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Protein Bodies of the Soybean

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Summary. Some microscope observations of the protein bodies of the cotyledon cells of the soybean (*Glycine max*) are described, together with changes in their appearance which occur on germination. Density gradient centrifugation permits the isolation of protein bodies from soymeal. They contain about 70 % of the protein of the bean. Only 1 protein could be detected in them: glycinin, the major soybean protein.

The protein bodies were fractionated to light and heavy fractions. The former contained 97.5 % protein, the latter 78.5 %. RNA, phytic acid and lipids were also present. The 2 fractions probably differ only in the extent of contamination by other cell fragments.

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It has been known for many years that seeds, especially those with a high protein content, have numerous globular inclusions in the cells. These are rich in protein by histological techniques and are the protein bodies. The protein almost certainly acts as a storage material.

More recently there have been attempts to isolate these particles from wheat (7, 10, 13) from groundnut (1, 2) from pea (18) and from cotton seed (20) by using differential sedimentation in a variety of media. The reported composition always includes a predominance of protein, justifying the name of protein body.

In investigating soybeans the major protein, glycinin, was difficult to extract, and this seemed to be connected with the fact that a large part of the protein was contained in protein bodies. We have therefore investigated what proportion of the total protein occurred in the protein bodies, which protein or proteins, and which other components occurred associated with them, in order to try and clarify the status of glycinin.

Materials and Methods

Soybeans and Meal. These were American No. 2 grade commercial samples, which were dehulled and hexane extracted, milled, and the material passing a 350 mesh sieve used. Some Nigerian beans were also used; these were an ordinary commercial sample.

Density Gradient Centrifugation. A continuous sucrose density gradient was made by a machine similar to that of Choules (8) in 35 ml centrifuge tubes, for the 3 × 35 ml swing out head of the M.S.E. SS50 centrifuge. The sucrose solutions (70 % w/v and 90 % w/v) were chosen to give a gradient from 1.25 to 1.34, with 1.3, the anticipated density of the protein body, near the centre of the tube.

Meal (100 mg) was homogenized with 5 ml of 20 % (w/v) sucrose, 50 mM citrate (pH 5), in a mortar and pestle, and layered on top of the tube. Tubes were then centrifuged at 24,000 g average for 30 minutes. Further spinning caused no change in the zones. Zones were visible after centrifuging and the tubes were photographed with a Polaroid camera. Then layers, either of 1.5 ml or 3 ml volume were removed by suction through a hypodermic needle. This method caused very little disturbance of the layers, as judged by the appearance of the visible zones, possibly because the high viscosity of the sucrose solutions caused layered streaming towards the needle.

The density of each fraction was found by refractometry, previously calibrated with standard sucrose solutions.

Analytical Methods. Nitrogen was determined by micro-Kjeldahl, and converted to protein by a factor of 5.8. This factor was calculated from the amino-acid composition of soy protein.

Phosphorus was found by a slight modification of the method of Murphy and Riley (14). Chloroform:methanol soluble phosphorus was regarded as lecithin and converted to total phospholipid by a factor of 25. Non-RNA, non-phospholipid phosphorus was assigned to phytic acid, which was assumed to have a phosphorus content of 28.2 %.

RNA was determined by perchloric acid extraction (9), assuming that 31 μg gave an absorbance of 1.0 in a 1 cm cuvette at 260 mμ.

Nitrogen and phosphorus values shown in tables I to III are the mean of 6 determinations with an estimated error of ± 2 %. RNA values are the mean of 3 with an estimated error of ± 5 %. Total lipids, and moistures are the mean of 2

gravimetric determinations. It was not possible to do all the analyses on single preparations, owing to a shortage of material. Three main preparations were used, as shown in table II.

Carbohydrate hydrolysis was carried out by mixing 66 mg of protein body with 50 ml of 0.01 N HCl and 10 g of Biorad AG2 resin and refluxing for 36 hours. The resin was then removed, and the supernatant concentrated. It contains all the neutral and acidic sugars. This method (adapted from ref 6) avoids losses due to reaction with amino-acids and peptides which remain attached to the resin. The hydrolysate was analysed by chromatography in ethyl acetate 12: pyridine 5: water 3 parts, or in 10 isopropanol 90 parts water, with suitable authentic samples as standards.

Microscopy. Samples for electron microscopy were fixed in 1 % buffered osmium for 2 hours, embedded in Epon 812, sectioned on a Porter-Blum MT2 microtome at 500 Å and then stained for 2 minutes with lead citrate. Sections for optical microscopy were stained with either $HgCl_2$/bromphenol blue or Morell-Sisley stain, with equivalent results.

Electrophoretic analysis was as described by Tombs (17) in polyacrylamide gels.

Results

Obviously, to separate particles by density gradient centrifugation the particles must be free and unaggregated. It proved impossible to obtain such a system from intact cotyledons. They were extremely difficult to homogenize: grinding with sand, Potter homogenizers, ultrasonics and blade homogenizers all failed to break more than a small proportion of the cells and furthermore intractable lipid emulsions were formed, particularly between fat globules and protein bodies which made any kind of separation based on density impossible. Other species such as groundnut also have this problem, though not so severely (unpublished data).

