

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ABSTRACT

A number of methods have been used to extract, purify and characterise the major storage proteins of dry seeds of Phaseolus vulgaris L. cv. 'Seafarer'. Four fractions, which together account for the majority of the proteins solubilised by alkaline salt extractants and separated by polyacrylamide gel electrophoresis, are described. The predominant protein, accounting for about 60% of the total protein content of the seed, is a vicilin-like protein, and is composed of two subunits, with molecular weights 50,000 and 47,000. A second protein fraction, prepared by zonal isoelectric precipitation, is a legumin-like protein; a third fraction was found to agglutinate erythrocytes. Amino acid compositions are presented for these protein fractions and their identity with storage proteins prepared in previous studies is discussed. The fourth fraction was not characterised.

The protein content of protein bodies prepared by two methods was compared; the method of preparation was found to have a significant effect on the apparent distribution of both storage proteins and trypsin inhibitor activity. Variations in the distribution of storage proteins within the cotyledon are also described.

The formation of storage protein during seed development and its subsequent mobilisation during seed germination was followed by SDS gel electrophoresis. Changes in proteolytic activity during germination were determined and attempts were made to identify the *in vivo* location of storage protein hydrolysis and the possible effect of endogenous inhibitors on this process.

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ABBREVIATIONS

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 :-

α-N-Benzoyl-DL-arginine-p-nitroanilide HCl	BAPNA
Cetyltrimethylammonium bromide	CTAB
Diisopropylfluorophosphate	DFP
Dimethyl sulphoxide	DMSO
2-mercaptoethanol	2-Me
N-ethylmaleimide	NEM
N,N^1-methylbisacrylamide	bisacrylamide or bis.
N,N,N^1,N^1-tetramethylethylenediamine	TEMED
Periodate-acid-Schiff	PAS
Phenylmethylsulphonylfluoride	PMSF
Sodium dodecyl sulphate	SDS

INTRODUCTION

The development of the seed habit produced an efficient method for the dispersal of the progeny of the parent plant and a means whereby unfavourable environmental conditions, for example climatic, could be temporarily tolerated. The structure of the seed bears witness to the requirements of these functions: a resistant outer layer within which tissues with a low rate of metabolic activity (permitting, if necessary, a lengthy period of dormancy) can be maintained, together with a supply of stored food to nourish the developing seedling when conditions improve and germination occurs.

While the structure and composition of the seed reflect the requirements made upon it in the context of the viability of the seed, our interest in seeds stems from their fundamental place in stable agricultural systems. In particular, two groups of plants, the cereals (Gramineae) and the legumes (Leguminosae) are of the greatest importance. Their seeds are rich in reserve materials in the form of nitrogen and carbon compounds which are later metabolised for use by the developing seedling; the nitrogen is mainly stored in proteins, carbon is present as carbohydrate (starch) or oil. Agricultural practices have increased the size of the seed and the amount of these reserves greatly. Cereals on average contain 10-15% protein (on a dry weight basis) while legumes contain 20-25% protein in many wild varieties, but may contain markedly more (up to 50%) in cultivated varieties; for example, Smith *et al.* (1959) state that *Lupinus luteus* seeds contain 44% protein by dry weight.

Protein present in the seed may be broadly described as metabolic and structural protein or storage protein. Metabolic

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proteins tend to be present in relatively small quantities of a very large number of different types and are concerned in the metabolism of the seed during its development - and hence in the synthesis of the storage protein - and subsequent germination. Storage protein may be defined as that protein which is synthesised in the developing seed, and is subsequently degraded during germination, acting as a source of nitrogen for the developing seedling. In contrast to metabolic proteins, storage proteins often display no enzymatic activity and the very large amounts that are present (in the dry seed storage protein frequently constitutes 80% or more of the protein present) tend to be composed of a relatively small number of types.

Current interest is particularly concerned with attempts at improving both the yield and the nutritional quality of the storage protein, as a protein source for human consumption. Thus the amino acid composition of natural varieties show serious inadequacies: some cereals are deficient in lysine, while the protein of legume seeds contains relatively low amounts of the sulpho-amino acids, methionine and cysteine. The use of mixed diets can help to compensate for these deficiencies (Bressani, 1973). Although soybean meal is used as an animal feed, it is unsuitable for human consumption; however, the use of textured vegetable protein (TVP) has increased markedly recently, using protein extracted from soybeans and other legumes (for example, the Kesp manufactured in the United Kingdom from field bean). The use of analytical data of the type described here on storage proteins in the breeding of improved nutritional varieties will be considered later.

A-number of reviews on seed proteins have been published from time to time; these include those by Osborne (1924); Chibnall (1939); Vickery (1945); Brohult and Sandegren (1954); Danielsson (1956); Stahman (1963); and Altschul *et al.*, (1966).

Historically the work and influence of Osborne is very important. Osborne and Campbell (1898a) extracted proteins from seeds of *Pisum sativum* with salt solutions and fractionated them by precipitation, either by dilution or by heat treatment. They also described proteins extracted from other legumes, for example, lentil (Osborne and Campbell, 1898b), vetch (Osborne and Campbell, 1898c), and soybean (Osborne and Campbell, 1898d). They could describe two major globulin fractions: the first, legumin, was less soluble in dilute salt solutions than the second, vicilin; legumin was thus precipitated by stepwise dilution of salt extracts. Legumin was not coagulated by heating to 100°C, whereas solutions of vicilin coagulated at 95°C. Osborne and Harris (1907) later fractionated legumin and vicilin by fractional precipitation with ammonium sulphate.

The results obtained from his studies of the proteins of legume seeds and other plant sources formed the basis of the classification of plant proteins, proposed by Osborne (1924), that has since been widely used. As outlined by Yemm (1958), these consisted of :-

Albumins, soluble both in water and in dilute salt solutions.

Globulins, soluble in neutral salt solutions, but only sparingly soluble in water.

Prolamins, soluble in 70-80% ethanol, but insoluble in water or pure ethanol.

Glutelins, insoluble in the above solvents, but soluble in dilute acids or alkalis.

While distinction between prolamins and glutelins, which predominate in cereals, is clear cut, the distinction between the albumins and globulins, which constitute the majority of the seed proteins of legume seeds, is less well defined. Indeed, Osborne and Campbell (1898a) prepared a third fraction from pea seeds, legumelin, which, in contrast to legumin and vicilin, was only partially precipitated by dialysis and which they considered to be more an albumin than a globulin protein.

Osborne's analysis of seed proteins was limited to solubility studies and elemental analysis of the fractions obtained. The advent of the ultracentrifuge and the use of electrophoresis permitted further investigation. Danielsson (1949) separated two globulins from *Pisum sativum* by ammonium sulphate fractionation and isoelectric precipitation at pH 4.7; these he equated, on the basis of their solubility, nitrogen content and behaviour on heating, with the legumin and vicilin prepared by Osborne and Campbell (1898a). While both ammonium sulphate fractionation and dilution of solutions of the globulins in buffer (pH 7.0) at 0°C proved partially successful in separating the two globulins, only isoelectric precipitation yielded preparations that were homogeneous in the ultracentrifuge; legumin sedimented with a sedimentation coefficient of 12.64S, while vicilin was lighter, with $S^{\circ}_{20,w}$ 8.1S; molecular weights were calculated as 331,000 and 186,000, respectively (Danielsson, 1949). Electrophoretic analysis (Danielsson, 1950) confirmed their homogeneity and indicated, as determined by zero mobility in free flow electrophoresis in the Tiselius apparatus (Tiselius, 1937), that the isoelectric point of legumin was pH 4.8 and that of vicilin pH 5.5.

Danielsson (1949) also examined the seed globulins of 33 other species of the Leguminosae; with a few exceptions, they were all found to contain two globulins with sedimentation coefficients of approximately 7S and 11S, which, solely on the basis of their sedimentation coefficients, he called vicilin and legumin. In some cases, globulins of both higher (18-20S) and lower (2S) sedimentation coefficients were observed, which were respectively attributed to complex formation between legumin and vicilin and to a degradation product of legumin. The only species examined which did not contain an 11S component were some Acacia species, for example A.longifolia, A.verticillata, and Trifolium repens, although Acacia saligna and Trifolium pratense did contain an 11S component. However, the low sensitivity of the method may be responsible for these observations.

Danielsson also pointed out that while the proportions of the two components varied widely between genera (for example, legumin predominates in Lathyrus and Vicia species, while vicilin is present in greater amount than legumin in species of Lupinus and Phaseolus), related species tended to contain similar proportions of the two globulins.

Because of its nutritional and commercial importance the protein composition of the soybean (Glycine max) has been studied in greater detail than any other legume. The major globulin fraction, glycinin, was first isolated by dialysis of a salt extract (Osborne and Campbell, 1898d); however, this preparation was shown to be heterogeneous by both electrophoresis (Briggs and Mann, 1950) and ultracentrifugation (Naismith, 1955). It is now known to be composed of four components, having sedimentation coefficients of approximately 2S, 7S, 11S and 15S (Naismith, 1955; Wolf and Briggs, 1956), of which only the 11S component is now referred to as glycinin. Glycinin is present to the greatest extent; the 11S and 7S components together account for 70% of the protein (Wolf and Smith, 1961).

Cooling water extracts of soybean meal results in the precipitation of a cryoprotein (Briggs and Mann, 1950) which was shown to be composed mainly of 11S protein (Briggs and Wolf, 1957). Cryoprecipitation has been used as the initial step in the purification of this protein (Eldridge and Wolf, 1967). A number of other workers have also purified the 11S globulin (Mitsuda _et al._, 1965; Shvarts and Vaintraub, 1967; Catsimpoolas _et al._, 1967; Koshiyama, 1972a). The purified protein was found to consist of twelve subunits, with three different N-terminal amino acid residues (glycine, phenylalanine and either leucine or isoleucine) (Catsimpoolas _et al._, 1967). Six different subunits were isolated (Catsimpoolas, 1969) by preparative isoelectric focusing in urea-2-mercaptoethanol and found to fall into two categories, basic subunits with isoelectric points 8.0, 8.25 and 8.5, and acidic subunits with isoelectric points of 4.75, 5.15 and 5.4; SDS gel electrophoresis (Catsimpoolas _et al._, 1971) showed the basic subunits to have molecular weights of about 22,300; the acidic subunits were larger, with molecular weights 37,500: however, greater heterogeneity was observed by the increased resolution afforded by analytical scanning isoelectric focusing in urea - dithiothreitol (Catsimpoolas and Wang, 1971). Okubo _et al._, (1969) also separated the 11S globulin into acidic and basic subunits by chromatography in urea on DEAE-cellulose; the basic subunits had glycine as the N terminal amino acids. Amino acid analysis of the basic subunits showed that while they did not have significantly larger amounts of lysine or arginine than the undissociated 11S protein, they contained about 35% less glutamic acid.

Two three-dimensional models for the structure of glycinin, based on electron microscopy, have been proposed. One consists of two doughnut-like structures, each composed of six subunits, stacked one on top of the other (Catsimpoolas, 1969), while the second (Saio et al., 1970) consists of two split-rings facing each other; both models contain a hole in the centre of the molecule. Glycinin is also known to be able to form disulphide polymers (Briggs and Wolf, 1957); its quaternary structure is disrupted by a variety of conditions, for example detergents, high concentrations of urea, acid pH and changes in ionic strength (see Wolf, 1970a).

The 7S fraction is more heterogeneous, for only half of this is globulin, the remainder comprising a haemagglutinin, lipoxygenase and β-amylase (Wolf and Smith, 1961). Subsequent investigations have shown this globulin fraction to be even more complex. Hasegawa et al., (1963) separated the water extractable proteins into nine components by gel filtration on Sephadex G-200. In the ultracentrifuge, four of these had sedimentation coefficients of approximately 7S; one of these was eluted with the 11S globulin and could not be separated from it by rechromatography. Vaintraub separated three 7S proteins, with different N-terminal amino acid residues by chromatography on calcium phosphate and gel filtration. Roberts and Briggs (1965) and Koshiyama (1965) both purified 7S globulin proteins, which could associate to a 9S form. Koshiyama's preparation contained eight different N-terminal amino acids (Koshiyama, 1968a) and, like the 11S, could be dissociated by acid and alkali; the acid dissociation was dependent on the salt concentration (Koshiyama, 1968b). Koshiyama (1970) subsequently showed that only one component of molecular weight 22,500 was formed by dissociation in 8M urea, and that the subunits, unlike those of the 11S, were not held together by disulphide bonds (Koshiyama, 1971).

Koshiyama (1969) isolated a glycopeptide from the 7S globulin which contained a single polysaccharide unit of 51 sugar residues (composed of 39 mannose and 12 glucosamine residues) which accounts for the sugar composition of the complete 7S. Kitamura _et al._ (1974) made use of this to purify the 11S globulin by removing the 7S globulin by specific absorption onto a column of Con-A-Sepharose 4B, with subsequent purification of the 11S by gel filtration on Sepharose 6B.

More recently Hill and Breidenbach (1974a) separated the major storage proteins of soybean seeds by sucrose density gradient centrifugation into three fractions with sedimentation coefficients 2.2S, 7.5S and 11.8S; by analysis of the formation during seed development of these ultracentrifugal components and their electrophoretic characteristics, at least three 7S proteins were found to be present (Hill and Breidenbach, 1974b).

Catsimpoolas _et al._ (1968c) used immunoelectrophoresis to demonstrate the presence of four major antigenic components, designated A, B, C and D. The use of monospecific antisera demonstrated component A to be equivalent to glycinin. By preparing 7S fractions by the methods of Roberts and Briggs (1965) and Koshiyama (1965), components B and C, referred to as β- and γ-conglycinin respectively, were equated with these preparations. The fourth component (D), referred to as α-conglycinin was equated by Catsimpoolas and Ekenstam (1969) with the 2S globulin seen as a contaminant in the 7S preparation of Koshiyama (1965). However, the 2.2S fraction prepared by Hill and Breidenbach (1974a), could be separated by electrophoresis into two components, one of which migrated with the same mobility as commercially obtained soybean trypsin inhibitor. Subunit analysis shows α-conglycinin to be composed of one subunit, β-conglycinin of four subunits and γ-conglycinin to contain nine subunits (Catsimpoolas, 1970).

The preceding description of some of the structural analyses of the major soybean globulins is intended to demonstrate the complexity of storage proteins, in that they are generally large molecules, often with complex subunit structures; they also undergo association and dissociation reactions with alterations of pH and ionic strength.

The proteins of peanuts (Arachis hypogaea) have been studied in similar detail. Classically they were separated by ammonium sulphate fractionation, the fraction precipitating at 40% saturation being termed arachin and that precipitating over the range 40-85% saturation conarachin (Jones and Horn, 1930). Complex patterns result from analysis of these proteins by ion-exchange chromatography and polyacrylamide gel electrophoresis (for example, Dechary et al., 1961; Tombs, 1965; Neucere, 1969; Dawson, 1971; Cherry et al., 1973). Tombs (1965) found four different types of subunits in arachin and three forms of arachin could be prepared in which the occurrence of these subunits differed; however, the number and size of the subunits present in Tombs' arachin preparations differ from the results of Singh and Dieckert (1973). The original conarachin fraction is also heterogeneous (Daussant et al., 1969a).

Similar investigations have been undertaken on the storage proteins of other legumes, including peas (Pisum sativum) (Vaintraub and Gofman, 1961; Grant and Lawrence, 1964), broad beans (Vicia faba) (Bailey and Boulter, 1970, 1972; Wright and Boulter, 1974), vetch (Vicia sativa) (Vaintraub et al., 1962; Vaintraub and Shutov, 1964), lupins (Lupinus species) (Joubert, 1956; Blagrove and Gillespie, 1975), mung beans (Phaseolus aureus) (Ericson and Chrispeels, 1973) and various cultivars of Phaseolus vulgaris, for example Bourdillon (1949), Jaffé and Hannig (1965) and Racusen and Foote (1971).

Pusztai has purified and characterised several seed proteins from Phaseolus vulgaris (Pusztai, 1966, 1968, Pusztai and Duncan 1971a; Pusztai and Watt, 1970, 1974); Hall and his co-workers have also investigated the seed storage proteins of this species (McLeester et al., 1973; Sun et al., 1974; Sun and Hall, 1975); an 11S storage protein from Phaseolus vulgaris has recently been described (Derbyshire and Boulter, 1975). The results of these investigations will be considered in detail in the discussion, in that they are of greater relevance in the context of the results to be presented here than the results obtained from soybeans and peanuts.

A number of factors present in legume seeds are nutritionally deleterious. While some of these are not proteins, for example the lathyrogen β- (γ-L-glutamyl) aminopropionitrile, isolated from Lathyrus odoratus (see review by Sarma and Padmanaban, 1969), phytohaemagglutinins and protease inhibitors are. Phytohaemagglutinins (agglutinins, lectins) may be considered as storage proteins both on the basis of the definition previously mentioned or, as will be demonstrated, by that proposed by Altschul et al. (1964), i.e. that storage proteins are those seed proteins, termed aleurins, found in discrete membrane bound particles, now called protein bodies. Agglutinins have now been isolated from a large number of sources, mainly from the Leguminosae, and have been reviewed by Jaffé (1969) and Lis and Sharon (1973); Liener (1974) has reviewed their nutritional significance; the best characterised is concanavalin A from seeds of Canavalia ensiformis, for which the complete amino acid sequence and three-dimensional structure at 2Å resolution has been published (Wang et al., 1975; Cunningham et al., 1975; Becker et al., 1975). Agglutinins have been purified and characterised from seeds of Phaseolus vulgaris by numerous workers (Jaffé and Hannig, 1965; Takahashi et al., 1967; Dahlgren et al., 1970; Allan and Crumpton, 1971; Weber et al., 1972; Andrews, 1974; Pusztai and Watt, 1974).

Trypsin inhibitors were initially isolated in crystalline form from soybean by Kunitz (1945) and later characterised by him (Kunitz, 1947); they and other proteolytic enzyme inhibitors have subsequently been prepared from a variety of sources, mainly from the Leguminosae, Gramineae and Solanaceae. Their properties have been reviewed recently (Ryan, 1973; Tschesche, 1974). Trypsin inhibitors have been purified and characterised from seeds of Phaseolus vulgaris (Pusztai, 1966, 1968, 1972); Wilson and Laskowski (1973) obtained three iso-inhibitors, for one of which (trypsin inhibitor II) they have published a partial amino acid sequence (Wilson and Laskowski, 1975). Complete amino acid sequences and three-dimensional structures have been published for a number of inhibitors (see Tschesche, 1974). Pusztai (1972) obtained a number of iso-inhibitors from Phaseolus vulgaris seeds differing in their isoelectric points. The possible involvement of these and other inhibitors in the control of seed proteolysis in vivo will be considered later. Their relevance as a toxic constituent of legumes for human consumption appears to depend to a large extent on the source of the inhibitor; Feeney et al. (1969) showed that while lima bean trypsin inhibitor strongly inhibited human trypsin, that from kidney bean inhibited only weakly; both strongly inhibited bovine trypsin.

Storage proteins are located in membrane-bound particles called protein bodies and as mentioned previously, their localisation forms the basis of one definition of storage protein (Altschul et al., 1964). While early preparations were often made in non-aqueous media e.g. in Carbowax (polyethylene glycol) (Altschul et al., 1961) and in cotton seed oil (Dieckert et al., 1962) they are more usually prepared by centrifugation in sucrose density gradients (Tombs, 1967; Schnarrenberger et al., 1972). They have been studied from numerous sources, including soybeans (Tombs 1967; Catsimpoolas and Ekenstam, 1969; Wolf, 1970b; Koshiyama, 1972b) peanuts (Daussant et al., 1969a; Neucere and Ory, 1970) barley (Ory and Henningsen, 1969; Tronier et al., 1971), cottonseed (Yatsu and Jacks, 1968), peas (Varner and Schidlovsky, 1963), broad beans (Morris et al., 1970,) and mung beans (Ericson and Chrispeels, 1973). Using fluorescent antibodies to vicilin and legumin, Graham and Gunning (1970) showed that both these proteins occur in the same protein bodies, although both proteins were not necessarily present in each protein body and some protein bodies were not labelled. Vogel and Wood (1971) used histochemical techniques to show that subepidermal protein bodies contained a larger amount of protein bound sulphydryl groups than those found elsewhere in the cotyledon.

Suggestions that protein synthesis occurred directly in protein bodies (Graham et al., 1962; Morton and Raison, 1963, 1964) have since been shown to be the result of bacterial contamination (Wilson, 1966; Wheeler and Boulter, 1967); protein bodies do not contain ribosomes (Opik, 1968). The storage protein which accumulates in storage proteins is synthesized in the cytoplasm, on membrane-bound or free cytoplasmic polysomes (Payne and Boulter, 1969) and is then channelled through the endoplasmic reticulum (Bailey, et al., 1970). In view of the glycoprotein nature of many storage proteins, for example, the soybean 7S (Koshiyama, 1969) and storage proteins of Phaseolus aureus (Ericson and Chrispeel, 1973) and Phaseolus vulgaris (Pusztai, 1966; Pusztai and Watt, 1970), the Golgi apparatus may subsequently be implicated (see Northcote, 1971).

Early investigations on the course of seed development concentrated mainly on the gross changes in nitrogen, starch-sugar, and metabolic aspects such as respiratory activity (e.g. Loewenberg (1955) on Phaseolus vulgaris); early work on the accumulation of storage protein and nitrogen in developing pea seeds was carried out by Danielsson (1952) and by Raacke (1957). The origin and transfer of nitrogen and carbon compounds to developing pods and seeds has been examined (Boulter and Davis, 1968; Pate and Flinn, 1973; Kipps and Boulter, 1974). Morphological changes in developing Phaseolus vulgaris (Opik, 1968) and Vicia faba seeds (Briarty et al., 1969) have been described. Various parameters concerned with the protein synthesising capacity of developing seeds have been investigated (Cruz et al., 1970; Beevers and Poulson, 1972; Poulson and Beevers, 1973). Changes in the nucleic acid composition have been studied in Phaseolus vulgaris (Walbot, 1973), Vicia faba (Millerd and Whitfield, 1973; Millerd and Spencer, 1974), and in Pisum sativum (Scharpé and Van Parijs, 1973) and Pisum arvense (Smith, 1973). A characteristic feature is that at least some of the parenchymatous cotyledonary cells become highly polyploid during seed development; in both Pisum arvense (Smith, 1973) and Pisum sativum (Scharpé and Van Parijs, 1973) nuclei with up to 64C DNA were observed by Feulgen histophotometry. These authors also followed the development of protein.

The composition of the storage protein fraction of legume seeds changes during development. Danielsson (1952) found that vicilin was synthesised prior to legumin in ripening pea seeds; this pattern has been confirmed in *Vicia faba* (Wright and Boulter, 1972).Millerd *et al.*, (1971) used microcomplement fixation to determine the timing of legumin synthesis during development; the order of synthesis of three components was later established (Millerd and Spencer, 1974). Changes in the rates of synthesis of proteins have also been observed in soybeans(Hill and Breidenbach, 1974b), and in peas (Klimenko and Pinegina, 1964); the electrophoretic patterns of developing seeds and pods of *Phaseolus vulgaris* have been recorded (Hall *et al.*, 1972).

In the case of *Vicia faba*, not only do the relative timings and rates of synthesis of vicilin and legumin differ, but subunit ratios in the vicilin fraction altered during development, implying the presence of several vicilins (Wright and Boulter, 1972). The ratio of three individual electrophoretic components of the soybean 7S fraction also vary during development (Hill and Breidenbach, 1974b).

During seed germination and subsequent growth of the seedling, marked changes occur in the nitrogenous constituents of the seed. It has been known for some time that during germination there is a decrease in the protein content of the seed and a concomitant increase in free amino acids and amides (Chibnall, 1939). Numerous proteolytic activities are found in germinating seeds: their properties have been reviewed (Ryan, 1973) and further consideration of them, in relation to the results presented here, will be continued later. During germination, protein bodies swell and late in germination have increased markedly in volume (Opik, 1966; Briarty *et al.*, 1970); Smith (1974) has also studied morphological changes in germinating *Phaseolus vulgaris* seeds.

The storage proteins of peas, beans and peanuts (Basha and Beevers, 1975; Kloz *et al.*, 1966; Daussant *et al.*, 1969b) are modified early in germination to form proteins of greater electrophoretic mobility. Legumin and vicilin are degraded at a similar rate in peas (Danielsson, 1951) but differential rates of breakdown have been reported, for example in soybeans (Catsimpoolas *et al.*, 1968a). It has been suggested (Daussant *et al.*, 1969b), that a progressive deamidation of the storage proteins (which would account for the increased electrophoretic mobility observed) takes place prior to extensive degradation.

There has been interest in the specificity of seed proteolytic enzymes. Garg and Virupaksha (1970a) purified an acid protease from germinating sorghum; its hydrolytic activity was found to be confined to the peptide bond succeeding an aspartyl or a glutamyl residue; the enzyme was inactive on peptide bonds following the corresponding amides (Garg and Virupaksha, 1970b). Virupaksha has subsequently succeeded in purifying this enzyme by affinity chromatography on Sepharose 4B-D-glutamyl-D-glutamic acid, a competitive inhibitor (Virupaksha and Wallenfels, 1974). Whereas earlier studies on the proteolytic enzymes employed casein or acid denatured haemaglobin as substrate, the use of storage protein from the species under investigation (the 'natural' substrate) is now preferred (Hobday *et al.*, 1973; Basha and Beevers, 1975).

There is also considerable interest in the possible role of some of the proteinase inhibitors in seeds in the regulation of proteolytic activity. Shain and Mayer (1965, 1968) reported the presence in lettuce seeds of a proteinase inhibitor that inhibited an endogenous, 'trypsin-like' endopeptidase (i.e. the enzyme was inhibited by diisopropylfluorophosphate (DFP) and was hence considered to be a serine proteinase)in ungerminated seeds. The inhibitors disappeared as enzyme activity increased during germination.

Ungerminated barley contains three types of inhibitors (Kirsi and Mikola, 1971), which inhibit trypsin, microbial proteinases and endogenous endopeptidases respectively. Other suggested roles for inhibitors include protection against insect pests and as storage proteins (see Ryan, 1973).

A wide variety of methods have been used in attempting to purify and characterise seed proteins. Both this variety and the fact that some of the methods, for example ultracentrifugation, do not unequivocally identify a particular protein, have meant that the identification of similar proteins prepared in different laboratories has often been difficult. It is also often unclear what proportion a particular purified protein represents of the total protein of the seed. With these criteria in mind, this investigation was intended to identify, prepare and partially characterise the major storage proteins of Phaseolus vulgaris L. cv. 'Seafarer', using a variety of techniques. Preparative methods included the classical procedures of precipitation by dilution, dialysis and at the isoelectric point, as well as cryoprecipitation from water extracts, ion-exchange chromatography and centrifugation of proteins in sucrose density gradients. Analysis of protein fractions was carried out by electrophoresis. The protein composition of protein bodies was investigated. The pattern of the development of the main storage proteins during seed development was followed, and changes in protein composition and the activities of certain proteolytic enzymes during germination were analysed.

MATERIALS

I. Biological Materials

Seeds of *Phaseolus vulgaris* L. cv. 'Seafarer' were obtained from H.J. Heinz Co. Ltd., Eastern Avenue, Team Valley Trading Estate, Gateshead 11. Seeds of *Phaseolus vulgaris* L. cv. 'Canadian Wonder' were obtained from the Tyneside Seed Company, Gateshead.

II. Chemicals and Reagents

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

Sephadex G50, medium, was obtained from Pharmacia Ltd., 75 Uxbridge Rd., London, W.5.

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

Ampholine carrier ampholytes, pH ranges 3-10 and 5-8, were obtained from LKB Ltd., Addington Rd., Selsdon, South Croydon.

Gelatin (Difco bacto Gelatin) was obtained from Difco Laboratories, P.O. Box 14B, Central Avenue, West Molesey, Surrey.

Vermiculite was obtained from J. Bentley Ltd., Hull.

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

Methyl green was obtained from Searle Diagnostic (Gurr Products) Lane End Rd., High Wycombe, Bucks.

Albumin, bovine (Bovine serum albumin); fraction V powder;

Antifoam A emulsion;

Azo-Albumin (Sulphanilic acid-Azoalbumin);

α-N-Benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA);

Carboxypeptidase A; Type I, from bovine pancreas;

Catalase;

N-Ethyl maleimide;

γ-globulin, bovine, Cohn fraction II;

L-leucine-p-nitroanilide;

Myoglobin, Type II;

Ovalbumin, Grade V;

Phenylmethylsulphonylfluoride;

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

Human Group O erythrocytes were obtained from a local hospital.

METHODS

I. Growth of Biological Materials.

A. Developing seeds.

Seeds of Phaseolus vulgaris L. cv. 'Seafarer' were grown in a greenhouse in the University of Durham Botanic Gardens during the summer. Some flowers were labelled with jewellers tags on the day of flower opening. Seeds were harvested according to total seed fresh weight; they were frozen in liquid nitrogen and stored at $-20^{o}C$. Only uniform material was collected and no account was taken of the position of the pod on the plant. Seeds were also dried at $105^{o}C$ for 24 h to determine the dry weight. The percentage dry weight was then calculated i.e.

$$\% \text{ Dry weight} = \frac{\text{Dry weight} \times 100}{\text{Fresh weight}} .$$

B. Germinating seeds.

Seeds of P. vulgaris L. cv. 'Seafarer' were surface sterilised by soaking in 1% (w/v) sodium hypochlorite for 15 min; after soaking overnight in running tap water, they were sown in seed trays in moist sterilised vermiculite and allowed to germinate in a greenhouse in natural daylight. Germinating seeds were harvested at 2 day intervals from the morning of planting; the testas and embryo axes were removed and the cotyledons were stored at $-20^{o}C$. Only material showing no signs of surface infection or discolouration was collected.

II. Preparation of Seed Meals.

A. Whole seeds.

Mature, dry seeds of P. vulgaris cvs 'Seafarer' and 'Canadian Wonder' were finely ground in a Janke and Kunkel water-cooled mill (type 10).

B. Cotyledons

To determine the protein composition of different regions of the cotyledons, meals were prepared from the abaxial and adaxial surfaces (both to a depth of up to 1 mm) and also from the central part of the remainder of the cotyledon, by grinding in a pestle and mortar. For analysis of the proteins of developing seeds by SDS extraction (Section IIId) and electrophoresis (Section VII A(ii)) samples of the frozen cotyledons were lyophilised and carefully ground in a mortar and pestle.

III. Extraction of Proteins

A. Alkaline salt extraction

(i) From meal. 6g of meal was stirred with 60 ml of 0.5M NaCl, 0.05M sodium phosphate buffer, pH 7.5, for 30 min at 4°C. The slurry was clarified by centrifugation at 38,000 xg for 90 min at 4°C. When performed, re-extractions were carried out by dispersing the pellet from the centrifugation in a further 60 ml of solvent and repeating the above procedure. The total protein content and trypsin inhibitor content of different regions of the cotyledon were determined on alkaline salt extracts of the meals prepared from these regions.

(ii) From cotyledons. All procedures were carried out at 0-4°C. 50 cotyledon pairs were ground, with a small amount of acid-washed sand, in a chilled mortar with 30 ml 0.5M NaCl, 0.05M sodium phosphate buffer, pH 7.5. The extracts were stirred occasionally for 1 h and clarified by centrifugation at 38,000 xg for 90 min. The pellet was re-extracted twice, as described above; the last extraction removed less than 2% of the protein obtained in the first extract. Only the first extract from germinating cotyledons was used for enzyme assays. Extracts were dialysed against excess extractant before the protein concentration was determined.

B. Water extraction

6 g of meal was stirred with 60 ml of distilled water for 30 min at room temperature (18-20°C). After clarification by centrifugation at 38,000 xg for 90 min (at 20°C) the supernatant fluid was cooled in an ice bath for 90 min. The precipitate which formed during cooling was collected by centrifugation at 23,000 xg for 30 min at 4°C.

C. Acidic extractions and fractionation.

All procedures were performed at 4°C. Extraction from meal and fractionation of the clarified extract were carried out as described by McLeester *et al.* (1973) using 0.5M NaCl, 0.25M ascorbic acid (measured pH value, 2.4); a similar procedure was followed in the absence of ascorbate, using 0.5M NaCl adjusted to pH 2.0 with dilute HCl as the extractant. The solvent to meal ratio was 10:1 (v/w). The material precipitated by the addition of two volumes of distilled water to the clarified initial supernatant was collected by centrifugation, and is referred to as the F-I fraction. Overnight dialysis of the supernatant fluid, after removal of the F-I fraction, against running tap water resulted in the formation of a precipitate; this was collected by centrifugation and is referred to as the F-IIa fraction. A further precipitate was obtained by dialysis of the remaining supernatant fluid against several changes of distilled water; this was also collected by centrifugation and is referred to as the F-IIb fraction.

D. SDS extraction.

20 mg samples of meal were stirred with 4 ml of 0.01M sodium phosphate buffer pH 7.0, 1.0% (w/v) SDS and 1.0% (v/v) 2-mercaptoethanol for 60 min at 37°C; the suspensions were then heated in a boiling water bath for 3 min and allowed to cool at room temperature;

the extracts were clarified in a Piccolo bench-top centrifuge and analysed by SDS gel electrophoresis (Section VII A(ii)).

IV. Preparation of Protein Bodies.

A. By sucrose density gradient centrifugation.

(i) Preparation of sucrose gradients. Linear 50-90% (w/v) sucrose gradients in 0.025M sodium phosphate, 0.025M citric acid, pH 7.5, were prepared in 23 ml polycarbonate centrifuge tubes by the successive addition of 3 ml each of 90%, 80%, 70%, 60% and 50% (w/v) sucrose solutions to the tubes. The interface between each layer was broken by pushing a glass rod through the interfaces to the bottom of the tube 5 times. The gradients were kept at 0-4°C for 36 h , by which time a linear gradient had formed.

(ii) Sample preparation. The same procedure was used with seeds that had been soaked overnight in running tap water and those that had been germinated for longer periods; all procedures were carried out at 0-4°C. After removal of the testas and embryo axes, 3 g of cotyledons were finely sliced and extracted with 10 ml of 35% (w/v) sucrose in 0.025M sodium phosphate, 0.025M citric acid, pH 7.5, by gentle grinding for 1 min in a chilled mortar. The extracts were stirred for 10 min and then centrifuged at 165 xg for 5 min, to remove cell debris.

(iii) Centrifugation and fractionation of gradients. Following the removal of cell debris, approximately 1.5 - 2.0 ml of the supernatant fluid was carefully layered onto the previously prepared sucrose gradients. The gradients were centrifuged for 3 h in the 3 x 23 aluminium swing-out rotor in an MSE Super Speed 65 centrifuge, operating at 4°C and 30,000 rev./min (R av. 94,000 xg). Gradients were fractionated by inserting a thin capillary tube through the gradient to the bottom of the tube and pumping the contents out, from the bottom, using a LKB Varioperpex 12000 peristaltic pump. The gradient was monitored at 280 nm using an IsCo optical unit, model UA, and recorded on a Servoscribe IS potentiometric recorder, model 541.20. Sucrose concentration was measured using a Bellingham and Stanley sugar refractometer. Samples were taken for electron microscopy, SDS gel electrophoresis and protein estimation.

B. By centrifugation in glycerol solutions.

The method used was communicated by Chrispeels(1974). 3 g of meal was gently blended with 30 ml of 80% (v/v) glycerol in 0.05M sodium phosphate, 0.05M citric acid, pH 5.0, containing 0.1% (v/v) 2-mercaptoethanol, at room temperature. The suspension was centrifuged in a MSE bench centrifuge for 10 min at half-maximum speed to remove cell debris. When the supernatant fluid was layered onto 8 ml of 90% (v/v) glycerol in water in a 50 ml centrifuge tube and centrifuged at 38,000 xg for 60 min at 15°C, protein bodies were obtained as a pellet. Samples of some pellets were taken for electron microscopy and for SDS gel electrophoresis; pellets were also resuspended in 0.5M NaCl, 0.05M sodium phosphate buffer, pH 7.5, for protein estimation. When protein bodies (and the resulting supernatant fluid) were being prepared for enzyme distribution studies, 2-mercaptoethanol was omitted. The procedure was also carried out in the glycerol-phosphate-citrate buffer adjusted to pH 7.5 with NaOH.

V. Protein Fractionation.

A. Dialysis of alkaline salt extracts.

Samples of globulin proteins extracted by alkaline salt solutions were prepared by dialysis overnight against running tap water, then against several changes of cold distilled water, and finally against several changes of cold deionised water. To obtain maximum precipitation of protein, especially when analysing extracts from developing seeds, it was found to be necessary to remove the precipitate formed by overnight dialysis against running tap water, by centrifugation, prior to dialysis of the supernatant fluid against distilled or deionised water.

B. Isoelectric precipitation.

(i) Repetitive manual isoelectric precipitation. Repeated isoelectric precipitation of alkaline salt extracts at pH 4.7 was carried out as described by Bailey and Boulter (1970).

(ii) Zonal isoelectric precipitation. This was based on the method described by Shutov and Vaintraub (1965) and Wright and Boulter (1974). The whole procedure was carried out at room temperature and will be briefly described. 6 g of meal was stirred with 60 ml of 0.5M NaCl, 0.05M sodium phosphate buffer, pH 7.5 made 0.1% (v/v) in 2-mercaptoethanol, for 30 min. After clarification by centrifugation, ammonium sulphate was added to 50 ml of the supernatant fluid to 70% saturation; after 40 min stirring, precipitated protein was collected by centrifugation at 23,000 xg for 30 min. This precipitate was dispersed in 0.2M NaCl, 0.05M sodium phosphate buffer, pH 8.0, made 0.1% (v/v) in 2-mercaptoethanol; after extensive dialysis against this buffer, it was applied to a column (3.5 x 44 cm) of Sephadex G-50 equilibrated in 0.2M NaCl, 0.05M citric acid, adjusted to pH 4.7 with dilute sodium hydroxide. Elution was carried out with phosphate buffer of the same composition as that used for dialysis. Fractions of 6 ml were collected using a LKB Ultrorac fraction collector; absorption at 280 nm of eluted fractions was determined in a Hilger and Watts Uvispek H700 spectrophotometer, and pH was determined using a Pye model 291 pH meter.

C. DEAE - cellulose ion-exchange chromatography.

Ion-exchange chromatography was carried out on columns of Whatman DE 52 cellulose equilibrated in 0.025M sodium phosphate buffer, pH 7.5. Samples for chromatography were dialysed against this buffer prior to application. Elution was commenced with buffer of the same composition, then with a linear sodium chloride gradient (routinely 0-0.5M) in the same buffer. The absorption of the eluate at 280 nm was monitored using an IsCo optical unit, model UA, and recorded on a Servoscribe potentiometric recorder.

D. Centrifugation in sucrose density gradients.

(i) Preparation of sucrose gradients. Linear 5-20% (w/v) sucrose gradients in 0.5M NaCl, 0.05M sodium phosphate buffer, pH 7.6, were constructed in 23 ml polycarbonate centrifuge tubes using the apparatus described by Britten and Roberts (1960); the gradients were 14 ml in length and rested upon 2 ml of 40% (w/v) sucrose in the same buffer.

(ii) Preparation of samples. Sample solutions were prepared from meal as described in Section III. Following extraction and clarification, samples were dialysed against 2.2 l of 0.5M NaCl, 0.05M sodium phosphate buffer, pH 7.6, at 4°C for at least 5 h before centrifugation. Additional procedures used in the preparation of samples, prior to the dialysis step described, are described with the results.

(iii) Centrifugation and fractionation of gradients. About 1.5 ml of sample solution was carefully layered onto each gradient. Gradients were centrifuged for 18 h at 4°C and 30,000 rev./min (R av. 94,000 xg) in the 3 x 23 ml swing-out rotor in a MSE Super Speed 65 centrifuge; no braking was used during deceleration. Gradients were fractionated in a manner similar to that used for protein body preparation (Section IV A); fractions of 1 ml were collected. Absorption at 280 and 260 nm of diluted samples (in buffer of the same composition) was measured in a Hilger and Watts Uvispek H700 spectrophotometer.

VI. Estimation of Proteins.

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Crude globulin preparations, prepared by the lyophilisation of the precipitate formed by overnight dialysis against running tap water of an alkaline salt extract of *P. vulgaris*, gave a similar calibration curve up to a concentration of 200 µg/ml. The Calibration graph is shown in Fig. 1. Protein concentration was also estimated using absorption at 280 nm and 260 nm, and the equation described by Layne (1957) i.e. protein concentration (mg/ml) = $1.55\ E_{280} - 0.76\ E_{260}$

VII. Polyacrylamide Gel Electrophoresis.

In the electrophoretic systems described below, % acrylamide refers to the % concentration (w/v) of acrylamide alone, and not to the total amount of acrylamide and bis-acrylamide. In each system, the length of time described for electrophoresis was such that the marker used migrated about 5 cm; gels were normally 7 cm long.

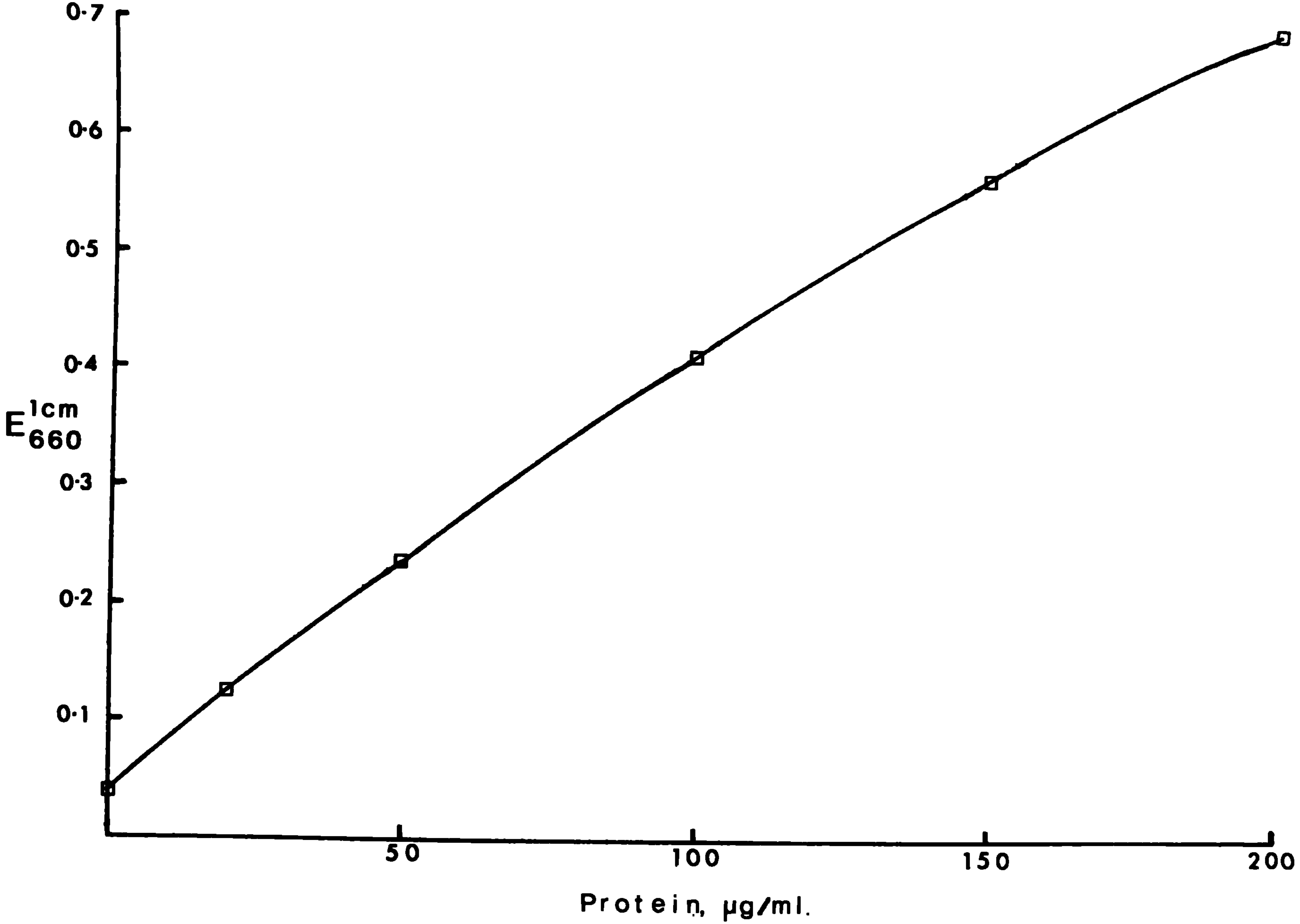
A. Gel systems.

The following gel systems were used :-

(i) Non-dissociating. The disc electrophoretic procedure of Ornstein (1964) and Davis (1964) was used to analyse undissociated proteins; 7% (w/v) acrylamide gels were used routinely. Spacer and sample gels were omitted; samples were dissolved in spacer gel buffer

Fig. 1

A calibration graph for the estimation of protein in solution by the method of Lowry *et al*. (1951). Bovine serum albumin was used as the standard and was dissolved in 0.5M sodium chloride, 0.05M sodium phosphate, pH 7.5. Standard solutions were routinely analysed at the same time as solutions of unknown protein content. Similar results were obtained when protein was dissolved in 0.1N sodium hydroxide. Absorbance was measured in 1 cm cuvettes at 660 nm.


0·7
0·6
0·5
0·4
E_{660}^{1cm}
0·3
0·2
0·1
50
100
150
200
Protein, μg/ml.

and density stabilised with sucrose prior to electrophoresis; bromo-phenol blue was included as a marker. Electrophoresis was carried out at 2.5 mA/gel until the sample had entered the gel (15 min), then at 4 mA/gel for 1.75 h. After electrophoresis, the total length of the gels and the distance of migration of the bromo-phenol blue band, which remained very sharp throughout electrophoresis, were measured. Gels were stained in 0.2% (w/v) amido black (Naphthalene Black 12 B) in 7% (v/v) acetic acid for 90 min and destained by diffusion in 7% (v/v) acetic acid.

(ii) Dissociating. (a) With SDS. The method of Weber and Osborn (1969) was used to determine the apparent molecular weights of protein dissociated with SDS. Gels were normally of 7.0% and 10.0% acrylamide, with a bis-acrylamide : acrylamide ratio of 1:37; when gels of other concentrations were used, this ratio was kept constant. Samples of freeze-dried proteins were dissolved in 0.01M sodium phosphate buffer, pH 7.0, containing 1.0% (w/v) SDS and 0.2% (v/v) 2-mercaptoethanol (Incubation buffer) by incubation at 37°C for 3 h, followed by heating in a boiling water bath for 3 min. Samples already in solution, for example from sucrose density gradient centrifugation and from extracts of germinating seeds, were extensively dialysed against 0.01M sodium phosphate buffer, pH 7.0 containing 0.1% (w/v) SDS and 0.2% (v/v) 2-mercaptoethanol, before heating in a boiling water bath for 3 min. No differences in band patterns or mobilities were apparent between samples prepared and applied in 1.0% (w/v) or 0.1% (w/v) SDS. Bromo-phenol blue was added as a marker and samples were density stabilised with sucrose. Electrophoresis was carried out at 5mA/gel for 20 min, then 10mA/gel for 4.5 h. Following measurement as described for disc gels, gels were stained for 2 h in 0.2% (w/v) amido black in 7% (v/v) acetic acid and destained as described before; gels were occasionally stained in 0.1% (w/v) Coomassie brilliant blue R250 in methanol : acetic acid : water (90 : 7 : 43 by vol.) and diffusion destained in the same solvent.

(b) With CTAB, pH 5.7.

Stock solutions used were :-

(i)	Buffer :	0.2M sodium dihydrogen phosphate, adjusted to pH 5.7 with dil. NaOH
(ii)	Monomer :	22.2 g acrylamide 0.6 g bis-acrylamide Water to 100 ml

7.0% acrylamide gels were prepared by mixing 15 ml of gel buffer, 9.45 ml of monomer solution, 4.0 ml of distilled water, 0.05 ml of Temed and 1.5 ml of freshly prepared ammonium persulphate (10 mg/ml (w/v) in water). After polymerisation, gels were pre-electrophoresed with 0.1M sodium phosphate pH 5.7 containing 0.1% (v/v) thioglycollic acid for 60 min at 8mA/gel. This electrode buffer was removed and pre-electrophoresis was continued with 0.1M sodium phosphate pH 5.7 containing 0.2% (w/v) CTAB for 60 min at 8mA/gel.

Samples were dissolved in 0.01M sodium phosphate pH 5.7 containing 1% (w/v) CTAB and 0.2% 2-mercaptoethanol, by incubation at 35°C for 2 h, followed by heating in a boiling water bath for 2 min. Samples were made dense with sucrose and methyl green was added as a marker. Electrophoresis was carried out, in the direction of the cathode, for 15 min at 5mA/gel, then at 10mA/gel for 2 h. After measuring, gels were stained in 0.1% (w/v) Coomassie brilliant blue R250 in methanol: acetic acid: water (50:7:43, by vol.) and diffusion destained in the same solvent.

