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Structural and genetical studies on legumin of pisum sativum L. and vicia faba L.

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STRUCTURAL AND GENETICAL STUDIES ON LEGUMIN OF

PISUM SATIVUM L. AND VICIA FABA L.

A THESIS SUBMITTED IN ACCORDANCE WITH

THE REQUIREMENTS OF THE UNIVERSITY

OF DURHAM FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

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Thesis
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To

My Father

ABSTRACT

Studies have been made on the structure and
genetics of the major seed storage protein, legumin,
in Pisum sativum L. and Vicia faba L.. Two dimensional
gel electrophoresis, using non-reducing conditions in
the first dimension and reducing conditions in the
second dimension, used in conjunction with other
different combinations of various electrophoretic
techniques, has been found to be of great value in
elucidating the structure of legumin.   In both these
legumes, legumin has been shown to be composed of a
number of subunit pairs of different molecular weights,
Vicia legumin (subunit pairs of mol. wt. 37,000-79,000)
being more heterogeneous in this respect than the
Pisum legumin (subunit pairs of mol. wt. 35,000-58,000).
The subunit pairs are associated into hexameric
molecules by non-covalent interactions; there exist a
number of different molecular forms containing the
subunit pairs in different combinations.   Each subunit
pair contains a large, relatively acidic subunit
(mol. wt. 22700-58,000 in Vicia and 24,500-43,000 in
Pisum; pI range 4.6-6.1 in Vicia and 4.85-6.15 in
Pisum) and a smaller, relatively basic subunit (mol. wt.
21,000-23,000 in Vicia and 20,700-22,700 in Pisum; pI
range 8.2-8.5 in Vicia and 6.2-8.0 in Pisum) linked
covalently by one or more disulphide bonds.

Genetic studies on Pisum sativum have shown that
various legumin subunits all followed monogenic inheritance,

the alleles showing co-dominance with respect to each other. The genes for some but not all different types of legumin subunits have been shown to be inherited independently. The gene(s) for the acidic subunits of the major legumin subunit pair(s) is located 15 map units distant from the tl locus on the side away from the r locus on chromosome 7. A further storage protein gene has been mapped in that the gene for the convicilin subunits is strongly linked to the locus k on chromosome 2.

CONTENTS

Section I   <u>INTRODUCTION</u>

A very simple classification of reserve proteins
of fruits and seeds is based on the pioneering work of
T.B. Osborne with various plant proteins. This
classification in spite of subsequent modifications,
retains its importance by defining the following four
groups of proteins on the basis of their solubility
characteristics(Osborne, 1924):

1. <u>Albumins</u> : These proteins are soluble in water
   and the group is mainly made up by enzymes.

2. <u>Globulins</u> : These are insoluble in water but
   soluble in salt solution and storage proteins
   of legumes are the predominant members.

3. <u>Prolamins</u> : These are proteins which are soluble
   in 70-80% ethanol and insoluble in water and salt
   solutions. Members of Gramineae e.g. maize,
   wheat and barley have prolamins as their storage
   proteins although some cereals e.g. oats and
   rice do not.

4. <u>Glutelins</u> : These are insoluble in all the above
   solvents but soluble in dilute acids or dilute
   alkalies. Cereals like rice are rich in glutelins;
   they also occur in the other cereals but to a
   lesser extent than prolamins.

While investigating 'proteids' (a term used previously for proteins) of pea, Osborne and Campbell (1898) separated two types of globulins which they designated as legumin and vicilin. Studies by repeated dilution of salt solutions showed that vicilin is soluble in dilute salt solutions and coagulates when heated to 100°C whereas legumin is less soluble in dilute salt solutions and does not coagulate on heating. Also legumin was found to be richer in sulphur content (0.42%) than vicilin (0.18%). Later, Osborne and Harris (1907) separated these fractions by ammonium sulphate fractionation.

Osborne's separatory techniques have been replaced by the modern superior methods of ultracentrifugation, electrophoresis and chromatography. Ultracentrifugation was introduced by Svedberg and Lysholm (1927) who made some studies on seed proteins. Later, extensive studies of seed proteins by ultracentrifugation were performed by Danielsson (1949) and from his work on 34 leguminous species he concluded that legume seeds generally consist of two types of globulins with sedimentation coefficients approximately 11S and 7S. The mol.wt. of the heavy fractions was calculated to be 331,000 and that of the lighter fraction as 186,000. He also equated the 11S and 7S proteins with Osborne's legumin and vicilin respectively by coagulation studies on these proteins. Later on, with the help of electrophoretic techniques, Danielsson (1950) determined the isoelectric points of legumin and

vicilin to be 4.8 and 5.5 respectively. By carrying
out some studies on the chemical composition of the two
proteins, Danielsson and Lis (1952) showed that legumin
was richer in acidic and basic amino acids than vicilin
and vicilin had a higher content of neutral amino acids.

However, in considering the values given above
for legumin and vicilin, it is important to realize that
they have been obtained using 'impure' preparations,
which in the case of vicilin consisted of several different
proteins and in the case of legumin contained small
amounts, less than 5% of impurities, usually vicilin.
However, few results obtained by modern, technologically
advanced methods of protein chemistry have been reported
which challenge the basic findings of the earlier workers.
Rather, use is still made to a considerable extent of the
fundamental knowledge gained by way of their experiments.

As mentioned, originally the terms legumin and
vicilin were coined for the globulins of Pisum sativum
(Osborne and Campbell, 1898) but were extended to similar
proteins in other members of the family Leguminosae
(Danielsson, 1949). However, further investigations
have shown that these terms should perhaps be restricted
to the members of the tribe Vicieae e.g. Vicia, Pisum,
Cicer, Lens and Lathyrus (Derbyshire et al., 1976). Until
comparatively recently, modern techniques of protein
separation were mostly employed to study the globulin
proteins of legumes of other tribes e.g. glycinin in
soyabeans and arachin in peanuts. However, lately,

considerable work has also been done on the globulins of <u>Pisum</u> <u>sativum</u> and <u>Vicia</u> <u>faba</u> of the tribe <u>Vicieae</u>.

<u>Pisum</u> <u>sativum</u> and <u>Vicia</u> <u>faba</u> provide a favourable food for a considerable part of the world's population. Their seed protein content falls in the range of 20-30% and the two crops are thus a rich source of protein. In most varieties, legumin is the dominating protein fraction of their globulins and constitutes as much as 60-80% of the total protein (Gatehouse <u>et</u> <u>al.</u>, 1979). Like other legumes, the major seed proteins of pea and broad bean are nutritionally first limiting in the sulphur amino acids, methionine and cysteine. Legumin is the protein fraction richer in these amino acids (Boulter <u>et</u> <u>al.</u>, 1973) and thus it was decided to devote the present studies to the legumin of <u>Pisum</u> <u>sativum</u> and <u>Vicia</u> <u>faba</u>.

After the initial separations by Osborne and Campbell (1898) and Osborne and Harris (1907), legumin of <u>Pisum</u> <u>sativum</u> and <u>Vicia</u> <u>faba</u> has been separated from vicilin by various workers employing different methods. Crude legumin was isolated by isoelectric precipitation (Danielsson, 1949), by zonal isoelectric precipitation (Wright and Boulter, 1974), by ion-exchange chromatography (Grant and Lawrence, 1964), by sucrose density gradient centrifugation (Casey 1979a; Utsumi and Mori, 1980) and by hydroxylapatite column chromatography (Gatehouse <u>et</u> <u>al.</u>, 1980). Highly purified legumin has recently been prepared by Casey (1979a) by immunoaffinity chromatography using antilegumin antibodies.

Molecular weights of legumin reported so far fit well into the hexameric model given by Wright and Boulter

(1974). Using the Svedberg equation, Danielsson (1949) determined the mol.wt. of legumin to be 331,000 in the ultracentrifuge. Later on, larger values were reported by the same technique for legumin of Pisum sativum (Brand et al.,1955; Brand and Johnson, 1958; Casey, 1979a). These workers showed that the mol. wt. values of pea legumin varied between 390,000 and 410,000. Gel filtration has also been employed by Casey (1979a) and Croy et al.(1979) to determine mol. wt. They reported the mol. wt. value to be 395,000 and 390,000 respectively. Recently, a somewhat lower mol. wt. (350,000^{+}10,000) for pea legumin has been reported by Blagrove et al.(1980) who instead of using the Svedberg equation used the 'meniscus depletion sedimentation equilibrium method' in the ultracentrifuge. The mol. wt. of Vicia faba legumin has been calculated to be lower than that of Pisum sativum. It was determined as 328,000 by sedimentation studies (Wright, 1973), as 347,000 by gel filtration (Croy et al.,1979) and as 320-400,000 on polyacrylamide gradient gels (Utsumi and Mori, 1980). The latter workers obtained four different molecular forms of legumin whose mol. wt. varied from 320,000 to 400,000.

There have been conflicting reports in the literature as to whether legumin is glycosylated or not. Reports by Basha and Beevers (1976) suggested that legumin was a glycoprotein where amino sugars and neutral sugars were associated with this protein. These reports were confirmed by Browder and Beevers (1978) who claimed

to have demonstrated the occurrence of glucosaminyl asparagine in glycopeptides isolated from legumin. On the other hand, other authors considered that legumin is not associated with carbohydrates (Bailey and Boulter, 1970; Casey 1979a; Gatehouse et al., 1980). A fluorescent labelling technique and a sensitive radiochemical labelling technique both failed to show any carbohydrate bound to Pisum legumin and it was concluded by Gatehouse et al. (1980) that legumin was not a glycoprotein. This controversy has been ended by Hurkman and Beevers (1981) who showed that the earlier reports of legumin glycosylation by their group were actually due to the presence of some low mol. wt. glycopolypeptides which co-purified with legumin and not to legumin as such. Thus legumin of Vicia faba and Pisum sativum is not a glycoprotein.

Legumin synthesis starts quite late during seed development (Wright and Boulter, 1972). They showed that legumin synthesis in Vicia faba starts after vicilin synthesis but that the rate of its synthesis is faster so that by the time the seed completes its development, legumin content is usually found to be much higher in the mature seed. However, using in vitro developmental studies of the cotyledons, Millerd et al. (1975) and Guldager (1978) showed that legumin synthesis starts in very young embryos as soon as cell division has ceased. These workers measured the amount of legumin synthesized by immunoelectrophoresis

which is a more sensitive method than that used by Wright and Boulter (1972). However, using the highly sensitive technique of 'enzyme-linked immunosorbent assay' (ELISA), Domoney et al. (1980) have shown that legumin synthesis starts from the initial stages of embryo development. They could detect legumin in embryos weighing as little as 2×10^{-3}g. According to these authors, legumin may be synthesized in the embryo when DNA endo-reduplication is still occurring but only in cells which have stopped dividing.

The overall amino acid composition of Pisum and Vicia legumin shows a clear similarity between the two (Jackson et al., 1969; Bailey and Boulter, 1970; Casey and Short, 1981). The storage role of legumin is characterized by the presence of very high percentage of amides i.e. asparagine, arginine and glutamic acid. These findings are similar to the amino acid composition of legumin like proteins in many other leguminous and non-leguminous seeds (Derbyshire et al., 1976).

Resemblance between the legumin of Pisum sativum and Vicia faba has also been shown by immunological studies performed by a number of workers. Using antiserum prepared against Pisum legumin, Danielsson (1949) showed that legumin of Vicia faba cross-reacted with that of Pisum sativum. Later on with the help of a semiquantitative serological method, Kloz and Turkova (1963) showed that the intensity of the Vicia

legumin cross-reaction was about 74% of the homologous reaction to _Pisum_ legumin. Antigenic identity between the two legumins using antilegumin antibodies prepared against _Vicia_ legumin was also shown to occur by Dudman and Millerd (1975) and Croy et al. (1979); the latter workers showed that _Pisum_ legumin had one or more antigenic determinants in addition to those of _Vicia_ legumin.

The very high mol. wt. of _Pisum_ and _Vicia_ legumin is self-indicative of the subunit structure of this protein. Vaintraub and Gofman (1961) isolated legumin of _Pisum_ _sativum_ by isoelectric precipitation and proposed that it consisted of 12 subunits of mol. wt. 33,000 each. They also found the N-terminals to be glycine and leucine. However, Jackson et al. (1969) showed the presence of N-terminal threonine in addition to the glycine and leucine reported by Vaintraub and Gofman (1961). The occurrence of these three N-terminals has been recently confirmed by Casey (1979a). Bailey and Boulter (1970) determined the N-terminals of _Vicia_ legumin to be glycine, leucine and threonine also. They proposed that _Vicia_ legumin was composed of three types of subunits of mol. wt. 56,000, 42,000 and 23,000. By using densitometric scanning and _in_ _vivo_ radiochemical labelling of these legumin subunits, they further showed that these subunits were present in the ratio of 1:3:6. Thus _Pisum_ and _Vicia_ legumin were shown to be heterogeneous.

The well known hexameric model of legumin was proposed by Wright and Boulter (1974) using <u>Vicia</u> <u>faba</u>. They showed that legumin consists of two types of subunits, (i) α , acidic subunits of mol. wt. about 40,000 and (ii) β , basic subunits of mol. wt. about 20,000. It was shown that the two types of subunits (one each) were joined by disulphide bonds to form an intermediary of about 60,000 mol. wt. . Six subunit pairs were proposed to comprise the legumin molecule. They determined the acidic subunits to be of mol. wt. 37,000 and found three different basic subunits of mol. wt. 23,800, 20,900 and 20,100. It was also shown that the acidic subunits were richer in aspartic acid, glutamine and arginine than the basic subunits which were richer in alanine, valine and leucine.

It has been demonstrated that the higher mol. wt. subunits are acidic and the lower mol. wt. subunits basic, by electrophoresis in urea (Thomson <u>et</u> <u>al</u>., 1978; Croy <u>et</u> <u>al</u>.,1979) and by isoelectric focusing (Krishna <u>et</u> <u>al</u> 1979; Casey, 1979b). Electrophoresis on urea-polyacrylamide gels (Thomson <u>et</u> <u>al</u>.,1979) and on cellulose acetate membranes (Croy <u>et</u> <u>al</u>.,1979) has confirmed these findings by showing that in alkaline pH large subunits move towards anode and the small subunits move towards cathode.

Heterogeneity in subunit molecular weight and charge has been studied in detail in <u>Pisum</u> <u>sativum</u> (Thomson and Schroeder, 1978; Croy <u>et</u> <u>al</u>.,1979; Krishna

et al.,1979; Gatehouse et al, 1980). Thomson and
Schroeder (1978) reported a complex system of
classification in which they differentiated legumin
into five groups (LA, LB, LC, LD and LE). Groups LA,
LB and LC correspondended to acidic subunits of mol. wt.
about 40,000, LD to the basic subunits of mol. wt.
20,000 and LE was constituted by subunits of mol. wt.
25-27,000 of undecided identity. Similar conclusions
were reached by Casey (1979b) who divided acidic
subunits of different concentrations into major
(corresponding to LA and LC of Thomson and Schroeder)
and minor subunits (equivalent to LB of Thomson and
Schroeder). Isoelectric points of the major subunits
were in the range of 6.0-6.5 and those of minor
subunits 5.6-5.8. These values for isoelectric points
for acidic subunits are very different from those
determined by Krishna et al. (1979) as 4.55-4.9 and
by Gatehouse et al. (1980) as 5.1-5.3 for acidic
subunits. Use of 6M urea (Krishna et al.,1979) and
50% formamide (Gatehouse et al.,1980) did not cause a
significant difference in the pI values of basic
subunits which were determined to be 8.45 - 8.8 and
8.3 - 8.7 respectively by these workers.

In addition to the heterogeneity found at the
subunit level, charge and molecular weight heterogeneity
at the molecular level has been observed in Vicia faba.
This has been recently shown by Utsumi and Mori (1980)
who suggested 7 molecular forms of molecular weight

320,000, 350,000, 380,000 and 400,000 as measured by
gradient gel electrophoresis. Acidic subunits of mol.
wt. 61,700, 59,800, 48,000 and basic subunits of mol.
wt. 23,000, 20,500 and 19,000 were observed.

Heterogeneity in the primary structure has been
revealed by amino acid sequence studies of basic
subunits carried out by Gilroy and Boulter (1979) in
Vicia faba and Casey et al.(1981) in Pisum sativum.
A comparison of these results shows the occurrence of
sequence microheterogeneity at three of the same
positions in both the molecules. Thus, this heterogeneity
seems to have arisen as a result of point mutations in
the nucleotide sequence.

Few studies have been done regarding the
inheritance of Pisum legumin. Thomson and Schroeder
(1978) found that acidic subunits (classified by them
as LA and LC legumin groups) were each controlled by a
single pair of genes. The genes responsible for these
subunits were co-dominant and segregated independently
of each other. Basic subunits represented by LD group
were found to be under multigenic control.

Casey (1979b) used two-dimensional electrophoretic
analysis to study the inheritance of legumin subunits
and showed that the major acidic subunits of legumin
followed a 1:2:1 segregation in F_2 generation i.e. were
controlled by a single pair of co-dominant genes.

Improvement of a character by breeding can be

expedited if the genetics of the character in question

is well studied. Furthermore it would appear that

qualitative and quantitative improvement of pea protein

could be effected through changes in the legumin

fraction. The legumin fraction is important due to

it constituting a comparatively high proportion of the

seed proteins and due to its higher content of sulphur-

amino acids than other storage proteins. For these

reasons, studies on the 'structure and genetics of

legumin' was selected as the topic for this thesis.

Section II   MATERIALS AND METHODS

I. MATERIALS

A. Biological materials

The various studies made use of the following lines of (a) Vicia faba L. and (b) Pisum sativum L.

(a) Vicia faba L.

Seeds of the lines 'Wierboon', 'Felix' and 'Minica' were kindly provided by Dr. G. Dantuma, Centre for Agrobiological Research, Wageningen, The Netherlands.

(b) Pisum sativum L.

Seeds of the varieties English Wonder, Kelvedon Wonder, Peter Pan, Pioneer, Little Marvel, Meteor, Mangtout and Feltham First were purchased from Tyneside Seed Stores, Gateshead, Co. Durham. Lines 110, 180, 360, 611, 806, 807, 808, 851, 1238, 1249, 1256, 1263, 1293, 1552 and 2162 were kindly provided by Dr. S. Blixt, Weibullsholm Plant Breeding Institute, Sweden.

B. Chemicals

Most of the chemicals used were from BDH Ltd., Poole, Dorset, U.K. and were of the purest grade available. Trizma base [Tris (Hydroxymethyl) amino methane] and Coomassie brilliant blue R250 were from Sigma Chemical Company, Poole, Dorset, U.K. Carrier ampholytes for isoelectric focusing were manufactured by Pharmacia Fine Chemicals, Uppsala, Sweden and LKB-Produkter A.B.,

Bromma-Stockholm, Sweden. Hydroxylapatite was from
BioRad Laboratories Ltd., Watford, Herts, U.K.; DEAE-
cellulose was manufactured by Whatman Ltd., Springfield
Mill, Maidstone, Kent.

Visking tubing was obtained from Medical
International, 49 Queen Victoria St., London.

Cellulose acetate membranes were supplied by
Beckman Instruments Inc., Fullerton.

Uncoated cellophane for drying gels was obtained
from W.E. Cannings, Avonmouth way, Avonmouth, Bristol.

II. METHODS

A. Growth of biological materials

Seeds of pea varieties were germinated in
Alkathene polyethylene beads in a dark spray room at
28°C with water misting at regular intervals for
5-6 days. Seedlings were then transferred to plastic
pots containing compost (Levington : John Innes
No.2 = 1:3 parts) in the Botanic Gardens, University of
Durham. Plants were grown under 12h light per day and
at a minimum temperature of 14°C. Plants were also
grown in culture bottles of nutrient solution in a
'Warren Sherer model CEL 511-38' growth cabinet under
controlled environmental conditions of light, temperature
and humidity, as described by Evans et al. (1979). Pods
were harvested when dry-ripe and the seeds stored in a
cold room at 4°C.

B. <u>Preparation of single seed extracts</u>

The testa was removed from a part of the seed and a quarter of seed (half of the cotyledon) was cut off taking care that the radicle and the other parts of embryo were not injured. The cotyledon was ground and a known weight of seed meal (assumed to have 20% globulins on dry weight basis) was finely suspended by whirlmixing and sonication, in a known volume of extraction buffer to get a final protein concentration of 10 mg/ml of extract. The extraction buffer consisted of 0.125M Tris-HCl pH 6.8 with 2% (w/v) Sodium dodecyl sulphate (SDS) and 1% (v/v) 2-Mercaptoethanol. Suspended seed meal was heated in 1.5 ml centrifuge tubes with frequent shaking in a boiling water bath for 30 to 40 min. The extract was clarified by centrifugation in a Haematocrit centrifuge at full speed and 10% (v/v) glycerol was added to the supernatant to make it dense for subsequent loading on to the gels. For electrophoresis under non-reducing conditions, 2-mercaptoethanol was omitted from the extraction buffer and for electrophoresis under non-dissociating conditions, extraction buffer consisted of only 0.125M Tris-HCl pH 6.8 and the suspended meal instead of being heated, was left at room temperature for 3-4h.

C. Extraction of Globulins

Salt soluble proteins (globulins) were extracted by the method of Croy (1977). The testas were removed from dry seeds and the cotyledons were ball-milled to a fine powder. All subsequent operations were carried out at 7^OC. Seed meal was defatted twice with hexane (30ml g^{-1}) and then extracted with 50mM sodium borate buffer pH 8.0 (20ml g^{-1}). After 2 h of stirring, the extract was centrifuged at 16,000 g for 1h. The supernatant which contained dissolved globulins and albumins was dialysed overnight against 33mM sodium acetate buffer pH 5.0. The precipitated globulins were collected by centrifugation at 16,000g at 4^OC for 30 min. The pellet was washed and suspended in distilled water and then freeze-dried to give the globulin preparation. Albumins left in the supernatant were dialysed against water at 4^OC and freeze-dried.

D. Hydroxylapatite column chromatography

Legumin was purified from the globulins or the total protein extract by hydroxylapatite column chromatography following the method given by Gatehouse et al.(1980). The column (3.2cm x 20cm) was equilibrated with 50mM potassium phosphate buffer pH 8.0. The total extract prepared by stirring seed meal in equilibrating buffer, or globulins dissolved in the equilibrating buffer, were loaded on top of the column and the column

washed with equilibrating buffer until the non-bound
materials were fully eluted as the first peak. Further
elution was effected by either of the following methods :
i) Stepwise elution - using 0.3M-phosphate buffer for
the second peak and 1M-phosphate buffer for the third
peak, ii) Gradient elution by using a linear gradient
of phosphate buffer pH 8.0 with molarity increasing
from 50mM to 1M. 300 ml each of 50mM and 1.0M
phosphate buffer was used for the purpose. The eluant
was monitored automatically at 280nm by the detector
unit Type 8303A of an LKB UVICORD II UV Absorptiometer.
The flow of solutions was regulated by using an LKB
Varioperspex 12000 peristaltic pump and the salt concent-
ration of fractions was measured on a refractometer.
Fractions representing various peaks were pooled,
dialysed against distilled water for two days and then
freeze-dried on an Edwards Modulyo freeze dryer. All
buffers used in this column chromatography contained
0.05% sodium azide and were sterilized by filtration
through a 0.45 μm pore size cellulose acetate filter.

E. Ion-exchange chromatography

Ion-exchange chromatography was performed on a
DEAE-cellulose column (23 x 1.6cm) equilibrated with
50mM-Tris HCl, pH 8.0 containing 0.05% sodium azide.
Legumin dissolved in the equilibrating buffer was
loaded on top of the column and the column washed with
the same buffer till the non-bound materials were eluted

under the first peak. Further elution was carried out
by using a linear concentration gradient of sodium
chloride from O.OM to O.3M. 5ml fractions were
collected at 280nm using an LKB fraction collector
at a flow rate of 50ml h^{-1}. The required fractions
were pooled together, dialysed against distilled water
and freeze dried for analysis by various electrophoretic
techniques. Molar concentration at which a particular
protein was eluted was calculated from the refractive
index of that fraction using a calibration curve.

F. Ultrogel column chromatography

Globulins were chromatographed on a column of
ultrogel AcA22 (70 x 22cm) equilibrated with 0.1M Tris-HCl
pH8.0 containing 0.25M-sodium chloride and 0.05% sodium
azide. The column was calibrated for mol. wt. by using
the following standard proteins: rabbit immunoglobulins
(150,000), catalase (232,000), glutamate dehydrogenase
(340,000), ferritin (440,000) and thyroglobulin (669,000).
The sample, in about 2ml of buffer, was applied to the
column and eluted by upward flow at the rate of 6ml h^{-1}.
The effluent was monitored automatically at 280nm as
described for hydroxylapatite column chromatography. The
apparent mol. wt. of a protein was calculated by measuring
the elution volume of protein peak relative to the
void volume.

Distribution of the subunits in the elution
profile was estimated by following the method given by

Croy et al.(1980b). One ml of each fraction was allowed

to precipitate after addition of trichloracetic acid

to a final concentration of 12%, for 2 h at 4°C.

Precipitate was centrifuged, washed twice with ethanol

and dried by evaporation. This precipitated protein

was dissolved in sample buffer containing 2-ME and

analysed by SDS-gel electrophoresis. Quantitative

measurement of the amount of protein represented by

each band was made by scanning the stained gel strip

on a Gilford spectrophotometer and by integrating

the areas under the peak corresponding to protein bands.

G. Polyacrylamide gel electrophoresis

a. Under dissociating conditions:

SDS-polyacrylamide gel electrophoresis was carried

out in the discontinuous buffer system of Ornstein (1964)

and Davis (1964) using the formulations of Laemmli (1970).

Twelve and a half percent gel slabs were made in Raven

glass plates by using the following stock solutions:

a-1. Separation gel

    (i) Acrylamide stock   25 ml

         (containing 30 g acrylamide

         and 135 mg NN' -Methylene-

         bisacrylamide (Bis) to

         100ml with water)

    (ii) 1M Tris-HCl pH 8.8   22.5 ml

    (iii) Water   9.9 ml

(iv) 1% Ammonium persulphate   1.5 ml

—— degassed ——

(v)   SDS   0.6 ml

(vi) TEMED – NNNN′ Tetramethyl   20 µl
      ethylene diamine

a-2. <u>Stacking gel</u>

(i)   Acrylamide stock   3 ml
      (containing 30g acrylamide
      and 435 mg Bis to 100 ml
      with water)

(ii) 1M Tris-HCl pH 6.8   2.5 ml

(iii) Water   13.8 ml

(iv) 1% Ammonium persulphate   0.5 ml

—— degassed ——

(v)   SDS   0.2 ml

(vi) TEMED   10 µl

<u>Standard reservoir buffer</u>

(i)   Tris   3.0 g

(ii) Glycine   14.1 g

(iii) SDS   1.0 g

made to 1 lt with water.

