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INVESTIGATION INTO THE HARDBEAN PHENOMENON

IN PHASEOLUS VULGARIS L.

By

PETER M. B. JONES

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

Botany Department.

July 1982.



To Mum and Dad.

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ABSTRACT

The textural deterioration of dry beans during storage was investigated. The primary symptoms of this hardbean phenomenon were found to be reduced viability, reduced water uptake and reduced cooking rate. It was found that the basic cause of the beans failing to cook - or soften - was the failure of the cotyledon cells to separate during the cooking process.

From these initial observations it was hypothesised that reduced cell separation rate was due to either reduced turgor pressure due to reduced water uptake, or reduced pectin solubility, or both.

Techniques used included light microscopy, transmission and scanning electron microscopy, thin-layer chromatography and spectrophotometry and it was found that reduced cell separation rate was due to a combination of both reduced water uptake and reduced pectin solubility. By inducing the hardbean symptoms artificially their relevance to textural deterioration were determined.

The significant events within the beans during storage were found to be phytin breakdown, membrane deterioration, cation leakage, pectin demethylation and pectin calcification.

By running a storage trial and thus allowing hardness to develop within the beans the sequence of events was elucidated.



## INTRODUCTION

The significance of the use of legumes in many diets is their abundance and high protein content. Hence their predominance in the diet of poor people in Latin America. Legumes have the highest protein content of the vegetable foods. Phaseolus vulgaris has a protein content of approximately 23% while soybeans (Glycine max) have up to 37% protein.

Although there is a minimum requirement for protein intake by man, 570 mg Kg<sup>-1</sup> body weight per day in a young adult male (Scrimshaw<sup>and Young</sup>, 1978), not only is the quantity of protein consumed important but also its quality, i.e. amino-acid composition, and the percentage protein in the diet. A young adult male requires 14% protein in the diet and this cannot, for example, be attained by eating just cereal grains which are at best 12% protein (Pirie, 1976). Protein deficiency diseases such as kwashiorkor and marasmus are regarded as an important cause of malnutrition in most low income countries and commonly lead to increased susceptibility to disease.

Bressani et al. (1961a) analysed the essential amino-acid content of four legumes, including two varieties of P. vulgaris and found that although the protein content was high (20-25%) they were deficient in methionine, ½-cystine, leucine and tryptophan. If one or more of the essential amino-acids are partially missing the utilisation of all others is reduced accordingly. Consequently total amino-acid utilisation in the legumes studied was not possible. Substitution of the bean diet with 0.2% methionine significantly increased the protein efficiency ratio (PER) (Bressani et al., 1963). The PER is a measurement of the percentage conversion of food protein into the protein of the animal consuming it. A similar effect was achieved with an isoproteic mixture of maize and beans because the maize protein amino-acids compensated for the limiting



amino-acids of the bean protein and vice-versa. (The limiting amino-acid in maize being lysine (Bressani et al., 1961b) ). It is a common practice in Latin America for the indigenous population to mix maize and beans together - thereby fortuitously improving their diet. Similarly in China, rice is supplemented with a protein rich cabbage (Borgstrom, 1973).

Further analysis by de Moraes and Angelucci (1971) proved beans to be a good source of dietary iron, calcium, magnesium and phosphates. Beans are also rich sources of niacin (Bressani et al., 1954), riboflavin and vitamin E (Bunnell et al., 1965).

Vegetable crops must be stored, if not used immediately, in order to secure food supplies from one harvest to the next and also to form the basis of distribution between areas of population. If storage conditions are not adequate, quality changes may occur in four main ways. One is loss of viability which has been recorded in many species, for example wheat (Glass et al., 1959), lettuce (Harrison and McLeish, 1954; Koostra & Harrington 1969) perennial rye grass and crimson clover (Ching and Schoolcraft, 1968), etc. Toole et al. (1948) investigated fifteen different fruit and vegetables and found that all of them lost viability if stored at high temperature and high relative humidity (RH).

Secondly, poor storage can result in microbial infestation. Dexter et al. (1955) found that on white pea bean, storage at between 21°C and 38°C at above 75% RH created rapid mould growth. Other quality defects were discoloration and testa splitting which affects their commercial acceptability. Bottomly et al. (1950) found identical mould growth effects under such conditions on stored yellow corn.

Taste can also deteriorate on storage as reported by Morris and Wood (1956) and Muneta (1964) on stored beans. Both instances involved storage at high temperature and high moisture content. Morris and Wood attributed the organoleptic deterioration in beans stored at 25°C

and 13% moisture content to an increase in lipid acid value. Shah and Edwards (1976) found that dehydrated peas maintained flavour best if stored at  $-10^{\circ}\text{F}$ .

The hardbean phenomenon is included in the fourth mode of quality deterioration - texture. This phenomenon is a condition whereby beans which usually take between twenty and forty minutes to cook, depending on variety, may instead take in excess of five hours before softening sufficiently to eat. A cooked bean will yield easily to pressure and "soften" when squeezed, but the texture of a hardbean does not change during cooking or when submitted to external force. The hardbean phenomenon has been cited in the records for over two thousand years.

Textural changes have been reported in other vegetables. For example, it has been reported that the pH of storage of canned sweet potatoes has a profound influence on texture. As the pH rose from 3-8, the canned potatoes softened due to the effect of pH on carbohydrate transformation (Sistrunk, 1971). Potatoes also undergo such a change during storage known as hardcore which is a hardening of patches of the tuber and may well have much in common with the hardbean phenomenon. Hardcore is triggered by storage of tubers at  $2^{\circ}\text{C}$  (Daines *et al.*, 1974) and can also be initiated by frost damage in the field (Sistrunk, 1977). Reddy and Sistrunk (1980) found that carbohydrate composition, including decreases in water soluble pectin was associated with quality changes in the stored tubers. This corroborated the work of Heinze and Appleman (1943) who also found pectin solubility decreased as the incidence of hardcore increased.

Pectins are a group of complex colloidal carbohydrates rich in galacturonic acid units. The solubility is influenced by chain length and cross-linkages. They are commonly found in the middle lamella and primary cell walls of plant tissue. The pectin chemistry is more fully explained in the discussion.

The hardbean phenomenon is commonly reported in legumes which have been stored at high relative humidity (>70% RH) and high temperature (>20°C). If such conditions prevail for long enough, just a few months, then hardness will develop throughout the whole of the bean crop. In Guatemala, for example, where there are two bean crops a year (in December/January and June/July) the beans need to be stored between harvests and conditions are perfect for hardbean to develop, the climate is right and there is enough time. The consequences of this condition, which coincides with reduced viability are:-

a) inconvenience, it is inconvenient to have to boil beans for five hours when thirty minutes should be adequate:

b) commercial, hardbeans are unsaleable, especially when in competition with beans that cook properly:

c) economics, fuel costs will be proportionately higher for the cooking of hardbeans than for normal beans. In arid areas where firewood may be scarce this problem is magnified:

d) crop loss, hardbeans are non-viable and therefore are useless as sowing seed:

e) political instability, for example, General Efraim Rios Montt who came to power in Guatemala in March 1982 was quoted as saying that the solution to Guatemala's guerilla problem was, "... a policy of beans and rifles to help the people to develop." (Aguliar, 1982) referring in part to dissatisfaction among the population due to persistent food shortages:

f) nutritional, Bressani et al. (1963) have shown that excessive cooking of beans reduces their PER. He found that the weight gain of growing rats was highest on a diet of beans pressure-cooked for between 10 and 30 min., beyond this time the weight gain of the rats begins to drop. These results were interpreted as due to a drop in the nutritive

value of the bean protein. Molina et al. (1975) found that protein digestibility and PER of beans was decreased by six months storage despite an increase in available methionine and lysine. It follows therefore that hardbeans which would require extremely long cooking times would be less nutritious. <sup>and Sgarbieri</sup> Antunes (1979) also found that storage of beans under adverse conditions reduced the PER and said that it was mainly due to a decrease in the biological availability of methionine and lysine. This was overcome by supplementing the cooked product with methionine.

In the developed world where meat is generally plentiful the availability of vegetable protein is of less importance but in parts of the world where meat is not a regular component of the diet and where legumes supply some 30% of the dietary protein as in Central America (Tandon, <sup>et al</sup> 1957) the consequences are potentially severe, especially if on a nutritionally minimal diet. Many millions of people in Latin America live on such a diet and it is therefore most important to control storage conditions. This is an increasing problem since in most developing countries the population is growing at a faster rate than food production (Bhattacharjee, 1976). Borgstrom (1973) stated that the existence of protein deficiency in poor countries was not necessarily due to insufficient protein production but due to insufficient protein consumption caused by either the dietary structure, the selling of produce to supplement a low income or protein loss during harvest, storage or cooking.

The hardbean condition should not be confused with another phenomenon called hardshell. Both are common in legumes and can develop during storage. Gloyer (1921) clarified the difference when he stated that hardshell is due to the testa being impermeable to water caused by storage in a heated room of low relative humidity, while hardbean (which he called sclerema) was probably an enzymically created condition of the cotyledons due to storage of beans in a warm humid atmosphere. He found

that hardshell could be overcome by a short immersion in boiling water as did Morris et al. (1950) while Nelson (1926) found that abrasion of the testa worked, and Davies (1928a) found that hardshell in Melilotus alba and Medicago sativa could be relieved by high pressure (2000 atm) though this tended to reduce the final germination rate due to embryo damage (Davies, 1928b). The cause of hardshell has been attributed to a resinous layer (Nelson, 1926), a cuticular layer (Harrington, 1916), adpressed cells in the staphiolar cleft (Hamly, 1932) and contraction of the walls of the palisade layer (Corner, 1951) depending on species. Quenzer et al. (1978) has found that a late frost can cause hardshell in pinto beans (P. vulgaris).

Leggatt (1931) suggested that a benefit of hardshell (in alfalfa) was the creation of a reserve seed store in the soil which would germinate only after an extended period of waterlogging which may have killed seedlings from previously germinated permeable seeds. Pollock and Toole (1966) said that the restriction of imbibition by lima beans (Phaseolus lunatus) by the testa below 25°C was to prevent chilling injury to the embryonic axis. Rejection of the smallest 1% of imbibed beans has proved to be an efficient method of removing hardshell beans from a batch for canning (Bourne, 1967).

Returning to a consideration of hardbean, Burr et al. (1968) found that hardbean could be avoided by keeping the moisture content of the beans - which is governed by the ambient conditions, below 10%. Otherwise, he found, storage for twelve months at 13-14% moisture content and 90°F increased the cooking time by 11 to 17 times depending on variety. He said that "Beans would be more acceptable .... if care was taken to insure low to moderate moisture contents and temperatures and to avoid prolonged storage." Muneta (1964) announced that moisture content of the beans during storage, although not the actual cause of hardbean, was

probably correlated with the cause.

After six months adverse storage conditions, Antunes<sup>and Sgarbieri</sup> (1979) reported up to a five-fold increase in the cooking time of P. vulgaris. In beans stored at 25°C and 65-70% RH for six months the cooking time increased from 60 min. to 116 min. while beans which were stored at 37°C and 76% RH over the same period had a cooking time of 300 min. They also recorded a moderate increase in the cooking time of beans stored for six months at 12°C and 52% RH.

Ozawa (1978) investigated hardbean in adzuki beans (Phaseolus angularis) and found that adverse storage conditions doubled the cooking time, and reduced the hydration rate and seed viability.

Ideally the simplest way to prevent the development of hardbean is to keep the beans cooled, by refrigeration for example, but as this is not possible for people who live below the poverty line then it means that the hardbean phenomenon will remain a problem. The aim of this research was not to find out how to prevent the development of hardbean but to determine how it develops.

Although much work has been done on the changes in texture of various fruits and vegetables and the effects of storage on bean nutrition and cooking rate, the changes within the bean actually relevant to the hardbean phenomenon have not yet been conclusively proved.

The aim of this work is to analyse the actual characteristics of hardbean in Phaseolus and to determine which ones actually influence the textural deterioration and which ones are coincidental. If as seems likely, middle lamella solubility is a key factor, then the cause of the change must be determined. As pectin is the main component of the middle lamella, its solubility during the cooking process was analysed in order to determine if this could be equated with changes in the

cooking rate. Visual surveys of cell separation were performed by light microscopy and transmission electron microscopy to determine if it was due to middle lamella dissolution or general cell wall breakdown, and also if its rate or form was different in hard beans. If pectin solubility can be related to the cooking rate then the pectin chemistry would be investigated to determine what changes have occurred. Other factors such as testa and/or cotyledon impermeability, water uptake, swelling power of the cells or their components and the cell structure were also investigated.

Once the cause of the hardbean phenomenon had been determined, the theory was tested by the induction of these influences artificially and further elucidated by setting up a storage trial to follow various characteristics as they change and thus determine which ones are relevant to hardbean and the sequence of events involved.

MATERIALS AND METHODS

Biological Material

Seeds of Phaseolus vulgaris var. S-19-N were obtained from Dr. Ricardo Bressani, INCAP (instituto de Nutricion de Centro America Y Panama), Guatemala.

From this initial batch a crop of 200 plants of P. vulgaris was cultivated in the University greenhouses producing 1 Kg of seeds and subsequently a further 350 plants were cultivated to produce 2 Kg of black beans.

The beans were stored at 4°C until required.

The Determination of Seed Viability and Vigour

Twenty-five large black beans ( $\approx 8$  mm in length) were placed on filter paper in Petri dishes and 10 ml water was added. The percentage germination was the percentage of seeds which had shown radicle emergence after 4 days. Care was taken not to confuse seeds whose radicles had emerged due to embryonic activity with seeds which had split the testa due to passive expansion of the cotyledons.

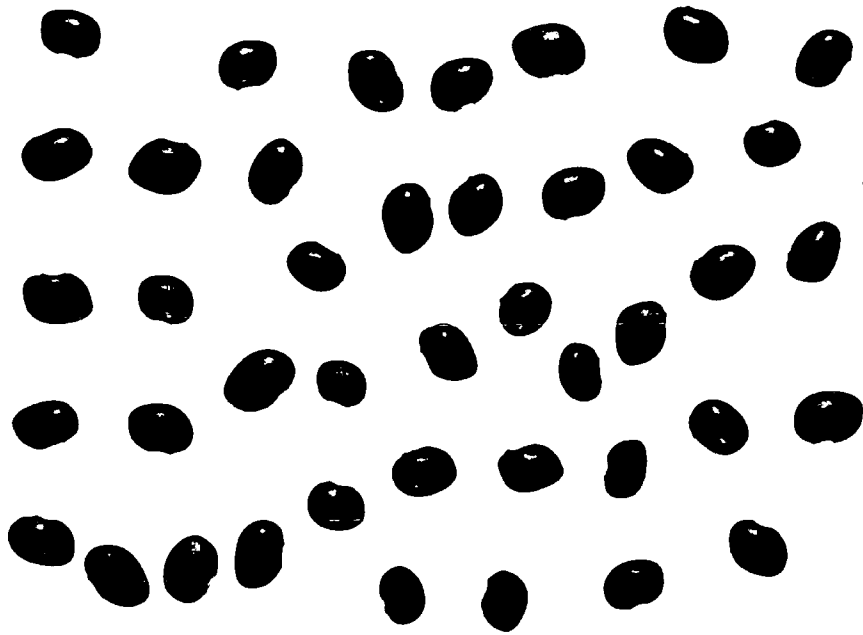
Seed vigour was determined using a simple points system.

Assuming that any viable seed will have germinated by the fourth day of treatment the points awarded were worked thus:-

4	points	for	each	seed	to	germinate	within	24	h.
3	"	"	"	"	"	"	"	between	24 and 48 h.
2	"	"	"	"	"	"	"	"	48 and 72 h.
1	"	"	"	"	"	"	"	"	72 and 96 h.

Seed vigour was expressed as a percentage of the maximum points possible (i.e. 4 points for each seed) gained after 4 days.





X 1

fig. 1. Black beans Phaseolus vulgaris var S-19-N.

### Determination of Moisture Content

The testas were removed and the cotyledons and embryo weighed. The material was then dried in an oven at 105°C for 24 h., placed in a desiccator until cooled to room temperature (approximately 10 min.) and then weighed.

The moisture content was calculated as:-

$$\frac{\text{fresh wt.} - \text{dry wt.}}{\text{fresh wt.}} \times 100\%$$

### Cooking Rate

10-15 g. of whole beans were soaked in 100 ml. water for approx. 18 h. at room temperature. After soaking, the beans were drained and put into approx. 200 ml. boiling water. After 20, 35, 45 and 60 min. (and if necessary at 15 and then 30 min. intervals for up to 4 h.) the beans were drained and then squeezed individually between forefinger and thumb - a technique also used by Sistrunk (1977) with potato - to determine if the beans were cooked. A bean was considered cooked if the cotyledons yielded to only slight pressure. If the cotyledons did not soften they were boiled until they did.

To determine the total cooking time (CT<sub>100</sub>) the cooking figures were expressed as a percentage and from these figures the total cooking time in minutes of a batch of 100 black beans was calculated.

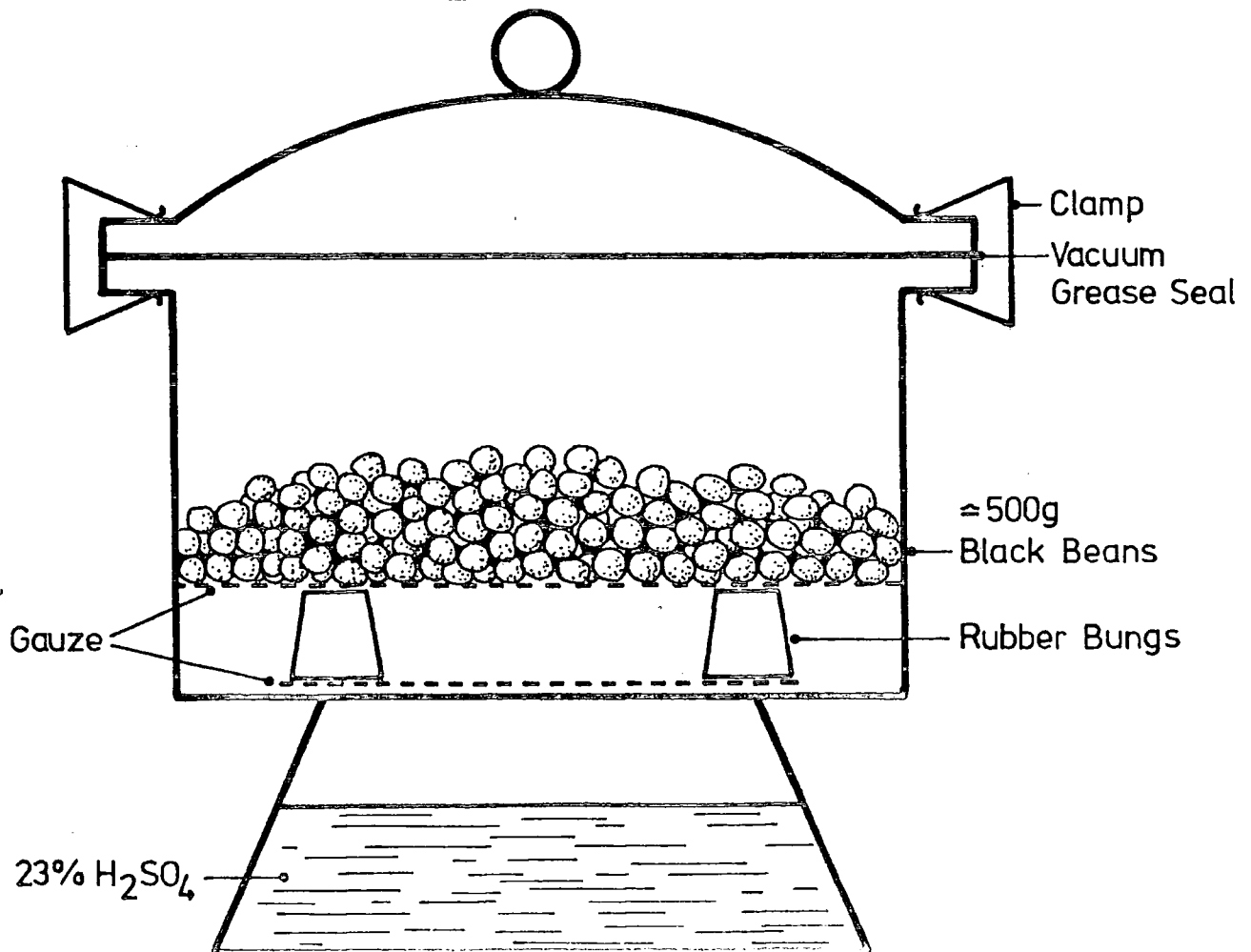
For example:-

<u>Time</u> <u>(min.)</u>	<u>No. cooked</u> <u>(Max. 50)</u>	<u>% Cooked</u>	<u>CT</u>
0	0	0	-
20	18	36	36 x 20 min. = 720 min.
35	48	96	(96-36) x 35 min. = 2100 min.
45	50	100	(100-96) x 45 min. = 180 min.

Therefore the CT<sub>100</sub> of this batch of beans would be 3000 min.

Storage to Induce Hardbean Development

500 g. of black bean seeds were surface sterilised for 30 sec. in ethanol, and after draining, for a further 30 sec. in a fungicidal solution (Benlate, benomyl fungicide, a product of E. I. du Pont de Nemours and Co. (Inc.), U.S.A.) at a concentration of  $300 \text{ mg. l}^{-1}$ . After draining the seeds were allowed to dry at room temperature overnight. When dry, the seeds were placed above 23% sulphuric acid in a sealed desiccator and stored at  $34^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . (See fig 2)

fig. 2. Bean storage vessel

Determination of the Imbibition Value of Bean Cotyledons and the Degree of Leakage from them

The testas were removed by hand from 15 beans, taking care not to break the outer surface of the cotyledons or snap off the embryonic axis. After weighing to within 0.1 mg. (and calculating the dry weight) the cotyledons were soaked in water for 18 h. at room temperature (20 ml. water to 1 g. of tissue fresh weight). After 18 h. the cotyledons were drained and the solution retained. The cotyledons were blotted dry with tissue paper, making sure to remove any water pocket trapped between the cotyledons, and weighed immediately. The imbibition value (I.V.) was determined as:-

$$\frac{\text{wet weight}}{\text{dry weight}}$$

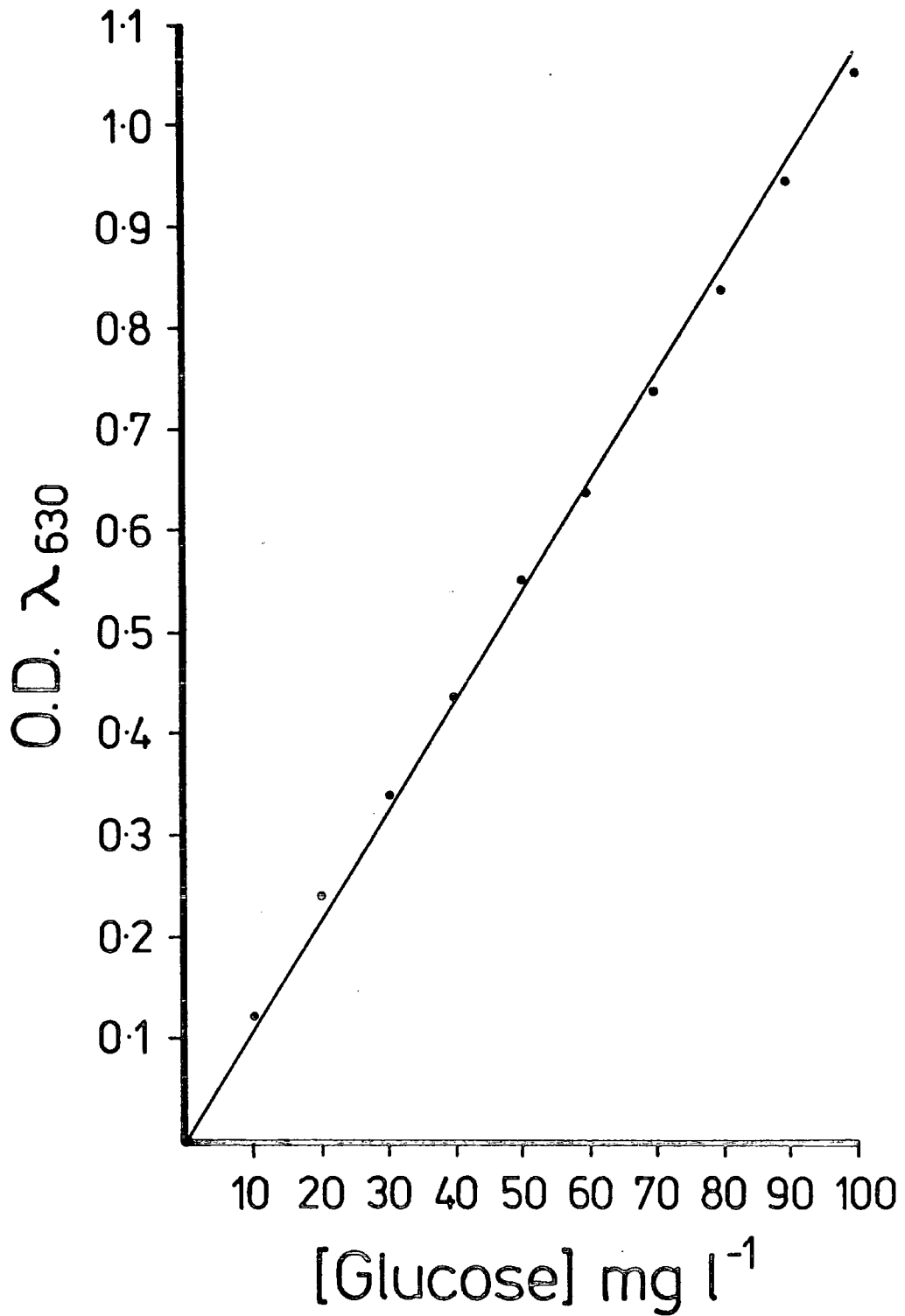
The volume of the leachate was measured to the nearest 0.5 ml. and a 1 ml. sample was removed and diluted to 10 ml. (When leakage was high the dilution was fifty-fold.)

The sugar content of this diluted extract was then determined by adding 3 ml. of anthrone reagent (0.2% in conc.  $\text{H}_2\text{SO}_4$ ) to each of five 1 ml. samples and boiling for 7.5 min. After cooling in ice the absorbance at 630 nm was measured using a Perkin-Elmer 402 Ultraviolet-Visible Spectrophotometer and the amount of sugar in the sample determined.

A calibration curve (Fig. 3) was prepared using known concentrations of sugar solutions.

The amount of sugar leakage from the beans was expressed as mg. glucose  $\text{g.}^{-1}$  d.wt. of bean tissue.

The remaining extract was poured onto a previously oven-dried and pre-weighed tin foil tray and allowed to dry by evaporation on the top of a drying oven overnight. After drying in an oven at  $105^\circ\text{C}$  for an

fig. 3 Calibration curve - Glucose.

additional 2 h. and cooling in a desiccator, the tin foil plus residue was weighed, and the total leakage from the bean cotyledons determined and expressed in  $\text{mg. g.}^{-1}$  d.wt. of bean tissue.

#### Analysis of Phytin

This was essentially according to the technique of Makower (1970):-

220 mg. samples of ball milled tissue ( $\pm$  200 mg. d.wt.) were placed in acid washed centrifuge tubes and extracted twice with 4.0 ml. 0.5 N HCl for 15 min. at 60°C. After each extraction the tissue was centrifuged for 7 min. at approx. 2500 r.p.m. on a bench centrifuge and the supernatants bulked.

The pH of the supernatants was then adjusted to 1.9-2.0 with NaOH and to this was added 2.0 ml.  $\text{FeCl}_3$  in 0.375 N HCl containing 8 mg.  $\text{ml.}^{-1}$   $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ .

The combined extracts were poured into tubes and boiled for 1 h. Boiling of the extracts precipitated the phytin present as a gelatinous iron phytate which was centrifuged down and washed three times with 0.1 N HCl.

The iron, a measure of the phytin present, was measured by digesting the residue in 1.5 ml. conc.  $\text{H}_2\text{SO}_4$  and 0.9 ml.  $\text{H}_2\text{O}_2$ ; and 400  $\mu\text{l}$ . samples were diluted to 4.0 ml. with deionised water and the iron content determined on a Perkin Elmer 403 atomic absorption spectrophotometer at 384 nm.

Iron phytate was assumed to be:-

$\text{Fe}_4\text{P}_6\text{C}_6\text{H}_6\text{O}_{24}$  with a molecular weight of 871.28 g. of which 223.4 g. is iron (25.64%).

The amount of phytin present was determined by multiplying the amount of iron present by 3.9 ( $\frac{100}{25.64}$ ). This figure was read as calcium magnesium phytate which has an almost identical molecular weight (872.4 g.).

The Quantitative Analysis of Calcium and Magnesium  
Adhering to the Cell Wall

The testa, inner integuments and embryonic radicles were removed from approx. 3 g. of beans and the cotyledons partially dried overnight over silica gel.

The cotyledons were then ball-milled for 40 sec. and immediately placed on ice. Two 200 mg. samples were removed for moisture content determination and the remainder ground in a pestle and mortar for about 1 min.

110 mg. samples ( $\approx$  100 mg. d.wt.) were then placed into small pre-weighed, acid washed, centrifuge tubes and washed in 8 ml. 0.33 M. mannitol to remove the mitochondria and other cell organelles without rupturing the membranes, and the tissue centrifuged down for 4 min. at 2000 r.p.m. The supernatant was discarded. The residue was washed in 8 ml. 1.0 m. mannitol to remove the bulk of any residual cytoplasmic debris followed by three further washes in 8 ml. deionised water, one wash in acetone and another three washes in deionised water. It was assumed that any remaining tissue was cell wall debris and starch grains which was confirmed by viewing under a light microscope.

The residue was digested as described for phytin analysis. 400  $\mu$ l. samples were then diluted to 4 ml. with deionised water. To each 4 ml. sample six drops of  $\text{LaCl}_3$  (10% w/v) were added to precipitate any phosphates present and the samples analysed for calcium and magnesium on a Perkin Elmer 403 atomic absorption spectrophotometer at 211 and 285 nm respectively. Each element was expressed as  $\mu\text{g. Ca/Mg mg.}^{-1}$  d.wt. whole tissue.

In order to determine the degree of phytin interference in the calcium magnesium analysis, tissue was prepared as for the calcium and magnesium cell wall analysis but not acid digested. The tissue

was then extracted twice with 10 ml. 0.5 N<sup>o</sup> HCl at 60°C to remove any residual phytin and the extracts bulked. The extract was then analysed for phosphate by the addition of the following reagent:-

- a. 14% H<sub>2</sub>SO<sub>4</sub>
- b. 3.75% Ammonium molybdate
- c. 5.4% Ascorbic acid
- d. 0.34% Potassium antimony tartrate

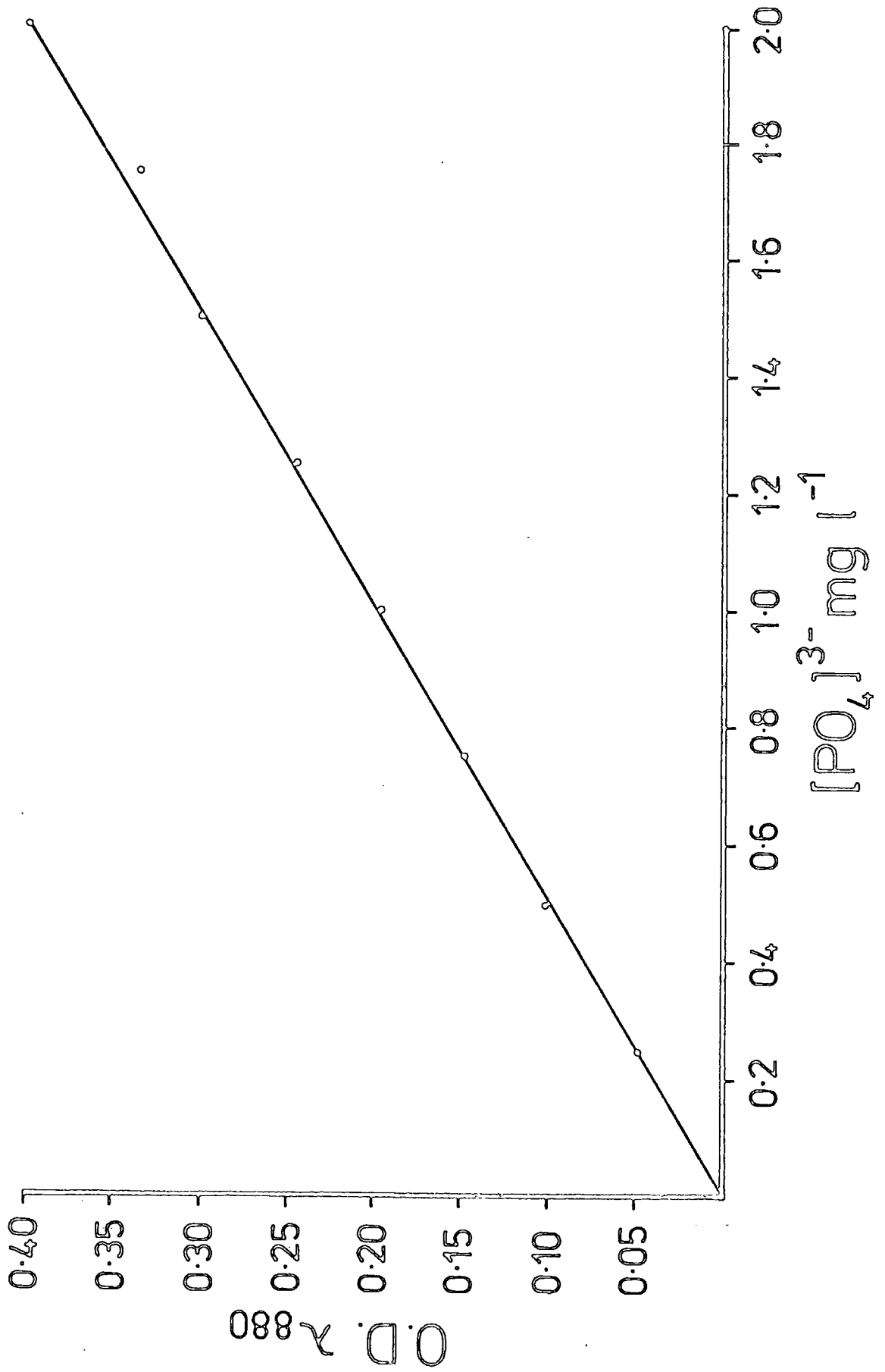
(Mixed in the ratio a:b:c:d, 5:2:2:1).

750 μl. was added to 3 ml. extract and the absorbance measured on a SP8-150 UV/VIS spectrophotometer at 880 nm.

The calculation of phytin interference was as follows:-

	<u>Soft bean extract</u>	<u>Hard bean extract</u>
O.D 880	0.026	0.020
μg. PO <sub>4</sub> <sup>3-</sup> l <sup>-1</sup>	133	102
μg. PO <sub>4</sub> <sup>3-</sup> in extract (19 ml.)	2.53	1.94
Phytin (PO <sub>4</sub> <sup>3-</sup> x 1.42) μg.	3.61	2.77
Calcium (μg.) (12% of Phytin)	0.433	0.332
Magnesium (μg.) (1.5% of Phytin)	0.054	0.042
Tissue d.wt. (mg.)	95.7	100.4
Ca. g. μmg. <sup>-1</sup>	5 x 10 <sup>-3</sup>	3 x 10 <sup>-3</sup>
Ca. interference	<u>≈4%</u>	<u>≈2%</u>
Mg. μg. mg. <sup>-1</sup>	6 x 10 <sup>-4</sup>	4 x 10 <sup>-4</sup>
Mg. interference	<u>≈3%</u>	<u>&lt;2%</u>



fig.4 Calibration curve - Phosphate.

