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THE EXTRACTION AND CHEMICAL COMPOSITION OF ALEURONE GRAINS (PROTEIN BODIES) ISOLATED FROM SEEDS OF *VICIA FABA*

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Abstract—A procedure for the isolation of aleurone grains from seeds of *Vicia faba* is described which gives a preparation essentially free of starch grains, nuclei, mitochondria and ribosomes. The stability of the grains was studied over a range of concentrations of sucrose, phosphate and magnesium chloride. The isolated grains were found to contain 14·1 per cent by weight of non-dialysable nitrogen and 1·4 per cent by weight of phosphorus, the majority of which was phytate. No convincing evidence was obtained to suggest that the grains contain RNA. The major proteins present in the grains were the storage proteins, vicilin and legumin, but in addition to these were several different albumins. The albumins of aleurone grains isolated from 24-hr soaked seeds possessed proteolytic activity and acid phosphatase activities. Phytase activity, however, was only shown by albumins isolated from aleurone grains of seeds germinated for periods longer than 24 hr.

INTRODUCTION

HARTIG[1] first isolated sub-cellular particles from seeds and showed that these particles contained protein. He called these particles aleurone grains. More recently, aleurone grains have been isolated from seeds of *Arachis hypogaea* by Dieckert *et al.*;[2] from grains of *Triticum vulgare* by Graham, *et al.*;[3] from seeds of *Pisum sativum* by Varner and Schidlovsky;[4] and from seeds of soybean by Saio and Watanabe.[5]

It has been known since the time of Sachs[6] that even those aleurone grains occurring in a single cell in some species are heterogenous with respect to size and to the presence or absence of inclusions. Similarly, the presence of various types of inclusions in aleurone grains led Paleg and Hyde[7] to suggest that the term aleurone grain had been applied to a "heterogenous collection of bodies . . . and that there may be more than one basic structure with more than one basic mode of evolution and/or dissolution". Evidence for heterogeneity amongst aleurone grains has also been obtained with sub-cellular preparations of these particles. Dieckert *et al.*[2] isolated aleurone grains from seeds of *A. hypogaea*, separating them into two fractions with different nitrogen and phytate contents. Graham *et al.*[3] were able to separate an aleurone grain preparation from wheat grain into small and large particles by centrifugation through a sucrose density gradient. Furthermore, these workers were able to

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[1] T. HARTIG, *Botan. Z.* **13**, 881 (1855); *Botan. Z.* **14**, 257 (1856).
[2] J. W. DIECKERT, J. E. SNOWDON, A. G. MOORE, D. C. HEINZELMAN and A. M. ALTSCHUL, *J. Food Sci.* **27**, 321 (1962).
[3] J. S. D. GRAHAM, R. K. MORTON and J. K. RAISON, *Australian J. Biol. Sci.* **16**, 375 (1963).
[4] J. E. VARNER and G. SCHIDLOVSKY, *Plant Physiol.* **38**, 139 (1963).
[5] K. SAIO and T. WATANABE, *Agri. Biol. Chem.* **30**, 1133 (1966).
[6] J. SACHS, *Text Book of Botany*, p. 51, Oxford University Press, Oxford (1882).
[7] L. PALEG and B. HYDE, *Plant Physiol.* **39**, 673 (1964).

show that the proteins of the two types of aleurone grain had different amino-acid compositions.

Altschul *et al.*[8] were able to separate aleurone grains, extracted from seeds of *A. hypogaea* into two fractions, a heavy fraction and a second fraction stable at pH 6·0. These fractions behaved similarly in the ultra-centrifuge and gave similar protein band patterns when extracts of them were examined by electrophoresis. Differences in the elution patterns were detected, however, when the proteins of the two types of particle were separated by DEAE-cellulose column chromatography. Further differences between the two types of grain were detected when their ATPase and lipid contents were examined.

