Characterisation of the major storage proteins of Pisum Sativum L.

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CHARACTERISATION OF THE MAJOR STORAGE PROTEINS OF *PISUM SATIVUM* L.

BY

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A THESIS SUBMITTED IN ACCORDANCE WITH THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE UNIVERSITY OF DURHAM

BOTANY DEPARTMENT NOVEMBER 1981

To my father

ABSTRACT

Several types of purification techniques have been investigated with a view to isolating the storage proteins of *Pisum sativum*: vicilin and legumin. A third storage protein, distinct from vicilin and legumin, has been purified from seed extracts using a combination of ammonium sulphate fractionation, gel filtration and hydroxylapatite chromatography. Hydroxylapatite chromatography has also been used to show that a protein corresponding to convicilin may be isolated from *Vicia faba*. Convicilin has been characterised and its properties compared to those of the other storage proteins. It has been shown by SDS-polyacrylamide gel electrophoresis that convicilin has a subunit mol. wt. of 71,000 and gel filtration studies indicate the mol. wt. of its native form to be 290,000. Convicilin is antigenically dissimilar to legumin, but gives a reaction of identity with vicilin when tested against antibodies raised against both proteins. Convicilin has been so named because of this latter property. Convicilin contains no vicilin subunits and is clearly separated from vicilin by non-dissociating techniques. Unlike vicilin, convicilin does not interact with concanavalin A, and contains no significant amounts of carbohydrate. Convicilin also differs from vicilin in its amino acid composition, one of the most important differences being that convicilin contains small amounts of sulphur amino acids, while vicilin contains none. Limited heterogeneity in convicilin has also been demonstrated

by isoelectric focussing, N-terminal analysis and CNBr cleavage.

Changes that occurred in the subunit composition of the storage proteins during pea seed development have been investigated. Two forms of vicilin 50,000 mol. wt. and 47,000 mol. wt. subunits have been detected at early stages. The disappearance of the 47,000 mol. wt. vicilin subunit and appearance of lower mol. wt. vicilin subunits at later stages of development has also been demonstrated.

The subunit composition, size and charge of the storage proteins of pea, during germination have been investigated. The storage proteins appear to undergo an initial modification, possibly nicking, and there is depletion of most protein subunits with increasing germination time.

ACKNOWLEDGEMENTS

I would like to thank Professor Donald Boulter, my supervisor and head of department, for his valuable advice and guidance during the course of this work and also for allowing me to use the departmental facilities for this research.

I am grateful to Dr. Ron Croy and Dr. John Gatehouse for their helpful discussions and sustained interest in this work, as well as for allowing me to include some of their unpublished results.

I would also like to acknowledge, with gratitude, the assistance of Mr. C. Alcock in performing the carbohydrate analysis and of Dr. A. Pusztai in performing confirmatory amino acid analyses.

I am thankful to Margaret Burn for typing this thesis and to Dr. Angharad Gatehouse for her helpful suggestions for the presentation of this thesis.

A Science Research Council grant is thankfully acknowledged and finally I want to thank my husband for being so patient.

ABBREVIATIONS

The following abbreviations have been used in this thesis:

Bisacrylamide	=	N, N' - Methylenebisacrylamide
TEMED	=	N, N, N', N' - Tetramethylethylenediamine
SDS	=	Sodium dodecyl sulphate
TRIS	=	Tris (hydroxymethyl) aminomethane
TCA	=	Trichloroacetic acid

CONTENTS

Section I INTRODUCTION

The importance of legumes in agriculture arises from their use as a high protein seed crop, for human and animal consumption. Another fact to be taken into consideration is the essential role legumes play in crop rotation and improving soil fertility by fixing atmospheric nitrogen. Legume seeds in general contain a high proportion of protein, usually 20-30% of the dry weight and up to 50% of the dry weight in the case of some varieties of soyabean (Glycine max). Legumes play a significant role in supplying dietary protein in many developing countries, and when used for fodder, the whole plant is sometimes utilised. By comparison, the major source of dietary protein in developed countries is of animal origin. This pattern, involving a process of inefficient conversion of plant protein into meat, will have to alter, as the world population increases, to one in which there is an increased consumption of plant proteins by humans. However, as it is unlikely that established dietary habits in the developed countries will change immediately, there will also be a need to improve the quality of plant proteins which are fed to monogastric animals. The main driving force leading to the change in dietary habits will be cost, since it is generally accepted that animal protein is a far more expensive commodity than plant protein, as the animal is a poor 'converter' of food. Many different values for conversion rates are available. Mellanby (1975) maintains that taking into account the wastage in the carcase and the high percentage of water in the meat, twelve pounds of feed are required to produce a one

pound gain in broiler house birds, on the same basis, conversion ratios for cattle and pigs are 30:1 and 20:1 respectively. However, even when all these considerations are taken into account, meat still has enormous appeal to the consumer world wide and, unless there are religious or other taboos, people will eat meat if they can afford to buy it.

The world food situation was regarded as satisfactory in the post war period until 1972, when a series of crop failures led to soaring prices as food production declined. This had the effect of greatly stimulating research into agricultural production and initiating the trend to make better use of the available food supplies. An example of this is the present-day exploitation of oil seed protein in the developed countries, in particular the use of textured soya-protein as meat replacements, at a considerably reduced cost to the consumer. It has been pointed out (Derbyshire et al., 1976) that proteins of other legumes have similarities to those of soyabean, and this raises the question as to whether other legumes could be used as protein sources for vegetable based meat substitutes for human consumption. This however would depend on improving the protein yield and quality of these other legumes, which are either too low or too unreliable for continuous exploitation by the seed meal market.

As cereals provide about 70% of the world's protein (Jalil and Tahir, 1973) it would at first appear that the best strategy might be to improve the yield and/or protein content of cereals. However, Sinclair and de Wit (1975) have shown that 1 unit of glucose gives rise to 0.83 units of carbohydrate and only 0.4 units of protein, as more energy is required to

synthesize protein than polysaccharide. Therefore for the same quantity of photosynthate transported to the seed, there is, in most cases, an inevitable inverse relationship between seed protein content and seed weight. Thus in cereals, where the harvest index is high, increased seed protein content is usually accompanied by decreased yield, unless high protein lines also generate an increased partitioning of photosynthate into their seeds. Generally it is easier, in the case of cereals, to increase protein yield by increasing overall yield per hectare. An alternative strategy is to improve the protein yield from legumes, by increasing their overall yield, since these have a higher protein content that cereals to start with.

World production of legumes is about one-tenth that of cereals (Burr, 1975) and in 1973 the P.A.G. of the United Nations recommended urgent research attention be given to eight major food legumes (Phaseolus vulgaris, Cajanus indicus, Vigna unguiculata, Cicer arietinum, Vicia faba, Pisum sativum, Glycine max, and Arachis hypogaea) which account for over 90% of total world legume production. As in the case of cereals, increase in protein yield in legumes can be brought about by increasing the overall yield and/or increasing the actual protein content. In legumes the harvest index is such as to suggest that the point has not been reached where increasing protein content would necessarily decrease yield, lines of Phaseolus vulgaris (Leleji et al., 1972) and Vicia faba (Bond, 1970) have been obtained that support this theory as they exhibit higher protein content without total yield decrease. Soyabeans have the highest protein content of all the food

legumes, ranging on average from 37-50% of the dry weight. Most attempts to improve the protein content of soyabeans have resulted in an unwanted drop in oil content, but lines have been found (Panton, 1975) which show a 10% increase in protein content with no apparent loss of oil content. Unfortunately a breeding programme for soyabeans in this country is not feasible until lines that give high yields in the cool British growing season are developed. An alternative to altering the relative synthesis of carbohydrate and protein in the seed in order to obtain a higher protein yield, might be increasing the input of nitrogen and photosynthate to the seed. It has been suggested that a form of self-destruction may take place in legumes (Sinclair and De Wit, 1976) as the energy requirement for protein synthesis in the seeds is high and requires so much photosynthate, that leaves and root nodules senesce during the pod-fill stage (Hardy and Havelka, 1975) as they try to meet the nitrogen requirements of the developing seeds. This has the effect of further restricting nitrogen uptake as nitrogen fixation is dependant on a supply of photosynthate from the leaves. The end result is a serious limitation in seed yield. A possible solution to this problem might be breeding for protein lines with a lower level of nitrogen rich amino acids i.e. amides and basic amino acids which occur in large amounts in some legume storage proteins (Boulter and Derbyshire, 1971). However the extent to which it is possible to alter the amino acid composition of storage proteins and still retain seed viability is determined by various possible constraints (Derbyshire et al., 1976). Not only is yield limited by

intrinsic characters under genetic control which determine the developmental, physiological characteristics of the plant, such as photosynthetic efficiency, nutrient utilisation and partitioning of resources between vegetative and reproductive structures but also environmental parameters such as soil composition, climate, light quality and intensity as well as by managerial practises such as pest-control, fertilisation, irrigation and planting density. Variation in these conditions can lead to samples from the same variety showing considerable variation in protein content. However with adequate replications in various seasons, conditions and locations, these difficulties due to gene-environment interactions may be overcome by the plant breeder.

While it is necessary to distinguish between yields in terms of protein per hectare and actual protein content of the seed, protein quality is also of extreme importance in human diet or animal feed. Often a smaller content of high quality protein may be a more desirable breeding objective than a larger amount of less nutritious protein. There are two main aspects to the consideration of protein quality from a nutritional point of view. Firstly in comparison with the W.H.O. reference amino acid pattern (W.H.O. 1973) the first limiting amino acids of legumes are the sulphur amino acids (F.A.O. 1970) either cysteine or methionine and sometimes both. It is usually necessary to consider both of these amino acids together, as evidence from rat feeding trials with cowpeas suggests cysteine can spare methionine as the latter can give rise to the former but not vice versa (Boulter *et al.*, 1973). Therefore any breeding programme concerned with protein

quality will involve screening for the sulphur amino acids. A potential screen is a total seed sulphur assay for some legumes (Evans and Boulter, 1974; Boulter *et al.*, 1973; Porter *et al.*, 1974) but for many legumes it is first necessary to remove the S-methyl-L-cysteine, found in some legume seeds (Evans and Bandemer, 1967), although this may not be necessary for legumes where relatively little of this compound occurs e.g. *Pisum sativum*, *Cajanus cajan*, *Vicia faba* minor, *Vicia faba* major, *Glycine max*, *Lupinus albus* and *Sphenostylis stenocarpa* (Evans and Boulter, 1975). However the case is further complicated by these latter examples containing other soluble non-amino acid sulphur compounds which may interfere with assays. The nutritional significance of these latter compounds and S-methyl-L-cysteine in some legume meals is not completely understood, though it has been suggested that they may function as antimetabolites for cystine or methionine (Evans and Bandemer,1967). On the other hand, S-methyl-L-cysteine was found to have no harmful effect on rats and at high concentrations of methionine it was found to inhibit the metabolic pathway of methionine by which a toxic product is apparently formed (Benevenga, 1974).

A negative correlation between protein sulphur amino acid content and percentage protein in legume seeds has been found (Evans and Boulter, 1974) and this may be explained by the fact that relatively low sulphur containing proteins will constitute a higher proportion of the protein of higher protein content seeds. The response of legumes to increased sulphur fertilisation appears to be favourable. It has been shown that sulphur fertilisation increases sulphur amino acid content

of some lupin varieties up to a certain level and this increase is associated with a change in the proportions of actual storage proteins (Blagrove, Gillespie and Randall, 1976). Changes in the sulphur amino acid content of seed meals of some cowpea varieties have also been induced by increased levels of sulphur fertilisation (Evans et al., 1977). Similar results for Phaseolus (Arora and Luthra, 1971) and soyabean (Sharma and Bradford, 1973) have been observed. Thus there is a strong gene-environmental interaction, although whether the effect is directly on gene expression at the transcription or translation levels, or whether it operates by a changed physiology is not yet clear.

The second aspect of protein quality that must be taken into account is the presence of antimetabolic compounds and digestability. Most legumes contain a range of these substances (Liener, 1969), although they have not been the subject of investigation in this thesis.

Many legumes contain protease inhibitors although their biological role in the seed is uncertain, however it has been suggested (Ryan, 1973) that they may be involved in pest and disease protection and possibly metabolism regulation. Of the protease inhibitors most work has been carried out on trypsin inhibitors and one of the first to be isolated came from raw soyabeans (Kunitz, 1945). Ingestion of the trypsin inhibitor in the form of raw beans, has a growth inhibitory effect accompanied by pancreatic hypertrophy in experimental animals (Rackis, 1974). It is thought that pancreatic hypertrophy leads to an excessive loss of endogenous protein secreted by the pancreas (Booth et al., 1960). Since this

protein consists largely of pancreatic enzymes, it is quite rich in cystine, the resulting effect is a net loss of sulphur-containing amino acids from the body. This would explain why the need for methionine, which is inherently limiting in soyabean protein is even more acute in diets containing raw soyabeans. There is also evidence that trypsin or chymotrypsin in the intestine suppresses pancreatic enzyme secretion by feedback inhibition and that trypsin inhibitors evoke increased enzyme secretion by counteracting the suppression produced by trypsin (Green and Lyman, 1972; Niess et al., 1972). Investigations into the adverse effect of trypsin inhibitors have mainly been carried out on animals and it is therefore important to note that a major part of human trypsin fails to be inhibited by soyabean trypsin inhibitor (Travis and Roberts, 1969; Mallory and Travis, 1973; Figarella, Negri and Guy, 1975). Therefore it is essential to establish the correct processing procedure depending on whether soyabean products are for human or animal consumption. Trypsin inhibitors have also been found in a large number of other legumes including Arachis hypogeae, Vicia faba, Pisum sativum, etc., in fact all legumes appear to contain trypsin inhibitors to varying degrees (Liener and Kakade, 1969).

Another substance which appears to be present in most legumes is a protein that has the property of being able to agglutinate red blood cells. The phytohaemagglutinins or lectins (Lis and Sharon, 1973; Liener, 1974) are a significant factor contributing to the poor nutritive value of many beans which enjoy popular consumption in many lesser developed countries. From his work on rats fed Phaseolus vulgaris

haemagglutinin, Jaffe (1969) postulated that the haemagglutinin combined with cells lining the intestinal wall and caused a non-specific interference with the absorbtion of nutrients leading to growth inhibition. Furthermore, Pusztai *et al.*, (1979) using gnotobiotic rats, has recently shown that lectin toxicity in *Phaseolus vulgaris* is also due to the leakage of bacterial toxins through the damaged intestinal wall.

Fortunately most protease inhibitors and phytohaemagglutinins are heat labile and under the correct cooking conditions become non-inhibitory. Although most legumes are eaten cooked, it should be borne in mind that in developing countries the amount of fuel available for cooking is often limited and furthermore, since animals are fed raw seeds, large amounts of these anti-nutritional factors may influence animal nutrient utilisation.

Legumes have also been shown to contain many other antimetabolites such as the goitrogens found in soyabeans and peanuts (Van Etten, 1969) and glycosides from which HCN may be released by hydrolysis and cause cyanide poisoning if improperly cooked. Lima beans in particular contain high levels of glycosides (Montgomery, 1969). In addition, antivitamin factors, metal binding constituents, polyphenolics and saponins have all been found in some legumes. These latter examples are of particular importance when considering protein quality as they are not always heat labile and therefore removed by cooking. Nevertheless, taking into account all these toxic constituents, legumes still provide a valuable source of protein.

Generally, digestability of raw legumes is improved by cooking and this is probably due to inactivation of protease inhibitors and lectins as well as making the essential amino acids of these proteins more available. It has been shown, for example, that trypsin inhibitors have high levels of sulphur amino acids, especially cysteine (Pusztai, 1966), while protein complexing substances such as polyphenolics are important in reducing digestability (Bressani, 1975). In breeding for protein quality, the possible implications of changes must be taken into account, for example the reduction of an antimetabolite might increase digestability but would this also affect the resistance of the seed during growth and storage, to pests and diseases.

Having outlined the major nutritionally important legume proteins, attention is now turned to the storage proteins, the proteins investigated in detail in this investigation. Legume seeds contain several thousand different proteins (Boulter and Derbyshire, 1971), which can be arbitrarily divided into (i) metabolic proteins such as enzymes and structural proteins and (ii) storage proteins. The metabolic proteins occur in too small amounts to be of great significance nutritionally, however the enzymes (albumins) have a higher percentage of essential amino acids than do the legume storage proteins (globulins), (Boulter and Derbyshire, 1971). They also occur in greater amounts in the embryo axis than in the cotyledons and screening lines for those with a higher proportion of embryo axis to cotyledons might be feasible (Boulter, 1977).

Storage proteins are proteins laid down in the seed in large amounts to be used at a later stage in the life cycle.

Manipulation directed at changing the content of essential amino acids of the storage proteins offers the most promise however, for legume quality improvement. This follows from the fact that the storage proteins are synthesised in large amounts in seeds and any change in their composition will have a large effect on the quality of the seeds. Several different storage proteins occur and individually they differ in their nutritional status (Derbyshire *et al.*, 1976).

Seed development begins by fertilisation of the ovule, then the embryonic axis and cotyledons develop by active cell division, followed by cell elongation. Storage compounds such as carbohydrates, fats and proteins are deposited only after cell division has ceased. The storage proteins are synthesised in the cotyledon cells on the rough endoplasmic reticulum (Bailey *et al.*, 1979; Bollini and Chrispeels, 1979) which is assembled at the appropriate time in large amounts during seed development (Millerd, 1975). They are then transported and deposited in membrane bound protein bodies.

The precise number of different storage proteins is still not known and the relative proportions of different storage proteins vary considerably in different legumes, since they have very different amino acid compositions and hence nutritional status, a knowledge of their occurrence and characterisation is fundamental to any plant breeding work aimed at changing the protein content or quality.

The storage proteins of legumes were first separated by Osborne and Campbell (1898) into two fractions; vicilin and legumin. Osborne demonstrated that similar protein fractions could be extracted from many different legume seeds

(Osborne, 1894; Osborne and Campbell, 1898). Later, Danielsson (1949), examined the globulin fraction of 34 species of legumes from 8 tribes of the leguminosae, using the technique of ultracentrifugation and found that they virtually all contained two globulin fractions with sedimentation coefficients of 7S (vicilin) and 11S (legumin). Added evidence for the widespread existence of these two protein types in the leguminosae comes from immunoelechophoretic experiments carried out by Kloz (1971) and Dudmand and Millerd (1975). As differences exist in the amino acid composition of these two proteins, for example, legumin of Vicia faba and Pisum sativum contains larger amounts of sulphur amino acids than vicilin, (Derbyshire et al., 1976) it might be possible, since these two proteins are formed at different times and are under separate genetic control (Wright and Boulter, 1972; Millerd, 1975), to breed lines with changed proportions of storage protein and so obtain proteins with better amino acid profiles. Such improvements in protein quality and ultimately yield may be brought about more readily if detailed chemical and physiochemical knowledge of the storage proteins exists. With this knowledge it is possible to define the extent to which the functional requirements of seed storage proteins such as: high nitrogen content, location in protein bodies, ability to withstand dessication, ability to be rapidly hydrolyzed by the proteolytic enzymes of the germinating seed, can be varied and changed for nutritional improvement, before seed viability is impaired.

Characterisation of these proteins is also essential in

order to understand the details of their biosynthesis and the controlling mechanisms involved. To date the legumin fraction is well characterised. The hexameric model for legumin, still in use today, was proposed by Wright and Boulter (1974) using Vicia faba. They postulated that legumin consists of two main types of subunit, (i) acidic subunits of molecular weight about 40,000 and (ii) basic subunits of molecular weight about 20,000. One type of subunit is joined to the other type by disulphide bonds, to give an intermediate subunit pair of about 60,000 molecular weight. Six subunit pairs are thought to comprise the legumin molecule. A similar structure has been proposed for Pisum sativum legumin (Croy et al., 1979). It has been shown for pea legumin, that the higher molecular weight subunits are acidic and the lower molecular weight subunits are basic, by isoelectric focussing (Krishna et al., 1979; Casey, 1979b) and electrophoresis in urea (Thomson et al., 1978; Croy et al., 1979). Furthermore, these results have been confirmed by electrophoresis on urea-polyacrylamide gels (Thomson et al., 1978) and on cellulose acetate membranes (Croy et al., 1979). Both the acidic and basic subunits have been demonstrated to be heterogenous with respect to molecular weight and isoelectric point (Casey, 1979b; Krishna et al., 1979) and recently legumin of Pisum sativum has been partially separated into different forms (Matta, 1981). These results have given rise to a modified model for legumin, in which each hexameric molecule is formed by the association of individual subunit pairs of one or more types (Matta, 1981). Therefore legumin is probably not a single protein (with a

defined amino acid sequence) but a series of closely related proteins.

Molecular weights for legumin from Pisum sativum have been obtained by various workers using a variety of techniques and taking into account subunit heterogeneity, they fit the hexameric model proposed by Wright and Boulter (1974). Estimates vary from 330,000 (Danielsson, 1949) to 410,000 (Brand and Johnson, 1958).

The carbohydrate content of legumin has also been investigated and was the subject of much controversy. Basha and Beevers (1976) showed that acid hydrolysates of legumin, isolated by isoelectric precipitation (Danielsson, 1949), from developing pea seeds contained amino sugars and neutral sugars. Also using this preparation method, Browder and Beevers (1978) were able to demonstrate the occurrence of glucosaminyl-asparagine in glycopeptides isolated from legumin. More recently Davey and Dudman (1979) have also shown that pea legumin isolated from protein bodies by isolectric precipitation was also glycosylated and the amounts of glucosamine and neutral sugars that they were able to detect in their preparations were in good agreement with the results previously reported by Basha and Beevers (1976). However, other workers using a combination of techniques for the preparation of legumin from pea seeds (Casey, 1979a; Gatehouse et al., 1980) were not able to detect carbohydrate and concluded that legumin was not a glycoprotein. These conflicting sets of results have been explained by Hurkman and Beevers (1981), who demonstrated that previous reports of legumin glycosylation were due to the presence of some low

molecular weight glycopeptides, which co-purified with legumin isolated by the method of Danielsson (1949). Thus it is now generally accepted that legumin of Pisum sativum is not a glycoprotein.

In contrast to legumin, vicilin usually occurs in smaller amounts in the seed and has been less extensively characterised. The vicilin fraction is thought to contain more than one protein (Derbyshire et al., 1976; Millerd, 1975) and evidence for the heterogenous nature of this protein fraction comes from ion exchange chromatography (Vaintraub and Shutov, 1964), carbohydrate affinity chromatography (Davey and Dudman, 1979), ultracentrifugal analysis (Joubert, 1955) and fractionation by solubility under varying conditions of pH and ionic strength (Ghetie and Buzila, 1968; Thomson et al., 1978). Seven major polypeptides of approximately 70,000, 50,000, 30,000, 24,000, 18,000, 14,000 and 12,000 molecular weight have been identified in the pea vicilin fraction (Thomson et al., 1978). Such an array of constituent polypeptides also provides additional evidence for the vicilin fraction to be composed of more than one protein. At the time of this study no precise models for the subunit composition of vicilin proteins were in existence, thus confirming the need for further work in this area.

The purpose of this study was to further characterise the storage proteins, especially vicilin. Most of this work has been carried out on the pea or Pisum sativum, a species in the genera Pisum, one of five genera in the tribe Vicieae of the family Leguminosae (Heywood, 1971). The pea itself is

an important legume crop, being cultivated in virtually every temperate region, including high altitude locations in tropical countries such as Nigeria and Kenya. It has many advantages for biochemical work, including uniform growth leading to synchronised developmental physiology, as well as lesser amounts of interfering substances such as polyphenolics.

Initial studies involved isolation and purification using ammonium sulphate fractionation and a variety of chromatographic techniques. This was followed by characterisation of separated proteins using several types of electrophoresis and chromatography, as well as carrying out N-terminal analysis, amino acid analysis, sugar analysis and immunological experiments. By using a combination of these techniques, the variation in the structure and number of the storage proteins, which takes place during the process of seed development and seed germination was also investigated.

Section II MATERIALS AND METHODS

MATERIALS

A. Biological materials

Seeds of *Pisum sativum* L. (var. Feltham first), *Vicia faba* L. (var. Maris bead) and *Phaseolus vulgaris* L. (var. Processor) were obtained from Suttons Seeds Ltd., Torquay, Devon TQ2 7QJ.

B. Chemicals and reagents

Chemicals and reagents, apart from those listed below, were obtained from BDH Ltd., Poole, Dorset BH12 4NN, U.K. and were of analytical grade or the best available.

Beckman Instruments Inc., Fullerton, California
Cellulose Acetate Membranes

BioRad Laboratories Ltd., Watford, Herts., U.K.
Hydroxylapatite (Bio-Gel, HT Grade)

ICI Plastics Division, Welwyn Garden City, Herts., U.K.
Alkathene polyethylene granules

Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.
Trichloroacetic Acid

LKB Instruments Ltd., South Croydon, Surrey, U.K.
Ultrogel AcA 22

Medical International, 49 Queen Victoria Street, London
Visking dialysis tubing

Pharmacia Fine Chemicals, Uppsala, Sweden
Dextran Blue 2000
Pharmalyte Carrier ampholytes pH 3.5-10
Sephadex G-200
Sephadex G-50

Sigma London Chemical Company Ltd.
Bovine serum albumin
Catalase
Carboxypeptidase A

Concanavalin A (from Jack bean)
Cytochrome C
Ferritin
Immunoglobulin G
Methyl-α-D-mannoside
Myoglobin
Ovalbumin
Phosphorylase b
Soyabean trypsin inhibitor
Thyroglobulin
Transferrin
Trizma Base (Tris)

Glycoprotein II, Pisum sativum lectin and albumin, Vicia faba vicilin and legumin preparations were kindly supplied by Dr. R.R.D. Croy (University of Durham).

Buffers used for column chromatography were routinely made 0.05% with sodium azide.

METHODS

I. GROWTH OF BIOLOGICAL MATERIALS

A. Germinating seeds

Seeds of Pisum sativum (var. Feltham First) were grown as described by Evans et al., (1979) for 72 h. The addition of water to the dry seeds was taken as zero time germination. Seeds germinated in this way were then grown under controlled environmental conditions, as follows:- lighting: 16 h total (including morning and evening phases), temperature: day 28°C, night 23°C, humidity: 75-80% rel. hum. The seedlings were supported by a 4 cm layer of alkathene polyethylene granules in bowls containing distilled water and harvested at intervals of 3, 5, 7 and 10 days.

B. Developing seeds

Seeds were germinated as described above. Seedlings were transferred to water culture bottles of nutrient solution after 4-5 days in the spray room, to be grown under the controlled environmental conditions described above. Harvesting of the pods was carried out at intervals of 11, 13, 17, 21 and 33 days after flowering. The cotyledons were separated aseptically from the testas, radicles and hypocotyls immediately prior to extraction.

C. Determination of water uptake by germinating seeds

One hundred seeds of average dry weight were germinated in a spray room as described above, then blotted dry to remove surface moisture prior to weighing at intervals of 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 25 h, 48 h and 72 h. See Fig.1.

II. PREPARATION OF MEALS

A. Pisum sativum

Seeds were freeze-dried for 30min. to remove surface moisture, then ground to a fine flour using a Janke and Kunkel water-cooled mill. The flour was passed through a 365 µm mesh endecott sieve. Defatting was carried out by two hexane extractions (10 ml hexane/g meal), for 30 min. at 0°C. After filtering off the hexane, the meal was air-dried under vacuum.

B. Vicia faba

As for Pisum sativum except that removal of the testa

was facilitated by milling for 2-5 seconds in a ball mill, prior to grinding seed fragments in the Janke and Kunkel water-cooled mill.

C. Phaseolus vulgaris

As for Pisum sativum

III. PREPARATION OF EXTRACTS

A. Pisum sativum

(i) From meals

Defatted meal from air-dried seeds was extracted with 0.1 M potassium phosphate buffer pH 8.0, containing 0.4 M NaCl (10 ml buffer/g meal) at 4°C for 1 h, then centrifuged at 23,000 g for 20 min. and the precipitate discarded. These extracts were used for Sephadex chromatography, ammonium sulphate fractionation, gel electrophoresis or to prepare the globulin fraction.

Extracts for Ultrogel chromatography were prepared as described above with 0.1 M Tris-HCl buffer, pH 8.0 containing 0.2 M NaCl (12.5 ml buffer/g meal).

For non-dissociating polyacrylamide gel electrophoresis, meal was extracted in 0.125 M Tris made pH 6.8 with concentrated HCl (20 ml buffer/g meal).

(ii) From developing seeds

15 g of developing seeds were homogenised with 50 m M potassium phosphate buffer, pH 8.0 (40 ml), using a polytron macerator at fast speed, with 5-10 sec. bursts. The extract

was centrifuged at 10,000 g for 10 min. to remove large debris which was re-extracted twice. The pooled supernatents were then clarified by centrifugation at 38,000 g for 1 h, followed by filtration through GF/C, GF/F glass fibre filters and finally 0.22 µm pore size cellulose acetate membrane filters. These extracts were used for hyroxylapatite chromatography. When seeds 33 days or older were extracted, the following procedure was used:- defatted meal was extracted with 50 m M potassium phosphate buffer, pH 8.0 (10 ml buffer/g meal for mature seeds and 4 ml buffer/g meal for seeds extracted 33 days after flowering) at 4°C for 1 h, centrifuged at 23,000 g for 20 min. and the precipitate discarded. Filtration and hydroxylapatite chromatography of the extract was then carried out as described above.

(iii) From germinating seeds

The extraction conditions used were similar to those for developing seeds except the ratio of material to extractant was varied to take into account the increasing water content of the germinating seeds at different days (See Fig.1). For non-dissociating polyacrylamide gel electrophoresis, germinating seeds were extracted (10-5 ml buffer/g meal) with 0.125 M Tris made pH 6.8 with concentrated HCl.

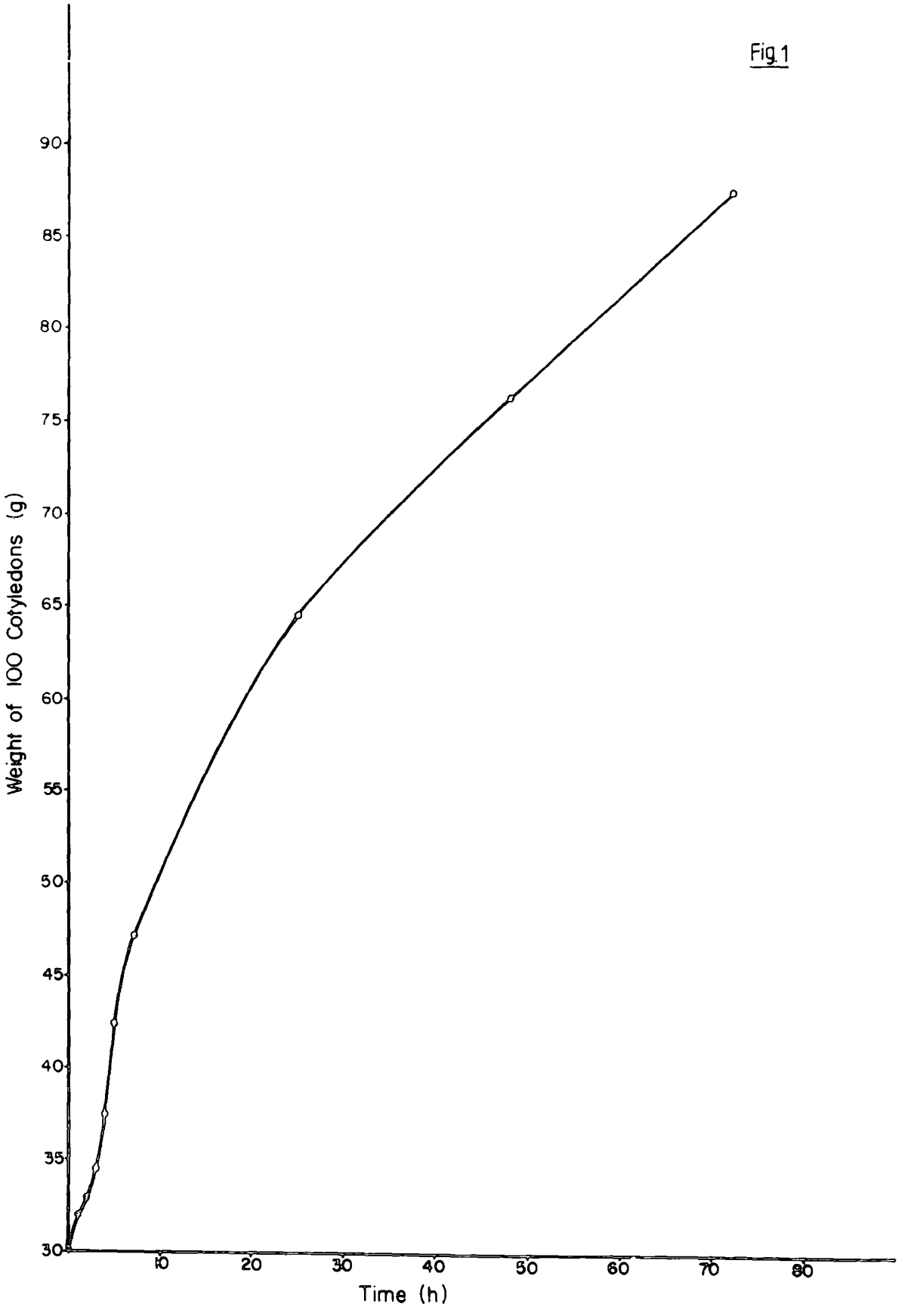
B. Vicia faba

Defatted meal was extracted with 0.05 M potassium phosphate buffer, pH 7.8, with 0.4 M NaCl (10 ml buffer/ g meal) at 4°C for 1 h, then centrifuged at 38,000 g for 30 min. The supernatent was clarified by filtration through GF/C, GF/F glass fibre filters and finally 0.22 µm pore size cellulose

FIG. 1

The determination of water uptake by germinating seeds.

Fig.1


Weight of 100 Cotyledons (g)
90
85
80
75
70
65
60
55
50
45
40
35
30
10
20
30
40
50
60
70
80
Time (h)

acetate membrane filters.

C. Phaseolus vulgaris

10 g of defatted meal from air-dried seeds was extracted with 200 ml 0.05 M sodium borate buffer, pH 8.0 at 4°C for 1 h, then centrifuged at 15,000 g for 30 min. The supernatent was re-centrifuged at 38,000 g for 1 h and the resulting precipitate discarded.

IV. SEPARATION OF THE GLOBULIN FRACTION

A. Pisum sativum

Total extract (III.A (i)) was extensively dialysed against 33 m M sodium acetate buffer, pH 4.7. The precipitated globulin proteins were separated from albumin proteins still in solution by centrifugation at 38,000 g. The precipitate was redissolved by suspending the pellet in water, then titration to pH 8.0 - 9.0 with 0.1 M NaOH. This solution was buffered at pH 8.0 with 1.0 M sodium borate buffer, pH 8.0, to give a final concentration of 0.05 M borate, then dialysed against 0.05 M sodium borate buffer, pH 8.0, prior to freeze-drying.

B. Vicia faba

Supernatant proteins in the total extract prepared as described by Scholz et al., (1974) were precipitated with ammonium sulphate at 90% ralative saturation and separated by centrifugation at 23,000 g for 20 min. The precipitated

protein was redissolved in sodium phosphate buffer, pH 7.0 and dialysed against 25 m M sodium acetate buffer pH 4.7. The precipitated globulin proteins were separated by centrifugation at 23,000 g then redissolved by the procedure described above in IV.A. The globulin fraction was dialysed against McIlvaine (citrate-phosphate) buffer, pH 7.0, for zonal isoelectric precipitation. (See Flow diagram, Fig.2).

C. Phaseolus vulgaris

A total extract (III.C) was dialysed against 33 mM sodium acetate buffer, pH 5.0. The precipitated globulin proteins were separated by centrifugation at 38,000 g for 1 h, then redissolved by the procedure described in IV.A.

V. AMMONIUM SULPHATE PRECIPITATION

A. Pisum sativum

Total extract (III.A (i)) was fractionated by ammonium sulphate precipitation at 40, 50, 60, 70, 80, 90 and 100% relative saturation. The precipitates were collected by centrifugation at 23,000 g, then redissolved in 20 ml 0.1 M sodium borate buffer, pH 8.0. The supernatent at 100% relative saturation with ammonium sulphate and the redissolved precipitates were extensively dialysed against 0.05 M sodium borate buffer, pH 8.0, then freeze-dried prior to analysis by SDS-polyacrylamide gel electrophoresis.

B. Phaseolus vulgaris

Globulin protein solution was fractionated by ammonium

FIG. 2

Flow diagram for the preparation of the globulin fraction of *Vicia faba*. Abbreviation used: rel. sat., relative saturation.

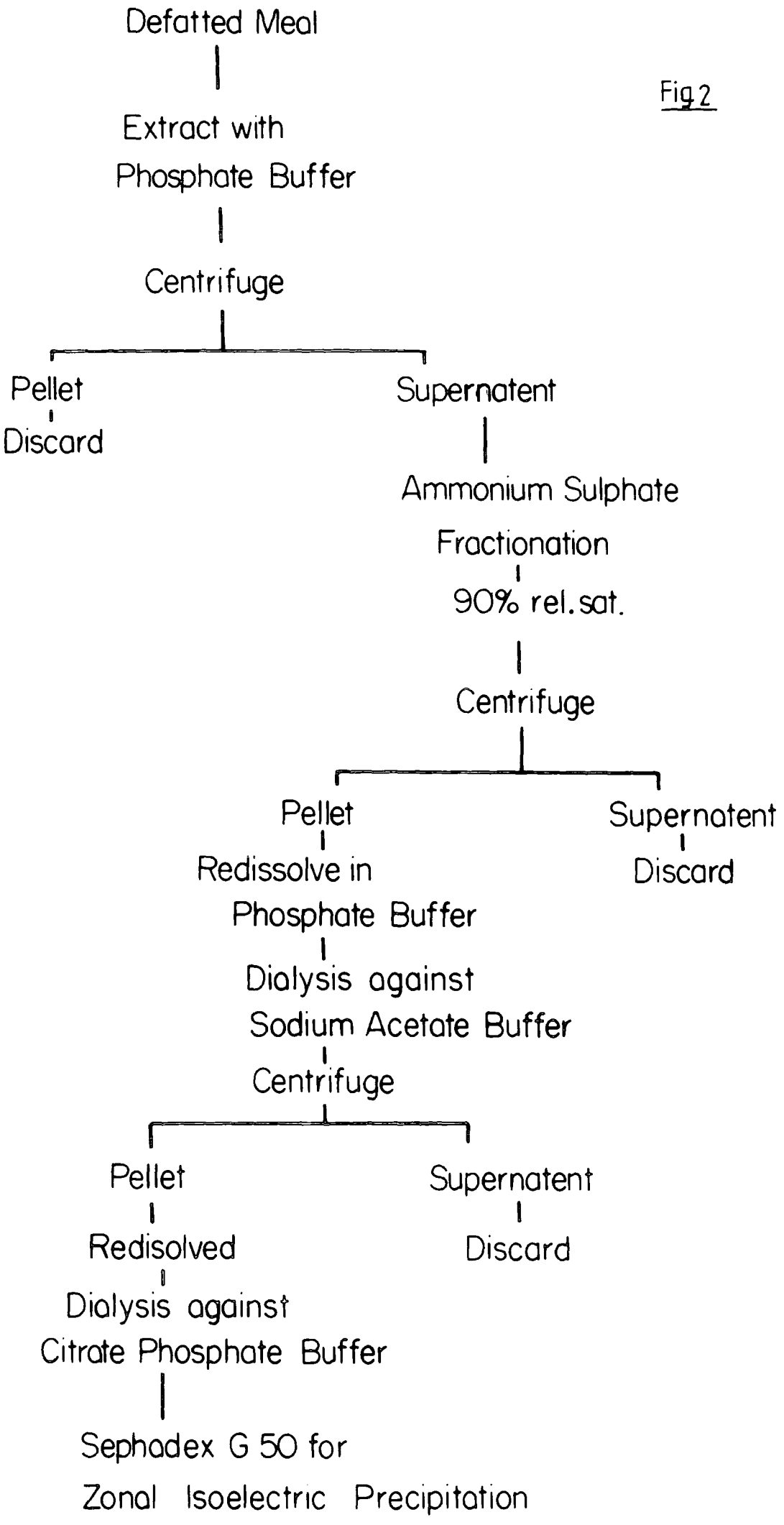

Defatted Meal
Extract with
Phosphate Buffer
Centrifuge
Pellet
Discard
Supernatent
Ammonium Sulphate
Fractionation
90% rel. sat.
Centrifuge
Pellet
Redissolve in
Phosphate Buffer
Dialysis against
Sodium Acetate Buffer
Centrifuge
Supernatent
Discard
Pellet
Redisolved
Dialysis against
Citrate Phosphate Buffer
Supernatent
Discard
Sephadex G 50 for
Zonal Isoelectric Precipitation

Fig 2

sulphate precipitation at 50, 70, 90 and 100% relative saturation. The resulting precipitates and supernatent were processed as described above in V.A.

VI. SEPARATION OF PROTEINS OF THE VICILIN FRACTION

A. Convicilin (See Fig.3)

(i) Ammonium sulphate fractionation

A total protein extract was prepared from 40 g of defatted pea meal. This was fractionated by ammonium sulphate precipitation at 4°C. The material precipitating in the range 50-80% relative saturation was collected by centrifugation at 23,000 g for 20 min. and redissolved in 20 ml 50 m M sodium borate buffer, pH 8.0.

(ii) Sephadex G-200 chromatography

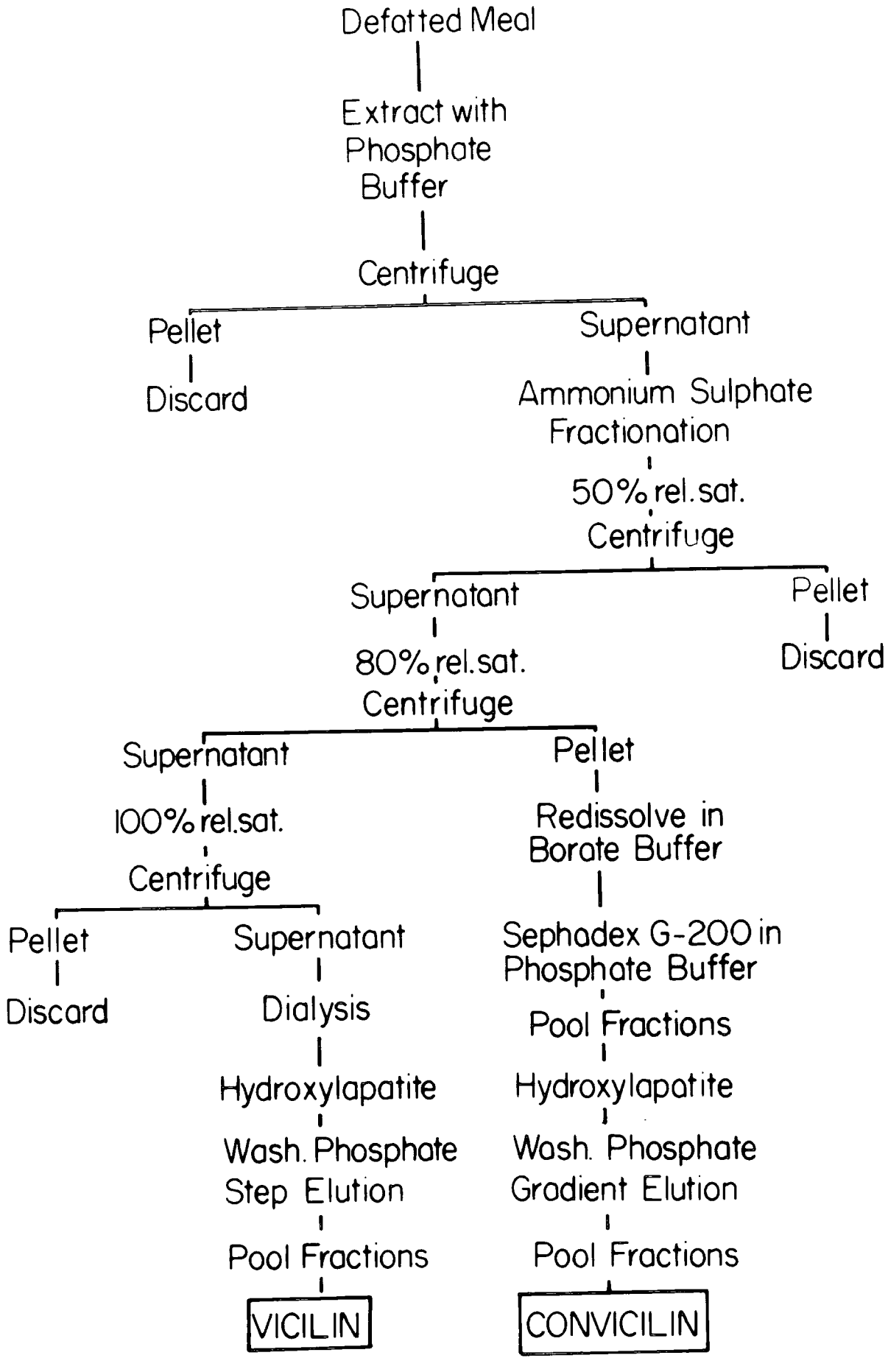
The prepared protein solution, normally either a total extract or the material precipitating at 50-80% relative saturation with ammonium sulphate, was applied to a column of 5.5 cm. diam. with 950 ml Sephadex G-200 equilibriated with 0.1 M potassium phosphate buffer, pH 8.0. Elution was effected by the same buffer at a flow rate of 50 ml/h and 10 ml fractions were collected. Column eluant was continuously monitored at 280 nm using an LKB 8300 Uvicord II detector and control unit, with an LKB chart recorder. Fractions were collected using an LKB ultrorac 7000 fraction collector.

Samples from fractions (0.5 ml) were made 12.5% with respect to TCA at 0°C and the precipitate was centrifuged, washed with ethanol twice then dried by evaporation. The precipitated protein was dissolved in sample buffer containing

FIG. 3

Flow diagram for the preparation of convicilin and vicilin of *Pisum sativum*. Abbreviation used: rel. sat., relative saturation.

Fig.3


Defatted Meal
Extract with Phosphate Buffer
Centrifuge
Pellet
Discard
Supernatant
Ammonium Sulphate Fractionation
50% rel.sat.
Centrifuge
Supernatant
Pellet
Discard
80% rel.sat.
Centrifuge
Supernatant
Pellet
100% rel.sat.
Centrifuge
Redissolve in Borate Buffer
Pellet
Discard
Supernatant
Dialysis
Sephadex G-200 in Phosphate Buffer
Pool Fractions
Hydroxylapatite
Hydroxylapatite
Wash. Phosphate Step Elution
Wash. Phosphate Gradient Elution
Pool Fractions
Pool Fractions
VICILIN
CONVICILIN

2-mercaptoethanol and analysed by SDS-polyacrylamide gel electrophoresis.

(iii) Hydroxylapatite chromatography

Thos fractions containing the highest concentration of protein with a subunit of 71,000 mol. wt. were pooled and applied to a column (3.5 cm. diam., 140 ml vol.) of hydroxylapatite equilibriated with 0.1 M potassium phosphate buffer, pH 8.0. The hydroxylapatite column was extensively washed with the same buffer, then eluted with a linear concentration gradient of potassium phosphate buffer, pH 8.0 (0.1 M - 0.75 M, 300 ml + 300 ml). The flow rate was 40 ml/h and 10 ml fractions were collected. The salt concentration of fractions was determined by refractive index measurements using a Bellingham and Stanley Ltd. (No.424132) refractometer, (see calibration graph, Fig.4). Fractions were analysed as described in VI.A (ii) for 71,000 mol. wt. protein subunits, these were pooled and either concentrated by ultrafiltration or dialysed against 50 m M sodium borate buffer, pH 8.0, or distilled water and then freeze-dried.