B. Additional procedures.

(i) Glycoprotein staining. SDS gels were stained for the presence of glycoprotein using the periodic-acid Schiff (PAS) procedure of Zacharius et al. (1969). To ensure no background staining, particular care was taken over the washing step after incubation in periodic acid (1% (v/v) in 3% (v/v) acetic acid) and prior to staining with commercial Schiffs reagent.

(ii) S-carboxymethylation. This was carried out by the method of Crestfield et al. (1963).

(iii) Direct analysis of bands from disc gels. After electrophoresis of undissociated proteins in disc gels (Section VII A(i)), the region corresponding to a particular band was cut from several gels in which the same sample had been electrophoresed; the pooled gel sections were mashed and extracted with 0.025M sodium phosphate buffer, pH 7.0, containing 2% (w/v) SDS and 2% (v/v) 2-mercaptoethanol. The region taken was based on comparison with either (a) the position of precipitated protein in a similar gel incubated in 25% (w/v) trichloracetic acid for 15 min; or (b) the known Rm of the band (determined on previously stained gels). Extraction and analysis of gel sections was carried out as for SDS extractions from meal (Section III D).

(iv) Recording of electrophoretic patterns and determination of subunit ratios. After gels which had been stained for protein using either amido black or Coomassie brilliant blue were fully destained, they were scanned in transmission at 620 nm using a Joyce-Loebl Chromoscan. The Chromoscan integrator values were used for the estimation of peak areas. The values for all the resolved peaks were added together and each peak was then calculated as the percentage of the total. After PAS staining, gels were scanned at 490 nm. Electrophoretic patterns were also recorded as line drawings; in some cases gels were photographed.

(v) Estimation of mobility and determination of molecular weights. Electrophoretic mobilities were calculated relative to the mobility of the bromo-phenol blue marker band (methyl green band in the CTAB-containing system). The position of the marker band after staining was calculated by assuming linear swelling of the gel in the fixative i.e.

$$\text{distance of marker from top of gel, after staining} = \frac{\text{distance the marker had migrated before staining} \times \text{length of gel after staining}}{\text{length of gel, before staining}}$$

In the disc system, results are expressed as the relative mobility (Rm) of a protein band to that of the bromo-phenol blue marker. Results from the SDS system are expressed as apparent molecular weights, determined by comparison of the Rm's of seed proteins with the Rm's of standard proteins of known molecular weights.

VIII. Gel Isoelectric Focusing in 7M Urea.

The method was based on that of Wrigley (1968). 11 cm gels were cast in 12 x 0.6 cm perspex tubes from the following solutions:

		Final Concentration
40% Ampholine carrier ampholytes	1.8 ml	2% (w/v)
Acrylamide monomer solution (7.5 g acrylamide, 0.25 g bisacrylamide, made up to 25 ml with freshly prepared 8M urea	6.0 ml	5% (w/v)
8M Urea (freshly prepared)	25.3 ml	7M
1% (w/v) ammonium persulphate in 8M Urea (freshly prepared)	2.1 ml	
Distilled water to 36.0 ml i.e.	0.8 ml	

Samples were dissolved in 2% (w/v) carrier ampholytes, 7M urea, 10% (w/v) sucrose and applied to the upper (cathodic) end of the polymerised gels under a protective layer of 2% (w/v) carrier ampholytes, 5% (w/v) sucrose. The lower electrode compartment (anode) contained 0.2% (v/v) sulphuric acid; the upper electrode compartment (cathode) was filled with 0.4% (v/v) ethanolamine. Focusing was carried out at 2mA/gel for about 90 min (until the total voltage had risen to 250 V), then at 250 V (total) for a further 10 h. Gels were extensively washed in 25% (w/v) trichloracetic acid (8 changes of 50 ml/gel over the course of 3-4 days) to remove carrier ampholytes and then stained in 0.2% (w/v) amido black in 7% (v/v) acetic acid. The bromo-phenol blue staining method described by Awdeh (1968) was also used, without prior removal of carrier ampholytes, but rapid fading of bands occurred. The pH gradient in the gel was determined by measurement of the pH of water extracts of slices of an unstained gel.

IX. Amino Acid Analysis of Proteins.

Duplicate samples of proteins in 6N-HCl were hydrolysed *in vacuo* at 105°C for 24 h and 72 h; their amino acid compositions were determined on a Locarte automatic-loading amino acid analyser. The cysteine-cystine content was determined as cysteic acid following performic acid oxidation (Moore, 1963).

X. N-terminal Amino Acid Analysis.

N-terminal amino acids were determined by the dansyl method of Gros and Labouesse (1969). After overnight hydrolysis in 6N HCl at 105°C, chromatography of dansyl-amino acids was carried out on polyamide sheets (Woods and Wang, 1967) using the solvent systems described by Ramshaw *et al.* (1970).

XI. Electron Microscopic Analysis of Protein Body Preparations.

Fixation and all subsequent procedures were carried out by Dr. N. Harris. Samples of protein bodies were fixed in 2.5% (v/v) glutaraldehyde, 1.0% (w/v) osmic acid, 0.05M sodium cacodylate buffer pH 7.0 in 80% sucrose (for samples from sucrose gradients) or in 80% glycerol (for samples from the glycerol procedure) for 3 h at 4°C. They were dehydrated in an alcohol series and embedded in Spurr's resin. Thin sections were post-stained with uranyl acetate and alkaline lead citrate and examined in an A.E.I. EM66 electron microscope.

XII. Agglutination Test.

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

XIII. Enzyme and Trypsin Inhibitor Assays.

All assays were carried out at 35°C and unless stated otherwise were in 0.05M phosphate, 0.05M citrate buffer, at the pH specified; determination of enzyme activity with respect to pH was carried out in buffer of the same composition adjusted to the required pH at 35°C. The conditions used for all the assays were such that hydrolysis of substrate was linear with respect to the duration of the assay and to the amount of extract used. When the effects of potential inhibitors or activators were investigated, they were made up at 10 X the required concentration. Extracts were incubated with the inhibitor/activator for 1 h at 0°C prior to assay, and it was also included in the assay at the same concentration as that used in the preincubations.

A. Leucine amino peptidase.

Substrate :	41.7 mg L-leucine-p-nitroanilide was dissolved in 1 ml dimethyl sulphoxide (DMSO) and made up to 20 ml with water.
The assay contained:	1.0 ml 0.1M sodium phosphate, 0.1M citric acid, pH 6.5
	0.5 ml substrate solution
	0.2 ml extract (usually a 10-fold dilution, in 0.025M phosphate, 0.25M NaCl pH 7.5)

Water (or inhibitor, etc) to 2 ml.

After equilibration at 35°C for 5 min, the assay was started by the addition of substrate. After a period of 40 - 60 min, 2 ml 15% (v/v) acetic acid was added; the incubations were left in ice for 60 min, centrifuged at 2000 xg for 20 min and the absorption of the supernatant fluid was measured at 410 nm. Results were corrected for enzyme (no substrate) and substrate (no enzyme) controls.

One unit of enzyme activity was defined as that amount of enzyme that caused the hydrolysis of 1 μ mole of L-leucine-p-nitroanilide per min; the extinction coefficient of the p-nitro aniline (the hydrolysis product) was taken as 8,800 at 410 nm; at this wavelength there is no contribution to the overall absorbance from unhydrolysed anilide (Erlanger et al. 1961).

B. BAPNA-ase.

Substrate :	37.0 mg, BAPNA (α-N-Benzoyl-DL-arginine-p-nitroanilide hydrochloride) was dissolved in 0.8 ml warm DMSO; the volume was made to 21.0 ml with water.
The assay contained:	1.0 ml 0.1M sodium phosphate, 0.1M citric acid, routinely at pH 7.5 and 5.0.
	0.5 ml substrate solution.
up to	0.2 ml extract.
Water (inhibitor) to	2.0 ml.

The assay was carried out as described for leucine amino peptidase. Incubations were routinely of 60 min duration. The assay was terminated and hydrolysis estimated as for leucine amino peptidase.

One unit of activity was defined as that amount of enzyme that resulted in the hydrolysis of 1 μ mole of BAPNA per min.

C. Trypsin inhibitor.

Trypsin inhibitor activity was determined by the inhibition by seed extracts of the trypsin-catalysed hydrolysis of BAPNA.

5 mg trypsin was dissolved in 50 ml water (at 0°C)

BAPNA was dissolved as described previously.

The assay contained : 1.0 ml 0.1M sodium phosphate, 0.1M citric acid.
0.5 ml BAPNA solution.
0.2 ml Trypsin solution.
up to 0.3 ml suitably diluted extract.
water to 2.0 ml

The trypsin solution was added 3 min before the assay was started by the addition of the BAPNA solution. Incubations were usually of 40 min duration. Termination of the assay and determination of the amount of hydrolysis were as described for leucine amino peptidase. Inhibitor activity was either expressed as the percentage inhibition of the control trypsin activity or as units of inhibitor activity. One unit of inhibitor activity was defined as that amount that caused 50% inhibition of the control trypsin activity. Assays were carried out so that the total inhibition did not exceed 60% of the control trypsin activity; Fig. 2 shows the % inhibition of the control trypsin activity caused by the addition of extracts of meal; inhibition is linear up to approximately 70% inhibition.

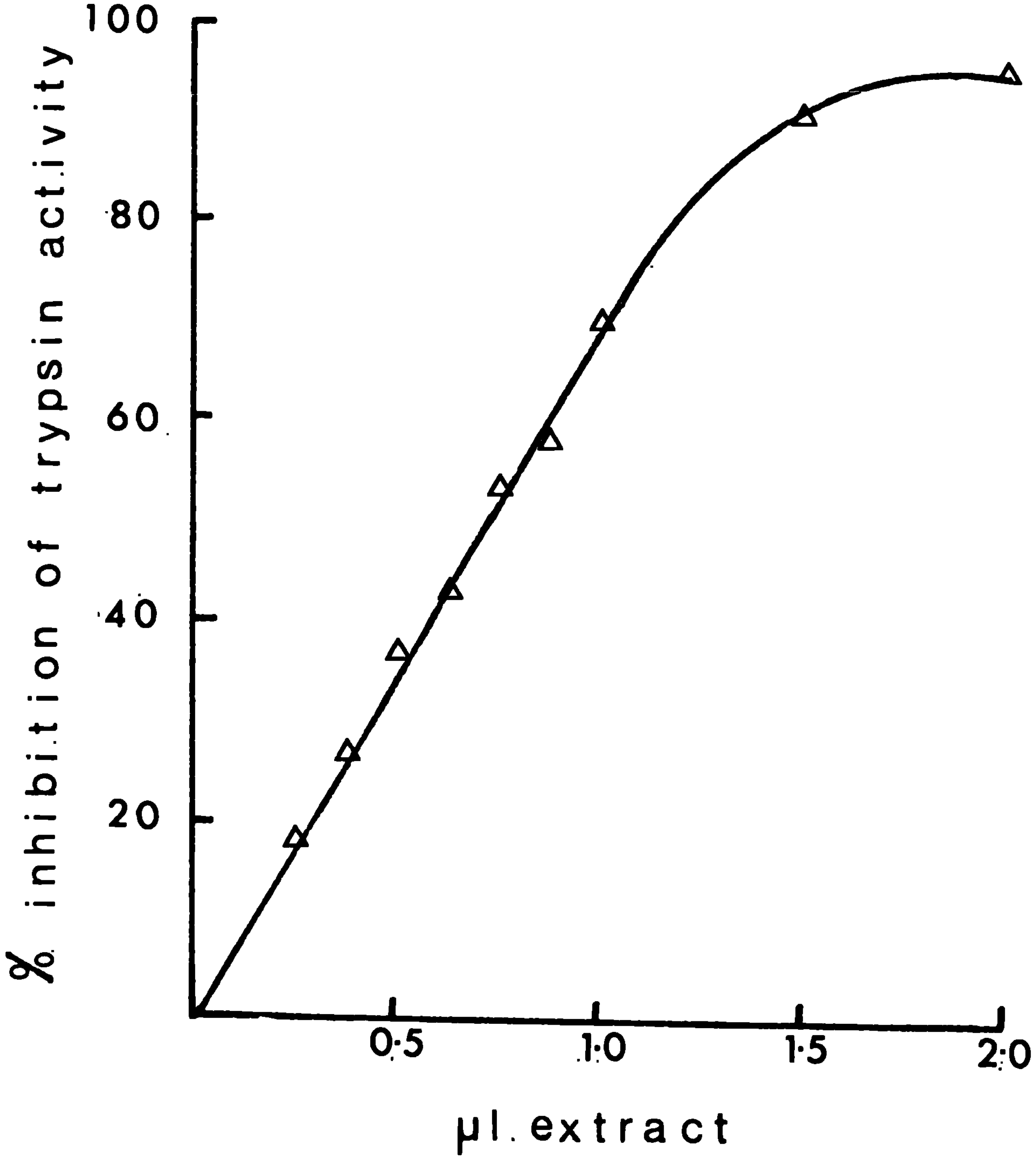
D. Protease assays.

(i) Azoglobulin hydrolysis. The preparation of azoglobulin was based on the method used by Hobday *et al*. (1973) and was communicated by Thurman (1974). The method of preparation used here is briefly described.

(a) Preparation of diazosulphanilic acid. 19.2 mg of sulphanilic acid and 26.7 mg of potassium bromide were dissolved in 2.81 ml of N hydrochloric acid with 2.3 ml of distilled water. After cooling to 0°C, 1.12 ml of 1M sodium nitrite, also at 0°C, was slowly added over the course of 10 min. The solution was allowed to stand at 0°C for 15-30 min before use.

Fig. 2

Inhibition of trypsin activity by increasing amounts of seed extracts. The extract was from seed meal; although the percentage inhibition is presented as for undiluted extract, the extract was in fact diluted (50- and 200- fold, in 0.25M sodium chloride, 0.025M sodium phosphate, pH 7.5) before assay. In all assays of trypsin inhibitory activity, controls were carried out, in the absence of trypsin, to allow for the effect of endogenous BAPNA-ase activity.


% inhibition of trypsin activity
100
80
60
40
20
0·5
1·0
1·5
2·0
µl. extract

(b) Preparation of azoglobulin. The protein used to prepare azoglobulin was freeze-dried cryoprotein, obtained as a precipitate by cooling a water extract of meal (Section III B). 2.5 g was dissolved in 125 ml of 0.01N sodium hydroxide, and was cooled to $0^{\circ}C$; the pH was adjusted to pH 10. The diazosulphanilic acid solution was slowly added, at $0^{\circ}C$, over the course of 10 min; this procedure was carried out in an ice bath on a magnetic stirrer and the pH was maintained throughout at pH 10 by the addition of cold 0.2N sodium hydroxide. Coupling was allowed to proceed for a further 15 min and the reaction was then stopped by adjusting the pH to 7.0, using 0.2N hydrochloric acid.

In the method used by Hobday et al. (1973) the azoglobulin (prepared from Pisum sativum globulin) was recovered by precipitation at pH 4.0 - 4.5; with the preparation described here this procedure was only partially effective. The most convenient method of recovery of the azoglobulin was by addition of ammonium sulphate to 100% saturation (at $0^{\circ}C$); after stirring for 45 min, the precipitate was collected by centrifugation at 23,000 xg for 20 min at $4^{\circ}C$. The precipitate was resuspended in a few ml of distilled water and dialysed overnight against running tap water, then against deionised water; it was then freeze-dried.

(c) Assay. A 2% (w/v) solution of azoglobulin was prepared by dissolving 0.5 g of the freeze-dried azoglobulin in 10 ml 0.02N sodium hydroxide; it was then adjusted to pH 7.0 with 0.2N hydrochloric acid, and made up to 25 ml with distilled water.

The assay contained : 1.0 ml 0.1M sodium phosphate, 0.1M citric acid, routinely pH 5.5.
0.5 ml substrate solution.
up to 0.5 ml extract.
water to 2.0 ml.

The assay was carried out for 2 h, and was terminated by the addition of 2 ml of 20% (w/v) trichloracetic acid; after ageing in ice for 2 h, the assays were clarified by centrifugation at 2,000 xg for 30 min. 1.5 ml of the supernatant fluid was mixed with 1.5 ml of 4N sodium hydroxide and the absorption at 430 nm was determined. Change in absorption was corrected for enzyme and substrate blanks by incubating samples of each separately (at pH 5.5) and only mixing them (in appropriate proportions) after the addition of trichloracetic acid. One unit of azoglobulytic activity was defined as that amount of enzyme which caused a change in the absorption at 430 nm, as measured, of 0.001 optical density units per min.

The absorption spectrum of azoglobulin, determined in a Perkin-Elmer model 402 spectrophotometer, is shown in Fig. 3.

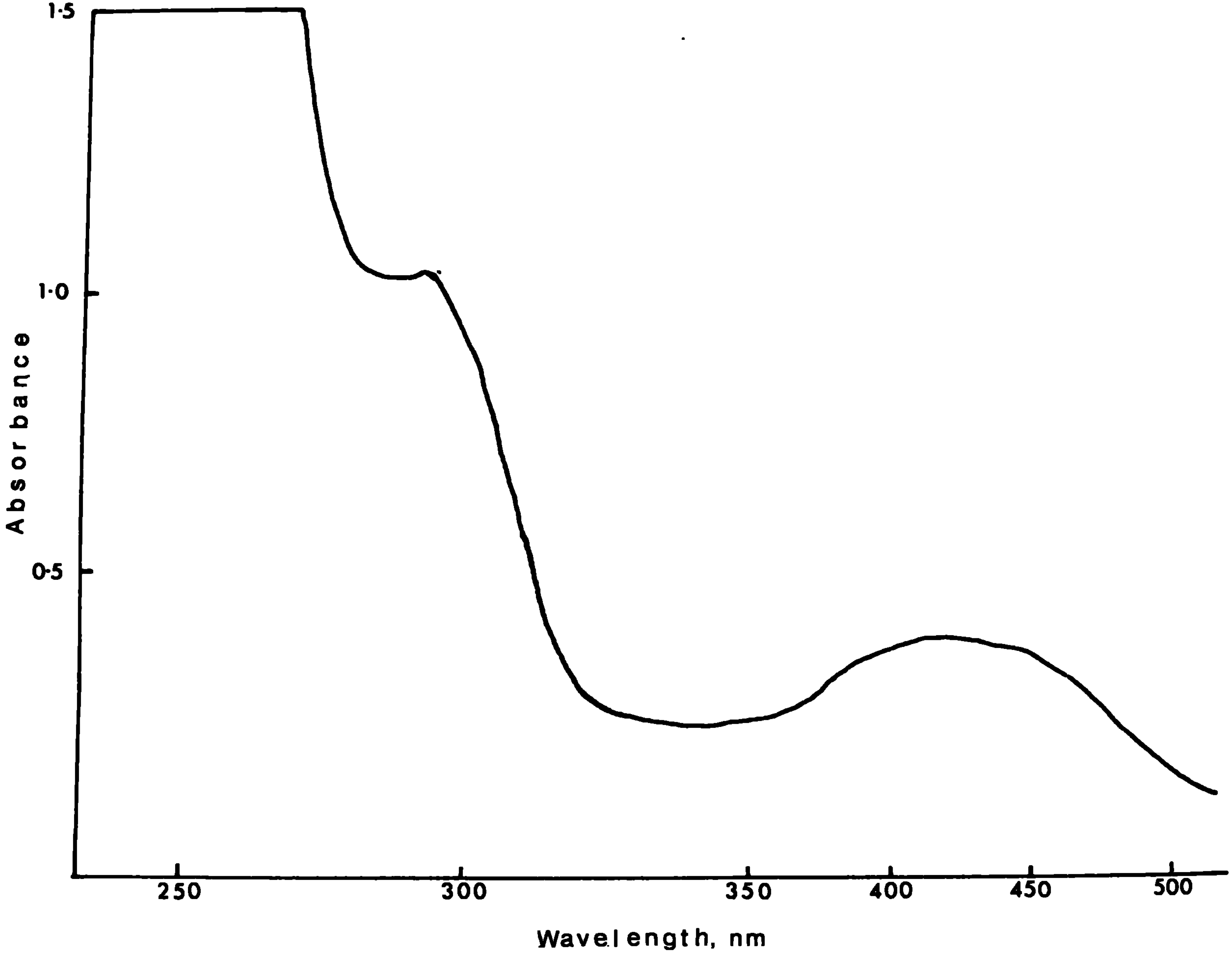
(ii) Azoalbumin hydrolysis. Protease activity was determined in exactly the same way as by azoglobulin hydrolysis, using commercial azoalbumin of the same concentration (% w/v) as substrate; in this case absorption was measured at 440 nm. One unit of azoalbumin-hydrolysing activity was defined as that amount of enzyme which caused a change in the absorption at 440 nm of 0.001 optical density units per min, as measured.

With both azo-substrates the percentage of each substrate solubilised during the assays was determined from the extinction coefficients measured on suitably diluted substrate solutions treated as for the incubation samples.

(iii) Gelatin hydrolysis, determined by viscometry. Protease activity was also determined by the reduction, resulting from hydrolysis, of the viscosity of gelatin solutions; the method

Fig. 3

The absorption spectrum of azoglobulin, prepared from the cryoprotein derived from a water extract of *Phaseolus vulgaris* cv 'Seafarer' seed meal. The cuvette (1 cm light path) contained 0.25 ml 2% (w/v) azoglobulin solution, 0.75 ml water, 1.0 ml 20% (w/v) trichloracetic acid and 1.0 ml 4N sodium hydroxide. Note change of wavelength scale at 350 nm.


1·5
1·0
0·5
Absorbance
250
300
350
400
450
500
Wavelength, nm

used was described by Sundblom and Mikola (1972).

Substrate : 5 g of Difco gelatin was dissolved in 100 ml 0.05M sodium phosphate, 0.05M citric acid by heating to 70 - 80°C, with stirring. When dissolved, it was cooled to 35°C and the pH was adjusted at this temperature to the required pH (routinely pH 5.5). The substrate was made up fresh and was not stored.

Assays were carried out in an Ubbelohde capillary viscometer. 10 ml substrate (at 35°C), 1 ml distilled water, 2 drops of Antifoam emulsion A, and 1 ml extract were mixed in the viscometer. The first reading was taken 2.5 min after these additions had been made. Subsequent readings were at 3 min intervals and were continued for 15 - 20 min. When the effect of 2-mercaptoethanol on activity was being determined, 1 ml of freshly prepared 10% (v/v) 2-mercaptoethanol was used in place of the 1 ml of distilled water.

The flow time is the time taken (in seconds) for the meniscus to pass the two measuring marks. Extracts were diluted such that the maximum reduction in the flow time (initially about 85 seconds) was less than 0.5 second per min; under these conditions the reduction in flow time was linear.

One unit of proteolytic activity was defined as that amount of enzyme which caused a reduction in the flow time of the substrate-sample mixture of 1 second per minute.

(iv) 'In vitro' storage protein hydrolysis, analysed by SDS-gel electrophoresis. One ml of samples of extracts, protein bodies and sample layer regions of sucrose density gradients were mixed with 1 ml of 0.05M sodium phosphate, 0.05M citric acid and the pH was adjusted to pH 5.5 at 30°C; 100 µl of 2% (w/v) sodium azide was added. Incubations were carried out in a shaking water bath at 30°C; at zero time, 20 h and 48 h samples were withdrawn, dialysed against 0.01M sodium phosphate buffer, pH 7.0, containing 0.2% (w/v) SDS and 0.2% (v/v) 2-mercaptoethanol and analysed by SDS gel electrophoresis.

Some incubations contained only one sample, for example protein bodies from 3 day germinated cotyledons, while others contained mixtures, for example both 1 ml of protein bodies and 1 ml of the sample layer region from a sucrose gradient.

RESULTS

I. Proteins of Mature Seeds

A. Extraction and Fractionation of the Main Storage Proteins.

(i) Alkaline salt extraction and isoelectric precipitation.

Table 1 shows the total amount of protein extracted from 10 g of seed meal by successive aliquots of extractants. More than 90% of the total protein extracted was salt soluble at pH 7.5. When the supernatant fluid from the first extraction was acidified to pH 4.7, 89% of the protein originally present remained in solution. Sequential dialysis of the alkaline salt extract against running tap water, distilled water and deionised water resulted in the precipitation of over 80% of the protein present.

When the alkaline salt extract was analysed by electrophoresis in the non-dissociating gel system at pH 8.3 (hereafter referred to as disc gel electrophoresis), a diffuse major band, Rm 0.35 - 0.4, was obtained, together with slower moving components with Rm's 0.19, 0.1 and several other minor components. A more clearly defined pattern was obtained when samples of this extract were subjected to SDS gel electrophoresis. Fig. 4 shows a densitometric trace of the protein band pattern obtained from this extract. There are two major components, molecular weight 50,000 and 47,000, but four other components, molecular weights 60,000, 32,000, 23,000 and 20,000 are resolved. Fig. 5a presents the result of direct SDS extraction and electrophoretic analysis of meal; a similar pattern to that obtained from the alkaline salt extract was obtained. Fig. 5b shows the result of similar SDS extraction and electrophoresis of meal of _P. vulgaris_ cv. 'Canadian Wonder'. In place of the 50,000 and 47,000 molecular weight subunits of

TABLE 1

Extraction of protein from 10g of seed meal

The amount of protein extracted from 10g seed meal by successive extractions with the alkaline salt extractant, dilute sodium hydroxide and 70% (v/v) ethanol. Protein concentration was determined using the procedure of Lowry *et al.* (1951).

Extractant	Vol. recovered ml	Protein mg/ml	Total protein mg
100 ml 0.5M NaCl 0.05M Sodium phosphate pH 7.5	94	19.1	1,795
100 ml "	97	1.26	122
100 ml "	102	0.37	37.7
100 ml "	101	0.28	28.3
100 ml 0.01N NaOH	103.5	1.32	137
100 ml 70% (v/v) Ethanol	102	.205	20.9
			2,140

Fig. 4

The protein band pattern resulting from electrophoresis of an alkaline salt extract of meal in the SDS gel system; the acrylamide concentration was 7.0% (w/v). Numbers above peaks refer to the molecular weights (x 10^{-3}) of the indicated peaks. These were estimated using the calibration graphs shown in Fig. 6.

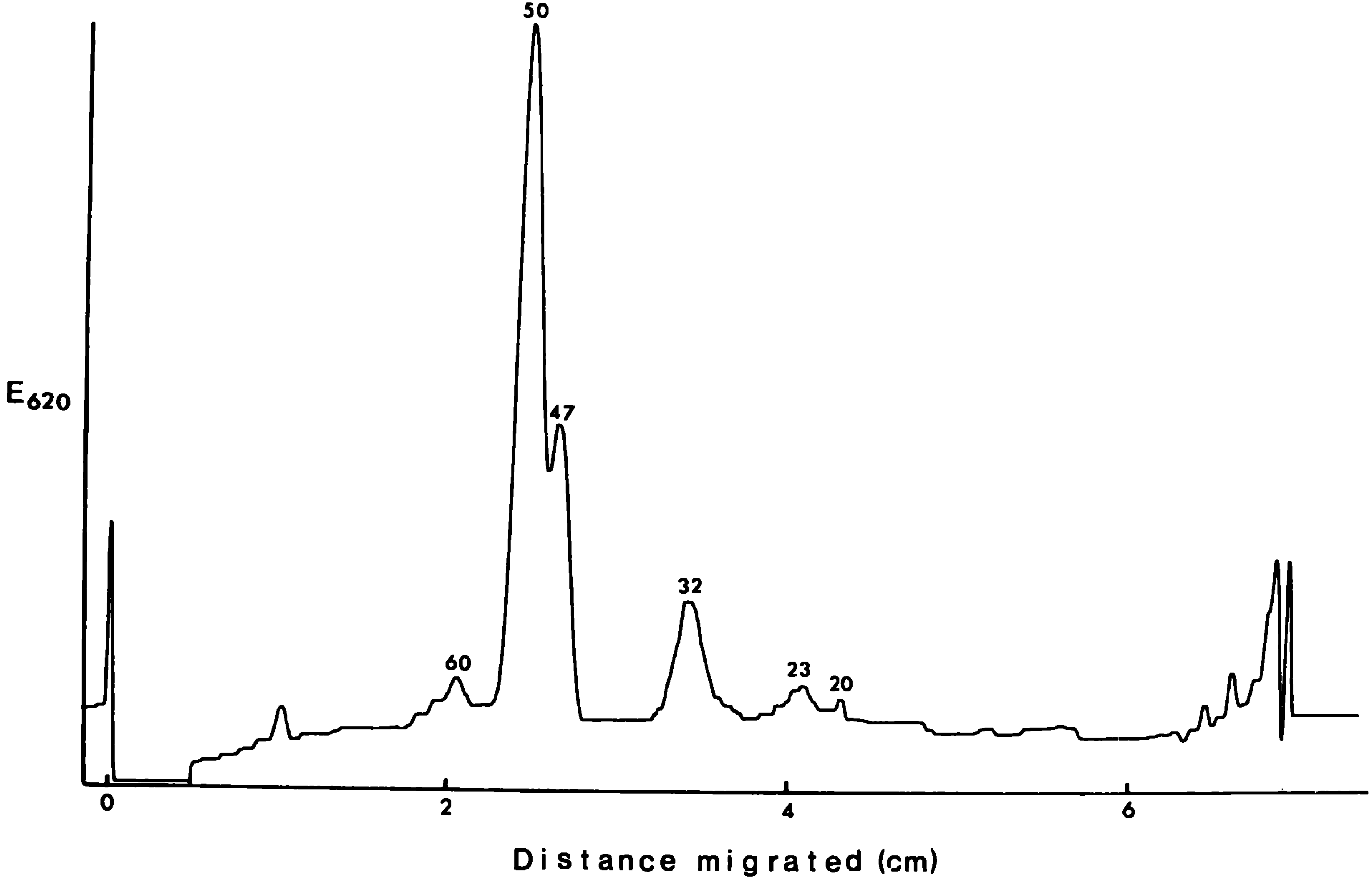
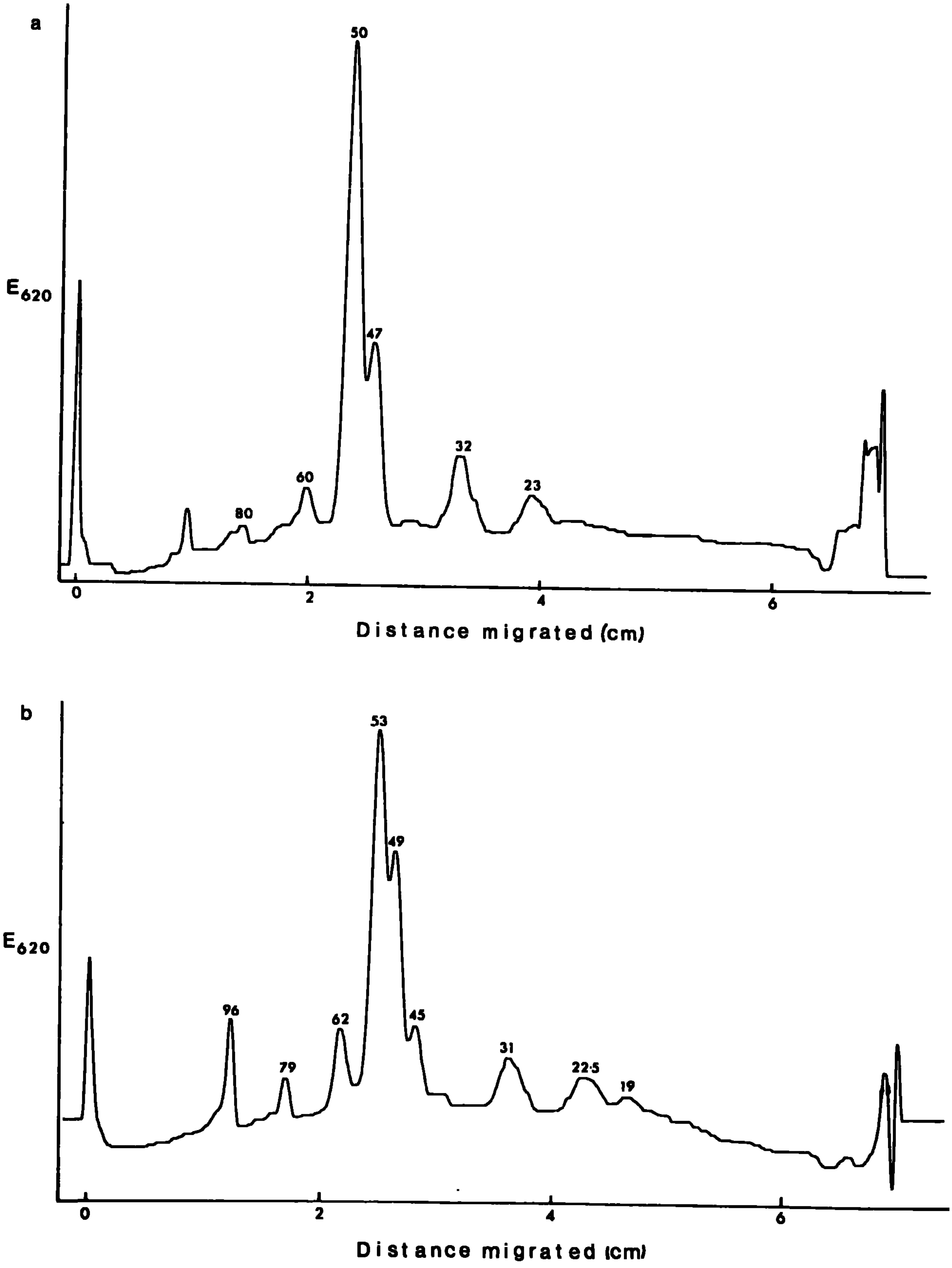

E620
50
47
60
32
23
20
0
2
4
6
Distance migrated (cm)

Fig. 5

Fig. 5 a The protein band pattern resulting from electrophoresis of an SDS extract of meal of *Phaseolus vulgaris* L. cv 'Seafarer' in the SDS gel system (7.0% (w/v) acrylamide). The numbers refer to the molecular weights (x 10^{-3}) of the associated peaks.

Fig. 5 b The protein band pattern resulting from similar SDS extraction and electrophoresis (in a 7.0% (w/v acrylamide gel) of meal of *Phaseolus vulgaris* L. cv 'Canadian Wonder'. Numbers above peaks are the apparent molecular weights (x 10^{-3}) of the peaks indicated.


a
E620
50
47
60
80
32
23
0
2
4
6
Distance migrated (cm)
b
E620
53
49
96
62
45
79
31
22·5
19
0
2
4
6
Distance migrated (cm)

the cultivar 'Seafarer', two major subunits molecular weight 53,000 and 49,000, and a minor subunit molecular weight 45,000, were found. Unless otherwise stated, all experiments were conducted on seed of the cultivar 'Seafarer'. Molecular weights were estimated using the calibration graphs shown in Fig. 6.

Fig. 7a presents the results of disc electrophoresis of the fractions obtained by manual isoelectric precipitation. The fraction which was soluble at pH 4.7 contained an intense, poorly defined band, Rm 0.35 - 0.4, together with a subsidiary band, Rm 0.19. The pH 4.7 insoluble fraction, which was poorly soluble in the electrophoresis sample buffer, migrated as a single band Rm 0.1. Subunit analysis of the pH 4.7 insoluble fraction by SDS gel electrophoresis showed it to be mainly composed of subunits of molecular weight 60,000 and 20,000, but a significant quantity of the major subunits, molecular weights 50,000 and 47,000, were also present (Fig. 7b, gel 2). The pH 4.7 soluble fraction (Fig. 7b, gel 1) contained subunits of molecular weights 50,000, 47,000, 32,000 and 23,000; however, the relative amounts of the lower molecular weight subunits, i.e. 32,000 and 23,000, varied in amount with respect to each other and also to the amount of the 50,000 and 47,000 molecular weight subunits at different stages of purification.

The precipitate obtained from alkaline salt extracts by addition of ammonium sulphate to 70% saturation was enriched in the 60,000, 32,000, 23,000 and 20,000 molecular weight subunits. Two fractions were obtained by zonal isoelectric precipitation of the redissolved precipitate (Fig. 8). The first eluted at and immediately after the void volume, in citrate buffer, pH 4.7,

Fig. 6

Molecular weight calibration graphs for the SDS gel system. Molecular weights were routinely estimated in gels of 7.0 and 10.0% (w/v) acrylamide concentration. Standard proteins used, together with their molecular weights (as described by Weber and Osborn, 1969) were :-

bovine serum albumin	68,000
catalase	60,000
γ-globulin, H-chain	50,000
ovalbumin	43,000
carboxypeptidase A	34,600
γ-globulin, L-chain	23,500
myoglobin	17,200

10.0% (w/v) acrylamide gel △————△

7.0% (w/v) acrylamide gel ○————○

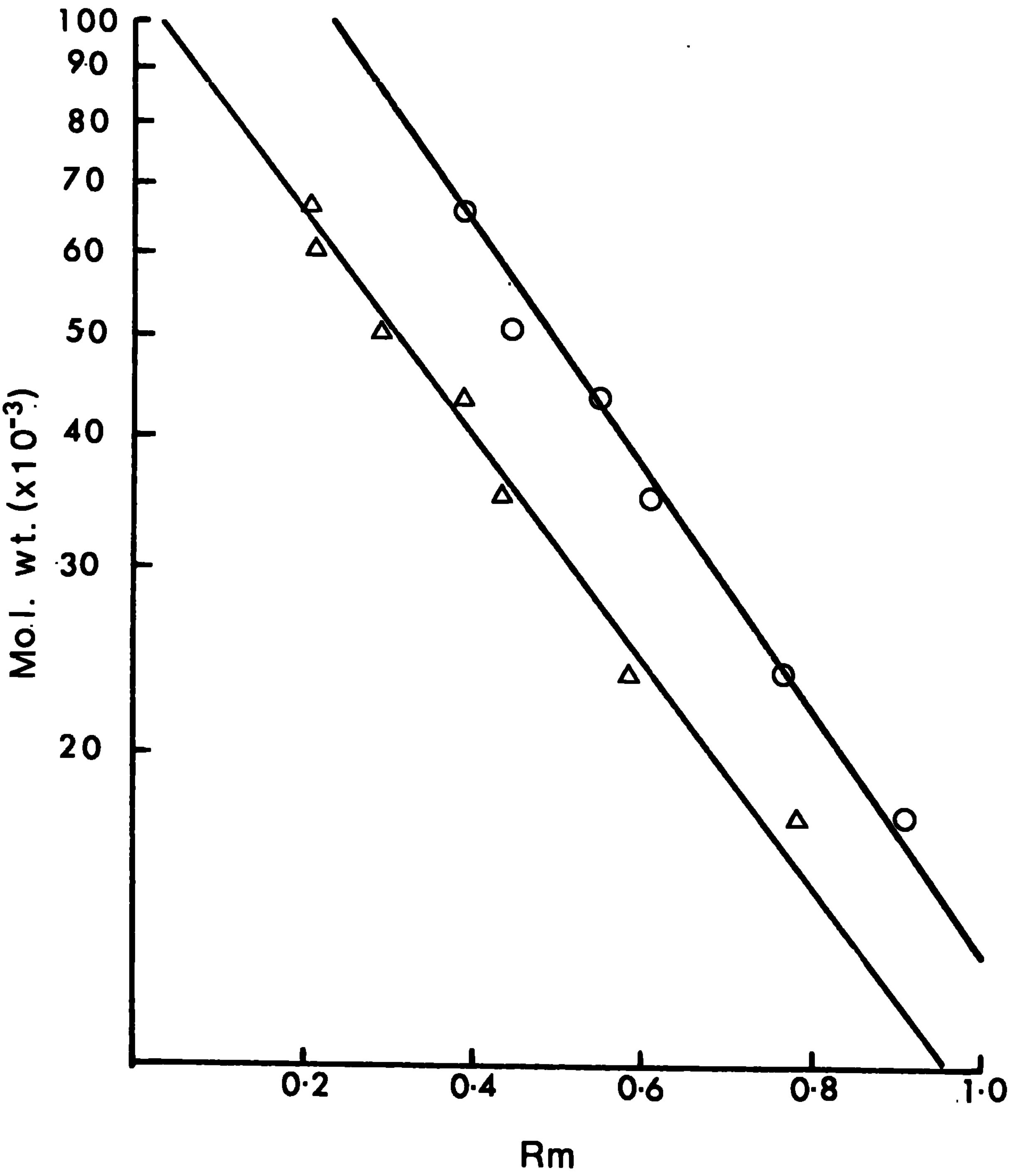

100
90
80
70
60
50
40
30
20
Mol. wt. (x10^{-3})
0·2
0·4
0·6
0·8
1·0
Rm

Fig. 7

Fig. 7 a Disc electrophoretic patterns of fractions obtained by manual isoelectric precipitation (repeated three times).

Gel A : the pH 4.7 soluble fraction.

Gel B : the pH 4.7 insoluble fraction.

Numbers refer to the mobilities of the bands relative to that of the bromo-phenol blue marker.

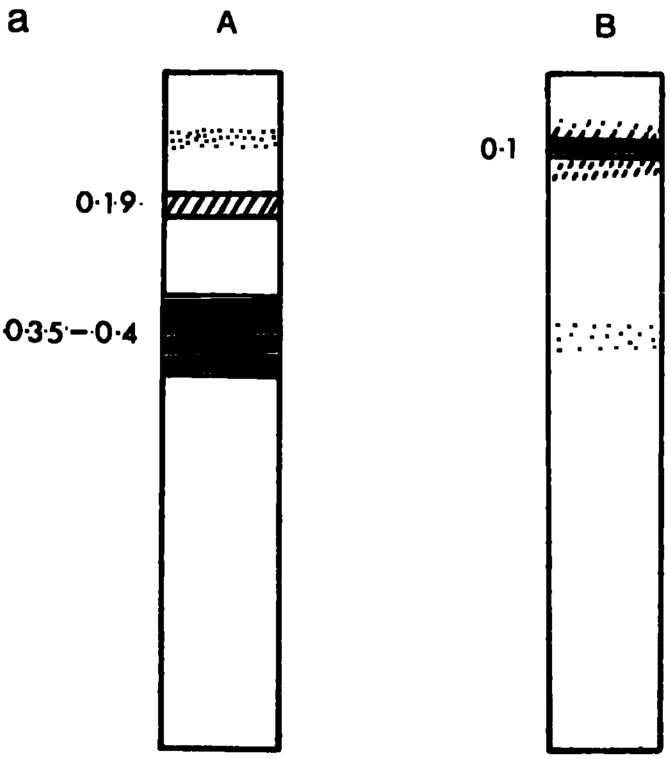
Fig. 7 b SDS gel elctrophoretic analysis of fractions prepared by isoelectric precipitation.

Gel 1 : the pH 4.7 soluble fraction, prepared manually.

Gel 2 : the pH 4.7 insoluble fraction, prepared manually.

Gel 3 : the pH 4.7 insoluble fraction, prepared by zonal isoelectric precipitation.

Numbers represent the molecular weights (x 10^{-3}) of the bands indicated. 7.0% acrylamide gels were used.


a
A
B
0·1·9·
0·3·5·–0·4
0·1

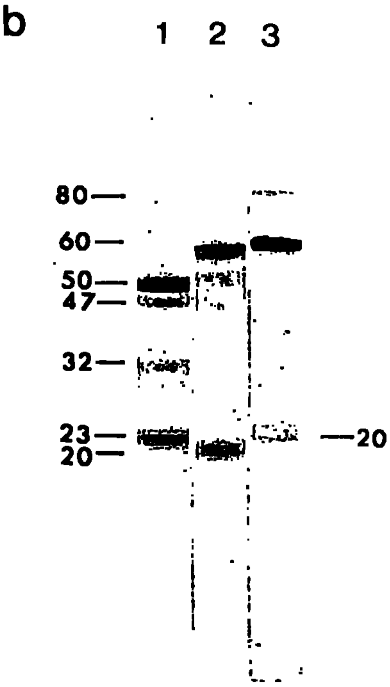
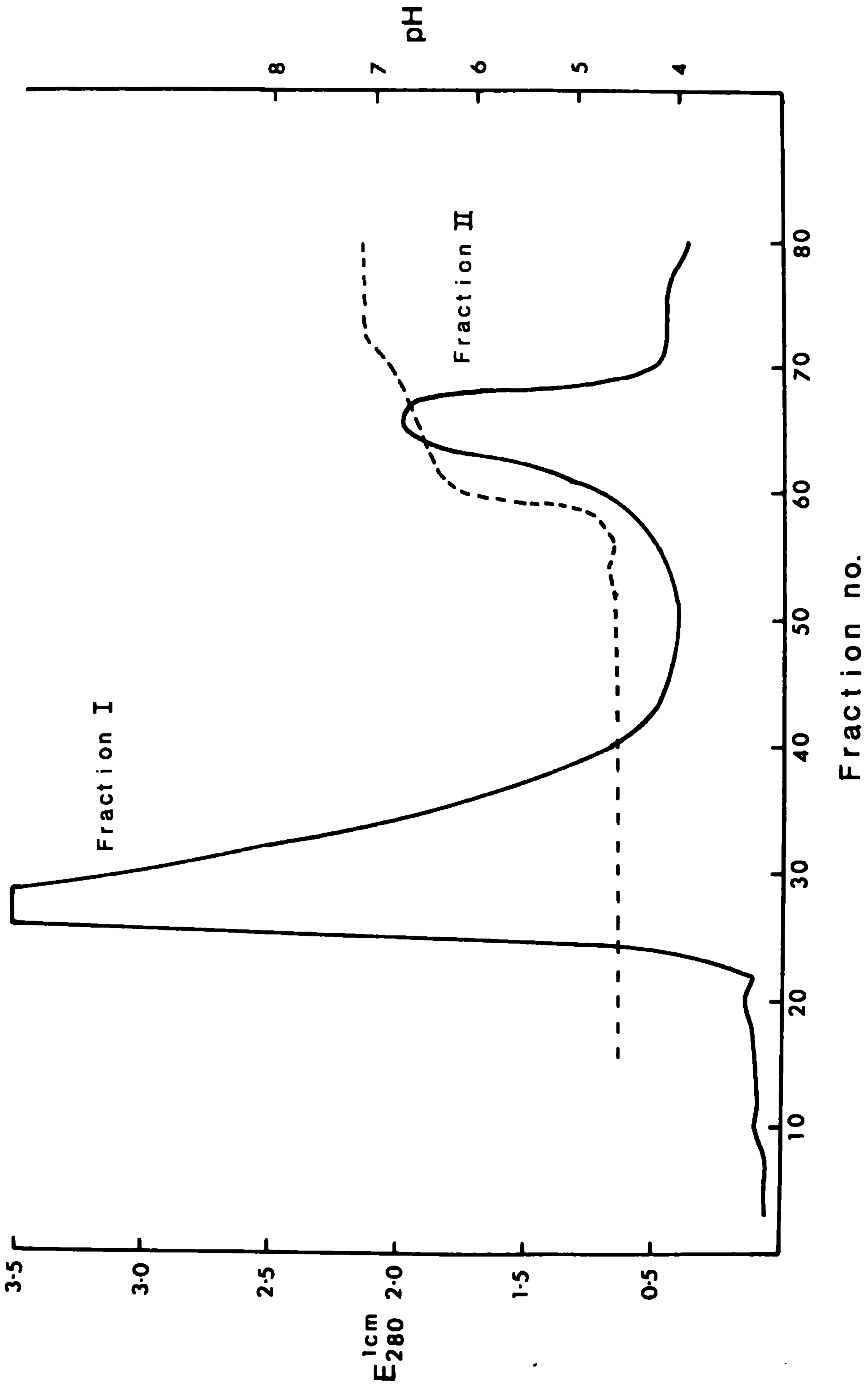

b
1
2
3
80
60
50
47
32
23
20
20

Fig. 8

The elution profile for the zonal isoelectric precipitation procedure described and carried out as detailed in Methods (Section V B.(ii)). The flow rate was 12 ml/h and fractions of 6.0 ml were collected. The continuous line presents the absorbance at 280 nm; the dashed line indicates the pH. Fraction I is the unretarded fraction, soluble at pH 4.7, referred to in the text; fraction II is the retarded fraction, which elutes as the pH increases above pH 5.5 - 6.0.


E^{1cm}_{280}
3·5
3·0
2·5
2·0
1·5
0·5
pH
8
7
6
5
4
Fraction I
Fraction II
10
20
30
40
50
60
70
80
Fraction no.

and was soluble at this pH; the second fraction, which accounted for approximately 20% of the protein applied to the column, was insoluble at pH 4.7 and was retarded during chromatography. The pH 4.7 soluble fraction was shown by SDS gel electrophoresis to contain subunits of molecular weight 50,000, 47,000, 32,000 and 23,000. However, as shown in Fig. 7b (gel 3), the pH 4.7 insoluble fraction from the zonal procedure was more pure than that prepared manually and contained only three subunits of molecular weight about 80,000, 60,000 and 20,000.

