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STRUCTURAL STUDIES ON THE
MAJOR RESERVE PROTEINS OF VICIA FABAE (L)

by

D.J. Wright, B.Sc. (Dunelm)

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

November 1973



To my parents

The single plant seed, the ultimate source of all foodstuffs, is one of the most important organisms with which mankind is concerned.

H.B. Vickery (1945).

ABSTRACT

The storage globulins of Vicia faba were extracted and the component proteins separated using various procedures. The sub-unit structures of the resulting globulin fractions were investigated and, in the case of legumin, the isolation of the constituent sub-units examined. These sub-units were characterised using gel electrophoresis, isoelectric focussing, amino acid composition, N-terminal amino acid and N-terminal sequence data. Possible causes of artifactual heterogeneity and anomalous behaviour in gel electrophoresis and gel isoelectric focussing were discussed. The onset of synthesis and composition of the globulins during seed development were investigated.

Legumin was prepared in a very pure form by extraction in a neutral saline buffer followed by zonal isoelectric precipitation; in contrast, vicilin, prepared by this method, was contaminated with legumin.

Structural studies on legumin indicated that it was composed of equimolar proportions of two types of sub-unit, acidic (α) and basic (β), with molecular weights of 36,200 and 22,000 respectively. Isoelectric focussing, sequence data and electrophoresis in a variety of gel systems showed these sub-units to be heterogeneous, and, on the basis of electrophoretic information five sub-units were positively identified viz, α_1 , α_2 , β_1 , β_2 and β_3 . It was proposed that legumin consisted of twelve sub-units, six acidic and six basic, but because of the heterogeneous nature of the α and β sub-units, the exact composition of the native legumin molecule could not be ascertained.

The possibility of naturally-occurring variants of legumin was suggested. The proposed sub-unit model was compared with those published for other legumin-like seed proteins.

Vicilin was formed prior to legumin during seed development, although the rate of synthesis of the latter was faster, so that in the mature seed the ratio of legumin to vicilin was between 3:1 and 4:1 by weight. While the sub-unit structure of legumin, as examined by SDS gel electrophoresis, remained reasonably constant during seed development, that of vicilin changed, suggesting the existence of more than one protein in the vicilin fraction. Together with this evidence, results from electrophoretic studies of various preparations of the 4.7 soluble globulin (vicilin) indicated that the latter probably consisted of at least three distinct proteins.

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INTRODUCTION

Today seeds of legumes represent one of the principal food sources in the world; they are also important as fodder plants. Legumes are not only important as a major source of protein and other nutrients, but also play an essential role in crop rotation. The seeds are rich in protein, containing 20-30% by weight in wild, and as much as 50% by weight in cultivated varieties. Apart from forming the protein rich part of the basic diet of many developing countries, the seeds are also used as a source of protein for processing into meat substitutes. Because of the increased use envisaged of legume seeds in both these respects, in the future, many research programmes have been initiated to investigate the possibility of increasing their yield, generally lower than that of cereals, and also of improving their protein concentration and nutritional status. The latter is particularly concerned with the content of methionine and cysteine which are usually the first-limiting amino acids of the seeds of legumes. The relatively low concentration of sulphur amino acids in meals is reflected in a similar deficiency in the meal proteins. Investigations into the structure of these nutritionally-important proteins were considered necessary in view of its possible effect on digestibility, processing and stability to storage of the proteins. Although the more significant developments in the field of legume protein chemistry have emerged during the past twenty years, it is important to realise that many of the experimental results obtained by the early investigators are still valid to this day.



The pioneer in the field of plant protein studies was undoubtedly Osborne, who described methods for the separation and purification of proteins from various fruits and seeds, and also formulated the following classification of reserve proteins (Osborne, 1924):

1. Albumins: soluble in water and dilute salt solutions.
2. Globulins: sparingly soluble in water, but soluble in neutral salt solutions.
3. Prolamins: soluble in 70-80% ethanol but insoluble in water or pure ethanol.
4. Glutelins: soluble in dilute acids or alkalis, but insoluble in the solvents mentioned above.

Prolamins and glutelins predominate in cereals, whereas the major reserve proteins of legume seeds are globulins, constituting about 90% by weight of the total protein. Even though the above classes were later found to be not totally exclusive, and the validity of a classification scheme, which depended on a concept that was not fundamental to proteins (i.e. solubility), was questioned (Altschul et al., 1964), these solubility criteria are still incorporated into many of the modern methods developed for protein purification.

In his studies Osborne separated the globulins of pea into two components, initially by stepwise dilution of salt solutions of the proteins (Osborne and Campbell, 1898a), and later by $(\text{NH}_4)_2\text{SO}_4$ fractionation (Osborne and Harris, 1907). The two globulins, which he called legumin and vicilin, were characterised by the following properties: (i) legumin did not coagulate when a solution of it was heated to 100° , and (ii) vicilin coagulated upon heating to 95° to

100° and was also soluble in more dilute salt solutions than legumin. Chemical analyses also revealed that legumin possessed a higher sulphur content than vicilin.

The introduction of ultracentrifugation (Svedberg and Lysholm, 1927) and electrophoresis (Tiselius, 1930) as analytical tools for high molecular weight substances, led to a reinvestigation of Osborne's results. Ultracentrifugation analysis of seed proteins was performed initially by Svedberg (1937) and Loring et al. (1938) and then later and more extensively by Danielsson (1949); electrophoretic studies were conducted by Wetter and McCalla (1949) and Danielsson (1950). Danielsson (1949) examined over 30 species from various genera of the Leguminosae in the ultracentrifuge, and from his data, concluded that apart from a few exceptions, all species examined contained the same two globulin components, with sedimentation coefficients of approximately 7S and 11S. Notable exceptions were Acacia longifolia, A. penninervis, A. verticillata and Trifolium repens, all of which contained only vicilin. Working with Pisum sativum, Danielsson experimented with various methods in an attempt to separate these globulin components. As judged by the ultracentrifugal patterns of the various fractions obtained, isoelectric precipitation at pH 4.7 proved a far more efficient method of separation than either $(\text{NH}_4)_2\text{SO}_4$ fractionation or precipitation by dilution of globulin solutions at 0°. Using the criteria of solubility and stability to heat, he equated his fractionated pea globulins with the legumin and vicilin described by Osborne. Molecular weight calculations gave values of 331,000 and 186,000 for legumin and vicilin (Danielsson, 1949), and their

isoelectric points, as determined from electrophoretic experiments, were 4.8 and 5.5 respectively (Danielsson, 1950).

Research on other legume proteins was also proceeding during this period. Thus, Johns and Jones (1916, 1917) separated peanut (Arachis hypogaea) globulins into two fractions, called arachin and conarachin, which they reported differed in sulphur, nitrogen and amino acid content. Jones and Horn (1930) isolated arachin by two procedures, one involving heat treatment of the protein extract and the other using $(\text{NH}_4)_2\text{SO}_4$ fractionation. Using the latter method, Johnson (1946a) obtained an arachin preparation that was almost homogeneous by ultracentrifugation. He determined the sedimentation coefficient of this protein as 14.6S, and a corresponding molecular weight of 396,000. He also observed that dilution of the protein solution with water and simultaneous acidification to pH 5.0 resulted in dissociation of the arachin molecules into components with sedimentation coefficients of 9.5S (Johnson, 1946b). Arachin was, in fact, the first example of a reversible association-dissociation system, but reports of similar observations on the globulins of both soyabean (Naismith, 1955; Kretovich, et al., 1958) and lupin (Joubert, 1955a; Petri et al., 1955) soon followed. On the other hand, in Vicia faba, the 11.5S and 7.0S globulins were shown to be devoid of any dissociation-association properties, at least between pH 7 and pH 9 and over the range of ionic strength $\mu = 0.1$ to 0.31 (Joubert, 1957). Also the 12.3S globulin of Pisum sativum was stable to changes in pH and ionic strength, although association of the 7.2S component to a 10.7S species was observed at low pH and low ionic strength (Joubert, 1955b). These observations are

very important as regards our understanding of the nature of seed globulins, and in particular the physical state in which they exist in the seed. It is also important to recognise that the determined characteristics of those globulins which undergo such reactions will depend largely on the conditions employed for extraction and analysis.

The major protein fraction of soyabean, called glycinin, was originally prepared as a precipitate by dialysis of a 10% NaCl meal extract (Osborne and Campbell, 1898b). This preparation and others obtained by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ (Jones and Csonka, 1932) were shown to be heterogeneous both by electrophoresis (Briggs and Mann, 1950) and ultracentrifugation (Naismith, 1955). The latter observed three components in crude glycinin in the ultracentrifuge, with sedimentation coefficients of 6.6S, 10.6S and 15.0S. Alternative methods for preparing the major globulin component (11S) were reported; thus, Hartman and Cheng (1936) employed isoelectric precipitation at pH 5.0 and Briggs and Mann (1950) used precipitation by cooling a solution of the crude globulin preparation. The latter method, producing what has been termed the cold-insoluble fraction, is still used today, as the initial step in many procedures developed for the purification of the 11S globulin.

The other major contribution, from this period, to our knowledge of seed proteins was a series of investigations carried out on the globulins of the lupins. Preliminary studies had been conducted by Osborne and Campbell (1897) and also Danielsson (1949), but the more important work was due to Joubert (1955a,c, 1956). Using fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$, he achieved a fairly good separation of the

two globulin components of the blue lupin (Lupinus angustifolius) (Joubert, 1955a). In the ultracentrifuge, these proteins sedimented with $s_{20,w}$ values of 11.6S and 7.8S, which corresponded to molecular weights of 336,000 and 181,000, values in very good agreement with those reported earlier for pea globulins (Danielsson, 1949). Similar investigations (Joubert, 1955c; 1956) on the globulins of white (Lupinus albus) and yellow (L. luteus) lupin indicated the presence of 12.1S, 8.3S and 2.8S components for the former and 11.6S, 7.4S and 2.0S components for the latter. The 11-12S globulin from each species was found to undergo reversible association-dissociation to a 7S form at low ionic strength and high pH. Under the same conditions the 7S globulins of L. luteus and L. angustifolius were stable but the 8S globulin of L. albus reversibly associated to a 12.5S species.

Joubert's investigations heralded the end of what could be described as the classical period of legume protein studies, in which progress was somewhat restricted by the inadequacy of the techniques available at that time. Nevertheless the expertise of the early workers must be recognised in view of the fact that even with today's technological advances in the field of protein chemistry, few results have emerged which conflict with their experimental observations. In fact the present-day preparative and separatory procedures still make considerable use of much of the fundamental knowledge gained by them. With the advent of column chromatography, however, it became possible to isolate these macromolecules in a sufficiently homogeneous state to warrant a comprehensive investigation into their sub-molecular structure.

A considerable volume of work, largely biochemical in nature, on legume seed globulins has emerged during the past two decades. As a measure of both its economic and nutritional importance, by far the greater proportion of this research has been concerned with the globulins of soyabean. The original glycinin fraction isolated by Osborne and Campbell (1898b) is now known to consist of four components, having sedimentation coefficients of approximately 2S, 7S, 11S and 15S (Naismith, 1955; Wolf and Briggs, 1956). The 7S and 11S proteins account for some 37% and 31% of the total water-extractable soyabean proteins but whilst the 11S protein consists chiefly of the 11S globulin, the 7S globulin corresponds to only 50% of the 7S protein, the remainder being represented by an hemagglutinin, lipoxygenase and β -amylase (Wolf and Smith, 1961). The 11S globulin was shown to be capable of forming disulphide polymers (Naismith, 1955; Briggs and Wolf, 1957) and also of undergoing dissociation in acid medium (Kretovich et al., 1958; Wolf et al., 1958) and in solutions containing detergent or urea (Wolf and Briggs, 1958). These properties make the selection of suitable isolation and purification conditions of paramount importance. In recent years the preparation of the 11S globulin has been achieved using mainly chromatographic procedures (Mitsuda et al., 1965; Shvarts and Vaintraub, 1967; Eldridge and Wolf, 1967; Catsimpoilas et al., 1967). Using gel filtration on Sephadex G-100 and G-200, Koshiyama (1972) reported the isolation, from the cold-insoluble fraction, of an 11S globulin which was homogeneous by ultracentrifugation, disc electrophoresis and isoelectric focussing, and determined its molecular weight as between 309,000 and 322,000 by three different methods. The purified 11S globulin was shown to contain three different N-terminal amino acids,

namely glycine, phenylalanine and leucine or isoleucine (Mitsuda et al., 1965; Catsimpoilas et al., 1967; Vaintraub, 1967), results which suggested the existence of a complex sub-unit structure. Using the sedimentation equilibrium method, Vaintraub and Shutov (1971) determined the molecular weight of the 11S globulin in acid media and in solutions of 4M urea, as 63,000 and 31,000 respectively. They postulated the following stepwise dissociation scheme to explain their results:

$$11S \rightarrow 2 \times 7S \rightarrow 6 \times 3S \rightarrow 12 \times 2S.$$

Catsimpoilas (1970) observed six different sub-units when glycinin was dissociated and electrophoresed in the solvent system phenol-acetic acid-2-mercaptoethanol-urea. These sub-units were isolated using preparative isoelectric focussing in an urea-2-mercaptoethanol medium (Catsimpoilas, 1969). Three of them, termed the acidic or A sub-units, had pI values of 4.75, 5.15 and 5.4, and the remaining three basic or B sub-units had pI values of 8.0, 8.25 and 8.5. The two groups of sub-units (A and B) were later shown to have molecular weights of 37,500 and 22,300 respectively, as determined by SDS gel electrophoresis (Catsimpoilas et al., 1971). An even greater heterogeneity of the glycinin sub-units was observed when glycinin was subjected to analytical scanning isoelectric focussing in urea-dithiothreitol media (Catsimpoilas and Wang, 1971). The multiplicity of components was attributed mainly to differences in the primary structure of the sub-units, although no conclusive evidence was presented to substantiate this statement. This heterogeneity has also been reported by other workers. Thus, Vaintraub (1967) obtained 18 bands when the 11S globulin was electrophoresed in 2-8M urea on polyacrylamide gels. Chromatography of the urea-dissociated protein on DEAE-cellulose resulted in the separation of four fractions, each consisting of two

sub-fractions, and characterised by different N-terminal amino acids (Vaintraub, 1967). These separated fractions each gave several bands when electrophoresed on gels, a fact the author attributed to isomerisation of the sub-units by the urea.

Okubo et al. (1969a) have also separated the 11S globulin into acidic and basic sub-units using chromatography in urea on DEAE-cellulose. The basic sub-units were found to have glycine as N-terminal amino acid and the acidic sub-units, phenylalanine and leucine or isoleucine. A molecular weight of 36,000 was cited for the basic sub-units (Okubo et al., 1969a), a value appreciably higher than those obtained by Catsimpoilas et al., (1971) and Vaintraub and Shutov (1971). The former value, however, represents an average sub-unit molecular weight determined on the urea-dissociated 11S protein and not on the separated sub-units (Okubo et al., 1969b).

As mentioned previously, the 7S component of soyabean proteins consists of several proteins, a fact which is readily apparent from the results of chromatographic studies. Thus, Hasegawa et al. (1963) separated water-extractable soyabean proteins into nine protein components by gel filtration on Sephadex G-200. When examined in the ultracentrifuge, four of these fractions contained "7S" components with $s_{20,w}$ values of 7.8S, 7.5S, 7.2S and 6.0S. The 7.8S component was eluted with the 11S protein and could not be separated from the latter by rechromatography on Sephadex G-200. This fact was taken to indicate that it had a similar molecular weight to the 11S protein despite the distinct difference in sedimentation coefficients. Using both chromatography on calcium phosphate and gel filtration, Vaintraub (1965)

with the results obtained by Johnson and Naismith (1953), this protein was shown to undergo association from a 7.8S form (molecular weight 147,000) to a 12.6S form (molecular weight 295,000) at low ionic strengths (Dechary et al., 1961).

Soyabean and peanut are semi-tropical crops and have probably been worked upon extensively because they are two of the major cash crops of the world. They contain oil as well as protein reserves and it is for the former that they have, in the past, been mainly grown, although with the present shortage of protein they are being cultivated on an ever-increasing scale as a protein crop. They do not grow well in the cooler climate of Europe, where the major legume crops are broad bean and the various forms of Phaseolus spp. Recently, therefore, several research programmes have been instituted on these crops in European countries.

The extent of our biochemical and biophysical knowledge on the broad bean proteins is much more limited than in the case of soyabean or peanut proteins. Legumin and vicilin, the reserve globulins, of the broad bean were partially characterised in the early part of this century, but the most significant progress in this respect has been made during the past twenty years. Originally, the terms legumin and vicilin, as used by Osborne, referred only to the globulins he isolated from seeds of Pisum sativum (Osborne and Campbell, 1898a). However, because he found globulins with the same approximate sedimentation coefficients as pea globulins (11S and 7S) in species of several genera of the Leguminosae, Danielsson (1949) referred to them all as legumin and vicilin. As our knowledge increased, so it became apparent that legumin, as well as vicilin, isolated from seeds of different legumes were far from identical. Nowadays these terms are, in the main, reserved

separated three 7S proteins from water-extracted soyabean proteins, which were characterised by different N-terminal amino acids, whilst Wolf and Sly (1965) isolated two 7S proteins by chromatography on hydroxylapatite. Roberts and Briggs (1965) prepared a 7S protein that was 90% pure, and showed that it was capable of reversible dimerisation to a 9S form. The apparent molecular weight of this 7S protein was strongly dependent on the protein concentration, and extrapolation to zero concentration resulted in a true molecular weight of 330,000. An electrophoretically, ultracentrifugally and chromatographically pure 7S protein prepared by Koshiyama (1965) also underwent reversible association to a 9S form (Koshiyama, 1968a), but the molecular weight of this 7S preparation, determined by four different methods, was between 180,000 and 210,000 (Koshiyama, 1968b). N-Terminal amino acid analysis of the 7S globulin preparations indicated the existence of a complex sub-unit structure; thus, Koshiyama (1968b) found a total of nine (eight different) and Okubo *et al.* (1969b) ten (nine different) moles of N-terminal amino acids per mole of 7S globulin. When dissociated in 8M urea the 7S globulin produced only one component with a molecular weight of 22,500 (Koshiyama, 1970). Experimental evidence would seem to indicate that the sub-units of the 7S globulin are held together by non-covalent forces i.e. no disulphide bonds (Koshiyama, 1971; Wolf, 1956). By means of immunoelectrophoresis Catsimpoolas *et al.* (1968) demonstrated the presence of four major antigenic components designated A, B, C and D, in soyabean seed. The use of monospecific antisera indicated that component A was equivalent to the 11S globulin (glycinin). Components B, C and D, termed β -, γ - and α -conglycinin respectively, were easily distinguishable from one another by their mobility in

immuno-electrophoresis experiments. Immuno-electrophoretic examination of the 7S globulin, as prepared by the methods of Koshiyama (1965) and Roberts and Briggs (1965) showed that the major component of each preparation was immunochemically distinct; Koshiyama's 7S preparation represented mostly γ -conglycinin, whilst that of Roberts and Briggs (1965) consisted mainly of β -conglycinin. Hence, the indications are that soyabean proteins contain two 7S globulins, β -conglycinin and γ -conglycinin, with molecular weights of 330,000 and 192,000 (average) respectively. The difference in molecular weight of these two globulins is reflected in their contrasting chromatographic behaviour. Thus, whereas γ -conglycinin (molecular weight 192,000) could be separated from both glycinin (molecular weight 350,000, Wolf and Briggs, 1959) and β -conglycinin (molecular weight 330,000) by gel filtration on Sephadex G-150, using the same experimental procedure β -conglycinin could not be separated from glycinin (Catsimpoilas et al., 1968). Similarly, Hasegawa et al. (1963) found the 11S globulin to be unseparable from a 7S component by gel filtration, although at the same time good separation from other 7S proteins was achieved.

The fourth major antigenic component of soyabean proteins, D or α -conglycinin, was immuno-electrophoretically identified as the principal contaminant present in the crude 7S globulin, as prepared by Koshiyama (1965) (Catsimpoilas and Ekenstam, 1969). As Koshiyama (1965) had already reported the presence, in his 7S preparation, of a 2S protein contaminant, Catsimpoilas and Ekenstam (1969) equated this 2S protein with their α -conglycinin. Identification of the latter with the 2S soyabean protein isolated by Vaintraub (1965) was also tentatively suggested.

The α -, β - and γ -conglycinins were isolated from crude soyabean protein preparations by a combination of gel filtration and DEAE-Sephadex chromatography (Catsimpoilas and Ekenstam, 1969), and their sub-unit compositions examined by dissociation and electrophoresis in the solvent system, phenol-acetic acid-2-mercaptoethanol-urea (Catsimpoilas, 1970). Only one sub-unit was observed for α -conglycinin, whereas β -conglycinin gave four and γ -conglycinin gave nine sub-units. These results are consistent with those of Vaintraub (1965) and Koshiyama (1968b) in that the former observed only one N-terminal amino acid (aspartic acid) for his 2S protein (α -conglycinin) and Koshiyama (1968b) detected nine moles of N-terminal amino acids per mole of 7S globulin (γ -conglycinin).

Investigations have also continued into the structure of the peanut globulins, arachin and conarachin. However, in this case, progress has been hindered by the complexity of the system, a situation apparent from the complex patterns produced by these proteins in DEAE-cellulose chromatography (Neucere and Ory, 1970; Neucere, 1969; Cherry et al., 1973; Dechary et al., 1961) and polyacrylamide gel electrophoresis (Evans et al., 1962; Dawson, 1971; Cherry et al., 1973).

The idea of a sub-unit structure for arachin was suggested by Cater et al. (1957), based on the results of N-terminal analysis and denaturation studies (Johnson and Shooter, 1950; Cater and Naismith, 1958). Isolation of the sub-units was achieved by Tombs (1965), who described four different kinds of sub-unit, two with molecular weights of 35,000 (α , β) and two with molecular weights of 10,000 (γ , δ). Three forms of arachin, A, B and A1, were found in which the sub-unit composition differed (Tombs, 1965; Tombs and Lowe, 1967); arachin A was represented

by $\alpha_4\beta_4\gamma_2\delta_2$, B by $\beta_8\gamma_2\delta_2$ and A1 by $\alpha_8\gamma_2\delta_2$. Arachin could be dissociated into these sub-units by 10M formamide at pH 8-9. Because similar electrophoretic sub-unit patterns were produced irrespective of whether 2-mercaptoethanol was present or not, Tombs (1965) concluded that any disulphide bonds were confined to intramolecular bonding only. Although later experiments with urea-mediated dissociation indicated the presence of some disulphide bonding between sub-units, this was attributed to disulphide-interchange reactions, occurring after dissociation, in which disulphide bonds, initially intramolecularly, were cleaved and then reformed intermolecularly (Tombs and Lowe, 1967).

Recent studies on arachin preparations include those of Neucere (1969), Dawson (1971) and Singh and Dieckert (1973a & b). The latter prepared an arachin fraction, called arachin P6, principally by heat treatment and $(\text{NH}_4)_2\text{SO}_4$ fractionation, and reported it homogeneous by immunodiffusion, immunoelectrophoresis, and chromatography on DEAE-cellulose and Sephadex gels. Minor components, visible in gel electrophoresis and in the ultracentrifuge, were attributed to monomeric and tetrameric forms of arachin. SDS gel electrophoresis separated arachin P6 into nine sub-units with molecular weights ranging from 15,000 to 65,000 (Singh and Dieckert, 1973b). There were six principal sub-units with molecular weights of 43,104, 41,326, 37,161, 29,019, 23,557 and 15,069. The cause of the discrepancies in the number and molecular weights of the arachin sub-units as determined by Singh and Dieckert (1973b) and by Tombs (1965) and Tombs and Lowe (1967) has still to be resolved.

The major component of the conarachin fraction, α -conarachin, was isolated from a crude protein extract by repeated chromatography on DEAE-cellulose (Dechary *et al.*, 1961; Evans *et al.*, 1962). In agreement

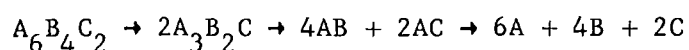
for the globulins of some species belonging to the tribe Viciaeae, e.g.

Pisum sativum, Vicia faba and Cicer arietinum.

Legumin and vicilin of pea have been separated both by the isoelectric precipitation procedure of Danielsson (1949) (Vaintraub and Gofman, 1961) and by chromatography on DEAE-cellulose (Grant and Lawrence, 1964).

Eight N-terminal glycine and four N-terminal leucine were found for legumin, and assuming a molecular weight of 380,000, it was concluded that legumin consisted of twelve polypeptide chains of average molecular weight 33,000 (Vaintraub and Gofman, 1961). The N-terminal amino acids of vicilin were found to be aspartic acid (2) and serine, indicating three sub-units of average molecular weight 58,000 (Vaintraub and Gofman, 1961).

Legumin isolated from species of Vicia contained three N-terminal amino acids, namely glycine, leucine and threonine (Vaintraub et al., 1962; Bailey and Boulter, 1970). No dissociation-association reactions were apparent in legumin of V. sativa when subjected to changes in ionic strength in the pH range 7 to 9, but irreversible dissociation to a 2S component in acid medium was demonstrated (Shutov and Vaintraub, 1966). This legumin was separated into three sub-units (A, B and C) using chromatography on DEAE-cellulose in 4M urea (Vaintraub and Nguyen Thanh Thien, 1968) and these were later shown to have molecular weights of 24,300(A), 37,600(B) and 32,600(C) by ultracentrifugation (Vaintraub and Nguyen Thanh Thien, 1971). Based on this information the structure $A_6B_4C_2$ was proposed for legumin, and using data obtained from dissociation studies, the following stepwise dissociation scheme was postulated:



In contrast to these results, legumin from V. faba was reported to

consist of three sub-units with molecular weights of 56,000, 42,000 and 23,000 (Bailey and Boulter, 1970). These results were obtained using SDS gel electrophoresis, SDS having previously been shown to effect dissociation of legumin (Grant and Lawrence, 1964; Brand and Johnson, 1956). Using a dye-binding method and in vivo radiochemical labelling, these sub-units were found to be present in the legumin molecule in the approximate ratio of 1 (56,000):3 (42,000):6(23,000).

It is apparent from various investigations that the vicilin fractions isolated from Vicia spp. are far less homogeneous and also display less similarity, than the corresponding legumin fractions. Thus, the N-terminal amino acids determined for various vicilin preparations were as follows: glutamic acid, lysine and serine (V. sativa), glutamic acid, lysine, serine and valine (V. ervilia) (Vaintraub et al., 1962) and leucine, threonine, serine and lysine (V. faba) (Bailey and Boulter, 1972). Moreover, the presence of three and four components was demonstrated in the vicilin fractions of globulins of V. sativa and V. ervilia respectively (Vaintraub et al., 1962); those of V. sativa were later separated by chromatography on DEAE-cellulose (Vaintraub and Shutov, 1964). The sub-unit structure of vicilin of V. faba was investigated by Bailey and Boulter (1972). Using SDS gel electrophoresis, they separated vicilin into four sub-units, with apparent molecular weights of 66,000, 60,000, 56,000 and 36,000. From dye-binding studies they concluded that the sub-units were present in equimolar proportions in the vicilin molecule.

This represented the limit of our present knowledge concerning the structural characteristics of the two globulins in the broad bean, and it was therefore the purpose of this present study to continue the

investigations into the sub-unit structures of these proteins and, if possible, to correlate the findings with those obtained by other workers. This entailed firstly finding or developing suitable techniques for both the isolation of these proteins in homogeneous form and also the separation of their component sub-units and secondly, the characterisation of the isolated sub-units, including determination of their primary structures. It was also considered of interest to investigate the globulins of the seed during development, in order to examine possible differences in their synthetic behaviour and sub-unit composition during this period.

MATERIALS

I. Biological Materials

Seeds of Vicia faba L. (var. Triple White) were purchased from the Tyneside Seed Company, Gateshead, Co. Durham.

II. Chemicals and Reagents

Except for those listed below, chemicals were obtained from British Drug Houses (BDH) Ltd., Poole, Dorset, or Hopkin and Williams Ltd., Chadwell Heath, Essex. They were of analytical grade where necessary.

Sephadex G50, medium, was obtained from Pharmacia Ltd., Uppsala, Sweden.

Dowex AG 1-X2 (200 to 400 mesh), was obtained from Bio-Rad Laboratories, Richmond, California, U.S.A.

Ampholine carrier ampholytes, pH ranges 3-10, 3-6 and 8-10, were obtained from LKB-Produkter A.B., Stockholm-Bromma, Sweden.

Bovine serum albumin; fraction V

Cytochrome c (horse heart)

Ovalbumin; grade V

Creatine phosphokinase (rabbit muscle)

β -lactoglobulin

Alcohol dehydrogenase (Yeast)

Lysozyme (egg white); grade I

PTH-norleucine

were obtained from Sigma Chemical Company, St. Louis, U.S.A.

PTH-amino acid standards were obtained from Mann Research Labs., New York, U.S.A.

Sequencer reagents were obtained from the Pierce Chemical Co., Illinois, U.S.A.

Chromosorb W, AW-DMCS

SP400

were obtained from Phase Separations Ltd. Queensferry, Flintshire.

Trypsin

Pepsin

were obtained from Worthington Biochemical Corporation, New Jersey, U.S.A.

METHODS

I. Growth of Biological Materials

Seeds of Vicia faba L. (var. Triple White) were grown in the University of Durham botanic gardens during the summer. Flowers were labelled when fully open, and the age of the developing seed was calculated from that day. Studies conducted on the developing seed used beans of an average cotyledon weight (Fig.1).

II. Preparation of Bean Meal

Dried mature beans were firstly ground in a Janke and Kunkel water-cooled mill (type 10) and then in a Glen Creston Micro-Hammer Mill, fitted with a grid of 1 mm mesh size.

III. Determination of Moisture Content

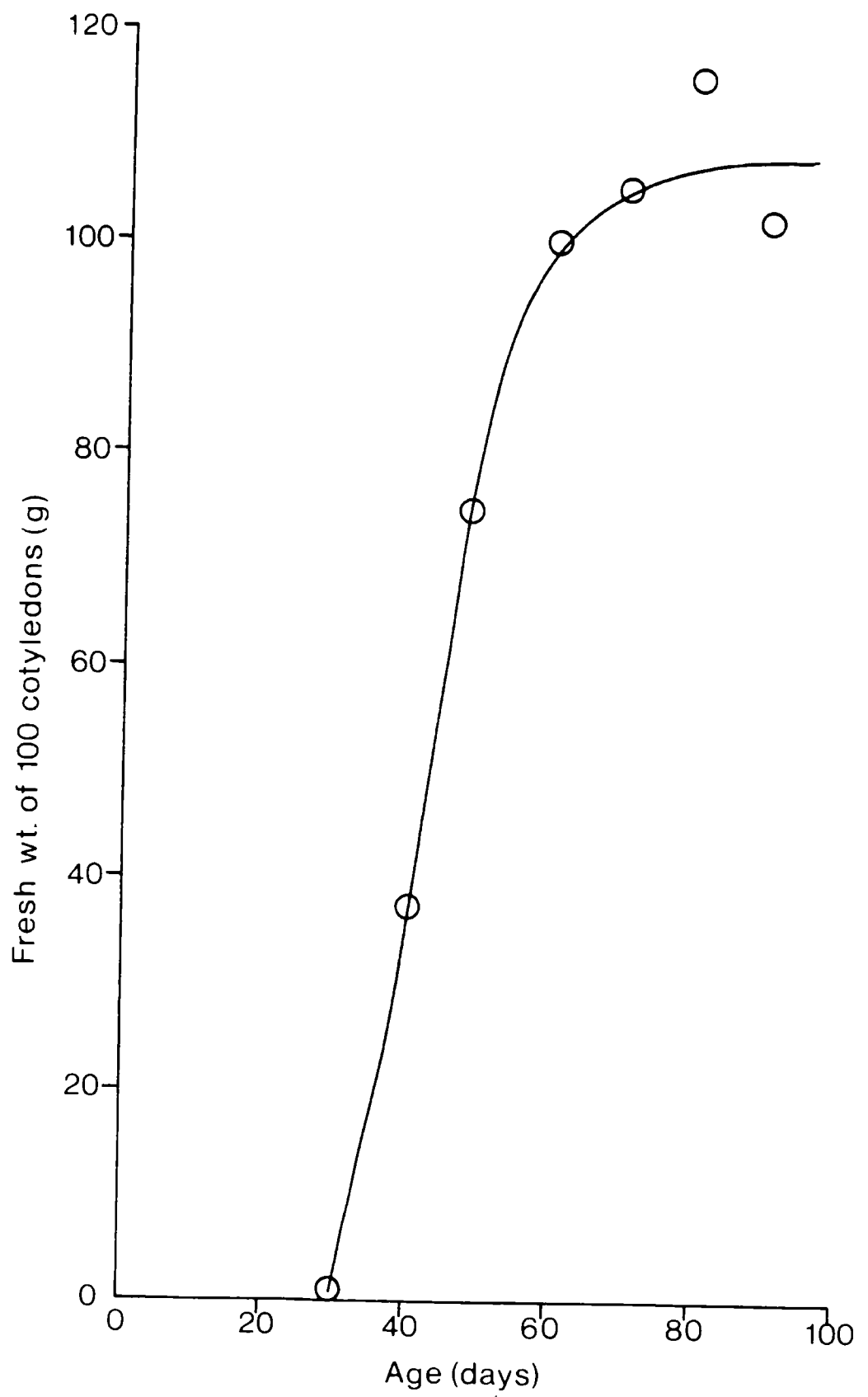
Weighed duplicate samples were dried for 2 h at 105^o, allowed to cool in a dessicator and then re-weighed to determine their water content.

IV. Determination of Total Nitrogen Content

The total nitrogen content of material was determined by a micro-Kjeldahl technique using the method of Varley (1966), as modified by T. Whitmore (private communication). Duplicate protein samples (10 to 20 mg.) were weighed out into paper cups and then dried at 105^o for 2 h to determine their moisture content. For the digestion, the sample, together with a couple of glass beads and one Kjeldahl tablet, were placed in a Kjeldahl flask. Then 5 ml. of 95% (v/v) H₂SO₄ - 5% (v/v) H₃PO₄ and 3 ml. H₂O₂ were gradually added and the mixture digested on a Gallenkamp micro-Kjedahl digestion stand until the solution was clear (approximately 30 min.). Flasks were removed, allowed to cool, and the contents

FIG.1.

The average increase in the fresh weight of developing cotyledons harvested during the 1969 season.



transferred to a volumetric flask and made up to 100 ml. Duplicate analyses were performed using a Carlo Erba autoanalyser. Nitrogen values were obtained from a calibration graph constructed using standard solutions of tyrosine.

V. Protein Determination

Protein in solution was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

VI. Extraction of Proteins

A. Extraction at pH 7.0

Extraction with phosphate-buffered salt solutions at pH 7.0 was carried out as described by Bailey and Boulter (1970), being basically the method used by Danielsson (1949).