B. Vicilin

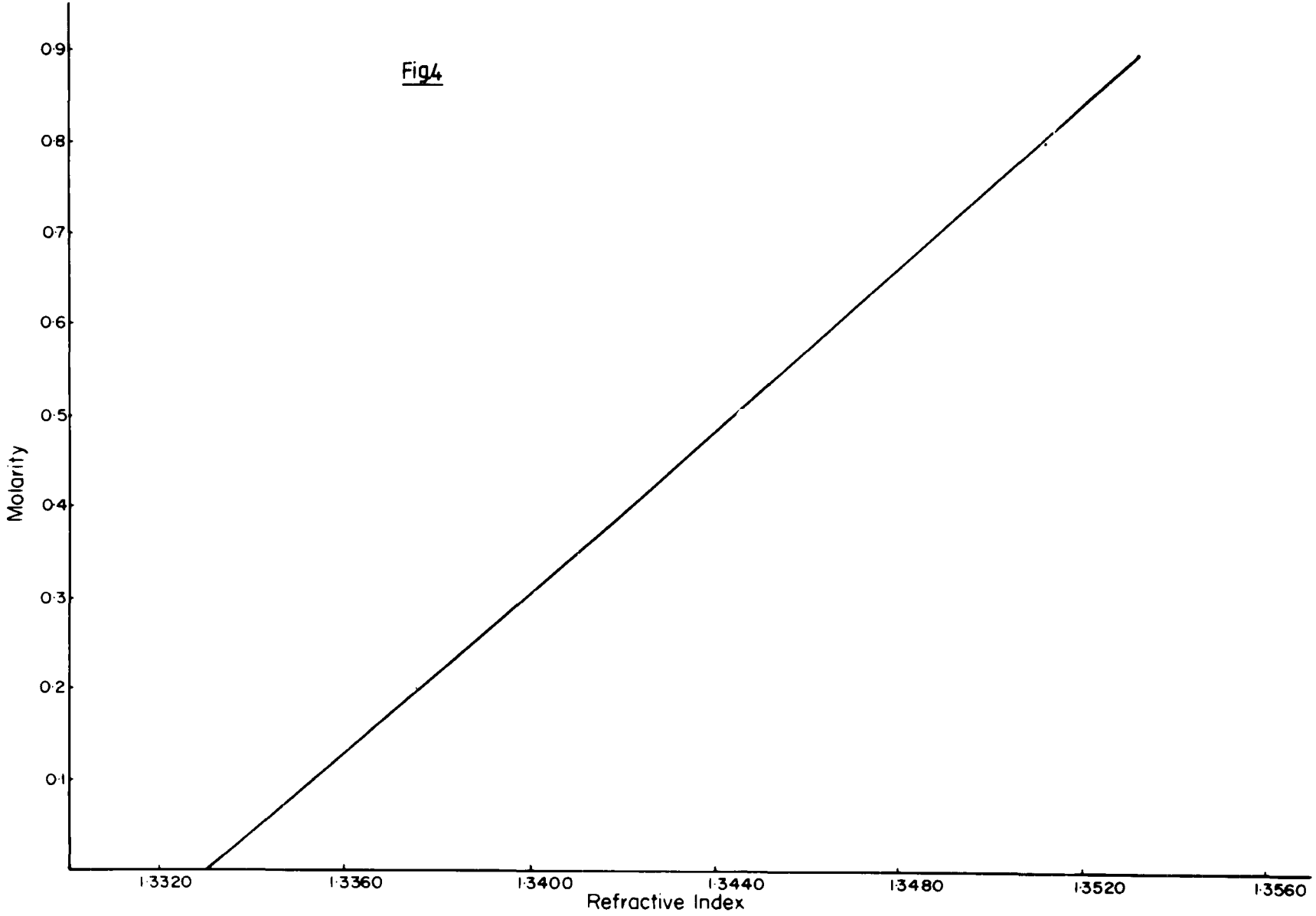
(i) Ammonium sulphate fractionation

A total protein extract was prepared from 40 g of defatted pea meal as described in III.A (i). Ammonium sulphate was added to give 100% relative saturation. The soluble material was collected by centrifugation at 23,000 g for 20 min. This was extensively dialysed against 0.1 M potassium phosphate buffer, pH 8.0 for complete removal of salt.

FIG. 4

Calibration curve relating values of refractive index with the concentration of potassium phosphate buffer.

Fig4


0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Molarity
1·3320
1·3360
1·3400
1·3440
1·3480
1·3520
1·3560
Refractive Index

(ii) Hydroxylapatite chromatography

The solution prepared in VI.B (i) was applied to a column of hydroxylapatite as in VI.A (iii), after washing, the column was eluted with 0.75 M potassium phosphate buffer, pH 8.0. Fractions containing vicilin were pooled and processed as for convicilin.

VII. HYDROXYLAPATITE CHROMATOGRAPHY

Twenty-five ml of extract was applied to a column of hydroxylapatite (dimensions as for VI.A (iii)) equilibriated with 50 m M potassium phosphate buffer, pH 8.0. After washing, the column was eluted with a linear concentration gradient of potassium phosphate buffer, pH 8.0 (50 m M - 1 M, 300 ml + 300 ml) at a flow rate of 40 ml/h, 10 ml fractions were collected.

VIII. ULTROGEL CHROMATOGRAPHY

A. Convicilin

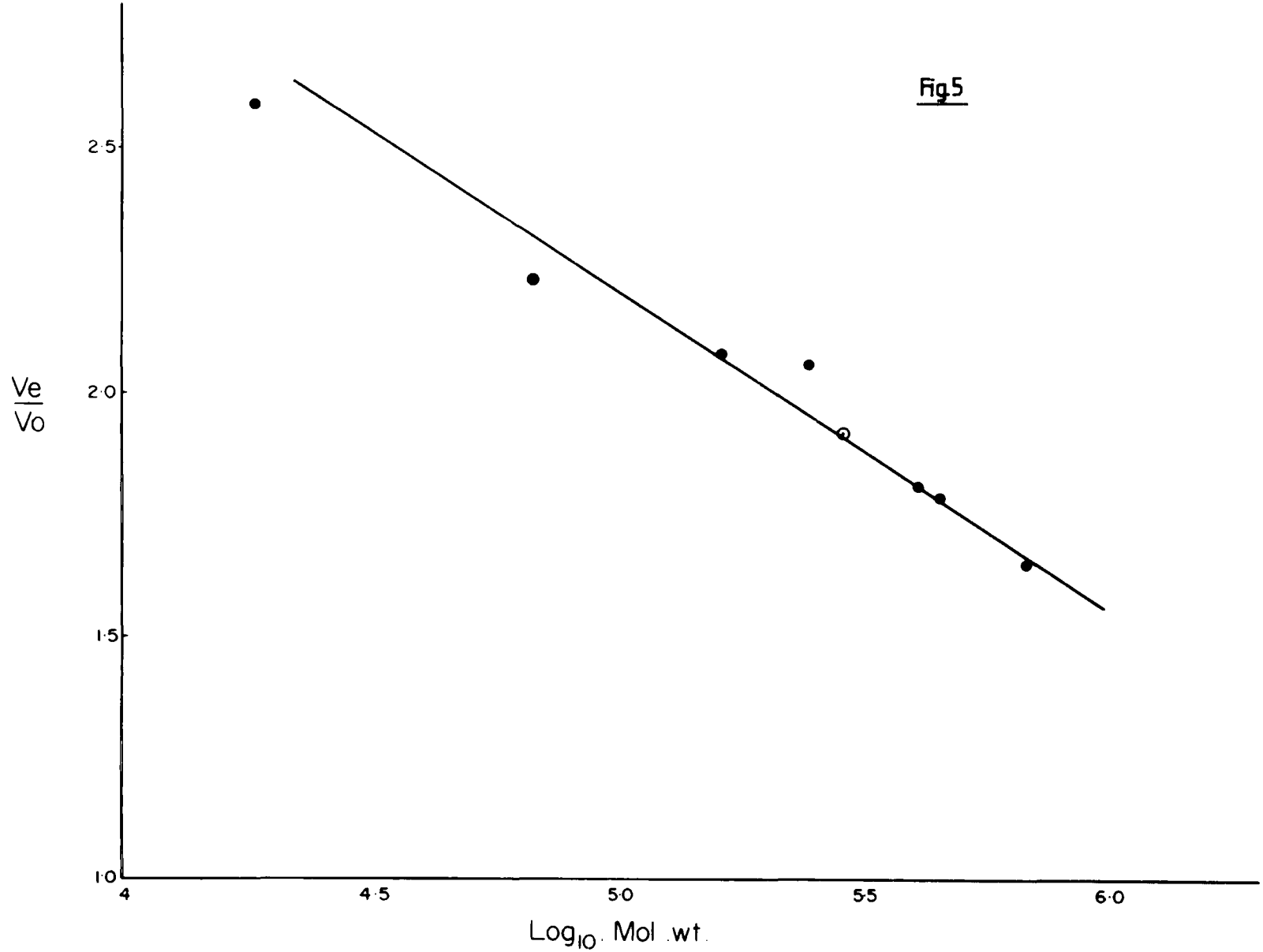
A plot of Ve/Vo against log mol. wt. (See Fig.5) was obtained by chromatographing the following standard proteins: thyroglobulin (669,000 mol. wt.), ferritin (440,000), Pisum legumin (400,000), catalase (240,000), immunoglobulin G (160,000), bovine serum albumin (68,000), and myoglobin (18,000), on a column of Ultrogel AcA 22 (2.2 cm. diam., 320 ml vol.). The column was equilibriated by upward flow using 0.1 M Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl. It was then loaded with sample and eluted with the same buffer,

FIG. 5

Calibration curve relating Ve/Vo values of standard proteins, chromatographed on Ultrogel AcA 22, with their molecular weights. Protein standards in order of decreasing size, are: thyroglobulin, ferritin, Pisum legumin, catalase, immunoglobulin G, bovine serum albumin and myoglobin.

○ = convicilin

Fig.5



with a flow rate of 8.0 ml/h, 5 ml fractions were collected and the elution volumes of standards and sample calculated.

B. Mature and germinating pea extracts

A plot of Ve/Vo against log mol. wt. (See Fig.6) was obtained by chromatographing the following standard proteins: ferritin (440,000 mol. wt.), catalase (240,000), immunoglobulin G (160,000), bovine serum albumin (68,000), ovalbumin (43,000), myoglobin (18,000) and cytochrome C (12,700). Two ml of total extract was applied to the Ultrogel AcA 22 column (dimensions and buffer as for convicilin, VIII.A). The flow rate was 6 ml/h and 3 ml fractions were collected. The fractions were made 12.5% with respect to TCA and the precipitated protein was analysed by SDS-polyacrylamide gel electrophoresis. The molecular weights of the various proteins in the total extracts were determined by comparing elution volumes with those of the standard proteins in the Ve/Vo against log mol. wt. plot (Fig.6).

IX. ZONAL ISOELECTRIC PRECIPITATION

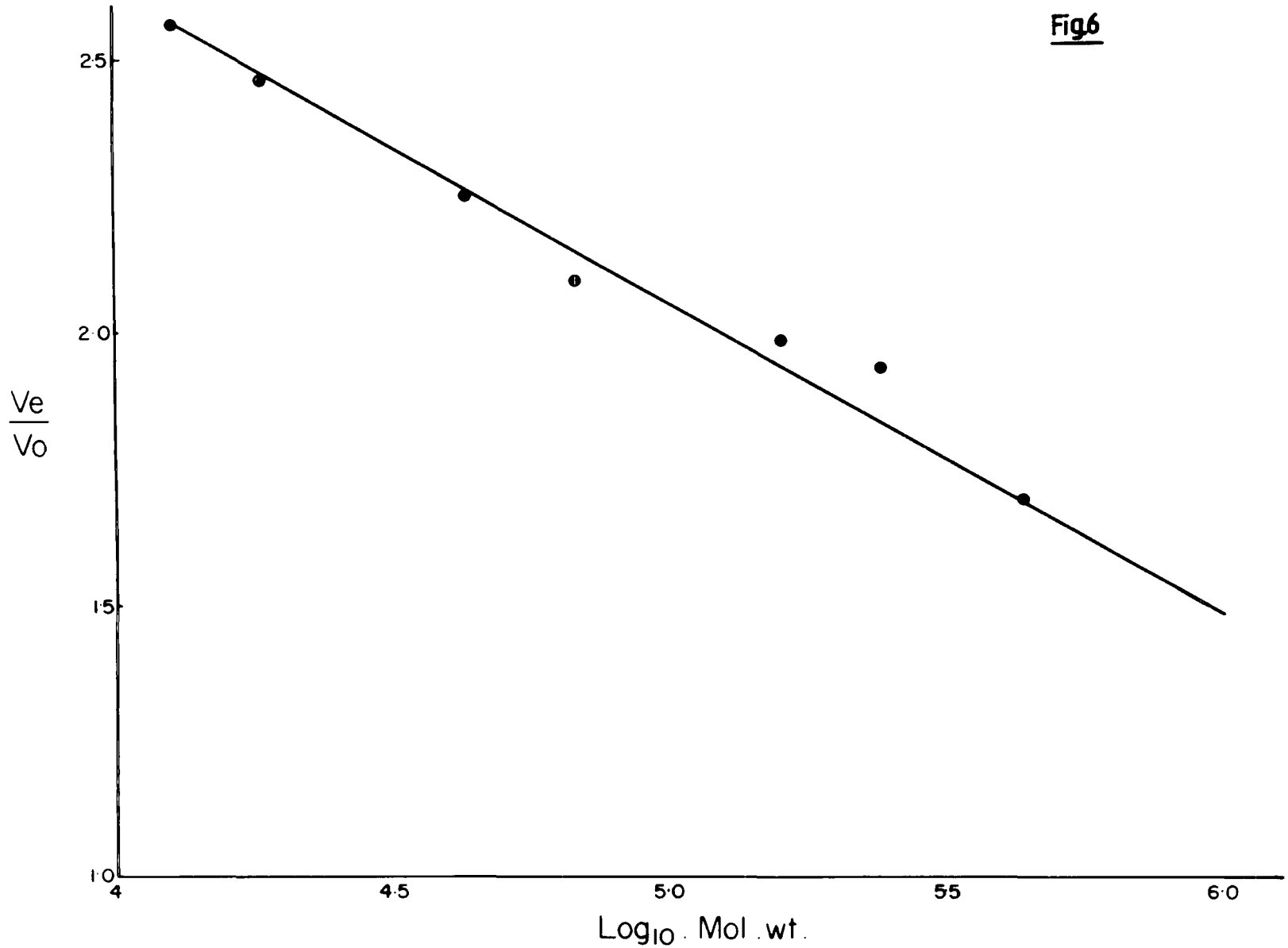
A. Pisum sativum extract

Total extract (5 ml) was applied to a Sephadex G-50 column (3.5 cm diam., 250 ml vol.) equilibriated with 0.25 M sodium acetate buffer, pH 4.7. After washing with 10-20 ml of the same buffer, the column was eluted with 0.5 M sodium borate buffer, pH 8.0, at a flow rate of 56 ml/h, 14 ml fractions were collected. Those containing protein were

FIG. 6

Calibration curve relating Ve/Vo values of standard proteins, chromatographed on Ultrogel AcA 22, with their molecular weights. Protein standards in order of decreasing size, are: ferritin, catalase, immunoglobulin G, bovine serum albumin, ovalbumin, myoglobin and cytochrome C.

Fig6



pooled according to the elution profile, dialysed against distilled water and freeze-dried, then analysed by SDS-polyacrylamide gel electrophoresis.

B. Vicia faba globulin fraction

The globulin fraction (25 ml) was applied to a Sephadex G-50 column (dimensions as for IX.A) equilibriated with McIlvaine (citrate-phosphate) buffer pH 4.8. The column was eluted with McIlvaine buffer, pH 7.0, at a flow rate of 36 ml/h and 9 ml fractions were collected. Fractions containing protein were pooled, dialysed against 0.02 M sodium borate buffer, pH 8.0, then freeze-dried.

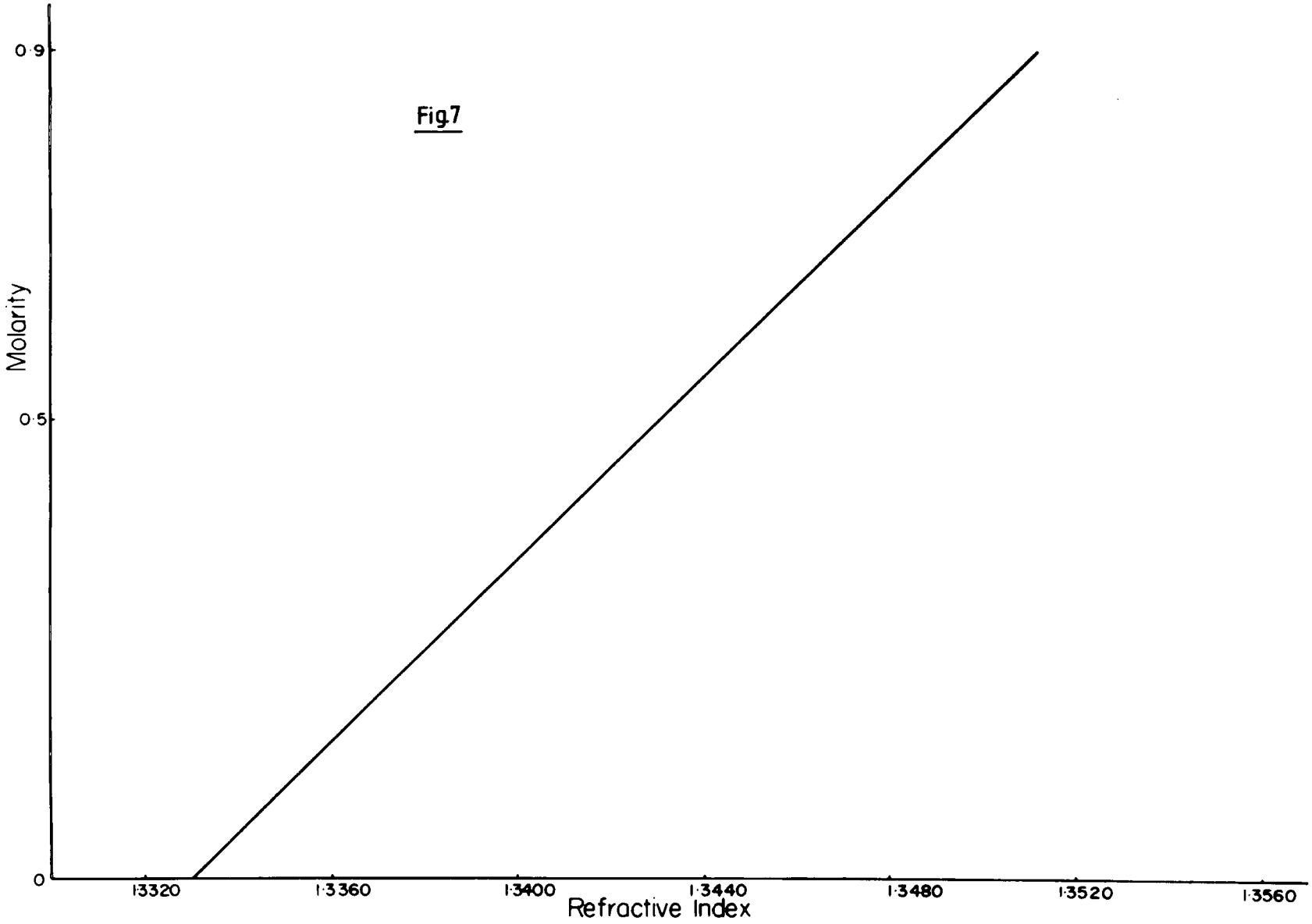
X. ION EXCHANGE CHROMATOGRAPHY

A solution of 450 mg freeze-dried globulin protein in 0.1 M Tris-HCl buffer, pH 7.7, was applied to a column of DEAE cellulose (3 cm diam., 250 ml vol.) equilibriated with 0.1 M Tris-HCl buffer, pH 7.7 and washed in with 150 ml of the same buffer. Elution was effected with a linear concentration gradient of Tris-HCl buffer pH 7.7 (0.1 M Tris-HCl-0.1 M Tris-HCl containing 0.75 M NaCl, 400 ml + 400 ml) at a flow rate of 60 ml/h with 15 ml fractions being collected. The salt concentration of fractions was determined by refractive index measurements as described above (VI.A (iii), see calibration graph, Fig.7.

FIG. 7

Calibration curve relating values of refractive index with the concentration of NaCl in 0.1M Tris-HCl buffer.

Fig.7


0·9
0·5
0
Molarity
1·3320
1·3360
1·3400
1·3440
1·3480
1·3520
1·3560
Refractive Index

XI. GEL ELECTROPHORESIS

Gel concentrations refer to total monomer (i.e. acrylamide and bis acrylamide).

A. 17% and 10% SDS slab polyacrylamide gel electrophoresis

Adapted from Laemmli (1970).

(i) Reagents

(a) 1 M Tris pH 8.8 (adjusted with concentrated HCl).

(b) 1 M Tris pH 6.8 (adjusted with concentrated HCl).

(c) Main gel acrylamide stock solution

12 g acrylamide
54 mg bisacrylamide
) 40 ml H_2O

(d) Stacking gel acrylamide stock solution:

0.6 g acrylamide
8.6 mg bisacrylamide
) 2 ml H_2O

(e) SDS 10%

(f) Ammonium persulphate (10 mg/ml)

(g) TEMED

(ii) Gel preparation

Glass plate assembly and procedure as described by Payne (1976). Main gels of 17% acrylamide concentration were prepared by mixing 22.5 ml (a) and 34.5 ml (c), then degassing this solution. To this 0.6 ml of (e), 1.5 ml (f) and 20 µl (g) were added. This gave sufficient gel mixture for one slab gel. The 10% main gels were prepared as described above after 19.5 ml (c) had been made up to 34.5 ml with distilled

water. Stacking gels (3% acrylamide) were prepared by adding 2.5 ml of (b) and 14.8 ml of distilled water to (d) then degassing. Following this 0.2 ml of (e), 10 µl of (g) and 0.5 ml of (f) were added.

(iii) Sample preparation

Protein samples were dissolved at a concentration of about 2 mg/ml (unless otherwise stated) in sample buffer made up as follows:

Sample buffer formulation - stock solution

10 ml glycerol

6.25 ml 1 M Tris pH 6.8 (adjusted with concentrated HCl)

12.05 ml distilled water

2 g SDS

Working solution

2.83 ml stock solution
0.5 ml 2-mercaptoethanol
} made up to 10 ml with distilled water

Samples were incubated at 100°C for 2 min. prior to gel application.

(iv) Electrophoresis

Electrophoresis buffer consisted of glycine 141.1 g, Tris 30 g and SDS 10 g made up to 1l (pH 8.3) and was diluted 1:10 before use. Samples (10 µl unless otherwise stated) were loaded on to gels and marker dye (0.1% bromophenol blue) added to the upper reservoir. Electrophoresis was carried out using a Raven slab gel apparatus with a Shandon Vokam SAE 2761 power supply, at 25 mA/gel (constant current), for approximately 6 h per gel. The gels were then stained (Reid and Bielski, 1968) as required with 0.025% Coomassie

Blue in 50% methanol, 7% acetic acid and destained using 50% methanol, 7% acetic acid solution.

B. Subunit molecular weight estimation

The method of Weber and Osborn (1969) was used to determine the apparent molecular weights of proteins dissociated with SDS. Electrophoretic mobilities were calculated relative to the mobility of the bromophenol blue marker band in 17% and 10% polyacrylamide slab gels. The following proteins were used as molecular weight standards: phosphorylase b (100,000 mol. wt.), transferrin (76,600), bovine serum albumin (68,000), catalase (60,000), ovalbumin (43,000), carboxypeptidase A (34,300), soyabean trypsin inhibitor (21,000) and cytochrome C (12,700). The Rm values were then plotted against the log of the molecular weight (See Fig.8 and Fig.9).

C. Non SDS-polyacrylamide slab gel electrophoresis

Non dissociating polyacrylamide gel electrophoresis was carried out as described above for gel electrophoresis in XI.A. SDS was omitted from gel preparations and replaced by the same volume of distilled water. In the case of running buffer, SDS was not added. Sample buffer was prepared without SDS and in dilution of the stock solution, 2-mercaptoethanol was replaced by the same volume of distilled water. Main gels with a polyacrylamide concentration of 8.5% were obtained by diluting 17.25 ml of (c) to 34.5 ml with distilled water.

FIG. 8

Calibration curve relating the molecular weight of standard proteins with their electrophoretic mobilities relative to bromophenol blue, on 17% SDS-polyacrylamide gels. Standard proteins in order of decreasing size, are: phosphorylase b, transferrin, bovine serum albumin, catalase, ovalbumin, carboxypeptidase A, cytochrome C (dimer), trypsin inhibitor and cytochrome C.

◁ = convicilin and vicilin subunits

Fig 8

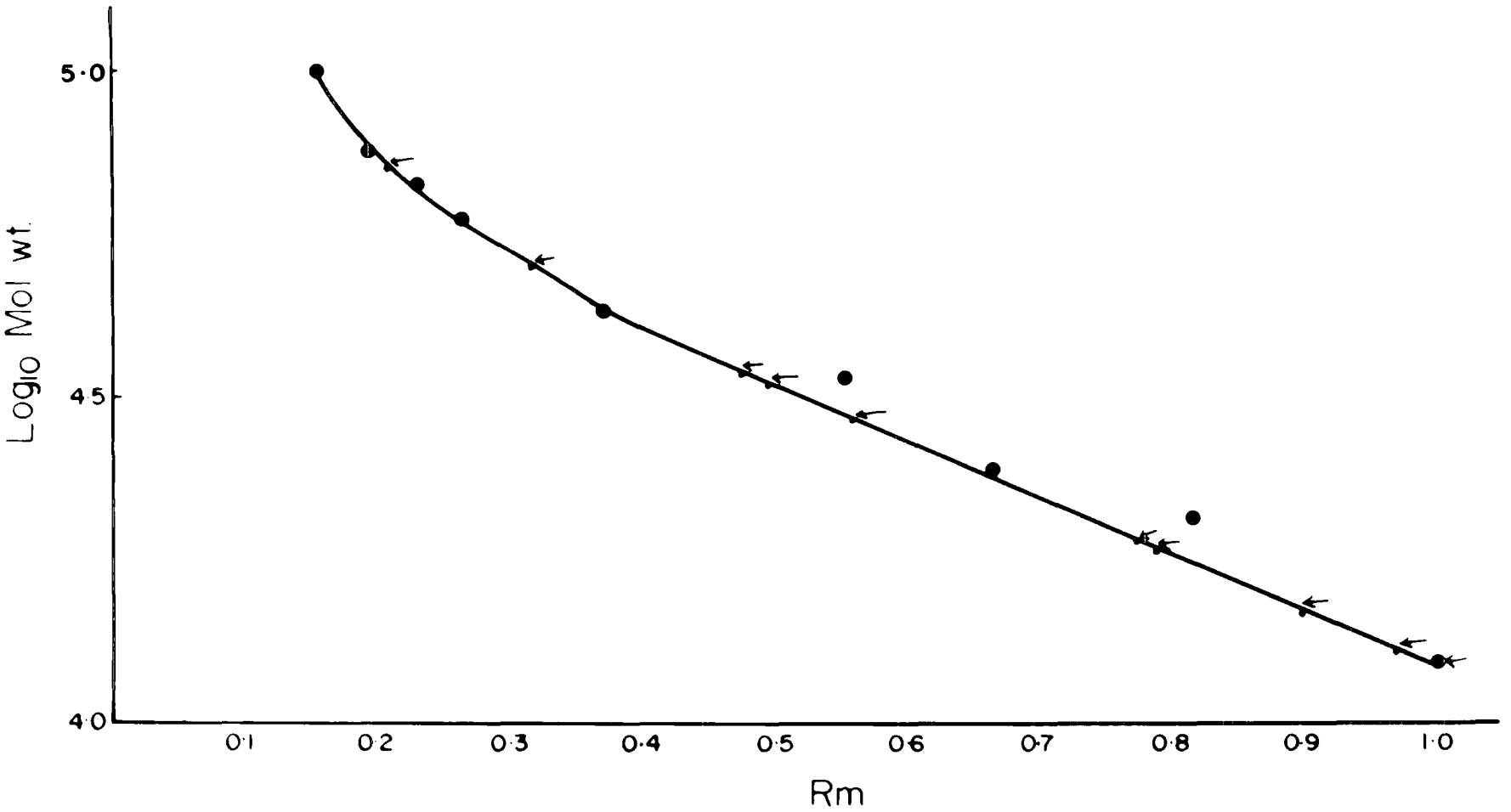
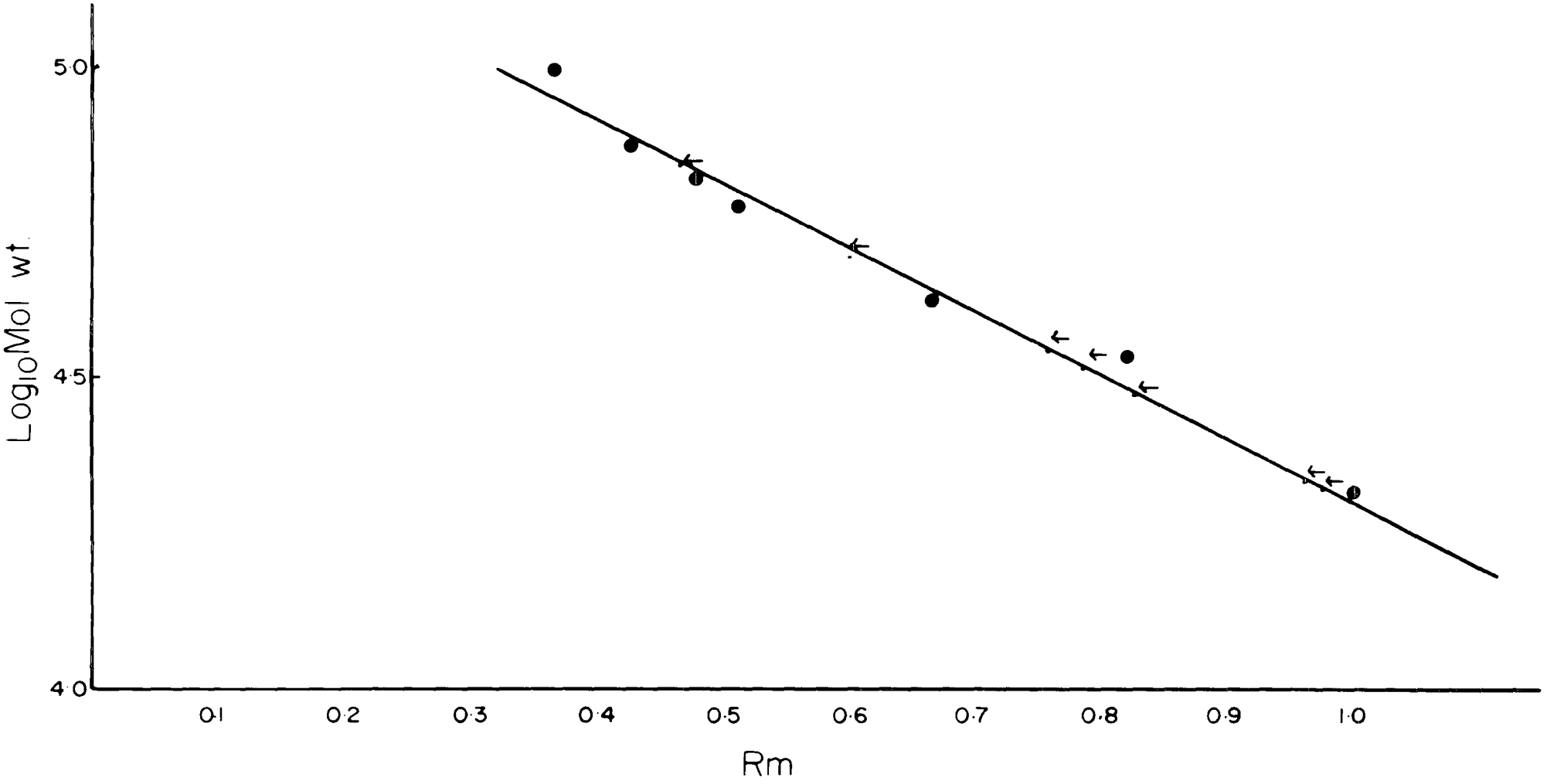


FIG. 9

Calibration curve relating the molecular weight of standard proteins with their electrophoretic mobilities relative to bromophenol blue, on 10% SDS-polyacrylamide gels. Standard proteins in order of decreasing size, are: phosphorylase b, transferrin, bovine serum albumin, catalase, ovalbumin, carboxypeptidase A and trypsin inhibitor.

<- = convicilin and vicilin subunits

Fig.9



D. Two-dimensional polyacrylamide gel electrophoresis

Samples were electrophoresed in 8.5% non SDS-polyacrylamide slab gels for separation in the first dimension. After staining and drying of the slab gel, sample tracks were excised in the form of long strips. These were incubated in 0.2 M Tris-HCl buffer, pH 6.8, containing 2% SDS and 2% 2-mercaptoethanol, at room temperature for 1 h. The gel strip was then inserted between the glass plates on to a 17% SDS-polyacrylamide main slab gel with 1 cm, 3% SDS-polyacrylamide stacking gel. (No well-former was used and the stacking gel was overlaid with water prior to polymerisation, to obtain a flat surface.) In order to hold the gel strip in position and facilitate formation of an extra sample well for standards, 1% agarose in sample buffer was poured into the remaining space surrounding the gel strip and an extra, single well-former. On setting of the agarose, the standard sample was applied to the gel and electrophoresis was carried out in the second dimension under the same conditions as described above in XI.A.

E. Densitometric scanning of slab gels

Sample tracks were cut as slices from slab gels using a sharp blade, then placed in a long cuvette containing destaining solution. Gels were scanned using a Giford 2000 spectrophotometer with gel scanning attachment at 1 cm/min at 580 nm.

F. Isoelectric focussing

Isoelectric focussing was carried out on 5% acrylamide slab gels, using a Shandon U77 flat bed electrophoresis

apparatus with cooled platen and LKB 3371E power supply.

Gel formation

acrylamide, 1.21 g

bis acrylamide, 37.5 mg

sucrose, 3.125 g

distilled water, 22.5 ml

ammonium persulphate (12 mg/ml H_2O), 1.25 ml

40% ampholine carrier ampholytes pH 3.5-10, 1.25 ml

with deionised urea solution to give a final urea concentration of 8 M.

The anode was formed by 0.1 M phosphoric acid, pH 1.5 and the cathode by 1 M ethylenediamine, pH 11.5. Samples for isolectric focussing were prepared by dissolving freeze-dried material in distilled water (2 mg/ml). The protein standard was used at a concentration of 10 mg/ml and contained the following proteins: ferritin (pI 4.4), albumin (pI 4.7), B lactoglobulin (pI 5.34), conalbumin (pI 5.9), horse myoglobin (pI 7.3), whale myoglobin (pI 8.3), ribonuclease (pI 9.45) and cytochrome C (pI 10.65). Prior to loading, the gel was pre-run at approximately 260 volts for 10 min. to establish a pH gradient. All samples, except the haemoglobin marker (4.5 mg/ml) were loaded centrally; the marker was loaded at both poles. The gel was run at approximately 15 watts until the two marker samples had merged together and become focussed, this took about 2 h.

After isoelectric focussing the gel was extensively washed in 15% TCA to remove carrier ampholytes and then stained with 0.025% Coomassie Blue in a solution of 7% acetic acid, 50% methanol in water.

XII. CELLULOSE ACETATE MEMBRANE ELECTROPHORESIS

Sartorius cellulose acetate membranes were used in a Beckman microzone 2-cell apparatus with a continuous 0.05 M sodium phosphate buffer, pH 7.0, system (Gillespie and Blagrove, 1974). Membranes were soaked for 5-10 min. by gently lowering on to the buffer surface, without trapping air. They were then dried between sheets of filter paper and transferred to the cells. Using the applicator in the positioning groove, the protein samples (3-4 mg/ml electrophoresis buffer) were loaded on to the membranes, at the cathode end. The cover was placed on the apparatus and a current of 8 mA (at 300 volts) per membrane was applied for 12 min. The membranes were then quickly removed and stained by immersion in a 0.025% solution of Coomassie Blue in 25% methanol, 7% acetic acid. Excess stain was removed by several washes in a solution of 25% methanol, 7% acetic acid. Finally the membrane was blotted and pressed dry.

XIII. AMINO ACID ANALYSIS

Amino acid analysis of proteins was carried out according to the method of Moore and Stein (1963), on a Locarte single-column amino acid analyser, after hydrolysis of the protein for various times in 6 M HCl at 105°, in Vacuo.

The sulphur amino acids: methionine (as sulphone) and cysteine (as cysteic acid) were determined by analysis of performic acid-oxidized protein (Hirs, 1956).

XIV. N-TERMINAL AMINO ACID DETERMINATION

The N-terminal amino acids were determined by the SDS/dansyl technique of Gray (1972). The N-terminal dansyl-amino acid was identified by the two-dimensional chromatography on polyamide thin layer sheets (Woods and Wang, 1967).

XV. SUGAR ANALYSIS

Sugars present in protein samples that had been precipitated from solution by the addition of TCA to give a final concentration of 10% (w/v) TCA, and washed extensively with 10% TCA, were determined after hydrolysis and gas-liquid chromatography of their derivatives (Sweeley et al., 1966).

XVI. CLEAVAGE WITH CYANOGEN BROMIDE

Protein samples dissolved in 70% (v/v) formic acid were incubated with a 100-fold molar excess of CNBr (2 g/ml solution in acetonitrile) for intervals of 4 h, 8 h, 24 h and 48 h (Kasper, 1970). The solutions were evaporated to dryness under vacuum and the residues analysed by SDS-polyacrylamide gel electrophoresis.

XVII. IMMUNODIFFUSION

Immunodiffusion of convicilin and vicilin against purified antibodies raised against an extract containing both these proteins was carried out by standard methods (Ouchterlony and Nilsson, 1978), on 1% agarose gels. The precipitin arcs

formed after 48 h diffusion were either pressed, washed and stained with staining solution (0.025% Coomassie Blue, 7% acetic acid, 50% methanol in water), or excised and washed thoroughly in 50mM sodium borate buffer, pH 8.0, containing 0.15 M NaCl to remove soluble protein. This was followed by analysis on SDS-polyacrylamide gels. Samples were dissolved in SDS sample buffer containing no 2-mercaptoethanol, in order to prevent immunoglobulin G heavy and light subunits, interfering with the band patterns of convicilin and vicilin.

XVIII. REACTION WITH CONCANAVALIN A

The reaction of convicilin and vicilin with concanavalin A was tested by a similar diffusion system to that described in XVII, using 1% agarose gels. In a negative control test, 0.1 M α methyl D mannoside was incorporated into the agarose gel.

Section III RESULTS

I. CHARACTERISATION OF THE VICILIN TYPE PROTEINS

A. Preliminary Experiments

(i) Ammonium sulphate fractionation

(a) Pisum sativum

Ammonium sulphate fractionation of a total extract prepared from Pisum sativum at 40, 50, 60, 70, 80, 90 and 100% relative saturation gave the separation of protein subunits, seen on analysis by SDS-polyacrylamide gel electrophoresis in Fig.10. Protein precipitating in the range 60-90% relative saturation of ammonium sulphate contained predominantly subunits with mol. wts. of 71,000, 51,000, 40,000, 33,000 and 20,000. The protein soluble and precipitating at 100% relative saturation of ammonium sulphate consisted mainly of 51,000, 33,000 and low mol. wt. subunits.

(b) Phaseolus vulgaris

Ammonium sulphate precipitation of globulin proteins prepared from Phaseolus vulgaris at 50, 70, 90 and 100% relative saturation gave the separation of protein subunits, seen on analysis by SDS-polyacrylamide gel electrophoresis in Fig.11. The soluble protein at 100% relative saturation with ammonium sulphate consisted of three subunits with approximate mol. wts. of 53,000, 47,000 and 43,000. Fractions precipitated at lower ammonium sulphate concentrations contained increasing amounts of other subunits present in the globulin fraction.

FIG. 10

SDS-polyacrylamide gel electrophoresis of different fractions obtained after ammonium sulphate fractionation of a total protein extract of Pisum sativum.

Track 1	:	total extract
Track 2	:	0-40% cut of ammonium sulphate saturation
Track 3	:	40-50% " " " " "
Track 4	:	50-60% " " " " "
Track 5	:	60-70% " " " " "
Track 6	:	70-80% " " " " "
Track 7	:	80-90% " " " " "
Track 8	:	90-100%" " " " "
Track 9	:	100% ammonium sulphate saturated solution
Track 10	:	total extract

FIG. 11

SDS-polyacrylamide gel electrophoresis of different fractions obtained after ammonium sulphate fractionation of proteins in a globulin extract of Phaseolus vulgaris.

Track 1	:	0-50% cut of ammonium sulphate saturation
Track 2	:	50-70% " " " " "
Track 3	:	70-90% " " " " "
Track 4	:	90-100%" " " " "
Track 5	:	100% ammonium sulphate saturated solution
Track 6	:	globulin fraction
Track 7	:	Glycoprotein II - standard preparation
Track 8	:	molecular weight - standard proteins (bovine serum albumin, ovalbumin and cytochrome C)

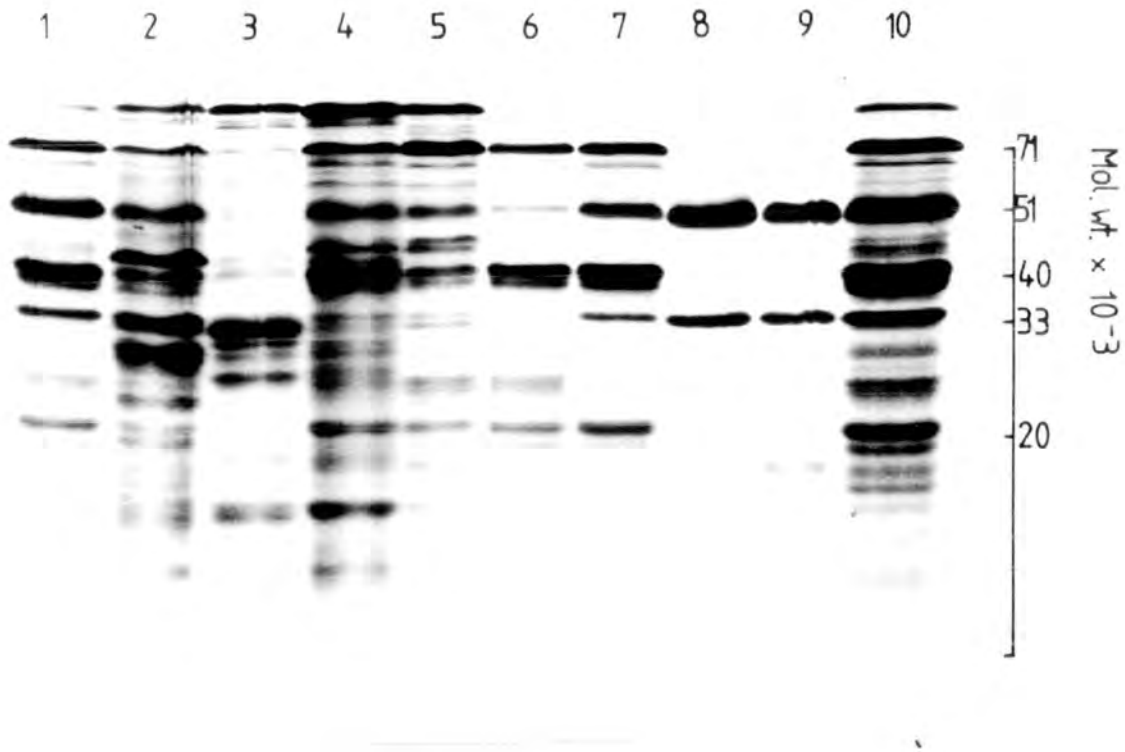

1
2
3
4
5
6
7
8
9
10
71
51
40
33
20
Mol. wt. × 10-3

Fig.10

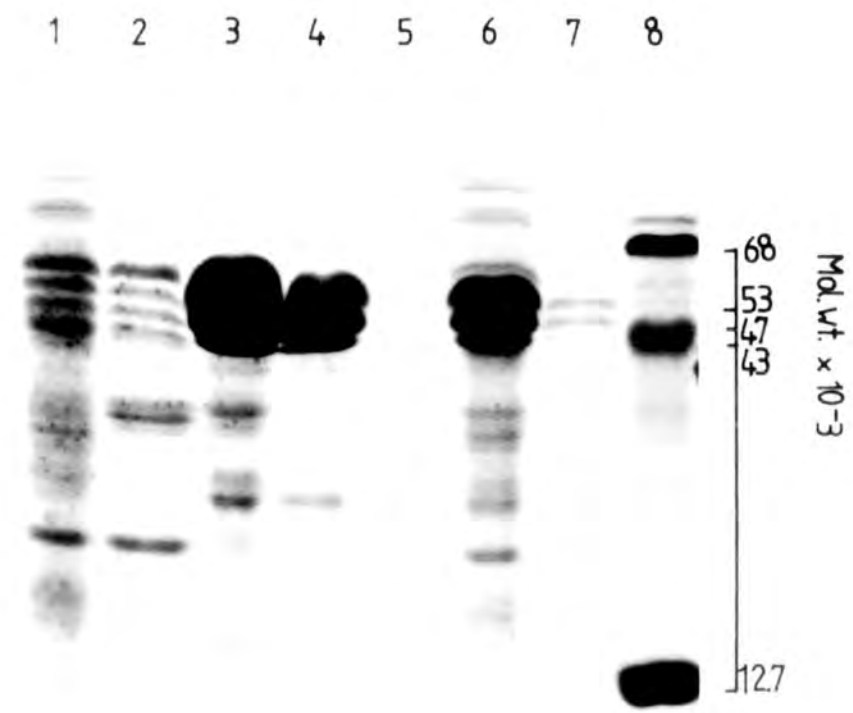

1
2
3
4
5
6
7
8
68
53
47
43
12.7
Mol.wt. × 10-3

Fig.11

(ii) Ion-exchange chromatography

The elution profile of DEAE chromatography of Pisum sativum globulin protein featured three peaks, on elution with a salt gradient, see Fig.12. On analysis of fractions by SDS-polyacrylamide gel electrophoresis, Fig.13, the leading edge of the first peak was shown to contain mainly 51,000 and 33,000 mol. wt. subunits. The trailing edge of the first peak contained predominantly 71,000 and 51,000 mol. wt. subunits. The area between the first and second peak consisted of 71,000, 51,000, 40,000, 33,000 and 20,000 mol. wt. subunits. The second peak consisted mainly of 40,000 and 20,000 mol. wt. subunits. Material present in fractions from the third peak was not precipitated by TCA.

B. Separation of the vicilin type proteins

(i) Convicilin

Convicilin was separated from pea meal proteins as shown in Fig.3. After extraction, the proteins precipitating in the range 50 to 80% relative saturation with ammonium sulphate were redissolved and chromatographed on a column of Sephadex G-200. The elution profile is shown in Fig.14. When fractions from the leading edge of the large asymmetrical peak of protein were analysed by SDS-polyacrylamide gel electrophoresis, Fig.15, they were found to contain mainly subunits of 40,000 and 20,000 mol. wt. Fractions from the mid-area of the peak were found to contain 71,000 mol. wt. subunits in addition, while fractions from the trailing edge of the peak had a subunit pattern containing 51,000, 33,000, 21,000, 20,000 and

FIG. 12

Elution profile obtained after ion-exchange chromatography of *Pisum sativum* globulin proteins on a column of DEAE cellulose.

FIG. 13

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.12. Tracks 1-11 : fractions 1-11. Track G : *Pisum sativum* globulin protein standard.