Electrophoresis was carried out at a current of 15mA for 1h followed by 25mA till the tracking dye (0.1% bromophenol blue) reached the gel bottom. Power was supplied by a Shandon Southern SAE 2761 constant current source. Protein bands were stained overnight with 0.05% Coomassie brilliant blue in methanol, acetic acid and water in the ratio 50:7:43 (v/v). Gels were destained by diffusion in the solvents as given above for the staining solution. Gels, where required, were scanned by using/Gilford 2000 spectrophotometer. Molecular weights of different polypeptides were measured by using the method given by Weber and Osborn (1969). A number of standard proteins were used to calibrate gels by measuring mobility of the protein with respect to the reference marker — bromophenol blue and by plotting these values against the log of mol. wt. Following standard proteins were used for the purpose : myoglobin (17,500), chymotrypsinogen (25,000), ovalbumin (43,000), bovine serum albumin (67,000), transferrin (76,000) and phosphorylase b (97,000).

b. **Under non-dissociating conditions**

Polyacrylamide gel electrophoresis under non-dissociating conditions was carried out by the method of Gabriel (1971) modified to suit slab gels. Ten percent and 7.5% slabs were formed using the stock solutions and buffers as described for SDS-polyacrylamide gel electrophoresis except that SDS was omitted from all the solutions.

c.   SDS-polyacrylamide gradient gel electrophoresis

SDS-polyacrylamide gradient gel electrophoresis
was performed according to the method given by
O'Farrell (1975). Gradients between 7.5% (light
acrylamide solution) and 22.5% (heavy acrylamide
solution) concentration were generated by the use of
a Pharmacia P-3 peristaltic pump. Heavy acrylamide
was poured into the light solution and the light
solution with constantly increasing concentration was
pumped into the gel assembly from the bottom. Following
was the composition of light and heavy acrylamide
solutions for the separation gel:

Light acrylamide solution

(i)	Acrylamide stock (29.2g of acrylamide and 0.8g of Bis made to 100ml with water)	6.0 ml
(ii)	1.5M Tris-HCl pH 8.8 containing 0.4% SDS	6.0 ml
(iii)	Water	12.0 ml
(iv)	10% Ammonium persulphate	20 µl

—— degassed——

(v)	TEMED	20 µl

<u>Heavy acrylamide solution</u>

     (i)   Acrylamide stock   18.0 ml

          (29.2g of acrylamide

          and 0.8g of Bis made

          to 100ml in 75% glycerol)

     (ii)   1.5M Tris-HCl pH 8.8   6.0 ml

          containing 0.4% SDS

    (iii)   10% Ammonium persulphate   10   µl

          —— degassed ——

     (iv)   TEMED   20   µl

Stacking gel constitution and other conditions for
electrophoresis were the same as described for SDS-PAGE.
Myoglobin, chymotrypsinogen, ovalbumin, bovine serum
albumin and transferrin were used for mol. wt. calibration
of the gels.

d.   <u>Polyacrylamide gradient gel electrophoresis</u>

     These gels were prepared by the same method as
for the SDS-gradient pore gels except that SDS was
omitted from all the solutions and that concentration
gradient was from 5% to 15%. Light and heavy acrylamide
solutions had the following composition:

<u>Light acrylamide solution</u>

     (i)   Acrylamide stock   4.0 ml

          (29.2g of acrylamide

          and 0.8g of Bis made

          to 100 ml with water)

(ii)	1M Tris pH 8.8	9.0 ml
(iii)	Water	11.0 ml
(iv)	10% Ammonium persulphate	20 µl

—— degassed ——

(v)	TEMED	20 µl

Heavy acrylamide solution

(i)	Acrylamide stock (23.4g of acrylamide and 0.64g of Bis made to 100 ml with 75% glycerol)	15 ml
(ii)	1M Tris-HCl pH 8.8	9 ml
(iii)	10% Ammonium persulphate	10 µl

—— degassed ——

(iv)	TEMED	20 µl

Gels were run at a constant voltage of 100V for 70h till the ferritin marker stopped moving since it had reached the pore size limit. Gels were stained and destained as usual. Thyroglobulin (mol. wt. 669,000), ferritin (440,000) and catalase (232,000) were used as standard proteins for mol. wt. calibration of the gels.

H. Isoelectric focusing

The methods used were based on comments and suggestions made by Vesterberg (1975) and Gatehouse et al. (1980). The technique employed made use of a Pharmacia

flat bed apparatus FBE 3000 and samples were loaded on

filter paper pieces (5 x 10mm) after the slab gels were

prefocused for half an hour. A constant power of

10 watts was maintained throughout and the completion of

electrofocusing was marked by sharp focusing of the

standard protein. Gels were fixed overnight in 15%

trichloroacetic acid, washed with distilled water and

stained in 0.05% Coomassie brilliant blue in methanol,

acetic acid and water in/the ratio of 25:7:43 (v/v). A

solvent mixture in the ratio as given above was used

to destain the gels. Urea solution and formamide for

electrofocusing of acidic and basic subunits

respectively were deionized by stirring for 1h with

Amberlite MB-1 mixed bed resin(1g per 100 ml solution).

Details regarding the gel systems and other conditions

for electrofocusing of different subunits were as

follows:

a.   <u>Acidic subunits</u>

10 x 20 cm gels of 6% acrylamide concentration

with 6M-Urea and 2% (v/v) carrier ampholytes of major

buffering capacity in the pH range of 4 - 6.5 were

used for acidic subunits of legumin. Lysis buffer for

preparing the sample solutions consisted of : (i) 9M Urea

(ii) 2% Nonidet P40 and (iii) 1% 2-mercaptoethanol.

0.03M glutamic acid and 1M ethylenediamine were used

as anolyte and catholyte respectively. Sample protein

and the reference protein (ferritin in this case) were

loaded on the acidic end of the gel. The following constituted the gel recipe made to 35 ml with water :

(i)	Urea		12.6 g
(ii)	Acrylamide		1.96g
(iii)	Bis		0.14g
(iv)	Carrier ampholytes		
		pH range 4-6.5	1.4 ml
		pH range 3-10	0.35ml
		—— degassed ——	
(v)	10% Ammonium persulphate		50 μl
(vi)	TEMED		25 μl

b. <u>Basic subunits</u>

Isoelectric focusing for basic subunits was performed in gels of 8% acrylamide concentration containing 75% formamide, 10% glycerol and 3% (v/v) carrier ampholytes. Protein samples in the lysis buffer (75% formamide, 2% NP40, 1% 2-mercaptoethanol) along with the marker protein (cytochrome C) were loaded on the acidic end of the gel. Electrode buffers used were 0.1M sulphuric acid for anode and 1M Ethylenediamine for cathode.The Following were used to make a 10 x 20cm gel:

(i)	Acrylamide	2.626 g
(ii)	Bis	84 mg
(iii)	Glycerol	3.5 ml
(iv)	Formamide	26.25 ml

(v)   Water   1.4 ml

(vi) Carrier ampholytes

      pH range 6-8   0.7 ml

      pH range 8-10   0.7 ml

      ── degassed ──

(vii) 3.6% Ammonium persulphate   1.75ml

I. Two dimensional electrophoreses

These experiments involved the combined use of polyacrylamide gel electrophoresis (PAGE), SDS-polyacryl-amide gel electrophoresis (SDS-PAGE), polyacrylamide gradient gel electrophoresis (PAGGE) and isoelectric focusing (IEF) under the conditions already described. These were performed in different combinations as follows:

a. Polyacrylamide gel electrophoresis ⟶ SDS-polyacrylamide gel electrophoresis

Ten percent or 7.5% gels were run under non-dissociating conditions in the first dimension. Gel strips were incubated separately in SDS-sample buffer with or without 1% 2-ME. A number of washings were given during the 2h period of incubation. Gel strips were slid, with the help of a thin spatula, onto the top of a single or separate SDS-polyacrylamide gels of the dimensions (including thickness) similar to that of the gel for first dimension. The second dimension gels were then run under the conditions as described before for SDS-polyacrylamide gel electrophoresis.

b. <u>SDS-polyacrylamide gel electrophoresis under non-</u>
   <u>reducing conditions</u> → <u>SDS-polyacrylamide gel</u>
   <u>electrophoresis under reducing conditions</u>

Gel dimensions and electrophoretic conditions
for both the runs were essentially the same. The only
difference was that the unreduced samples were run in
the first dimension on 10% or 12.5% gels and then gel
strips were incubated in sample buffer containing
1% 2-ME to reduce the protein subunits before loading
onto the second dimension gel and re-electrophoresed.

c. <u>Polyacrylamide gradient gel electrophoresis</u> →
   <u>SDS polyacrylamide gel electrophoresis</u>

The first dimension gel was run as described
under gradient pore gel electrophoresis. Incubation
in sample buffer with and without 2-ME and electrophoresis
in the second dimension were similar to that described
for PAGE → SDS-PAGE.

d. <u>SDS-polyacrylamide gel electrophoresis</u> →
   <u>Isoelectric focusing</u>

SDS-polyacrylamide gel electrophoresis in the first
dimension was followed by isoelectric focusing in order
to study the isoelectric points of polypeptides
representing the large and small subunits of legumin.
Gel pieces cut from the first dimension gels of reduced
and unreduced samples were incubated in respective lysis
buffers as given before for isoelectric focusing of the

acidic and the basic subunits. After incubation, gel
pieces were placed on the acidic end of the prefocused
gel and electrofocusing carried out as described
earlier.

e. <u>Isoelectric focusing</u> ⟶ <u>SDS-polyacrylamide</u>
   <u>gel electrophoresis</u>

Isoelectric focusing performed in the first
dimension was followed by SDS-polyacrylamide gel
electrophoresis. The gel strip was incubated in the
sample buffer containing 1% 2-ME and run in the second
dimension as usual.

J. <u>Three dimensional electrophoresis</u>

The various electrophoretic techniques with
incubation buffer and conditions differing in each
dimension were used in the combinations as summarised
below:

a. PAGE $\xrightarrow{\text{Sample buffer - 2-ME}}$ SDS-PAGE

SDS-PAGE $\xleftarrow{\text{Sample buffer+2\% 2-ME}}$

b. PAGE
   ↓
   Sample buffer
   + 2-ME
   ↓   9.5M Urea+2%NP4O+ 2%2-ME
   SDS-PAGE IEF ⟨ ⟶ Acidic subunits

                      75% Formamide+2% NP4O+2% 2-ME Basic subunits

K.   Cellulose acetate membrane electrophoresis

Cellulose acetate membrane electrophoresis was
carried out using 50mM-phosphate buffer pH 7.0 on a
Beckman microzone apparatus. Cellulose acetate membranes
were presoaked in the phosphate buffer and dried in
filter paper folds by applying even pressure. About
2.5 µl of the sample (10mg ml^{-1}) was applied to the
membrane with the help of an applicator and a current
of 8mA applied to the membrane for 15 min. Membranes
were stained and destained like acrylamide gels.
Dried membranes were scanned in the reflectance mode on
a Joyce Loebl Chromoscan.

L.   Immunodiffusion

Immunoprecipitation by double diffusion was
carried out by standard methods (Ouchterlony and Nilsson,
1978). Antibodies were prepared by precipitation with
ammonium sulphate and chromatography on QAE-A50
(Quaternary amino ethyl) Sephadex following the method
of Joustra and Lundgren (1969) or were affinity
purified by immunoaffinity chromatography by the methods
given by Bokhout and Van Tiggele (1977) and March
et al. (1974). Precipitin arcs were cut off and washed in
50mM-sodium borate buffer pH 7.2 (containing 0.15M NaCl)
to remove soluble proteins and analysed by SDS-polyacryl-
amide gel electrophoresis following the method of Croy
et al. (1980b). For this, samples after washing were
dissolved in the SDS-sample buffer without 2-ME.

M. Laurell 'rocket' immunoelectrophoresis

This immunoelectrophoresis was performed in 1% agarose gels (10 x 20 x 0.15cm) using a Shandon flat-bed electrophoresis apparatus according to the method of Laurell (1966) as modified by Weeke (1973). 0.3g of agarose was dissolved in 15 ml water by boiling for 2 min and then was kept in a water bath at 60°C. 15 ml of 0.5M Tris-EDTA borate buffer (0.5M Tris, 1.62mM EDTA, 0.075M borate, pH 8.6) equilibrated to 60°C was mixed with the agarose solution and the appropriate antiserum (400 µl of antilegumin or 600 µl of antivicilin antibodies). The mixture was poured into the gel plate assembly and left for 1h to set. Samples were prepared by extracting seed meal (2.5 mg/ml) in 0.5M Tris-EDTA borate buffer for 2h at room temperature. These samples were loaded along with standards of the appropriate protein (legumin or vicilin) into wells made in the gel. The gel was run overnight at a constant voltage of 100V. Gels were pressed onto agarose coated glass plates and washed extensively in 0.02M saline phosphate buffer followed by washing in water. The gels were then pressed, dried, stained in Coomassie brilliant blue (2 min) and destained. The amounts of legumin and vicilin were determined from their rocket heights by comparison with the calibration curves prepared by plotting the rocket heights against amounts of the standard proteins.

N.   Statistical methods

a.   Chi-square values were calculated by following the
formula given by Fisher (1925)

$$\chi^2 = \Sigma \frac{d}{e}^2$$

Where 'd' stands for deviation of observed value from
the expected value, 'e' represents the corresponding
expected value and 'Σ' is the symbol for 'summation of'.

b.   Percentage of crossing over and the probable error
was calculated by the 'product ratio' method of
Immer (1930).   One of the co-dominant factors in the
allelic pair was considered as dominant so that in a
dihybrid cross involving 'dominant and co-dominant'
factors, factors were in a coupling phase.

Nomenclature

        The various types of legumin and legumin subunits
are represented by the following abbreviations in
this thesis :

a.   'Conventional' legumin:- Legumin   Lg-C
     subunit pairs of conventional legumin
     model (Wright and Boulter, 1974).

b.   'Big' legumin:- legumin subunit   Lg-B
     pairs of mol. wt. higher than
     that of Lg-C.

c.   Small legumin:- legumin   Lg-S
     subunit pairs of mol. wt. smaller
     than that of Lg-C.

d. Acidic subunits of legumin.   Lg-1

e. Basic subunits of legumin.   Lg-2

Thus acidic subunits of 'conventional', 'big' and 'small' legumin are called as Lg-1C, Lg-1B and Lg-1S respectively and the basic subunits as Lg-2C, Lg-2B and Lg-2S respectively.

Section III   RESULTS

Part I   PRELIMINARY INVESTIGATIONS

A. Molecular weight calibration of gel systems

Molecular weights of the polypeptides on SDS-
polyacrylamide gels were calculated from a calibration
curve prepared by running a number of standard proteins
on 12.5% single pore and 7-22% gradient pore gels
(Figs. 1.1 and 1.2). The mobilities of the proteins
relative to the bromophenol blue dye front, run on
two types of gels, were as follows:

| S.No. | Protein | Mol.Wt. | Relative mobilities | |
			Single pore gel	Gradient pore gel
a	Myoglobin	17,500	0.984	0.790
b	Chymotrypsinogen	25,000	0.715	0.615
c	Ovalbumin	43,000	0.520	0.470
d	Bovine serum albumin	67,000	0.350	0.330
e	Transferrin	76,000	0.309	0.310
f	Phosphorylase b	97,000	0.260	–

A linear separation according to mol. wt. was
observed in the region of mol. wt. 25,000 and 76,000 in
both the systems whereas at lower values, relative
mobilities were not linear. Also relative mobilities
of the standard proteins on gradient pore gels were
lower as compared to those on the single pore gels.

Fig. 1.1 : Calibration curve for mol.wt. of

standard proteins (a = myoglobin,

b = chymotrypsinogen, c = ovalbumin,

d = bovine serum albumin,

e = transferrin, f = phosphorylase b)

on single pore 12.5% SDS-gels.

Fig. 11

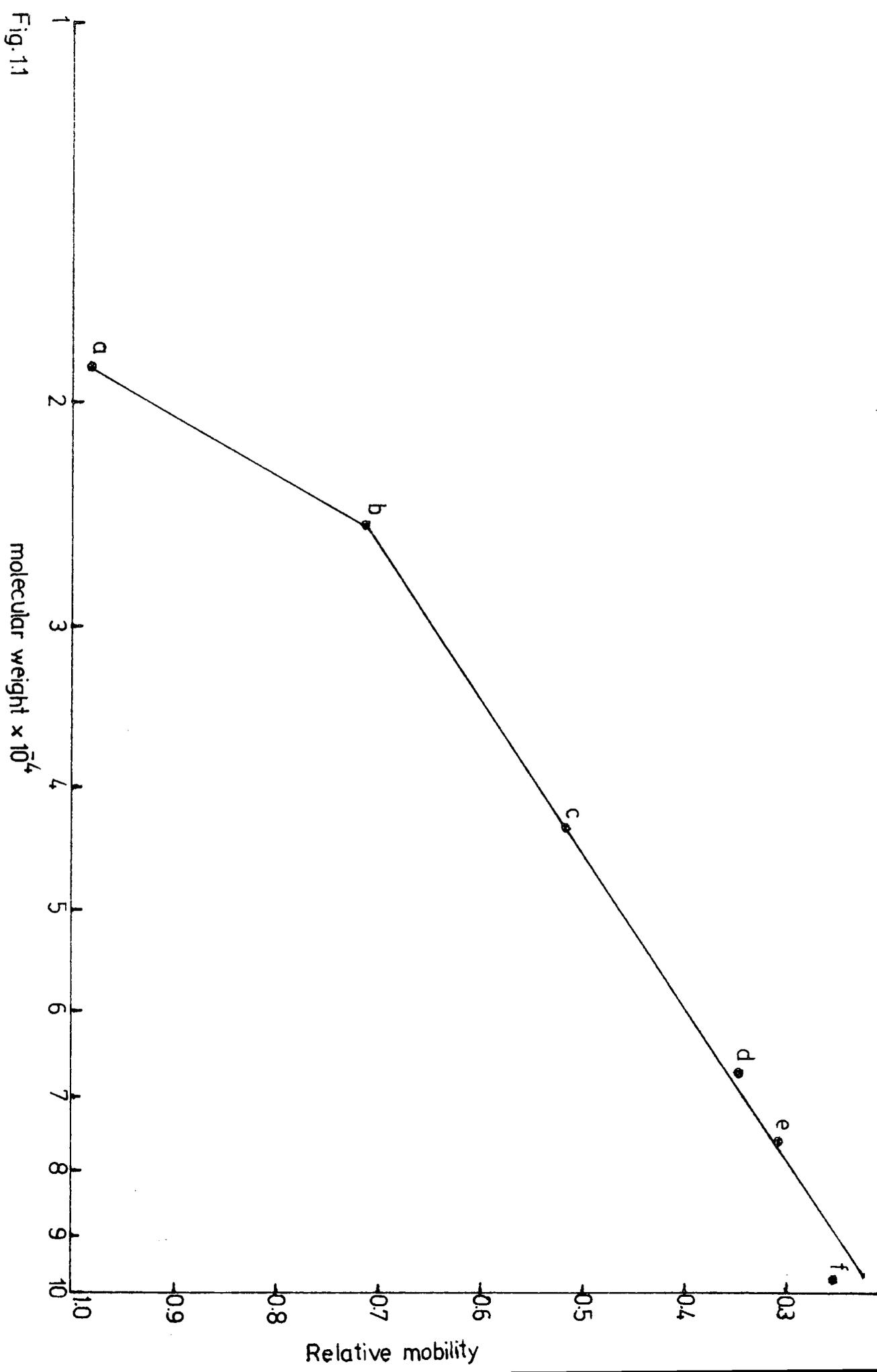molecular weight × 10⁻⁴

Relative mobility

Fig. 1.2 : Calibration curve for mol. wt. of

standard proteins (a = myoglobin,

b = chymotrypsinogen, c = ovalbumin,

d = bovine serum albumin,
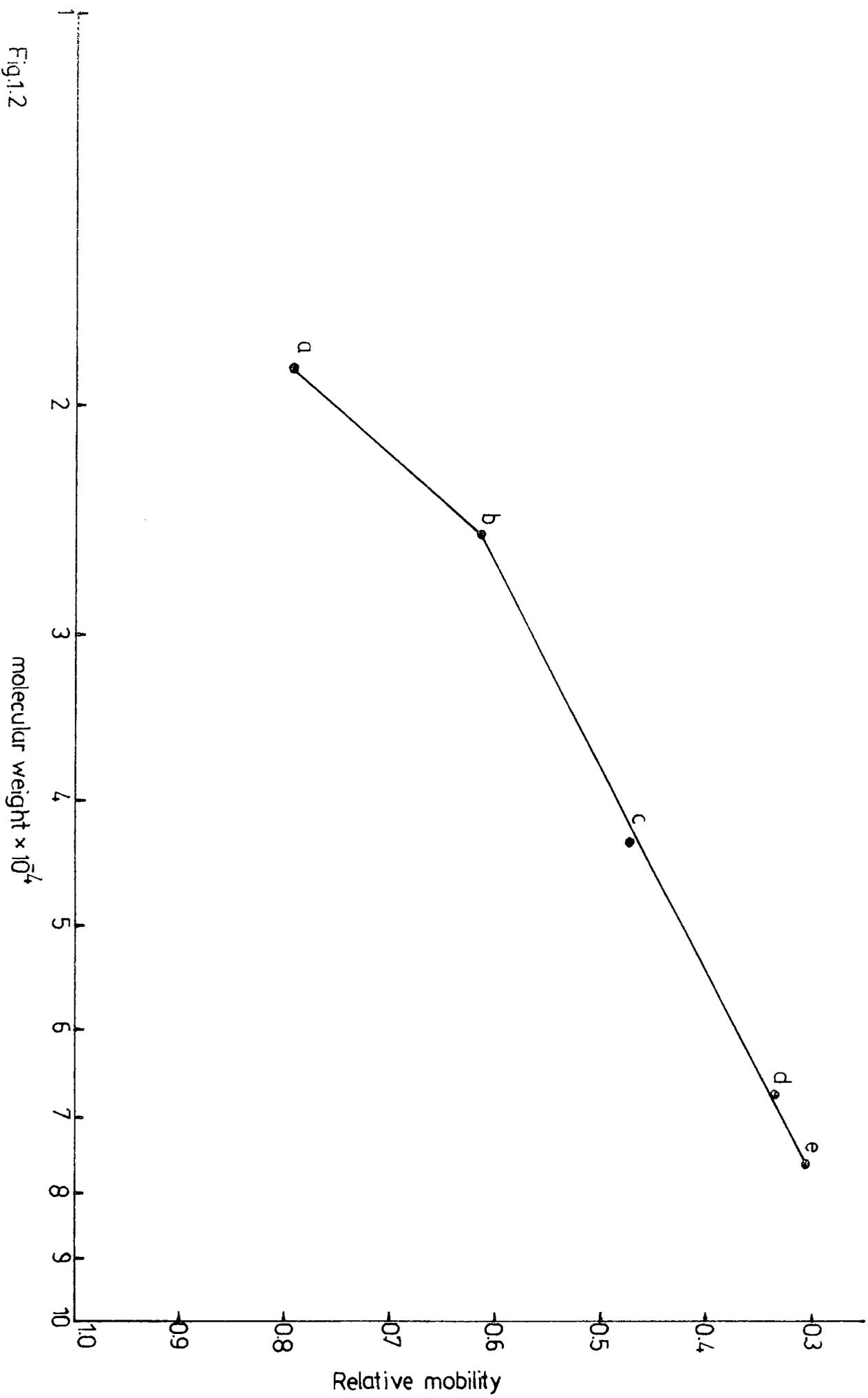
e = transferrin) on gradient pore

gels.

Fig.1.2

molecular weight × 10⁻⁴

Relative mobility

B. Analysis and screening for variation of
        storage proteins

A large number of Pisum lines (see materials
and methods section) were examined using single pore
and gradient pore gel electrophoresis by investigating
total protein extracts or globulin preparations (Fig.1.3).
Based on the results of this analysis, the following
storage protein bands were identified : (i) bands of
mol. wt. 70,000 and 65,000 representing convicilin
(Croy et al.,1980b), (ii) bands in the region of mol.
wt. 50,000, 30,000 and 18,000 representing vicilin
and (iii) legumin bands in the region of mol. wt.
40,000 and 20,000. Considerable variation among
different lines was observed for acidic subunits of
conventional legumin (Lg-1C) but very little variation
was seen for vicilin, convicilin and basic subunits
of legumin.

When globulins of different lines of Vicia faba
were examined, the mol. wts. of all these components
were found to be slightly lower except legumin basic
subunits (Lg-2) which had a mol. wt. somewhat higher
than similar components in Pisum. More variation with
respect to the number of bands was seen in Pisum. A
few bands lying between mol. wt. 50,000 and 70,000,
later identified as bands of heterogeneous legumin
which were found in Vicia, were absent in Pisum (Fig.1.4).
In contrast to the considerable variation of Lg-1C
subunits in Pisum, only one type of pattern represented

Fig. 1.3 : SDS-polyacrylamide gel electrophoresis

of total protein extracts prepared from

single seeds of various lines

(180, 360,------2162) of _Pisum_ _sativum_.

CV stands for convicilin, V for vicilin,

Lg-1 and Lg-2 for acidic and·basic

subunits of legumin and Lg-1S for acidic

subunits of small legumin subunit pair.

Fig. 1.4 : SDS-polyacrylamide gradient gel

electrophoresis of globulins of lines

a) Kelvedon Wonder, b) English Wonder

and c) Feltham First of _Pisum_ _sativum_

and lines d) Minica and e) Wierboon

of _Vicia_ _faba_. CV stands for

convicilin, V for vicilin, Lg-1C

for acidic subunits of conventional

legumin, and Lg-2 for basic subunits

of legumin.