For this reason, it was essential to extract at least the bulk of the lipid with ether or hexane before separation, so that all our experiments were performed on meal. Figure 1 shows electron micrographs of intact cotyledon cells and micrographs of meal. The protein bodies are clearly visible, and in these 2 samples range in size from 2 to 20 μ. They are contained in a single unit membrane. The Nigerian bean shows a granular appearance which is probably atypical of the dry mature bean, but the membrane can be seen more clearly. The numerous densely staining cytoplasmic inclusions are lipid. Large numbers of free, apparently intact protein bodies can be seen in the meal. When meal is suspended in water, the protein bodies can be seen to swell, often doubling in diameter. Eventually they rupture, releasing numerous small granules of less than 0.5 μ diameter. Buffering the medium to pH 5, the pH of minimum solubility of glycinin, prevents this and is the reason for our choice of homogenizing medium. Osmic acid stains the protein bodies lightly and also prevents swelling.

These observations, as well as direct electron microscopic observation on meal, suggested that the limiting membrane of the protein body survives hexane extraction of the lipid.

Changes on Germination. Beans (American sample) were germinated in beakers at room temperature by placing them on wet filter paper. At suitable intervals, using mainly the length of shoot as a criterion, samples were taken for electron and optical microscopy. Some typical sections are shown in figure 2. Sampling for electron microscopy was difficult because beans showed cells at different stages of development almost adjacent to each other. Samples were usually taken as nearly at the geometrical centre of the cotyledon as possible.

Germination seemed to be accompanied by 2 early changes: the protein bodies became more granular in appearance, and the limiting membrane could no longer be seen. Eventually the bodies became irregular in appearance and sometimes coalesced into a single mass. At the same time the number of lipid globules decreased and immature plastids, together with what seem to be starch grains appeared. Sections of the whole cotyledon were taken for optical microscopy, to see if disruption of the protein bodies was localised. Generally, disruption and coalescence of the protein bodies occurred in the epidermis layer, and round the vascular bundles, particularly at the embryo end. Some typical sections are shown in figure 3.

Distribution of Protein and RNA. Results of analyses of fractions from density gradient tubes are shown in figure 4 together with the appearance of the tube. The nitrogen distributed into 3 main fractions, the uppermost layer H, the protein body zones, PB, and the bottom layer B. Two protein body zones were always visible in the tubes and, judged by the appearance of the tube, were of roughly equal amounts. The protein body zone centered on a density of 1.30, extending from a density of 1.28 to 1.32. It was always skew towards the heavy side, and in the example shown, shows signs of resolution. In some experiments a small peak (unknown) was found just below the H layer, at a density of about 1.26.

In a run where 3 ml fractions were removed the microscopic appearances were as follows.

(Volumes from top of tube). 1 to 3 ml Small number of particles, 3 to 6 ml large number of very small particles 0.5 μ, 6 to 15 ml few fragments, whole cells, 15 to 18 ml clumped and unclumped partly disintegrated protein bodies, 18 to 27 ml very large number of protein bodies: some clumping, cell wall fragments attached to clumps, 27 to 30 ml cell wall fragments, protein bodies

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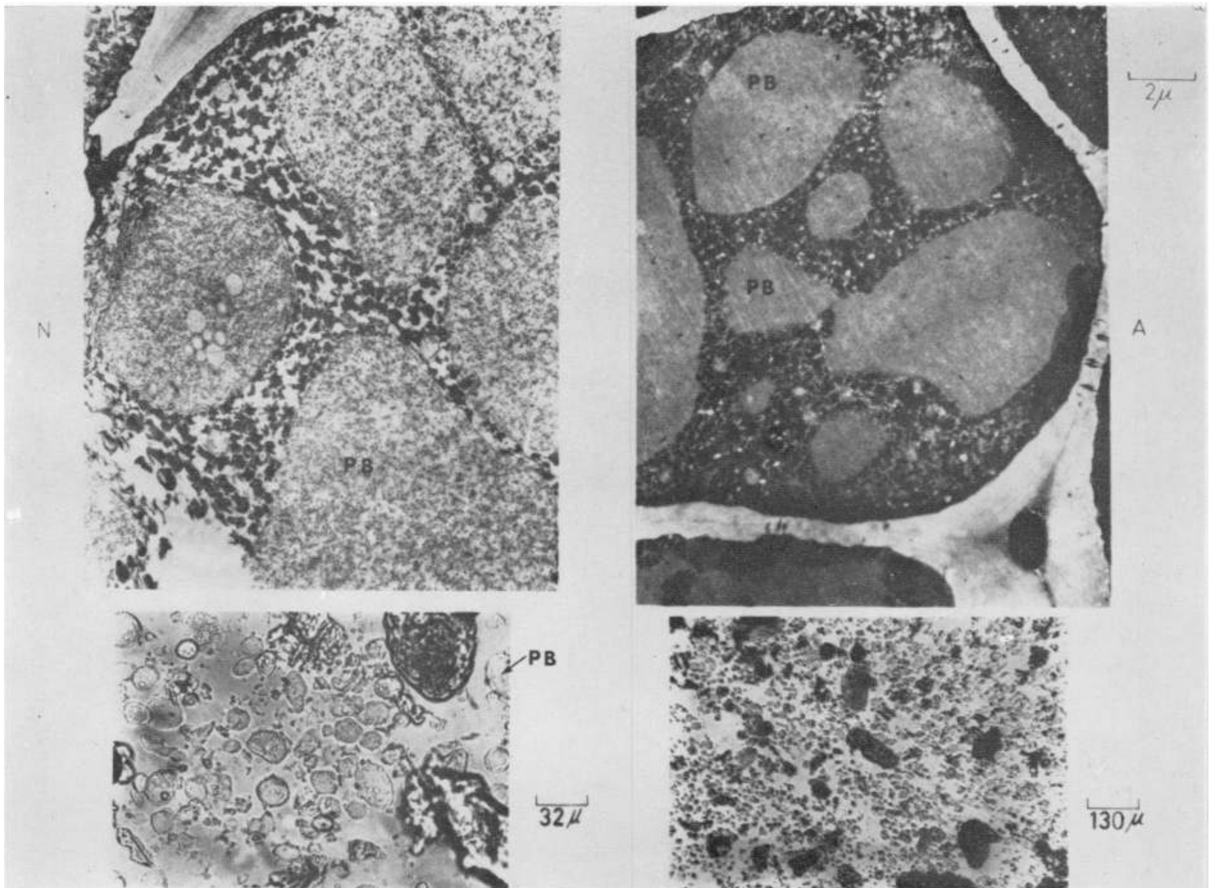