(ii) Water extraction and cryoprecipitation.

When water extracts of meal were cooled a substantial cryoprecipitate formed. Analysis by disc electrophoresis showed this precipitate to be composed of a main component which migrated as a wide band, Rm 0.35 - 0.4, together with a subsidiary band Rm 0.2. The supernatant fluid after removal of the cryoprecipitate contained approximately equal amounts of components with Rm's 0.37 and 0.1.

Fig. 9 contains densitometric traces of the analysis of these two fractions by SDS gel electrophoreses. While the cryoprecipitate is composed mainly of subunits of molecular weight 50,000 and 47,000, together with traces of subunits of lower molecular weight, the supernatant fluid after removal of the cryoprotein contained an increased proportion of subunits of molecular weight 80,000, 60,000, 32,000, 23,000 and 20,000.

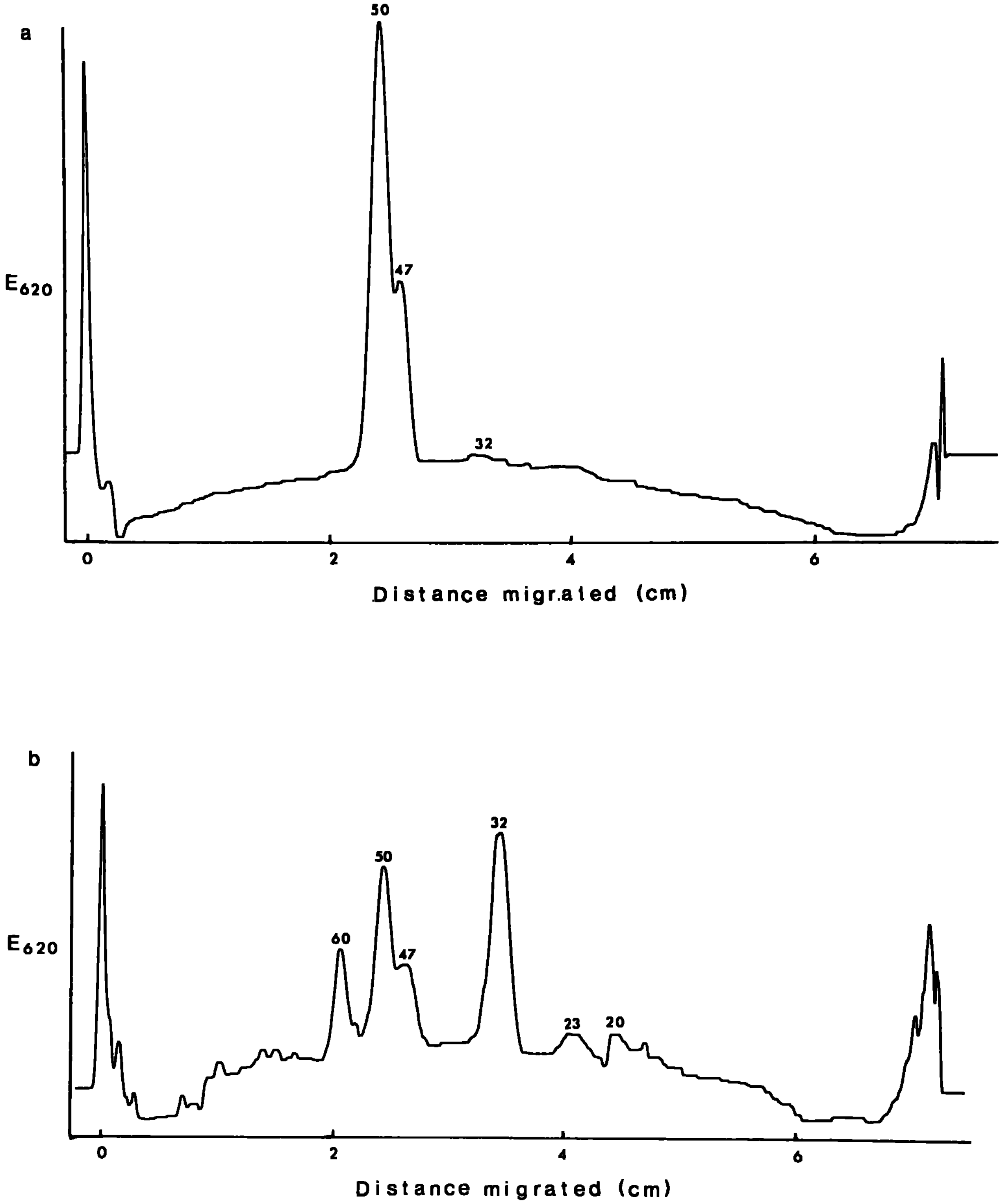
(iii) Acidic extractions and fractionation.

Extraction and fractionation was carried out with acidic extractants as described in Methods (Section III C). When these acidic extracts were examined by disc gel electrophoresis, similar

Fig. 9

Fig. 9 a The electrophoretic pattern resulting from SDS gel electrophoresis of the cryoprotein prepared by cooling a water extract of meal. Molecular weights (x 10^{-3}) of peaks are also presented.

Fig. 9 b The electrophoretic pattern resulting from SDS gel electrophoresis of the supernatant fluid remaining after the removal of the cryoprotein from a water extract. Molecular weights (x 10^{-3}) of peaks are as indicated. The acrylamide concentration in both gels was 7.0% (w/v).


a
50
47
32
E_{620}
0
2
4
6
Distance migrated (cm)
b
32
50
60
47
23
20
E_{620}
0
2
4
6
Distance migrated (cm)

patterns to those obtained from alkaline salt extracts were obtained. The initial supernatant, F-I fraction and F-IIa fraction disc electrophoretic patterns of the acidic extraction, in the absence of ascorbate, are shown in Fig. 10. The lack of definition of bands in the initial supernatant is typical of all extractions; the diffuse edges of the main component, Rm 0.37, are seen in all samples, whereas the electrophoresis of bovine serum albumin gave rise to bands with very sharp leading edges. The F-I fraction, consisting mainly of a slow moving band, Rm 0.1, resembles the pH 4.7 insoluble fraction, while the F-IIa fraction is composed almost entirely of the main component of the initial extract, together with a subsidiary band Rm 0.2.

The results of SDS gel electrophoresis of the fractions obtained from the acidic extracts are shown in Fig. 11. In the ascorbate-containing extract (Fig. 11a), subunits of molecular weight 60,000 and 20,000 were confined to the F-I fraction, but those of molecular weight 50,000 and 47,000 were found in all three fractions. The 23,000 molecular weight subunit was enriched in the F-I fraction, while subunits of molecular weight 32,000 were only found in the F-II fractions.

When acidic extracts were prepared and fractionated in the absence of ascorbate (Fig. 11b) the F-I fraction contained mainly the same subunits as the corresponding fraction from the ascorbate-containing extract, but there was markedly less of the 50,000 and 47,000 molecular weight subunits, i.e. the 80,000, 60,000 and 20,000 molecular weight subunits were enriched. Whilst the presence or absence of ascorbate had less effect on the composition of the F-II fractions, nevertheless, in the

Fig. 10

Disc electrophoretic patterns of fractions from extraction of meal with 0.5M NaCl, adjusted to pH 2.0 with dilute HCl (i.e. no ascorbate).

Gel 1 : Initial supernatant, after removal of cell debris by centrifugation.

Gel 2 : F - I fraction.

Gel 3 : F - IIa fraction.

The numbers represent the Rm's of the bands indicated; electrophoresis was in 7.0% (w/v) gels. The terminology of the fractions is described in Methods, Section III C.

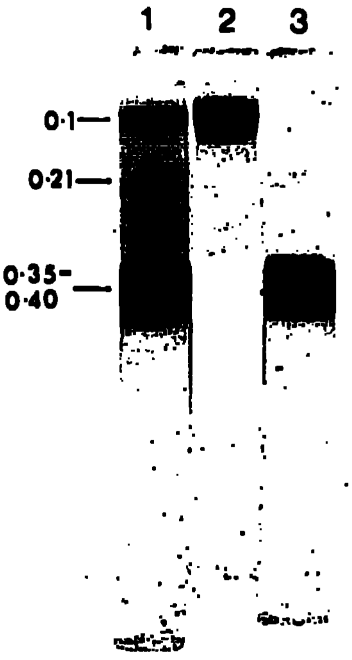

1
2
3
0·1
0·21
0·35-
0·40

Fig. 11

SDS gel patterns of fractions prepared from acid extracts of seed meals. All gels are 7.0% (w/v) acrylamide, and numbers refer to the molecular weights ($\times 10^{-3}$) of the bands indicated.

Fig. 11 a Extraction with 0.5M NaCl, 0.25M ascorbic acid, pH 2.4.

Gel 1 : Initial supernatant

Gel 2 : F - I fraction

Gel 3 : F - IIa fraction

Gel 4 : F - IIb fraction

Fig. 11 b Extraction with 0.5M NaCl, adjusted to pH 2.0 with dilute HCl.

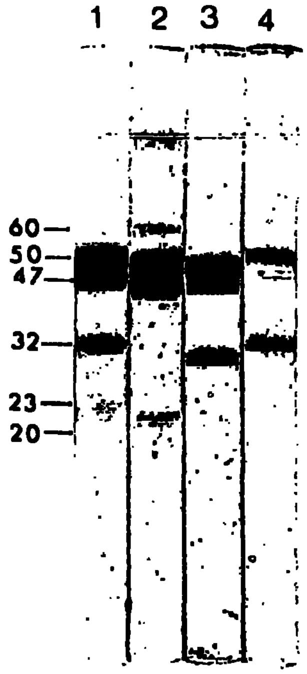
Gel 1 : Initial supernatant

Gel 2 : F - I fraction

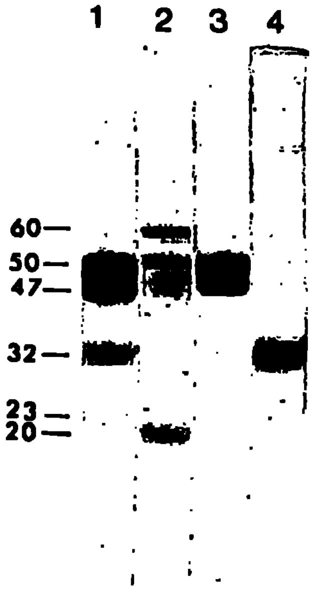
Gel 3 : F - IIa fraction

Gel 4 : F-IIb fraction

a


1
2
3
4
60
50
47
32
23
20

b


1
2
3
4
60
50
47
32
23
20

absence of ascorbate the F-IIa fraction consisted almost entirely of the 50,000 and 47,000 molecular weight subunits, and the F-IIb fraction of the 32,000 molecular weight subunit. Using either extractant, the F-II fractions agglutinated human group O erythrocytes.

In contrast to the situation with acidic extracts, protein was not precipitated by two-fold dilution of alkaline salt extracts. When the latter were dialysed overnight against running tap water, a fraction with subunit pattern similar to that of the F-I fraction from the ascorbate-containing extract was precipitated.

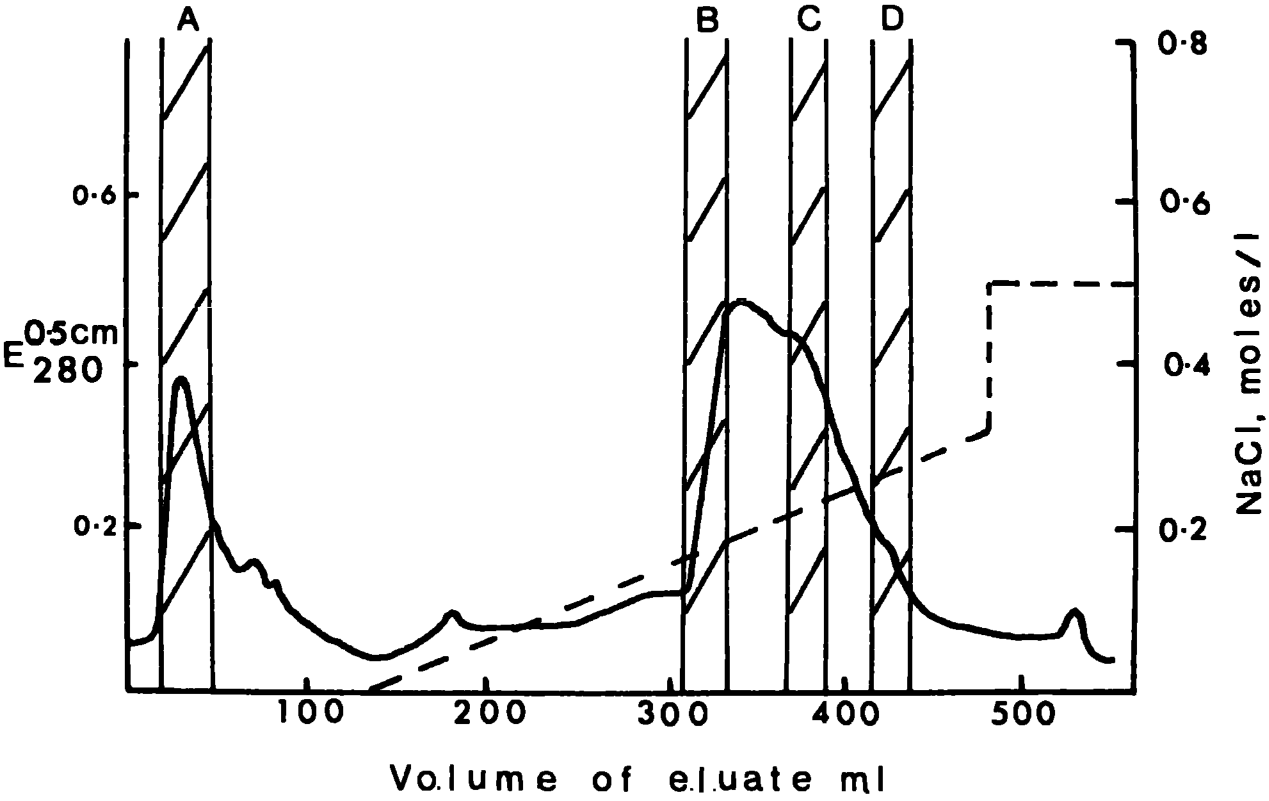
(iv) Fractionation of alkaline salt extracts by ion exchange chromatography.

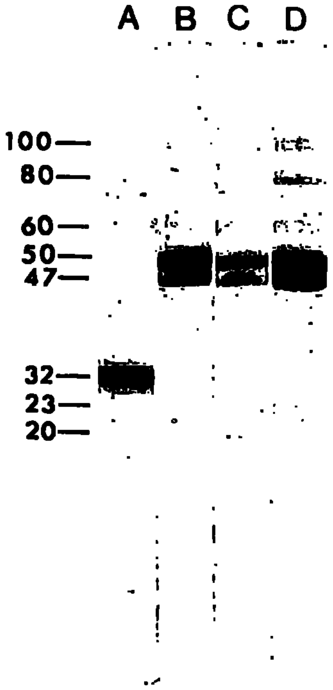
When alkaline salt extracts were chromatographed on DEAE-cellulose, the protein was separated into two fractions; the elution profile is shown in Fig. 12a, and experimental details are described in the legend to this figure. Non-adsorbed protein was eluted as a small peak with the starting buffer while adsorbed protein was subsequently eluted as a large peak with 0.17M sodium chloride. While the leading edge of this peak was sharp, a shoulder occurred on the trailing edge. Disc electrophoretic analysis of the non-adsorbed protein resulted in a weak diffuse band, Rm 0.27 - 0.39, together with a band migrating with the bromo-phenol blue marker, Rm 1.0. The leading edge of the main, adsorbed, peak was composed of a major component Rm 0.37 with a weak band Rm 0.21; the trailing edge contained a slow moving band, Rm 0.1, in addition to the components found in the leading edge fraction.

Fig. 12

Ion-exchange chromatography of protein extracted from meal by the alkaline salt extractant. The extract was made to 100% saturation with ammonium sulphate and the precipitate formed collected by centrifugation and dispersed in 0.025M sodium phosphate pH 7.5; after dialysis against 2 changes of 1,200 ml of buffer of the same composition, the slightly opaque solution was clarified by centrifugation, and about 150 mg was applied to a 2.5 x 15 cm column of Whatman DE52 cellulose, equilibrated in buffer of the same composition; preparation of the ion exchange cellulose was carried out as described in the Whatman Laboratory manual. The flow rate was approximately 1.5 ml/min. After initial elution with the starting buffer (130 ml), elution was continued with a linear sodium chloride gradient in buffer of the same composition. The continuous line indicates absorption at 280 nm, the dashed line the approximate sodium chloride concentration.

Samples of the hatched regions of the elution profile designated A, B, C and D were analysed by SDS gel electrophoresis in 7.0% (w/v) gels; the results are shown below. Numbers refer to the molecular weights of the bands indicated.


A
B
C
D
0·6
0·2
E 0·5cm 280
0·8
0·6
0·4
0·2
NaCl, moles/l
100
200
300
400
500
Volume of eluate ml


A
B
C
D
100
80
60
50
47
32
23
20

The results of SDS gel electrophoresis of samples from the hatched regions of the elution profile (Fig. 12a) are shown in Fig. 12b. The non-adsorbed fraction was mainly composed of a subunit of molecular weight 32,000. The leading edge of the adsorbed peak was composed mainly of subunits of molecular weight 50,000 and 47,000, although a trace of a 23,000 molecular weight subunit is apparent. The middle region of the peak contained subunits of molecular weight 60,000, 50,000, 47,000 and 20,000; the trailing edge contained, in addition to these subunits, subunits of molecular weight 80,000 and 23,000. Subunits of molecular weight 32,000 were not observed in this adsorbed peak.

Rechromatography of fractions from the leading edge of the adsorbed peak failed to completely remove the trace amount of the 23,000 molecular weight subunit present. When samples of the trailing edge of this peak were rechromatographed, fractions enriched in the 60,000 and 20,000 molecular weight subunits, relative to the 50,000 and 47,000 molecular weight subunits, were obtained, but the latter subunits were still the major components on SDS gels.

While adjustment of some of the experimental parameters of the chromatography resulted in differences in the shape of the main peak, no marked improvement in resolution was obtained. In attempting to increase resolution, longer columns (1 x 30 cm and 1 x 53 cm), lower pH (pH 7.2 and 6.8), a steeper sodium chloride elution gradient (0 - 1.0M sodium chloride in a total gradient volume of 300 ml), slower flow rate (approx 1.0ml/min for the 2.2 x 15 cm column), and applying a smaller amount of protein (approx 80 mg) were all tried without marked success.

(v) Sucrose density gradient centrifugation of proteins.

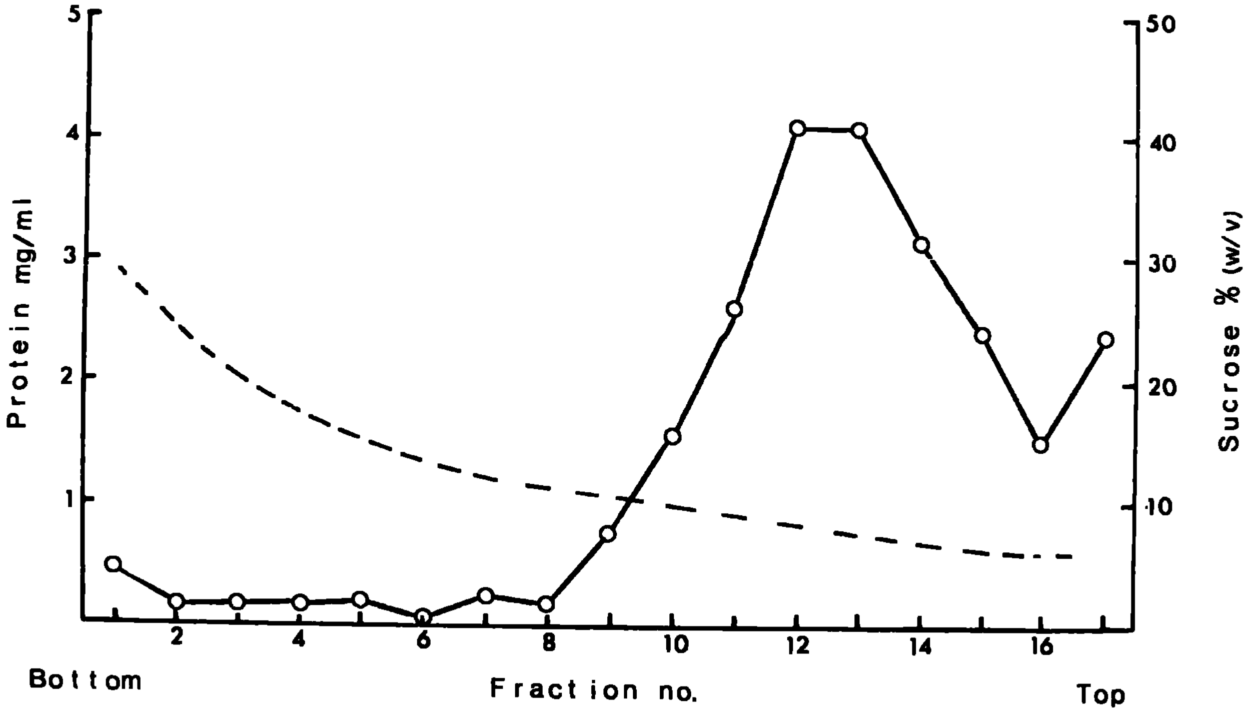
When samples of alkaline salt extracts were centrifuged in sucrose density gradients a single broad peak was obtained, although some protein remained at the top of the gradient. The protein distribution of the fractionated gradient is shown in Fig. 13 and the results of SDS gel electrophoresis of fractions from the gradient are shown below. It is apparent that (a) the pH 4.7 insoluble fraction subunits, molecular weight 60,000 and 20,000, are confined to the lower edge of the peak; (b) as judged by SDS gel electrophoresis, most of the 50,000, 47,000 and 32,000 molecular weight subunits are found in the broad peak, although some protein composed of these subunits remained at the top of the gradient; (c) while the protein which remained at the top of the gradient is enriched in subunits of 23,000 molecular weight, traces of subunits of this size are also seen in the SDS gels in which fractions from the main peak have been electrophoresed.

The results of similar centrifugation of a sample enriched in the pH 4.7 insoluble fraction are presented in Fig. 14. The preparation of the sample is described in the legend to this figure. The main peak sedimented further than that obtained from alkaline salt extracts, and SDS gel electrophoresis of fractions from this region of the gradient show that the 60,000 and 20,000 molecular weight subunits are most concentrated, as judged by SDS gel electrophoresis, in the lower part of the peak (fractions 7 and 9), while the 50,000 and 47,000 molecular weight subunits are only clearly defined higher in the gradient (fraction 11).

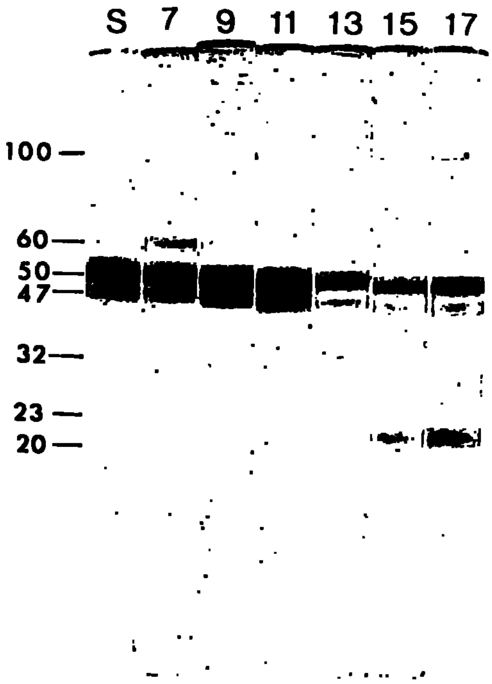
Fig. 13

Fractionation of an alkaline salt extract by sucrose density gradient centrifugation. After centrifugation, fractions of 1 ml were collected. Protein concentration (continuous line) was determined from the absorption (measured in 1 cm light path cuvettes) at 280 nm and 260 nm, using the equation described by Layne (1957); the dashed line indicates the sucrose concentration.

Underneath the fractionation profile of the gradient are presented the results of SDS gel electrophoretic analysis of samples of fractions from the gradient. The first gel (labelled 'S') is a sample of the extract that was not subjected to centrifugation; the remaining gels (7, 9, 11, 13, 15, 17) demonstrate the subunit composition of the protein recovered from each of these fractions. Gels were 7.0% (w/v) acrylamide; molecular weights ($\times 10^{-3}$) are also presented.



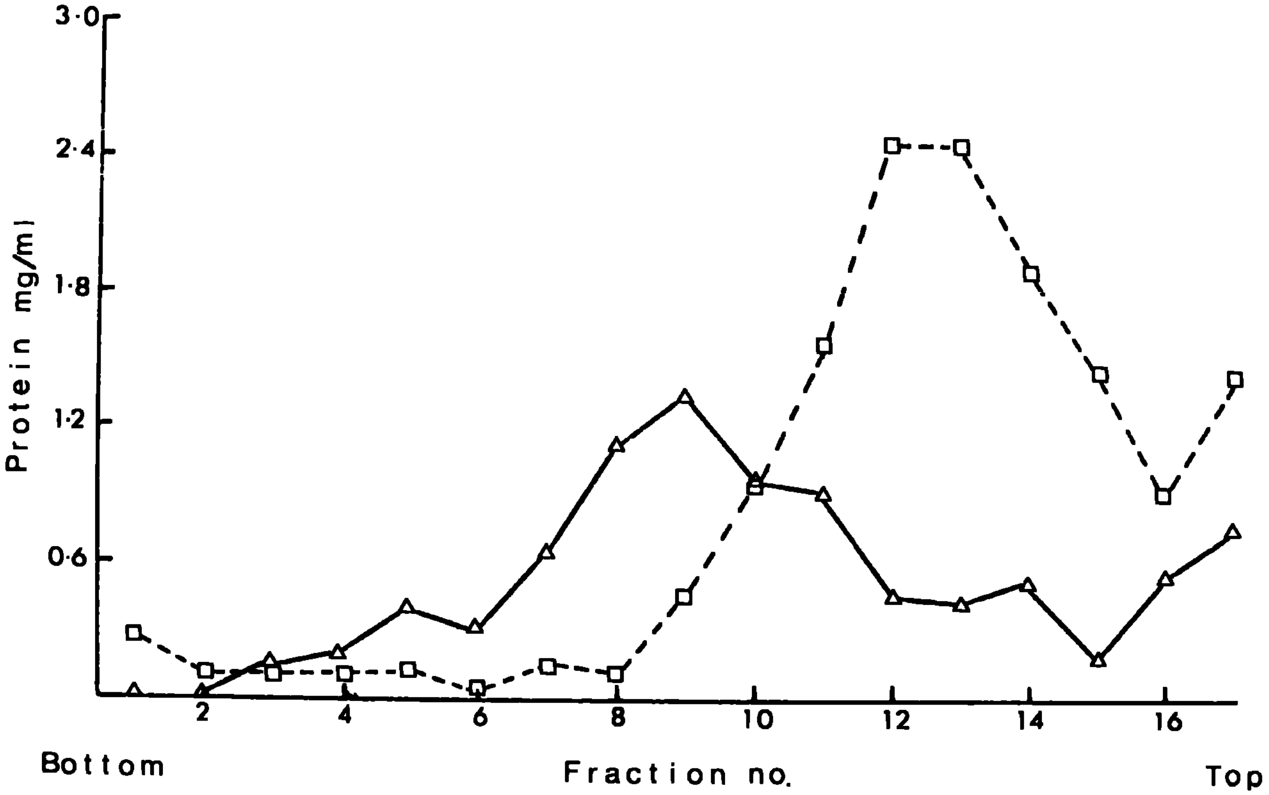
5
4
3
2
1
Protein mg/ml
50
40
30
20
10
Sucrose % (w/v)
2
4
6
8
10
12
14
16
Bottom
Fraction no.
Top

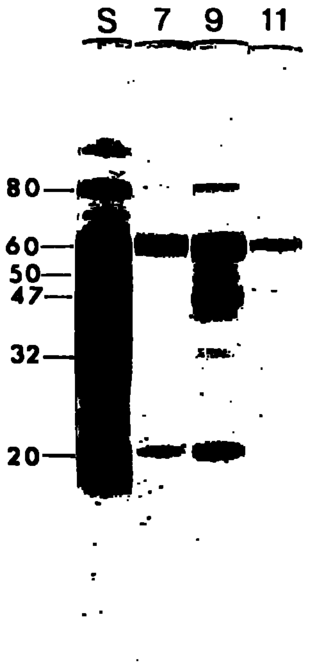


S
7
9
11
13
15
17
100
60
50
47
32
23
20

Fig. 14

Fractionation by centrifugation of an extract enriched in the pH 4.7 insoluble fraction protein. The supernatant fluid after the removal of the cryoprecipitate from a water extract was made 0.2M with respect to NaCl and 0.25% (v/v) with respect to 2-mercaptoethanol and acidified to pH 4.7 with dilute HCl. The resulting precipitate was collected by centrifugation, resuspended in 0.5M NaCl, 0.05M sodium phosphate, pH 7.5, and dialysed against buffer of the same composition before centrifugation in a sucrose gradient. The solid line shows the protein concentration after centrifugation, determined as described for the fractionation of the alkaline salt extract, shown in Fig. 13; for comparison, the dotted line shows the profile from the alkaline salt extract. The sucrose gradient was similar to that shown in Fig. 13. The SDS gels shown underneath are of the sample prior to centrifugation (S) and samples of the protein recovered from fractions 7, 9 and 11 respectively.


3·0
2·4
1·8
1·2
0·6
Protein mg/ml
2
4
6
8
10
12
14
16
Bottom
Fraction no.
Top


S
7
9
11
80
60
50
47
32
20

(vi) Summary of the fractionation of the main storage proteins.

From the results presented, four fractions can be described: (a) insoluble at pH 4.7, and consisting of three subunits, molecular weights 80,000, 60,000 and 20,000; (b) soluble at pH 4.7 and composed of two subunits, molecular weights 50,000 and 47,000, although traces of a 23,000 molecular weight have not been completely removed; (c) soluble at pH 4.7 and composed of subunits of molecular weight 32,000; and (d) a protein with subunits of 23,000 molecular weight, which was also soluble at pH 4.7.

B. Characterisation of Protein Fractions.

(i) Correspondence between non-dissociated (disc) and dissociated (SDS) gel electrophoretic patterns.

The main component (Rm 0.35 - 0.4) after separation by disc gel electrophoresis of alkaline salt extracts, the pH 4.7 soluble fraction and the cryoprotein from a water extract was cut out, extracted with SDS and analysed by SDS gel electrophoresis. Fig. 15 presents densitometer traces of SDS gels of the pH 4.7 soluble fraction main band (Fig. 15a) and the cryoprotein (from a water extract) main band (Fig. 15b); the main band from an alkaline salt extract was similar in subunit composition to that from the pH 4.7 soluble fraction. In each case they were mainly composed of subunits of molecular weight 50,000 and 47,000; the 23,000 molecular weight subunit was present in all three samples but the cryoprotein main band appeared to completely lack subunits of 32,000 molecular weight.

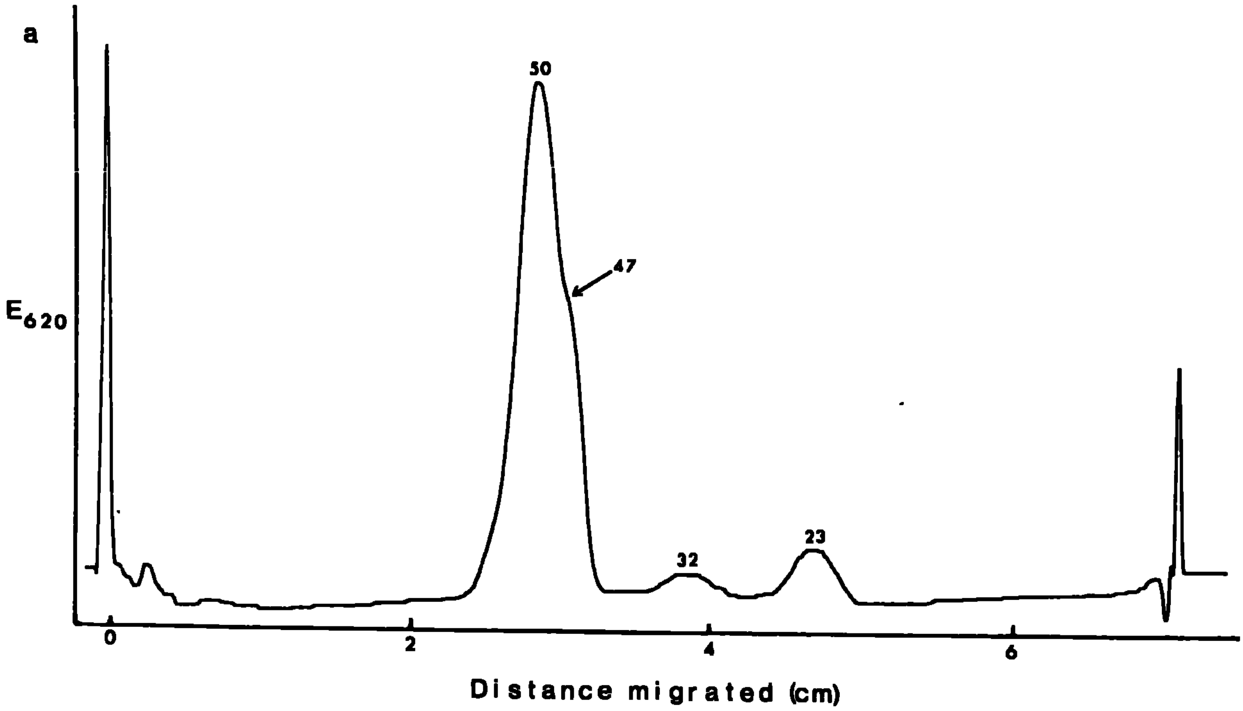
When the subsidiary band present in the pH 4.7 soluble fraction samples after disc electrophoresis (Rm 0.19) was

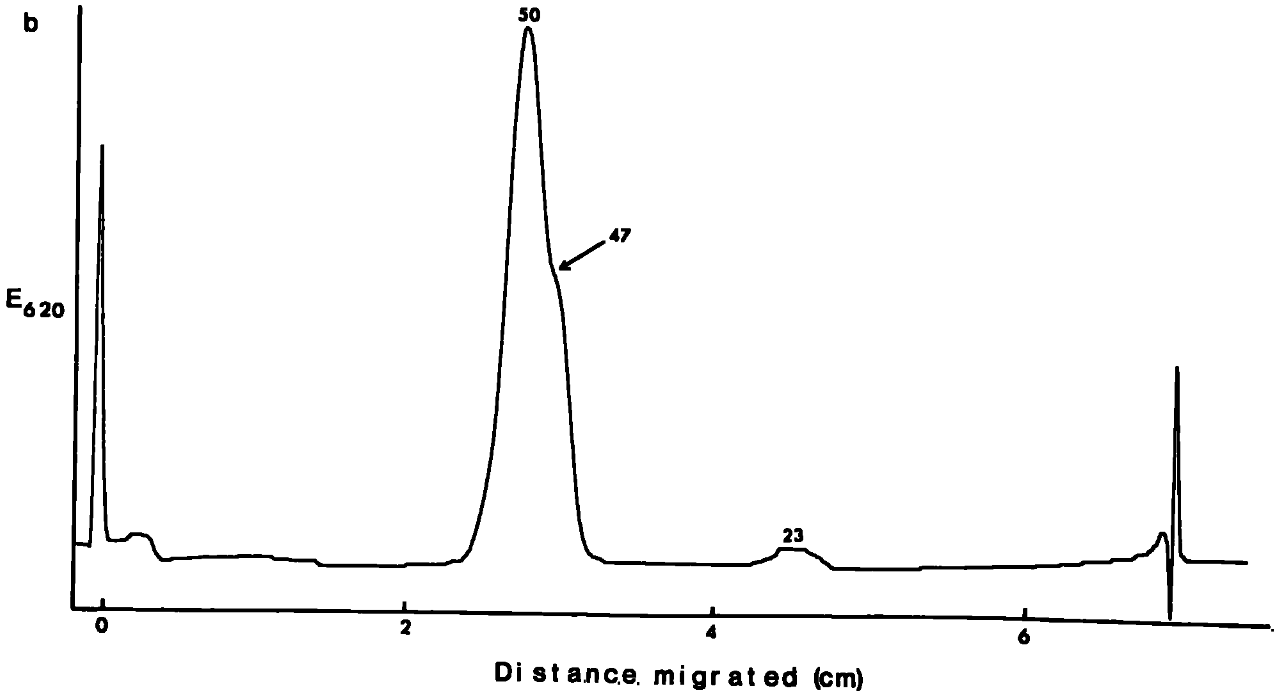
Fig. 15

SDS gel electrophoresis, in 7.0% (w/v) acrylamide gels, of the main band cut from gels of the disc electrophoretic analysis of (a) the pH 4.7 soluble fraction.

(b) the cryoprotein derived from a water extract.

Molecular weights (x 10^{-3}) are indicated as previously.


a
E620
50
47
32
23
0
2
4
6
Distance migrated (cm)


b
E620
50
47
23
0
2
4
6
Distance migrated (cm)

similarly cut out and analysed, subunits of 50,000, 47,000 and 32,000 molecular weight were all found.

(ii) Effect of boiling, 2-mercaptoethanol and carboxymethylation on subunit patterns.

Incubation of samples in 1% SDS, 0.2% 2-mercaptoethanol at 37°C for several hours resulted in low yields of the 50,000 and 47,000 molecular weight subunit, while a slow-moving band, molecular weight about 95,000, was prominent. After heating in a water bath for 30 secs, nearly complete loss of this slow moving component occurred, with the concomitant appearance of the 50,000 and 47,000 molecular weight subunits. This effect is shown in Fig. 16. The molecular weight of this slow-moving component was highly dependent on acrylamide concentration; when analysed in gels over the acrylamide concentration range 5.0% (w/v) - 12.5% (w/v), the apparent molecular weight was altered from 120,000 in 5% (w/v) gels to a wide band of approximately 90,000 molecular weight in 12.5% (w/v) gels. However, these estimates of molecular weights are obtained by extrapolation of the calibration graphs, and should therefore be viewed with caution.

In the absence of 2-mercaptoethanol, the pH 4.7 insoluble fraction (prepared by zonal isoelectric precipitation)formed mainly a single band of about 80,000 molecular weight; treatment with 0.2% 2-mercaptoethanol resulted in breakdown of this band to form bands of molecular weight 60,000 and 20,000. The pH 4.7 soluble fraction gave similar banding patterns in the presence or absence of 2-mercaptoethanol. Carboxymethylation did not affect the mobility or proportions of the subunits of either of these fractions.

Fig. 16

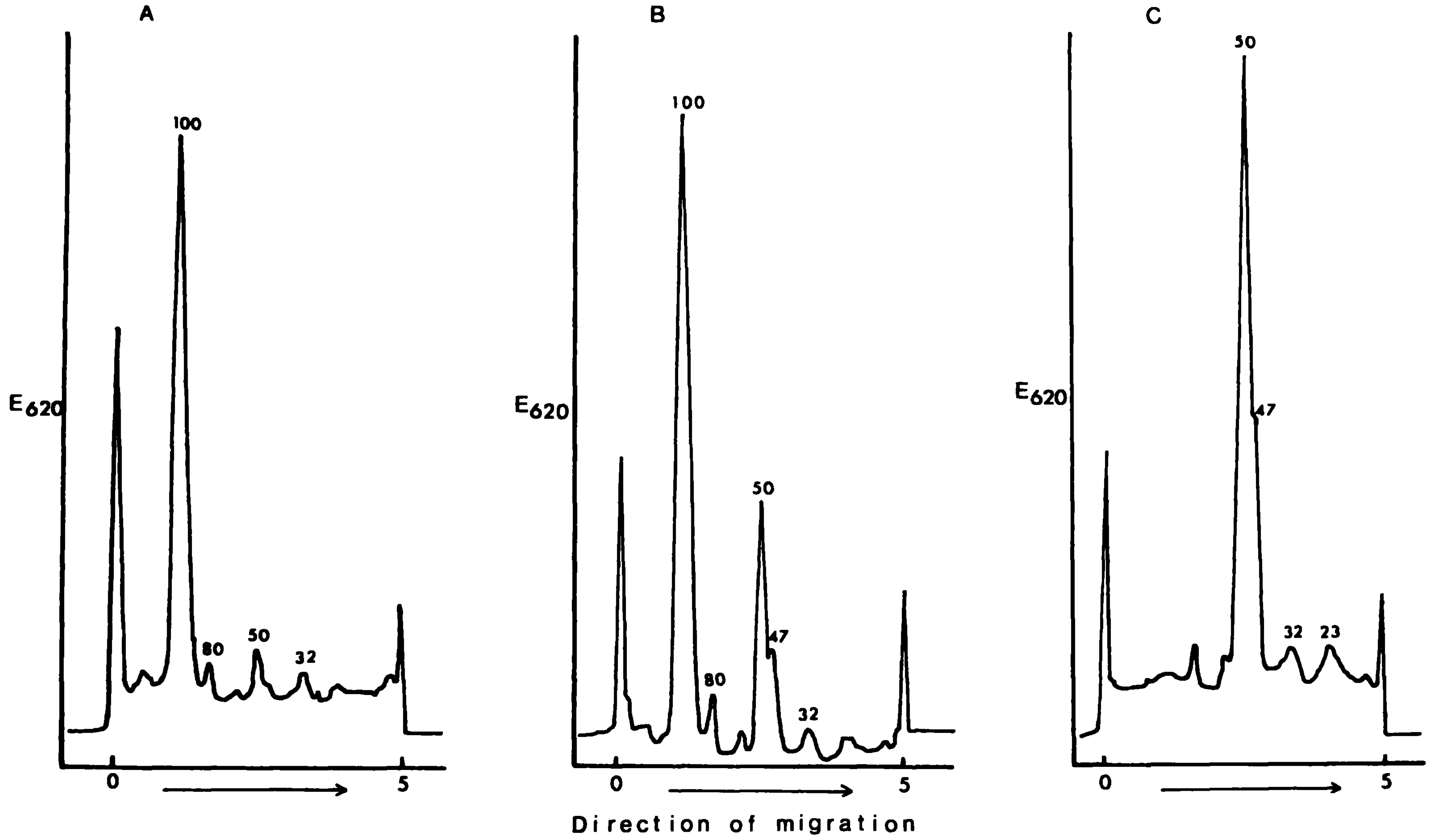
The effect of heating at 100°C on the subunit pattern of a cryoprotein preparation from a water extract. Samples were incubated in 0.01M sodium phosphate buffer pH 7.0, 1.0 % (w/v) SDS, 0.2% (v/v) 2-mercaptoethanol, for 2 h at 37°C.

Gel A : the sample was not treated further.

Gel B : the sample was placed in a water bath at 100°C for 20 sec.

Gel C : the sample was placed in a water bath at 100°C for 1 min.

Gels were 7.0% (w/v) acrylamide; in this case they were cut at the bromo-phenol blue marker band prior to staining; the top and bottom of each gel is indicated and numbers on peaks refer to their molecular weights (x 10^{-3}).


A
B
C
E620
100
80
50
32
47
23
0
5
Direction of migration

(iii) Molecular weight estimation of subunits with CTAB.

The molecular weights of subunits were estimated in a cationic detergent system at pH 5.7. The calibration graph for this system is shown in Fig. 17. Electrophoresis of the pH 4.7 soluble fraction in this system resulted in a similar band pattern and apparent molecular weights to those obtained with the SDS system; these are presented with Fig. 17. However, CTAB appeared to be less effective than SDS as a dissociating agent, in that components presumed to be dimers of myoglobin and ovalbumin, as well as several bands of low mobility in the γ-globulin samples, were always present.

(iv) Isoelectric focusing in 7M urea.

The result of gel isoelectric focusing in 7M urea, over the pH range 3 - 6, of the cryoprotein derived from a water extract is shown in Fig. 18. At least eighteen bands were present over the pH range 5.0 - 5.75; the subunit profile of this sample, when analysed by SDS gel electrophoresis, was shown in Fig. 9a. Similarly complex patterns were also obtained from the pH 4.7 soluble fraction on isoelectric focusing in 7M urea gels.

(v) Glycoprotein staining.

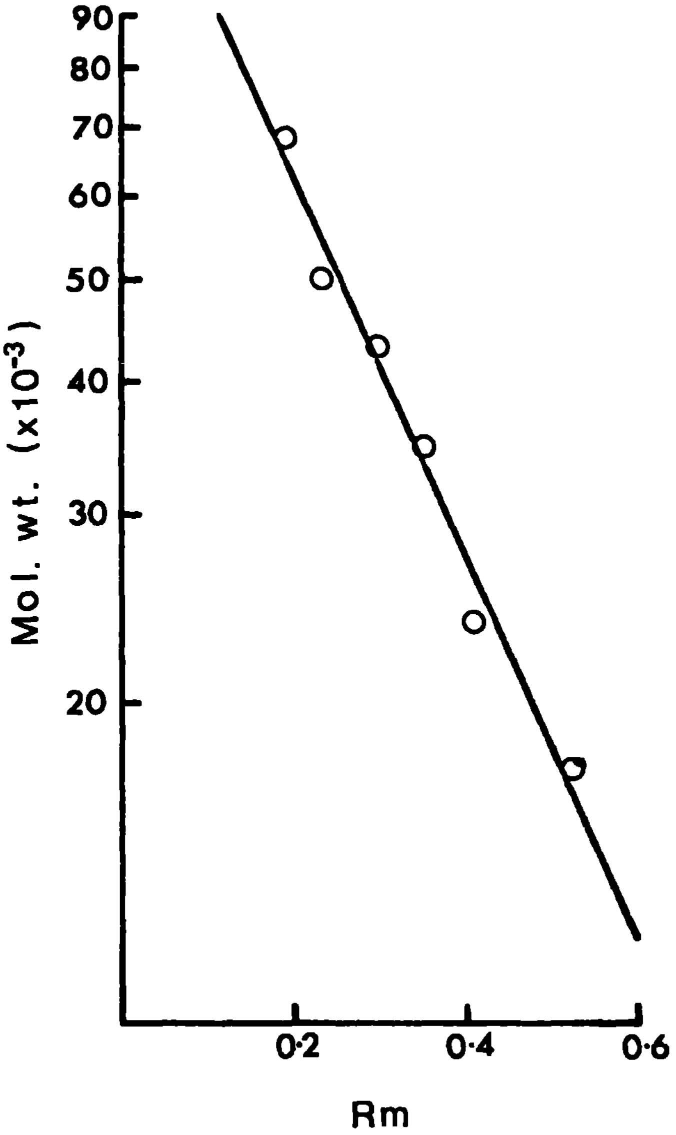
When SDS gels were stained for the presence of glycoprotein, subunits of molecular weight 50,000 and 32,000 took up stain (Fig. 19); the 32,000 molecular weight subunit stained relatively strongly. A subunit of 36,000 molecular weight, which was only weakly apparent after protein staining, was also stained. When gels of SDS extracts of *P. vulgaris* cv. 'Canadian Wonder' were stained for the presence of glycoproteins, the 53,000, 49,000 and 32,000 molecular weight subunits were stained (Fig. 20).

Fig. 17

The calibration graph for the CTAB gel system (7.0% (w/v) gels). Proteins used, together with their molecular weights, were :-

bovine serum albumin	68,000
γ-globulin, H-chain	50,000
ovalbumin	43,000
carboxypeptidase A	34,600
γ-globulin, L-chain	23,500
myoglobin	17,200

The molecular weights obtained by electrophoresis of a pH 4.7 soluble fraction in both CTAB and SDS gel systems are also shown; the 60,000 molecular weight subunit, which is the major component of the pH 4.7 insoluble fraction, was visible in this preparation.



Gel system	Mol. wt. (x10^{-3})					
CTAB	approx. 100	58	49	46	31	23·5
SDS	90–100	60	50	47	32	23

Fig. 18

A densitometric trace of the band pattern resulting from isoelectric focusing of the cryoprotein, derived from a water extract, in acrylamide gels (5.0% w/v) containing 7M urea. The gel was stained with amido black; the continuous line represents absorbance at 620 nm, while the pH gradient, determined on water extracts of slices of an unstained gel, is shown by the dashed line.