Seed (50 g.), after soaking overnight and removal of testas, was homogenised for 2 min. with 500 ml. M NaCl, 0.05M phosphate, pH 7.0. The homogenate was stirred for 1 h. and filtered through muslin and centrifuged at 20,000 xg for 30 min. $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 70% saturation, and after 1 h. the precipitate was collected by centrifugation at 20,000 xg for 30 min. This precipitate was dispersed in 75 ml. 0.2M NaCl, 0.05M phosphate, pH 7.0, the solution clarified by centrifugation at 35,000 xg for 2h., and then dialysed against tap water overnight. The resulting globulin precipitate was collected by centrifugation at 20,000 xg for 20 min. The initial extraction from soaked seed was sometimes replaced by an extraction from finely ground bean meal. In this case, bean meal (10%, w/v) in buffer was stirred for 1 h. to effect extraction of the proteins.

B. Ascorbic acid extraction:

This was carried out as described by Wright and Boulter (1973). All operations in this procedure were performed at 5°C. Approximately 5 g. of cotyledons were homogenised with 50 ml. of a 0.5M NaCl, 0.25M ascorbic acid solution. The homogenate was filtered through muslin and then centrifuged at 25,000 xg for 30 min. The supernatant was decanted, clarified again at 25,000 xg for 30 min, and then stirred during the addition of an equal volume of deionised water. The resultant precipitate (fraction I) was centrifuged down at 25,000 xg for 30 min. The supernatant was dialysed for 4 h. firstly against tap water, then glass distilled water and finally deionised water. The precipitate (fraction II) was collected by centrifugation at 25,000 xg for 30 min.

C. Sodium dodecyl sulphate extraction:

Four types of extractants were employed:

- (i) 3% (w/v) SDS, 0.05M NaH₂PO₄ adjusted to pH 7.0 with dilute NaOH.
- (ii) As (i) but including 1% (v/v) 2-mercaptoethanol.
- (iii) 4% (w/v) SDS, 0.125M Tris-HCl, pH 6.8.
- (iv) As (iii) but including 1% (v/v) 2-mercaptoethanol.

2% (w/v) suspensions of bean meal in extractant were stirred for 2-3 h. at room temperature. Insoluble debris was removed by centrifugation.

D. Extraction at pH 4.7:

A 5% (w/v) suspension of bean meal in 0.1M NaCl, maintained at pH 4.7 by the addition of 1M HCl, was stirred for 1 h, and then centrifuged at 23,000 xg for 20 min. The clear supernatant was decanted off the residue and the proteins precipitated either, (a) by dialysing against water, or (b) by adjusting the pH to 8 with dilute NaOH.

VII. Separation of Globulin Components

A. Isoelectric precipitation:

Isoelectric precipitation was performed as described by Bailey and Boulter (1970).

B. Zonal isoelectric precipitation:

The method, with some small modifications, was that used by Shutov and Vaintraub (1965), and is outlined below.

(i) Buffers

The following buffers were used:

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

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

(ii) Preparation of Sephadex G50:

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

(iii) Preparation of column:

Columns were poured at room temperature, and the resin allowed to settle under gravity. Hydrostatic pressure was used to pack the column.

(iv) Elution of column:

The sample, dissolved in phosphate buffer, was applied to the column and was washed in with the same buffer. Elution of the column was continued with phosphate buffer. Column eluant was monitored continuously at 280 nm using an Isco Ultraviolet Analyzer in conjunction with a Servoscribe potentiometric chart recorder. Fractions were collected using an LKB Ultrorac fraction collector.

VIII. Carboxymethylation of Proteins

This was performed as described by Bailey and Boulter (1970). Protein (500 mg.) was dissolved in 25 ml. of 6M guanidine hydrochloride, 1M Tris-Cl pH 8.7, containing 1mM EDTA, and the solution flushed with nitrogen. 2-Mercaptoethanol (0.25 ml.) was added and the solution incubated at 35° for 4-5 h. Iodoacetic acid (1.2 g.) was added and the reaction allowed to proceed in the dark. After 15 min., when a negative nitroprusside reaction was obtained, the solution was dialysed against tap water, then distilled water and finally freeze-dried.

IX. N-Terminal Amino Acid Analysis

The N-terminal amino acids of proteins were determined by the dansylation procedure of Gros and Labouesse (1969).

X. Acid Hydrolysis and Amino Acid Analysis of Proteins

Duplicate samples in 6N HCl (approx. 2mg/ml.) were hydrolysed in vacuo at 105° in sealed Pyrex tubes. Hydrolysates were dried in vacuo over solid NaOH and then analysed on a Locarte automatic loading amino acid analyser.

XI. Analytical Ultracentrifugation

This was performed using a Christ Omega II 70000 Ultracentrifuge. Runs were carried out at 40,000 rev/min and 20°. Sedimentation coefficients and $S_{20,w}$ values were determined as described by Svedberg and Pedersen (1940), and molecular weights were calculated according to Halsall (1967). Serial dilutions of some samples were also run to examine the effect of protein concentration on sedimenting species.

XIII. Polyacrylamide Gel Electrophoresis

Acrylamide and bisacrylamide were recrystallised according to the method of Loening (1967). Throughout this thesis, gel concentrations (i.e. 5%, 7.5% and 10%) are referred to on the basis of acrylamide concentration only, and not concentration of total monomer (i.e. acrylamide and bisacrylamide).

A. Analytical scale gel electrophoresis:

(i) Gel system

The following gel systems were employed, with the modifications listed:

(a) pH 8.3 (Ornstein, 1964; Davis, 1964). 5% and 7.5% gels.

The reservoir buffer was used undiluted. For some experiments, 6M urea was incorporated into all gel solutions.

(b) pH 4.5 (Reisfeld et al., 1962). 5% and 7.5% gels.

In some experiments 6M urea was incorporated into all gel solutions.

(c) pH 8.9 (modification of Akroyd, 1968). 10% gels.

The gel and reservoir buffers were prepared according to Akroyd (1968). The monomer solution consisted of 40% (w/v) acrylamide and 0.8% (w/v) Bis. The gel solution was prepared by mixing 6 ml. of the monomer solution, 3 ml. of the gel buffer, 2 ml. of 1% (w/v) ammonium persulphate solution, 8.65 gm. urea to a final volume of 24 ml with distilled water.

(d) SDS-phosphate, pH 7.0 (Schapiro et al., 1967; Weber and Osborne, 1969). 10% gels.

The stock solutions required were prepared as follows:

- Solution A: 20 g. acrylamide
0.54 g. bisacrylamide
0.20 g. SDS
0.15 ml. Temed
50 ml. 0.2M NaH_2PO_4 (adjusted to pH 7.0 with dilute NaOH)
Water to 100 ml.
- Solution B: 0.15 g. ammonium persulphate
50 ml. 0.2M NaH_2PO_4 , pH 7.0
Water to 100 ml.

Gels were prepared by mixing 1 volume each of solutions A and B. Electrophoresis buffer was 0.1M NaH_2PO_4 , 0.1% (w/v) SDS adjusted to pH 7.0 with dilute NaOH. Samples were dissolved at a concentration of approximately 2 mg/ml. in 0.2M NaH_2PO_4 , 0.2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol adjusted to pH 7.0 with dilute NaOH, and were then incubated at 37°C for 3 h. Sample solutions were diluted with 1 volume of 50% (v/v) glycerol prior to their application to gels. Electrophoresis was carried out at 10 volts cm^{-1} for 3 h. Gels were stained in either 0.5% (w/v) amido black in methanol/acetic acid/ H_2O (5:1:5, by vol.), or more usually 1% (w/v) amido black in 7% (v/v) acetic acid for 1 h. then diffusion destained in 7% (v/v) acetic acid.

(e) SDS- pH 8.3. 10% gels.

This system was used in both discontinuous and continuous forms. The discontinuous system is that described by Laemmli (1970), incorporating a stacking gel and used without a pre-run prior to electrophoresis. The continuous system is an analytical scale version of that used for preparative electrophoresis and is described in detail later (Methods XII (c)(ii)). No stacking gel was employed in this system and the gels were pre-electrophoresed.

After electrophoresis, gels were fixed overnight in 50% (w/v) trichloroacetic acid, rinsed for 3 h. in several changes of 7% (v/v) acetic acid and stained for 1 h. with 1% (w/v) amido black in 7% (v/v) acetic acid.

(f) SDS-citrate, pH 7.0 10% gels.

The following stock solutions were required:

Monomer solution: 30 g. acrylamide

0.8 g. bisacrylamide

Water to 100 ml.

Gel buffer: 48 ml. M NCl

5.9 g. Tris

0.46 ml. Temed

Water to 100 ml.

Reservoir buffer (pH 7.0): 16.15 g. Tris

8.95 g. citric acid

2 gm. SDS

Water to 2L.

Gels containing 10% acrylamide were prepared by mixing 8 ml. of monomer solution, 6 ml. of gel buffer, 4 ml. of 0.6% (w/v) SDS and 6 ml. of 0.28% (w/v) ammonium persulphate solution.

Gels were electrophoresed at 6 volts cm^{-1} for 3-4 h. and were then stained in 1% (w/v) amido black in 7% (v/v) acetic acid.

(ii) Recording of electrophoretic patterns:

Gels were scanned in transmission at 620 nm using a Joyce-Loebl Chromoscan. Peak areas were measured using a Technicon integrator/calculator.

Gel patterns were also recorded in the form of line drawings.

(iii) Molecular weight determinations:

Molecular weights of polypeptide chains were determined on 10% gels containing SDS, using the methods of Weber and Osborne (1969) and Laemmli (1970). Gels were calibrated using the following protein standards (molecular weights are given in parentheses): lysozyme (14,300), β -lactoglobulin (18 400), trypsin (23,300), pepsin (35,000), alcohol dehydrogenase (37,000), creatine phosphokinase (40,000), ovalbumin (43,000), γ -globulin (25,000 and 55,000) and serum albumin (64,000).

In the system of Weber and Osborn (1969), relative mobilities were calculated relative to cytochrome c. Using the discontinuous system of Laemmli (1970), the bromophenol blue marker band remained very sharp throughout the course of the experiment, and so mobilities could be measured relative to this.

B. Preparative scale gel electrophoresis

(i) Apparatus:

A Quickfit 'Prep-P.A.G.E.' apparatus was employed in these studies. Reservoir buffer recirculation was achieved using an LKB Varioperpex peristaltic pump. An LKB Recychrom peristaltic pump was used to pump elution buffer through the elution chamber. Eluant was monitored at 280 nm by an Isco Ultraviolet Analyzer connected to a Servoscribe chart recorder. Fractions were collected in an LKB Ultrorac fraction collector.

(ii) Procedure:

The principal gel system employed was a modification of that of Ornstein (1964) & Davis (1964) and is described below. The SDS-phosphate, pH 7.0 and SDS-citrate, pH 7.0 systems, as described previously, were also used on occasions.

(a) Preparation of gel

The following solutions were required:

A: 30 g. acrylamide

0.8 g. bisacrylamide

add distilled water to 100 ml.

B: 0.8% (w/v) SDS

C: 36.3 g. Tris

0.46 ml. Temed

48 ml. 1M HCl

add distilled water to 100 ml.

D: 0.28% (w/v) ammonium persulphate solution.

The gel solution was prepared as follows:

8 vol. A + 3 vol. B + 3 vol. C + 4 vol. water + 6 vol. D

This recipe produced a 10% gel, 0.1% in SDS.

(b) Buffer solutions

Reservoir buffer (pH 8.4):

6 g. Tris

28.8 g. glycine

1 g. SDS

0.1 g. dithiothreitol (if required)

add distilled water to 1L.

Elution buffer (pH 8.6):

52 g. Tris

14 ml. acetic acid (glacial)

1 g. SDS

0.1 g. dithiothreitol (if required)

add distilled water to 1L.

(c) Operation

After polymerisation of the gel, the apparatus was assembled as described in the manufacturer's manual. Pre-electrophoresis was carried out until the ultra-violet-absorbing material (catalysts, residual acrylamide, etc.) had been eluted. The sample, dissolved in approximately 2 ml. of ten-fold diluted reservoir buffer and density stabilised with glycerol, was layered onto the gel surface using fine bore Teflon tubing attached to a peristaltic pump. Pooled fractions were concentrated using an Amicon ultrafiltration cell (Model 12) fitted with a PM30 membrane.

In order to obtain good, reproducible results when using the preparative electrophoresis apparatus, it was found necessary to observe the following precautions:

(a) Levelling of the apparatus

During both gel polymerisation and subsequent electrophoresis, the elution chamber should be in a perfectly horizontal position. This ensures that the sample migrates through the gel parallel to both the surface of the gel and the axis of the elution chamber, resulting in less trailing of eluted components and thus better resolution.

(b) Level gel surface

Any irregularities in the gel surface or upper gel layers will clearly affect the resolution. To avoid this, water is layered on top of the gel solution, prior to polymerisation, using fine bore Teflon tubing connected to a peristaltic pump. Careful control during this operation results in little, if any, mixing with the gel solution. The gel surface will be uniform and there should be no concentration discontinuities present in the gel.

On occasions when it was necessary to repeat the separations of proteins or protein sub-units under identical conditions, it was found more convenient and less time-consuming to use the same gel, than to disassemble the apparatus and all its associated equipment in order to polymerise a fresh gel. This was feasible providing, (i) the gel surface had not been damaged (e.g. during sample application), (ii) the gel had not separated from the sides of the apparatus, possibly as a result of overloading, (iii) samples had been centrifuged before application to remove insoluble debris that might otherwise block the pores at the gel surface, and (iv) the reservoir buffer was replaced with fresh buffer.

XIII. Protein Extraction from Stained Gel Bands

The procedure of Wada and Snell (1972) was used to extract proteins from stained sections of SDS gels. The gels were stained and destained as outlined in Methods, and then corresponding slices from several gels were combined and homogenised with 5 ml. of 70% (v/v) formic acid in an MSE homogeniser at full setting. A further 5 ml. 70% (v/v) formic acid was added and the gels allowed to soak at -20°C with occasional shaking for about 24 h. The homogenate was filtered through a medium pore glass sinter and the residue extracted once more. Combined extracts were applied to a column of Dowex 1 that had previously been equilibrated with 70% (v/v) formic acid. The amido black was retained at the top of the resin, while the proteins were eluted from the column free of dye. Protein was recovered either by lyophilisation or rotary evaporation of the eluant.

XIV. Ion-exchange Chromatography

Dowex AG1-X2 was prepared as outlined by Weber and Kuter (1971), with slight modifications. The resin was successively washed with 2M NaOH, distilled water, 4M acetic acid, distilled water and finally 50mM Tris-acetate, pH 8.0. Just before use the resin was equilibrated on a glass sinter with 50 mM Tris-acetate, pH 8.0, 6M in urea. The same buffer was used for sample application and also for initial elution. Subsequent elution was performed with 50 mM acetic acid, 6M in urea, adjusted to pH 4.5 with dilute NaOH. Column eluant was monitored at 280 nm with an Isco Ultraviolet Analyzer.

XV. Gel Isoelectric Focussing

The method adopted was basically that of Wrigley (1968) and is outlined below:

A. Preparation of gel

6 cm. gels, cast in 90 x 6 mm perspex tubes, were prepared from the following solutions:

40% carrier ampholine ampholytes:	1 ml.
Monomer solution (50 g. acrylamide, 1.25 g. bisacrylamide, 200 μ l Temed to 100 ml):	3 ml.
8M urea (deionised):	15 ml.
1% (w/v) ammonium persulphate:	0.5 ml.
Distilled water to 20 ml.	

B. Procedure

Protein samples were either incorporated directly into the gel solution (1-2 mg) before polymerisation, or applied to the top of the gel (50-100 μ g) under a protective layer of ampholine ampholytes. Upper reservoir buffer (cathode) contained 0.01M NaOH and the lower reservoir (anode) 0.005M H₂SO₄. Isoelectric focussing was performed at 2mA per tube until the voltage reached 350 V, then at a constant voltage of 350 volts for the remainder of the experiment. Gels were removed at intervals in order to observe the progress of the focussing bands.

Focussed bands were made visible by two methods:

- (i) the bromophenol blue staining procedure of Awdeh et al. (1968).
- and (ii) precipitation with 10% (w/v) trichloroacetic acid, with subsequent staining with 1% (w/v) amido black in 7% (v/v) acetic acid after the carrier ampholytes had diffused out of the gel.

XVI. Determination of Sub-unit Ratios

Two methods were employed to determine the ratios of sub-units present in legumin and vicilin. One involved the quantitative measurement of the amount of dye bound to each sub-unit on SDS gels, and the other was

based on amino acid composition data (Burgess, 1969). In the case of the dye-binding procedure, the relative amounts of dye bound to each sub-unit were obtained by integrating the densitometric traces of the gels. Alternatively, by using the amino acid compositions determined for the isolated sub-units and the intact protein, it was possible to calculate weighting coefficients which, in effect, represented the relative proportions of each sub-unit required to produce a protein of the desired amino acid composition. The "best fits" for these coefficients were obtained by regression analysis.

XVII. Deionisation of Urea Solutions

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

XVIII. Sequence Analysis

Amino acid sequence investigations were performed on a Beckman Model 890C Sequencer using the fast protein-quadrol programme.

A. Detection of PTH-amino acids using thin-layer chromatography

Unknown PTH-amino acids were chromatographed together with standard mixtures of PTH-amino acids on silica gel sheets (Eastman Chromagram). Sheets were developed firstly in the solvent system: heptane/propionic acid/dichloroethane (58:17:25, by vol.) and then, after drying and examination, in the same dimension, using the system, heptane/butan-1-ol/75% (v/v) formic acid (50:30:9, by vol.).

B. Detection of PTH-amino acids using gas chromatography

A Varian Aerograph (Series 1400) gas chromatograph was used to analyse the PTH-amino acids, which in some instances, were run after

conversion to their TMS derivatives by treatment with NO-bis (tri-methylsilyl)acetamide. PTH-norleucine was used as an internal standard for quantifying certain PTH-amino acids.

The column employed for these investigations was composed of 10% SP400 on Chromosorb W, AW-DMCS and was 4ft in length. A linear temperature programme of 160^o to 270^o at a rate of 8^o/min was used.

C. Detection of PTH-arginine and PTH-histidine

The presence of these derivatives was established using the phenanthrenequinone (Yamada and Itano, 1966) and Pauly spot tests respectively.

RESULTS

I. Moisture content of bean meal

The average loss in weight of meal samples after drying at 105° for 2 h. was 13.4 ± 0.8%.

II. Nitrogen content of bean meal

The total nitrogen content of Vicia faba meal, determined by the micro-Kjeldahl method, was 5.53 ± 0.08% after correcting for water content.

III. Characterisation of extracted and fractionated proteins of Vicia faba

A. Components separated by isoelectric precipitation

(i) Separation

The crude globulin preparation obtained by extraction at pH 7.0 was fractionated into two components by isoelectric precipitation at pH 4.7. The majority of the protein was precipitated at this pH (legumin), while the 4.7 soluble protein (vicilin) was obtained as a precipitate after dialysis of the supernatant against water. Unless proteins were required for immediate use, they were lyophilised and stored at -20°.

(ii) Analytical ultracentrifugation

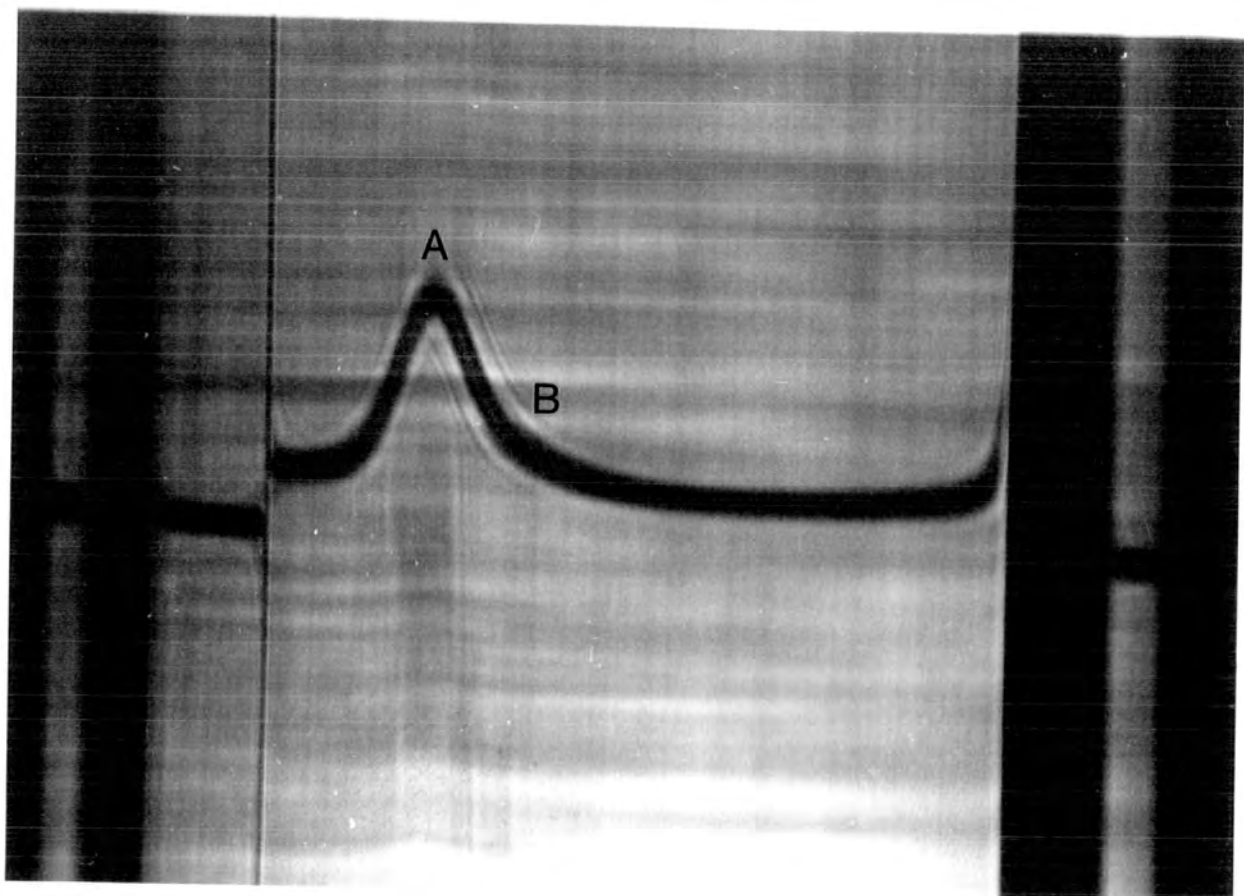
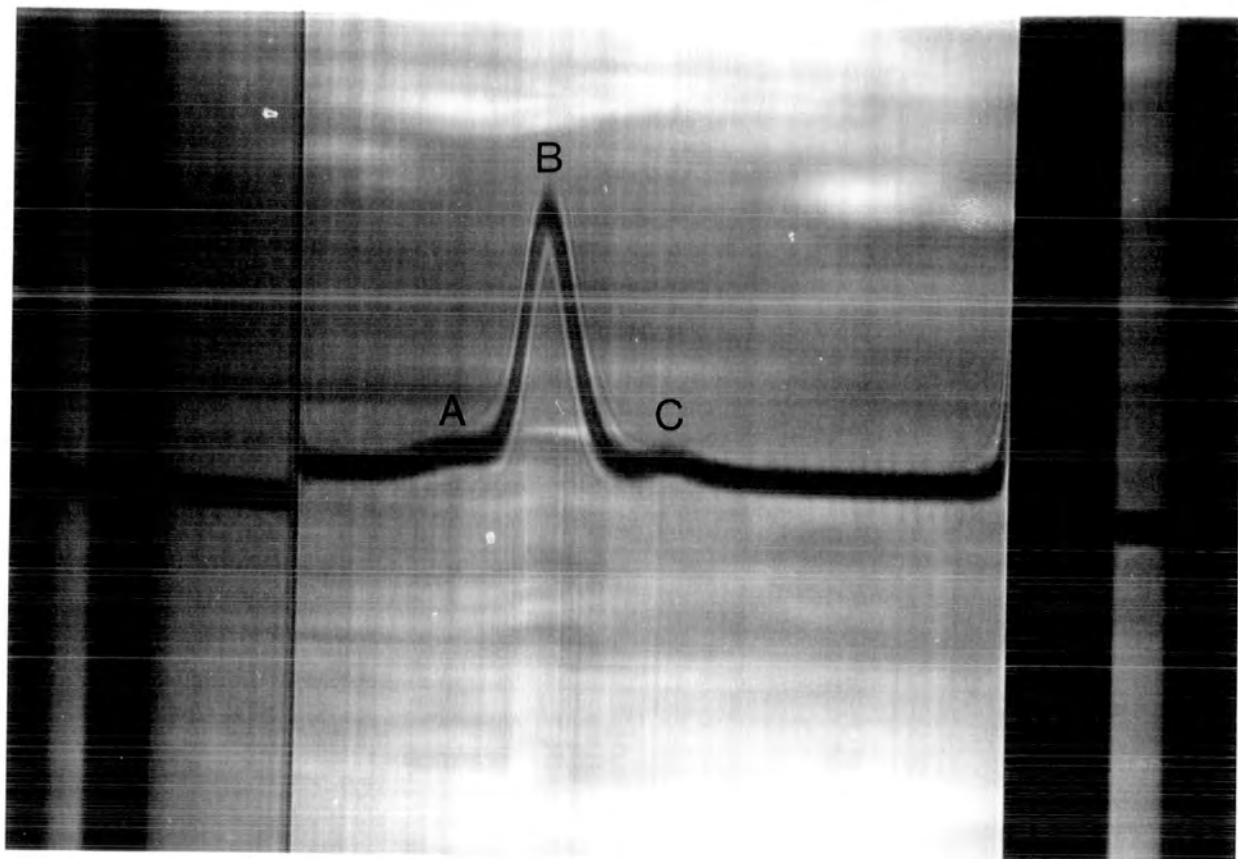
The homogeneity of the two fractions was examined by ultracentrifugation analysis. The results of this showed that legumin was composed of one major and two minor components (Plate 1), and vicilin of one major and one minor component (Plate 2). Using partial molar volumes of legumin (0.722) and vicilin (0.733), calculated from the amino acid compositions (Bailey and Boulter, 1970 and 1971), the $s_{20,w}$ values of the legumin ultracentrifuge components were found to be 8.6S(A), 11.7S(B) and 16.9S(C), and those of the vicilin fraction, 7.1S(A), and 8.6S(B). The effect of protein concentration on the sedimentation

PLATE 1

Ultracentrifuge pattern of legumin, separated from total globulins of V. faba by isoelectric precipitation. Protein is dissolved in 0.5M NaCl. Photograph was taken 60 min. after centrifuge had reached a speed of 40,000 rev/min. Bar angle, 70°. Direction of sedimentation: left to right.

PLATE 2

Ultracentrifuge pattern of vicilin, separated from total globulins of V. faba by isoelectric precipitation. Protein is dissolved in 0.5M NaCl. Photograph was taken 65 min. after centrifuge had reached a speed of 40,000 rev/min. Bar angle, 60°. Direction of sedimentation: left to right.



behaviour of the major (11.7S) legumin component is shown in Fig.2. Extrapolation to zero concentration resulted in an $s_{20,w}$ value of 11.4S. The molecular weight of the major component of each fraction was $328,000 \pm 50,000$ for legumin (B) and $150,000 \pm 25,000$ for vicilin (A).

(iii) Electrophoretic Analysis

The electrophoretic patterns produced by legumin and vicilin when analysed in the pH 8.3 gel system are shown in Fig.3. While the major band in legumin ($R_m = 0.14$) is well-defined, that of vicilin is diffuse, stretching from $R_m = 0.18$ to $R_m = 0.26$. Other bands present on the gels may be due to polymeric forms of the proteins or cross-contamination of the preparations. The sub-unit structures were investigated using the SDS-phosphate, pH 7.0 gel system. The molecular weight calibration graph for this gel system is shown in Fig.4. Using this, the molecular weights of the three main legumin bands are 21,500, 35,000 and 46,800, and of the two minor bands 56,500, and 62,500 (Fig.5a). After carboxymethylation, the molecular weights of the three main components were calculated as 48,500, 37,000 and 23,000. When vicilin was dissociated with SDS and electrophoresed the band pattern was much more complex than that of legumin (Fig.5b). The major vicilin components have molecular weights ranging from 40,000 to 60,000.

(iv) N-Terminal analysis of legumin

Using the dansylation procedure, the N-terminal amino acids of legumin were qualitatively identified as leucine, glycine and threonine.

B. Components separated by zonal isoelectric precipitation

(i) Chromatography results

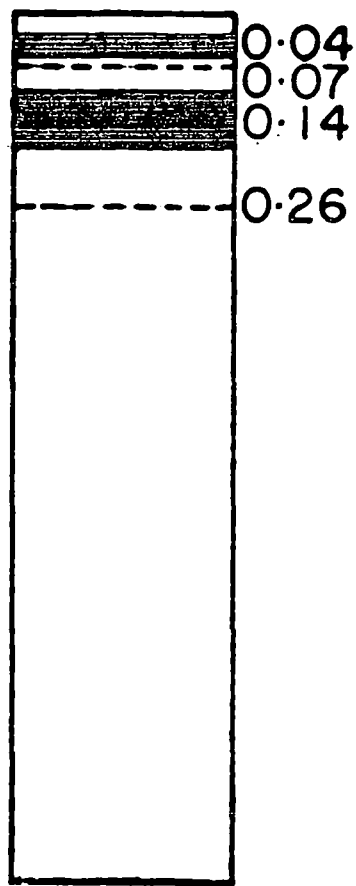
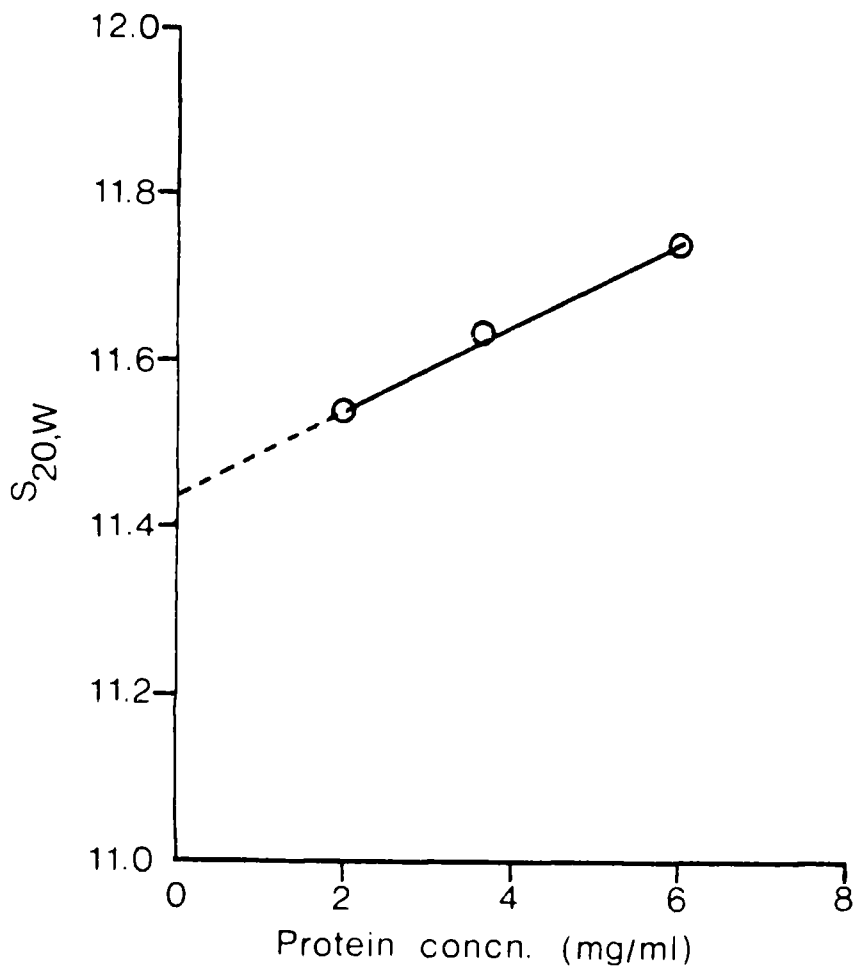
Crude proteins to be fractionated by zonal isoelectric

FIG.2

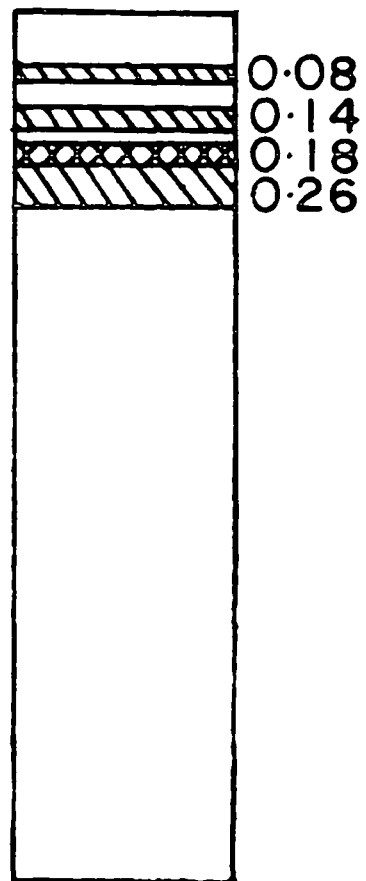
The effect of protein concentration on the $s_{20,w}$ value of legumin.