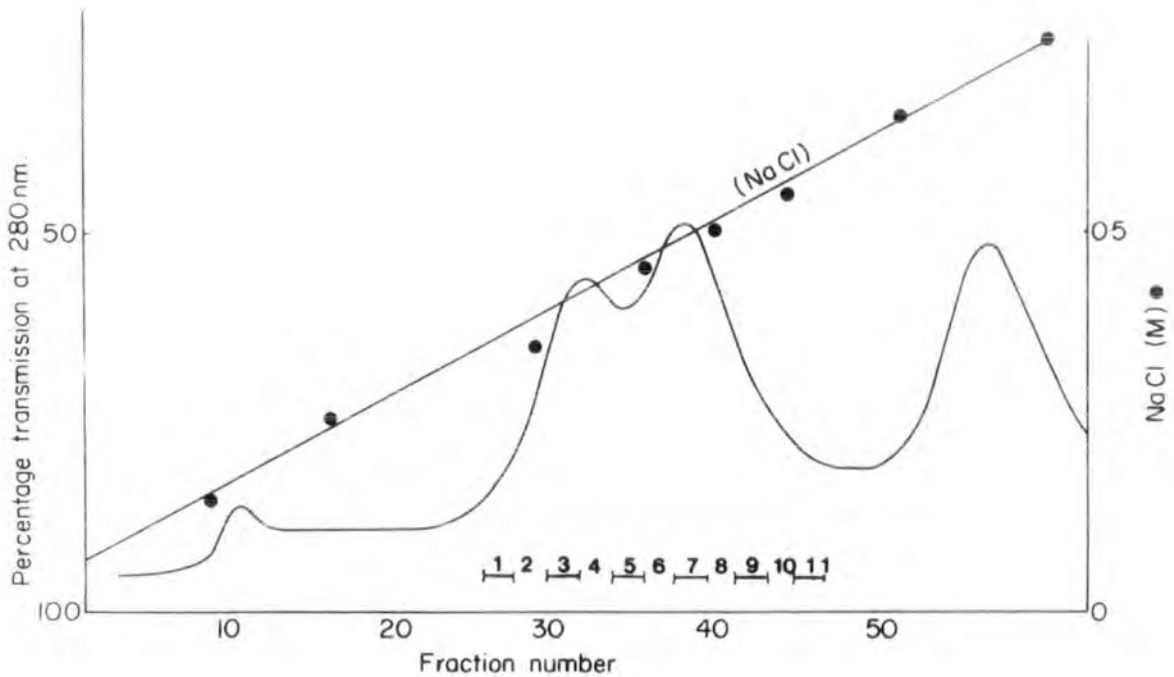

Percentage transmission at 280nm
50
100
(NaCl)
1 2 3 4 5 6 7 8 9 10 11
10
20
30
40
50
Fraction number
05
0
NaCl (M)

Fig. 12

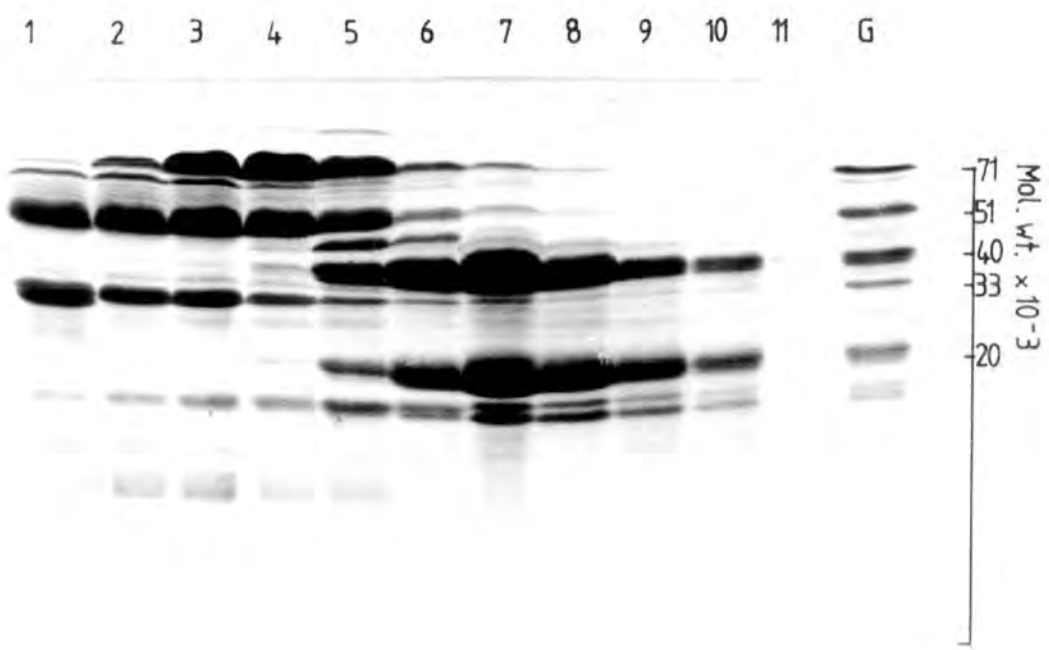

1 2 3 4 5 6 7 8 9 10 11 G
71
51
40
33
20
Mol. wt. x 10-3

Fig. 13

FIG. 14

Elution profile obtained after Sephadex G-200 chromatography of the proteins precipitating in the 50-80% cut of ammonium sulphate saturation of a total protein extract of Pisum sativum.

FIG. 15

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.14. Tracks 1-6 : fractions in areas 1-6. Track Mw : Molecular weight - standard proteins 1-3 represent bovine serum albumin, ovalbumin and cytochrome C. Track G : Pisum sativum globulin protein standard.

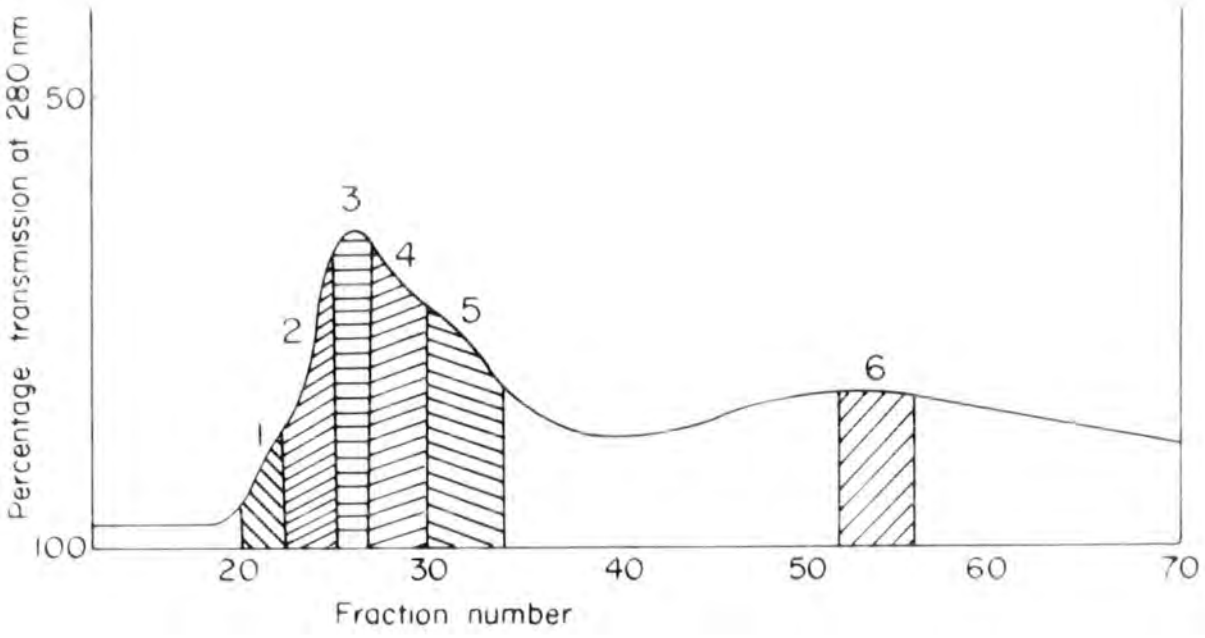

Percentage transmission at 280 nm
50
100
1
2
3
4
5
6
20
30
40
50
60
70
Fraction number

Fig.14.

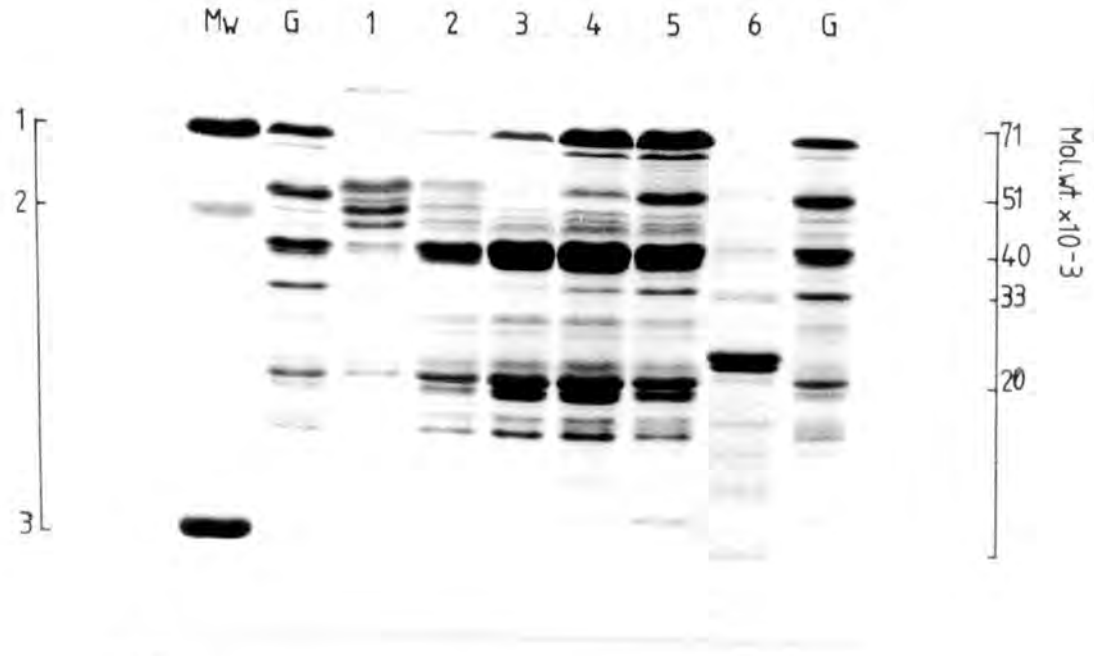

Mw
G
1
2
3
4
5
6
G
1
2
3
71
51
40
33
20
Mol.wt. x10-3

Fig.15

lower mol. wt. subunits. Finally a small peak containing the very low molecular weight proteins was eluted.

Hydroxylapatite chromatography was employed in order to further purify the fractions containing the 71,000 mol. wt. subunit. The pooled fractions containing the 71,000 mol. wt. subunit (areas 3 and 4 in Fig.14) were applied to the column and eluted by linear concentration gradient of phosphate buffer. The elution profile is shown in Fig.16. The initial large peak of protein eluted at 0.2 - 0.25M potassium phosphate was shown to contain 40,000 and 20,000 mol. wt. subunits by SDS-polyacrylamide gel electrophoresis, Fig.17. The leading edge of the second peak, eluted at 0.28M - 0.35M potassium phosphate, contained 71,000 mol. wt. subunits with only traces of lower mol. wt. subunits, subsequent fractions were increasingly contaminated with 51,000, 33,000, 21,000, 20,000 and lower mol. wt. subunits as phosphate concentration increased. On the gel shown in Fig.17 the 51,000 mol. wt. subunit appeared as a double band, the reason for this is not clear.

Densitometric scanning of the pooled fractions containing the 71,000 mol. wt. subunits on gels, indicated 90% purity for this protein.

(ii) Vicilin

Vicilin was prepared by making a total extract of pea meal proteins and separating the fraction soluble at 100% relative saturation of ammonium sulphate, see Fig.10. After extensive dialysis, this fraction was concentrated by hydroxylapatite chromatography. Elution with a single step of 0.75M potassium phosphate buffer produced the elution

FIG. 16

Elution profile obtained after hydroxylapatite chromatography of protein in fractions, from areas 3 and 4 of the Sephadex G-200 elution profile, Fig.14.

FIG. 17

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.16. Tracks 1-6 : fractions in areas 1-6.

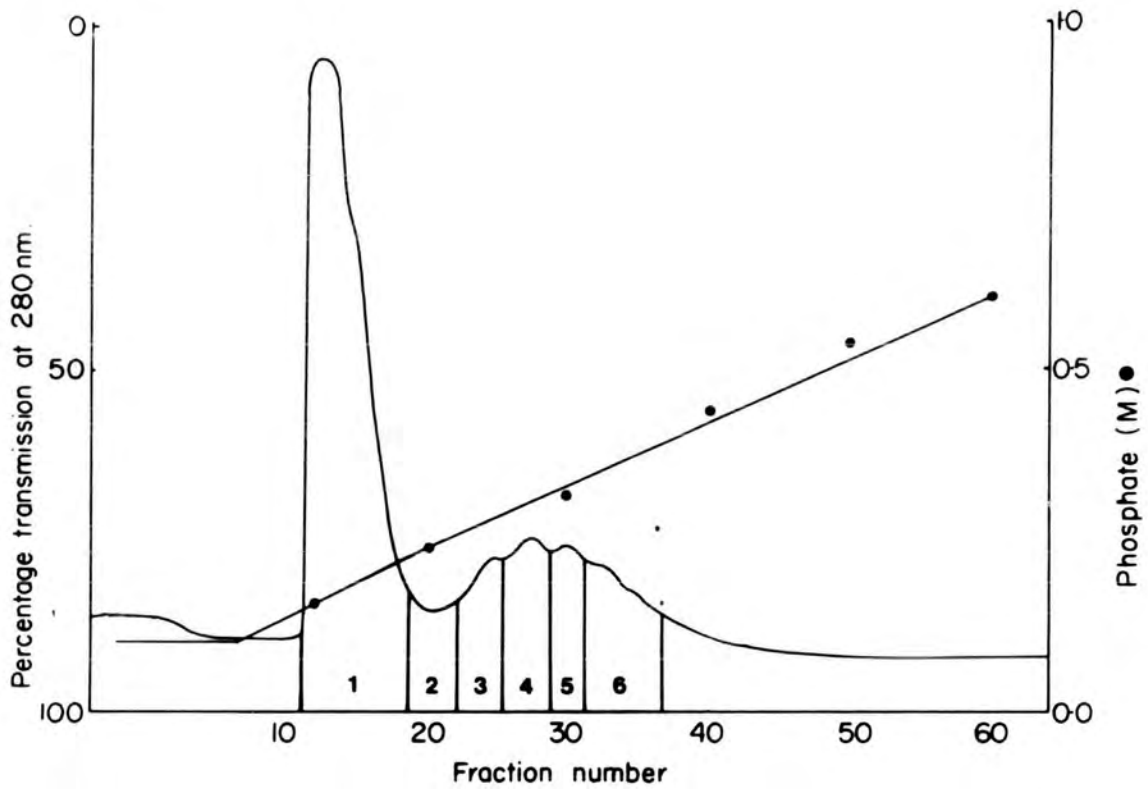

0
50
100
Percentage transmission at 280nm.
1·0
0·5
0·0
Phosphate (M) ●
1
2
3
4
5
6
10
20
30
40
50
60
Fraction number

Fig. 16

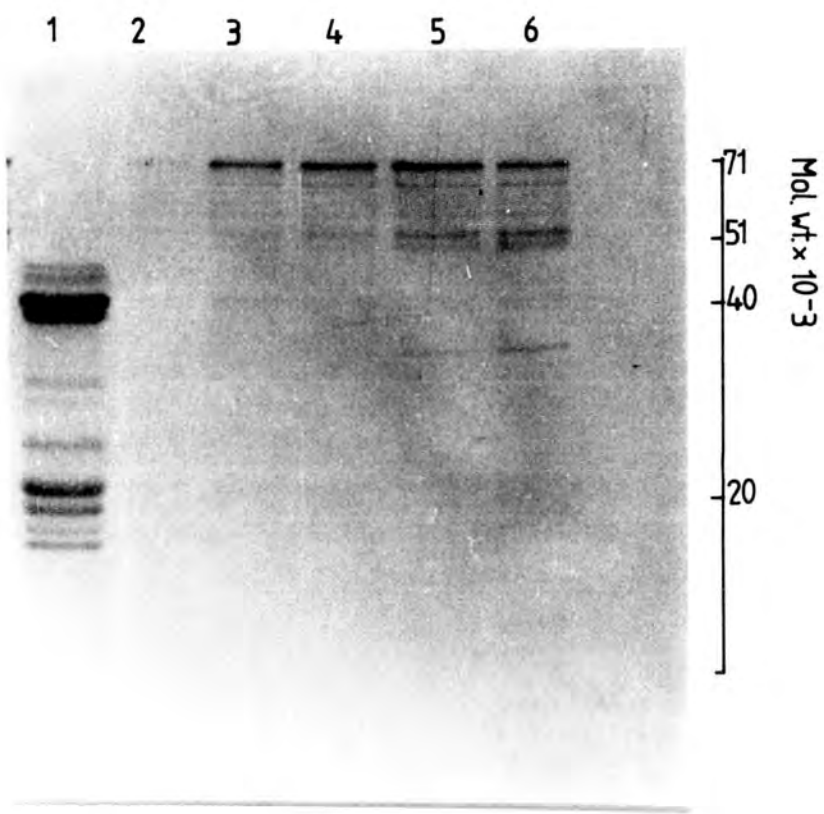

1
2
3
4
5
6
71
51
40
20
Mol. wt. x 10-3

Fig.17

profile seen in Fig.18. This consisted of a single symmetrical peak. On analysis by SDS-polyacrylamide gel electrophoresis, this peak was shown to consist of major subunits of mol. wts. 51,000, 33,000, 21,000, 20,000 and minor subunits of mol. wts. 35,000, 30,000, 15,000, 13,000 and 12,000 (see Fig.19 and Fig.20).

From the results of these separation experiments, the 71,000 mol. wt. subunits were assigned to a separate protein: convicilin (see discussion). The 40,000 and 20,000 mol. wt. subunits represented legumin (Higgins and Spencer, 1980; Croy et al., 1979) and the remaining 51,000, 33,000, 30,000, 35,000 and lower mol. wt. subunits were assigned to vicilin (Gatehouse et al., 1981), see discussion. The proteins described above were analysed on a separate gel for comparison, see Fig.30.

C. Molecular weight estimation of proteins

(i) Subunit molecular weight of convicilin

A subunit molecular weight of 72,000 $\pm$ 3,000 was determined for convicilin in 17% SDS-polyacrylamide gels and a value of 70,000 $\pm$ 2,500 was obtained in 10% SDS-polyacrylamide gels by comparison with standard proteins, see Fig.8 and Fig.9, also Fig.19 and Fig.20. A mean value of 71,000 was adopted as the mol. wt. of convicilin subunits.

(ii) Subunit molecular weight of vicilin

Subunits of vicilin were found to have mol. wts. of 51,000, 35,000, 34,000, 29,000, 19,000, 18,500, 15,000, 13,000

FIG. 18

Elution profile obtained after hydroxylapatite chromatography of the protein soluble, at 100% relative saturation with ammonium sulphate, in a total protein extract of *Pisum sativum*.

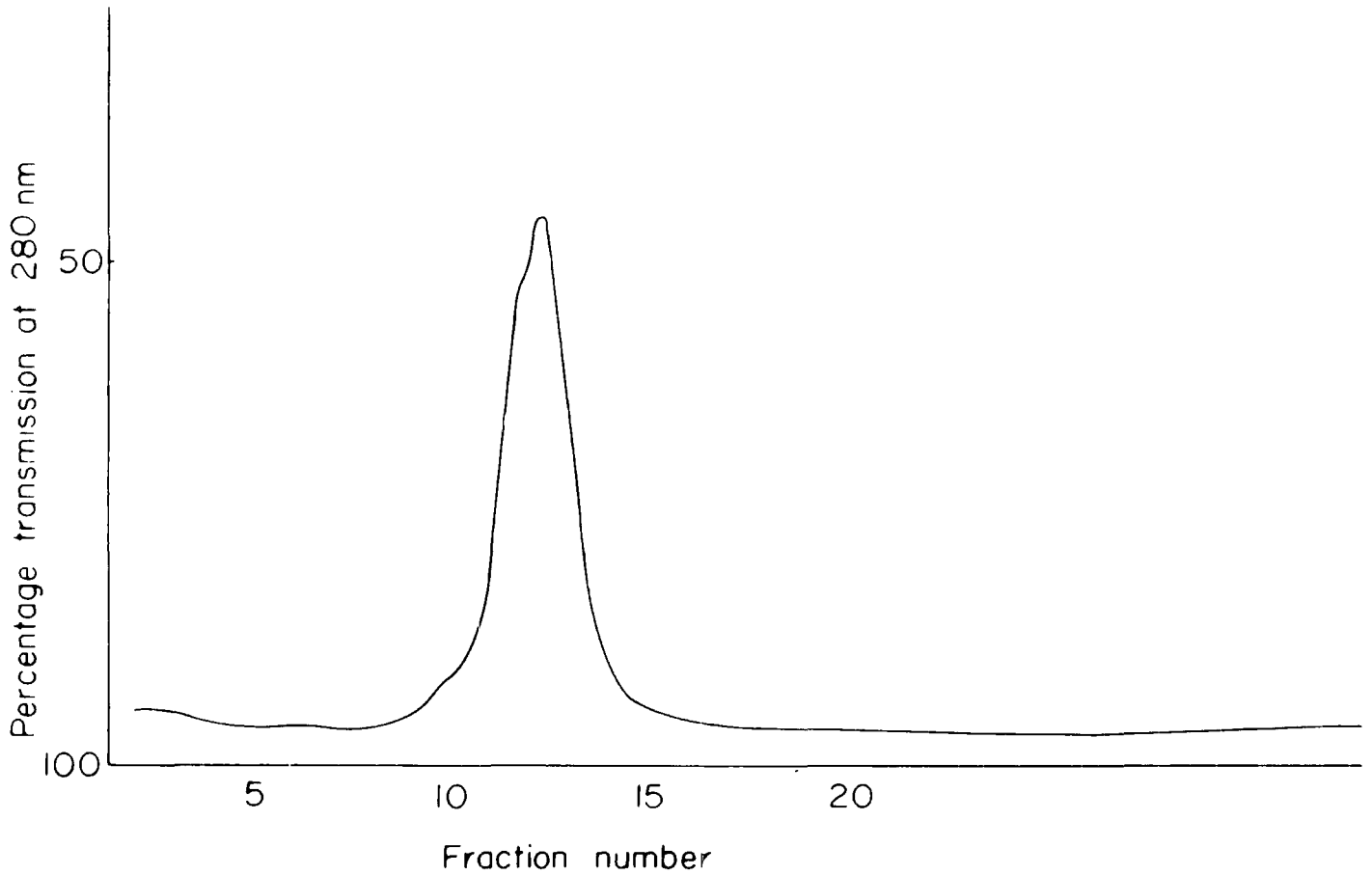

Percentage transmission at 280 nm
50
100
5
10
15
20
Fraction number

Fig. 18

FIG. 19

Determination of the subunit molecular weight(s) for vicilin and convicilin by 17% SDS-polyacrylamide gel electrophoresis.

Standard proteins
Track 1 : phosphorylase b
Track 2 : transferrin
Track 3 : bovine serum albumin
Track 4 : catalase
Track 5 : ovalbumin
Track 6 : carboxypeptidase A
Track 7 : trypsin inhibitor
Track 8 : cytochrome C

Track V : vicilin
Track C : convicilin

FIG. 20

Determination of the subunit molecular weight(s) for vicilin and convicilin by 10% SDS-polyacrylamide gel electrophoresis.

Standard proteins
Track 1 : phosphorylase b
Track 2 : transferrin
Track 3 : bovine serum albumin
Track 4 : catalase
Track 5 : ovalbumin
Track 6 : carboxypeptidase A
Track 7 : trypsin inhibitor

Track C : convicilin
Track V : vicilin

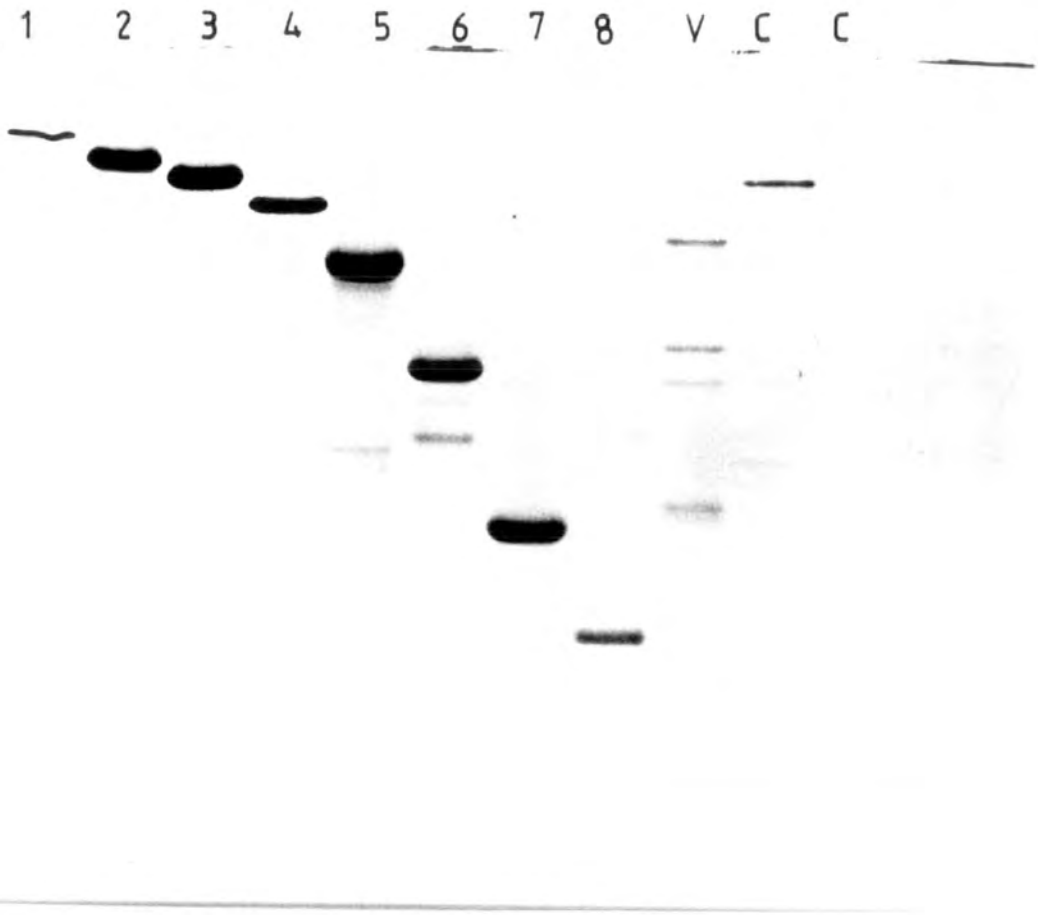


Fig.19

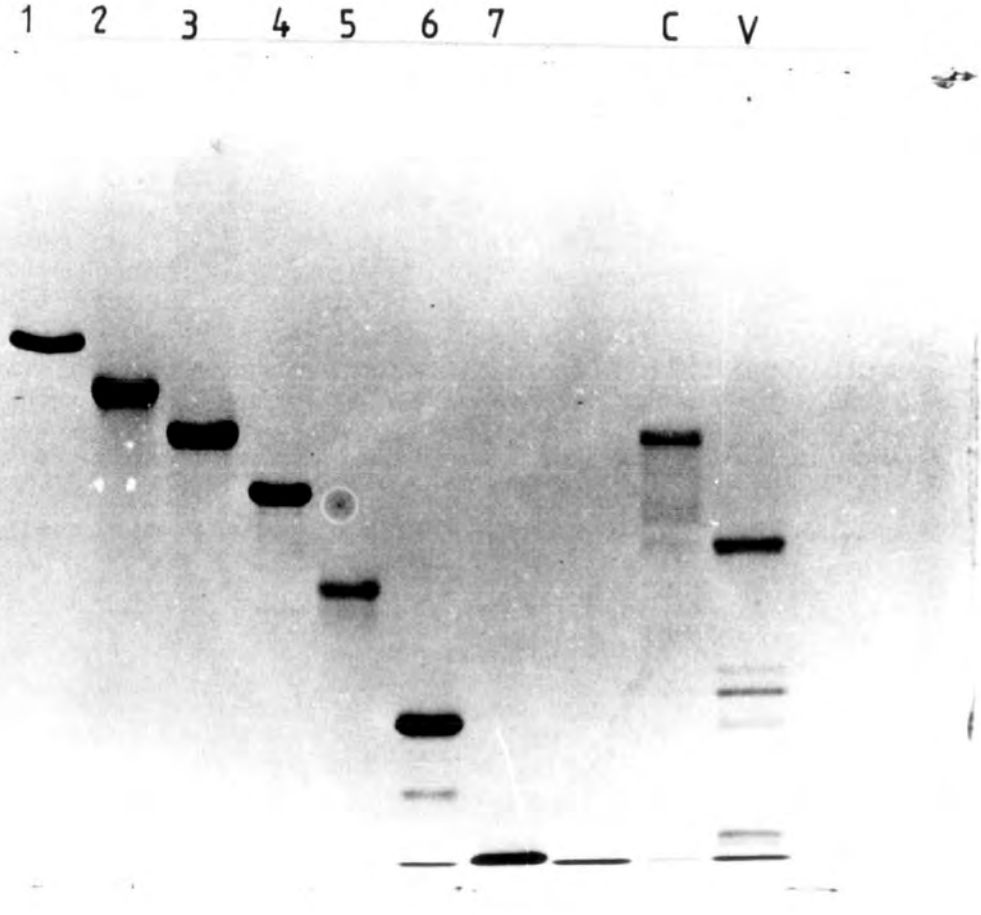


Fig. 20

Amino acid composition of convicilin and vicilin

Values are means of three or more separate determinations. The S.E.M. was less than 5% in all cases.

Amino acid	Composition (residues/100 residues)		
	Convicilin	Vicilin	Legumin‡
Asp	11.64	18.87	13.07
Thr	2.55*	2.69*	3.08
Ser	6.39*	7.67*	5.77
Glu	22.08	19.18	19.74
Pro	5.47	1.99	5.50
Gly	5.90	4.85	6.89
Ala	4.23	4.13	6.01
Val	4.46	4.84	4.97
Met	0.13+	0.00+	0.66
Ile	3.85	5.11	3.95
Leu	8.71	9.83	7.60
Tyr	2.59	1.91	3.64
Phe	3.30	4.67	2.30
His	2.22	1.61	1.75
Lys	8.18	8.10	4.24
Arg	8.15	4.57	9.99
Cys	0.13+	0.00+	1.21

* Corrected for decomposition from 22, 48 and 72h hydrolyses.

+ From analysis of performic acid-oxidized protein.

‡ From Casey (1979a).

Table 1.

and 12,000 on 17% SDS-polyacrylamide gels and values of 50,000, 35,000, 33,000, 30,000, 22,000, 21,000 were obtained with 10% SDS-polyacrylamide gels when compared to standard proteins, see Fig.8 and Fig.9, also Fig.19 and Fig.20. Mean values of 51,000, 35,000, 33,000, 30,000, 21,000, 20,000, 15,000, 13,000 and 12,000 mol. wt. were adopted.

(iii) Protein molecular weight of convicilin

Gel filtration chromatography of a total protein extract of mature pea seeds on a column of Ultrogel AcA 22 showed that purified convicilin eluted as a single peak, see Fig.21. A molecular weight of 290,000 $\pm$ 40,000 was determined for convicilin by comparison with standard proteins.

D. Amino acid analysis

The amino acid compositions of convicilin and vicilin are given in table 1 and compared with that of legumin of Pisum sativum. The amounts of methionine and cysteine in convicilin correspond to 0.9 residue per 71,000 mol. wt. subunit for both amino acids.

E. N-Terminal amino acid determination

The N-terminal amino acids of convicilin were determined by using the dansyl-SDS technique (Gray, 1972) and were found to be aspartic acid and leucine in comparable amounts.

FIG. 21

Gel filtration of a total protein extract of pea seeds on a column of Ultrogel AcA 22.

(a) Elution profile obtained after Ultrogel chromatography of a total protein extract

(b) Elution profile obtained after Ultrogel chromatography of purified convicilin

(c) Distribution of legumin (40,000 mol. wt. subunits) (◎), vicilin (50,000 mol. wt. subunits (○) and convicilin (71,000 mol. wt. subunits) (△) relative to the elution profile in (a), as determined from densitometric scans of SDS-polyacrylamide-gel-electrophoretic analyses of each fraction from the column. Legumin values have been plotted on half the indicated scale for clarity. Electrophoretic analyses of the representative fractions are shown in (a)

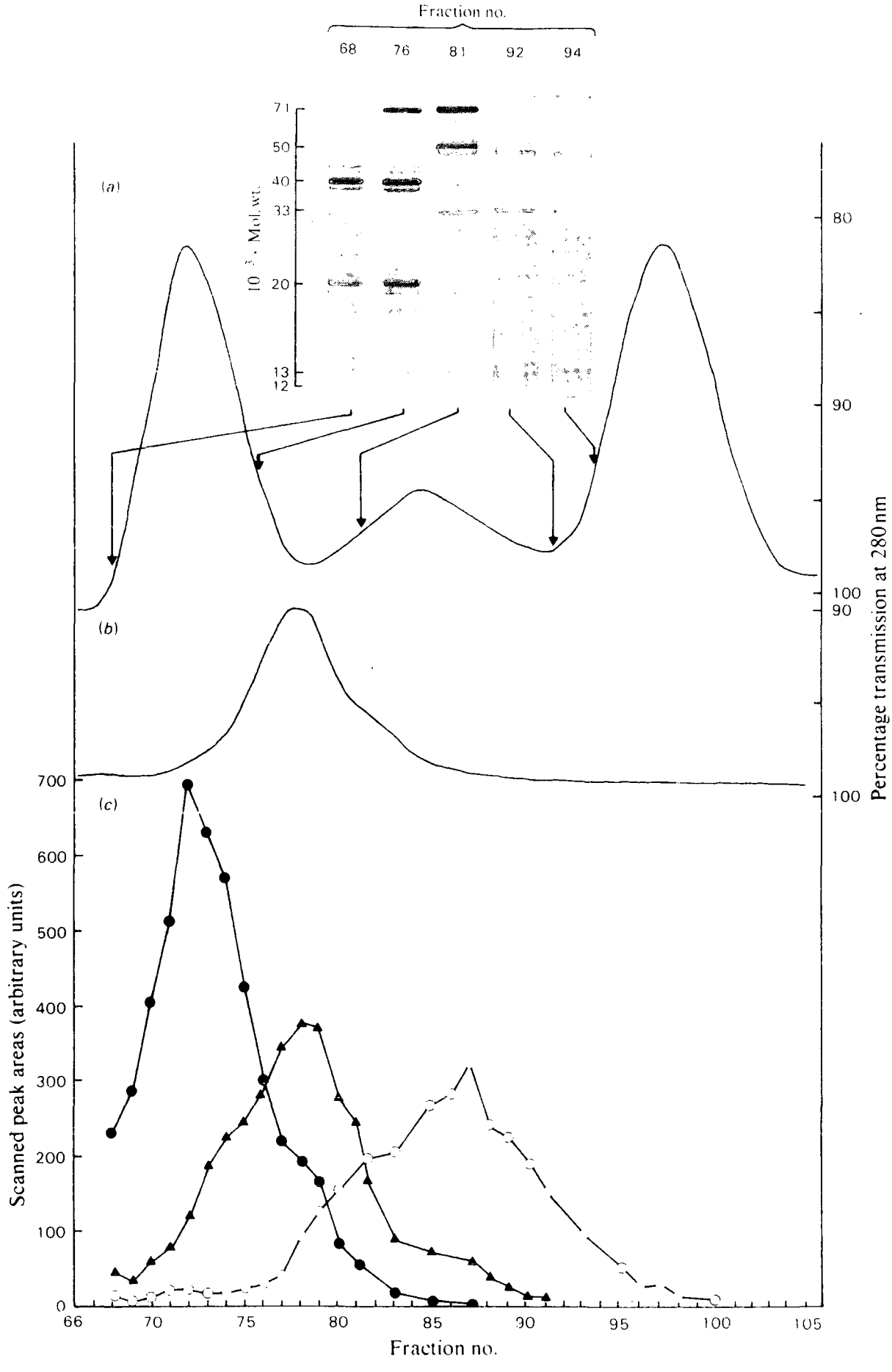

Fraction no.
68
76
81
92
94
71
50
40
33
20
13
12
10^{-3} · Mol. wt.
(a)
(b)
(c)
80
90
100
90
100
Percentage transmission at 280 nm
700
600
500
400
300
200
100
0
Scanned peak areas (arbitrary units)
66
70
75
80
85
90
95
100
105
Fraction no.

Fig. 21

F. Sugar analysis

Small amounts of glucose, galactose and mannose were detected in convicilin, but these amounts were comparable with those found in a known non-glycoprotein: bovine serum albumin, and were significantly less than one sugar residue per 71,000 mol. wt. subunit.

G. Cleavage with cyanogen bromide

Two major fragments were produced on incubation of convicilin with CNBr with approximate mol. wts. of 55,000 and 14,000, there were also a number of cleavage products with an intermediate mol. wt. see Fig.22. However, it can be seen that some material remained undigested even after 48h incubation. In the same experiment vicilin appeared to be uncleaved by cyanogen bromide.

H. Serological properties

When convicilin and vicilin (free of cross-contaminating subunits) were allowed to diffuse against an antiserum prepared against an extract containing both in an Ouchterlony double immunodiffusion test, the two proteins gave a reaction of identity, see Fig.23a. The precipitin arcs were excised and analysed by SDS-polyacrylamide gel electrophoresis to demonstrate that the immunoprecipitates produced by the reaction of convicilin with the above antibodies contained only 71,000 mol. wt. subunits with no trace of vicilin subunits.

FIG. 22

SDS-polyacrylamide gel electrophoresis of convicilin and vicilin before and after reaction with CNBr.

Track 1 : convicilin before reaction
Track 2 : convicilin after 4h reaction
Track 3 : convicilin after 8h reaction
Track 4 : convicilin after 24h reaction
Track 5 : convicilin after 48h reaction
Track 6 : vicilin before reaction
Track 7 : vicilin after 4h reaction
Track 8 : vicilin after 8h reaction
Track 9 : vicilin after 24h reaction
Track 10 : vicilin after 48h reaction

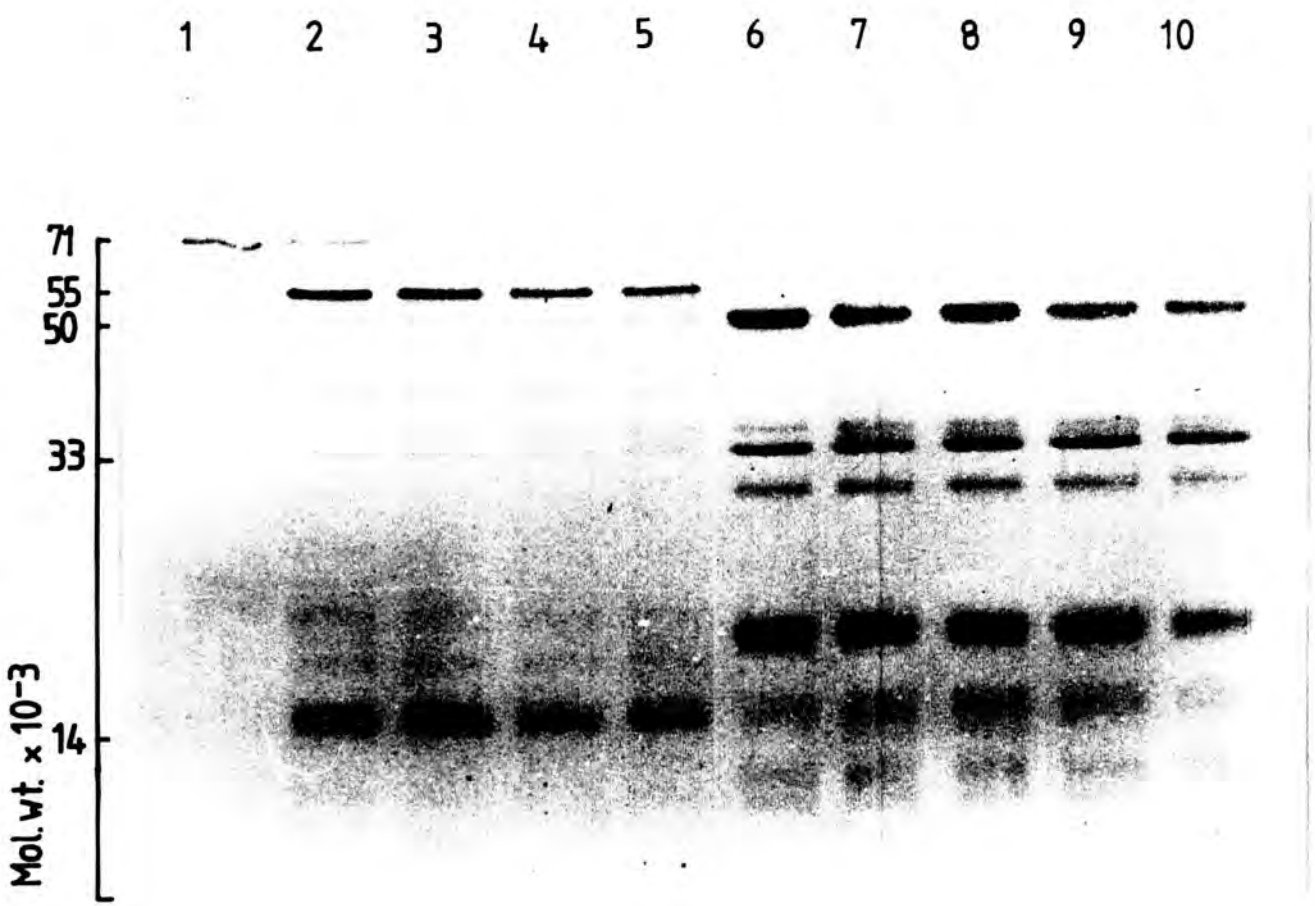


Fig. 22

FIG. 23

Cross-reactivity of convicilin and vicilin.

(a) Comparison of convicilin (C) and vicilin (V) by double immunodiffusion against antibodies (immunoglobulin G) raised against a protein fraction containing both proteins

(b) Double diffusion of convicilin and vicilin against concanavalin A (Con A)

FIG. 24

SDS-polyacrylamide gel electrophoresis of precipitin arcs seen in Fig.23(a).

Track 1 : convicilin precipitin arc
Track 2 : vicilin precipitin arc
Track 3 : convicilin standard preparation
Track 4 : convicilin and vicilin standard preparations
Track 5 : total protein extract standard preparation

Ig G : immunoglobulin G subunits

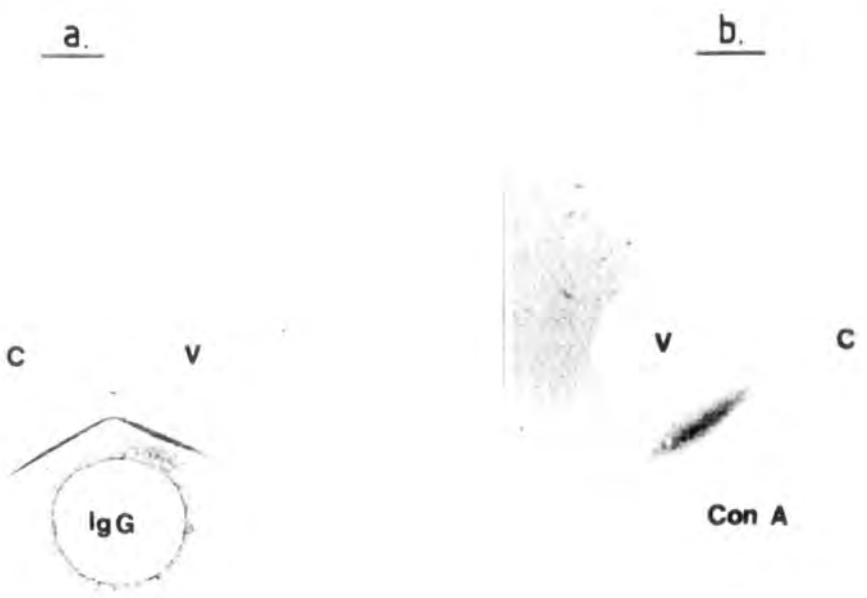

a.
b.
C
V
IgG
V
C
Con A

Fig. 23

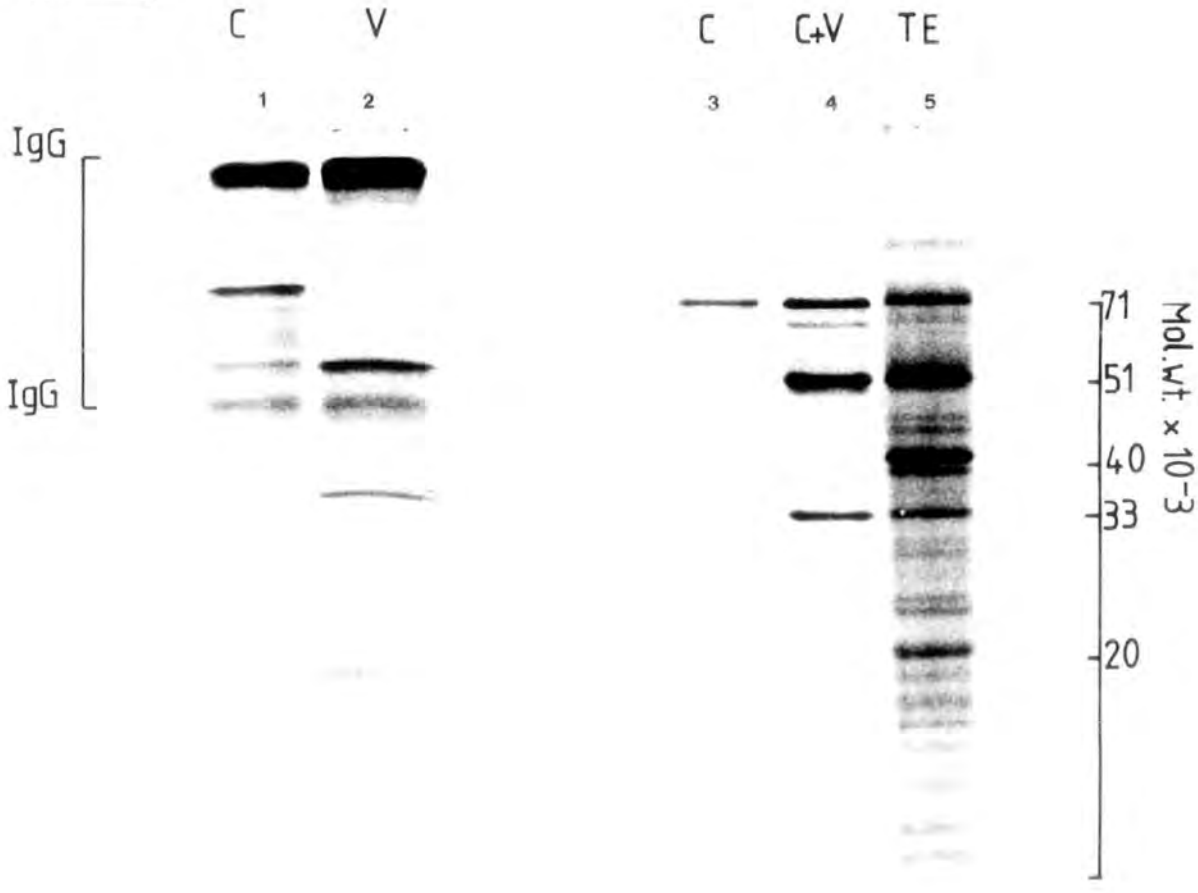

C
V
C
C+V
TE
1
2
3
4
5
IgG
IgG
71
51
40
33
20
Mol.wt. x 10-3

Fig. 24

Conversely those of vicilin contained only vicilin subunits and no 71,000 mol. wt. subunits, see Fig.24.

When tested against antiserum prepared against total pea proteins, convicilin gave a reaction of non-identity with legumin (data not presented).

I. Reaction with concanavalin A

Convicilin gave no precipitin reaction with concanavalin A in a double immunodiffusion experiment, see Fig.23b. However, vicilin gave a positive reaction with concanavalin A in the same experiment.

J. Isoelectric focussing

Convicilin focussed to a series of closely spaced, slightly blurred bands in the pI range of 5.6 to 5.8, when subjected to isoelectric focussing in a polyacrylamide gel slab containing 8 M urea.

K. Zonal isoelectric precipitation

(i) *Pisum sativum* total protein extract

The elution profile obtained on zonal isoelectric precipitation of a total extract of pea is shown in Fig.25. Three small peaks were eluted prior to two larger close peaks of protein. Fractions representing areas 1 - 5 were analysed by SDS-polyacrylamide gel electrophoresis, Fig.26.

FIG. 25

Elution profile obtained after zonal isoelectric precipitation of a total protein extract of *Pisum sativum* on a column of Sephadex G-50.

FIG. 26

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig. 25.