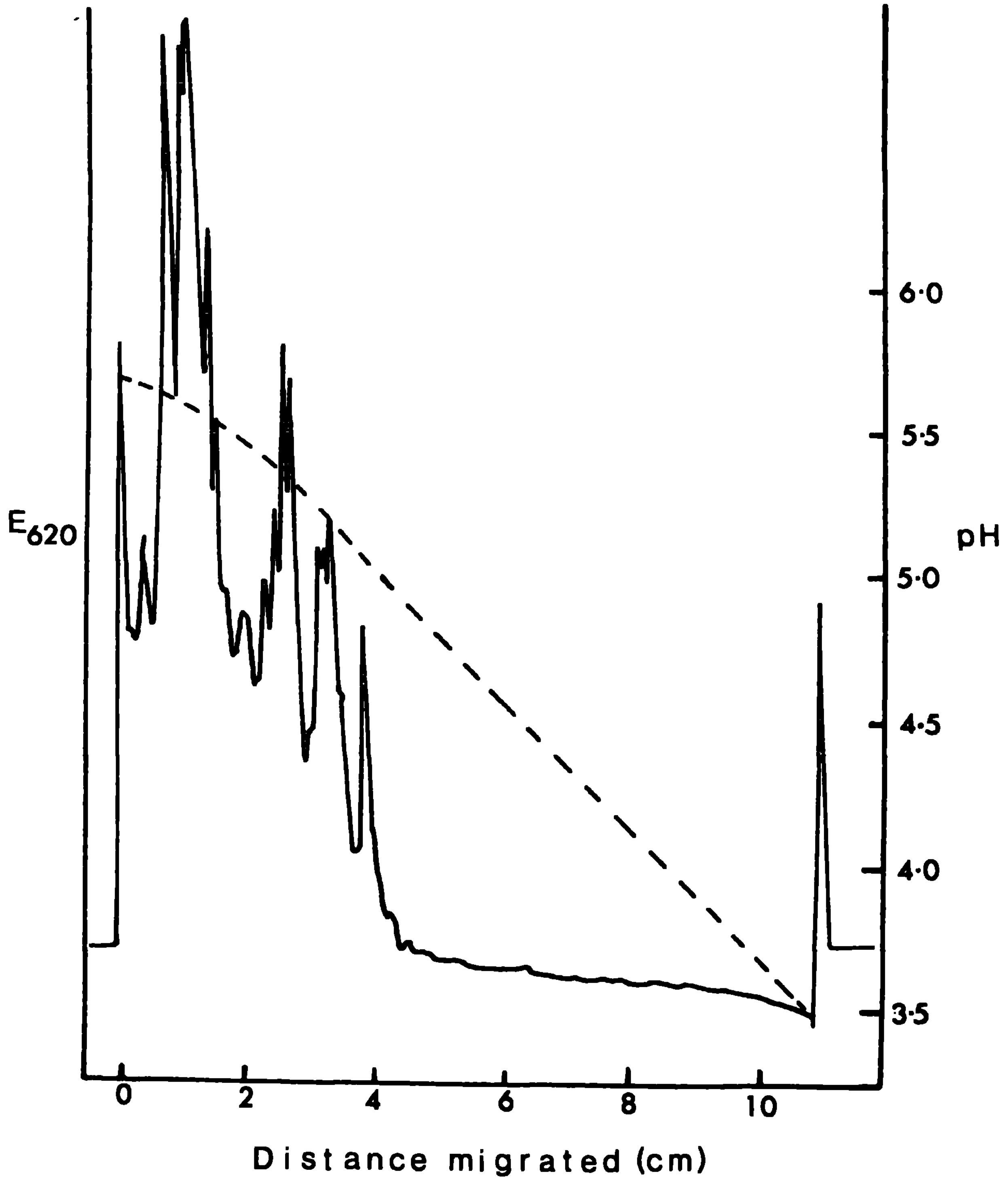

E620
pH
6·0
5·5
5·0
4·5
4·0
3·5
0
2
4
6
8
10
Distance migrated (cm)

Fig. 19

Comparison of the band pattern of an alkaline salt extract of meal of *Phaseolus vulgaris* L. cv 'Seafarer' analysed by SDS gel electrophoresis and stained for protein and glycoprotein. The continuous line represents absorption at 620 nm of a gel stained for protein with amido black; the dashed line is the pattern resulting from staining a similar gel for the presence of glycoprotein, using the procedure of Zacharius *et al.* (1969). In this case, absorbance was measured at 490 nm. 7.0% (w/v) acrylamide gels were used, and molecular weights (x 10^{-3}) are presented as previously.

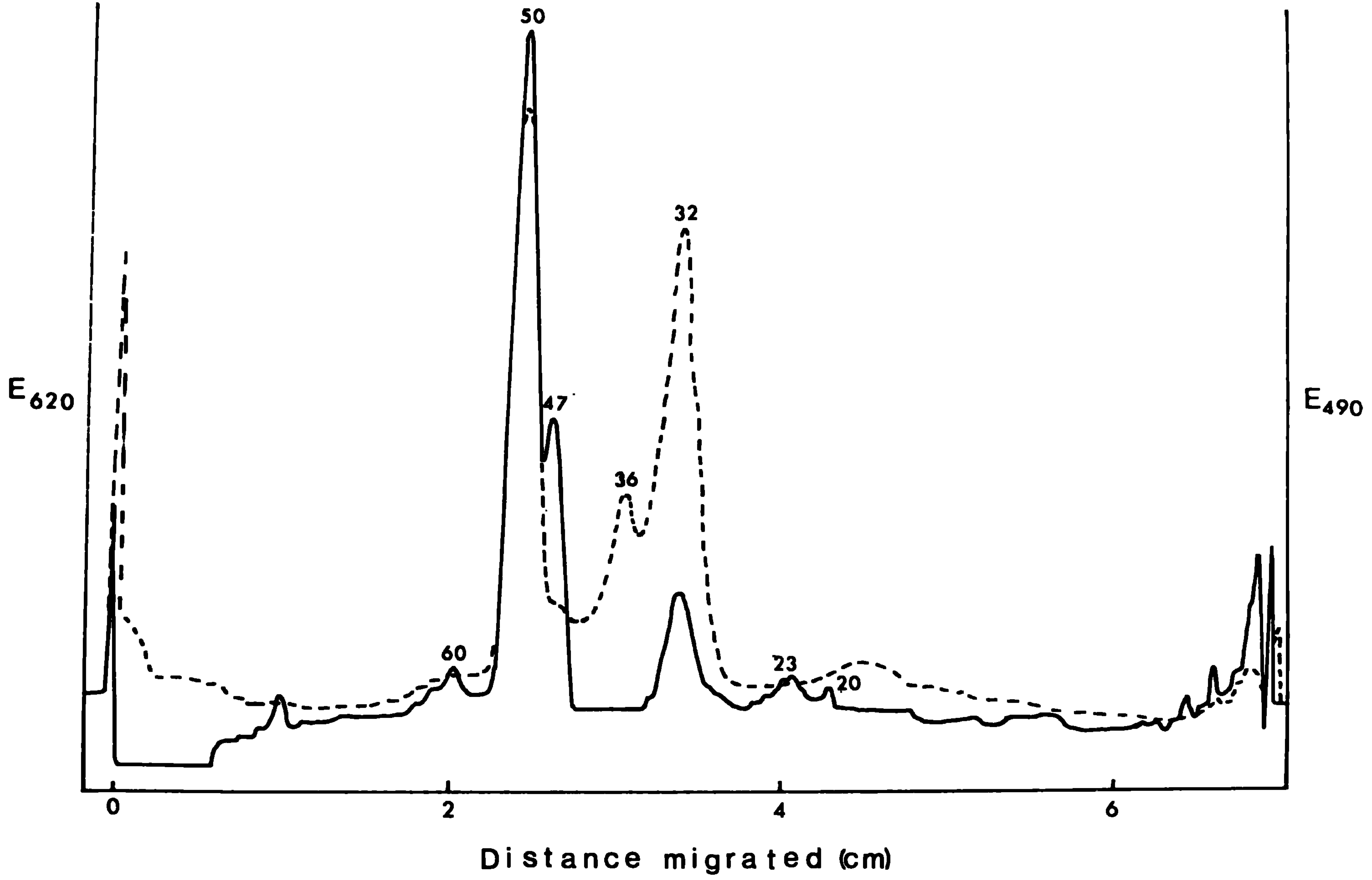
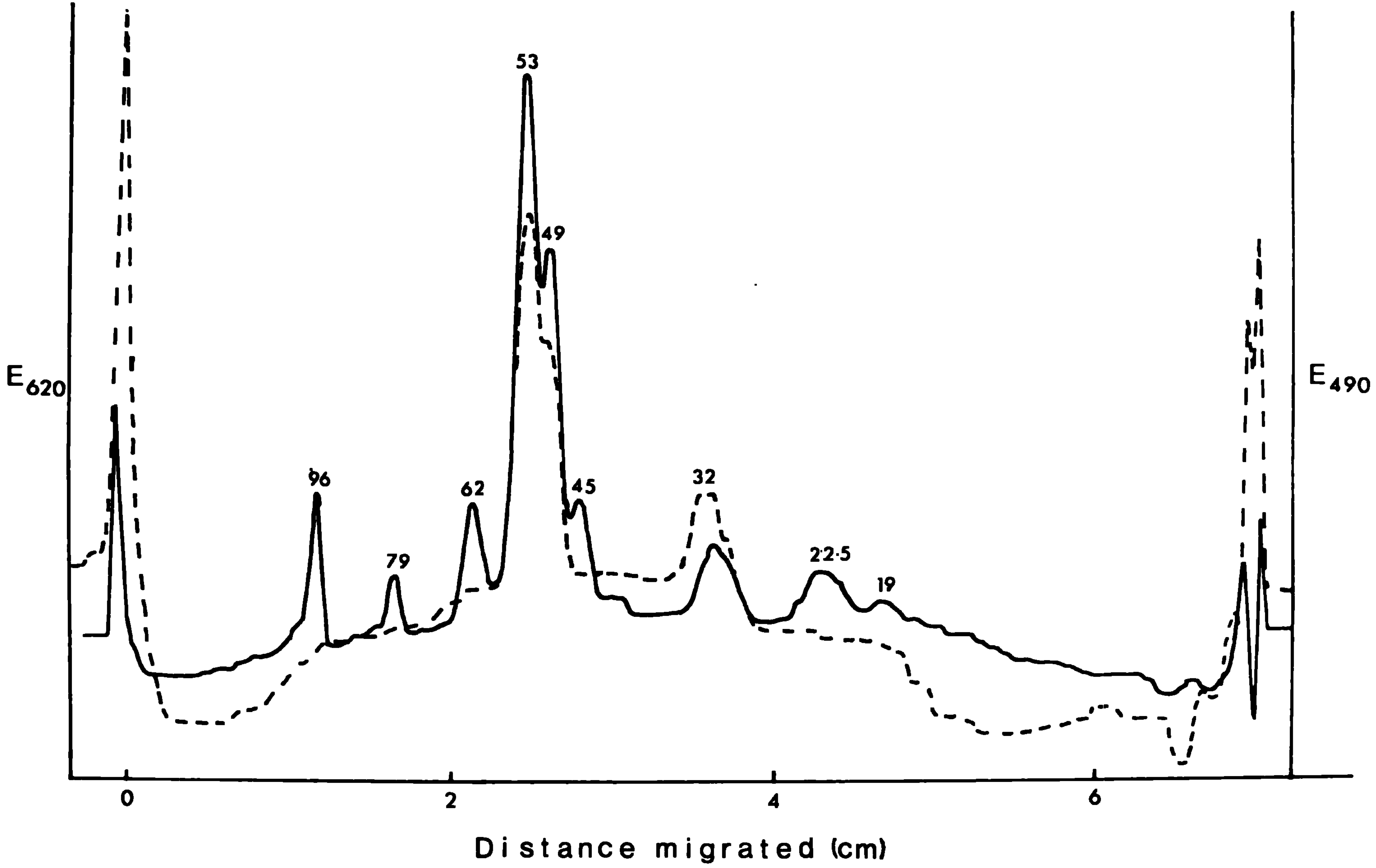

50
32
47
36
60
23
20
E_{620}
E_{490}
0
2
4
6
Distance migrated (cm)

Fig. 20

Comparison of the band pattern of an SDS extract of meal of *Phaseolus vulgaris* L. cv 'Canadian Wonder' electrophoresed in 7.0% gels and stained for protein (continuous line, E_{620}) with that of a similar gel stained for glycoprotein (dashed line, E_{490}). Molecular weights (x 10^{-3}) are indicated.


53
49
96
62
45
32
79
2.2·5
19
E620
E490
0
2
4
6
Distance migrated (cm)

(vi) Amino acid compositions and N-terminal amino acid residues.

The amino acid compositions of the pH 4.7 insoluble fraction, prepared by zonal isoelectric precipitation (60,000 and 20,000 molecular weight subunits), the non-adsorbed fraction from ion-exchange chromatography (32,000 molecular weight subunit), and the fraction comprising the leading edge of the adsorbed peak eluted during ion-exchange chromatography (50,000 and 47,000 molecular weight subunits) were determined. The results of the duplicate hydrolyses at 24 h and 72 h of these fractions are presented in Tables 2, 4 and 6 respectively, while the amino acid compositions, calculated from these results and expressed as moles per 10^5g protein and as moles %, are shown in Tables 3, 5 and 7 respectively. N-terminal amino acid residues are also presented.

For the analysis of the two fractions prepared by ion-exchange chromatography, the four required samples of each fraction (for the duplicates of both 24 h and 72 h hydrolysis) were individually weighed directly into hydrolysis tubes. Due to shortage of material, the pH 4.7 insoluble fraction was first dissolved in 6N HCl and aliquots of this solution were then transferred to the hydrolysis tubes. It is possible that the low overall recoveries for this fraction and the higher variation in recovery between duplicate hydrolyses may have been caused by incomplete solubilisation of the sample prior to transfer to the hydrolysis tubes; some of the protein may also have become adsorbed to the glass walls of the vial used to dissolve the sample.

When the amino acid compositions of the three fractions are compared the most obvious difference is in the ratio of glutamic

TABLE 2

Amino acid analyses of the pH 4.7 insoluble fraction, prepared by zonal isoelectric precipitation

Amino acid	Recovery g amino acid/100 g protein	
	24 h hydrolysis	72 h hydrolysis
Aspartic acid	5.26 ± 0.78	4.91 ± 0.32
Threonine	1.49 ± 0.11	1.43 ± 0.12
Serine	2.12 ± 0.35	2.17 ± 0.09
Glutamic acid	8.59 ± 0.68	8.84 ± 0.55
Proline	2.28 ± 0.16	2.41 ± 0.25
Glycine	1.72 ± 0.06	1.83 ± 0.07
Alanine	1.40 ± 0.07	1.47 ± 0.09
Cysteine	N.D.	N.D.
Valine	2.27 ± 0.14	2.71 ± 0.23
Methionine	0.37	0.45 ± 0.08
Isoleucine	1.55 ± 0.06	1.87 ± 0.20
Leucine	3.31 ± 0.17	3.60 ± 0.37
Tyrosine	1.40	1.69 ± 0.23
Phenylalanine	1.56 ± 0.04	1.66 ± 0.16
Histidine	1.96 ± 0.25	1.88 ± 0.13
Lysine	3.83 ± 0.20	4.09 ± 0.25
Arginine	2.79 ± 0.09	2.90 ± 0.22
	41.90	43.91

N.D. Not determined.

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

TABLE 3

The amino acid composition of the pH 4.7 insoluble fraction

Amino acid	Amino acid content		
	g amino acid /100g protein	moles amino acid /10^5g protein	mole %
Aspartic acid	5.08	44.2	11.9
Threonine [a]	1.52	15.0	4.1
Serine	2.14	24.6	6.6
Glutamic acid	8.71	67.5	18.3
Proline	2.34	24.1	6.5
Glycine	1.77	31.1	4.7
Alanine	1.44	20.2	5.5
Cysteine	N.D.	N.D.	N.D.
Valine	2.49	25.2	6.9
Methionine	0.41	3.1	0.8
Isoleucine	1.71	15.1	4.1
Leucine	3.45	30.5	8.2
Tyrosine	1.54	9.5	2.6
Phenylalanine	1.61	10.9	2.9
Histidine	1.92	14.0	3.7
Lysine	3.96	30.9	8.3
Arginine	2.85	18.2	4.9
	42.94	384.1	100.0

N-terminal amino acids : Glycine, Threonine.

a Extrapolated to zero time.

N.D. Not determined.

TABLE 4

Amino acid analyses of the non-adsorbed fraction from DEAE-ion-exchange chromatography (32,000 molecular weight subunits)

Amino acid	Recovery g amino acid/100g protein	
	24 h hydrolysis	72 h hydrolysis
Aspartic acid	11.12 ± 0.00	11.97 ± 0.06
Threonine	5.14 ± 0.11	5.78 ± 0.26
Serine	4.81 ± 0.04	5.05 ± 0.10
Glutamic acid	5.67 ± 0.05	6.63 ± 0.10
Proline	2.76 ± 0.06	3.13 ± 0.03
Glycine	2.79 ± 0.03	3.21 ± 0.02
Alanine	3.15 ± 0.05	3.59 ± 0.05
Cysteine [a]	0.95 ± 0.05	N.D.
Valine	4.36 ± 0.10	5.03 ± 0.00
Methionine	0.33 ± 0.00	0.31 ± 0.03
Isoleucine	3.61 ± 0.08	4.33 ± 0.06
Leucine	7.05 ± 0.00	8.44 ± 0.30
Tyrosine	2.52 ± 0.03	3.07 ± 0.16
Phenylalanine	4.98 ± 0.05	5.68 ± 0.04
Histidine	1.12 ± 0.01	1.31 ± 0.02
Lysine	4.15 ± 0.06	4.81 ± 0.09
Arginine	2.84 ± 0.02	3.25 ± 0.01
	66.46	75.59

[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 5

The amino acid composition of the non-adsorbed fraction from DEAE-ion exchange chromatography

Amino acid	Amino acid content		
	g amino acid /100g protein	moles amino acid /10^5g protein	mole %
Aspartic acid	11.97	104.0	14.7
Threonine	5.78	57.2	8.1
Serine	5.05	58.0	8.2
Glutamic acid	6.63	51.4	7.3
Proline	3.13	32.2	4.6
Glycine	3.21	56.2	8.0
Alanine	3.59	50.5	7.1
Cysteine	0.95	5.6	0.8
Valine	5.03	50.8	7.2
Methionine	0.31	2.4	0.3
Isoleucine	4.33	38.3	5.4
Leucine	8.44	74.6	10.6
Tyrosine	3.07	18.8	2.7
Phenylalanine	5.68	38.6	5.5
Histidine	1.31	9.5	1.3
Lysine	4.81	37.5	5.3
Arginine	3.25	20.8	2.9
	76.54	706.4	100.0

TABLE 6

Amino acid analyses of the fraction comprising the leading edge of the main peak eluted during DEAE-ion exchange chromatography (50,000 and 47,000 molecular weight subunits)

Amino acid	Recovery g amino acid/100 g protein	
	24 h hydrolysis	72 h hyrdolysis
Aspartic acid	9.58 ± 0.08	11.03 ± 0.24
Threonine	2.15 ± 0.02	2.47 ± 0.17
Serine	3.64 ± 0.04	3.98 ± 0.06
Glutamic acid	11.60 ± 0.04	13.49 ± 0.22
Proline	2.03 ± 0.04	2.26 ± 0.00
Glycine	1.94 ± 0.01	2.26 ± 0.07
Alanine	2.05 ± 0.01	2.38 ± 0.12
Cysteine [a]	0.43 ± 0.02	N.D.
Valine	3.03 ± 0.06	3.79 ± 0.06
Methionine	0.65 ± 0.01	0.68 ± 0.00
Isoleucine	2.98 ± 0.05	3.90 ± 0.12
Leucine	5.87 ± 0.01	6.90 ± 0.18
Tyrosine	2.34 ± 0.00	2.72 ± 0.06
Phenylalanine	4.36 ± 0.01	5.11 ± 0.15
Histidine	1.88 ± 0.03	2.22 ± 0.05
Lysine	4.61 ± 0.06	5.27 ± 0.08
Arginine	4.10 ± 0.02	4.80 ± 0.26
	63.24	73.26

[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 7

The amino acid composition of the 50,000 and 47,000 molecular weight subunits, obtained as the leading edge of the main peak eluted during DEAE-ion exchange chromatography

Amino acid	Amino acid content		
	g amino acid /100g protein	moles amino acid /10^5g protein	mole %
Aspartic acid	11.03	95.8	14.8
Threonine	2.47	24.4	3.8
Serine	3.98	45.7	7.1
Glutamic acid	13.49	104.5	16.1
Proline	2.26	23.3	3.6
Glycine	2.26	39.6	6.1
Alanine	2.38	33.5	5.2
Cysteine	0.43	2.6	0.4
Valine	3.79	38.2	5.9
Methionine	0.68	5.2	0.8
Isoleucine	3.90	34.5	5.3
Leucine	6.90	61.0	9.4
Tyrosine	2.72	16.7	2.6
Phenylalanine	5.11	34.7	5.4
Histidine	2.22	16.2	2.5
Lysine	5.27	41.1	6.3
Arginine	4.80	30.7	4.7
	73.69	647.7	100.0

N-terminal amino acids : Threonine, Serine, Leucine, together with a trace of Glutamic acid.

acid to aspartic acid. The pH 4.7 insoluble fraction contained a high proportion of glutamic acid and aspartic acid (18.3 and 11.9 mole %, respectively; total 30.2 mole %); the ratio (mole/mole) of these amino acids was 1.54. The fraction composed of 50,000 and 47,000 molecular weight subunits also contained similarly large amounts of these amino acids (16.1 mole % glutamic acid, 14.8 mole % aspartic acid; total 30.9 mole %) but in this case the mole/mole ratio was 1.09. The non-adsorbed fraction from ion-exchange chromatography contained less of these amino acids (7.3 mole % glutamic acid, 14.7 mole % aspartic acid, total 22.0 mole %) and their mole/mole ratio was completely different (0.50). All three protein fractions contained relatively low amounts of the sulphur amino acids, cysteine(cystine) and methionine.

The N-terminal amino acid residues found for the pH 4.7 insoluble fraction were glycine and threonine; those obtained for the fraction composed of the 50,000 and 47,000 molecular weight subunits were threonine, serine and leucine, together with a trace of glutamic acid.

C. Localisation of Storage Proteins.

(i) Protein composition of protein bodies.

(a) Preparation of protein bodies on sucrose density gradients. After centrifugation in linear 50 - 90% (w/v) sucrose gradients, as described in Methods (Section IV A), protein bodies were obtained as an opaque band centred at approximately 80% (w/v) sucrose. Fig. 21 shows the optical density trace (at 280 nm) for the fractionation of a typical preparation from cotyledons imbibed for 16 h. Fig. 22 presents densitometric traces of the results

Fig. 21

The fractionation profile of a 50-90% (w/v) sucrose density gradient in which an extract from cotyledons soaked for 16 h had been centrifuged. Absorbance was monitored at 280 nm. The dashed line indicates sucrose concentration (% (w/v)). When referred to in the text, the sample region corresponds to the top 2-3 ml of the gradient; the opaque region referred to below the sample region, is found 10-12 ml from the bottom of the gradient.

PB	protein bodies

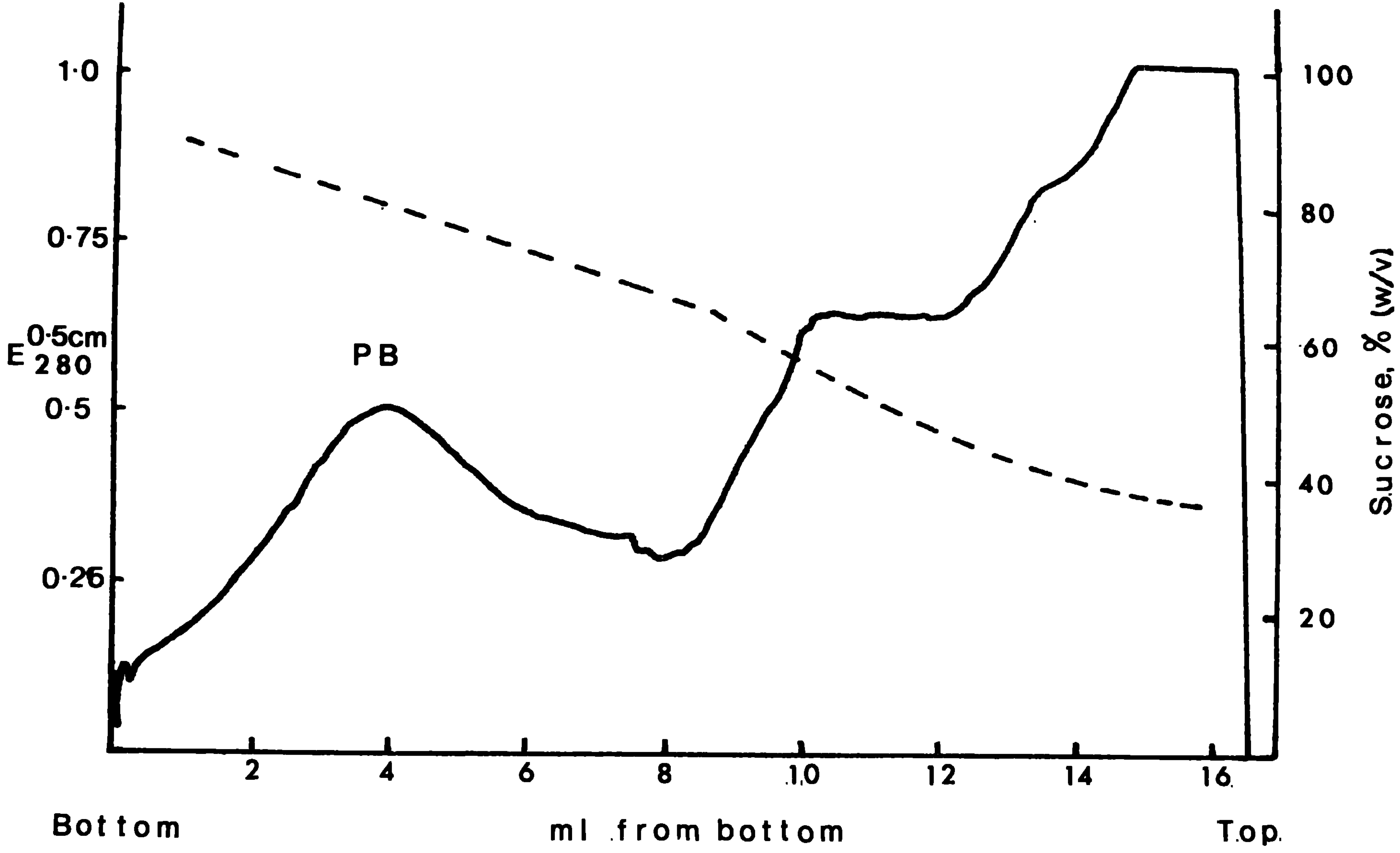
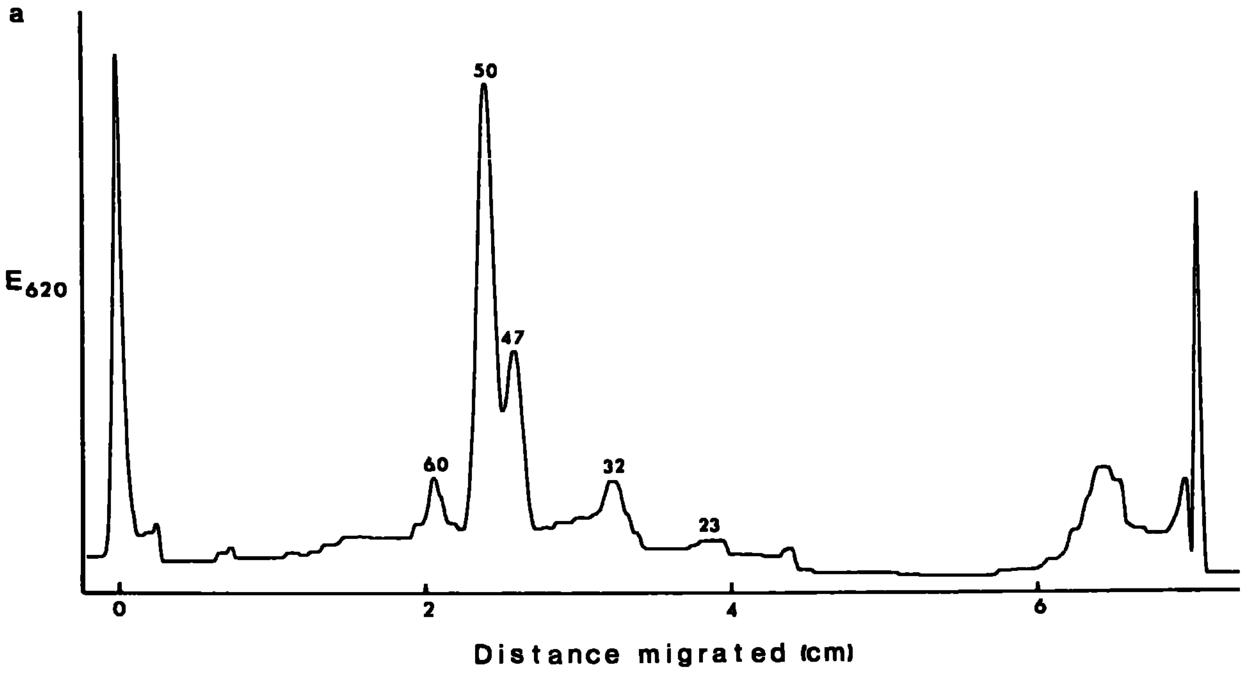

1·0
0·75
$E^{0·5cm}_{280}$
0·5
0·26
PB
100
80
60
40
20
Sucrose, % (w/v)
2
4
6
8
10
12
14
16
Bottom
ml from bottom
Top.

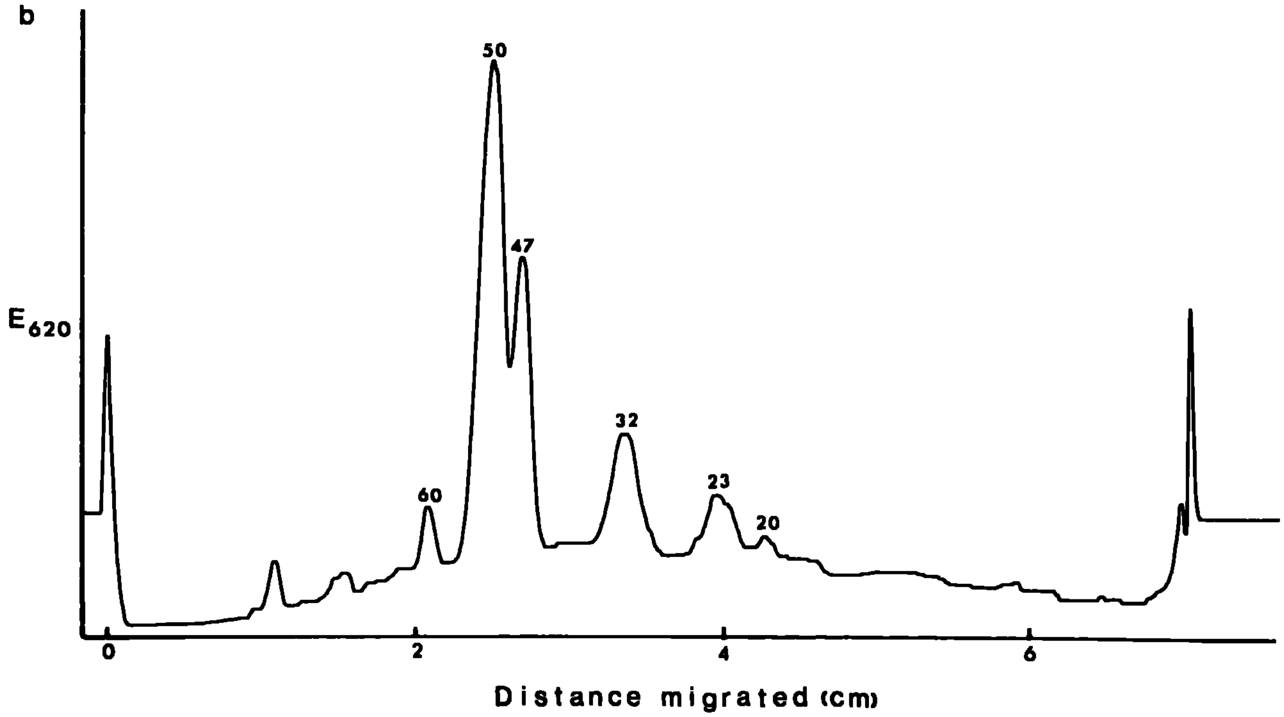
Fig. 22

SDS gel electrophoretic analysis of samples from a 50-90% (w/v) sucrose density gradient in which an extract from cotyledons soaked for 16 h had been centrifuged. 7.0% gels were used; numbers above peaks refer to the molecular weights (x 10^{-3}) of the associated peak.

(a) Protein bodies

(b) The sample region


a
E620
50
47
60
32
23
0
2
4
6
Distance migrated (cm)


b
E620
50
47
32
60
23
20
0
2
4
6
Distance migrated (cm)

of SDS gel electrophoresis of samples of the protein body region and the sample layer region at the top of the gradient. All the main protein subunits were found in the protein body fraction, and human group O erythrocytes were agglutinated by protein body preparations. Beneath the sample layer, which was usually slightly yellow, there was an opaque white layer; when samples of this region were analysed by SDS gel electrophoresis, all the main subunits were found to be present but, as judged by the intensity of staining, relatively small amounts of protein were present.

Although the protein body peak was usually symmetrical with respect to absorption at 280 nm, shoulders of apparently more or less dense particles were occasionally observed. However, no differences in the subunit pattern were detected when these regions were analysed by SDS-gel electrophoresis. Only about 10% of the protein applied to the gradients was recovered in the protein body fraction. All the subunits present in the protein body fraction were also found at the top of the gradients.

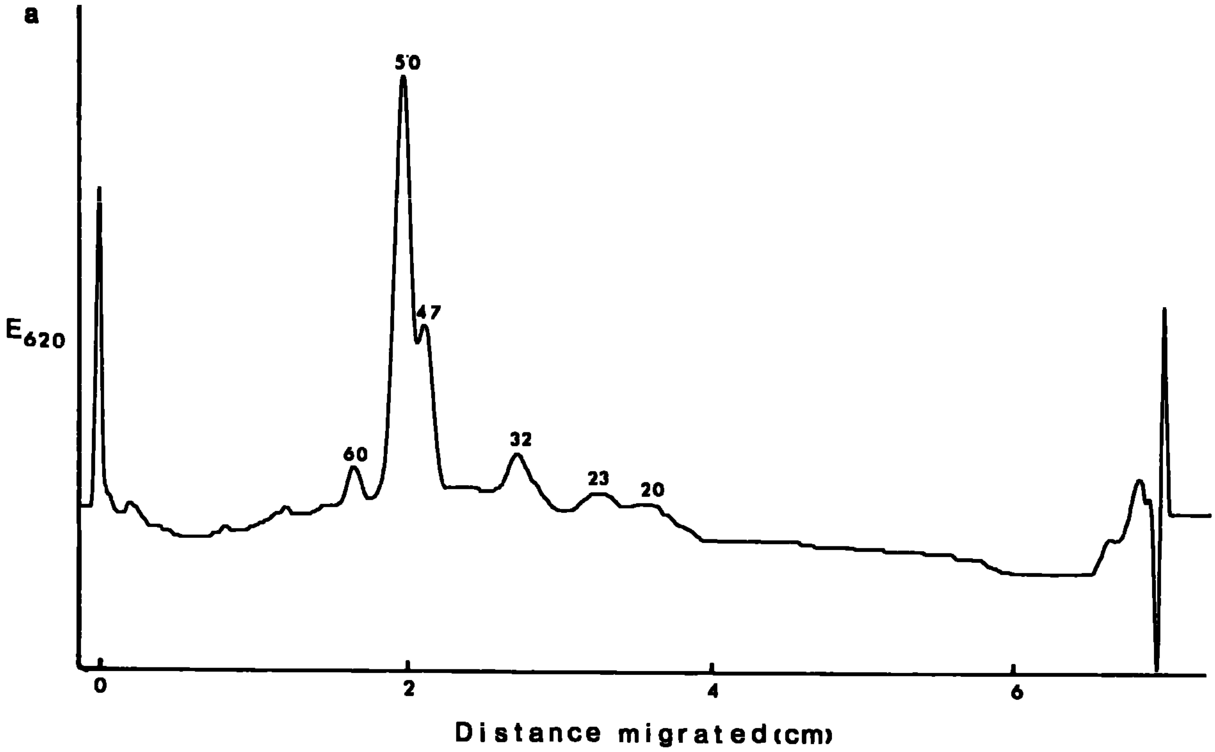
(b) Preparation of protein bodies in glycerol solutions. Isolation of protein bodies with glycerol at pH 5.0 gave a protein body fraction which accounted for approximately 40% of the total protein extracted from meal. The subunit composition of this protein body preparation and the supernatant fluid is shown in Fig. 23. While the subunit composition of the protein bodies resembled that of those isolated on sucrose gradients, none of the pH 4.7 insoluble fraction subunits were found in the supernatant. The results of preparing protein bodies in glycerol at pH 7.5 are shown in Fig. 24; although at this pH some of the pH 4.7 insoluble fraction subunits were

Fig. 23

SDS gel patterns of protein body and supernatant fluid fractions from the preparation of protein bodies using glycerol solutions at pH 5.0. The gels were 7.0% (w/v) acrylamide and molecular weights are indicated as previously.

(a) Protein body fraction.

(b) Supernatant fluid.


a
E620
50
47
60
32
23
20
0
2
4
6
Distance migrated (cm)

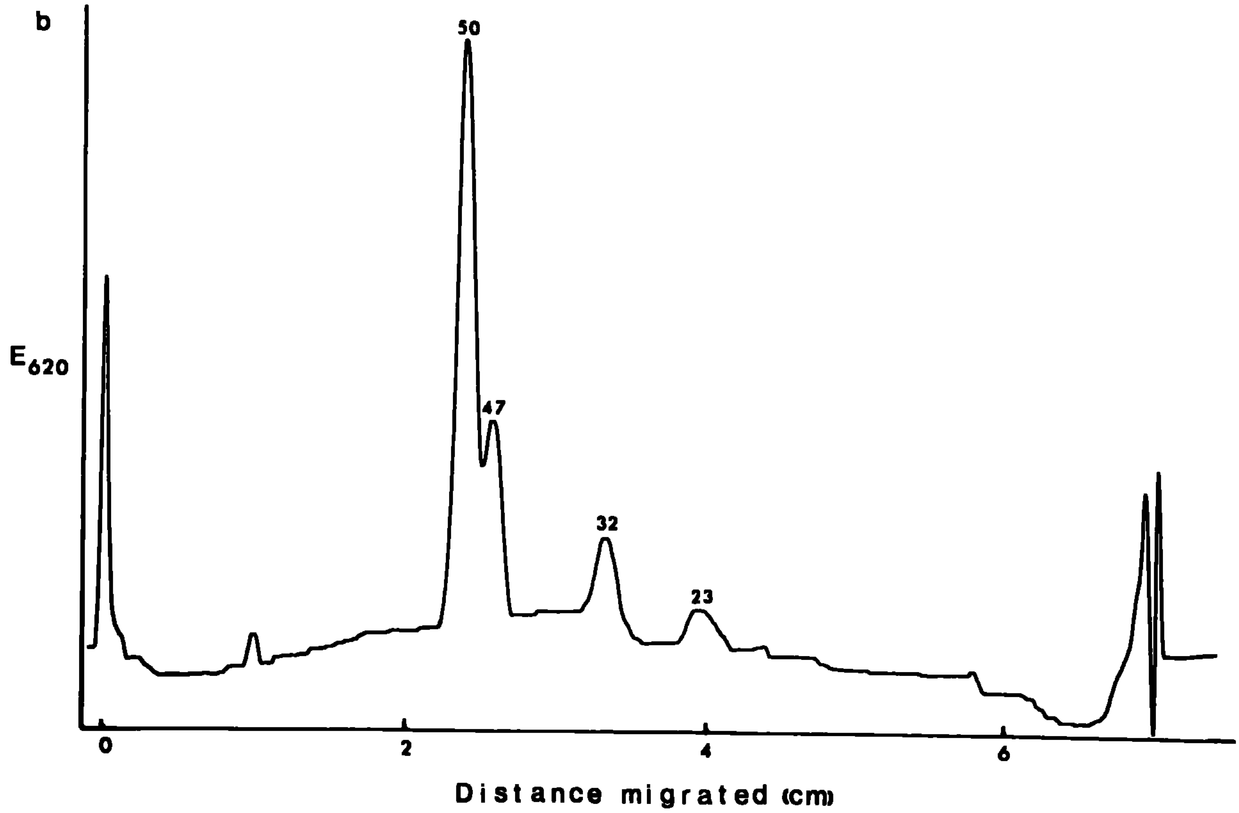
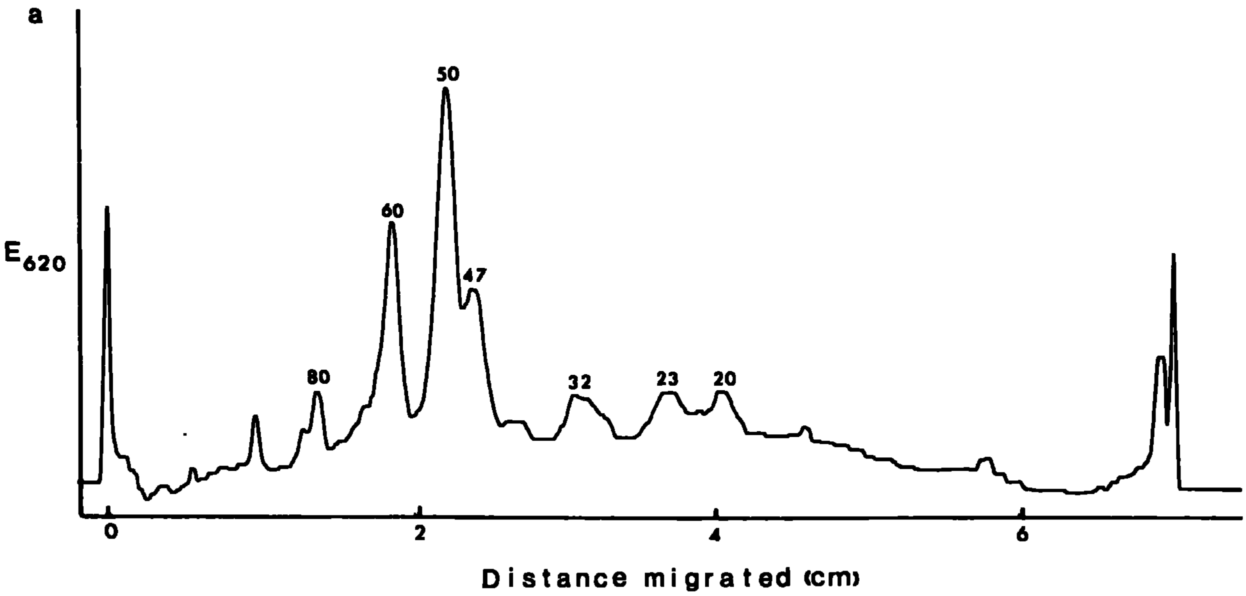

b
E620
50
47
32
23
0
2
4
6
Distance migrated (cm)

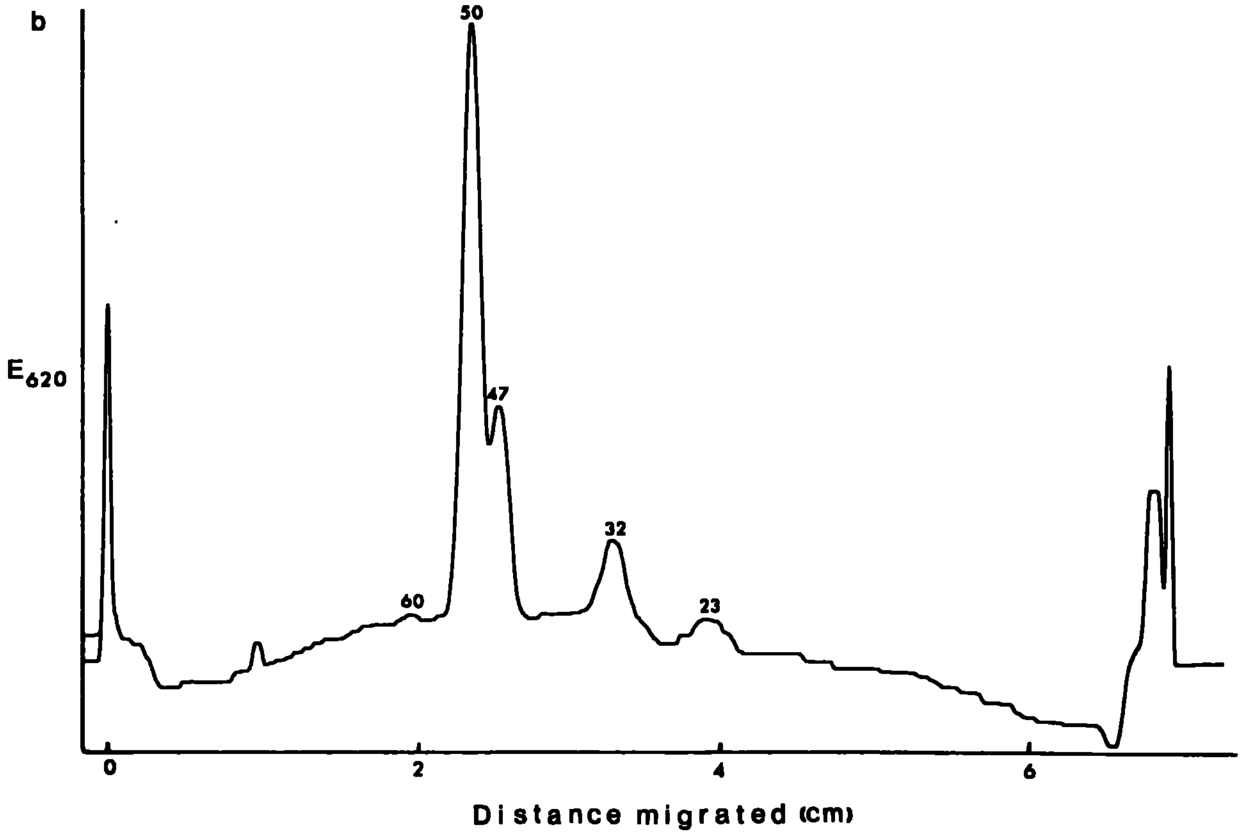
Fig. 24

SDS gel patterns of protein body and supernatant fluid fractions prepared using glycerol solutions at pH 7.5. Molecular weights are indicated as before (7.0% gels).

(a) Protein body fraction.

(b) Supernatant fluid.


a
E$_{620}$
50
60
47
80
32
23
20
0
2
4
6
Distance migrated (cm)


b
50
E$_{620}$
47
32
60
23
0
2
4
6
Distance migrated (cm)

found in the supernatant, the yield of protein bodies was lower (about 25% of the protein present in the pH 5.0 protein body fraction) and the protein body fraction contained an even higher proportion of the pH 4.7 insoluble fraction subunits relative to the total.

Fig. 25a is an electron micrograph of protein bodies prepared by sucrose gradient centrifugation. The protein-bodies are membrane-bound, although the membranes of several have been ruptured and leakage of their contents is apparent. In some protein bodies, distinct and often sharp protuberances (arrowed) are found. Protein bodies prepared by the glycerol method at pH 5.0 were more intact (Fig. 25b) and their membranes were more clearly defined. However, small fragments of cell wall were present and protein bodies were frequently present in clusters, surrounded by cytoplasmic material.

(ii) Protein distribution within the cotyledon.

Alkaline salt extracts of meal prepared from the abaxial regions of the seed contained 20% more protein than similar extracts from meal from the central part of the cotyledon; the extracts from the abaxial regions also contained 60% more trypsin inhibitor activity (per g meal) than those from the centre of the cotyledon.