FIG.3

Electrophoretic patterns of (a) legumin and (b) vicilin separated by isoelectric precipitation. Electrophoresis was performed in the pH 8.3 gel system, using 7.5% gels. Mobilities were calculated relative to bromophenol blue (not shown).



a



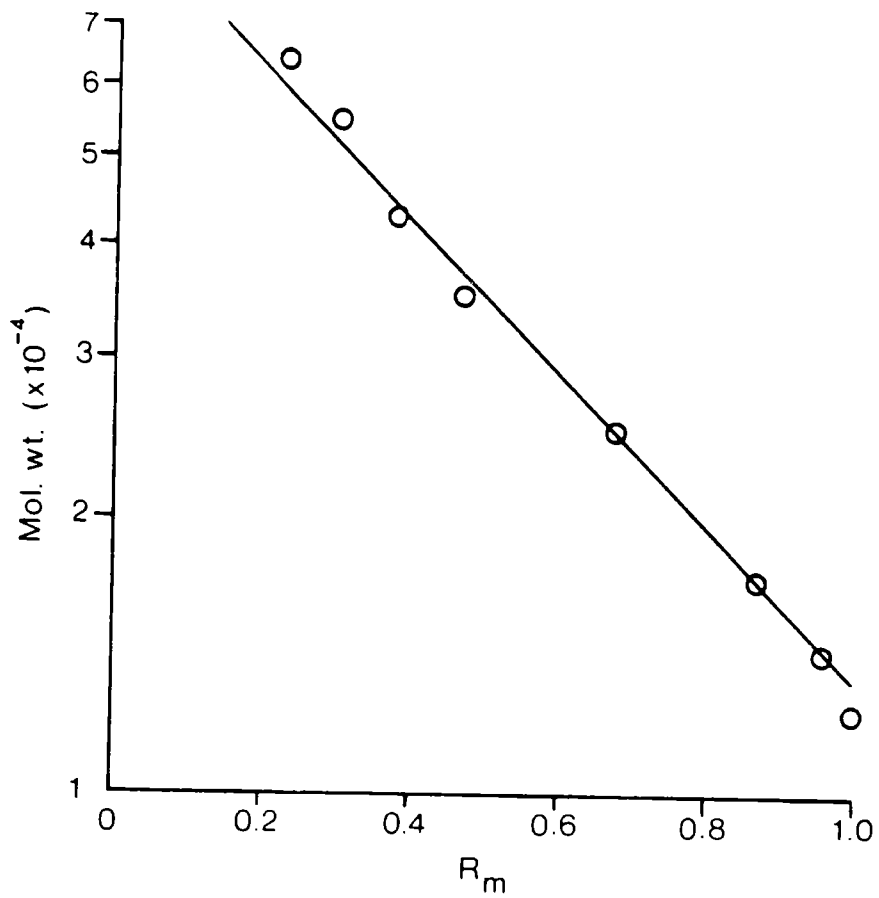
b

FIG.4

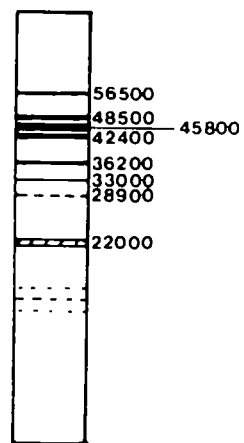
Molecular weight calibration graph for the SDS-phosphate, pH 7.0 gel system. Protein standards, in order of decreasing molecular weights, are: serum albumin, γ -globulin (H-chain), ovalbumin, pepsin, γ -globulin (L-chain), myoglobin, lysozyme and cytochrome c.

FIG.5

SDS Electrophoretic patterns and sub-unit molecular weights of (a) legumin and (b) vicilin separated by isoelectric precipitation. Electrophoresis was performed in the SDS-phosphate, pH 7.0 gel system, using 10% gels. Protein samples were dissolved in buffer containing SDS and 2-mercaptoethanol as described in Methods (section XII.A(i)(d)). The molecular weights of the polypeptide chains were obtained from the calibration graph in Fig.4.



a



b

precipitation were normally prepared by the addition of $(\text{NH}_4)_2\text{SO}_4$ to 100% saturation to the initial phosphate, pH 7 extracts. This procedure resulted in a protein precipitate which dissolved very readily in the column elution buffer (phosphate, pH 8.0). Before application to the column, the sample was dialysed against the elution buffer to remove all traces of $(\text{NH}_4)_2\text{SO}_4$.

A typical elution profile for zonal isoelectric precipitation is illustrated in Fig.6. The globulins were recovered from the two fractions by dialysis against water, and were then freeze-dried. Fraction II (4.7 insoluble) was pure white in colour, while fraction I (4.7 soluble) was slightly creamy.

Fraction I was purified further by conventional isoelectric precipitation carried out at reduced ionic strength (i.e. in 0.1M NaCl). The results of this purification were assessed by gel electrophoresis.

Rechromatography of fraction II under identical conditions resulted in the separation of only a small amount of impurity, which eluted in the void volume (Fig.6). This contaminant was retained for analysis on SDS gels.

Two fractions, designated IA and IIA, were also obtained when zonal isoelectric precipitation of crude globulin was performed at lower ionic strength (0.1M NaCl). The composition of these two fractions was also examined by SDS gel electrophoresis.

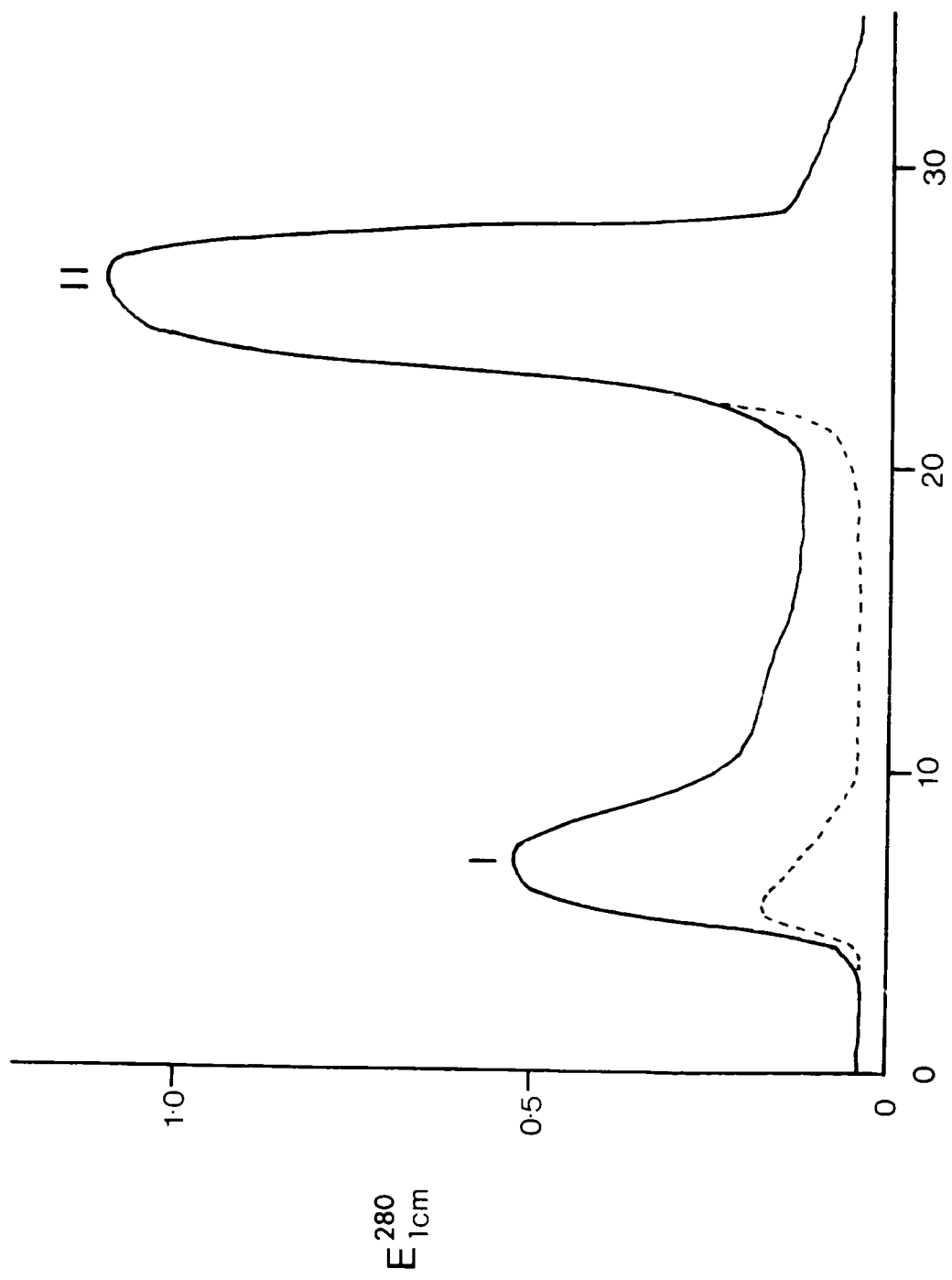
(ii) Moisture content of column fractions

The average moisture content of fractions I and II were found to be $4.9 \pm 1.3\%$ and $6.2 \pm 0.1\%$ respectively. Carboxymethylated fraction II contained $7.5 \pm 0.2\%$ moisture.

FIG.6

Elution profile for zonal isoelectric precipitation of V. faba globulins. Elution was performed on a 2.2 x 53 cm. column at a rate of 36 ml/h. 4.8 ml. fractions were collected.

..... elution profile obtained when fraction II was rechromatographed.



Fraction no.

(iii) Nitrogen content of column fractions

The total nitrogen content of fractions I and II, after correcting for the water content, were $17.5 \pm 0.3\%$ (I) and $17.8 \pm 0.2\%$ (II). Carboxymethylated fraction II contained $16.4 \pm 0.1\%$ nitrogen.

(iv) Amino acid composition of carboxymethylated fraction II

The amino acid analysis of 20 h. and 72 h. hydrolysates of duplicate samples of CM fraction II are collected together in Table 1. Sample weights were corrected for a moisture content of 7.5%. The calculated amino acid composition of this protein is given in Table 2. Values for serine, threonine and tyrosine were obtained by extrapolation to zero time. Tryptophan, cysteine and methionine were not determined.

(v) Polyacrylamide gel electrophoresis of column fractions

When analysed in the pH 8.3 gel system, fractions I and II gave very similar patterns, with one major and one minor band (Fig.7). When electrophoresed in a dissociating gel system (SDS-phosphate, pH 7) fraction II displayed two major and one minor band, and fraction I, five major and three minor bands. The molecular weight of these component polypeptides are presented alongside the electrophoretic patterns in Fig.8.

The SDS gel pattern of the contaminant, separated from fraction II after rechromatography, is shown, as a densitometric trace, in Fig.9. The two main components have molecular weights of 20,800 and 28,400.

The column fractions were also analysed in the discontinuous SDS-pH 8.3 gel system, the molecular weight calibration curve for which is shown in Fig.10. Fractions were electrophoresed after treatment with either SDS and 2-mercaptoethanol (Fig.11 band d) or SDS alone (Fig.11a and c). The results from both SDS gel systems indicate that fraction I

TABLE 1

Amino acid analyses of hydrolysates of CM fraction II (legumin)

Amino acid	Recovery	
	20 h.	72 h.
	g*/100 g. protein	
Aspartic acid	11.42 ± 0.15	10.84 ± 0.29
Threonine	2.53 ± 0.01	2.51 ± 0.06
Serine	4.13 ± 0.08	3.45 ± 0.0
Glutamic acid	17.96 ± 0.08	16.40 ± 0.57
Proline	4.61 ± 0.70	4.01 ± 0.19
Glycine	3.01 ± 0.14	3.04 ± 0.07
Alanine	3.24 ± 0.13	3.18 ± 0.03
Valine	3.58 ± 0.07	3.99 ± 0.0
Isoleucine	3.09 ± 0.02	3.61 ± 0.0
Leucine	7.05 ± 0.23	6.78 ± 0.08
Tyrosine	3.29 ± 0.07	3.12 ± 0.07
Phenylalanine	3.86 ± 0.52	3.41 ± 0.03
Histidine	1.99 ± 0.15	2.07 ± 0.02
Lysine	4.73 ± 0.10	4.98 ± 0.06
Arginine	10.13 ± 0.54	10.91 ± 0.73
	<u>85.4</u>	<u>83.04</u>

* Amino acid residues

The figures are the mean values and errors from duplicate analyses. The small amount of methionine present in the hydrolysates made its determination inaccurate and so the results are not quoted.

TABLE 2

The amino acid composition of CM fraction II (legumin)

Amino acid	Content ^a		
	g/100 g. protein	moles/10 ⁵ g. protein	mole%
Aspartic acid	11.42	99.2	12.96
Threonine ^b	2.54	25.1	3.28
Serine ^b	4.43	50.9	6.65
Glutamic acid	17.96	139.1	18.17
Proline	4.61	47.5	6.20
Glycine	3.04	53.3	6.96
Alanine	3.24	45.6	5.96
Valine	3.99	40.2	5.25
Isoleucine	3.61	31.9	4.17
Leucine	7.05	62.3	8.14
Tyrosine ^b	3.36	20.6	2.69
Phenylalanine	3.86	26.2	3.42
Histidine	2.07	15.1	1.97
Lysine	4.98	38.8	5.07
Arginine	10.91	69.8	9.12
	<u>87.85</u>	<u>765.6</u>	

a. Values refer to amino acid residues

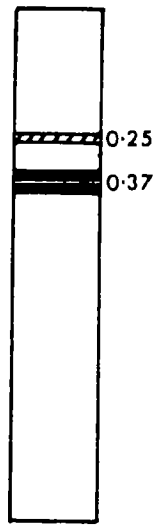
b. Extrapolated to zero time

FIG.7

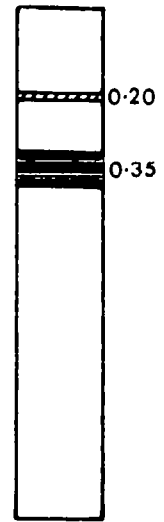
Electrophoretic patterns of (a) fraction I and (b) fraction II obtained by zonal isoelectric precipitation of V. faba globulins. Electrophoresis was performed in the pH 8.3 gel system, using 5% gels. Mobilities were calculated relative to bromophenol blue.

FIG.8

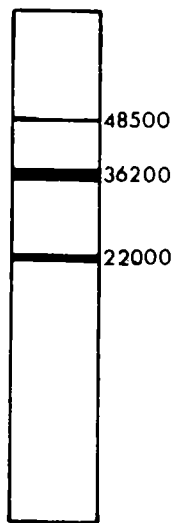
SDS electrophoretic patterns and sub-unit molecular weights of (a) fraction II and (b) fraction I, obtained by zonal isoelectric precipitation of V. faba globulins. Electrophoresis was performed in the SDS-phosphate, pH 7.0 gel system (10% gels).



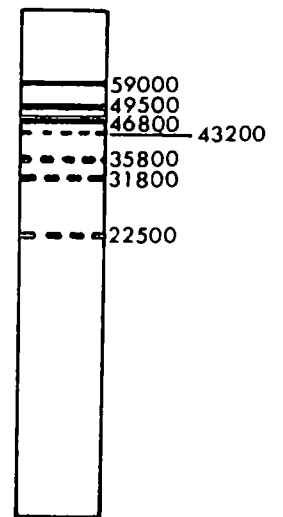
a



b



a



b

FIG. 9

SDS electrophoretic pattern of contaminant isolated from fraction II after second cycle of zonal isoelectric precipitation (see Fig.6). Electrophoresed in the SDS-phosphate, pH 7.0 gel system (10% gels). Numbers above peaks refer to the molecular weights ($\times 10^{-3}$) of the associated polypeptide chains.

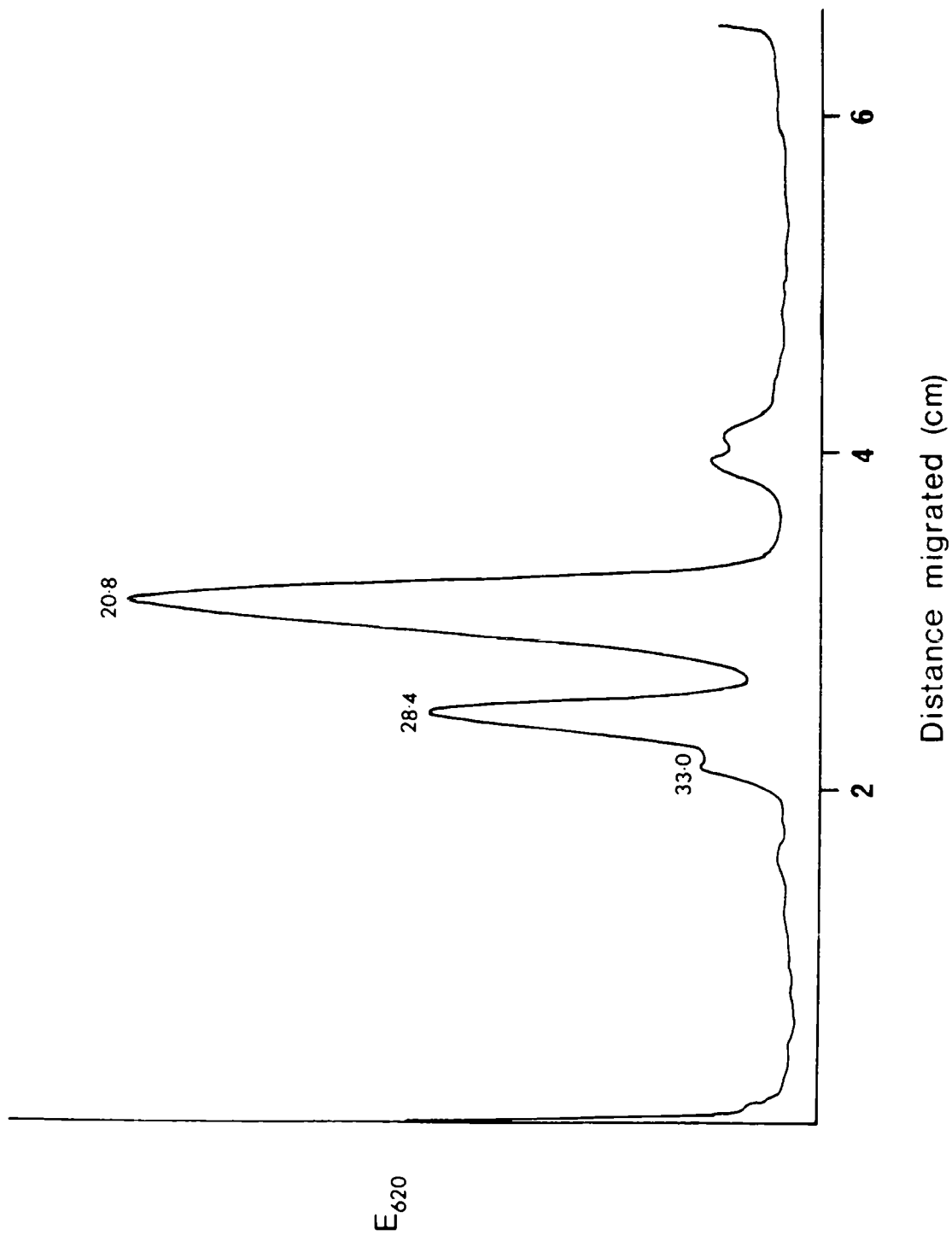


FIG. 10

Molecular weight calibration graph for the SDS-pH 8.3 gel system. Protein standards, in order of decreasing molecular weight are: serum albumin, γ -globulin (H-chain), ovalbumin, creatine kinase, alcohol dehydrogenase, pepsin, trypsin and β -lactoglobulin.

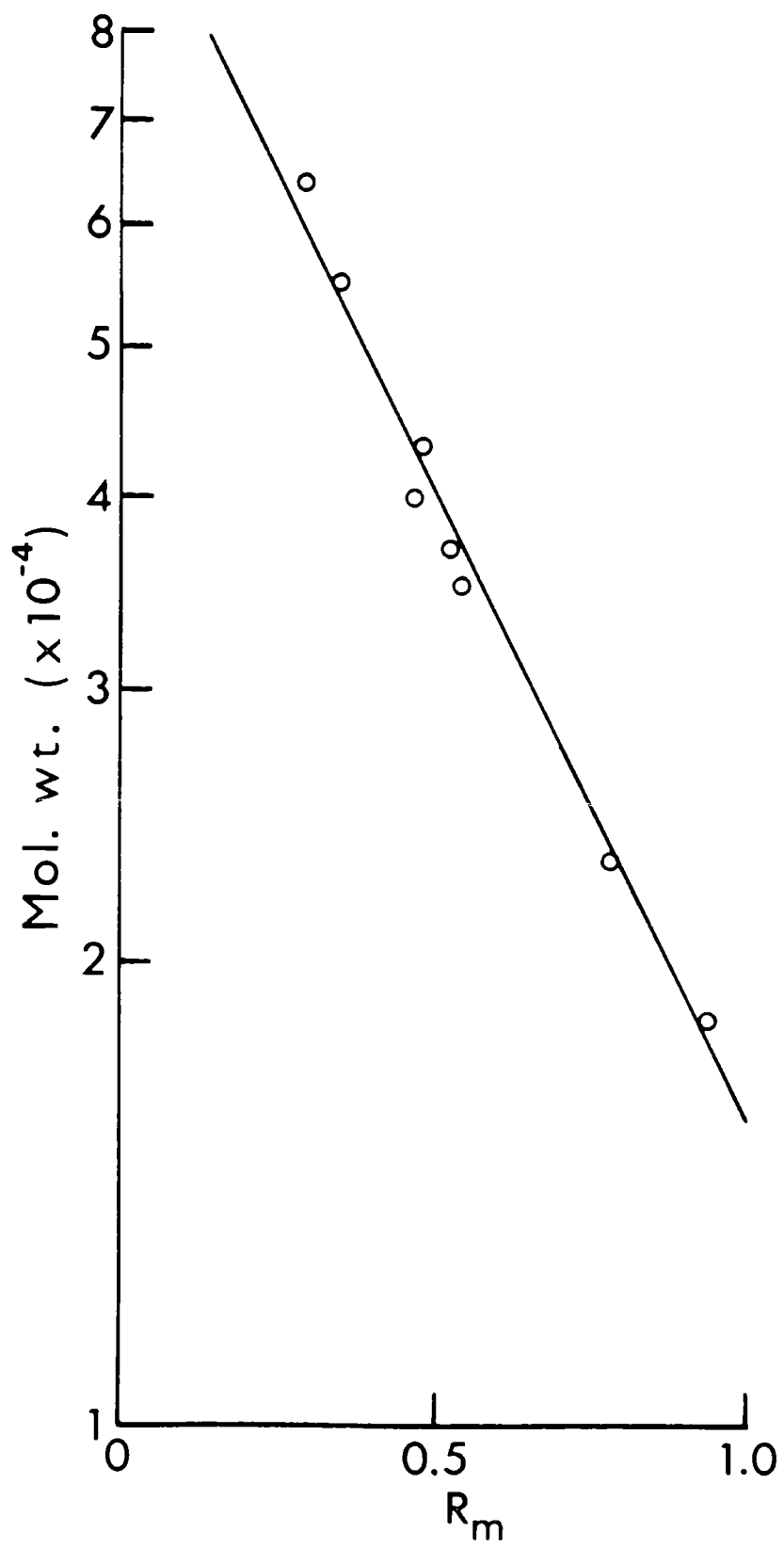


FIG.11

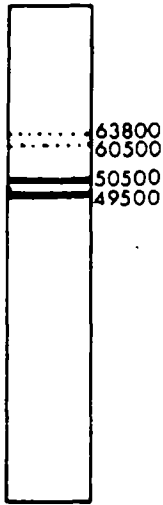
SDS electrophoretic patterns of fractions obtained by zonal isoelectric precipitation of V. faba globulins. (a) Fraction II in SDS only; (b) fraction II in SDS and 2-mercaptoethanol; (c) fraction I in SDS only; (d) fraction I in SDS and 2-mercaptoethanol. Electrophoresed in the discontinuous SDS-pH 8.3 gel system (10% gels). Molecular weights of component polypeptide chains were obtained from the calibration graph illustrated in Fig.10.

FIG.12

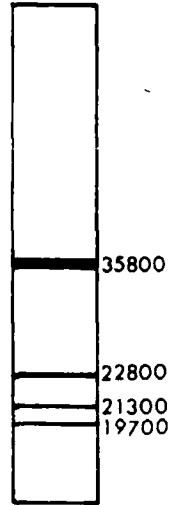
(a) SDS electrophoretic pattern of the pH 4.7 soluble fraction obtained from isoelectric precipitation of fraction I.

(b) SDS electrophoretic pattern of the water-insoluble protein of the pH 4.7 extract of bean meal.

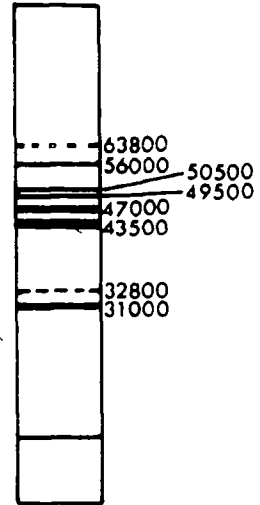
In both cases electrophoresis was conducted in the discontinuous SDS-pH 8.3 gel system, using 10% gels.



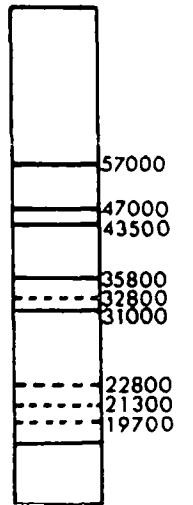
a



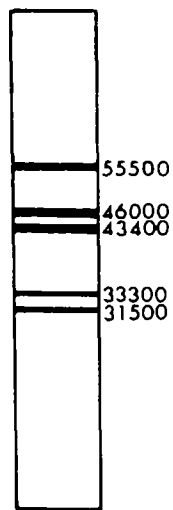
b



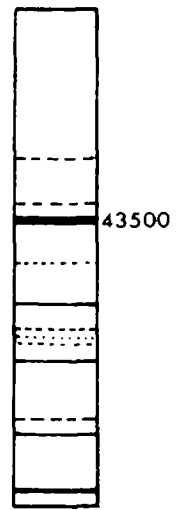
c



d



a



b

was contaminated by fraction II. However, as illustrated in Fig.12a, this contaminating protein could largely be removed by conventional isoelectric precipitation at pH 4.7 at reduced ionic strength (0.1M NaCl).

The SDS electrophoretic pattern of fraction IA, produced by zonal isoelectric precipitation in 0.1M NaCl, is shown in Fig.12b. Although many minor bands are in evidence, there is only one principal component and this has an apparent molecular weight of 43,500. The band patterns of fraction II and fraction IIA proved more or less identical.

(vi) Analytical ultracentrifugation

When examined in the ultracentrifuge, fraction I gave two components, corresponding to approximately 7S(A) and 11S(B) species (Plate 4), while fraction II gave only one, an 11S species (Plate 3).

(vii) N-Terminal amino acid analysis

Only fraction II was examined by the dansylation procedure. Three N-terminal amino acids were identified, viz. glycine, leucine and threonine.

C. Ascorbic Acid Extraction

(i) Extraction results

Using the acidic extraction procedure outlined in Methods (section VI.B), two globulin fractions were obtained. Fraction I precipitated out when the ionic strength was reduced by one-fold dilution with deionised water, and fraction II was obtained as a precipitate after dialysing the remaining supernatant.

(ii) Analytical ultracentrifugation

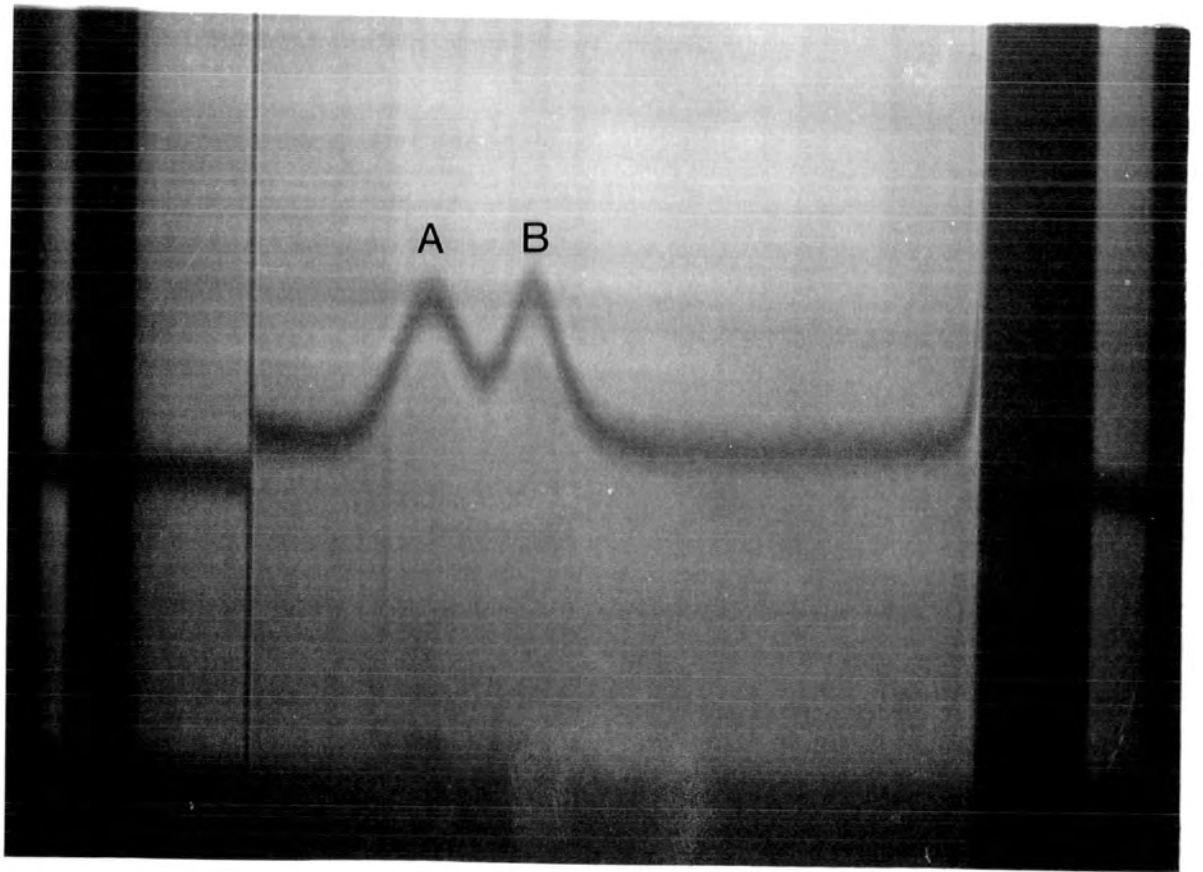
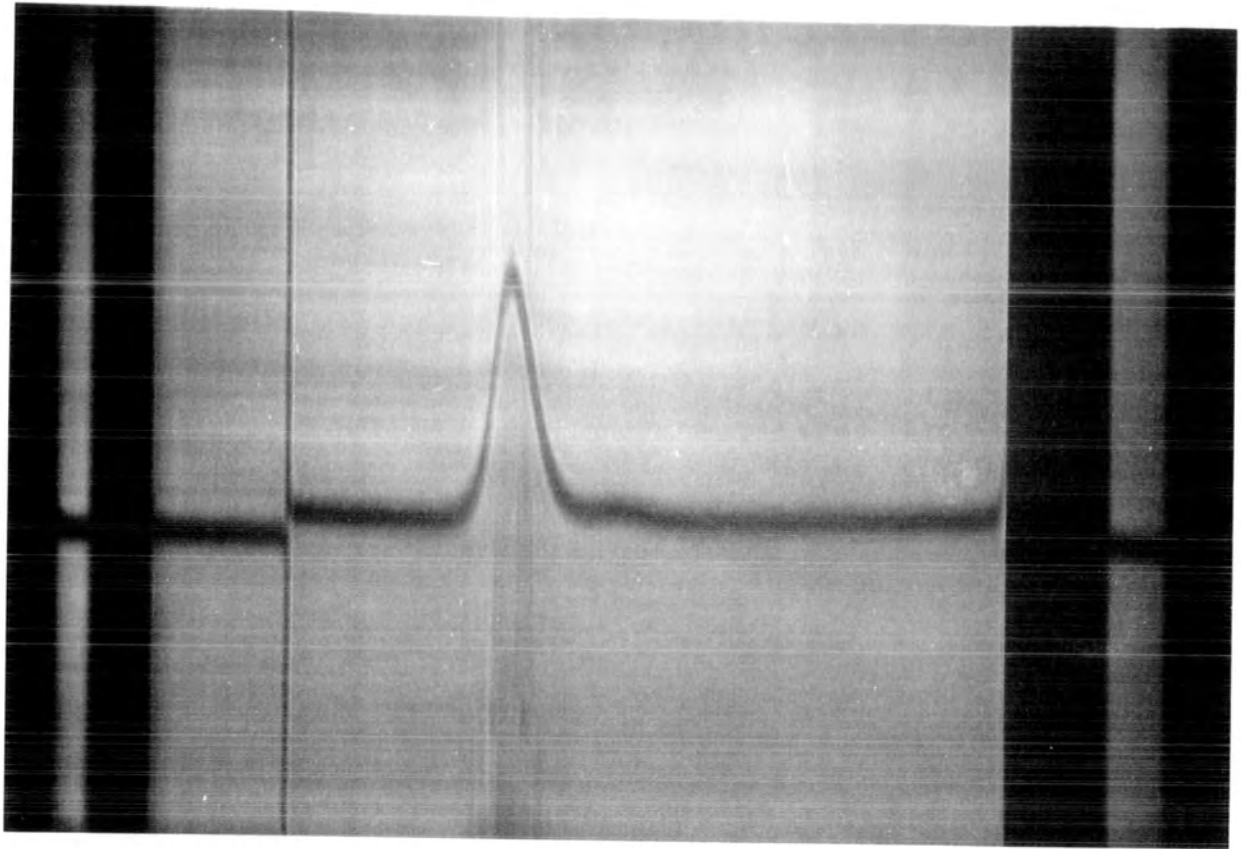
Fractions I and II were examined in the ultracentrifuge after dissolving in either 0.5M NaCl or 0.5M NaCl, 0.25M ascorbic acid.

PLATE 3

Ultracentrifuge pattern of fraction II, obtained by zonal isoelectric precipitation of globulins of V. faba. Protein is dissolved in 0.5M NaCl, 0.05M phosphate, pH 8.0. Photograph was taken 65 min. after centrifuge had reached a speed of 40,000 rev/min. Bar angle, 80°. Direction of sedimentation: left to right.

PLATE 4

Ultracentrifuge pattern of fraction I, obtained by zonal isoelectric precipitation of globulins of V. faba. Protein is dissolved in 0.5M NaCl, 0.05M phosphate, pH 8.0. Photograph was taken 85 min. after centrifuge had reached a speed of 40,000 rev/min. Bar angle, 70°. Direction of sedimentation: left to right.



Fraction I in 0.5M NaCl gave a single sedimenting species, with an $s_{20,w}$ value of 7.3S (Plate 5). As this value was found to be independent of the protein concentration, it also corresponds to the $s_{20,w}^0$ value for that species. This is equivalent to a molecular weight of $167,000 \pm 25,000$. However, when dissolved in 0.5M NaCl containing 0.25M ascorbic acid, no single peak was in evidence. Instead, a sedimenting 'plateau' was observed, with its origin at the meniscus of the solution (Plate 6). Even after 2 h. in the ultracentrifuge there was still no sign of peak formation. Similar results were obtained with fraction II dissolved in either 0.5M NaCl or 0.5M NaCl, 0.25M ascorbic acid. The sedimentation coefficient determined for the leading edge of the 'plateau' was 3.1S, which corresponds to a molecular weight of $46,000 \pm 7,000$.