Tracks 1-5	:	fractions in areas 1-5
Track V	:	vicilin standard preparation
Track C	:	convicilin standard preparation
Track L	:	legumin standard preparation

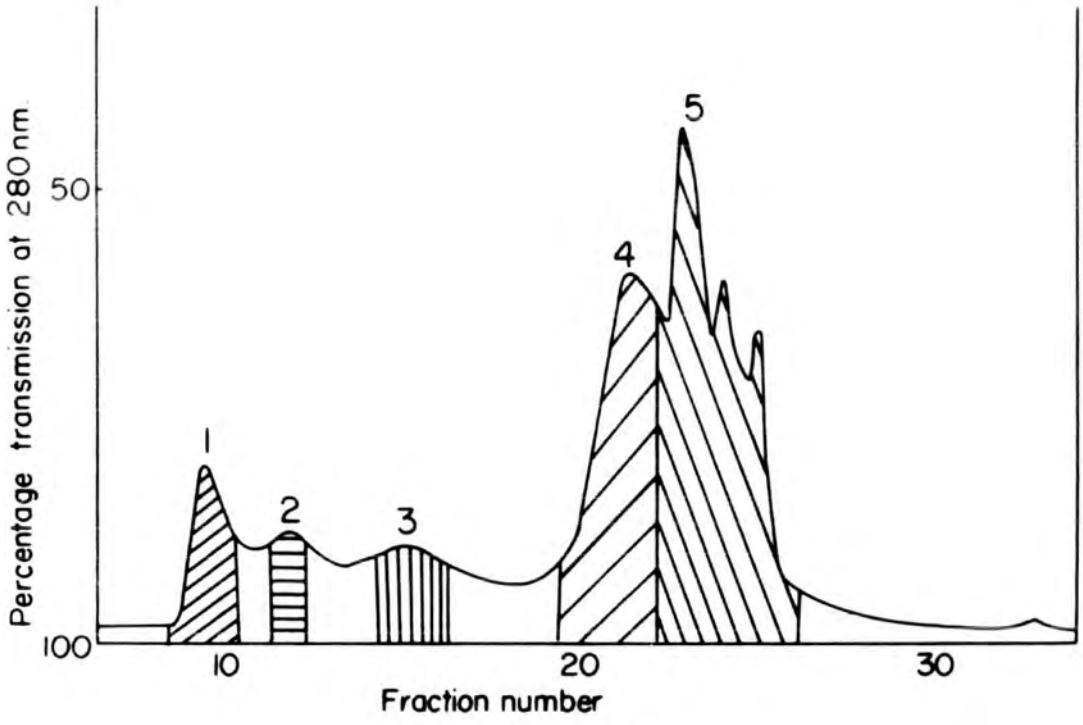

Percentage transmission at 280nm
50
100
10
20
30
Fraction number
1
2
3
4
5

Fig. 25

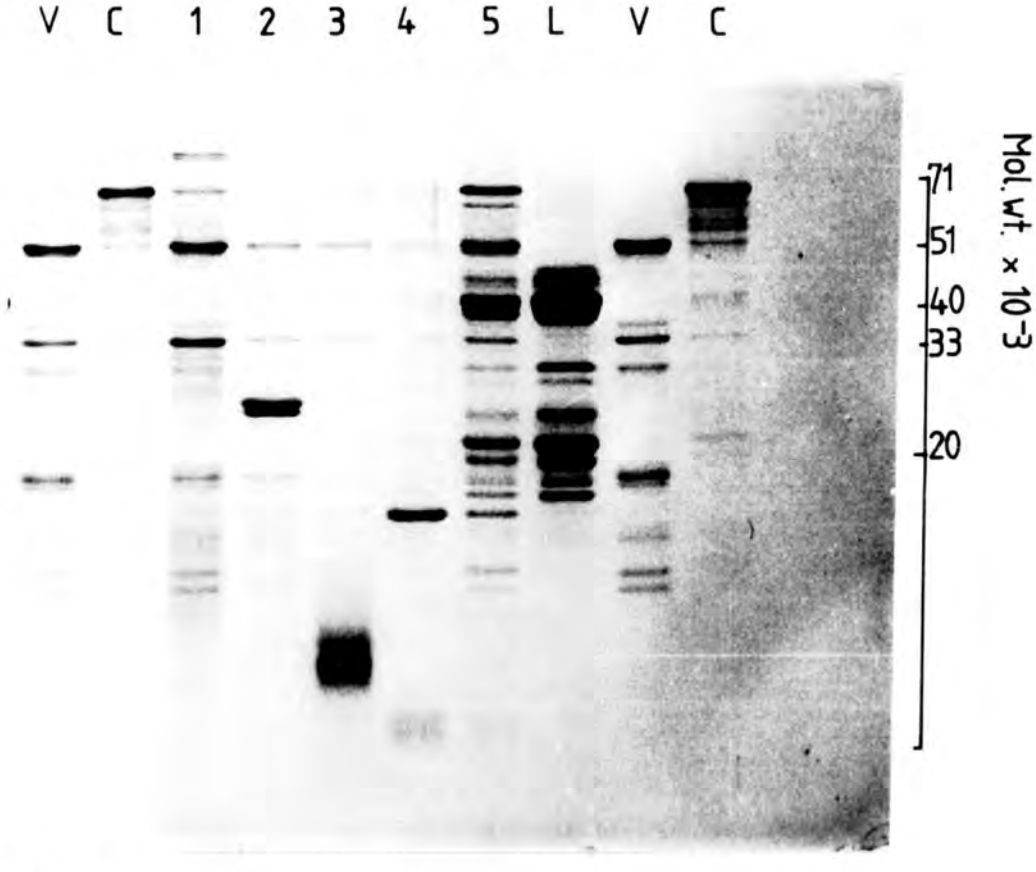

V
C
1
2
3
4
5
L
V
C
71
51
40
33
20
Mol.wt. x 10-3

Fig. 26

It was found that area 1 consisted mainly of 51,000 and 33,000 mol. wt. subunits, area 2 contained 24,000 mol. wt. subunits, following this, area 3 contained low mol. wt. proteins. Area 4 consisted of protein subunits of approximately 17,000 mol. wt. and finally area 5 contained mainly 71,000, 40,000 and 20,000 mol. wt. subunits.

(ii) Vicia faba globulin fraction

Vicilin was separated from legumin of Vicia faba according to the method of Scholz et al., (1974) as seen in Fig.2. An elution profile consisting of two, well separated peaks was obtained on zonal isoelectric precipitation of a globulin extract prepared from Vicia faba meal, see Fig.27. Using cellulose acetate membrane electrophoresis the first peak was shown to consist mainly of protein with a relative mobility similar to that of Vicia faba standard vicilin, while the second peak was shown to consist mainly of a protein with a similar relative mobility to that of Vicia faba standard legumin.

L. Cellulose acetate membrane electrophoresis

Cellulose acetate membrane electrophoresis of vicilin and convicilin indicated that convicilin was slightly less negatively charged than vicilin under these conditions.

M. Preparation of convicilin from Vicia faba

The elution profile shown in Fig. 28 was obtained when a

FIG. 27

Elution profile obtained after zonal isoelectric precipitation of *Vicia faba* globulin protein fraction on a column of Sephadex G-50.

1 : vicilin

2 : legumin

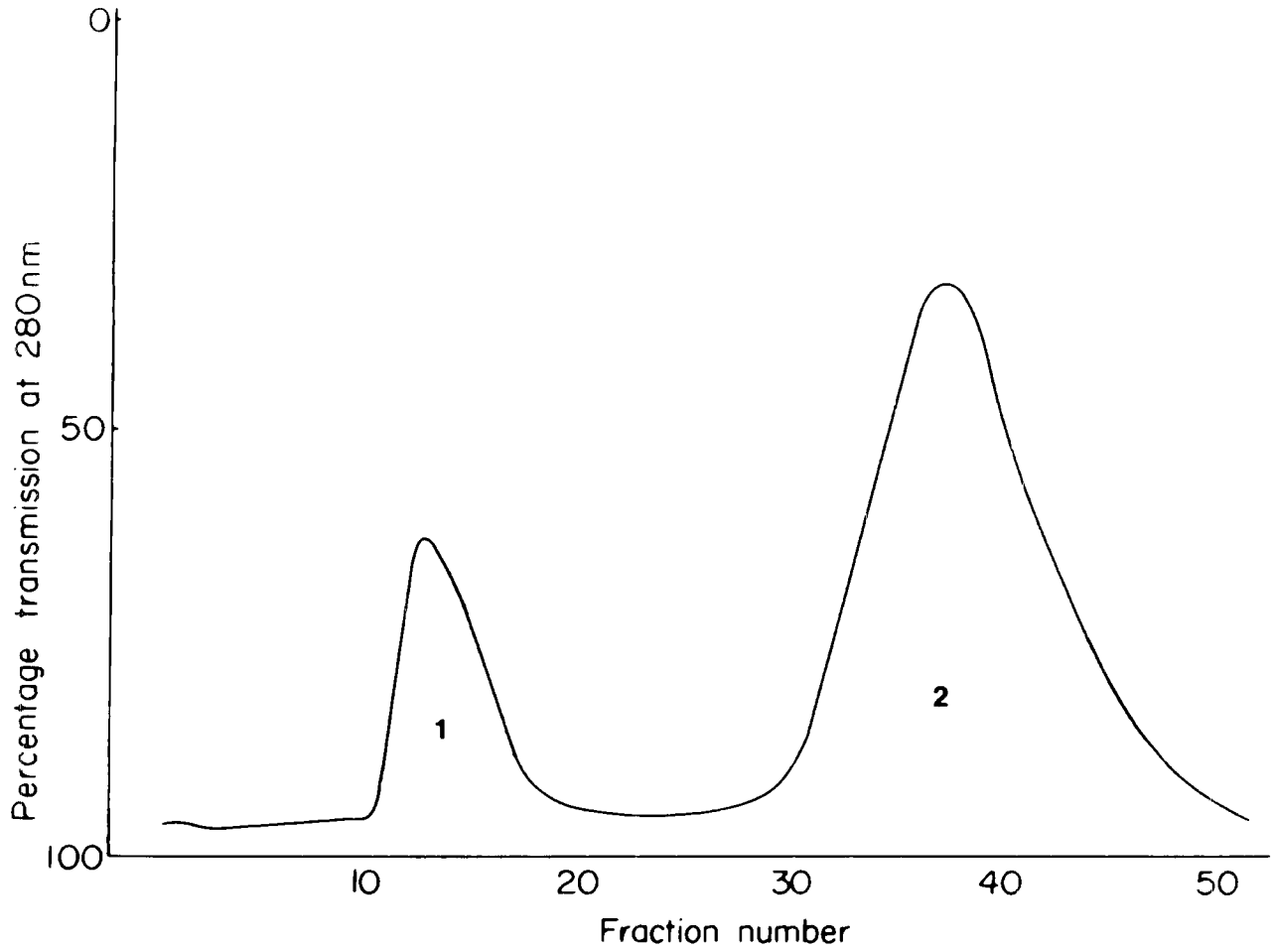

0
50
100
Percentage transmission at 280nm
1
2
10
20
30
40
50
Fraction number

Fig. 27

total extract of Vicia faba meal was chromatographed on a column of hydroxylapatite. A large peak of non-protein material was eluted before a linear concentration gradient of phosphate buffer was applied. Following this, three symmetrical peaks of protein were eluted. Alternate fractions were analysed by SDS-polyacrylamide gel electrophoresis, see Fig.29. The initial peak eluted at 0.1M potassium phosphate contained approximately 32,000 and 33,000 mol. wt. subunits, subunits with a mol. wt. greater than 70,000 were also present in this peak. The second and largest peak that eluted at 0.15M - 0.25M potassium phosphate buffer contained 37,000 and 20,000 mol. wt. subunits similar to those described for legumin of Vicia faba (Wright and Boulter, 1974). The leading edge of the third and smallest protein peak that eluted at 0.3 to 0.375M potassium phosphate buffer consisted mainly of 68,000 mol. wt. subunits becoming more contaminated with lower mol. wt. subunits with increasing potassium phosphate concentration. Finally the trailing edge of the third peak contained mainly 48,000 and 32,000 mol. wt. subunits, similar to those identified as components of vicilin of Vicia faba (Wright, 1974) with only a trace of 68,000 mol. wt. subunits.

FIG. 28

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of *Vicia faba*.

FIG. 29

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.28.

Track TE : *Vicia faba* total protein extract

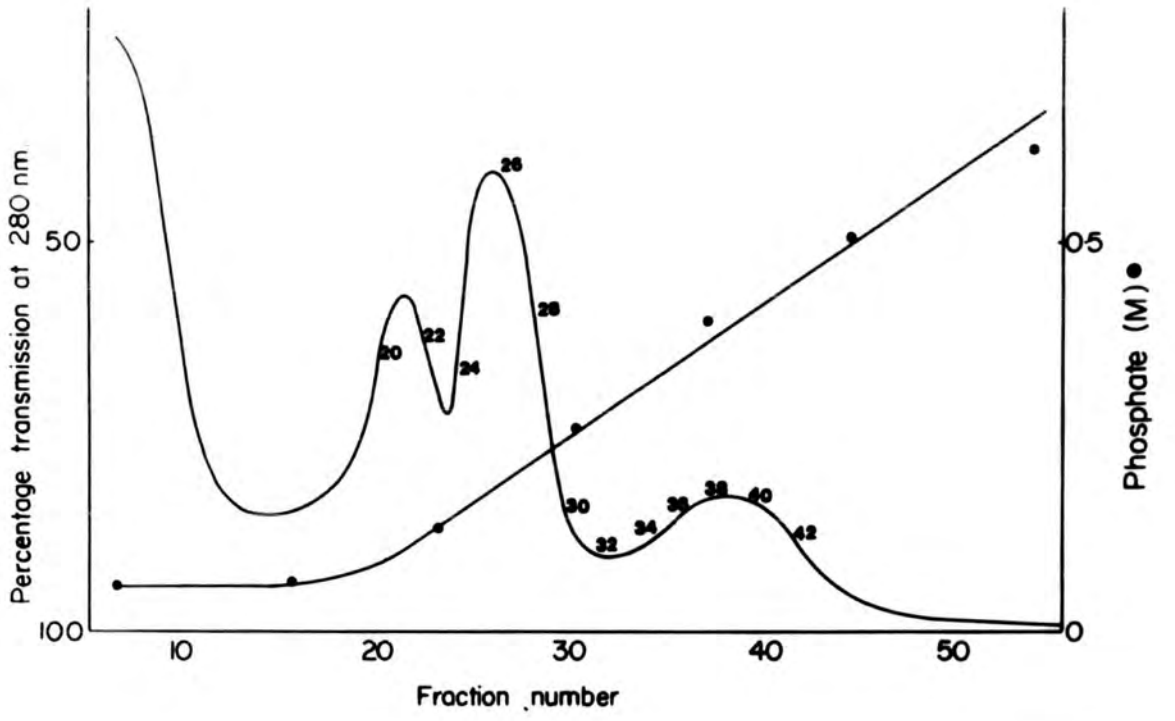

Percentage transmission at 280 nm
50
100
10
20
30
40
50
Fraction number
0·5
0
Phosphate (M)
20
22
24
26
28
30
32
34
36
38
40
42

Fig. 28

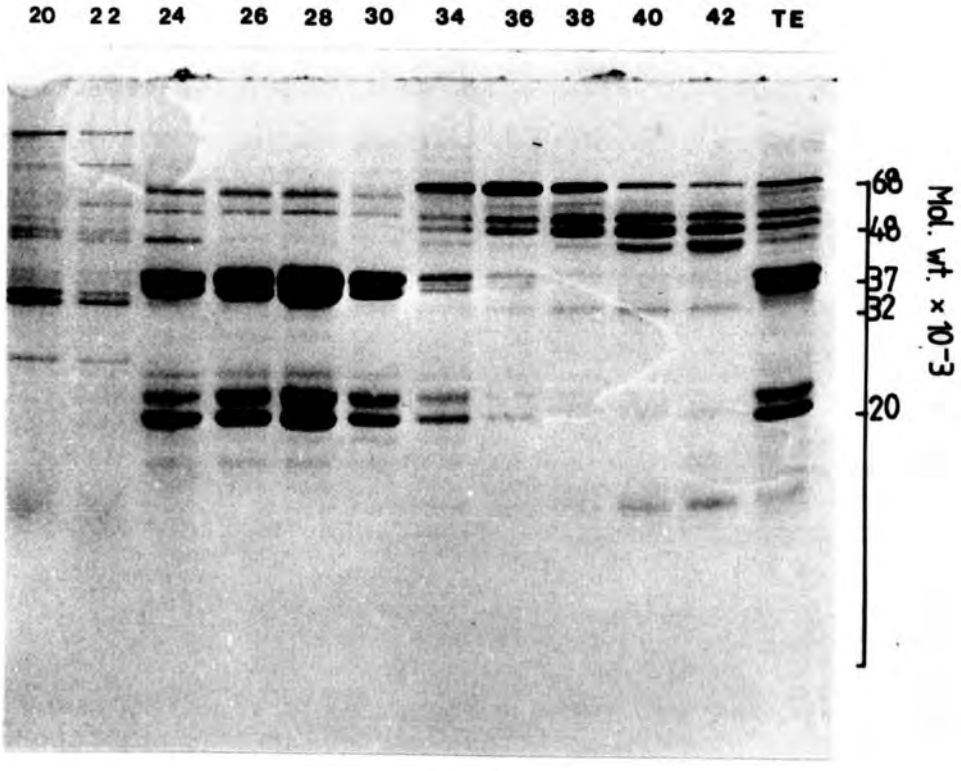

20
22
24
26
28
30
34
36
38
40
42
TE
68
48
37
32
20
Mol. wt. × 10-3

Fig.29

FIG. 30

SDS-polyacrylamide gel electrophoresis of various protein fractions and convicilin.

Track TE : Total protein extract
Track L : purified legumin
Track V : purified vicilin
Track C : purified convicilin

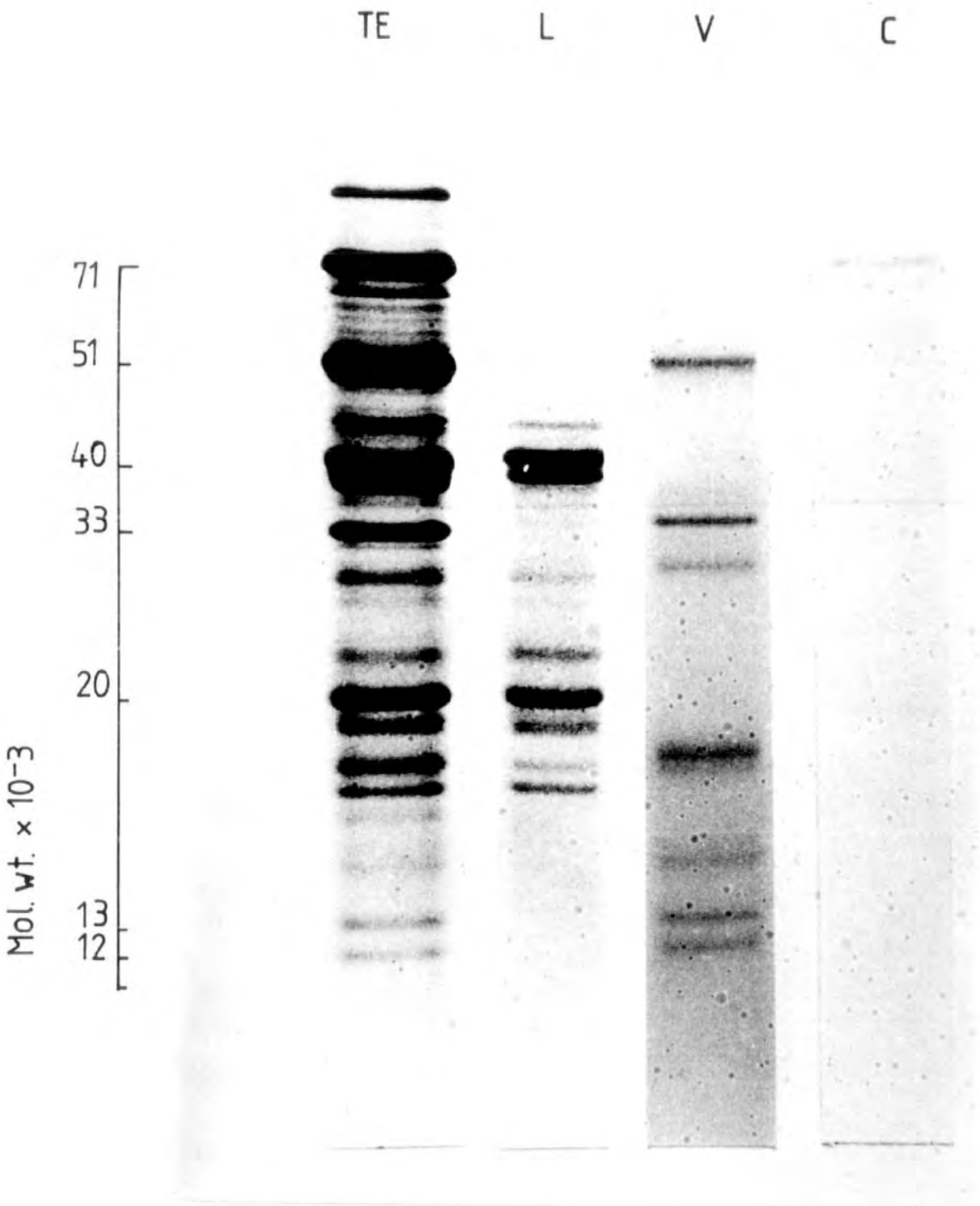


Fig. 30

II. MAJOR STORAGE PROTEIN CHANGES DURING SEED DEVELOPMENT

When a total protein extract of Pisum sativum meal, from mature seeds, was chromatographed on a column of hydroxylapatite, the elution profile shown in Fig.31 was obtained. A large peak of non-protein, pigment containing material was eluted prior to application of a linear salt gradient. Following this, four peaks of protein were eluted. On analysis by SDS-polyacrylamide gel electrophoresis (Fig.32), it was found that the first peak eluting between 0.1M - 0.175M phosphate contained 24,000 and 17,500 mol. wt. subunits. The second peak eluting between 0.175M - 0.275M phosphate consisted of predominantly 40,000 and 20,000 mol. wt. subunits. The third, small, peak contained mainly 71,000 mol. wt. subunits with 51,000 and 33,000 mol. wt. subunits and eluted over the range 0.3M - 0.375M phosphate. Finally the fourth peak eluting between 0.375M to 0.4M phosphate contained predominantly 51,000 and 33,000 mol. wt. subunits with 71,000 mol. wt. subunits.

This method was then used to analyse the major storage proteins at different stages of seed development.

Hydroxylapatite chromatography of a total protein extract prepared from peas, harvested 11 days after flowering, gave the elution profile shown in Fig.33. After application of a linear salt gradient, three peaks of protein were eluted. The first of these was asymmetrical and the largest, followed by two much smaller peaks. It was shown that the trailing edge of the first peak consisted of protein with subunits of approximately 32,000 mol. wt. (Fig.34). The second peak

FIG. 31

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of *Pisum sativum* meal from mature seeds.

FIG. 32

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.31.

Tracks 1-4	:	fractions in areas 1-4
Track MW	:	molecular weight - standard proteins (bovine serum albumin, ovalbumin and cytochrome C)
Track A	:	standard albumin fraction
Track L	:	standard legumin preparation
Track Le	:	standard lectin preparation
Track C+V	:	standard convicilin and vicilin preparations

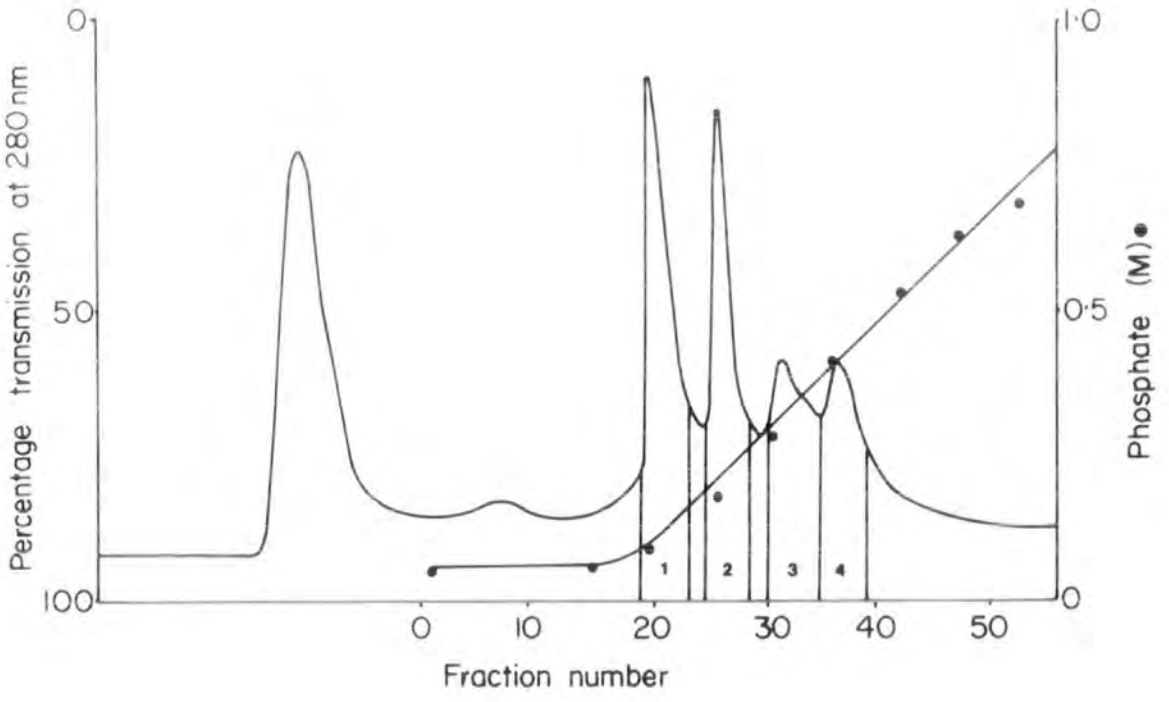

Percentage transmission at 280nm
0
50
100
1·0
0·5
0
Phosphate (M)●
0
10
20
30
40
50
Fraction number
1
2
3
4

Fig. 31

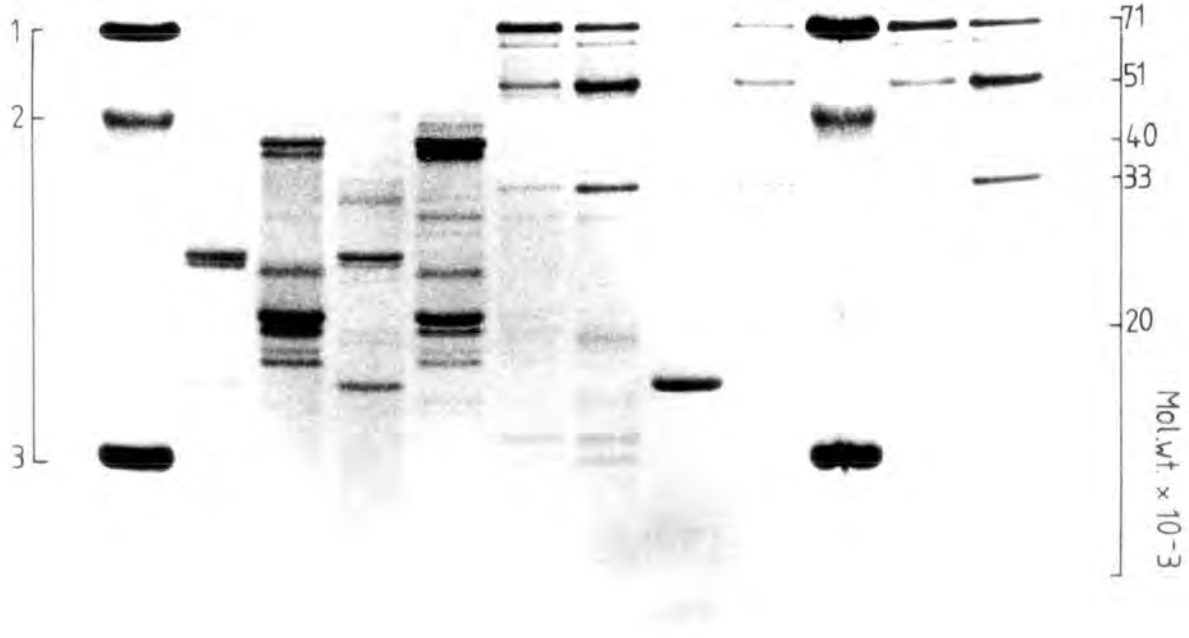

Mw
A
L
1
2
3
4
Le
C+V
Mw
3
4
1
2
3
71
51
40
33
20
Mol.wt × 10-3

Fig. 32

FIG. 33

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of Pisum sativum, 11 days after flowering.

FIG. 34

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.33.

Tracks 1-8	:	fractions in areas 1-8
Track 11/TE	:	unchromatographed total protein extract of peas, 11 days after flowering
Track A	:	standard albumin fraction
Track L	:	standard legumin preparation
Track Le	:	standard lectin preparation
Track V+C	:	standard vicilin and convicilin preparations
Track TE	:	standard total protein extract of mature peas

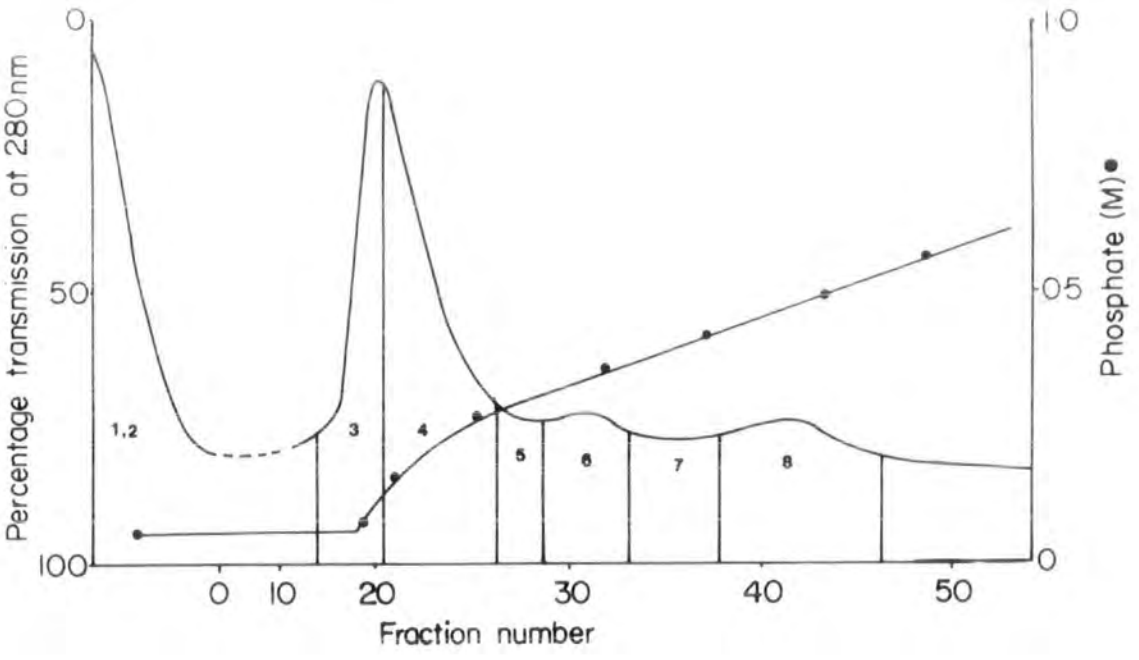

Percentage transmission at 280nm
0
50
100
1.0
0.5
0
Phosphate (M)•
1,2
3
4
5
6
7
8
0
10
20
30
40
50
Fraction number

Fig. 33

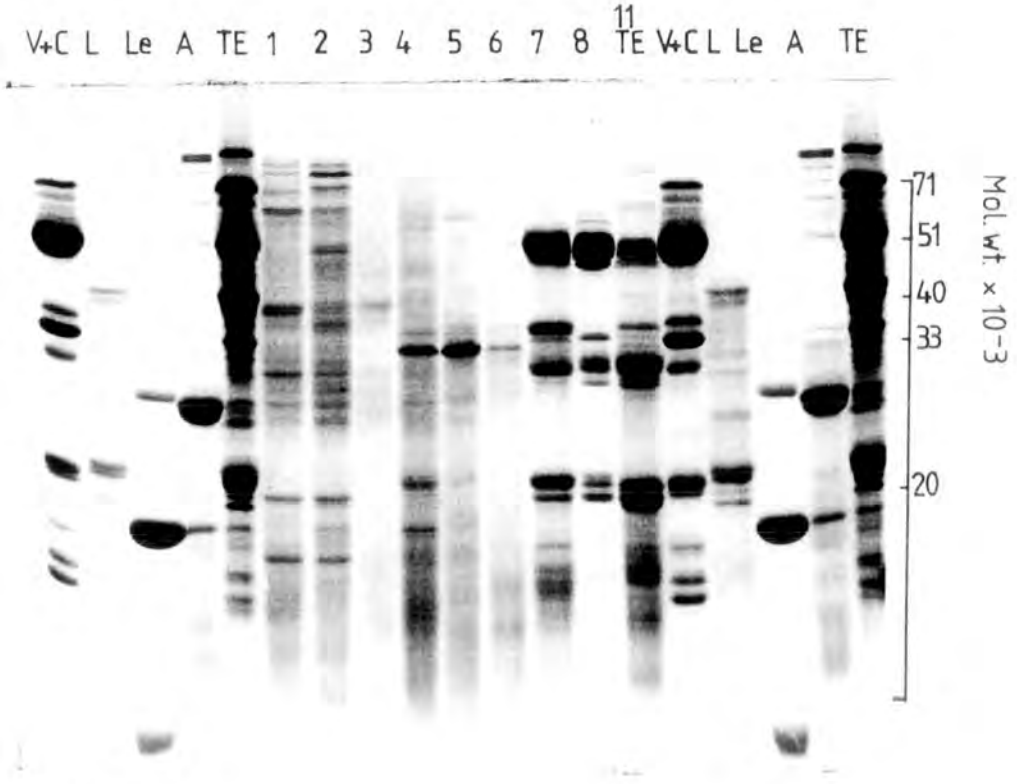

V+C L Le A TE 1 2 3 4 5 6 7 8 11 TE V+C L Le A TE
71
51
40
33
20
Mol.wt. x 10-3

Fig. 34

contained very little protein material as shown by the gel. Following this, protein with subunits of approximately 50,000, 47,000, 35,000, 30,000, 21,000, 20,000 and lower mol. wts. was eluted. The third peak contained mainly 50,000, 47,000, 34,000, 30,000, 21,000, 20,000 and lower mol. wt. subunits. A non-chromatographed total protein extract was shown to consist of mainly 50,000, 47,000, 35,000, 30,000, 21,000, 20,000 and lower mol. wt. protein subunits.

The difference in relative mobility of the protein subunit seen in the trailing edge of the large asymmetrical peak (see areas 4 and 5 of Fig.33), compared to that of the 33,000 mol. wt. subunit seen in mature vicilin, was demonstrated by running this sample alongside a standard vicilin preparation on a separate SDS-polyacrylamide gel, see Fig.46.

The elution profile for peas harvested 13 days after flowering is shown in Fig.35. As with the 11 day interval, three main protein peaks were in evidence. The first peak was almost symmetrical and still the largest. The second peak showed at least a threefold increase in size and the third peak was twice as large as that seen at 11 days, even though the protein from the same weight of extracted material was loaded on to the hydroxylapatite column in each case. On gel analysis, Fig.36, the first peak was shown to consist mainly of 24,000 mol. wt. protein subunits, with a trace of 17,500 mol. wt. protein subunits in the trailing edge of this peak. The second peak contained 32,000 mol. wt. protein subunits, similar on analysis by gels, to those

FIG. 35

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of *Pisum sativum*, 13 days after flowering.

FIG. 36

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.35.

Tracks 1-9	:	fractions in areas 1-9
Track 13/TE	:	unchromatographed total protein extract of peas, 13 days after flowering
Track A	:	standard albumin fraction
Track L	:	standard legumin preparation
Track Le	:	standard lectin preparation
Track V+C	:	standard vicilin and convicilin preparations
Track TE	:	standard total protein extract of mature peas

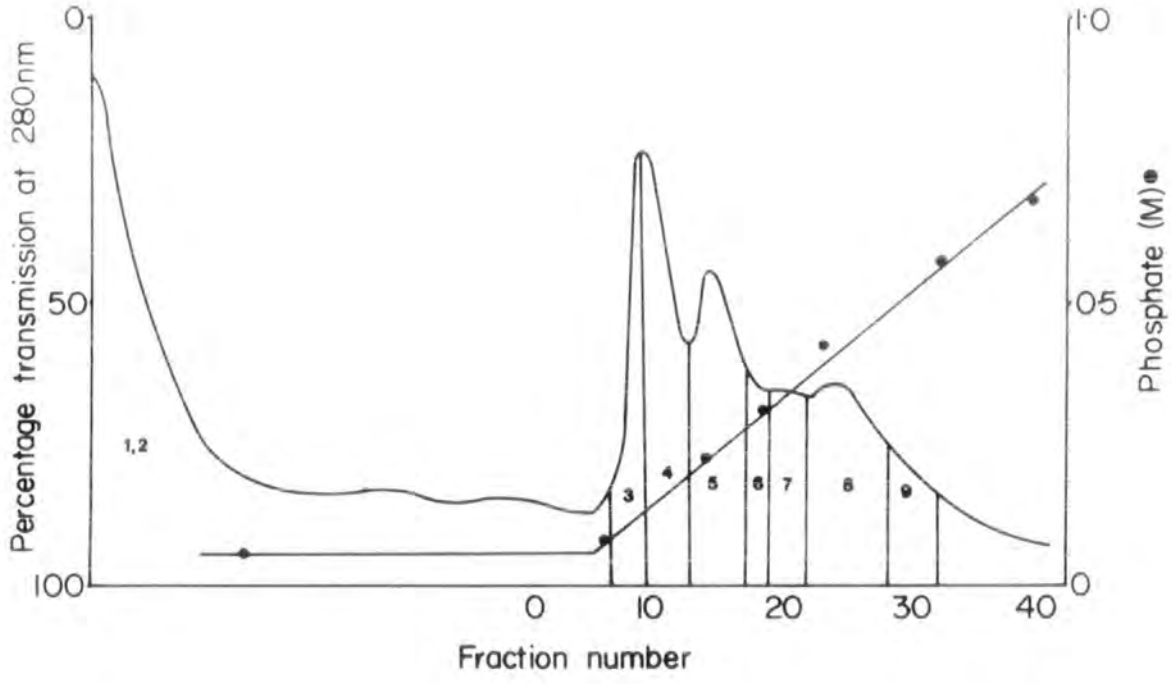

Percentage transmission at 280nm
0
50
100
1,2
3
4
5
6
7
8
9
0
10
20
30
40
Fraction number
1·0
·5
0
Phosphate (M)•

Fig. 35

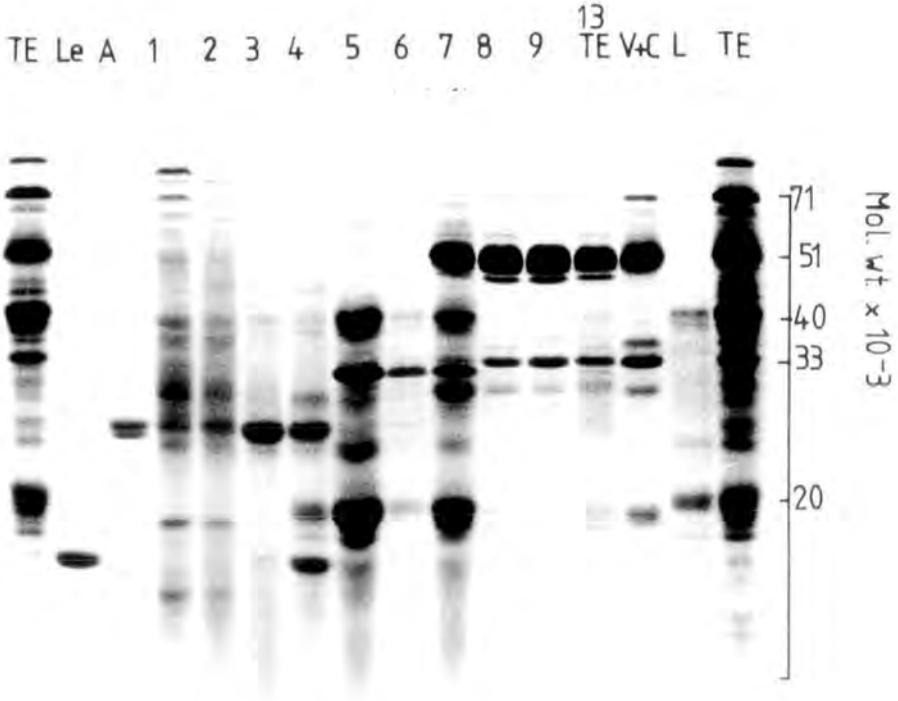

TE Le A 1 2 3 4 5 6 7 8 9 13 TE V+C L TE
71
51
40
33
20
Mol. wt x 10-3

Fig. 36

observed at day 11, see above, and in addition several protein subunits of approximately 40,000 and 20,000 mol. wt. The trough between the second and third peaks contained a 32,000 mol. wt. protein subunit. Area 7 (Fig.35) before the third peak was shown to contain various protein subunits of mol. wts. - 50,000, 40,000, 32,000, 30,000 and 20,000. Finally the centre and trailing edge of the third peak had a similar protein subunit pattern consisting of a trace band of 71,000 mol. wt. and main protein subunits of 50,000, 47,000, 33,000 and small amounts of 20,000 mol. wt. subunits. The unchromatographed total protein extract for peas, 13 days after flowering, had three main protein subunits of 50,000, 47,000 and 33,000 mol. wt.

The elution profile for peas, harvested 17 days after flowering, is shown in Fig.37. Four peaks of protein were apparent at this stage. The first and second were approximately the same height as seen at day 13, these were followed by a third small peak, then a larger fourth peak. The first peak on SDS-polyacrylamide gel analysis, Fig.38, consisted of two close protein subunit bands with an approximate mol. wt. of 24,000. The smaller of these subunits was predominant in the leading edge of the first peak, while increased amounts of the larger of the two subunits were located in the trailing edge. The second peak contained a large number of protein subunits of varying mol. wts., the most prominant were those with 40,000 and 20,000 mol. wts. Subunits of 32,000, approximately 24,000 and lower mol. wts. were also present in this peak. The

FIG. 37

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of *Pisum sativum*, 17 days after flowering.

FIG. 38

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig. 37.

Tracks 1-8	:	fractions in areas 1-8
Track 17/TE	:	unchromatographed total protein extract of peas, 17 days after flowering
Track A	:	standard albumin fraction
Track L	:	standard legumin preparation
Track Le	:	standard lectin preparation
Track V+C	:	standard vicilin and convicilin preparations
Track TE	:	standard total protein extract of mature peas

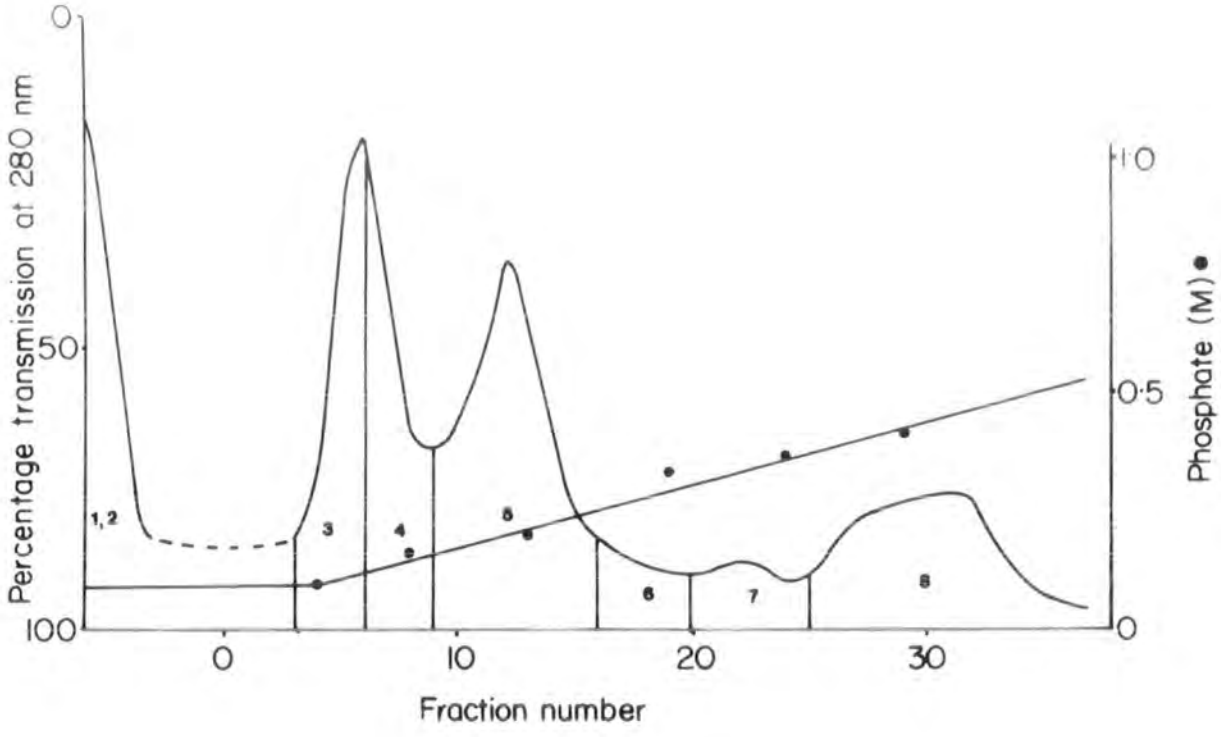

Percentage transmission at 280 nm
0
50
100
1,2
3
4
5
6
7
8
0
10
20
30
Fraction number
1·0
0·5
0
Phosphate (M)

Fig. 37

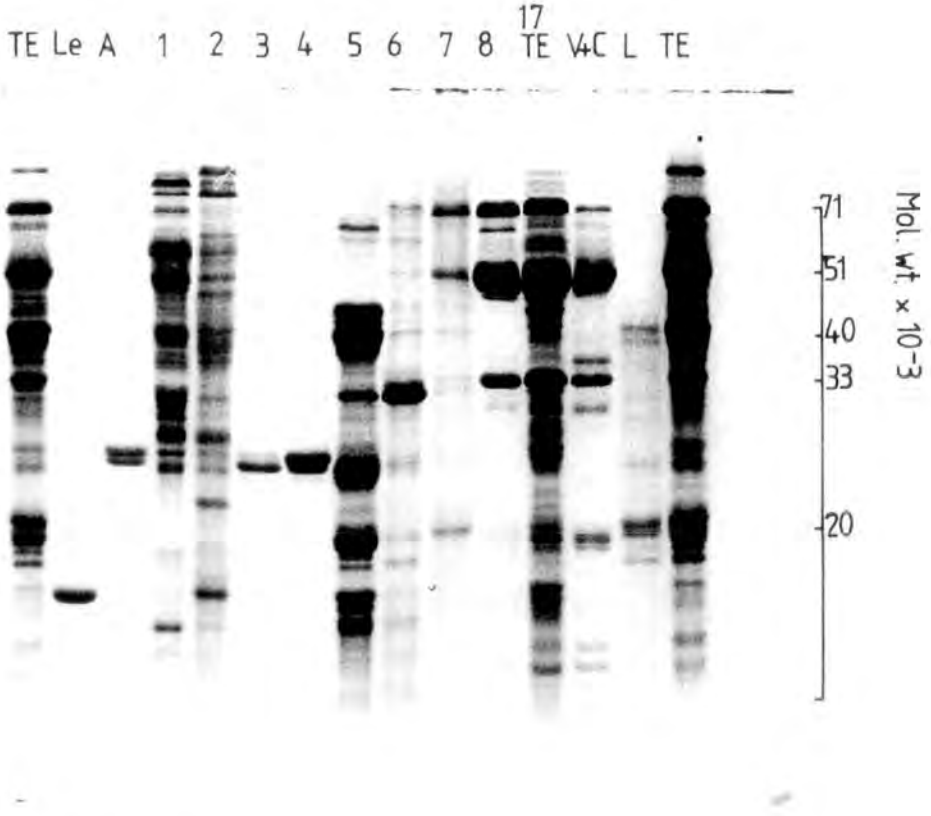

TE Le A 1 2 3 4 5 6 7 8 17 TE V4C L TE
71
51
40
33
20
Mol. wt. x 10-3

Fig. 38

trailing edge of the second peak was shown to consist of 32,000 mol. wt. protein subunits. Only traces of 71,000 and 50,000 mol. wt. subunits were detected in the third small peak. Finally the fourth peak was shown to consist of three main protein subunits of mol. wt. 71,000, 50,000 and 33,000 mol. wt. The unchromatographed total protein extract for peas, 17 days after flowering, had main protein subunits of 71,000, 50,000 and 33,000 mol. wt. with additional subunits of intermediate and lower mol. wts.

Hydroxylapatite chromatography of a total protein extract prepared from peas, 21 days after flowering, gave the elution profile shown in Fig.39. This featured three closely associated peaks, followed by a broader fourth peak of protein. In this elution profile the second peak was the largest. On analysis by SDS-polyacrylamide gel electrophoresis, Fig.40, the first peak contained mainly 24,000 mol. wt. protein subunits and the trough between the first and second peak contained mainly 17,500 mol. wt. protein subunits. The second peak was shown to consist of predominantly 40,000 and 20,000 mol. wt. protein subunits. The third peak also contained 40,000 and 20,000 mol. wt. protein subunits with a trace of 32,000 mol. wt. protein subunit. The leading edge of the broader fourth peak consisted of mainly 71,000 mol. wt. protein subunits with 68,000, 50,000 and 33,000 mol. wt. subunits present in lesser amounts. Finally the trailing edge of the fourth peak contained decreased amounts of 71,000 and 68,000 mol. wt. subunits and increased amounts of 50,000 and 33,000 mol. wt.

FIG. 39

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of *Pisum sativum*, 21 days after flowering.

FIG. 40

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.39.