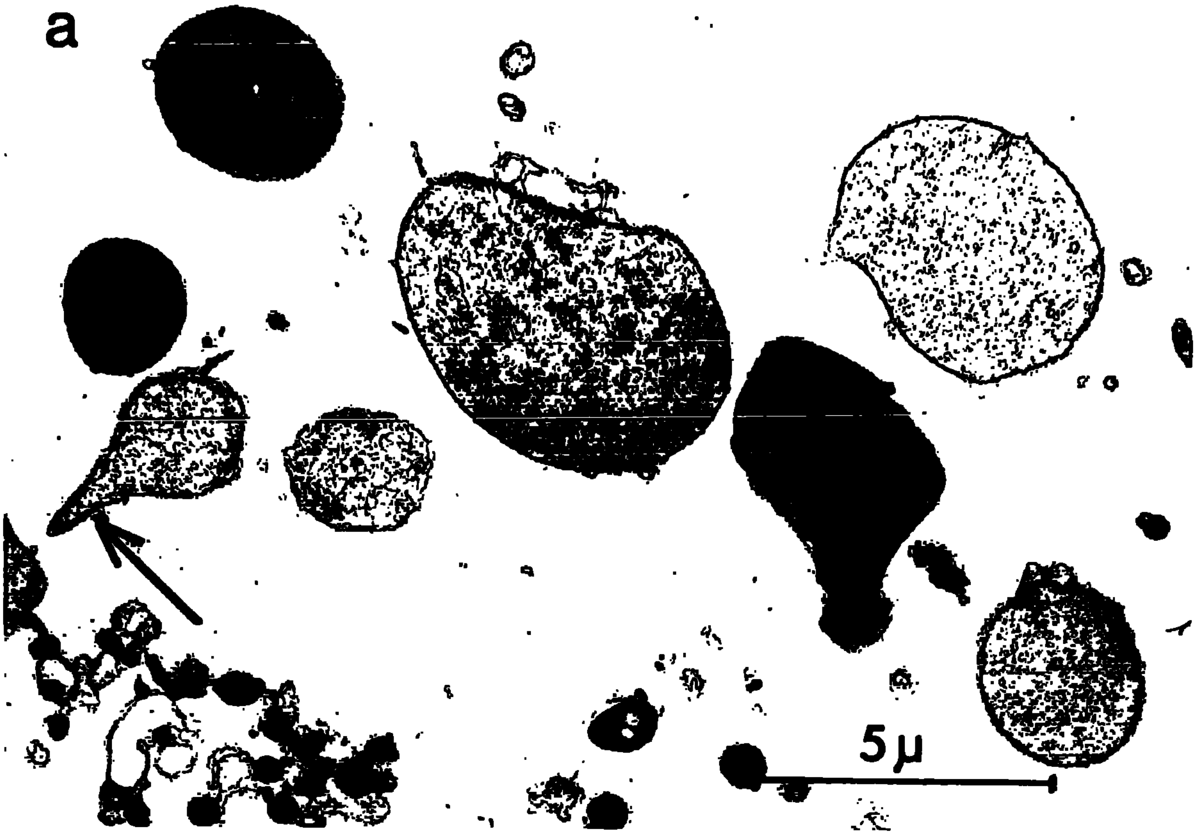
SDS extraction and gel electrophoretic analysis of meals from different regions of the seed showed that the proportions of the different subunits varied. Densitometer traces of SDS gels of extracts of the abaxial and central parts of the cotyledon are compared in Fig. 26. The abaxial region is enriched relative to the central part in 60,000 and 20,000 molecular weight subunits, and is impoverished in those of molecular weight 32,000 and 23,000;

Fig. 25

Electron micrographs of protein body preparations.

Fig. 25 a Prepared by sucrose density gradient centrifugation on 50-90% (w/v) sucrose gradients from cotyledons soaked for 16 h. The arrow indicates one of the protuberances noted in the text. The bar in the lower right corner represents 5 µm.

Fig. 25 b Prepared by the glycerol procedure at pH 5.0. The bar in the lower right corner represents 4 µm.


a
5µ

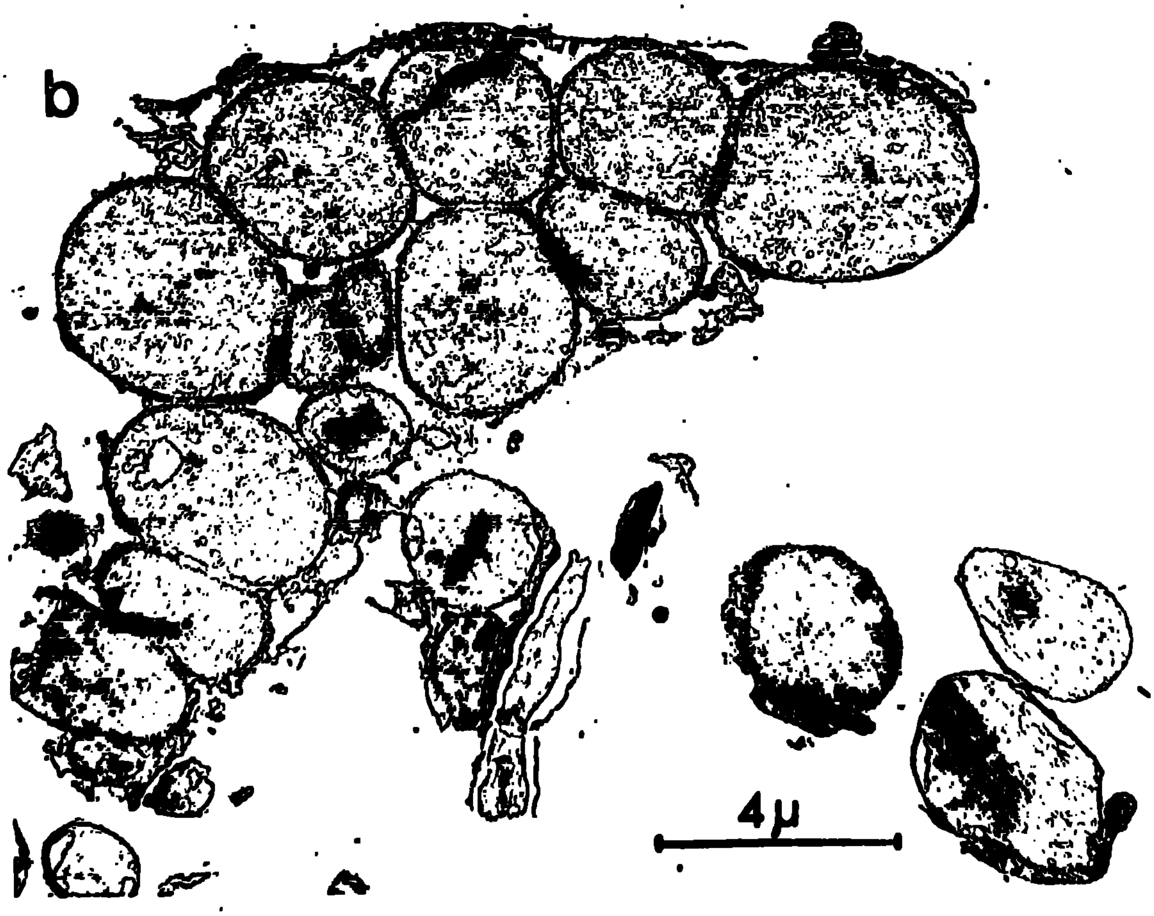
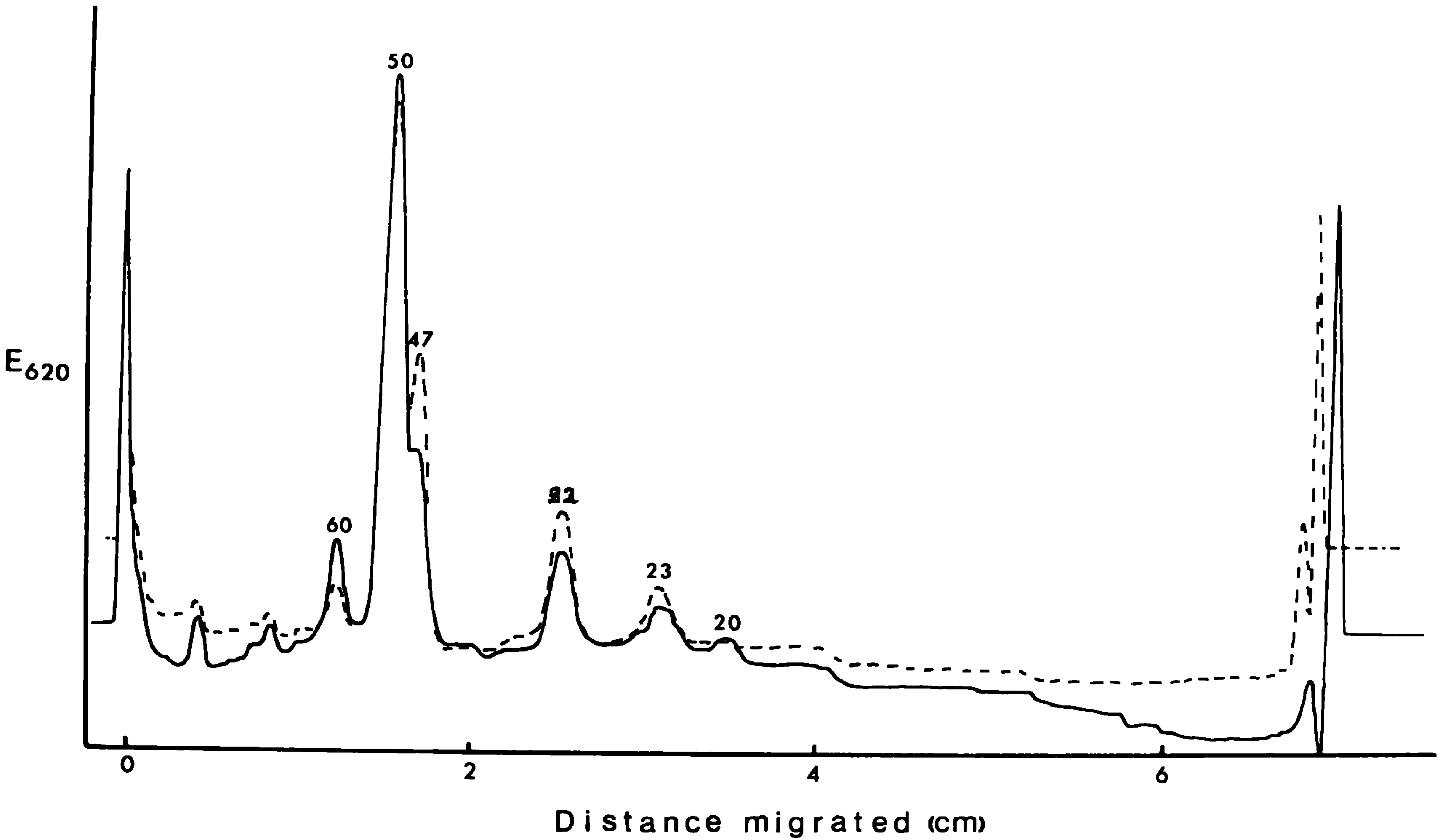

b
4µ

Fig. 26

SDS extraction and electrophoresis (in 7.0% gels) of meal prepared from different regions of the cotyledons of *Phaseolus vulgaris* L. cv 'Seafarer'. The dashed line is the densitometric trace of a gel in which an extract from meal prepared from the central region of the cotyledon had been electrophoresed; the continuous line is the trace of a gel in which an extract of meal derived from the abaxial surface of the cotyledon had been electrophoresed.

i.e. abaxial region extract ————————————

central region extract - - - - - - - - - - - - - - -


50
47
60
32
23
20
E620
0
2
4
6
Distance migrated (cm)

it also contains less 47,000 molecular weight subunit relative to the amount of the 50,000 molecular weight subunit. In contrast, the adaxial region contains relatively large amounts of 32,000 and 23,000 molecular weight subunits when compared with either the central region or with the subunit pattern derived from control (complete) seed meal (Fig. 27).

Protein bodies were isolated from the abaxial surface region using the glycerol procedure at pH 5.0; the subunit pattern is compared with that of protein bodies of the whole seed, isolated in the same way, in Fig. 28. As with the direct analysis of SDS extracts, the abaxial region contains less of the 47,000 molecular weight subunit than the remainder of the seed.

Fig. 27

SDS extraction and electrophoresis of meal prepared from the adaxial region, compared with meal similarly prepared and analysed from whole cotyledons. (7% gels).

whole cotyledon extract ————————

adaxial region extract -------------

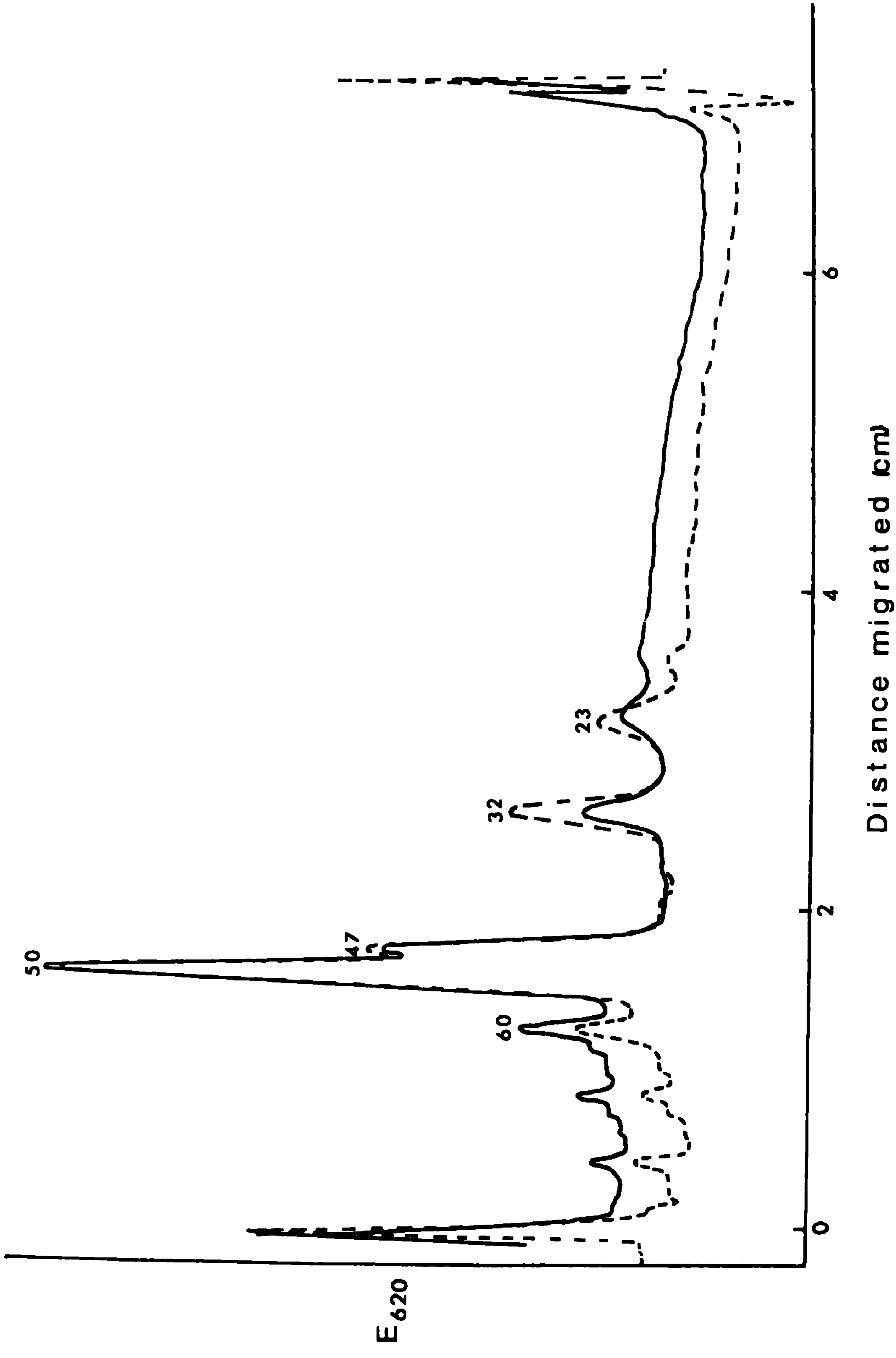
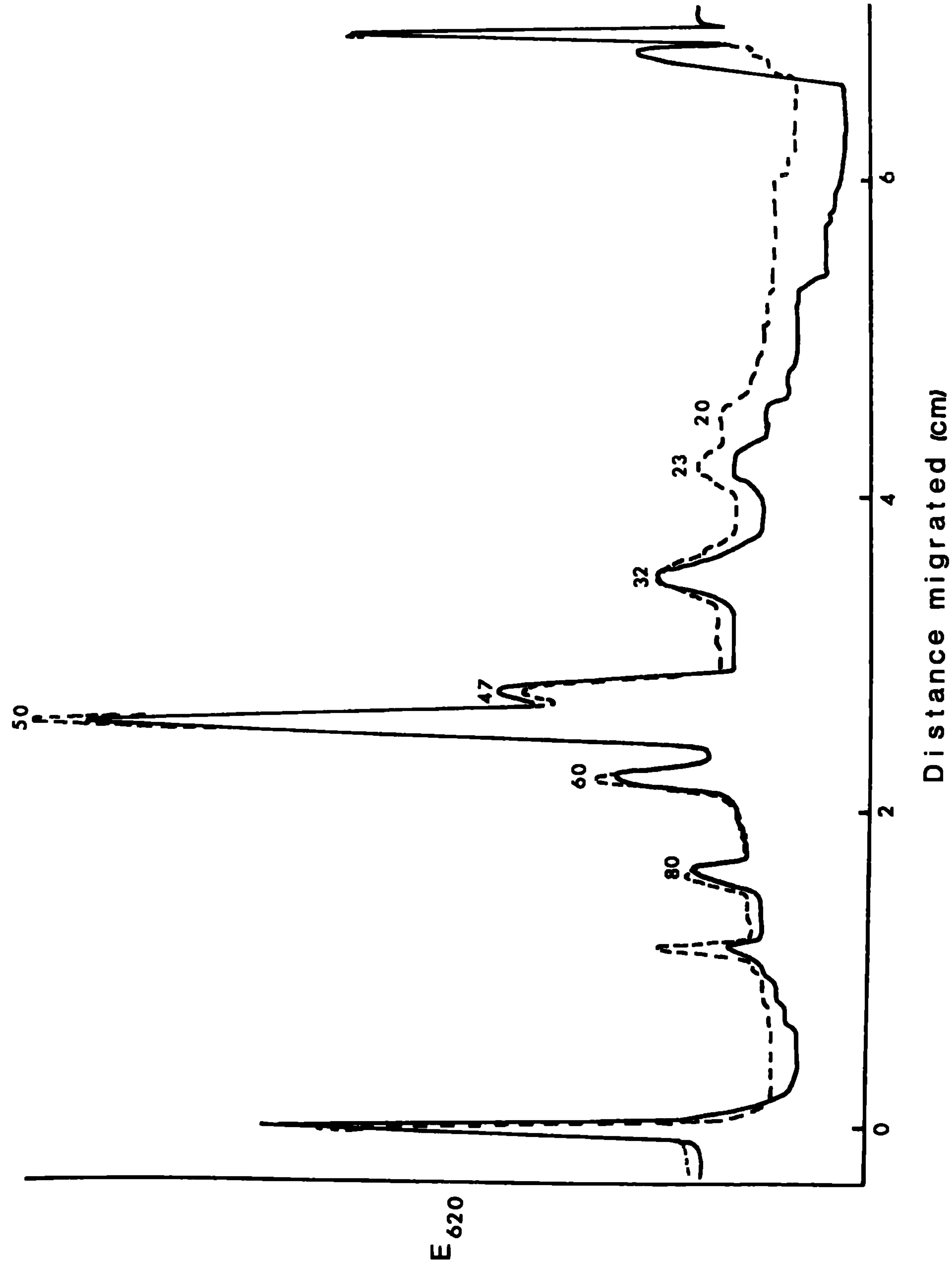

E$_{620}$
50
47
60
32
23
0
2
4
6
Distance migrated (cm)

Fig. 28

SDS gel electrophoretic analysis, in 7% gels, of protein bodies prepared by the glycerol procedure at pH 5.0, from meal of the abaxial region (dashed line) and of whole cotyledons (continuous line). As in Fig. 26 and Fig. 27, molecular weights are indicated as previously.

protein bodies from the whole cotyledon ——————

protein bodies from the abaxial region ----------


E620
Distance migrated (cm)
0
2
4
6
80
60
50
47
32
23
20

II. Proteins of Developing Seeds.

A. Changes in Fresh Weight.

Fig. 29 shows the increase in fresh weight of the whole seed with age, in days, from the day of flower opening. After a period of approximately 20 days, in which there is little increase in the fresh weight of the seed and during which time the testa accounts for most of the seed fresh weight, there is a steady increase in fresh weight for approximately 20 days, accounted for mainly by growth of the cotyledons. After this time dehydration occurs, and seeds are mature (c 15% water) by 45 - 50 days after flowering. In the course of obtaining this data, it was found that only 18% of the flowers tagged on the day of flower opening set fruit, although it is not known whether approximately 80% of all flowers fail to set fruit.

The proportion of the developing seed that is accounted for by dry matter was determined on seeds of increasing fresh weight. While the percentage dry matter was initially low (less than 20% of the total fresh weight) it increased with increasing fresh weight until at the maximum fresh weight, dry matter accounted for 45% of the total seed weight. Fig. 30 shows the increase in fresh weight and dry weight plotted against the percentage dry weight and demonstrates that increase in dry weight effectively ceases at the same time as dehydration begins.

B. Changes in Extractable Protein during Development.

The increase in the amount of protein extracted by alkaline salt solutions with increasing seed fresh weight is shown in Fig.31. Initially, the amount of protein is low (up to 150 mg seed fresh

Fig. 29

The increase in fresh weight of the whole seed with time, in days, from the day of flower opening, of seeds grown in the summer of 1972. Vertical lines indicate the 95% confidence limits.

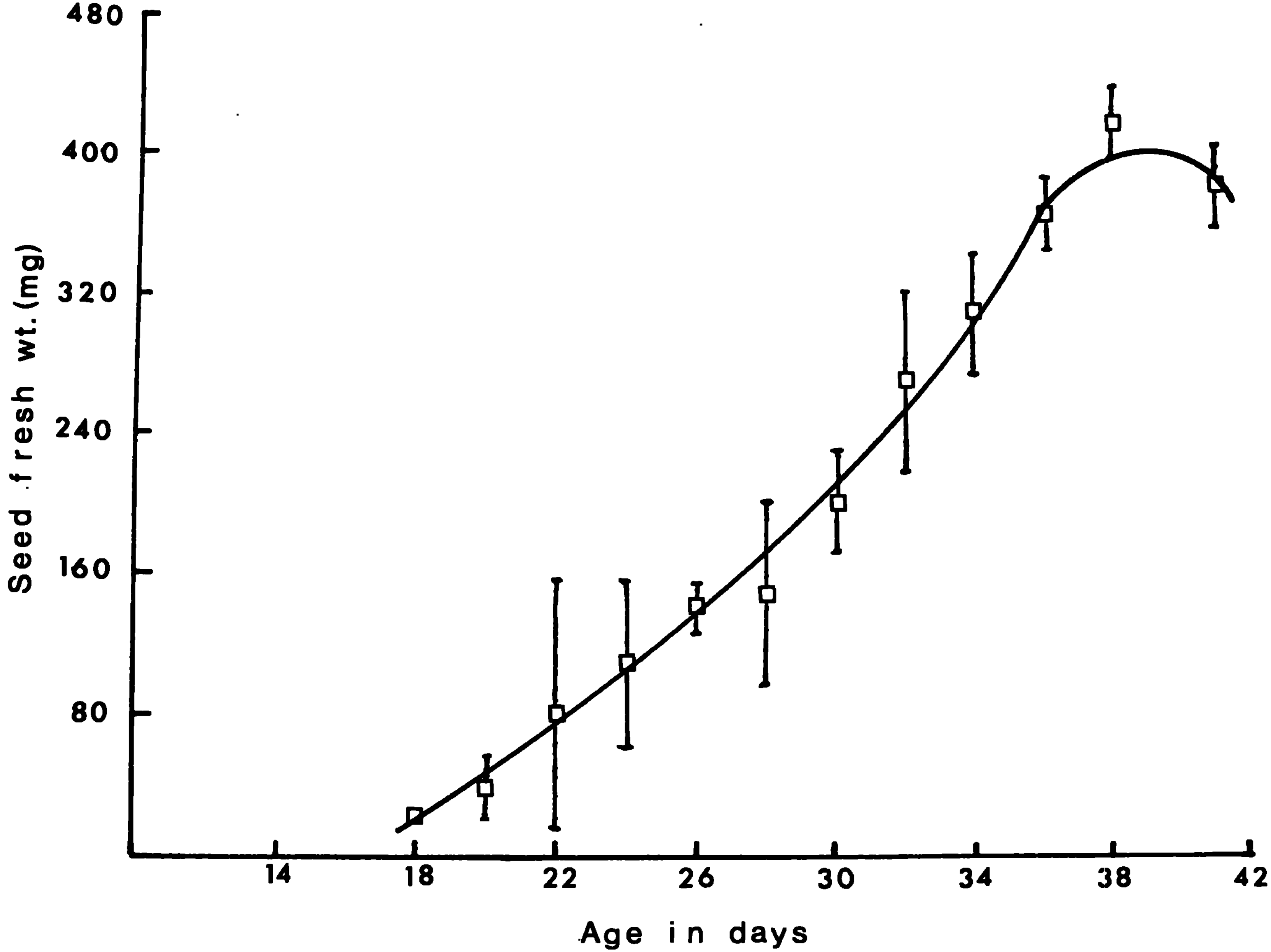

480
400
320
240
160
80
Seed fresh wt. (mg)
14
18
22
26
30
34
38
42
Age in days

Fig. 30

The increase in both fresh weight and dry weight of developing seeds are plotted against the percentage dry weight. The vertical lines represent the 95% confidence limits.

seed fresh weight (mg) △——△

seed dry weight (mg) ○——○

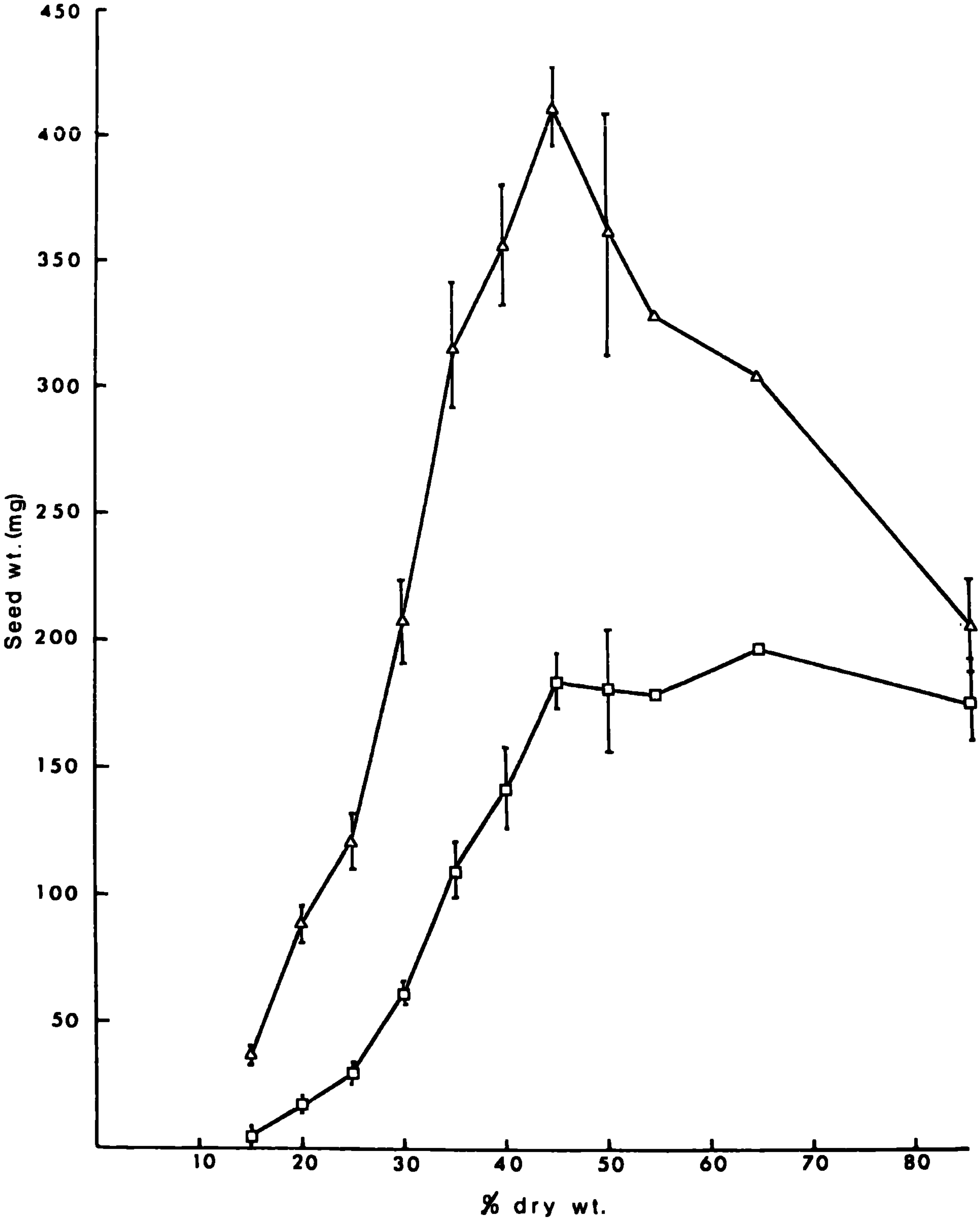
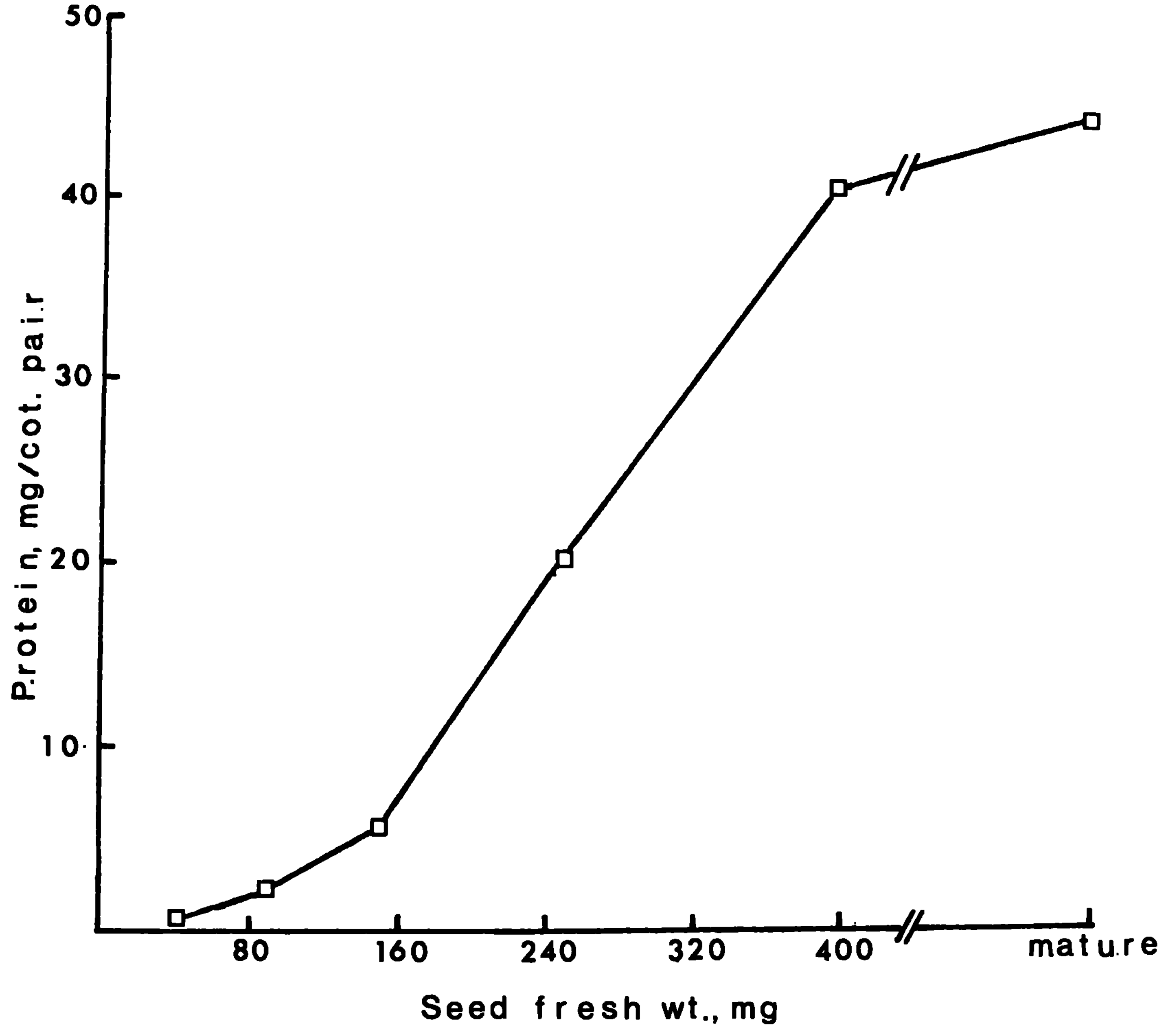

450
400
350
300
250
200
150
100
50
Seed wt. (mg)
10
20
30
40
50
60
70
80
% dry wt.

Fig. 31

The amount of protein extracted by alkaline salt solutions from seeds of increasing fresh weight. Extracts were dialysed against excess extractant prior to protein estimation, which was determined using the Lowry procedure (Lowry *et al.* 1951); the amounts presented are the sum of the first two extractions. The amount of protein extracted is expressed as mg per cotyledon pair. Also shown is the amount extracted from mature seeds.


50
40
30
20
10
Protein, mg/cot. pair
80
160
240
320
400
mature
Seed fresh wt., mg

weight, approximately 27 days from flowering) but then increases steadily, finally accounting for over 20% of the total seed weight.

The alkaline salt soluble protein extracted at different stages of development was fractionated into albumins and globulins by dialysis, initially against running tap water and then against deionised water. Precipitated material was removed by centrifugation after each dialysis and freeze-dried, and the amount of protein remaining in the supernatant fluid (both before and after each dialysis) was determined. The results are presented in Table 8.

The results in Table 8 show that dialysis against running tap water is less effective as a globulin precipitant than this dialysis followed by dialysis of the supernatant fluid against deionised water. While in the early stages of development (40 and 90 mg seed fresh weight) more protein is precipitated by running tap water (column 4, Table 8) than by the subsequent dialysis against deionised water (column 5, Table 8), with more mature seeds (250 and 400 mg seed fresh weight) the reverse was the case. As a percentage of the total protein, globulins represent about 84% of the protein at each stage of development, except in 40 mg fresh weight seeds, where virtually all the protein extracted was apparently precipitated by dialysis.

With all extracts protein precipitated by dialysis against running tap water was removed prior to further dialysis against deionised water. If this precipitate was not removed, but the dialysis continued directly against deionised water (for the same length of time, and with the same number of changes) protein

TABLE 8

Fractionation of the alkaline salt extracts of developing seeds into albumins and globulins.

All results are expressed as mg per cotyledon pair. The figures in column 2 represent the total amount of protein extracted from the cotyledons of seeds of the total fresh weight given in column 1. The protein remaining in solution after dialysis against running tap water (for 40 h) and then deionised water (8 changes over a period of 40 h, at 4°C) is shown in columns 3 and 5 respectively, while columns 4 and 6 respectively show the amount of protein precipitated by these dialyses, obtained by difference. The total amount of globulin is shown in column 7; the amount of albumin is presented in column 5.

TABLE 8

Fresh Wt. mg/seed (1)	Total Protein (2)	Soluble protein after running tap water dialysis (3)	Protein precipitated by running tap water dialysis (4)	Soluble protein after deionised water dialysis (5)	Protein precipitated by deionised water dialysis (6)	Total globulin protein (7)
40	0.65	0.22	0.43	0.01	0.21	0.64
90	2.32	1.28	1.04	0.32	0.96	2.00
150	5.66	4.19	1.47	1.13	3.06	4.53
250	19.9	13.1	6.8	2.7	10.4	17.2
400	40.2	26.4	13.9	5.9	20.5	34.3

precipitation was less complete than that obtained by similar dialysis against deionised water after the removal of material precipitated by the dialysis against running tap water. However, if the precipitated material from the continuous dialysis was removed by centrifugation and the clear supernatant redialysed against deionised water, rapid formation of a precipitate occurred; no further precipitate could be obtained by redialysis of the supernatant fluid remaining after the dialysis procedure normally used.

C. Estimation of Subunit Proportions during Development.

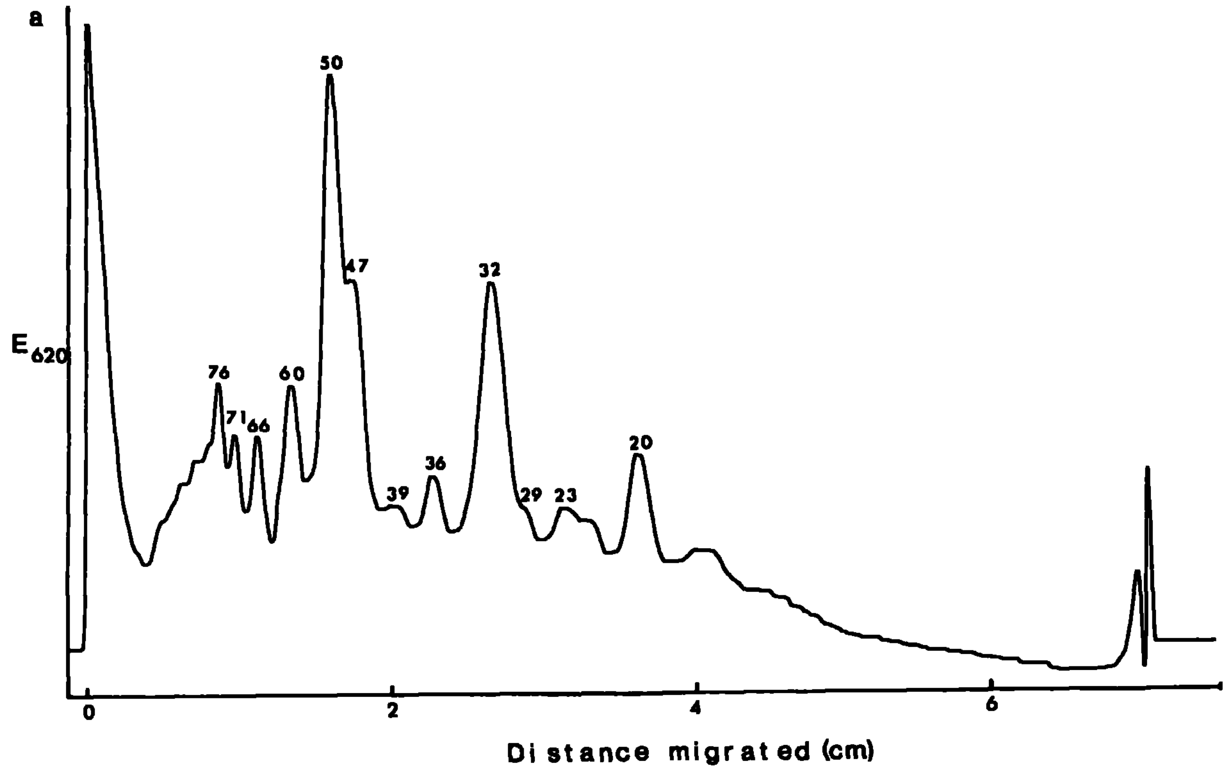
Globulins precipitated by dialysis against running tap water and deionised water were separately analysed by SDS gel electrophoresis. Densitometer traces of the results of SDS gel electrophoresis of globulins precipitated by the dialysis of extracts from seeds of fresh weight 90 mg and 400 mg against running tap water are shown in Fig. 32; Fig. 33 presents the patterns derived from the globulin precipitated by subsequent dialysis against deionised water, from the same stages of development. At each stage of development, the Chromoscan integrator values for each resolved peak were totalled and the proportion that each peak represented of the total was calculated; by assuming that all the protein applied was obtained as resolved peaks, the amount of a particular subunit, in µg, was calculated for each fraction. By combining the results from both running tap water and deionised water dialysis precipitates, the percentage that the different molecular weight subunits represent at each stage of development was determined. The results are collected in Table 9.

Fig. 32

SDS gel electrophoretic patterns of the globulin precipitated by dialysis against running tap water of alkaline salt extracts of the cotyledons of developing seeds. 10.0% (w/v) gels were used; the numbers above peaks represent molecular weights (x 10^{-3}).

(a) globulin from seeds of 90 mg fresh weight.

(b) globulin from seeds of 400 mg fresh weight.


a
E$_{620}$
50
47
32
76
71
66
60
36
39
29
23
20
0
2
4
6
Distance migrated (cm)

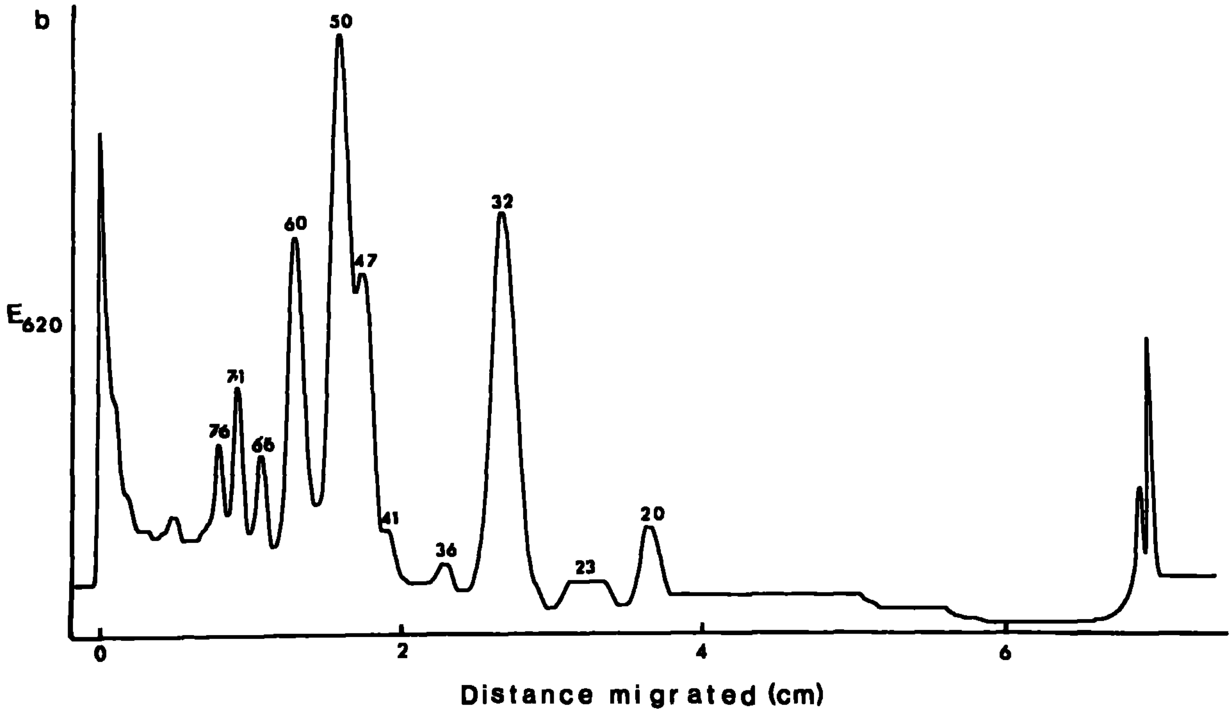
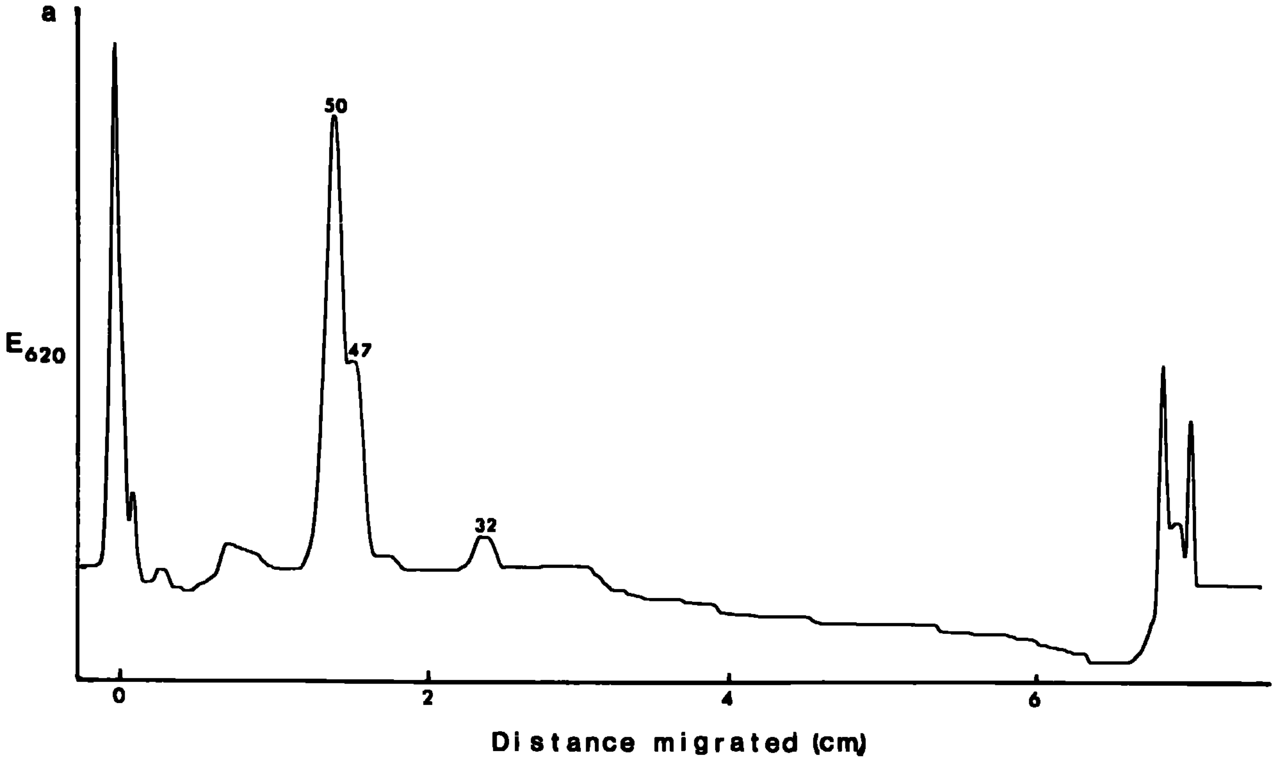

b
E$_{620}$
50
32
60
47
71
76
66
41
36
23
20
0
2
4
6
Distance migrated (cm)

Fig. 33

SDS gel electrophoretic patterns of globulin precipitated by dialysis of alkaline salt extracts of developing seeds against deionised water (subsequent to the removal of material precipitated by dialysis of the extracts against running tap water). Molecular weights (x 10^{-3}) are indicated as previously; the gels were 10% (w/v) acrylamide.

(a) from seeds of 90 mg fresh weight.

(b) from seeds of 400 mg fresh weight.


a
E$_{620}$
50
47
32
0
2
4
6
Distance migrated (cm)

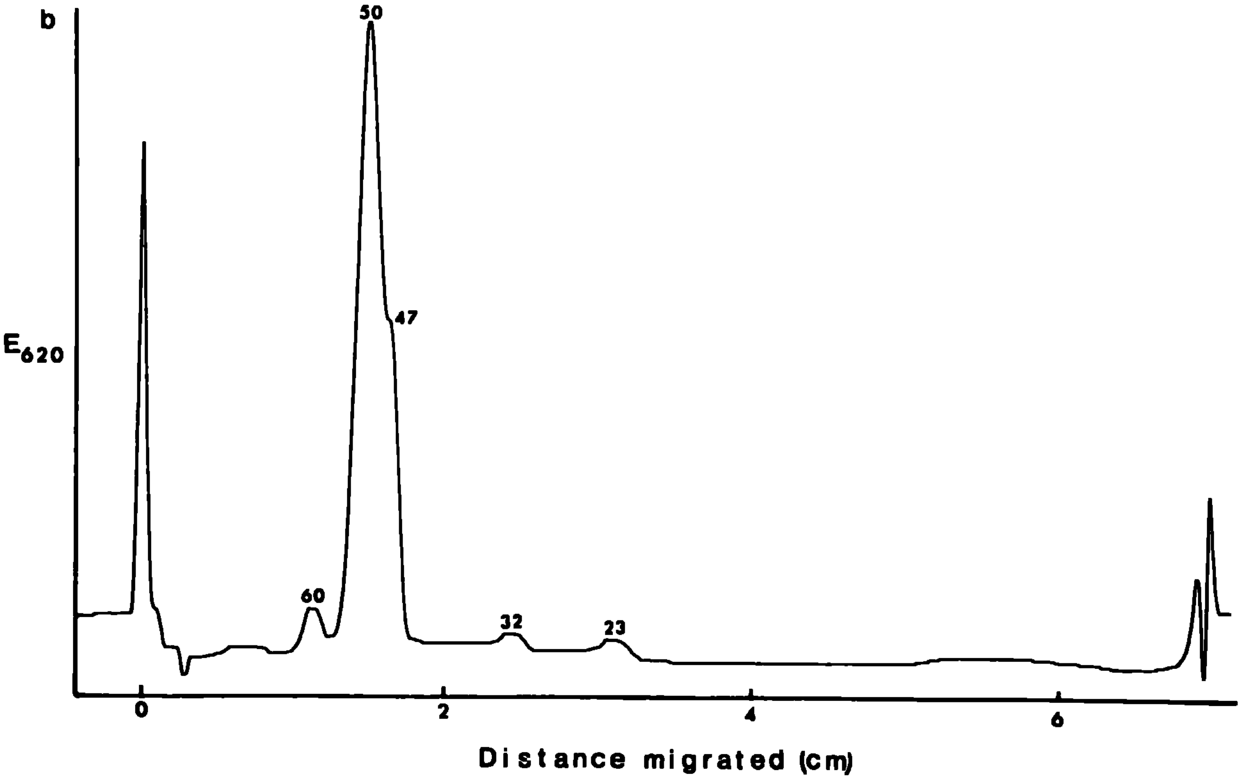

b
E$_{620}$
50
47
60
32
23
0
2
4
6
Distance migrated (cm)

TABLE 9

The percentage of the total globulin fraction comprised by each molecular weight subunit, at 4 stages of seed development. The procedure used to determine these percentages is described in the text.