FIG. 1. A and N are electron micrographs of sections of cotyledon cells of American and Nigerian soybeans respectively. The membrane surrounding the protein bodies (P.B.) can clearly be seen in N. At the bottom are 2 photomicrographs of meal, suspended in 0.1 % osmic acid. The protein bodies are visible and apparently intact. These photographs also give an impression of the numbers and discreteness of the protein bodies. Large cell wall debris and apparently intact cells can also be seen.

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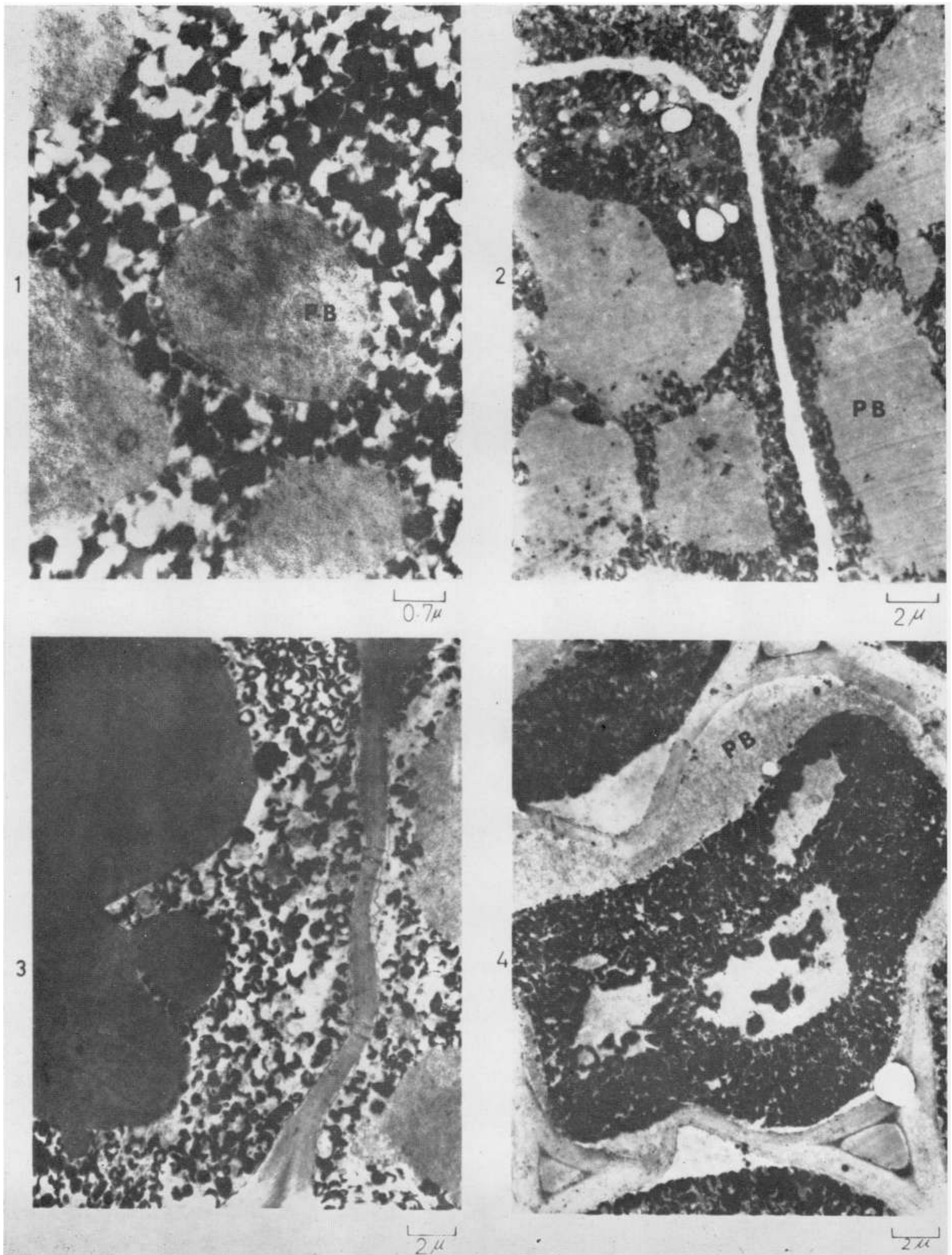

FIG. 2. Electron micrographs of cotyledon cells from germinating soybeans. All sections are of cells from the center of the cotyledons. 1) Shows the initial stages, about 2 hours after wetting. Note the granular appearance of the body. 2) 6 Days after germination: note the irregular shapes and apparent coalescence of the bodies, which also become less dense in appearance. 3) 3 Days. Note the marked difference between the cell on the right, and the dense appearance of the protein body mass on the left. 4) 7 Days. There now appears to be a mass of protein in the upper perimeter of the cell.