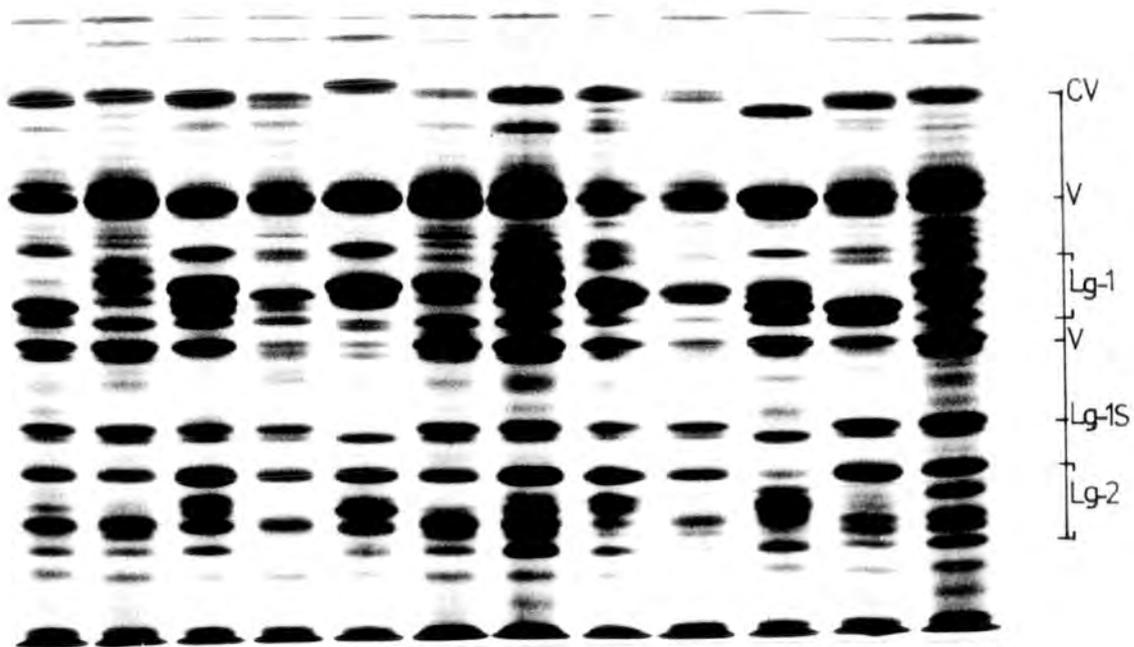180 360   611   806   808 851 1238 1249 1263   1293   1552 2162

Fig.1.3

a   b   c   d   e
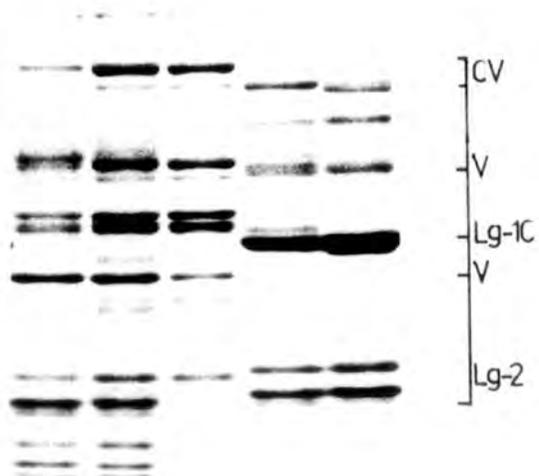
Fig.1.4

by a band of mol. wt. 35,000 was seen in *Vicia* *faba*.

The pattern of Lg-1 subunits in *Vicia* and Lg-2 subunits in *Pisum* in a given line was not constant always. Single seeds of any given generation of a *Vicia* line had the same pattern for legumin acidic subunits, but when selfed and analysed in the next generation, although all the single seeds examined showed the same pattern, this was different to that of the previous generation. However, these differences only involved quantitative differences in the bands. Two possibilities were thought to be responsible for this effect, either the developmental stage at which seeds were harvested or some storage effects.

C. Purification of storage proteins

a. Globulin preparation

The globulin fraction of *Pisum* *sativum* and *Vicia* *faba* seeds prepared by the procedure given in the materials and methods section, consisted of vicilin, convicilin and legumin as described previously.

b. Hydroxylapatite column chromatography

Various fractions of seed proteins were separated by hydroxylapatite column chromatography using the following types of elution techniques.

(i) Step-wise elution : This method was used to separate legumin and vicilin fractions of the globulins. The

first peak was obtained by elution with equilibrating buffer (0.05M phosphate buffer, pH 8.0), the second peak was eluted by using a buffer of higher concentration (0.3M) and the third peak by using a still higher concentration (1M) of phosphate buffer pH 8.0. Analysis by SDS-gel electrophoresis showed that no proteins were eluted in the first peak, legumin was obtained in the second and vicilin in the third peak. However, this method did not prove effective enough because it did not give a pure separation of any of the protein fractions.

(ii)   Linear gradient elution : Hydroxylapatite column chromatography by linear gradient elution was found to be a better method for fractionation of the proteins in the total protein extracts. All the proteins were eluted in the fractions obtained using a gradient between 0.05M to 1M phosphate buffer (molar concentrations were determined by measuring the refractive indices of various fractions; these values were used to ascertain the molar concentration from a calibration curve already prepared). Molar concentrations and the proteins eluted under different peaks are given in Table 1.1 and Fig. 1.5.

Fig.1.5 : Elution profile from chromatography
on hydroxylapatite column using
phosphate-gradient elution. For
details of the peaks see
table 1.1.
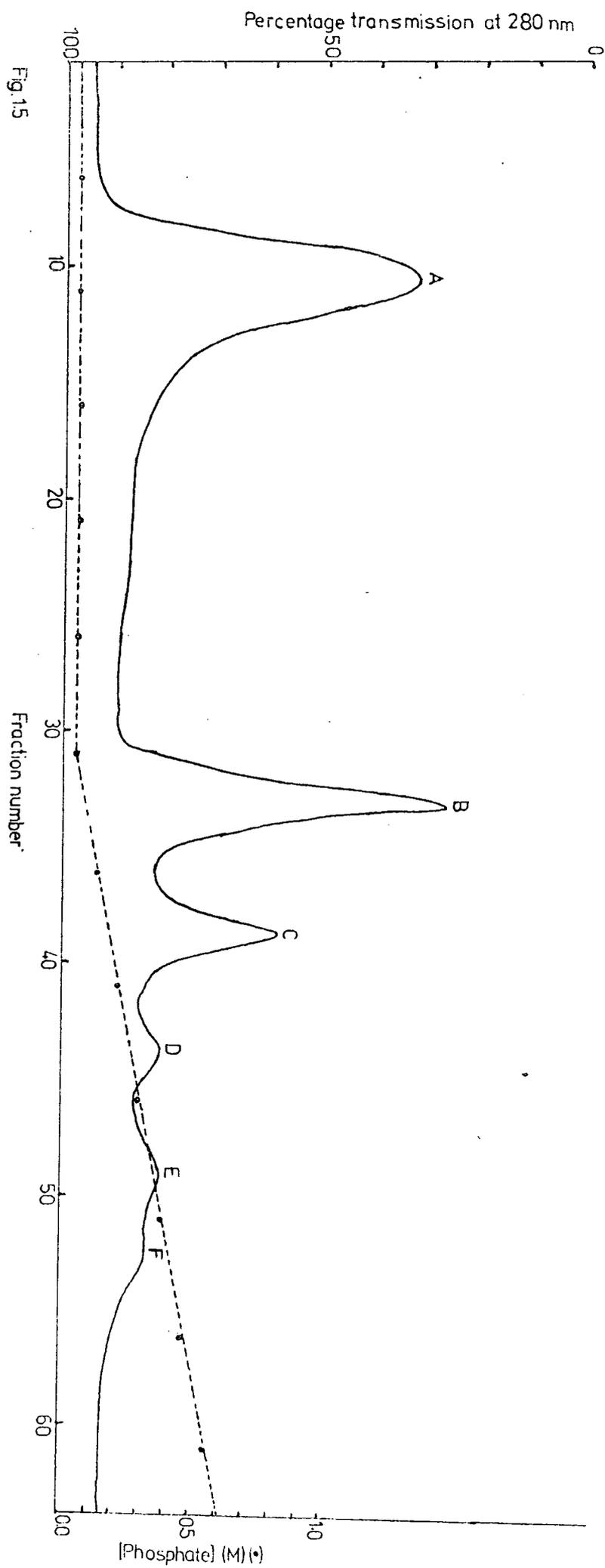
Fig.15

Table 1.1   Major proteins and the molar concentration
at which they were eluted using hydroxylapatite
column chromatography

Peak	Molar Concent-ration	Major proteins (mol. wt.)
A	0.05	Nil
B	0.05-0.1	Albumins (80,000, 50,000, 30,000, 25,000, 22,000 and 18,000).
C	0.13-0.21	Legumin (40,000, 25,000, 20-22,000)
D	0.21-0.28	Legumin, vicilin and convicilin
E	0.32-0.37	Convicilin (70,000, 65,000) and vicilin
F	0.4 -0.45	Vicilin (50,000, 30,000, 18,000).

D.   Quantitative determination of legumin and vicilin

Estimations of the legumin and vicilin fractions in globulins were carried out by various electrophoretic and chromatographic techniques as follows:

a)   Quantitation by electrophoresis

(i) Gel electrophoresis and scanning : The globulins from bulked seeds of seven commercial lines (English Wonder, Kelvedon Wonder, Little Marvel, Meteor, Peter Pan, Pioneer and Feltham First) grown in different years were extracted and studied on SDS-gradient pore gels. All the

lines were observed to be quite similar in the
qualitative pattern of legumin and vicilin. However,
the ratio of the concentration of legumin and vicilin
on the basis of intensity of staining of subunits
showed variation amongst lines for seeds harvested in
the same year and variation for the same line in seeds
harvested in different years (Table 1.2). As there
was overlapping of different polypeptide bands, actual
amounts of the legumin subunits was found by running
the globulins under reducing and non-reducing conditions.
Densitometric measurements of these gels confirmed that
although the total concentration of legumin in these
lines showed variation from year to year, the ratio
of the acidic and basic legumin subunits in all the
lines was the same i.e. 1:1 (after correction is made
for the difference in the size of legumin subunits).
'Feltham first' and 'Meteor' were found to have a
greater legumin to vicilin ratio in harvests of both
the years whereas other five lines had lower legumin
contents relative to vicilin. Similar variations were
also observed in the seed extracts of these lines.

Table 1.2 Densitometric analysis of legumin, vicilin and their subunit contents in pea seeds of different harvests

Pea variety	Harvest	Vicilin (area under the peak)	Legumin (area under the peak)	Lg-1 (acidic subunits)	Lg-2 (basic subunits)	$\frac{Lg-1}{Lg-2}$	Legumin, vicilin ratio
English Wonder	year I	45.42	18.11	12.4	5.71	2.17:1	0.4:1
	year II	28.52	19.03	12.74	6.29	2.03:1	0.7:1
Kelvedon Wonder	year I	49.57	36.38	25.88	10.5	2.4:1	0.7:1
	year II	17.69	9.81	6.6	3.2	2.06:1	0.6:1
Feltham First	year I	69.72	117.47	84.4	33.07	2.5:1	1:0.6
	year II	19.1	21.68	14.79	6.89	2.15:1	1:0.9

(ii) <u>Rocket immunoelectrophoresis</u> : Legumin and vicilin
were determined by using appropriate antisera in the
immunoelectrophoretic method given by Laurell (1966).
Legumin and vicilin contents of each line were estimated
from the calibration curves prepared for the 'rocket'
heights of standard legumin and vicilin samples
respectively. Results in Table 1.3 show the variation
of legumin and vicilin in these <u>Pisum</u> lines. Lines
1238 and JI 181 had a very high content of legumin and
the lines 1263 and 1238 were rich in vicilin. On the
other hand, lines Mangtout and 1263 were low in legumin
and lines JI 181 and Meteor low in vicilin content.

<u>Table 1.3</u> Quantitative analysis of legumin and vicilin
in total protein extract of pea seeds by
immunoelectrophoresis

S.No	Pisum line	% age of legumin	% age of vicilin	Legumin: vicilin ratio
1	Feltham First	24.8	8.48	2.92:1
2	Meteor	21.6	5.9	3.66:1
3	Mangtout	11.6	6.3	1.84:1
4	JI 181	34.4	5.3	6.50:1
5	807	22.0	8.0	2.75:1
6	851	20.8	9.8	2.12:1
7	1238	50.8	22.0	2.31:1
8	1263	12.4	10.2	1.20:1
9	110	31.6	8.0	3.95:1

(iii) <u>Cellulose acetate membrane electrophoresis</u> : Legumin

and vicilin in globulins and total protein extract were

also quantified by cellulose acetate membrane electro-

phoresis. Vicilin and legumin protein bands were

identified by running these purified proteins along

with the samples. Mobility of vicilin was lower than

that of legumin but in low legumin lines the legumin band

was not clearly resolved from the vicilin band. These

results were thus inferior to those obtained on gels

and were not in quantitative agreement.

b) <u>Quantitation by column chromatography</u>

Chromatographic separation by stepwise elution

on hydroxylapatite column was used to measure the legumin

and vicilin in <u>Vicia</u> globulins. Optical densities of

the fractions under the second and third peak were measured and fract-

ions pooled to get the relative amounts of proteins eluted

in these fractions. The second peak contained about

5 times more protein than that in the third peak.

However, this method of quantitation of legumin was also

found not to give reproducible results and also the

proteins eluted under the peaks were not pure legumin

and vicilin.

E. <u>Conclusions</u>

Single pore gels were found to be the best system

for qualitative analysis. These gels were very easy

to make and gave a wide and good separation of legumin

subunits. On the basis of differing patterns for

various subunits, a number of lines were selected for further studies on the structure and genetics of legumin. These lines were 'Felix' of _Vicia faba_ and 110, 807, 1238, 1263, 'Feltham First' and 'Meteor' of _Pisum sativum_. SDS-polyacrylamide gel electrophoresis suitably modified was found to be most suitable for studies on structure and genetics of legumin. Variety 'Felix' of _Vicia faba_ was selected for studies on the structure of legumin only. Genetical studies on _Vicia_ legumin could not be carried out as the pattern for Lg-1 subunits was not constant from generation to generation and differences in the subunit were more quantitative than qualitative.

Part II. STRUCTURE OF LEGUMIN OF <u>VICIA FABA L</u>.

A. SDS-Polyacrylamide gel electrophoresis of <u>Vicia</u>
<u>faba</u> globulins

Globulins of <u>Vicia faba</u> variety 'Felix' isolated as
in the Methods section were analysed on 12.5%
SDS-polyacrylamide gels. Under reducing conditions
(Fig.2.1d) legumin subunits were identified by reference
to the Wright and Boulter (1974) model of legumin. Acidic
subunits were represented by two bands of mol. wt. 37,500
and 35,000 and basic subunits by bands of mol. wt. 23,000,
21,600, 21,200 and 21,000. Bands representing polypeptides
previously identified as convicilin (CV), mol. wt.64,000
(Croy et al., 1980b) and as vicilin (V), mol. wt. 48,000 and
32,000 (Wright, 1973) as well as a number of other bands,
were also present. When examined under non-reducing
conditions (Fig.2.1a), the protein banding pattern was
found to be different with new bands of mol. wt. 79,000,
73,000, 67,000, 53,000 and 40,000-37,000. One of these,
the band H/I representing the subunit pair of conventional
legumin model (Wright and Boulter, 1974) had a mol. wt. 53,000.
The bands (A-J) under non-reducing conditions were found to
be due to components undergoing reductive cleavage i.e. a
series of disulphide bonded subunit pairs, by two dimensional
SDS-polyacrylamide gel electrophoresis, employing non-reducing
conditions in the first dimension and reducing conditions
in the second dimension (Fig.2.2). The mol. wts. (in
brackets) of two components of each of the bands 'A-J'

were as follows:

$$A(79,000) = (58,000) + (21,000)$$

$$B(73,000) = (54,000) + (21,200)$$

$$C(70,000) = (52,000) + (21,200)$$

$$D(67,000) = (50,000) + (21,200)$$

$$E(61,500) = (46,500) + (21,000)$$

$$F(60,000) = (43,500) + (21,000)$$

$$G(56,500) = (37,500) + (23,000)$$

$$H/I(53,000) = (35,000 + 21,600), (35,500 + 23,000)$$
$$\text{and } (35,500 + 21,600)$$

$$J(40,000 - 37,000; \text{ three bands})$$

$$= (22,700 - 23,100) + (21,200 - 21,000)$$

Thus, whereas the mol.wts. of the larger subunits, depending on the size of the subunit pair, varied from 23,100 to 58,000, those of the smaller subunits of all the pairs lay approximately in the same range (mol.wt. 21,000-23,000). Also, whereas mol.wts. of the larger subunits decreased linearly from band 'A' to 'H', band 'I' was peculiar in having a mol.wt. (35,500) greater than that of 'H' (35,000). Another characteristic of the subunit pairs 'I' was that in comparison to one smaller subunit contained in all other pairs, there were two smaller subunits of mol.wt. 23,000 and 21,600 respectively associated with subunit pair of the type 'I'.

Fig. 2.1 : SDS-polyacrylamide gel electrophoresis of
globulins and legumin of <u>Vicia</u> <u>faba</u>
variety 'Felix'. Track 'a' : globulins
under non-reducing conditions; track 'b' :
immunoprecipitated legumin obtained by
double diffusion of <u>Vicia</u> <u>faba</u>
globulins and affinity purified
anti-<u>Pisum</u> <u>sativum</u> legumin; track 'c' :
legumin purified by hydroxylapatite
column chromatography; track 'd' :
globulins under reducing conditions.
A-J represent legumin subunit pairs
as defined in the text. CV, V and
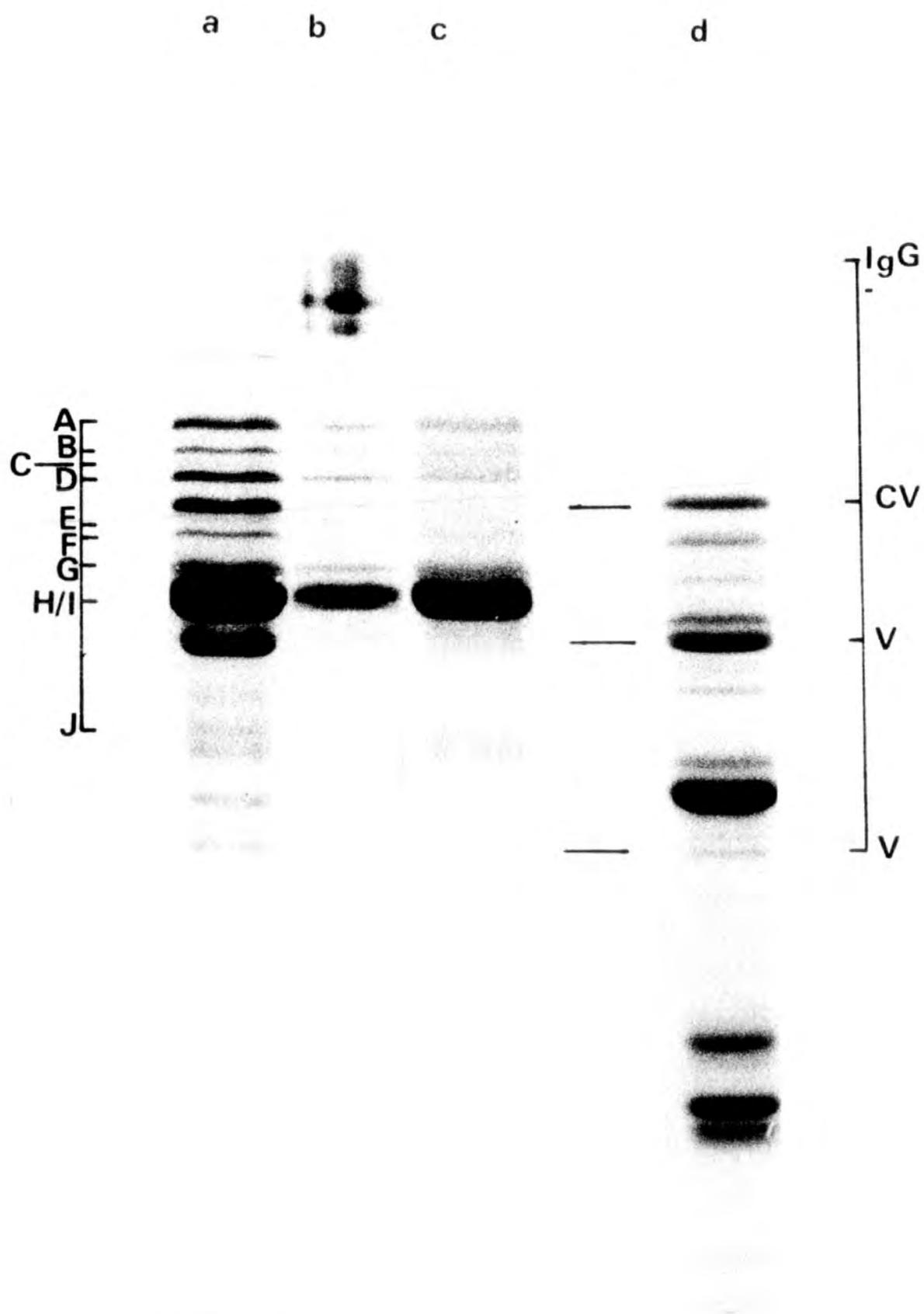IgG represent convicilin, vicilin
and immunoglobulins respectively.

Fig. 2.1

B.   Legumin purification by hydroxylapatite column Chromatography

Legumin of <u>Vicia</u> <u>faba</u> variety 'Felix' was fractionated on hydroxylapatite columns and examined under non-reducing conditions on 12.5% gels (Fig.2.1c). It contained all the bands of fig.2.1a except those representing convicilin and vicilin; all the bands present corresponded to ones containing disulphide bonded subunits i.e. bands 'A-J'. Densitometric scanning of the stained gels showed that the subunit pair H/I constituted approx. 80% of the total legumin whereas subunit pairs 'A-G' and 'J' were each present as minor components.

C.   Immunodiffusion studies

Immunoprecipitates were formed by double diffusion of <u>Vicia</u> <u>faba</u> globulins against the affinity purified antibodies directed against legumin of <u>Pisum</u> <u>sativum</u>, which is known to cross-react with <u>Vicia</u> <u>faba</u> legumin (Croy <u>et</u> <u>al</u>.,1979). The sharp precipitin arcs were excised, washed and when run on SDS-gels (Fig.2.1b), contained all the bands 'A-J' except 'F' seen in the purified legumin in Fig.2.1.c. This showed that all the subunit pairs except 'F' had determinants in common with the pea legumin.

Fig. 2.2 : Two dimensional SDS-polyacrylamide gel
electrophoresis of <u>Vicia</u> <u>faba</u>
globulins. 1D : analysis under
non-reducing conditions; band
pattern given as track (i) in the
first dimension. 2D : analysis under
reducing conditions in the second
dimension; band pattern given as
track (ii). A-J represent legumin
subunit pairs and their components
as defined in the text CV and V
represent convicilin and vicilin
respectively.

1D(−2ME)

2D (+ME)

A BCD EF G H/I   J
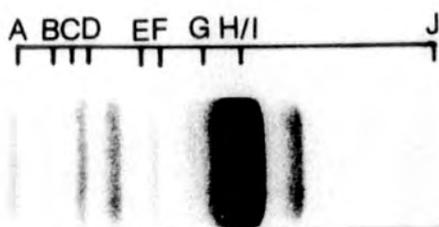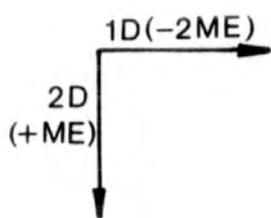
(i)

(ii)

Fig. 2.2

D. <u>Two dimensional electrophoreses</u>

(i) <u>Polyacrylamide gel electrophoresis/SDS-polyacrylamide</u>
<u>gel electrophoresis</u>

Globulins were run in the first dimension under
non-dissociating conditions on 10% polyacrylamide gels
(Fig.2.3). Three bands 'DLg', 'F' and 'Lg' of relative
mobilities 0.09, 0.11 and 0.19 respectively were observed,
the last being the major band. A smear was also observed
along the sides of the gel track beyond band 'Lg'. Gel
slices from the first dimension were run under reducing
and non-reducing conditions in the second dimension on
12.5% SDS-polyacrylamide gels (Fig.2.3). Bands 'DLg'
and 'Lg' were found to contain all the bands except 'F'
of Fig.2.1d and Fig.2.1a under reducing and non-reducing
conditions respectively. Band 'DLg' was considered to
be due to dimers of legumin molecules. Band 'F'
corresponded to band 'F' of Fig.2.1a under non-reducing
conditions and to the subunit products of band F of
Fig.2.2 under reducing conditions. Components of smears
corresponded to the bands of vicilin and convicilin.

(ii) <u>Polyacrylamide gradient gel electrophoresis/SDS-</u>
<u>polyacrylamide gel electrophoresis</u>

The first dimension electrophoresis of globulins
was carried out on 5-15% polyacrylamide concentration
gradient gels. Three bands of relative mobilities
0.275, 0.337 and 0.520 were seen (Fig.2.4). From the
mol.wt. calibration curve of these gels, the mol.wt.

Fig. 2.3 : Two dimensional analysis of <u>Vicia</u> <u>faba</u>

globulins. 1D : analysis by gel

electrophoresis (non-dissociating

conditions) in the first dimension.

Bands DLg, F and Lg of increasing

mobility shown in tracks (i) are

defined in the text. 2D : analysis

by SDS-polyacrylamide gel electrophoresis

in the second dimension under non-

reducing (b) and reducing (a) conditions.

Band patterns shown in tracks (ii)b

(non-reducing) and (ii)a (reducing).