Analysis of Available Sugar and Total Starch  
Present in Beans of *P. vulgaris*

Approx. 2.5 g. of ball-milled bean cotyledons were placed in a pre-weighed extraction thimble and treated with hot 80% ethanol in a soxhlet apparatus for 10 h.

The hot ethanol washed any soluble sugars out of the tissue and into the flask containing approx. 150 ml. 80% ethanol. A 10 ml. sample was taken and diluted to 100 ml. with water and the sugar content was determined as previously described (p.14) and expressed as mg. sugar  $\text{g.}^{-1}$  d.wt. tissue.

After drying the residual tissue at room temperature overnight the soxhlet thimble and its contents were reweighed and three 100 mg. samples removed to determine the moisture content.

The starch content was then analysed as follows:-

20 mg. samples were weighed into small test tubes and moistened with two or three drops of ethanol.

3.0 ml. of phosphate buffered saline (PBS) pH 7.1 were added and the suspension was boiled for 3 min. to initiate starch hydrolysis. To this was added a further 7 ml. PBS and 100 units of  $\alpha$ -amylase. The contents were transferred to a small beaker and stirred overnight at room temperature.

The suspension was then filtered and the filtrate treated with an equal volume of 9.2 N perchloric acid. The residue was stirred for 15 min. in 20 ml. 4.6 N perchloric acid and then filtered. The residue was then washed with water for a further 15 min. and again filtered. All three filtrates were bulked and the sugar content was determined by anthrone and expressed as mg. equivalents of glucose  $\text{g.}^{-1}$  d.wt. tissue.

### Extraction and Analysis of Uronic Acid Fractions

#### (a) Cold water soluble

A known weight (approx. 4 g.) of whole bean cotyledons were soaked for 18 h. in approx. 100 ml. water at room temperature. The cotyledons were then drained and the extract stored at 4°C.

#### (b) Hot water soluble

The cotyledons from (a) were placed in approx. 200 ml. boiling water for 20 min. and then drained (or if tissue breakdown was extensive, centrifuged). The extract was stored at 4°C until required.

#### (c) Ammonium oxalate soluble

The tissue from (b) was suspended in 150 ml. 0.5% ammonium oxalate and refluxed at 85-90°C for 5 h. The suspension was continuously stirred.

After 5 h. the suspension was allowed to cool and then centrifuged. The residue was washed with water and the extract bulked, and stored at 4°C.

#### (d) NaOH soluble

The residue from (c) was stirred for 15 min. in 50 ml. 0.05 N NaOH at room temperature and then centrifuged, washed in 0.05 N NaOH and the bulked extracts stored at 4°C.

### Analysis of Uronic Acids

This was according to the technique of Blumenkrantz and Asboe-Hansen (1973) except that  $\sigma$ -hydroxy diphenyl was used instead of m-hydroxy diphenyl.

2.4 ml. of 0.0125 M di-sodium tetraborate in conc.  $H_2SO_4$  was added to 400  $\mu$ l. of extract, cooled in ice and then placed in a boiling water bath for 5 min.

After cooling, 40  $\mu$ l. of 0.15%  $\sigma$ -hydroxy diphenyl in 0.5% NaOH

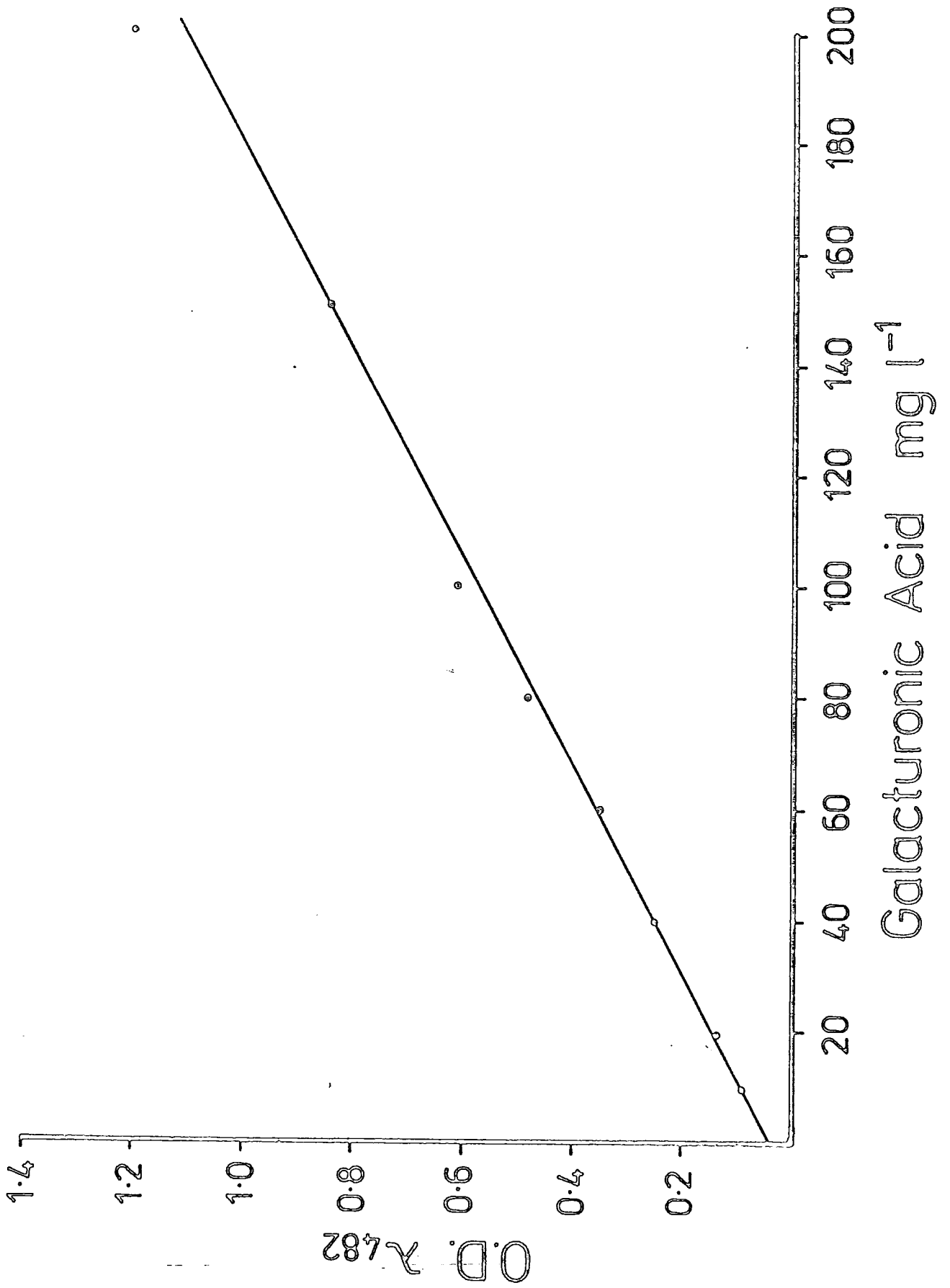
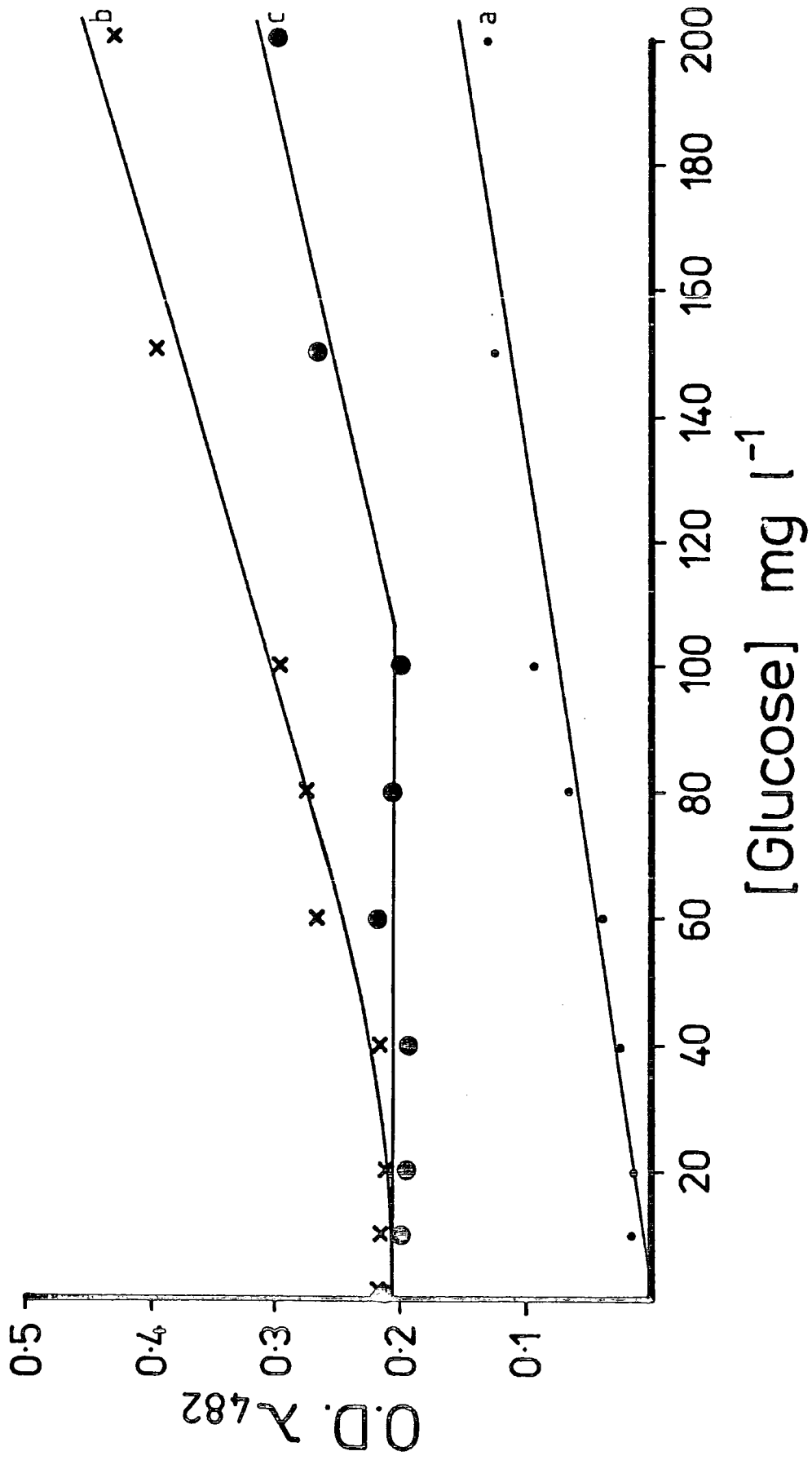


fig. 5. Calibration curve - Galacturonic acid.

fig. 6 The influence of glucose (a) on the absorbance of a galacturonic acid-glucose mixture (b).



Line c represents the corrected value for galacturonic acid.

was added and the absorbance at 482 nm measured in a Perkin Elmer 402 spectrophotometer within 5 min. For each extract analysed a zero was set up using 40  $\mu$ l. of 0.5% NaOH without 0.15%  $\sigma$ -hydroxy diphenyl to determine the quantity of other non-uronic acid sugars present. Subtraction of the latter from the former gave the uronic acid content. It was found that other sugars did not interfere if their optical density was kept below 0.05 by suitable dilution (see Figs. 5 and 6).

#### Extraction of Lipids from Whole Cotyledons

(From Priestley and Leopold, 1979)

Approximately 6 g. of bean cotyledons were ball-milled and immediately placed in a cooled 250 ml. polythene bottle and covered by 50 ml. chloroform:methanol 2:1 v/v. Oxygen free nitrogen was bubbled through this for five minutes and then the tissue and the chloroform methanol mixture was further maccerated with a polytron PCU-2 for three minutes while still under nitrogen. This mixture was centrifuged and the process repeated with fresh chloroform: methanol and centrifuged again.

The supernatants were bulked and fractionated against 0.2 vol. 0.9% w/v NaCl and twice against 0.9% w/v NaCl in 50% methanol.

The purified extract was then transferred to a pre-weighed McCartney bottle and the chloroform evaporated while nitrogen was bubbled through until the extract reached constant weight (approx. 18 h.). The bottle was kept in a desiccator during evaporation. After weighing, the sample was suspended in chloroform at a concentration of 10 mg. ml.<sup>-1</sup> lipid.

#### Phospholipid Analysis

Phospholipids in the lipid extract were separated and identified

by thin-layer chromatography. 10  $\mu$ l. extract (10 mg. ml.<sup>-1</sup> lipid) was loaded onto a TLC plate (Kieselgel 60 G) and was run for 50 min. in the following phase:-

Chloroform	:	Methanol	:	Acetic Acid	:	Water
85		15		10		3.6

The chromatogen was as used by Raheja et al. (1973):-

Solution a : 80 ml. 13.3% ammonium molybdate.  
40 ml. conc. HCl containing 10 ml. mercury.  
Shake for 30 min.

Solution b : 40 ml. 13.3% ammonium molybdate.  
200 ml. conc. H<sub>2</sub>SO<sub>4</sub>.  
Solution "a".  
Allow to cool.

Solution b was then mixed with methanol, chloroform and water in the ratio 5:9:1:4.

203 ml. of this solution was diluted to 660 ml. with water and used as a spray. Phospholipids on a TLC plate showed up immediately as blue spots. The colour developed more fully over the next 4 h.

To cancel out the background yellow colour the plate was lightly sprayed with water.

#### Measurement of Polar Lipids

A 20 mg. sample of lipid extract was placed in a pre-weighed tube and resuspended in 200  $\mu$ l. chloroform. To this was added 4 ml. of acetone and the precipitate (polar lipids) was allowed to form overnight at 4°C.

The suspension was then centrifuged and the acetone discarded. The white polar lipid precipitate was allowed to dry at room temperature and weighed and expressed as a percentage of the total polar lipid extract.

Non-Aqueous Extraction of Whole Starch Grains

40 g. of black beans were shelled and the embryos removed. The inner integuments were removed by soaking for 15-20 sec. in water.

The cotyledons were then placed in 100 ml. ethanol and liquefied in an Osterizer for 30 sec. After allowing large pieces of cotyledon tissue to settle out for 10 sec. the supernatant was poured off and 100 ml. of fresh ethanol was added to the residue. This process was repeated four or five times until only a small amount of tissue remained unmacerated. The supernatants were bulked and consisted of predominantly isolated starch grains, broken cells and clumps of tissue consisting of no more than 10 cells. These clumps were further broken up by macerating the supernatant in 50 ml. batches with a PCU-2 polytron for one minute.

The macerate was then placed in a pot with a tap at its base and the volume brought up to 1 l. with ethanol. This macerate was continuously stirred and poured via a tap down a strip of aluminium guttering (3.36 m. x 5 cm. x 2.5 cm.) at a rate of approximately 1 ml. sec.<sup>-1</sup>. This guttering was held securely in a sloping position of approximately 1.6%. The surface of the aluminium guttering was polished with a mildly abrasive pad and soapy water.

As the tissue macerate ran down the guttering the tissue particles sedimented out. Any clumps of cells settled out immediately within the first 5 cm. Starch grains settled out in the first 40 cm. and large cell debris, mainly cell walls, settled out in the first 1.5 m.

By analysing samples under the light microscope it was found that the sediment between 5 and 40 cm. was 60-90% starch grains.

By re-running all the sediment which deposited between 5 and 60 cm. four or five times it was possible to obtain a preparation of pure starch grains.



It is interesting to note that by using the same principal but taking samples beyond the 2.5 m. mark one can obtain a pure preparation of protein bodies.

#### The Imbibition Value of Starch Grains

The moisture content of the starch grains was determined as for whole beans.

A 400 mg. sample was placed into a 5 ml. bottle and moistened with two drops of ethanol. Water was then added in a ratio 0.5 ml. to 100 mg. of starch. One drop of toluene was added to the surface of the water to inhibit microbial action and the sample was rotated slowly for 5 h. After 5 h. the sample was centrifuged in pre-weighed centrifuge tubes and the supernatant poured off. Once any excess water was removed from the sides of the tube the sample was weighed and the imbibition value of the starch determined as:-

$$\frac{\text{wet weight}}{\text{dry weight}}$$

#### The Swelling Power of Starch at Elevated Temperatures

(From Schoch, 1964)

To 500 mg. of starch was added 20 ml. water plus 20 ml. water at the elevated temperature under investigation.

Maintaining the sample at this temperature the sample was stirred for 30 min. When complete the stirrer was rinsed into the sample and the starch centrifuged down in pre-weighed centrifuge tubes at 3000 r.p.m. for 15 min. The supernatant was then poured off into a pre-weighed aluminium foil tray and evaporated to determine the quantity of soluble starch. The starch paste residue was weighed and the swelling power determined as:-

$$a = \frac{\text{wt. soluble starch}}{\text{d.wt. starch}} \times 100$$

$$\text{Swelling power} = \frac{\text{wt. of paste} \times 100}{\text{d.wt. of starch} \times (100-a)}$$

Readings for both hard and soft bean starch were taken at 52, 85 and 95°C.

#### The Determination of Pectin Esterification

(From Reeve, 1959)

Dried beans were macerated and divided into four 200 mg. lots, and treated independantly as follows:-

- Sample 1        - Washed in deionised water.  
                  "        " methanol x 3.  
                  Left overnight in methanol containing 0.5 N HCl.
- Samples 2 & 3 - Washed in deionised water.  
                  "        " methanol x 3.  
                  "        " acetone x 2  
                  Left overnight in acetone.
- Sample 4        - (To be performed 10 min. before analysis).  
                  Washed in deionised water.  
                  Left in 14% NaOH in 60% ethanol for 10 min.

The pectin in Sample 1 was 100% esterified while the pectin in Sample 4 was totally de-esterified. The degree of pectin esterification in Samples 2 and 3 was unaltered.

All samples were then centrifuged and washed in methanol. To each tube was added 5 ml. 14% NaOH in 60% ethanol and 5 ml. 14% hydroxylamine hydrochloride in 60% ethanol. At this stage vigorous shaking on an omnimixer is recommended to prevent clumping of tissue which would prevent the total infusion of the iron chloride.

After 5 min., while stirring vigorously 5 ml. of 1 vol. conc. HCl and 2 vol. of 60% ethanol were added followed by 15-20 ml. of 25% FeCl<sub>3</sub> in 60% ethanol containing 0.1 N HCl. The samples were then

allowed to stand for 15 min. Methylated pectin picks up the iron stain and becomes dark red in colour. After 15 min. the tissue was washed three times in 0.1 N HCl in 60% ethanol, freeze-dried, ground in a pestle and mortar, then washed a further three times in acid. After drying and grinding the tissue was ready for iron analysis. 5 mg. samples were digested in sulphuric acid and hydrogen peroxide as previously described (p.16). The iron content was determined on a Perkin Elmer 403 atomic absorption spectrophotometer at 384 nm. The readings for Samples 1 and 4 were taken to represent 0% and 100% pectin esterification respectively and thus the percentage esterification in Samples 2 and 3 could be determined.

#### Preparation of Sections for Light Microscopy

Cotyledon section 10 $\mu$  . thick were cut on a hand-cranked microtome. The cotyledons were sectioned directly without embedding. If the cotyledons were too dry for adequate sectioning then they were placed above water in a sealed container at 30°C for 1-2 h. to increase the moisture content slightly.

#### Preparation for Samples for Transmission Electron Microscopy

(Performed by Dr. N. Harris)

2 mm. cubes of cotyledon tissue were rotated in the following compounds:-

2.5% glutaraldehyde	)	
1.5% formaldehyde	)	
0.1 M. cacodylate buffer	)	2 days
pH 7.2	)	
0.1 M. cacodylate buffer		2 h. (wash)
1% OsO <sub>4</sub>		2 h. (post fix)
0.1 M. cacodylate buffer		2 x 15 min. (wash)

The tissue was then dehydrated by successive 10 min. washings in 25, 50, 75, 100 and 100% ethanol and then rotated overnight in

ethanol:resin, 1:1, followed by rotation for 8 h. in pure resin.

The samples were then cured in fresh resin at 80°C for 7 h.

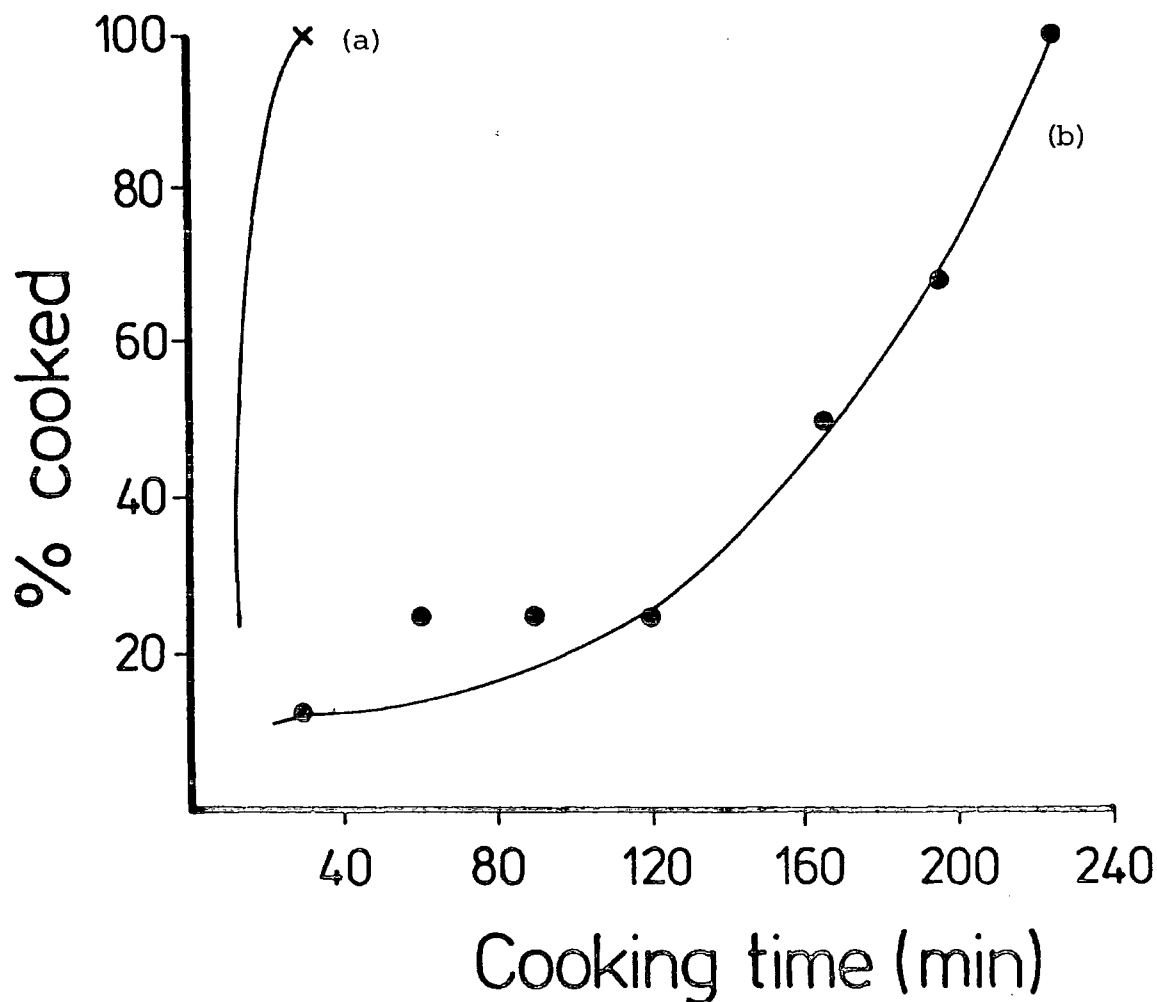
Resin:

- 10 g. Vinylcyclohexane dioxide
- 6 g. Diglycidyl ether of polypropyleneglycol
- 26 g. Nonyl succinic anhydride
- 0.4 g. Dimethylaminoethanol

RESULTSSection 1. Initial Observations

The experiments in this section were performed only on small quantities of beans because of the limited number ( $\approx 120$  soft, 120 hard) available from Guatemala. Despite this it was found that the differences between hard and soft beans were large enough for significant results to be obtained with small samples. The results presented in fig.7 show that the soft beans supplied cooked in 30 min and the hard beans cooked in 225 min.

fig.7 The cooking rate of soft (a) and hard (b) black beans.



Each sample was 8 beans.

TABLE 1.

The total cooking times (CT<sub>100</sub>) of black beans stored at different temperatures with different moisture contents

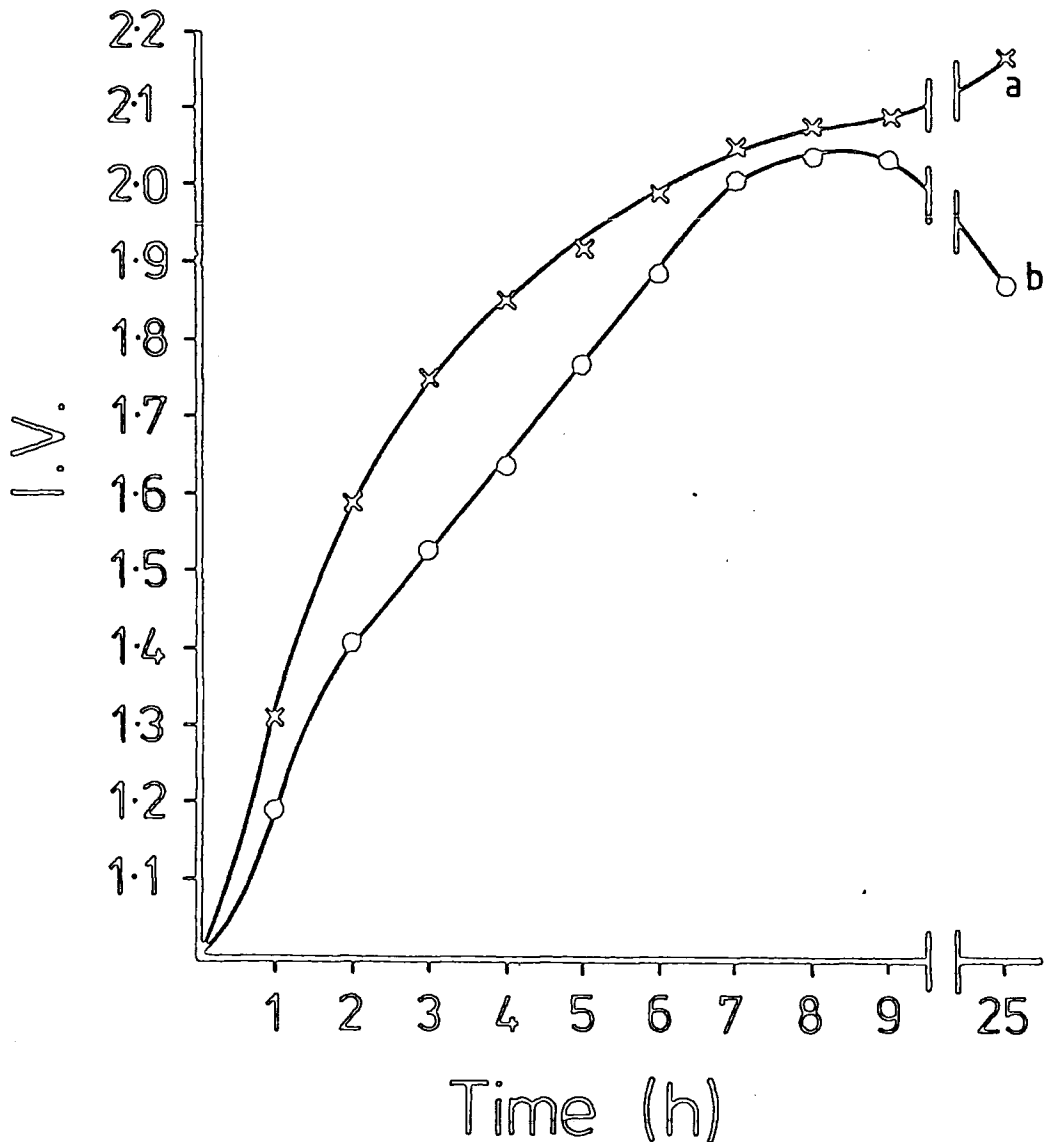
Sample	Temperature °C	Moisture Content %	Length of Storage (Months)	CT <sub>100</sub> (min.)	<i>p</i>
A	4	9	2	2000	-
B	4	9	4	2000	-
C	4	9	6	2015	-
D	4	13	4	2015	-
E	4	13	6	2030	-
F	4	17	2	2000	-
G	4	17	4	2015	-
H	20	9	2	2000	-
I	20	9	4	2000	-
J	20	9	6	2000	-
K	20	13	2	2000	-
L	20	13	6	2190	< 0.05
M	20	17	2	3075	< 0.005
N	36	9	2	2000	-
O	36	9	4	2000	-
P	36	9	6	2015	-
Q	36	13	6	5210	< 0.005
R	36	17	2	2335	< 0.05

- = Not significant.

Temperature, moisture content and time of storage data were as supplied by Dr. R. Bressani (INCAP).

These beans were a separate later batch to the first supply, supplied by Dr. R. Bressani.

fig.8 The imbibition value (I.V) of soft (a) and hard (b) black beans



Each reading was an average of ten weighings.

When the imbibition value of the beans was studied it was found to be much greater in the soft beans (2.17) than in the hard beans (1.88) as demonstrated in figure 8. Evenso, the imbibition value of hardbeans did reach 2.05 in 8h before receding due to leakage of free water back out through the testa. This is explained by figure 10 which illustrates the condition of beans after the completion of imbibition. The soft bean cotyledons (a) had swollen and filled the space within the expanded testa. The hard bean cotyledons (b) though had not filled the entire space

within the expanded testa and thus a "water jacket" developed which effectively made the bean appear heavier than it really was. During repeated handling the testa tended to rupture and consequently released the water within, thus giving lower readings later in the experiment.

The difference in cooking rates of hard and soft beans was still apparent despite the removal of the testa (fig.9 below).

Another difference between hard and soft beans is illustrated in fig.11 which shows that the soft beans had a germination capacity of 100% but the hardbeans were completely inviable. However respiration was still detectable in hard beans at  $\frac{1}{5}$  the rate in soft beans. (see fig.12 ).

fig. 9. The softening rate of shelled soft (a) and hard (b) beans.

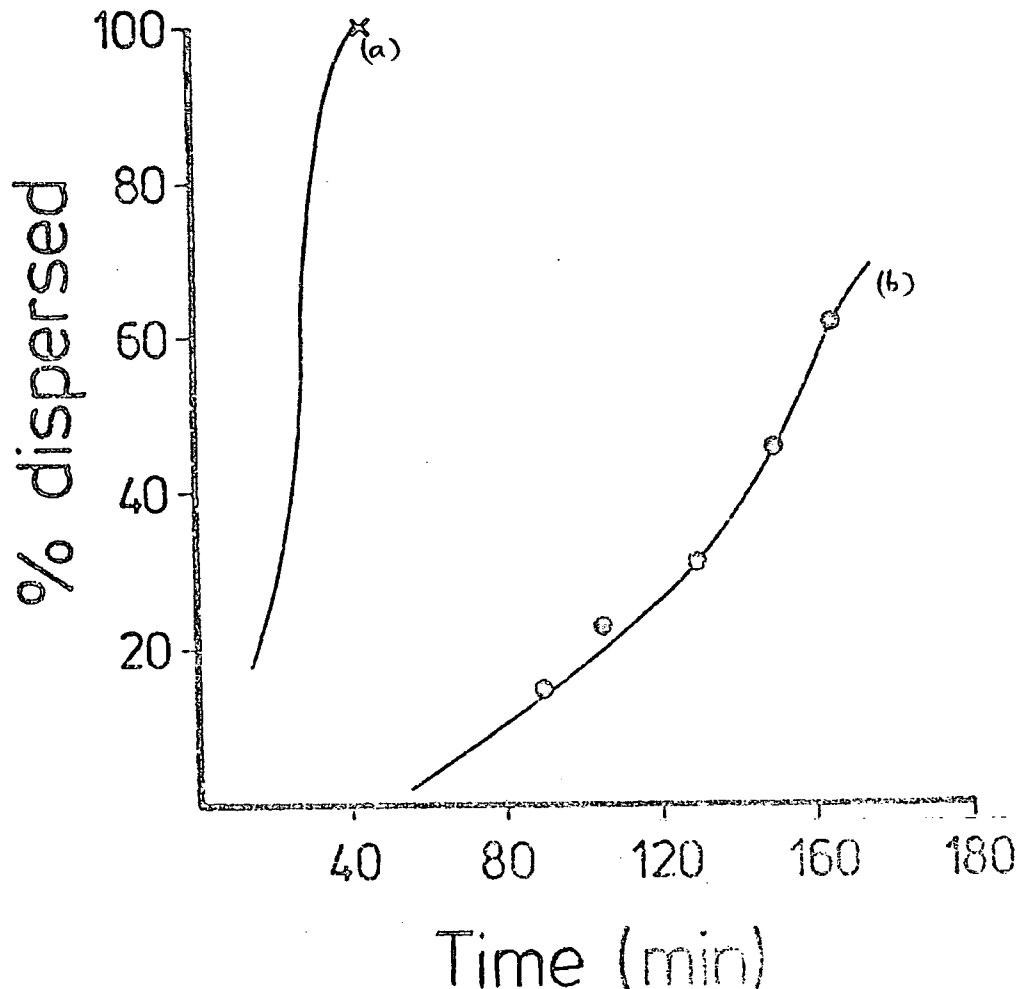




fig.10 Longitudinal diagrams of soaked black beans.