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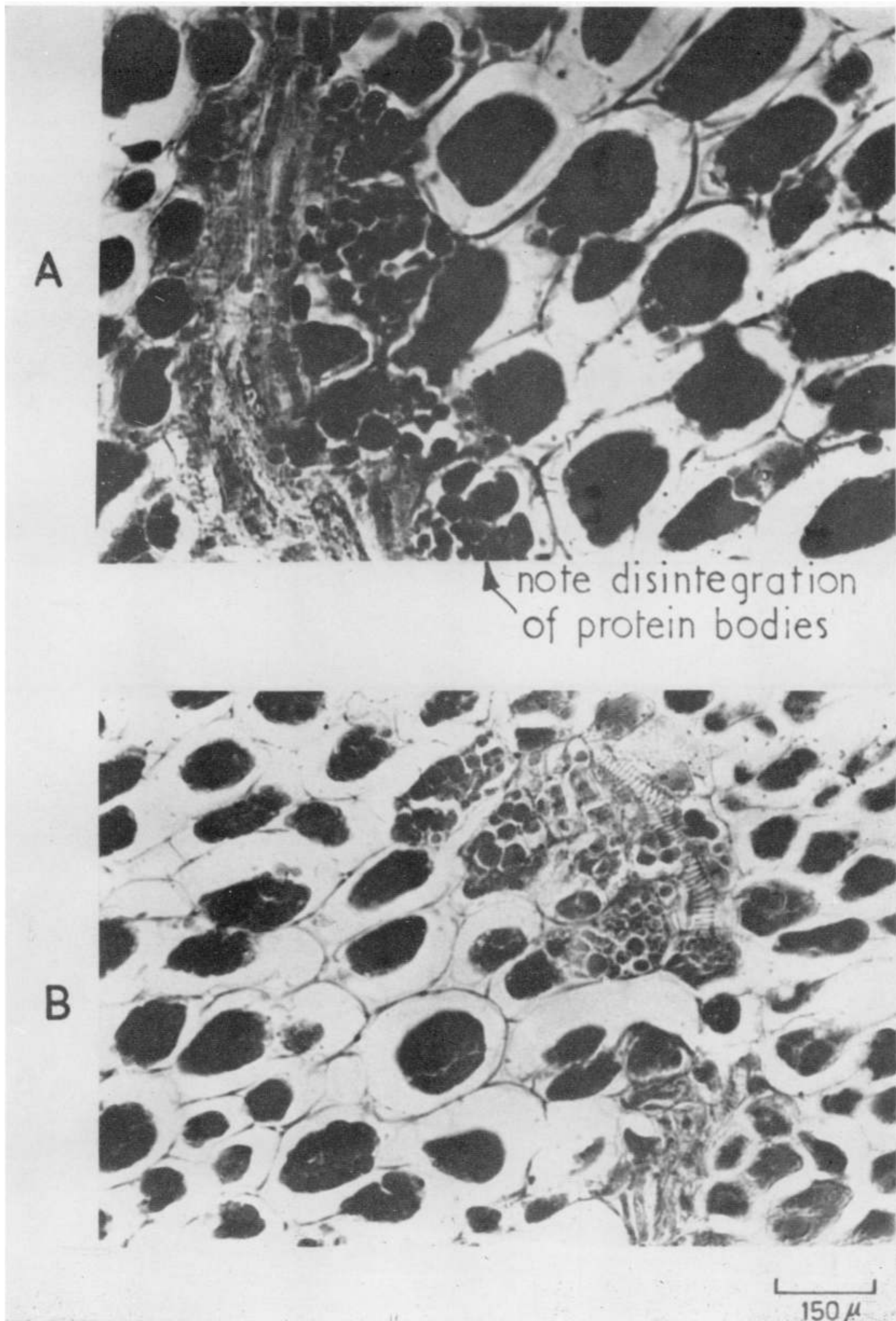

FIG. 3. Photomicrographs of sections of cotyledons. The disruption of the protein bodies near the vascular bundles is clear. A) after 6 days, B) after 5 days.

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attached, 30 to 33 ml very few particles, 33 to 36 ml cell wall debris, intact cells containing protein bodies, a few starch grains. RNA occurred mainly in the homogenate and bottom fractions.

Because intact cells were found in the bottom layer, in 1 experiment this zone, and the protein body zone were isolated, ground with sand, and re-run (fig 5). The protein body zone occupied the same position as in the initial separation, though it was more symmetrical than previously. The bottom zone was more diffuse, and shows partial resolution into 2 zones: a small amount of nitrogen also occurred in the homogenate layer.

Assuming all the nitrogen to be of protein origin, the percentage distribution in the various fractions is shown in table I. It includes the results of 2 experiments, with different homogenates. The bottom zone shows the largest difference between the 2 sets of results, apart from the small unknown fraction already mentioned.

Isolated Protein Bodies. Protein bodies were isolated by the same technique, usually in batches of about 100 mg by running 3 gradient tubes, removing the zones, combining them, and sedimenting the particles, washing twice with water and finally drying over P_2O_5.