Morton *et al.*[9] have analysed an aleurone grain preparation isolated from wheat grain and found it to contain proteins, lipids, nucleic acids, phytate and free amino acids. In a study of the biochemical activity of these grains Morton and Raison[10] showed that the particles were capable of incorporating amino acids into storage protein. The amino acyl-RNA synthetases and soluble-RNA associated with this incorporation were found to be localized in the grain. It was suggested[11] that ATP, used in this incorporation of aminoacids into storage protein, was generated from phytate by an ADP-phosphoinositol phosphotransferase, the latter enzyme being located in the aleurone grains. However, similar preparations made from maize did not incorporate amino acids into peptidyl material; amino acid incorporation by such preparations being due to contamination by bacteria.[12]

In describing the methodology of isolating aleurone grains from seeds, inadequate information is often given about either the degree of contamination of the aleurone grain preparation by unbroken cells, bacteria, other kinds of sub-cellular particles, or the stability of the isolated grains. Both these factors may, of course, affect the biochemical activity and chemical composition of the isolated preparations. This paper describes the sedimentation characteristics of aleurone grains isolated from soaked seeds of *Vicia faba* in relation to other cell organelles in order to obtain some measure of the degree of contamination of the aleurone grain preparation. The stability, heterogeneity and chemical composition of the isolated aleurone grains were also investigated.

RESULTS

A Comparison of the Sedimentation Behaviour of Starch Grains, Aleurone Grains and Mitochondria

An homogenate of soaked *Vicia faba* seeds was subjected to differential centrifugation and the results are given in Fig. 1. For details of the justification for the use of *g*–min (the force–time integral) and S_{min} (the sedimentation constant of the lightest spherical particles which are completely sedimented under the conditions used), see De Duve and Berthet.[13] Pellets sedimented by forces greater than 800 *g*–min ($S_{min} = 3410 \times 10^3$) contained negligible amounts of starch. The pellet obtained after centrifuging by 6400 *g*–min ($S_{min} = 4260 \times 10^2$) contained a large proportion of the cell protein. Cytochrome oxidase activity was sedimented between the integrated forces of 25,600 *g*–min ($S_{min} = 1065 \times 10^2$) and 409,600 *g*–min ($S_{min} = 31{\cdot}9 \times 10^2$). The major particles comprising the 6400 *g*–min pellet ranged in size

[8] A. M. ALTSCHUL, N. J. NEUCERE, A. A. WOODHAM and J. M. DECHARY, *Nature* **203**, 501 (1964).
[9] R. K. MORTON, B. A. PALK and J. K. RAISON, *Biochem. J.* **91**, 522 (1964).
[10] R. K. MORTON and J. K. RAISON, *Biochem. J.* **91**, 528 (1964).
[11] R. K. MORTON and J. K. RAISON, *Nature* **200**, 429 (1963).
[12] C. M. WILSON, *Plant Physiol.* **41**, 325 (1966).
[13] C. DE DUVE and J. BERTHET, *Nature* **172**, 1142 (1953).

from 2 to 4 μ in diameter, stained with safranin and corresponded morphologically to the large number of aleurone grains seen in sections of cotyledon when examined by light and electron microscopy.[14] Electron micrographs of sections of the isolated aleurone grain pellet showed that the grains still possessed a limiting membrane as seen *in vivo*; no internal structures were present.

As a result of the above analysis the pellet sedimenting between 400 and 14,000 *g*–min was considered to constitute the aleurone grain fraction. The stability and homogeneity of this fraction was then examined.

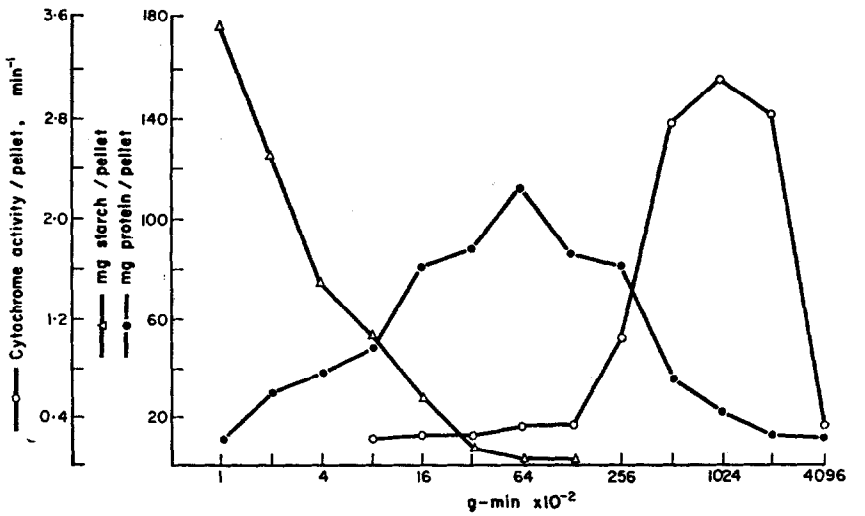

FIG. 1. ILLUSTRATION OF THE SEDIMENTATION OF STARCH, PROTEIN AND CYTOCHROME OXIDASE ACTIVITY WHEN A HOMOGENATE OF SOAKED *Vicia faba* SEEDS WAS SUBJECTED TO DIFFERENTIAL CENTRIFUGATION.