(iii) Electrophoretic analysis

Electrophoretic patterns obtained for the two fractions are shown in Fig.13. Fraction I dissolved in 0.5M NaCl gave one main band with $R_m = 0.18$, and several minor bands, notably one at $R_m = 0.22$ (Fig. 13). When dialysed overnight against 0.5M NaCl before electrophoresis, these minor bands disappeared, leaving only that at $R_m = 0.18$. In the presence of ascorbic acid, one major component of fraction I was present at $R_m = 0.22$, with four other components of greater mobilities (Fig.13b). Several well-defined components separated out on gels when fraction II, dissolved in 0.5M NaCl, was electrophoresed (Fig.13c). R_m 's of major bands are 0.28, 0.62, 0.75, 0.91 and 1.0. In the presence of ascorbic acid, a similar pattern was produced, although the slower moving bands were much less in evidence.

The sub-unit compositions of fractions I and II were examined by SDS gel electrophoresis. The results are presented in Fig.14, together with

PLATE 5

Ultracentrifuge pattern of fraction I, obtained from ascorbic acid extraction of V. faba proteins. Protein is dissolved in 0.5M NaCl. Photograph was taken 60 min. after the centrifuge had reached a speed of 40,000 rev/min. Bar angle, 45°. Direction of sedimentation: left to right.

PLATE 6

Ultracentrifuge pattern of fraction II, obtained from ascorbic acid extraction of V. faba proteins. Protein is dissolved in 0.5M NaCl. Photograph was taken 100 min. after the centrifuge had reached a speed of 40,000 rev/min. Bar angle, 35°. Direction of sedimentation: left to right.

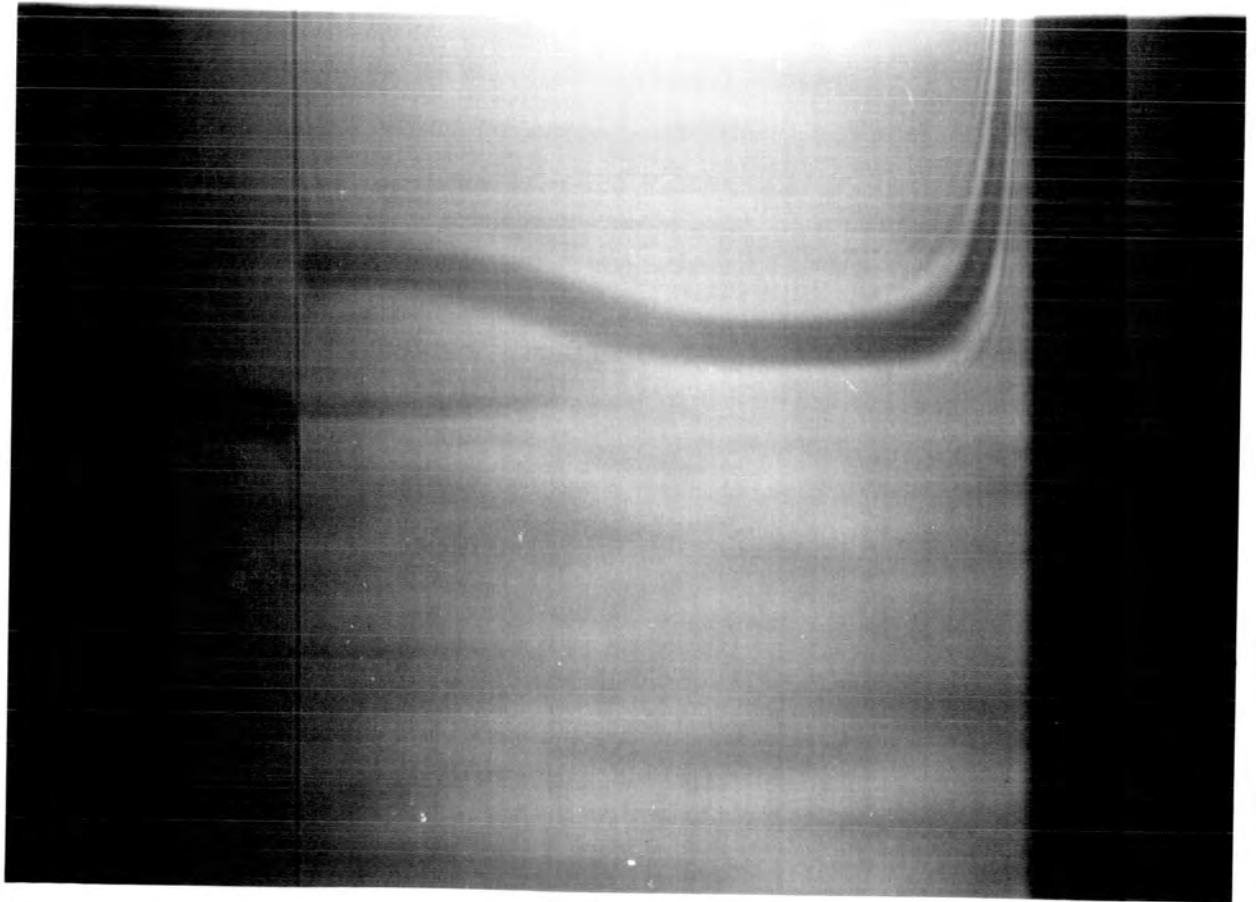
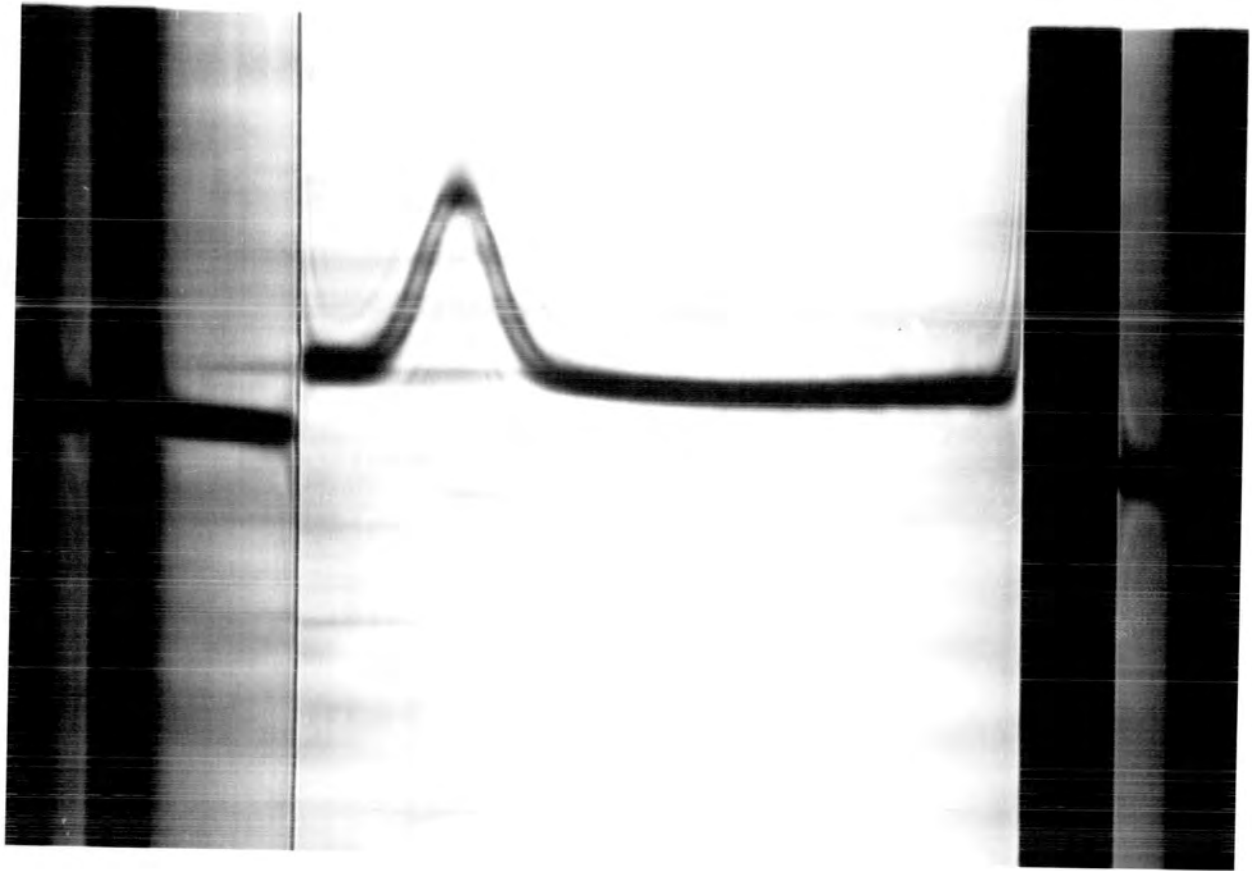
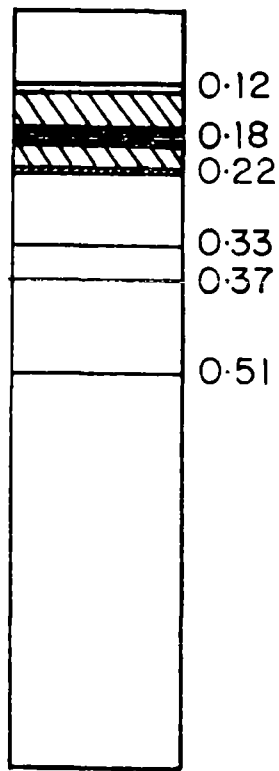


FIG.13

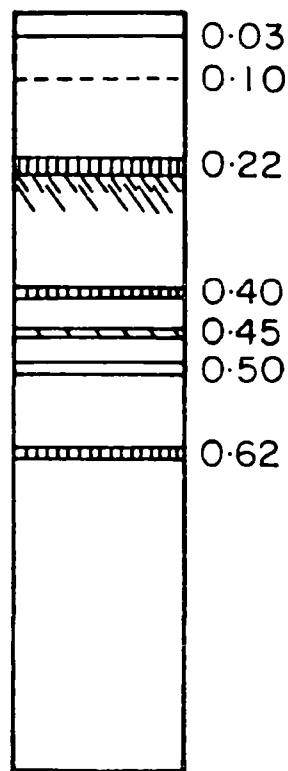
Electrophoretic patterns of fractions obtained by ascorbic acid extraction of V. faba proteins: (a) fraction I in 0.5M NaCl, (b) fraction I in 0.5M NaCl, 0.25M ascorbic acid and (c) fraction II in 0.5M NaCl. Electrophoresis was conducted in the pH 8.3 gel system using 7.5% gels. Mobilities were calculated relative to bromophenol blue (not shown).

FIG.14

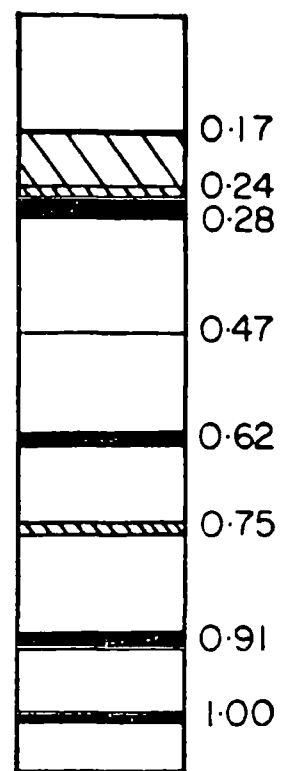
SDS electrophoretic patterns of fractions obtained by ascorbic acid extraction of V. faba proteins: (a) carboxymethylated fraction I, (b) carboxymethylated fraction II and (c) fraction II in SDS without 2-mercaptoethanol. Electrophoresis was performed in the SDS-phosphate, pH 7.0 gel system using 10% gels.



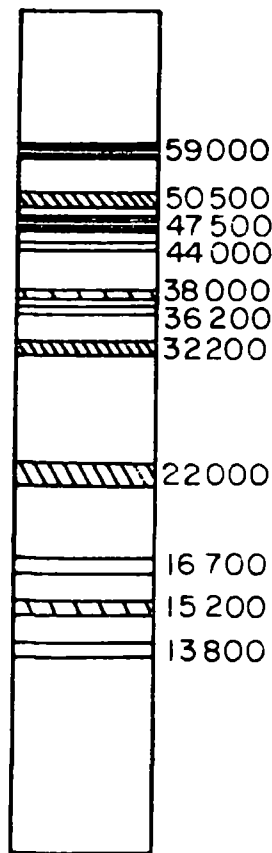
(a)



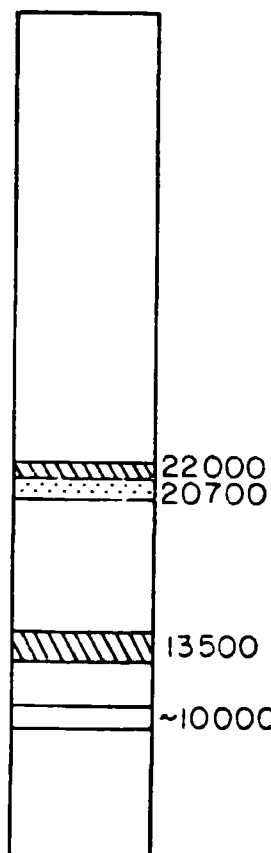
(b)



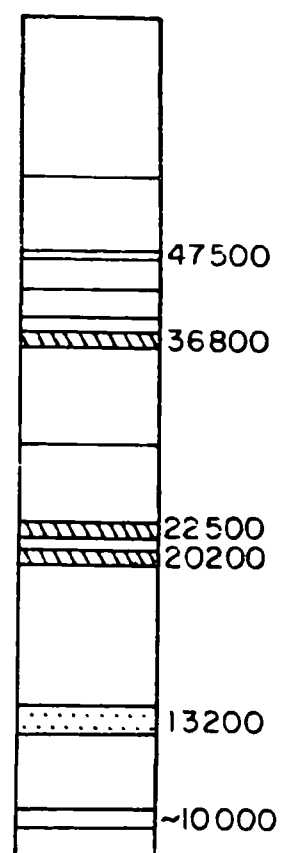
(c)



(a)



(b)



(c)

the calculated molecular weights for the individual components. Similar electrophoretic patterns were produced by fraction I, (a) after carboxymethylation, (b) after incubation in 0.2% (w/v) SDS for 3 h. and (c) after incubation in 0.2% (w/v) SDS containing 2% (v/v) 2-mercaptoethanol for 3 h. (Fig.14a). Fraction II gave very diffuse bands when electrophoresed after reduction in 0.2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, only after carboxymethylation were the separate bands identifiable (Fig.14b). The pattern produced by fraction II after incubation in SDS alone is shown in Fig.14c. The low molecular weight bands seen in Fig. 14b are still in evidence, but the presence of higher molecular weight species probably indicates incomplete dissociation.

(iv) N-Terminal amino acid analysis

N-Terminal analyses were performed on fractions I and II, and the following N-termini were identified. Fraction I: leucine, glycine and alanine with strong traces of aspartic acid, glutamic acid, serine, threonine and phenylalanine. Fraction II: leucine, valine, alanine, glycine and a strong trace of threonine.

D. Sodium dodecyl sulphate extraction

Aliquots of the clarified supernatants from the SDS extracts of bean meal were electrophoresed directly on gels using the appropriate buffer system. Thus, the phosphate extracts were analysed in the SDS-phosphate, pH 7.0 system, and the tris-glycine extracts in the SDS-pH 8.3 system. The resultant gel patterns are shown in Figs. 15 and 16. Comparison of Fig.15b with Fig.5a and Fig.16b with Fig.11b, indicates that the principal components extracted using either SDS buffer are sub-units derived from legumin. Many of the minor bands also present can be attributed to vicilin sub-units e.g. those with molecular weights of 32,800, 44,200, 46,000 and 57,700 in Fig.16b.

FIG.15

SDS-extracted proteins of V. faba meal, electrophoresed in the SDS-phosphate, pH 7.0 gel system (10% gels). Numbers above peaks refer to the molecular weights ($\times 10^{-3}$) of the associated polypeptide chains.

(a) Meal proteins extracted with 3% (w/v) SDS, 0.05M NaH_2PO_4 , pH 7.0.

(b) Meal proteins extracted with 3% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.05M NaH_2PO_4 , pH 7.0.

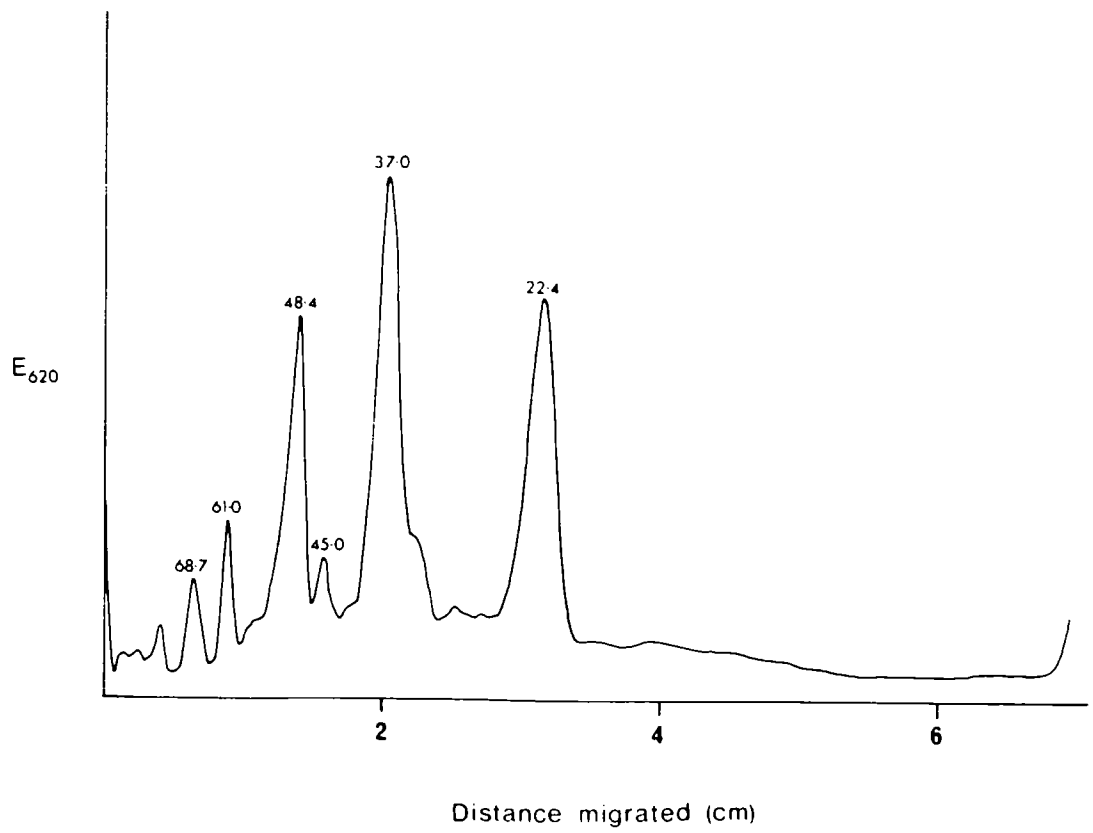
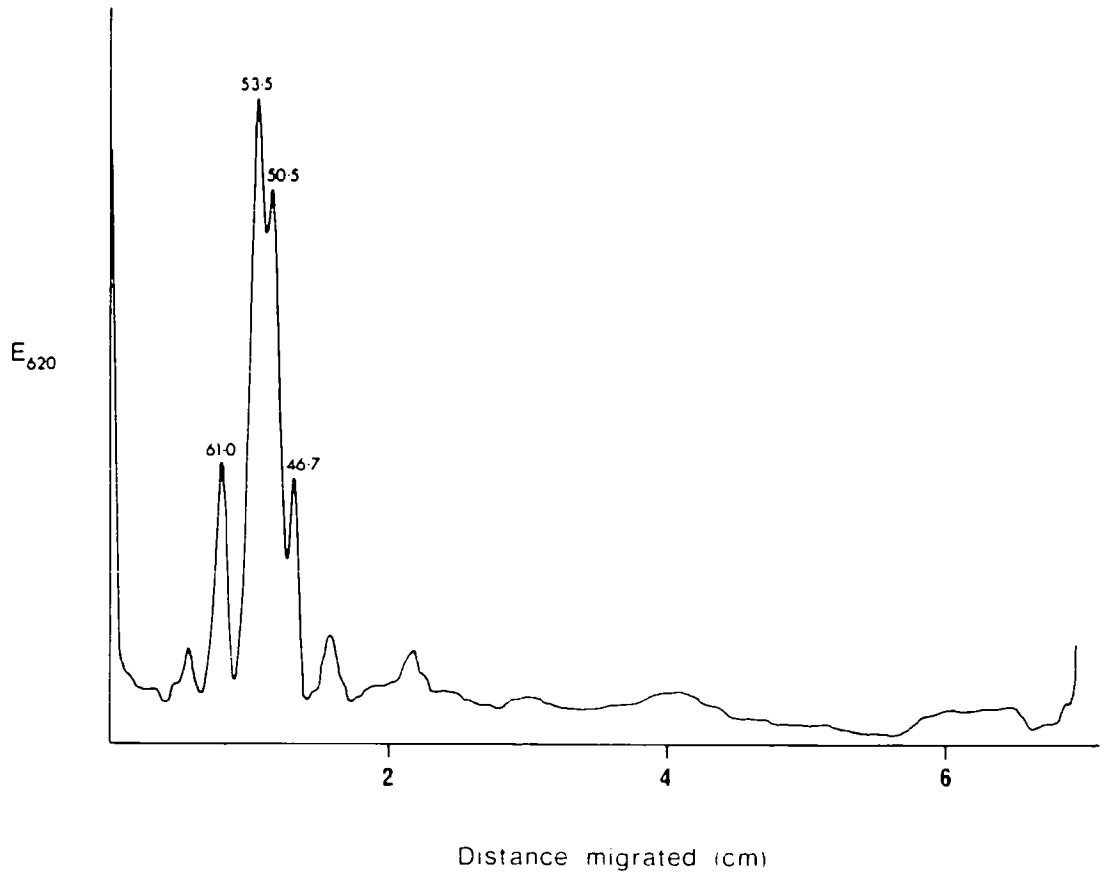
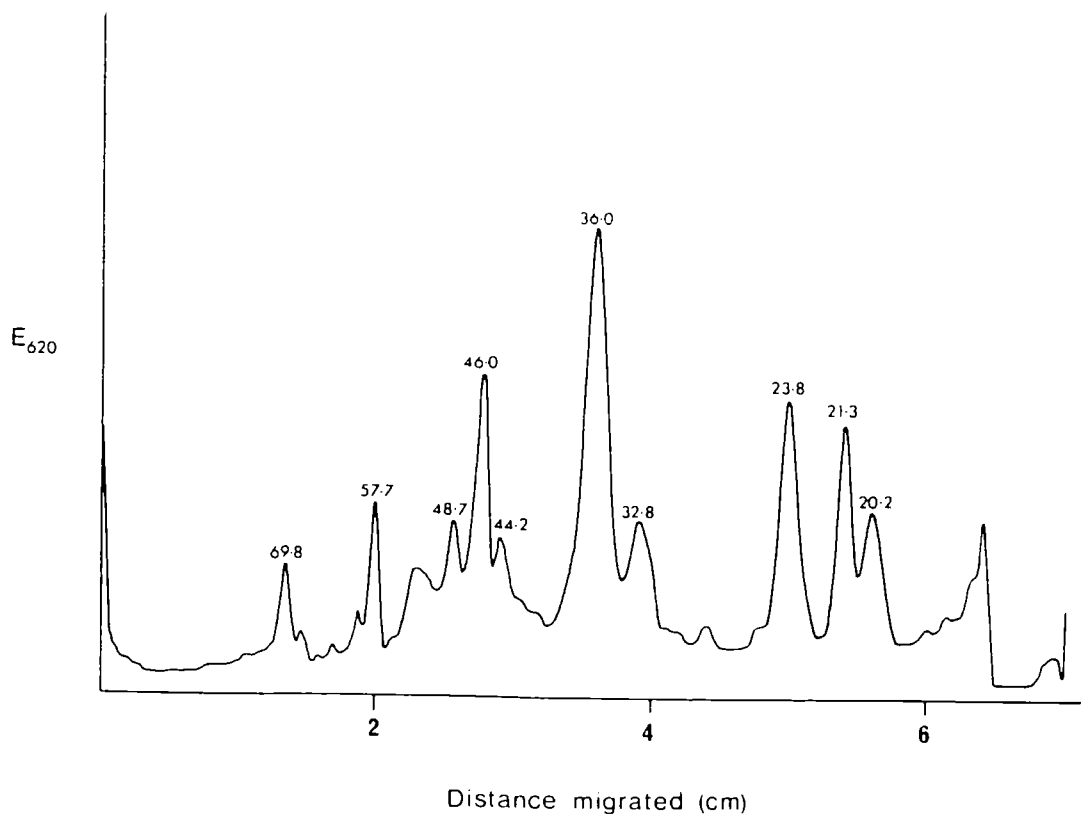
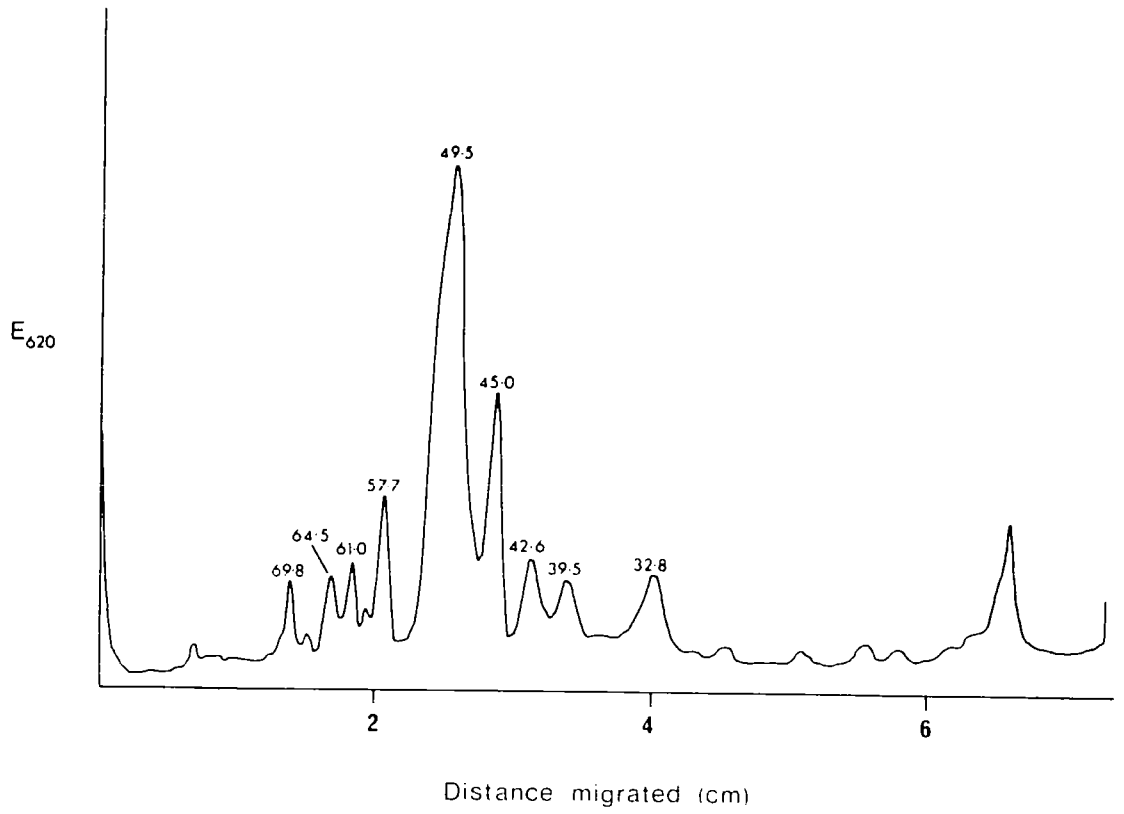


FIG.16

SDS-extracted proteins of V. faba meal electrophoresed in the SDS-pH 8.3 gel system (10% gels). Numbers above peaks refer to the molecular weights ($\times 10^{-3}$) of the associated polypeptide chains.

(a) Meal proteins extracted with 4% (w/v) SDS,
0.125M Tris-HCl, pH 6.8.

(b) Meal proteins extracted with 4% (w/v) SDS,
1% (v/v) 2-mercaptoethanol, 0.125M Tris-HCl,
pH 6.8.



E. Extraction at pH 4.7

Two protein preparations were obtained from the pH 4.7 extract of bean meal, one as a precipitate by dialysing the extract against water (water-insoluble fraction), and the other as a precipitate by adjusting the pH of the extract to 8 (pH 8-insoluble fraction). Both fractions were investigated by SDS gel electrophoresis. In Fig.17 is illustrated the SDS electrophoretic pattern of the water-insoluble fraction. The two principal components have molecular weights of 42,000 and 20,700. The gel pattern (not shown) of the pH 8-insoluble fraction contained only the 42,000 molecular weight component.

IV. Characterisation of Legumin and Vicilin During Seed Development

The relative amounts of the two storage proteins extracted from seeds of different ages are shown in Table 3. Vicilin was formed in the developing seed before legumin could be detected, but legumin was synthesised at a faster rate than vicilin. In the mature seed (90 days), legumin predominated over vicilin by about 4:1 on a weight basis.

The sub-unit compositions of the extracted globulins were examined on SDS gels using the SDS-phosphate, pH 7.0 gel system. Legumin extracted from seeds at different stages of development gave the same characteristic band pattern illustrated in Fig.5a. Relative mobilities of the sub-units, calculated relative to bromophenol blue, were 0.17, 0.21, 0.25, 0.36 and 0.56. When vicilin, extracted from mature seed, was electrophoresed it gave five major bands with R_m 's of 0.17, 0.23, 0.25, 0.29 and 0.38. While these components were present more or less throughout the course of development, considerable changes in their relative staining intensities were apparent. Changes in the ratios of vicilin and legumin sub-units during development were examined more

FIG.17

The water-insoluble proteins (globulins) of a pH 4.7 extract of V. faba meal. Electrophoresis was performed in the SDS-pH 8.3 gel system, using 10% gels. Numbers above peaks refer to the molecular weights ($\times 10^{-3}$) of the associated polypeptide chains.

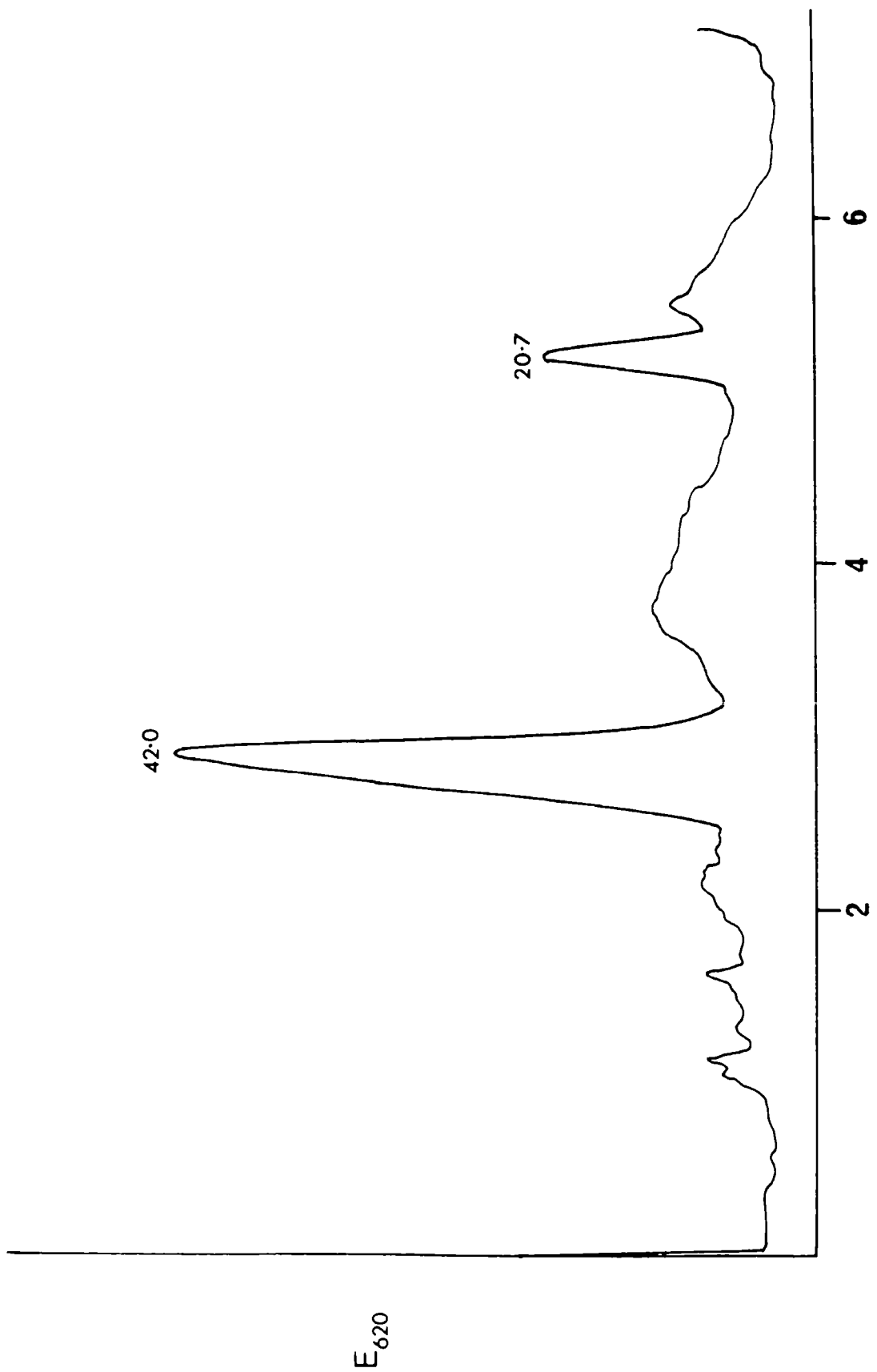


TABLE 3

Amounts of vicilin and legumin extracted from seeds of
Vicia faba at different stages of development

Sample age (days)	Total globulin ^a (mg)	Protein	Amount ^b (mg)	% total globulin
30	0.193	legumin ^c vicilin	- 0.193	- 100
41	212	legumin vicilin	93 119	44 56
49	1330	legumin vicilin	1000 330	75 25
60	2380	legumin vicilin	1700 680	71 29
70	3690	legumin vicilin	2900 790	79 21
90	2870	legumin vicilin	2200 670	77 23

a. Extractable protein from 100 cotyledons.

b. Values for legumin and vicilin were assigned on the basis that legumin is precipitated at pH 4.7, whereas vicilin remains in solution (Danielsson, 1949).

closely by the dye-binding method, in which the ratio of dye bound to each sub-unit on the gels was used as a measure of the amount of protein. This procedure showed that the ratio of the sub-units, one to another, remained reasonably constant in legumin extracted at various stages of development. In contrast the ratios of vicilin sub-units changed during seed development (Fig.18). The trend in the results is best illustrated when the sub-unit ratios are calculated relative to one standard sub-unit (Fig.19). From this it can be seen that relative to the sub-unit with R_m 0.25, all other sub-units steadily increase in amount during development.