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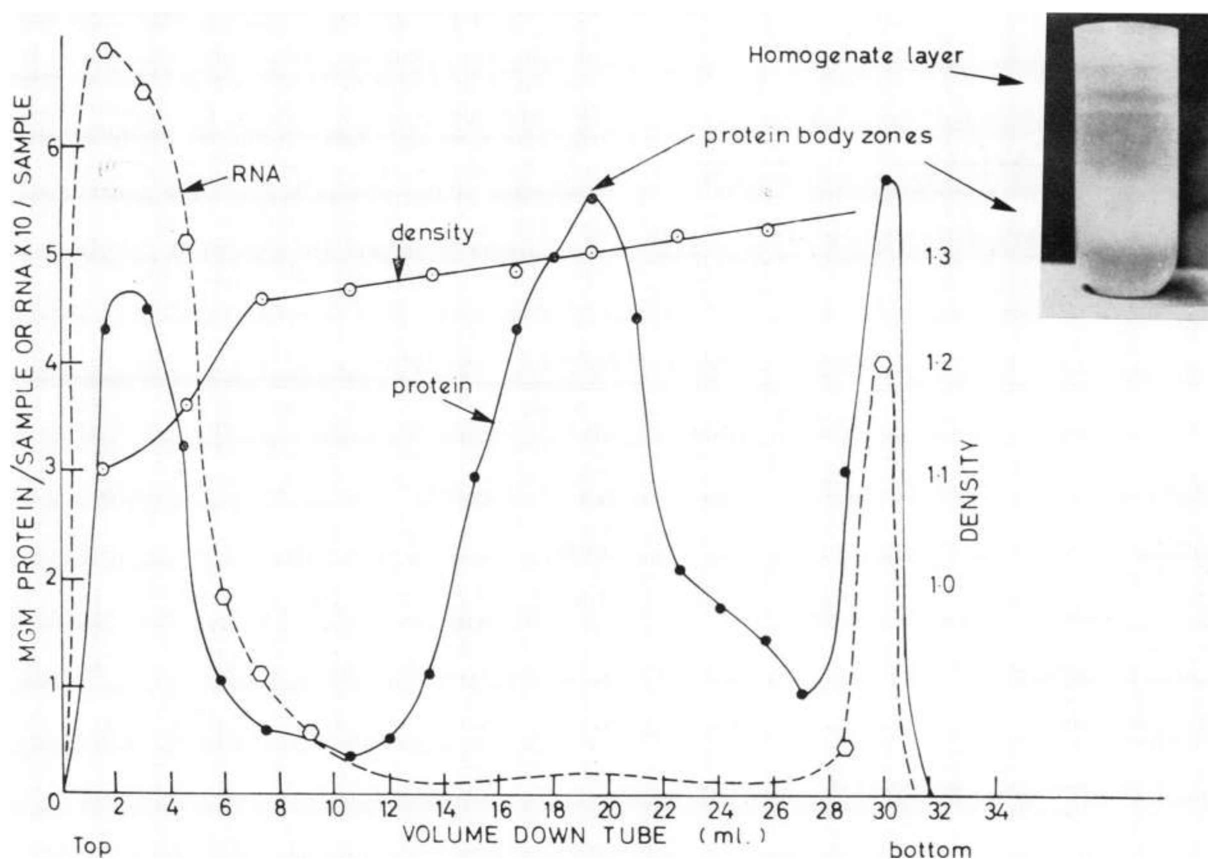

FIG. 4. Distribution of protein and RNA in density gradient tubes after centrifugation.

Table I. *Distribution of Protein in Density Gradient Tubes*

Fraction	I Protein	% Of total	II Protein	% Of total
	mg		mg	
Homogenate	12.48	25.6	14.31	26.0
Unknown	1.19	2.5	...	...
Protein body	30.1	62.2	30.30	57.5
Bottom	5.15	10.8	8.64	16.5
Total	48.5		53.27	
Applied as homogenate	50.0		55.0	
Recovery	97 %		97 %	

They were then analysed for moisture, by drying at 105°, for total phosphorus, RNA and chloroform:methanol soluble phosphorus, for nitrogen, for carbohydrate after hydrolysis and for total chloroform:methanol extractable lipid, (table II). Similar analyses were also made on meal.

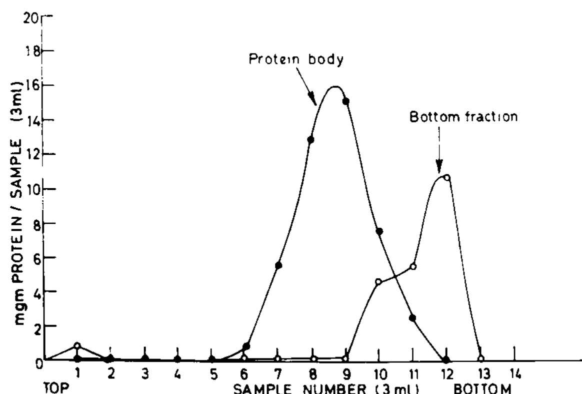

FIG. 5. The result of re-running protein-body zones, and bottom fractions in sucrose density gradient tubes. Details as for figure 4.