Effects of Different Concentration of Sucrose, Phosphate and Magnesium Chloride on the Stability of the Isolated Aleurone Grains

The stability of the aleurone grains was measured by determining the amount of soluble protein liberated when the grains were exposed, for 18 hr at 5°, to a range of concentrations of sucrose from 0·05 M to 0·5 M, of phosphate from 0·02 M to 0·1 M and of magnesium chloride from 1 to 6·0 mM. Magnesium chloride had little effect upon the liberation of soluble protein from the grain and 0·5 M sucrose and 0·1 M phosphate appeared to confer maximum stability.

Investigations into the Homogeneity of the Aleurone Grain Preparation

(a) *By density gradient centrifugation.* An aleurone grain suspension was suspended in 0·5 M sucrose/0·1 M phosphate buffer, pH 7·0, and aliquots of this suspension were applied separately to the following linear sucrose gradients; 25–40 per cent, w/w, 35–55 per cent, w/w and 50–70 per cent, w/w which were then centrifuged for 144,000 *g* for 12 hr. The gradients were then fractionated and the protein content of these fractions was then determined using the Lowry modification of the Folin method; sucrose solutions of the appropriate concentration were used as blanks. Any particulate protein present in the fractions collected from the

[14] L. G. BRIARTY, D. A. COULT and D. BOULTER, *J. Exptl. Botany* **20**, 358 (1969).

sucrose gradients was solubilized by the $NaOH–Na_2CO_3$ solution used in the Lowry method. The results of this experiment are presented in Fig. 2 where the protein contents of the collected fractions are plotted together. About two-thirds of the total protein applied to the 50–70 per cent gradient sedimented between sucrose concentrations of 58–66 per cent, w/w, the remaining one-third remained in the sample layer of this gradient. No other major protein-containing bands were detected on the gradients used. Microscopic examination of the protein-containing band sedimenting between sucrose concentrations of 58–66 per cent, w/w revealed the presence of particulate material identified as aleurone grains.

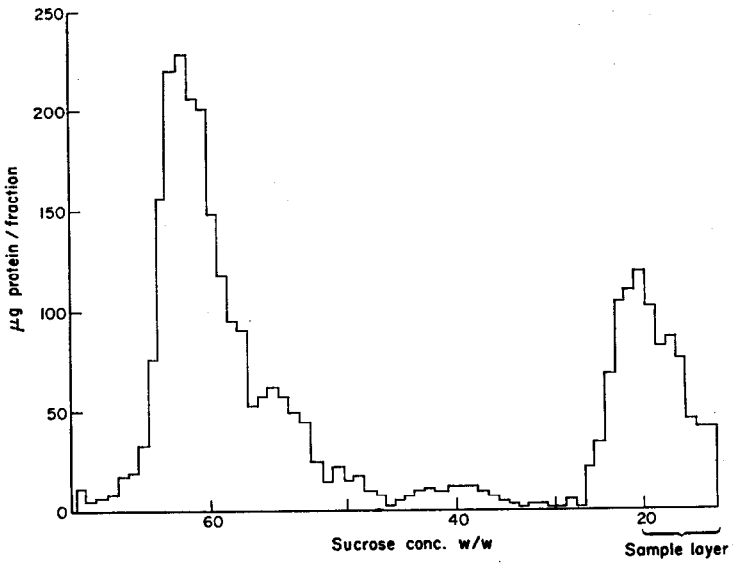

FIG. 2. THE DISTRIBUTION OF PROTEIN, DETECTED ALONG THREE SEPARATE LINEAR SUCROSE GRADIENTS (20–40%, 35–55% and 50–70%, w/w SUCROSE) PRESENTED AS A SINGLE HISTOGRAM.

A suspension of aleurone grains, isolated from soaked seeds of *Vicia faba*, was applied to each gradient and centrifuged for 144,000 *g* for 12 hr. The distribution of protein along the complete 20–40 per cent gradient plus sample layer is shown. The distributions of protein in the sample layer and in the 35–40 per cent region of the 40–55 per cent gradient are not shown, likewise for the sample layer and the 50–55 per cent region of the 50–70 per cent gradient. Each point on the figure represents the amount of protein in a ten-drop fraction of a gradient. The sucrose concentrations indicated on the figure are inferred from the fraction number.