A-J represent legumin subunit pairs

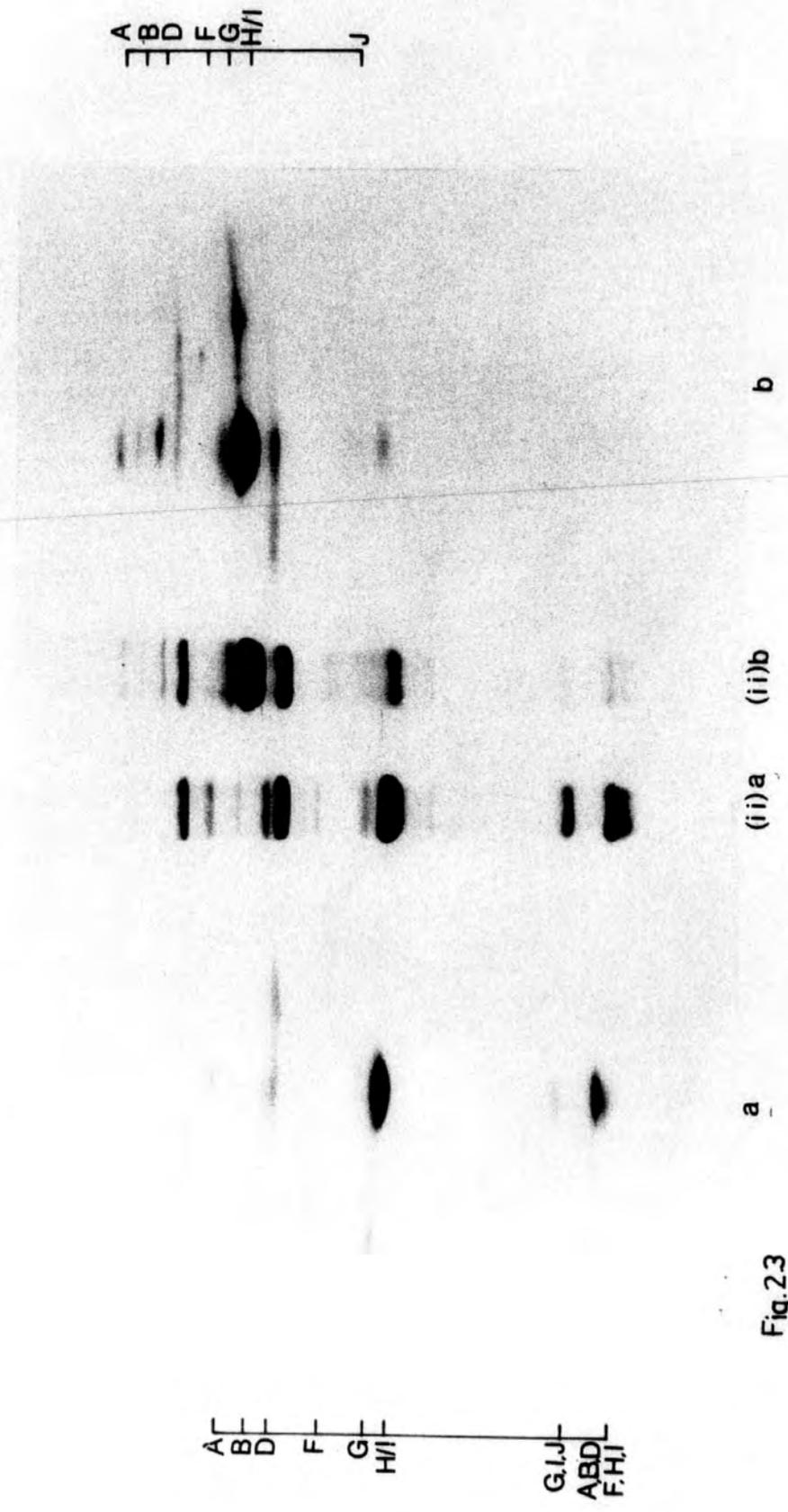and their components as defined in

the text.

Fig.2.3

Fig. 2.4 : Two dimensional gel analysis of
Vicia faba globulins. 1D : analysis
by gradient pore gel electrophoresis
under non-reducing conditions in
the first dimension. 2D : analysis
by SDS-polyacrylamide gel
electrophoresis in the second
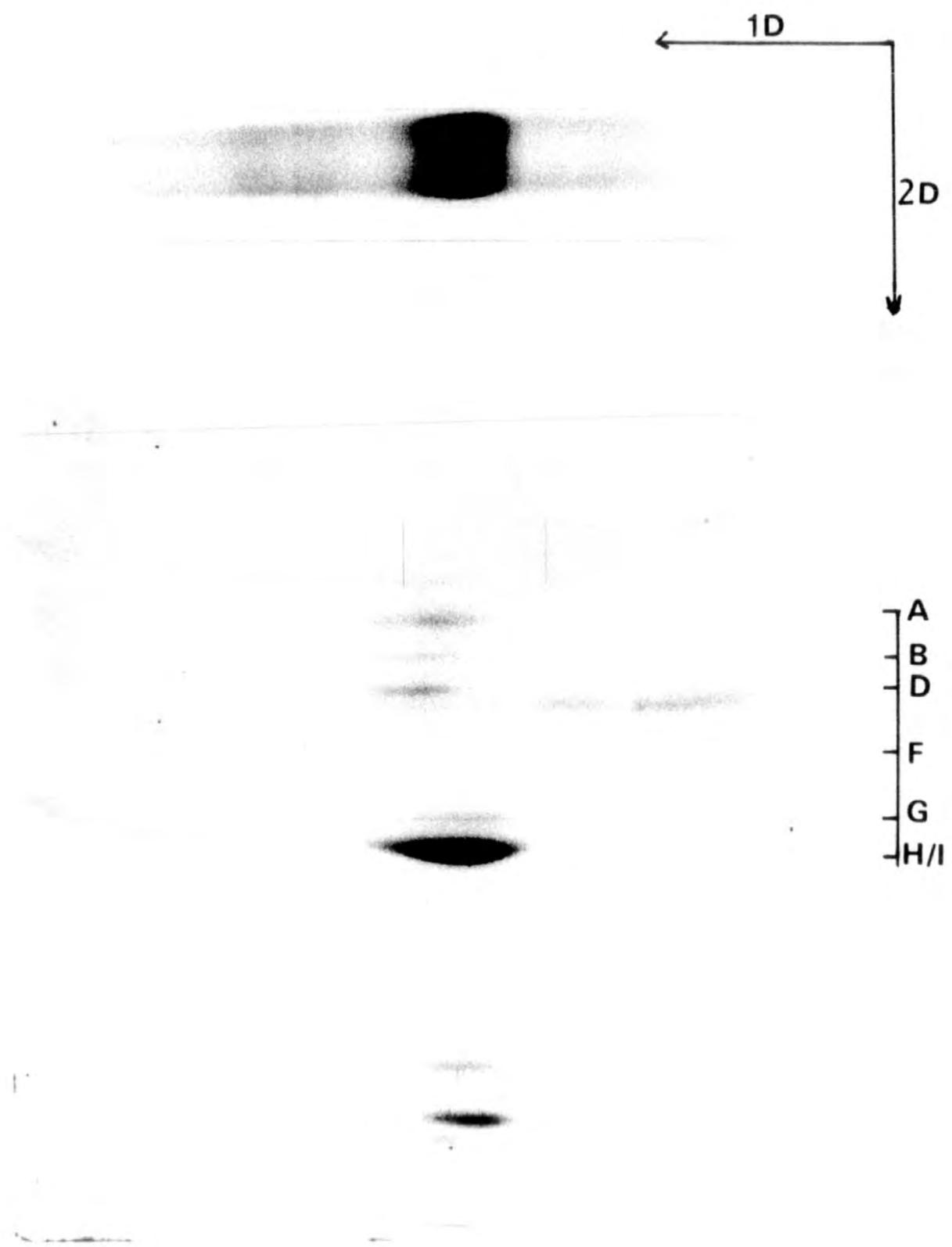dimension. A-J represent large
acidic subunits as defined in the
text.

Fig. 2.4

values of these bands were found to be 630 - 650,000,
560,000 and 330,000 respectively. Mol. wt. 330,000 of the
third band represented the mean value of a large number
of closely placed dark bands (as many as 10 in number) in
the mol. wt. range of 280,000 to 380,000. The subunit
pairs present in these bands (mol.wt. 280 - 380,000) were
revealed by analysis in the second dimension by using
SDS-polyacrylamide gel electrophoresis under reducing
conditions (Fig.2.4). Although the resolution in the
first dimension did not allow complete analysis of species
present in the major band, subunit pair A, B, C, D were
partially separated and had a lower mobility in the first
dimension whereas 'F' was partially separated to a higher
mobility. The legumin subunit pairs 'H/I' and the subunit
pair 'G' were found in all the legumin species analysed.
Subunit pair 'J' was also found to be associated with the
subunit pairs 'H/I'. The minor bands at lower mobility
in the first dimension also contained legumin subunits
with a distribution similar to the major legumin bands.

(iii) SDS-polyacrylamide gel electrophoresis/Isoelectric
focusing

This technique involved the analysis of Vicia
globulins on SDS-polyacrylamide gels in the first dimension
followed by isoelectric focusing in the pH range 4-6.5
(for acidic subunits) and pH 6-10 (for basic subunits).
Results are shown in Fig.2.5a and 2.5b for acidic and
basic subunits respectively. All the bands 'A-J' of Fig.2.1a
separated in the first dimension under non-reducing

Fig. 2.5 : Two dimensional analysis of Vicia faba

legumin. 1D : analysis by SDS-

polyacrylamide gel electrophoresis

under non-reducing conditions in the

first dimension. 2D : analysis by

isoelectric focusing in the second

dimension in the acidic range

(a) and in the basic range (b).

A-J represent legumin subunit pairs

as defined in the text.

(a)
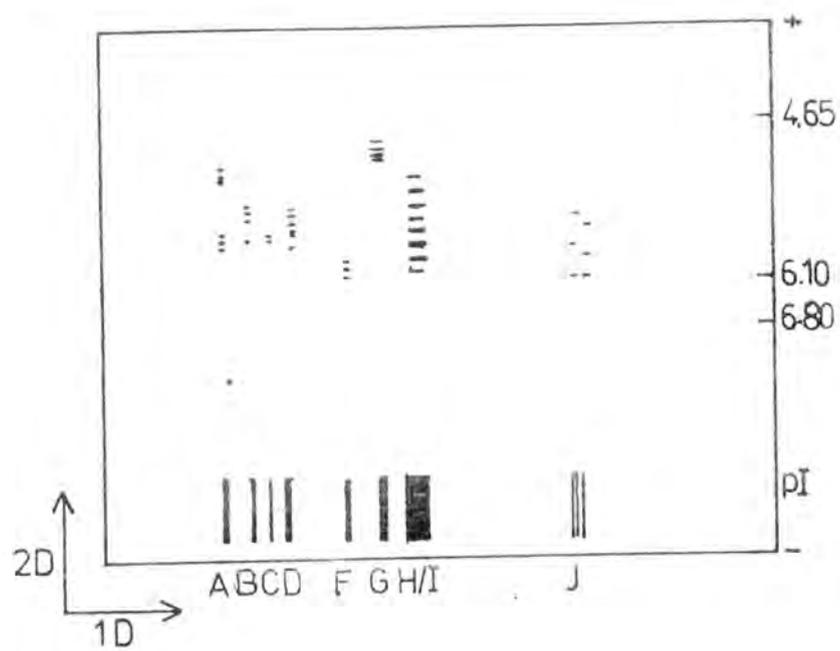
(b)
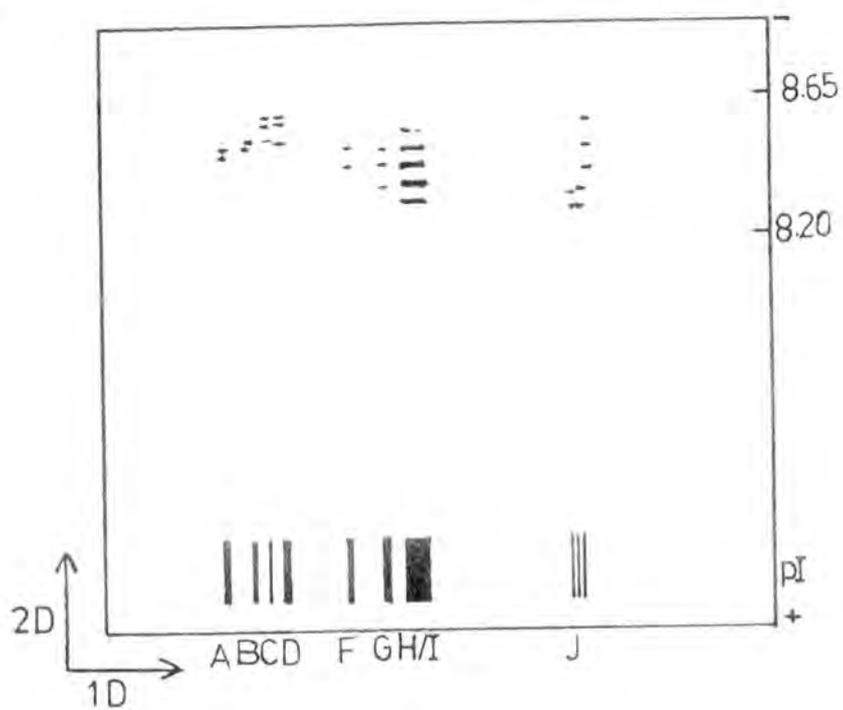
Fig. 2·5

conditions were shown to consist of acidic and basic subunits in the second dimension. The pI range of acidic subunits was 4.6-6.1 and of basic subunits 8.2-8.5. When subunit-pair-products separated in the first dimension under reducing conditions were subjected to isoelectric focusing in the second dimension (results not shown) it was observed that in each case the large subunit of a pair was acidic and the smaller subunit basic. All the subunits showed charge heterogeneity and the pI range of the subunit pairs 'A-G' and 'J' was similar to those of the subunits of conventional legumin H/I.

E. **Ultrogel column chromatography**

Fig. 2.6 shows the elution profile of _Vicia_ _faba_ total proteins on a column of ultrogel ACA 22. Proteins in fractions (No.31-47) representing globulins in the total protein extract were precipitated individually by adding trichloroacetic acid to a final concentration of 12%. The precipitates were analysed on 12.5% SDS-gels under reducing conditions. Products of subunit pairs 'A-J' of Fig.2.1d, representing legumin subunits, dominated the fractions 31-44 whereas vicilin and convicilin subunits were present in fractions 40-47. Stained gel strips of individual fractions were scanned densitometrically and the peak areas of acidic subunits of pairs A, B, D, F and G and the basic subunits of all the subunit pairs taken collectively were plotted to

Fig. 2.6 : Elution profile from ultrogel

column chromatography of <u>Vicia</u>

<u>faba</u> total proteins. Lg represents

the fractions in which legumin

is eluted; Vo stands for void

volume.
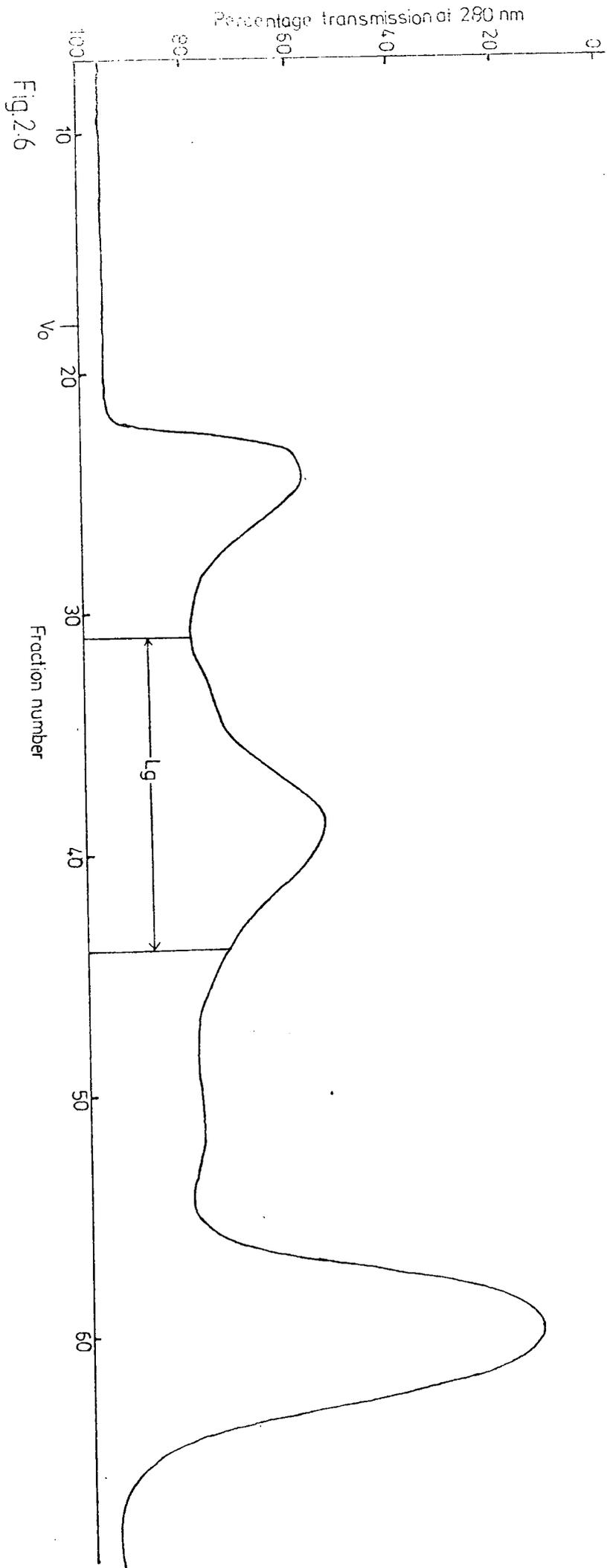
Fig.2.6

get the elution pattern relative to that of the acidic subunits of pair H/I (Fig.2.7). Fractions 32-33, representing higher mol.wt. contained significant amounts of all the subunit pairs and were due to dimerised legumin. Acidic subunit 'G' followed the distribution of conventional legumin subunits H/I in being present in higher amounts in the fractions 37 and 38. Acidic subunits A, B and D though eluting throughout the legumin fractions were concentrated relative to H/I in the faster-eluting fractions 35 and 36. Acidic subunit 'F' showed the anomalous distribution by eluting predominantly only in the fraction No. 33, between monomer and dimer peaks of H/I.

Thus all the bands 'A-J' in the globulin fraction of <u>Vicia</u> <u>faba</u> which represented disulphide bonded subunits were considered as representing legumin as they were eluted by hydroxylapatite column chromatography along with H/I, were antigenically similar and ran together under non-dissociating conditions on polyacrylamide gels and on ultrogel column.

Fig. 2.7 : Distribution of legumin subunits
after gel filtration chromatography
determined by densitometric scanning
of stained SDS-polyacrylamide gel
electrophoretic analysis of
individual fractions. Elution
volume increases with fraction
number. Curve H/I : distribution
of large acidic subunits of subunit
pairs H/I. Curves A, B, D, F,
G : distribution of large acidic
subunits of subunit pairs A, B, D,
F, G respectively relative to H/I.
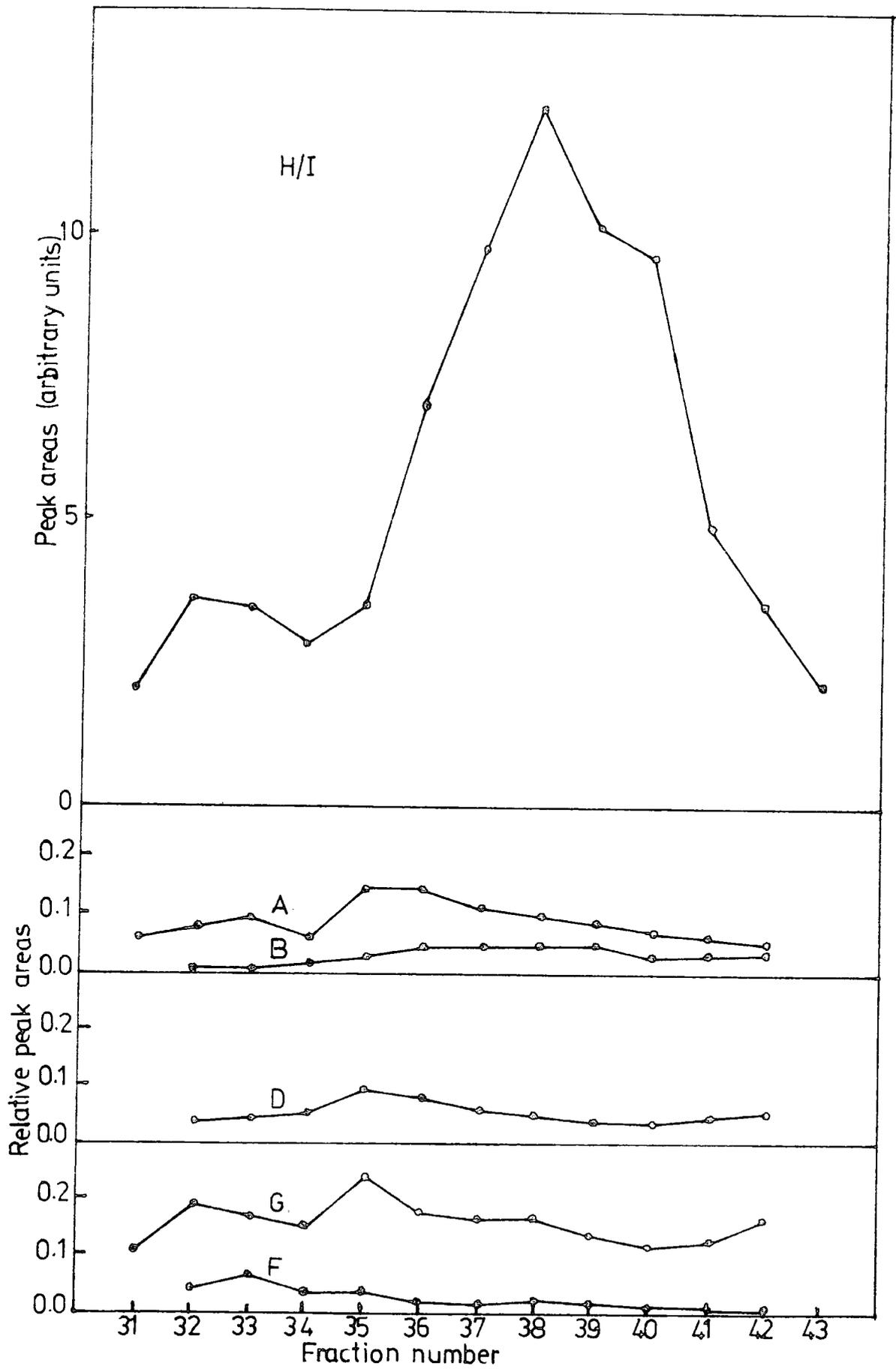
Fig.2.7

Part III   STRUCTURE OF LEGUMIN OF <u>PISUM SATIVUM L</u>.

The legumin fraction of seed proteins of <u>Pisum</u> <u>sativum</u> was purified by hydroxylapatite chromatography and analysed by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions(Fig.3.1a and 3.1b respectively). Under reducing conditions, mol. wts. of the components varied from 20,700 to 43,000. Bands of mol. wt. 35,000 to 43,000 represented acidic and those of mol. wt. 20,700 to 22,700 represented basic subunits of legumin (Gatehouse <u>et</u> <u>al</u>.,1980). A number of bands in mol. wt. range of 35,000 to 58,000 were observed under non-reducing conditions. Faint bands at mol. wt. 26,500 and 26,000 were also observed in the absence as well as presence of 2-mercaptoethanol and thus these bands represented the subunits that were not disulphide bonded. These subunits (mol. wt. 26,000 and 26,500) and subunit pair of mol. wt. 35,000 have previously been reported as impurities (Gatehouse <u>et</u> <u>al</u>, 1980).

A . <u>Immunodiffusion studies</u>

The total protein extract of pea seeds was immunoprecipitated against purified anti-legumin antibodies of <u>Pisum</u> <u>sativum</u> by double diffusion (Fig.3.2). The sharp precipitin arc when excised and examined on SDS-gel under non-reducing conditions (Fig.3.1c) showed subunit components similar to those of legumin purified by hydroxylapatite column chromatography. Hence all

Fig. 3.1 : Polyacrylamide gel electrophoresis

of pea seed proteins under dissociating

conditions (tracks 'a-d') and under

non-dissociating conditions (track 'e').

Tracks 'a' and 'b' : purified legumin

under reducing and non-reducing

conditions respectively; track 'c' :

immunoprecipitated legumin under

non-reducing conditions; track 'd' :

total protein extract under non-

reducing conditions; track 'e' :

purified legumin. A, B, C and D

represent legumin molecular forms

and impurities as defined in

the text; IgG stands for immuno-

globulins.
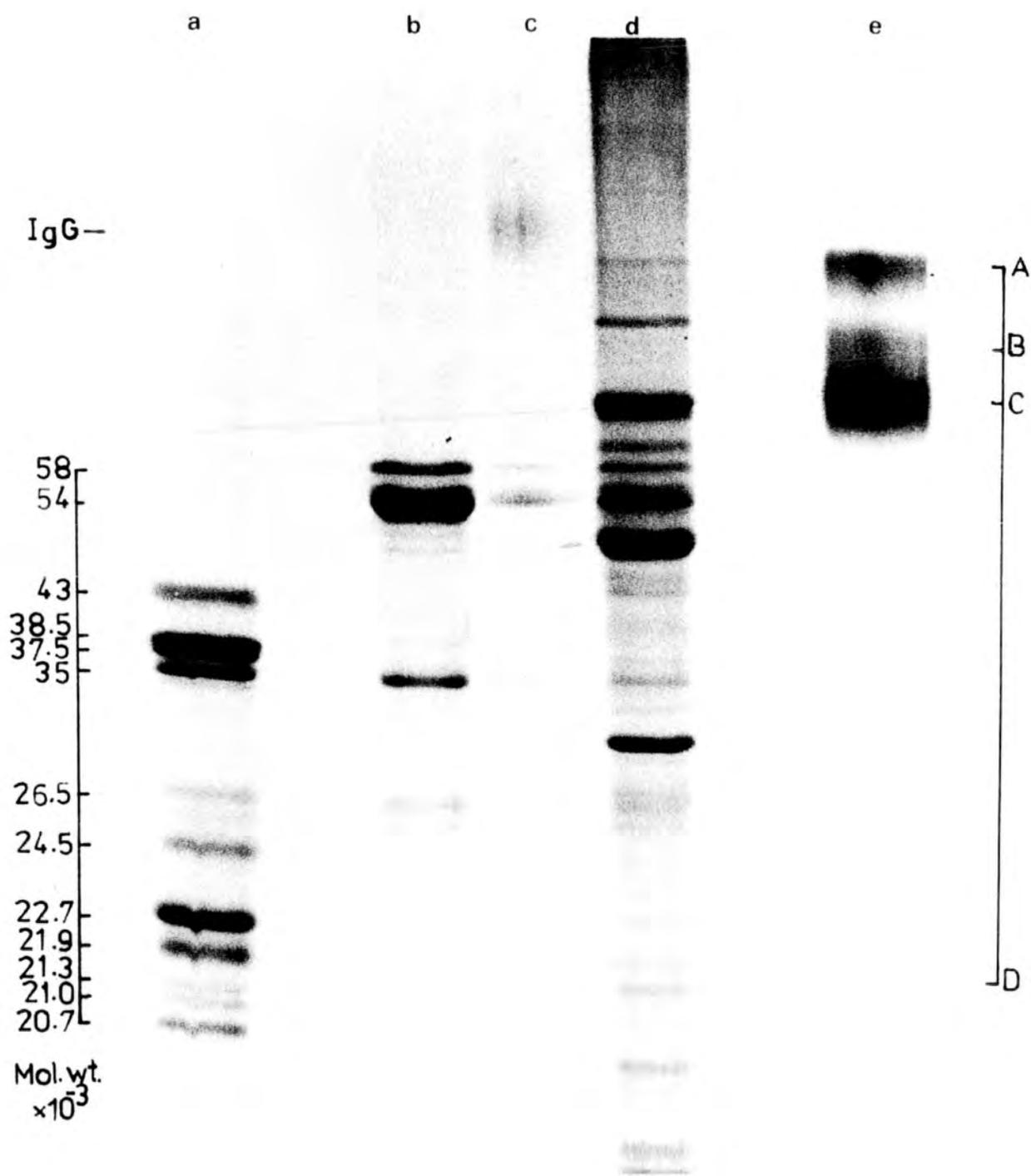
Fig. 3.1

Fig. 3.2 : Ouchterlony double-diffusion experiment.