Carbohydrate detected after hydrolysis, included traces of mannose and fructose, glucose, galactose, some arabinose and ribose. There was no sucrose and the presence of only traces of fructose proves that it was effectively removed by washing the isolated protein bodies. Glucose was the main sugar present. Clearly an accurate estimate was impossible but comparison with markers suggested that 3 to 5 % of the protein bodies was carbohydrate. Inositol was also found, presumably deriving from phytic acid.

The ether extractable solids were analysed by thin layer chromatography. 5 % Of a protein body preparation was lipid, of which 0.75 % was triglyceride, 1.5 % free fatty acid and the remainder phospholipid (lecithin). Electrophoretic analysis is illustrated in figure 6. Total soy proteins by this technique always show 2 heavy zones, which are the monomer and dimer forms of glycinin (unpublished data). These were the only components detectable in the protein bodies. The other components, such as trypsin inhibitor appeared in the homogenate layer, and are presumably cytoplasmic. The bottom layer contained all components.

Table II. *Analysis of Isolated Protein Bodies*

	Prepn 1		Prepn 2		Prepn 3		Meal
	(i)*	(ii)**	(i)	(ii)	(i)	(ii)	(i)
Moisture	12.07	...	9.67	...	3.87	...	...
Protein	67.7	76.0	74 3	82.5	...	...	50
Total P	..	...	0.43	...	...	...	...
Lecithin Phosphorus	...	...	0.039	...	...	...	...
RNA	0 98	1.11	1.16	1.29	...	...	1.66
Lecithin	...	...	...	1.00	...	...	2.25
Phytate	...	...	...	1.35	...	...	2.24
Chloroform :methanol Extractable solids	...	...	...	...	10.9	11.3	8.6
Ether extractable solids	...	...	...	...	4.5	4.5	...

* (i) Expressed as a percentage of total weight.
** (ii) Are calculated to a moisture free basis.

Table III. *Composition of Light and Heavy Protein Bodies*

	Light		Heavy	
	(i)*	(ii)*	(i)*	(ii)*
	%	%	%	%
Moisture	8.4	...	8.3	...
Protein	90 9	97.5	72.0	78.5
Total P	0.79	0.84	0.83	0 90
RNA	0.43	1.7	1.86	
Chloroform :methanol extractable solid	1.40	1.50	5.2	5.6
Phytic acid**	...	2.6	...	2.3
Unknown components	...	...	...	12 0
Total		100.0		100.0

* (i) Percentage of total weight.
* (ii) Calculated on a moisture free basis.
** See text

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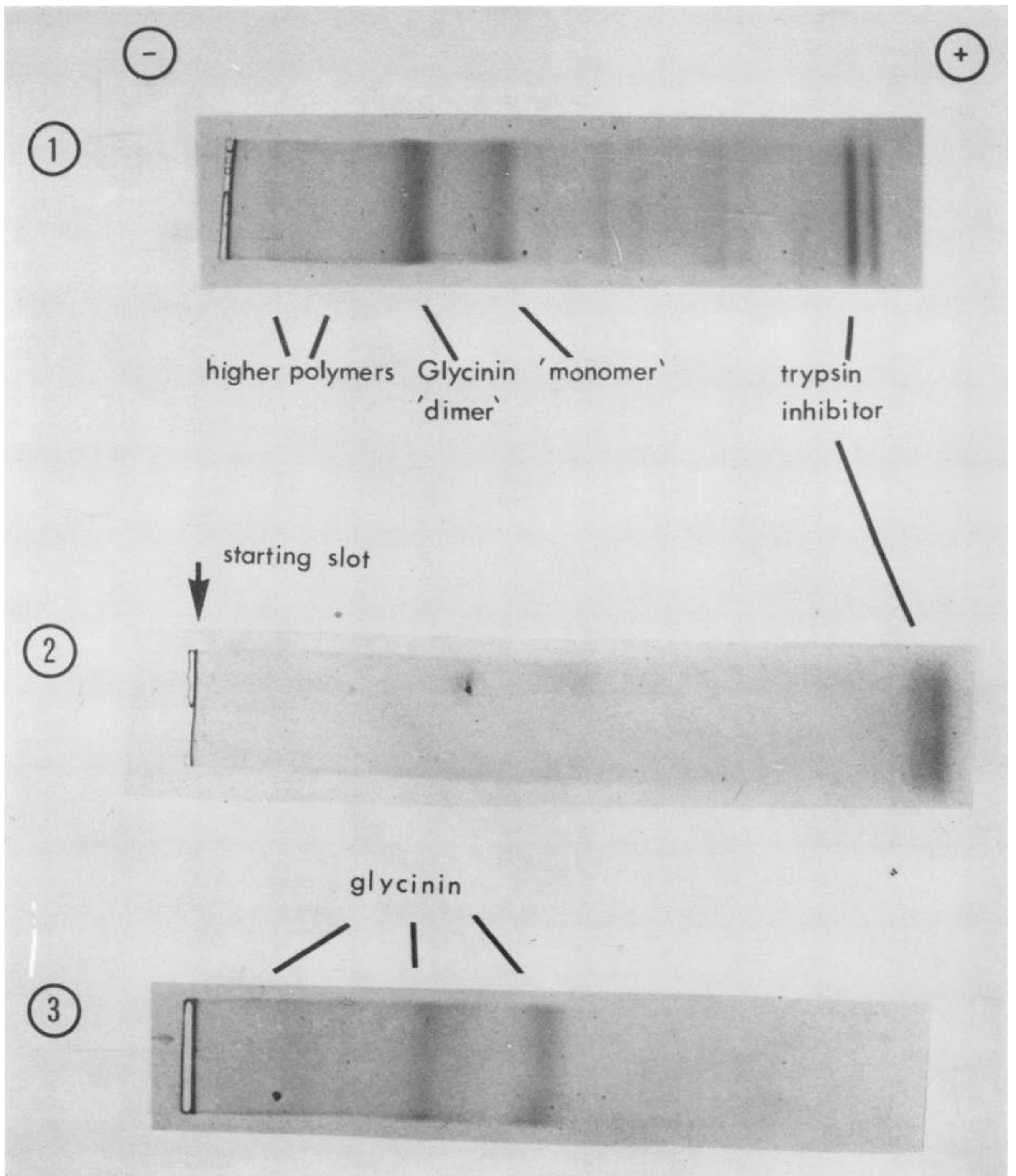