Tracks 1-10	:	fractions in areas 1-10
Track 21/TE	:	unchromatographed total protein extract of peas, 21 days after flowering
Track A	:	standard albumin fraction
Track L	:	standard legumin preparation
Track Le	:	standard lectin preparation
Track V+C	:	standard vicilin and convicilin preparations
Track TE	:	standard total protein extract of mature peas

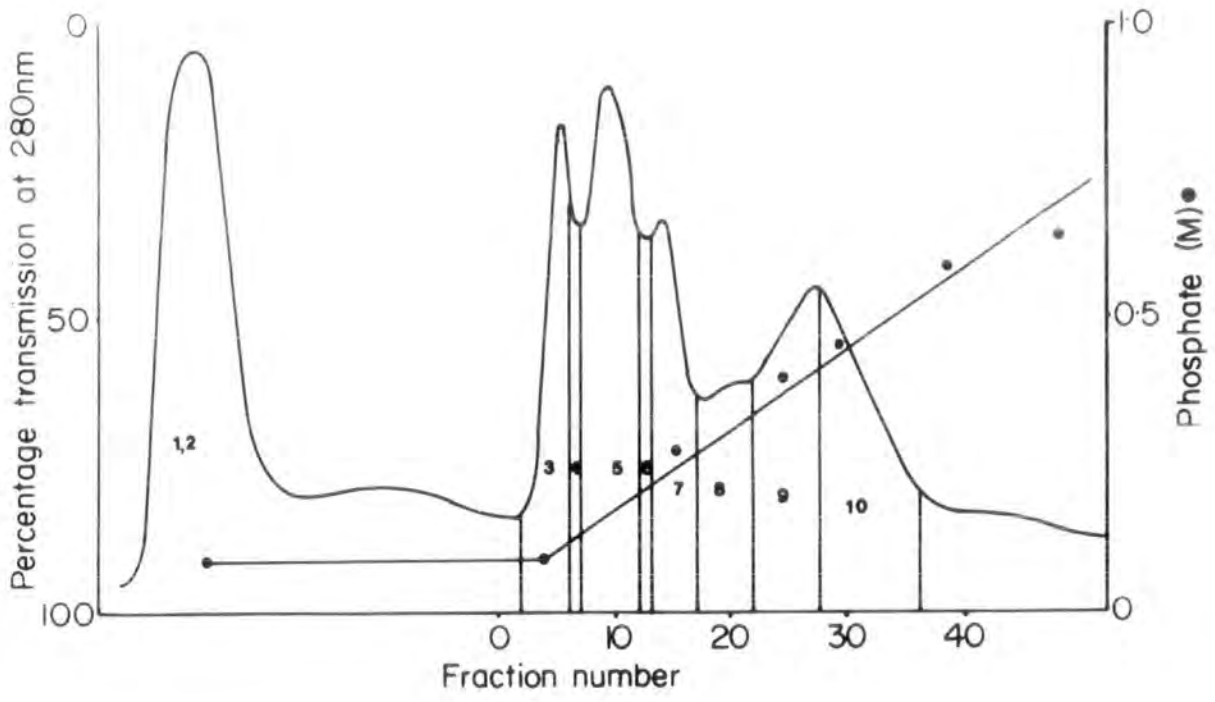

Percentage transmission at 280nm
0
50
100
1,2
3
4
5
6
7
8
9
10
0
10
20
30
40
Fraction number
1·0
0·5
0
Phosphate (M)

Fig. 39

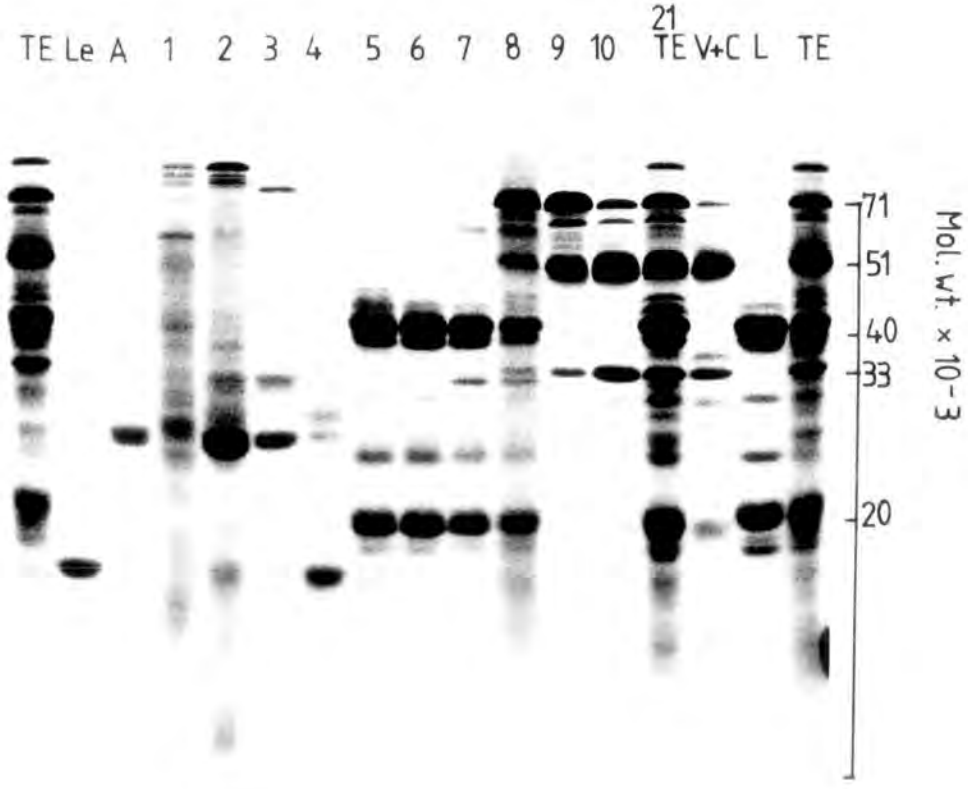

TE Le A 1 2 3 4 5 6 7 8 9 10 21 TE V+C L TE
71
51
40
33
20
Mol. wt. × 10-3

Fig. 40

protein subunits. The unchromatographed total protein extract resembled the standard total protein extract in containing a range of protein subunits of mol. wts. - 71,000, 68,000, 50,000, 40,000, 33,000, 20,000 and lesser amounts of other protein subunits with intermediate and lower mol. wts. than this range.

At 33 days after flowering, the elution profile of a total protein extract from peas,as shown in Fig.41, still featured four peaks of protein, eluted after application of a linear salt gradient, as seen for 21 days after flowering. The first three peaks were still closely associated, followed by a broader fourth peak. The peaks were all larger than seen for the 21 day interval but the third peak was the largest, compared to the second peak being the largest at day 21. Analysis of the peaks by SDS-polyacrylamide gel electrophoresis (Fig.42) showed that the first peak contained protein subunits of 24,000 mol. wt. The second peak consisted of 17,500 mol. wt. subunits. The trough between the second and third peak was shown to have 40,000, 20,000 and 17,500 mol. wt. protein subunits. The third peak consisted mainly of 40,000 and 20,000 mol. wt. subunits. The trough between the third and fourth peak had a mixture of 71,000, 50,000, 40,000, 20,000 and lesser amounts of other protein subunits. The leading edge of the fourth peak contained a large amount of 71,000 mol. wt. protein subunit, as well as 50,000 and 33,000 mol. wt. subunits. Finally the trailing edge of the fourth peak had predominantly 50,000 and 33,000 mol. wt. protein subunits with a decreased amount of 71,000 mol. wt.

FIG. 41

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of *Pisum sativum*, 33 days after flowering.

FIG. 42

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.41.

Tracks 1-9	:	fractions in areas 1-9
Track 33/TE	:	unchromatographed total protein extract of peas, 33 days after flowering
Track A	:	standard albumin fraction
Track L	:	standard legumin preparation
Track Le	:	standard lectin preparation
Track V+C	:	standard vicilin and convicilin preparations
Track TE	:	standard total protein extract of mature peas

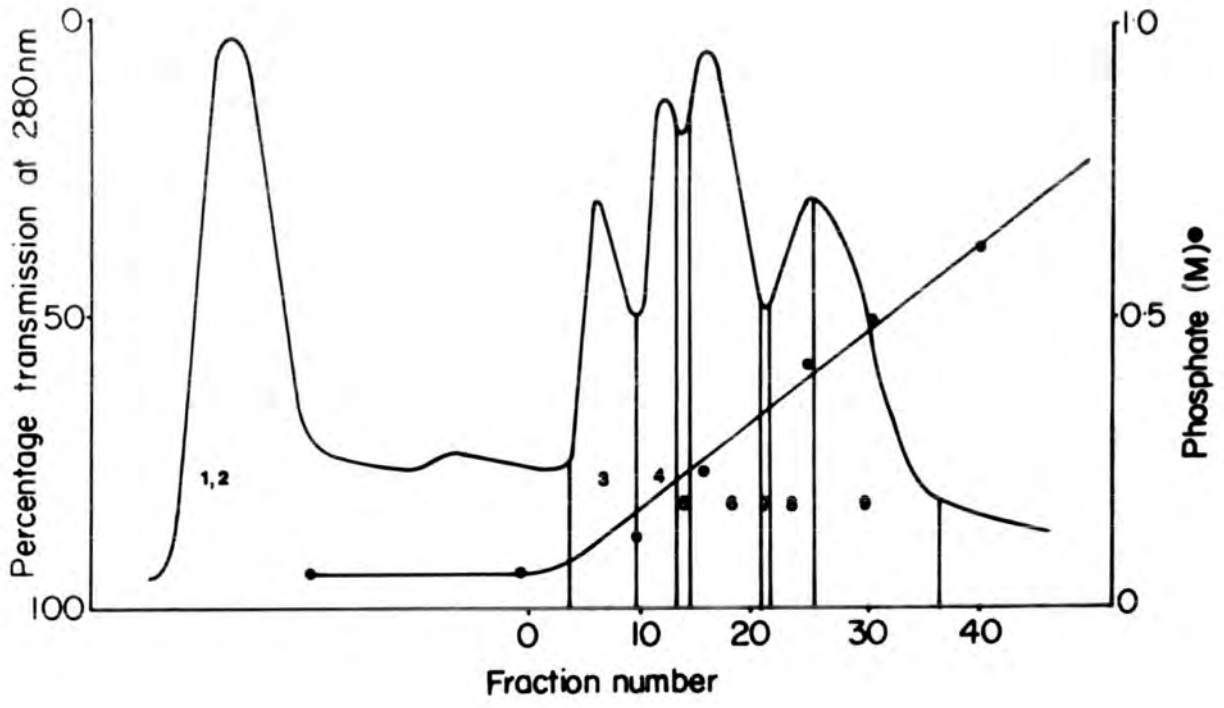

Percentage transmission at 280nm
0
50
100
1,2
3
4
0
10
20
30
40
Fraction number
1·0
0·5
0
Phosphate (M)

Fig. 41

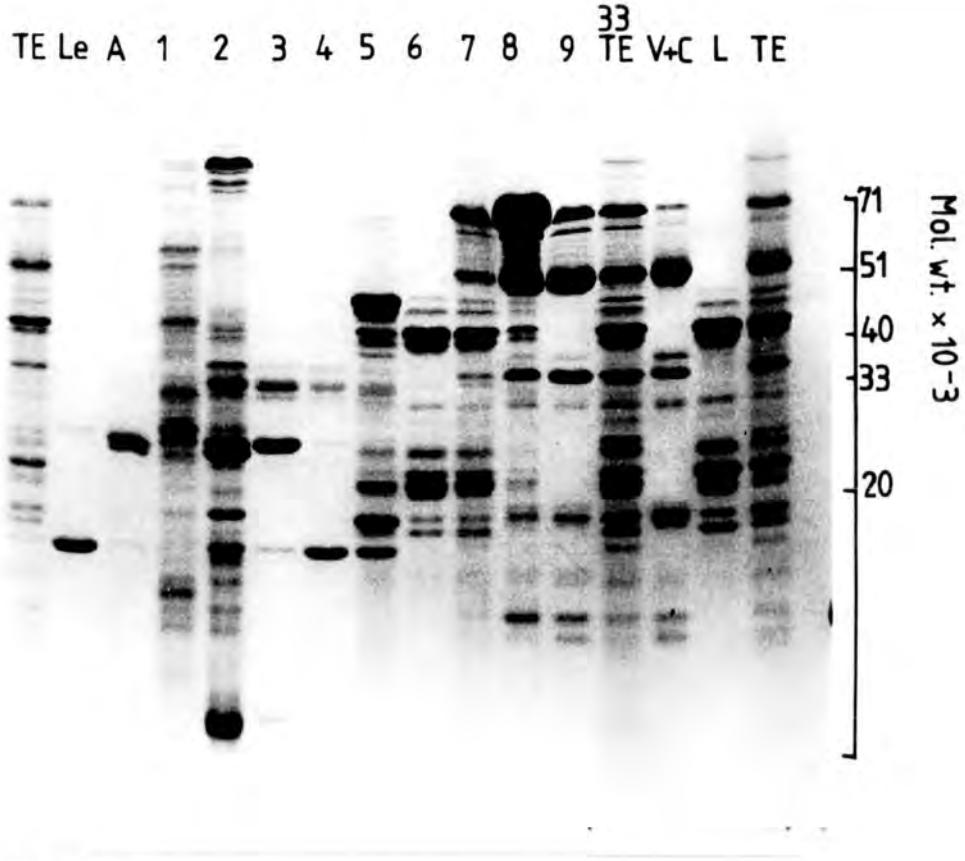

TE Le A 1 2 3 4 5 6 7 8 9 33 TE V+C L TE
71
51
40
33
20
Mol. wt. x 10-3

Fig. 42

protein subunit as compared to the leading edge of this peak. The unchromatographed total protein extract at 33 days after flowering contained all the protein subunits seen in a standard total protein extract i.e. 71,000, 68,000, 50,000, 40,000, 33,000, 20,000 and lower mol. wt. subunits.

For convenience of comparison the vicilin containing fractions from peas harvested at 11, 13, 17, 21 and 33 days after flowering, were analysed on separate gels, at two protein concentrations, see Fig. 43 and Fig.44. With the increased resolution obtained at lower sample loadings, (Fig.43), it was possible to observe a double subunit band pattern of about 50,000 mol. wt. and an additional protein subunit of approximately 47,000 mol. wt. at 11 days after flowering. While at the higher sample loading (Fig. 44), several protein subunits of 33,000 and lower mol. wts.were evident, with a different pattern compared to that seen for a standard mature vicilin preparation. Trace amounts of a 71,000 mol. wt. protein subunit were detected in area 13.9 (Fig.35) of the elution profile obtained after hydroxylapatite chromatography of a total protein extract of peas 13 days after flowering. The protein subunit pattern of vicilin at 17 days after flowering resembled that of the standard, mature vicilin. Vicilin tracks were cut from this slab gel (Fig.44) and densitometric scans obtained in order to demonstrate the changing pattern of vicilin subunits with development time and also to determine the 51,000/33,000 subunit area ratios (Table 2).

Similarly selected legumin containing fractions or those

FIG. 43

SDS-polyacrylamide gel electrophoresis of selected vicilin containing fractions, obtained by hydroxylapatite chromatography of developing pea total protein extracts.

Track 1 : fractions in area 8 (Fig.33),11 days after flowering

Track 2 : fractions in area 9 (Fig.35),13 days after flowering

Track 3 : fractions in area 8 (Fig.37),17 days after flowering

Track 4 : fractions in area 10 (Fig.39),21 days after flowering

Track 5 : fractions in area 9 (Fig.41),33 days after flowering

Track V+C : standard vicilin and convicilin preparations

Track V : standard vicilin preparation

Tracks 1-5 were loaded with sample at 1/4 normal concentration.

FIG. 44

SDS-polyacrylamide gel electrophoresis of selected vicilin containing fractions, obtained by hydroxylapatite chromatography of developing pea total protein extracts.

Tracks 1-5 : as for Fig.43 but at normal sample concentration

Track V+C : standard vicilin and convicilin preparations

Track V : standard vicilin preparation

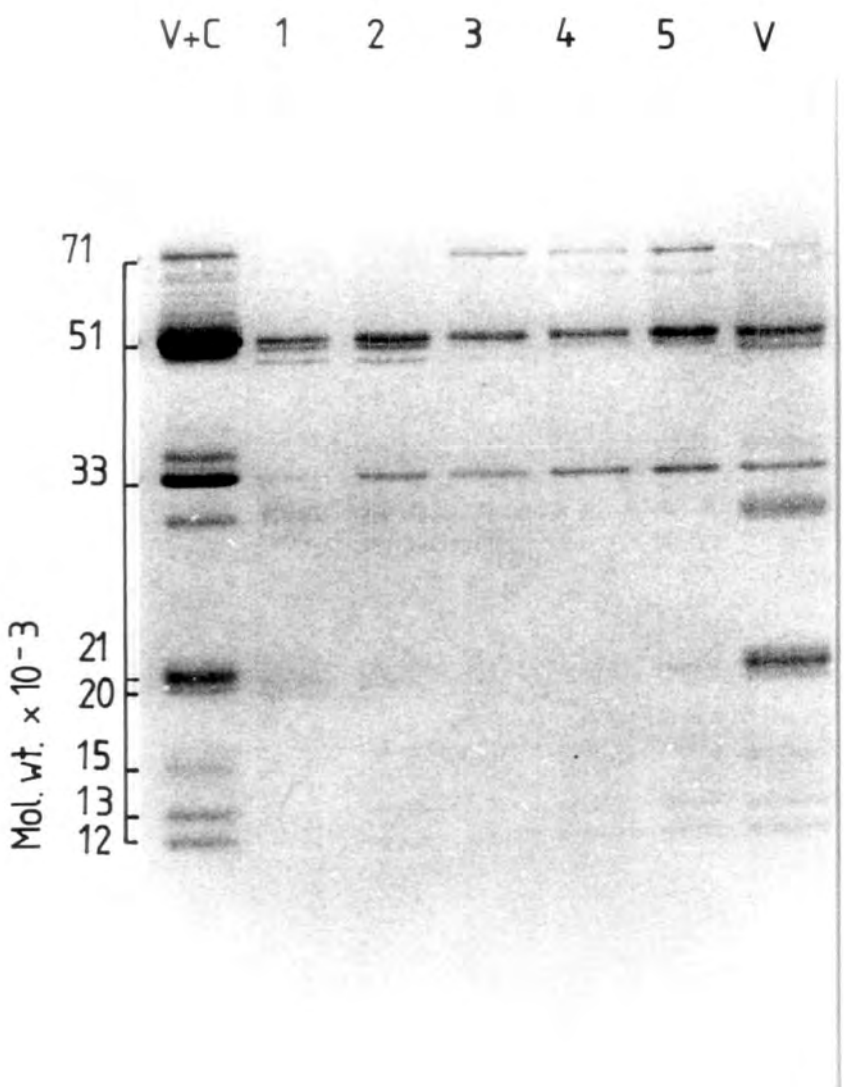

V+C
1
2
3
4
5
V
71
51
33
21
20
15
13
12
Mol. wt. x 10-3

Fig. 43

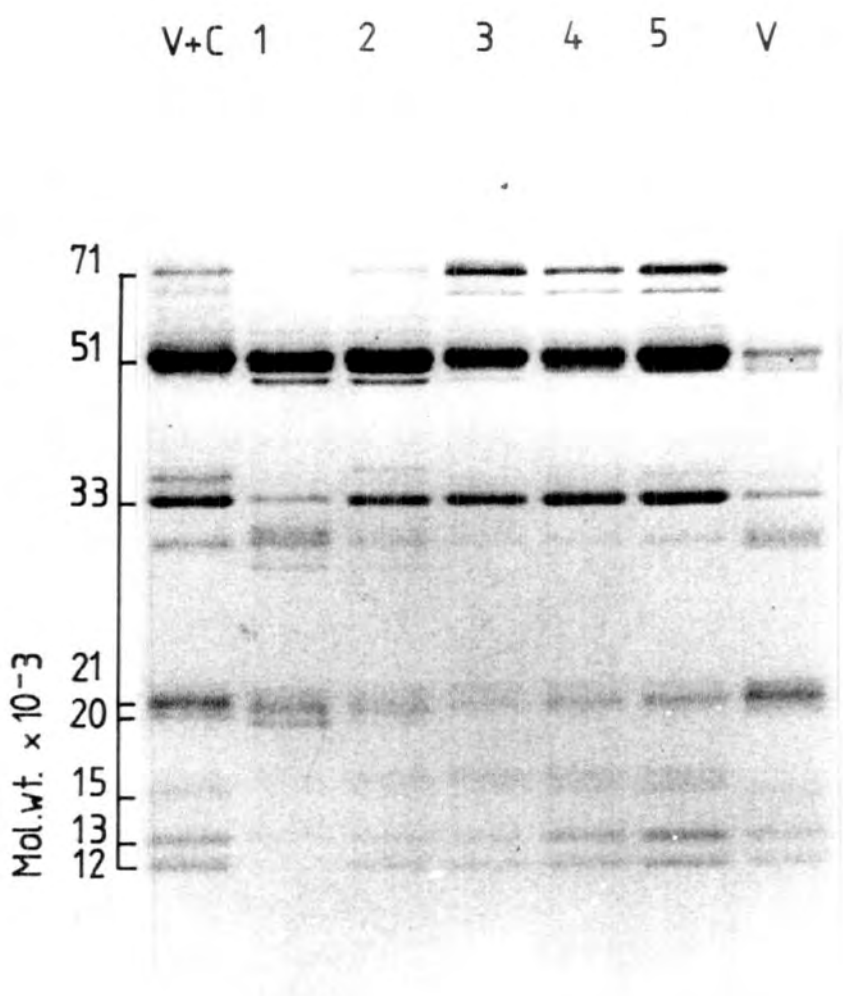

V+C
1
2
3
4
5
V
71
51
33
21
20
15
13
12
Mol.wt. x 10-3

Fig. 44

Time after flowering	Area peak A	Area peak B	B/A
11 days	0.53	4.73	8.92
13 days	2.01	5.45	2.71
17 days	1.98	5.15	2.60
21 days	2.58	3.92	1.52
33 days	3.57	5.23	1.46

Table 2

Determination of 51,000/33,000 subunit area ratios from densitometric scans of vicilin fractions obtained at various stages of development (Fig.48).

protein fractions from developing seeds, which eluted at approximately the same phosphate concentration required to elute legumin from protein extracts of mature seeds, were analysed on a single SDS-polyacrylamide slab gel (Fig.45).

Lastly, the unchromatographed total protein extracts of peas 11, 13, 17, 21 and 33 days after flowering were analysed on a single SDS-polyacrylamide slab gel (Fig.47) with various protein standards.

FIG. 45

SDS-polyacrylamide gel electrophoresis of selected legumin containing fractions (or fractions eluted at the approximate phosphate concentration required to elute legumin from mature seed total protein extracts), obtained by hydroxylapatite chromatography of developing pea total protein extracts.

Track 1 : fractions in area 4 (Fig.33),11 days after flowering
Track 2 : fractions in area 5 (Fig.35),13 days after flowering
Track 3 : fractions in area 5 (Fig.37),17 days after flowering
Track 4 : fractions in area 5 (Fig.39),21 days after flowering
Track 5 : fractions in area 6 (Fig.41),33 days after flowering

Track L : standard legumin preparation
Track A : standard albumin fraction preparation
Track Le : standard lectin preparation
Track V+C : standard vicilin and convicilin preparations

FIG. 47

SDS-polyacrylamide gel electrophoresis of unchromatographed total protein extracts of peas at various stages of development.

Track 1 : total protein extract of peas,11 days after flowering
Track 2 : total protein extract of peas,13 days after flowering
Track 3 : total protein extract of peas,17 days after flowering
Track 4 : total protein extract of peas,21 days after flowering
Track 5 : total protein extract of peas,33 days after flowering

Track V+C : standard vicilin and convicilin preparations
Track A : standard albumin fraction preparation
Track L : standard legumin preparation
Track Le : standard lectin preparation

FIG. 46

SDS-polyacrylamide gel electrophoresis of the fractions in area 4 of the elution profile shown in Fig.33 (11 days after flowering).

Track 1 : fractions in area 4 (Fig.33),11 days after flowering
Track V+C : standard vicilin and convicilin preparations

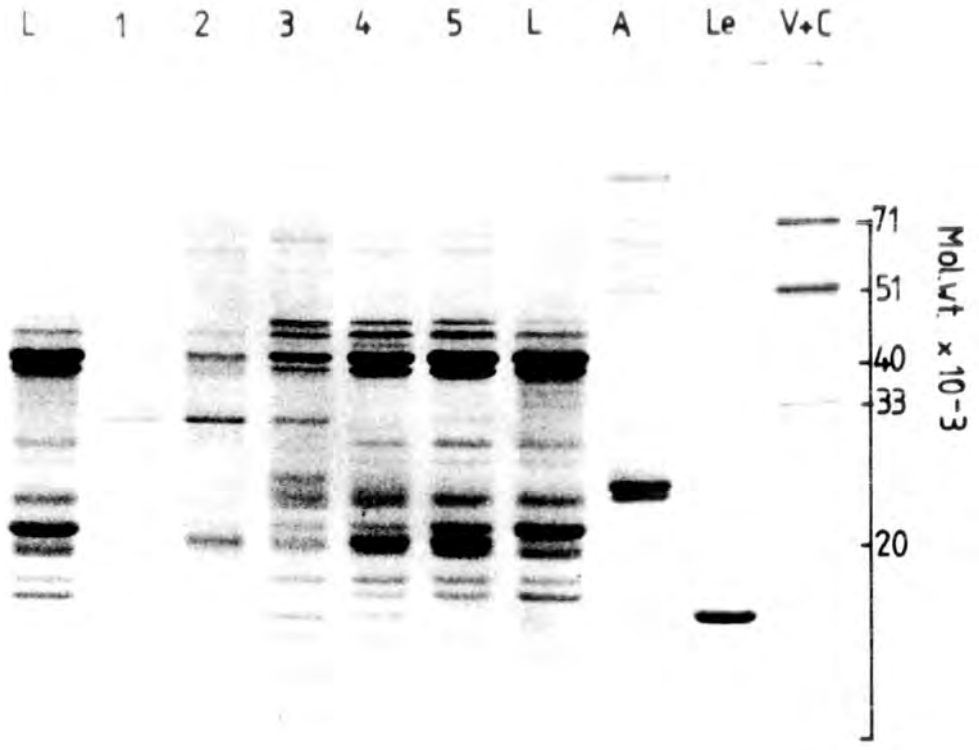


Fig. 45

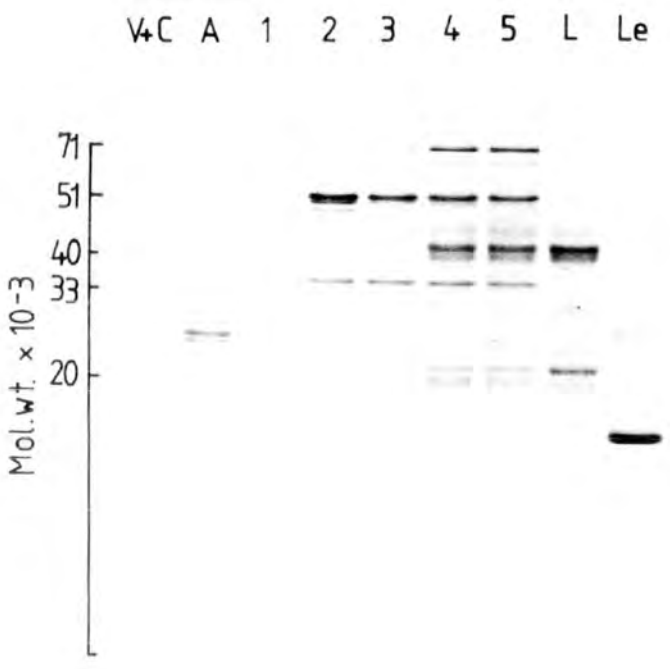


Fig. 47

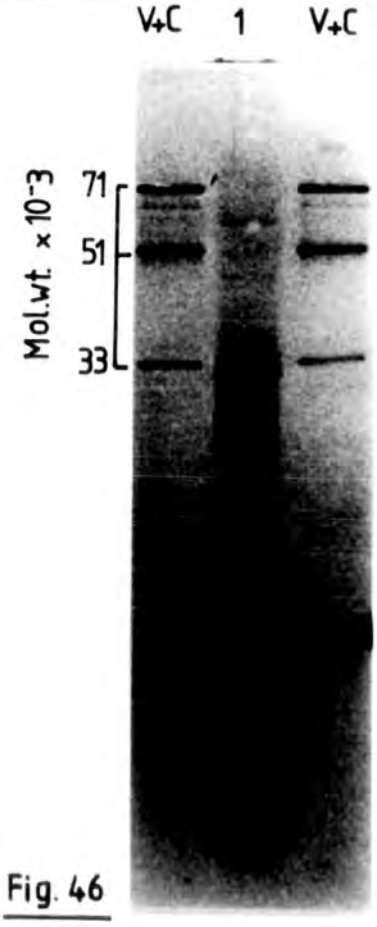


Fig. 46

FIG. 48

Densitometric scans of the gel tracks 1-5 shown in Fig.44. A, B and C represent the 33,000, 51,000 and 71,000 mol. wt. protein subunits respectively.

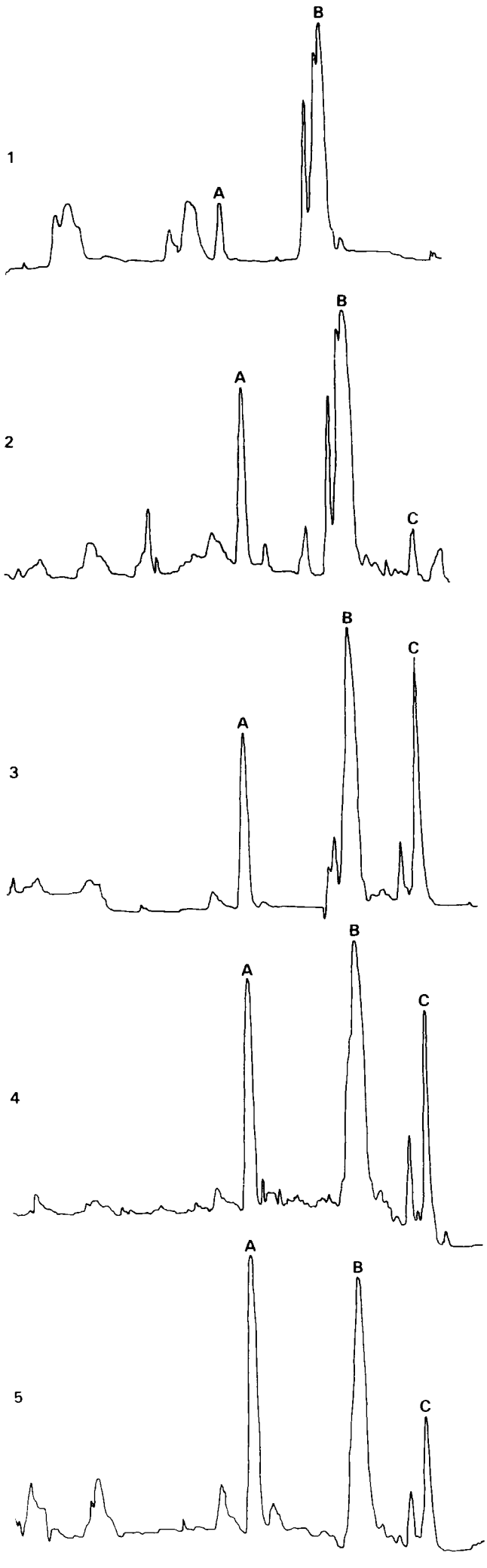


Fig 48

III. MAJOR STORAGE PROTEIN CHANGES DURING SEED GERMINATION

A. Hydroxylapatite chromatography of germinating seed extracts

The results of hydroxylapatite chromatography of a total protein extract of mature seeds have been described in the previous section (II).

Hydroxylapatite chromatography of a total protein extract prepared from peas, three days after germination, gave an elution profile with four peaks of protein, after application of the linear salt gradient, see Fig.49. The first peak was shown by SDS-polyacrylamide gel electrophoresis (Fig.50), to contain protein subunits with approximate mol. wts. of 24,000 and 17,500. The second and largest peak contained mainly 40,000 and 20,000 mol. wt. subunits. Following this, the third smaller peak also contained protein subunits of 40,000 and 20,000 mol. wt. with increased amounts of additional protein subunits of approximately 33,000, 30,000 and 23,000 mol. wt. as compared to the second peak. The leading edge of the fourth broad peak was shown to contain 71,000 mol. wt. subunits, several protein subunits with a mol. wt. range of 65 - 50,000 and at least two protein subunits of about 33,000, 30,000 and 20,000 mol. wt. The unchromatographed total protein extract was shown to consist mainly of protein subunits with mol. wts. of 71,000, 51,000, 40,000, 33,000, 30,000, 23,000 and 20,000. The 51,000 and 40,000 mol. wt. subunits occurred in more than one form which differed slightly in mol. wt. However, resolution of these varied and was often

FIG. 49

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of Pisum sativum, after 3 days of germination.

FIG. 50

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.49.

Tracks 1-8	:	fractions in areas 1-8
Track 3/TE	:	unchromatographed total protein extract of Pisum sativum after 3 days of germination
Track G	:	standard globulin protein preparation
Track L	:	standard legumin preparation
Track A	:	standard albumin fraction preparation

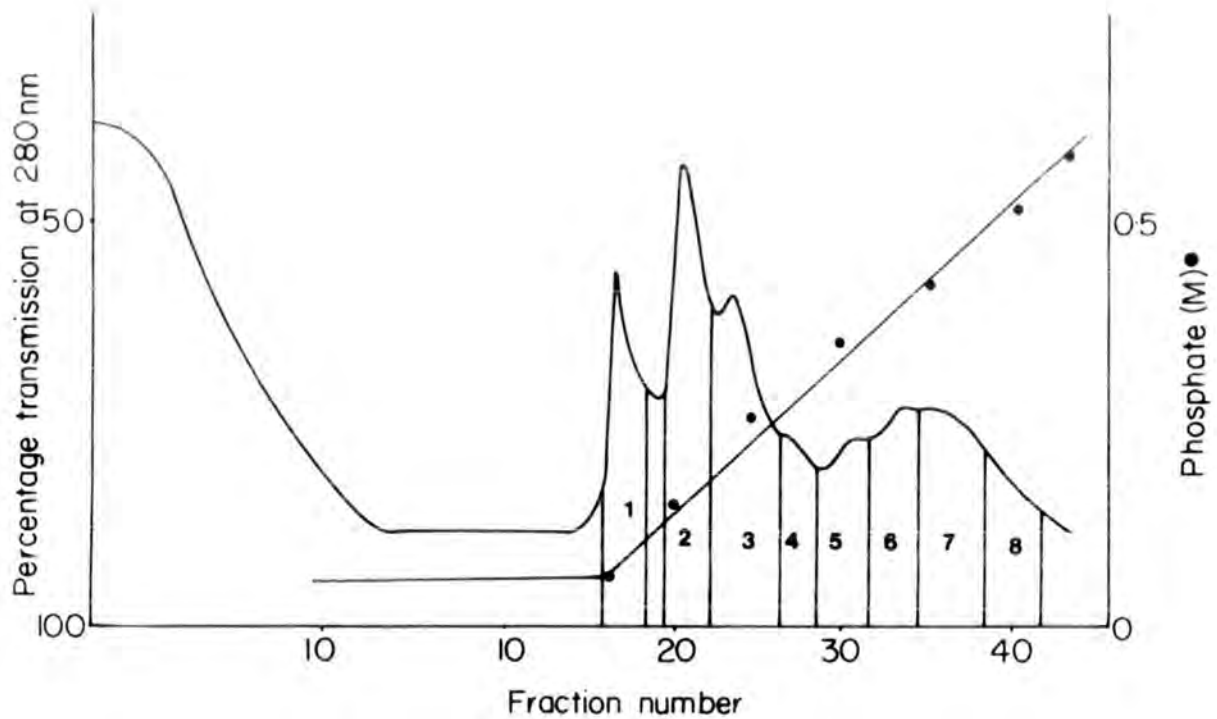

Percentage transmission at 280nm
50
100
10
10
20
30
40
Fraction number
0·5
0
Phosphate (M)
1
2
3
4
5
6
7
8

Fig. 49

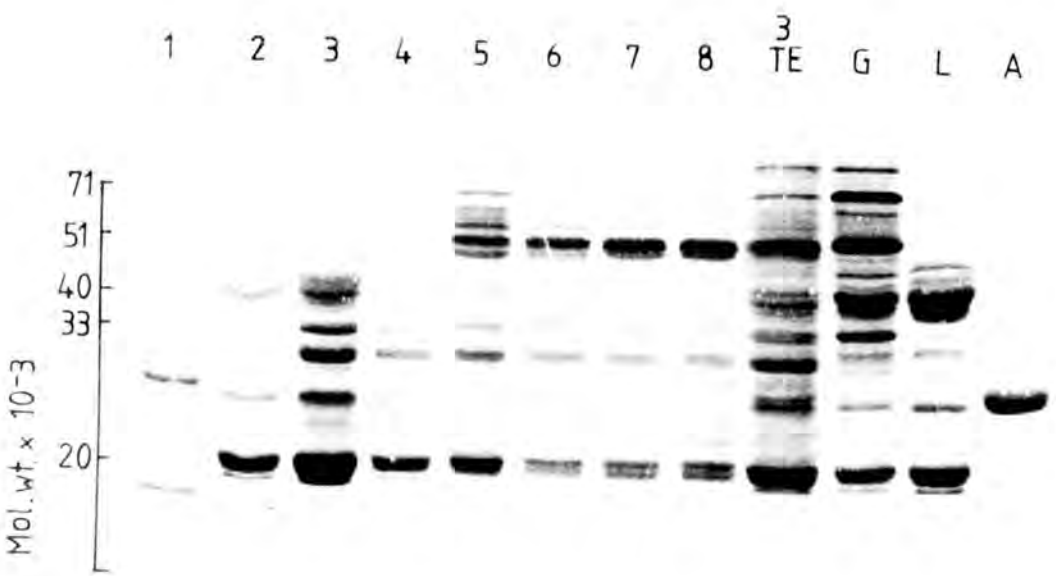

1
2
3
4
5
6
7
8
3
TE
G
L
A
71
51
40
33
20
Mol. wt. x 10-3

Fig. 50

incomplete. Decreased amounts of the 71,000, 40,000 and 33,000 mol. wt. subunits were present in the total protein extract, 3 days after germination, as compared to the amounts seen in the mature seed total protein extract.

Hydroxylapatite chromatography of a total protein extract prepared from peas, after 5 days of germination, gave the elution profile shown in Fig.51. With increasing time of germination, larger amounts of material failed to bind to the column and came through in the void volume. Three main peaks of protein were eluted after application of the linear salt gradient. The leading edge of the first and largest peak was shown to contain protein subunits of 17,500 mol. wt. and traces of 24,000 mol. wt. protein subunits, see Fig.52. The remainder of the first peak and the whole of the second peak were shown to consist of protein subunits with a mol. wt. of approximately 30,000 and 20,000. The broad third peak contained protein subunits of approximately 51,000, 30,000 and 20,000 mol. wt. An unchromatographed total protein extract featured a main protein subunit band of about 20,000 mol. wt. and lesser bands of protein subunits with 51,000, 30,000 and 23,000 mol. wts.

Fractions in areas 2 and 3 of the elution profile obtained after hydroxylapatite chromatography of a total protein extract from peas, after 5 days of germination, were also analysed on a separate gel under reducing and non-reducing conditions, see Fig.66. A protein subunit band of slightly less than 60,000 mol. wt. was detected when both samples were electrophoresed under non-reducing conditions

FIG. 51

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of *Pisum sativum*, after 5 days of germination.

FIG. 52

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.51.

Tracks 1-8	: fractions in areas 1-8
Track 5/TE	: unchromatographed total protein extract of *Pisum sativum* after 5 days of germination
Track TE	: standard globulin protein preparation
Track L	: standard legumin preparation
Track A	: standard albumin fraction preparation

(Correction: TE should read G)

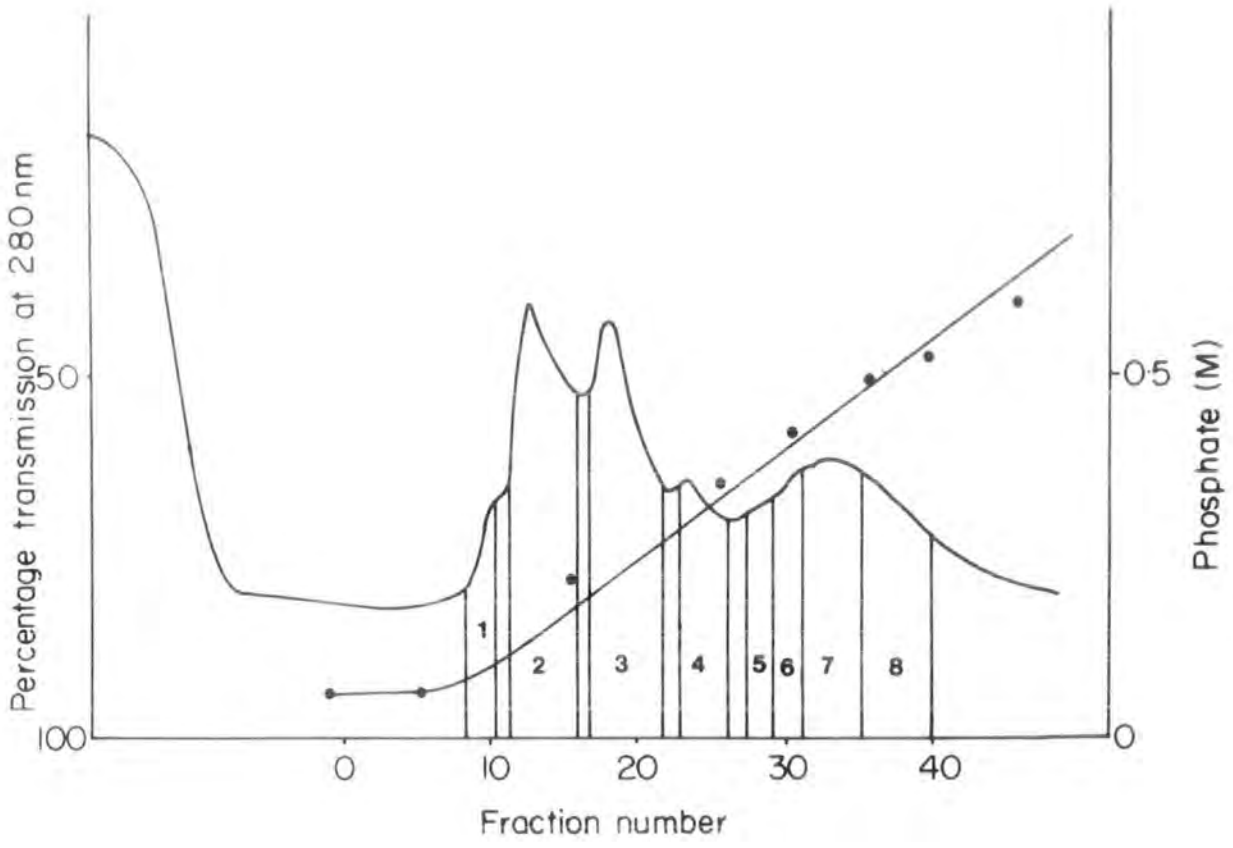

Percentage transmission at 280 nm
50
100
0
10
20
30
40
Fraction number
0·5
0
Phosphate (M)
1
2
3
4
5
6
7
8

Fig. 51

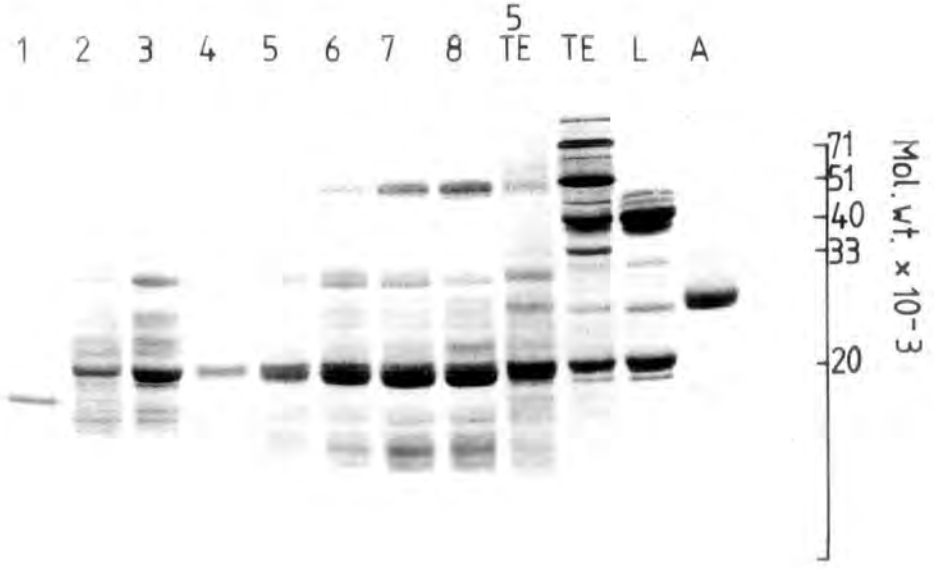

1
2
3
4
5
6
7
8
5 TE
TE
L
A
71
51
40
33
20
Mol. wt. × 10⁻³

Fig. 52

Similarly, fractions from area 3 and fractions from area 7 of the elution profiles obtained after hydroxylapatite chromatography of a total protein extract from peas, 3 and 5 days after germination respectively (Fig.49 and Fig.51), were analysed on a separate gel, under reducing and non-reducing conditions, see Fig.67. Under non-reducing conditions, fractions from area 3 contained subunits of 60,000 and slightly lower mol. wt. The subunit pattern of fractions from area 7 remained the same under reducing and non-reducing conditions, with subunits of approximately 51,000, 30,000 and 20,000 mol. wts.

The elution profile for hydroxylapatite chromatography of a total protein extract from peas after 7 days of germination is shown in Fig.53. Four main peaks, reduced in size compared to those seen at day 3 and day 5, were obtained after application of the linear salt gradient. On analysis, Fig.54, the first peak contained only traces of protein subunits with a mol. wt. of approximately 33,000. The second and largest peak, and the third peak, were shown to consist of protein subunits with an approximate mol. wt. of 30,000 and 20,000. The broad fourth peak had 30,000 and 20,000 mol. wt. subunits and, in addition, traces of protein subunits with an approximate mol. wt. of 51,000 were detected. The unchromatographed total protein extract consisted of protein subunits with approximate mol. wts. of 30,000, 23,000 and 20,000.

Hydroxylapatite chromatography of a total protein extract of peas after 10 days of germination gave the elution profile

FIG. 53

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of Pisum sativum, after 7 days of germination.

FIG. 54

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.53.