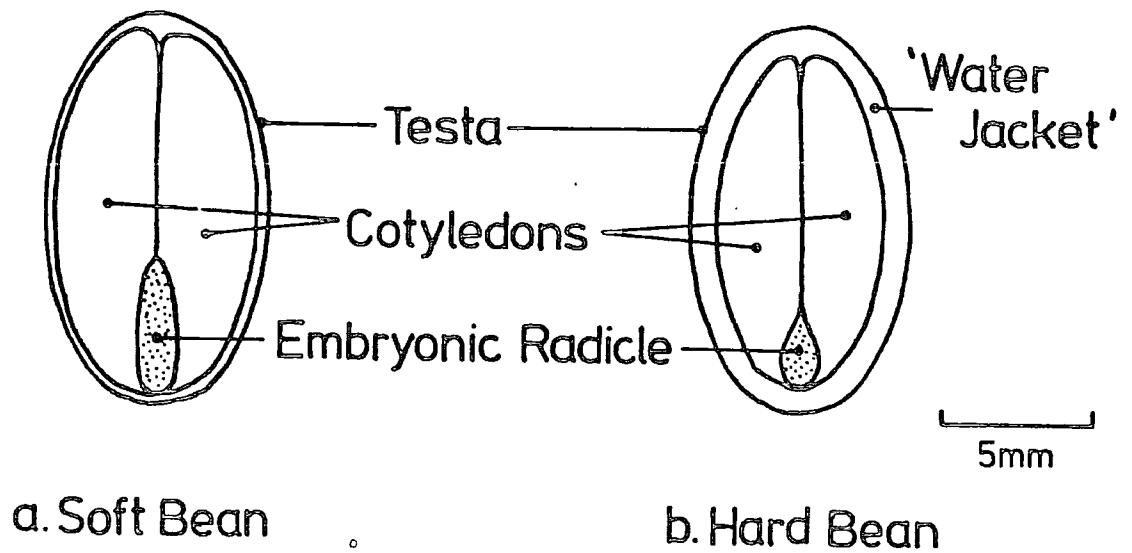
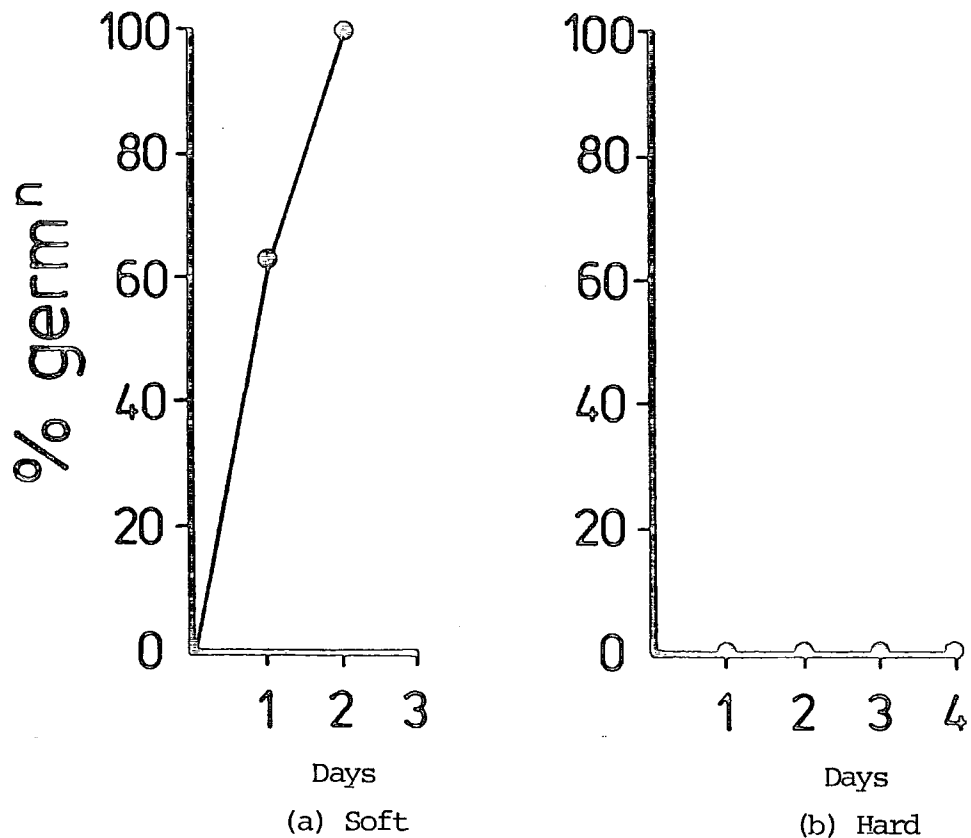
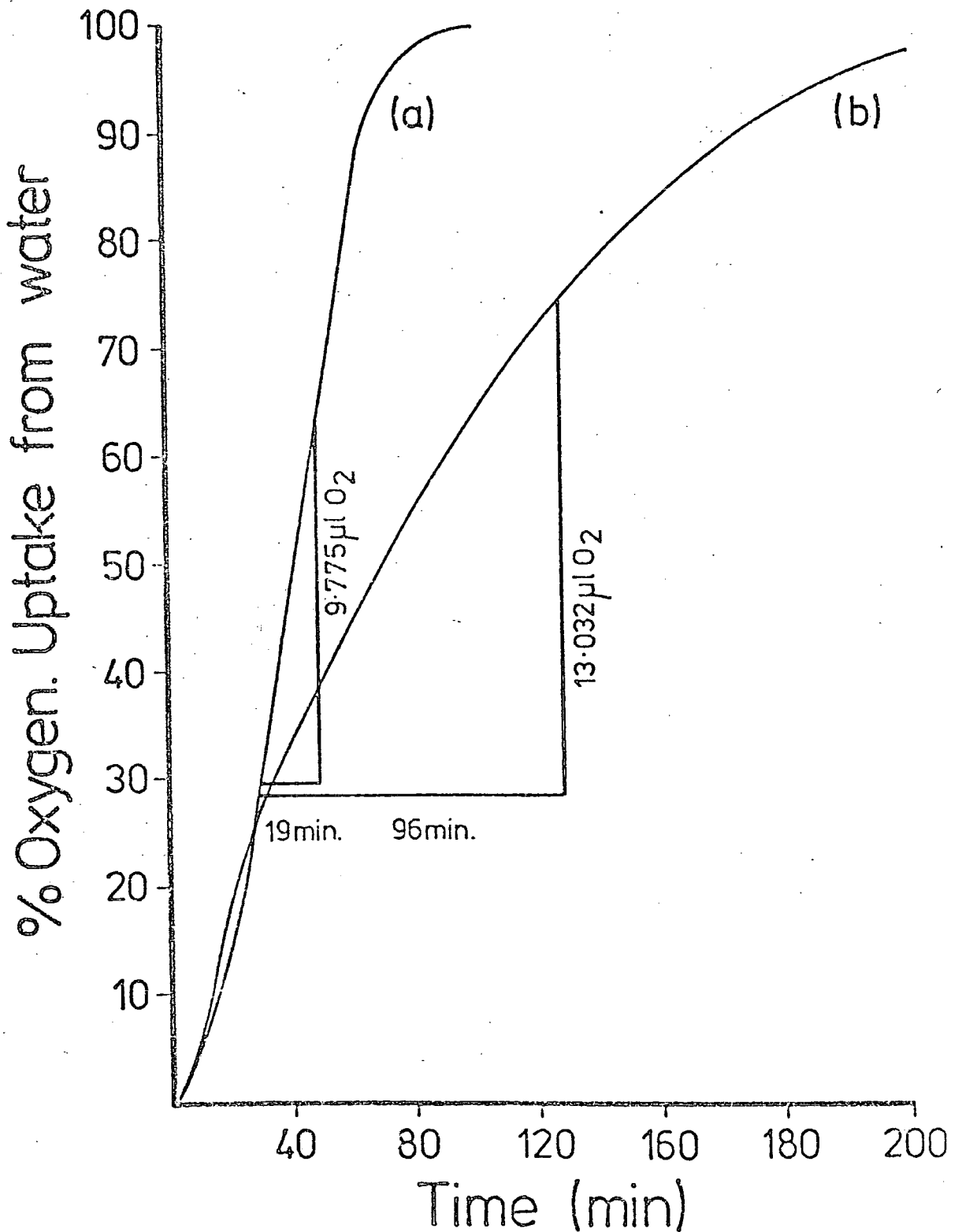


fig.11 The germination capacity of black beans.



Each germination trial was carried out on 10 beans.

fig. 12. The respiration rate of soft (a) and hard (b) bean cotyledons



Weight of soft bean sample 200.3 mg - respiration rate  $2.57 \mu\text{l O}_2 \text{min}^{-1} \text{g}^{-1}$   
 " " hard " " 231.2 mg " " 0.52  $\mu\text{l}$  " " "

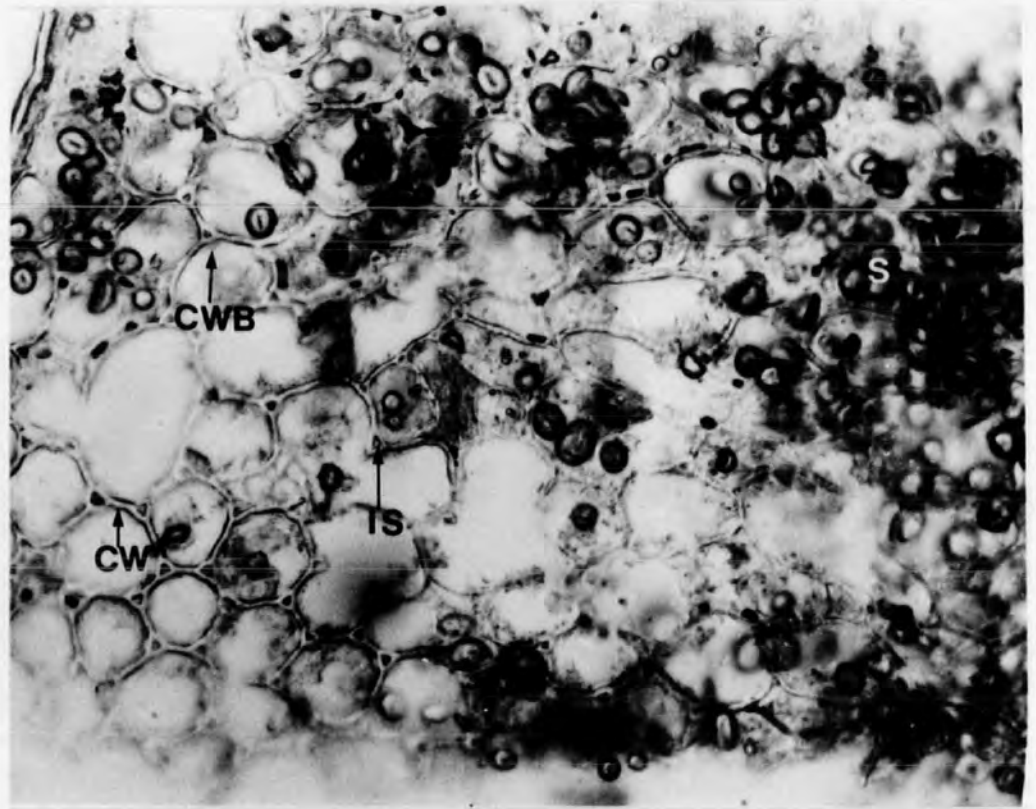
figure 12 is a direct copy from a chart recorder trace link to an oxygen electrode and represents two typical readings obtained.

When water was added to dry bean sections, both hard and soft samples expanded instantaneously (figs.13 and 14 ). There was no measurable difference in cell size between hard and soft beans.

Light micrographs of soft and hard beans after 30 min boiling are presented in figures 15 and 16 respectively and it was clear from these micrographs that the soft bean cells had separated without cell rupture while the hard bean cells had failed to separate, remaining firmly cemented along the middle lamella.

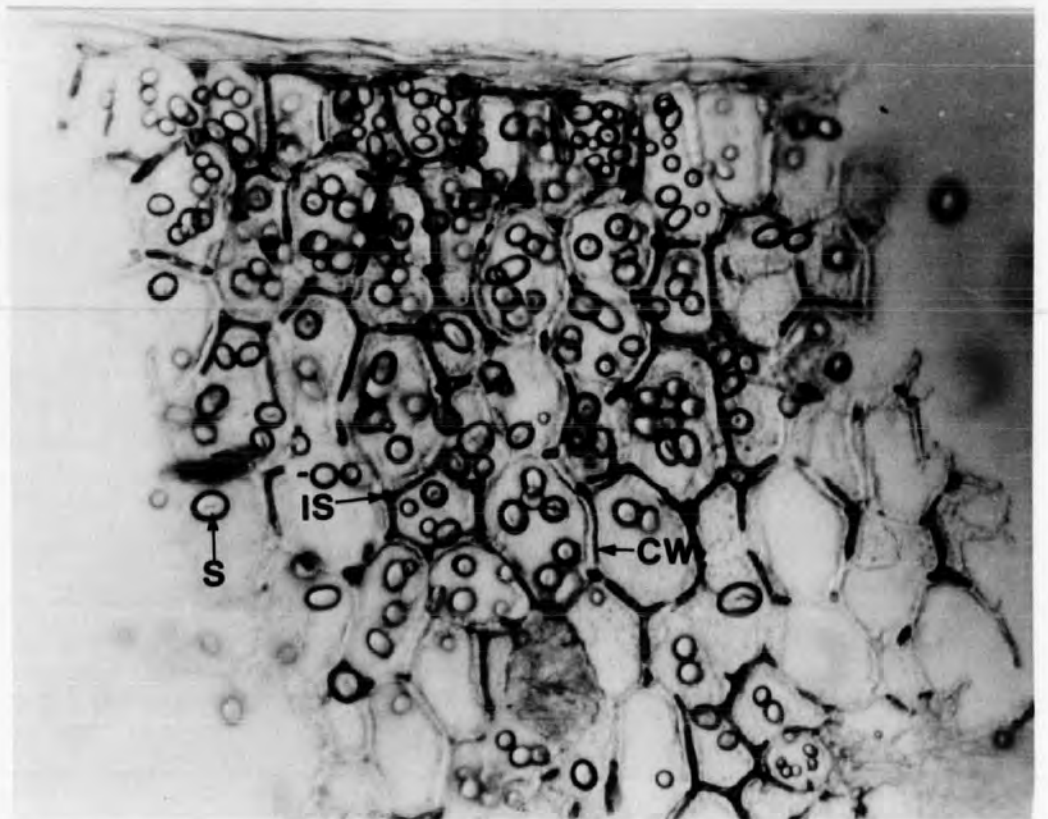
FIGS.13 and 14. Dry sections of soft (Fig.13 ) and hard (Fig.14 ) bean sections after the addition of water.

Fig.13



x 100

Fig.14



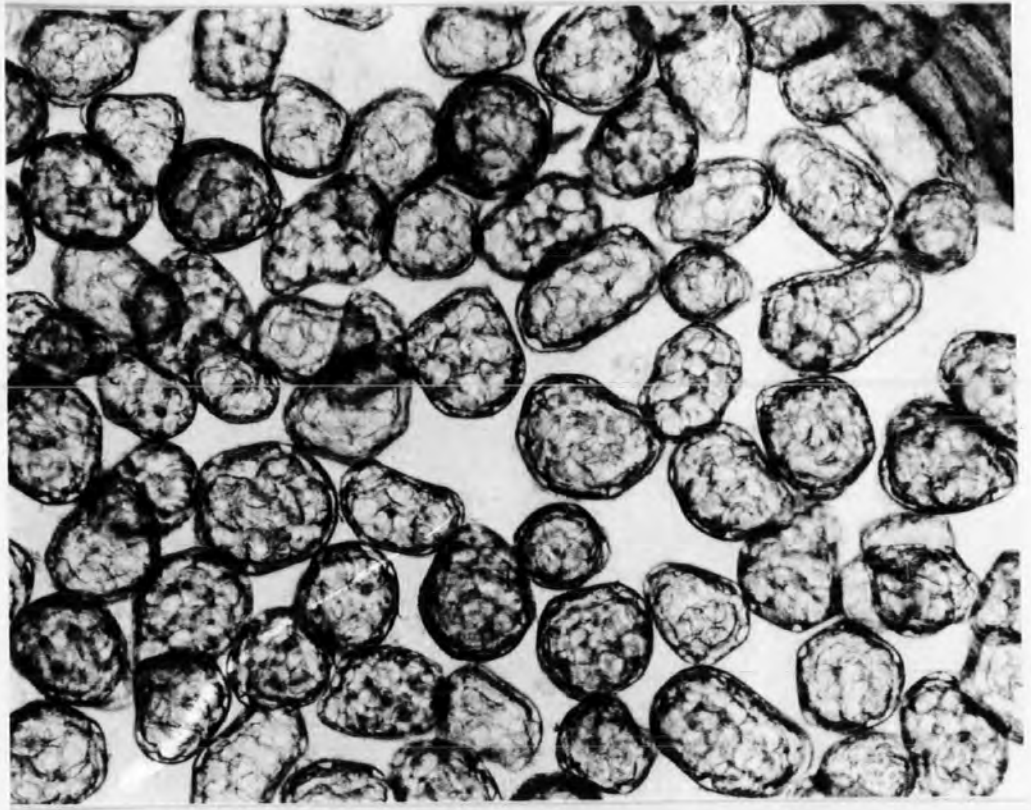
x 100

CW - Cell wall

S - Starch grain

CWB - Cell wall body

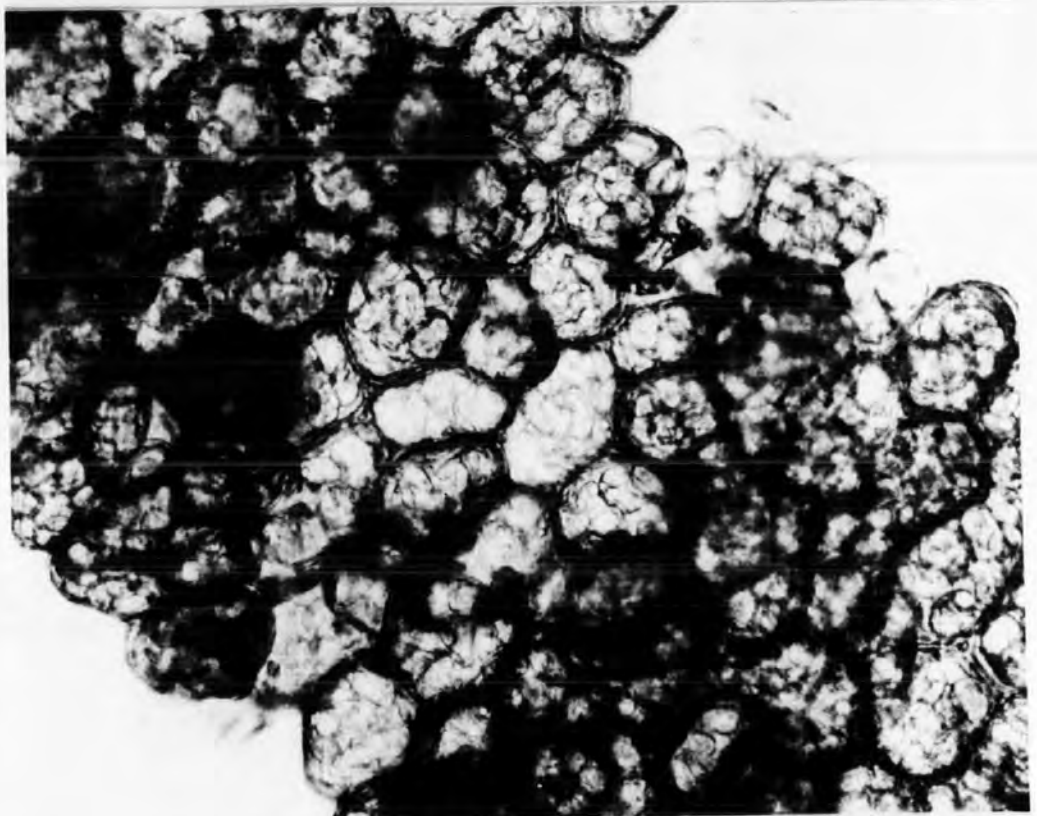
IS - Intercellular space



X 100

Fig.15 . Light micrograph of cells from a soft bean cotyledon after 30 min. boiling.

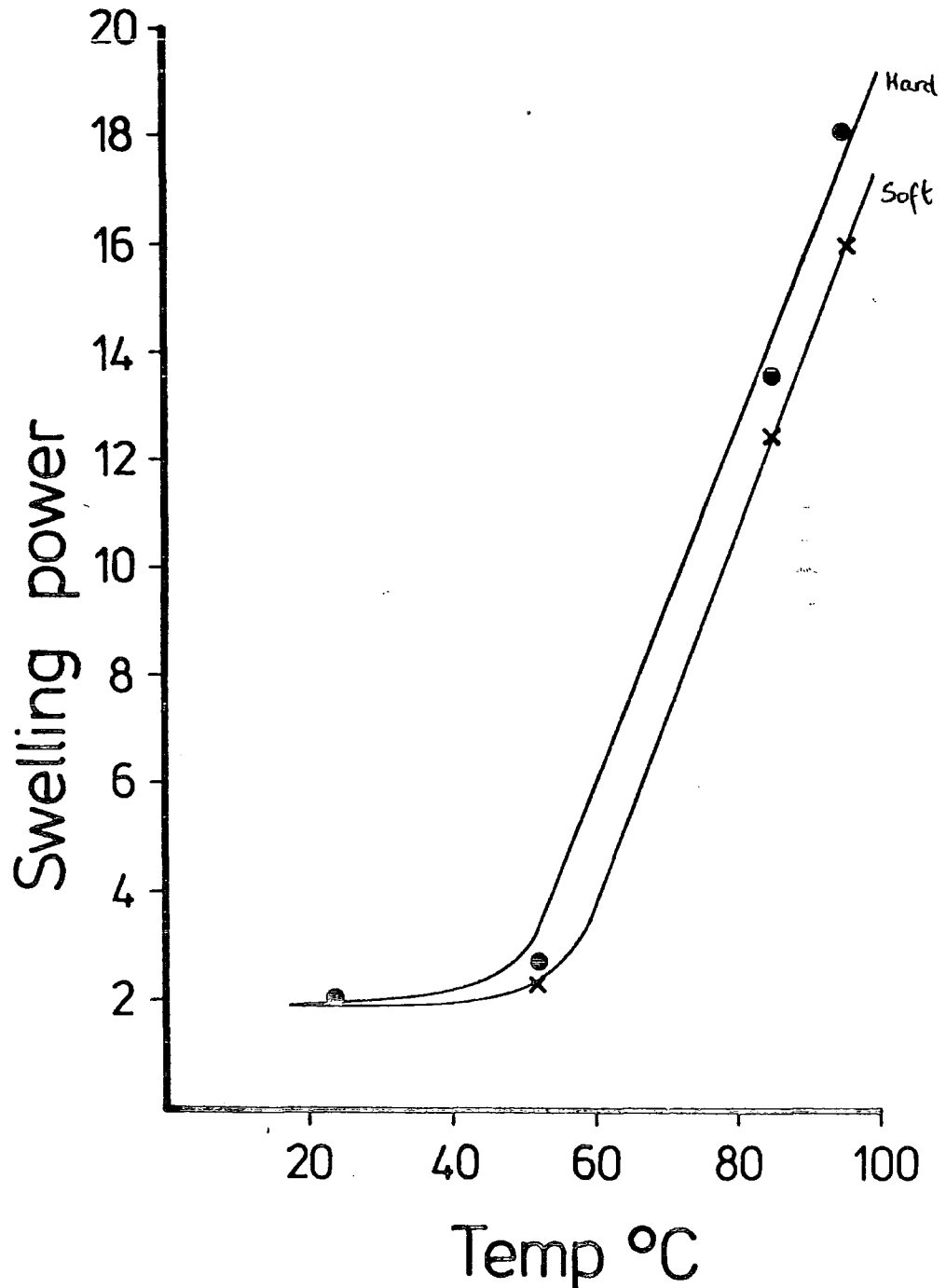
Fig.16 Light micrograph of cells from a hard bean cotyledon after 30 min. boiling.



X 100

Section 2. Investigation into the cause and effects of reduced imbibition value.

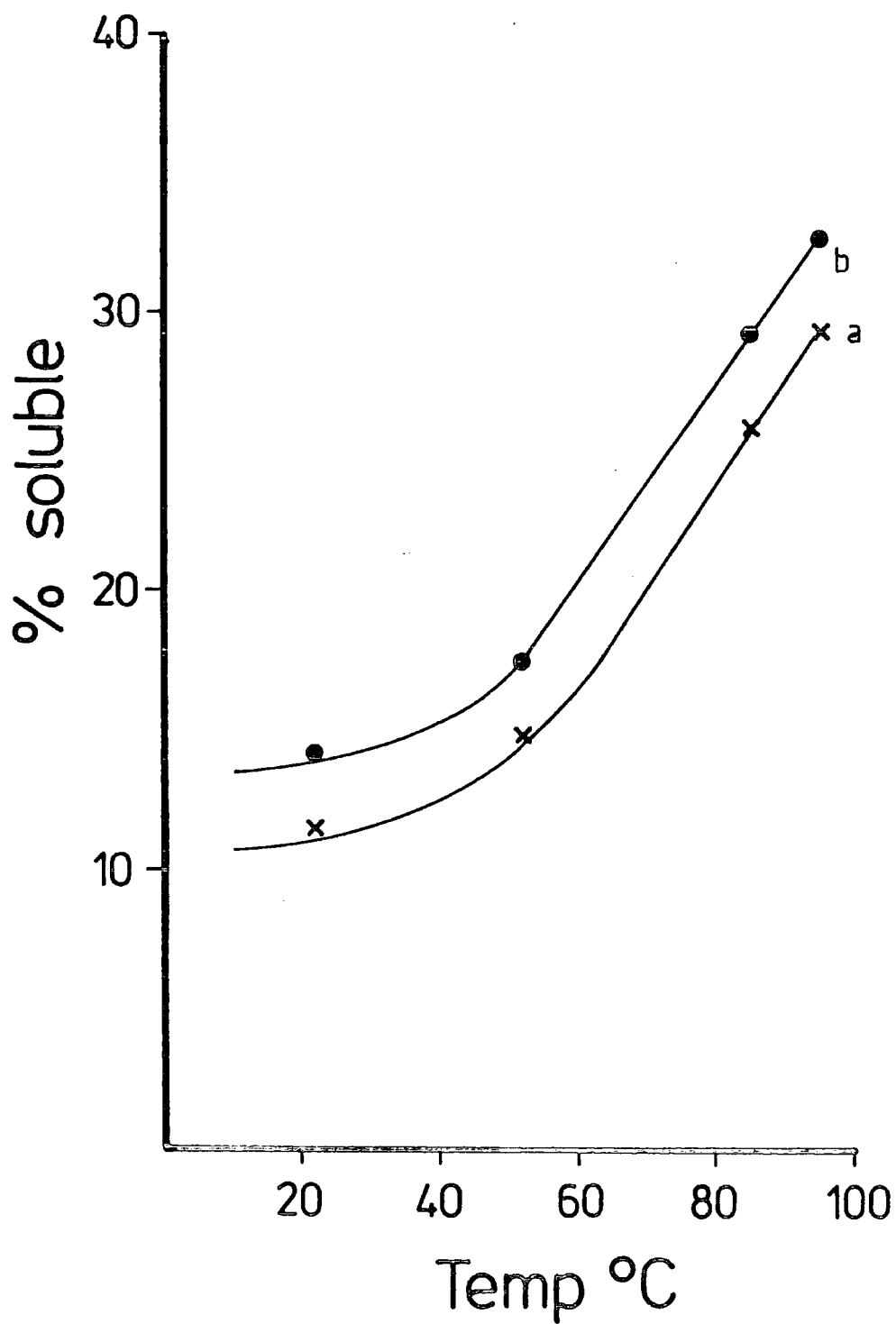
fig. 17. The swelling power of starch grains between 20°C and 100°C.



The swelling power units represent the water holding capacity of the starch grains and are expressed as multiples of the starch samples dry weight.

The experiments in chapters 2 & 3 were performed on a second generation of beans produced in the university greenhouse and stored in the department at 4°C (control) or at 34°C for 5 months to develop hardness.

fig.18 The solubility of extracted soft (a) and hard (b) bean starch grains.



The swelling power of starch grains in water was investigated at room temperature, 52°C, 85°C and 95°C. As Fig. 17 shows this property was greater in hard bean starch grains than in soft bean starch grains at all temperatures. Additionally, hard bean starch was 2.6 to 3.3% more soluble at all temperatures (Fig. 18).

Leakage of material out of the cotyledons was much higher from hard beans than soft beans, the data for which is shown below. Sugar leakage had increased seven-fold and total leakage had increased nine-fold.

TABLE 2.

The leakage of solids and electrolytes from  
black beans

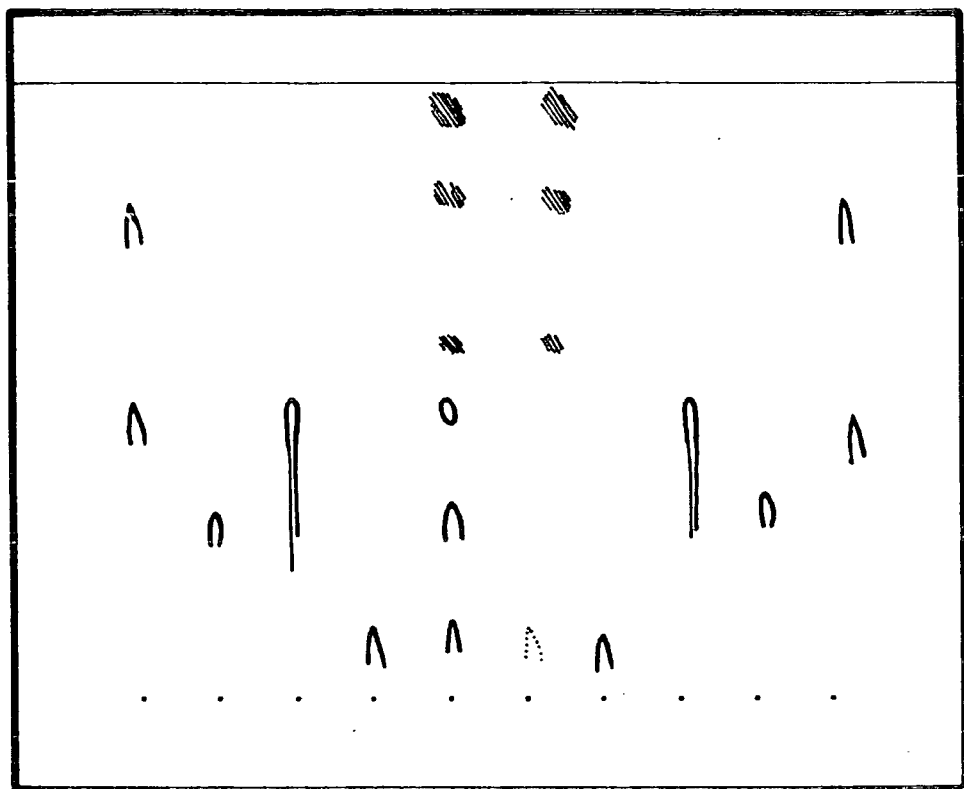
	<u>Soft Beans</u>	<u>Hard Beans</u>
Sugars. mg. gm. <sup>-1</sup>	10	66
Total solids. mg. gm. <sup>-1</sup>	21	195
Electrolytes*.	263	2311

\*Electrolytes expressed as mhos gm.<sup>-1</sup>  
d.wt. in 20 ml. deionised water.


Fig. 19 is a diagram of a TLC plate of phospholipids extracted from dry hard and soft bean cotyledons. It is clear that while phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine were present in the soft bean sample, they were absent from the hard bean sample except for a trace of phosphatidylinositol.



fig.19 TLC plate of phospholipids extracted from soft and hard beans.



PA PC PE PI S H PI PE PC PA

PI	Phosphatidylinositol	$1.4 \text{ mg ml}^{-1}$
PC	Phosphatidylcholine	$1.0 \text{ mg ml}^{-1}$
PE	Phosphatidylethanolamine	$1.4 \text{ mg ml}^{-1}$
PA	Phosphatidic acid	$1.4 \text{ mg ml}^{-1}$
S	Soft bean lipid extract	$10 \mu\text{l } 20 \text{ mgml}^{-1}$
H	Hard bean lipid extract	$10 \mu\text{l } 20 \text{ mgml}^{-1}$
	Galactolipids	

A negative correlation ( $r = -0.98$   $p < 0.001$ ) is demonstrated between the imbibition value (I.V.) of black beans and leakage of solids from them in fig. 20 below. In healthy beans the I.V. varied between 2.15 and 2.30 and the leakage between 14 and 21  $\text{mg g}^{-1}$  dwt. As the I.V. dropped to 1.72 the leakage increased to 195  $\text{mg g}^{-1}$  dwt.

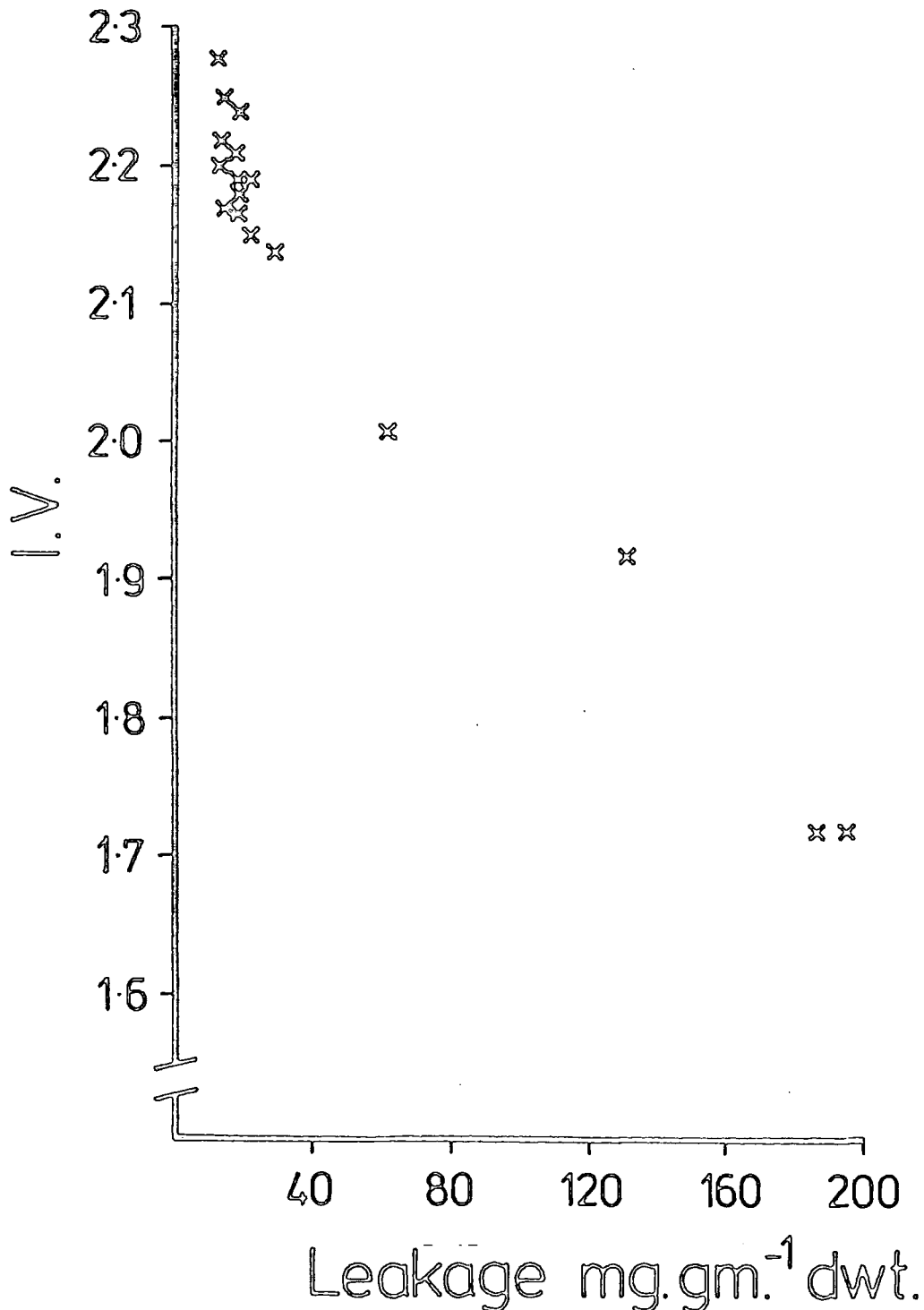
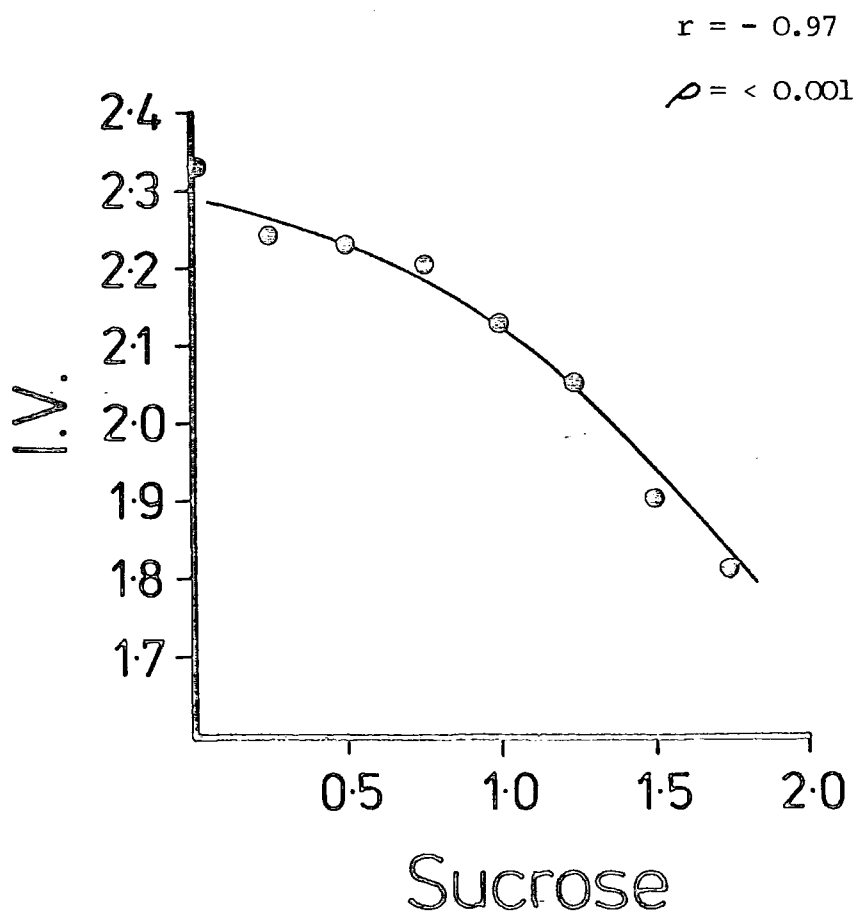


fig. 20 The relationship between imbibition value (I.V.) and total solids leakage from black beans.