FIG. 6. Acrylamide gel (7.5 % gels) electrophoresis of 1) total soy protein, 2) homogenate layer, after centrifuging, 3) protein body protein. The runs were in a *tris*-citrate-sodium borate discontinuous buffer pH 8.6.

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FIG. 7. Electron micrographs of representative isolated protein bodies. Note the apparent absence of membrane in A, and its presence in B. B also shows typical cytoplasmic attachments. In both cases holes are present in the bodies.

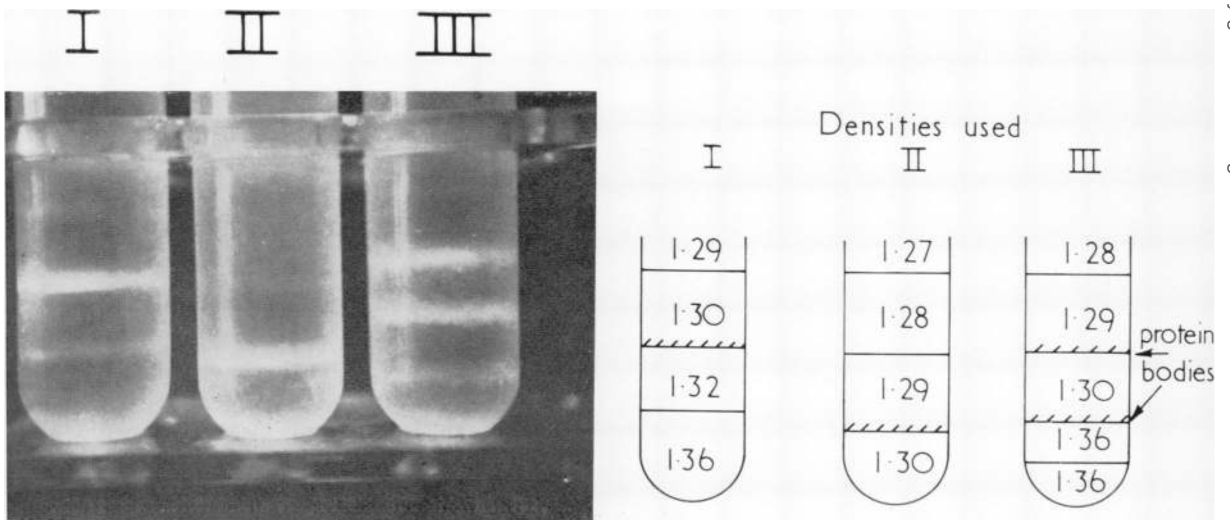

FIG. 8. Fractionation of protein bodies. Sucrose layers were formed in the tubes of the densities shown to the right. After spinning the tubes had the appearance shown to the left.

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Numerous observations of the isolated protein bodies were made by microscopy (fig 7).

Sub-fractionation of Protein Bodies. Since 2 zones were often seen, and the protein bodies were clearly heterogenous with respect to density an attempt was made to fractionate them further. In this experiment layers of sucrose were used rather than a continuous gradient. The arrangement and densities used are shown in figure 8. Protein bodies collected at the interfaces, and 2 fractions were obtained of approximately equal amounts, less than density 1.30 (lighter fraction) and less than density 1.32, but heavier than 1.30 (heavy fraction).

These were isolated and analysed as above; the results are shown in table III. As seen under the microscope, the light fractions were little aggregated and very little cell debris, such as cell walls, was present. The heavy fraction contained more cell wall fragments and clumped protein bodies, often with cytoplasmic attachments.

The phytate value in table III has been calculated from the non-RNA, non-phospholipid phosphorus as usual, but in this case it has been assumed that all the chloroform:methanol extractable material in the light fraction is phospholipid, and that the same value holds for the heavy fraction. Even if this assumption is wrong the error introduced in the phytate value is small and probably exceeded by errors involved in the assumption that no other phosphorus containing compounds are present.

By electrophoresis the protein in the 2 fractions was identical.

Discussion

In any experiment designed to isolate a cell inclusion such as protein bodies 2 main questions must be considered. Were the isolated protein bodies the same as those seen in sections of the intact cell, and to what extent was the isolated fraction contaminated by other parts of the cells.

The first question must rely mainly on microscopic evidence. From electron micrographs some of the bodies were damaged during isolation, holes appeared in them and the membrane could not be seen. However, many if not most of the isolated particles have an appearance similar to those in the intact cell, and retained their membrane.