Track A	:	standard albumin fraction preparation
Tracks 1-9	:	fractions in areas 1-9
Track 7/TE	:	unchromatographed total protein extract of Pisum sativum after 7 days of germination
Track TE	:	standard globulin protein preparation
Track L	:	standard legumin preparation

(Correction: TE should read G)

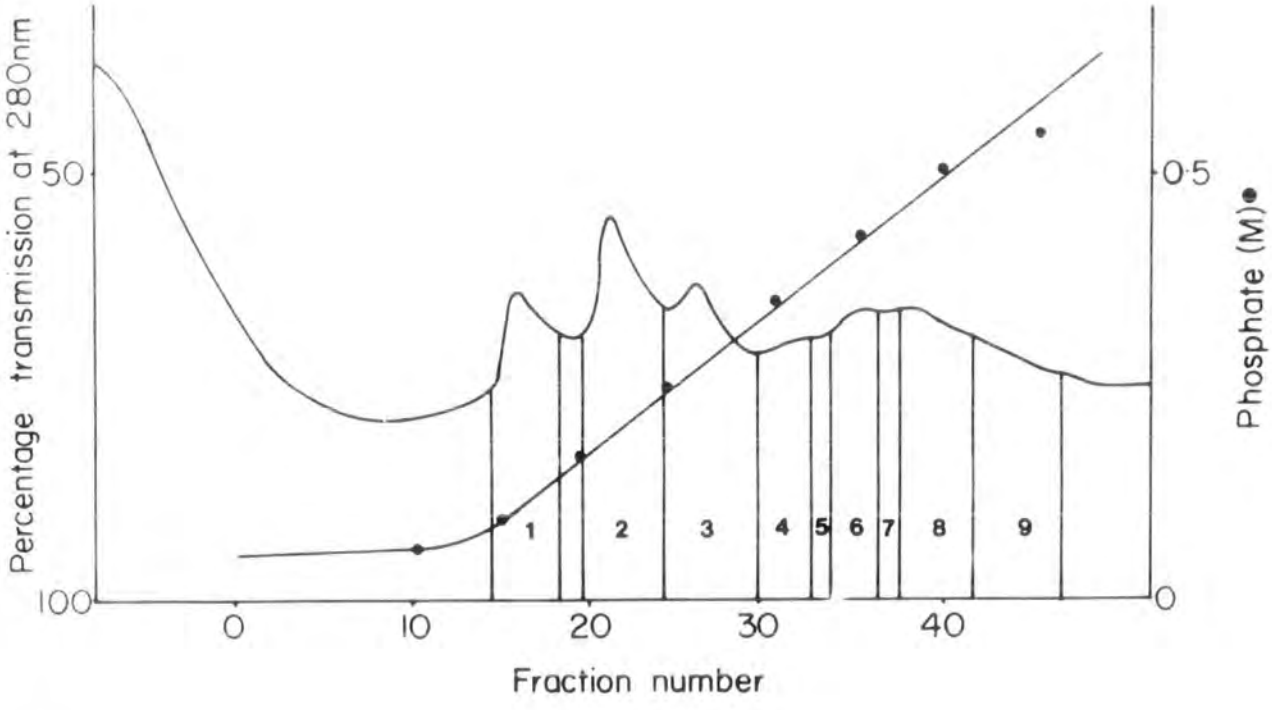

Percentage transmission at 280nm
50
100
0
10
20
30
40
Fraction number
0·5
0
Phosphate (M)•
1
2
3
4
5
6
7
8
9

Fig. 53

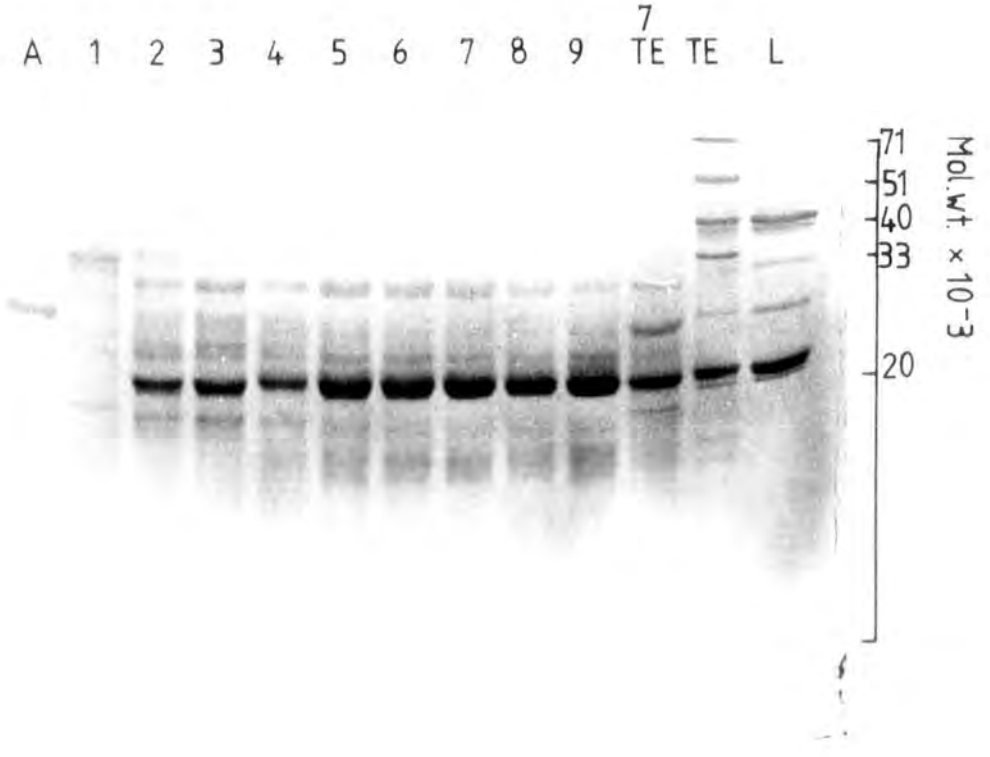

A
1
2
3
4
5
6
7
8
9
7
TE
TE
L
71
51
40
33
20
Mol.wt. × 10-3

Fig. 54

shown in Fig.55. No protein subunits were detected in the two main peaks, which eluted after application of the linear salt gradient. The area following the two main peaks was shown to contain mainly 20,000 mol. wt. protein subunits, Fig.56. The unchromatographed total protein extract contained protein subunits of 23,000 and 20,000 mol. wt. This sample was also analysed on a separate gel with a standard albumin protein preparation (Fig.66).

B. Ultrogel chromatography of germinating seed extracts

When a total protein extract of mature dried peas was chromatographed on a column of Ultrogel AcA 22, the elution profile shown in Fig.57 was obtained. The first peak to be eluted after the void volume was shown by SDS-polyacrylamide gel electrophoresis, Fig.58, to contain mainly 40,000 and 20,000 mol. wt. protein subunits. The highest concentration of these subunits (as judged by staining intensity) was found in fraction 34, the Ve/Vo ratio for this fraction was 1.72, corresponding to a protein mol. wt. of 450,000 (see Fig.6 for calibration curve). Fractions 34-38 in the trailing edge of this peak were shown to contain a 71,000 mol. wt. protein subunit and varying amounts of protein subunits with mol. wts. 51,000, 40,000, 33,000 and 20,000. The highest concentration of 71,000 mol. wt. protein subunits was found in fraction 36. The Ve/Vo ratio for this fraction was 1.82, indicating a mol. wt. of 290,000 for the protein containing this subunit. Fractions 37-42 eluting after this peak, consisted mainly of 51,000, 33,000 and 20,000 mol. wt. protein subunits. The

FIG. 55

Elution profile obtained after hydroxylapatite chromatography of a total protein extract of Pisum sativum, after 10 days of germination.

FIG. 56

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig. 55.

Track A	:	standard albumin fraction preparation
Tracks 1-8	:	fractions in areas 1-8
Track 10/TE	:	unchromatographed total protein extract of Pisum sativum after 10 days of germination
Track TE	:	standard globulin protein preparation
Track L	:	standard legumin preparation

(Correction: TE should read G)

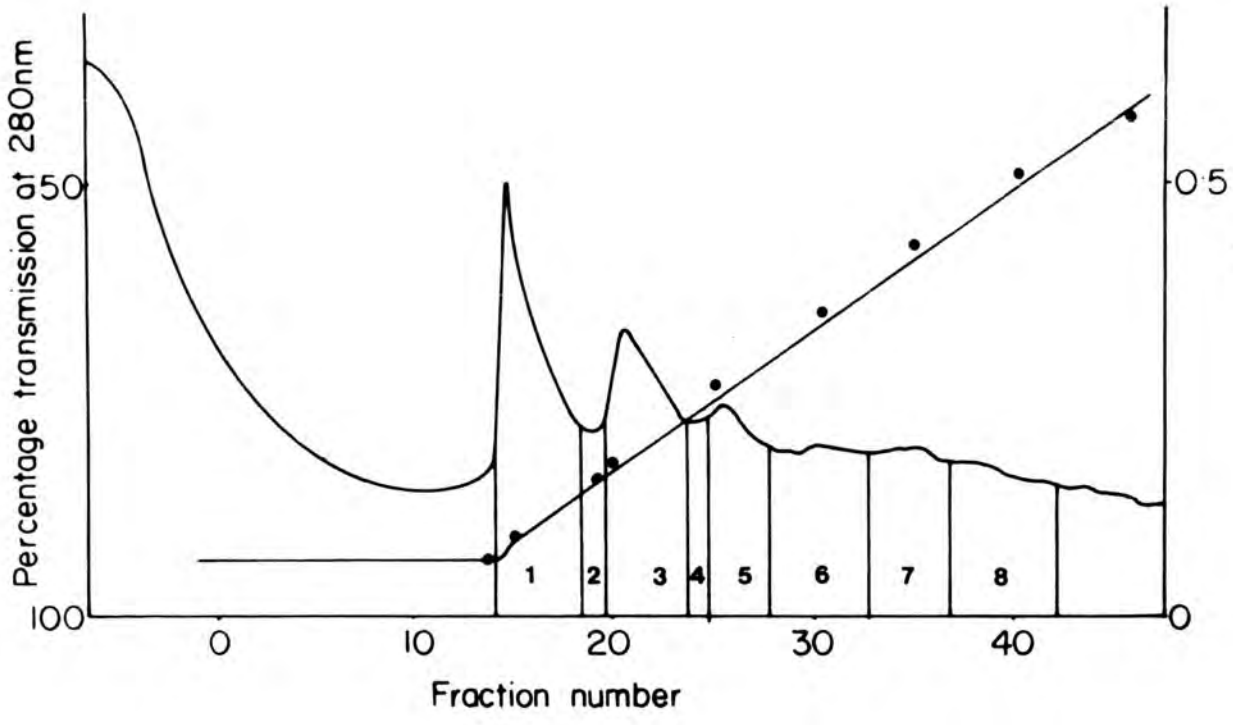

Percentage transmission at 280nm
50
100
0
10
20
30
40
Fraction number
0·5
0
1
2
3
4
5
6
7
8

Fig. 55

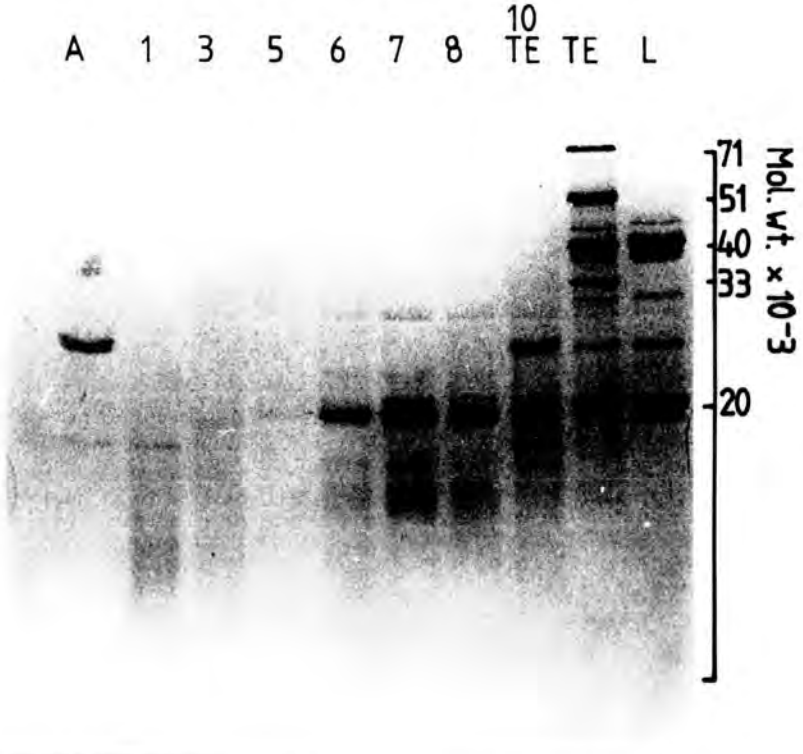

A
1
3
5
6
7
8
10
TE
TE
L
71
51
40
33
20
Mol. wt. × 10-3

Fig. 56

FIG. 57

Elution profile obtained after gel filtration of a total protein extract of *Pisum sativum* on a column of Ultrogel AcA 22.

FIG. 58

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig. 57.

Track G : standard globulin protein preparation
Tracks 31-41: fractions 31-41

FIG. 59

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig. 57.

Tracks 42-52: fractions 42-52
Track G : standard globulin protein preparation

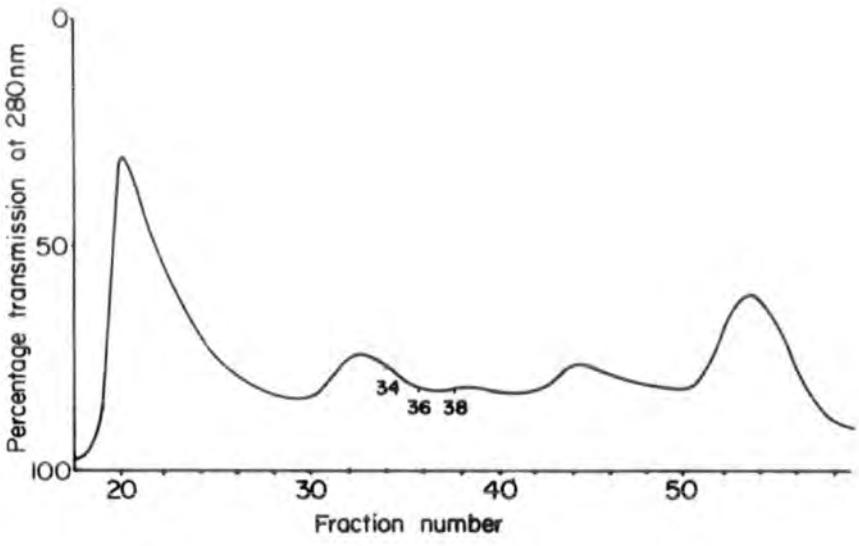


Fig. 57

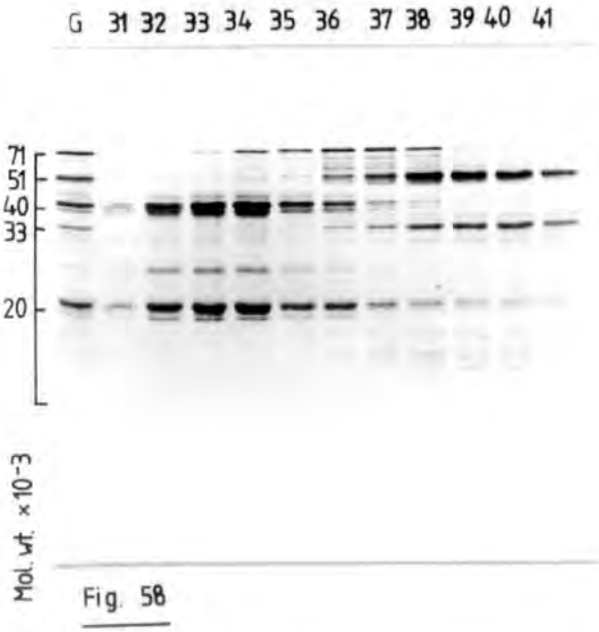


Fig. 58

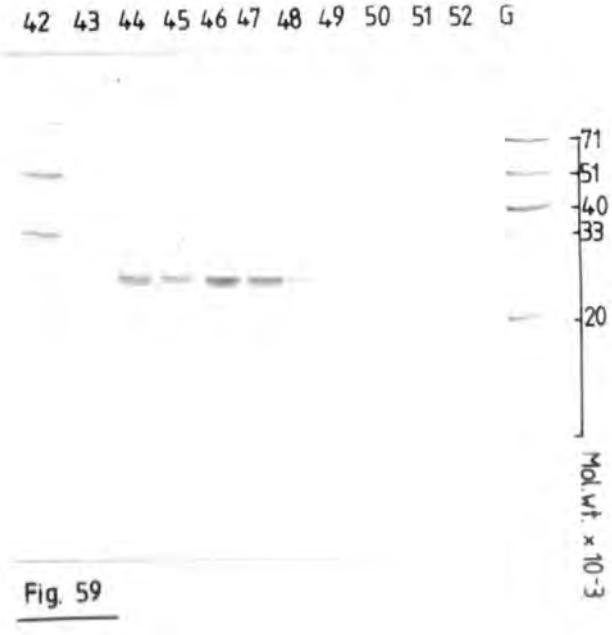


Fig. 59

highest concentration of these subunits was found in fraction 38; the Ve/Vo ratio for this fraction corresponded to a protein with a mol. wt. of 190,000. A small peak of protein was eluted from fraction 43 to fraction 50, Fig.59; this contained protein subunits of 24,000 and 17,500 mol. wt. Following this, fractions 50-60 contained low mol. wt. material, which was not TCA precipitable.

The elution profile for Ultrogel AcA 22 chromatography of a total protein extract, from peas 3 days after germination, is shown in Fig.60. The shape of this elution profile was different to that obtained when a mature pea total extract was chromatographed under the same conditions; the second and third peaks eluting after the void volume were larger. Fractions 32-37 were shown on analysis, Fig.61, to contain mainly 40,000 and 20,000 mol. wt. protein subunits. The highest concentration of these subunits was found in fraction 34, and as with Ultrogel chromatography of mature seed total protein extract, the Ve/Vo ratio for this fraction of 1.72 corresponded to a protein with a mol. wt. of 450,000. Traces of 71,000 mol. wt. protein subunit were detected in fractions 34-38, the highest concentration of this subunit being found in fraction 36, giving a Ve/Vo ratio of 1.82 and indicating again, a protein mol. wt. of 290,000. Fractions 37-43 contained mainly protein subunits with a mol. wt. of 51,000, approximately 30,000 and 20,000. The highest concentration of these subunits was seen in fraction 39, giving a Ve/Vo ratio of 1.97; indicating a protein mol. wt. of 150,000. Fractions 43-50 contained protein subunits with a mol. wt.

FIG. 60

Elution profile obtained after gel filtration of a total protein extract of Pisum sativum, after 3 days of germination, on a column of Ultrogel AcA 22.

FIG. 61

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.60.

Track G : standard globulin protein preparation
Tracks 32-41: fractions 32-41

FIG. 62

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.60.

Track G : standard globulin protein preparation
Tracks 42-51: fractions 42-51
Track A : standard albumin fraction preparation

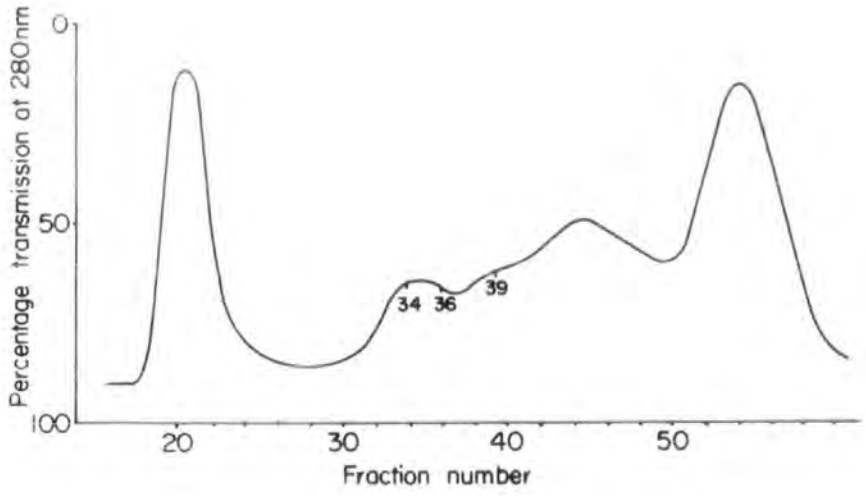


Fig. 60

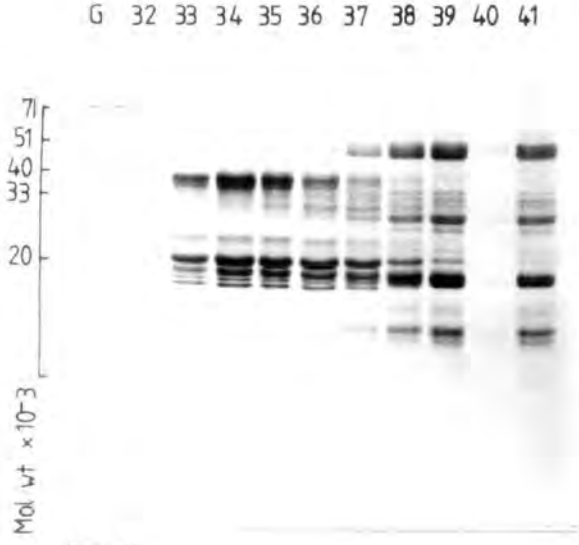


Fig. 61

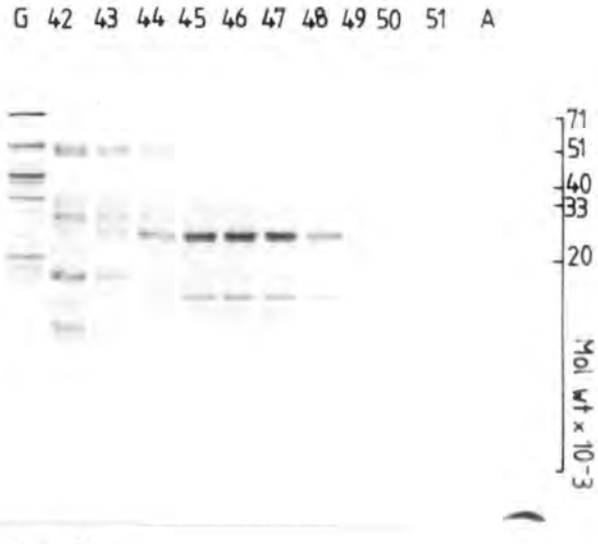


Fig. 62

of 24,000 and 17,500. The large peak from fraction 51 onwards contained low mol. wt. material.

The elution profile for Ultrogel AcA 22 chromatography of a total protein extract from peas, 5 days after germination, is shown in Fig.63. The shape of the elution profile was different yet again when compared to those obtained when total protein extracts of mature and 3 day germinated seeds were chromatographed under the same conditions. Fractions 32-37 consisted of several protein subunits with a mol. wt. range of about 40,000 to 30,000 and also subunits of 20,000 mol. wt. Fraction 34 (as in the chromatography of mature and 3 day germinated pea extracts) contained the highest concentration of these subunits and the Ve/Vo ratio for this fraction corresponded to a protein with a mol. wt. of 450,000. Fractions 32-37 were also analysed on a separate gel under non-reducing conditions, see Fig.68. Three protein subunit bands of 60,000 and slightly lower mol. wts. were detected in all fractions, in addition to minor bands of about 40,000 mol. wt. Fractions 37-43 contained protein subunits with mol. wts. of approximately 51,000, 30,000 and 20,000. These subunits appeared to be most concentrated in fraction 40. The Ve/Vo ratio of 2.02 for this fraction indicated a protein mol. wt. of 120,000. As found in Ultrogel chromatography of mature and 3 day germinated pea extracts, fractions 43-50 contained 24,000 and 17,500 mol. wt. protein subunits. Finally a large peak containing low mol. wt. material was eluted. Due to different column loadings, to allow for the water uptake of germinating seeds, 0, 3 and

FIG. 63

Elution profile obtained after gel filtration of a total protein extract of Pisum sativum, after 5 days of germination, on a column of Ultrogel AcA 22.

FIG. 64

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.63.

Track A : standard albumin fraction preparation
Tracks 32-41: fractions 32-41
Track G : standard globulin protein preparation

FIG. 65

SDS-polyacrylamide gel electrophoresis of fractions indicated in Fig.63.

Track A : standard albumin fraction preparation
Tracks 42-51: fractions 42-51
Track G : standard globulin protein preparation

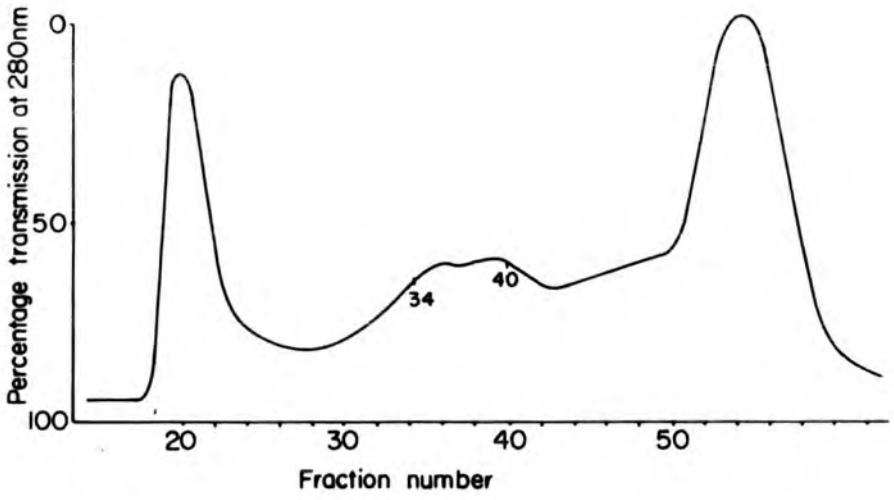


Fig. 63

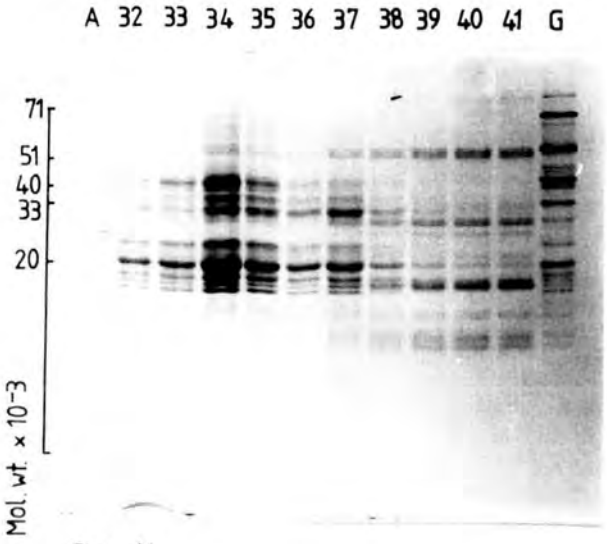


Fig. 64

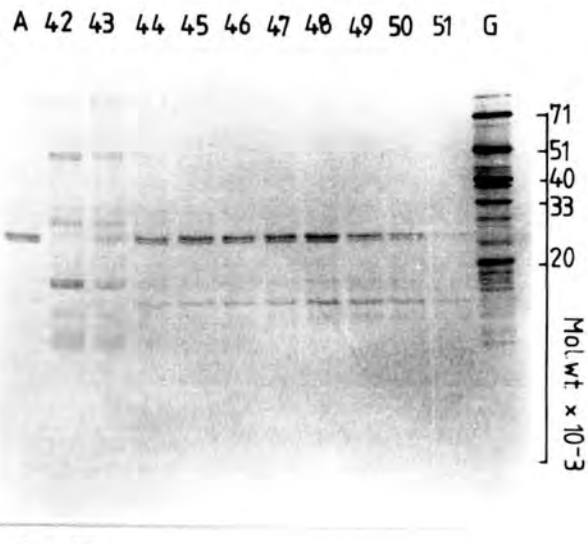


Fig. 65

FIG. 66

SDS-polyacrylamide gel electrophoresis of fractions obtained after hydroxylapatite chromatography of germinating seed extracts

reducing conditions	(Track A	: standard albumin fraction preparation
	(Track 10/TE	: unchromatographed total protein extract of Pisum sativum after 10 days of germination
	(Track L	: standard legumin preparation
	(Track 2	: fractions from area 2 of Fig.51
	(Track 3	: fractions from area 3 of Fig.51
non-reducing conditions	(Track L	: fractions from area 2 of Fig.51
	(Track 2	: fractions from area 3 of Fig.51
	(Track 3	: standard legumin preparation

(Correction: L23 should read 23L for the second part of this gel)

FIG. 67

SDS-polyacrylamide gel electrophoresis of fractions obtained after hydroxylapatite chromatography of germinating seed extracts

reducing conditions	(Track L	: standard legumin preparation
	(Track 3	: fractions from area 3 of Fig.49
	(Track 7	: fractions from area 7 of Fig.51
non-reducing conditions	(Track 7	: fractions from area 7 of Fig.51
	(Track 3	: fractions from area 3 of Fig.49
	(Track L	: standard legumin preparation

FIG. 68

SDS-polyacrylamide gel electrophoresis of fractions obtained after gel filtration of a total protein extract of Pisum sativum after 5 days of germination.

reducing conditions	(Track G	: standard globulin protein preparation
	(Track L	: standard legumin preparation
non-reducing conditions	(Track L	: standard legumin preparation
	(Tracks 32-37	: fractions 32-37 from Fig.63

Abbreviation 2ME = 2-Mercaptoethanol

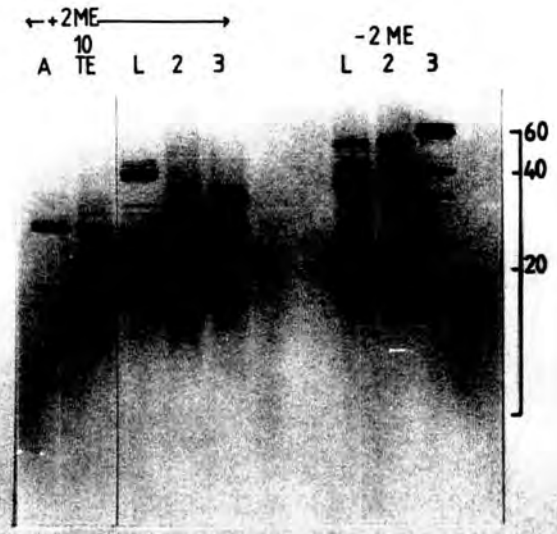


Fig. 66

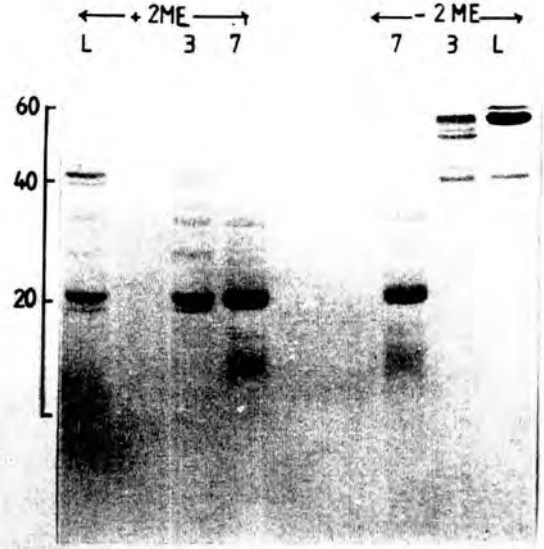


Fig. 67

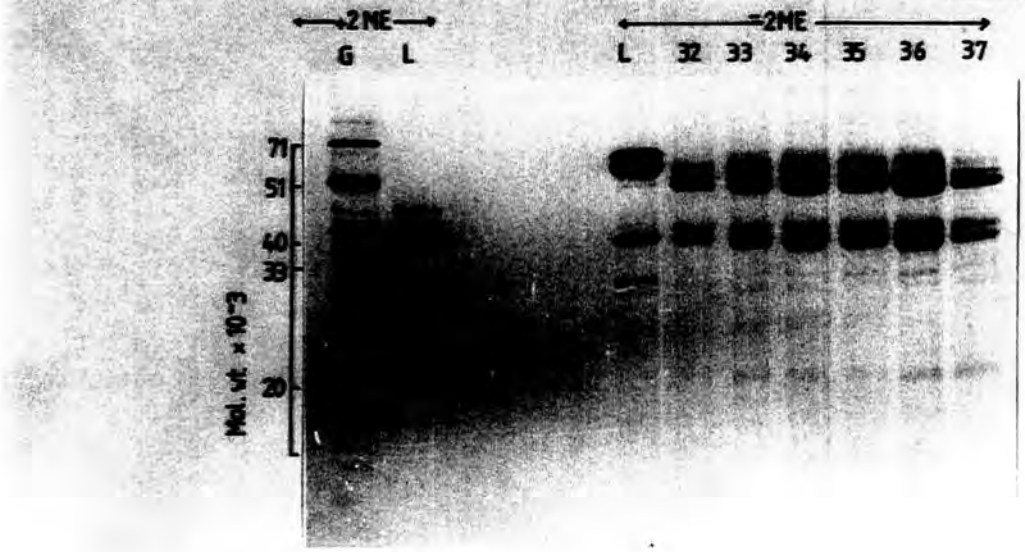


Fig. 68

5 day results are only approximately quantitative.

C. Two-dimensional polyacrylamide gel electrophoresis of germinating pea extracts

The results of two-dimensional polyacrylamide gel electrophoresis of total protein extracts from peas 0, 3 and 5 days after germination are shown in Figs. 69-71. It is not possible to compare the result obtained for mature seed total protein extract to those obtained for 3 and 5 days after germination, as the former gel was electrophoresed for a slightly longer time in the first dimension. For the stages of 3 and 5 days after germination, it can be seen that the mobility of the major components in the first dimension appears to be unchanged, indicating little change in overall charge with increasing germination time.

FIG. 69

Two-dimensional polyacrylamide gel electrophoresis of a total protein extract of Pisum sativum.

Track G : standard globulin protein preparation

FIG. 70

Two-dimensional polyacrylamide gel electrophoresis of a total protein extract of Pisum sativum, after 3 days of germination.

Track G : standard globulin protein preparation

FIG. 71

Two-dimensional polyacrylamide gel electrophoresis of a total protein extract of Pisum sativum after 5 days of germination.

Track G : standard globulin protein preparation

Additional gel tracks of standard globulin protein preparations are shown in Fig.70 and Fig.71.

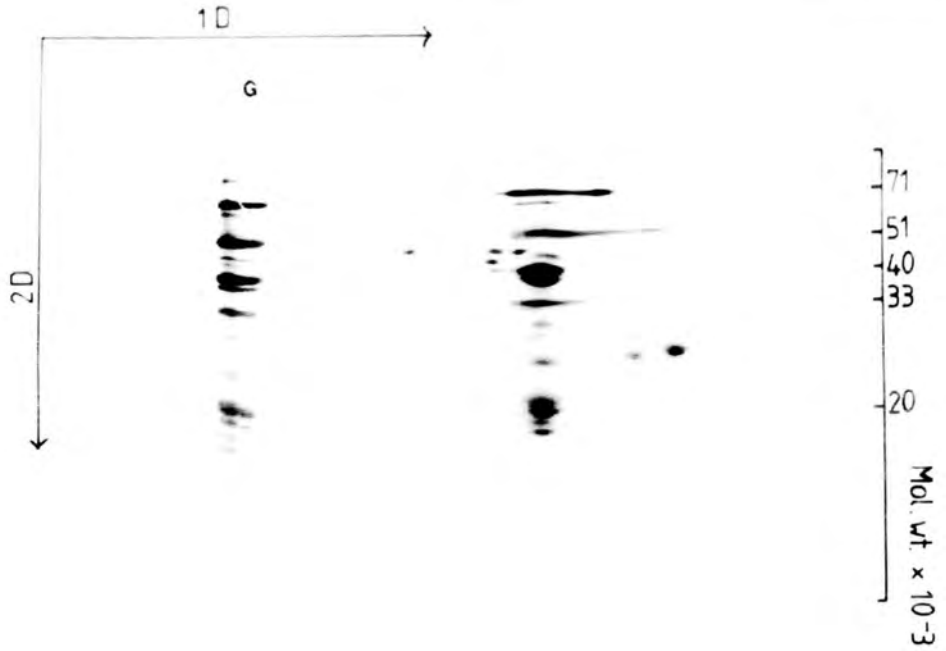


Fig. 69

Fig. 70

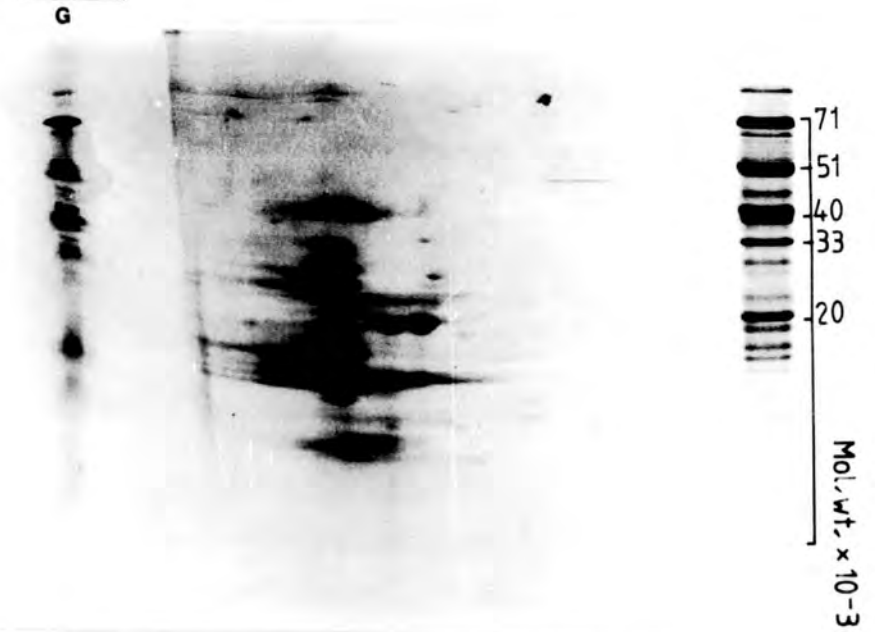


Fig. 71

Section IV DISCUSSION

A prerequisite for all protein characterisation studies is that the protein in question is homogenous. Previous work (Derbyshire *et al.*, 1976) has shown that seed storage proteins of legumes, however, are not easily isolated in a pure form. The storage proteins of peas have been separated into two fractions, vicilin and legumin, by many previous workers (Derbyshire *et al.*, 1976). The preparation of these fractions normally makes use of the differential solubility of the component proteins in them. This property was first exploited by the pioneers of seed protein purification, Osborne and Campbell, (1898) and later by Danielsson (1949). Subsequent work has shown the legumin fraction to be composed of a single, main type of protein, called legumin, whereas vicilin consists of several proteins (see later). In any case, legumin and vicilin have the classical globulin properties as defined by Osborne (1924), in that they are soluble in dilute salt solutions and insoluble in water. Hence a typical preparation of seed proteins involves extraction of the seed meal with salt solutions buffered to pH 7.0 or 8.0.

Prior to extraction, in this study, air-dried seeds which had been stored at $4^{\circ}C$, were freeze-dried for a short time to remove superficial moisture, in order to facilitate milling. When necessary, as in the case of *Vicia faba*, the testa was removed before milling, in order to eliminate difficulties that could be caused in later purification stages by the

presence of pigments and other constituents of seed coats. With Pisum sativum, testas were not removed since this was too difficult an operation, however, it was found necessary to defat the meal prior to extraction, in order to remove oils and pigments which otherwise interfered with the subsequent purification steps.

Milling of seeds was carried out immediately prior to extraction when possible, as it has been shown (Nash et al., 1971; Nash and Wolf, 1967) that there is a significant decrease in the amount of protein that can be extracted on storage of soyabean meals. In this study, extractions of meals were normally carried out at, or around, pH 8.0.

These are standard conditions for the extraction of seed proteins, since low yields are obtained in the pH 4 - 5 range, as this corresponds to the isoelectric points of the storage proteins and high yields have been obtained when extraction has been carried out in the pH range above 7; this is also true in the region of pH 2. However, dissociation of globulin proteins when extracted under acidic conditions has been demonstrated in Vicia faba (Wright and Boulter, 1973) and therefore acidic extraction conditions were not employed here with Pisum. Nevertheless, good separations of GI and GII globulin proteins from Phaseolus vulgaris have been achieved under acidic extraction conditions (Sun and Hall, 1975), indicating that procedures effective in extracting proteins in one legume species may not be effective for other legume species. All extractions of protein from air-dried, stored seeds were carried out at low temperatures as a precautionary

measure to minimize proteolytic degradation. However, it has been shown (Casey, 1979a) that the inclusion of proteinase inhibitors, such as di-isopropyl phosphorofluoridate in the extraction of Pisum meals does not significantly alter the SDS-polyacrylamide gel electrophoresis pattern of the extracts. However, there are several reports (see Abstracts of 14th Feb.'s meeting 1981) indicating the difficulty of inhibiting some proteolytic enzymes. Casey (1979a) also found that increasing the extraction temperature to 25°C or 37°C and prolonging the extraction time to 4 h at these temperatures did not alter the composition of Pisum extracts. Thus it can be tentatively concluded that proteinases were not important. Similarly Casey (1979a) has shown that sulphydryl reagents such as dithiothreitol at low concentrations, thought to increase the amount of protein extracted, had no effect on the degree of extraction of Pisum meal, and they were therefore not included in extraction media for this study.

The first purification step used after extraction was the fractionation of extracts by the addition of ammonium sulphate. This is a useful step in the preparation of homogenous proteins, as it serves to remove other non-protein materials from the extract, and gives rise to a crude fractionation of the proteins present by precipitation. Precipitates can be redissolved immediately for further purificiation or stored in a relatively stable condition. Although ammonium sulphate fractionation is normally used as one of several purification steps (see for example the

isolation of α arachin; Neucere,1969, or lupin globulins; Blagrove and Gillespie, 1975), in some cases a pure protein can be obtained by its use alone. The method for the preparation of Glycoprotein II from Phaseolus vulgaris involves only ammonium sulphate fractionation of the globulin proteins. Thus the results of Fig.11 show that a single ammonium sulphate fraction gave rise to a Glycoprotein II preparation which contained only 53,000, 47,000 and 43,000 mol. wt. subunits, known from the work of Pusztai (1980) and Croy (unpublished results) to be those of the exhaustively purified protein obtained by using many fractionation steps.

An alternative method for the preparation of the globulin storage proteins, which also utilises their differential solubility characteristics, is the use of the technique of zonal isoelectric precipitation as described by Porath (1962). Zonal isoelectric precipitation has been used to purify legumin of Vicia faba, separating it from vicilin by repeated precipitation at around pH 4.7 (the isoelectric point of legumin), and so leaving vicilin in solution (Wright and Boulter, 1974; Scholz et al., 1974 and Shutov and Vaintraub, 1965). The procedure as used by Scholz et al., was therefore repeated in this study with both Vicia faba and Pisum sativum globulin extracts (results of the latter not presented). A good separation of the two protein fractions was obtained in the case of Vicia faba (see Fig.27), and this was confirmed by cellulose acetate membrane electrophoretic analysis of the separated fractions (data not presented). However, with Pisum sativum a poor separation of proteins was obtained.

The exact reason for the poor resolution of Pisum sativum storage proteins was not established, but it was thought to be due to the ionic strength of the precipitating buffer, relative to the amino acid composition of the pea proteins involved, which differ somewhat from Vicia. In a separate experiment (see later), the zonal isoelectric precipitation of a total protein extract from Pisum, under different conditions, gave improved results.

Although zonal isoelectric precipitation is an effective separation method for legume storage proteins when correct conditions are established, it also has serious limitations. One major disadvantage of this technique is the co-precipitation of impurities and also of small amounts of the other protein which should remain in solution (Millerd, 1975). It is necessary, therefore, to repeat the process or include further purification steps, such as ion-exchange chromatography (Scholz et al., 1974). Another problem is also encountered when the precipitated protein is to be redissolved, as precipitation at the isoelectric point of many storage proteins can lead to aggregation and subsequent insolubility.

Bearing these facts in mind, it was decided that the equally powerful method of ammonium sulphate fractionation provided a better alternative to zonal isoelectric precipitation for the purification of the storage proteins of Pisum. The result of ammonium sulphate fractionation of a total protein extract (see Fig.10), was the separation of a subunit with a mol. wt. of 71,000, this subunit being normally

associated with the vicilin fraction (Croy et al., 1980a). This subunit was present in the fractions precipitating from 60 to 90% relative ammonium sulphate saturation, while the other protein subunits of the vicilin fraction, that is: 51,000, 33,000, 20,000 and lower mol. wt. subunits (subunit mol. wts. are only approximate $\pm$ 1500 and can vary when determined in different gel systems) were present alone in the precipitate and supernatent, at 100% relative ammonium sulphate saturation. This result indicated that under these conditions it is possible to separate a protein with a subunit of 71,000 mol. wt. from the other protein subunits that are usually assigned to the vicilin fraction.

The additional evidence for the protein containing 71,000 mol. wt. subunits being a separable protein of the vicilin fraction, and not merely a selection of molecules containing 71,000 mol. wt. subunits from a population containing 71,000 mol. wt. subunits in association with others during purification, comes from the fractionation of a total protein extract by gel filtration on Ultrogel AcA 22, under non-dissociating conditions (see Fig.21). Three discrete but overlapping protein peaks consisting of legumin, the 71,000 mol. wt. subunit containing protein and vicilin were obtained in addition to the lower mol. wt. proteins which eluted last.

The heterogeneity of the classical vicilin fraction has been well documented for some time; Derbyshire et al., (1976) claimed that the vicilin fraction contained more than one major protein, while Millerd (1975) mentioned that in some cases the 7 - 8S fraction, i.e. vicilin, contained two

proteins, in other cases three or more.

Additional evidence for the heterogeneity of the vicilin fraction comes from the findings of Wright and Boulter, (1972) who extracted vicilin and legumin from seeds at different stages of development. On analysis of the subunit patterns of the reduced proteins by SDS-polyacrylamide gel electrophoresis, it was shown that the subunit composition of vicilin changed during development, while that of legumin did not. Vicilin was therefore not considered to be a single protein. This evidence is supported by the crossed immunoelectrophoresis experiments of Millerd et al., (1978), who found that the polypeptide composition of the predominant precipitin peak corresponding to vicilin (peak 4), varied at different stages of seed development. However, the results they presented indicated further complexity, since the vicilin antiserum used detected four distinct non-crossreacting antigenic species belonging to their vicilin fractions.

Recently Thomson et al., (1978) attempted a fractionation of the storage proteins based on differential solubility at varying pH values and ionic strengths. Starting with protein body extracts they obtained three vicilin fractions, which they analysed in the ultracentrifuge and on SDS-polyacrylamide gradient gels. Their vicilin fraction 2 contained a 75,000 mol. wt. subunit, that appeared to be similar to the 71,000 mol. wt. subunit described above, plus small amounts of the other vicilin fraction subunits (approximately 50,000, 30,000 and lower mol. wts.). Vicilin fraction 3 contained a decreased amount of 75,000 mol. wt. subunit and increased

amounts of the other vicilin subunits. Vicilin fraction 4 was composed of 50,000, 30,000 and lower mol. wt. subunits alone, this resembled the subunit composition of the 100% ammonium sulphate fraction of a total protein extract (Fig.10). When examined in the ultracentrifuge the vicilin 4 fraction showed a single peak (7S) while the vicilin 2 fraction contained one peak sedimenting at 7S and distinct minor peaks, one of which sedimented at 9S. The vicilin 3 fraction gave a sedimentation profile with two roughly equal components at 7S and 9S. Clearly vicilin 2 and vicilin 3 contain more than one protein. These results are in agreement with those obtained in the ammonium sulphate fractionation of the total protein extract presented in this thesis; that it is possible to isolate the vicilin fraction subunits of 51,000, 33,000 and lower mol. wts. from a protein with subunits of 71,000 mol. wt., although this is not usually the case.

However, Thomson et al., (1980), continuing their studies on the heterogeneity of the vicilin fraction, obtained four vicilin fractions, (II2 II3 II4 and III) by subfractionation of the 'vicilins' by differential solubility. On analysis by two gel systems these were shown to contain variable amounts of all the typical major vicilin subunits, and in addition fraction II3 contained the major 75,000 mol. wt. subunit. The same workers also obtained three sub-fractions of vicilin, (IIA IIB and IIIC) by Sephacryl S-200 chromatography of whole vicilin preparations, of which subfraction IIB contained all the typical lower mol. wt.

vicilin subunits plus the 75,000 mol. wt. subunit, while the other two; IIA, IIC, contained all the vicilin subunits but no major 75,000 mol. wt. subunit. Thus these workers have proposed extremely complicated structures for vicilin, or the vicilin group of proteins to accommodate the presence in them of the major 75,000 mol. wt. subunit (similar to the 71,000 mol. wt. subunit in question). They suggest the existence of two general classes of vicilin 'holoproteins'; one class with the major 75,000 mol. wt. subunit and the other class without this subunit but still containing all the other lower mol. wt. subunits usually assigned to the vicilin fraction. Once again these results are in agreement with the results obtained in this study, in that by ammonium sulphate fractionation of a total protein extract it is possible to isolate typical vicilin lower mol. wt. subunits with or without the major 71,000 mol. wt. subunit, depending on the ammonium sulphate saturation.

However, this present study has gone on to separate a protein consisting of subunits of 71,000 mol. wt. away from the vicilin fraction, while Thompson et al., (1980) did not. Therefore, some of the vicilin types suggested by Thompson et al., (1980) are, in fact, mixtures of vicilin proteins and a separable vicilin type protein with a subunit mol. wt. of around 71,000 (called convicilin, see later).

From the results of Fig.10 it can be seen that at 100% relative saturation with ammonium sulphate, the supernatent solution contained a protein fraction with major subunits of approximate mol. wts. of 51,000, 33,000 and additional

subunits of about 35,000, 30,000, 21,000, 20,000, 15,000, 13,000 and 12,000 mol. wts. This protein or protein fraction has a similar subunit composition to some of the vicilin fractions obtained by Thomson et al., (1978), (1980). However, it was not the present purpose to characterise this protein extensively and it was only briefly investigated.

The method used to establish the evidence presented earlier, for the existence of a separate vicilin type protein, was not of sufficient resolution to be used to prepare samples of convicilin for characterisation. In order to isolate convicilin completely pure from the vicilin fraction, a combination of three purification techniques was necessary.

The ammonium sulphate fractionation of a total extract provided an early concentration step, and enriched convicilin relative to the other storage proteins.

The second purification step on Sephadex G-200 led to the removal of most of the contaminating vicilin, and also some legumin (see Fig.14 and Fig.15). Thus by virtue of their differing mol. wts., these proteins were eluted from the Sephadex G-200 column in order of decreasing size. Legumin was eluted first; its mol. wt. has been calculated as approximately 400,000: (330,000, Danielsson, 1949; 383,000, Brand, Goring and Johnson, 1955; 400,000, Brand and Johnson, 1958; 390,000, Croy et al., 1979; 400,000, Casey, 1979a; 350,000, Blagrove, Lilley and Davey, 1980). Convicilin was the next protein to elute (mol. wt. 280,000, see later). Finally, vicilin was eluted in the trailing edge of the large asymmetrical peak, its mol. wt. has recently been calculated

as approximately 170,000 (Gatehouse et al., 1981). At low protein sample loadings on gels the pea albumin fraction typically shows two close bands of 25,000 mol. wt. (Grant et al., 1976). This pattern was seen in the low mol. wt. elution fraction.

Thus this second purification step indicated that convicilin is intermediate in size between legumin and vicilin; this was confirmed by other methods (see later).

These results show that the resolution of the protein mixture by the Sephadex G-200 column was poor since no separate peaks of protein were obtained.