	Subunit molecular weight, x10^{-3}								
Seed fresh weight, mg	20	23	29	32	36	39	47 + 50	60	66+71+76
90	1.1	2.6	0.5	7.0	1.7	1.7	71.0	4.2	10.1
150	1.85	3.3	N.R.	8.2	1.25	0.9	71.7	5.5	6.4
250	2.5	3.4	0.16	8.8	0.23	0.43	74.6	5.1	4.7
400	2.83	3.15	N.R.	7.7	0.17	0.15	76.7	5.7	3.6

N.R. Not resolved.

Although the main subunits, molecular weights 50,000 and 47,000, were present in the protein obtained from cotyledons of seeds of 40 mg fresh weight, the band patterns were too weak to allow determination of proportions. The major subunits of mature seeds, molecular weights 50,000 and 47,000, predominated throughout development. Three bands with molecular weights above 60,000 declined in amount during development; however, these are believed to be polymers, presumably due to incomplete dissociation, and were not seen in SDS extracts of developing seeds made directly from freeze-dried ground cotyledons. Subunits of molecular weight 36,000 and 41,000 declined in amount during development, while those of molecular weight 60,000 and 20,000 showed slight increases. All changes, however, were slight.

Direct SDS extraction of freeze-dried cotyledons confirmed the overall pattern established using the two globulin preparations, but owing to the preponderance of the 50,000 and 47,000 molecular weight subunits, detailed comparisons were unsatisfactory and minor bands seen in the globulin preparations were not resolved. The characteristic storage protein subunits extracted from cotyledons were not found when SDS extracts of testas of developing seeds were examined by SDS gel electrophoresis.

III. Proteins of Germinating Seeds.

A. Changes in Fresh Weight.

Fig. 34 shows the change in fresh weight of the cotyledons of germinating seeds with the passage of time from the onset of germination. Imbibition from the dry seed fresh weight of approximately 210 mg to the hydrated condition takes 6-10 h. There is a slight increase in cotyledon fresh weight until day 6, followed by a steady and more rapid decline. This fall in fresh weight is accompanied by the partial collapse of the internal cellular structure, resulting in surface wrinkling.

B. Changes in Protein Content and its Electrophoretic Behaviour.

Fig. 35 shows the change in the amount of extractable protein with the passage of time during germination. There is a steady decline during the first 6 days of germination, followed by a more rapid loss of protein. Table 10(a) compares the values obtained for protein estimations before and after dialysis of the extracts against excess extractant. During the first 6 days of germination, protein estimates are higher after dialysis, but in the latter stages of germination, dialysis appears to result in the loss of material. Little protein remains after 12 days. Table 10(b) shows the amount of protein obtained by re-extracting the residue after removal of the supernatant fluid derived from the first extraction. In all cases the amount extracted represents less than 10% of that obtained from the first extraction. The values for protein content shown in Fig. 35 are the sum of the average of the dialysed and non-dialysed protein determinations, and the second extraction.

Fig. 34

The change in the fresh weight, in mg per cotyledon pair, of the cotyledons of germinating seeds with the passage of time from the onset of germination.

Fig. 35

The change in the amount of extracted protein with the passage of time during germination. Protein concentrations were determined by the method of Lowry _et al_. (1951). Values shown are the sum of the protein content of the second extraction and the average of the values obtained for the first extract before and after dialysis against excess extractant.

Fig. 34

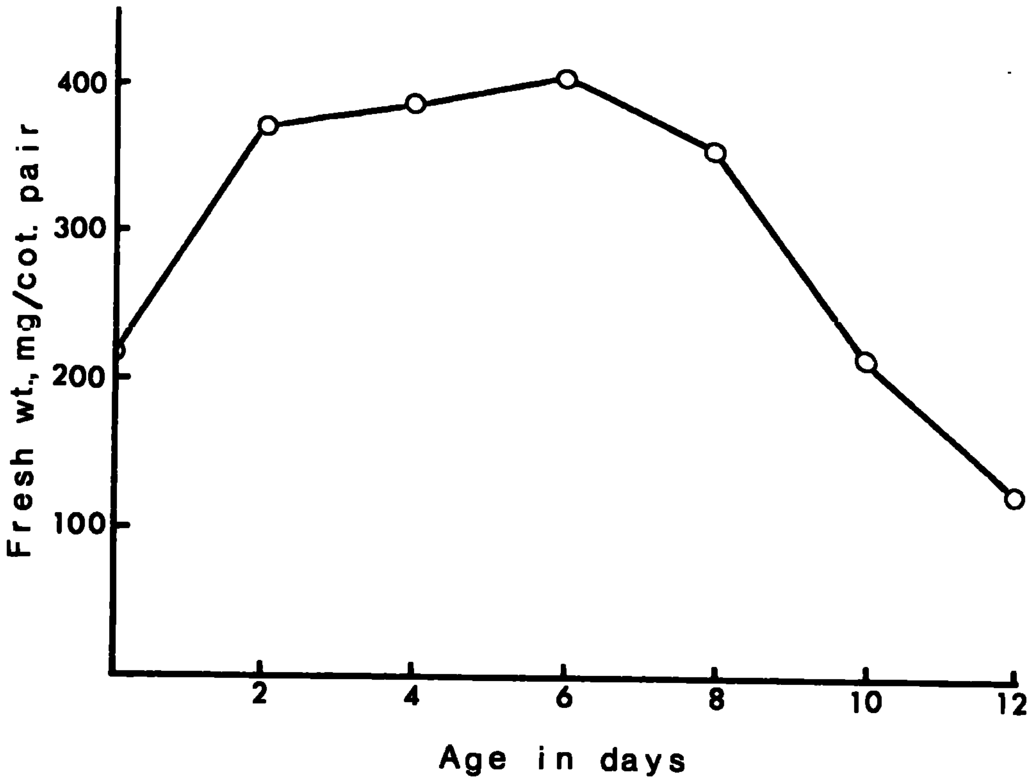

Fresh wt., mg/cot. pair
400
300
200
100
2
4
6
8
10
12
Age in days

Fig. 35

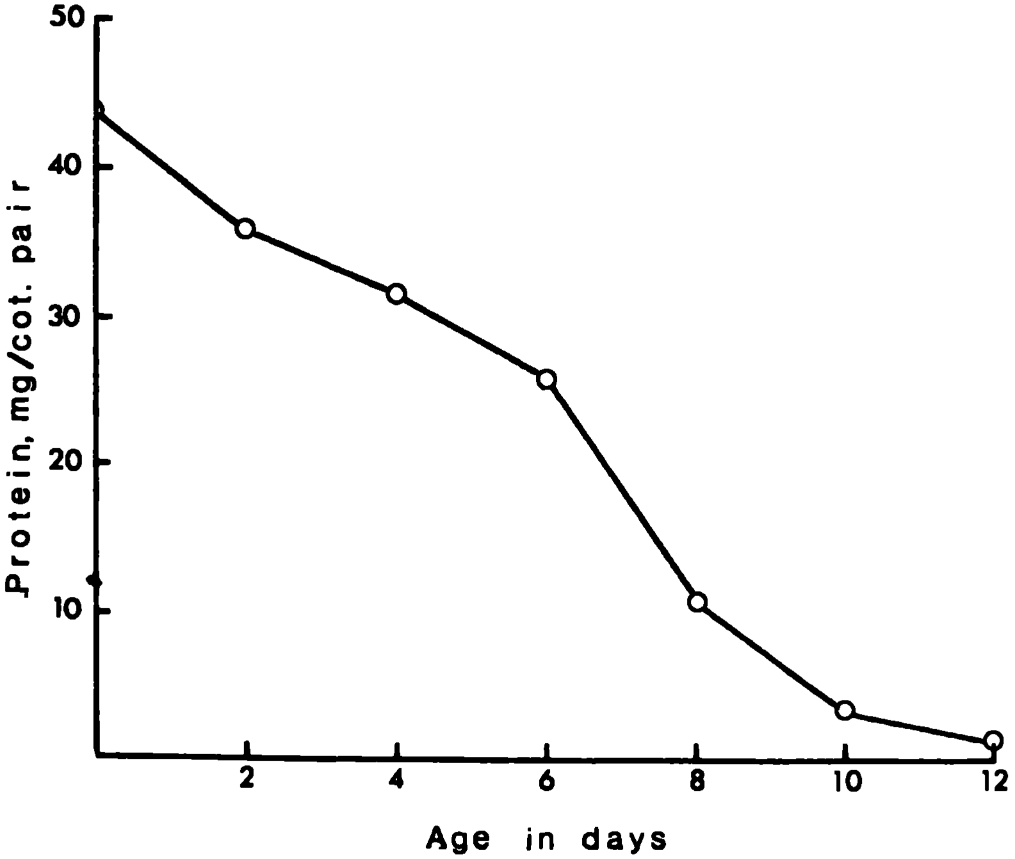

Protein, mg/cot. pair
50
40
30
20
10
2
4
6
8
10
12
Age in days

TABLE 10

Table 10(a) presents the amount of protein extracted, determined using the method of Lowry _et al_. (1951), before and after dialysis against excess extractant. Results are expressed as mg protein per cotyledon pair, for each age from the start of germination; the figures before dialysis are also shown as the percentage of those obtained after dialysis.

Table 10(b) shows the amount of protein extracted by the second extraction, for each age. This is also expressed as the percentage of that obtained in the first extraction.

TABLE 10

Table 10(a)

	Age, in days, from Onset of Germination						
	0	2	4	6	8	10	12
Amount of protein determined before dialysis against excess extractant	38.2	31.1	27.6	24.1	10.3	3.08	N.D.
Amount of protein determined after dialysis against excess extractant	44.8	37.0	32.2	25.8	10.0	2.9	1.2
Amount of protein before dialysis, as percentage of the amount after dialysis	85.2	84.0	85.7	93.4	103.0	106.5	N.D.

Table 10(b)

	Age, in Days, from Onset of Germination						
	0	2	4	6	8	10	12
Protein concentration, mg/ml, of SN_2^a, without dialysis against excess extractant	N.D.	1.85	1.75	0.83	0.60	0.30	0.05
Protein concentration of SN_2, expressed as the % of that of the first extract, before dialysis	-	5.95	6.35	3.40	5.80	9.70	N.D.

[a] SN_2 : the supernatant fluid from the second extraction.

Extracted protein was further analysed by polyacrylamide gel electrophoresis. Fig. 36 shows the band patterns resulting from disc electrophoresis of extracts of germinating seeds; while some minor bands (e.g. Rm 0.26, 0.77) are lost fairly rapidly, the major band (Rm 0.35 - 0.39) is degraded more slowly becoming indistinct by the eighth day; as the prominence of this band decreases, a diffuse band of increased Rm (approx. 0.47) increases in relative intensity and is present throughout the remaining period of germination. A band migrating with the bromo-phenol blue marker (Rm 1.0) increases in relative intensity throughout germination.

Fig. 37 presents densitometric traces of the SDS gel electrophoretic analysis of extracts of germinating seeds. The most significant change is the increase in relative importance of a band of approximately 21,000 molecular weight. It is also noticeable that the disappearance of the main band found in the disc electrophoretic system (Rm 0.35 - 0.39) appears to be approximately simultaneous with the loss of the main subunits, molecular weights 50,000 and 47,000. Later in germination there is a considerable quantity of low molecular weight material which does not form discrete bands. These densitometric traces are intended to provide comparison of the molecular weight distribution of the protein present at different stages of germination; they are not representative of the total amount of protein present.

C. Changes in some Enzyme Activities.

Fig. 38 shows changes in the activity of BAPNA-hydrolysing and leucine-p-nitroanilide-hydrolysing (leucine amino peptidase, LAP-ase) enzymes. BAPNA-ase activity at pH 7.5 shows an initial

Fig. 36

The results of disc electrophoretic analysis of alkaline salt extracts from seeds soaked for 16 h and from those allowed to germinate for 2, 4, 6, 8 and 10 days; the gel pattern from the 16 h soaked seeds is referred to as 0 day. The position of bands are described by their mobilities relative to that of the bromo-phenol blue marker (Rm.); 7% (w/v) acrylamide gels were used.

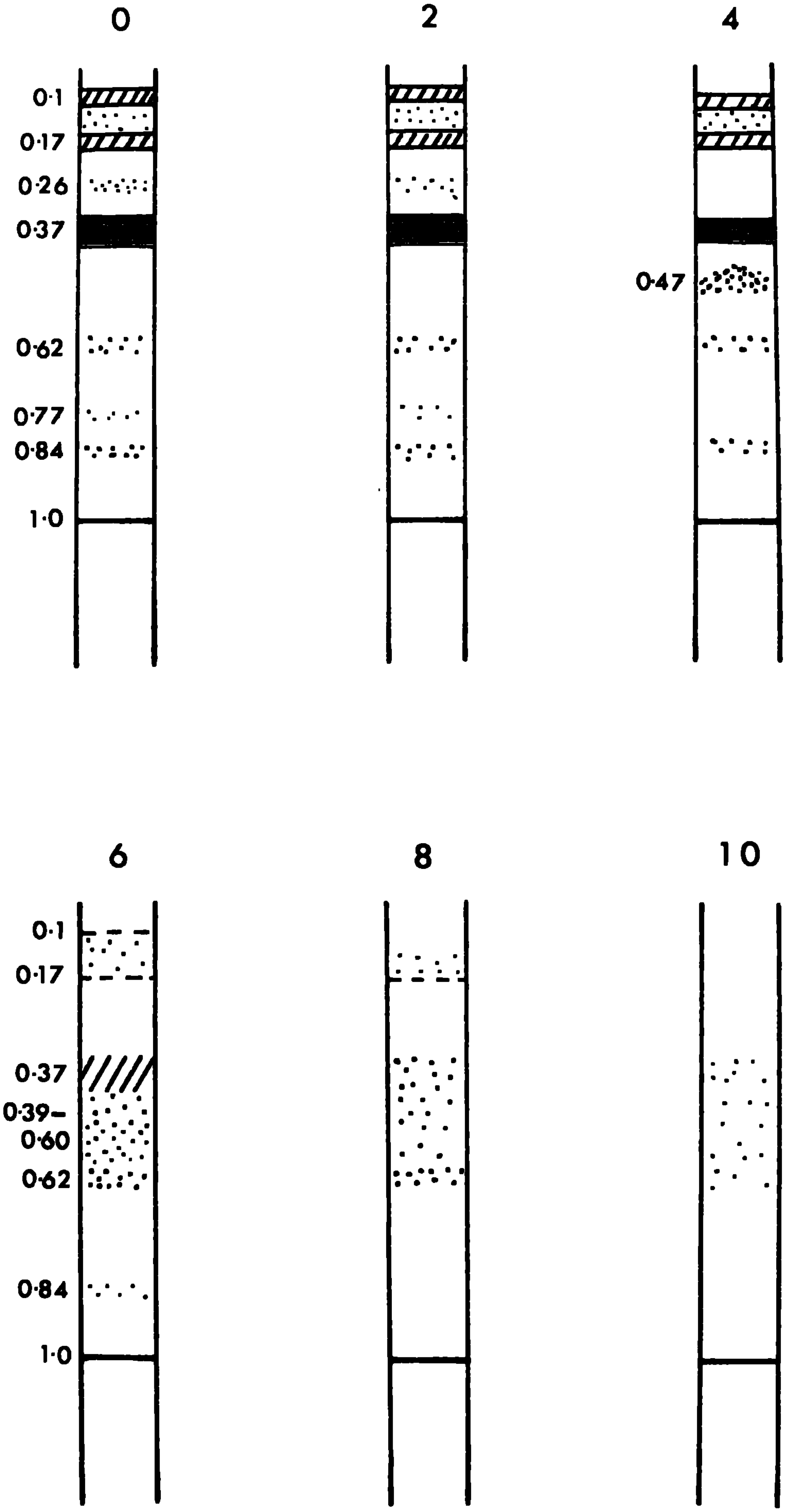

0
2
4
0·1
0·17
0·26
0·37
0·47
0·62
0·77
0·84
1·0
6
8
10
0·1
0·17
0·37
0·39–
0·60
0·62
0·84
1·0

Fig. 37

Densitometer traces of SDS gels (7.0% (w/v) acrylamide) in which extracts of germinating seeds have been electrophoresed and stained for protein (with amido black). Figures above peaks refer to the molecular weights (x 10^{-3}).

a) 0 day (16 h soaked seed).

b) 4 day germinated.

c) 6 day germinated.

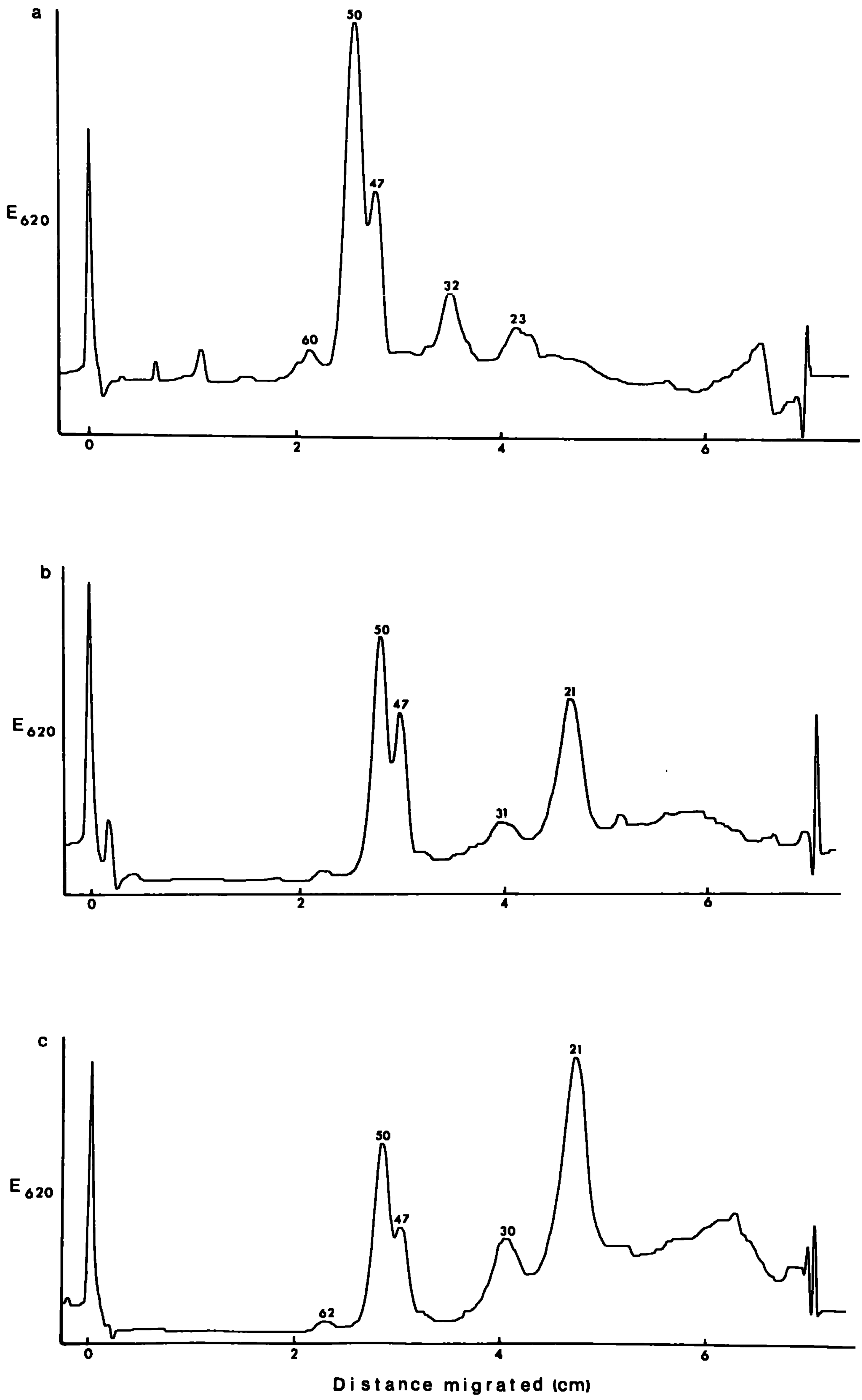
a
E620
60
50
47
32
23
0
2
4
6
b
E620
50
47
31
21
0
2
4
6
c
E620
62
50
47
30
21
0
2
4
6
Distance migrated (cm)

Fig. 37 (cont.)

d) 8 day germinated.

e) 10 day germinated.

f) 12 day germinated.

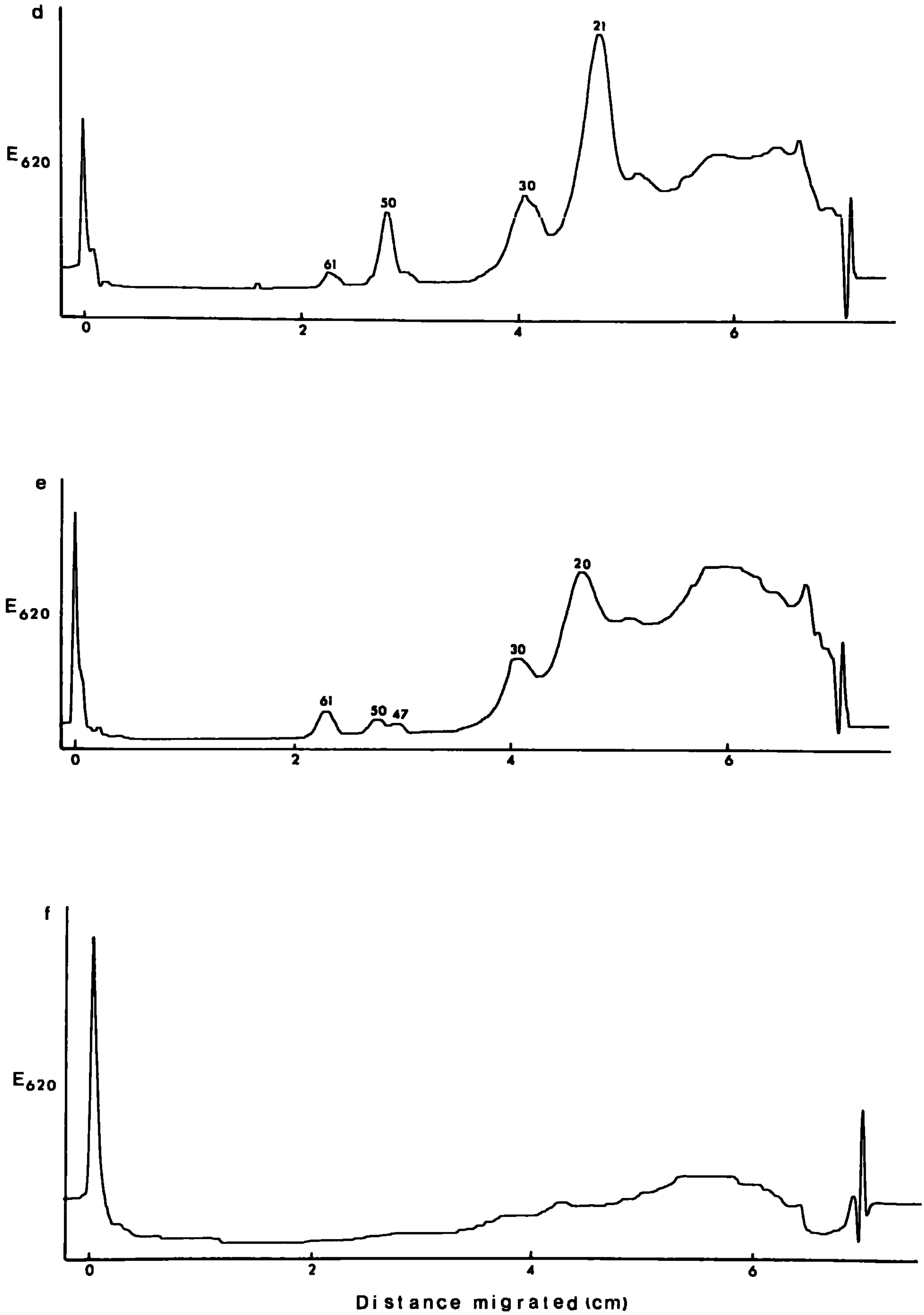
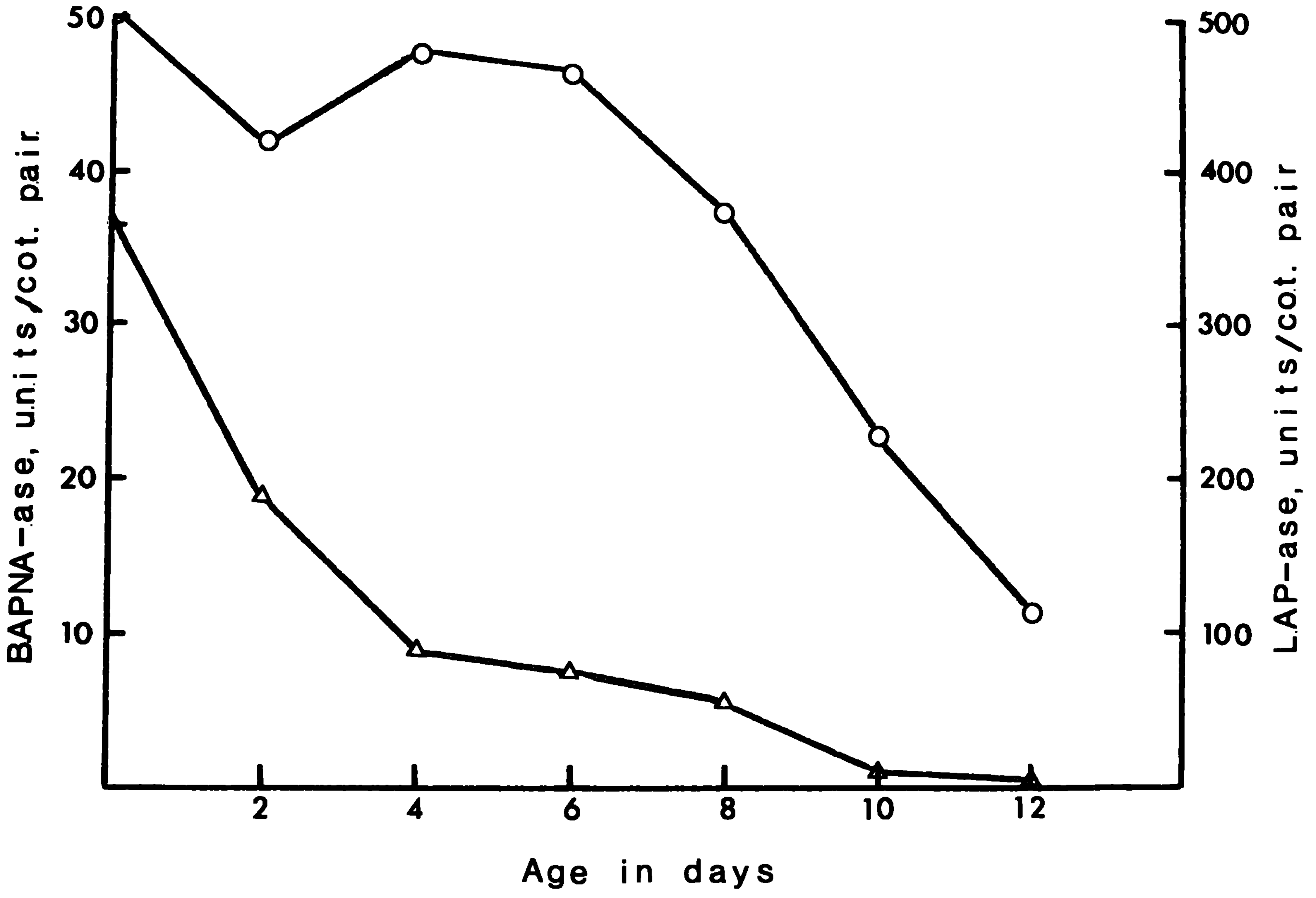

d
E$_{620}$
61
50
30
21
0
2
4
6
e
E$_{620}$
61
50
47
30
20
0
2
4
6
f
E$_{620}$
0
2
4
6
Distance migrated (cm)

Fig. 38

Changes in the activity of BAPNA-ase and leucine amino peptidase (LAP-ase) enzymes during germination. Results are expressed as units per cotyledon pair. The assay of BAPNA-ase activity was carried out at pH 7.5; leucine amino peptidase was assayed at pH 6.5.

BAPNA-ase ○————○

LAP-ase △————△


50
40
30
20
10
BAPNA-ase, units/cot. pair
500
400
300
200
100
LAP-ase, units/cot. pair
2
4
6
8
10
12
Age in days

fall, followed by a slight increase around days 4-6; this is succeeded by a steady decline in activity; BAPNA-ase activity at pH 5.0 (see Fig. 41 for the effect of pH on activity) shows similar changes. Leucine amino peptidase activity falls rapidly from the start of germination.

Changes in the trypsin-inhibitory activity are shown in Fig. 39; there is a slight decrease in the first 6 days, which is succeeded by a rapid decline. Fig. 40 shows changes in proteolytic activity measured by hydrolysis of both azoalbumin and azoglobulin substrates, and also by the reduction in the viscosity of a gelatin substrate resulting from its hydrolysis. In each case there is a marked rise in activity during germination: approximately 10-fold in the case of the viscometric assay, approximately 15-fold for both the azo-substrates.

Table 11 shows the specific activity of each enzyme at the selected stages of germination. The protein concentration used in the calculation is the average of the values obtained for dialysed and non-dialysed extracts, shown in Table 10(a). The specific activities show that, of the enzymes studied, only leucine amino peptidase declines in activity more rapidly than the fall in the overall amount of protein, and emphasises the very marked increase in protease activity.

D. Some Properties of the Enzyme Activities.

(i) BAPNA-ase and leucine amino peptidase.

Fig. 41 shows the BAPNA-ase and leucine amino peptidase activity of extracts from ungerminated seeds over the pH range 4.5 - 7.5 in the phosphate-citrate buffer. The pH profiles were similar for extracts from 4 day germinated cotyledons,

Fig. 39

The change in the amount of trypsin-inhibitory activity with age from the beginning of germination. Activity is expressed as units per cotyledon pair.

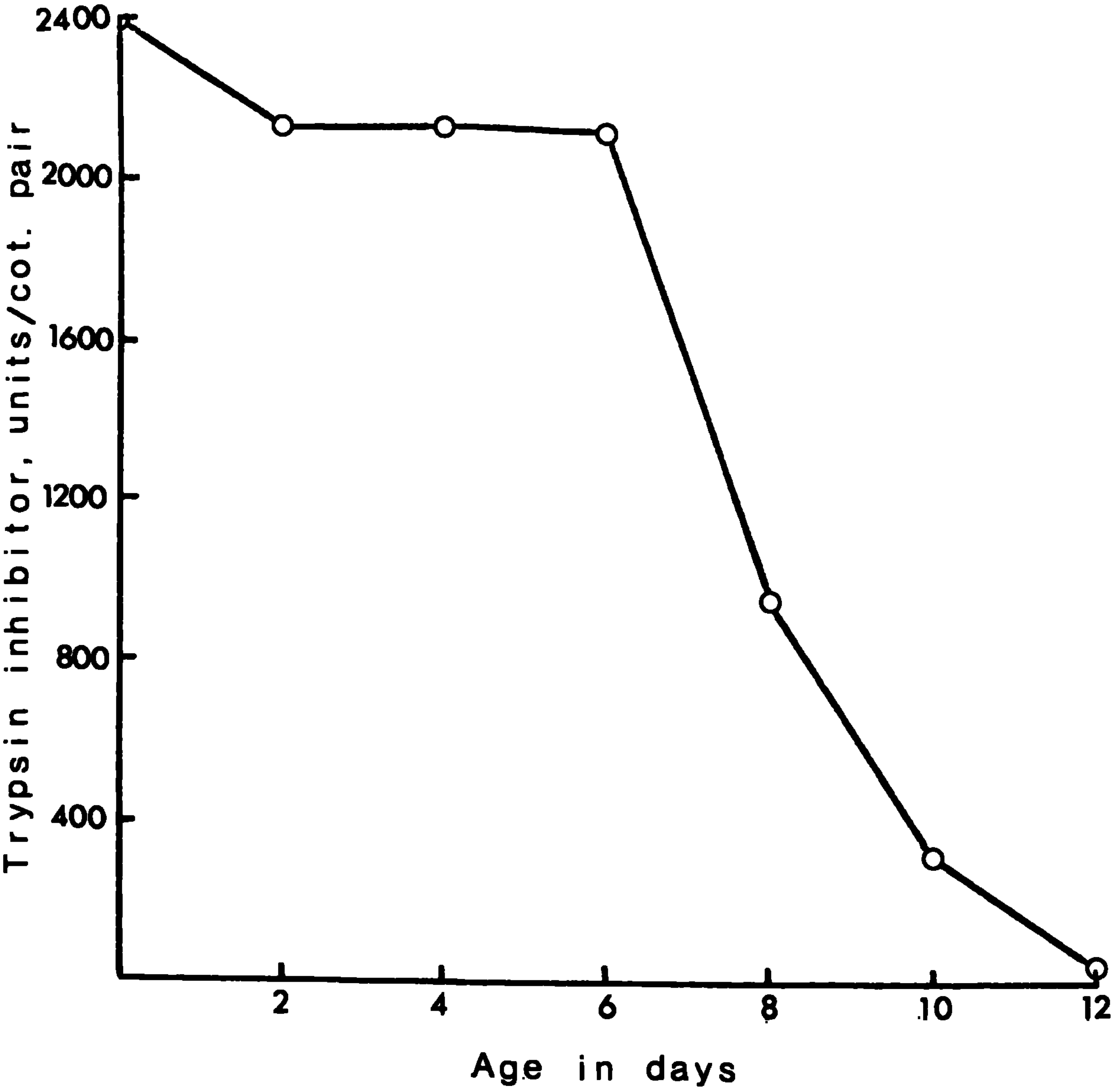

2400
2000
1600
1200
800
400
Trypsin inhibitor, units/cot. pair
2
4
6
8
10
12
Age in days

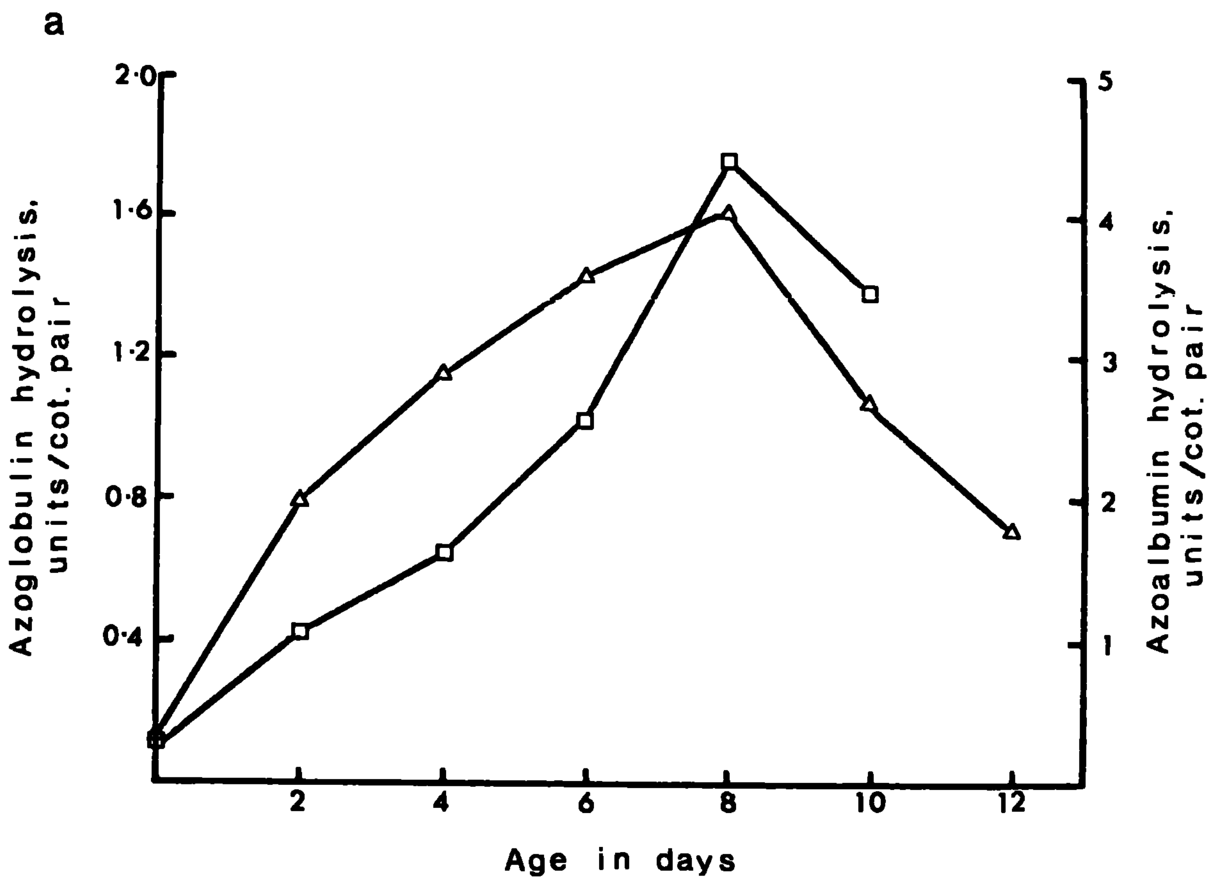
Fig. 40

Changes in proteolytic activity during germination.

Fig. 40 a Changes in proteolytic activity determined using azoalbumin and azoglobulin as substrates. Assays were carried out at pH 5.5 and results are expressed as units of activity per cotyledon pair.

Proteolytic activity measured by azoalbumin hydrolysis	□——□
Proteolytic activity measured by azoglobulin hydrolysis	△——△

Fig. 40 b Changes in proteolytic (endopeptidase) activity assayed by the decrease in the viscosity of gelatin solutions. Assays were carried out at pH 5.5 and proteolytic activity is expressed as units per cotyledon pair.


a
Azoglobulin hydrolysis, units/cot. pair
2·0
1·6
1·2
0·8
0·4
Azoalbumin hydrolysis, units/cot. pair
5
4
3
2
1
2
4
6
8
10
12
Age in days

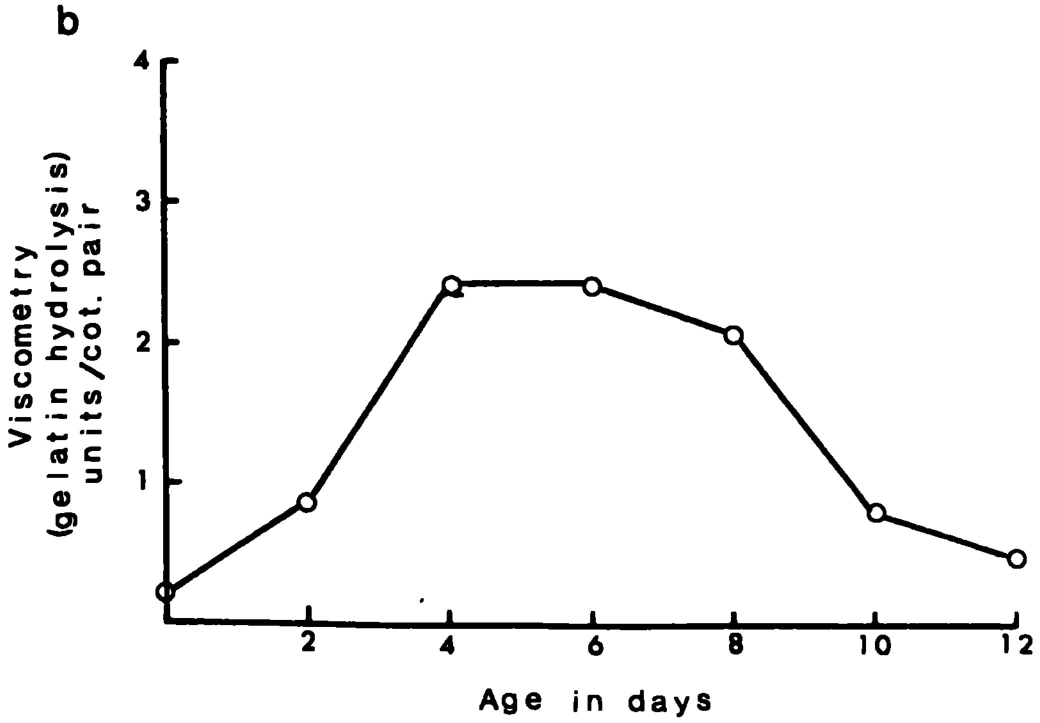

b
Viscometry (gelatin hydrolysis) units/cot. pair
4
3
2
1
2
4
6
8
10
12
Age in days

TABLE 11

Changes in Specific Activities of Enzymes during Germination

The results of the enzyme assays presented in Figs. 38, 39 and 40 are here expressed as specific activities, i.e. units of enzyme activity per mg protein, for each age.

	Age, in days, from Onset of Germination						
	0	2	4	6	8	10	12
Protein, mg/cot.pair	41.5	34.1	29.9	25.0	10.2	3.0	1.2
BAPNA-ase, units/mg	1.2	1.2	1.6	1.9	3.7	7.6	9.8
LAP-ase, units/mg	8.9	5.5	3.0	3.0	3.2	3.3	6.0
Trypsin Inhibitor, units/mg	57.6	62.2	70.8	84.2	92.6	103.2	34.5
Azoglobulin hydrolysis, units $x10^2$/mg	0.3	2.3	3.9	5.7	15.9	35.7	61.2
Azoalbumin hydrolysis, units $x10^2$/mg	1.0	3.7	6.0	10.8	46.0	122.0	N.D.
Viscometry (gelatin hydrolysis), units $x10^2$/mg	0.5	2.5	8.2	9.8	19.9	27.5	43.2

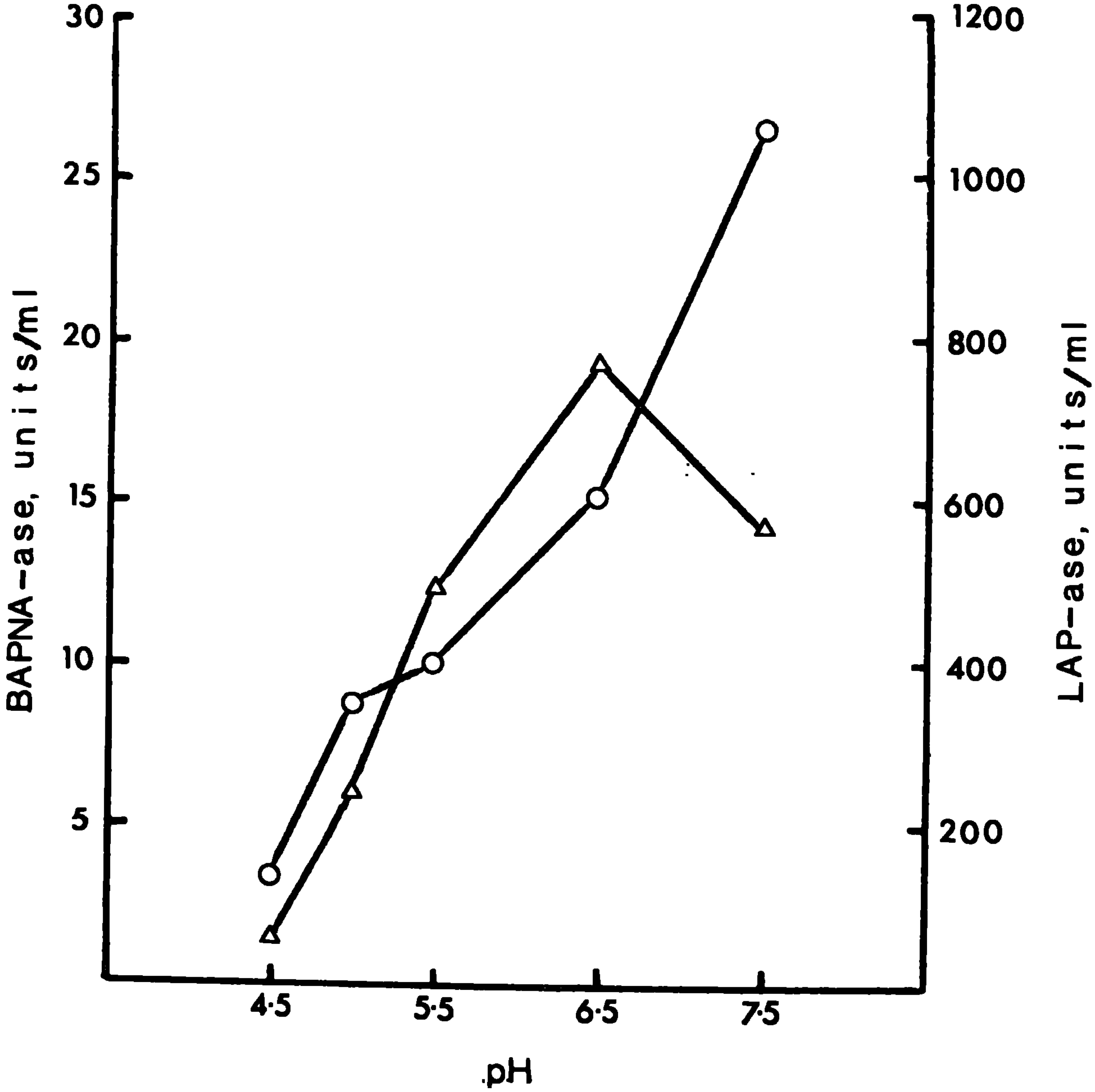
N.D. Not determined.

Fig. 41

The effect of pH on the activity of BAPNA-ase and leucine amino peptidase activities. Assays were carried out in 0.05M sodium phosphate, 0.05M citric acid buffer at the indicated pH s. Activity is expressed as units per ml.

BAPNA-ase ○———○

LAP-ase △———△


30
25
20
15
10
5
BAPNA-ase, units/ml
1200
1000
800
600
400
200
LAP-ase, units/ml
4·5
5·5
6·5
7·5
pH

although differences were less pronounced for leucine amino peptidase, probably due to the markedly lower overall activity; the pH optimum of leucine amino peptidase is between pH 6.0 and 7.0. When the pH range was extended using 0.1M borate buffers (final concentration), the apparent pH optimum of the alkaline BAPNA-ase activity was above pH 9.0.

When assayed in 0.1M phosphate buffer at pH 7.5, BAPNA-ase activity could be partially reduced by the addition of heated (75°C for 60 min) extracts; thus the incorporation of 0.02 ml of heated extract in the determination of the activity of 0.1 ml of unheated extract resulted in a 15% reduction of activity, after correction with the appropriate blanks. Complete inhibition was not obtained. No inhibitory activity could be obtained when using phosphate-citrate buffer, at any pH.

Inclusion of N-ethylmaleimide (NEM) at a final concentration of 10 mM resulted in 70% inhibition of BAPNA-ase activity at pH 7.5 and 90% inhibition at pH 5.0, compared to the water control; however, neither 2-mercaptoethanol (1% v/v) nor EDTA and cysteine (2 mM and 5 mM, respectively) had any effect on the activity at either pH. N-ethylmaleimide also completely inhibited leucine amino peptidase activity.

(ii) Trypsin inhibitor.