V. Investigation of the Sub-unit Structures of Legumin and Vicilin

The work on legumin is far more extensive and not all procedures were used with both legumin and vicilin preparations, but for reasons of brevity, where appropriate, results for both are given in the same subsection.

A. Sub-unit fractionation using preparative gel electrophoresis

(i) Gel systems

Three different dissociating gel systems were investigated in an attempt to optimise the separation of the legumin sub-units. The results obtained in each system are detailed below.

(a) SDS-pH 8.3

In this buffer system, legumin, after dissociation and reduction with SDS and 2-mercaptoethanol respectively, gave the elution profile illustrated in Fig.20. The rising baseline was due to the gradual production of the oxidised form of 2-mercaptoethanol. This oxidation product absorbs at 280 nm. Three main uv-absorbing components (I, II and III) were evident, apart from those due to bromophenol blue

FIG. 18

The percentage of each vicilin sub-unit present in vicilin extracted from seeds of V. faba at different stages of development. R_m values refer to mobilities of component sub-units, relative to bromophenol blue, as determined after electrophoresis in the SDS-phosphate, pH 7.0 gel system (10% gels).

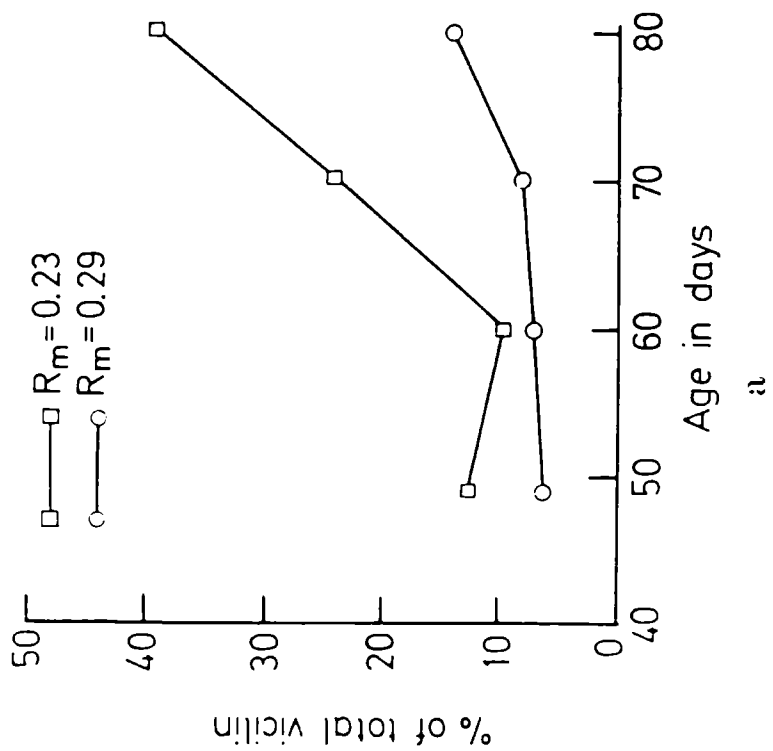
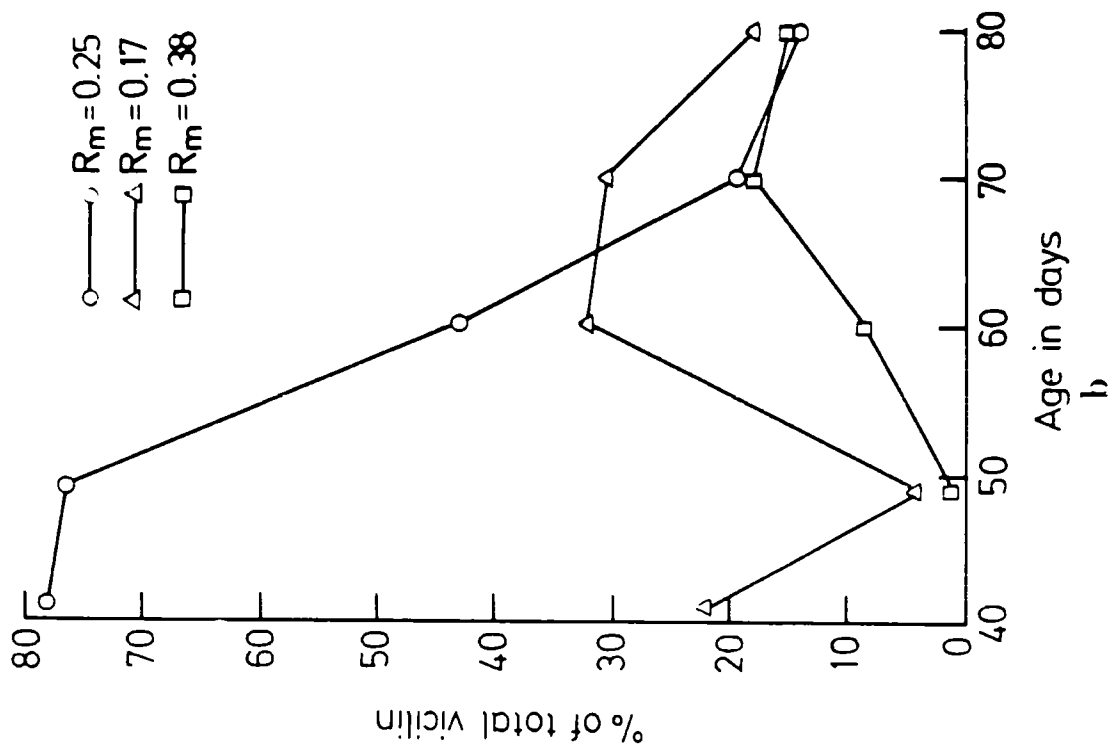


FIG. 19

The ratios of vicilin sub-units in vicilin extracted from seeds of V. faba at different stages of development. Ratios are calculated relative to one standard sub-unit ($R_m = 0.25$).

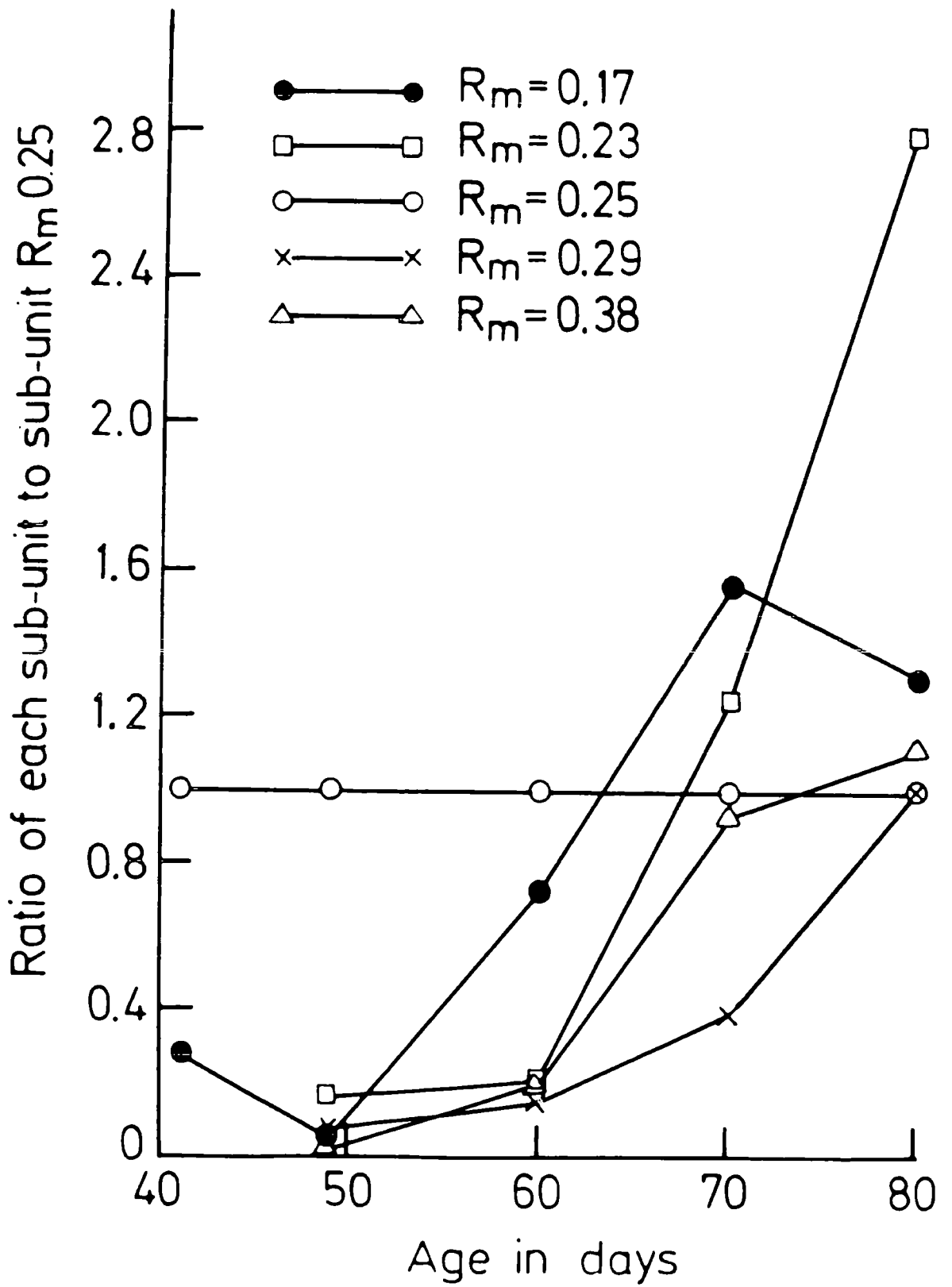
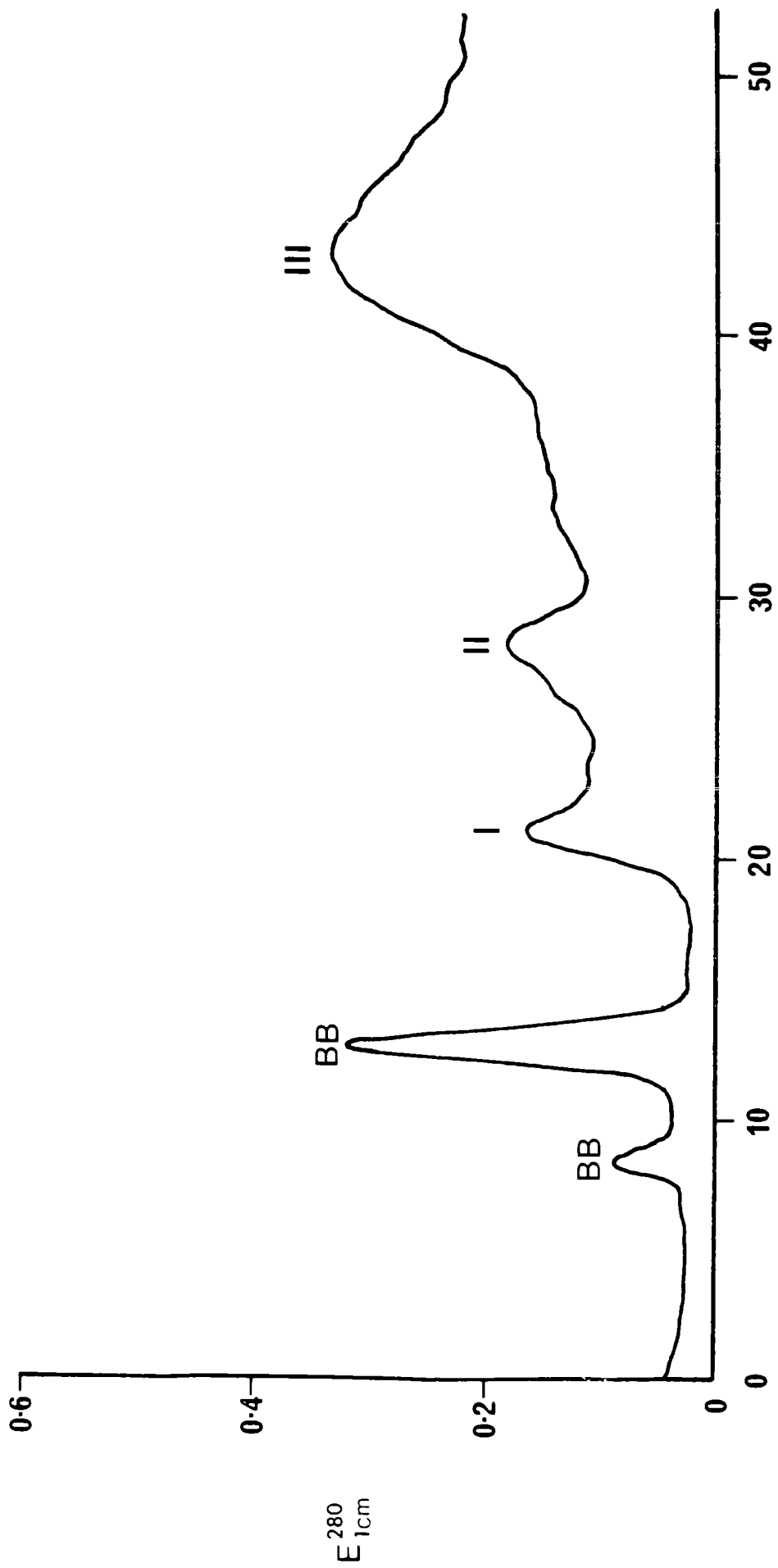


FIG. 20

Preparative gel electrophoresis elution profile for dissociated legumin, using the continuous SDS-pH 8.3 gel system. 20 mg. legumin, dissociated and reduced with SDS and 2-mercaptoethanol respectively, were applied to a 6 cm. 10% gel column. Elution buffer flow rate: 26 ml/h. 4 ml. fractions were collected. BB:bromophenol blue.



Fraction no.

(BB). The fractions corresponding to each of these three components were pooled and then re-electrophoresed on analytical scale gels to examine the efficiency of the preparative separation. The results are shown in Figs.21,22 and 23. Whilst fractions I and II appear almost homogeneous, fraction III is seen to contain all three components present in the initial sample.

Fig.24 shows the electrophoretic pattern of fraction III, after incubation in SDS and 2-mercaptoethanol for approximately four weeks. The fastest moving component now appears as a doublet. If fresh 2-mercaptoethanol was added after storage, prior to electrophoresis, a further change in the electrophoretic pattern was observed. In this case (Fig. 25), the slowest moving component was absent, although no additional bands were detected.

When samples of CM legumin were electrophoresed in the preparative apparatus, 2-mercaptoethanol (or dithiothreitol) were omitted from the sample solution and both the reservoir buffers, thus overcoming the problem of rising baselines. A typical elution profile is shown in Fig. 26. There were only two main protein-containing fractions. The electrophoretic patterns of the proteins recovered from these fractions are shown in Fig.27. When fraction II was re-electrophoresed in the apparatus under the same conditions, the elution profile (Fig.28) indicated that it was essentially free from contamination by fraction I.

(b) SDS-phosphate, pH 7.0

Attempts to use this system in the preparative apparatus were unsuccessful. This was due to the production of unstable high voltages during the course of the experiment. The heat dissipated probably exceeded the cooling capacity of the apparatus, eventually leading to gel distortion.

FIG. 21

Fraction I obtained by preparative gel electrophoresis of dissociated legumin (see Fig.20). Electrophoresis was performed in the continuous SDS-pH 8.3 gel system using 10% gels.

FIG. 22

Fraction II obtained from preparative gel electrophoresis of dissociated legumin (see Fig.20). Electrophoresis was performed in the continuous SDS-pH 8.3 gel system using 10% gels.

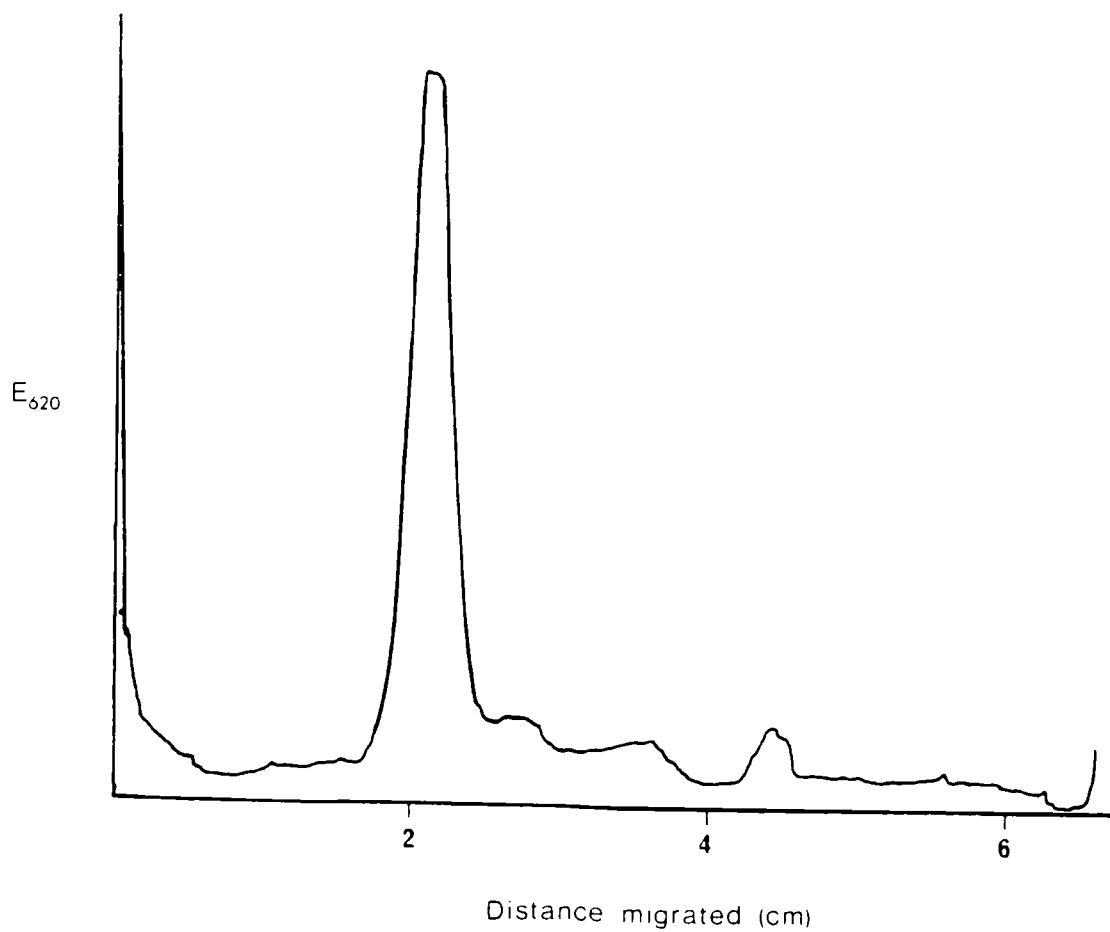
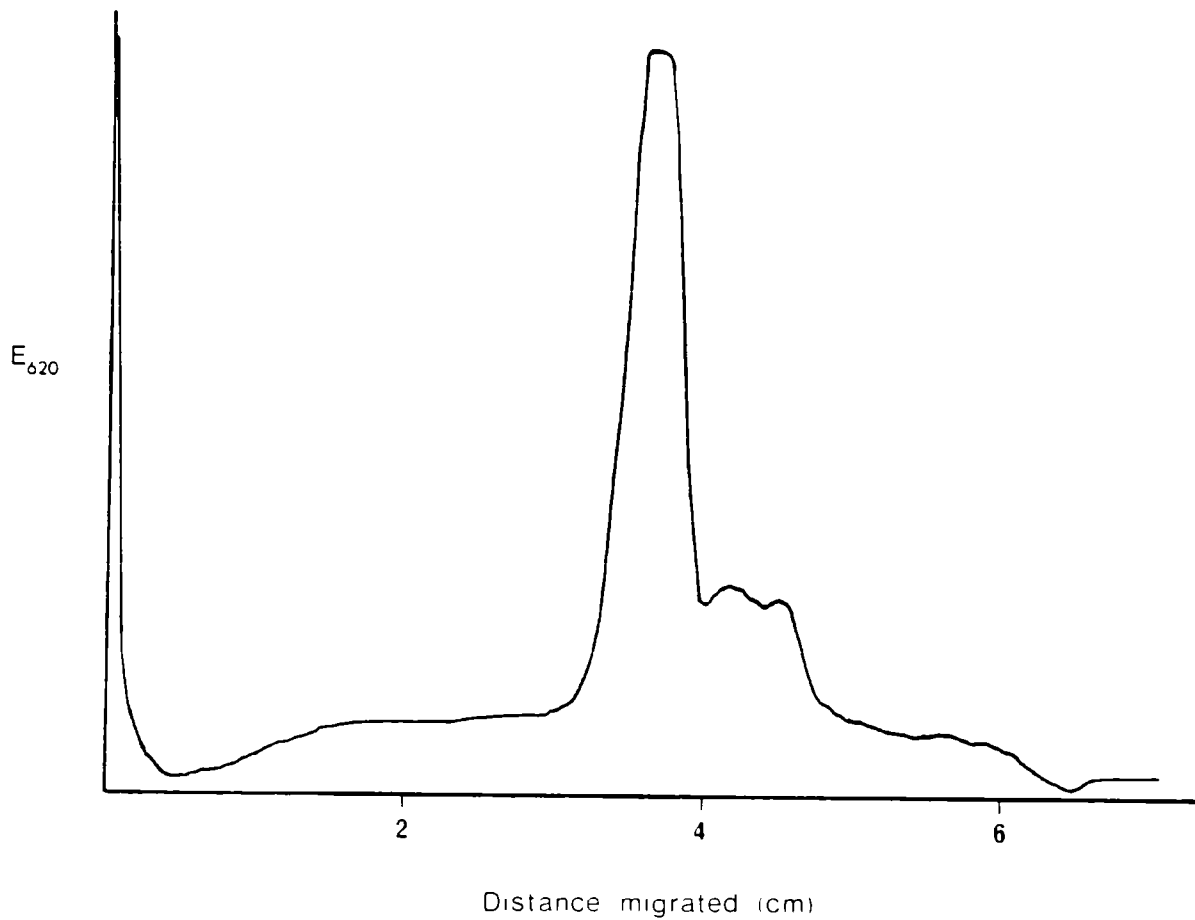
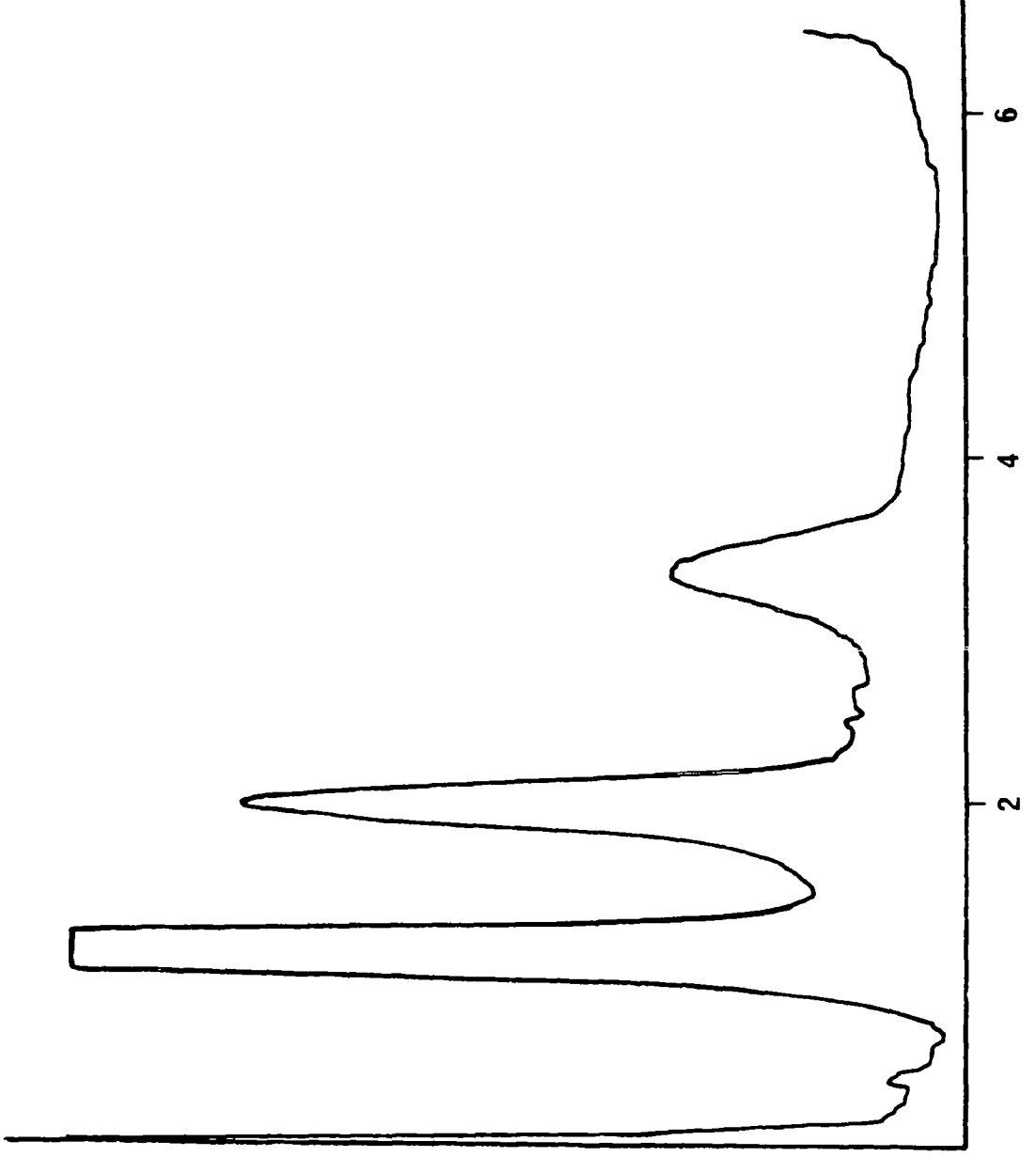


FIG. 23

Fraction III obtained from preparative gel electrophoresis of dissociated legumin (see Fig. 20). Electrophoresis was performed in the continuous SDS-pH 8.3 gel system using 10% gels.



E₆₂₀

Distance migrated (cm)

FIG. 24

As in Fig. 23, except sample containing fraction III was electrophoresed after incubation in SDS and 2-mercaptoethanol for approximately four weeks.

FIG. 25

As in Fig. 24, except fresh 2-mercaptoethanol was added to the sample prior to electrophoresis.

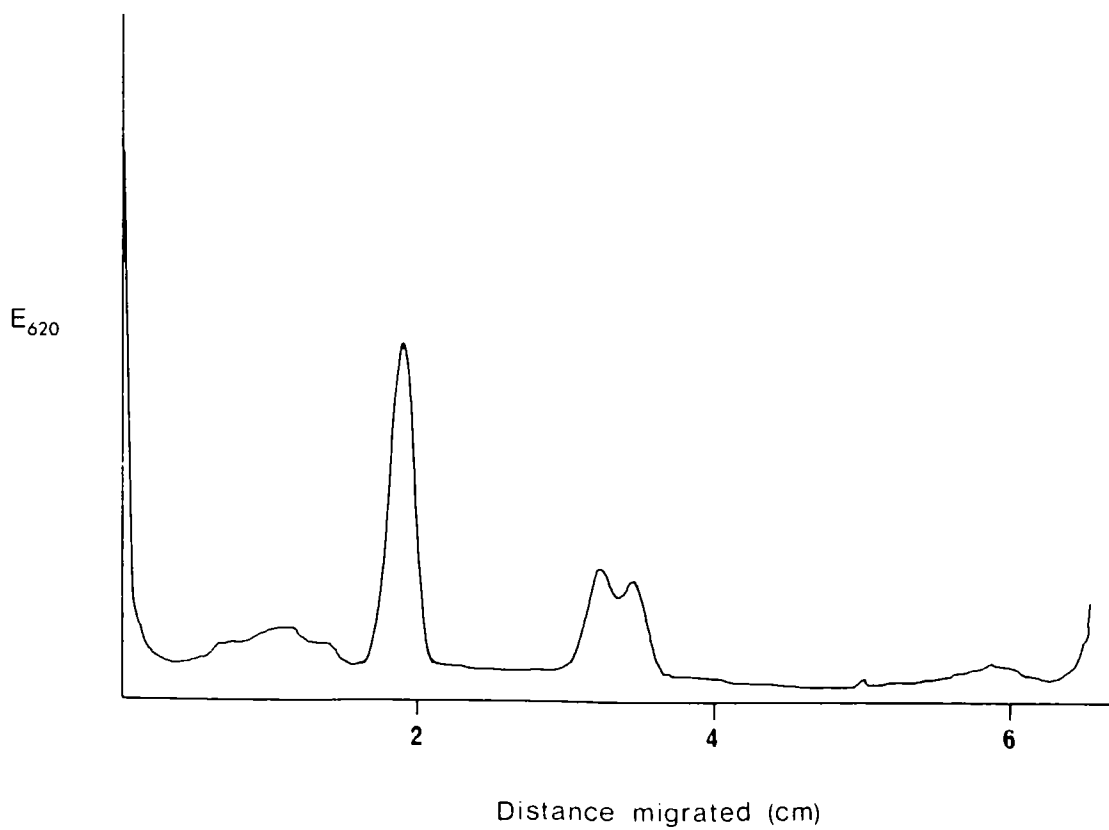
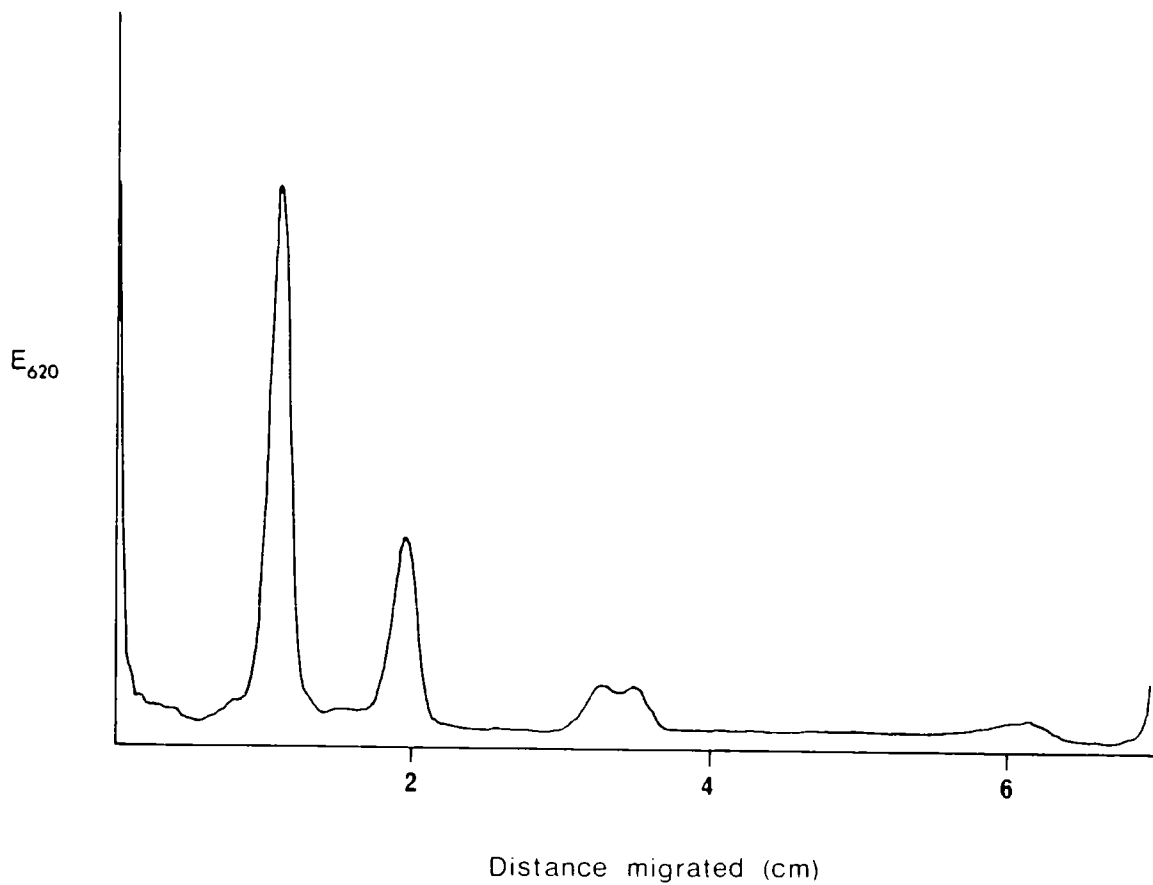
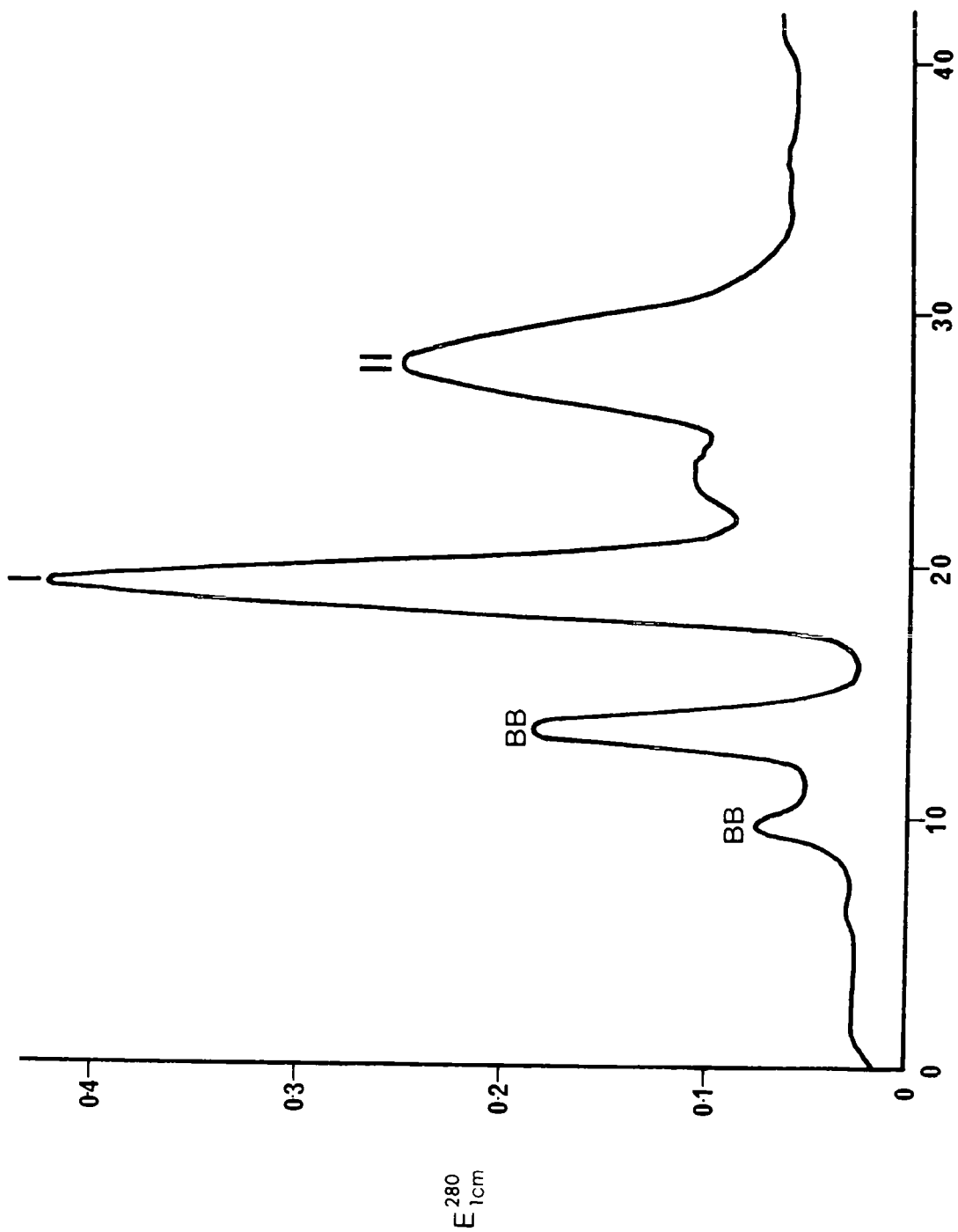


FIG. 26

Preparative gel electrophoresis elution profile for CM legumin, using the continuous SDS-pH 8.3 gel system. 25 mg. CM legumin, dissociated in SDS, were applied to a 6 cm. 10% gel column. 5 ml. fractions were collected. Elution buffer flow rate was 25 ml/h. BB: bromophenol blue.