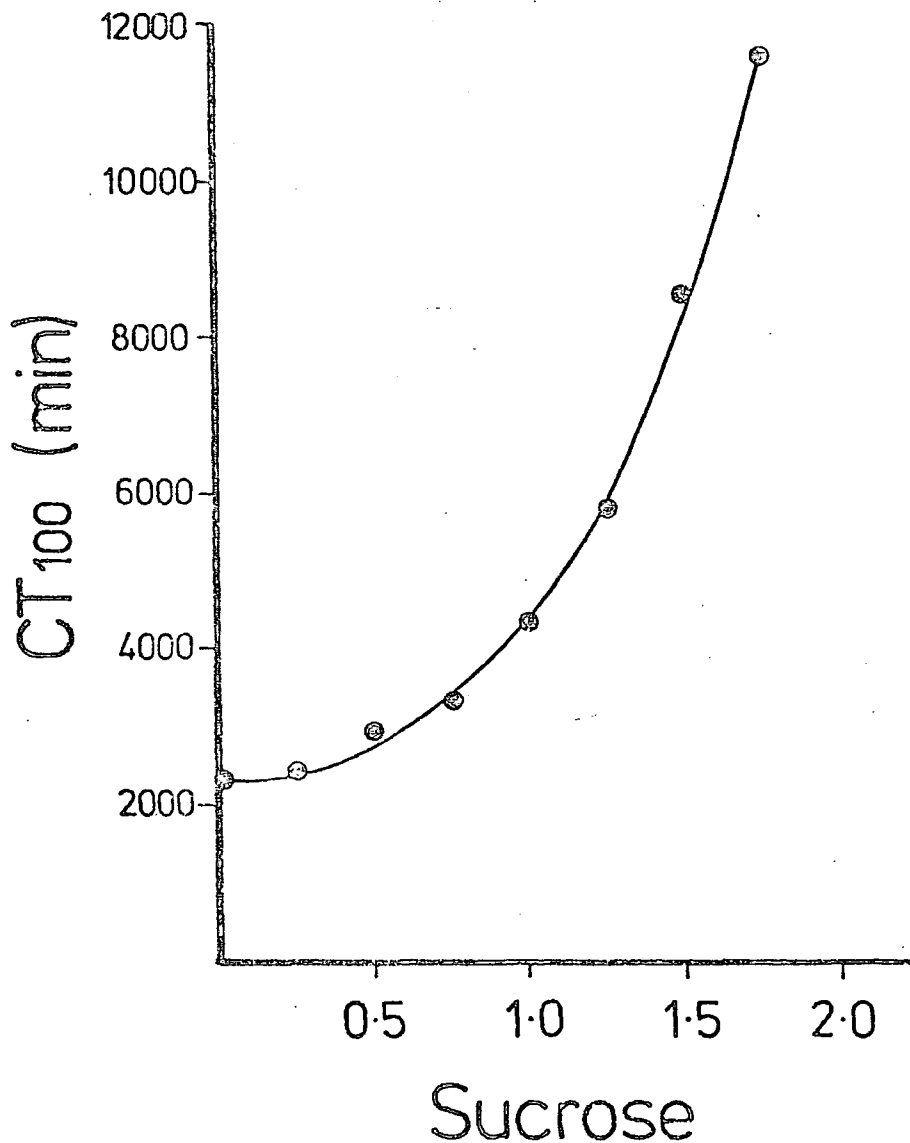
The effect of sucrose concentration during soaking and cooking was examined and the results given in figs. 21 and 22. As the sucrose concentration increased to 1.75M the imbibition value dropped from 2.32 to 1.81 and the total cooking time concomitantly increased from 2300 min to nearly 12000 min in an almost logarithmic relationship. By compounding the information from figures 21 and 22 a relationship can be graphically illustrated between artificially reduced imbibition value and the total cooking time (see fig.23). The relationship took the form of a strong negative correlation, the lower the imbibition value, the higher the cooking time.

fig. 2 1 The effect of sucrose concentration during soaking on the imbibition value (I.V) of black beans.



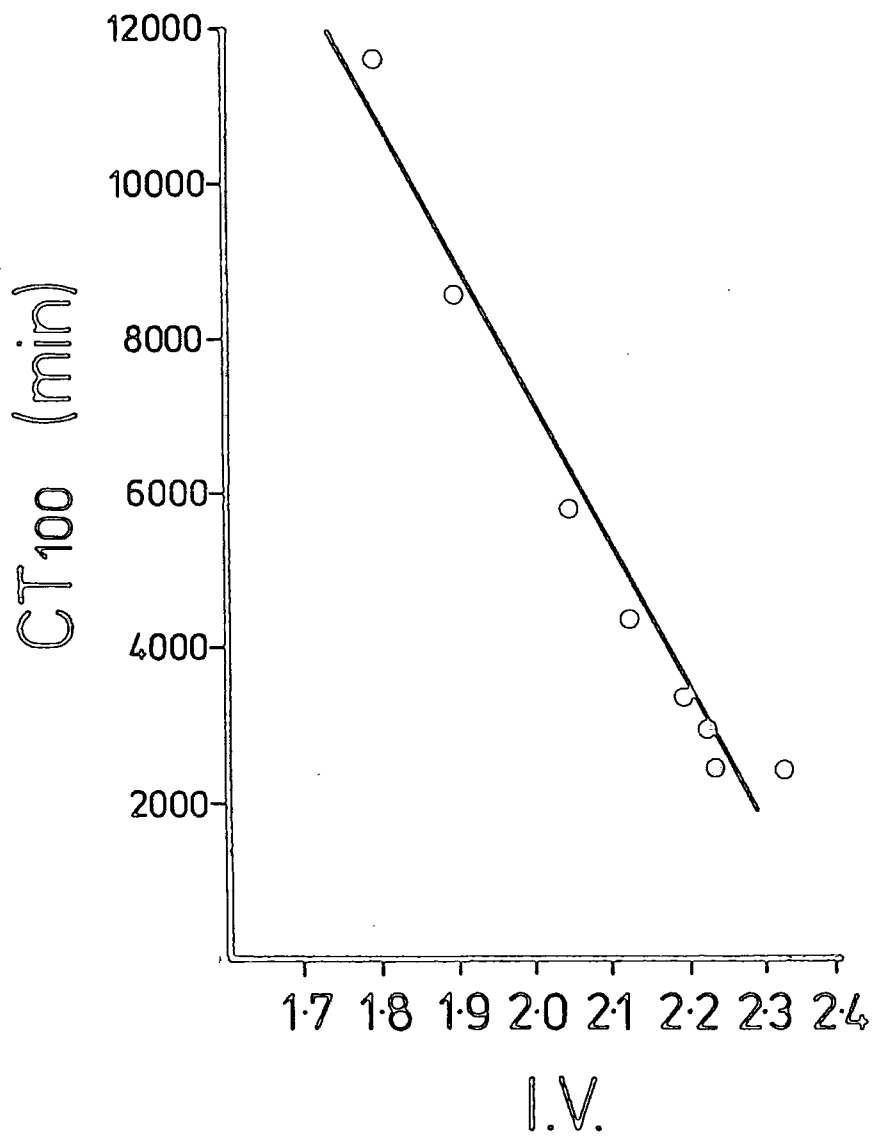
Evidence of a direct link between imbibition value and cooking time is further corroborated by figures 24, 25 and 26. During the 20 min it took to cook a batch of soft beans the imbibition value rose from 2.36 to 2.44 (fig.24) while the imbibition value of hard beans failed to exceed 2.10 (fig.25) throughout the 90 min required to soften the beans. In addition, the eventual softening of beans whose imbibition value had been restricted to 2.10 by soaking in 1.25M sucrose coincided with the imbibition value reaching 2.34 (see fig.26).

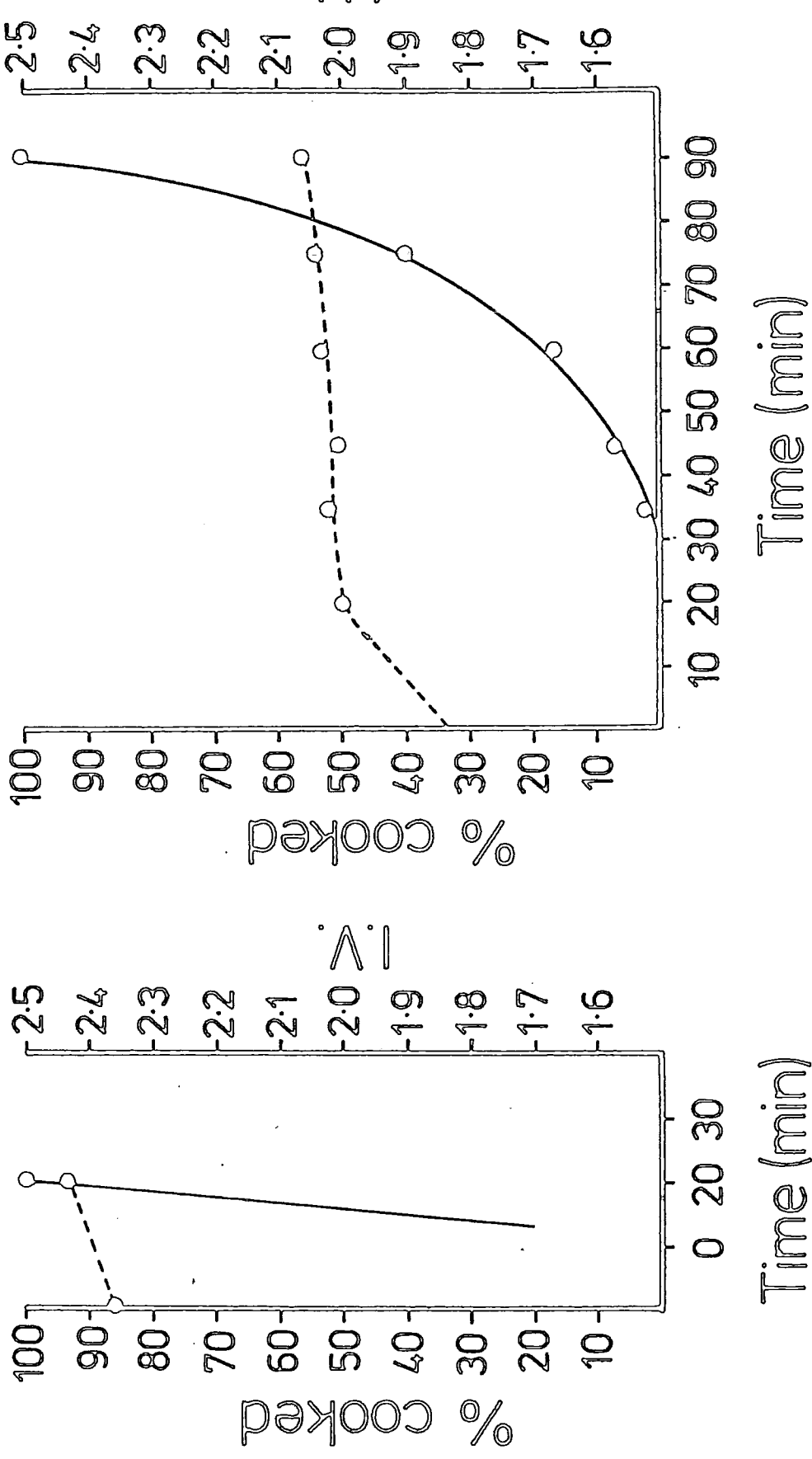
fig. 22. The influence of sucrose concentration on total cooking time.



Both the I.V measurements (fig.21) and the CT<sub>100</sub> measurements (fig.22) were carried out on the same beans. Each sample comprised of approximately 70 beans.

fig. 23 The correlation between artificially reduced imbibition value (I.V.) and total cooking time (CT<sub>100</sub>) of black beans.

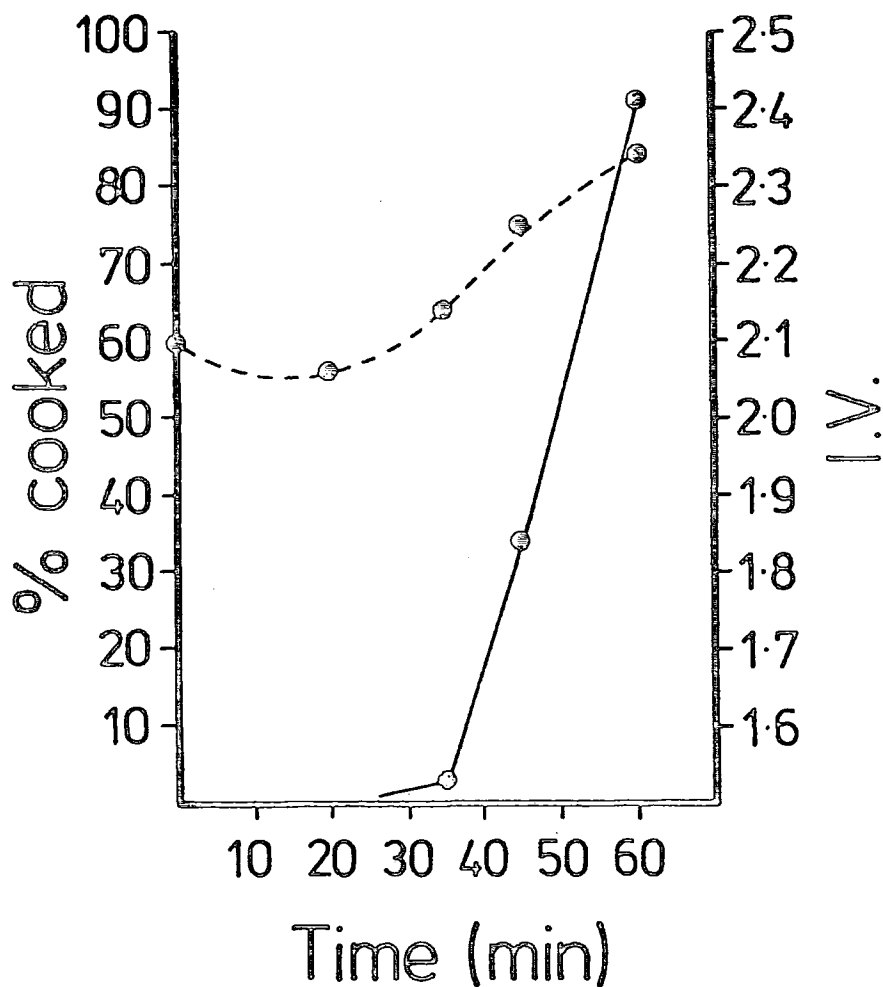




figs. 24 & 25 . The cooking rate (—) and imbibition value during cooking (---) of soft (fig. 24 left) and hard (fig. 25 right) black beans.

Sample sizes were 62 and 58 beans respectively.

fig.26 The effect of 1.25M sucrose on the imbibition value and cooking rate of soft black beans.



--- imbibition value

— cooking time

Sample size 59 beans

Beans were soaked and cooked in 1.25 m sucrose.

When the 8 batches of beans whose imbibition value had been restricted with increasing concentrations of sucrose were cooked, the imbibition value was measured on the completion of cooking as well as before cooking. From this data, presented in Table 3, it was found that in all these batches the imbibition value reached or exceeded 2.38 as the beans softened.

Table 3.

The imbibition value of beans before and  
after cooking

<u>Sucrose M</u>	<u>I.V Before cooking</u>	<u>I.V After cooking</u>
0	2.33	2.44
0.25	2.24	2.38
0.50	2.23	2.43
0.75	2.20	2.47
1.00	2.13	2.46
1.25	2.05	2.43
1.50	1.90	2.43
1.75	1.80	2.45

Sucrose refers to the concentration of sucrose in the soaking and cooking water.

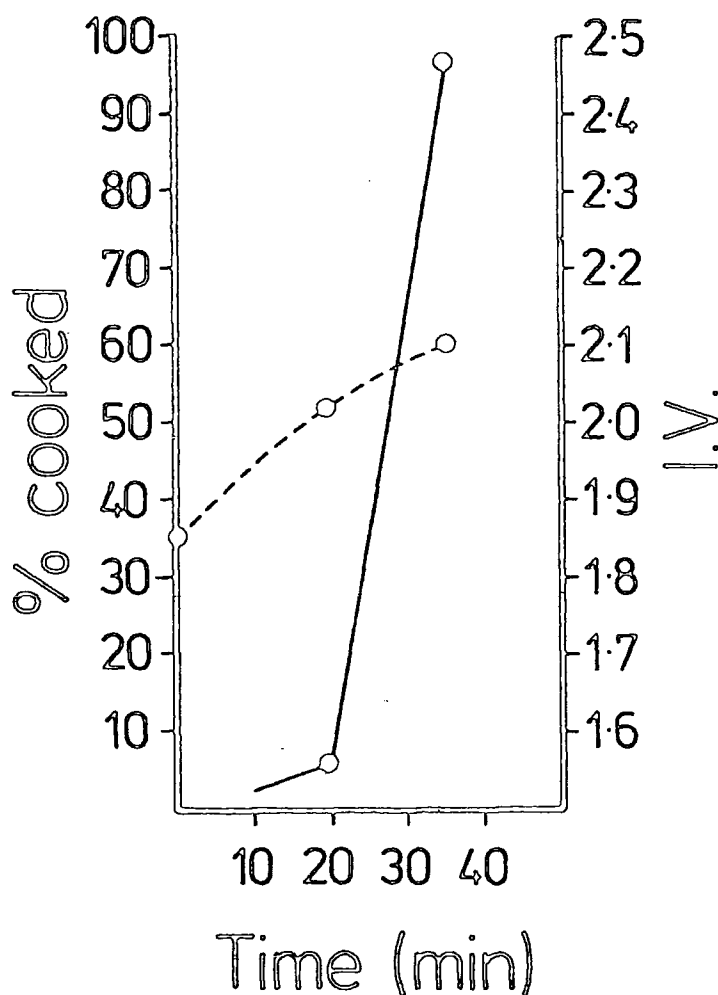
I.V values after cooking were taken within 5 min. of cooking.

All samples were between 65. and 70 beans.



By treating soft beans with hot 80% ethanol leakage can be induced. Beans treated thus had an imbibition value of 1.85 in water which rose to 2.10 during the 35 min required to cook the beans. See fig. 27

fig.27 The imbibition value and cooking rate of soft black beans after Soxhlet treatment.



--- imbibition value

— cooking time

The bean sample (32 beans) was treated with hot 80% ethanol for 15 h and allowed to dry before treatment. Imbibing and cooking were performed as normal in distilled water.

An investigation was also carried out into the rate of cell separation during boiling of ball-milled cotyledon tissue. figures 28 and 29 are scanning electron micrographs of macerated soft bean tissue after 30 min and 120 min boiling respectively. It is evident from the micrographs that the cells failed to separate and remained adhering along the middle lamella, though after 120 min there appears to be indications of general cell wall breakdown.

When cotyledon sections were stained with toluidine blue and viewed under the light microscope it was possible to see semi-circular bodies adjacent to the cell wall running the length of the intercellular space. figures 30 and 31 clearly show that these structures were greatly reduced in size in cells of hard bean cotyledons.

Further analysis of these structures under the transmission electron microscope (figures 32 and 33 ) revealed the presence of fine fibrillar and/or vesicular structures within these cell wall bodies which were much more apparent and tightly packed in hard bean sample. The cytoplasm of soft and hard bean cotyledons was also examined and the transmission electron micrographs are shown in figures 34 and 35 respectively. In both samples the dominance of protein bodies was noticeable but membranous organelles such as mitochondria, tubular endoplasmic reticulum and vesicular bodies were only apparent in the soft bean sample.

FIGS. 28 and 29 . Scanning electron micrographs of macerated bean cotyledon cells after 30 min. (Fig. 28) and 120 min. (Fig. 29) boiling.

Fig. 28 .

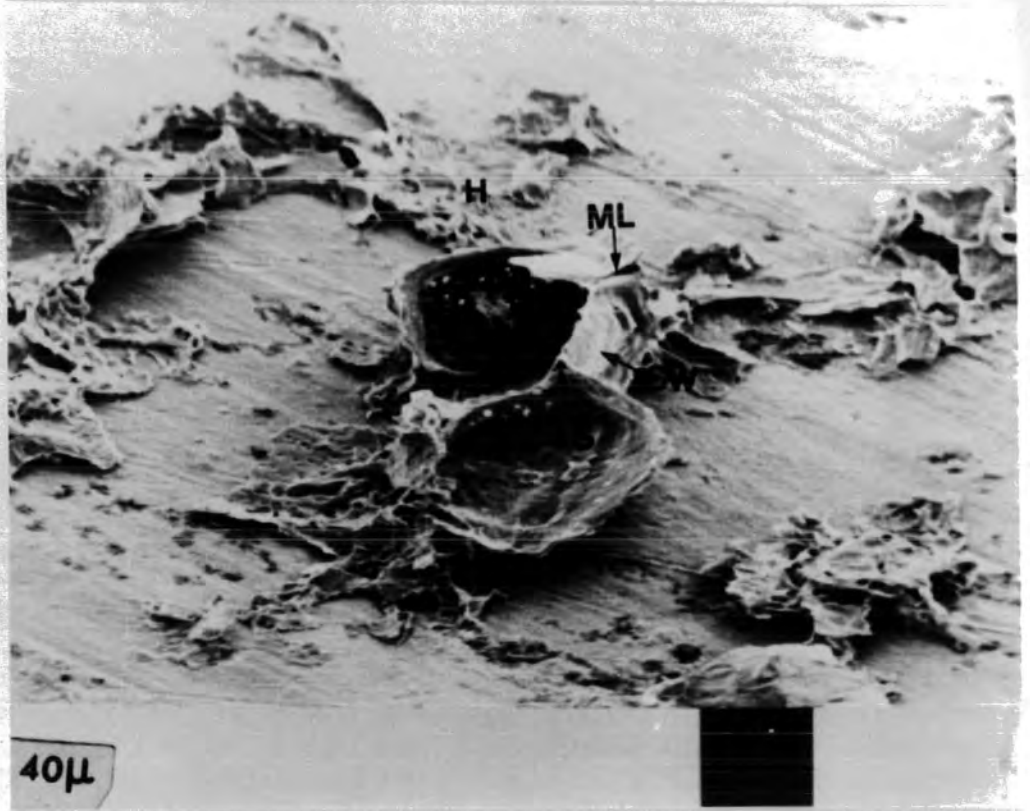


Fig. 29 .



CW - Cell wall  
IS - Intercellular space  
ML - Middle lamella

P - Plasmodesmata pit  
field  
H - Hydrolysed cellular  
contents

FIGS. 30 and 31. Cell wall and intercellular space detail of soft (Fig.30) and hard (Fig.31) bean cotyledons.

Fig.30 .

X 350

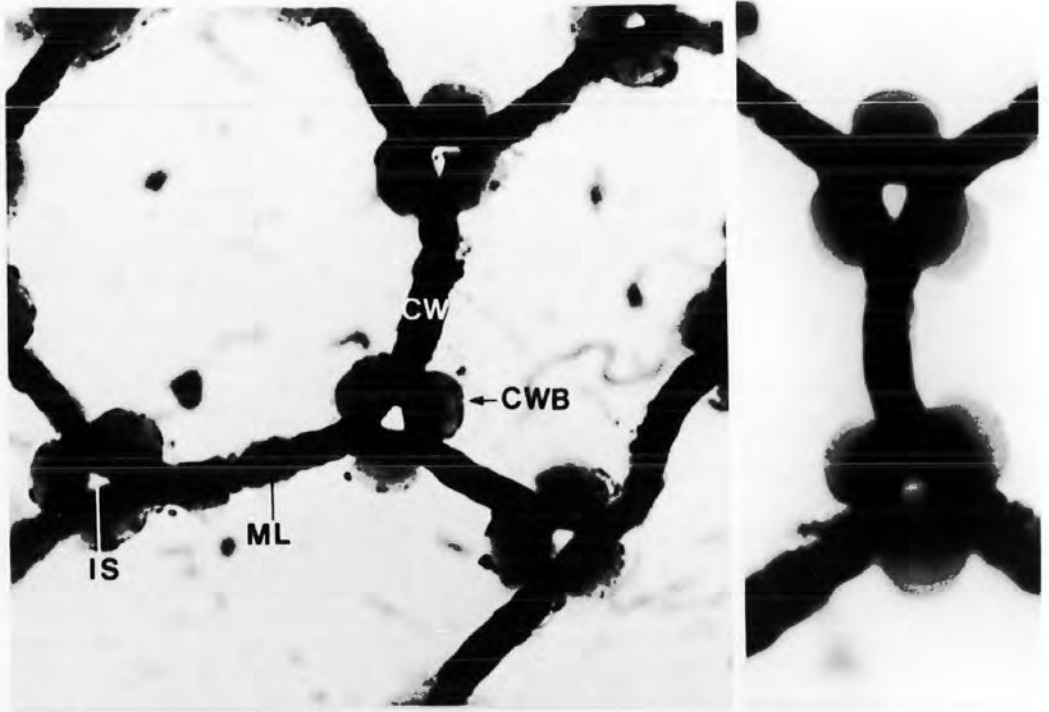
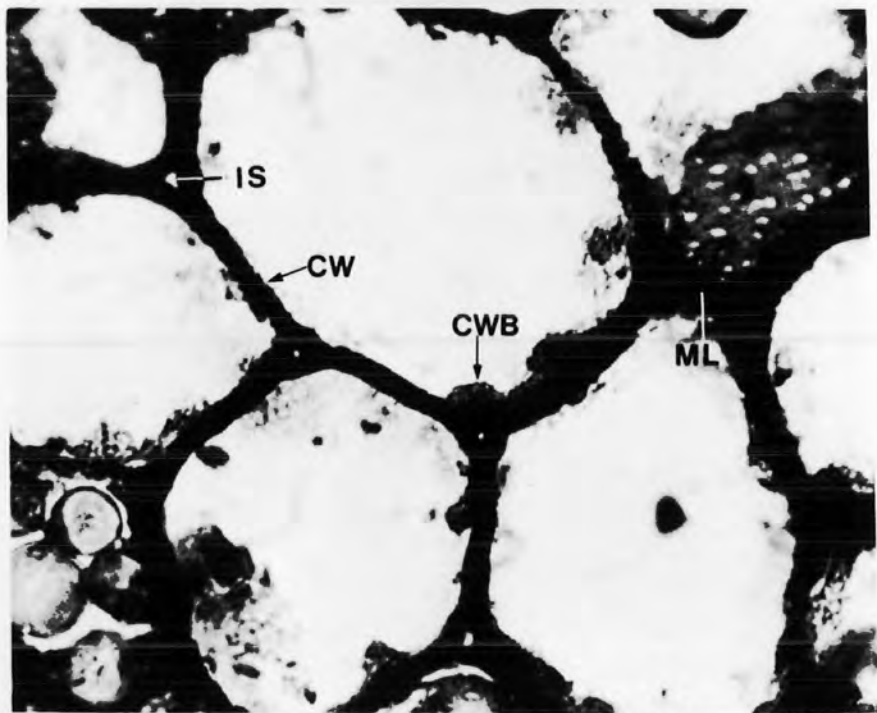


Fig.31 .

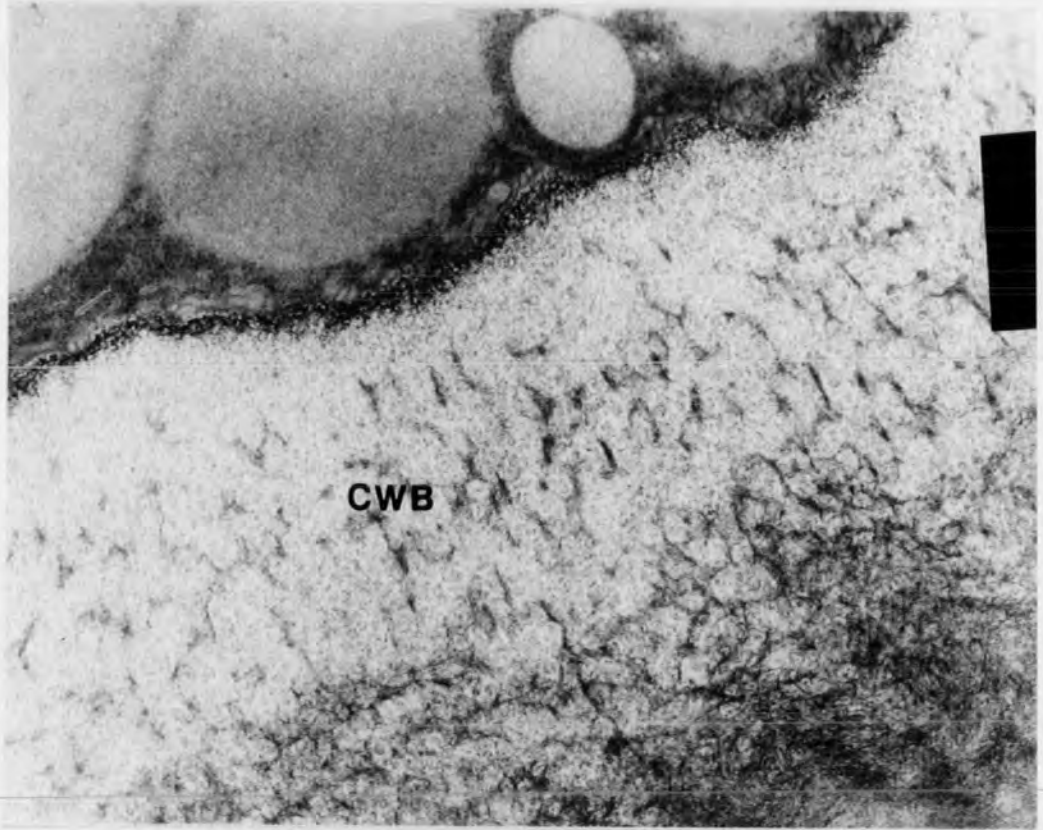
X 350



CW - Cell wall  
IS - Intercellular space

CWB - Cell wall body  
ML - Middle lamella

Note the lamellar structure of the cell wall body in soft beans (see inset) and also the thicker cell wall than that in hard beans.



X 29500

Transmission electron micrographs of cell wall body adjacent to intercellular space in soft (Fig. 32 above) and in hard (Fig. 33 below) bean cotyledons.



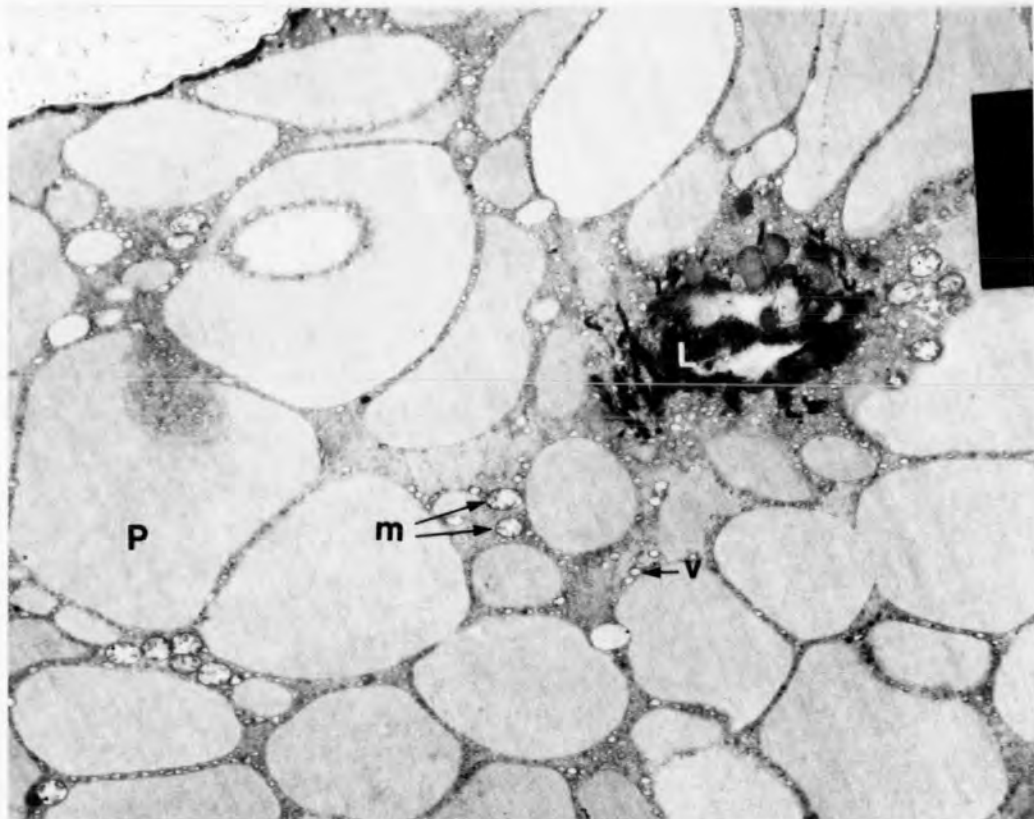
CW - Cell wall  
CWB - Cell wall body

IS - Intercellular space  
ML - Middle lamella

X 13500

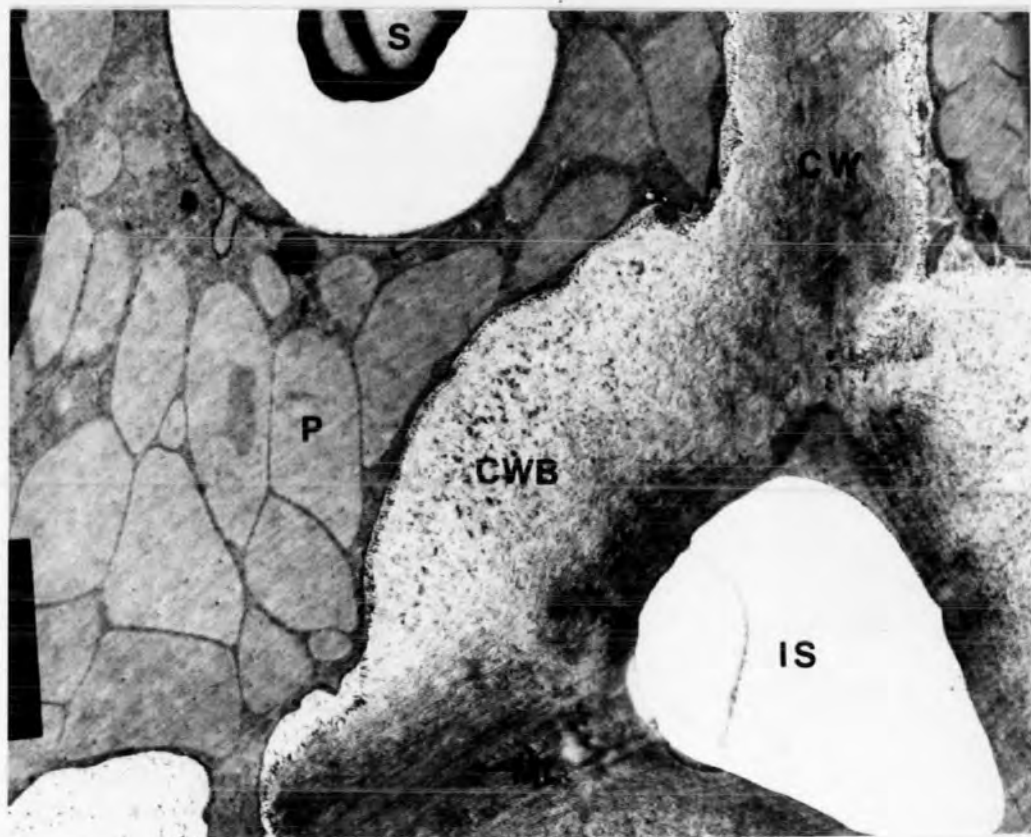


Fig.34



X 10500

Fig.35



X11000

Transmission electron micrographs of soft (Fig. 34) and hard (Fig.35) bean cytoplasm.