(a) - Outer wells : 1, 4 _Pisum_ _sativum_

total protein extract; 2, 5 _Pisum_

sativum legumin; 3, 6 _Pisum_ _sativum_

vicilin (b) - Outer wells : 1 - 6

Pisum _sativum_ total protein extract.

(a) and (b) - inner wells : affinity

purified anti-_Pisum_ legumin IgG.

Fig. 3.2

(a)

(b)
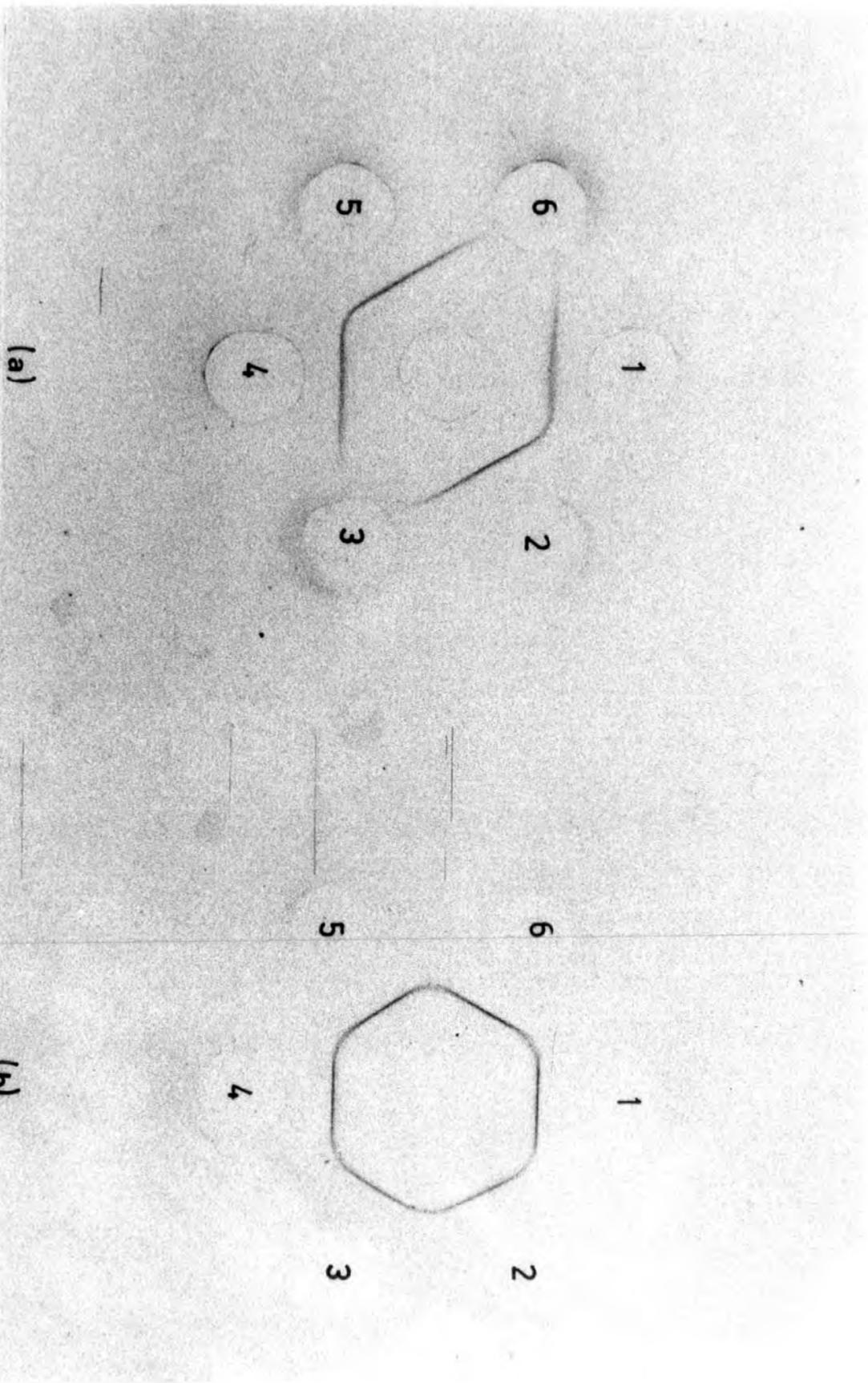
these subunit pairs and their products were considered as representing different constituents of legumin.

B . <u>Composition of subunit pairs</u>

Two dimensional SDS-polyacrylamide gel electrophoresis of legumin separating subunit pairs under non-reducing conditions in the first dimension followed by separation of respective subunits of each pair after reduction by 2-mercaptoethanol in the second dimension was employed to understand the relationship of subunit pairs to their respective subunits (Fig.3.3).The following subunit pairs (mol. wt. in brackets) were deduced :

1. (58,000) = (43,000) + (21,300)

2. (58,000) = (43,000) + (21,900)

3. (55,000) = (35,000) + (21,900)

4. (54,000) = (38,500, 37,500 and 35,000) + (22,700)

5. (35,000) = (24,500) + (21,000 and 20,700)

The subunits lacking disulphide bonds ran to the same mol. wt. in both the dimensions.

C . <u>Composition of legumin molecules</u>

Legumin when analysed under non-dissociating conditions on 7.5% polyacrylamide gels, showed separation into a number of components as shown in Fig. 3.1e. A number of faint bands of very high mobility (0.8-0.86) and three incompletely separated bands 'A-C' of fairly low mobility (0.27-0.37) were obtained (Table 3.1).

Fig. 3.3 : Two dimensional SDS-polyacrylamide gel

electrophoresis of _Pisum_ legumin.

1D : analysis under non-reducing

conditions in the first dimension;

band pattern given as track (i).

2D : analysis under reducing

conditions in the second dimension;

band pattern given as track (ii).

1, 2, 3, 4 and 5 represent

components of subunit pairs as

defined in the text.
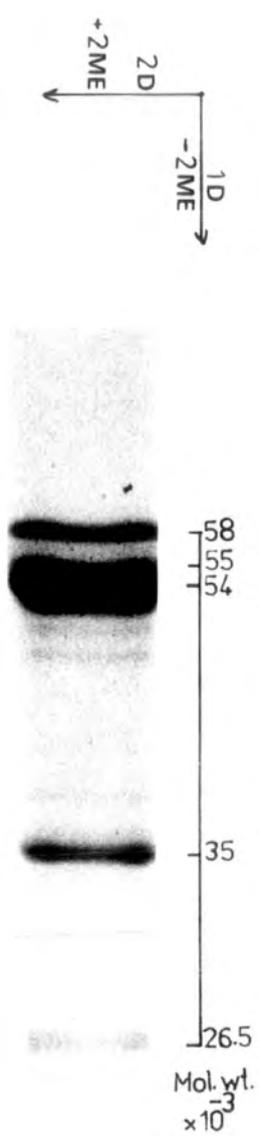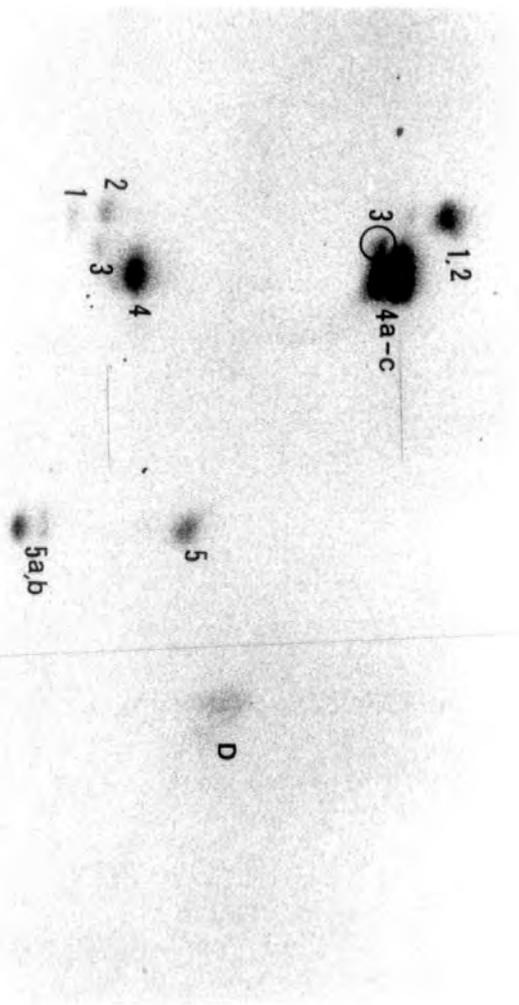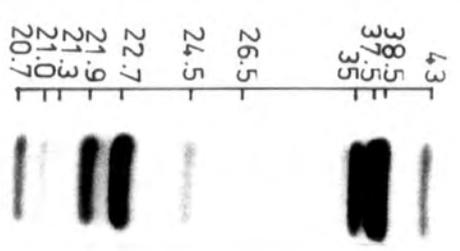
Fig. 3.3

Table 3.1   Composition of molecular species

S.No	Band (Fig 3.1e)	Relative Mobility	Percent-age	Subunit pairs and percentage ( )
1	A	0.27	23	1 : 4 : 5 (45) (52) (3)
2	B	0.32	20	2 : 3 : 4 : 5 (4) (4) (90) (2)
3	C	0.37	47	2 : 3 : 4 : 5 (15) (32) (47) (6)
4	D	0.8-0.86	10	Not legumin subunits (impurity)

Densitometric measurements of these stained bands showed
that legumin form represented by the band 'C' constituted
about half of the total legumin.

The subunit composition of these bands 'A-D' was
established by running the separated bands in second
dimension in the absence and presence of 2-mercaptoethanol
(Fig. 3.4a and 3.4b respectively). This analysis clearly
showed that bands of high mobility   (D) contained only
the polypeptides of mol. wt. 26,500 and 26,000
demonstrating thereby that these polypeptides belong to
a protein impurity in the legumin fraction. The
remaining bands of lower mobility (A-C) contained
subunit pairs and their respective subunits in different
combinations as detailed in Table 3.1. Whereas, the
subunit pair of mol. wt. 54,000 was common to all the
bands, the subunit pair of mol. wt. 58,000 was present

Fig. 3.4 : Two dimensional gel electrophoresis of

purified Pisum legumin. 1D : (a) and

(b) - polyacrylamide gel electrophoresis

under non-dissociating conditions in

the first dimension, see text for

A, B, C and D.

2D : SDS-polyacrylamide gel electro-

phoresis in the second dimension.

(a) : under non-reducing conditions.

Boxed areas excised for 'third dimension'

analysis shown in Fig. 3.5.(b) : under

reducing conditions. Boxed areas excised

for 'third dimension' analysis shown in

Fig. 3.6.
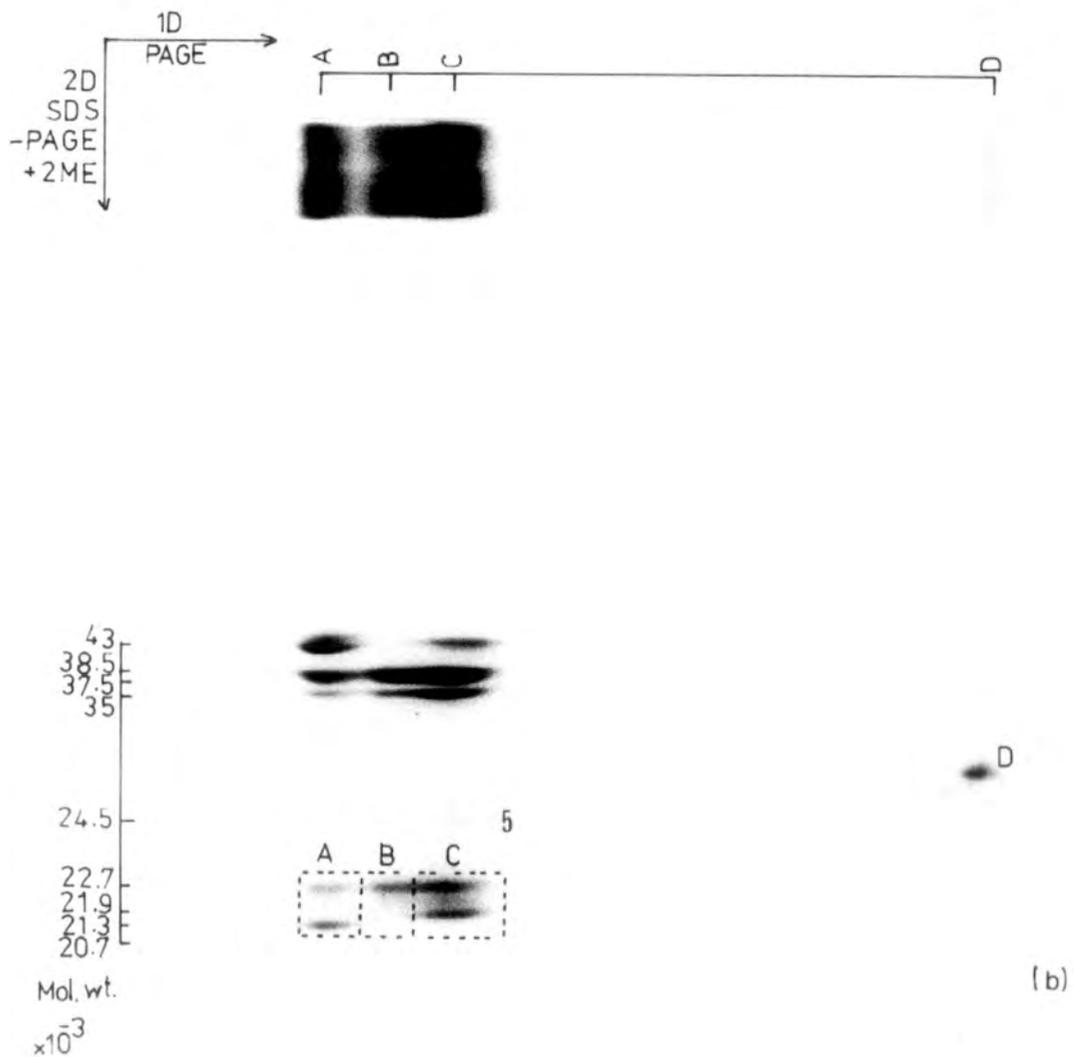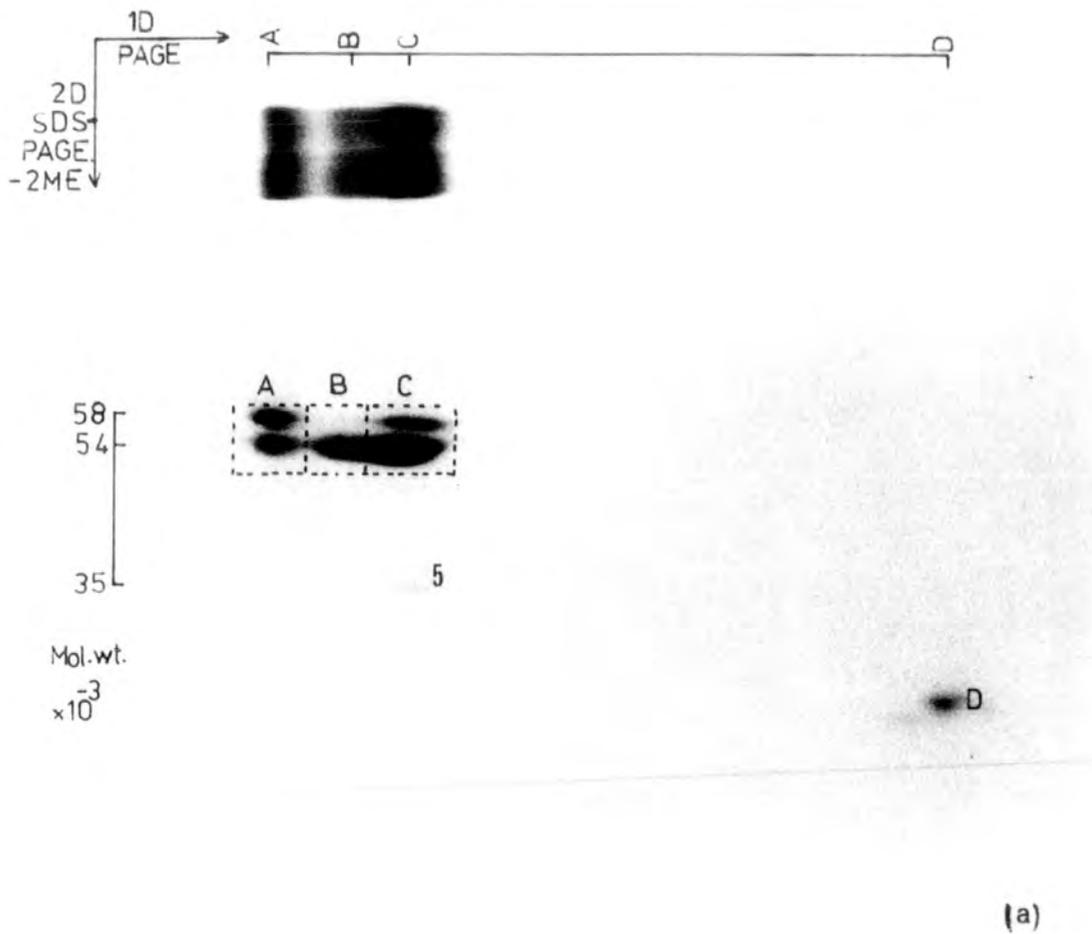
Fig. 3.4

predominantly in bands A and C and those of mol. wt. 55,000 and 35,000 in band C only. Traces of polypeptides of mol. wt. 26,500 and 26,000 were also found in band C. The constitution of the three bands became clearer when run under reducing conditions. The subunit pair of mol. wt. 58,000 which appeared to be common to A and C, was actually represented by two different types which differed in their subunit composition. Thus, the 58,000 pair had subunits of mol. wt. 43,000 + 21,300 and 43,000 + 21,900 in band A and C respectively.

Assignment of the subunit pairs to molecular forms of legumin A, B and C obtained on non-dissociating gels was confirmed by taking the subunit pairs obtained after second dimension under non-reducing conditions and running these into a 'third dimension' SDS-gel under reducing conditions. Thus, this analysis made apparent the presence of an additional subunit pair (mol. wt. 55,000) in form C. It consisted of a large subunit of mol. wt. 35,000 and a small subunit of mol. wt. 21,900 (Fig. 3.5).

D. Isoelectric points of the legumin subunits

Isoelectric focusing (IEF) of the acidic and basic subunits of legumin subunit pairs was carried out by second dimension IEF after separation of the subunits by SDS-polyacrylamide gel electrophoresis under reducing conditions in the first dimension. The observed band patterns were then compared with band patterns obtained

Fig. 3.5 : 'Third dimension' SDS-polyacrylamide
gel electrophoresis (under reducing
conditions) of legumin molecular
forms A, B and C separated by two
dimensional gel analysis shown in
Fig. 3.4a. 1, 2, 3, 4 represent
components of subunit pairs as
defined in the text.
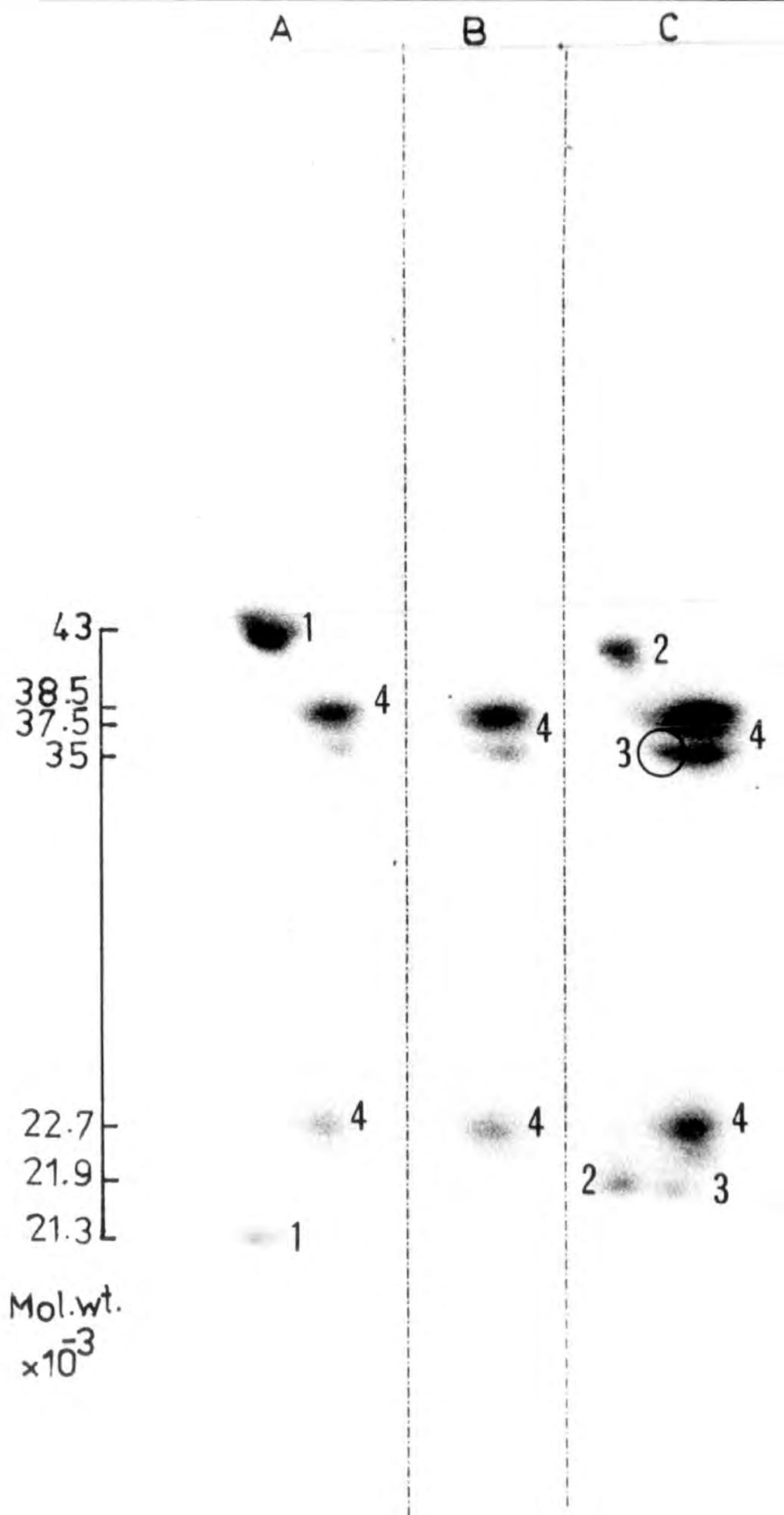
Fig.3.5

by isoelectric focusing in the third dimension of
component subunits of molecular forms separated in two
dimensions by electrophoreses under non-dissociating
and dissociating conditions in the presence of
2-mercaptoethanol (Fig. 3.6). Table 3.2 lists the
isoelectric points of the subunits. In all subunit
pairs, the large subunits had lower pIs i.e. were more
acidic. Most acidic subunits fall in the pI range
5.0-5.8 (excepting those belonging to subunit pair 2
and subunit pair 5) and most basic subunits fall in
the pI range 7.4-8.0. The subunit pair 1 had pIs of
its basic subunits below 7 (6.2-6.4). Twenty two
separate acidic subunits and 11 separate basic subunits
were identified.