(b) *By enzymic and chemical assay.* The degree of contamination by mitochondria and starch grains was measured by estimating the cytochrome oxidase activity and starch content of the aleurone grain preparation (Table 1). The aleurone grains were found to contain only 1·3 per cent of the total amount of starch found in the homogenate and only 7·4 per cent of the total cytochrome oxidase activity.

(c) *Bacterial contamination.* When aleurone grains were prepared from unsterilized beans the preparations contained about 5×10^7 to 10^8 bacteria per ml. Various types of bacteria were present including mainly gram-negative rods and cocci and some gram-positive cocci. From a range of antibiotic tests (Oxoid multidisc) only polymixin-B completely inhibited bacterial growth. Chloramphenicol only partially inhibited the growth of bacteria. Attempts to separate bacteria by density gradient centrifugation were only partially successful although bacterial density was reduced to 2×10^3 bacteria per ml.

TABLE 1. CYTOCHROME OXIDASE, STARCH AND PROTEIN CONTENTS OF VARIOUS FRACTIONS PREPARED FROM AN HOMOGENATE OF SOAKED *Vicia faba* SEEDS

Fraction	Cytochrome oxidase activity expressed as 1st order rate constant (min^{-1})	Protein (mg per fraction)	Starch (mg)
Strained brei	112	2500	418
Starch pellet	Nil	Nil	412
Aleurone grain			
Pellet	8·3	870	5·5
Supernatant	104	1380	Nil

Chemical Analysis of the Aleurone Grain Preparation

(a) *Nitrogen content.* In order to compare the nitrogen content of our aleurone grain preparation with those of other workers[3,5,8] dialysed and freeze-dried samples of aleurone grains were analysed for nitrogen content. A value of 14·1 per cent was obtained for the non-dialysable nitrogen content of the preparation when corrected for water content.

(b) *Phosphorus content.* Aleurone grains were prepared in 0·1 M Tris/HCl buffer, pH 7·0, before being used for the determination and fractionation of phosphorus compounds. The aleurone grain preparation was found to contain 1·4 per cent phosphorus when expressed as a percentage of the dry weight. Fractionation of this phosphorus revealed that 86 per cent of the total phosphorus present was acid soluble and of this 74 per cent occurred as phytate which was identified by paper chromatography.[15] Analysis of the aleurone grains for RNA-phosphorus, using the method of Smillie and Krotkov,[16] indicated that 10 per cent of the total phosphorus was represented by "RNA phosphorus". However, the presence of nucleotides in hydrolysates of this fraction, after chromatography on Dowex 1 resin, could not be detected.

Investigations into the Proteins of the Aleurone Grains

Aleurone grains which had been washed twice with sucrose/phosphate extractant were ruptured by freezing and thawing. The proteins in the resulting extract were then separated into globulins and albumins by repeated dialysis and subsequently freeze-dried. Aliquots of these two fractions were then analysed by disc electrophoresis. Figure 3a is a photograph of an electrophoregram of the globulins and shows two major amido-black staining bands. Figure 3b is a photograph of an electrophoregram of the albumin fraction of the aleurone grains and shows that this fraction is composed of a greater number of proteins than the globulin fraction, some of these proteins have high electrophoretic mobilities when run as anions.

Assay of Albumins of Aleurone Grains for Protease, Phytase and Phenylphosphatase Activities

The results of Table 2 show that the albumins of aleurone grains isolated from seeds soaked for 24 hr show protease and acid phosphatase activities. Phytase activity could not be detected in these preparations but was detectable in the albumins of aleurone grains isolated from seeds germinated for 4 days.

[15] A. M. SOBOLEV, *Fiziol. Rast.* **11**, 1069 (1964).
[16] R. M. SMILLIE and G. KROTKOV, *Can. J. Bot.* **38**, 31 (1960).

TABLE 2.