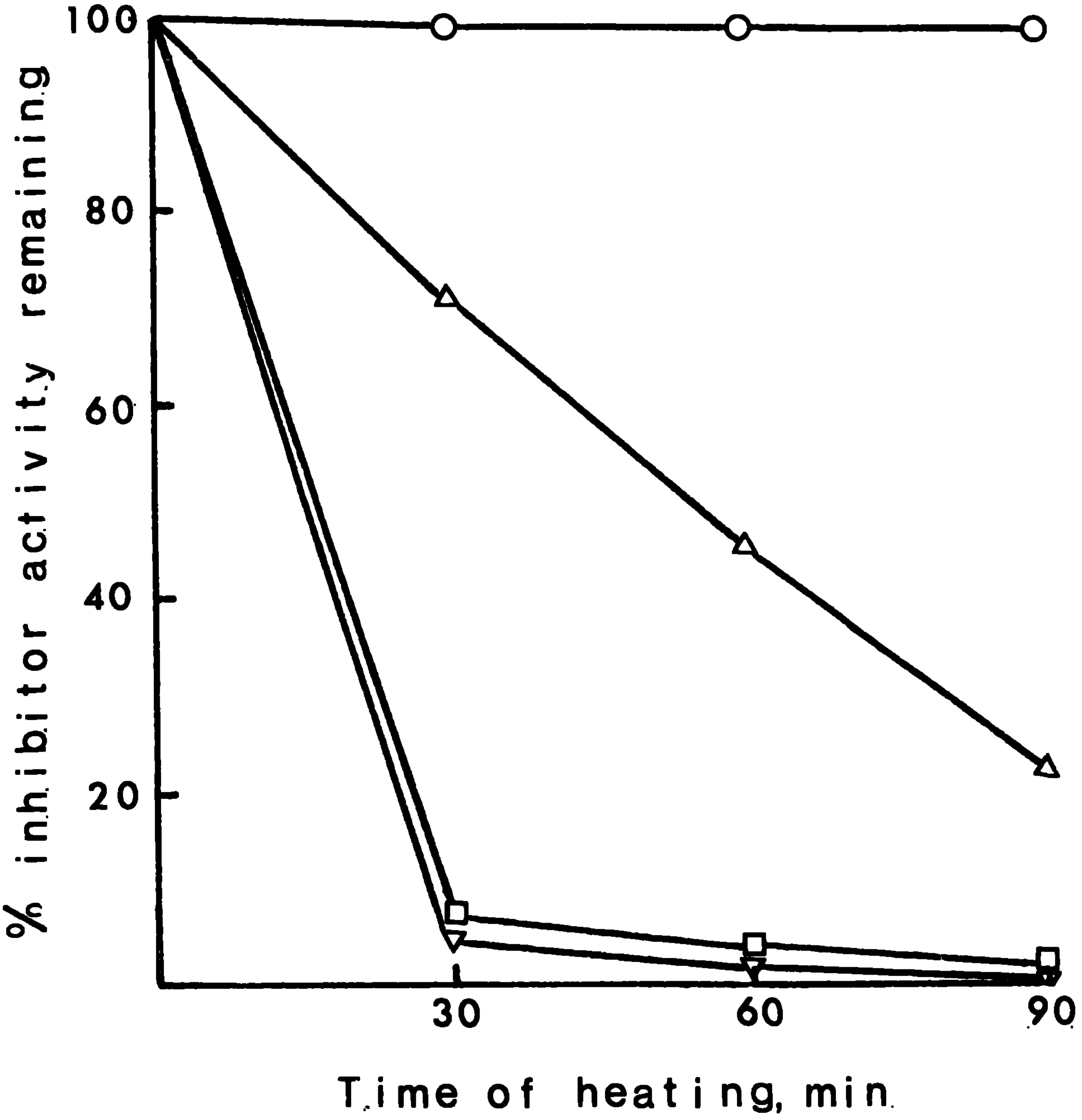
Fig. 42 presents the amount of trypsin inhibitory activity remaining after various heating treatments. While the inhibitory activity appears stable at 60°C, loss of activity occurs at higher temperatures, and increases with the duration of the heating treatment; inhibitory activity is rapidly lost at 90°C and 100°C. The inclusion of 2-mercaptoethanol at a final

Fig. 42

The amount of trypsin inhibitor activity remaining after heating an alkaline extract from seed meal (in 0.5M NaCl, 0.05M sodium phosphate buffer, pH 7.5) in a water bath for the lengths of times and the temperatures indicated; after removal of precipitated protein by centrifugation, inhibitory activity was determined on the supernatant fluid in the standard assay system.

Temperature of heating treatment :-

60°C	○———○
75°C	△———△
90°C	□———□
100°C	▽———▽


100
80
60
40
20
% inhibitor activity remaining
30
60
90
Time of heating, min

concentration of 1% (v/v) in the assay caused a 20% reduction in the apparent activity of trypsin but completely prevented inhibition of trypsin by seed extracts.

(iii) Protease activities.

(a) pH optimum and stability. Several determinations of the pH optima of the azoalbumin, azoglobulin and gelatin hydrolysing activities (assayed by viscometry) were carried out. Although minor variations occurred between different determinations, the optimum was always rather broad, pH 5.0 - 5.5, and activity was low at pH 7.5. Typical results for azoglobulin and gelatin substrates are shown in Fig. 43.

The gelatin-hydrolysing activity was particularly unstable to storage. About 40% of activity was lost during overnight storage, at 5°C and pH 7.5. When incubated at 35°C, pH 7.5, for 60 min, 80% of the initial activity was lost; losses were greater when the incubation was carried out in 0.1M citrate buffer at pH 2.5. The inclusion of 2-mercaptoethanol did not appear to prevent loss of activity at 35°C. Hydrolysis of azoglobulin was reduced by approximately 25% by overnight storage at 5°C, pH 7.5, although the activity of an extraction made at pH 2.5 (in 0.5M NaCl, 0.1M citrate) was more stable to storage.

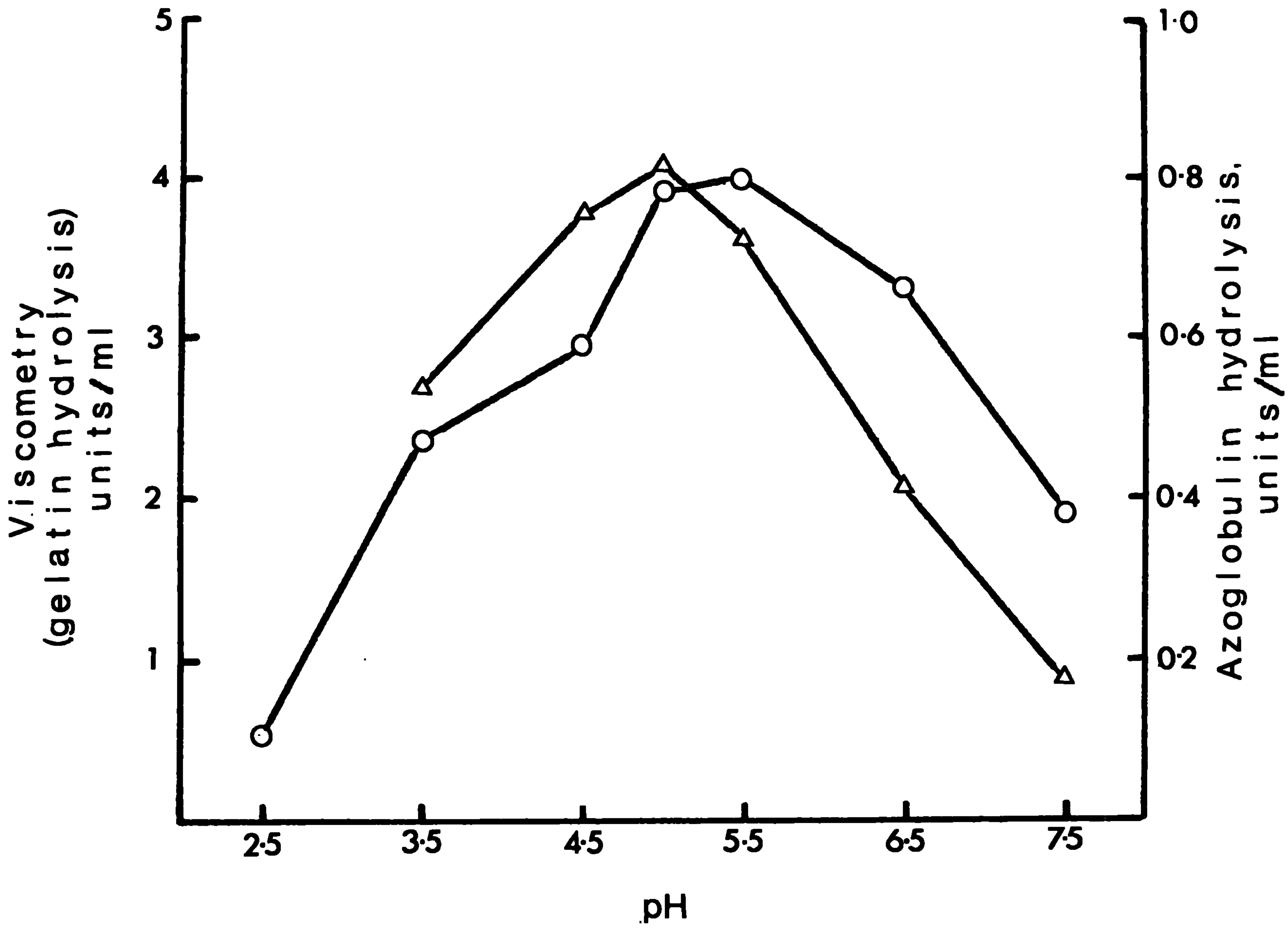
(b) Effect of selected compounds. The inclusion of 2-mercaptoethanol at 1% (v/v) final concentration in the assays had a marked effect on protease activity. With all 3 assays, increased activity occurred, although in the azoglobulin hydrolysis assay there was an apparent reduction in the amount of E_{430} - absorbing material that was solubilised. This was caused by the reduction, over the course of an hour, of the extinction

Fig. 43

The effect of pH on proteolytic activity, using azoglobulin and gelatin as substrates. For the azoglobulin assays, 0.1M sodium phosphate, 0.1M citric acid buffers were prepared, at 35°C, at the required pH; for the viscometric assay, the gelatin substrate was dissolved in 0.05M sodium phosphate, 0.05M citric acid and the pH was adjusted to the required value, at 35°C, prior to assay. Results are expressed as units per ml.

Substrate : Azoglobulin △———△

Gelatin ○———○


Viscometry
(gelatin hydrolysis)
units/ml
5
4
3
2
1
2·5
3·5
4·5
5·5
6·5
7·5
pH
1·0
0·8
0·6
0·4
0·2
Azoglobulin hydrolysis,
units/ml

coefficient of azoglobulin to a stable value of 40% of the initial value. 2-mercaptoethanol had no effect on the extinction coefficient of azoalbumin.

Table 12(a) compares the protease activity, measured with the 3 substrates, in the presence and absence of 2-mercaptoethanol, at different stages of germination. To ease comparison, the azoglobulin hydrolysis values in the presence of 2-mercaptoethanol have been scaled up to compensate for the change in the extinction coefficient. Also shown for the azo-substrates are the percentages of the total amount of substrate present that have been solubilised (calculated from the extinction coefficients). A greater proportion of the azoglobulin substrate was solubilised than of the azoalbumin.

Although it does not seem to apply to the azoglobulin assay, the influence of 2-mercaptoethanol in the other two assays increases during germination. This is clearly shown in Table 12(b), where the data presented in Table 12(a), is replotted as the ratio of activity in the presence and absence of 2-mercaptoethanol.

The influence of N-ethylmaleimide (NEM) and phenyl-methyl sulphonyl fluoride (PMSF) on the azoalbumin hydrolysing activity are shown in Table 13. Results are expressed as percentages of the control incubation, for each age, in the presence of water in place of the inhibitor. PMSF apparently reduces azoglobulin hydrolysis by a similar amount throughout germination whereas the effect of NEM decreased later in germination; however, the determination was not carried out in the presence of 2-mercaptoethanol.

TABLE 12

The effect of 2-mercaptoethanol on protease activity

Table 12(a). The effect of 2-mercaptoethanol on protease activity, measured by viscometry and azoglobulin and azoalbumin hydrolysis. Results are expressed as units of activity per ml, in the presence and absence of 1% (v/v) 2-mercaptoethanol. The figures in parentheses for the determinations of hydrolysis of the azosubstrates by the extracts of 4 day and 8 day germinated cotyledons are the percentages of the total amount of substrate present that have been solubilised.

Table 12(b) The ratio of protease activity in the presence of 2-mercaptoethanol to that in the absence of 2-mercaptoethanol.

TABLE 12

The effect of 2-mercaptoethanol on protease activity

Table 12(a)

Age, in days	Substrate					
	Azoglobulin		Azoalbumin		Gelatin(Viscometry)	
	Without	With	Without	With	Without	With
0	0.03	N.D.	0.18	0.31	N.D.	N.D.
2	0.21	0.32	0.52	1.06	0.55	1.05
4	0.33 (7.0%)	0.53 (11.0%)	0.77 (1.1%)	2.25 (3.3%)	0.89	3.62
6	0.44	0.77	1.31	5.0	N.D.	N.D.
8	0.52 (10.9%)	0.84 (17.7%)	2.24 (3.3%)	8.65 (12.7%)	0.73	4.16
10	0.49	0.87	2.14	8.75	0.25	2.16

Table 12(b)

Age (days)	Substrate		
	Azoglobulin	Azoalbumin	Gelatin
0	N.D.	1.71	N.D.
2	1.51	2.04	1.91
4	1.60	2.91	4.06
6	1.74	3.83	N.D.
8	1.63	3.87	5.78
10	1.77	4.1	8.65

N.D. Not determined

TABLE 13

Influence of NEM and PMSF on azoalbumin hydrolysing activity

	Age, in days, from Onset of Germination				
	2	4	6	8	10
Treatment					
5mM PMSF	49%	43%	N.D.	48%	47%
10mM NEM	43.5%	52.0%	N.D.	80.4%	78%

Results are expressed as percentages of the uninhibited activity, for each age. PMSF was dissolved (at 50mM) in isopropanol, and used at 5mM during the incubation; activity was compared with that of controls containing 10% (v/v) isopropanol. NEM was dissolved in water; the concentration used during incubation was as shown (10mM); activity was compared with water controls.

(c) Inhibition of azoalbumin hydrolysis by seed extract. The inclusion of extracts of ungerminated and 2 day germinated cotyledons in the assay of extracts from cotyledons germinated for longer periods of time reduced the protease activity. Table 14 shows the effect of adding extract of 2 day germinated seed to the assay of 4 day and 8 day germinated material, using azoalbumin as substrate. There is progressive reduction of activity of 8 day extracts by increasing amounts of 2 day extracts, but inhibition of the proteolytic activity of 4 day extracts only becomes apparent on addition of 0.5 ml of 2 day extract. In the presence of 2-mercaptoethanol slightly less inhibition occurred, as shown for the 8 day extract. The addition of heated (75°C for 60 min) 4 day extract, itself inactive, had no effect on activity of ungerminated and 2 day germinated extracts.

E. Analysis of Protein Bodies during Germination.

Protein bodies were isolated on linear sucrose gradients from germinating seeds in the same way as for cotyledons soaked for 16 h. Fig. 44 shows the fractionation profiles of 50 - 90% sucrose gradients in which extracts of 16 h imbibed, and 3 and 6 day germinated seeds have been centrifuged. The white band seen below the sample region in gradients in which extracts of cotyledons imbibed for 16 h have been centrifuged was no longer apparent in gradients from 3 - 4 day germinated seeds. After 6 days no protein bodies can be isolated for analysis. Fig. 45 presents densitometric traces of SDS gels in which protein bodies isolated from 16 h and 3 day germinated

TABLE 14

Effect of extracts of 2 day germinated cotyledons on the azoalbumin hydrolysing activity of extracts of 4 day and 8 day germinated cotyledons.

Sample	Net ΔE_{440}	ΔE_{440} after deduction of absorption attributable to activity of 2 day extract	
0.2ml 2d alone	0.018	0	% of activity of 4d/8d extract alone
0.5ml 2d alone	0.036	0	
0.5ml 4d alone	0.034	0.034	-
0.5ml 4d + 0.1ml 2d	0.044	0.035	103%
0.5ml 4d + 0.2ml 2d	0.055	0.037	109%
0.5ml 4d + 0.5ml 2d	0.058	0.022	65%
0.5ml 8d alone	0.148	0.148	-
0.5ml 8d + 0.1ml 2d	0.124	0.115	77%
0.5ml 8d + 0.2ml 2d	0.111	0.093	63%
0.5ml 8d + 0.5ml 2d	0.098	0.062	42%
In the presence of 2-mercaptoethanol			
0.5ml 8d alone	0.525	0.525	-
0.5ml 8d + 0.2ml 2d	0.405	0.370	70%

Results are expressed as the net change in absorbance (1 cm light path) at 440 nm. 2d, 4d refer to the extracts from cotyledons of seeds allowed to germinate for 2 days, 4 days etc.

Fig. 44

Preparation of protein bodies in sucrose gradients from cotyledons of germinating seeds. Fractionation profiles are presented of 50 - 90% (w/v) sucrose density gradients in which extracts from cotyledons soaked for 16 h and from those allowed to germinate for 3 and 6 days had been centrifuged. Absorbance was monitored at 280 nm. For clarity, the sucrose concentration, which was similar to that shown in Fig. 21, has been omitted.

16 h soaked cotyledons	————————
3 day germinated cotyledons	—— —— —— ——
6 day germinated cotyledons	---------------

PB Protein bodies

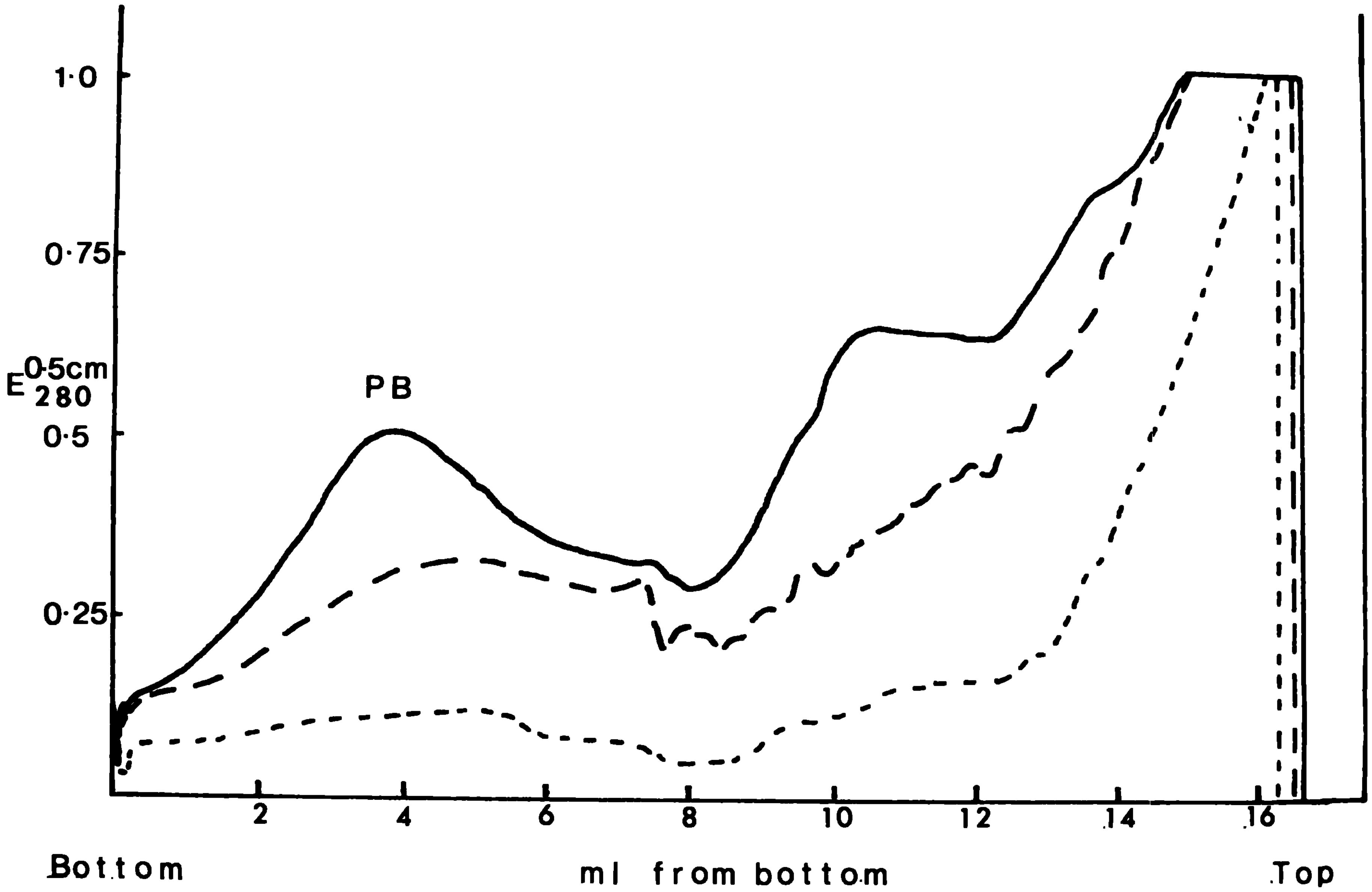
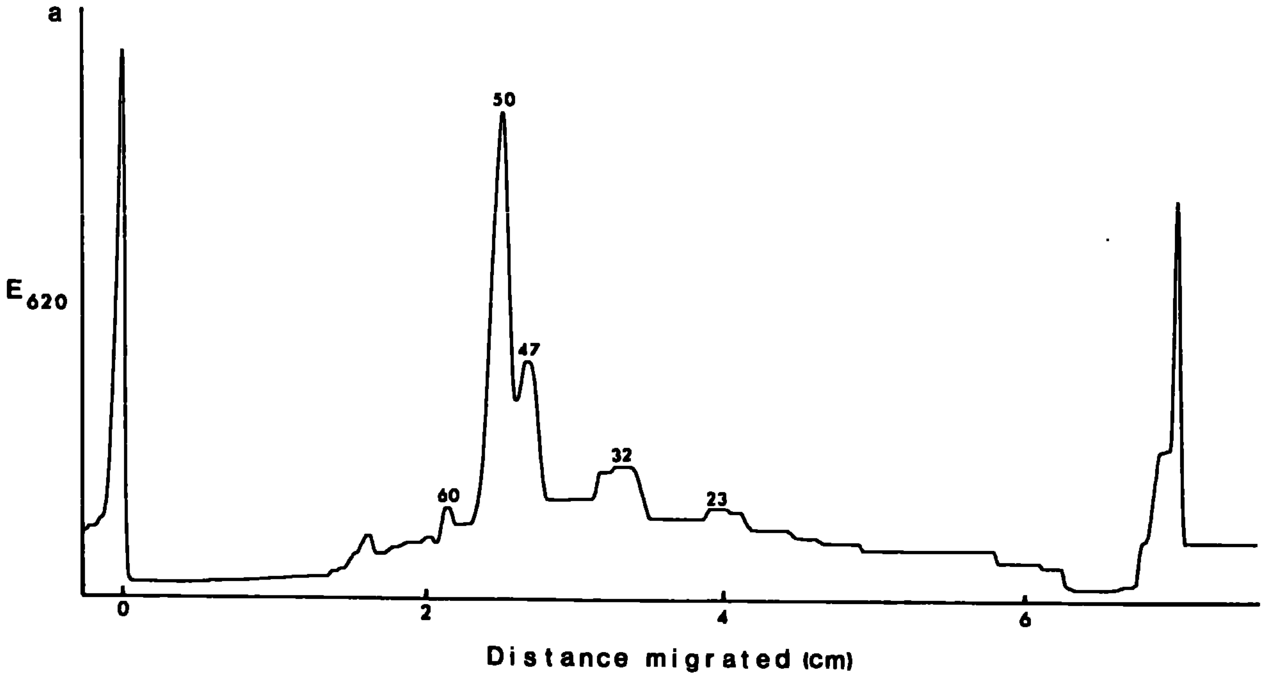

1·0
0·75
$E_{280}^{0·5cm}$
0·5
0·25
PB
2
4
6
8
10
12
14
16
Bottom
ml from bottom
Top

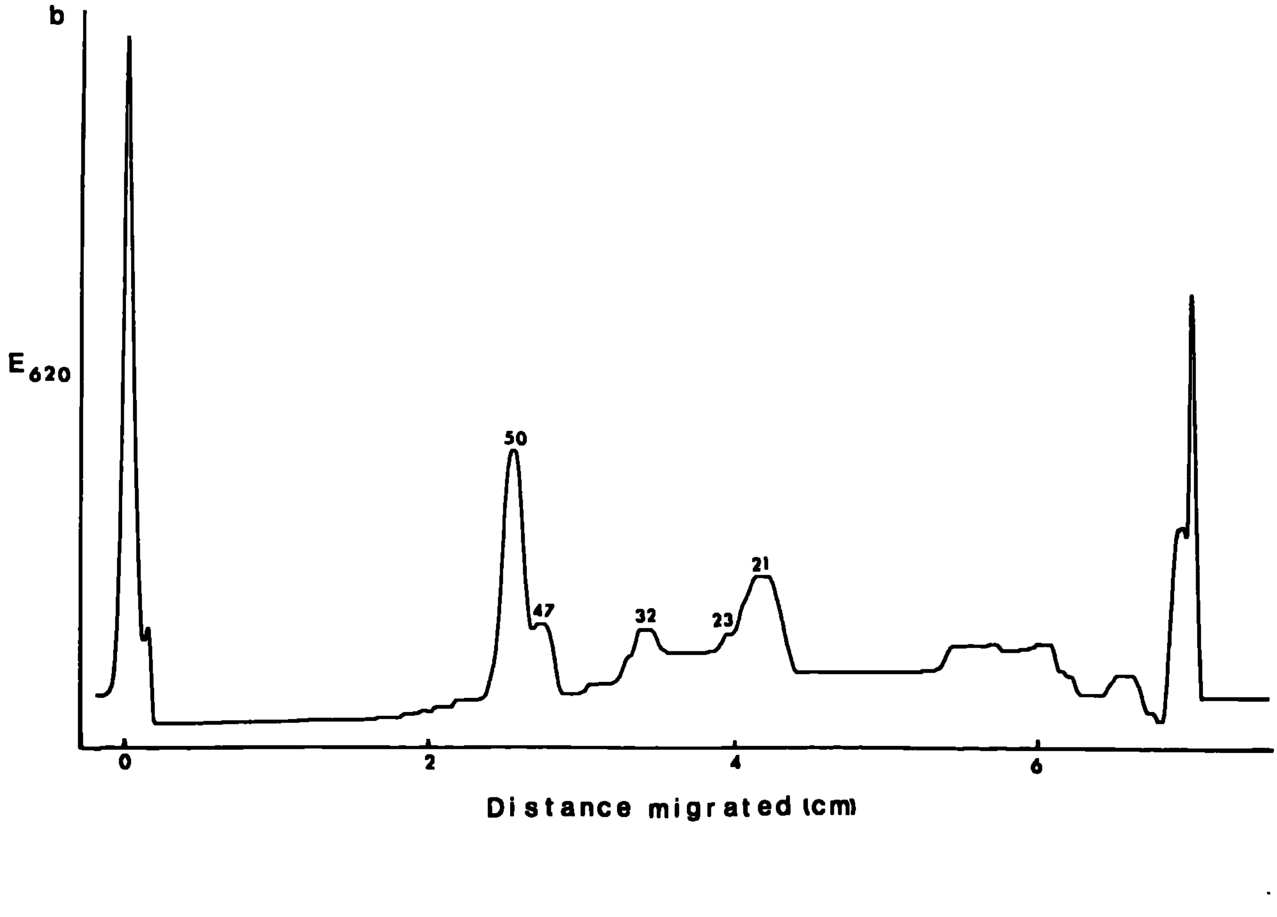
Fig. 45

SDS gel electrophoretic analysis of protein bodies prepared by sucrose density gradient centrifugation of extracts of cotyledons soaked for 16 h and also from the cotyledons of seeds allowed to germinate for 3 days. 7.0% (w/v) acrylamide gels were used. The molecular weights (x 10^{-3}) of the resolved peaks are presented.

(a) Protein bodies from 16 h soaked cotyledons

(b) Protein bodies prepared from the cotyledons of seeds allowed to germinate for 3 days.


a
E620
50
47
60
32
23
0
2
4
6
Distance migrated (cm)


b
E620
50
47
32
23
21
0
2
4
6
Distance migrated (cm)

seeds have been electrophoresed; similar analyses of the corresponding sample regions, together with this region from the 6 day germinated preparation, are shown in Fig. 46. The low molecular weight component (molecular weight 21,000) prominent later in germination was found to be present both in protein bodies and in the sample layer.

F. Localisation of Enzymes.

(i) 'In vitro' storage protein hydrolysis, analysed by SDS-polyacrylamide gel electrophoresis.

Incubation experiments were carried out to try to determine the origin of the 21,000 molecular weight component. Samples from sucrose density gradient centrifugation of extracts from 16 h soaked and 3 day germinated cotyledons were incubated (at pH 5.5 and 30°C) in the presence of sodium azide; changes in subunit composition were determined by SDS gel electrophoresis. Sample treatments and the results obtained are presented in Table 15. Only those samples containing the sample layer from sucrose gradients in which extracts of cotyledons germinated for 3 days had been centrifuged showed any changes. Fig. 47 presents densitometric traces of the subunit patterns on SDS gels of samples taken from the incubation of protein bodies and the sample region from a gradient in which an extract from cotyledons germinated for 3 days had been centrifuged; the samples were taken after 0, 20 and 48 h incubation, and changes resemble those seen in extracts from seeds allowed to germinate for increasing periods of time. Similar results were obtained when the sample region from the gradient in which cotyledons allowed to germinate for 3 days had been centrifuged were incubated with cryoprotein obtained from a water extract of meal (Fig. 48).

Fig. 46

SDS gel electrophoretic analysis of the sample regions from 50 - 90% (w/v) sucrose density gradients. The gels were 7.0% (w/v) acrylamide and molecular weights (x 10^{-3}) are presented as before.

a) The sample region from a gradient in which an extract from 16 h soaked cotyledons had been centrifuged.

b) The sample region from a gradient in which an extract from cotyledons of seeds allowed to germinate for 3 days had been centrifuged.

c) The sample region from a gradient in which an extract from cotyledons of seeds allowed to germinate for 6 days had been centrifuged.

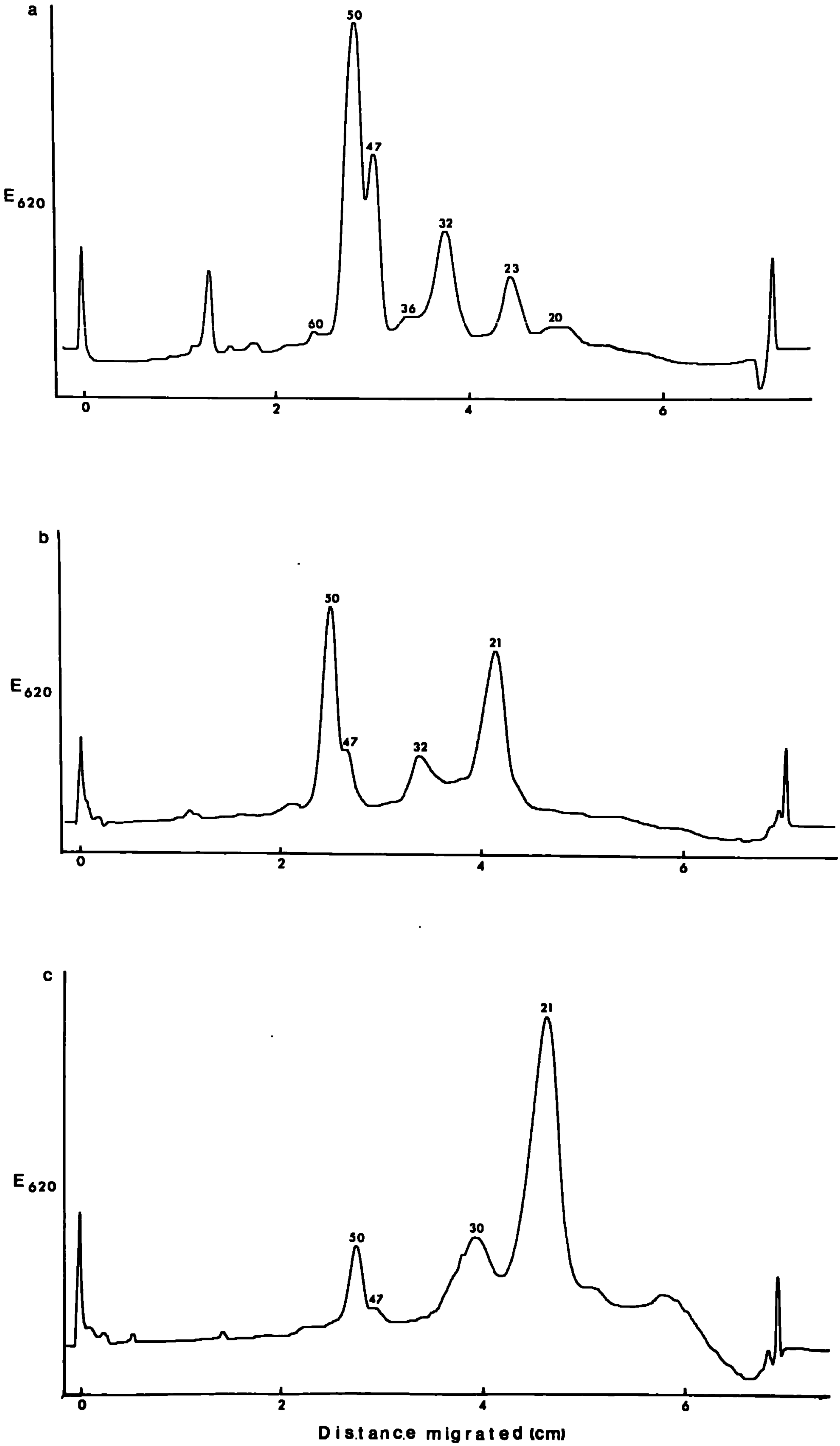

a
E620
50
47
32
36
23
20
60
0
2
4
6
b
E620
50
47
32
21
0
2
4
6
c
E620
21
50
47
30
0
2
4
6
Distance migrated (cm)

TABLE 15

Sample treatments and the results of '*in vitro*' storage protein hydrolysis. Incubations were at pH 5.5 and 30°C, as described in Methods, Section XIII D(iv).

Incubation	Result
0 day protein bodies, alone	No detectable change
0 day sample region, alone *or* 0 day sample region + 0 day protein bodies	No detectable change
3 day protein bodies	No detectable change
3 day sample region, alone *or* 3 day sample region + 0 day protein bodies *or* 3 day sample region + 3 day protein bodies	Increase in 21,000 molecular weight subunit. Decrease in 50,000 and 47,000 molecular weight subunits and of the diffuse, low molecular weight material.

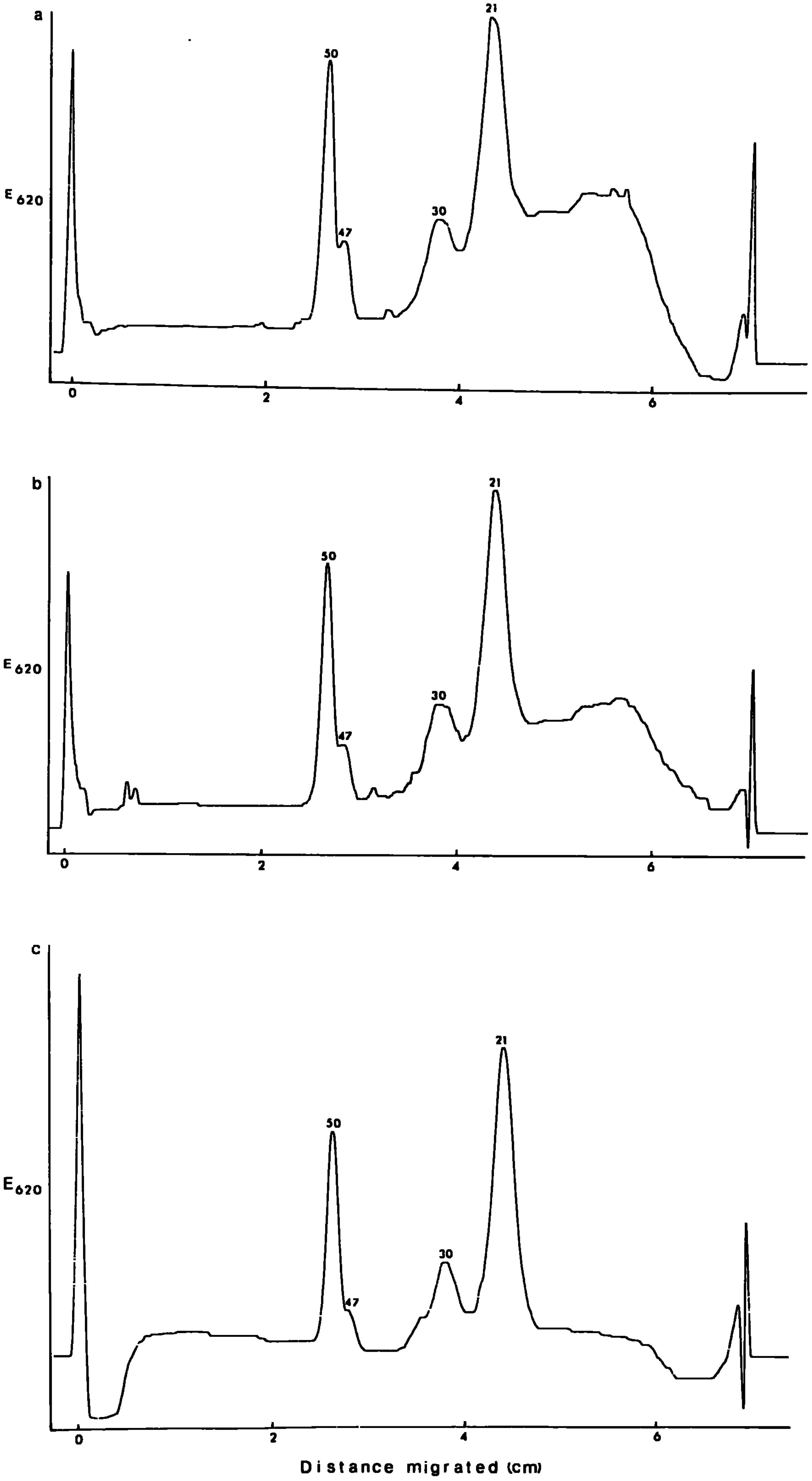
Fig. 47

SDS electrophoretic analysis of in vitro storage protein hydrolysis. An extract from cotyledons of seeds allowed to germinate for 3 days was fractionated by centrifugation in a 50 - 90% (w/v) sucrose density gradients. One ml samples of the protein body fraction and the sample region were incubated together at pH 5.5 and 30°C, in the presence of 0.06% (w/v) sodium azide. Samples were withdrawn at the start of the incubation and after 20 and 48 h and analysed by SDS gel electrophoresis in 7.0% (w/v) gels.

a) Sample taken at start of incubation.

b) Sample taken after 20 h incubation.

c) Sample taken after 48 h incubation.


a
E 620
50
47
21
30
0
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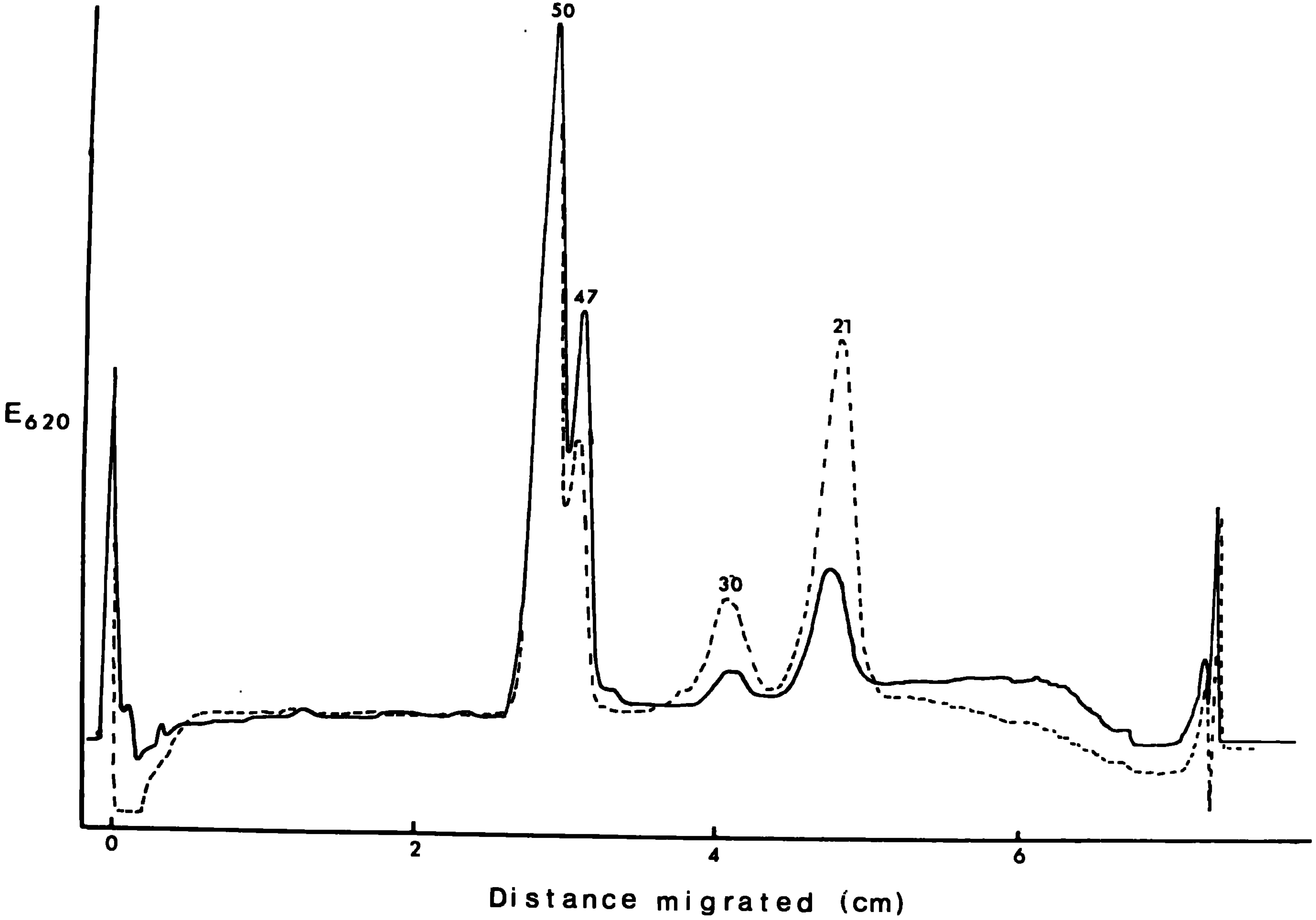
4
6
b
E 620
50
47
21
30
0
2
4
6
c
E 620
50
47
21
30
0
2
4
6
Distance migrated (cm)

Fig. 48

SDS electrophoretic analysis of *in vitro* storage protein hydrolysis. One ml of the sample region from a 50 - 90% (w/v) sucrose density gradient in which an extract from cotyledons of seeds allowed to germinate for 3 days had been centrifuged was mixed with 1 ml of a 1% (w/v) solution of cryoprotein (from a water extract of seed meal) and incubated at pH 5.5 and 30°C in the presence of 0.06% (w/v) sodium azide. Samples were withdrawn and analysed by SDS gel electrophoresis in 7.0% (w/v) gels.

Sample taken at the start of incubation ————————

Sample taken after 48 h incubation - - - - - - - - - -


50
47
30
21
E$_{620}$
0
2
4
6
Distance migrated (cm)

(ii) Protease activity.

In the viscometric assay, only the sample layer of sucrose gradients of extracts of 2 day germinated cotyledons contained activity; no change in viscosity was obtained with 2 day germinated protein bodies. Similarly, in azoglobulin assays of protein bodies and sample layers from 3 and 4 day germinated cotyledons, activity could only be found in the sample layer.

(iii) BAPNA-ase, Leucine amino peptidase and trypsin inhibitor.

The distribution of BAPNA-ase, leucine amino peptidase, and trypsin inhibitor activity between protein body and supernatant/sample layer fractions, for the two methods of preparation, are shown in Table 16. The sucrose gradient fractions are from 16 h soaked cotyledons, while those from the glycerol method were from dry meal. After both preparative methods it is apparent that the majority of all three activities is in the supernatant fraction. The crucial comparison, however, is of the specific activities. The ratios of the specific activities confirm that leucine amino peptidase is almost entirely in the supernatant, that BAPNA-ase is mainly in the supernatant, and that the location of the trypsin inhibitor is apparently dependant on the method of preparation. Protein bodies prepared by sucrose density gradient centrifugation had a higher specific activity than the sample layer region, whereas after preparation in glycerol the specific activity was markedly higher in the supernatant.

TABLE 16

The distribution of BAPNA-ase, leucine amino peptidase and trypsin inhibitor activity in protein body and supernatant fluid/sample region fractions, prepared by the two methods used to isolate protein bodies.

Notes		
	SN	Supernatant fluid
	PB	Protein bodies

In the results presented in Table 16(a), 88% of the BAPNA-ase present in the initial extract, prior to separation into protein bodies and supernatant fluid, was subsequently recovered in these fractions. Of the total protein extracted, 68.6% was recovered in the supernatant fraction. Protein concentration was estimated by the method of Lowry *et al.*, (1951).

TABLE 16

Table 16(a) Fractions prepared by the glycerol method, at pH 5.0.

Assay	Fraction	Units/ ml	Vol. ml	Total Units	% of Total	Protein Conc. mg/ml	Specific Activity	Ratio of Specific Activities
BAPNA-ase	SN	24.8	28	694	91.3	8.9	2.79	4.75
	PB	2.2	30	66	8.7	3.8	0.58	
Leucine amino peptidase	SN	388	28	10850	97.0	8.9	43.6	14.8
	PB	11.3	30	338	3.0	3.8	2.96	
Trypsin Inhibitor	SN	1040	28	29150	95.7	8.9	117.4	10.3
	PB	43.3	30	1300	4.3	3.8	11.4	

Table 16(b) Fractions prepared by sucrose density gradient centrifugation.

Assay	Fraction	Units/ml	Protein mg/ml	Specific Activity	Ratio of Specific Activities
BAPNA-ase	Sample region	20.4	12.5	1.63	2.78
	PB	0.54	0.92	0.59	
Leucine amino peptidase	Sample region	515	12.5	41.2	12.07
	PB	3.13	0.92	3.4	
Trypsin Inhibitor	Sample region	735	12.5	58.8	0.69
	PB	78.5	0.92	85.3	

DISCUSSION

A number of extraction and fractionation procedures were employed to determine the number of different storage proteins present in seeds of Phaseolus vulgaris cv. 'Seafarer',and their relative importance. These methods were a combination of the classical procedures of precipitation by dilution, dialysis or at the isoelectric point, and more modern procedures, based on separation by size (for example, zonal centrifugation and gel filtration) and charge (ion-exchange chromatography), neither of which result in insolubilisation of the proteins. It seems worthwhile considering the status of the results obtained and the methods used in detail, in order to attempt to identify the fractions obtained in this investigation with those prepared by previous workers.

Disc electrophoretic analysis of alkaline salt extracts revealed one major, poorly defined band, with several other also poorly defined bands. SDS gel elctrophoresis demonstrated the presence of two major components with molecular weights 50,000 and 47,000, together with four other clearly defined subunits, molecular weights 60,000, 32,000, 23,000 and 20,000. From the results obtained four protein fractions can be defined. The predominant protein present, representing the major disc electrophoretic band, 60-70% of the total protein and composed of the 50,000 and 47,000 molecular weight subunits, was soluble at pH 4.7 and, as described by Derbyshire and Boulter (1975), sedimented as a 7S protein at pH 7.0 (thus, on the basis of size and solubility at pH 4.7, it is a vicilin-type protein). At pH 6.2 it partially associated to an 18S form. Association of this kind was previously observed to occur with the major component of several cultivars of

Phaseolus vulgaris by Joubert (1957), either by reducing the ionic strength at pH 7.0 (from I = 0.31 to I = 0.11) or by reducing the pH to 6.2; the process was reversible. This association behaviour was a characteristic of the protein Glycoprotein II isolated by Pusztai and Watt (1970) from Phaseolus vulgaris; the major glycoprotein of Phaseolus vulgaris isolated by Racusen and Foote (1971), representing about 46% of the soluble protein, was equated by them with Glycoprotein II on the basis of its amino acid composition and sedimentation coefficient. The amino acid compositions of the major pH 4.7 soluble fraction, composed of subunits of molecular weight 50,000 and 47,000, prepared in this investigation, and of Pusztai and Watt's Glycoprotein II, recalculated to mole %, are presented in Table 17. In general, there is good agreement, particularly in view of the differences known to occur between different cultivars. On the basis of sedimentation coefficient and association behaviour, amino acid composition, and similarity with the disc electrophoretic pattern described by Racusen and Foote (1971), the protein prepared here is considered to be equivalent to the Glycoprotein II isolated by Pusztai and Watt (1970) and will be referred to as glycoprotein II. Pusztai and Watt (1970) determined the subunit molecular weight as 35,000 - 40,000 by gel filtration in 5M guanidinium hydrochloride; no estimates of subunit molecular weight by SDS gel electrophoretic analysis have previously been published.