CW - Cell wall  
 ML - Middle lamella  
 m - Mitochondria  
 T - Tubular endoplasmic  
     reticulum  
 L - Lipid body

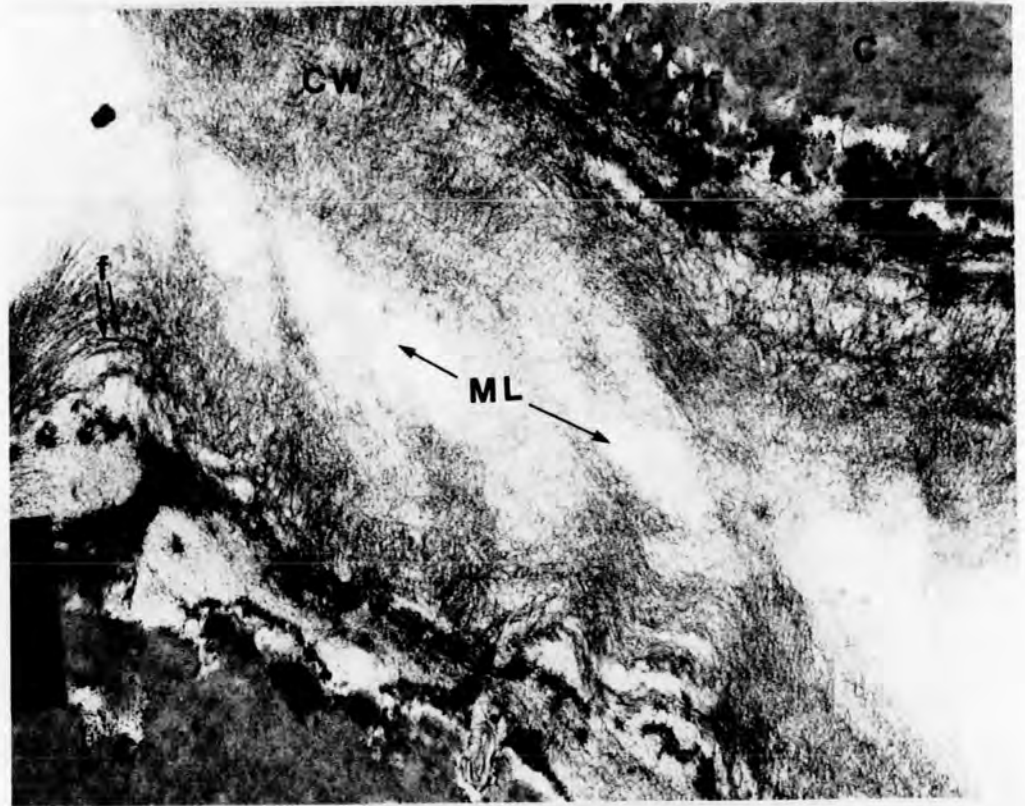
CWB - Cell wall body  
 IS - Intercellular space  
 v - Vesicles  
 P - Protein body  
 S - Starch grain

Section 3Investigation into the cause and effects of pectin solubility changes

Transmission electron micrographs of adjacent cell walls from soft and hard bean tissue are shown in figure 36 and 37 respectively. The cells of the soft bean had nearly separated, the cell walls were frayed and individual cellulose fibrils were clearly visible. In the hard bean samples the two adjacent cell walls were still lightly adherant and there was no indication of cell wall disruption.

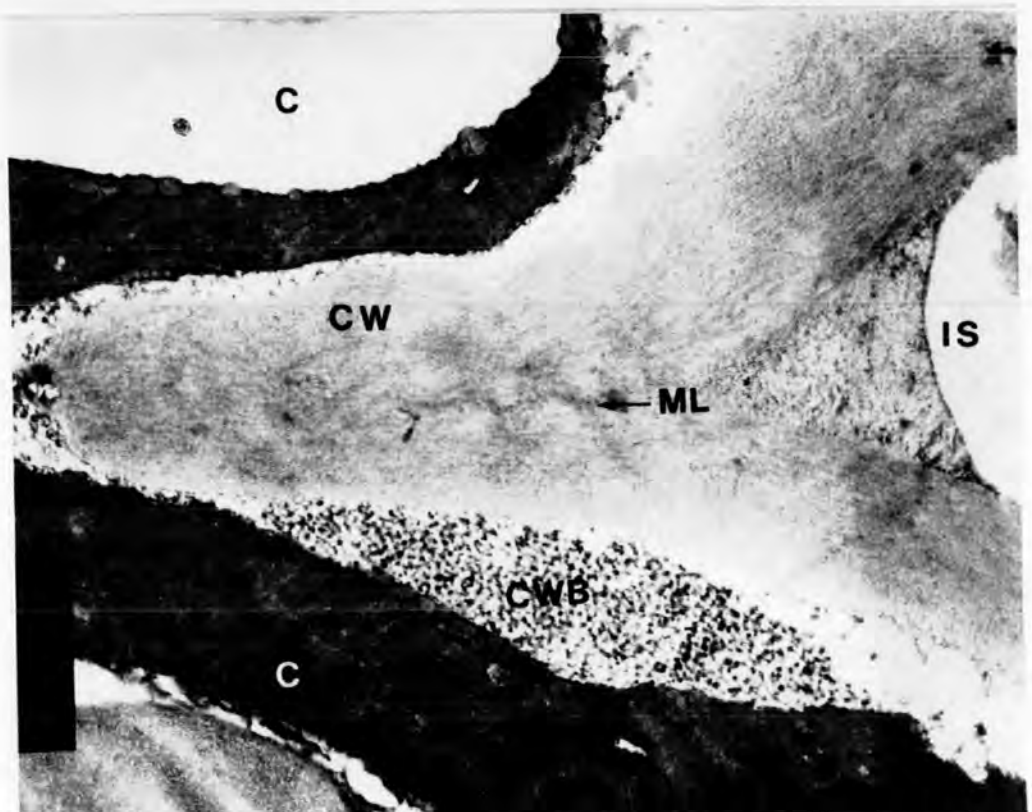
FIGS. 36 and 37. Transmission electron micrographs of soft (Fig.36) and hard (Fig.37) cooked tissue. Middle lamella detail.

Fig. 36 .



X46000

Fig. 37 .

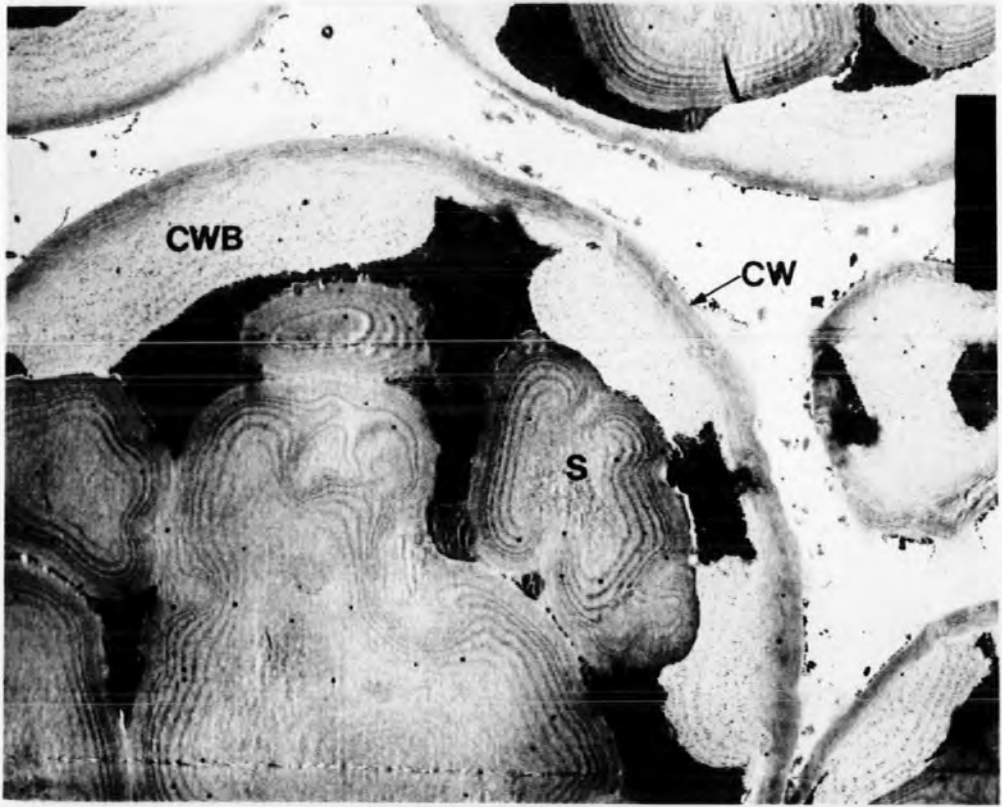


X 17000

CW - Cell wall  
CWB - Cell wall body  
ML - Middle lamella

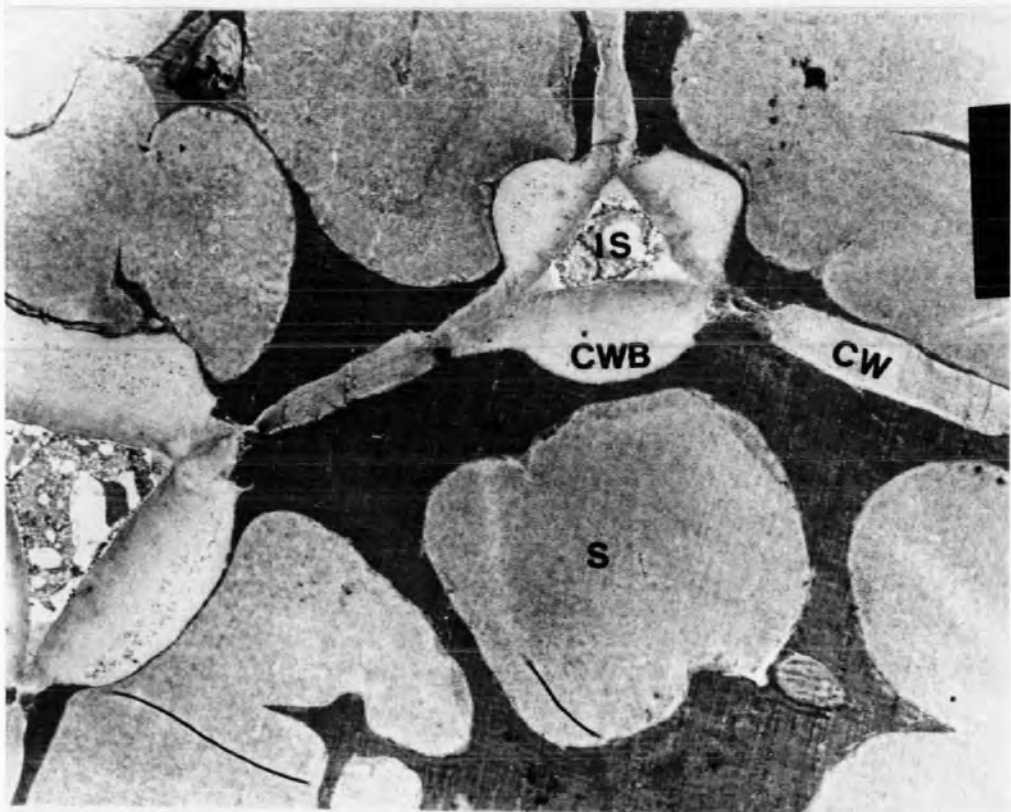
f - Cellulose fibrils  
C - Position of cell  
IS - Intercellular space





X 2300

Transmission electron micrographs of cooked soft (Fig. 36a above) and hard (Fig. 37a below) bean cotyledons.



X 2900

CW - Cell wall  
CWB - Cell wall body

IS - Intercellular space  
S - Starch

When beans were boiled in alkali solutions the rate of cell separation was accelerated. The effects of  $\text{NaHCO}_3$  and  $\text{KOH}$  compared with water are listed in Table 4. 0.25%  $\text{KOH}$  is shown to decrease the time of cell separation from over 40 min. to 30 min. and 1%  $\text{KOH}$  to decrease the time further to 10 min.  $\text{NaHCO}_3$  is shown to have a similar effect.

Table 4.

The effect of  $\text{NaHCO}_3$  and  $\text{KOH}$  on Cotyledon  
breakdown

---

<u>Solution.</u>	<u>Comments on effects.</u>
Water.	Partial breakdown of soft bean cotyledons in 40 min.
0.05 M $\text{NaHCO}_3$ .	Hard beans had failed to breakdown after 90 min. boiling in water. The sample was divided into two lots. Sample (a) was boiled in water and required a further 175 min. before cotyledon breakdown was established. Sample (b) was boiled in 0.05 M $\text{NaHCO}_3$ and breakdown of the cotyledons was complete in 15 min.
1% $\text{KOH}$ .	Total breakdown of soft bean cotyledons in 10 min.
0.25% $\text{KOH}$ .	Total breakdown of soft bean cotyledons in 30 min.

Each experiment was carried out on cotyledons with the testa removed. Overnight soaking was done in water as always.

Each sample was 10 beans.

No squeezing of beans was performed - cotyledons were left to fall apart on their own.

Table 5 displays the different solubilities of hard and soft bean pectin. Approximately 40% of the soft bean pectin was soluble in water with the bulk of the remainder solubilising in 0.5% ammonium oxalate. Hard bean pectin was only approximately 14% soluble in water. An estimated 22% of the hard bean pectin still stuck to the cell wall even after mild saponification.

Table 5.

The solubilities of hard and soft bean pectin during sequential extraction (expressed as uronic acid g.<sup>-1</sup> d.wt. bean)

	<u>Soft Bean</u>	<u>Hard Bean</u>
<u>Cold Water Soluble.</u>	0.4)	1.7)
	13.4)	4.7)
<u>Hot Water Soluble.</u>	13.0)	3.0)
	19.5)	21.0)
<u>Ammonium Oxalate Soluble</u>	18.0)	12.5)
<u>NaOH Soluble</u>	1.5)	8.5)
<hr/>		
<u>Total.</u>	32.9	25.7
<u>Insoluble.</u>	-	7.2

This is a selected typical result of three replicates.

The sample size was approximately 5 gm.

The different levels of calcium and magnesium associated with the cell walls of hard and soft beans are listed in Table 6. In each case the cation levels are higher in the hard bean, rising by 45% and 66% respectively before, during or after the development of hardbean. The data supplied also shows that the degree of pectin esterification dropped from 51% to 15% and the quantity of phytin within the beans dropped from 29 to 18 mg. gm.<sup>-1</sup> d.wt. over the same period.

Table 6.

Analysis of phytin levels, pectin esterification  
and cell wall cation levels

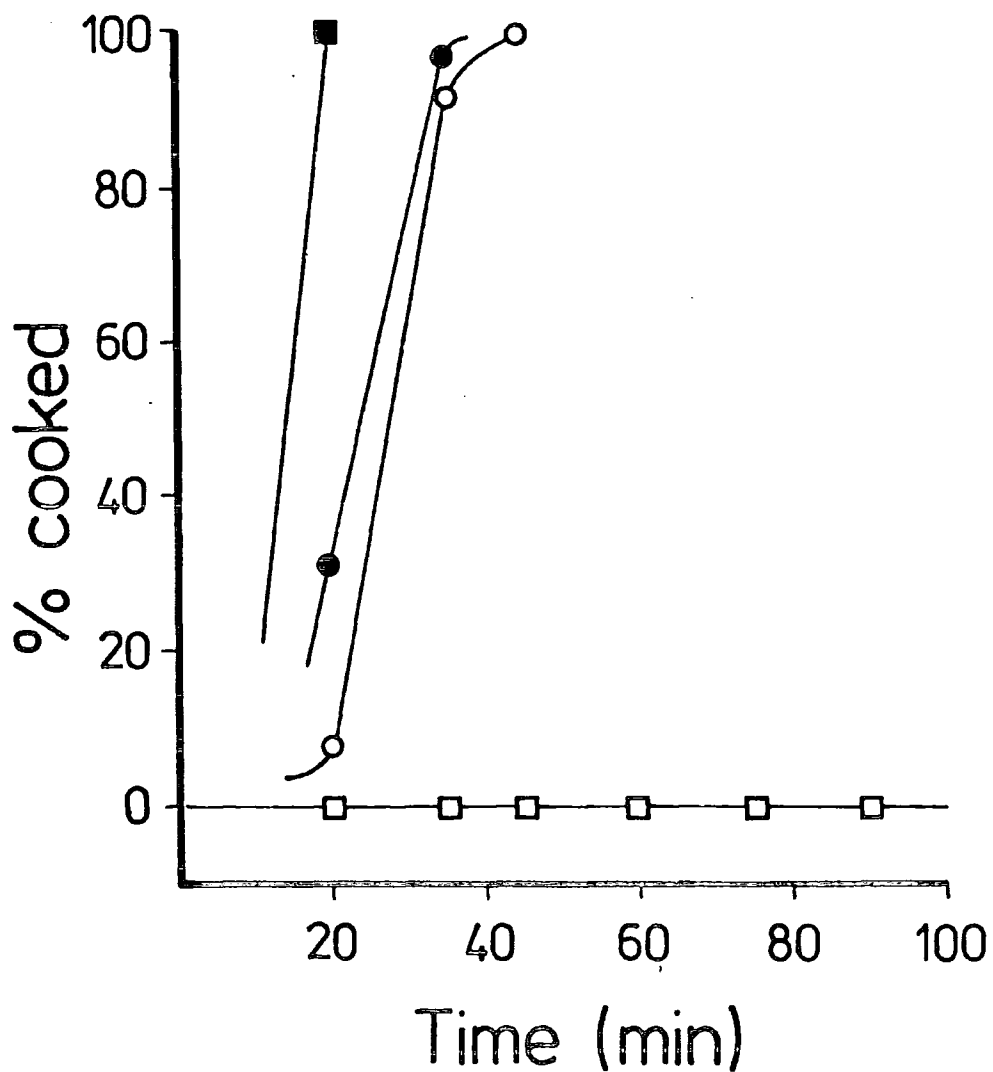
	<u>Soft Bean</u>	<u>Hard Bean</u>
Ca. $\mu\text{g. gm.}^{-1}$	120	173
Mg. $\mu\text{g. gm.}^{-1}$	210	354
Phytin. $\text{mg. gm.}^{-1}$	29	18
Pectin Esterification.	51%	15%

Calcium and magnesium values are the average of three replicates.

The readings are expressed as  $\mu\text{g. Ca/Mg}$  adhering to the cell wall per gram dry weight of whole bean meal.

fig. 38 (below) shows the effect of calcium ions on the cooking rate of black beans. The  $CT_{100}$  increases from 2000 min to > 3000 min and when combined with exposure of PME increases to > 3200 min. If the beans are pre-treated in a Soxhlet apparatus with 80% ethanol the  $CT_{100}$  exceeds 15000 min.

fig. 38 The combined effects of  $CaCl_2$ , PME and Soxhlet pretreatment on the cooking rate of black beans.



■—■ control

●—● " + 0.03 m  $CaCl_2$  pH 7.0

○—○ " + " " " " + PME

□—□ " + " " " " + " + Soxhlet pretreatment

PME = Pectin Methyl Esterase

## Section 4

### Storage trial

The development of sixteen characteristics of hard beans was followed at 10 day intervals during an 84 day storage trial at  $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and 75% relative humidity during which time hardness developed within the beans. The changes which occurred and illustrated in figures 39-56. Each batch of 16 analyses was carried out on a total sample of approximately 35 g.

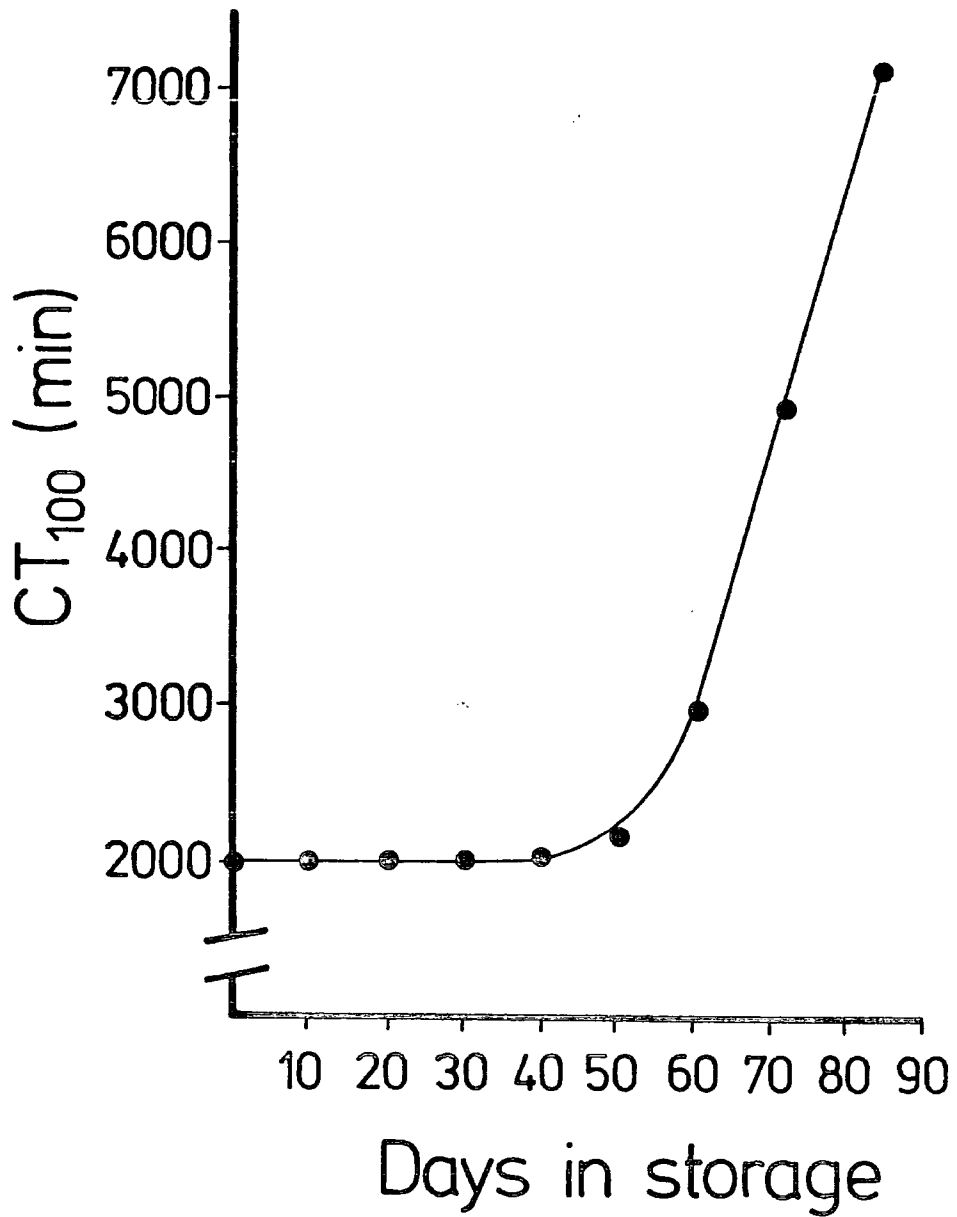
During the storage trial the total cooking time of the beans increased from 2000 min to 7000 min though as demonstrated in figure 39 this change was not linear. No significant increase in the total cooking time occurred until after day 50, thereafter the increase was logarithmic. Representative graphs of individual cooking tests (days 0 and 84) are presented in figures 40 and 41 respectively.

There was a daily average increase of 0.13% in the moisture content of the beans during the storage trial, and as shown in figure 42, this stood at nearly 23% when the trial was terminated.

Both seed viability and seed vigour were found to drop rapidly after 40 days storage and both parameters were at nought after 72 days.

(see fig. 43 ).

fig. 39 The change in total cooking time ( $CT_{100}$ ) of beans during storage.



Each sample was 50 beans except at day 84 where the sample size was 63 beans.

figs.40 and 41 . The cooking rate of beans at day 0 (fig.40 left) and day 84 (fig.41 right) of storage. Sample sizes 50 and 63 beans respectively.

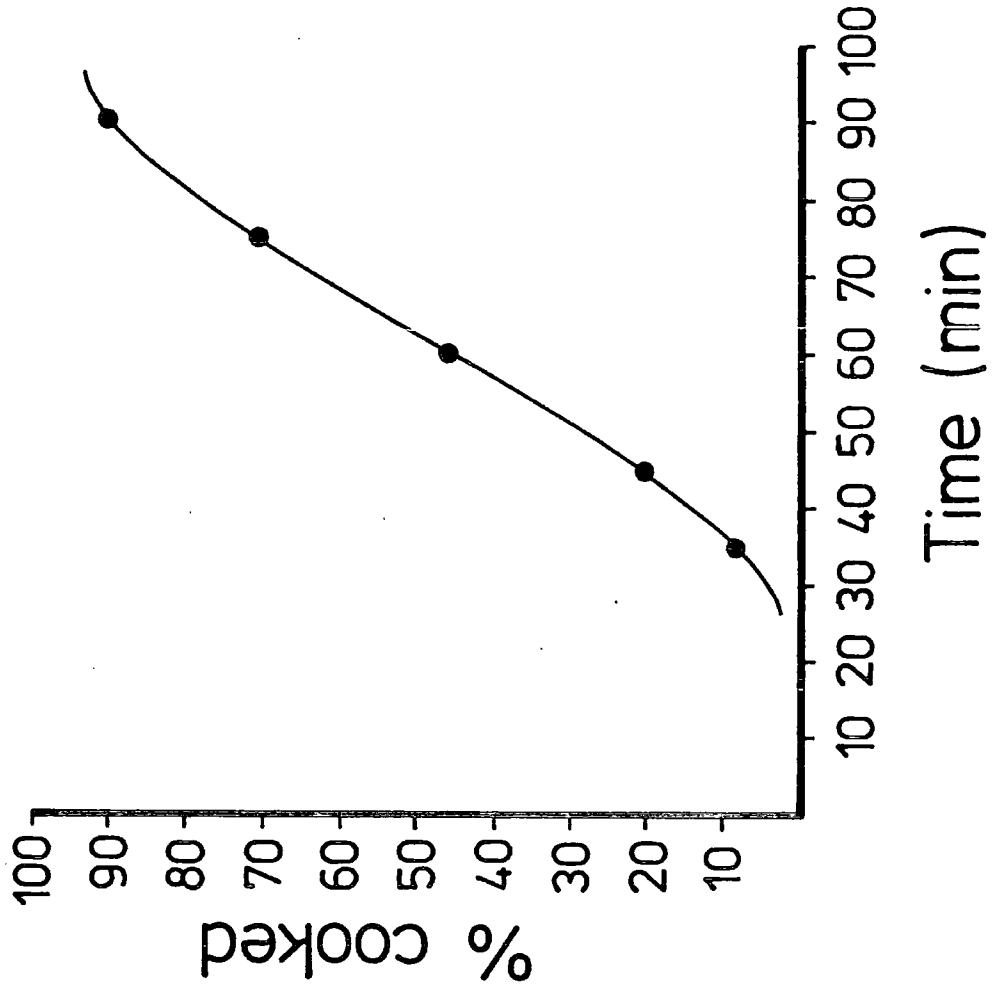
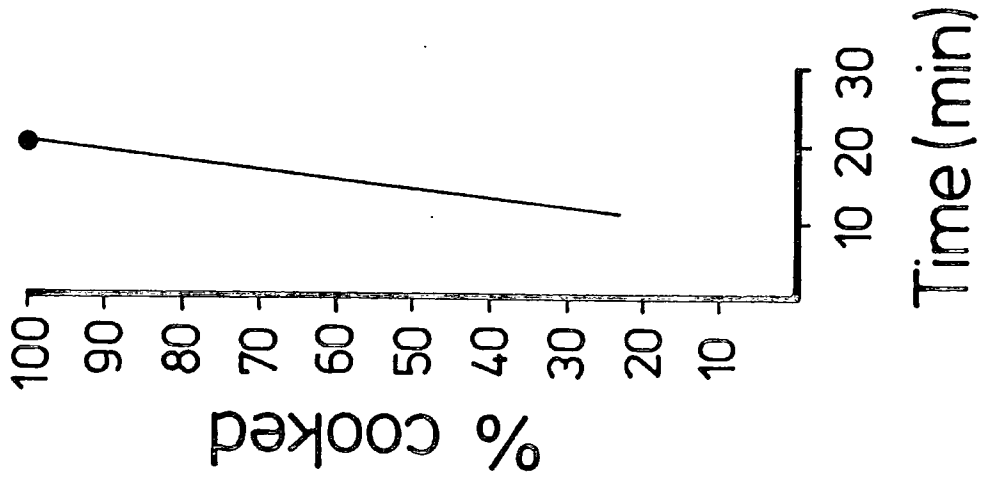
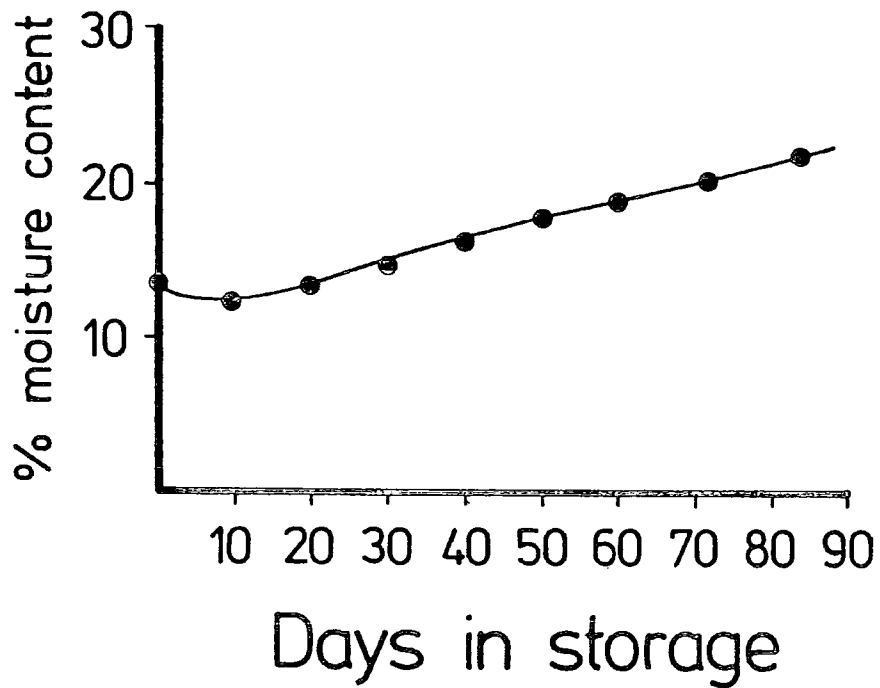


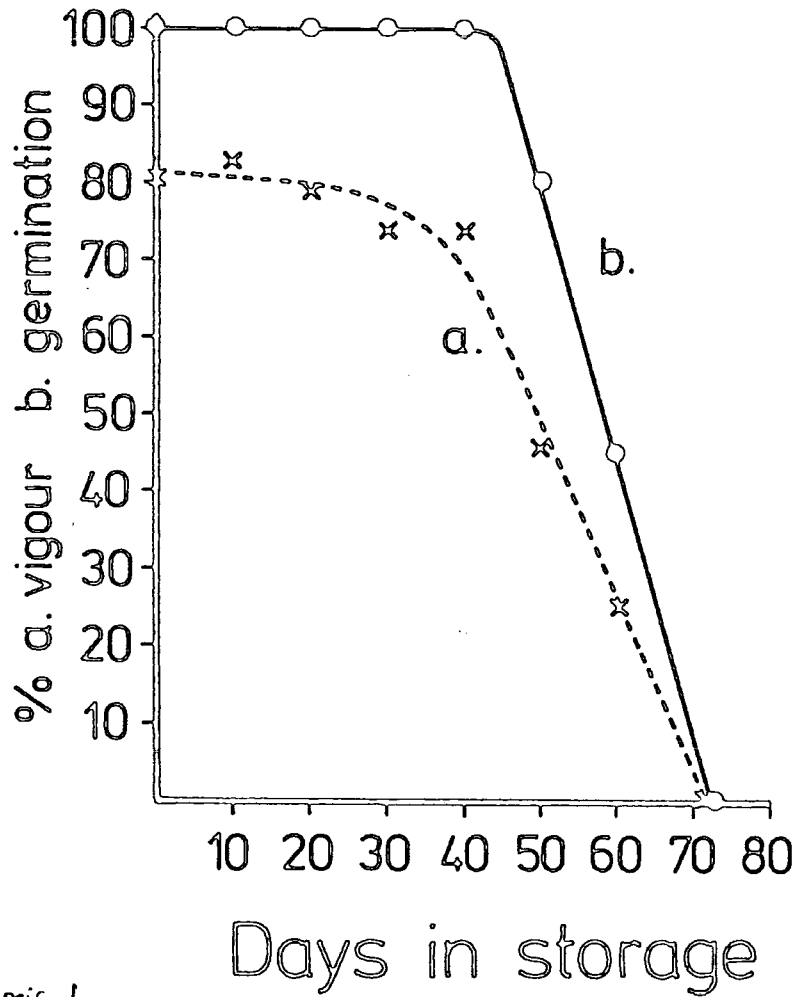


fig.42 The moisture content of black beans during storage



Each analysis was an average of 5 beans, and varied on average by 0.7 percentage points (minimum variance 0.3, maximum 0.9).