It is known that certain molecular types do not display typical elution behaviour and are eluted earlier or later than predicted from their molecular size. This could be due to gel-solute interactions and adsorption phenomena which are thought to be of two main types:- 1. Electrostatic interactions, which may be due to the small number of ionized carboxyl groups on Sephadex, leading to the adsorption of some basic substances and exclusion of acid substances from the gel matrix, at low ionic strength (Gellotte, 1960). This effect is most prominant on the more tightly cross-linked Sephadex types (Janson, 1967). 2. Aromatic adsorption due to the interaction of heterocylclic and homocyclic compounds with Sephadex (Janson, 1967). Once again this effect is more pronounced with high density matrix, Sephadex gel types and low mol. wt. solutes (Janson, 1967; Brook and Housley, 1969b). The interaction is thought to be the result of hydrogen bonding between aromatic substituents and the

hydroxy-ether linkage between dextran chains (Williams, 1972; Brook and Munday, 1970; Determan and Walter, 1968; Brook and Housley, 1969a).

However, since the proteins to be fractionated in this case were of reasonably high mol. wt. and did not have a high aromatic amino acid content, plus the fact that all buffers used contained electrolytes and that Sephadex G-200 has a low degree of cross-linkage, it can be assumed that the above considerations did not apply.

Probably the main reason for the poor separation of these proteins on the column used was that chromatography was carried out on a preparative scale, using a large column and high sample load, in order to obtain an enrichment of a specific protein fraction, i.e. convicilin. Inevitable trailing effects occurred due to the various appreciable protein-protein interactions usually encountered at high protein concentrations.

The third and final purification step for the preparation of convicilin involved hydroxylapatite chromatography. This form of chromatography was found to be an excellent final purification step, or convenient preliminary fractionation step of extracts in this study.

It is thought, that in the case of acidic proteins, adsorption on to hydroxylapatite is a result of interaction between the carboxyl groups at the protein surface and the calcium sites of the hydroxylapatite crystals (Bernadi and Kawasaki, 1968). Desorption occurs on increasing the molarity of the eluting phosphate buffer, as this progressively reduces the interaction to zero. This decrease in the

interaction appears to be due to a specific competition between the phosphate ions of the eluting buffer and the carboxyl groups of the protein for the calcium sites of the hydroxylapatite (Bernadi and Kawasaki, 1968). Elution appears to be independent of ionic strength (Bernadi and Kawasaki, 1968; Bernadi, 1971). Studies on copolymers containing different percentages of glutamic acid residues have shown that the elution molarity was lowered when the percentage of glutamic acid residues in the copolymer was smaller (Bernadi and Kawasaki, 1968) confirming the fundamental importance of carboxyl groups in the protein-hydroxylapatite interaction process.

The storage globulins of pea contain characteristically high amounts of aspartic and glutamic acids and/or their amides (Derbyshire et al., 1976; Hurich et al., 1977; Jackson et al., 1969; Konopska, 1975). Legumin has the lowest amount of acidic amino acids and/or amides compared to vicilin and convicilin, and this may have been reflected in the elution profile, in which legumin was eluted first (see Fig.16 and Fig.17). Incidentally, the legumin obtained by this method was composed mainly of 40,000 and 20,000 mol. wt. subunits, therefore this step also provided a source of fairly pure legumin. Convicilin was found to have a lower level of acidic amino acids and/or amides than vicilin (see Table 1) and was the second protein eluted. Finally vicilin with the highest amount of acidic amino acids and/or amides was eluted closely after convicilin. Vicilin was shown to have a comparatively higher aspartic acid and/or amide

content and it is of interest that Bernadi and Kawasaki (1968) have shown that aspartic acid binds more strongly than glutamic acid. Although vicilin and convicilin displayed a similar affinity for hydroxylapatite, pure convicilin could be obtained by selecting fractions in the leading edge of the second peak. This result may reflect the similarity of the secondary and tertiary structure of these proteins, as it has been suggested that the stability of the protein-hydroxylapatite interaction is associated with a local concentration of carboxyl groups and/or a local distribution of carboxyl groups fitting the distribution of adsorbing sites (Bernadi and Kawasaki, 1968). As hydroxylapatite chromatography therefore gave a better separation of convicilin from legumin, than convicilin from vicilin, the fractions containing convicilin least contaminated with vicilin from the previous Sephadex G-200 step were selected for the final purification.

Thus using this outlined method it was possible to completely isolate a separate protein of the vicilin fraction, under non-dissociating conditions. Variations of this purification strategy have also been previously used to isolate homogenous preparations of soyabean globulin proteins. Wolf and Sly (1965) were able to separate soyabean proteins into four major fractions, by gradient elution on hydroxylapatite alone, while Mitsuda (1964) obtained pure 11S globulin by Sephadex G-200 chromatography of soyabean proteins, followed by a further purification step of hydroxylapatite chromatography. Koshiyama (1965) obtained

a homogenous 7S soyabean preparation with the use of Sephadex chromatography, and later (Koshiyama, 1972) went on to isolate the 11S globulin of soyabean seeds with this simple gel filtration method. Similarly working with vetch globulin proteins, Shutov and Vaintraub, (1966), found zonal isoelectric precipitation unsatisfactory for the production of pure vicilin and legumin and so resorted to the use of hydroxylapatite chromatography for the final purification of legumin and vicilin, from the minor components of total seed extracts.

Not only was the purification procedure described above an excellent means of obtaining pure convicilin from Pisum sativum but from preliminary investigations using a total extract from Vicia faba and subjecting this to hydroxylapatite chromatography, it would appear that an equivalent protein to convicilin, with a subunit mol. wt. of 68,000, can be isolated from Vicia faba (variety Maris Bead), in a similar way (see Fig.28 and Fig.29). A vicilin subunit from Vicia faba with a mol. wt. of 66,000 has been described by Bailey and Boulter (1972) and a subunit of 68,000 mol. wt. has also been demonstrated in the GI fraction of Vicia faba obtained by McLeester et al., (1973) using an acidic extraction procedure. However, to date, there are no reports of the complete isolation of this 68,000 mol. wt. subunit containing protein, from the other proteins of the globulin fraction in Vicia faba. Thus future work could be directed towards the purification and characterisation of this protein.

Returning to Pisum, the properties of convicilin and in

some instances, those of vicilin were investigated and compared. The amino acid analyses of the purified proteins revealed important differences (see Table 1). It can be seen that the glutamic acid/aspartic acid/or amide ratio in convicilin is high (approx. 2:1), and this distinguishes it from vicilin (approx. 1:1), and also legumin (approx. 1.5:1). The proline and arginine content of convicilin is also higher, compared to that of vicilin, although it is similar to that of legumin. An even more important difference between convicilin and vicilin is their sulphur amino acid content. Convicilin has one cysteine residue and one methionine residue per subunit, while vicilin contains no sulphur amino acids. Legumin, by comparison, is richer in sulphur amino acids, with four methionine and seven cysteine residues per 60,000 mol. wt. subunit.

This difference in sulphur amino acid content was reflected in the results of the CNBr cleavage (Fig.22). Convicilin was cleaved into two fragments, (of approx. 55,000 and 14,000 mol. wt.) while vicilin appeared to remain intact. However, these results also indicated some sequence heterogeneity in convicilin, as most of the 71,000 mol. wt. subunit was cleaved rapidly, leaving a resistant fraction, which remained uncleaved even after prolonged incubation. It is possible that this resistant form of convicilin may have another amino acid substituted for the methionine.

Another indication of sequence heterogeneity comes from the results of the N-terminal determination, in which comparable amounts of aspartic acid and leucine were detected as N-terminals. Heterogeneity of convicilin was also

suggested by the results obtained on isoelectric focussing the purified protein, as a single band was not obtained.

Vicilin and convicilin were also distinguished by their relative sugar content. The sugar analysis of convicilin detected small amounts of hexose sugars, but these amounted to less than one sugar residue per 71,000 mol. wt. subunit. Vicilin is apparently richer in sugar (Basha and Beevers, 1976) and this is demonstrated by the results of the immunodiffusion experiment (see Fig.23b), in which convicilin gave no reaction with concanavalin A. On the other hand, vicilin was shown to give a positive reaction with concanavalin A, the latter known to bind α Dmannopyranose, α Dglucopyranose, D fructofuranose, their glycosides and sterically related structures (Lis and Sharon, 1973). This result is in agreement with the findings of Davey and Dudman, (1978, 1979) who investigated the extent of glycosylation of their four protein body fractions; legumin, vicilin 2, vicilin 3 and vicilin 4. These workers found that all four classes were glycosylated, with 6 - 12 µg hexose per mg protein, but while legumin contained mainly glucose and some mannose, their vicilin fractions contained varying proportions of mannose, glucose and galactose. Using a concanavalin A Sepharose 4B column, they separated their vicilin 2 fraction (consisting of vicilin and convicilin, see above) into two fractions. This result would be expected if one fraction (convicilin) was not glycosylated to the same extent as the other fraction (vicilin). It is also interesting that on investigating the localisation of the carbohydrate to various

subunits, these workers found that in their vicilin 2 and vicilin 3 fractions, most of the carbohydrate was attached to the 50,000 and 14,000 mol. wt. subunits, with only a trace on the 70,000 mol. wt. subunit.

Although convicilin has been shown to have distinctive properties from vicilin, it gives a serological reaction of identity with the vicilin fraction, indicating that convicilin and vicilin share the same antigenic determinants. Thus convicilin must be closely related in structure to vicilin, since normally one would expect that there would be several antigenic sites, each consisting of a few amino acid residues, for a protein of this size. Hence convicilin was named on the basis of its serological cross-reactivity with vicilin but no homology to other 'Con' - proteins e.g. conglycinin or conarachin is implied. However, it must be borne in mind that the antibodies used in the immunodiffusion experiments were raised against a protein fraction containing a mixture of vicilin and convicilin (approx. 4:1). Thus the chances of the antibodies detecting additional determinants on the convicilin molecules, even if the extra amino acid sequence of convicilin was exposed, could have been diminished.

These results, like the close affinity of convicilin and vicilin for hydroxylapatite confirm the similarity of these protein structures.

Convicilin is usually obtained as a contaminant of the vicilin fractions (Thomson et al., 1978, 1980). However, it has also been isolated with the legumin fraction (Przbylska et al., 1978; Davies, 1976; Casey, 1979a)

during isoelectric precipitation. In this study it was shown that it is possible, with the technique of zonal isoelectric precipitation, to isolate the majority of convicilin and legumin from a total extract, in a single fraction (see Fig.25 and Fig.26). This separation was achieved with the use of a sodium acetate buffer system at pH 4.7, as described by Davies (1976). This result provides a contrast to the poor separation of globulin proteins from Pisum sativum obtained using the citrate-phosphate buffer system described earlier, and successful in the case of Vicia faba (Scholz et al., 1974). Convicilin has also been eluted from a zonal isoelectric precipitation column separately from vicilin and legumin by a linear salt gradient at pH 5.0 (R.R.D. Croy unpublished work). The zonal isoelectric precipitation technique employed non-dissociating conditions throughout and successfully separated convicilin completely from vicilin and legumin. Therefore, these results also provide strong evidence for convicilin being a separate, distinct protein with solubility properties midway between those of legumin and vicilin, depending on the conditions employed for separation.

During the brief investigation of DEAE chromatography (Fig. 12 and Fig.13) as an alternative method for the separation of the globulin proteins, it was found that vicilin was eluted prior to the majority of convicilin, which in turn eluted before legumin. Similar elution profiles, featuring two main peaks of protein, with vicilin eluting before legumin, have been reported for the storage proteins of

peas (Grant and Lawrence, 1964), vetch (Shutov and Vaintraub, 1964) and Phaseolus aureus (Ericson and Chrispeels, 1973) although convicilin was not described. It is interesting to note that in this study, a partial separation of vicilin into different forms was obtained, as analysis of fractions revealed that the leading edge of the first peak contained all the typical vicilin subunits, while the trailing edge was depleted in the 33,000 mol. wt. subunit. This result has been studied in greater detail (Gatehouse et al., 1981).

Returning to this study, the result of DEAE chromatography of the globulin proteins, indicated that convicilin was more negatively charged than vicilin under the conditions used. However, during cellulose acetate membrane electrophoresis of vicilin and convicilin at pH 7.0, convicilin appeared less negative than vicilin. These contradicting results may be explained by virtue of the fact that the isoelectric point convicilin is probably higher than that of vicilin, so that at pH 7.0 convicilin is more positive than vicilin and at pH 7.7 the converse is true.

Finally to complete the characterisation of convicilin in this study a mol. wt. for purified convicilin of 290,000 $\pm$ 40,000 was determined by comparison with standard proteins. This would suggest that convicilin is a tetrameric molecule composed of four 71,000 mol. wt. subunits. However, Casey and Sanger (1980) have proposed a possible trimeric structure for a 7S protein, similar to convicilin, based on mol. wt. estimates of 220,000 - 230,000 obtained by gel filtration of this 7S protein on Sepharose 4B. Clearly several methods

must be employed before the quaternary structure for convicilin can be agreed on.

Thus a third storage protein with distinct properties from vicilin and legumin has been identified in peas. This protein can be considered a storage protein as it is a major component of protein-body proteins (Croy et al., 1980b) and also a major seed protein. Convicilin contains sulphur amino acids while vicilin does not and therefore the former will be of greater nutritional significance than the latter.

A knowledge of the sequence of accumulation and changing subunit patterns of the storage protein fraction, during seed development, is a prerequisite for the analysis of the developmental controls involved. This knowledge could eventually help the plant breeder formulate strategies aimed at improving seed protein yield and quality.

In studies of this kind, various criteria have been used to define the developmental stage of the immature seed at harvest, including fresh weight of the cotyledon (Raacke, 1957a,1957b; Millerd et al., 1975) and cotyledon length (Millerd et al., 1971). In this investigation 'days after flowering' was adopted; however, it must be appreciated that not all cotyledons of the same age feature the same developmental stage of protein accumulation (Millerd et al., 1975; Carasco et al., 1978; Domoney et al., 1980; Guldager, 1978).

Hydroxylapatite chromatography of seed extracts has been demonstrated to be a convenient method for the preparation of four protein fractions: albumins and low mol. wt. proteins, legumin, convicilin and vicilin (see Fig.31 and Fig.32),

although the latter two show slight cross-contamination. Therefore this technique was used to isolate the storage proteins of developing seed extracts. Other workers (Millerd et al., 1978; Thomson et al., 1979) have used immunoelectrophoretic methods which have the disadvantage of only being able to detect storage proteins with antigenic determinants, represented in extracts of protein bodies from mature seed, against which the antibodies were raised. The techniques used in this study provided direct information about the subunit composition of storage proteins in developing seeds.

The plants were grown under controlled environmental conditions; this had two advantages: firstly seeds matured earlier than if grown under normal field conditions (Millerd and Spencer, 1974) and secondly, synthesis of the storage proteins in seeds, grown in this way, occurs at precise, predictable stages of seed development (Millerd and Spencer, 1974; Millerd et al., 1975). The developing seeds were examined at 5 stages, these were at: 11, 13, 17, 21 and 33 days after flowering.

The cotyledons of developing legume seeds exhibit an initial, relatively short phase of cell division, followed by an extended phase of cell expansion (Millerd et al., 1971; Millerd and Spencer, 1974; Pate 1974; Murray and Collier, 1977). It has been shown, in earlier studies with Vicia faba, that 95% of the total seed protein is synthesised during this second growth phase of cell expansion (Briarty et al., 1969; Millerd et al., 1971). Since Millerd and Spencer

(1974) considered growth by cell expansion (from the results of cell counts in developing cotyledons) in the case of Pisum, grown under controlled environmental conditions, to start about 10 days after flowering, the first stage of 11 days after flowering was chosen in this study. This stage represented the onset of storage protein synthesis. Days 13 and 17 were selected to represent maximum synthesis stages of vicilin and legumin respectively, as it has been reported that legumin synthesis reaches a maximum rate after the synthesis of vicilin has begun (Raacke, 1957b; Wright and Boulter, 1972).

The decrease in fresh weight of the seed begins about 21 days after flowering (Millerd and Spencer, 1974), therefore this stage marked the onset of seed maturation. The stage of 33 days after flowering represented the completion of seed maturation (Beevers and Poulson, 1972).

In agreement with other studies on the sequence of legume storage protein accumulation (Danielsson, 1952; Raacke, 1957b; Graham and Gunning, 1970; Wright and Boulter, 1972; Millerd et al., 1978; Millerd and Spencer, 1974), vicilin was the first storage protein to be detected. However, in this study vicilin was first detected at 11 days after flowering, while other workers using more sensitive immunological techniques have been able to detect vicilin 9 days after flowering (Millerd and Spencer, 1974; Millerd et al., 1975) and even earlier (Craig et al., 1980). It was not possible in this study to analyse earlier stages of development, due to the lack of sufficient material for chromatography.

The subunit pattern of vicilin isolated at 11 days after

flowering did not resemble that of vicilin isolated from mature seeds, in that the 51,000 mol. wt. subunit had a double band pattern and an additional band of 47,000 mol. wt. was also present and occasionally seen as a doublet on some gels. Similar results have been reported for the subunit pattern of vicilin, synthesised 11 days after flowering, *in vivo*, using a cotyledon labelling technique (Spencer *et al.*, 1980; Gatehouse *et al.*, 1981; Higgins and Spencer, 1980, 1981). Higgins and Spencer (1981, 1980) were also able to demonstrate the presence of a 75,000 mol. wt. polypeptide in addition to the 50,000 and 47,000 mol. wt. polypeptides at eleven days after flowering, while in this study no convicilin was detected until two days later. This result probably reflects the relative sensitivity of the detection methods employed.

The results of *in vitro* studies are also in agreement with the results obtained here, for the subunit pattern of vicilin at eleven days after flowering. Higgins and Spencer (1980, 1981) were able to obtain polypeptides of 75,000, 50,000 and 47,000 mol. wt., with their cell-free translation systems programmed by polysomal RNA (isolated from cotyledons at eleven days after flowering), provided microsomal membranes were added to the cell-free systems at the beginning of translation. In similar experiments Evans *et al.*, (1979) and Croy *et al.*, (1980a) were able to obtain similarly sized polypeptides with their translation systems alone.

The apparent double nature of the 51,000 and 47,000 mol. vicilin subunit bands (seen in this study) supports the

suggestion of Gatehouse *et al.*, (1981) that vicilin synthesised *in vitro* on polysomes, represents a mixture of polypeptides which have, or have not, been co-translationally processed. Evidence for this theory was supplied by their results of obtaining only the upper bands of the doublets when poly A RNA or polysomes treated with methyl-mercuric hydroxide were translated, and predominantly the lower bands when microsomes or polysomes plus endoplasmic reticulum were translated. They concluded that processing occurred on the endoplasmic reticulum concomitantly with translation and consisted of the removal of a small polypeptide (less than 1,000 mol. wt.).

The vicilin analysed in this study at eleven days after flowering featured no major 33,000 mol.wt. subunit as seen in vicilin from mature pea extracts; however, a complex pattern with at least seven subunits with a mol. wt. range of 35,000 to 30,000 were detected (See Fig.34, Track 11/TE). Once again, an explanation for the existence of these subunits can be found in the suggestion of Gatehouse *et al.*, (1981) that the smaller polypeptides found in deposited vicilin arise from post-synthetic proteolysis of some of the $\sim$ 50,000 mol. wt. subunits already assembled into vicilin, with the 47,000 mol. wt. subunits being completely cleaved, in a specific manner, giving rise to subunits of predictable lower mol. wts. It is possible, here, that this post-translational proteolysis has been 'interrupted' at an incomplete stage and that various partially processed forms of vicilin have been separated by hydroxylapatite chromatography. This 'interruption' would

also explain the slight size difference of the vicilin subunits with mol. wts. of less than 33,000 when compared to mature vicilin.

The basis for the complex picture of vicilin subunits undergoing the various post-translational changes described, became clearer on analysis of storage proteins at later stages of development. At 13 days after flowering the subunit pattern, i.e. the 33,000 mol. wt. subunit and the relative mobilities of other minor vicilin subunits, resembled those of mature vicilin. Furthermore, the relative amounts of these subunits, as judged by staining intensity, increased with development time, while there was a decrease in the amount of 47,000 mol. wt. subunit and this result supports the suggestion of Gatehouse et al., (1981), that the 33,000 mol. wt. subunit is derived from the 47,000 mol. wt. subunit by postsynthetic proteolysis. The results obtained in this study indicate that the vicilin subunit pattern does not change after 17 days after flowering. This is correlated with the fact shown, by the isolation of translation products of polysomes obtained at different stages of development (J.A. Gatehouse, personal communication), that the rate of vicilin synthesis slows down at 16 days after flowering.

Although not the major topic of this thesis, it was also possible to observe some of the sequential changes that occurred in the legumin subunit pattern, during the protein accumulation phase of seed development. A 'legumin type' subunit pattern with evidence of 40,000 and 20,000 mol. wt. subunits was first detected at 13 days after flowering;

similarly, Spencer *et al.*, (1980) using *in vivo* labelling of cotyledons, were able to detect legumin, 40,000 mol. wt. subunits by 14 days after flowering. However, other workers (Domoney *et al.*, 1980; Craig *et al.*, 1980) using the highly sensitive technique of 'enzyme-linked immunosorbent assay' (ELISA) were able to show that legumin synthesis starts from the initial stages of embryo development. Clearly the ability to detect legumin in developing cotyledons depends on the sensitivity of the technique or assay employed. It was not possible however, to demonstrate clearly the presence of a legumin 60,000 mol. wt. precursor as described by Croy *et al.*, (1980c) due to its rapid turnover.

The legumin acidic subunits "assumed" the general pattern seen in mature legumin at 17 days after flowering, although the relative amounts of these acidic subunits were different compared to those of mature legumin. The basic subunit pattern did not resemble that of mature legumin until considerably later.

In this study, an interesting change in the relative amounts of the basic 19,000 and 20,000 mol. wt. subunits, as judged by staining intensity, was detected after storage. On storage the 19,000 mol. wt. subunit decreases and the 20,000 mol. wt. subunit increases, although some change in this direction was also noticed in the last phase of development (Fig.45). This change has also been observed by Spencer *et al.*, (1980), who attributed it to a delayed post-translational modification of a long-lived precursor for the legumin major small subunit, the 19,000 mol. wt. subunit of

'immature' legumin being converted to the 20,000 mol. wt. subunit several weeks after its synthesis. Recently, Matta (1981) has suggested that this change is a storage effect due to non-enzymic chemical alteration of legumin rather than a directed enzyme catalysed process, possibly due to the deamidation of some asparagine or glutamine residues.

The identity of the ~32,000 mol. wt. polypeptide eluted at low phosphate concentration on hydroxylapatite chromatography of developing seed extracts is unclear. Millerd et al., (1978) also reported the presence of an antigenically active component that appeared at earlier stages in developing cotyledon extracts (Peak P and 6), with unique polypeptides of about 33,000 and 35,000 mol. wt., and suggested that it might be the 'third protein' described by Dudman and Millerd (1975). This suggestion is made unlikely by the fact that Dudman and Millerd (1975) report that the sequence in which the storage proteins become detectable by immuno-electrophoresis is vicilin, legumin and finally the 'third protein'. A 30,000 mol. wt. polypeptide has been detected by Spencer et al., (1980) at 8 days after flowering, in pulse labelled cotyledons, by fluorography and staining of gels. As shown in this study (Fig.46) their 30,000 mol. wt. polypeptide did not coincide with the 'mature' vicilin 30,000 mol. wt. subunit. Since results in this thesis have demonstrated that it is possible to isolate this polypeptide in a single fraction from developing seeds by hydroxylapatite chromatography, future work could be directed towards investigating its nature.

Thus from these results it would appear that not all the components of vicilin and legumin are synthesised simultaneously and that the component composition of these proteins varies with the developmental stage of the cotyledon. Changes in the subunit composition may even occur during storage of mature seeds. This may be due to a combination of:

(1) differential gene expression

(2) translational control through either:

  (i) stability of mRNA

  (ii) efficiency of translation

(3) post-translational modification

Having investigated the changing component composition and pattern of accumulation of the storage proteins during seed development, it was decided to complete this study by examining the changes in component composition, size and charge of the storage proteins that occur on seed germination.

At the first stage examined: 3 days of germination, the total protein extract showed only a slight decrease in the amounts of higher mol. wt. protein subunits of legumin (40,000 mol. wt.), convicilin (71,000 mol. wt.) and vicilin (51,000 and 33,000 mol. wt.), see Fig. 50. It was possible, on isolation of individual storage protein fractions, by hydroxylapatite and Ultrogel chromatography, to demonstrate the 'multibanded' appearance of the subunits mentioned above. These fine bands, which differed only slightly in mol. wt., could be modified

subunits that have been enzymatically 'nicked' at certain points in preparation for the formation of smaller peptides at a later stage in germination, as proposed by various workers (Basha and Beevers, 1977; Shutov and Vaintraub, 1977; Bollini and Chrispeels, 1978). This nicking could be due to the excision of only a few amino acid residues and if this is the case, the protein molecule would be left relatively intact, until later cleavage. This possibility is supported by the results of Ultrogel chromatography of germinating seed extracts, which showed that, within the limits of experimental error, the molecular weight of the storage proteins; legumin, convicilin and vicilin, did not decrease in the period up to 5 days of germination. Another indication that the storage proteins remain intact after 'nicking', prior to later breakdown, comes from the fact that the surface properties of these proteins in extracts of 3 and 5 day germinated seeds, as determined by hydroxylapatite chromatography, appeared to be virtually unchanged. It was also shown that the major (60,000 mol. wt.) subunit pairs of legumin remained intact, with little decrease in mol. wt. by electrophoresing legumin, obtained by hydroxylapatite and Ultrogel chromatography of germinated seed extracts, under non-reducing conditions, see Figs. 66, 67 and 68. However, some heterogeneity was observed; this could be explained by the fact that legumin has been shown to exist in a number of different molecular forms, containing subunit pairs in different combinations (Matta, 1981). Thus different forms of legumin might undergo different initial modifications.

After the initial modification, there is at first a gradual depletion of the storage proteins; this process becomes more rapid at about 5 days after germination and appears to be more or less complete at 10 days, as can be seen from the results of electrophoretic analysis of total protein extracts (Figs. 50, 52, 54 and 56) which show the disappearance of the 71,000, 51,000, 40,000 and 33,000 mol. wt. subunits with increasing germination time. At 10 days of germination, the only protein subunits, or polypeptides, to be detected by this method were those of 20,000 and 23,000 mol. wt. It was demonstrated that the 23,000 mol. wt. subunits had a similar appearance and relative mobility to a standard albumin protein fraction, when both samples were electrophoresed together on a separate gel. Although this is not substantial proof that these subunits are those of the albumin fraction, the breakdown of the globulins before the albumins has been reported elsewhere (Danielsson, 1951; Basha and Beevers, 1977). On the other hand Murray (1979), who also detected a persistent polypeptide of 23,000 mol. wt. in germinating seed extracts, has suggested that these resistant polypeptides may have a key function as structural components of cellular organelles.

The nature of the 20,000 mol. wt. polypeptide(s) is not clear and it is difficult to say whether this is a breakdown product of larger subunits or a legumin basic subunit. It is of interest that Basha and Beevers (1977) claim to have detected a resistant low mol. wt. component of legumin at 14 days of germination, but these workers were also unable

to confirm whether this was a legumin component or a hydrolysis product.

Returning to this study, it was found that during the depletion period (up to 10 days of germination), the remaining modified protein retained the mature storage protein properties with respect to elution molarity for hydroxylapatite chromatography, and size, as determined by Ultrogel chromatography. This may be explained by the fact that not all the storage protein is degraded at the same time, hence these are the properties of the undegraded protein. It has been shown in Pisum arvense (Smith and Flynn, 1967) that there is a 'wave' of storage protein depletion which begins at the periphery of the cotyledons and spreads inwards. Similar zonation of reserve protein depletion has also been demonstrated in Vicia faba (Briarty et al., 1970): protein-body degradation begins nearest to the vascular bundles and proceeds outwards and also from the epidermis of the cotyledons inwards. Although, in Phaseolus vulgaris breakdown has been observed to start in the cells furthest from the vascular bundles (Opik, 1966). The gradual disappearance of the protein bodies with increasing germination time has also been demonstrated (Varner and Schidlovsky, 1963), while more detailed studies have been able to reveal the gradual loss of protein body contents (Bain and Mercer, 1966; Harris and Chrispeels, 1975).

Investigations into whether the protein bodies of mung beans undergo autolysis or not have demonstrated that they contain a number of proteolytic enzymes (Chrispeels and

Boulter, 1975). It has also been shown that the incubation of isolated protein bodies results in the release of amino acids. However, on analysis it was found that very little storage protein, if any, was being hydrolysed. Yet hydrolysis of the storage proteins in protein bodies was greatly accelerated by the addition of extracts from cotyledons of 4 day germinated seeds (Harris and Chrispeels, 1975). This may be explained by the findings of Shutov and Vaintraub (1977) who claim that reserve proteins must be modified by a specific protease (protease A), synthesised during germination, before the other proteolytic enzymes already present can go on to hydrolyze the modified proteins completely. They suggested that the resistance of the storage proteins to the enzymes already present (in the protein bodies) is a protection against premature hydrolysis. The results presented in this thesis fit this theory in that:

(a) an initial modification, attributed to 'nicking', of the storage proteins was shown to occur in the early stages of seed germination.

(b) depletion of the storage proteins with increasing germination time was demonstrated.

It was not possible, from the results, however, to say whether the storage proteins were degraded in any sequence, even though it has been reported that vicilin is mobilised before legumin (Konopska, 1979) and conversely legumin before vicilin (Basha and Beevers, 1977).

Finally, unlike the results of other seed germination studies on various legumes, which showed a change in

electrophoretic mobility of the proteins of germinated seed extracts with increasing germination time and attributed this to progressive deamidation (Catsimpoolas et al., 1968; Daussant et al., 1969; Shutov and Vaintraub, 1977; Konopska, 1979), no change in charge was detected by two-dimensional polyacrylamide gel electrophoresis of 3 and 5 day germinated seed extracts. It would be interesting if future work included other techniques such as two-dimensional crossed immunoelectrophoresis to confirm this result.

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ochem J. (1980) 191, 509–516
inted in Great Britain

The purification and characterization of a third storage protein (convicilin) from the seeds of pea (*Pisum sativum* L.)

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(*Received 8 April 1980/Accepted 30 May 1980*)

A third storage protein, distinct from legumin and vicilin, has been purified from the seeds of pea (*Pisum sativum* L.). This protein has been named 'convicilin' and is present in protein bodies isolated from pea seeds. Convicilin has a subunit mol.wt. of 71000 and a mol.wt. in its native form of 290000. Convicilin is antigenically dissimilar to legumin, but gives a reaction of identity with vicilin when tested against antibodies raised against both proteins. However, convicilin contains no vicilin subunits and may be clearly separated from vicilin by non-dissociating techniques. Unlike vicilin, convicilin does not interact with concanavalin A, and contains insignificant amounts of carbohydrates. Limited heterogeneity, as shown by isoelectric focusing, *N*-terminal analysis, and CNBr cleavage, is present in convicilin isolated from a single pea variety; genetic variation of the protein between pea lines has also been observed.

The storage proteins of the seeds of pea (*Pisum sativum* L.) have been divided into two fractions on the basis of solubility (Osborne & Campbell, 1898). Although both fractions have been shown to be heterogeneous (Derbyshire *et al.*, 1976), the legumin fraction contains a major component, legumin, which has been purified by several workers and has been shown to be an essentially homogeneous protein that displays subunit heterogeneity (Krishna *et al.*, 1979). The other fraction, the vicilin fraction, is not so simple in its composition, and a recent review (Derbyshire *et al.*, 1976) has presented some evidence that it contains more than one major protein. Millerd (1975) has claimed that a third storage protein is also present in peas, although it is as yet uncharacterized.

In the course of investigations into the synthesis of pea seed storage proteins, it became necessary to define the protein species present more closely than in previous work. To this end the present paper describes the purification and characterization of a homogeneous protein from pea seeds that is considered to be a third storage protein. In view of the lack of a systematic nomenclature for seed storage proteins, and for reasons to be described, it has been named 'convicilin'.

Experimental

Materials

Pea (*Pisum sativum* L.) seeds, variety Feltham First, were obtained from Suttons Seeds Ltd., Torquay, Devon TQ2 7QJ, U.K. Other genetic lines of peas were kindly given by Dr. S. Blixt, Weibullsholm, Sweden. Air-dried mature pea seeds were used. Ultrogel AcA 22 was obtained from LKB Instruments Ltd., South Croydon, Surrey CR2 8YD, U.K.; hydroxyapatite (Bio-Gel HT grade) was from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K. Sephadex G-200 and G-75 (Superfine grade), Blue Dextran and Pharmalyte Ampholines (pH ranges 3.5–10 and 4–6) were purchased from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. The marker proteins used were from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K., and were the purest grade available. All other chemicals were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K., and were of AnalaR grade, or the best available.

Abbreviations used: SDS, sodium dodecyl sulphate.

Methods

Buffers. Buffers used in the present work contained 0.05% sodium azide unless otherwise stated. Phosphate buffers were sterile-filtered through a 0.22 μM-pore-size cellulose acetate (Oxoid) membrane filter before use and their concentration determined by measurement of their refractive index at 20°C.

Purification of convicilin (*see Fig. 1 below*). Seeds were dried under vacuum for 1 h before being milled for 30s in a ball mill. The flour was sieved through a 365 μm mesh and material not passing through was discarded. The meal was extracted twice with hexane

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at 0°C for 30min (10ml of hexane/g of meal) and each time the hexane was decanted after centrifugation. The pea meal was then dried under vacuum. A portion (40g) of defatted meal was extracted with 500ml of 0.1M-potassium phosphate buffer, pH 8.0, containing 0.4M-NaCl at 4°C for 1h, centrifuged at 23000*g* for 20min and the precipitate discarded. The protein solution was subsequently fractionated by $(NH_4)_2SO_4$ precipitation at 4°C and the material precipitating in the range 50–80% relative saturation was collected by centrifugation at 23000*g* for 20min and retained. This material was redissolved in 20ml of 50mM-sodium borate buffer, pH 8.0, and loaded on to a column (5.5cm diam., 950ml vol.) of Sephadex G-200, equilibrated, and eluted with 0.1M-potassium phosphate buffer, pH 8.0, at a flow rate of 50ml/h; 10ml fractions were collected. Fractions containing convicilin were pooled and applied directly to a column (3.5cm diam., 140ml vol.) of hydroxyapatite that had been equilibrated with 0.1M-potassium phosphate buffer, pH 8.0. The hydroxyapatite column was washed, then eluted with a linear concentration gradient of potassium phosphate buffer, pH 8.0 (0.1M–0.75M, 300ml + 300ml) at a flow rate of 40ml/h, 10ml fractions being collected. Fractions containing convicilin were pooled and either concentrated by ultrafiltration or dialysed against 50mM-sodium borate buffer, pH 8.0, or distilled water, and then freeze-dried.

Gel electrophoresis. Protein samples were analysed by SDS/polyacrylamide-gel electrophoresis in 17% (w/v) polyacrylamide slab gels with the system of Laemmli (1970). Subunit molecular weights were determined in 17% polyacrylamide and 10% polyacrylamide gel slabs, the following standard proteins being run on the same slabs: phosphorylase *b* (mol.wt. 100000), transferrin (76600), bovine serum albumin (68000), catalase (60000), ovalbumin (43000), carboxypeptidase A (34300), soya-bean trypsin inhibitor (21000), cytochrome *c* (12700).

Amino acid analysis. Amino acid analysis of proteins was performed by the methods of Moore & Stein (1963) on a Locarte single-column amino acid analyser after hydrolysis of the protein for various times in 6M-HCl at 105°C *in vacuo*. The sulphur amino acids methionine (as sulphone) and cysteine (as cysteic acid) were determined by analysis of performic acid-oxidized protein (Hirs, 1956).

Determination of N-terminal amino acids. The *N*-terminal amino acids of convicilin were determined by the SDS/dansyl technique of Gray (1972). The *N*-terminal dansyl-amino acid was identified by two-dimensional chromatography on polyamide thin-layer sheets (Woods & Wang, 1967).

Sugar analysis. Sugars present in a sample of convicilin that had been precipitated from solution by adding trichloroacetic acid to a final concentration of 10% (w/v) and washed extensively with 10% trichloroacetic acid were determined after hydrolysis and gas-liquid chromatography of their derivatives (Sweeley *et al.*, 1966).

Cleavage with CNBr. Convicilin dissolved in 70% (v/v) formic acid was made to react with a 100-fold molar excess of CNBr (2g/ml solution in acetonitrile) for various times (Kasper, 1970). The solutions were evaporated to dryness under vacuum and the residues were analysed by SDS/polyacrylamide-gel electrophoresis.

Molecular-weight determination. A column (2.2cm diam., 320ml vol.) of Ultrogel AcA 22 was equilibrated by upward flow (flow rate 8.0ml/h) with 0.1M-Tris/HCl buffer, pH 8.0, containing 0.2M-NaCl. The following standard proteins were chromatographed on the column and their elution volumes measured: thyroglobulin (669000mol.wt.), ferritin (440000), *Pisum* legumin (400000), catalase (240000), immunoglobulin G (160000), bovine serum albumin (68000) and myoglobin (18000). The molecular weight of convicilin was determined by comparing its elution volume with the standard proteins on a plot of V_e/V_0 against log(mol.wt.). A total protein extract (prepared as in Fig. 1) of pea seeds was chromatographed under the same conditions; 5ml fractions were collected. The fractions were made 10% with respect to trichloroacetic acid and the precipitated protein was analysed by SDS/polyacrylamide gel electrophoresis followed by staining with Coomassie Blue and densitometric scanning of the stained gel at 580nm.

Immunodiffusion. Immunodiffusion of convicilin and vicilin against antibodies raised against protein fractions and purified by $(NH_4)_2SO_4$ precipitation and chromatography on QAE (quaternary aminoethyl)-Sephadex (Livingstone, 1974; Joustra & Lundgren, 1969) was carried out by standard methods (Ouchterlony & Nilsson, 1978). Precipitin arcs formed after 48h diffusion were cut out of the gel, washed thoroughly in 50mM-sodium borate buffer, pH 8.0, containing 0.15M-NaCl to remove soluble protein, and analysed by SDS/polyacrylamide-gel electrophoresis. Samples were dissolved in SDS sample buffer containing no 2-mercaptoethanol in order to prevent immunoglobulin G heavy and light subunits interfering with the band patterns of convicilin or vicilin.

Reaction with concanavalin A. The reaction of convicilin or vicilin with concanavalin A was tested by a diffusion system analogous to that used for immunodiffusion, with concanavalin A replacing the antibodies. Positive controls of known glycoproteins and negative controls in the presence of 0.1M-α-methyl mannoside were carried out.

Isoelectric focusing. Convicilin was subjected to isoelectric focusing in polyacrylamide-gel slabs containing 8M-urea in the pH ranges 3.5–10 and

-6 by the methods of Vesterberg (1975). The pH radient in the slab was determined by standard ıethods (Vesterberg, 1975).

Protein-body separation. Protein bodies were repared by a modification of the procedures escribed by Croy (1977). All operations were arried out at 4°C. Cotyledons (5g) from mature ea seeds before commencement of drying out were omogenized by gentle chopping in a domestic food ıixer (slow speed, 60s) in 50ml of 50mM-sodium hosphate buffer, pH7.5, containing 0.5M-sucrose. 'he homogenate was centrifuged for 5min at 300**g** nd the pellet discarded, and then centrifuged for 0min at 10000**g** and the supernatant discarded. 'he crude protein-body pellet was resuspended in ml of buffer as described above and purified by entrifugation through a step gradient prepared by ayering 5ml of 90% (w/v) sucrose, 8ml of 70% ucrose and 8ml of 30% sucrose in buffer. The rotein body sample was layered on to the gradient nd centrifuged at 60000**g** for 2h. Protein bodies vere removed from the 70/90% sucrose interface ınd subsequently analysed by SDS/polyacryl-ımide-gel electrophoresis.

Biosynthesis in vivo and in vitro during seed levelopment. Seed materials have been described reviously (Evans *et al.*, 1979) and methods in Croy *t al.* (1980).

Results

Purification of convicilin

Convicilin was purified from pea meal as shown in he flow diagram (Fig. 1). After extraction and $(NH_4)_2SO_4$ fractionation, the appropriate precipi-ate was redissolved and passed through a column of Sephadex G-200. The elution profile is shown in Fig. 2(*a*). When fractions from the large asymmetrical eak of protein were analysed by SDS/polyacryl-amide-gel electrophoresis, those from the leading dge of the peak were found to contain exclusively egumin subunits (40000 and 20000mol.wt.) (Wright & Boulter, 1974), those from the middle of he peak were found to contain 71000-mol.wt. subunits in addition, whereas those from the trailing dge of the peak had a typical vicilin pattern (50000, 3000, 17000 and lower-molecular-weight sub-nits: Croy *et al.*, 1980). The fractions containing a igh proportion of 71000-mol.wt. subunits were ooled and further purified by chromatography on ydroxyapatite. Protein was applied to the column quilibrated with 0.1M-potassium phosphate buffer, H8.0, and eluted by linear concentration gradient f this salt. The elution profile is shown in Fig. 2(*b*). he initial large peak of protein eluted at 0.25M-otassium phosphate was shown to be legumin by DS/polyacrylamide-gel electrophoresis. The eading edge of the second peak (at 0.28–0.35M-

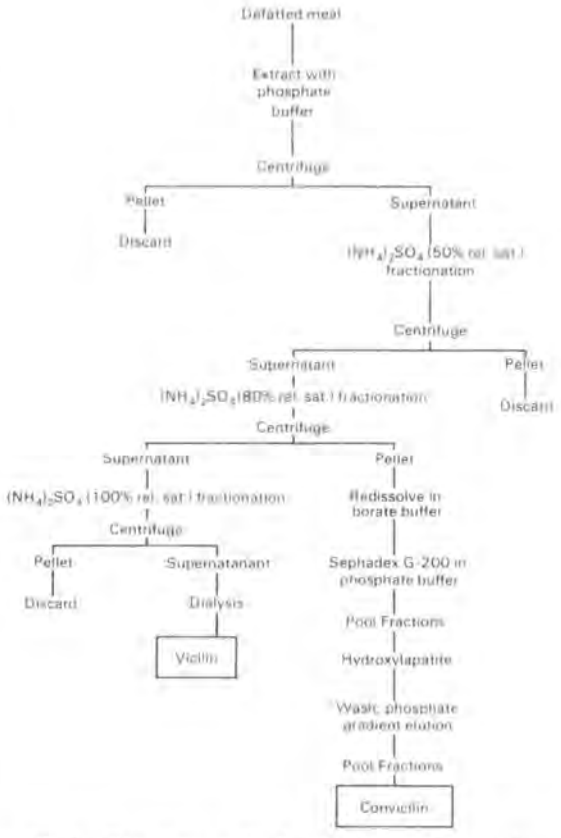

Fig. 1. *Flow diagram for the purification of convicilin*
Abbreviation used: rel. sat., relative saturation.

potassium phosphate) was convicilin, containing 71000-mol.wt. subunits with only traces of lower-molecular-weight subunits; subsequent fractions were contaminated with vicilin (shown in separate experiments to elute at 0.5M-potassium phosphate). Analysis of the purified convicilin by SDS/poly-acrylamide-gel electrophoresis is shown in Fig. 3; the presence or absence of 2-mercaptoethanol made no difference to the observed band pattern. Densito-metric scanning of the stained gel showed that the convicilin was at least 90% pure.

Properties of convicilin

Amino acid composition. The amino acid com-position of convicilin is given in Table 1, and compared with vicilin and legumin from *Pisum sativum*. The amounts of methionine and lysine in convicilin correspond to 0.9 residue per 71000 mol.wt. subunit for both.

N-Terminals. The *N*-terminal amino acids of convicilin, determined by using the dansyl/SDS technique (Gray, 1972) were aspartate and leucine in comparable amounts.

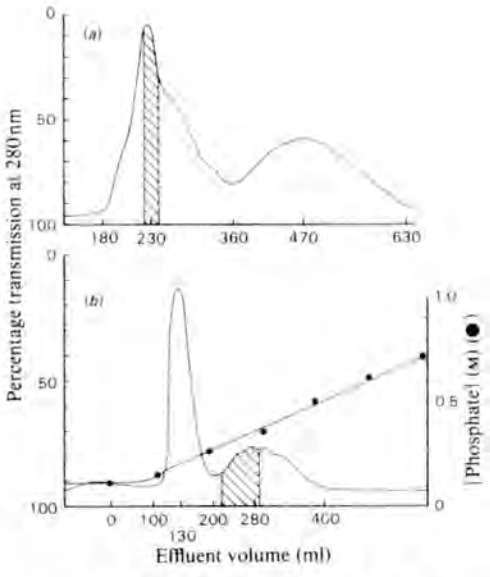

Fig. 2. *Purification of convicilin*
(*a*) Elution profile from chromatography on Sephadex G-200. (*b*) Phosphate-gradient-elution profile from chromatography on hydroxyapatite. Hatched areas show the fractions containing convicilin.

Table 1. *Amino acid composition of convicilin and vicili*
Values are means of three or more separate determinations. The S.E.M. was less than 5% in all cases.

	Composition (residues/100 residues)		
Amino acid	Convicilin	Vicilin	Legumin‡
Asp	11.64	18.87	13.07
Thr	2.55*	2.69*	3.08
Ser	6.39*	7.67*	5.77
Glu	22.08	19.18	19.74
Pro	5.47	1.99	5.50
Gly	5.90	4.85	6.89
Ala	4.23	4.13	6.01
Val	4.46	4.84	4.97
Met	0.13†	0.00†	0.66
Ile	3.85	5.11	3.95
Leu	8.71	9.83	7.60
Tyr	2.59	1.91	3.64
Phe	3.30	4.67	2.30
His	2.22	1.61	1.75
Lys	8.18	8.10	4.24
Arg	8.15	4.57	9.99
Cys	0.13†	0.00†	1.21

* Corrected for decomposition from 22, 48 and 72h hydrolyses.
† From analysis of performic acid-oxidized protein.
‡ From Casey (1979).

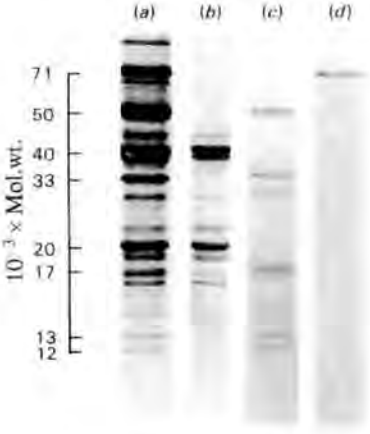

Fig. 3. *Analysis by SDS/polyacrylamide-gel electrophoresis of various protein fractions and convicilin*
(*a*) Total soluble protein fraction (see Fig. 1); (*b*) purified legumin; (*c*) purified vicilin; (*d*) purified convicilin.

Absorbance. The $A^{1\%}_{280,1\,cm}$ for convicilin was 7.3.

Sugar analysis. Small amounts of glucose, galactose and mannose were detected in convicilin, but the amounts were comparable with those found in a known non-glycoprotein (bovine serum albumin) and were significantly less than one sugar residue per 71000-mol.wt. subunit; it was therefore concluded that convicilin contains no significant amounts of carbohydrate.

Cleavage with CNBr. Two major fragments were produced on CNBr cleavage; they had approx. mol.wts. 55000 and 14000 (see Fig. 4). This indicates one methionine residue per subunit, in agreement with the amino acid analysis. However, some material remained undigested, even after 48h reaction, and there were a number of further minor cleavage products.

Subunit molecular weight. Molecular weights of 72000 ± 3000 in 17% acrylamide gels, and 70000 ± 2500 in 10% acrylamide gels, were determined for the convicilin subunit(s) on SDS/polyacrylamide-gel electrophoresis by comparison with standard proteins. A mean value of 71000 was adopted.

Molecular weight. Purified convicilin was eluted as a single peak after gel-filtration chromatography (see Fig. 6*b* below) A mol.wt. of 290000 ± 40000 was determined for convicilin by comparison with standard proteins, suggesting a tetrameric molecule consisting of four 71000-mol.wt. subunits.

Serological properties. When convicilin and vicilin (free of cross-contaminating subunits) were allowed to diffuse against an antiserum prepared against an extract containing both in an Ouch-

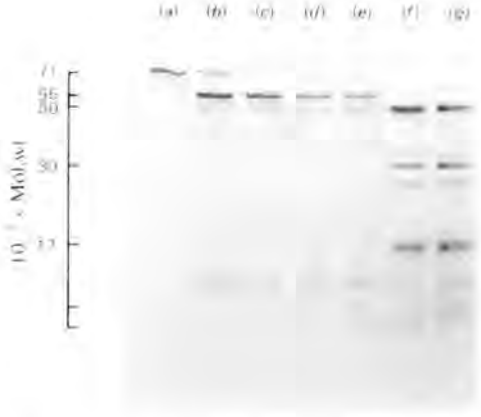

Fig. 4. *Analysis by SDS/polyacrylamide-gel electrophoresis of convicilin and vicilin before and after reaction with CNBr*

(*a–e*) Convicilin before (*a*) and after 4h (*b*), 8h (*c*), 24h (*d*), 48h (*e*) reaction. (*f* and *g*) Vicilin before (*f*) and after 48h (*g*) reaction.