No. of days' germination	Enzyme	Units of enzyme activity (*a*) Phosphatases, μg P released/hr/mg protein (*b*) Protease, ΔOD 280 nm/hr/mg protein	
		Aleurone grain fraction	Supernatant fraction
	(*a*) Phosphatases		
1	Phytase	Nil	Nil
4		17·4	19·3
1	Acid phosphatase	50·1	42·8
4		74·5	66·8
1	(*b*) Protease	0·04	0·01

DISCUSSION

By carefully observing the sedimentation characteristics of aleurone grains, mitochondria and starch grains, it has been possible to isolate an aleurone grain preparation from seeds of *Vicia faba* essentially free from the above-mentioned organelles. When prepared from sterilized beans or when separated on sucrose gradients, the aleurone grain preparations possessed a bacterial content of 10^3 bacteria per ml. This number of bacteria would not be expected to contribute significantly to the results of chemical and enzymic analysis. Wheeler and Boulter[17] have shown that these preparations incorporate only very small amounts of amino acid into peptidyl material under *in vitro* conditions and concluded that the aleurone grains do not synthesize protein *in vivo*.

Since it is known that aleurone grains are somewhat unstable in aqueous media, their stability was examined in different concentrations of the constituents of the isolation medium. Some protein was lost but this loss was minimized by the use of 0·5 M sucrose and 0·1 M phosphate. Sucrose, phosphate and magnesium chloride at different concentrations have often been included in media used for the isolation of aleurone grains. The fact that Graham *et al.* isolated aleurone grains from wheat, using a medium devoid of sucrose may mean that considerable losses of soluble protein may have occurred.

There is evidence that aleurone grains are heterogenous for size, presence of inclusions,[2,3,8,7] type of protein and phosphorus content. These findings have been confirmed in the work described here. For example, when an aleurone grain preparation was subjected to differential centrifugation, most of the particulate protein sedimented over the range 1000–8000 *g*–min. This pattern of sedimentation suggested that more than one type of particle was being sedimented. However, analysis of the aleurone grain preparations by density gradient centrifugation revealed that the original sample contained particles all having a similar density.

Nitrogen contents of dialysed samples of the isolated aleurone grains were found to be 14·1 per cent of the dry weight. Altschul *et al.*[8] reported a value of 10·7 per cent for aleurone grains of *Arachis hypogaea*, Graham *et al.*[3] a value 12·6 per cent for aleurone grains of wheat and Saio and Watanabe[5] a value of 11·2 per cent for aleurone grains of soybeans. All of the above determinations were carried out on dialysed samples of protein bodies and all are consistently lower than those reported here. This may be due to a difference in protein composition of the grains studied or to the presence of contaminating material. For instance,

[17] C. T. WHEELER and D. BOULTER, *J. Exptl. Botany* 18, 229 (1965).

the grains studied by Sai and Watanabe[5] contained 9·2 per cent, by wt. carbohydrate which might represent contamination by starch grains, though the glycoprotein nature of many seed proteins has now been recognized.

The phosphorus content of the aleurone grains was found to represent 1·4 per cent of their dry weight, a higher value than that found for grains isolated from other seeds. The majority of this phosphorus occurred as by phytate. Claims that RNA occurs in aleurone grains of wheat and soybean have been made by Morton *et al.*[9] and by Saio and Watanabe.[5] Both groups of workers determined the RNA contents of the grains by absorbance at 260 nm. The determination of the RNA in crude extracts by this method is beset with many difficulties[18] and further confirmation of their results is required before they can be accepted as evidence for the occurrence of RNA in aleurone grains of wheat and soybean. Analysis of the "RNA phosphorus" fraction of the aleurone grains studied here failed to reveal the presence of nucleotides, which suggests that RNA is absent from the isolated grains.

Investigation into proteins of the isolated aleurone grains confirmed that the grains contained the two major storage proteins characteristic of the seeds, vicilin and legumin. These two proteins were identified by comparing their behaviour on electrophoregrams with that of vicilin and legumin, prepared by *iso*electric precipitation, from seeds of *V. faba*. In addition, the aleurone grains contained an albumin fraction which was resolved by disc electrophoresis into a number of protein components. The demonstration that the grain contains this albumin protein fraction and the fact that the enzymic activities of the whole seed reside in the albumins of the seed raises the question of whether the albumins of the aleurone grains possess enzymic properties concerned in aleurone grain metabolism. The two major components detected in the aleurone grains prepared here were storage proteins and phytate. It would not be unreasonable therefore to suppose that the grain might contain enzymes involved in the metabolism of these compounds. This was confirmed to some degree by demonstrating that the albumins of the grains extracted from 24-hr soaked seeds possessed protease activity towards a substrate of storage protein isolated from the same seed. No phytase activity was found to be associated with these aleurone grain albumins, although they did show acid phosphatase activity when phenyl disodium orthophosphate was used as a substrate. However, aleurone grain albumins isolated from seeds which had been allowed to germinate for 4 days, did show phytase activity though the specific activity of the aleurone grain albumins was less than that of the supernatant albumins. Obviously, further confirmatory evidence is required to support the contention that these protease and phytase activities are located in the grains.