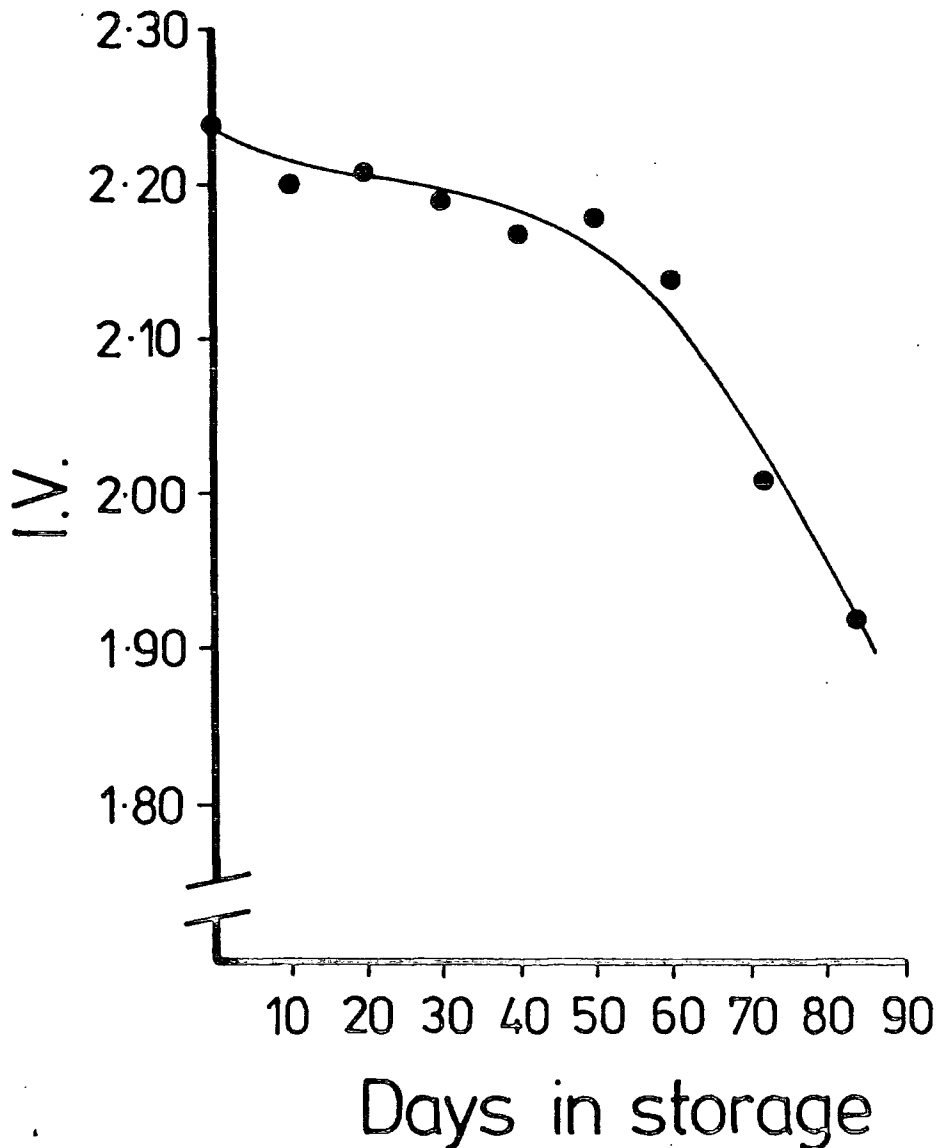
Fig.43 vigour (a) and viability (b) of black beans during storage.



Each <sup>pair of</sup> readings was determined from the same sample of 20 seeds.

The imbibition value of the beans dropped by only 10 l.V units during the first 60 days of storage (2.24 → 2.14) but then dropped rapidly, by 22 units, in the next 24 days (2.14 → 1.92). As shown in figure 44 this represents 69% of the imbibition value decay in the last 25% of the storage trial.

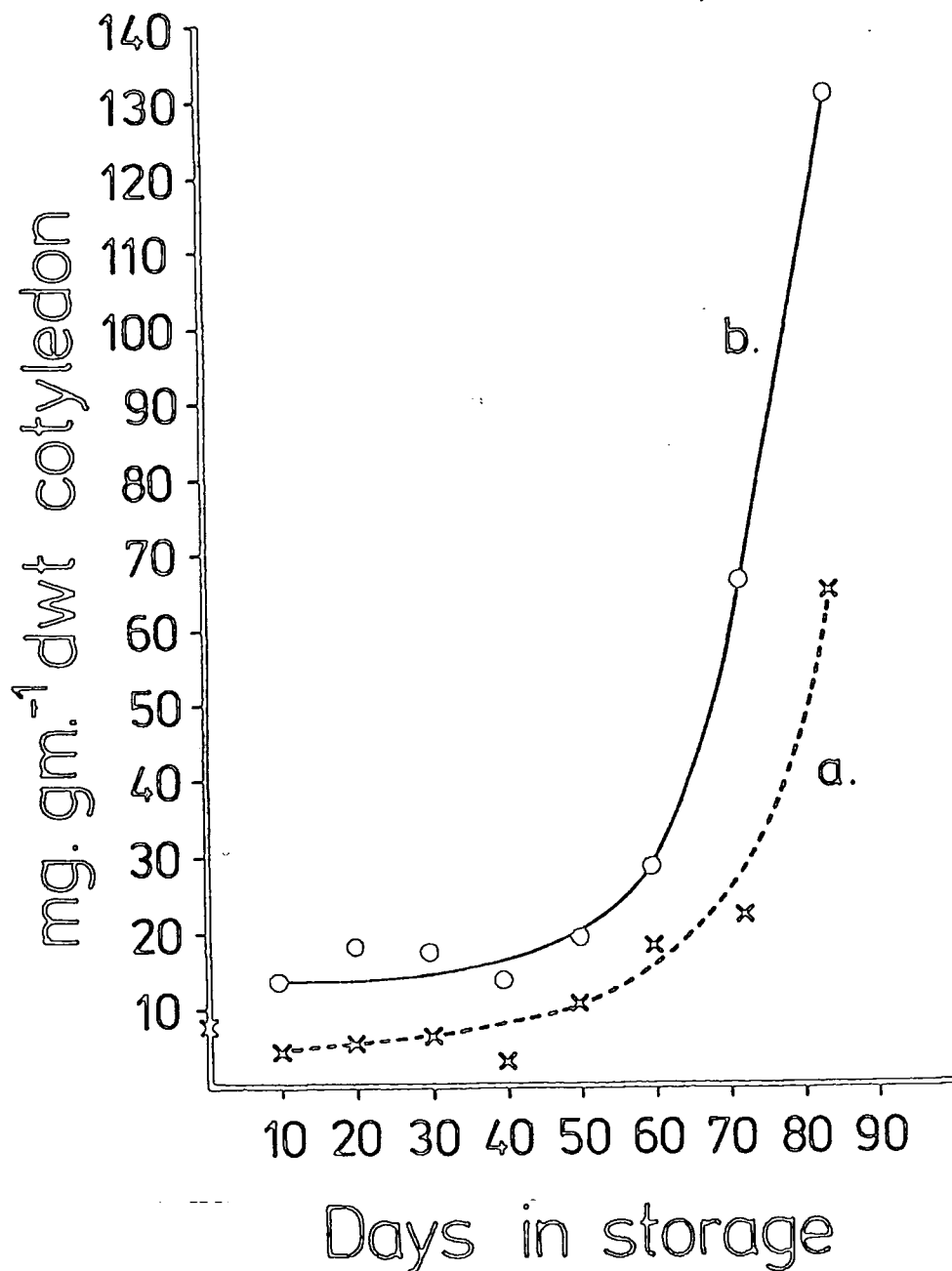
fig. 44 The imbibition value of black beans during storage.



Each reading was a bulk reading from 15 beans.

The rate of increase in leakage from beans is shown in fig.45 below. For the first 50 days there is little change but the rate accelerates thereafter and by day 84 the total leakage and sugar leakage have both increased approximately 8-fold. These values doubled in the last 12 days.

fig.45 The leakage of soluble sugars (a) and total leakage (b) from imbibed beans after increasing periods of storage.



Each sample consisted of 15 shelled beans.

Figure 46 describes the fluctuations in the quantities of soluble sugars extracted from macerated beans by hot 80% ethanol in a Soxhlet apparatus during the storage trial. Initially the amount of soluble sugars dropped from nearly  $70 \text{ mg g}^{-1}$  to  $58 \text{ mg g}^{-1}$  and then rose to nearly  $71 \text{ mg g}^{-1}$  over the next 40 days. After day 60 the quantity again dropped.

Concomitantly figure 47 shows that the levels of starch dropped by approximately  $0.5 \text{ mg g}^{-1} \text{ day}^{-1}$  for the first 50 days and then the rate of decrease slowed down. When the quantities of starch and soluble sugar are added together then we get the graph shown in figure 48. This shows that the drop in total sugar content stopped for the period 40-72 days and then continued.

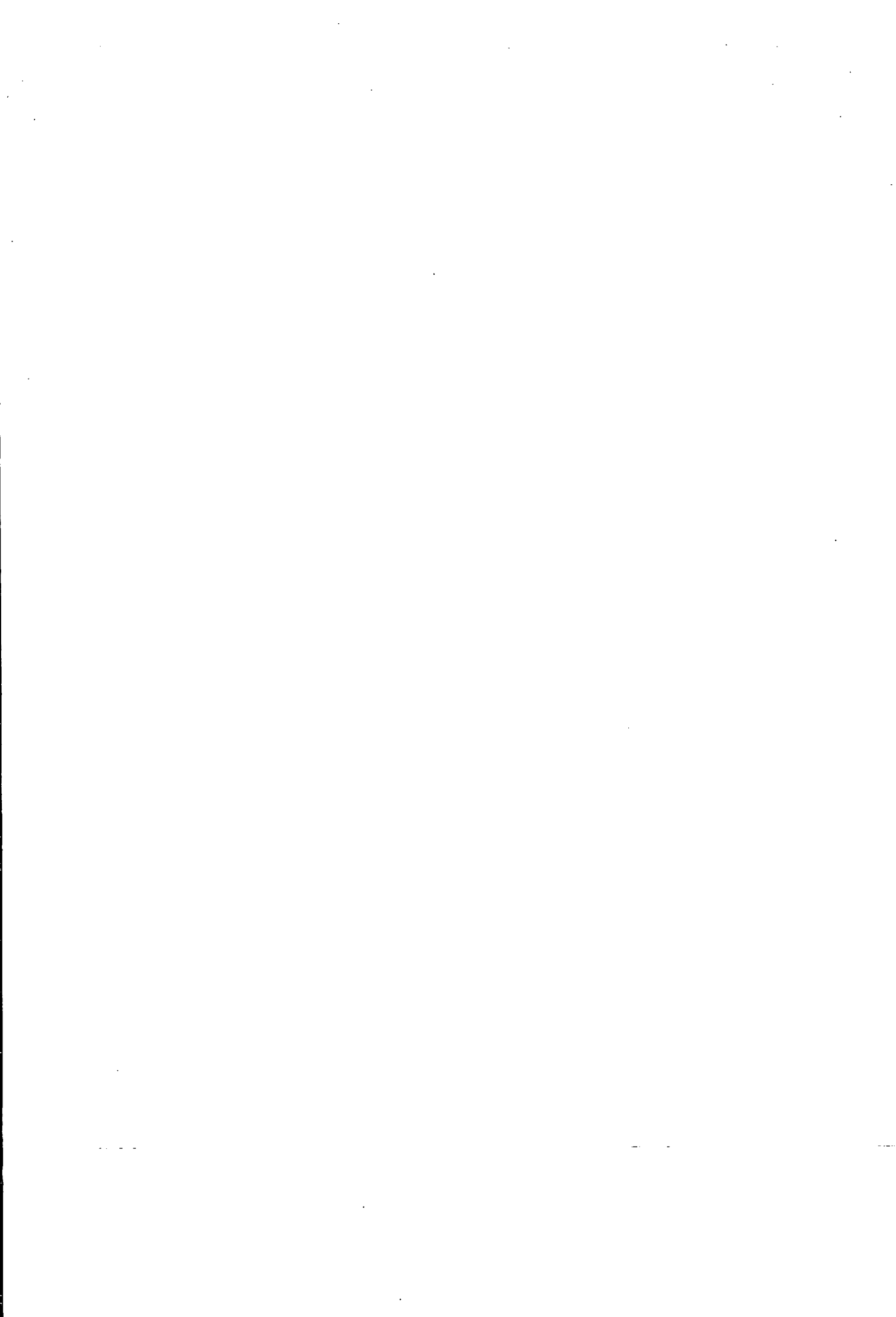


fig. 46

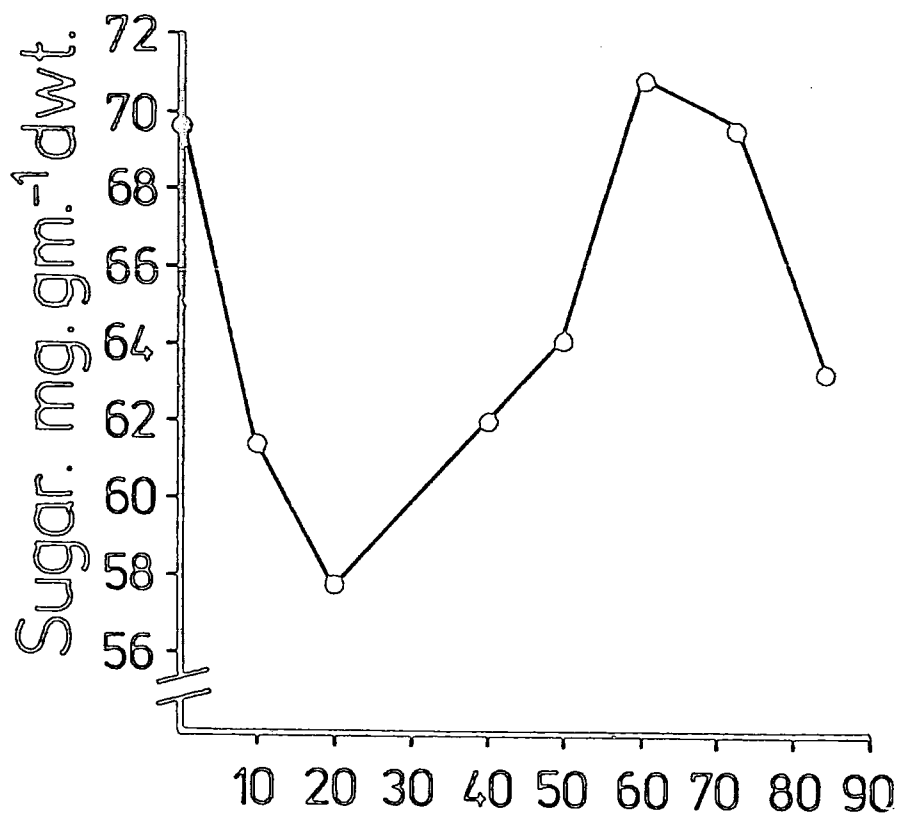


fig. 47

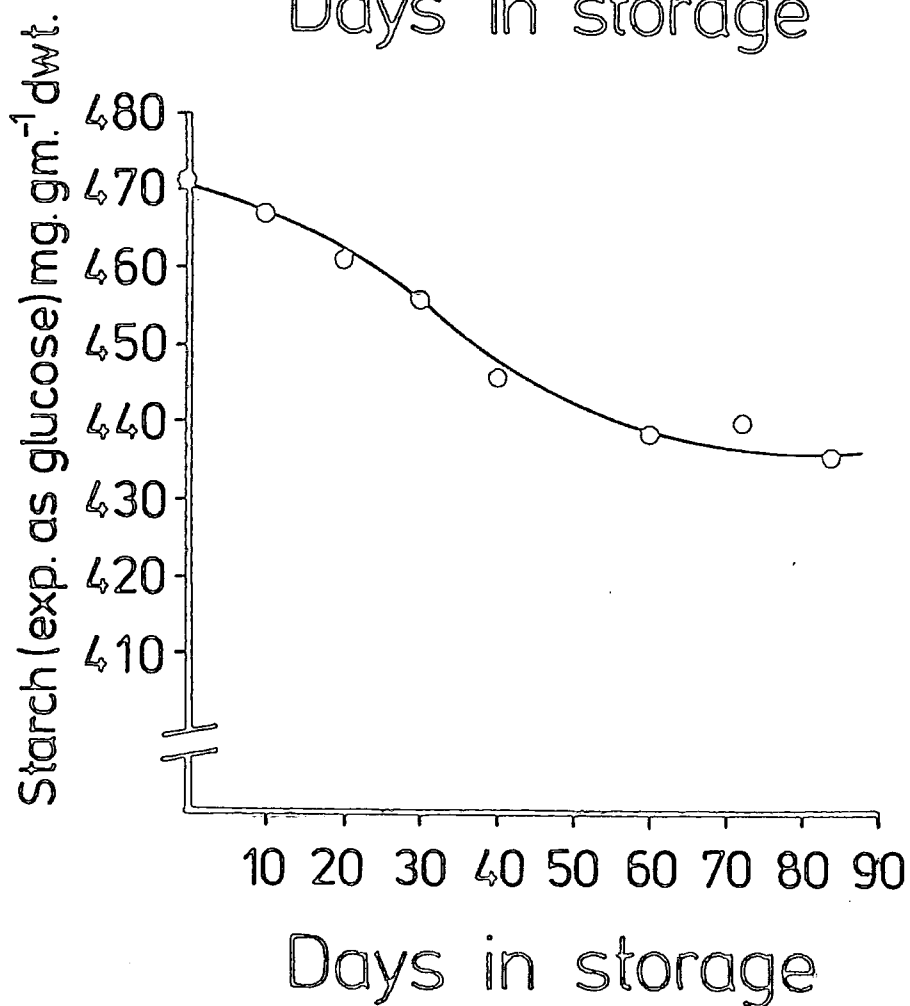
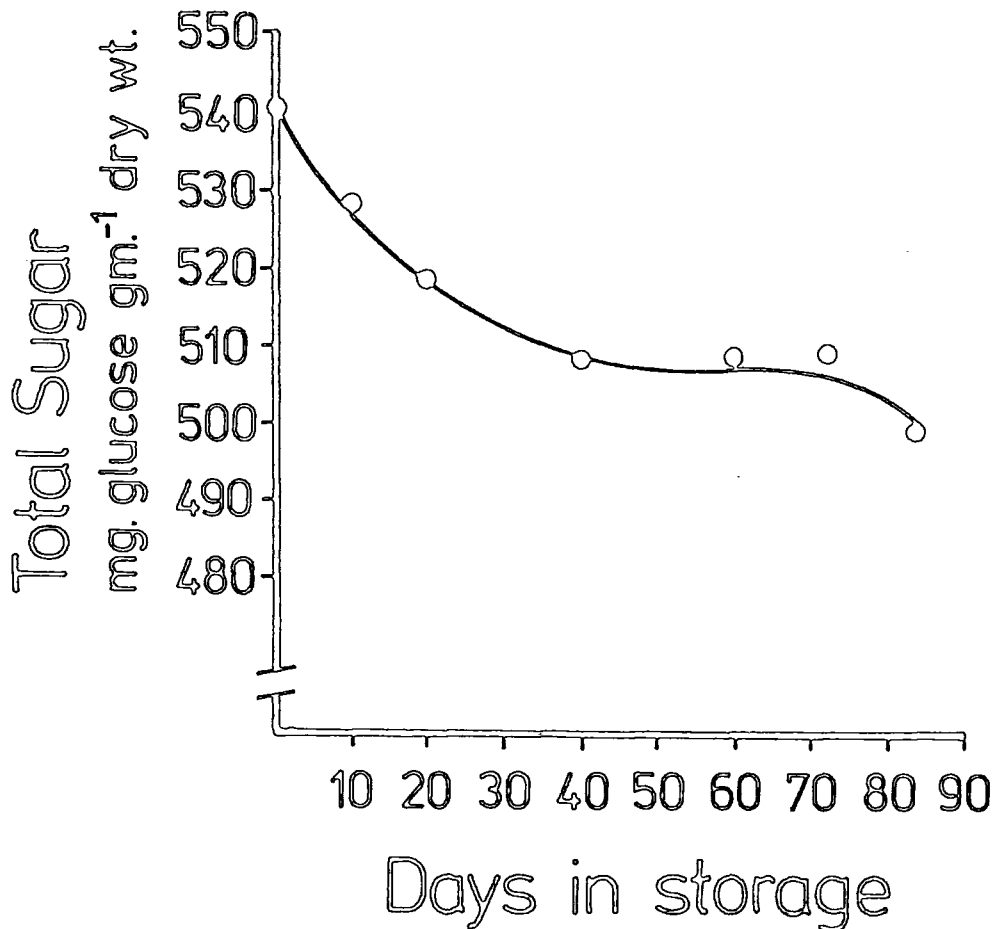


fig.48 The amount of total sugar available within black bean cotyledons during storage.



Expressed as mg glucose gm<sup>-1</sup> dwt tissue.

The results of the cell wall cation study are displayed in figures 49 (calcium) and 50 (magnesium). Calcium increased slightly and the change in magnesium levels appeared to be byphasic. The quantity of magnesium dropped from 270 mg to 240 mg g<sup>-1</sup> in the first 30 days and then increased to 315 mg g<sup>-1</sup> between days 40 and 50. Thereafter it increased to nearly 360 mg g<sup>-1</sup> at 84-days.





fig.49 Calcium

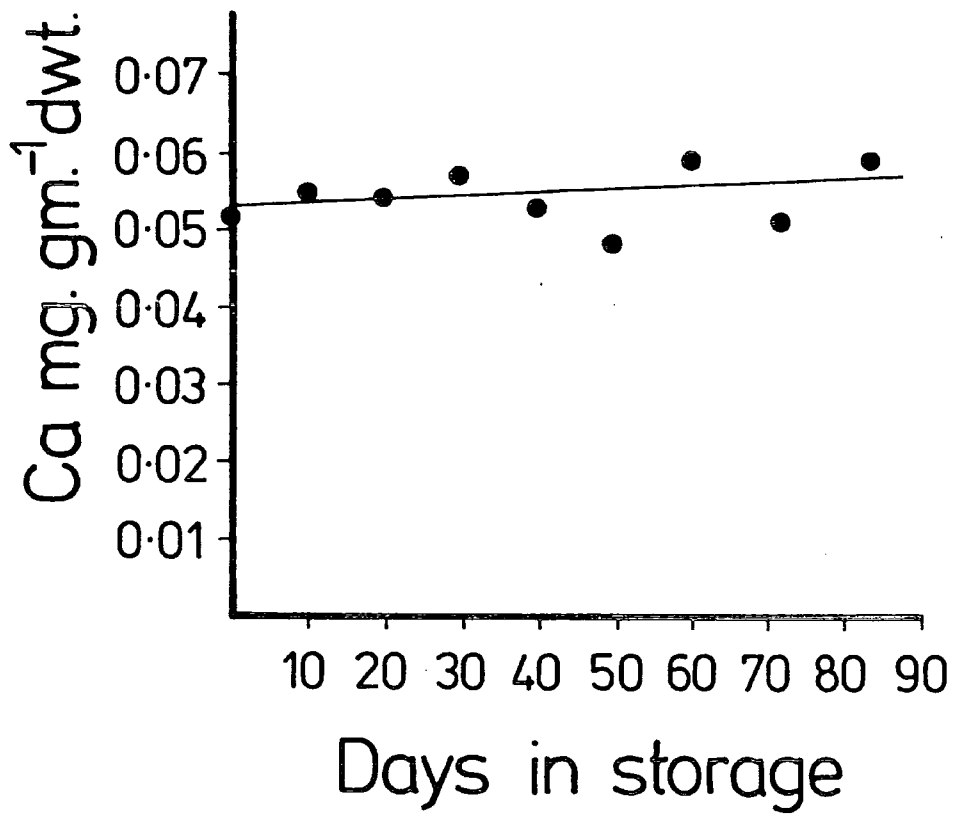
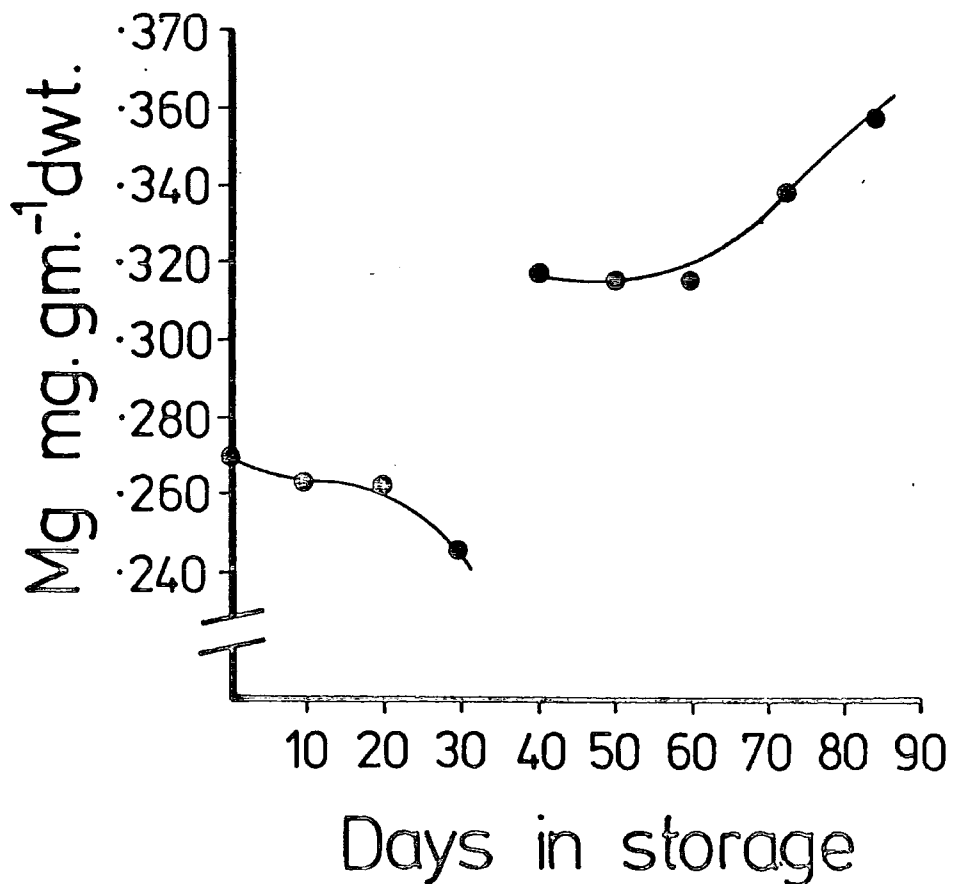
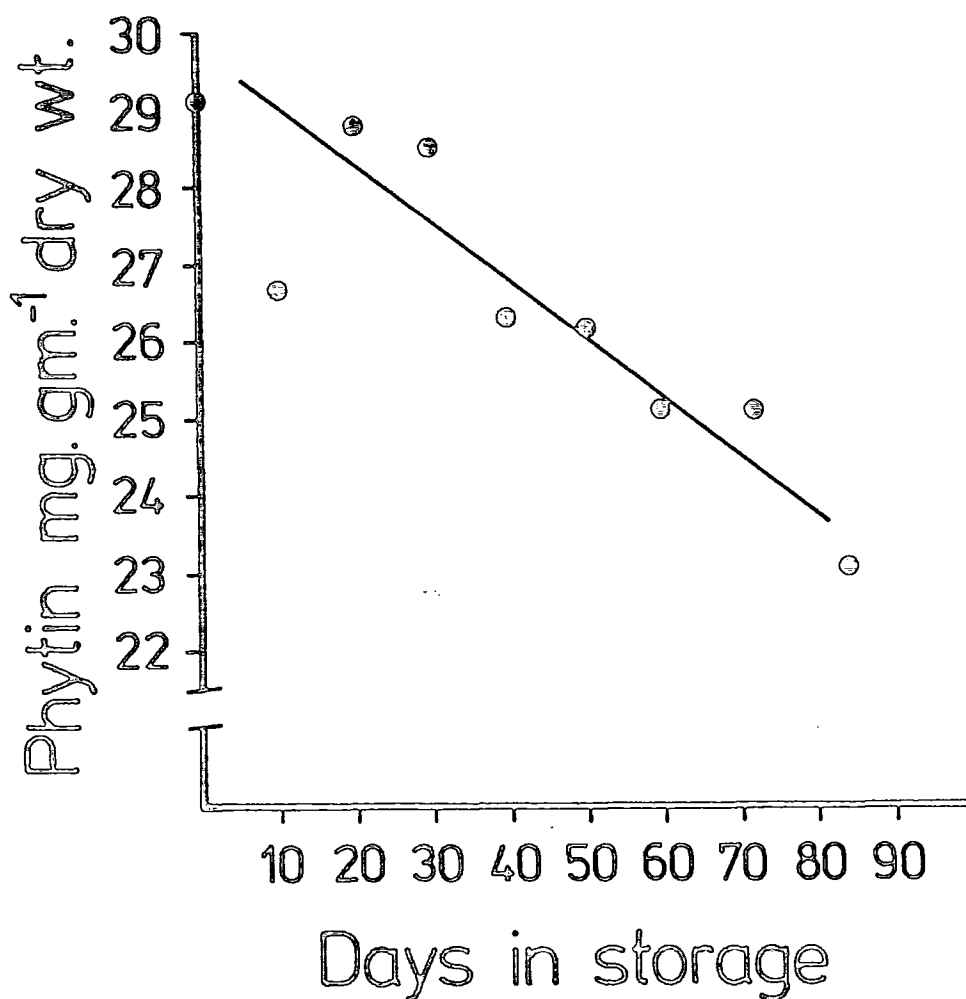


fig.50 Magnesium



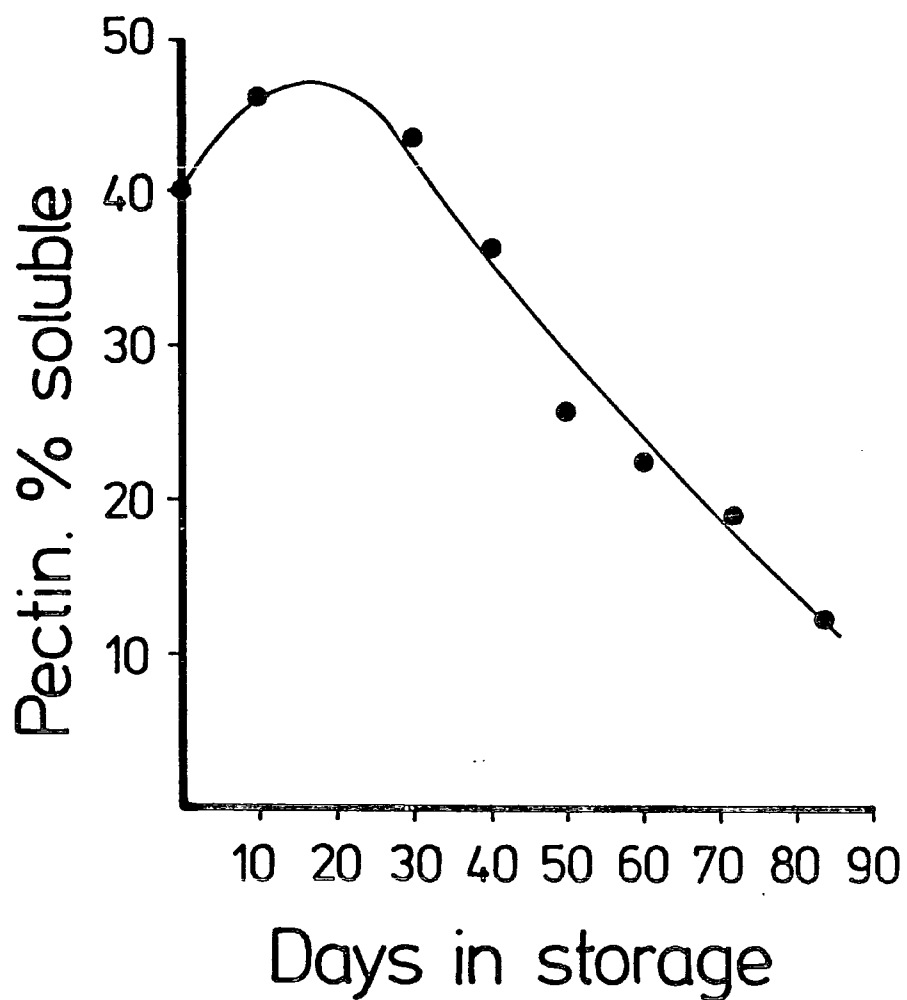
The levels of phytin during the storage trial dropped by approximately 20% (from 29 mg gm<sup>-1</sup> to 23 mg gm<sup>-1</sup>). See fig.51

fig.51 The level of phytin in bean cotyledons during storage.



Each determination was performed in duplicate on 100 mg tissue dry weight samples from 10 macerated beans and 3 analyses carried out on each sample.

When the relative levels of water soluble and insoluble pectin were measured (fig.52) it was found that for the first 20 days the pectin solubility increased from 41% to 47% and then dropped to 12% by day 84.

fig.52 The solubility of bean pectin during storage

In each analysis the total amounts of pectin (soluble + insoluble) was  $33 \text{ mg gm}^{-1} \pm 1.0 \text{ mg}$ . Sample size was 4 gm.

fig. 53 The correlation between cell wall magnesium and pectin insolubility.

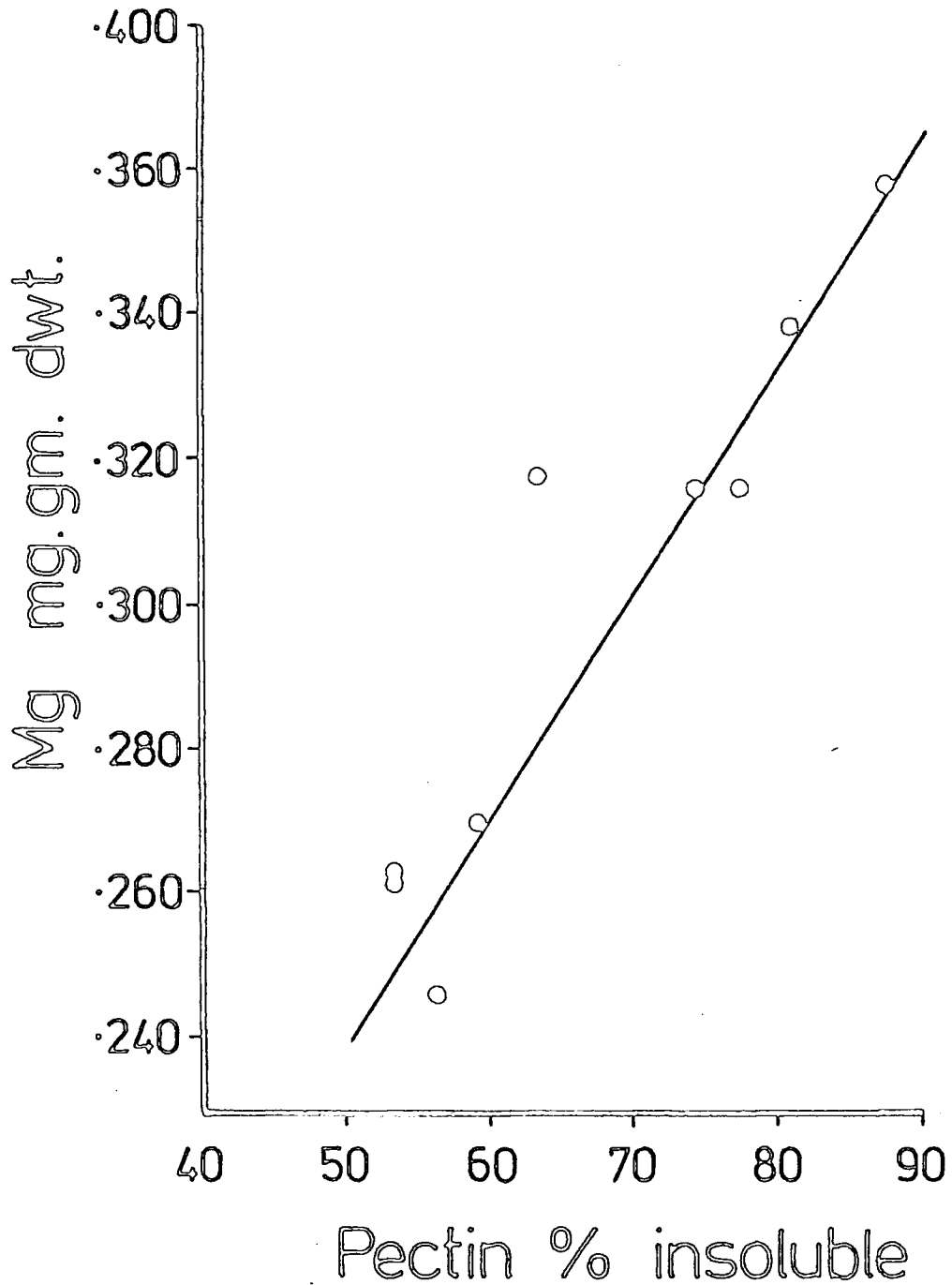
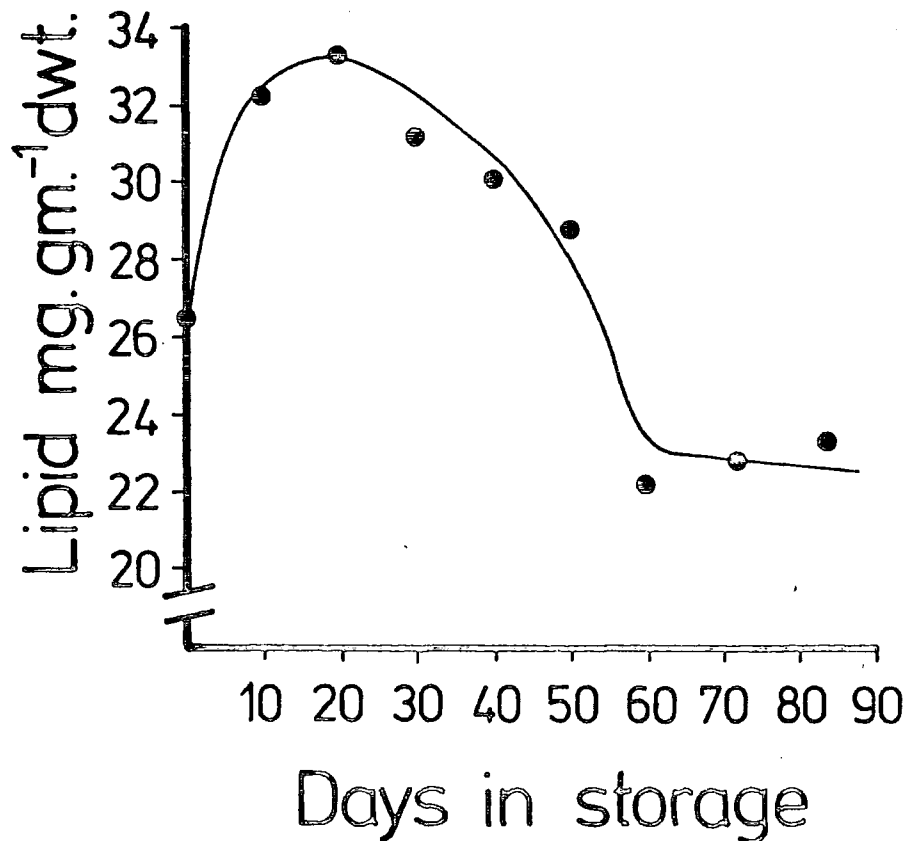


fig. 53 above shows there is a positive correlation between insolubility and the levels of cell wall magnesium.  
 $r = + 0.91$  ( $p < 0.001$ ).