Fraction no.

FIG. 27

Electrophoresis of fractions obtained from the preparative gel electrophoresis of CM legumin (see Fig. 26).

Gel system: SDS-pH 8.3 (continuous), 10% gels.

(a) Fraction I

(b) Fraction II

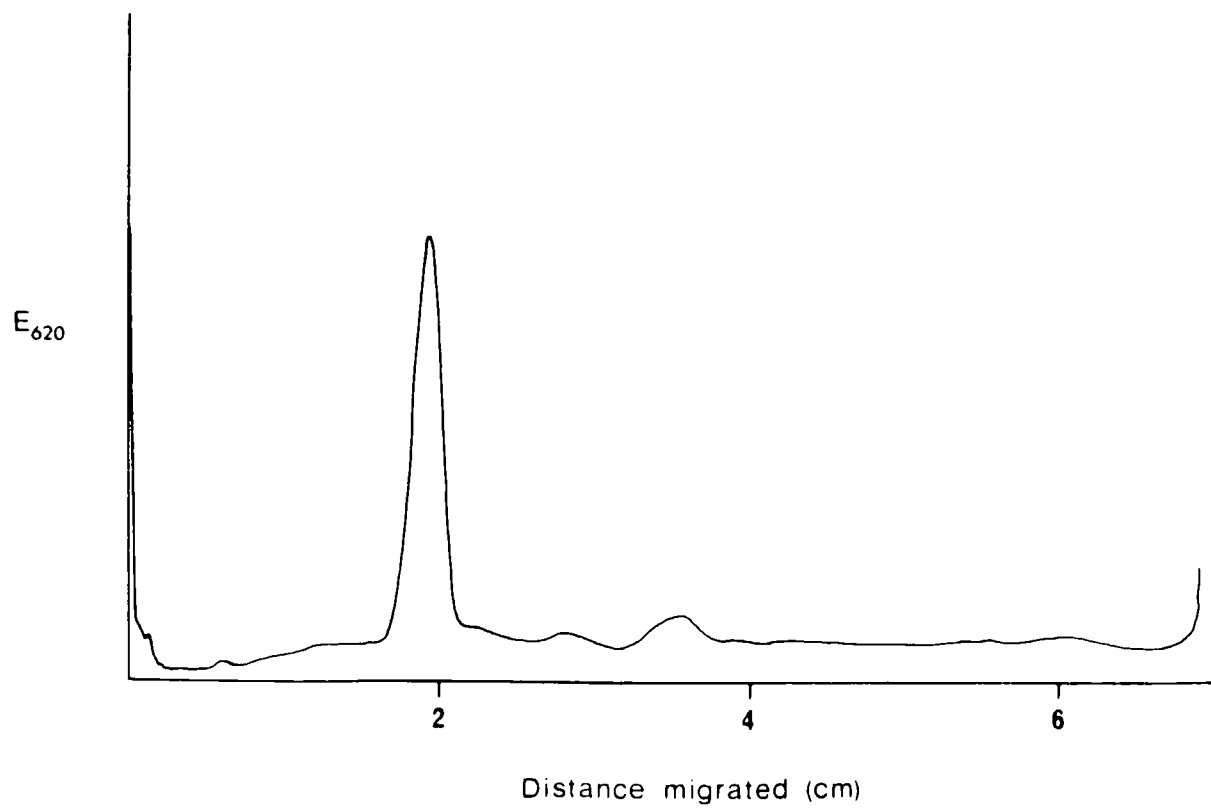
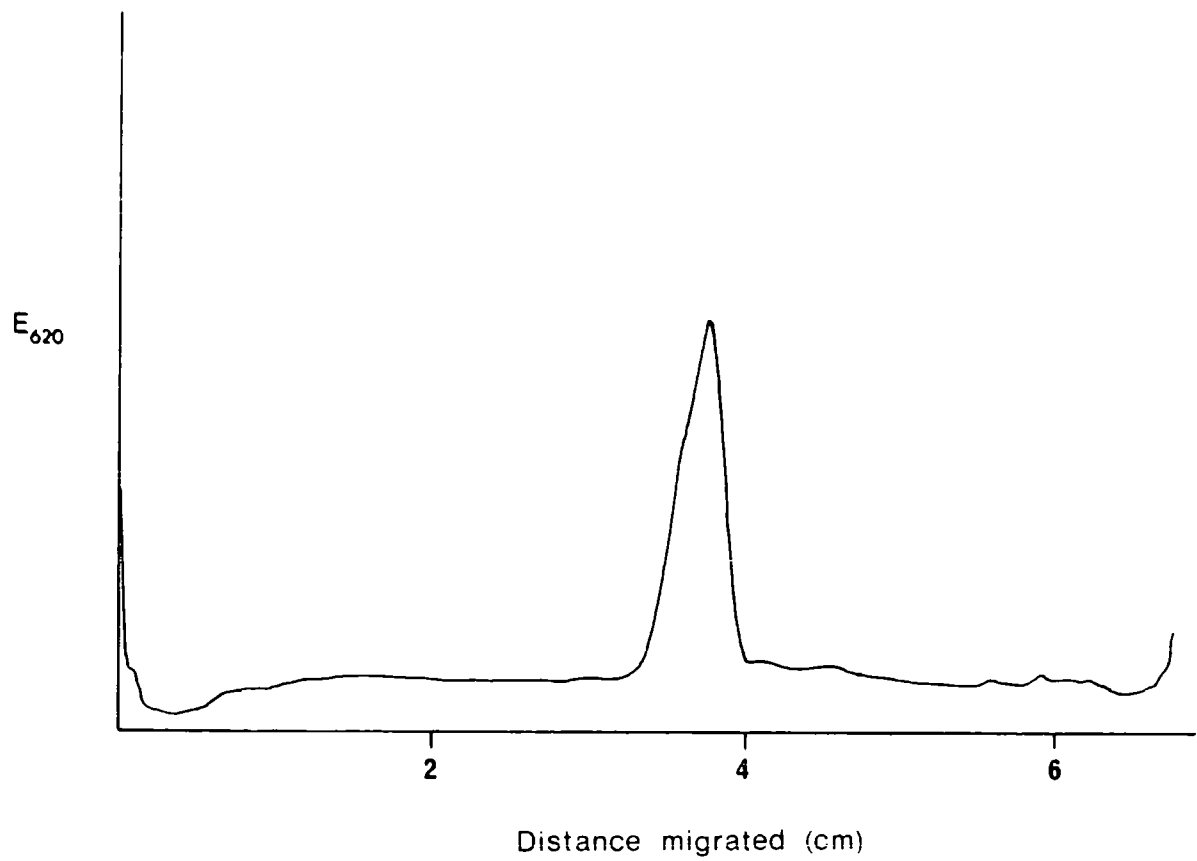
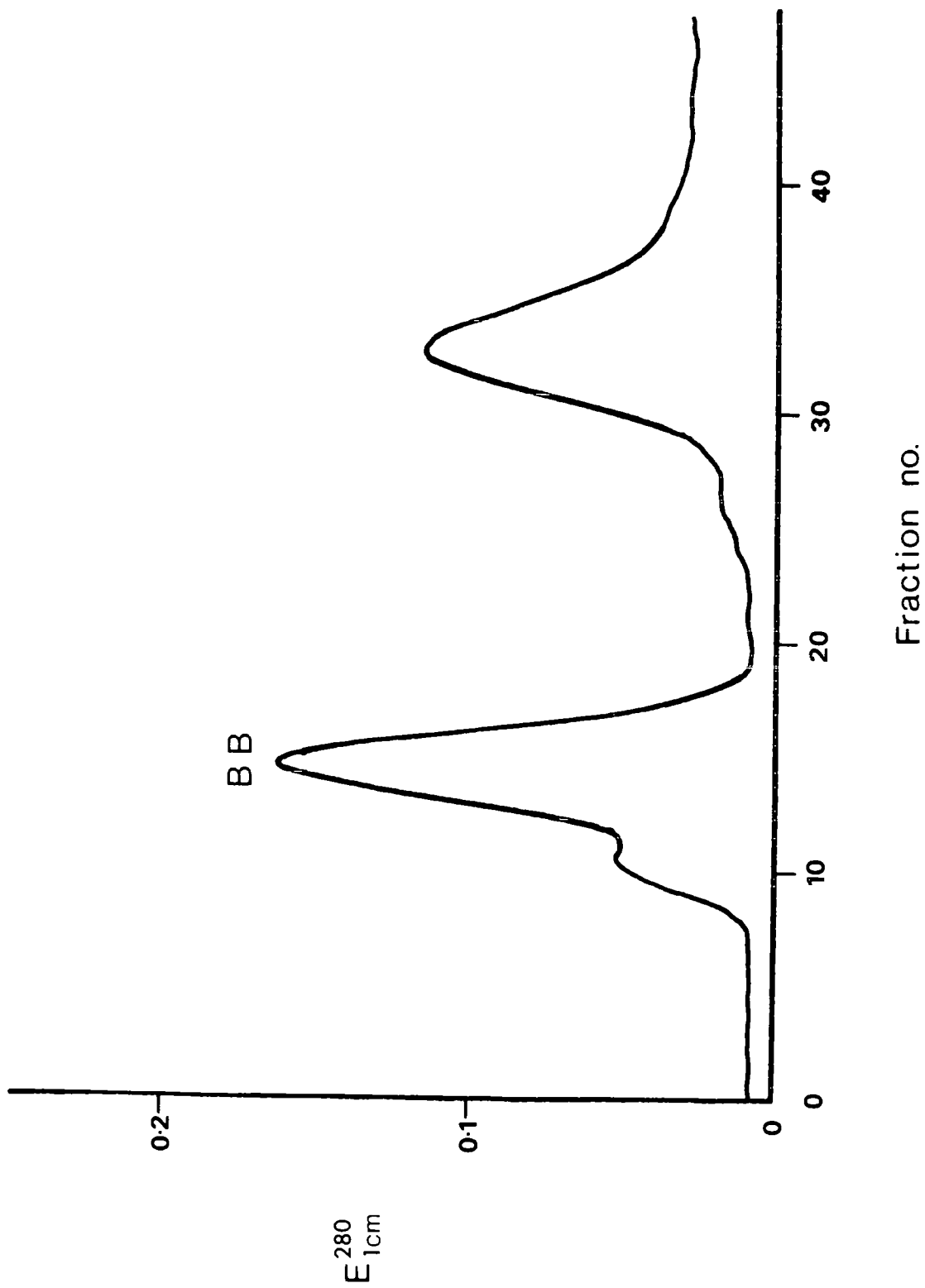


FIG. 28

Preparative gel electrophoresis elution profile of fraction II obtained by electrophoretic fractionation of CM legumin (see Fig. 26). Experimental conditions as in legend to Fig. 26.



(c) SDS-citrate, pH 7.0

This system was developed to overcome the difficulties encountered with the phosphate system described above. The results obtained were essentially the same as those from the SDS-pH 8.3 system (Fig.29). In order to examine the degree of resolution achieved, fractions were not pooled, but instead they were analysed individually by electrophoresis. The results (Fig.30) show that a partial separation of the components comprising fraction I was accomplished.

(ii) N-Terminal amino acids of sub-units

The N-terminal amino acids of the separated sub-units of legumin (see Fig.26) were found to be glycine (I) and leucine and threonine (II).

B. Extraction of Sub-units from Gel Slices

Fig.31 illustrates the effectiveness of this technique for isolating small quantities of sub-units from analytical scale gels; when re-electrophoresed, the extracted sub-unit was essentially free from contamination by the other sub-units.

Preliminary experiments were performed in order to test the feasibility of conducting amino acid analyses on the extracted material. After elution from the gel slices and removal of bound dye, the protein, in 70% (v/v) formic acid, was dialysed against water to remove all traces of electrophoresis buffer and polyacrylamide decomposition products. The solution was evaporated to dryness on a rotary evaporator, the residue taken up in a small volume of 70% (v/v) formic acid and transferred to Pyrex tubes for hydrolysis. The formic acid was removed in vacuo and 6M HCl added. After hydrolysis the sample was analysed as usual. The results of the amino acid analysis showed that the sub-units

FIG. 29

Preparative gel electrophoresis elution profile of CM legumin, using the SDS-citrate, pH 7.0 gel system. 25 mg. CM legumin, dissociated in SDS, were applied to a 5 cm. 10% gel column. 3 ml. fractions were collected. Elution buffer flow rate was 24 ml/h. BB: bromophenol blue.

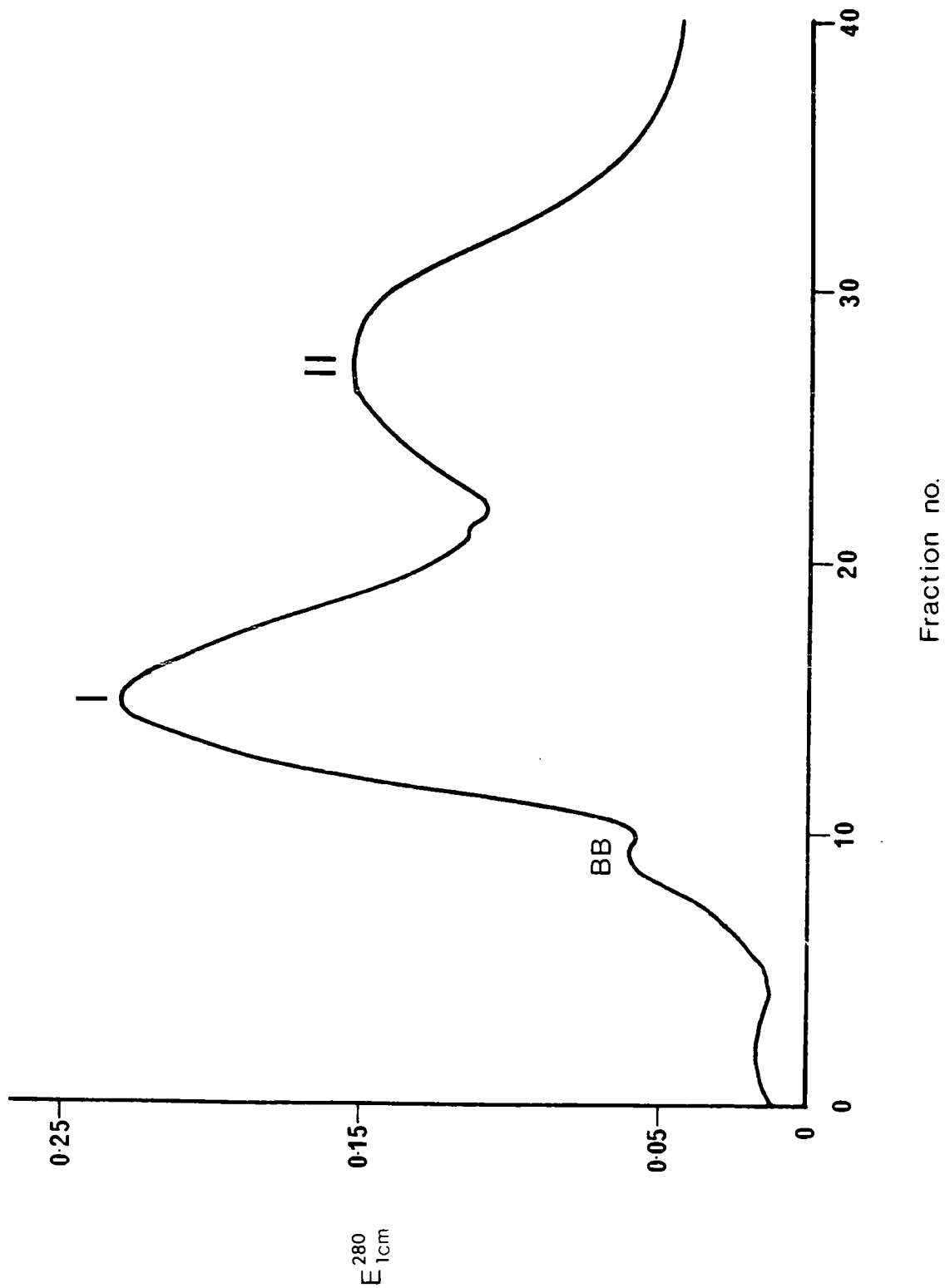


FIG. 30

Electrophoretic patterns of (a) fraction 12, (b) fraction 14, (c) fraction 16, (d) fraction 18 and (e) fraction 27 obtained by preparative gel electrophoresis of CM legumin (see Fig.29). Electrophoresis was performed in the SDS-citrate, pH 7.0 gel system (10% gels).



e



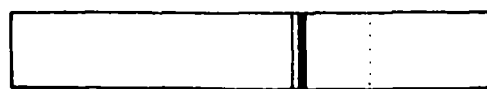
d



c



b

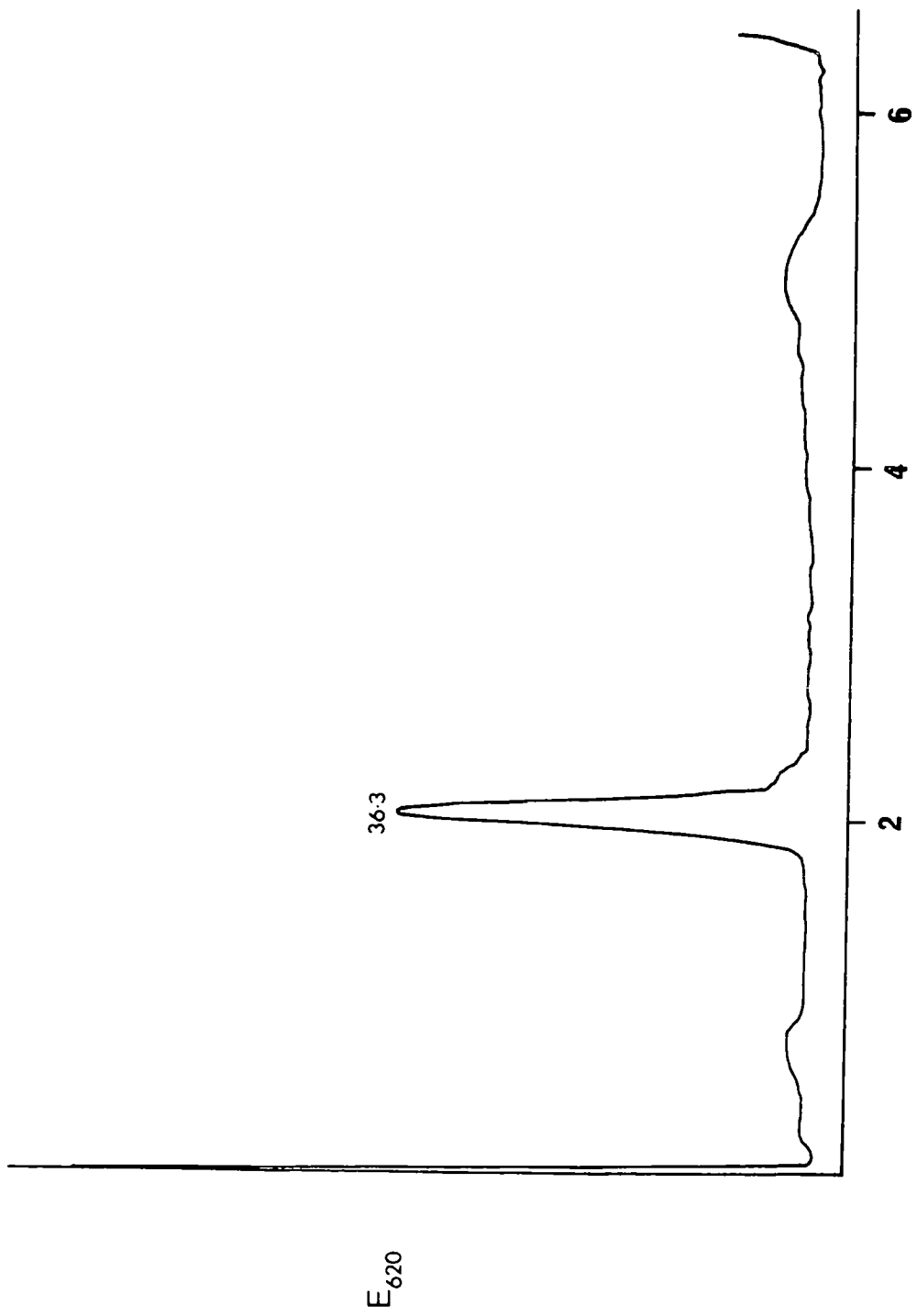


a

FIG. 31

Electrophoretic pattern of the 36,300 molecular weight sub-unit of CM legumin, obtained by extraction from gel slices.

Electrophoresed in the SDS-phosphate, pH 7.0 gel system. The number above the peak refers to the molecular weight ($\times 10^{-3}$) of the associated sub-unit.



contained a much higher content of glycine, glutamic acid and serine than expected, presumably due to contamination at some stage of the extraction procedure.

C. Separation of Sub-units using Ion Exchange Chromatography

(i) Chromatography

Two fractions were obtained when CM legumin, dissolved in 6M urea, was chromatographed on the anion exchange resin, AG1X2. The first (basic fraction) was not bound to the column at pH 8 and consequently passed straight through, the other (acidic fraction) was bound to the resin, and was removed by elution with a pH 4.5 buffer.

When CM vicilin (CM fraction I) was chromatographed, three fractions were obtained; the first (V1) did not bind to the resin, the second (V2) was eluted with pH 4.5 buffer and the third (V3) was eluted with M acetic acid containing 6M urea.

(ii) Electrophoretic analysis of fractions

(a) Analysis

The homogeneity and purity of the two fractions of CM legumin, obtained as outlined above, were examined using the SDS-phosphate, pH 7.0 and SDS-pH 8.3 gel systems and the following systems incorporating 6M urea: pH 4.3, pH 8.3 and pH 8.9. The electrophoretic patterns obtained for the two fractions are illustrated in Figs. 32 and 33. In both SDS systems, the acidic fraction gave only one major component (Figs. 33b and d), whereas the basic fraction gave one principal component at pH 7.0 (Fig. 33a) and three at pH 8.3 (Fig. 33c). In all the urea-containing systems the acidic fraction separated into two closely-spaced components (Fig. 32a, b and d). The basic fraction failed to

FIG.32

Electrophoretic patterns of CM legumin and its component sub-units separated by ion exchange chromatography. Electrophoresis was performed in various urea-containing gel systems. (a) Acidic sub-units, urea - pH 8.3 gel system, 7.5% gels; (b) acidic sub-units, urea - pH 8.9 gel system, 10% gels; (c) basic sub-units, (d) acidic sub-units and (e) CM legumin, all in the urea - pH 4.3 gel system, 7.5% gels. In all cases mobilities were measured relative to bromophenol blue.

FIG.33

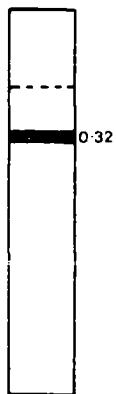
SDS electrophoretic patterns of CM legumin sub-units, separated by ion exchange chromatography. Electrophoresis was performed in the SDS-phosphate, pH 7.0 (a and b) and the discontinuous SDS-pH 8.3 (c and d) gel systems using 10% gels. Samples: (a), (c) basic sub-units; (b), (d) acidic sub-units.



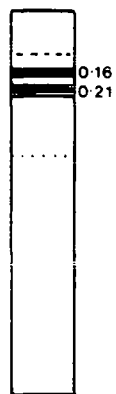
a



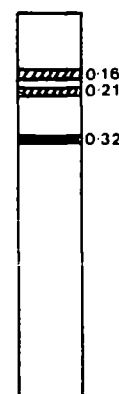
b



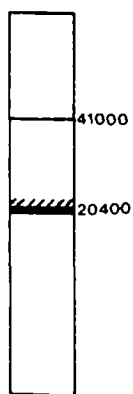
c



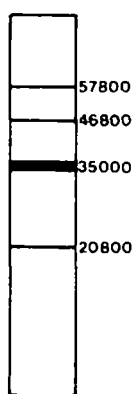
d



e



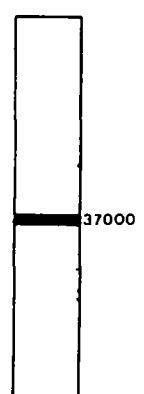
a



b



c



d

migrate into the gel when electrophoresed in the alkaline systems, but at pH 4.3 it migrated as a single band (Fig.32c).

Analysis of the three vicilin fractions (V1, V2 and V3) on discontinuous SDS-pH 8.3 gels indicated that partial separation of the vicilin sub-units had been achieved using ion exchange chromatography (Fig.34).

(b) Reproducibility of electrophoretic technique

Since estimates of the molecular weights of polypeptides were obtained using the technique of SDS gel electrophoresis, some measure of the accuracy and reproducibility of this technique was required.

Samples of CM legumin were electrophoresed in the SDS-pH 8.3 gel system and the relative mobilities of the component sub-units compared between gels in the same experiment and between gels from different experiments. For a group of seven gels electrophoresed together, the average R_m 's of the three basic sub-units were 0.786 ± 0.006 , 0.854 ± 0.005 and 0.879 ± 0.004 . When the results from three different experiments were compared, the average R_m 's were found to be 0.781 ± 0.009 , 0.847 ± 0.012 and 0.872 ± 0.009 . In every case the error is less than 2%. The greater error involved in the second group of results probably reflects the volumetric error involved in the preparation of the different series of gels. In the last analysis, the accuracy of molecular weight estimates by this method is dependent on the construction of a reasonably linear calibration graph. The graph for this particular gel system is illustrated in Fig.10. From this, the molecular weights of the legumin sub-units are 23,500, 20,700 and 19,800 (basic sub-units) and 37,000 (acidic sub-unit, $R_m = 0.544$).

FIG. 34

SDS electrophoretic patterns of fractions V1, V2 and V3 obtained from fraction I by ion exchange chromatography. Electrophoresis was performed in the discontinuous SDS-pH 8.3 gel system (10% gels). Numbers above peaks refer to the molecular weights ($\times 10^{-3}$) of the associated polypeptide.

(a) Fraction V1

(b) Fraction V2

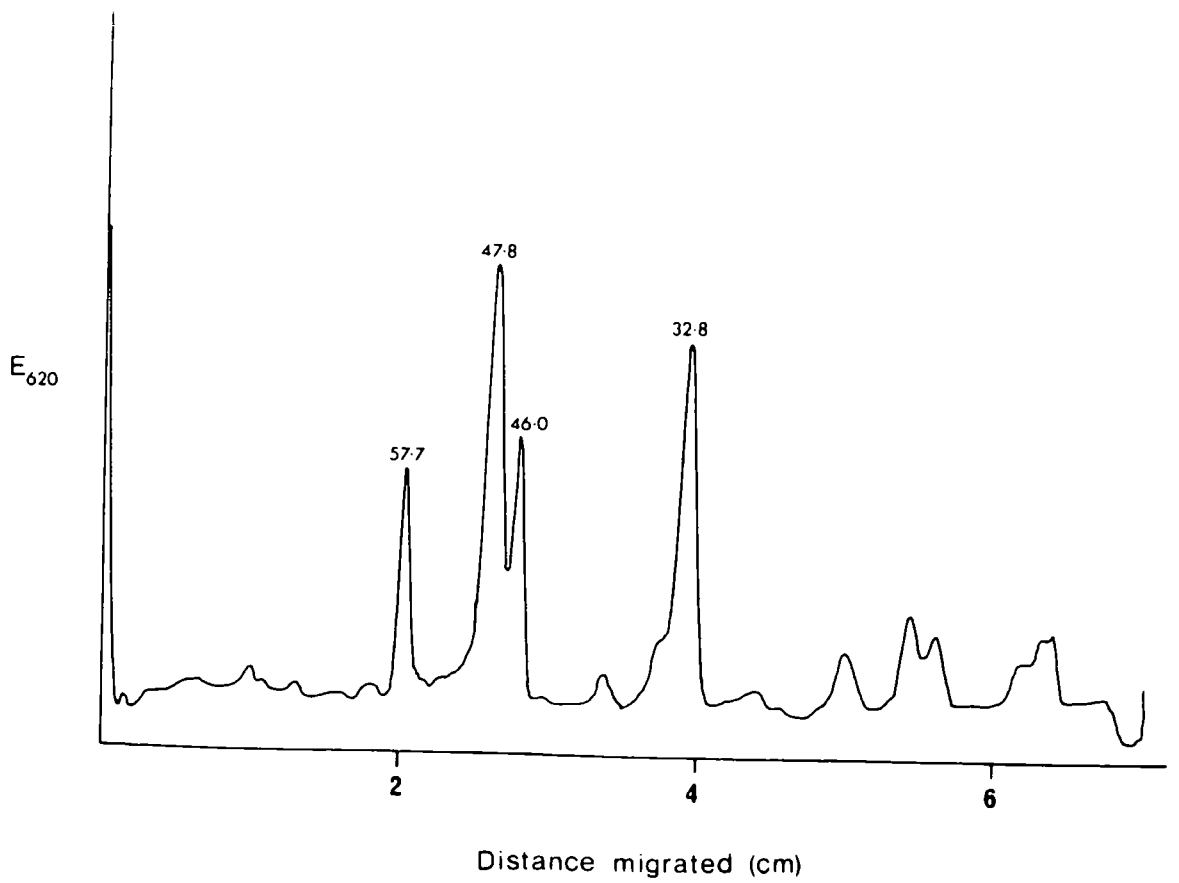
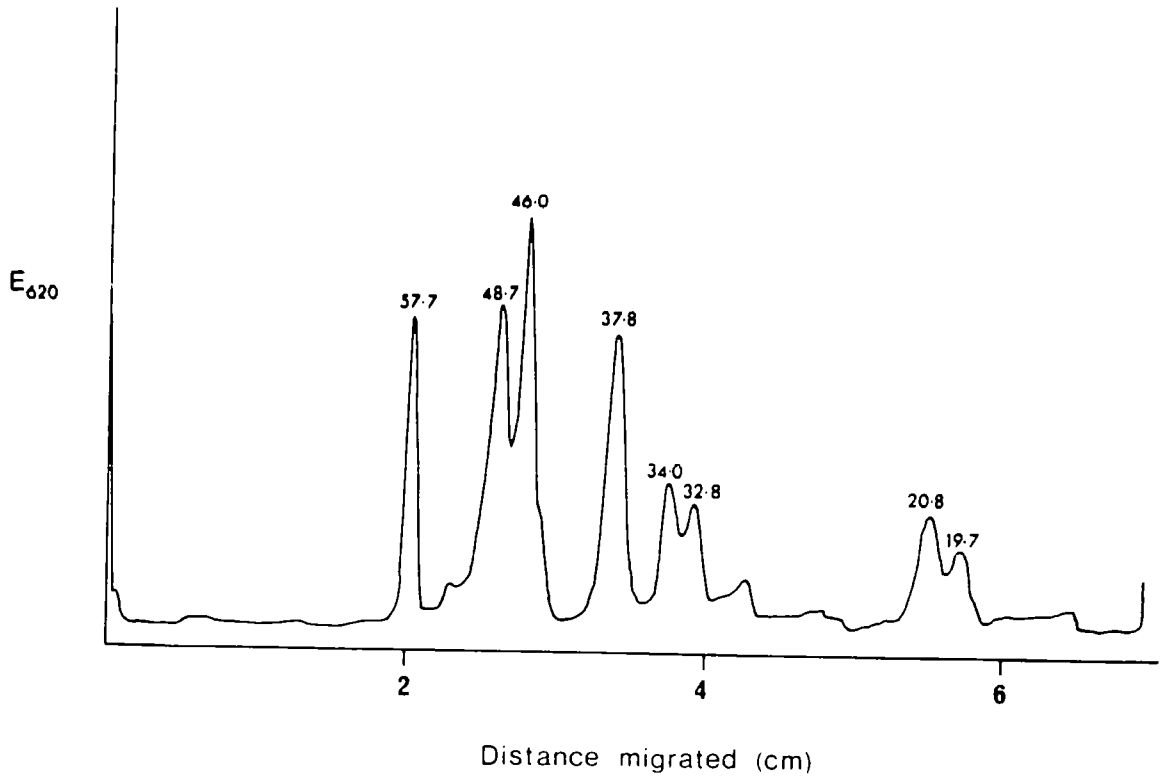
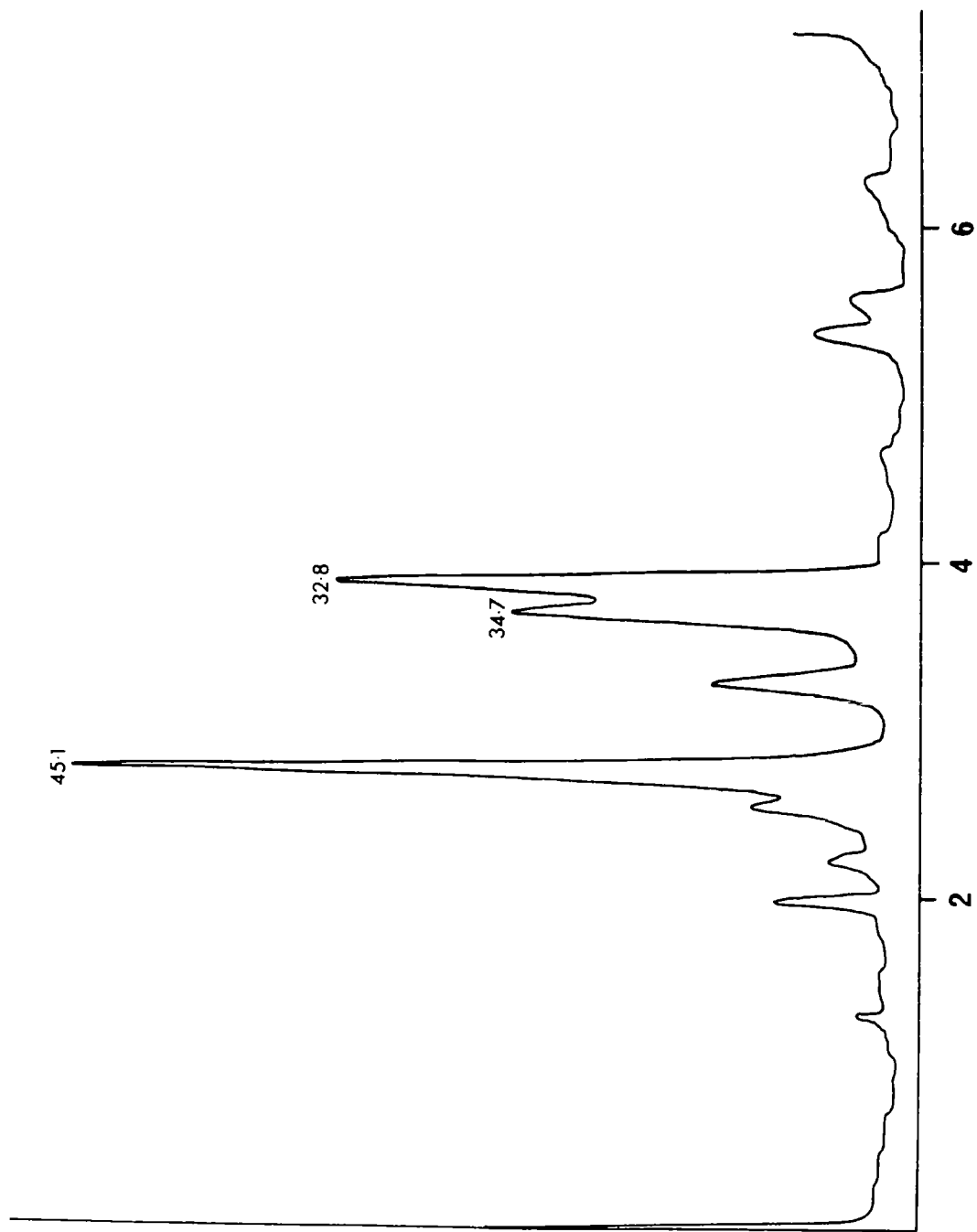


FIG. 34

(c) Fraction V3



Distance migrated (cm)

(iii) Gel isoelectric focussing of sub-unit fractions

The composition of the acidic and basic sub-unit fractions of CM legumin was further investigated using the powerful technique of gel isoelectric focussing, in which proteins migrate in a pH gradient to their isoelectric points. Urea was incorporated into the supporting gel in order to maintain the solubility of fractions at their isoelectric points. 6M urea proved satisfactory in this respect. At lower concentrations, 4M for example, precipitation of the acidic sub-units occurred. In both the pH 3-6 and pH 3-10 gradients, the acidic sub-units focussed into several distinct bands (Fig.35). The same pattern was produced irrespective of whether the sample was incorporated into the gel prior to polymerisation, or was layered onto the gel surface. Assuming a linear pH gradient throughout the gel, these components had isoelectric points in the approximate range of 4.7 to 5.2.