Three N-terminal amino acid residues were found for this preparation of glycoprotein II, serine, leucine and threonine; these amino acids, together with lysine, were the most prominent

TABLE 17

Comparison of the amino acid composition , expressed as moles %, of the major storage protein of *Phaseolus vulgaris* cv. 'Seafarer', composed of subunits of molecular weight 50,000 and 47,000, with that of Glycoprotein II prepared by Pusztai and Watt (1970).

Amino acid	Amino acid composition, mole %	
	50,000 and 47,000 Mol.wt. subunits	Glycoprotein II (Pusztai and Watt 1970)
Aspartic acid	14.8	13.4
Threonine	3.8	4.2
Serine	7.1	9.6
Glutamic acid	16.1	14.6
Proline	3.6	3.7
Glycine	6.1	5.8
Alanine	5.2	5.2
Cysteine	0.4	0.2
Valine	5.9	6.5
Methionine	0.8	0.7
Isoleucine	5.3	6.2
Leucine	9.4	10.0
Tyrosine	2.6	2.7
Phenylalanine	5.4	5.6
Histidine	2.5	2.4
Lysine	6.3	5.4
Arginine	4.7	4.0

N-terminal amino acids found by Bailey and Boulter (1972) for vicilin from *Vicia faba*; aspartic acid and serine were the predominant N-terminal amino acids of vicilin from *Pisum sativum* (Grant and Lawrence, 1964) but as with vicilin from *Vicia faba* other amino acid residues were present. A purified 7S globulin from *Glycine max* contained eight different N-terminal amino acids (Koshiyama, 1968a).

A second fraction, insoluble at pH 4.7 and thus a legumin-type protein, was prepared by isoelectric precipitation. While impure preparations, still containing glycoprotein II subunits when analysed by SDS gel electrophoresis, could be prepared by repeated manual isoelectric precipitation (Bailey and Boulter, 1970), markedly purer preparations were prepared by the zonal isoelectric precipitation procedure described by Shutov and Vaintraub (1965) and Wright and Boulter (1974). This is a modification of the zonal solubilisation of ammonium sulphate precipitates in columns of Sephadex gel, first described by Porath (1962), and now more widely used in conjunction with Celite (King, 1972). The sample is chromatographed on a Sephadex gel which completely excludes the proteins; those that are soluble in the buffer in which the gel has been equilibrated (pH 4.7) migrate into buffer of this pH and are eluted at and close to the void volume of the column; those proteins which are insoluble in this buffer are subjected to a transitory insolubilisation/solubilisation cycle as they migrate and are eluted at and following the pH interface between the two buffers used. The frequency of the cyclic insolubilisation probably accounts for the increased purity obtained. In the manual procedure, considerable losses occur because the precipitate is only partially soluble; the presence of 2-mercaptoethanol only partially alleviates this problem (Nash and Wolf, 1967); this insolubility may be caused by the production, during the manual procedure, of local regions of low pH, which do not occur with the zonal procedure; it is also probably because of the increased duration for which the protein is insolubilised.

Subunit analysis by SDS gel electrophoresis in the presence of 2-mercaptoethanol showed this fraction to be composed of two subunits, with molecular weights 60,000 and 20,000; in the absence of 2-mercaptoethanol only one component, molecular weight about 80,000, was found; this behaviour was observed whether the legumin examined had been prepared in the presence or absence of 2-mercaptoethanol: it is therefore considered likely that the subunits of this fraction are disulphide-bonded *in vivo*. This fraction has been described in greater detail by Derbyshire and Boulter (1975): in the ultracentrifuge it sedimented as an 11S; breakdown of the 60,000 molecular weight subunit was observed, yielding two subunits of molecular weight 37,000 and 34,000; in this respect it more closely resembles the legumin prepared by zonal isoelectric precipitation from *Vicia faba* (Wright and Boulter, 1974).

The amino acid composition, in moles %, of the fraction described here is compared in Table 18 with legumin of *Vicia faba* (Wright and Boulter, 1974) and from *Phaseolus vulgaris* cv 'Streamline' (Derbyshire and Boulter, 1975). The major difference with the legumin from *Vicia faba* is in the arginine content, which is markedly lower in legumins prepared from *Phaseolus vulgaris*; these are also lower in arginine content than legumin from *Pisum sativum* (Grant and Lawrence, 1964), but this legumin is similar in other respects to the legumin prepared from *Phaseolus vulgaris* cv. 'Seafarer', particularly in the higher ratio of glutamic acid to aspartic acid in legumin, compared with vicilin, and the proportion of the total amino acid composition that these amino acids represent (30.2% in *Phaseolus vulgaris* cv. 'Seafarer' legumin, 32.0% in

TABLE 18

The amino acid compositions, expressed as moles %, of legumin prepared from *Phaseolus vulgaris* cv. 'Seafarer' (this investigation), that prepared from *Phaseolus vulgaris* cv. 'Streamline' (Derbyshire and Boulter, 1975) and legumin from *Vicia faba* (Wright and Boulter, 1974).

Amino acid	Amino acid composition, mole %		
	Legumin, *Phaseolus Vulgaris* cv. Seafarer	Legumin *Phaseolus vulgaris* cv. Streamline (Derbyshire and Boulter, 1975)	CM-Legumin *Vicia faba* (Wright and Boulter,1974)
Aspartic acid	11.9	9.5	12.9
Threonine	4.1	5.0	3.3
Serine	6.6	7.2	6.6
Glutamic acid	18.3	12.6	18.0
Proline	6.5	5.0	6.2
Glycine	4.7	8.0	6.9
Alanine	5.5	6.9	5.9
Cysteine	N.D.	0.6	N.D.
Valine	6.9	7.1	5.2
Methionine	0.8	1.4	0.8
Isoleucine	4.1	5.1	4.1
Leucine	8.2	8.6	8.1
Tyrosine	2.6	2.9	2.7
Phenylalanine	2.9	4.4	3.4
Histidine	3.7	2.9	2.0
Lysine	8.3	7.9	5.0
Arginine	4.9	4.8	9.1

that from *Pisum sativum*).Only two N-terminal amino acid residues (glycine and threonine)were found in this legumin preparation, compared with the three (glycine, leucine and threonine) found in *Vicia* legumin. However, Derbyshire and Boulter (1975) describe four N-terminal amino acids (the three previously mentioned, together with methionine); the presence of only two in this preparation may be related to the apparently greater stability of the 60,000 molecular weight component: this was not found to be altered by reduction and carboxymethylation in 8M urea. In a comparison of different species of *Lupinus* and cultivars of *Lupinus angustifolius*, Gillespie and Blagrove (1975) reported differences in the apparent importance of disulphide bonding between subunits of the same fractions from different sources; a similar situation may be responsible for the different results obtained here.

A second fraction soluble at pH 4.7 was obtained as non-adsorbed material during ion-exchange chromatography on DEAE-cellulose; it was composed of subunits of molecular weight about 32,000 which stained for glycoprotein. During sucrose density gradient centrifugation it was sedimented at a similar or slightly slower rate than glycoprotein II. Fractions containing this subunit (for example, the F-II fractions from the acidic extracts) agglutinated erythrocytes.

The amino acid composition of this fraction is compared in Table 19 with that of the lectin (subunit molecular weight 30,000 - 32,000) isolated by Andrews (1974) from *Phaseolus vulgaris*; there is good agreement especially with respect to the low content of glutamic acid as compared to the other storage proteins, and marked difference to the second protein prepared by Andrews (1974), called Component 1; the amino acid composition of this protein is also included in Table 19.

TABLE 19

The amino acid compositions, expressed as moles %, of the non-adsorbed protein from ion-exchange chromatography, composed of subunits of molecular weight 32,000, and of the two proteins prepared by Andrews (1974): a lectin (subunit molecular weight 30,000 - 32,000) and Component 1 (subunit molecular weight 36,000 - 39,000).

Amino acid	Amino acid composition, mole %		
	32,000 mol.wt. subunit(s)	'Lectin' sub-unit mol. wt. 30,000-32,000	Component 1 subunit mol. wt., 36,000 - 39,000
		(Andrews, 1974)	
Aspartic acid	14.7	15.5	13.5
Threonine	8.1	9.5	3.6
Serine	8.2	10.7	8.9
Glutamic acid	7.3	6.4	15.8
Proline	4.6	4.1	3.8
Glycine	8.0	7.7	6.3
Alanine	7.1	6.7	5.2
Cysteine	0.8	Trace	0
Valine	7.2	8.1	6.1
Methionine	0.3	0	0.8
Isoleucine	5.4	4.9	5.2
Leucine	10.6	9.1	9.8
Tyrosine	2.7	2.7	2.8
Phenylalanine	5.5	6.1	5.8
Histidine	1.3	1.6	2.5
Lysine	5.3	4.7	5.7
Arginine	2.9	2.1	4.2

All four haemagglutinating fractions prepared by Jaffé and Hannig (1965) had similarly low glutamic acid contents, compared to other fractions. Pusztai and Watt (1974) have recently separated and partially characterised a number of agglutinins from Phaseolus vulgaris which differ in their isoelectric points but consist of subunits of about 32,000 molecular weight and are also characterised by low glutamic acid contents. Thus, although it is apparent that the fraction described here represents an agglutinin, its precise relationship to the isolectins described by Pusztai and Watt (1974) is not clear. Similarly, while the erythroagglutinating fraction purified by Weber et al. (1972) had an isoelectric point of 6.5 (and thus would not be adsorbed during ion exchange chromatography at pH 7.0-7.5), isoelectric focusing in 8M urea resulted in the appearance of several bands; in this case the subunits were estimated to have molecular weights of 36,000.

The presence of at least a fourth protein is suggested by the occurrence of subunits of molecular weight 23,000 which cannot be assigned to any of the three previously described proteins. After sucrose density gradient centrifugation of alkaline salt extracts, the majority of the subunits of this size were recovered in fractions close to the sample region, but a small amount, as judged by the SDS gel patterns, sedimented to the same position in the gradient as the glycoprotein II subunits. Similarly, during ion exchange chromatography, some subunits of this size were recovered from the leading edge of the adsorbed peak, but they were also eluted at a markedly higher salt concentration. On the basis of the results from ion exchange chromatography, the agglutinin fraction described possessed the highest isoelectric point(s); legumin had a lower isoelectric point than at least part of the glycoprotein II fraction (as judged by the elution of the 50,000 and 47,000 molecular weight subunits) and the 23,000 molecular weight subunits had a lower isoelectric point than the legumin fraction.

The protein composed of subunits of molecular weight 23,000 was not isolated and characterised. It was not possible to completely remove traces of subunits of this size from glycoprotein II, and direct analysis of the main glycoprotein II band from disc gel electrophoresis showed subunits of molecular weight 23,000 to be present; they were not, however, present in stoichiometric quantities, as judged by staining intensity, and this subunit is not considered to be a subunit of glycoprotein II.

The identity of the two proteins prepared from seeds of Phaseolus vulgaris by Osborne (1894) is not clear; the most abundant, phaseolin (accounting for about 20% of the seed), was prepared by a variety of dilution and dialysis procedures and was a 'typical' globulin; it is probably equivalent to glycoprotein II. The second component, phaselin, was not completely precipitated even by prolonged dialysis in distilled water.

By acidification of an extract to pH 5.5 and addition of ethanol (50%, v/v) Bourdillon (1949) prepared a crystalline protein, in a yield of 4g per 100 g meal, which, by osmotic pressure measurements, was estimated to have a molecular weight of 170,000 at pH 7.2 in 0.2M phosphate buffer; it was only precipitated by ammonium sulphate concentration of greater than 80% saturation. On the basis of its apparent isoelectric point, molecular weight, and solubility in ammonium sulphate solutions, this also is probably equivalent to glycoprotein II prepared by more modern procedures.

The pH of the extractant has a marked effect on the solubilisation of seed proteins. Smith and Circle (1938) showed that maximum extraction was achieved between pH 6.5 and 12, but that extraction was low in the pH range 3 - 5, coinciding with the isoelectric points of the storage protein; good solubilisation was achieved below pH 2.5. Fontaine _et al._ (1946) confirmed the low extractability of protein in the pH range 3.8 - 5.8, using peanut meal, and also showed that the presence of phytic acid in meals reduced the solubility of seed proteins at pH values below the isoelectric point of the proteins; pH 3.5 was found to be the optimum pH for the formation of protein-phytate complexes in peanut meal extracts. Phytic acid is present in _Phaseolus vulgaris_ seeds; Bourdillon (1951) prepared a crystalline protein preparation in combination with phytic acid. In general, the complexing of phytate with protein increases the problem of isolation of pure proteins, altering both the electrophoretic mobility as well as decreasing the solubility of the proteins (Smith and Rackis, 1957).

Low pH may also modify glycoproteins. Bourdillon's original protein preparation (Bourdillon, 1949) was extracted from meal with dilute hydrochloric acid, before raising the pH to 7.5 and then precipitating at pH 5.5; Bourdillon's preparation was very similar to a globulin prepared by Jaffé and Hannig (1965) by ammonium sulphate precipitation except that it contained less carbohydrate. Rigas and Osgood (1955) prepared a phytohaemagglutinin that was a glycoprotein at alkaline pH, but lost the carbohydrate moiety at pH 2.0.

van Megen (1974) and Sun and Hall (1975) have recently investigated and defined the conditions of pH and ionic strength for solubility of _Glycine max_; and _Phaseolus vulgaris_ globulins. These confirm earlier results with respect to the low extraction yields and solubility in the pH region of the isoelectric points at low ionic strength but demonstrate that high ionic strength markedly increases solubility of both unpurified globulins from soybeans and the partially purified proteins from Phaseolus vulgaris.

Smith et al. (1959) carried out a comprehensive survey of the effectiveness of different extractants for the extraction of nitrogenous compounds from seeds of a large number of species. In the case of Phaseolus vulgaris,water (at room temperature) was as effective as 0.5M NaCl, extracting 75% of the nitrogenous constituents of the seed meal. However, water is a poor extractant for some other legumes; Smith et al. (1959) could only extract 25% of the nitrogenous compounds of Lupinus angustifolius with water; Blagrove and Gillespie (1975) removed albumins by water extraction prior to the extraction of the globulins of this species. Osborne (1894) also noted that while salt soluble proteins were the predominant type in Phaseolus vulgaris seeds these same proteins could be extracted quite efficiently with water.

As mentioned in the Introduction, cooling water extracts of soybean meal to 0-4°C results in the formation of a cryoprecipitate which is largely composed of glycinin (the 11S, legumin-type globulin) (Briggs and Wolf, 1957). A substantial cryoprecipitate was obtained by cooling water extracts of meal of Phaseolus vulgaris, which was found to be almost entirely composed of glycoprotein II. Legumin remained in the supernatant fluid and could be obtained as a precipitate by dialysis against water. In contrast, overnight dialysis of an alkaline salt extract against running tap water resulted in virtually complete precipitation of legumin, while only part of the glycoprotein II was precipitated; similarly, in the fractionation of acidic extracts by dilution and dialysis all of the legumin was precipitated before at least part of the glycoprotein II.

Cryoprecipitation of protein has been observed before from extracts of Pisum sativum. Buzila (1967) demonstrated immunochemical and physico-chemical similarity between the cryoprotein and vicilin prepared by the method of Danielsson (1949); however, not all vicilin was cryoprecipitable. Although immunoelectrophoresis, using the Osserman procedure, showed complete antigenic identity, and there were no differences in mobility on electrophoresis between the cryoprotein and vicilin, the two fractions behaved differently during ion exchange chromatography on DEAE-cellulose; while about 80% of the cryoprotein was not adsorbed the majority (85%) of vicilin was adsorbed. Differences were detected in the molecular weights of the adsorbed and non-adsorbed fractions.

The main globulin component (a vicilin) of the common bean was also obtained as a cryoprecipitate from water extracts by Sayanova and Vysokos (1972); the legumin fraction remained in the supernatant fluid.

In this investigation, analysis of cryoprotein by ion-exchange chromatography on DEAE-cellulose and by centrifugation in sucrose density gradients showed no differences in the distribution of the characteristic subunits of glycoprotein II; they were eluted by the same sodium chloride concentration and were recovered from the same position in the gradient as those derived from an alkaline salt extract.

The use of acidic extraction has been advocated by Hall and his co-workers; ascorbic acid was incorporated because of its anti-oxidant properties (Polter and Müller-Stoll, 1970). Two fractions were obtained; the first, precipitated by the addition of two to five volumes of water (McLeester et al., 1973; Sun and Hall, 1975) and designated the G-1 fraction, was initially described as an 11S globulin and called legumin (McLeester et al., 1973), but was subsequently equated with Glycoprotein II on the basis of its sedimentation coefficient and association behaviour (Sun *et al.*, 1974); it was found to be composed of three subunits when analysed by SDS gel electrophoresis, with molecular weights 53,000, 47,000 and 43,000, which appear to be equivalent to the three main subunits obtained by SDS extraction and electrophoresis of *Phaseolus vulgaris* cv. 'Canadian Wonder'. The second fraction (G-2) obtained by dialysis of the supernatant fluid remaining after removal of the G-1 fraction, was composed of subunits of molecular weight 32,000, with a sedimentation coefficient of 5.8S. When analysed by disc electrophoresis, the G-1 fraction consisted of the main band seen in the total extract, Rm c 0.4, while the G-2 contained a number of bands, Rm 0.2 - 0.3.

When this procedure was repeated on the cultivar 'Seafarer', the first fraction precipitated, called the F-I, contained all of the subunits associated with the legumin fraction and a substantial amount of the glycoprotein II subunits. In the absence of ascorbate, the F-I fraction contained markedly less glycoprotein II, while still containing all of the legumin subunits. The F-II fraction was subdivided into protein precipitated by dialysis against running tap water and that subsequently precipitated by dialysis against distilled water.

It seems likely that the G-II fraction prepared by McLeester *et al.* (1973) is the same as the F-IIb fraction prepared in this investigation in the absence of ascorbate and represents agglutinins. The fact that McLeester *et al.* (1973) observed a number of bands on disc electrophoresis, while only one subunit size was obtained, would be compatible with this fraction representing a mixture of the isolectins described by Pusztai and Watt (1974).

It also seems likely that the G-1 fraction of McLeester *et al.* (1973) contains any legumin present in this cultivar (Tendergreen), and is thus an impure preparation of Glycoprotein II. Although variations in the amounts of different storage proteins occur between cultivars (for example Gillespie and Blagrove (1975) *Lupinus angustifolius*, and Adriaanse *et al.* (1969) *Phaseolus vulgaris*), it is unlikely that legumin is completely absent. Moreover, bands of low mobility on disc electrophoresis were present in the total globulin fraction which were not represented in the diagrams of the disc gels of the separated G-1 and G-2 fractions (McLeester *et al.*, 1973); the legumin fraction prepared from the cultivar 'Seafarer' had low mobility in disc electrophoresis.

Although acid extraction and fractionation can yield some purification, in view of the possibility of interactions with phytate (Fontaine *et al.*, 1946) and of deglycosylation (Rigas and Osgood, 1955) at acid pH, and the fact that the use of an acid extraction method with *Vicia faba* led to the formation of lower molecular weight polypeptides (Wright and Boulter, 1973), the method cannot be recommended for general use with legumes.

Centrifugation of solutions of seed proteins in sucrose density gradients has previously been used to separate legumin and vicilin of *Vicia faba* (Millerd *et al.*, 1971) and *Phaseolus aureus* (Ericson and Chrispeels, 1973); Hill and Breidenbach (1974a) separated soybean (*Glycine max*) proteins into 2S, 7S and 11S fractions by this method. Incomplete separation of the small amount of the 11S (legumin) from the preponderant 7S (glycoprotein II) was obtained in this case, partly because of the rather large sample volumes required to allow identification of separated components, and partly because of the very large proportion of glycoprotein II present in alkaline salt extracts. Better resolution was obtained when the amount of this component present was reduced.

During disc electrophoresis, glycoprotein II always migrated as a broad band, with diffuse edges, whereas electrophoresis of bovine serum albumin resulted in bands with very sharp leading edges. In the case of Phaseolus aureus vicilin, this diffuse pattern has been attributed (Ericson and Chrispeels, 1973) to microheterogeneity of the carbohydrate portion of the glycoprotein (Schmid, 1968). Blagrove and Gillespie (1975) separated α-conglulin from Lupinus angustifolius into cold insoluble and cold soluble fractions by allowing solutions to stand in phosphate buffer (pH 7.0) for 5 days at 4°C; these fractions differed very slightly in amino acid composition. Thus there may be heterogeneity of the protein portions of the glycoprotein as well; either or both of these types of heterogeneity might account for the elution of glycoprotein II over a rather broad range of sodium chloride concentration during ion exchange chromatography, and the diffuse band resulting from disc electrophoresis.

The results of gel isoelectric focusing in 7M urea of cryoprotein derived from a water extract also suggest microheterogeneity. Similar isoelectric focusing of glycinin from soybean resulted in the demonstration of more protein components than expected from the subunit composition based on molecular weights (Catsimpoolas, 1969; Catsimpoolas and Wang, 1971). The acidic subunits of legumin from Vicia faba, for which two N-terminal amino acids were determined, yielded 8 bands when analysed by gel isoelectric focusing in urea (Wright, 1973). It is not possible to say whether some of the components seen here are artefacts, for example caused by deamidation, which is known to occur at pHs above neutrality, especially in phosphate buffers (McKerrow and Robinson, 1971).

All gel isoelectric focusing experiments were carried out in the presence of urea, solutions of which are known to exist in equilibrium with cyanate ions, which can cause carbamylation of amino groups (Stark et al., 1960; Cejka et al., 1968); Bobb and Hofstee (1971) used isoelectric focusing to follow the successive carbamylation of the fourteen ϵ-NH_2 groups of lysine and the N-terminal-amino group present in chymotrysinogen A by heating in urea at 100°C. Cole and Mecham (1966) earlier observed progressive changes in the electrophoretic mobility of casein and wheat proteins as a result of incubation in urea. It is customary to deionise urea solutions prior to use; however, in view of the rapidity with which the urea - cyanate equilibrium is re-established (Hagel et al., 1971; Gerding et al., 1971) compared to the duration of the experiments, this was not carried out. The carrier ampholyte concentration was raised to 2% (w/v), partly to counteract the 'plateau phenomenon' (Finlayson and Chrambach, 1971; Miles et al., 1972), in which the pH gradient progressively flattens with time and which is more marked at low ampholyte concentrations, and it seems unlikely that in the presence of this concentration of ampholyte, protein carbamylation will occur to any marked extent.

Another source of possible artefacts is the formation of protein-ampholyte complexes (Hayes and Wellner, 1969) which, although not irreversible, could give rise to additional bands. In addition, the occurrence of artefactual bands in gel electrophoresis as a result of the presence of the persulphate used during gel polymerisation have been described (Fantes and Furminger, 1967; Mitchell, 1967; King, 1970).

There is also the possibility of proteolytic degradation during extraction and fractionation. Gray and Kekwick (1973) obtained multiple bands on isoelectric focusing of ribulose-diphosphate carboxylase prepared in the absence of diisopropylfluorophosphate (DFP) from Phaseolus vulgaris leaves. Proteolytic inhibitors, such as DFP and PMSF, have been included in extraction media to minimise proteolytic degration, especially in the preparation of yeast enzymes (Lazarus et al., 1969; Clarke and Jakoby, 1970). Moreover, Pringle (1970), and Porter and Preston (1975) have shown that proteolytic enzymes are active in SDS solutions, unless heated at 100°C for three minutes or treated with appropriate inhibitors. The similarity of the band patterns obtained by SDS gel electrophoresis of both alkaline salt extracts and direct SDS extraction of meal, combined with the low level of proteolytic activity determined in ungerminated seeds, suggest that proteolysis has not occurred with the preparations described here. Although the normal preparation procedure for SDS gel electrophoresis involved incubation at 37°C prior to heating at 100°C, the same results were obtained when the order was reversed. Direct SDS extraction of meal seemed to be extremely efficient, in that no protein staining material could be detected by re-extraction and electrophoresis of the residue.

Much of the analysis of protein fractions described here is based on SDS gel electrophoresis, both because of its high resolution, and because it can be used to identify proteins on the basis of a property which is presumed to be fundamental (i.e. subunit molecular weight) rather than a more variable property, such as the molecular weight, estimated by ultra-centrifugation, which is usually dependent on the pH, ionic strength and sometimes previous treatment. Molecular weights were estimated by the method of Shapiro et al. (1967) as described by Weber and Osborn (1969); the general applicability has been confirmed by other workers, for example Dunker and Rueckert

(1969), although these workers' findings on the effect of the intrinsic charge of the protein have been questioned (Tung and Knight, 1971). Reliable results are obtained providing disulphide bonds are disrupted by reduction and a binding ratio of 1.4 g SDS per g protein is attained (Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970; Trayer *et al.*, 1971); no differences were obtained whether the sample was prepared by direct solubilisation in 1.0% (w/v) SDS or by dialysis against excess 0.1% (w/v) SDS; as observed with the lac repressor (Hamada *et al.*, 1973) no differences resulted from applying the sample in 1.0% or 0.1% (w/v) SDS.

Katzman (1972) found SDS inadequate for the complete dissociation of brain proteins and glycoproteins; Grant and Lawrence (1964) obtained more than one N-terminal amino acid residue from single bands obtained from pea globulins by preparative SDS gel electrophoresis. However, as demonstrated in this work, the solubilisation conditions vary for different proteins, the glycoprotein II subunits requiring brief treatment at 100°C for apparently complete dissociation. Variation in the estimation of molecular weights was routinely within a range of ± 5%; in the interests of consistency, where the identity of a particular band was unequivocal, for example by its particular appearance, average molecular weights have been presented.

Glycoproteins are known to behave anomalously during SDS gel electrophoresis (Segrest *et al.*, 1971; Russ and Polakova, 1973;), because of reduced binding, per gram of protein, of SDS. The apparent molecular weight is observed to decrease with increasing acrylamide concentration, presumably because sieving exerts a greater influence at higher acrylamide concentrations, and the effect of the relatively low charge: mass ratio is reduced. However, this effect is only seen strongly with glycoproteins containing large amounts of glycoprotein, for example the erythrocyte membrane glycoprotein, which is 60% carbohydrate (Segrest _et al._, 1971), and analysis of the major storage protein subunits over the acrylamide range 5.0 - 12.5% (w/v) revealed only a very slight change in the molecular weight of the 32,000 molecular weight subunit (approx. 33,500 molecular weight in 5% gels, 30,500 molecular weight in 12.5% acrylamide gels).

Williams and Gratzer (1971) reported the use of a cationic detergent, CTAB, in place of SDS in the pH 7.0 phosphate buffer system. Modifications to the described system were found necessary: due to reaction between the ammonium persulphate catalyst and CTAB, it was necessary to polymerise the gel in the absence of CTAB, subsequently introducing CTAB by electrophoresis after removal of residual persulphate with thioglycollic acid. Low mobilities were obtained at pH 7.0, but satisfactory results were obtained at pH 5.7; both the band pattern and the apparent molecular weights were similar to those observed with SDS. Fairbanks and AVruch (1973) have used CTAB detergent electrophoretic systems at pH 2.4 and 8.3 to study membrane proteins, and another cationic system has recently been described (Schick, 1975); the use of CTAB as an alternative to SDS has,however, been questioned on theoretical grounds (Nozaki _et al._, 1974).

Previous studies on protein bodies from legume seeds have demonstrated that much or all of the globulin proteins are localised in these organelles. Varner and Schidlovsky (1963) found the majority of the pea globulinsto be present in their protein body fraction while albumins were mainly in the supernatant fluid. Tombs (1967) isolated protein bodies from soybeans: one fraction contained 98% protein, while a second also contained RNA, phytic acid and lipid; 70% of the seed protein was recovered in protein bodies and glycinin was confined to this fraction. Koshiyama (1972b) found no difference between the protein of protein bodies and soybean globulins in comparisons by ultracentrifugation, gel filtration and isoelectric focusing; the 7S and 11S globulins were the main component of both; this was confirmed by Catsimpoolas *et al.* (1968c), using mono-specific antisera to the globulins. Daussant *et al.* (1969a) and Neucere and Ory (1970), however, found differences in the distribution of peanut globulins; while α-arachin (the major immunoelectrophoretic component of the arachin fraction) was found in the protein bodies, the conarachin fraction, although homogeneous by ultra-centrifugation and zone electrophoresis, was separable into two fractions (α_1- and α_2-conarachin) by immunoelectrophoresis, of which only α_2-conarachin was found in the protein bodies (Daussant *et al.*, 1969a). Protein bodies from *Vicia faba* (Morris *et al.*, 1970) and from *Phaseolus aureus* (Ericson and Chrispeels, 1973) contained both vicilin and legumin; those from *Vicia faba* also contained an albumin fraction which was resolved into several protein components by disc electrophoresis.

Protein bodies prepared from *Phaseolus vulgaris* cv. 'Seafarer' resembled those prepared from other sources in size and appearance (for example, the preparation from *Phaseolus aureus* by Ericson and Chrispeels (1973)), and were similar to those seen *in situ* (Opik, 1968); the protuberances found on some protein bodies prepared in sucrose density gradients are probably the result of osmotic stress during fixation (N. Harris, personal communication). All four storage protein fractions described earlier were found in the protein body fraction prepared by this method, although considerably larger quantities of these proteins were recovered from the sample region of the gradients.

Yatsu and Jacks (1968) isolated protein bodies from cotton seed using glycerol solutions. With the glycerol procedure used here, while the four storage proteins were all recovered in the protein body fraction, the yield and composition (i.e. the relative proportions of the four proteins) were dependent on the pH. When isolated at pH 5.0, high yields were obtained but the legumin fraction was confined to the protein body fraction; extraction was carried out at pH 7.5 to determine if this distribution was a result of the pH of the extractant: although some legumin was recovered in the supernatant fluid from preparations at pH 7.5, the yield of the protein body fraction was lower; the protein bodies also contained a markedly higher proportion of legumin, relative to the other storage proteins.

Enzyme distribution studies showed that both BAPNA-ase and leucine amino peptidase were cytoplasmic, little activity being found in protein bodies prepared by sucrose density gradient centrifugation or by the glycerol method at pH 5.0. However, although trypsin inhibitor activity was greater in the sample region/supernatant fluid for both methods of preparation, the specific activity, as shown in Table 16, was higher in the protein body fraction prepared by sucrose density gradient centrifugation than in the sample region of the gradient; in contrast, the glycerol method yielded a higher specific activity for trypsin inhibitor activity in the supernatant fluid than in the protein bodies.

The apparent differences in protein composition of protein bodies prepared by different procedures and at different pHs may be accounted for in several ways. There may be several types of protein bodies, perhaps synthesised at different times during seed development, which contain different amounts of each storage protein; Graham and Gunning's observation of protein bodies which only reacted to antisera to legumin or vicilin, as well as those which reacted to both (Graham and Gunning, 1970) would be compatible with this hypothesis. Protein bodies of differing protein content may show differential susceptibility to bursting; thus those containing predominantly legumin may be relatively more stable in glycerol solutions at pH 7.5, and hence a protein body fraction enriched in legumin is prepared at this pH. Protein bodies may vary in density, so that a particular procedure may tend to isolate only part of the total; however, no differences in protein composition were detected in protein bodies isolated from different regions of the protein body fraction from sucrose density gradients.

The higher specific activity for trypsin inhibitor found in supernatant fluid from glycerol preparations could arise either from a completely cytoplasmic origin, or from the presence of inhibitor in both fractions; the higher specific activity found in the protein bodies prepared by sucrose density gradient centrifugation implies the location of the inhibitor in the protein bodies, or that trypsin inhibitors are preferentially adsorbed. Hobday *et al*. (1973) found that trypsin inhibitor activity in *Pisum sativum* protein bodies could be leached out, by washing, more rapidly than overall protein i.e. the specific activity of the inhibitor decreased in the protein body fraction with washing;they concluded that the inhibitor was mainly cytoplasmic in location. Alternatively, this may demonstrate that protein body membranes are more 'porous' to small proteins, such as trypsin inhibitors, than to larger protein molecules.

No firm conclusions may be drawn on the location of the trypsin inhibitor in *Phaseolus vulgaris*. While the glycerol method of protein body preparation is rapid and effective, the results obtained are dependant on the pH used. It was not possible, using either method of preparation, to establish whether all the protein described hitherto as storage protein, was confined to protein bodies *in situ* or whether some was cytoplasmic in origin.

Differences in the amount of protein in different parts of legume and cereal seeds have previously been reported (Normand *et al.*, 1965; Zimmermann *et al.*, 1967). Vogel and Wood (1971) demonstrated histochemically that aleurone grains from the outer regions of the cotyledons of *Phaseolus vulgaris* were richer in protein-bound sulphydryl groups than those of the inner regions; Wood and Cole (1973) subsequently showed that individual disc electrophoretic components were found in different ratios in the outer and central regions of the cotyledon. The higher levels of protein content and trypsin inhibitor activity, described by Zimmermann *et al.* (1967) in the outer regions of the cotyledon have been confirmed, and differences in the distribution of the storage protein subunits established. Although the protein-sulphydryl content of different regions was not analysed, the presence of greater amounts of both trypsin inhibitor and legumin subunits in the abaxial region of the cotyledon is compatible with the results of Vogel and Wood. (1971).

A striking result was the difference in the ratio of the two subunits of glycoprotein II (molecular weights 50,000 and 47,000) observed between the abaxial surface and the central regions of the cotyledon. Differences in relative staining intensity were first noted visually and were confirmed by densitometric analysis. Kruski and Narayan (1968) found that sample volume and acrylamide concentration were crucial factors in determining band widths: only gels onto which the same sample volume had been applied were compared. The fact that this difference in the staining intensity of the two subunits was also seen when the protein subunits of protein bodies derived from these regions were analysed by SDS gel electrophoresis suggests that the difference is not simply an extraction artefact.

The method of extraction used does not differentiate between those subunits which are dissociated and those which are associated together, representing the 7S component observed in the ultracentrifuge. Thus, the synthesis of the subunits may be independent, such that either excess or insufficient synthesis of one or other subunit could occur. The isolation of homogeneous proteins may obscure the presence of non-associated subunits. Alternatively, the difference in ratio may be caused by some associated compound, perhaps non-proteinaceous, which alters the staining intensity of one of the subunits, and whose distribution throughout the cotyledon is non-uniform. Further work will be necessary to clarify whether this difference in ratio of these subunits is upheld in purified preparations of glycoprotein II derived from these regions, and thus represents polymorphism.

Identification of individual storage proteins, and characterisation of their properties (subunits, amino acid compositions, and nutritional value) is vital for the nutritional improvement of legume crops. The differences found in relative preponderance of different proteins and their subunits in different cultivars (Hynes, 1968; Adriaanse et al., 1969; Gillespie and Blagrove, 1975) may allow selection of breeding strains containing improved characteristics. The isolation of γ-conglutin from Lupinus angustifolius by Blagrove and Gillespie (1975) and the demonstration that it contains a relatively high methionine and cysteine content has obvious implications, especially as not all the Lupinus species examined contained this protein. The significance of the apparent occurrence of glycoprotein II with either two or three subunits in different cultivars of Phaseolus vulgaris is not known. This could give rise to considerable differences in the amino acid composition of this protein from different cultivars, but until the subunits have been purified and more detailed analysis carried out, for example of amino acid compositions, peptide maps and even amino-acid sequence studies, the position will remain unclear.

The increase in fresh and dry weight of the seed during development was similar to that observed in previous studies (Opik, 1968; Briarty et al., 1969; Smith, 1973); three main phases could be distinguished: cell division, expansion and deposition of reserve materials (starch and protein; possibly nucleic acid), and dehydration. Various parameters have previously been used to determine the stage of development of the seed. The classical days from flowering is subject to variations from climatic factors and the position of the pod on the plant, and seed weight (Kloz et al., 1966) seed colour (Racusen and Foote, 1973) and seed length (Millerd et al., 1971; Hall et al., 1972) have all been used as alternative indices. In this case, seeds were harvested according to total seed fresh weight. The determination of the percentage dry weight of seeds of increasing fresh weight (Fig. 30) showed a fairly linear increase in dry weight until dehydration began, after which no further increase in dry weight was found; the increasing percentage dry weight may thus represent a good indication of the 'physiological' age of the seed, but the acquisition of this information requires the drying of the seed, and is not suitable for use during the collection of material.

While the pattern of accumulation of protein was similar to that found in previous studies, and the amount of protein present increased rapidly during the phase of rapid increase in fresh weight, the proportion of globulin to albumin did not appear to alter. Previous studies on peas (Danielsson, 1952; Beevers and Poulson, 1972) demonstrated that globulins were synthesised in larger amounts later in development than albumins. However, Phaseolus vulgaris globulins seem atypical in that the proteins comprising the majority of the storage protein fraction (glycoprotein II and agglutinins) are both fairly soluble in water.

Differences in the rate of synthesis of vicilin and legumin are well established in peas (Danielsson, 1952), broad beans (Wright and Boulter, 1972; Millerd and Spencer, 1974) and soybeans (Hill and Breidenbach, 1974b). Marked differences were not detected in this case in developing seeds of Phaseolus vulgaris, either by direct analysis of SDS extracts of the seeds, or by the analysis of partially fractionated globulins. This is considered, at least in part, to be a result of the relative insensitivity of the method used, compared to, for example, the immunoelectrophoretic procedure used by Millerd and Spencer (1974), and also to the great preponderance of glycoprotein II found at all stages of development. The values obtained for each subunit probably do not represent absolute amounts of protein, because of the variation in the amount of amido black bound by different proteins (Racusen, 1973); however, the determination of relative changes in the amount of individual subunits is not affected by this metachromicity.

Kloz et al. (1966) detected the appearance of new immunoelectrophoretic components during seed dehydration; however, no changes in subunit pattern were observed here at this stage of seed development.

Changes in the protein content and composition, and proteinase and some peptidase activities were followed during the course of seed germination. Before considering these, the results of morphological and histochemical analyses of germinating seeds will be considered. Two patterns of degradation of reserve materials in germinating cotyledons have been described: in Vicia faba, degradation starts in the cells nearest to the vascular bundles (Briarty et al.,1970), whereas in Arachis hypogaea (Bagley et al., 1963) and in Phaseolus vulgaris (Opik, 1966; Smith, 1974) breakdown of reserves commences in cells furthest away from the vascular bundles and moves towards them. In both patterns, and as is particularly emphasised by the histochemical localisation of proteolytic enzyme activity in germinating Phaseolus vulgaris cotyledons demonstrated by Yomo and Taylor (1973), there are areas in which there is no proteinase activity, as well as areas which contain active proteinase. Extraction of proteins from whole cotyledons thus gives rise to a mixture of proteins derived from degraded cells, those in which active degradation is occurring, and those in which no changes may yet have taken place.

Disc electrophoretic analysis showed that while there is a progressive loss of the major electrophoretic component, Rm 0.35 - 0.4, together with the appearance of diffuse material of increased mobility, the increase in mobility described by Daussant et al. (1969b) as a preliminary to degradation was not clearly observed; electrophoresis did not demonstrate whether an initial dissociation into subunits took place, as suggested by Ghetie (1966) and Catsimpoolas et al. (1968b). SDS gel electrophoretic analysis showed the progressive loss of the major storage protein subunits with the concomitant formation of diffuse, low molecular weight material, as well as a more discrete band of molecular weight about 21,000. Basha and Beevers (1975) analysed the subunit composition of legumin and vicilin isolated from germinating seeds of *Pisum sativum*; with both storage proteins the proportion of low molecular weight components increased during germination. However, with neither storage protein did there appear to be formation of greater amounts of these components, whereas the 21,000 molecular weight component observed here appeared to increase in amount before being completely degraded. The origin of this is unclear, but it seems unlikely that it is due to bacterial contamination, as the massive invasion that would be required to produce this amount of protein would, as noted by Pusztai and Duncan (1971b), almost certainly be accompanied by a large increase in peptidase activities, which were not observed.

Proteolytic activity increases in seeds of *Pisum sativum* (Beevers and Splittstoesser, 1968; Beevers, 1968; Hobday *et al.*, 1973) during germination; in *Phaseolus vulgaris*, Pusztai and Duncan (1971b) found that endopeptidase activity was initially quite high and decreased slowly until the eighth day of germination, when it rapidly increased, followed by a further decline. In contrast, Yomo and Taylor (1973) and Yomo and Srinivasan (1973) found that increased proteolytic activity developed during germination. In this study, proteinase activity measured using commercial azoalbumin, azoglobulin prepared from glycoprotein II extracted from dry seeds and endopeptidase assayed by the decrease in viscosity of gelatin solutions, was initially very low in the dry seed but increased steadily during germination. Many previous studies have used casein (for example, Beevers, 1968) or acid denatured haemoglobin (e.g Pusztai and

Duncan, 1971b) as substrates; however, the estimation of proteolytic activity by the increased absorption at 280nm of trichloracetic acid soluble material resulting from proteolysis (Kunitz, 1947) has been criticised; thus, changes in absorption at 280nm resulting from proteolytic activity may be masked by ribonuclease activity (Marrink and Gruber, 1966), which is known to increase during germination (Beevers and Splittstoesser, 1968); estimation of the extent of proteolysis by the ninhydrin reaction (Moore and Stein, 1954) is unsatisfactory in the presence of 2-mercaptoethanol. The use of commercial azosubstrates allow the change in absorption to be measured at wavelengths other than 280nm, for example 440nm with azoalbumin; the use of endogenous substrate, in the form of the azo-derivative, also allows comparison of specificity. The azoglobulin preparations described here had a low extinction coefficient ($E^{1\%}_{430nm}$ = 2.78), which was even lower in the presence of 2-mercaptoethanol; assays were more easily carried out using commercial azoalbumin (extinction coefficient $E^{1\%}_{440nm}$ = 42) as substrate. Comparison of the amount of these two substrates solubilised did, however, suggest that azoglobulin was hydrolysed more rapidly than commercial azoalbumin; the enzyme(s) responsible may therefore be partially specific for the endogenous substrate. In addition to the acid proteinase from sorghum which only hydrolysed peptide bonds following an aspartic acid or a glutamic acid residue (Garg and Virupaksha, 1974b), Spencer and Spencer (1974), using a fluorescence assay method, have described a proteinase from germinating pumpkin seeds that was highly active with pumpkin seed globulin as substrate but showed little activity with bovine serum albumin as substrate. Harvey and Oaks (1974) found that an acid proteinase from maize endosperm hydrolysed both gliadin and glutelin (the natural substrates) more rapidly than bovine serum albumin.

Korolyova et al. (1975) have recently shown that while proteolytic enzymes from dry seeds of vetch will hydrolyse both legumin and vicilin prepared from germinating seeds, they are inactive with these storage proteins prepared from dry seeds; no change in the molecular weights of the vicilin and legumin (prior to hydrolysis) were detected but one modification considered responsible was deamidation. The fact that extracts of germinating Phaseolus vulgaris seeds will hydrolyse azoglobulin prepared from storage protein from dry seeds suggests either that artefactual modification has taken place or that this 'activation' of the storage protein is unnecessary in this case.

While pea seeds contain proteolytic activities with pH optima at pH 5.0 - 5.5 and pH 7.0 (Beevers, 1968; Guardiola and Sutcliffe, 1971), the proteinase activity measured here was only active at pH 5.0 - 5.5. Activity was enhanced in the presence of 2-mercaptoethanol; both phenylmethylsulphonylfluoride, an inhibitor of 'serine' proteinases, and N-ethylmaleimide, which reacts irreversibly with sulphydryl groups, caused a reduction in activity. In contrast, endopeptidase activity in extracts from germinating seeds of Phaseolus aureus was not inhibited by phenylmethylsulphonylfluoride (Chrispeels and Boulter, 1975).

The intracellular location of the proteinases present in Phaseolus vulgaris during germination is not clear. Acid proteinases have been detected in the protein bodies of other seeds, for example hemp (St. Angelo et al., 1969), barley (Ory and Henningsen, 1969) and cotton seed (Yatsu and Jacks, 1968). In pea cotyledons, proteolytic activity has been found in the protein bodies: Matile (1968) showed that proteinase had a higher specific activity in protein bodies, which were free of both mitochondrial and cytoplasmic enzymes, than in the total extract;

he considered protein bodies to represent lysosomos. Hobday *et al.* (1973) also found the proteinase present in ungerminated pea seeds to be located in the protein bodies.

Attempts at isolating protein bodies from germinating seeds were partially successful, in that protein bodies were prepared from seeds allowed to germinate for three days, but it seems likely, in view of the ultrastructural changes associated with protein bodies during germination (Briarty *et al.*, 1969; Smith, 1974), that these were derived from regions of the cotyledon that had not begun to be degraded. No proteinase activity could be detected in these protein body preparations, using any of the proteinase assays described.

Attempts at purifying the azoalbumin-hydrolysing enzyme were unsuccessful. While some purification was achieved by ammonium sulphate precipitation and ion exchange chromatography, no means of adequately stabilising the enzyme was found; gel filtration on Sephadex G-100 resulted in elution of enzyme activity at and closely following the void volume.

Both leucine amino peptidase and BAPNA-ase activity declined during germination; the loss of leucine amino peptidase activity was faster than the overall loss of protein, and this enzyme was the only one of those assayed to show a decrease in specific activity. Again, this may be a result of the mixing, in the extraction, of components derived from autolysing and non-degraded cells.

Pusztai (1972) found that the content of trypsin inhibitory activity of *Phaseolus vulgaris* seeds increased during the first seven days of germination, declining slowly after the tenth day. The changes in activity described here are more similar to those described in *Pisum sativum* by Hobday *et al.* (1973) and in *Vigna sinensis* by

Filho (1973), in which activity declined, initially slowly, then rapidly, although unlike the situation in Vigna, an overall increase in specific activity was observed. Trypsin inhibitor from dry seeds was reasonably stable to heating; Mikola and Suolinna (1969) found that during the purification of a trypsin inhibitor from barley it tended to be adsorbed onto protein precipitates; some of the losses observed with heating may be the result of adsorption onto precipitated protein. The loss of activity in the presence of 2-mercaptoethanol could be expected from the high cysteine/cystine content known to be a characteristic of these inhibitors (Pusztai, 1968).

During germination, the nutritional quality of soybeans increases (Everson et al., 1944). More recently, germination has been shown to bring about a gradual increase in nutritive value of kidney beans (Palmer et al., 1973) although the trypsin inhibitor content rises during this period; trypsin inhibitors therefore do not appear to be the main toxic constituent of this species.

The inclusion of extracts of ungerminated and 2-day germinated seeds in the assay of azoalbumin hydrolysis by extracts of 8-day germinated seeds resulted in reduction of activity, whereas the activity of extracts of 4-day germinated seeds was not affected except with large amounts of extract. However, as suggested by Hobday et al. (1973), this may be the result of the increased protein present adsorbing solubilised azoalbumin, as well as acting as a competitive substrate. The reduction of the inhibition by 2-day extract of the azoalbumin hydrolysing activity of 8-day extract in the presence of 2-mercaptoethanol cannot be taken to imply that an inhibitor has been partially inactivated. It is not possible to decide if, as in ungerminated lettuce seeds (Shain and Mayer, 1965; 1968), proteinase activity is initially regulated by an inhibitor that is itself rapidly degraded during germination, or whether the proteinase inhibitors present are solely inhibitory towards exogenous proteinases.

A very large amount of data has already been published on structural, physiological and nutritional aspects of seed proteins. In conclusion, the present study, in which the main storage proteins of Phaseolus vulgaris cv. 'Seafarer' are described and partially characterised, and their formation during seed development and degradation during germination followed, is of value not only in terms of the results presented, but also in indicating which aspects of the study of seed proteins are amenable to successful analysis. Techniques for the rapid and unequivocal identification of individual proteins are now available, for example SDS gel electrophoresis and the use of immunological determinants, and the purification and characterisation of storage proteins in terms of their amino acid compositions and nutritive value may now be approached with confidence. However, meaningful results on the control of germination and the roles played in germination by the various enzymatic and inhibitory activities present in the seed appear to be less accessible.

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