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Protein Synthesis in Ripening Pea Seeds

1. ANALYSIS OF WHOLE SEEDS

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Under conditions conducive to protein synthesis, the amount of protein nitrogen increases at the expense of soluble nitrogen; this has been shown by a great number of workers to hold true for developing seeds (cf. Chibnall, 1939; Schulze, 1911; Schulze & Winterstein, 1910; Emmerling, 1880, 1887, 1900; Zaleski, 1905; Nedokutschajew, 1902, 1904; Boswell, 1924; Bisson & Jones, 1932), for leaves (Wood & Petrie, 1942) and for actively metabolizing potato disks (Steward & Preston, 1940, 1941). This fact, however, does not provide any decisive information about the path of protein synthesis, and can be interpreted in the light of several current theories. Wood & Petrie (1942), for example, considered the correlation between amino acid and protein nitrogen as strong evidence in favour of the hypothesis of direct condensation of amino acids, whereas Steward & Preston (1940, 1941), from their studies with metabolizing potato disks, thought that the amino acids were not utilized directly in protein synthesis, but were first deaminated.

It is not possible, at present, to decide between the two hypotheses of protein synthesis mentioned

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above; and although that of the direct utilization of amino acids seems to be preferred, there is not sufficient evidence to decide whether the condensation of the amino acids takes place in a single step, i.e. by a 'template' mechanism, or whether it takes place in a stepwise manner. If the latter is true, polypeptide precursors ought to be detectable.

The presence in plants of polypeptides and other relatively low-molecular components termed 'peptones', 'proteoses', or 'albuminoses' has been vaguely inferred by many workers, and some attempts have been made to include the last-named in the analysis of seeds. Nedokutschajew (1902, 1904) determined 'albuminose' in wheat seeds at different stages of development by precipitation with zinc sulphate, and found that the amount of albuminose decreased up to a certain point in the ripening seeds and increased again in the mature grain. Zaleski (1905) determined albuminoses in unripe peas by determining the difference between the nitrogen precipitated by zinc chloride and that precipitated by cupric hydroxide. He found that upon storage of the seeds the amount of albuminoses remained constant, but the amount of protein nitrogen increased by an amount equivalent to that of the albuminoses originally present. From this Zaleski concluded that protein was formed at the expense of the albuminoses and these latter were replenished from materials not precipitated by zinc chloride.

More specific data are required to determine whether these low-molecular substances are involved in the synthesis of proteins and it is the object of the present investigation to supply these.

No one has demonstrated peptides as intermediates in protein synthesis in animal tissues, possibly because of their rapid rate of metabolism. In animals there is also a continuous turnover of protein, and one has to take into consideration the outgoing as well as the incoming nitrogen. Plants, on the other hand, metabolize at a much slower rate, and have generally well-defined reserve proteins which, once formed, do not seem to undergo further metabolic changes until germination, so that one can assume that all the nitrogen which is taken in accumulates.

Previous work of Danielsson (1949*a*) in this laboratory on the pea proteins has shown that there are two well-defined globulins, namely, vicilin, with an S_{20} value of 8, and legumin, with S_{20} 12·5; and a less well-defined albumin fraction, showing two main components (*a* and *b*) with sedimentation coefficients in the neighbourhood of 1 and 4 respectively. The same author, using ultracentrifuging and electrophoresis as analytical tools, studied the breakdown of the high-molecular proteins during germination (Danielsson, 1951), and also the formation of these same proteins during ripening of the seeds (Danielsson, 1952).

Although the emphasis in the present work is placed upon the lower-molecular nitrogenous constituents of pea seeds, data on the formation of the high-molecular proteins seemed necessary to complete the picture of the overall nitrogen balance. Part of these latter data confirm and amplify results obtained in Danielsson's study, which was carried out at the same time as the present one but with a different variety of pea.

EXPERIMENTAL

Material. Two different varieties of seeds of *Pisum sativum* were used. For development of experimental methods, seeds of a green wrinkled garden pea at two different stages of development were taken. The peas designated as 'unripe' (U) were of the size generally used for canning; those designated as 'ripe' (R) were fully grown dried seeds.

For the analyses proper, a garden pea of the variety Fenomen was used. The peas were grown in season, under natural conditions. Because of the many irregularities in the Swedish climate, the different stages, designated I–VI, were selected on the basis of weight rather than age (Table 1). Owing to an unusually short summer the peas did not mature, and part of the original seed was used for analysis of ripe peas (stage VII). Samples were taken every 3 days, and after shelling the peas were sorted according to size, then ground in a Waring Blendor and lyophilized.

The dried material was finely ground and stored in glass bottles. It was sufficiently uniform to permit good reproducibility with Kjeldahl and Van Slyke analyses performed directly on the solids; there was no change when these analyses were repeated after several months' storage.

Analysis of the original seed material. Moisture determinations were made on the fresh peas and on the lyophilized material by drying at 75° for 3–4 days. If drying was continued for a longer time slight increases in weight were observed.