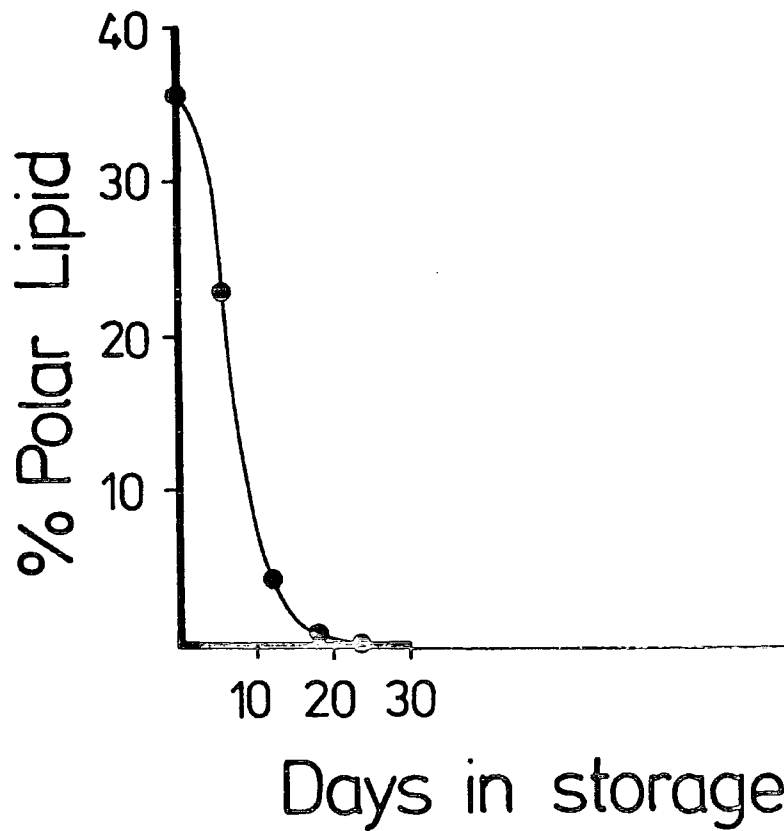
After a rapid increase during the first 10 days the level of lipid within the bean dropped from  $33 \text{ mg g}^{-1}$  to  $23 \text{ mg g}^{-1}$  - the biggest single drop occurring during days 50 and 60 (see fig.54 below). The decay of lipid polarity from 35.5% to  $< 1\%$  during the first 30 days of storage is shown in fig.55.

fig.54 The amount of lipid extracted from black bean cotyledons during storage.



Sample size approx. 6 gm beans.

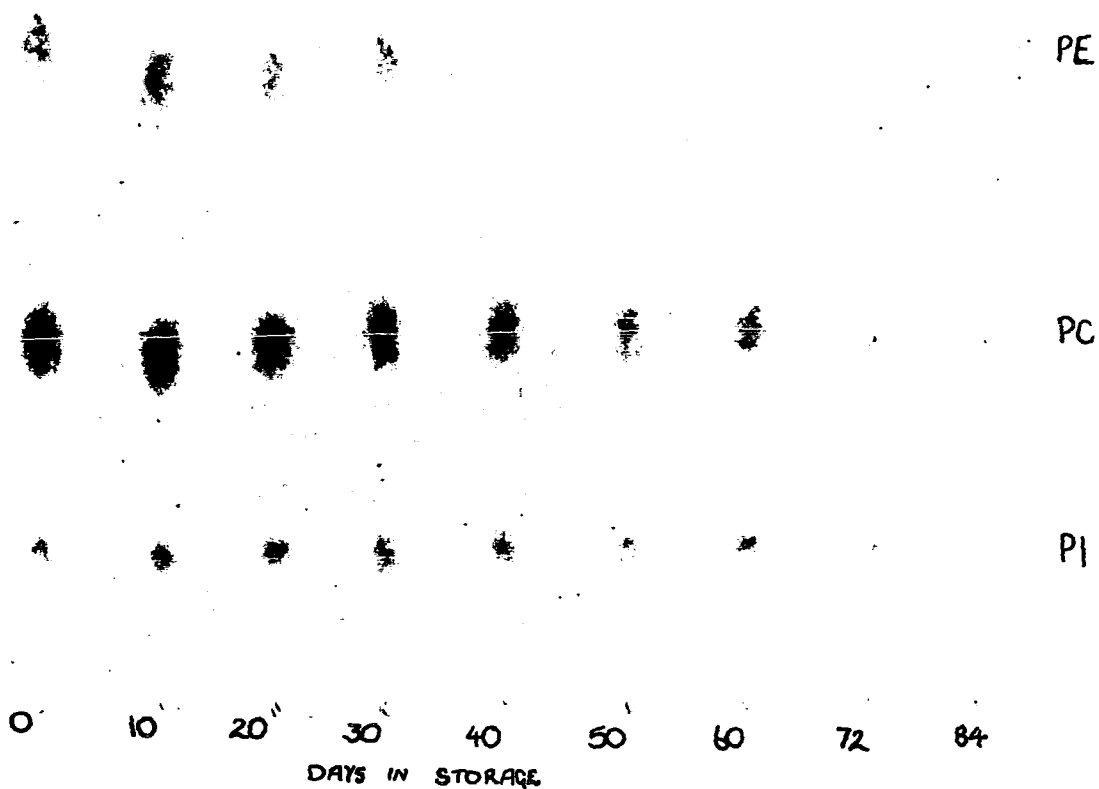
fig.55 The percentage of polar lipid in the lipid extract.



Analysis performed on a 20 mg sample of the lipid extract

When the lipid extract was developed on a TLC plate the three phospholipids phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol were visible - though with decreasing clarity as the period of bean storage increased - showing that these three phospholipids were being degraded during storage.

fig. 56. TLC plate of lipid extract.



Each spot size was 10  $\mu$ l containing 10 mg ml<sup>-1</sup>.

PE = phosphatidylethanolamine

PC = phosphatidylcholine

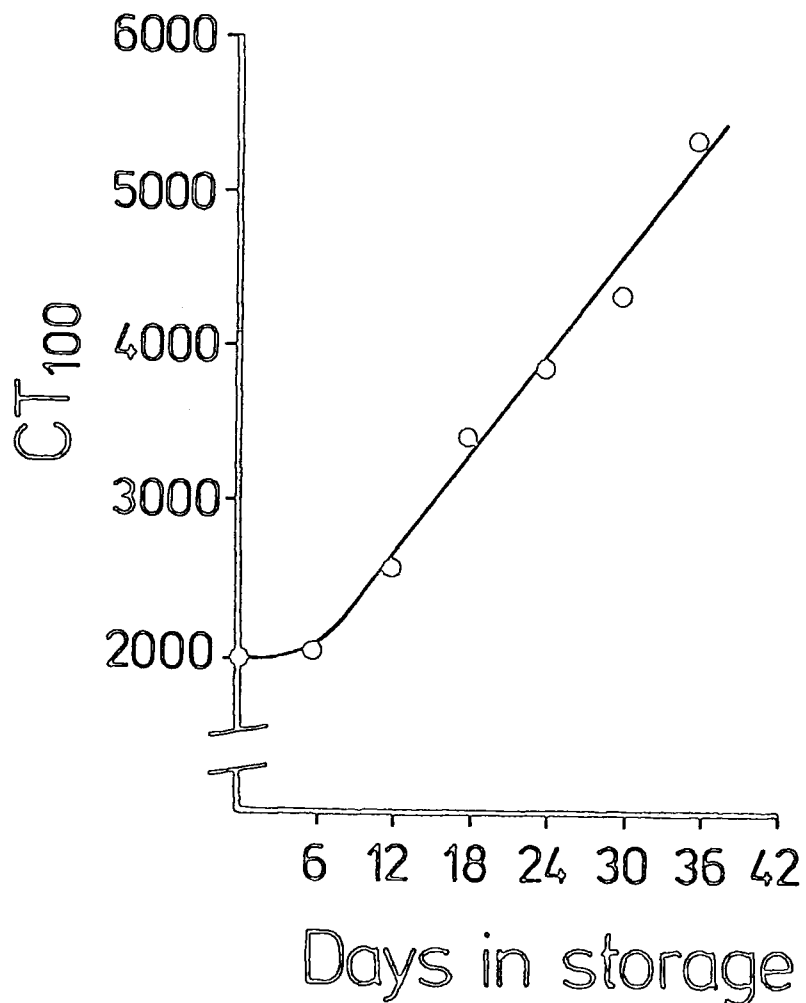
PI = phosphatidylinositol



Section 5The influence of wet bean blanching on the development of hardbean.

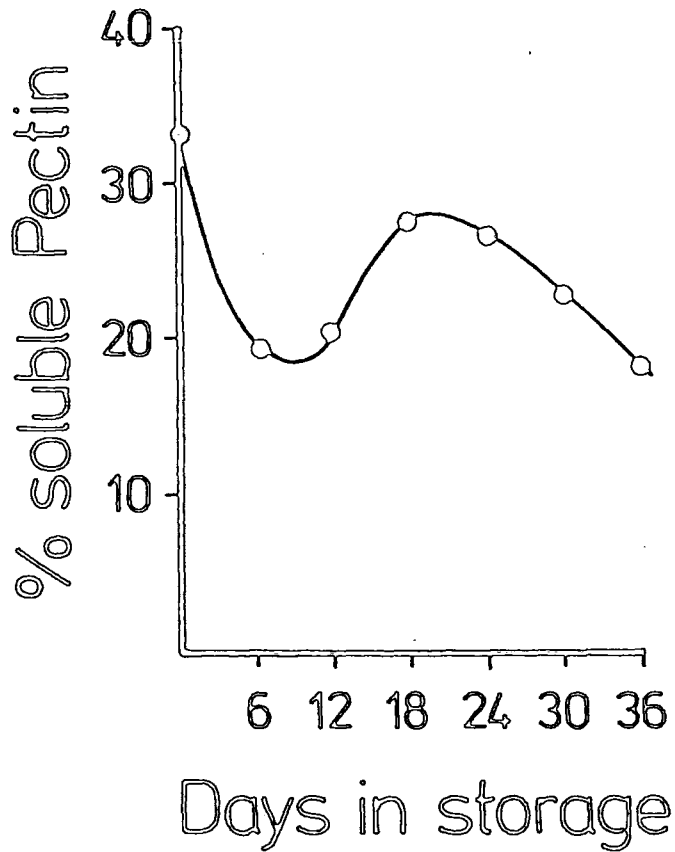
When wet blanched beans were stored at  $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and 75% relative humidity there was a rapid and linear increase in the total cooking time after 6 days storage as described in fig. 57 . After 36 days the total cooking time of the beans had reached 5300 min.

fig.57 The total cooking time ( $CT_{100}$ ) of blanched beans during storage.



Each sample was 50 beans.

fig. 58 The solubility of pectin in blanched beans during storage.



Each sample size was approx. 5 gm.

During the first 18 days of storage the solubility of the blanched bean pectin fluctuated and then steadily decreased as shown in fig. 58 above.

The data supplied in Table 7 showed that three parameters remained stable, imbibition value ( $2.20 \pm 0.03$ ), total leakage ( $105 \text{ mg. gm.}^{-1} \pm 6$ ) and phytin (average  $24.3 \text{ mg. gm.}^{-1} \pm 0.7$ ).

Table 7.

Imbibition value, leakage and phytin levels of  
blanched beans during storage

<u>Days in Storage</u>	<u>I.V.</u>	<u>Leakage (<math>\text{mg. gm.}^{-1}</math>)</u>	<u>Phytin (<math>\text{mg. gm.}^{-1}</math>)</u>
0	2.20	111	-
6	2.23	101	24.7
12	2.21	102	24.0
18	2.20	103	24.5
24	2.19	100	24.1
30	2.19	103	23.6
36	2.18	107	24.7

Sample sizes.	I.V.	15 beans.
	Leakage	15 beans.
	Phytin	2 x 100 mg. samples from 10 macerated beans.

## DISCUSSION

### Chapter 1. Initial Observations

In order to investigate the hardbean phenomenon it was necessary to start at a very basic level. This was because although much work had been carried out on cooked bean texture, whether resulting from storage or otherwise, no clear conclusions had been drawn and even the primary causes of hardbean had not been established. This may have been because different varieties and/or species of beans lose cooking quality for different reasons or just due to differing interpretations of similar results.

Some workers such as Molina et al. (1975) appear to confuse hardshell, the total inability to absorb water, with hardbean (or hard-to-cook) the reduced softening rate during cooking. Quenzer et al. (1978) classed hardbean (sclerema) as one of two types of hardshell and Snyder (1936) claimed that the main reason for reduced cooking rate of beans was an impermeable testa, i.e. hardshell was causing hardbean. Salih (1979) also reported this consequence of hardshell in Vicia faba. Muneta (1964) said that, "... at the time (of writing) it was thought that the seed coat did not influence the cooking time (of bean) ...", while Gloyer (1921) observed that reduced water uptake by hard cooking beans was due to the cotyledons and not the testa. El-Tabey Shehata and Youssef (1979) found that one of the factors influencing cooked legume texture was the amount of water absorbed. Snyder (1936) commented that pre-soaking the beans (non-hardshell beans) considerably reduced the cooking time but Tinkler and Masters (1932) claimed that overnight soaking does not greatly reduce the cooking time. Antunes (1979) found that storage of beans at elevated temperature and relative humidity caused reduced water uptake (due to hardshell) and a doubling of the cooking time over six months storage in one sample but in another sample stored at even

more extreme conditions caused a slight increase in water uptake over the control samples and yet still a five-fold increase in cooking time. Quenzer et al. (1978) and Sefa-Dedeh<sup>et al</sup> (1979) both noted reduced water uptake by beans of longer cooking time but said that the two factors did not correlate. Molina et al. (1975) found only a slight reduction in water uptake by hard beans though such measurements only appear to have been made during the first 4 h. of soaking. Burr et al. (1968) noted an increase in the rate of water uptake by hardbeans but not in the overall values. Both Molina<sup>et al</sup> and Burr<sup>et al</sup> agree with Quenzer<sup>et al</sup> and Sefa-Dedeh<sup>et al</sup> that the water uptake did not influence cooking rate. It must be noted that the work by Quenzer et al. concerned different varieties of beans with naturally differing cooking rates while the work by Burr<sup>et al</sup>, Molina<sup>et al</sup> and Sefa-Dedeh<sup>et al</sup> dealt with beans whose cooking times had increased during storage.

Another point of contention was pectin solubility. Both Kon (1968) and Molina et al. (1975) have stated that pectin solubility of the middle lamella was not a causative factor in the hardbean phenomenon. Kon stored beans at moisture contents of 8.1% and 13.3% for 4 years and found that the cooking times were 29 min. and 210 min. respectively, but he found that there was no significant difference in pectin solubility. Mattson (1946/7) though has found that the cooking rate of peas deteriorated during 10 days soaking primarily because the pectin solubility decreased. Likewise, many papers (Rockland & Jones 1974; Sefa-Dedeh et al., 1978, 1979) have shown that when beans have sufficiently softened during cooking it was because the middle lamella had dissolved.

Additionally, Mattson (1946/7) has claimed that phytin levels may influence the change in cooking quality of peas by altering the cation balance of the pectin thus changing its solubility, while Abu-Shakra

(1975) has found that phytin levels are not relevant to the cooking rate of lentils yet agreed that pectin solubility was involved due to cation changes.

Because of this general confusion and contradiction it was decided to start the investigation from initial personal observations. The preliminary investigation therefore involved determining the extent of cooked bean hardness in the samples received and taking note of any differences between the samples which may give an indication of the cause of reduced cooking rate.

For example, it was desirable to find the actual physical manifestation of hardness, i.e. were there any features immediately obvious in the dry beans that gave a clue to the possible cause of hardbean. This included determining if the beans supplied were actually displaying reduced cooking rate due to sclerema in the cotyledons or hardshell in the testa. The beans were soaked for 15 h. and then boiled until considered cooked whilst continually observing whether there were any differences between the "soft" and "hard" beans.

As a result of this preliminary investigation it was hoped that a decision could be made as to how the investigation was to be approached.

When the beans were examined for physical differences from the outside there appeared to be none except for a slight discolouration of the otherwise white hilum of the "hard" beans. When the testa was removed the cotyledons of the "soft" beans were pale and covered by a mottled brown or purple inner integument. The cotyledons of the hard beans were a darker brown and the inner integuments did not remain attached to them. The browning of the cotyledons was probably due to non-enzymic, or more specifically Maillard browning, a common phenomenon in stored fruit and vegetables and is due to an interaction between carbohydrates and proteins.

There were no visible indications in the dry beans that water uptake might be affected by, for example, a resinous layer on the testa or cotyledons, nor was there any visible evidence that the cotyledons would not soften during cooking at the usual rate. It was concluded therefore that no relevant information was obtained about the cause of hardbean from this initial enquiry.

The preparation of beans for cooking involves soaking in water though this makes no difference to the weight of the final product after cooking (Quast<sup>and Da Silva</sup>, 1977). In order not to influence the cooking rates of the beans they were, throughout this study, soaked overnight (15 h.) at room temperature. Dawson et al. (1952) found that a rapid method of soaking beans was to boil them for two minutes and then soaking for one hour - this resulted in fully rehydrated beans. Additionally, Iyer et al. (1980) showed that soaking beans at higher temperatures (37°C and 45°C) increased the rate of water uptake, but also resulted in a greater loss of solids (and therefore nutrients) due to leakage. A similar effect was reported by Kon (1979). Junek<sup>et al</sup>, (1980) stated that the temperature of the soaking solution can influence the overall cooking quality, 25°C being the optimum temperature though soaking at 35°C gave the lowest shear press values.

When the beans supplied were soaked it was found that the "soft" beans absorbed significantly more water than the "hard" beans and this suggested that water was being prevented from entering the cotyledons either by their reduced permeability or by an increased water potential due to solute changes within the beans. It was clear that the testa was not restricting water uptake because of the formation of a water jacket between the cotyledons and the testa of the "hard" beans as described in Fig. 10 i.e. the testa was permeable to water, there was

therefore no hardshell affect influencing the cooking rate of these beans. Therefore, the observation by Snyder (1936) on soaked Great Northern beans that testa permeability influenced cooking rate was not confirmed in this investigation and in contrast the reduced imbibition value was due to the cotyledons themselves.

The cooking experiments in this preliminary study proved that there was a more than five-fold increase in the total cooking time (3000 min. - 16000 min.) as defined of the hard beans supplied by Bressani, which had been stored at ambient conditions for 18-24 months instead of at 4°C. Since the sample size was only 8 beans this value should be considered an indication of hardness rather than an absolute measurement. The term "ambient conditions" was taken to mean temperatures in excess of 20°C and conditions of high humidity.

This preliminary cooking experiment also showed that the hardness was a property of the cotyledons as a whole, i.e. the cotyledon was uniformly hard and would not yield to pressure and soften. The texture, between the fingers, was not unlike that of a peanut. The texture of a fruit or vegetable as a single structure (in this case bean cotyledons) is only influenced by a small number of variables. Firstly, is the size of the plant cells and their intercellular spaces (Reeve, 1970). Secondly, is skin thickness and/or toughness, for example strawberries with their thin skins are soft and watermelons are firm because of their thick skins. Thirdly, is the properties of the cell contents. Bennet (1950) attributed hardness in uncooked corn to starch grain size. He found that hard dent corn had small starch grains, while soft dent corn had large starch grains. Finally, is the ease of cell separation. This last category is often relevant to cooked produce, where the pectin has been solubilised, but can also refer to fresh fruit. Bourne (1976) stated that tomato fruit softening was related to the breakdown of long



pectin chains into shorter, more soluble units which are not as strong as the original protopectin and therefore the tomato softens.

There are other classes of texture in plant tissues such as "crispness" which is determined by cell turgor (e.g. lettuce and celery) and "stringiness" which is influenced by the fibre (usually lignin) content. "Grittiness" due to cell clumping could be influenced by rate of cell separation if irregular within the tissue and hardness of individual cells would cause a finer form of "grittiness".

The results in Table 1 show that the cooking rate was only significantly affected when the beans were stored at high temperatures and with high moisture content, i.e. these two conditions acted synergistically to cause hardbean. This observation is in agreement with all other storage trials related to the hardbean phenomenon (Morris and Wood, 1956; Muneta, 1964; Ozawa et al., 1978; Burr et al., 1968; and many others). This observation was made unwittingly about 300 BC by Theophrastus (Hort, 1916), who stated that it was the climate that causes "this variation (in texture); a proof of which is the fact the same piece of land, tilled in the same manner produces seeds that are "cookable", sometimes seeds that are "uncookable". In the district of Philippi, if the beans, while being winnowed, are caught by the prevailing wind of the country they become "uncookable" having previously been "cookable." This is obviously due to the humidity of the prevailing wind influencing the moisture content of the beans.

The cooking rate was not significantly affected by testa. Shelled beans were boiled to determine the rate of cotyledon breakdown. As can be seen in Fig. 9 the rate of hardbean breakdown was still much slower than the rate of soft bean breakdown.

The method of determining if a bean was cooked, i.e. squeezing between forefinger and thumb, may appear to be subjective and inaccurate

but it was found to be very reproducible. A similar method was used by Snyder (1936) on cooked beans and by Sistrunk (1977) when investigating the softness of potato slices. It was also recommended by Bressani (pers. comm.). Many researchers have used shear presses (Quenzer et al., 1978; Iyer et al., 1980) or similar devices giving actual numerical values for bean texture (Bourne, 1972; Sefa-Dedeh et al., 1978; Nordstrom<sup>and Sistrunk</sup>, 1979). A review by Bourne (1976) has listed several puncturing devices. Burr et al. (1968) erected an experimental bean cooker wherein rods were positioned over individual beans during cooking. These rods would penetrate and fall through the beans when sufficiently soft and thus by watching for the rods to drop through the samples the cooking rate could be determined. During this project it was attempted initially to use a similar technique with weighted plungers positioned on top of beans and designed to deliver approximately  $3.5 \text{ kg. cm.}^{-2}$  ( $90 \text{ g. over } 2.5 \text{ m}^2$ ) the average force applied by teeth and jaw while chewing (Garrett et al., 1964), but found this too cumbersome - especially for statistically large enough samples to be used. A 100 bean sample would require a container of boiling water  $1600 \text{ cm.}^2$  and a similar sized heating plate. Muneta (1964) set up a taste panel of seven judges to determine if beans were cooked or not. Lamond (1976) produced a discussion on the theory, training and use of food tasting panels. Shehata and Youssef El-Tabey<sup>^</sup> (1979) used both penetrometers and a taste-testing panel and found that both methods gave similar results.

Over a 3-year investigation one cannot guarantee having the same members of a taste panel available and introduction of new members may make results inconsistent. An added inconvenience would be not being able to carry out cooking tests outside normal working hours.

The beans used in this investigation were very easily squashed into a mush if cooked, while uncooked or even partly cooked beans were

quite resistant to finger pressure; it was felt therefore that the finger pressure technique was quite adequate. In fact, hard beans when "cooked" still had a rather gritty texture - due probably to the cotyledons fracturing rather than the cells separating uniformly. This observation was re-enforced by using light microscopy (see Figs.15 and 16).

Smithies (1960) also noted that the softening of peas during cooking was due to cell separation and related this to the dissolution of the pectin in the middle lamella. Breakdown of the middle lamella in such cases was further confirmed by Rockland and Jones (1974) and Sefa-Dedeh et al. (1978) who used scanning electron microscopy.

Thus, it became clear that the prime reason for hardbean was the failure of cells to succumb to external pressure and separate.

It has been suggested by Youssef et al. (pers. comm.) that the cause of the hardbean phenomenon (in Vicia faba) is due to individual cells becoming harder, but this would not cause hardening of the cotyledons as a whole if the rate of cell separation was not reduced as is the case of the hardbean phenomenon in Phaseolus vulgaris, though it may cause a gritty or powdery texture to develop.

Having identified reduced cell separation rate as being the physical cause of hardbean it was necessary to determine why the cotyledon cells failed to separate.

Sterling and Bettelheim (1955) found that raw potato, when subjected to force broke across the cell walls as the middle lamella was stronger than the cell wall - but that the cooked potato breaks along the middle lamella because the cooking process has solubilised the pectin therein.

The middle lamella between cells is composed almost entirely of pectin ("polyuronides composed mostly of anhydrogalacturonic acid residues", Kertesz, 1951). The evidence from the light micrographs presented in this thesis as well as that from the papers already cited, suggested that the pectin in the soft beans had broken down during the

cooking process allowing the cells to separate and therefore the tissue to soften. Therefore, it could be a matter of reduced pectin solubility which accounted for the reduced cell separation rate and therefore firmer texture of the hard beans.

However, the cause of reduced middle lamella breakdown might not be due to the pectin being less soluble but to reduced forces on the middle lamella in the hard beans. These forces would be in the form of turgor pressure from the surrounding cells due to the inflow of water stretching the cell wall and transferring the pressure to the middle lamella. This pressure would then stretch the middle lamella and therefore weaken it. Muller (1967) mooted the idea of starch grain swelling being responsible for cell separation and analysed starch content and amylose/amylopectin ratios to determine this possibility but drew negative conclusions on this basis. He did not, however, investigate the actual swelling power and/or imbibition value of the starch grains themselves which would be the most direct method of determining the involvement, if any, of starch grains in the hardbean phenomenon.

It has already been shown that the hard beans absorb less water than the soft beans and therefore theoretically impart less turgor onto the middle lamella. It was necessary therefore to determine if the swelling power of the hardbean starch grains was reduced during storage thus causing there to be less turgor pressure on the middle lamella due to reduced imbibition value.

Abu-Shakra (1975) stated that the cooking rate of lentils was improved if the cell wall was plastic, thereby allowing cell expansion and reduced intercellular adhesion. A brief inspection of dry cotyledon sections after the addition of water (see Fig.13 & 14) however, showed that there was no difference in cell or intercellular

space size. Both hard and soft bean sections were fully expanded within one second after the addition of water to dry cotyledon sections proving there to be no restriction on cell wall elasticity in either hard or soft beans, although to investigate the effects of reduced water uptake per se would require using whole cotyledons.

Another primary observation was that the hard beans had lost their viability, proven by their inability to germinate (Fig.11 ) and although respiration is detectable (as oxygen absorption) almost immediately after the addition of water (Fig.12 ) the respiration rate was greatly reduced in hard beans. Spontaneous oxygen absorption by embryonic tissue was also reported by Wilson and Bonner (1971) in peanut embryos and by Collins and Wilson (1972) in whole bean<sup>cotyledons</sup> of *P. vulgaris*. This reduced viability may have some bearing on the cause of reduced water uptake because although this is initially passive there must come a point when water is actively drawn into the cell as a part of the mechanism of respiration and germination as discussed by Bewley and Black (1978). Therefore, reduced viability may mean reduced water uptake causing less turgor pressure on the middle lamella than in soft beans and therefore reduced rate of cell separation.

At this stage in the investigation therefore, it was considered that the cause of hard bean was due to one of the following causes:- reduced imbibition value creating less turgor pressure on the middle lamella, reduced pectin solubility, both reduced imbibition value and reduced pectin solubility, or neither of these two factors.

The research programme was therefore, carried out initially in two phases, investigating:-

- (1) the cause and effects of reduced imbibition value,
- and (2) the degree of pectin solubility, and if different in hard and soft beans, the cause of the change.

## Chapter 2. The Cause and Effects of Reduced Imbibition Value

A comprehensive investigation was carried out to determine the cause of reduced imbibition value in hard beans and to determine if this could create sufficiently large a drop in turgor pressure to significantly reduce the rate of cell separation.

Five possible causes for the reduction in imbibition value were considered:-

### (1) Impermeability of the cotyledons -

As already discussed there were no external visible indications that water would not be able to diffuse into the cotyledons. Furthermore, <sup>and Varriano Marston</sup> Jackson (1980) has shown that water passes into the cotyledons of black beans via all parts of its surface and not via any specific structure.

Initially, it was hypothesised during this present study, that the corner thickenings found adjacent to the intercellular spaces might restrict water uptake. These structures were first recorded in Phaseolus vulgaris by Opik (1966) though she did not suggest any function for them. Harris et al. (1975) found similar structures in the cowpea (Vigna unguiculata) but they do not appear to occur in all species of the Leguminosae. They were not evident in the study on developing pea cotyledons (Pisum sativum) by Bain and Mercer (1966). Smith (1974) suggested that these corner thickenings were a final food source to follow the depletion of the starch and protein stores.

It was because these corner thickenings were positioned adjacent to every intercellular space as well as at intervals along the cell wall, interrupted only by plasmodesmata pit fields, that it was thought that they could be involved in the regulation of water movement in and out of the cell. Light microscope studies (Figs. 30 & 31) showed that these structures were very much reduced or missing in hard beans - and transmission electron microscopy suggests that this reduction in size

was due to compaction. Higher magnification showed the presence of small vesicles and fibrils within the corner thickenings of both hard and soft bean samples though the hard bean corner thickenings appear to have undergone some form of internal structural change. As histological studies of the cell wall failed to prove the presence of any suberin or similar compounds analogous to the casparian strips of dicotyledon roots which would prevent the apoplastic passage of water into the cells, it was decided that these corner thickenings were not responsible for the regulation of cell water content. It was feasible that the cell wall bodies in hard beans had been metabolised as suggested by Smith (1974) (the cell walls are much thinner in hard beans due to a lack of lateral cell wall bodies) though as an initial energy source rather than a final one.

(2) Inelasticity of the cell wall -

The possible reduction in cell wall elasticity was disproved by the equally spontaneous expansion of dry bean sections upon the addition of water in both hard and soft bean samples, as already discussed.

(3) Drop in respiratory ability -

Bewley and Black (1978) have shown that the uptake of water by most seed types is in three phases. Phase I, imbibition, is passive, followed by a lag phase (phase II) and then by active water uptake of which only viable seeds are capable (phase III). As the imbibition value of soft beans reached 2.17 before entering any lag phase it was concluded that this was predominantly a passive (i.e. phase I) event and therefore active respiration or metabolism played no part in this reduced water uptake.

Consequently, it was considered that reduced respiration rate was

not responsible for the reduced imbibition value of hard beans.

(4) Increased osmotic potential -

In seeds of such high starch content (>40%) this factor might potentially have the greatest influence on the osmotic potential of the cell. The swelling power and/or water absorption capacity of extracted hard and soft bean starch grains was therefore examined. Figure 17 clearly shows that this is greater in hard bean than soft bean starch grains. The swelling power of both types of starch grains are much higher than that reported by Lai and Varriano-Marston (1979) for soft black bean starch grains though this present work agrees that the swelling pattern is a single stage process. The significant disagreement with starch solubility values compared with Lai and Varriano-Marston can be explained by the fact that Lai and Varriano-Marston used the extraction technique published by Schoch and Maywald (1968). This was an aqueous process and thus some of the soluble starch would be lost during the extraction. The wet bench technique used in this research used only ethanol and thus water soluble starch was not lost from the starch grain extract.

If starch grain swelling power was to be instrumental in the decrease in the imbibition value then the swelling power of hard beans should have been lower in hard beans. As the swelling power was higher in hard beans it was concluded that starch grain swelling power was not responsible for the drop in imbibition value. Although there was a drop in starch content in hard beans it was not enough to more than compensate for the increased swelling power of the starch grains.

Macerated hard bean cotyledons had a greater swelling power at room temperature than soft bean cotyledons (3.5 and 2.8 respectively) and although this cannot be fully accounted for in differences in starch grain swelling power, it suggests that the data for starch



grain swelling power was not an artefact of extraction.

There was also a 13.5% increase in the quantity of extractable ethanol soluble sugar in the hard beans and this should be responsible for drawing morewater into the hard beans than soft beans. The slight increase in solubility of hardbean starch is indicative of limited - amylase activity. Linehan and Hughes (1969a, b) suggested that in potatoes, solubilised starch could leach out of the cells and cement neighbouring cells together by hydrogen-bonding but Lai and Varriano-Marston (1979) investigated black bean starch and concluded that it was unlikely that sufficient amylose would leach out to bind bean cells together to cause hardbean.

Therefore, reduced imbibition value was not caused by an increase in osmotic potential or due to a change in starch grain properties.

(5) Leakage -

There was a nearly ten-fold increase in the quantity of leachates from whole hard bean cotyledons during imbibition (see Table 2 ) proving that there was a massive outflow of solutes from the hard beans. Chilling injury to the membranes reported during imbibition of lima beans (Phaseolus lunatus) by Woodstock and Pollock (1965) was an unlikely cause as both hard and soft beans were imbibed at over 20°C.

Loss of nutrients such as sugars and protein by leakage may help to explain the lower biological value of hard beans.

Ching and Schoolcraft (1968) measured leakage of electrolytes from crimson clover (Trifolium incarnatum) and perennial ryegrass (Lolius perenne) by conductivity and used this as an index of seed viability during adverse storage (though the correlation only appeared conclusive in the crimson clover samples) and related this to the degradation of cellular membranes and consequently the loss of control

of permeability. The moisture content during storage was directly responsible for the loss of viability and membrane damage. High temperature during storage was not necessary to assist the membrane break down.