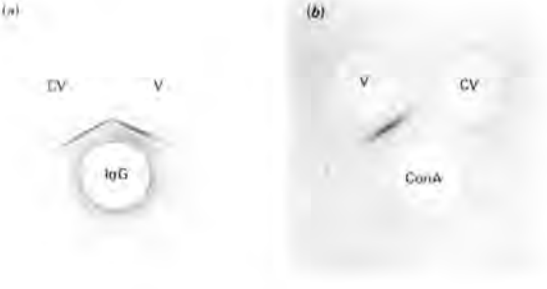

Fig. 5. *Cross-reactivity of convicilin and vicilin*

(*a*) Comparison of convicilin (CV) and vicilin (V) by double immunodiffusion against antibodies (immunoglobulin G) raised against a protein fraction containing both proteins. (*b*) Double diffusion of convicilin and vicilin against concanavalin A (Con A).

terlony double-immunodiffusion test, the two proteins gave a reaction of identity (Fig. 5*a*). The precipitin arcs produced were excised and analysed by SDS/polyacrylamide gel electrophoresis. The immunoprecipitate produced by the reaction of convicilin with the above antibodies showed only 71000 subunits, with no trace of vicilin subunits and vice versa. Convicilin gave a reaction of non-identity with legumin when tested against antiserum prepared against total pea proteins.

Reaction with concanavalin A. Concanavalin A gave no reaction with convicilin in a double immunodiffusion experiment (Fig. 5*b*) when vicilin, known to react with concanavalin A, was included as a positive control.

Isoelectric-focusing properties. Convicilin was subjected to isoelectric focusing in a polyacrylamide-gel slab containing 8M-urea. The protein focused to a series of closely spaced slightly blurred bands, indicating limited heterogeneity, in the pI range 5.6–5.8.

Evidence that convicilin is a separate storage protein

When a total protein extract or globulin protein fraction from peas, prepared under non-dissociating conditions, was subjected to zonal isoelectric precipitation on Sephadex G-75 (Wright & Boulter, 1974), convicilin was found associated with the legumin (retarded) fraction if the ionic strength was approx. 0.15, or with the vicilin (unretarded) fraction if the ionic strength was approx. 0.25 or higher. Convicilin was also eluted from a zonal-isoelectric precipitation column separately from vicilin and legumin by a linear salt gradient at pH5.0 (R. R. D. Croy, unpublished work). In addition, the purification procedure above employs only non-dissociating conditions throughout and as such successfully separates convicilin completely from vicilin and legumin. These results strongly support the existence of a separate and distinct protein containing only 71000-mol.wt. subunits.

Since the existence of a separate convicilin protein when purified may be argued as being due to selection of molecules containing only 71000-mol.wt. subunits from a population of molecules containing 71000-mol.wt. subunits in association with others during purification, a further experiment was carried out. A total protein extract from peas was fractionated by gel filtration on Ultrogel AcA 22 under non-dissociating conditions. Fig. 6 shows the distribution of the proteins in the elution profile from the gel-filtration column deduced from the SDS/polyacrylamide-gel-electrophoresis subunit patterns of each fraction.

The results show discrete but overlapping peaks of legumin, convicilin and vicilin in order of decreasing molecular weight. All three proteins eluted as symmetrical peaks without undue spreading: the centres of the peaks were at estimated mol.wt. values (±10%) of 380000, 280000 and 160000 respectively, indicating that convicilin in the extract has the same molecular weight as purified convicilin. The final large peak eluted from the Ultrogel column (Fig. 6*a*) represents the lower-molecular-weight proteins, including the lectin and major albumin as shown in gel 94 (Fig. 6*a*). (R. R. D. Croy, unpublished work).

Further evidence that the 71000-mol.wt. subunits

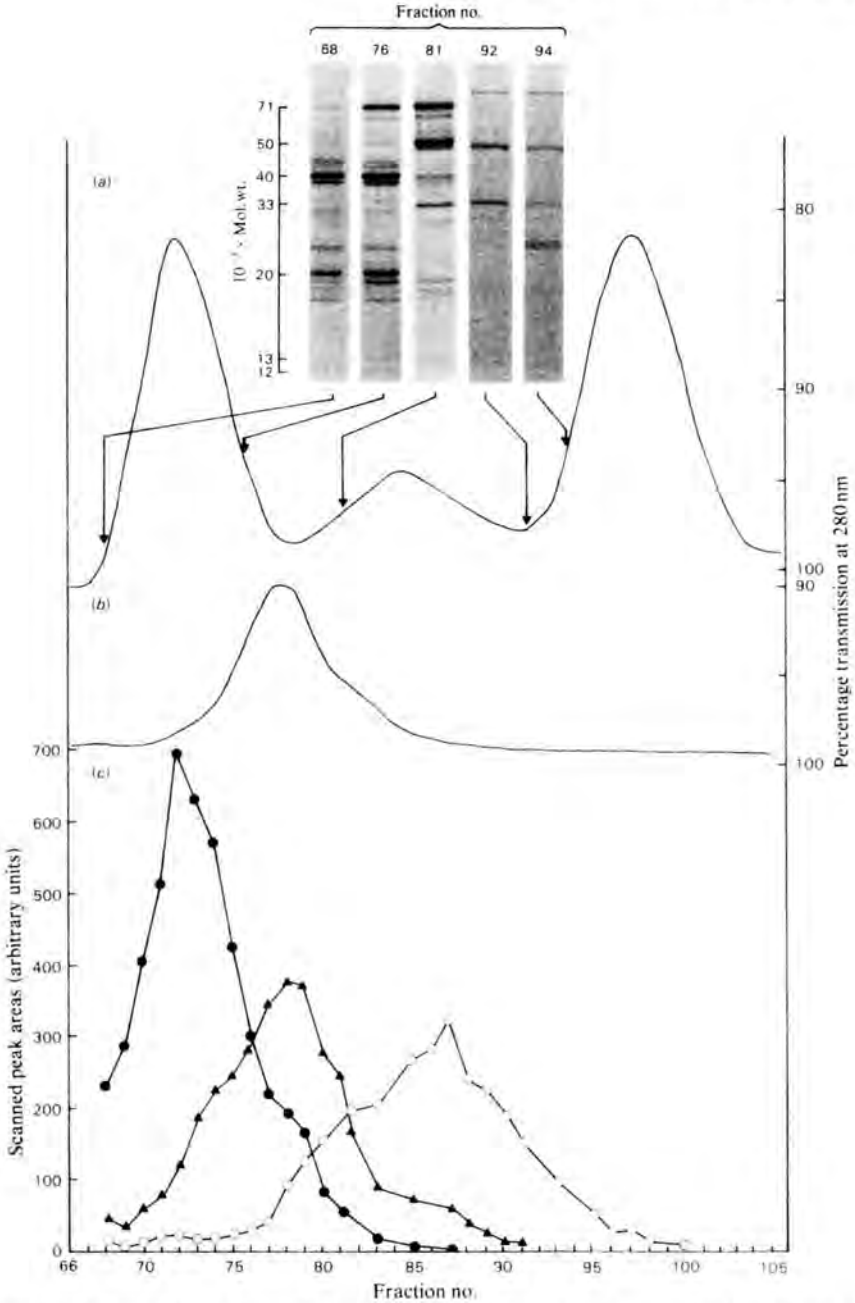

Fig. 6. *Gel filtration of a total protein extract (Fig. 1) of pea seeds on a column of Ultrogel AcA 22*
(*a*) Elution profile of a total protein extract chromatographed on the column monitored at 280nm; (*b*) the elution profile of purified convicilin chromatographed on the same column monitored at 280nm; (*c*) distribution of legumin (40000-mol.wt. subunits) (●), vicilin (50000-mol.wt. subunits) (○) and convicilin (71000-mol.wt. subunits) (▲) relative to the elution profile in (*a*), as determined from densitometric scans of SDS/polyacrylamide-gel-electrophoretic analyses of each fraction from the column. Legumin values have been plotted on half the indicated scale for clarity. Electrophoretic analyses of representative fractions are shown in (*a*).

belong to a protein distinct from vicilin (50000-mol.wt. subunits) follows from experiments in which the biosynthesis of the storage proteins has been monitored *in vivo* and *in vitro* during seed development. At an early stage in the laying down of the storage proteins during seed development, 50000-

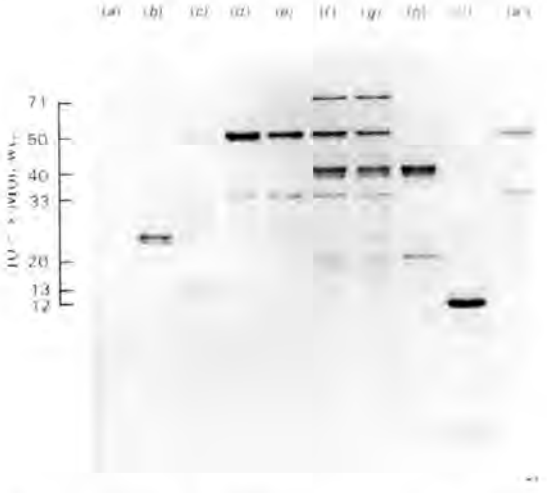

Fig. 7. *Analyses by SDS/polyacrylamide-gel electrophoresis of total soluble proteins from pea seeds harvested at different stages of development*

(*a* and *a'*) Vicilin standard (3 and 10 μg respectively). (*b*) Albumin fraction from mature seeds. (*c–g*) Total soluble proteins isolated from seeds 11 days (*c*), 13 days (*d*), 17 days (*e*), 21 days (*f*), 33 days (*g*) after flowering. (*h*) Legumin standard (10 μg). (*i*) Pea seed lectin standard (10 μg).

mol.wt. but not 71000-mol.wt. subunits are synthesized, whereas towards the end of the storage-protein-deposition stage, the 71000-mol.wt. subunits are accumulated (Fig. 7). Similar results are found in the translation products when polyribosomes at the two stages are translated in the wheat-germ and reticulocyte cell-free systems (Croy *et al.*, 1980). From the results of the protein-accumulation experiment (Fig. 7) it is noteworthy that there is a sequential accumulation of seed proteins in the order vicilin, legumin and convicilin.

Convicilin was shown to be present in the protein bodies of mature pea cotyledons by lysing isolated protein bodies and analysing their contents by SDS/polyacrylamide-gel electrophoresis. Convicilin (71000-mol.wt. subunits) was a major component of the protein-body proteins.

Discussion

A third storage protein distinct from vicilin and legumin has been identified in peas on the following evidence.

(i) Under non-dissociating conditions a protein containing only 71000-mol.wt. subunits can be separated (see Fig. 3).

(ii) The protein has a distinctive amino acid composition, particularly with respect to sulphur amino acids, having one cysteine residue and one methionine residue per subunit, whereas vicilin has no sulphur amino acids and legumin has approx. four methionine and seven cysteine residues per 60000-mol.wt. subunit. This difference is reflected in the results of CNBr cleavage: convicilin is cleaved into two fragments (approx. 55000 and 14000 mol.wt.), whereas the reagent has no apparent effect on vicilin (Fig. 4; Croy *et al.*, 1980). The glutamic acid/aspartic acid ratio in convicilin is high (approx. 2:1), distinct from vicilin (approx. 1:1) and legumin (approx. 1.5:1).

(iii) The protein gives a serological reaction of non-identity with legumin.

(iv) Experiments on biosynthesis *in vivo* and *in vitro*.

This protein has been named convicilin on the basis of its serological cross-reactivity with vicilin. Alternative names, e.g. vicilin 2, were rejected as confusing, and until such time as a systematic nomenclature for seed storage proteins is developed, we would propose the name 'convicilin' be used. However, no homology to other 'con-' proteins, e.g. concanavalin A, conglycinin or conarachin is implied. Furthermore, convicilin cannot be the third protein referred to by Millerd (1975) on the basis of the difference in subunit molecular weights and antigenic properties (Millerd *et al.*, 1978).

The serological results show that convicilin has all the vicilin determinants and therefore must be closely related to vicilin. The absence of additional determinants in convicilin shown by the immunodiffusion experiment may reflect the type of antisera used, since these were raised against a protein fraction containing a mixture of vicilin and convicilin (approx. 4:1).

Although convicilin is sometimes found as a contaminant in crude legumin preparations, as may be deduced from the results reported by Casey (1979), it is more often found in the vicilin fraction of pea globulins. Convicilin is difficult to purify from vicilin, and unless the purification procedure outlined in the present paper is used, most other procedures result in convicilin preparations heavily contaminated with vicilin. This is illustrated by the results of gel filtration of total pea seed proteins shown in Fig. 6, which clearly show vicilin contaminating many convicilin fractions. However, the subunit patterns of the earlier convicilin fractions with very high 17000-mol.wt./50000-mol.wt. subunit ratios (> 10 by densitometric scanning of gels) could not be explained by hybrid molecules containing both types of subunit, but only by vicilin contamination. Further, the normal distribution of 71000-mol.wt. subunits on elution gives no evidence for hybrid molecules, although the existence of such species cannot be ruled out on present evidence. The association of convicilin with vicilin is

implied in the results of other authors, who have commented on the heterogeneity of the vicilin fraction of pea (Derbyshire *et al.*, 1976) and by the C.S.I.R.O.'s group in Australia (Thomson *et al.*, 1978; Millerd *et al.*, 1978), who have noted repeatedly the presence of a major 75000-mol.wt. subunit in pea vicilin preparations that appears to be similar to the 71000-mol.wt. subunit described here. On the basis of crossed immunoelectrophoresis and agarose-gel electrophoresis, these authors have suggested the presence of several different vicilin types, some, but not all of which contain the 75000-mol.wt. subunit, but none of which contain only this type of subunit.

Although convicilin is homogeneous by SDS/polyacrylamide-gel electrophoresis, heterogeneity must be present in the protein, since it does not give a single band on isoelectric focusing and it has two *N*-terminal amino acids. Also, the results of cleavage with CNBr indicate that sequence heterogeneity may be present, since although most of the 70000-mol.wt. subunit is cleaved rapidly (within 4h), a significant proportion remains uncleaved even after prolonged (48h) reaction. This resistant fraction of convicilin may have another amino acid substituted for methionine. As well as heterogeneity in a single variety, genetic variation in convicilin has been observed among certain pea lines, e.g. in line 1552 (provided Dr. S. Blixt): the convicilin band appears to be of significantly lower molecular weight on SDS/polyacrylamide-gel electrophoresis (approx. 65000) (result not shown). Others have also presented evidence showing qualitative and quantitative variations in convicilin subunits (Przybylska *et al.*, 1978).

An equivalent protein to *Pisum* convicilin has also been isolated from field bean (*Vicia faba minor*) (variety Maris Bead) by the purification technique described in the present paper. Its molecular-weight on SDS/polyacrylamide-gel electrophoresis appears to be slightly lower than the *Pisum* protein subunit (approx. 68000) (R. R. D. Croy & M. Tyler, unpublished work).

Although no experiments designed to test the function of convicilin have been carried out, it is considered to be a storage protein, since it is not only a major seed protein but also a major component of protein bodies. As previously stated, convicilin contains sulphur amino acids, whereas vicilin does not, and is thus a component of pea storage proteins of greater nutritional value than vicilin.

We acknowledge with gratitude the assistance of Mr. C. Alcock in performing carbohydrate analysis, of Dr. A. Pusztai in performing confirmatory amino acid analyses, and of Miss H. Morton in preparing protein bodies.

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l. Biochem. *118*, 627–633 (1981)
:BS 1981

aracterisation and Subunit Structures of the Vicilin Storage Proteins of Pea *sum sativum* L.)

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eived January 26 / April 15, 1981)

Investigations of the vicilin fraction of the storage proteins of pea (*Pisum sativum* L.) have shown that its major components are a number of protein species of M_r 170000. Convicilin (M_r 280000, composed of 71000-M_r subunits) is a separable component of this fraction. The vicilin proteins are composed principally of $\approx$ 50000-M_r polypeptides, but also contain a number of smaller polypeptides. The sub-unit polypeptide composition of vicilin changes during seed development quantitatively and qualitatively. Vicilin sub-units have been shown to be synthesised as polypeptides of $M_r \approx 50000$ by means of pulse-labelling experiments *in vivo*, and synthesis of vicilin *in vitro* directed by mRNA, polysomes and microsomes extracted from pea cotyledons in cell-free translation systems. Polypeptides then undergo two distinct types of proteolytic modification: (a) co-translational removal of a small polypeptide (M_r less than 1000); (b) 'nicking' of polypeptide chains in assembled vicilin molecules, which occurs more than 4 h after their initial synthesis. The basic structure of the vicilin molecule is thus a multimer, possibly a trimer, of $\approx$ 50000-M_r subunits. The heterogeneity of the initially synthesised 50000-M_r subunits accounts not only for the several different 50000-M_r polypeptides found in vicilin, but also for the range of minor polypeptides, since the 'nicking' points will differ among subunits. It also accounts for the observed partial separation of vicilin into different molecular species, since different subunit combinations will give rise to molecules with different properties. Vicilin is also glycosylated and this is a source of further variation.

The storage proteins of pea (*Pisum sativum* L.) have been ıventionally divided into two fractions, legumin and vicilin Depending on the variety, vicilin is usually the quantiively smaller fraction and is thought to contain more than protein [2], since it is heterogeneous when analysed in ultracentrifuge [3] by ion-exchange chromatography [4] when fractionated by solubility under varying conditions pH, ionic strength and temperature [5,6] or by carbodrate affinity chromatography [7]. The vicilin fraction conns major polypeptides of M_r 71000, 50000 and 33000 with nor components of lower M_r [8]. Previous reports have imed to have isolated 'vicilins' of differing subunit comsitions, either varying slightly in the relative amounts of different polypeptides present, or in the absence of one more of the polypeptides [7,9–11], but in no case has a cilin' containing a single polypeptide, or even two or three ypeptides, been isolated.

Recently it was shown [12] that the polypeptide of M_r 000 was the subunit of a protein distinct from the rest the vicilin fraction. This protein, named 'convicilin', had M_r of 280000 (i.e. four 71000-M_r subunits) and although nunologically related to the other vicilin protein(s) could separated from them by non-dissociating techniques. In s paper the terminology used will be as follows: 'the vicilin ction' will refer to convicilin and vicilins, i.e. all the maal reacting with anti-vicilin antibodies; 'vicilin' will refer material of the vicilin fraction containing no convicilin; 'vicilin I', etc. will refer to individual molecular species king up vicilin.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphoacid; IgG, immunoglobulin G.

The complex mixture of polypeptides found in the vicilin fraction isolated from mature pea seeds is not reflected in the translation products *in vitro* of polysomes or mRNA isolated from developing pea cotyledons, since only polypeptides of M_r about 50000 and 71000 (the latter corresponding to convicilin) are immunoprecipitated from translation products by anti-vicilin antibodies [13,14]. No smaller vicilin polypeptides were observed in immunoprecipitates, or were apparent in the total translation products. Spencer et al. [15] have further reported that pulse labelling *in vivo* (2 h) of developing pea cotyledons with radioactive amino acids showed that initially radioactivity was not incorporated into vicilin polypeptides of M_r less than 50000. There is thus a discrepancy between the observed polypeptide content of vicilin as isolated from seeds (accumulated vicilin), and the polypeptides recognizable as vicilin translation products synthesised *in vitro* and *in vivo*.

The present paper describes work investigating the synthesis of vicilin polypeptides *in vitro* and post-translational modification of vicilin, in comparison with the structure of accumulated vicilin.

MATERIALS AND METHODS

Plant Materials

Seeds of *Pisum sativum* L. c.v. Feltham First (Suttons Seeds Ltd, Torquay, Devon, UK) were germinated and grown as described previously [13]. Developing seeds were harvested, frozen and lyophilised prior to protein extraction; mature seeds were air-dried. Cotyledons from developing seeds were separated under sterile conditions; those used for RNA ex-

traction were stored frozen at −80 °C, whereas cotyledons to be labelled *in vivo* were used immediately.

Purification of Vicilin

Pea seed meal (40 g) was prepared, extracted with 50 mM potassium phosphate buffer pH 8.0, and fractionated by hydroxyapatite chromatography as described previously [16]. The broad peak of protein eluting in the range of phosphate buffer concentration 0.3–0.5 M was collected, dialysed against 5 mM sodium borate buffer pH 8.0, and lyophilised. This contained vicilin and convicilin. To remove convicilin, the lyophilised material was redissolved in 0.1 M potassium phosphate and rechromatographed on a hydroxyapatite column as described by Croy et al. [12]. Eluent fractions were assayed by dodecyl sulphate/polyacrylamide gel electrophoresis. Fractions eluting at lower phosphate buffer concentrations, 0.3–0.37 M, contained convicilin and were discarded: fractions eluting at higher phosphate concentrations, 0.37–0.5 M, contained almost exclusively vicilin and were pooled, dialysed and lyophilised as before.

Alternatively, the extract of pea seeds was made 85% saturated with respect to $(NH_4)_2SO_4$ at 4 °C and the material precipitating was collected by centrifugation at 23000 × *g* for 20 min and discarded. The supernatant, which contained no convicilin, was dialysed and lyophilised as above, and the vicilin was purified by gel filtration on a column of Ultrogel AcA 22 (2.2 cm diameter, 320 ml. vol., flow rate 12.0 ml/h) equilibrated with 0.1 M Tris/HCl buffer, pH 8.0, containing 0.25 M NaCl. Two distinct peaks of protein were eluted: the first peak contained vicilin, and constituent fractions were pooled, dialysed and lyophilised as above. Analysis by two-dimensional gel electrophoresis showed that both these methods gave vicilin preparations depleted in the more acidic vicilin species as compared to material present in total extracts, although the overall subunit patterns were identical.

Gel Electrophoresis

Dodecyl sulphate/polyacrylamide gel electrophoresis was carried out in gels of 10%, 12% and 17% acrylamide concentrations by standard methods [17, 18]. Gels were calibrated for M_r determination as described by Croy et al. [12]. Polyacrylamide gel electrophoresis under non-dissociating conditions was carried out in gels of 7.5% and 10% acrylamide concentration by the Laemmli [17] system modified by the omission of sodium dodecyl sulphate. Gels were stained with Coomassie blue R-250 and destained as described [19]. Seed material was extracted in the appropriate sample buffer for 2 h at room temperature at 40 mg meal/ml extractant and the supernatant after centrifugation at 5000 × *g* for 5 min taken for analysis on gels. Two-dimensional gel electrophoresis using dodecyl sulphate/polyacrylamide gel electrophoresis in the second dimension was carried out by equilibration of a stained strip of first-dimension gel with second-dimension sample buffer, after which the strip of gel was laid on top of the stacking gel of the second-dimension gel slab, which was subsequently run as normal [20]. Two-dimensional dodecyl sulphate/polyacrylamide-isoelectric focusing gel analysis was carried out by similar methods [20].

Crossed immunoelectrophoresis was carried out as described by Weeke [21] using 300 µl antiserum per 15 ml second-dimension agarose gel. Immunoprecipitates were produced by double diffusion in agarose gels [22]: the precipitates were excised, washed four times for 20 min in 20 ml saline, then 30 min in 0.2 M Tris/HCl, pH 6.8; they were then incubated 20 min with sample buffer and analysed dodecyl sulphate/polyacrylamide gel electrophoresis, loa the agarose gel slice.

Gels were processed and fluorographed by the met of Bonner and Lasky [23] as described by Evans et al. [1.

Molecular Weight Determinations

The M_r of purified vicilin and of vicilin in total extr of pea seeds (40 mg/ml of column buffer) were determi on a calibrated column of Ultrogel AcA 22 as describec Croy et al. [12]. In addition, a column of Sephacryl S- superfine (1.6-cm diameter, 112 ml vol.), equilibrated v 0.1 M Tris/HCl, pH 8.0 containing 0.25 M NaCl, 0.1% sod azide was employed: the column was run with upward f at 5.8 ml/h. The following standard proteins were used calibrate the column: ferritin (M_r 440000), catalase (2400 immunoglobulin, IgG (160000), bovine serum albu (67000), ovalbumin (43000) and myoglobin (18000): vic samples and seed extracts were chromatographed. Fracti were collected, and the proteins were precipitated with chloroacetic acid (12% final concentration) and analysed dodecyl sulphate/polacrylamide gel electrophoresis.

Ion-Exchange Chromatography

Vicilin was chromatographed on a column of DEA cellulose (DE-52, 2.5-cm diameter, 100 ml. vol.) equilibra with 50 mM Tris/HCl buffer, pH 8.0. The column was elu with a linear 0–0.4 M gradient of NaCl in column buff Vicilin eluted as a broad peak containing several unresolv components in the salt concentration range 0.1–0.25 Fractions were precipitated with trichloroacetic acid a analysed by dodecyl sulphate/polyacrylamide gel elect phoresis as described.

Protein Synthesis in vivo

Cotyledons from peas at 11 and 14 days after floweri were transferred under sterile conditions to culture medi (1 ml medium per cotyledon) prepared as described by Mill et al. [24], but lacking amino acids. Radioactive amino aci either [^{3}H]leucine or ^{3}H-labelled total amino acid mixt (25 µCi per cotyledon), were then added, and the cotyledc incubated 4 h at 28 °C in light. Where applicable, cotyledc were transferred to medium containing unlabelled ami acids and incubated for further periods under the same c ditions. Cotyledons were immediately frozen at the end the incubation period and subsequently used for protein traction as described. Results from the two developmen stages were qualitatively similar.

Protein Synthesis in vitro

Total polysomes were extracted as described by Eva et al. [13]. Microsomes were extracted by a modification methods used by Cameron-Mills et al. [25, 26]. Cotyledc were homogenised by low-speed chopping in buffer A (0.2 Tris/HCl pH 9.5, 60 mM KCl, 30 mM $MgCl_2$, 0.2 M sucro 1 mM dithiothreitol). The homogenate was filtered throu two layers of Miracloth and centrifuged at 500 × *g* for 10 m The pellet was discarded and the supernatant was centrifug at 29000 × *g* for 10 min. The pellet of crude microsomes v resuspended in 2.26 M sucrose in buffer B (40 mM Tris/H

.5, 20 mM KCl, 10 mM $MgCl_2$). The suspension was ayed with equal portions of 1.75 M, 1.5 M, and 0.2 M ɔse in buffer B, and centrifuged at 96000 × *g* for 16 h in ing-out rotor. Two bands formed, one at the 1.75 M/ M sucrose interface, the second at the 1.5 M/1.75 M ɔse interface. The bands were both removed with a syringe, ed with equal volumes of buffer B and layered onto a ion of 1.3 M sucrose in buffer B. This was centrifuged 65000 × *g* for 1 h. The pellet formed was rinsed with M sucrose in buffer B and either resuspended in Hepes ɛr pH 7.6 for immediate use, or frozen as a pellet and ɛd at − 80 C. The absorbance was measured at 260 nm 280 nm to determine the amount of RNA present (A_{260} 1 mg/ml = 25).

ɔaration of Stripped Microsomes 'crosomal Membranes)

This was carried out by a method adapted from Cameron-.s and Ingversen [27]. A microsomal pellet was resuspended ɔuffer C (40 mM Tris/HCl, pH 8.5, 20 mM KCl). After ıbation at 0 C for 30 min, the suspension was layered r a two-step gradient comprising of 0.73 M and 0.3 M rose in buffer C, and centrifuged at 100000 × *g* for 1 h. The et was suspended in Hepes buffer, pH 7.6, and stored in ıid air. The absorbance at 260 nm and 280 nm was mea-ɛd.

'imycin-A-Insensitive NADH-Dependent ɔchrome c Reductase Assay

This was carried out as described by Bollini and Chrispeels]. The reaction mixture contained 8 mg cytochrome *c*, l 0.1 M KCN, 1 µl antimycin A at a concentration of 8 mg/100 µl ethanol, 0.8 ml 1 M potassium phosphate ffer, pH 7.5, 5 mg NADH in a total volume of 10 ml. mples of the various polyribosomal preparations were ded to 1 ml of reaction mixture and the change in ab-bance at 550 nm with time was measured.

nslation of Samples

Microsomes, polyribosomes, and polyribosomes plus pped microsomal membranes were used as templates in reticulocyte lysate cell-free system as described by Evans l. [13], using [^{3}H]leucine as radioactive amino acid.

Optimum concentrations of K^+, Mg^{2+}, and spermine were ermined for each preparation, but did not differ signifi-tly from conditions described previously [13]. Products e analysed by dodecyl sulphate/polyacrylamide gel electro-ɔresis and fluorography [13]. Immunoprecipitation was ried out by previously described methods [8, 13] or by an ıptation of those methods employing Sepharose-bound ɔtein A as a second 'antibody'.

SULTS

RIFICATION OF VICILIN

Vicilin was purified from extracts of pea seeds by hydroxy-tite chromatography [16] followed by rechromatography hydroxyapatite to remove convicilin. Alternatively, seed racts were fractionated by addition of ammonium sulphate 35% saturation, the supernatant being retained. This con-ɛd no convicilin. It was purified by gel filtration on a column of Ultrogel AcA 22. The vicilin preparations obtained were completely soluble of neutral pH. Vicilin was also prepared by the zonal isoelectric precipitation method of Wright and Boulter [29] but was found to be subsequently incompletely soluble, although of similar subunit composition to vicilin prepared as above.

CHARACTERISATION OF VICILIN

Dodecyl sulphate/Polyacrylamide Gel Electrophoresis

The polypeptide band patterns of vicilin isolated from mature and developing pea seeds are shown in Fig. 1. The band patterns were essentially independent of the three methods used of purify the protein, and of the gel system (continuous or discontinuous) used in its analysis. Polypeptides of the following M_r were observed for mature seeds: 50000, 35000, 33000, 30000, 19000, 15000, 13000 and 12500. In all cases the 50000-M_r components were most prominently stained followed by the 33000-M_r component. However, in agreement with results reported by Wright [30] for *Vicia faba*, the relative amounts of the subunits varied during development, subunits of M_r less than 50000 being present in relatively smaller amounts in earlier stages than later. In addition, a subunit of M_r 47000 found at earlier stages was not present later. The 50000-M_r band often showed resolution into more than one component, dependent on sample and loading.

Molecular Weight Determination

Vicilin isolated from mature seeds behaved as a single component of M_r 145000 ± 15000 in the ultracentrifuge (Pusztai, unpublished results). The same material behaved as a single component of M_r 170000 ± 20000 on gel filtration. Further gel filtration experiments showed that the molecular weights of vicilin in extracts of 11-day-old, 14-day-old and mature seeds and the molecular weights of vicilin purified from 11-day-old and mature seeds were all identical within experimental error, even though the subunit composition of the vicilin differs at these different developmental stages as described above. These results show that the M_r of vicilin is not altered on purification. In no case was any significant separation of vicilin into types containing differing subunits observed on gel chromatography.

Charge Heterogeneity

The charge heterogeneity of purified vicilin of mature pea seeds was investigated by ion-exchange chromatography on DEAE-cellulose at pH 8.0. This effected a partial separation into different forms: analysis of fractions across the peak of eluted protein showed that whereas the leading edge contained all types of vicilin subunits, the trailing edge lacked the 33000-M_r subunit. A parallel result was obtained by two-dimensional gel electrophoretic analysis of a total protein extract of pea seeds, employing non-dissociating conditions in the first dimension and dissociating conditions in the second (Fig. 2A). Vicilin 50000-M_r subunits were distributed over a series of components (i.e. molecular species), in the first dimension but the 33000-M_r subunits were only found in the slower moving predominant components. Two-dimensional analysis of protein extracts of peas at earlier developmental stages (Fig. 2B) showed a similar separation of vicilin,

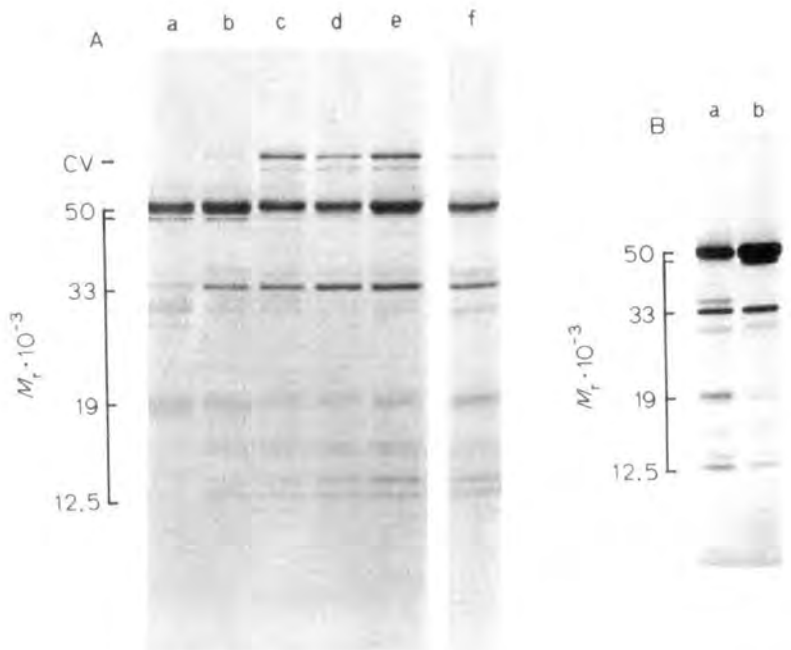


Fig. 1. *Dodecyl sulphate/polyacrylamide gel electrophoresis of vicilin preparations.* (A) Vicilin prepared from pea cotyledons at different stage development by hydroxyapatite chromatography. 20 µg protein loaded per gel track. Cotyledons harvested 11 days (a), 13 days (b), 17 days 21 days (d) and 33 days (e) after flower opening. Track f shows material of track e re-chromatographed to remove most convicilin (B) Tracks a and b: vicilin prepared from pea cotyledons by ammonium sulphate precipitation: (a) cotyledons 33 days after flowering (1 protein); (b) 11 days after flowering (30 µg protein)

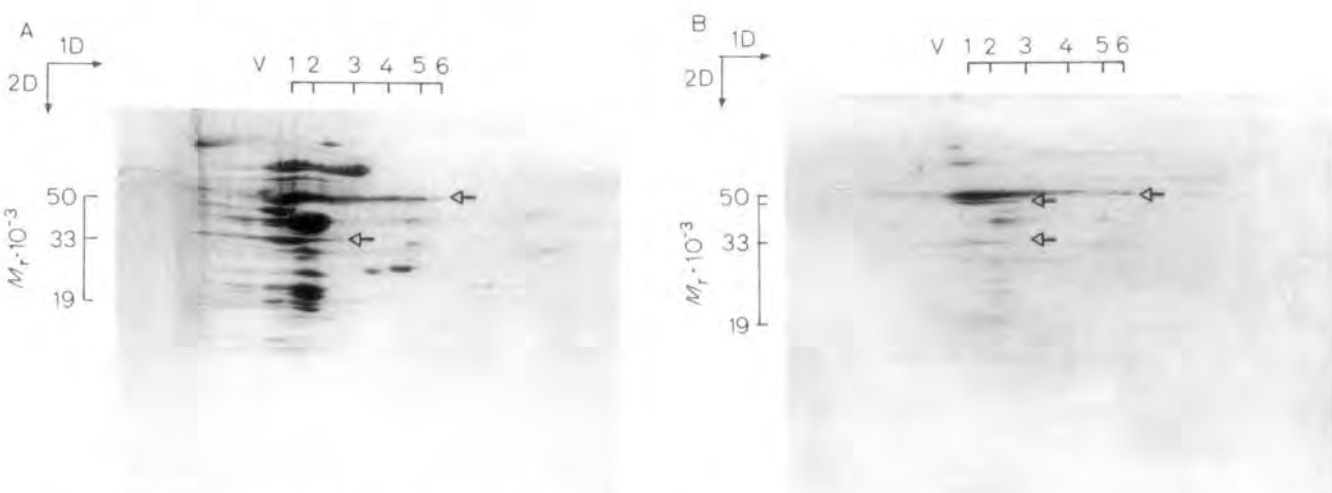


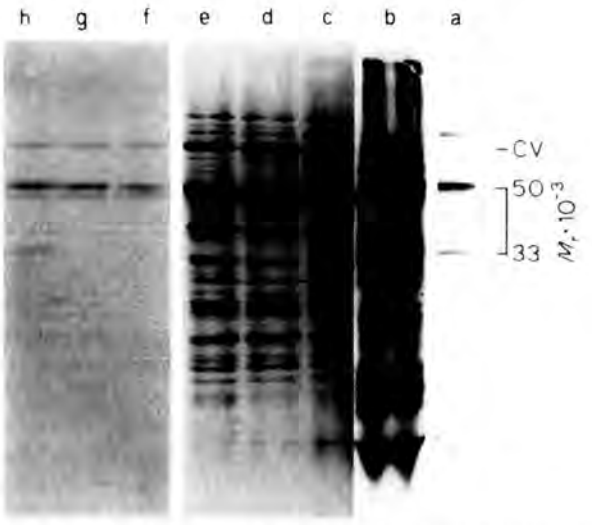
Fig. 2. *Two-dimensional gel analysis of total protein extracts of mature pea cotyledons (A) and cotyledons harvested 11 days after flowering* 1D = polyacrylamide gel electrophoresis at pH 8.8 (non-denaturing); 2D = dodecyl sulphate/polyacrylamide gel electrophoresis. Vicilin subu are indicated by M_r and arrows: vicilin molecular species by V1, V2 etc.

with the 47000-M_r and 33000-M_r subunits present only in the slower moving species.

The vicilin polypeptides of each M_r type were also shown to exhibit charge heterogeneity by two-dimensional dodecyl sulphate/polyacrylamide-isoelectric focusing gel analysis. A complex pattern was obtained (results not shown) with most polypeptide components focusing in the pI range 5–6, although the 12500-M_r polypeptide contained a major component of lower pI (about 4.5), and the 33000-M_r, 19000-M_r and 13000-M_r polypeptides also contained some components with pI values in the range 6–7.

Immunological Properties

Vicilin of mature pea seeds was reacted against antibod to total pea seed proteins by crossed immunoelectrophore A single broad peak of a single precipitin line was giv This indicated that the protein was homogeneous by se logical criteria. Antibodies were raised against this vic and affinity-purified; they were then used to immunopreci tate vicilin from extracts of mature and developing pea see Analysis of the immunoprecipitates showed that their s unit patterns were qualitatively identical with and qua



3. *Analysis by dodecyl sulphate/polyacrylamide gel electrophoresis fluorography of vicilin subunits labelled* in vivo *with radioactive amino* . (a) Radioactive standard protein; (b) polysomal translation prod- *in vitro*; (c–e) total labelled protein from cotyledons labelled *in* 4 h at 14 days after flowering; (c) no chase with non-radioactive ıo acids; (d) 6-h chase; (e) 24-h chase; approximately equal radio- ity was loaded in each of tracks c–e; (f–h) vicilin immunoprecip- d from total labelled protein *in vivo* as above; (f) no chase, (g) 6-h e, (h) 24-h chase. Convicilin (cv) is also immunoprecipitated. All cs are from the same gel

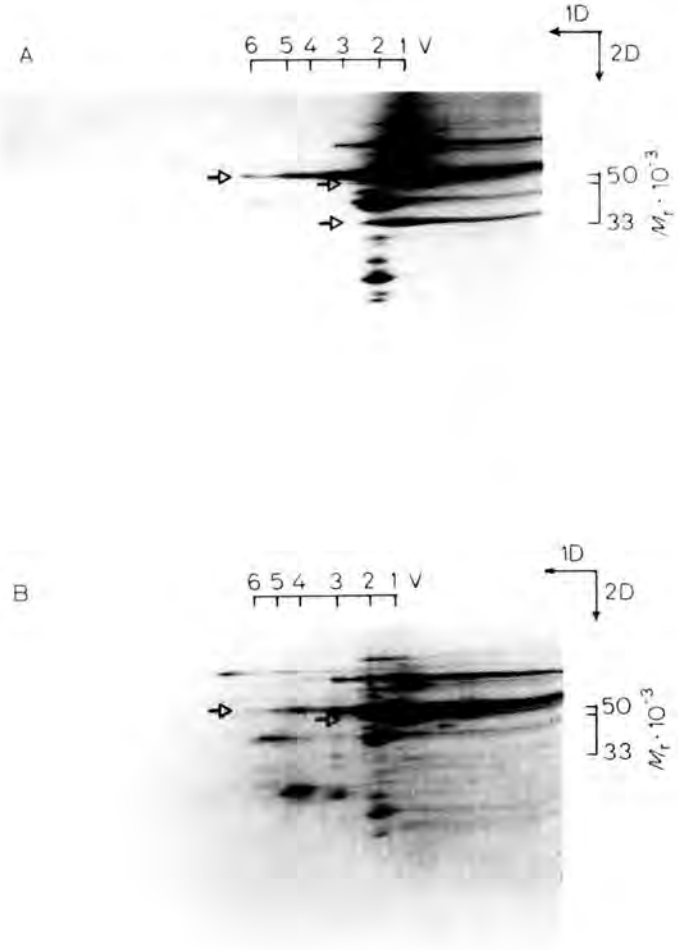


Fig. 4. *Two-dimensional gel analysis of total protein from cotyledons labelled* in vivo *14 days after flowering*. Cotyledons were labelled 4 h with radioactive amino acids. 1D = polyacrylamide gel electrophoresis at pH 8.8 (non-denaturing); 2D = dodecyl sulphate/polyacrylamide gel electrophoresis. (A) Coomassie blue staining of 2D gel. (B) Fluorograph of 2D gel showing distribution of radioactive label. Vicilin subunits indicated by M_r and arrows, vicilin species by V1, V2 etc. (Streaking on these gels is due to overloading in the first dimension)

vely very similar to that of vicilin isolated chemically, icating that the vicilin preparations were not non-represen- ve subsets of 'total' vicilin.

NTHESIS OF VICILIN IN VIVO AND IN VITRO

Synthesis of vicilin *in vivo* was investigated by a cotyledon elling technique. After 4 h of labelling with radioactive ino acids, only the 50000-M_r and 47000-M_r subunits of lin were labelled (Fig. 3). Only after a chase period of at st 6 h with non-radioactive amino acids was label seen in lower M_r subunits of vicilin: concomitantly label in the 00-M_r subunit decreased. Gel chromatography of a total tein extract of labelled material showed that distribution he labelled 50000-M_r and 47000-M_r vicilin subunits over olecular weight range was identical to that of the total lin subunits from unlabelled total extract (result not wn). Similarly, the vicilin synthesised *in vivo* in the 4-h elling period behaved very similarly to deposited vicilin two-dimensional gel analysis (Fig. 4). This indicates that lin labelled *in vivo* and deposited vicilin have similar rge heterogeneity and M_r despite their different subunit npositions (only 50000-M_r and 47000-M_r subunits were sent in the former).

The products of synthesis *in vitro* using either poly- nylated RNA, polysomes or microsomes (polysomes ached to endoplasmic reticulum, shown by the presence antimycin-insensitive cytochrome *c* reductase in a cell- synthesising system) were compared under highly resolv- gel analysis. Polysomal translation products contained lin polypeptides of M_r 50000 and 47000, each as two closely spaced bands; in the translation products of y(A)-rich RNA, only the upper bands of each doublet were present, whereas in the translation products of microsomes only the lower bands of each doublet were present (Fig. 5A). The differences in M_r between the bands of the doublets were less than 1000 in each case. Polysomes treated with methyl-mercuric hydroxide prior to translation gave only the upper band of each doublet, but when polysomes were treated with stripped microsomal membranes, the lower bands of each doublet became more intense (Fig. 5B).

Analysis of polysomal translation products by two-dimensional gel electrophoresis showed that both vicilin 50000-M_r and 47000-M_r products were heterogeneous in charge.

Comparison of Products Synthesised in vitro *and* in vivo

Within the limits of resolution of the gel systems used, products synthesised *in vivo* and those co-translationally processed *in vitro* both included 47000-M_r and 50000-M_r polypeptides (Fig. 3). However, an additional band of M_r 49000 was always present *in vivo* and not *in vitro*.

Fig. 5. *Analysis by dodecyl sulphate/polyacrylamide gel electrophoresis and fluorography of translation products* in vitro *of mRNA preparations from pea cotyledons harvested 14 days after flower opening.* All translations were carried out in the reticulocyte cell-free synthesising system with [^{3}H]leucine as radioactive label. Vicilin subunits are indicated by M_r. (A) (a) Polysomal translation products; (b) immunoprecipitate of A with anti-vicilin IgG; (c) microsomal translation products; (d) immunoprecipitate of c with antivicilin IgG (in tracks b and d convicilin (cv) is also precipitated); (e) polysomal translation products; (f) translation products of polyadenylated RNA; (g) translation products of polysomes treated with 10 mM CH_3HgOH; (h) translation products of polysomes after an equivalent amount of CH_3HgOH as in g added separately to translation system; (i) polysomal translation products. All tracks are from the same gels. (B) (a) Radioactive standard protein; (b) microsomal translation products; (c) polysomal translation products; (d) translation products of microsomes + microsomal membranes; (e) translation products of polysomes + microsomal membranes. All tracks from the same gel

DISCUSSION

The results presented in this paper lead to the following conclusions.

a) Vicilin subunits as initially synthesised *in vitro* and *in vivo* consist of polypeptides of M_r approximately 50000 (including the 47000-M_r subunit): no smaller polypeptides are produced.

b) Vicilin synthesised *in vitro* on polysomes represen mixture of polypeptides which have and have not been translationally processed. This is deduced from the dou nature of both 50000-M_r and 47000-M_r bands. When p adenylated RNA, or polysomes treated with methylmerc hydroxide to remove half-completed polypeptide chains ('r off product', Gatehouse and Croy, unpublished results), translated, only the upper bands of the doublets, unproces product, are seen; when microsomes or polysomes plus en plasmic reticulum are translated, the lower bands of doublets, processed product, predominate. The process must occur on the endoplasmic reticulum concomitan with translation, since the run-off product is already p cessed. Possibly this processing resembles that described Blobel and Dobberstein [31].

c) Vicilin polypeptides are assembled *in vivo* into molecu of $M_r \approx 170000$ initially containing only ≈ 50000-M_r s units.

d) The smaller polypeptides found in deposited, i.e. i lated, vicilin arise from post-synthetic proteolysis of so of the ≈ 50000-M_r subunits already assembled into vici molecules. The 47000-M_r subunits are completely cleav whereas others are not.

e) The post-synthetic proteolysis of vicilin does not sign cantly alter either its molecular weight, or its charge hete geneity, implying that the cleaved polypeptides remain as ciated.

The model for the structure of vicilin which we have adopt postulates therefore that the molecule is a multimer, probab a trimer, of ≈ 50000-M_r subunits but that these subun may be 'nicked' by post-translational proteolysis witho significantly altering the structure of the molecule as whole. As reported by Millerd [32] no evidence for p teolytic degradation of vicilin during extraction was found comparison with extractions carried out in the presence protease inhibitors or under dissociating conditions. Furth the lack of proteolysis of the vilicin labelled *in vivo* on extra tion after a short pulse (Fig. 3 and 4) indicates that degrad tion does not take place during extraction. However, t possibility that some of the minor vicilin subunits arise fr degradation not occurring *in vivo* cannot entirely be rul out. The post-translational proteolysis occurs some ti (> 4 h) after synthesis as in the case of concanavalin A [3 It thus follows that the apparently large number of differe 'subunits' found in vicilin as isolated does not reflect t underlying structure of the molecule. The specificity of p teolysic cleavage is shown by the derivation of the 33000- subunit ($+ 15000$-M_r subunit?) from the 47000-M_r subun shown by the pulse-chase experiment, and the similar prop ties of the molecules containing 47000-M_r subunits prior cleavage to those containing 33000-M_r ($+ 15000$-M_r) su units after cleavage on two-dimensional gel analysis. Howev a complete description of the further proteolytic cleavag responsible for producing the other polypeptides is not possible. The sequence heterogeneity implied by the cha heterogeneity of the vicilin polypeptides before proteoly cleavage may account not only for the variety of cleava products produced, but also for the heterogeneity of mol ular properties found in vicilin, since different combinatio of the initial polypeptides would be expected to give mo cules differing in their physical properties. Further, sin vicilin is a glycoprotein [34] it is possible that additio heterogeneity, as shown by the extra band seen in the init products *in vivo*, is produced by glycosylation of one of t vicilin 50000-M_r polypeptides. If so, the glycosylated po

ptide must then be completely cleaved since carbohydrate found predominantly, if not exclusively, in the 12000–000-M_r polypeptides of deposited vicilin [7,16]. We have en unable so far to demonstrate glycosylation *in vitro*.

The present model for the structure of pea vicilin is similar that proposed for the 7-S storage protein of *Phaseolus lgaris*, which has been shown to be a trimer of ≈ 50000-M_r bunits [35]. Similar structures have also been proposed for owpea 7-S globulin [36] and soybean 7-S globulin [37]. eliminary investigations of the vicilin of *Vicia faba* (Croy id Gatehouse, unpublished results) have shown it to possess ry similar properties to pea vicilin, including its band pattern dodecyl sulphate/polyacrylamide gel electrophoresis, so iat it is also likely to conform to this model.

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