The isolated fractions certainly contained some contaminants because fragments of cell wall and other particles could be seen associated with clumps of protein bodies, and by microscopy the light fraction was the least contaminated. It is clear, moreover, from the composition that the extent of contamination was low. In the unfractionated protein body preparation the carbohydrate content did not exceed 5 % of the total, and the light fraction achieved the remarkably high level of 97.5 % protein. The heavy fraction, on the other hand, certainly contained some cell wall debris which accounts for some of the 13 % composition not determined.

The question of the status of the small amounts of lipid found is more difficult to decide. The amount of neutral lipid was variable but the unfractionated protein bodies contained about 10 % extractable with chloroform:methanol. The beans had previously been extracted with hexane, which never removes all the lipid, and some at least of this 10 % probably represents residual contaminant lipid. This is almost certainly the source of the 5 % extractable with ether. Since ether does not disrupt the membrane, it must be concluded that ether extractable lipids are not part of the protein body itself. In this preparation, therefore, perhaps 5 % of the protein body might be neutral lipid. In the light fraction only 1.5 % lipid was found, and most of this must be phospholipid. On the whole it seems probable that neutral lipids are not an appreciable part of protein bodies, certainly in this fraction. It may be significant that in whole cotyledon homogenates the lipid attached itself tenaciously to the protein bodies.

Table IV shows our consolidated results, together with some data on other species. The re-

Table IV. *Composition of Protein Body Preparations from Various Sources*

	*Wheat (13)			Groundnut (2)		Groundnut (1)		Soybeans		
	i**	ii**	Cottonseed (20)	i	ii	i	ii	Total prep	Light prep	Heavy prep
Protein	68	72	60–70	77	66	55	56	82.5	97.5	78.5
RNA	5.5	10.4	...	0.11	0.29	...	...	1.2	0.43	1.9
Phospholipid	...	0.83	...	...	...	...	...	1.0	...	...
Total lipid	22.7	...	20–25	...	...	8	45	11.3	1.5	5.2
Phytic acid	4.0	8.4	...	0.5	5.7	...	...	1.35	2.6	2.25
Sucrose	...	...	...	4.3	9.5	...	...	0	...	...
Carbohydrate	...	...	...	...	...	...	...	3	...	...
Total	90.2	91.5	80–95	82	81.5	63	91	99.3	100.8	87.4
Not accounted for (approx.)	10	9	20	18	18	37	9	1	...	13

* Data taken from references cited : all expressed asa percentage of total weight.
** i and ii indicate different preparations.

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sults with soybeans are similar to those obtained with groundnut, though they seem to have higher protein contents than any case previously described. We have also been able to account for more of the total weight of the bodies than in previous cases. The light fraction cannot contain any other major component, and it appears that in the soybean at least, the main components other than protein are RNA, phospholipid and phytic acid. The analytical differences between the light and heavy fractions almost certainly represent differences in the degree of contamination. There is no evidence to show that there is any fundamental difference between the protein bodies of the 2 fractions.

The phospholipid content is similar to that reported for wheat, and can readily be accounted for as a membrane component: thus the membranes have a thickness of about 75 Å, and assuming a 50 % phospholipid content and a density of 1.0, protein bodies of about 5 μ diameter require about 1 % phospholipid to form the membrane.

Phytic acid interacts strongly with glycinin and is presumably protein bound in the body. There was no evidence for its localization, unlike wheat where it appears to be present in specific areas of the protein bodies.

The protein bodies contain at least 60 % of the protein of the bean. However they also contribute a substantial amount of the protein in the bottom fraction. Protein bodies could be seen in this fraction, particularly in intact cells. Now about 10 % of the RNA is also in the bottom fraction and it is difficult to imagine any RNA containing structure sufficiently dense except intact cells. This suggests that about 10 % of the cells were undisrupted and so about half of the protein in the bottom layer was due to protein bodies. This is supported by the re-running experiment, where more extensive grinding produced a shoulder moving towards a lower density.

The nitrogen in the homogenate layer was assumed to be of protein origin. In fact, about 5 % of the soybean nitrogen is usually non-protein (12). If allowance is made for this, and the failure to completely disrupt the preparation, about 70 % of the protein must have been in the protein bodies.

It follows that the protein which is called glycinin in this paper, must contribute 60 to 70 % of the total soybean protein, since it was the only one detectable in the protein bodies. This protein is substantially identical to the cold ppt and 11S proteins described by Wolf (19). A very similar situation exists in the groundnut, where arachin which also constitutes about 70 % of the groundnut protein, is confined to its protein bodies (unpublished data).

Some observations on the formation of soybean protein bodies have been reported (5) but changes on germination have not been described. Our results are similar to those for other species, such as groundnut (3) pea (4) Yucca (11), and *Phaseolus* (15) with the exception that in the case of soybean disruption of protein bodies does seem to be present in the early stages around the vascular bundles. This does not seem to be the case in other species (15).

While this paper was in preparation a report (16) appeared of analysis of isolated soybean protein bodies. The reported composition is similar to that described here, though the method of preparation was quite different.

These results reinforce the general conclusion that protein bodies are storage particles, containing predominantly protein, which disintegrate on germination. It also seems likely that protein bodies in general contain a specialized protein, which is adapted to its storage function, and may show similarities between related species [cf Altschul (1)]. In the case of soybeans the protein which is called glycinin in this paper is the storage protein concerned, and constitutes some 60 to 70 % of the total soy protein.

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