Membrane deterioration was confirmed as the cause of leakage in hard beans by phospholipid analysis (Fig. 19) and further proof was provided by transmission electron microscopy where membranous structures such as mitochondria, vesicles, endoplasmic reticulum and plasmalemma were conspicuous by their absence from hard bean cytoplasm but their presence in soft beans (Figs. 34 and 35). Koostra and Harrington (1969) found that during artificial ageing of cucumber seeds (Cucumis sativus) the membranes underwent oxidative deterioration which correlated with the loss of viability. Priestly and Leopold (1979) reported phospholipid decay, particularly phosphatidylcholine, in stored soybeans (Glycine max) and postulated that the increase in the soybean moisture content due to high relative humidity during storage permitted the activity of phospholipid hydrolysing enzymes but that as the beans were not fully hydrated membrane repair was not operable. Similarly, McKersie and Thompson (1975) postulated an imbalance between the mechanisms of cytoplasmic membrane maintenance and decay and claimed that this resulted from NADH-Cyt c reductase inactivation. Gilkes et al. (1979) found that phospholipid catabolism is in excess of phospholipid synthesis in mung bean cotyledons (Vigna radiata) for an appreciable period of time before senescence. The thin-layer chromatography analysis appears to confirm that there was metabolic activity during the storage of these black beans due to their high moisture content resulting in membrane catabolism without any concomitant membrane repair mechanism resulting in leakage of solutes from the cell.

The loss of 200 mg. gm.<sup>-1</sup> leachates from the cotyledons only accounts for 0.2 I.V. units which alone does not account for the drop in imbibition value in full. The rest can be accounted for in the amount of water these leachates would have been responsible for drawing into the cell. The correlation coefficient,  $r$ , between leakage and imbibition value was  $-0.98$  ( $\rho = < 0.001$ ) (Fig. 20) thus confirming that leakage was responsible for the reduced imbibition value. It was then necessary to determine if reduced imbibition value could be responsible for an increase in the total cooking time of the beans. This entailed the reduction of water uptake by the soft (control) beans by artificial means, i.e. the use of strong osmoticum. Soaking soft beans in concentrated sucrose solutions reduced the imbibition value of beans and ultimately increased their total cooking time as shown in Figs. 21 and 22. The increase in total cooking time was exponentially related to sucrose concentration. Statistical analysis showed that there was a correlation coefficient of  $r = -0.99$  between imbibition value and total cooking time under these conditions and appeared to confirm that water content of the bean cotyledons was responsible for increased cooking time.

The experiment was repeated with concentrated solutions of mannitol and NaCl creating the same effect.

When the imbibition value of beans was analysed after cooking it was found that the imbibition value of soft beans and soft beans whose total cooking time had been increased with sucrose solutions always exceeded 2.38 (see Table 3), i.e. the beans did not soften unless they had absorbed 1.4 times their own weight in water, lending credence to the cell expansion theory of cell separation. Furthermore, the imbibition value of hard beans after "cooking" was only 2.10 suggesting



that the rate of cell separation was reduced because of the inability of the cotyledons to hold sufficient water.

If the cell expansion theory was correct then nullifying the affect of imbibition value, i.e. breaking open the cells, should result in a decreased rate of middle lamella breakdown during boiling.

When macerated soft bean tissue was boiled it was found that the cell wall fragments remained attached along the middle lamella for more than 2 h. showing no sign of pectin breakdown (see Figs. 28 and 29).

This appeared to further confirm that cell separation during cooking was dependant on turgor pressure within the cell. To finally confirm the influence of imbibition value it was necessary to emulate the cause of reduced imbibition value in hardbeans as closely as possible by inducing leakage in soft beans. By treating soft beans with hot ethanol (80%) in a Soxhlet apparatus it was possible to dissolve out all the available sugars and destroy the membranes. After drying, the imbibition value of these beans (1.85) was comparable with that of hard beans (1.88) yet the beans all cooked in 35 min. ( $CT_{100} = 3435$ ) which bore closer comparison to the total cooking time of soft beans ( $CT_{100} = 3000$  min.).

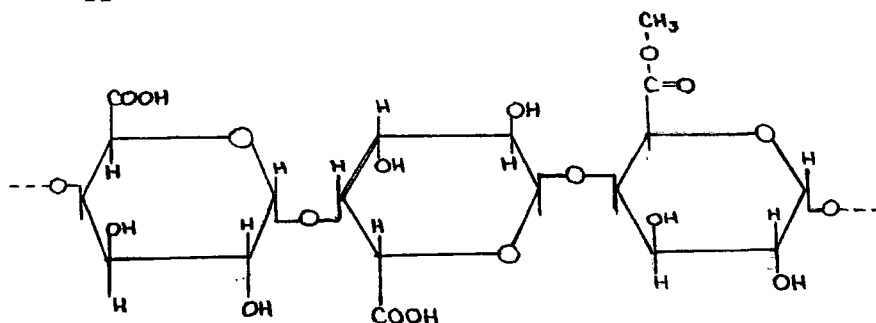
This experiment alone showed that although the artificially reduced imbibition value can increase the total cooking time of beans (to nearly 12000 min.) the imbibition value plays only a minor role in the rate of cell separation during the normal cooking process, and that a more significant change occurred during storage of these beans to reduce their cooking rate. The concentrated sucrose solutions when used to reduce the imbibition value of the beans were possibly also reducing the pectin solubility in the middle lamella and this suggests that when reduced pectin solubility and reduced imbibition value are operating together, then we have hard beans.

### Chapter 3. The Cause and Effects of Reduced Pectin Solubility

The transmission electron micrographs presented in Figs. 36 and 37 are confirmation of the light micrograph observations (Figs. 15 and 16) that the prime cause of hardbean was reduced cell separation rate. The electron micrographs clearly show that in the soft beans the middle lamella has dissolved and the outer cellulose fibrils of the primary cell wall are beginning to disperse because there is no matrix to hold them together. The middle lamella of the hardbean showed no evidence of dissolution and the cellulose fibrils remained in an orderly arrangement. There was also a greatly reduced corner thickening in the hardbean sample. The middle lamella is composed almost entirely of pectin, one of the five main plant polysaccharides. It is also found in the primary cell walls.

The main component of pectin is 1 - 4 linked  $\alpha$ -galacturonic acid units and due to its high uronic acid content is classed as a polyuronide. The DP may reach 300 000. The molecule is unbranched though side-chains of glucuronic acid, xylose, arabinose and rhamnose are commonly found at the C<sub>2</sub> and C<sub>3</sub> positions. Rhamnose is also found in small amounts within the main chain of the galacturonic acid units.

The typical structure is shown below:-



There is a structural twist at each (1 - 4) glycosidic link.

The C<sub>6</sub> carboxyl group may be free, esterified with methanol as shown in the third residue above, neutralised by a monovalent cation or H<sup>+</sup> or may be linked to another pectic carboxyl group via a cation bridge - usually calcium or magnesium. When there is a high proportion of these cation bridges the pectin solubility decreases in water. The presence of methyl groups or monovalent cations on the carboxyl groups prevents the formation of cross-linkages therefore maintaining the pectin solubility. Pectin may also be bound chemically to other cell wall constituents, predominantly hemicellulose, via the glycosidic linkages to glucuronic acid.

The pectin classification as outlined by Pilnik and Voragen (1970) is as follows:-

Pectic substances - a general term for the complex colloidal carbohydrate derivatives of plant origin which contain a large proportion of anhydrogalacturonic acid units in unbranched chains. The carboxyl groups may be partly esterified by methyl groups and partly or completely neutralised by one or more bases.

Protopectin - insoluble in water, restricted hydrolysis yields pectinic acid. The term protopectin is no longer in current usage.

Pectinic Acid - colloidal polygalacturonic acid containing more than a negligible amount of methyl ester groups. Pectinates are the normal or acid salt of pectinic acid.

Pectin - water soluble pectinic acid of varying methyl ester content and degree of neutralisation which are capable of forming gels with sugar and acid under suitable conditions.

Pectic acid - colloidal polygalacturonic acid essentially free from methyl ester groups. The salts of pectic acid are either normal or acid pectates.

As pectin composes the intercellular cement it is reasonable to assume that as the plasticity or solubility of the pectin changes then there will be a corresponding change in the texture of the plant tissue (in this case bean cotyledons). An increase in pectin plasticity will mean a softer fruit in the raw state (e.g. peaches) and an increase in pectin solubility will mean a softer product after boiling (e.g. beans). Both plasticity and solubility of pectin appear to be governed by the proportion of cation bridges within the polymer structure. Calcium has long been known to reduce pectin solubility (True, 1922) and to reduce the plasticity of the developing cell wall by increasing the rigidity of the pectin (Tagawa and Bonner, 1957; Cooil and Bonner, 1957).

Calcification is essential for the normal growth of the cell wall (Cormack<sup>et al</sup>, 1963) and enables the growth of more tightly packed cells by the development of calcium bridges within the middle lamella (Cormack, 1965). By breaking down the pectin one can separate plant cells. Pectic enzymes have been used for many years to macerate plant tissues (Chayen, 1952; Zaitlin, 1959) and are a common mechanism for tissue invasion by fungi (Bateman and Lumsden, 1965; Mutto et al., 1978). Chelating agents which can remove the calcium ions from pectin have also been used to macerate plant tissue, such as oxalate (Northcroft, 1951) and EDTA (Letham, 1960).

As pectin properties have therefore been shown to determine the ease with which neighbouring plant cells can be separated, and also to change in situ, pectin solubility in the hard and soft beans was investigated and is displayed in Table 5. In order to make the results relevant to the hardbean phenomenon soluble pectin was expressed as pectin solubilised during imbibing and cooking (i.e. cold and hot water soluble pectin) and insoluble pectin as that which remained adherent to the cell wall and removed by 0.5% ammonium oxalate and mild saponification

with NaOH. While 40% of the soft bean pectin was solubilised during the equivalent of the normal preparation and cooking process only 15% of the hardbean pectin solubilised during the same treatment. 18% of the hardbean pectin remained adherent to the cell wall after mild saponification.

A similar result was recorded by Buescher et al. (1976) who investigated the hardcore phenomenon in cold stored potatoes. They reported a decrease in water soluble pectin and a concomitant increase in SHMP (sodium hexametaphosphate) soluble pectin. It was hypothesised that the pectin solubility was decreased by phenolic substances and cations binding to the pectin due to enhanced permeability of the cell membranes. An increase in the absorbance of the hard core pectin fractions at 280 nm (the  $\lambda$  max of phenolics) supported this theory.

Hughes et al. (1975) found that the rate of softening of potato discs was directly related to pectin solubility - as the solubility increased then the potato discs softened at a faster rate, this process was speeded up by the addition of NaCl and SHMP, the sodium displacing the divalent cations from the salt bridges.

Postlmayr (1956) reported that as Elberta freestone peaches ripened (i.e. softened) the proportion of insoluble pectin in the middle lamella dropped and that the insoluble pectin could be solubilised by the removal of bivalent cations, i.e. the removal of the cations from the pectin allows for the softening of the peach.

A review by Bourne (1976) quoted figures for the firmness of carrots after soaking in one of six molar solutions of chlorides (Na, K, Ca, Mg, Zn, Al) and found that only soaking in the bivalent solutions (i.e. Ca, Mg or Zn) gave firmer texture of the carrots than soaking in distilled water. Trivalent cations had no significant effect because they were unable to bind to free carboxyl groups of the pectin and form cation bridges, and the monovalent cations (Na, K) would only have the effect of neutralising the  $\text{COO}^-$  groups.



The results expressed in Table 4 further suggested that pectin solubility was relevant to the hardbean phenomenon. It has been reported by many workers that  $\text{NaHCO}_3$  increases the cooking rate though with possible side effects such as over-softening (Tinkler and Masters, 1932), thiamine loss (Perry et al., 1976) and discoloration (Rizley and Sistrunk, 1979).  $\text{NaHCO}_3$  was used by Ku et al. (1976) to remove the oligo-saccharides responsible for flatulence in beans (stachyose and raffinose) but was reported to cause a three-fold increase in protein loss. Van Buren (1980) reported that  $\text{NaCl}$  in the cooking water can soften snap beans by substituting sodium for the calcium on the carboxyl binding sites. Varriano-Marston and de Omana (1979) found that each of four sodium salts ( $\text{NaCl}$ ,  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{CO}_3$ ,  $\text{Na}_5\text{P}_3\text{O}_{10}$ ) increased the cooking rate of black beans via substitution of calcium and/or magnesium. This agrees with the conclusions to be drawn from Table 4 where, for example, hard bean cotyledons broke down in 15 min. when boiled in 0.05 M  $\text{NaHCO}_3$  due to the substitution of  $\text{Ca}^{2+}$  with  $\text{Na}^+$  but this took more than 3 h. in boiling water. Similarly,  $\text{KOH}$  which solubilises pectin increased the rate of soft bean cotyledon breakdown. These results all suggested that the reduced rate of cell separation, and thus the hardbean phenomenon, was caused by reduced solubility of the pectin due to an increase in the number of cation bridges within it.

Muller (1967) devised a PCMP number (calculated as:-

$$\frac{\text{Pectin} \times (\text{Ca} + \frac{1}{2} \text{Mg})}{\text{Phytin}} \quad )$$

and found it correlated with the hardness of peas and therefore presumably inversely correlated with phytin levels. He further stated that hardness in four varieties of cooked soup-peas was due to calcium and magnesium from the testa but this did not apply in the wrinkled pea variety Cennia. Unusually the hardness tests were carried out on the cooked seeds after

they had been allowed to cool overnight. As discussed earlier, when black beans were shelled before soaking and cooking it was found that their cooking rate was not improved and thus it was considered that the testa played no part in the hardbean phenomenon. Muller also proposed that lignin in the cotyledon cell walls may be binding the cells together independantly of calcium and magnesium and thus influencing the cooking rate of peas. During this present study on the black beans, treatment of cotyledon sections with phloroglucinol and HCl failed to prove the presence of any cell wall lignin (except in the vascular elements) and therefore this possibility was discounted.

It was concluded at this point that the level of pectin solubility governed the rate of cell separation and that this rate was slower in hard beans due possibly to an increased amount of calcium and/or magnesium in the pectin. It was therefore necessary to analyse the levels of pectin calcium and magnesium in the hard and soft beans, and the quantities bound to the cell wall per se because pectin binding to hemicellulose and possibly to cellulose fibrils or binding between the three cell wall components would also restrict the dissolution of the cell wall. Ideally, cell wall prepared in the same way as were the starch grains should be used but the quantity of material available was insufficient to allow meaningful replication of this technique.

Fergusson et al. (1980) reviewed the problems of calcium fractionation and recommended that the best technique for the analysis of pectin and cell wall cations (from leaf material) was extraction with 80% acetic acid though this technique did not appear to take into account cytoplasmic cations. When the extraction was carried out on the hard and soft bean material it proved there was significantly more magnesium in the cell wall of hard beans than soft beans but the technique gave inconsistent results.

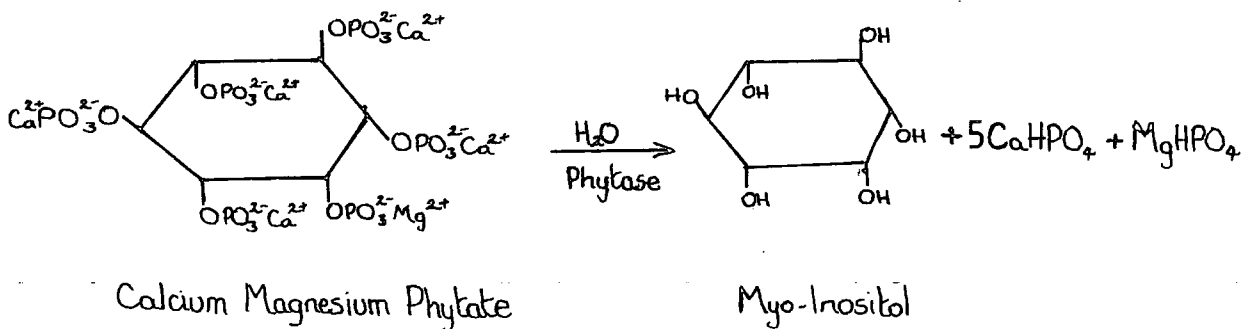
The technique adopted here was to successively wash bean meal in mannitol, water and acetone to remove all components except starch grains and cell wall fragments. By washing in mannitol, membrane bound organelles such as mitochondria and vesicles were removed without rupture and this was followed by the removal of water soluble compounds such as sugars and phytic acid. The acetone wash dissolved any extraneous membranous material. After further washing in deionised water, light microscope inspection confirmed that the remainder was cell wall fragments and starch grains. Nevins *et al.* (1967) used centrifugation in phosphate buffer and acetone as a method of cell wall extraction in *P. vulgaris* seedlings though there were no starch grains to contaminate the end product. As they were analysing cell wall sugars and not cations the phosphate buffer did not interfere with the cell wall cations. Lampport<sup>and Northcote</sup> (1960) washed his cell wall extracts with NaCl when analysing the cell wall protein. Analysis of phosphorous levels on the cell wall fraction in this current study proved that there was no significant interference by calcium magnesium phytate (<4%). An internal standard (control, cold stored beans) was extracted with each hard bean extraction to normalise the results.

The increased values for the cation levels in the hardbean cell wall supports the theory that the pectin in hard beans is less soluble because of the formation of cation bridges between free carboxyl groups.

Mattson (1946/7) noted that cooking peas in  $\text{CaCl}_2$  suppressed the cooking rate. He stated that it was the calcium content of the pea pectin which reduced the cooking rate and claimed that this was prevalent in peas of low phytin content. It was suggested that phytin was acting as a calcium/magnesium chelator, thus high phytin content meant low cation levels in the pectin and therefore soluble pectin. Similarly, he found that soaking peas in water for eight days caused a

decrease in cooking rate. He hypothesised, that the phytin lost its chelating ability due to hydrolysis by phytase to myo-inositol and  $\text{CaHPO}_4$  and  $\text{MgHPO}_4$  and was therefore unable to reduce the pectin cation levels. Haydar<sup>et al</sup> (1980) found that steeping potatoes in calcium or magnesium made them firmer but claimed that it was not only due to a reaction between the cations and pectin but also to the cations binding to starch phosphate groups and therefore decreasing the swelling power of the starch grains. Mattson (1950) later correlated the level of phytin within the peas to the level of  $\text{P}_2\text{O}_5$  in the soil. Muneta (1964) also noted that the origin of the beans may govern their cooking time. He found that identically stored pinto beans (Phaseolus vulgaris, stored at  $> 70^\circ\text{F}$  in sealed containers) had cooking times of 82 min. (Idaho variety) and 234 min. (Michigan variety) but as each variety had a different moisture content (8.9% and 12.6% respectively) it was not possible to tell if it was the moisture content<sub>or variety</sub> of the beans or the soil conditions at the place of origin that caused increased susceptibility to the development of hardbean.

Phytin is the calcium magnesium salt of phytic acid (myo-inositol hexaphosphate) and serves as a source of inorganic phosphate for the phosphorylation reactions in germinating seeds. The enzyme phytase converts phytin to myo-inositol by hydrolysis and thus calcium and magnesium are released into the cell.



Phytic acid, or its salt phytin is found in the seeds of many plant species and in the Leguminosae is associated with the protein bodies. Lott and Buttrose (1978) localised the <sup>calcium magnesium</sup> phytate in four legume species from three different sub-families and Lolas and Markakis (1975) confirmed the presence of a protein-phytic acid complex in P. vulgaris.

Smithies (1960) found a correlation between phytin content of peas and their cooking rate only when the average phytin content was low. Rosenbaum <sup>et al</sup> (1966) found a correlation between phytic acid content of field peas (Pisum sativum) and their cooking rates but not necessarily the calcium content, with peas of high phytic acid content cooking faster than peas of low phytic acid content (which implicates the chelating capacity of the phytic acid); but in a later paper (Rosenbaum <sup>and Baker,</sup> 1969) he showed that the peripheral region of the field peas cooked slower than the pea interior despite containing more phytic acid. Quenzer <sup>et al</sup> (1978) found a positive correlation ( $r < 0.05$ ) between calcium content of pinto beans and their shear values which suggests that phytin levels influence the cooking rate of these beans. The chelating capacity of phytic acid has often been blamed for the increased requirement for Mg, Mn, Zn and Ca in animals fed soybean protein.

In order to determine if the source of the pectic cations was phytin, the phytin levels of the hard and soft beans were measured (see Table 5) and found to have dropped by 28% during the development of hardbean. It was hypothesised that instead of this influencing the calcium content of the pectin by failing to chelate the cations it was because the hydrolysis of phytin, by phytase, made more soluble calcium and magnesium available for the creation of cation bridges.

The hardbean pectin cation levels increased during storage and therefore the only direction the calcium and magnesium could be moving was from the phytin to the cell wall and therefore the chelating capacity

of phytic acid as suggested by Mattson appears to be irrelevant to the hardbean phenomenon.

Further analysis of the pectin chemistry (see Table 6) showed that the degree of esterification dropped from 51% to 15% in hardbeans which means that more free carboxyl sites were available for cation bridges. Lee <sup>et al</sup> (1979) found that low temperature blanching made carrots firmer because of the activation of PME thus creating sites for cation bridges and therefore less soluble pectin, but high temperature blanching failed to do this because the PME was deactivated. Bartolome and Hoffe (1972) found the same effect with the low temperature blanching of potatoes and stated that the activation of PME at 60°C was due to plasmalemma breakdown allowing the outward diffusion of cations to activate the enzyme. Van Buren (1980) also found that the low temperature blanching<sup>h</sup> of snap beans increased the number of free carboxyl groups in the pectinaceous middle lamella which presented the correct conditions for the creation of cation bridges and a firm product.

Conversely, Reeve (1959) found that as the degree of esterification reached 100%, ripening peaches were at their hardest and then the peaches softened as the degree of esterification decreased, i.e. pectin demethylation increased the pectin solubility though it must be remembered that such changes may be coincidental and the true influence on the peach texture may be related to the cell contents.

Reeve also noted that as the peaches softened the cell walls became thinner. As already observed (p.97) the cotyledon cell walls became thinner during the development of hardbean but this coincides with the beans becoming harder.

In order to confirm the influence of cations in the hardbean phenomenon, beans were imbibed in 0.03 M CaCl<sub>2</sub> and as can be seen in Fig. 38 the cooking rate is reduced, an effect also recorded by Mattson (1946/7) and Haydar et al. (1980). The effect of calcium ions is

increased further by simultaneously incubating the beans with PME (in tris HCl buffer pH 7.5). This supports the theory that pectin demethylation in the beans during storage facilitates pectin calcification.

Further, when this treatment was applied over 24 h. to beans which had been pre-treated with hot 80% ethanol to induce leakage and therefore reduce cell wall turgor pressure then the beans failed to soften even after 3 h. boiling ( $CT_{100} > 18000$  min.) which confirms that when reduced water uptake and pectin calcification act synergistically extensive hardness develops within the bean.

Pectin solubilisation is now accepted to be the chemical change responsible for the softening of fruits during ripening though the mechanism is not the same in all tissues (Sawamura *et al.*, 1978; Wallner and Bloom, 1977; Awad and Young, 1979). Consequently, it is hypothesised here that the reverse process - the desolubilisation of pectin is responsible for the development of hardbean during storage. Data has been presented that shows a decrease in pectin solubility coinciding with an increase in pectin cation levels and a decrease in phytin levels from where it is suggested the calcium and magnesium originates due to phytin hydrolysis. Evidence of membrane breakdown and leakage out of the cell of solutes and electrolytes have already been presented and it is claimed that this is how the calcium and magnesium reach the cell wall and also possibly activate PME. Evidence is also presented of a reduction in pectin methyl esterification which facilitates an increase in cation bridges in the pectin by increasing the number of available free carboxyls.

The effect of increased cell adhesion is aggravated further by the reduced imbibition value of the beans due to leakage which reduces turgor pressure on the cell wall and therefore on the pectin also. This hypothesis is supported by the induction of hardness in soft beans by Soxhlet treatment followed by incubation in calcium salts and PME.

#### Chapter 4. Storage Trial.

An explanation of the hardbean phenomenon has been demonstrated and its validity supported by inducing the relevant symptoms (leakage and pectin calcification) in soft beans and thereby increasing their total cooking time.

In order to confirm this explanatory basis, soft beans were stored under conditions of high relative humidity (75%) and high temperature (34°C) to induce hardbean and to follow the relevant variables as hardness developed.

The first measurable change, induced by high relative humidity, was to increase the moisture content of the beans by approximately 0.13% per day (Fig.42), the consequence of which, at elevated temperatures, was increased metabolic activity. One would expect this to be reflected in seed vigour, but this did not increase during the storage trial. In a previous storage trial (unpublished data) the seed vigour did increase to nearly 100% (i.e. nearly all the beans germinated within 24 h.) and then dropped back to the original level (80%) over a period of 36 days before the trial was prematurely terminated due to incubator failure. The rise in moisture content increased the metabolic activity and hence seed vigour because biochemically the bean was more readily prepared for germination due to increased enzyme activity.

After 40 days seed vigour dropped rapidly and was accompanied by a drop in seed viability which had previously been 100%, reaching 0% after 72 days (Fig.43). As stated by Burr (1973) the United States Standard for Beans (1969) permits beans to contain up to 18% moisture without special labelling - but the first stages of degradation are seen here to occur below this moisture level and have also been recorded to occur below 18% by Morris and Wood (1956). Muneta (1964) calculated



a correlation coefficient of +0.81 between moisture content and cooking time.

When the level of soluble sugar was analysed it was found to drop markedly for the first 20 days because this is the first energy source to be utilised at the onset of germination. The level then increases to above the original value of  $69.9 \text{ mg. gm.}^{-1} \text{ d.wt.}$  by day 60 and is an indication of  $\alpha$ -amylase activity exceeding the respiratory activity of the bean. The total sugar content (starch + soluble sugar) levels off at day 40 (Fig. 48) suggesting that although  $\alpha$ -amylase activity is still proceeding at this point there is no more glucose hydrolysis. It is feasible that at the moisture content prevalent in the beans during storage there is sufficient moisture to initiate metabolic activity but insufficient to activate m-RNA activity and therefore enzyme synthesis. Marcus and Feeley (1964) found that in peanut cotyledons the entire apparatus necessary for protein synthesis was functional, but m-RNA was a limiting factor corrected by imbibition which activated the microsomes.

Consequently, the activity of any enzyme within the cell will be governed by the actual "life time" of the enzyme molecules as they are not renewed by enzyme synthesis. This explains why  $\alpha$ -amylase activity ceased after day 60, shown by the levelling off of starch levels in Fig. 47.

The leakage from the cotyledons on rehydration was stable for the first 50 days (fluctuating between  $14$  and  $20 \text{ mg. gm.}^{-1} \text{ d.wt.}$ ) but then increased rapidly, reaching  $131 \text{ mg. gm.}^{-1} \text{ d.wt.}$  after 84 days storage. This is an indication of plasmamembrane breakdown. Degradation of the mitochondrial membranes occurring at the same time will be the cause of seed death. Parrish and Leopold (1978) state that membrane decay is "... primary or causal rather than a secondary effect ..."

viability loss and related it to a loss in respiratory ability, leakage and reduced water uptake. The drop in imbibition value matches the rise in leakage as expected and confirms the relationship between these two variables.

The levels of lipid extracted from the seeds rises during the first 20 days of storage, but between days 50 and 60 there is a sudden drop from 28.8 to 22.2 mg. gm.<sup>-1</sup> d.wt. Priestley and Leopold (1979) found that during the ageing of soybeans (Glycine max) at 100% RH and 40°C over 5 days there was a slight increase in total lipid extracted though the phospholipid levels did drop slightly. Chapman and Robertson (1977) found that phospholipid content of soybeans stored at 85% RH and 35°C doubled over 10 days. TLC analysis of the extracted lipids during this study (Fig. 56) shows a continuous degradation of the three main phospholipid components of the extract, particularly after day 40. Koostra and Harrington (1969) claimed that during the ageing of lettuce seeds the phospholipids were peroxidised by free radicles from oxidised fatty acids and remained as a blue spot on the origin during TLC. No such spot was found on this study and therefore it was concluded that the membrane phospholipids had not been peroxidised.

Further evidence of membrane transformation was the rapid decrease in the percentage polar lipids in the lipid extract as shown in Fig. 55.

Cuning and Osborne (1978) studied membrane phospholipids in oats (Avena fatua) and stated that when the oats were fully imbibed, but still dormant rapid membrane turnover was occurring as a part of cell maintenance rather than repairing sites of localised membrane damage. Gilkes et al. (1979) also found that membrane turnover was a continuous process and reported that the half life of newly synthesised phospholipids in mung beans (Vigna radiata) was 2 to 2.5 days. In hard beans, if

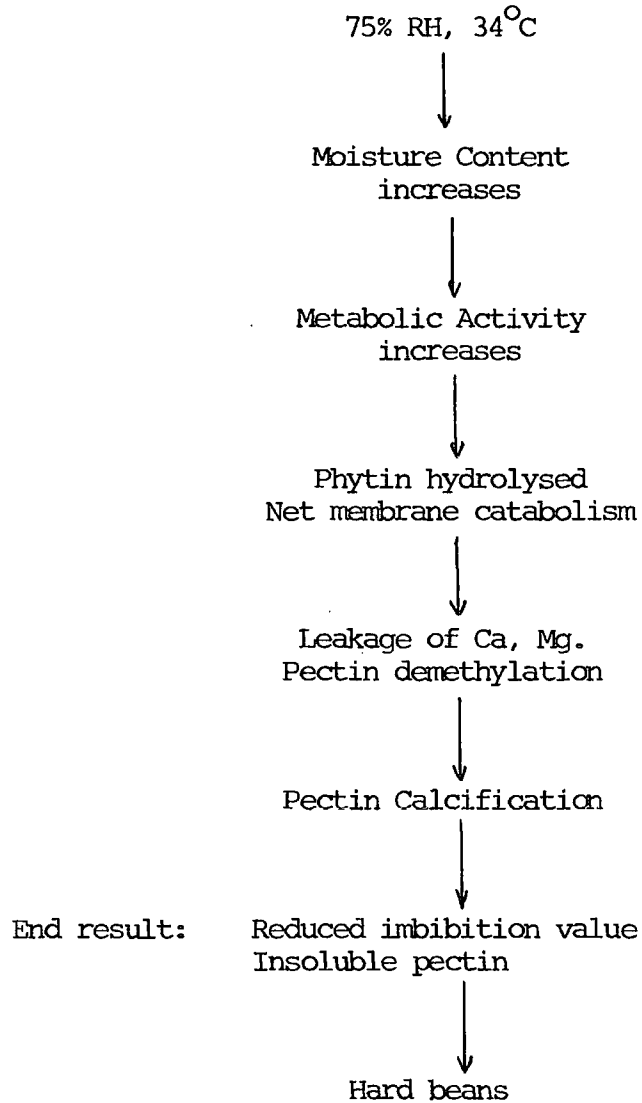
this system operates, as the beans are only partially imbibed it is possible that only half this system of membrane turnover is working - the membranes are being catabolised but the rate of replacement is inadequate to maintain intact membranes. Full imbibition can restore the enzyme levels via RNA activity and allow the seed to germinate, but if the elevated moisture content is allowed to persist under conditions of elevated temperatures for too long the metabolic breakdown within the cell becomes permanent and cell death occurs. Consequently, the membrane repair system ceases to function, the membranes breakdown and leakage occurs. Once this happens, the development of hardbean is inevitable and irreversible. This critical period appears to occur between days 50 and 60 in this storage trial and coincides with the onset of increased cooking time (Fig. 39) due to leakage of cations. During this same period the percentage of water soluble pectin dropped below 25% from a maximum of 47% (Fig. 52). The pectin solubility level rises and falls as the pectin magnesium level falls and rises (Fig. 50) which supports the hypothesis that the number of cation bridges within the pectin determines the pectin solubility and in turn influences the total cooking time of the beans. The correlation between cell wall magnesium levels and percentage insoluble pectin was  $r = +0.93$  ( $p < 0.001$ ) (Fig. 53). The calcium levels throughout the storage trial fluctuated though the general trend was upwards (Fig. 49).

Phytin levels dropped during this 84 days period by 20.9%, mainly after day 30, coinciding with the start in increases in the pectin cation levels.

The total cooking time began to increase rapidly during this critical stage (days 50 and 60) from 2180 min. to 7150 min. at day 84. Figures 40 and 41 demonstrate how the cooking rate has degenerated from being rapid from days 0 to 30, all beans softening within 20 min., to the

beans cooking slowly over a period of more than 90 min. by day 84.

Therefore, the sequence of events within Phaseolus vulgaris during storage which lead to the development of hardbean would appear to be:-



## Chapter 5. Blanching

It has been reported by several workers that the development of hardbean can be retarded by blanching before storage (Molina et al., 1976; Davis <sup>et al</sup>, 1980). As already stated, Lee et al. (1979) found that low temperature blanching can improve carrot texture during storage - in this case to prevent excessive softening - by the activation of PME.

The technique used in this investigation was to soak the beans overnight and then boil for 5 min. After drying (at room temperature for 36 h.) and surface sterilisation the beans were stored at 75% RH and 34°C. Analyses were performed at 6 day intervals.

It is immediately obvious that blanching, in this investigation, did not work. The total cooking time increased linearly after day 6 - reaching 5300 min. by day 36 (Fig. 57) and during the same period pectin solubility decreased (Fig. 58). The imbibition value remained stable at  $2.20 \pm 0.03$  which was the same as for soft control beans despite leakage being a consistent  $105 \text{ mg. gm.}^{-1} \text{ d.wt.}$  ( $\pm 6 \text{ mg.}$ ) - see Table 7 - and this was possibly due to chemical and/or structural changes in starch and protein properties during soaking and boiling which enabled them to hold more water on rehydration.

Due to extensive pectin calcification and possibly interference by starch with the cell wall the total cooking time increased despite the high imbibition values. Although Lai and Varriano-Marston (1979) have stated that interference of soluble starch, binding to the cell wall, was not a causative factor in the hardbean phenomenon, it is possible that in these blanched beans more starch has been solubilised and could be playing an adhesive role via hydrogen-bonding to the cell wall components, as was suggested by Lineham and Hughes (1969a, b)

who worked on hardcore in potato.

Phytin levels dropped during imbibition to approximately 24.5 mg. gm.<sup>-1</sup> d.wt., a drop of over 15%, and remained stable throughout the storage period (see Table 7). This was due to phytase activity during imbibition and despite consequent deactivation during boiling preventing further phytin hydrolysis, there was apparently sufficient free calcium and magnesium to desolubilise the pectin. Smithies (1960) also noted that prolonged soaking leads to hardness in pea texture due to phytin breakdown. Kon (1979) reported that the optimum temperature for phytase activity during imbibing by Californian small white beans was 40°C.

and Loh  
Tabekhia<sub>A</sub> (1980) however, reported only a slight decrease in the phytate levels of four dry bean varieties during 12 h. soaking and stated that during the first 2 days of germination phytase activity was not prevalent.

The reason why this blanching experiment accelerated rather than retarded the development of hardbean was, therefore, due to phytase activity during imbibition and membrane rupture during boiling which lead to leakage of solutes and pectin calcification.

The technique used by Molina et al. (1976) were effectively dry heat techniques such as heating in a retort at 15 psig (121°C) for 2 min. or under steam (98°C) without pressure for 10 min. Both techniques preserved the cooking quality of beans during storage. Thus, in that case, by heating beans without prior imbibition, phytase was deactivated before phytin hydrolysis could take place. Furthermore, Molina et al. found that the imbibition value was higher in heat-treated beans which would facilitate further the separation of the bean cells, though no correlation was found with the puncture force required to penetrate the cooked beans.

That imbibition before blanching accelerated the development of hard beans provides further support for the hypothesis described earlier. Imbibition had the same effect as increased moisture content by allowing metabolic activity, and boiling duplicated the effect of senescence by causing membrane breakdown thus the mechanism of hardbean development was allowed to operate.

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