Fig. 3.6 : 'Second' and 'third dimension' gel

analysis by isoelectric focusing of

legumin basic subunits separated

by SDS-PAGE under reducing conditions

(for 'second' dimension) and by

two dimensional gel analysis as

shown in Fig. 3.4b (for 'third'

dimension). 1, 2, 3, 4, 5 represent

basic subunits as defined in

Table 3.2.   2 and 3 in form B are

due to its incomplete resolution

from C.
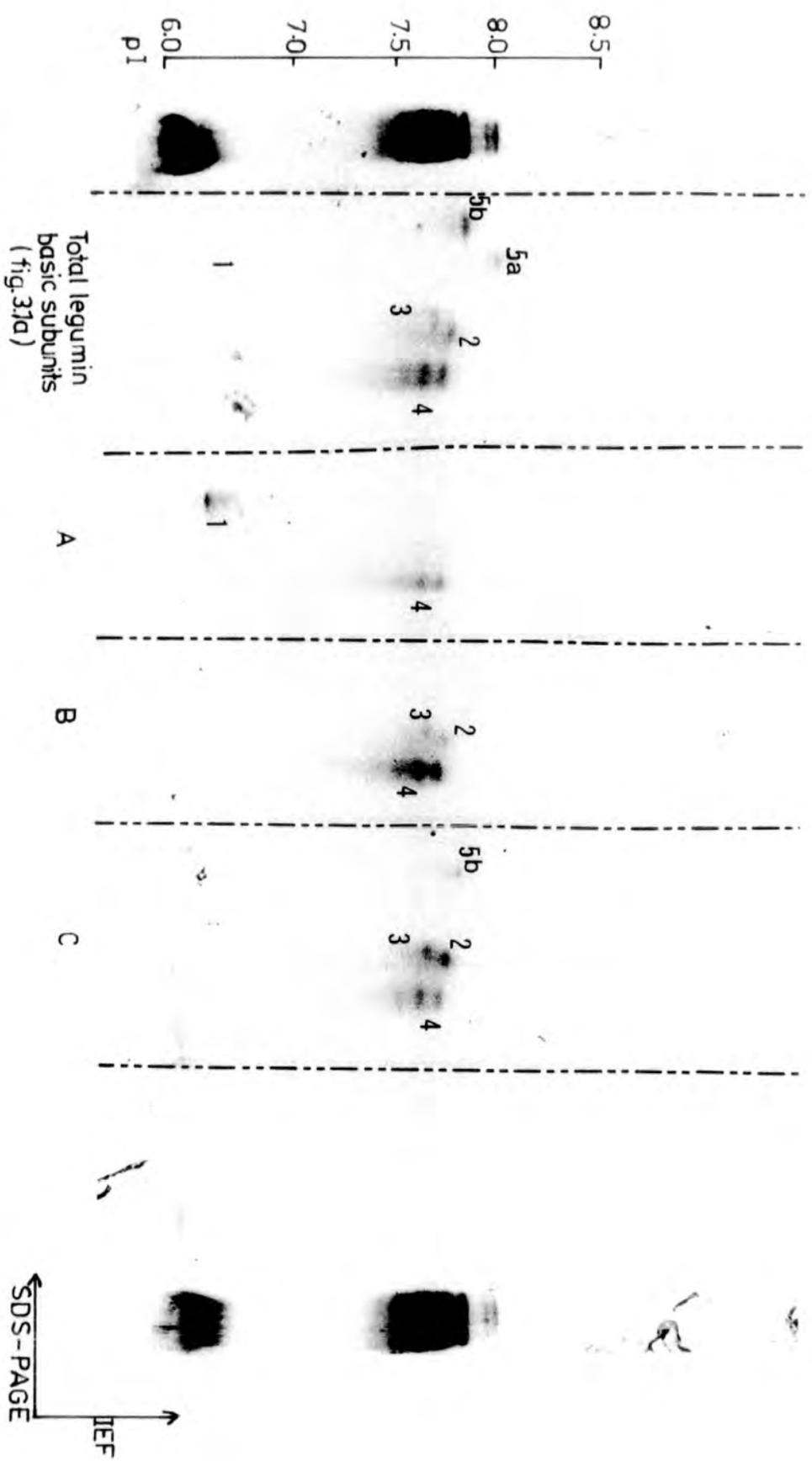
Fig. 3.6

Table 3.2 : Properties of subunit pairs of legumin

Subunit pair	Mol. wt.	Acidic subunits			Basic subunits		
			Mol. wt.	pI range ( )*		Mol. wt.	pI range ( )*
1.	58,000		43,000	5.48-5.76 (6)		21,300	6.22-6.35 (2)
2.	58,000		43,000	4.85-4.95 (2)		21,900	7.69 (1)
3.	55,000		35,000	5.1 -5.22 (2)		21,900	7.45-7.55 (2)
4.	54,000	a	38,500	5.25-5.52 (4)		22,700	7.4 -7.62 (3)
		b	37,500	5.05-5.18 (2)			
		c	35,000	5.28-5.55 (4)			
5.	35,000		24,500	5.95-6.15 (2)	a	21,000	7.92-8.0 (2)
					b	20,700	7.8 (1)

* Values within brackets denote the number of subunits

E. Molecular weight of the molecular forms

a) Polyacrylamide gradient gel electrophoresis

Legumin was run in the first dimension on 5-15%
polyacrylamide gradient gel for 70h at a constant
voltage of 100V. A band of relative mobility 0.425
(mol. wt. 420,000) and a group of bands of relative
mobilities 0.529-0.571 (mol. wt. 310-270,000) were
observed. An analysis of these bands in the second
dimension on SDS-polyacrylamide gels showed that the
bands of mol. wt. 420,000 corresponded to form A in
having subunit pairs 1 and 4 and the lower group of
bands contained the remaining subunit pairs 2, 3, 4
and 5 (Form C). Impurities of mol. wt. 26,500 and
26,000 were present in forms having mol. wt. in this
lower group of bands.

b) Ultrogel column chromatography

Analysis of the mol. wt. distribution of legumin
molecules carried out by gel filtration showed that most
of the legumin eluted with a mean mol. wt. of
400,000 ($\pm$40,000) i.e. similar to that previously
reported (Croy et al., 1979). However, subunits charac-
teristic of legumin molecular form A were distributed
about a significantly higher mol. wt. (450,000 $\pm$ 40,000).
No other separation of legumin molecular forms was
observed.

F. Fractionation of legumin molecular forms

The occurrence of legumin in three forms with
the subunit composition already observed was also
confirmed by ion-exchange chromatography of legumin
on DEAE-cellulose column. Legumin was eluted in a
broad peak and the fractions pooled in four parts were
analysed by PAGE and SDS-PAGE. Starting fractions of
the peak (elution molarity 0.16-0.18) contained
forms A and B (Fig. 3.7); the middle fractions (elution
molarity 0.19-0.23) contained A, B and C forms, form A
decreasing and C increasing in concentration; and the
later fractions (elution molarity 0.23-0.26) contained
no subunits characteristic of form A but contained
forms B and C. The impurity proteins in legumin
(mol. wt. 26,500 and 26,000) also eluted with the
later legumin fractions.

G. Legumin and storage effect

a) Molecular weights

In the recently harvested seeds of a number of
pea varieties, legumin basic subunits on SDS-
polyacrylamide gels were seen to have a pattern different
to that in the old seeds. An additional band of mol.
wt. 22,200 was observed in the new seeds. To study this
observation in detail, a line 'Meteor" was selected.
The normal 'Meteor' legumin had a pattern of acidic and
basic subunits similar to that described for 'Feltham
First' except that it had an extra acidic subunit of

Fig. 3.7 : Two dimensional gel analysis of Pisum

legumin fractions from ion-exchange

chromatography. 1D : polyacrylamide

gel electrophoresis of the initial

fractions (1), middle fractions

(2, 3) and last fractions (4) of

the legumin peak.   2D : SDS-

polyacrylamide gel electrophoresis

of A, B, C bands from 1D of 1, 2,

3 and 4.   A, B, C represent legumin

forms separated in 1D and various

components of these forms as defined

in the text.   Form C is absent in the

initial fractions (1) and form A

is absent in the last fractions (4).
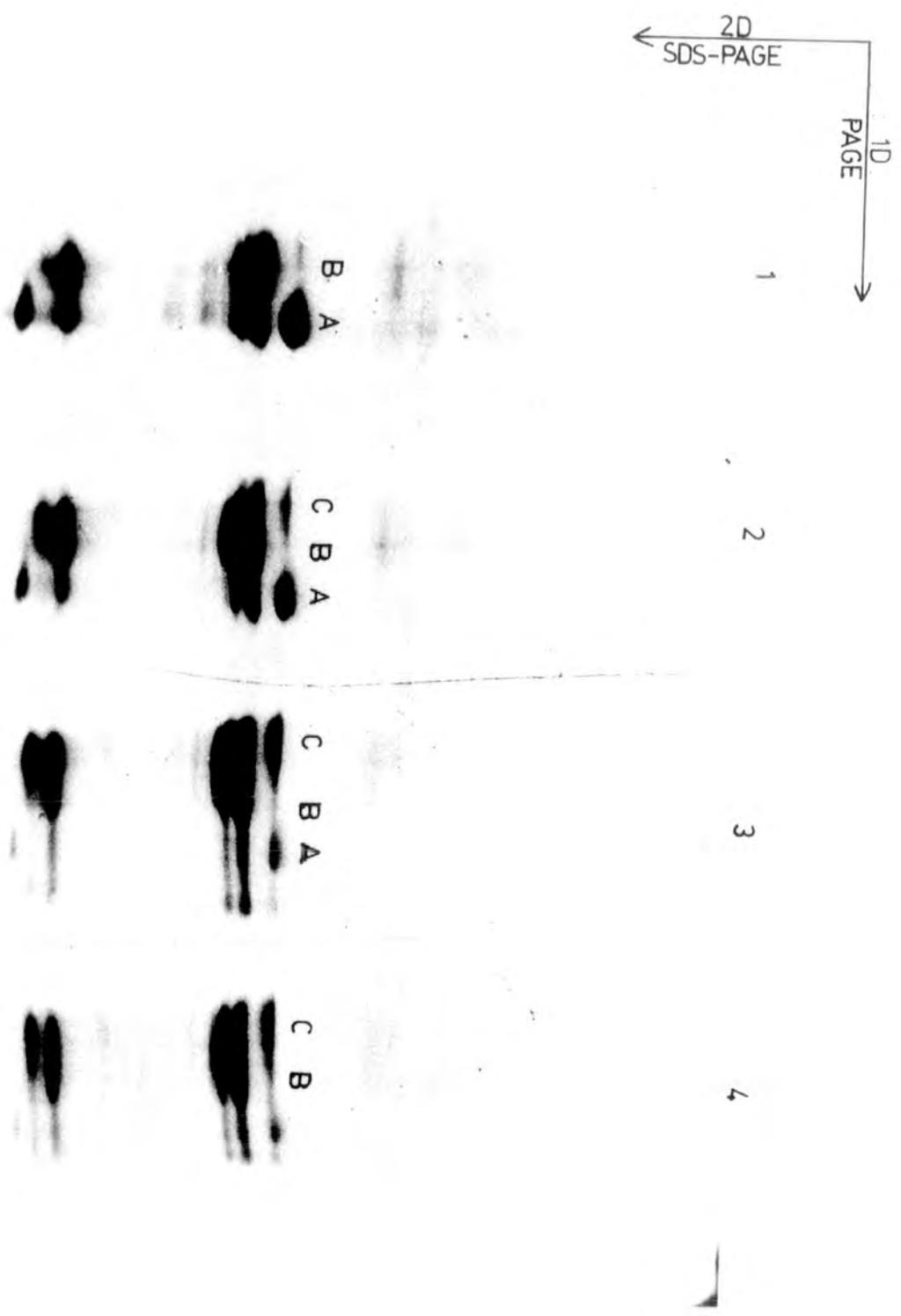
Fig.3.7

mol. wt. 40,000. A pod with dry mature seeds was harvested. Two seeds were frozen in liquid nitrogen immediately after harvest and one of these finely pulverized. Total protein extracts from these single seeds were prepared on the day of harvest, after a period of 2 weeks, 4 weeks and 6 weeks. An analysis of these extracts on SDS-polyacrylamide gel (Fig. 3.8) showed that the major basic subunit was represented by a dark band of mol. wt. 22,200 (white arrow) on the day of harvest (zero day of storage). A new band of mol. wt. 22,700 (black arrow) appeared in the 2 week old seeds. The concentration of this band increased and that of mol. wt. 22,200 decreased relatively with increase in storage period (4 and 6 week old seeds). This effect was more pronounced in eight months old seeds. In two year old seeds, basic subunit was represented by the band of mol. wt. 22,700 only and that of 22,200 had completely disappeared. Thus, mol. wt. 22,200 of the basic subunit appeared to be shifting to 22,700.

The basic subunit of mol. wt. 22,200 behaved in a similar way in the legumin purified by hydroxylapatite column chromatography of 6 week , 8 month and 2 year old seeds (Fig. 3.8). When run on non-dissociating polyacrylamide gel, legumin of the two year old seeds showed slight decrease in mobility as compared to 6 weeks old seeds. Also an alteration in the elution behaviour of legumin was observed on hydroxylapatite

Fig. 3.8 : SDS-polyacrylamide gel electrophoresis
          of total protein extract and purified
          legumin from seeds of pea variety
          'Meteor' stored for various periods.
          White arrows indicate legumin basic
          subunit band (mol. wt. 22,200)
          decreasing in amount and black
          arrows indicate the band (mol. wt.
          22,700) increasing in amount
          with storage.
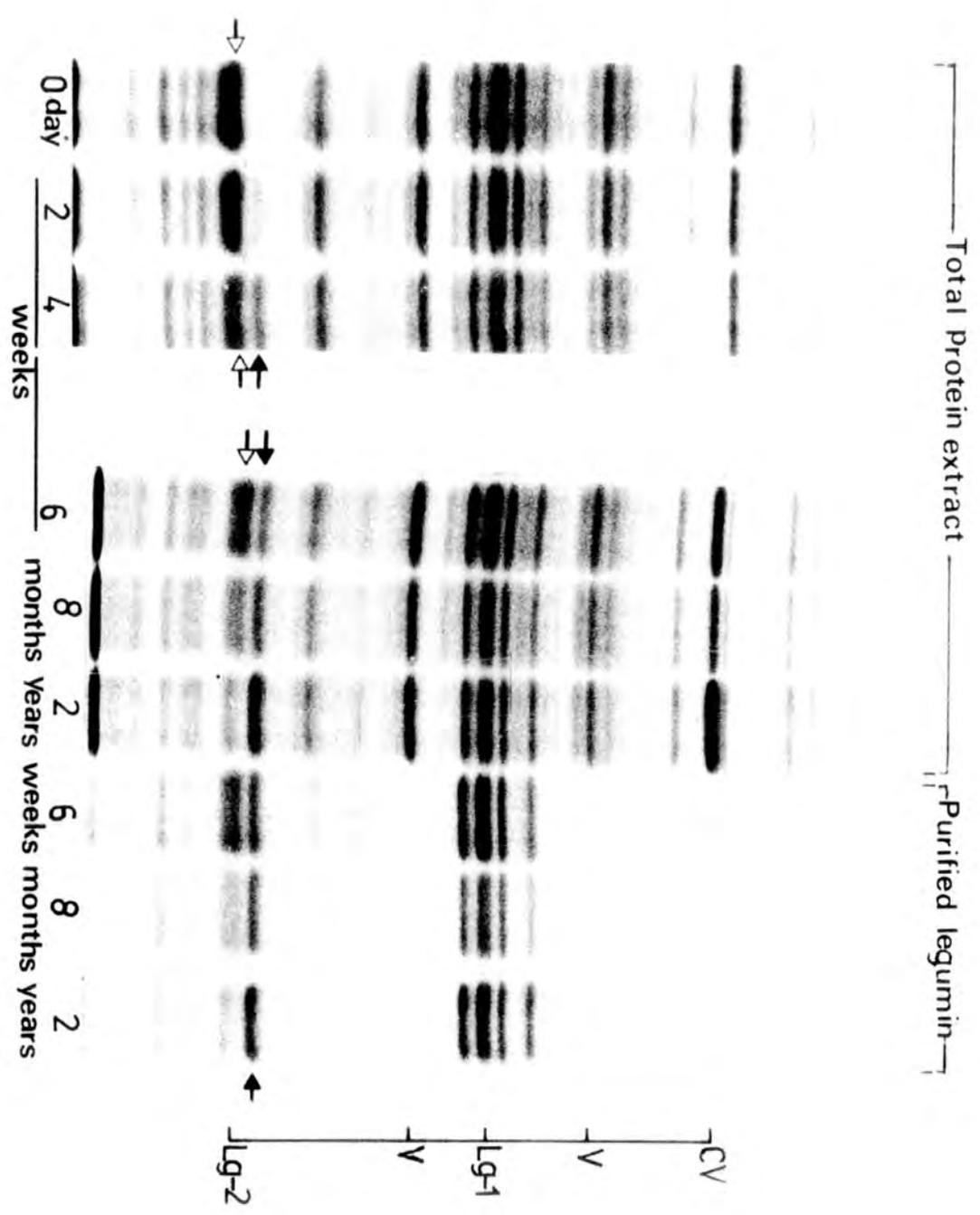
Fig. 3.8

column chromatography whereby legumin of the older seeds was eluted comparatively at higher molar concentration.

b) Isoelectric points

A two dimensional gel electrophoresis of the basic subunits separated on an SDS-gel in the first dimension and the isoelectric focusing in the second dimension showed that isoelectric points of all the basic subunit bands except those of mol. wt. 22,200 remained the same (Fig. 3.9). The 22,200 mol. wt. band consisted of three subunits and with the shift of this band to 22,700, isoelectric points of all the three subunits fell by 0.05 units each.

There is a possibility that this change in pIs is causing a shift in the basic subunit band on SDS-gel as these gels have been seen to give separation on the basis of charge as well.

Fig. 3.9 : Two dimensional electrophoretic analysis

and isoelectric focusing of legumin basic

subunits of 6 week (a), 8 month (b)

and 2 year (c) old seeds of pea variety

'Meteor'. 1D, IEF : isoelectric

focusing in the first dimension; a$'$

is the subunit pattern redrawn from a.

1D, SDS-PAGE (SDS-polyacrylamide gel

electrophoresis in the first dimension)

of a, b and c was followed by

2D, IEF (isoelectric focusing in the

second dimension). White arrows

indicate subunits decreasing in

amount with storage; black arrows

indicate subunits increasing in

amount with storage.
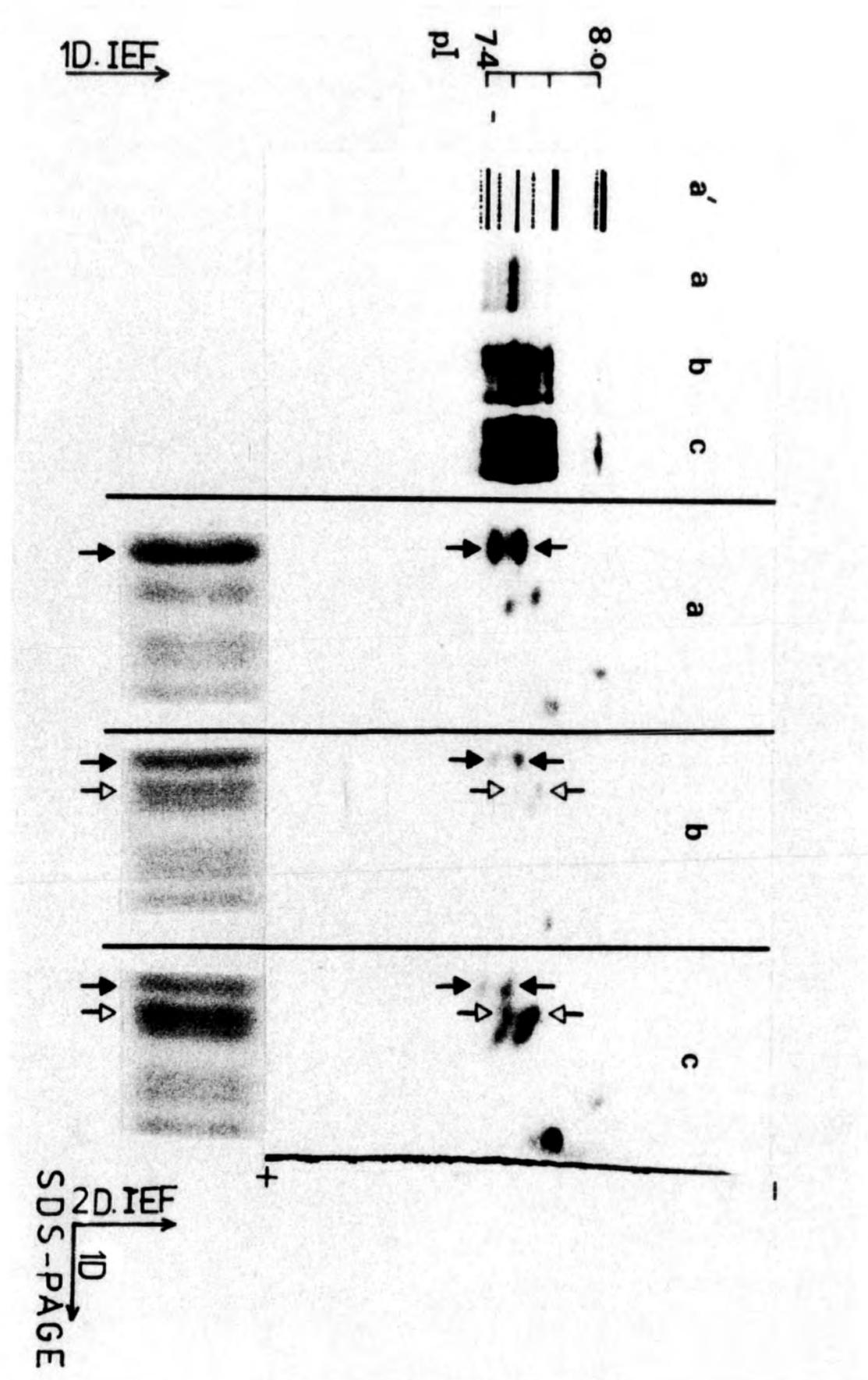
Fig. 39

Part IV.   GENETICS OF LEGUMIN SUBUNITS
IN PISUM SATIVUM L.

A number of Pisum lines were examined for legumin
subunit variation and four lines namely 110, 807, 1238
and 1263 were selected. Appropriate crosses were made
to study the inheritance of legumin subunits of various
types classified (see Materials and Methods section) as
conventional, big and small legumin. F_2 plants of the
cross 110 x 807 were analysed on 12.5% SDS-polyacrylamide
gels in order to study the inheritance of these subunits
and the linkage relationships of the genes controlling
them with respect to each other. The cross between
lines 1238 and 1263 was made to study the location of
the gene controlling acidic subunits of conventional
legumin (Lg-1C) in relation to convicilin gene and the
morphological markers selected for different linkage
groups.

A. Cross 110 x 807

a) Legumin banding patterns

Total protein extracts of single seeds of the
two Pisum lines 110 and 807 when examined on 12.5%
SDS-polyacrylamide gels under reducing conditions
differed in their legumin banding patterns. The bands
in the total protein extract were identified by running
the extracts under non-reducing conditions in the first
dimension followed by reducing conditions in the second
(Fig.4.1). Subunits obtained in the second dimension

Fig. 4.1 : Two dimensional SDS-polyacrylamide gel

electrophoresis of total protein

extracts of <u>Pisum</u> lines 110 and 807.

1D : electrophoresis under non-

reducing conditions in 10% gels in

the first dimension; band patterns

shown in tracks (i). 2D : electro-

phoresis under reducing conditions

in the second dimension; band patterns

shown in tracks (ii). A,B, C,D, R,S,

X,Y, Lg-B, Lg-C and Lg-S represent

various legumin species as defined

in the text. CV and V stand for

convicilin and vicilin respectively.
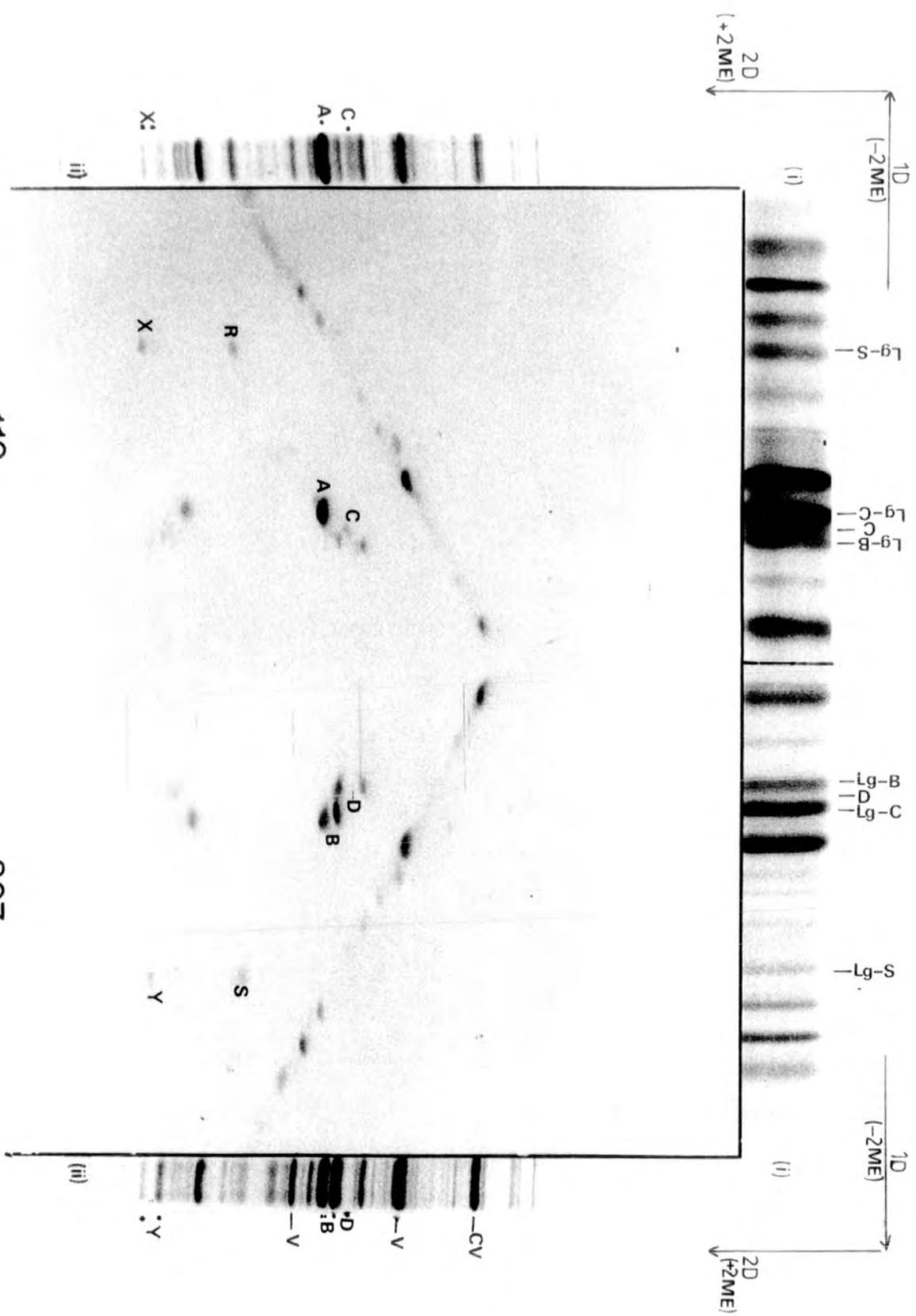
Fig. 4.1

and their mol. wt. were found as follows:

Subunit type	Pisum line 110		Pisum line 807	
	coded as	mol.wt.	coded as	mol. wt.
Lg-1B	1	43,000	1	43,000
Lg-1B	C	40,000	D	(absent)
Lg-1B	2	38,000	2	38,000
		37,500		
Lg-1C	A	35,500	B	37,500
				35,500
				35,000
Lg-1S	R	24,500	S	25,000
				24,500
Lg-2C	3	22,700	3	22,700
Lg-2B	4	22,300-21,300	4	22,300-21,900
Lg-2S	X	21,000	Y	21,300
		20,700		20,700

b) Inheritance of legumin subunits

Legumin subunits 1-1, C-D, 2-2, A-B, R-S, 3-3,
4-4 amd X-Y in the lines 110 and 807 respectively, were
assumed to be synthesized by alleles of the same gene
since these subunits had similar mol. wts. in the two
lines and were reduction products of similar subunit
pairs. As the two lines differed in the pattern of a
number of subunits only, inheritance of A and B, C
and D, R and S and X and Y only was studied.