EXPERIMENTAL

All chemicals used were of analytical grade quality. N determinations were made using the method described by Thurman and Boulter.[19] The moisture content of samples was determined by the method of Eastoe and Courts.[20] Starch determinations were made using the method of McCready[21] and protein determinations either by the method of Itzhaki and Gill[22] or Lowry *et al.*[23] Cytochrome oxidase activity was

[18] D. P. Holdgate and T. W. Goodwin, *Phytochem.* 4, 831 (1965).
[19] D. A. Thurman and D. Boulter, in *Techniques in Amino Acid Analysis*, p. 28, Technicon Instruments Co. Chertsey, England (1965).
[20] J. E. Eastoe and A. Courts, in *Practical Analytical Methods for Connective Tissue Proteins*, p. 34, Spon Ltd., London (1963).
[21] R. M. McCready, J. Guggolz, V. Silviera and H. W. Owens, *Anal. Chem.* 22, 1156 (1950).
[22] R. F. Itzhaki and D. M. Gill, *Anal. Biochem.* 9, 401 (1964).
[23] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* 193, 265 (1951).

assayed by the technique of Smith.[24] Total phosphorus was determined by the method of Marsh.[25] The fractionation of lipids, phytate, inorganic phosphorus, ester phosphorus and nucleic acid phosphorus was based on the methods described by Pons *et al.*[26] Ergle and Guinn[27] and Krotkov.[16]

Mature seeds of *Vicia faba* cv. Sharpes Conqueror were soaked for 20 hr in running tap-water. Testas and embryos were removed, cotyledons chilled to 4° and the following extraction procedure carried out at 4°. Cotyledons were sliced into 0·23-mm slices, using a sledge microtome and the slices placed into the extraction medium of 0·5 M sucrose and 0·1 M phosphate, pH 7·0. The extraction medium was then stirred gently for 15 min and the resulting brei squeezed through muslin before being centrifuged. Starch grains were removed by three successive centrifugations of 400 *g*–min and the aleurone grains were then collected as a pellet at 14,000 *g*–min. In some experiments, 0·1 M phosphate, pH 7·0, present in the extraction medium was replaced by 0·1 M Tris/HCl, pH 7·0.

Preparation of Albumins from Aleurone Grains

Aleurone grains were prepared as described above and washed twice with extractant, the grains were then ruptured by three successive freezings and thawings. The resulting extract was then repeatedly dialysed against distilled water adjusted to pH 7·0, for 12-hr periods until no pellet formed after centrifuging the contents of the dialysis bag at 20,000 *g* for 10 min. The final solution was freeze-dried and stored at −20°.

Proteolytic activity was assayed by measuring the increase in absorbance at 280 nm of a 5% (w/w) TCA-soluble fraction formed after incubation of the albumins with globulin substrate, prepared from seeds of *V. faba*, in *N*-ethylmorpholine, pH 7·0. Substrate and enzyme controls were conducted at the same time.

Phosphatase activity was assayed by measuring the release of inorganic phosphate from disodium phenyl phosphate or inositol hexaphosphate by aleurone grain albumins in acetate buffer, pH 5·0, according to the method of Gibbins and Norris.[28]

Disc electrophoresis was performed as described by Ornstein and Davis[29] except that proteins were added to the gels in 25 (w/v) sucrose. Proteins were detected by staining with Amido Black. Chromatography of inositol phosphates was performed as described by Sobolev.[15]

[24] L. SMITH, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. II, p. 732, Academic Press, New York (1955).
[25] B. B. MARSH, *Biochem. Biophys. Acta* **32**, 375 (1959).
[26] W. A. PONS, M. F. STANSBURY and C. L. HOFFPAUIR, *J. Assoc. Offic. Agn. Chemists* **36**, 492 (1953).
[27] D. R. ERGLE and F. W. H. GUINN, *Plant Physiol.* **34**, 476 (1959).
[28] L. N. GIBBINS and NORRIS, *Biochem. J.* **86**, 67 (1963).
[29] L. ORNSTEIN and B. L. DAVIS, *Disc Electrophoresis*, Preprint by Distillation Product Industries (Eastman Kodak Co.), Rochester, New York (1961).