Due to the "instability" of the alkaline section of the pH gradients, the basic sub-units could not be focussed in either the pH 3-10 or pH 8-10 ranges. As the run progressed, this fraction migrated to the end of the gel, where, because of the decreased urea concentration caused by diffusion into the reservoir, it precipitated out. Attempts to stabilise the pH gradient by supplementing the gel ampholytes with pH 7-10 ampholine ampholytes, inverting the gradient, incorporating urea into the reservoir solutions or adjusting the pH of the latter to the required pH limits, proved unsuccessful.

(iv) N-Terminal amino acid analysis

The N-terminal amino acids of the acidic and basic sub-units of CM legumin were found to be glycine for the basic, and

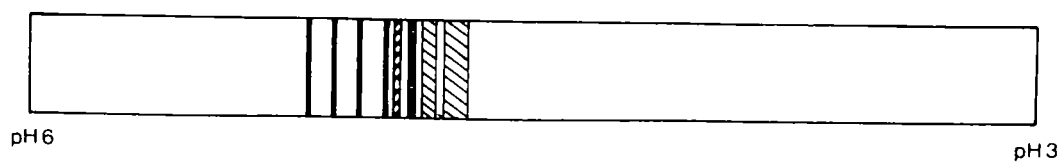
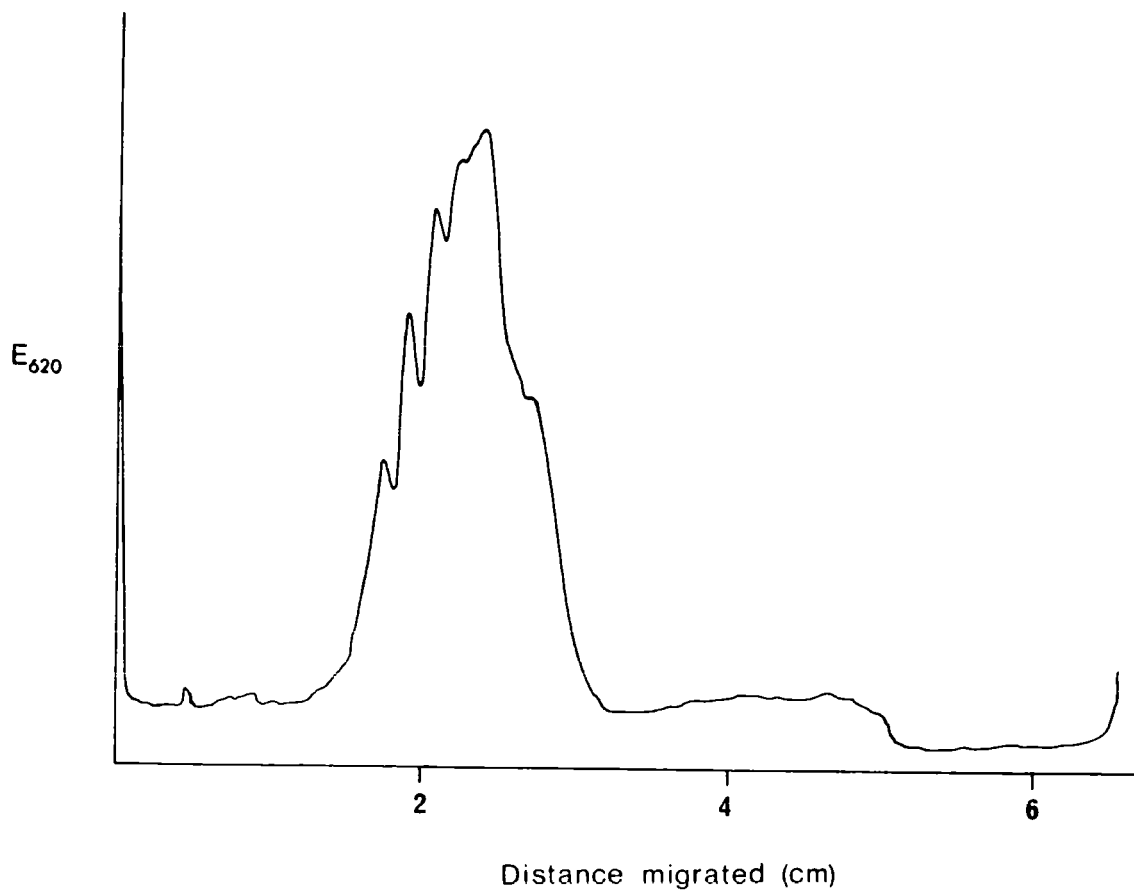
FIG. 35

Densitometric trace and band pattern obtained from gel isoelectric focussing of the acidic sub-units of CM legumin.

Nominal pH range of carrier ampholytes: pH 3-6

Sample: approximately 100 μ g α sub-units

Time of focussing: 4.5 h.



leucine and threonine for the acidic.

(v) Amino acid composition

The results of duplicate analyses of 20 h. and 72 h. hydrolysates of the acidic and basic sub-units of CM legumin are presented in Tables 4 and 5, and the amino acid composition (expressed as mole %) derived from them, in Tables 6 and 7. Values for serine, threonine and tyrosine were obtained by extrapolation to zero time. Tryptophan, cysteine and methionine were not determined.

(vi) Determination of sub-unit ratios in legumin

(a) Dye-binding method

Legumin was electrophoresed in both the SDS-phosphate, pH 7.0 and SDS-pH 8.3 gel systems and, using a dye-binding method, the ratio of acidic (α) to basic (β) sub-units determined. The results are summarised in Table 8. Measurements in both gel systems yielded the same acidic to basic sub-unit ratio in legumin of 4:5.

(b) Method based on amino acid composition

For any particular amino acid, if X, A and B represent the mole % of that amino acid in legumin, the acidic sub-units and the basic sub-units respectively, then

$$X = aA + bB$$

where a and b are weighting coefficients.

Using values for X, A and B given in Tables 2, 6 and 7, and applying regression analysis, the values obtained for a and b were 0.66 ± 0.02 and 0.34 ± 0.04 respectively, with a multiple correlation of 0.996. Values for methionine and proline were not included in the computation because of the greater analytical error involved in their determination.

TABLE 4

Amino acid analyses of hydrolysates of the α (acidic) sub-units
of CM legumin

Amino acid	Recovery	
	20 h.	72 h.
	g*/100 g. protein	
Aspartic acid	10.85 \pm 0.13	10.97 \pm 0.21
Threonine	2.21 \pm 0.04	2.28 \pm 0.11
Serine	3.85 \pm 0.02	3.21 \pm 0.0
Glutamic acid	20.92 \pm 0.84	19.59 \pm 0.56
Proline	3.89 \pm 0.04	3.57 \pm 0.51
Glycine	3.24 \pm 0.0	3.03 \pm 0.02
Alanine	1.97 \pm 0.03	1.88 \pm 0.06
Valine	2.37 \pm 0.02	2.48 \pm 0.09
Isoleucine	3.51 \pm 0.12	3.72 \pm 0.11
Leucine	5.36 \pm 0.15	5.41 \pm 0.34
Tyrosine	2.82 \pm 0.02	2.72 \pm 0.16
Phenylalanine	3.17 \pm 0.07	3.09 \pm 0.08
Histidine	2.64 \pm 0.10	2.48 \pm 0.09
Lysine	4.60 \pm 0.10	4.30 \pm 0.02
Arginine	10.25 \pm 0.20	11.83 \pm 0.71
	<u>82.23</u>	<u>81.27</u>

* Amino acid residues

The figures are the mean values and errors from duplicate analyses. The small amount of methionine present in the hydrolysates made its determination inaccurate and so the results are not quoted.

TABLE 5

Amino acid analysis of hydrolysates of β (basic) sub-units
of CM legumin

Amino acid	Recovery	
	20 h.	72 h.
	g*/100 g. protein	
Aspartic acid	10.72 \pm 0.03	10.55 \pm 0.39
Threonine	3.31 \pm 0.05	2.98 \pm 0.0
Serine	4.57 \pm 0.07	4.07 \pm 0.2
Glutamic acid	10.57 \pm 0.12	10.54 \pm 0.23
Proline	3.63 \pm 0.04	3.51 \pm 0.17
Glycine	2.76 \pm 0.04	2.68 \pm 0.03
Alanine	5.58 \pm 0.12	5.51 \pm 0.06
Valine	6.33 \pm 0.16	7.61 \pm 0.25
Isoleucine	3.10 \pm 0.03	3.21 \pm 0.07
Leucine	9.94 \pm 0.08	9.92 \pm 0.06
Tyrosine	3.98 \pm 0.04	3.68 \pm 0.13
Phenylalanine	3.69 \pm 0.06	3.88 \pm 0.04
Histidine	1.37 \pm 0.01	1.49 \pm 0.06
Lysine	6.02 \pm 0.10	6.12 \pm 0.2
Arginine	9.65 \pm 0.21	10.47 \pm 0.33
	<u>85.68</u>	<u>86.7</u>

* Amino acid residues

Legend as Table 4.

TABLE 6

The amino acid composition of the α (acidic) sub-units of CM legumin

Amino acid	Content ^a		
	g/100 g. protein	moles/10 ⁵ g. protein	mole%
Aspartic acid	10.97	95.3	13.1
Threonine	2.28	22.5	3.09
Serine ^b	4.13	47.4	6.52
Glutamic acid	20.92	162.0	22.27
Proline	3.89	40.0	5.5
Glycine	3.24	56.8	7.81
Alanine	1.97	27.7	3.81
Valine	2.48	25.0	3.44
Isoleucine	3.72	32.9	4.52
Leucine	5.41	47.8	6.57
Tyrosine ^b	2.85	17.5	2.41
Phenylalanine	3.17	21.5	2.96
Histidine	2.64	19.2	2.64
Lysine	4.60	35.9	4.94
Arginine	11.83	75.7	10.41
	<u>84.81</u>	<u>732.8</u>	

a. Values refer to amino acid residues

b. Extrapolated to zero time

TABLE 7

The amino acid composition of the β (basic) sub-units of CM legumin

	Content ^a		
	g/100 g. protein	moles/10 ⁵ g. protein	mole%
Aspartic acid	10.72	93.1	11.66
Threonine	3.45	34.1	4.27
Serine ^b	4.79	55.0	6.89
Glutamic acid	10.57	81.9	10.26
Proline	3.63	37.4	4.68
Glycine	2.76	48.4	6.06
Alanine	5.58	78.5	9.83
Valine	7.61	76.8	9.62
Isoleucine	3.21	28.4	3.56
Leucine	9.94	87.8	11.00
Tyrosine ^b	4.11	25.2	3.16
Phenylalanine	3.88	26.4	3.31
Histidine	1.49	10.9	1.36
Lysine	6.12	47.7	5.97
Arginine	10.47	67.0	8.39
	<u>88.81</u>	<u>798.6</u>	

a. Values refer to amino acid residues

b. Extrapolated to zero time.

TABLE 8

Determination of the sub-unit ratio in legumin

Gel system	SDS-phosphate pH 7.0		SDS-pH 8.3	
	α	β	α	β^a
Sub-unit				
Mol. wt. ^b	37,000	23,000	35,000	21,300
Optical ratio	1.28	1	1.32	1
Molar ratio	4	5	4	5

- a. Values were calculated for the total basic sub-unit fraction (i.e. $\beta_1 + \beta_2 + \beta_3$)
- b. Molecular weight as determined in the respective gel systems

Summing over all amino acids we have,

$$\begin{aligned}\Sigma X &= a\Sigma A + b\Sigma B \\ &= 0.66\Sigma A + 0.34\Sigma B\end{aligned}$$

where ΣX , ΣA and ΣB are equivalent to 100 moles of amino acids of legumin, the acidic sub-units and the basic sub-units respectively. From the amino acid composition data the molecular weights of the sub-units are 11,564 g. (acidic) and 11,065 g. (basic) per 100 moles of amino acids. As the molecular weights determined by SDS gel electrophoresis are 37,000 and 21,300, ΣA and ΣB thus represent 0.31 moles and 0.52 moles respectively of the acidic and basic sub-units. Hence the sub-unit ratio in the legumin molecule is given by $0.66 \times 0.31 : 0.34 \times 0.52 = 1.1:1.0$ or approximately 1:1. Thus the legumin molecule consists of an equimolar mixture of acidic and basic sub-units. Using the molecular weights determined for reduced (i.e. not carboxymethylated) legumin sub-units (35,000 and an average 21,300), a twelve polypeptide chain model for legumin would have a molecular weight of 337,800, in good agreement with the molecular weight of 328,000 obtained from sedimentation studies.

(vii) Results from preliminary sequence studies on the basic sub-units of legumin

It had not proved possible to separate the individual β_1 , β_2 , and β_3 sub-units present in the basic sub-units, in sufficient quantities to enable their primary structures to be investigated. Instead, however, the extent of sequence homology among the basic sub-units was examined by sequencing these sub-units together. The amino acid sequence of the first twenty-four residues was determined. Amino acids cleaved after each degradative cycle were identified as their PTH derivatives by

thin layer and gas chromatography. Fig.36 illustrates two examples of gas chromatographic identification of PTH amino acids present in the sequenator fractions. The chromatographs correspond to residue 6 (valine and leucine or isoleucine) and residue 23 (leucine or isoleucine). The complete amino acid sequence obtained is shown in Table 9. The blanks in the sequence correspond to cycles where no principal PTH amino acid(s) could be detected, possibly as a result of either breakdown of the extremely unstable sequenator products of serine and threonine, or the presence of arginine and histidine. The latter two were detected by the Phenanthrenequinone and Pauly spot tests respectively, but the results proved inconclusive and so no definite assignments could be made. As the degradation progressed, there was a considerable increase in the amount of background amino acids, resulting from non-specific acid cleavage and incomplete stepwise degradation. Because of this, the amino acids assigned to later positions in the sequence were those that had increased relative to the background. Even in the early stages of the degradation, there was pronounced carry over, for example, between residues 6 and 7, where there was approximately 25% carry over of leucine (isoleucine) and valine. Fig.37 illustrates the absolute stepwise yield, in nmoles, of those PTH derivatives, present in the sequence, which chromatograph particularly well and are easily quantitated. The low yields in positions 15 and 23 probably indicates, as in the cases of positions 6, 9 and 13, the presence of another amino acid which was not detected.

FIG. 36

Gas chromatographic analysis of the sequenator fractions obtained by sequential degradation of the basic (β) sub-units of CM legumin. Experimental details are given in Methods (section XVII.B).
I/L: isoleucine or leucine.

(a) Residue 6

(b) Residue 23

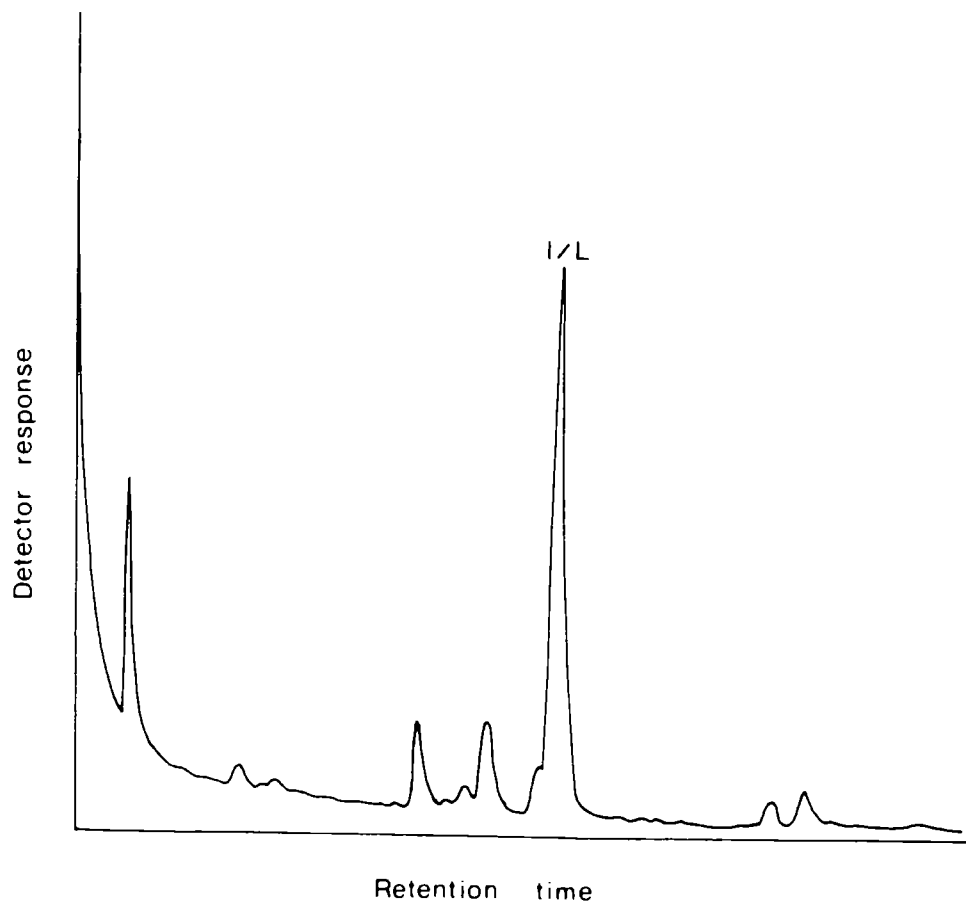
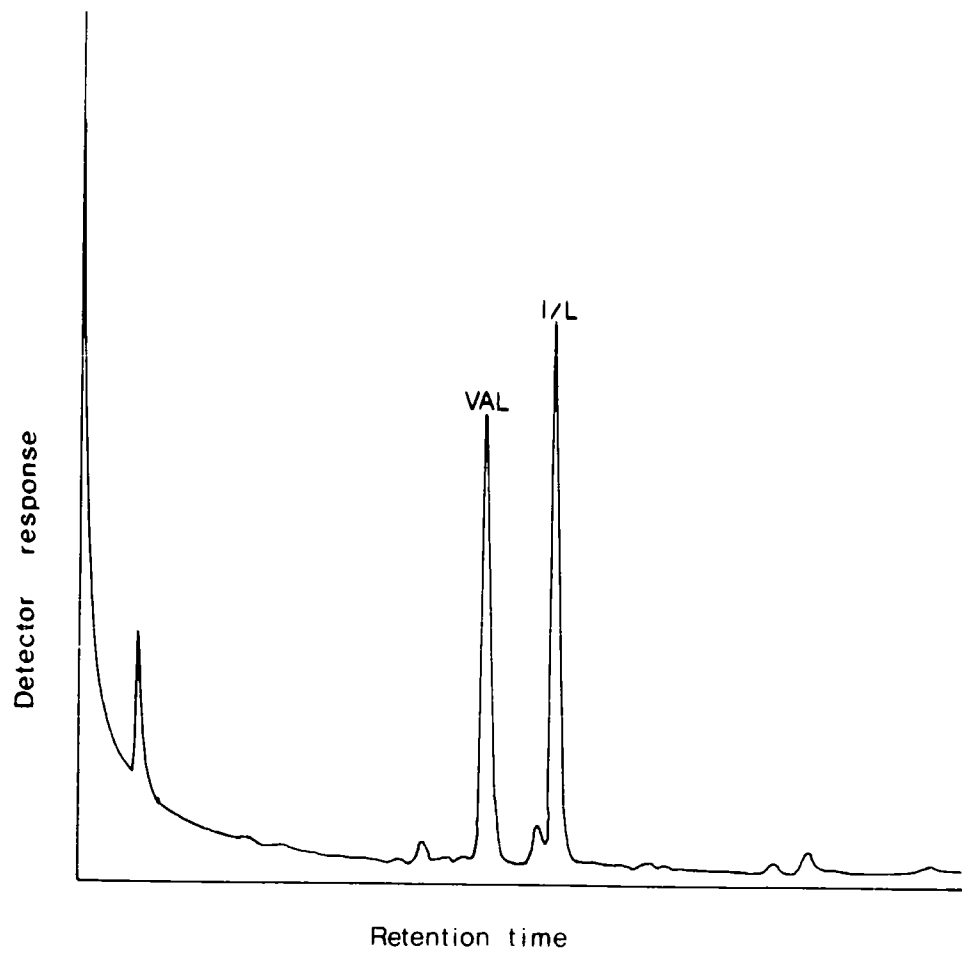


TABLE 9

The N-terminal amino acid sequence of the β sub-units of legumin

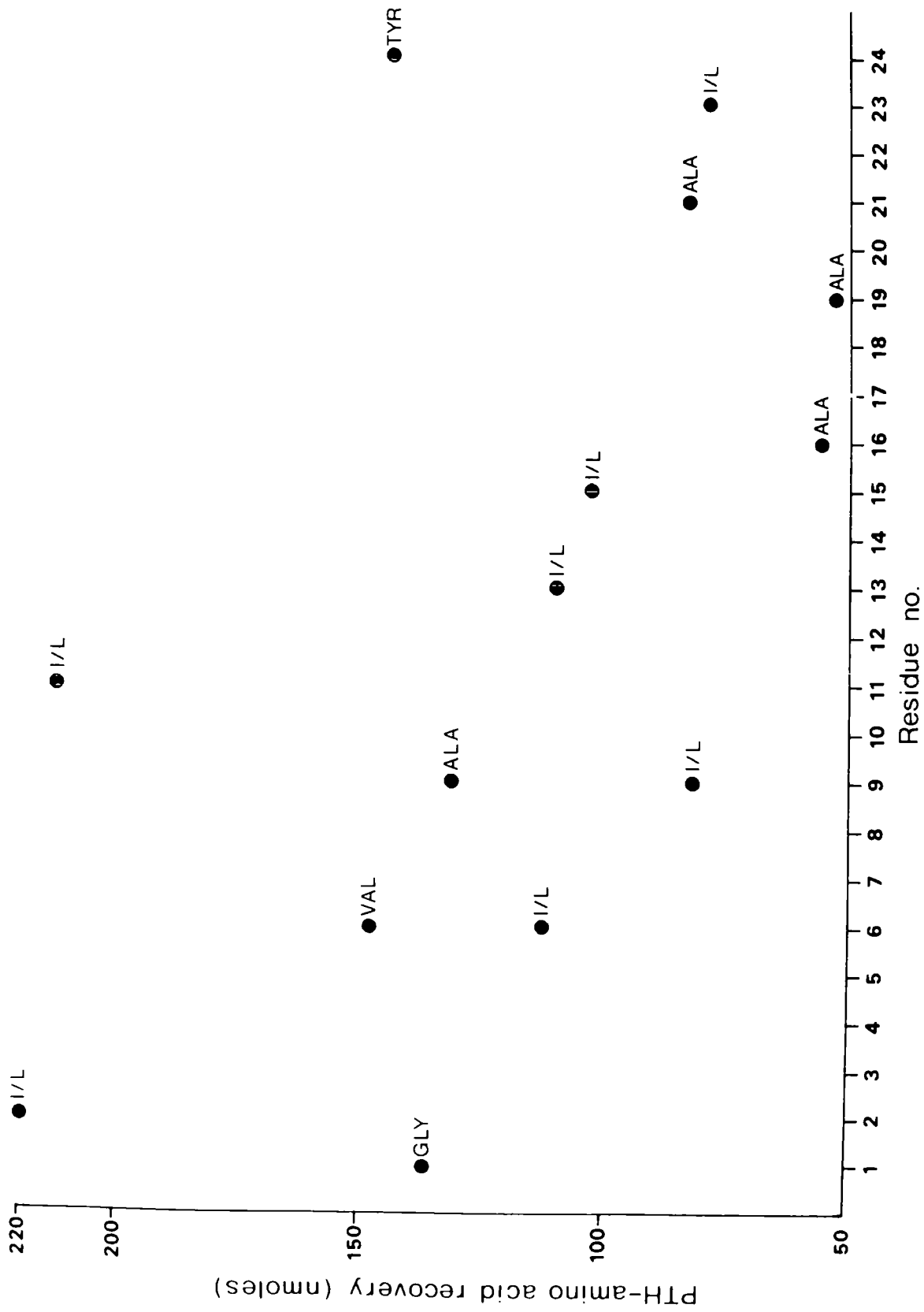
1	LEU/ILE	LEU/ILE	10	LEU/ILE
GLY-LEU/ILE-GLU-GLU-PRO-	VAL	-SER- *	- * -LEU/ILE- *	- * -GLU -
		ALA		
	20	24		
* -LEU/ILE-ALA- * - *	-ALA- *	-ALA-ASP-LEU/ILE-TYR		

FIG.37

Absolute yield of PTH-amino acid derivatives in the sequenator fractions of the basic (β) sub-units of CM legumin. Only those PTH-amino acids which were easily quantified are included.

Sample: 320 nmoles β sub-units

I/L: isoleucine or leucine



DISCUSSION

Generally speaking with each new advance in protein separatory techniques, some proteins previously thought to be homogeneous have been shown not so to be. Before starting to characterise a protein structurally, however, it is necessary for it to be homogeneous. In the past the isolation of homogeneous seed proteins has proved difficult and consequently, as a preliminary to the structural studies in the present work, a variety of extraction and separation procedures were examined and their effectiveness compared.

Our basic information on these storage proteins derives from the studies of Osborne (1924) and Danielsson (1949). However it is now recognised that these early preparations were far from homogeneous. Since then more sophisticated techniques have been applied to the problem of purification. Separation has been achieved by utilising differences in charge (electrophoresis, ion exchange chromatography) and differences in size/shape (gel filtration, gel electrophoresis, sucrose gradient centrifugation). The effect on the structural or functional integrity of the proteins must be taken into account when selecting a particular method. Proteins, being macro-molecules and containing so many different functional groups, are very susceptible to changes in environmental conditions. Variations in pH, ionic strength and temperature can result in deamidation, aggregation and dissociation. Normally the storage proteins of legume seeds are prepared by extraction into salt solutions buffered to pH 7 or thereabouts. However the pH

of the extractant is known to have a marked effect on the peptization of the protein (Schellman and Schellman, 1964; Fontaine et al., 1946). Smith and Circle (1938) demonstrated that, in the case of defatted soyabean meal, the amounts of protein extracted varied markedly with the pH. Low yields were obtained in the pH 4 to 5 region, which corresponded with the isoelectric points and hence minima in solubilities of the proteins, while 85% extraction was achieved above pH 7 and also in the region of pH 2. For this reason the preparation of globulins of Vicia faba using an acidic extractant was attempted. Ascorbic acid (McLeester et al., 1973) was used as this has also been shown to be an effective anti-oxidant for proteins during gel electrophoresis. (Polter and Mueller-Stoll, 1970). The extraction was thus carried out under reducing conditions, thereby minimising the possibility of disulphide formation leading to protein aggregates (Briggs and Wolf, 1957). Both 2-mercaptoethanol (Catsimpoilas et al., 1967; Tucker and Fairbrothers, 1970) and dithiothreitol (Hall et al., 1971) have been used previously to ensure reducing conditions during the extraction. The fractions obtained by this method were not homogeneous, as judged by ultracentrifugation and gel electrophoresis, and could not be positively identified with either legumin or vicilin. However the results would seem to indicate that the globulins of bean are, to some extent, irreversibly dissociated at very low pH, and so the validity of this extraction procedure is questionable. Reversible or irreversible dissociation of the 11-12S seed proteins of various legumes into the 8S half-molecules has been demonstrated by other workers (Johnson, 1946a; Joubert, 1956; Wolf and Briggs, 1958). Further dissociation

was shown to occur under acid conditions, thus Shutov and Vaintraub (1966) found that legumin was completely dissociated into a 2S form in pH 2.8 buffer, and a value of 1.9S has been reported by Danielsson (1949) when legumin was dissociated at pH 3. Vicilin was also shown to undergo partial dissociation into a 3.4S form at both pH 2.5 and 2.1 (Shutov and Vaintraub, 1966).

Another important factor to take into consideration during protein extraction is that of proteolytic degradation. Even if the extent of proteolysis is minimised, it can still result in the production of a microheterogeneous mixture of proteins, a fact which only becomes apparent in the later stages of investigation (Lederer and Jacq, 1971; Clark and Jakoby, 1970; Pringle, 1970). The multiple bands frequently observed when apparently homogeneous proteins were analysed by isoelectric focusing (Hayes and Wellner 1969; Drysdale 1970; Susor et al., 1969) have, in one particular case, been shown to be caused by the action of proteolytic enzymes during the extraction procedure (Gray and Kekwick, 1973). To overcome this problem proteinase inhibitors such as diisopropyl fluorophosphate and phenylmethylsulphonyl fluoride (Matsubara and Feder, 1971; Fahrney and Gold, 1963) have been incorporated into the extraction medium and throughout the purification stages (Gray and Kekwick, 1973; Lazarus et al., 1966; Lederer and Jacq, 1971; Clark and Jakoby 1970; Diezel et al., 1973). Sodium dodecyl sulphate can also be used to minimise the degree of proteolytic degradation, since it has been shown to inhibit enzymic activities (Calandra et al., 1951; Sugiura et al., 1964; Van Heyningen 1972; Viswanatha et al., 1955; Rogers and Yusko 1972).

However its powerful denaturing ability (Anson, 1939; Putnam, 1948) coupled with its tenacity in binding to proteins has limited its usefulness in regard to protein extraction. In the present work, however, an SDS extractant proved useful in demonstrating that the heterogeneity observed in the legumin basis sub-units when electrophoresed on SDS gels was not merely an artifact of the preparation. Whether this is also true of the multiple bands apparent on isoelectric focusing gels is impossible to say. In order to answer this question, resort must be made to chemical methods of analysis, such as N- and C-terminal amino acid analysis, amino acid composition and perhaps most important amino acid sequence determination.

In his studies on the pea globulins, Danielsson (1949) found that the two components could most satisfactorily be separated by using isoelectric precipitation. It is a modification of this technique, termed zonal isoelectric precipitation (Shutov and Vaintraub, 1965), that has been employed in the present studies for the separation of Vicia faba globulins. This procedure is based on the zone precipitation method described by Porath (1962), and, as the name implies, it achieves fractionation utilising isoelectric precipitation of proteins. Briefly the method operates in the following manner. The protein mixture to be fractionated is applied to a gel column equilibrated with buffer of a pH at which one protein component is insoluble (isoelectric point). The gel is chosen such that the proteins are excluded from the gel matrix and so, the former migrate through the column at a faster rate than the buffer front. This results in isoelectric precipitation of the protein, insoluble at pH 4.7, which then redissolves as it is

overtaken by the elution buffer. Thus, as this protein migrates down the gel column it undergoes a continuous cycle of precipitations and dissolutions. As a result, it is retarded, whilst the remainder of the proteins in the sample move unhindered and elute in the void volume of the column. In this way, the globulins of V. faba were fractionated into a pH 4.7 soluble and a pH 4.7 insoluble component. The latter (fraction II) was almost homogeneous by ultracentrifugation and gel electrophoresis, and was equated with classical legumin through comparisons of N-terminal amino acids and gel electrophoretic patterns. The elution profile obtained when fraction II was rechromatographed under the same conditions, indicates the presence of a small amount of impurity (Fig. 6), which eluted, like fraction I, in the void volume of the column. However, the SDS electrophoretic pattern of the impurity (Fig. 9), consisting of two major components with molecular weights of 20,8000 and 28,400, bears no relationship to that of fraction I (Fig. 8b) or even fraction II (Fig. 8a). It may represent a 4.7 soluble fraction that was co-precipitated, during the initial chromatography, with fraction II, but became dissociated from this fraction as a result of the subsequent dialysis and rechromatography steps. In any case, it was present in a sufficiently small amount to be considered as only a minor contaminant.