(i) Products of subunit pair Lg-C

This pair represents the conventional legumin subunit pair (mol. wt. 53,000) which on reduction gives rise to two groups of subunits. The acidic subunits in line 110 consisted of only one dark band of mol. wt. 35,500 (Pattern A) but those of line 807 gave three bands of mol. wt. 37,500, 35,500 and 35,000 (Pattern B). This is illustrated in Fig. 4.1 from two-dimensional SDS-polyacrylamide gel electrophoresis. Electrofocusing studies followed by SDS-polyacrylamide gel electrophoresis showed that the band in line 110 actually consisted of two subunits and 3 bands of line 807 consisted of one subunit each. One hundred and seventy plants were analysed (Fig. 4.3) to calculate the chi-square values (Table 4.1). Chi-square value for Lg-1C subunit ($\chi^2_{(2)}$ = 1.2) was found to be statistically insignificant and was in good agreement with the hypothesis that these subunits are controlled by a single pair of co-dominant genes.

Table 4.1 : Analysis of F_2 segregation of various legumin subunit patterns and their chi-square values

Subunit	Frequency of the segregating patterns in F_2 generation			χ^2 (n=2)
Lg-1C	A	AB	B	
Observed	48	84	38	1.2
Expected	42.5	85	42.5	
Lg-2S	X	XY	Y	
Observed	43	92	35	1.9
Expected	42.5	85	42.5	
Lg-1B	C	CD	D	
Observed	15	20	17	2.92
Expected	13	26	13	
Lg-1S	R	RS	S	
Observed	10	26	16	1.38
Expected	13	26	13	

Fig. 4.2 : Cut-out sectors from SDS-polyacrylamide
gel electrophoresis in the second
dimension under reducing conditions
(first dimension being under non-
reducing conditions) showing different
combinations of independently inherited
subunit patterns R,S and X,Y
(Lg-1S and Lg-2S respectively) in
the F_2 plants (No. 186, 169, ----177)
of the cross between _Pisum_ lines
110 and 807.
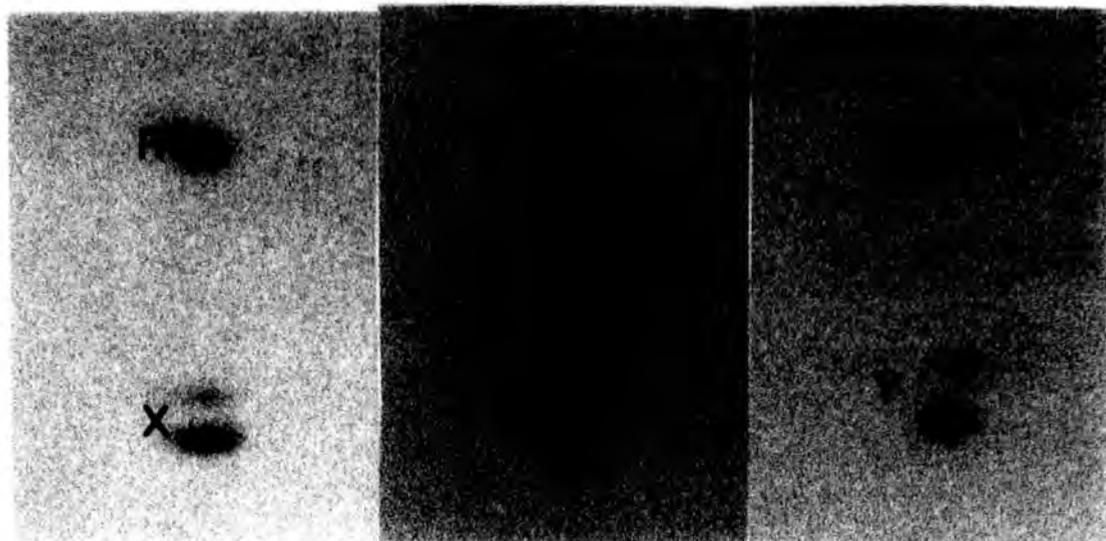
186 169 193
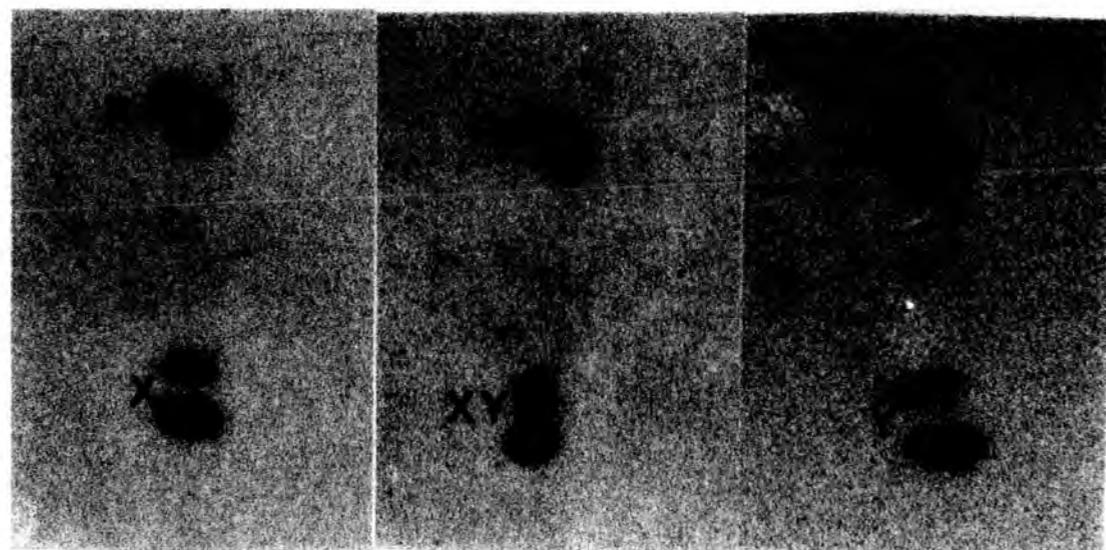
168 111 153
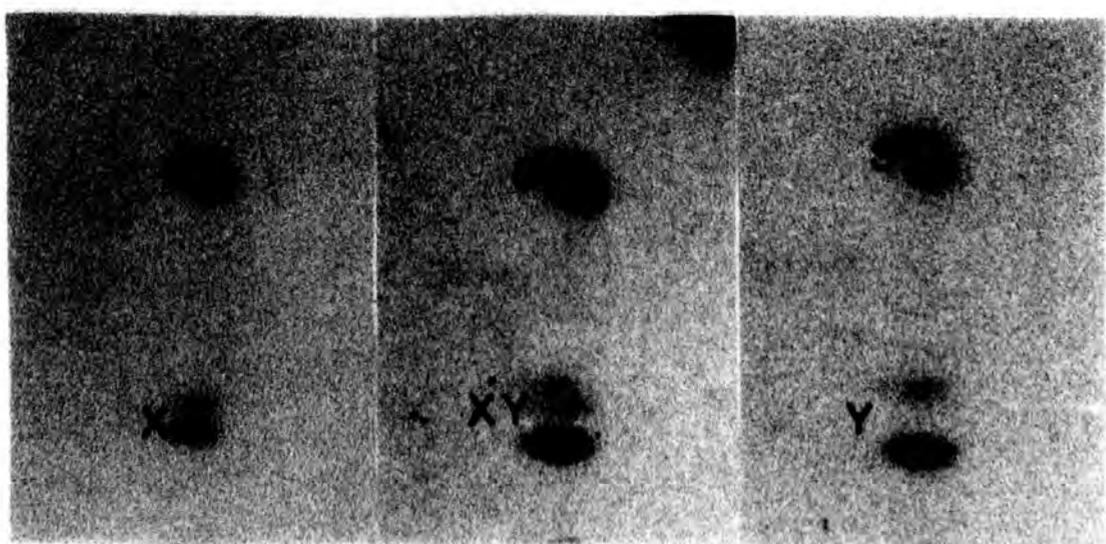
195 174 177

Fig.4.2

(ii) <u>Products of subunit pair Lg-B</u>

This subunit pair represents the legumin of mol. wt.
higher than that of conventional legumin - hence the
name Lg-B (Big legumin). A band of mol. wt. 55,000 was
present in the line 110 (Pattern C) and absent in the
line 807 (Pattern D) under non-reducing conditions. Two
dimensional electrophoresis showed that this subunit
pair on reduction gave rise to an Lg-1B subunit of mol. wt.
40,000 and Lg-2B subunit of mol. wt. 22,000 of which only
the acidic subunit was clearly analysed and studied. The
observed variation in the amount of protein present was
ascribed to a dosage effect,i.e. depended on the number
of genes. Thus, the pattern 'C', a dark spot, was
assumed to be due to a pair of homozygous alleles of the same
gene of the line 110, 'CD' - a fainter spot of half the
intensity of 'C' due to a single allele contributed by line 110 in
the heterozygote and 'D' - absence of this spot in the
absence of any effective allele was taken as contributed by
the line 807. Chi-square value, with the probability
of chance variation being 20-30%, was found to be
insignificant. Thus the subunit of big legumin was
also thought to be controlled by a single pair of
co-dominant alleles of the same gene.

(iii) <u>Products of subunit pair Lg-S</u>

This pair is called 'Lg-S' because it has a smaller
mol. wt. than that of conventional legumin. The acidic
and basic subunits differed in their pattern in the two

lines. Fifty two plants of the F_2 generation were analysed by two dimensional SDS-polyacrylamide gel electrophoresis for Lg-1S subunits represented by R and S in lines 110 and 807 respectively (Fig. 4.2) and 170 plants for Lg-2S represented by X and Y (Fig. 4.3). Chi-square values calculated (Table 4.1) were within the statistical bounds for monogenic segregation and co-dominance of the alleles responsible for these subunits.

c) Linkage relationships of the subunits

     Chi-square values were calculated to determine the status of the genes for various legumin subunits as to whether linkage occurred between the genes for various legumin subunits or if they were located on different chromosomes (Table 4.2). The assumption was made that they followed the mendelian law of independent assortment with respect to each other in the F_2 generation. The subunit combinations given below were studied for these linkage relationships:

       (i)   Lg-1C (AB) . Lg-2S (XY)

       (ii)   Lg-1C (AB) . Lg-1S (RS)

       (iii)   Lg-1C (AB) . Lg-1B (CD)

       (iv)   Lg-1S (RS) . Lg-2S (XY)

       (v)   Lg-1B (CD) . Lg-1S (RS)

       (vi)   Lg-1B (CD) . Lg-2S (XY)

Some of these combinations and their behaviour in F_2 generation are illustrated in Fig. 4.3 and 4.2.

Fig. 4.3 : SDS-polyacrylamide gel electrophoresis,
under reducing conditions, of total protein
extracts showing different combinations
of A,B and X,Y in F_2 plants of the
cross between <u>Pisum</u> lines 110 x 807.
A, B, C, D, X and Y represent the
legumin species as defined in the
text. CV and V stand for convicilin
and vicilin respectively.

Fig.4.3

F₂ plant number

AX	AX	AY	AXY	ABX	ABXY	ABY	BXY	BX	BY	BY
110	200	155	152	195	111	192	194	197	157	807

Table 4.2 : Subunit patterns of the dihybrid cross, their frequency and chi-square values in F₂ generation for various legumin subunit combinations

Subunit Combination	Pattern of subunit Combination	Expected ratio and frequency of subunit patterns in F₂ generation									χ² (n = 8)
		1	1	2	2	4	2	2	1	1	
Lg-1C.Lg-2S	$\frac{A\ X}{B\ Y}$	AX	AY	AXY	ABX	ABXY	ABY	BXY	BX	BY	4.51
	Observed	11	12	25	23	47	14	20	9	9	
Lg-1C.Lg-1S	$\frac{A\ R}{B\ S}$	AR	AS	ARS	ABR	ABRS	ABS	BRS	BR	BS	8.30
	Observed	4	4	6	3	18	9	2	3	3	
Lg-1C.Lg-1B	$\frac{A\ C}{B\ D}$	CA	CB	CAB	CDA	CDAB	CDB	DAB	DA	DB	7.13
	Observed	4	2	9	4	13	3	8	6	3	
Lg-1S.Lg-2S	$\frac{R\ X}{S\ Y}$	RX	RY	RXY	RSX	RSXY	RSY	SXY	SX	SY	12.71
	Observed	5	4	1	3	16	7	6	3	7	
Lg-1B.Lg-1S	$\frac{C\ R}{D\ S}$	CR	CS	CRS	CDR	CDRS	CDS	DRS	DR	DS	11.46
	Observed	6	5	4	2	13	5	9	2	6	
Lg-1B.Lg-2S	$\frac{C\ X}{D\ Y}$	CX	CY	CXY	CDX	CDXY	CDY	DXY	DX	DY	53.72
	Observed	8	2	5	3	15	2	3	0	14	

For all the aforementioned subunit combinations except
the last one, chi-square values were insignificant
and low enough not to indicate any linkage between various
subunits. However, acidic subunits of big legumin and
basic subunits of small legumin for which chi-square
values were highly significant, seemed to show a
considerable degree of linkage.

B. Cross 1238 x 1263

Reciprocal crosses were made between lines 1238
and 1263 in order to study the relative assignment of
the legumin gene to a particular linkage group. In
contrast to line 1263, line 1238 was double recessive for
the characters dwarfness, procumbent branches,
absence of maculum ring, absence of tendrils, pink
flowers, keel like wings, yellow pods, sticky seeds
due to tragacanth excretion on the outside, absence
of air pockets below the leaf epidermis, green
cotyledons and wrinkled seeds, thus of the genotype
le, pro, d, tl, b, k, gp, s, fl, i and r respectively.
On the other hand, line 1263 was characterised by the
absence of anthocyanin pigment, thus of genotype
recessive a. The two lines differed in the pattern of
Lg-1C subunits and convicilin subunits. Lg-1C subunits
were represented by three bands of mol. wt. 38,000,
37,000 and 35,000 in the line 1238 (pattern E) and by
two bands of mol. wt. 35,500 and 35,000 in the line
1263 (pattern F). Convicilin was represented by bands
of mol. wt. 70,000 and 68,000 (pattern U) in the

Fig. 4.4 : Two dimensional SDS-polyacrylamide gel

electrophoresis of total protein

extracts of pea lines 1263 and 1238.

1D : electrophoresis under non-

reducing conditions in 10% gel in the

first dimension; band patterns shown

in tracks (i). 2D : electrophoresis

under reducing conditions in the

second dimension; band patterns shown

in tracks (ii). E, F, Lg-1, Lg-2,

Lg-1S, Lg-B, Lg-C and Lg-S are the

legumin species and, U and V the

convicilin patterns as defined in

the text. CV and V stand for

convicilin and vicilin respectively.
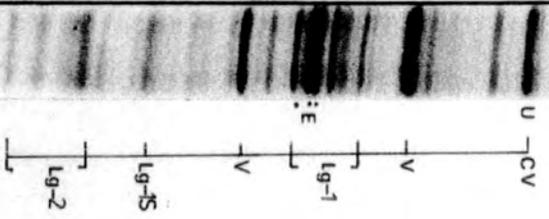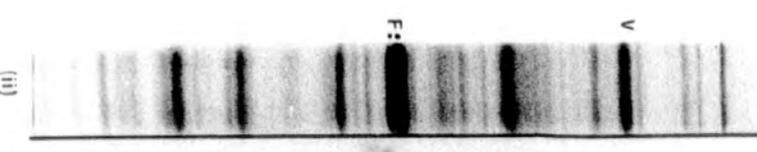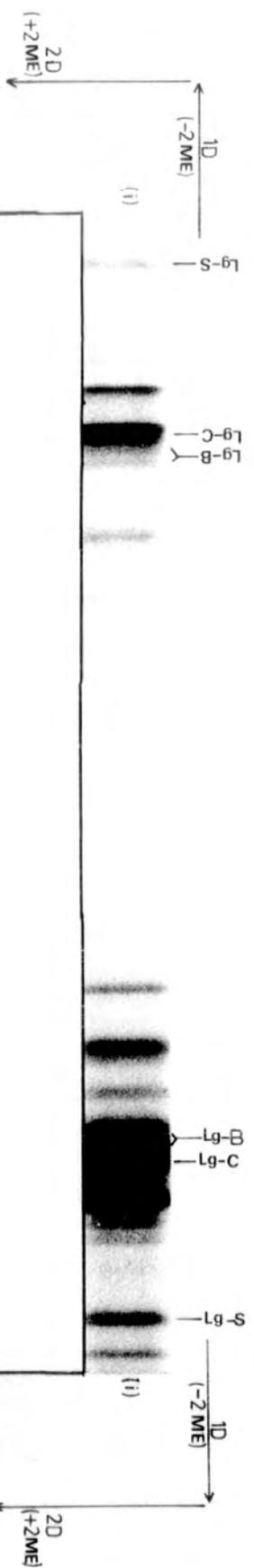
Fig.4.4

line 1238 and bands of mol. wt. 70,000 and 67,000

(pattern V) in the line 1263 (Fig. 4.4).

a) F_1 hybrids

F_1 plants of reciprocal crosses between the two

parental lines showed the expected dominant expression

for the genes Le, Pro, D, Tl, B, K, Gp, S, I, R and

A. The pattern of legumin acidic subunits and

convicilin in these F_1 plants was that of a hybrid

combination of the two parental lines (Fig. 4.5).

b) Segregation of legumin and convicilin subunit patterns

The observed frequency of F_2 plants with the

parental and hybrid patterns for acidic subunits of

legumin and convicilin subunits is given in Table 4.3.

For both of these polypeptides, chi-square values provided

support for the hypothesis that genes controlling these

subunits occur as a single pair of co-dominant alleles.

Table 4.3 : Analysis of F_2 segregation and chi-square
           values for legumin subunits Lg-1C and convicilin

Subunit	F_2 segregants and their frequency			x^2 (n = 2)
Lg-1C	E	EF	F	
Observed	35	67	26	1.55
Expected	32	64	32	
Convicilin	U	UV	V	
Observed	34	58	36	1.19
Expected	32	64	32	

Fig. 4.5 : SDS-polyacrylamide gel electrophoresis
(reducing conditions) of the total protein
extracts of $\underline{Pisum}$ parental lines 1238
and 1263, and the F_{1s} of the
reciprocal cross between them.
Lg-1, Lg-2, CV and V represent acidic
subunits of legumin, basic subunits
of legumin, convicilin and vicilin
respectively.
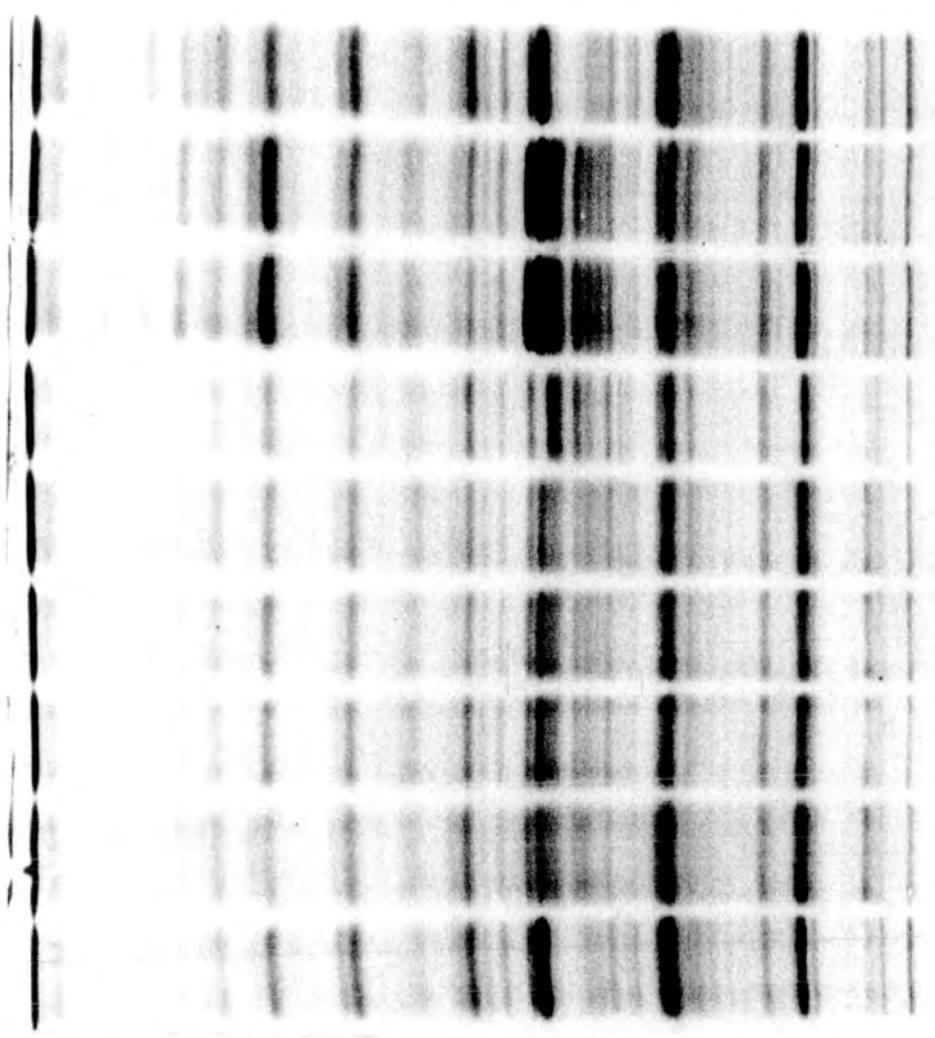
Fig. 4.5

c) <u>Testcross for linkage of legumin subunit genes</u>

To get an idea of linkage between the genes for the acidic subunits of legumin and the genes for the selected morphological markers, F_1 plants were backcrossed with the double recessive parent line 1238. The genotype of 19 plants as determined by this testcross made with respect to various characters is given in Table 4.4. As can be seen from the results of the table, genes for the acidic subunits of conventional legumin appeared to be linked with <u>tl-r</u> segment of the chromosome No.7. Plant No.2 and 8 show recombination between legumin and the tendril gene and plant No. 2, 8, 15 and 19 represent recombinants for legumin and seed surface gene <u>r</u>. A higher number of recombinants between <u>Lg-1C</u> and <u>r</u> genes points towards the location of <u>Lg-1C</u> gene being on the side of <u>tl</u> locus away from <u>r</u> locus.

Table 4.4 : Lg-1C pattern and genotype (tl and r markers
of linkage group 7) of testcross progeny
from a cross between F$_1$ of the cross 1238 x 1263
and double recessive parent line 1238

Plant No.	Lg-1C pattern	Genotype of the testcross plants	
	E/F	Tl/tl	R/r
1	F	Tl	R
2	F	tl	r
3	E	tl	r
4	F	Tl	R
5	F	Tl	R
6	F	Tl	R
7	F	Tl	R
8	E	Tl	R
9	E	tl	r
10	F	Tl	R
11	E	tl	r
12	E	tl	r
13	F	Tl	R
14	F	Tl	R
15	F	Tl	r
16	F	Tl	R
17	F	Tl	R
18	E	tl	r
19	F	Tl	r

d) <u>Linkage relationship of legumin subunit gene</u>

Patterns for acidic subunits of conventional legumin
in/F$_2$ generation assorted independently of the pattern for
the storage protein convicilin. The segregation of
patterns for these two storage proteins in/F$_2$ generation is
shown in fig. 4.6. Chi-square value (5.06 at 8 degree of
freedom) calculated from the observed number of different
F$_2$ classes showed that the genes for these polypeptides
were present on different chromosomes, or if present on
the same chromosome, were distantly located from each
other. However, when these legumin subunit patterns were
studied in relation to the F$_2$ segregation of seed surface
type (round 'RR' 'Rr' or wrinkled 'rr') and tendril
character (present 'TlTl', 'Tltl' or absent 'tl tl'), the
gene for acidic subunits of conventional legumin was
found to be linked with <u>tl-r</u> segment. The crossover
values calculated by the method of Immer (1930) were
18.3$\pm$2.6% between the legumin gene and the <u>r</u> locus and
15.1$\pm$2.3% between the legumin gene and <u>tl</u> locus (Table 4.5).
This confirms the location of legumin gene at 15 map units
from the <u>tl</u> locus on the side opposite to <u>r</u> locus since
<u>r</u> and <u>tl</u> loci are separated by 5 recombination units
according to Blixt (1972).

Fig. 4.6 : SDS-polyacrylamide gel electrophoresis,

under reducing conditions, of total

protein extracts showing different

combinations of U,V and E,F in F_2

plants of the cross between Pisum

lines 1238 and 1263. E, F and U,V

represent Lg-1C and convicilin

subunit patterns respectively as

defined in the text; Lg-1, Lg-1S

and Lg-2 represent different

legumin species as defined in the

text.
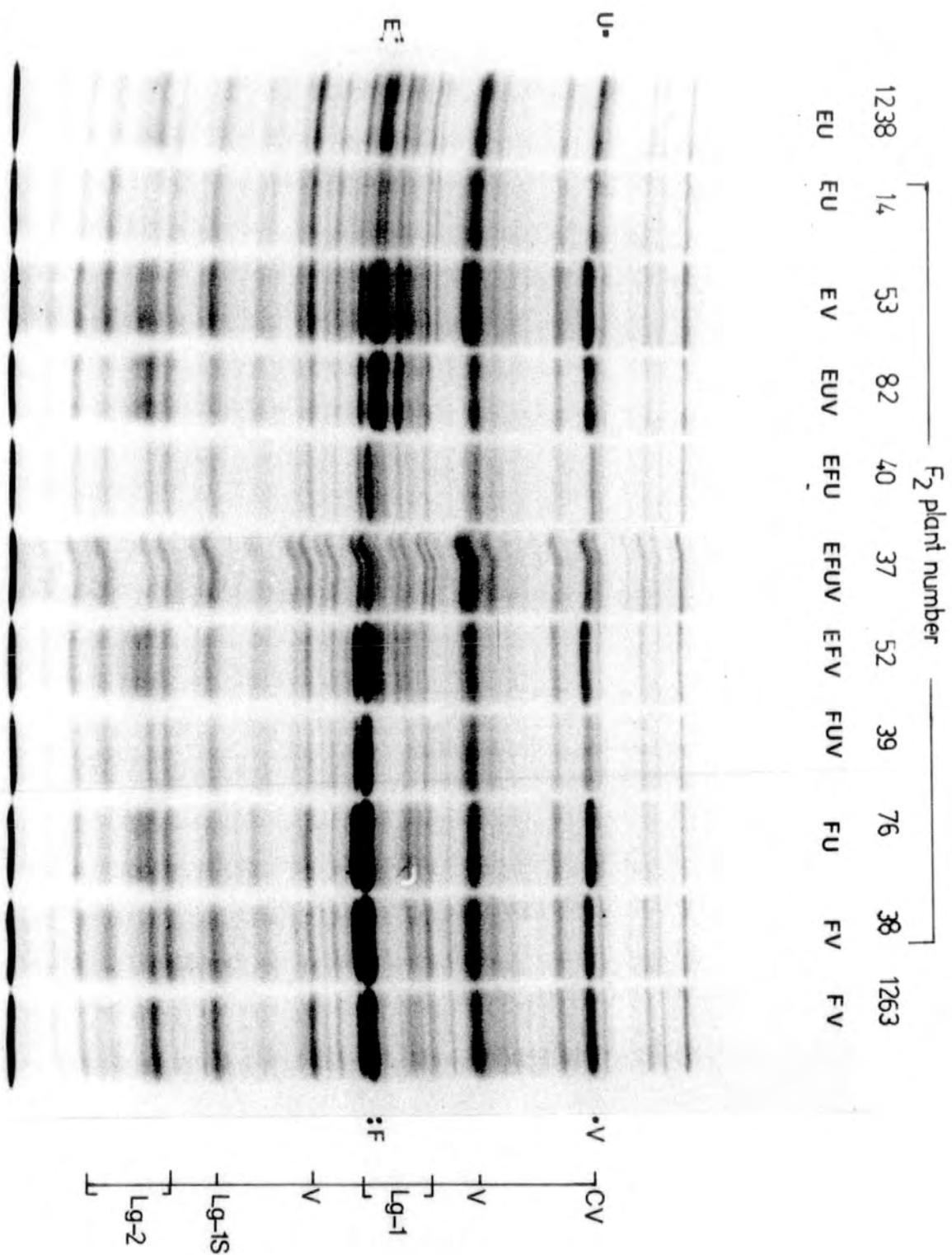
Fig. 4.6

Table 4.5 : Segregation of acidic subunits of legumin and convicilin subunits in F_2 generation with respect to the selected markers and their crossover values.

(Lg-lc^E and Lg-lc^F stand for the patterns E and F respectively of legumin subunits; 'R' for round and 'r' for wrinkled seeds, 'Tl' for presence and 'tl' for absence of tendrils. Cvc^U and Cvc^V stand for U and V, patterns of convicilin respectively, 'K' for normal wings and 'k' for keel like wings).

Character pair	F_2 classes and observed frequency				$p = \dfrac{bc}{ad}$	Crossover value $\pm$ probable error
	a	b	c	d		
Lg-lC. seed surface	Lg-lc^E.r 27	Lg-lc^E.R 7	Lg-lc^F.r 18	Lg-lc^F.R 76	0.0614	18.3$\pm$2.6%
Lg-lC. Tendrils	Lg-lc^E.tl	Lg-lc^E.Tl	Lg-lc^F.tl	Lg-lc^F.Tl	0.0397	15.1$\pm$2.3%
Cvc. Wingtype	Cvc^U.k 30	Cvc^U.K 2	Cvc^V.k 2	Cvc^V.K 90	0.0015	3.2$\pm$1.1%

e) Linkage relationships of convicilin gene

As stated earlier, the convicilin subunit patterns 'U' and 'V' were inherited independently of the Lg-lC subunit patterns, genes for which are located on chromosome 7. In/the F_2 generation, the Cvc patterns and the wing type in the flowers mostly showed the parental combinations and thus the gene Cvc for convicilin subunits showed a strong linkage with the locus k on chromosome 2. The crossover value between Cvc and k loci was found to be 3.2 $\pm$ 1.1% (Table 4.5). The data for segregation of k and Cvc loci with respect to s was not sufficient enough to give the exact location of Cvc locus in the linkage group. However, the detection of linkage between Cvc and s loci supports the location of Cvc in this segment of linkage group 2.

Section IV   DISCUSSION

Previous work on the structure of legumin has led to the general acceptance of the hexameric model of Wright and Boulter (1974) in which each legumin molecule is postulated to consist of six pairs of disulphide bonded acidic (approx. 40,000 mol. wt.) and basic (approx. 20,000 mol. wt.) subunits. However, there were already observations which indicated that the situation was more complicated than this. Bailey and Boulter (1970) had clearly demonstrated the presence of a 56,000 mol. wt. subunit in addition to the 40,000 and 20,000 mol. wt. subunits of the hexameric model in the legumin of Vicia faba and that the subunits (mol. wt. 56,000, 42,000 and 23,000) studied by them were present in the ratio of 1:3:6 respectively. The larger subunits were also subsequently noted by Utsumi and Mori (1980). This additional complexity of the legumin molecules has been thoroughly supported by the results presented in this thesis showing the presence of subunit pairs 'A-J' in Vicia legumin (Fig. 2.2) and '1-5' in Pisum legumin (Fig. 3.3). However, all these subunit pairs and the resultant legumin molecules can still be fitted easily into the operational definition of legumin given above. Subunit pairs H/I in Vicia and subunit pair 4 in Pisum constitute about 80% of the total legumin subunit pairs, i.e. represent the predominant subunit species. These are the subunit pairs previously considered in the hexameric

legumin model (Wright and Boulter, 1974; Croy et al.,
1979). We have, therefore, used the following nomen-
clature : subunit pairs H/I and No. 4 in Vicia and
Pisum respectively are called Lg-C (conventional);
subunit pairs 'A-G' in Vicia legumin and '1-3' in
Pisum legumin which have a mol. wt. greater than H/I
and 4 are called big legumin subunits i.e. Lg-B.
Subunit pairs of mol. wt. 37-40,000 (J) in Vicia and
mol. wt. 35,000 (5) in Pisum which have a lower mol.
are called Lg-S (small) legumin subunits. Pisum is
different to Vicia in that it does not have the subunit
pairs (Lg-B) with mol. wt. greater than that of the
convicilin subunits and with acidic subunits (Lg-1B) of
mol. wt. greater than 50,000. Polypeptides of mol. wt.
26,500 and 26,000 which always co-purify with Pisum
legumin but which are not disulphide bonded i.e. are
impurities, seem to be entirely absent in the case of
Vicia legumin.

Two dimensional electrophoresis employing
electrophoretic techniques in different combinations
is a powerful analytical method which has been used to
study the characteristics of many proteins by various
workers (Raymond, 1964; Margolis and Kenrick, 1969;
Kaltschmidt and Wittman, 1970; O'Farrell, 1975). In
this study, two dimensional SDS-polyacrylamide gel
electrophoresis with non-reducing conditions in the
first dimension and reducing conditions in the second
dimension has been shown to be of great value in studies

of the storage protein legumin. This is because legumin
is a protein containing disulphide-bonded subunits
which run as subunit pairs in the first dimension but
are separated by 2-mercaptoethanol used in the second
dimension, causing the two types of subunits to leave
the diagonal path whereas other proteins remain along
the diagonal. In this way, bands representing legumin
subunits in a complex mixture of seed proteins can
be recognised very easily; this is not the case with
other 2-D systems. However, this system, like others,
does not give a complete analysis of the heterogeneity
present and 2-D systems should be used complementary
to one another.

The mol. wts. of unreduced legumin subunit pairs
were lower than the sum of the mol. wts. of the
component reduced subunits. This discrepancy can be
accounted for by the presence of inter- and intrachain
disulphide bonds in the subunit pairs which can prevent
complete unwinding of the unreduced polypeptides
resulting in an apparent mol. wt. lower than the true
one. This has been very clearly seen in bovine serum
albumin which has an apparent mol. wt. of approx.
55,000 when unreduced as compared to 67,000 when reduced
(data not presented). Consequently the mol. wt. values
for reduced subunits are the more correct ones.

Molecular weights of the large subunits of all
the subunit pairs of _Vicia_ _faba_ legumin decreased
linearly according to mol. wt. of subunit pair, except

that of subunit pair 'I' which had a mol. wt. higher
than that of 'H' (Fig. 2.2). Thus, sum of the mol. wt.
of components of 'I' was higher whereas that of the
unreduced 'I' was lower when compared to 'H'. This was
also observed by Utsumi and Mori (1980) for the subunit
pair of composition 36,000 + 23,000 which had a lower
mol. wt. as compared to the one with subunit composition
36,000 + 20,500. Utsumi and Mori (1980) also reported
the presence of a subunit pair ('IV') equivalent to
subunit pair 'I' here,having one acidic subunit but
two basic subunits, thus confirming the present results.
These anomalies in the mol. wt. of subunit pairs relative
to their components can be attributed to the presence of
inter- and intrachain disulphide bonds.

Further studies of Vicia legumin showed greater
heterogeneity in the number of subunits when additional
separatory techniques were used. The 10 subunit pairs
seen under non-reducing conditions were shown to consist
of at least 21 components in a two-dimensional analysis
using both reducing and non-reducing conditions. Utsumi
and Mori (1980) detected 3 acidic and 3 basic subunits
and on the basis of their results with gradient pore
gel electrophoresis they proposed the existence of 7
molecular forms of the complete legumin molecule with
four different mol. wts. Studies carried out in this
investigation using ultrogel column chromatography and
gel electrophoresis also point to the existence of
several legumin molecular forms. However, as the 13

subunits (Fig. 2.1d) observed in first dimension under reducing conditions exist in a number of charge variants (Fig. 2.5a and 2.5b), the number of potential legumin molecules seems to be very large. The results of gel filtration and gel electrophoresis of Vicia legumin where subunit pair 'F' runs independently of the other subunit pairs can be explained by assuming that in this case subunit pairs 'F' assemble individually into hexameric molecules. This is also supported by the fact that the subunit pair 'F' was the only subunit pair not to be immunoprecipitated with the other subunit pairs by anti-legumin antibodies of Pisum sativum. It was not possible, however, to separate under non-dissociating conditions other legumin molecules consisting of only one type of subunit pair. Apart from the legumin with 'F' subunits, several other legumins were partly separated but most subunit pairs were represented in most molecular forms suggesting the possibility of random assembly of subunit pairs to form hexameric molecules. As the subunit pair H/I constitutes about 80% of the total legumin, it would be feasible to predict that the most common legumin species was 6 x (H/I) (although never isolated) followed by various 5 x (H/I) + 1 x (X) species of slightly different mol. wt. and charge. The partial separation of A, B, D from H/I on gel filtration and gradient pore gel electrophoresis suggests that certain combinations of subunit pairs in hexameric molecule are more likely

than others. These results thus do not entirely support or disprove the conclusions of Utsumi and Mori (1980).

Legumin of _Pisum sativum_ also shows considerable heterogeneity in the range of mol. wt. and isoelectric points of the subunits and has been partially separated into different molecular forms. As many as 13 subunits have been observed by two-dimensional analysis in contrast to 21 subunits seen in _Vicia_. The subunits of all the subunit pairs include charge heterogeneity, i.e. each subunit contains a number of charge isomers as specified in table 3.2. Subunit pair types are assigned firstly on the basis of their mol. wt. and the mol. wt. values of their component acidic and basic subunits and secondly, on the basis of the common subunits and their distribution in molecular species. For example, in subunit pair 4, the three acidic subunits a, b and c all have a common basic subunit (with three charge isomers) and occur in approximately equal proportions to each other in all molecular forms whereas subunit pairs 1 and 2 have no subunit in common, pair 1 occurs predominantly in legumin molecular form A and pair 2 predominantly in form C. Form B legumin containing subunit pair 4 seems to be the common form, its charge decreasing or increasing by its association with pair 1 or 2 respectively. Form A, of higher mol. wt. than the other legumin molecular types, contains subunit pairs 1 and 4 in approximately equal proportions, i.e. each hexameric molecule contains 3 subunit pairs each

of type 1 and 4 i.e. [3 x (1) + 3 x (4)] . From the gel electrophoresis and gel filtration studies it appears that form 'C' actually consists of different forms which have varying proportions of subunit pairs 2, 3 and 5; possibly these forms are [2 x (2) + 4 x (4)] , [2 x (3) + 4 x (4)] and [2 x (5) + 4 x (4)] . However, since a complete separation of the different molecular forms cannot be achieved, these conclusions may be considered as tentative.

Thus from these studies of <u>Vicia</u> and <u>Pisum</u> legumin a modified model for the legumin molecule which contains a number of subunit pairs other than that of conventional legumin (Wright and Boulter, 1974) may be put forward. In this case, each hexameric molecule is formed by the association of individual subunit pairs of one or more types, association being regulated by the relative abundance of subunit pairs and by structural constraints to give the observed selection of molecular species. Legumin is thus not a single protein species of defined amino acid sequence but is a generic name for a series of closely related proteins. This situation is analogous to that found in other proteins e.g. the isoenzymes of lactate dehydrogenase or immunoglobulins. We have adopted the operational definition of legumin outlined above.

Certain anomalies are raised by the results presented herein. For example, it has been demonstrated that separation on gradient pore gel is not solely

according to mol. wt. but a charge factor is also operative. Molecular weight values measured by this pore limit electrophoresis in the present work are lower for _Pisum_ legumin and higher for _Vicia_ legumin. But gel filtration (Croy et al., 1979) and ultra-centrifugation studies (Derbyshire et al., 1976) have shown that _Pisum_ legumin has a higher mol. wt. than the _Vicia_ legumin. The higher charge at pH 8.0 of _Pisum_ legumin in comparison to _Vicia_ legumin (Croy et al., 1979) may explain the higher mobility of _Pisum_ legumin in gradient pore gels. It has also been pointed out by Rodbard et al. (1971) that the concept of a 'pore limit' in gradient gels is theoretically invalid.

The terms acidic and basic for legumin large and small subunits respectively must be considered as relative since both the types of subunits of subunit pair 1 in _Pisum_ legumin have their pIs below 7, although the small (basic) subunits have higher pIs than the large (acidic) subunits. All other legumin basic subunits have pIs above 7. Small subunits of legumin (mol. wt. 21,300) with pIs below 7, i.e. in the acidic range, were reported by Casey (1979b) but were not assigned as components of any particular subunit pair.

Acidic subunits of subunit pairs 1, 2, 3 and 4 of _Pisum_ legumin can be compared with the 'LA-LC' groups of legumin as given by Thomson and Schroeder

(1978) and with minor and major α subunits given
by Casey (1979b). On the other hand basic subunits
of all the subunits represent 'LD' group of Thomson
and Schroeder (1978) and β subunits of Casey (1979b).
'LE' group of Thomson and Schroeder (1978) and γ
subunits of Casey (1979b) are represented by subunits
of mol. wt. 26,500, 26,000 and 24,500. Of these,
the subunits of mol. wt. 24,500 is actually the only
legumin acidic subunit i.e. of subunit pair 5 in
Pisum legumin. The polypeptides of mol. wt. 26,500
and 26,000 (pI 4.5 - 4.7) designated as 'D' bands on the
non-dissociating gel (Fig. 3.1e) are clearly not
legumin species since these are not disulphide bonded
and can be completely removed from legumin subunit
pairs under non-dissociating conditions. These
polypeptides are, therefore, associated with legumin
molecules by weak non-covalent bonds which are possibly
dependent on ionic strength. No further character-
ization of this protein was attempted and its functional
role is therefore unknown.

The high degree of heterogeneity observed in
the legumin of Vicia faba and Pisum sativum may be
expressed in terms of a hierarchial model as follows,
where the levels are given in ascending order.

(i)   Single substitutions in amino acid sequences
of polypeptides; these may be responsible for the
charge heterogeneity of individual subunits, although
many single substitutions may be unrecognised by the

procedure (isoelectric focusing) used.

(ii)   More extensive differences in sequence among polypeptides including differences in the total number of amino acids; these may be considered responsible for the size heterogeneity among subunits.

(iii)   The formation of legumin molecules containing subunit pairs in different combinations; this may be considered responsible for the charge and size heterogeneity of legumin under non-dissociating conditions.

It is possible that heterogeneity of types (i) and (ii) is a direct expression of genetic heterogeneity evident in the translation products synthesized in vitro on the polysomes purified from developing seeds of Vicia faba and Pisum sativum. Translation products were specifically immunoprecipitated against anti-legumin antibodies and were shown to be heterogeneous in mol. wt. when analysed by SDS-polyacrylamide gel electrophoresis and fluorography (Gatehouse, unpublished). The evidence that heterogeneity has a genetic basis also comes from the studies made by Thomson et al. (1978) who found the F_1 progeny to show a pattern additive of the two parental patterns. Muller and Gottschalk (1973) found some induced mutant lines with patterns of globulins different from those of the parent lines; this also suggests some mechanism at the primary level responsible for the heterogeneity in these seed proteins.

Heterogeneity may also arise as a result of some post-translational modification of the legumin

precursor which is synthesized as a 60,000 mol. wt.
polypeptide and is nicked in some way to give rise
to two types of disulphide bonded subunits after its
synthesis (Croy et al., (1980). Higgins and Spencer
(1980) attributed the increase in mol. wt. of legumin
basic subunit from 19,000 to 20,000 to the post-
translational modification of the long lived precursor
for legumin. A similar observation made by us appears
to be more of a storage effect rather than post-
translational modification. In the pea line 'Meteor',
during storage of the dry seeds, a basic subunit band
seemed to show an apparent increase in mol. wt. from
22,200 to 22,700. This apparent shift in mol. wt. is
accompanied by a decrease in the pIs of the three
subunits constituting the 22,200 mol. wt. band. It
seems likely that this change, as it occurs on storage
in the freeze dried and pulverized seeds, is due to a
non-enzymic chemical alteration of legumin rather than
a directed enzyme catalysed process. It may be
suggested that the most likely alteration in the legumin
basic subunits to account for these observations is
deamidation of asparagine or glutamine residues - this
will lower the pI of the subunit and decrease its
positive charge. This decrease in positive charge
might cause an upward shift of the subunit on SDS-gel
as SDS-gels have been shown to give separation on the
basis of mol. wt. as well as charge. Thus heterogeneity
may sometimes be artificial and generated during
storage.

Heterogeneity in mol. wt. and isoelectric points is greater for acidic subunits than that for basic subunits. This effect is more evident in *Vicia* legumin. Also, the treatment of the acidic and basic subunits belonging to various subunit pairs of *Vicia* legumin with cyanogen bromide showed the cleavage products of basic subunits to be more similar. On the other hand, the number and mol. wt. of the cleavage polypeptides for individual acidic subunits were much different from those of each other (data not presented). All these observations indicate that the structure of basic subunits is more constrained and that the genes responsible for the basic subunits have been more stable whereas those for acidic subunits seem to have been more prone to mutations and other changes during the course of evolution. From this assumption, a possible arrangement of the two types of subunits can also be deduced which is that basic subunits are situated towards the interior and acidic subunits towards the exterior of the hexameric molecule. This model is supported by the observation that the acidic subunits carry the antigenic determinants for the legumin molecule, and that the acidic subunits are proteolysed first on germination (Croy, unpublished results).

Recently a great deal of work has been undertaken regarding the inheritance of seed proteins of *Pisum sativum*. A number of proteins like 'vicilin' (Hynes, 1969), aminopeptidase (Scandalios and Espiritu, 1969),

acidic subunits of legumin (Thomson and Schroeder, 1978; Casey, 1979b), vicilin subunits (Thomson and Schroeder, 1978) and 'albumin a' (Blixt et al., 1980) have been found to be under the control of a single pair of co-dominant genes. Legumin basic subunits, however, have been reported to be under multigenic control (Thomson and Schroeder, 1978). These reports invalidate the earlier reports of maternal effects on the inheritance of storage proteins in pea (Davies, 1973). Results presented in table 4.1 and 4.3 of this thesis also support the monogenic control by co-dominant alleles for Lg-1B, Lg-1C, Lg-1S, Lg-2S and convicilin subunits. The subunit pattern for various legumin subunit types may consist of two or more than two subunit bands e.g. 3 bands in line 807 and 1238 and two bands in line 1263 for Lg-1C subunits, 2 bands for Lg-1S in line 807 and 2 bands for Lg-2S in lines 110 and 807. It may be assumed that each of these bands is controlled by one gene. Occurrence of either parental or the combination of two parental patterns in F_2 suggests that genes for these subunits are strongly linked. As the 2-D electrophoretic studies (SDS-PAGE $\longrightarrow$ IEF) have shown that each subunit band separated on the SDS-gel further consists of a number of charge variants, it is very likely that there exist a number of gene copies (more or less identical) for each subunit so closely located that there is no recombination at all among them and the genes for various subunits of a kind act as a single gene.

Four types of legumin subunits (Table 4.2) and, Lg-1C and convicilin ($\chi^2_{(n=8)}$ = 5.06) seem to be under independent control by the genes which are located either on different chromosomes or are syntenic,i.e. if located on the same chromosome are distantly located from each other so that they do not show any linkage. The only exception appears to be in the case of Lg-1B and Lg-2S which show a considerable degree of linkage with each other. The situation for most of the legumin subunit genes, thus, seems to be similar to zein polypeptide genes, which though being identical in effect are located on different chromosomes (Valentini et al., 1979).

Considerable evidence exists to support the hypothesis that legumin subunits are synthesized as precursor polypeptides of 60,000 mol. wt. (Croy et al., 1980; and unpublished results) containing both parts of the subunit pair. This implies an adjacent location of the genes for acidic and basic subunits on the same chromosome. (More correctly, each subunit pair is the product of a single gene). No evidence exists to show that this is not the case with the conventional legumin subunit pair i.e. 60,000 mol. wt. However, the results of independent inheritance of the genes for acidic and basic subunits of small legumin would suggest that a different pathway of synthesis takes place in this case. The absence of a 35,000 mol. wt. precursor in the translation products supports this suggestion.

Since the earliest days of classical genetics, Mendel's study material, <u>Pisum sativum</u>, has been one of the most extensively used plants for genetical studies. The first reports on linkage by Vilmorin and Bateson (1912) were followed by the extensive and outstanding work of H. Lamprecht (Lamprecht, 1948; Blixt, 1969). Nearly 200 genes, mostly for morphological traits, (Blixt, 1977; Marx and Provvidenti, 1979; Marx, 1980; Murfet, 1980; Gritton and Hagedorn, 1980 and Blixt <u>et al</u>., 1980) have been assigned to the seven linkage groups of pea. The <u>tl</u> and <u>r</u> genes linked to each other (Lamprecht, 1948) are located on the short arm of chromosome 7 (Blixt, 1972). The gene for the acidic subunit (Lg-1C) in the present studies has been found to show linkage with these loci and is located 15 map units from the <u>tl</u> locus on the side away from <u>r</u> locus. Recently there has been a controversy regarding the position of <u>tl-r</u> segment. Lamm (1978) on the basis of his studies on translocation lines has suggested the position of <u>tl-r</u> segment to be on the long arm of chromosome 5 rather than the short arm of chromosome 7. However, we have followed the gene map given by Blixt (1972) in considering the <u>Lg-1C</u> gene linked to <u>tl-r</u> segment as being on chromosome 7. As suggested under the rules for gene symbols (Annonymous, 1977) this <u>Lg-1C</u> gene maps as given below

Lg-1C --- 15 --- tl --- 5 --- r

The gene for convicilin subunits which shows
independent assortment with respect to Lg-1C gene
($\chi^2_{(n=8)}$ = 5.06) shows a strong linkage with the gene
k for keel like wings. It is about 3 recombination
units apart from k locus, k in turn being located on
chromosome 2. However, the exact location of the
convicilin gene (Cvc) could not be ascertained because
there was no other suitable marker for this linkage
group in the cross 1238 x 1263.

Lastly, it is appropriate to discuss the
implications of the results presented in this thesis
on the structure and genetics of legumin to the
importance of legumes as high protein crops.

Existing work on seed protein improvement in
cereals (Nelson and Burr, 1973) has indicated that
changing the ratio of major seed storage proteins may
well be the most easy route to protein improvement.
There are two requirements : (i) the existence of seed
proteins with improved amino acid profile, (ii) the
possibility of largely increasing the content of those
proteins relative to others. As pointed out in the
introduction, legumin potentially satisfies these
criteria. Its sulphur amino acid content is superior
to the other major storage protein vicilin and different
lines show varying proportions of legumin in the total
seed protein.

The complexity of the legumin molecule demonstrated
in these results would suggest that the sulphur amino

acid content of individual subunit pairs needs to be

determined in order to identify where improvement should

be made. Furthermore, although the genetic position

was not fully elucidated, it would appear complex and,

therefore, considerable work would be needed to accomplish

the desired results. On the positive side, however,

since no linkage occurs between the genes responsible

for different storage proteins, improving the protein

profile nutritionally would appear feasible in principle.

A discussion of the precise dietary situation in which

such improvements are desirable lies out of the scope

of this thesis.

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