Fraction I (pH 4.7 soluble globulin) was contaminated with legumin, but the latter could be removed by isoelectric precipitation at reduced ionic strength illustrating the fact that, even at its isoelectric point, the solubility of legumin is dependent on ionic strength. Recently, this has also been shown to be true for soyabean 11S protein

(Anderson et al., 1973). In this case, at zero NaCl molarity only the 2S and 7S proteins were extracted, but as the NaCl concentration increased, so did the solubility of the 11S protein, until a maximum was reached at 0.8M NaCl.

The total nitrogen content of 17.8% determined for legumin agrees reasonably well with the published values for other seed proteins (Boulter and Derbyshire, 1971). The decrease to 16.4% nitrogen on carboxymethylation probably reflects the increase in weight due to the attached carboxymethyl groups, or alternatively, actual loss of nitrogen due to deamidation of asparagine and glutamine (McKerrow and Robinson, 1971). The amino acid composition is in reasonable agreement with those published by other workers (Jackson et al., 1969; Bailey and Boulter, 1970; Millerd et al., 1971). Any apparent differences may, as suggested by Millerd et al., (1971), be attributable to the use of different cultivars or varieties, but a more probable explanation is that they arise from impurities in the different preparations. The composition is also similar to those reported for other seed storage globulins such as glycinin (Catsimpoolas et al., 1971), edestin (Kimmel and Smith, 1958) and arachin (Singh and Dieckert, 1973a), in so far as they contain a high proportion of glutamic acid (glutamine), aspartic acid (asparagine) and arginine, possibly reflecting one of their common functions, namely that of nitrogen storage.

Even to the early investigators the size of the legumin molecule, estimated to have a molecular weight in the range 300,000 to 400,000, indicated that it was unlikely to consist of only one component, but that it was probably an "aggregate" of several identical or non-identical units held together by covalent or ionic forces. Evidence

for the existence of a sub-unit structure came from experiments undertaken by Brand and Johnson (1956), Vaintraub and Gofman (1961) and Johnson and Richards (1962). Their findings were later confirmed and extended by Grant and Lawrence (1964), who used the powerful technique of polyacrylamide gel electrophoresis to demonstrate the breakdown of the legumin macromolecule by sodium dodecyl sulphate. The absence of any reducing agent in their experiments meant, however, that they failed to achieve complete dissociation, thus explaining the multiple N-terminal amino acids of single bands extracted from the gels. This phenomenon of partial dissociation is illustrated in Figs 11a and b, where the legumin "sub-units" with molecular weights of 50,500 and 49,500 were dissociated further by incubation with the reducing agent, 2-mercaptoethanol. The sub-unit structures of a number of seed globulins have now been investigated (Tombs and Lowe, 1967; Bailey and Boulter, 1970; Catsimpoolas et al., 1971; Vaintraub and Nguyen Thanh Thien, 1971; Singh and Dieckert, 1973b) and in some cases actual separation of the component sub-units has been achieved (Catsimpoolas et al., 1971; Vaintraub and Nguyen Thanh Thien, 1968). In order to obtain complete separation, the protein molecule must be fully dissociated and intermolecular interactions between sub-units eliminated. This can best be accomplished with the use of powerful denaturing agents such as urea, guanidine hydrochloride and sodium dodecyl sulphate (Reithel, 1963). The choice of a suitable denaturant will depend largely on experimentation. Although universally employed for its dissociating action, SDS is known to effect solubilisation without dissociation (Katzman, 1972) and in some cases actually causes

association (Johnson and Joubert, 1951; Ray, 1968). The use of urea can lead to carbamylation of amino groups (Stark et al., 1960; Cole, 1961; Manson, 1962; Cejka et al., 1968), due to the presence of cyanate ions (Marier and Rose, 1964). As the carbamylated residues are neutral, the proteins become less basic and as a consequence can exhibit modified electrophoretic mobilities (Cole and Meacham, 1966). In this work, all urea solutions were prepared from Analar grade reagent, and then deionised by passing through a column of mixed bed resin (Duesberg and Rueckert, 1965). According to Gerding et al., (1971), however, this procedure has limited usefulness as new cyanate formation occurs at pH values greater than 4. Hence, caution must always be exercised when assessing the results obtained from experiments in which urea has been employed. 2-Mercaptoethanol has its limitations as a reducing agent (Hu et al., 1959), and permanent protection of sulphhydryl groups by carboxymethylation is to be preferred.

The sub-units of legumin were separated using three different techniques. The first, a microscale method, involved extraction of the sub-units from stained gel slices, with subsequent removal of bound dye. The amount of protein (up to 20 $\mu\text{g/gel}$) limits the usefulness of this technique to the determination of N-terminal amino acids and amino acid composition. The results from the amino acid analyses of extracted protein proved disappointing, due to the presence of large amounts of contaminating amino acids. One possible way of overcoming the problem of contamination is to reduce the amount of manipulation. A recent method, described by Houston (1971), involves hydrolysis of the stained gel slice itself. Unfortunately the large amount of NH_3 present in the hydrolysate makes it necessary to remove it before application to an amino acid analyser.

The other two preparative techniques involved separation according to molecular weight (SDS preparative gel electrophoresis) and separation based on charge differences (ion exchange chromatography in 6M urea). The disadvantage of the electrophoretic method was that the separated sub-units still had detergent firmly bound to them. Before proceeding with any further studies, it was necessary to remove all the bound detergent. Two methods for the removal of SDS from proteins have been described (Lenard, 1971; Weber and Kuter, 1971), and both involve the use of ion exchange chromatography in 8M urea. However, as yet, there is no way of demonstrating the effectiveness of these methods for different proteins without recourse to expensive radiochemicals.

Excellent separation of the sub-units was achieved using ion exchange chromatography in 6M urea. The quality of the separation is, no doubt, partly due to the clear distinction between the two types of sub-units, acidic and basic. This property has also been reported for the sub-units of glycinin (Catsimpoolas, 1969). The main disadvantage of this method is the need to use 6M urea and hence, the associated dangers of carbamylation mentioned earlier. On the other hand, it has the advantage of being far less complex and time-consuming than preparative electrophoresis and also the size of the sample is only limited by the column dimensions; in the preparative apparatus 50 mg was the maximum feasible if the separation was to be maintained.

Up to this point in the discussion, the term "sub-unit" has been used operationally, i.e. to denote any chemically or physically identifiable sub-molecular component. Difficulties are encountered when attempts are made to describe the structure of legumin using the accepted terminology (Monod et al., 1965). The results indicate that

legumin is a polymeric protein and, being composed of non-identical sub-units it is thus termed a monomer. However, this helps little towards an understanding of the sub-molecular structure of the protein, for which new terms must be devised.

Legumin is dissociated by SDS into two entities with molecular weights of 50,500 and 53,500, henceforth referred to as the intermediary sub-units. If in addition to SDS, a reducing agent is present, legumin dissociates to give two species of molecular weights 37,000 and 22,400. These two components correspond to the acidic (α) and basic (β) fractions or sub-units obtained when legumin, dissociated in 6M urea, was fractionated by ion exchange chromatography. However, neither of these two fractions, α or β , is homogeneous. Using high resolution SDS gel electrophoresis, i.e. a discontinuous buffer system, the β fraction can be resolved into three components, β_1, β_2 and β_3 with molecular weights of 23,800, 21,300 and 20,200 respectively. It must be emphasised that these do not represent products of further dissociation, but merely result from the increased resolution afforded by the discontinuous gel system in the 15,000 to 30,000 molecular weight range (Weber et al., 1972). The α fraction separates into two distinct bands when electrophoresed on urea gels, a fact which agrees with the finding of two N-terminals (leucine and threonine) for this fraction. The elementary sub-units of legumin can thus be represented by $\alpha_1, \alpha_2, \beta_1, \beta_2$ and β_3 . The term elementary is used here in the sense that no further breakdown into smaller molecular weight components has been observed, and thus these sub-units represent the individual polypeptide chains of which legumin is composed. The results of electrophoretic experiments performed with reduced and unreduced legumin indicate that

these elementary sub-units are formed from the intermediary sub-units by reductive cleavage of intermolecular disulphide bonds. The similarity in molecular weight of the intermediary sub-units can only be explained by postulating a structure for them in which one α and one β sub-unit are combined. Any other combination of elementary sub-units would result in intermediary sub-units with widely differing molecular weights. The discrepancy in the apparent molecular weights of the proposed $\alpha\beta$ intermediary sub-units and the combined molecular weight of an α and β sub-unit (57,200 to 60,800) probably reflects the increased secondary and tertiary structure of the former, produced as a result of the disulphide bonding. The presence of inter- and intra-molecular disulphide bonds in proteins has been shown to affect their electrophoretic mobilities in SDS gels (Griffith, 1972), and thus can lead to erroneous estimates of molecular weights (Trayer et al., 1971; Dunker and Rueckert, 1969). The degree of error involved depends on two opposing factors. Firstly, as proteins containing intramolecular disulphide links bind much less SDS than their reduced counterparts (Pitt-Rivers and Impiombato, 1968), they should have a lower negative charge and thus be less mobile during electrophoresis. Secondly, as a result of both decreased SDS binding, and the presence of cross-links, unreduced proteins will have smaller Stokes radii (Fish et al., 1970) which in turn means less frictional resistance, and thus greater mobility in gels. Since the $\alpha\beta$ sub-units have apparent molecular weights lower than expected, it is clearly the second of these factors that predominates in this particular instance.

Six of the $\alpha\beta$ sub-units are required to form a legumin molecule with approximate molecular weight of 330,000 and, in view of the fact

that dissociation of the latter to the intermediary sub-units occurs in the absence of any reducing agents, the bonding between these sub-units must be of a non-covalent character. This investigation gave no indication as to the configuration of the $\alpha\beta$ sub-units in the legumin molecule or of the existence of any higher molecular weight intermediates between the $\alpha\beta$ sub-units and the intact legumin molecule. However, the dissociation, in acid or alkaline media, of legumin of V. sativa into identical 7S sub-units has been reported (Vaintraub and Nguyen Thanh Thien, 1971) and clearly the demonstration of analogous sub-units in legumin of V. faba would necessitate revision of the monomeric definition of this legumin.

An alternative model for the legumin molecule is one in which non-covalent bonding is the only force of attraction between sub-units, disulphide bridges being confined to intramolecular bonding. This would imply that in SDS complete dissociation to the elementary sub-units occurs initially, but that disulphide bond formation between sulphhydryl groups, exposed as a result of disruption of secondary and tertiary structure by the SDS, then results in association of these sub-units to form the intermediary sub-units. The latter are then merely artifacts of the analytical procedure. This explanation can however be discounted, as one would expect random disulphide bond formation to produce a whole range of molecular weight species and not just the two that are observed on SDS gels.

It is interesting to note that the content (moles/ 10^5 g) of basic amino acids (lysine, histidine and arginine) is approximately the same for both α (130.8) and β (125.6) sub-units. The acidic properties of

the α sub-units clearly stem from their far greater content of acidic amino acids (glutamic and aspartic acids), 257.3 moles/ 10^5 g compared with 175 moles/ 10^5 g for the β sub-units. It is also apparent that the β sub-units contain far more hydrophobic amino acids, such as leucine, valine and alanine, a fact which agrees well with experimental observations that they are much less soluble in aqueous media than the α sub-units. The amino acid compositions of the isolated A, B and C sub-units of legumin of V. sativa, determined by Vaintraub and Nguyen Thanh Thien (1971), are presented in Table 10, together with the amino acid compositions of the α and β sub-units as determined in the present work. For comparative purposes, the results of Vaintraub and Nguyen Thanh Thien (1971) were recalculated and expressed as mole percentages. The amino acid composition of the two low molecular weight sub-units (β and A) from both species of Vicia agree exceptionally well; in most cases the variation is considerably less than 10%. However, there is no similar correspondence between the amino acid compositions of the α sub-units and either the B or C sub-units. The best correlation is obtained between the α and B sub-units although, in most cases, the corresponding values differ by greater than 10% and sometimes by as much as 25%.

The heterogeneity observed in the α and β sub-units poses an interesting question, namely, is there a single legumin molecule composed of all the elementary sub-units, or are there several types of legumin, each one containing different α and β sub-units (polymorphism)? If the latter is the case, then there are some analogies with the situation described for arachin (Tombs, 1963; Tombs, 1965; Tombs and Lowe, 1967). In this case, the protein was found to contain four

TABLE 10

Amino acid composition of the sub-units of legumin from
Vicia faba (α and β) and *Vicia sativa* (A, B and C)

Sub-unit	A	β	B	α	C
Mol. wt.	24,300	21,300*	37,600	37,000	32,600
Amino acid	mole %				
Aspartic acid	12.57	11.66	13.38	13.1	9.69
Threonine	4.39	4.27	2.56	3.09	3.25
Serine	6.45	6.89	5.86	6.52	7.83
Glutamic acid	9.51	10.26	19.24	22.27	24.27
Proline	5.12	4.68	4.97	5.5	6.15
Glycine	6.17	6.06	7.93	7.81	7.04
Alanine	9.92	9.83	5.52	3.81	3.65
$\frac{1}{2}$ -Cystine	0.91	-	1.05	-	1.04
Valine	8.73	9.62	3.55	3.44	3.54
Methionine	0.46	-	0.96	-	0.11
Isoleucine	3.98	3.56	5.06	4.52	4.90
Leucine	10.15	11.00	6.04	6.57	6.94
Tyrosine	3.06	3.16	3.05	2.41	1.79
Phenylalanine	3.52	3.31	3.82	2.96	3.00
Histidine	1.60	1.36	2.47	2.64	3.18
Lysine	4.16	5.97	3.76	4.94	4.18
Arginine	8.28	8.39	9.81	10.41	8.01
Tryptophan	1.01	-	0.99	-	1.43

* Average molecular weight

different sub-units, α and β with molecular weight of 35,000 each and γ and δ with molecular weight of 10,000 each. In nature, arachin exists in three variant forms, arachin A, B and A1, which differ in their sub-unit composition and also in their relative proportions in different seeds. The most probable sub-unit structures for these proteins were given as $\alpha_4\beta_4\gamma_2\delta_2$ (arachin A), $\beta_8\gamma_2\delta_2$ (arachin B) and $\alpha_8\gamma_2\delta_2$ (arachin A1).

The ratio of the α to β sub-units present in the legumin molecule was determined by two methods, one based on dye-binding and the other on amino acid compositions. The molar ratios obtained were 4:5 and 1:1 respectively. The latter is considered the more reliable estimate because firstly, it was determined by regression analysis, in which a multiple correlation of 0.996 was obtained, and secondly, there are less assumptions involved in its derivation. Using the dye-binding method, meaningful results can only be obtained if (a) the different polypeptide chains have similar colour yields, i.e. they bind the same amount of dye per unit mass, (b) the intensity of stain is a linear function of the amount of protein over the range of concentration used and (c) the staining procedure is reproducible. Conditions can be adjusted such that the effect of (b) and (c) may be minimised, but the dye-binding ability mentioned in (a) is an inherent property of a particular protein, being dependent on the amino acid composition of that protein (Racusen, 1973). Differences in the latter will then result in different colour yields.

The results from the electrophoretic investigation of the legumin sub-units illustrate the importance of using more than one gel system when studying a particular protein system. It is possible for proteins migrating with the same apparent molecular weight to differ considerably in charge and vice versa, and only from observations in different systems can any definite conclusions be made as to the homogeneity of the proteins concerned (Ressler, 1973). Thus, the three legumin basic sub-units gave only one band on urea gels, whilst the acidic sub-unit, apparently homogeneous on SDS gels, produced two components when electrophoresed on urea gels. As mentioned earlier, the apparent discrepancy in the number of basic sub-units obtained from the two SDS systems probably reflects the better resolution afforded by the discontinuous gel system.

The heterogeneity of the acidic sub-units was further increased when they were analysed by isoelectric focusing. In this case, approximately eight components were visible (Fig. 35). A similar degree of heterogeneity was observed when glycinin sub-units, dissociated in a urea-dithiothreitol medium, were subjected to isoelectric focusing (Catsimpoolas and Wang, 1971). Exactly how many of these components were artifacts is impossible to judge, but in glycinin it was suggested that the observed microheterogeneity was mainly due to differences in the primary structure of the sub-units. Apart from this explanation, there are three ways in which artifactual heterogeneity would most probably arise in this study. Firstly, the proteins may have undergone proteolysis to a slight extent during extraction, secondly, they may have been carbamylated by cyanate present in the urea, and thirdly,

partial deamidation may have occurred during manipulation of the proteins (McKerrow and Robinson, 1971). The last two factors were shown to be responsible for the heterogeneity observed when insulin preparations were examined by isoelectric focusing (Welinder, 1971). Numerous other possible sources of artifacts have been invoked to explain the heterogeneity, whether artifactual or inherent. Formation of irreversible complexes between proteins and carrier ampholytes has been proposed on several occasions (Hayes and Wellner, 1969; Frater, 1970; Foster and Kaplan, 1971; Weller et al., 1973), but the results from other experimental investigations would seem to suggest otherwise (Hayes and Wellner, 1969; Vesterberg, 1970). Reaction of proteins with the persulphate, employed as a gel polymerising catalyst, can also give rise to artifacts (Brewer, 1967), usually as a result of the oxidative conversion of sulphhydryl to disulphide groups (Mitchell, 1967; Yasui et al., 1968). Other explanations include variation in the carbohydrate content of glycoproteins (Welinder et al., 1972) and the existence of stable protein conformers (Kaplan, 1968). Far more detailed accounts of protein heterogeneity are given by Kaplan (1968), Colvin et al., (1954) and more recently Williamson et al., (1973), and it is to these that the reader is referred.

Assuming a linear pH gradient throughout the gel, the isoelectric points of the α sub-units fell in the range pH 4.7 to pH 5.2, which compares favourably with the pI range of 4.75 to 5.4 determined for the A sub-units of glycinin (Catsimpoilas, 1969). These values can only be judged approximate because of two important factors. Firstly, incorporation of urea into the gel has been shown to alter the isoelectric points of some proteins (Ui, 1971; Salaman and Williamson, 1971),

as well as shifting the pH of the ampholyte gradient upwards (Josephson et al., 1971), and secondly, non-linearity of the pH gradient and its instability with respect to time have been reported (Miles et al., 1972; Chrambach et al., 1973). Both these factors may lead to erroneous estimates of isoelectric points.

One particular problem encountered during these investigations was that of failure to obtain focused bands of the β sub-units. In fact, during the course of the experiment, the latter simply migrated en bloc to the cathodic end of the gel, where precipitation eventually occurred. This effect may be associated with the cathodic migration of carrier ampholytes described by Righetti and Drysdale (1971), or alternatively, with the production, in the gel, of the less than nominal pH ranges reported by Percival et al., (1970). In any case, as reported in Results, attempts at overcoming this problem were unsuccessful.

Clearly, it would have been time-consuming, if not, impracticable to attempt to separate the number of sub-units seen in isoelectric focusing experiments, for sequence analysis. Also, as the β_1 , β_2 and β_3 components of the β sub-units had not been isolated in sufficient quantities, a preliminary sequence investigation was undertaken using the complete β sub-unit fraction. The results from the first twenty-four residues analysed would seem to indicate that the degree of heterogeneity is far less than the electrophoretic results implied. There is no obvious explanation for the surprisingly low recovery (43%) obtained for the N-terminal amino acid. The possibility of a second amino acid is ruled out in view of the fact that only one

N-terminal amino acid, i.e. glycine, was found using the dansylation procedure. It also seems improbable that the low recovery was caused by chemical modification of the N-terminal amino acid, since the subsequent amino acid (leucine or iso-leucine) was recovered in 70% yield.

Another feature of the sequencer results that requires comment was the high proportion of carry-over. This first became apparent in cycle 7, where there was a 25% carry-over of leucine/iso-leucine and valine from cycle 6, and may possibly be attributable to incomplete degradation of proline in cycle 5. The latter phenomenon has been reported by Hermodson et al., (1972), who observed that the effect was aggravated at temperatures lower than 50°, but could be minimised by raising the temperature of the reaction chamber to above 50°.

To date, the only other investigation undertaken on the primary structure of a seed globulin, was that by Dlouha et al., (1964). They determined the sequence of sixteen peptides separated from a tryptic hydrolysate of the A chain of S-sulphoedestin. The amino acid content of these peptides accounted for 35% of the total amino acids present in the protein.

When the sub-unit structure of legumin, as determined in the present work, is compared with those already published, and also with that of glycinin, the legumin-like protein of soyabean, some interesting differences emerge. A ten sub-unit model was proposed by Bailey and Boulter (1970) for legumin, composed of sub-units of molecular weights 56,000, 42,000 and 23,000 in the ratio of 1:3:6. This compares with the twelve sub-unit model proposed in the present investigation. Although the sub-unit electrophoretic patterns of both of these preparations on

SDS-phosphate, pH 7.0 gels appear similar, the determined molecular weights of the respective sub-units differ by about 10%. No reason for this discrepancy is apparent, although it should be noted that Weber and Osborn (1969) estimated the accuracy of molecular weight determination by SDS gel electrophoresis as only being within 10%. Probably more confidence can be placed on the values in this study, as almost identical results were obtained from determinations made in two different gel systems. A twelve chain model has also been proposed for legumin extracted from seeds of V. sativa (Vaintraub and Nguyen Thanh Thien, 1971). However, in this case the sub-units have molecular weights of 24,300, 37,600 and 32,600 and are present in the legumin molecule in the ratio 3:2:1. Glycinin was found to contain two types of sub-unit, acidic and basic, with molecular weights of 37,200 and 22,300 respectively (Catsimpoolas et al., 1971), a situation very similar to that described for legumin in this study. Furthermore, the molar ratio of the sub-units can be calculated from their results as being approximately 1:1.

Osborne and Campbell (1898a,c,d and e) isolated legumin from seeds of pea (Pisum sativum), horse-bean (Vicia faba), vetch (Vicia sativa) and lentil (Ervum lens). They also found another globulin, vicilin, in three of these legumes, namely pea, horse-bean and lentil, but not in vetch. However, Danielsson (1949) in his ultracentrifuge studies on legume globulins found that, as in most of the other legumes he studied, the globulin fraction of V. sativa consisted of two components, analogous to the legumin and vicilin of peas. His results also showed that sometimes vicilin (7S globulin) and sometimes legumin

(11S globulin) predominates in legume seeds. For example, in the genera *Lupinus*, *Phaseolus* and *Trifolium*, the 7S globulin predominates. In *Vicia faba*, on the other hand, the vicilin fraction accounts for only about 20% of the total globulins in the mature seed. Although it is with the major component, legumin, that the greater part of this thesis is concerned, a limited investigation on the vicilin component was also undertaken. This mainly involved electrophoretic characterisation of vicilin fractions obtained by the various extraction procedures.

Classically vicilin is that globulin remaining soluble at pH 4.7 during isoelectric precipitation of legumin (Danielsson, 1949). In regard to zonal isoelectric precipitation, vicilin (fraction I) is thus that fraction eluted in the void volume of the column. Prepared by both procedures, vicilin was contaminated by legumin, as judged both by ultracentrifugation analysis and by the presence of legumin sub-units on SDS gels of vicilin. The contaminating legumin could most effectively be removed by a further isoelectric precipitation carried out at reduced ionic strength. When analysed on SDS gels, this purified vicilin yielded five major components with molecular weights of 55,500, 46,000, 43,400, 33,300 and 31,500. The fact that the same components were observed whether or not a reducing agent was employed, indicates that sulphhydryl groups, if present, play no role in the bonding between sub-units. The absence of disulphide bonds between the component polypeptide chains of the 7S globulin of soyabean has also been reported (Koshiyama, 1971). As in the case of legumin, the molecular weight of the vicilin sub-units deviated considerably from those determined by Bailey and Boulter (1972). Arithmetic considerations make it clear that the five sub-units observed on SDS gels cannot be

present in stoichiometric amounts in a vicilin molecule, having a molecular weight of only $150,000 \pm 25,000$. As indicated by Bailey and Boulter (1972), this discrepancy is unlikely to be due to simple heterozygosity, in view of the different molecular weights of the sub-units. A more probable explanation is that of polymorphism. In fact, experimental evidence for the heterogeneous nature of vicilin has been accumulating for some time. Thus, Vaintraub et al., (1962) found three components in vicilin of V. sativa, which differed in their isoelectric points, N-terminal amino acids and electrophoretic behaviour. These three components were designated the G-, L- and S-components in accordance with the initial letters of their N-terminal amino acids. The G-component, which had glutamic acid as N-terminal amino acid and constituted approximately 50% of the total vicilin fraction, was found to have pigment bound to it. These three components were later separated using chromatography on DEAE-cellulose (Vaintraub and Shutov, 1964). Using the same technique, Buzila (1967) and Ghetie and Buzila (1968) also separated vicilin of Pisum sativum into two immunochemically identical components, one of which was shown to be a cryoprotein. Using chromatography on hydroxylapatite, the vicilin-like protein of mungbean was fractionated into four components, three of which had sedimentation coefficients of 7.48S, 7.58S and 7.72S (Sayanova et al., 1973).

The diffuse band obtained by Bailey and Boulter (1972), McLeester et al., (1973) and also in the present work, when vicilin was electrophoresed on gels, is also not typical of a homogeneous protein. One possible explanation for this phenomenon is that vicilin may be a glycoprotein, as the latter often appear as diffuse bands on gels.

This is thought to result from microheterogeneity of the carbohydrate portion of the glycoprotein (Schmid, 1968). Recent reports on the carbohydrate content of some 7S seed globulins would seem to justify their classifications as glycoproteins. Thus, Roberts and Briggs (1965) found 5.89% carbohydrate and Koshiyama (1966) 4% Mannose and 1.2% hexosamine in the 7S protein of soyabean, whilst Ericson and Chrispeels (1973) have shown that, in Phaseolus aureus the major storage proteins, which they identified with vicilin and legumin, are glycoproteins. Vicilin contained 0.2% glucosamine and 1% mannose, and legumin contained about 0.1% glucosamine. It was suggested that the vicilin component isolated was synonymous with the "glycoprotein II" described by Pusztai and Watt (1970). Vicilin of V. faba has also been shown to contain small but significant quantities of neutral sugars (0.5%) and also 0.2% hexosamines (Bailey and Boulter, 1972).

An important factor to take into consideration when studying glycoproteins is the effect of the carbohydrate moiety on the physical and chemical properties of the protein. Thus, it has been shown that glycoproteins bind less SDS per gram of protein than standard proteins (Pitt-Rivers and Impiombato, 1968; Segrest et al., 1971), a property that can lead to drastically lower electrophoretic mobilities on SDS gels, and consequently, anomalously high molecular weight estimates (Schubert, 1970; O'Daly and Cebra, 1971; Reid et al., 1972). However, since this mainly refers to proteins containing more than 10% carbohydrate (Segrest et al., 1971; Bretscher, 1971), it is doubtful whether the above-mentioned seed glycoproteins contain sufficient

carbohydrate to significantly affect their electrophoretic mobilities. Obviously, if the latter were not the case, it might explain the discrepancy between the molecular weights of the sub-units and the molecular weight of vicilin, and thus enable all the observed components to be included in a single vicilin molecule. However, the results obtained from other experiments, described below, would suggest otherwise.

In an attempt to obtain a legumin-free vicilin preparation, using the technique of zonal isoelectric precipitation, the NaCl concentration of the elution buffers was reduced to 0.1M, in order to decrease the solubility of the legumin. The 4.7 soluble globulin fraction obtained was electrophoresed on SDS gels, where, apart from many minor bands, one major band, with molecular weight of 43,500, was apparent. Furthermore, a similar-sized component was obtained when the water-insoluble proteins (globulins) of the pH 4.7 extracted meal proteins were electrophoresed on SDS gels. Since both these components were dissociation products of globulins prepared under similar conditions, i.e. at pH 4.7 and low ionic strength, it is reasonable to assume that they are identical. Also, as the preparation of this globulin fraction indicates it to be vicilin-like in character (i.e. soluble at pH 4.7), it can be concluded that its 43,500 dissociation product is equivalent to the 43,500 molecular weight sub-unit observed on SDS gels of vicilin. This being so, then the vicilin fraction of V. faba, defined by solubility in salt solutions at pH 4.7, consists of at least two distinct proteins, one corresponding to the 43,500 molecular weight sub-unit, and the other composed of the remaining four sub-units.

Apart from basic electrophoretic studies and a preliminary attempt at sub-unit separation (Fig. 34), no detailed study of the compositions or structures of the vicilin fractions was undertaken, because the latter were not considered sufficiently homogeneous to warrant such an investigation. However, several workers have obtained what they consider to be homogeneous preparations of the 7S globulin and have then proceeded to a study of its sub-unit structure. Thus, Catsimpoilas et al., (1968) demonstrated the presence of nine major and five minor sub-units, when the 7S globulin of soyabean (γ -conglycinin) was dissociated and then electrophoresed in the solvent system, phenol-acetic acid-2-mercaptoethanol-urea. At least nine sub-units were also found by Koshiyama (1971), when this same globulin was dissociated in urea. From their investigation on the dissociation of the 7S proteins of vetch and soyabean, in urea and guanidine hydrochloride, Vaintraub and Shutov (1972) concluded that dissociation to a 2S sub-unit occurred via a 4S intermediate. Using SDS gel electrophoresis, Bailey and Boulter (1972) separated vicilin of V. faba into four components with apparent molecular weights of 66,000, 60,000, 56,000 and 36,000, and using the same technique, the vicilin-like protein of Phaseolus aureus was also separated into four components, but these had molecular weights of 63,500, 50,000, 29,500 and 24,000 (Ericson and Chrispeels, 1973). It is apparent from the diversity of the results that further detailed studies of the 7S globulin fraction are needed, not only to clarify the situation as regards homogeneity and sub-unit structure, but also, perhaps more importantly, to seek justification for the wide application of the term vicilin to the 7S seed globulins.

Seeds of Vicia faba used in development studies were obtained from material grown outdoors during the summer months and were thus subject to considerable variation in environmental conditions. As a consequence of this, differences will occur in the size of seeds of a particular age and also in the seed maturation period (Robertson et al., 1962); these factors must be taken into consideration when a seed selection procedure is employed. One of the more frequently used parameters of seed development, the weighted age, was chosen for the present study. Seed samples were collected at several stages of development, small under-developed seeds discarded, and a mean weight for each age determined, the age of the seed being measured from the day the flower was considered fully open, to the day of harvesting. The construction of a graph of cotyledon weight against seed age then enabled seeds of specified ages to be selected for further study. An alternative parameter of seed development has been employed by Millerd et al., (1971). They found a good correlation between the length of the long axis of the cotyledon and the fresh weight, particularly in the early stages of development, and concluded that this was a more reliable criterion than days from flowering.

The synthesis of the reserve proteins during seed development was studied by measuring the amount of legumin and vicilin in the total globulins extracted from seeds of different ages. Legumin and vicilin were fractionated by repeated isoelectric precipitation and then determined in solution using the assay of Lowry et al., (1951). The results (Table 3) show that legumin did not become the major storage protein of the broad bean until between 41 and 49 days after the onset

of development; prior to this vicilin was the predominant protein. Furthermore, no legumin was detected in 30-day-old seeds confirming the results of Danielsson (1952) and Graham and Gunning (1970) that vicilin was the first of the two proteins to be synthesised in the seed. Previous investigations on both pea (Danielsson, 1952; Klimenko and Pinegina, 1964; Konarev et al., 1966) and V. faba (Davis, 1966; Klimenko and Berezovikov, 1963) have indicated that intensive vicilin synthesis occurs in the early stages of development, but that as the seeds ripen legumin synthesis predominates. This situation was also apparent in V. faba, where by the time the seeds had reached full maturity the ratio of legumin to vicilin was between 3:1 and 4:1 by weight. The major proportion of the globulins were synthesised during the period 40 to 50 days. This is consistent with previous data which showed that the protein nitrogen fraction of the seeds undergoes a rapid increase after 33 days (Grzesiuk et al., 1962), principally as a result of the laying-down of albumins and storage globulins (Boulter, 1965). As the absolute amounts of both globulins increase during the course of development, I would concur with the view of other workers (Danielsson 1952; Klimenko and Berezovikov, 1963; Klimenko and Pinegina, 1964) that their biosynthesis proceed along independent pathways. The questions of why synthesis of the two globulins occurs at different rates during development and, perhaps more importantly, of why the composition of the globulin fraction varies so dramatically in seeds of different members of the Leguminosae (Danielsson, 1952) remain to be answered. In regard to the latter, Kudryashova and Lafitskaya (1964) have suggested that vicilin predominates over legumin in the phylogenetically young plants. However,

as indicated by Boulter and Derbyshire (1971) this view cannot be totally reconciled with the data of Danielsson (1949).

The sub-unit compositions of both globulins were investigated during the course of development using SDS gel electrophoresis. The electrophoretic patterns of legumin samples prepared from seeds of different ages were all more or less identical to that obtained from legumin of mature seed (Fig. 8a). In contrast the sub-unit composition of vicilin was found to change throughout development of the seed. At 41 days the component with $R_m = 0.25$ (corresponding to a mol. wt. of 46,000) was the major sub-unit and although it remained prominent as ripening progressed, the amount of this sub-unit as a percentage of the total vicilin protein decreased (Fig. 19). These results add further weight to the conclusion reached earlier concerning the heterogeneous nature of vicilin. Thus the sub-units observed when vicilin is electrophoresed on SDS gels derive from at least three distinct proteins which differ in extractability and also in rates of synthesis in the developing seed. The proposed three "vicilins" would have the following sub-unit compositions: (i) 43,400 (ii) 46,000 and (iii) 55,000, 33,300 and 31,500.

In conclusion, although considerable progress has been made in the field of seed protein chemistry since the era of Osborne, there still remain some unanswered questions and unsolved problems, particularly with regard to the exact nature of vicilin. Evidence as to the heterogeneity of the latter has been presented here, but a far more extensive investigation is required to fully characterise the proteins of which vicilin is constituted. In the case of legumin, separation of the individual elementary sub-units (ie. α_1 , α_2 , β_1 , β_2

and β_3) should mean the elucidation of the complete primary structure in the near future. Although only partial separation (i.e. α and β sub-units) of the sub-units was achieved in this investigation, the indications are that further fractionation will be afforded by either preparative gel electrophoresis or by the relatively new technique of preparative isotachophoresis (Svendsen, 1973).

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ABBREVIATIONS

With the exception of those listed below, the abbreviations and conventions used throughout this thesis are those recommended by the Biochemical Journal (Biochem. J., 126, 1 (1972)).

N,N'-methylenebisacrylamide	Bisacrylamide or Bis
N,N,N',N'-tetramethylethylenediamine	Temed
Sodium dodecyl sulphate	SDS
Trimethylsilyl	TMS
Phenylthiohydantoin	PTH

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