Total nitrogen was determined by micro-Kjeldahl on samples (10 mg.) of the lyophilized material; deviations ranged from 2 to 5%. For amino nitrogen determinations samples (50 mg.) were washed directly into the Van Slyke reaction chamber and reacted for 20 min.; consecutive determinations agreed within 1–3%.

Table 1. *Physical characterization of peas at different stages of development*

Stage	Wet wt. of 100 peas (g.)	Dry wt. of 100 peas (g.)
I	1·30	0·20
II	6·08	0·93
III	14·7	2·24
IV	27·8	5·40
V	45·8	10·17
VI	50·7	15·10
VII	22·2	20·99
U	42·5	11·30
R	27	24·60

Comparison of methods of extraction. Since it was important to obtain representative extracts, a large number of extractions were performed to determine the best conditions. The extractions were carried out by placing an accurately weighed amount (0·25–1·5 g.) of lyophilized material in a dry beaker, then adding a measured volume (25 ml.) of extractant and stirring for the required time. For extraction periods of 20 min., the solutions were kept at room temperature (22°); overnight extractions were performed at 4°. The resulting suspension was centrifuged at 3500 rev./min. for 10–15 min. and the supernatant filtered through a dry sintered-glass filter. When the solutions obtained in this manner were still turbid, they were rapidly frozen, kept overnight at −16°, then allowed to thaw and centrifuged again at 10 000 rev./min. for 5 min. By this method completely clear extracts were always obtained. A minimum of three samples of each solution was used for Kjeldahl determinations.

The buffers which were used have already been described by Danielsson (1949*a*); their compositions are as follows. 'Standard buffer', pH 6·8–7: 0·2 M-NaCl; 0·03 M-Na_2HPO_4; 0·02 M-NaH_2PO_4. 'Extraction buffer', pH 6·8–7: M-NaCl; 0·06 M-Na_2HPO_4; 0·04 M-NaH_2PO_4. 'Borate buffer', pH 8: 0·6 M-NaCl; 0·02 M-H_3BO_3; 0·005 M-$Na_2B_4O_7$. 'Standard buffer' was found to extract 81 ± 1 % of the total nitrogen in unripe peas and 71 ± 1 % of the total nitrogen in ripe peas. The extraction was practically complete in 20 min., since the additional amount of nitrogen extracted over a period of 16 hr. was insignificant (Table 5). An increase in the concentration of salt from 0·3 to 1·1 M ('extraction buffer') had no significant effect on the extraction yield, but sometimes tended to lower it; the same was true when borate buffer was used. When the NaCl in the 'standard buffer' was substituted by KCl or LiCl, the extraction yield could be increased to 93 % of the total nitrogen. The relative distribution of the different nitrogenous constituents in the extracts, however, remained the same (Table 5).

Dilute alkali was found to give the largest yield; for this reason such solutions have been extensively used in plant analyses, despite the fact that alkali is known to alter to some extent plant protein preparations (see, for example, Osborne, 1907; Blish & Sandstedt, 1929; Neglia, Hess & Sullivan, 1938). The use of alkali is unjustifiable, however, since it results in a complete breakdown of the proteins in the seed, as can be seen from the solid line in Fig. 1, which represents an ultracentrifuge diagram of a 0·1 % KOH extract of unripe peas, dialysed against distilled water, lyophilized and redissolved in standard buffer. With this information in mind, analytical results obtained with alkali extracts should be accepted with reservation. Even the painstaking and frequently quoted work of Emmerling (1880, 1887, 1900), who determined legumin and albumin in KOH extracts of peas, must be read in this light.

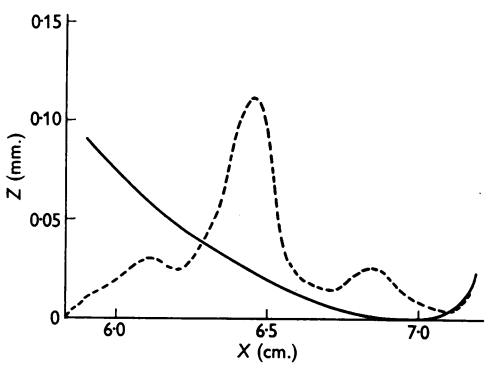

Fig. 1. Typical ultracentrifuge pattern of the proteins in extracts of the wrinkled green pea. – – –, 'Standard buffer'; ——, KOH. The ordinate is proportional to the gradient of refractive index; abscissa is distance from the centre of rotation.

Analysis of the extracts. The extracts, obtained by stirring the peas with 'standard buffer' for 20 min., were analysed for total nitrogen, protein nitrogen, total and 'protein' amino nitrogen, and total and 'protein' amide nitrogen. Total non-protein and peptide nitrogens were obtained by difference according to the following scheme:

Total N–Protein N = Total non-protein N,
Total amino N–'Protein' amino N = Amino N,
Total amide N–'Protein' amide N = Amide N.
Total non-protein N–(amino N + amide N) = Peptide N.

Four to six extracts of the peas in each developmental stage were analysed, except for stage I, for which only two extractions were made.

Total nitrogen (Kjeldahl) was determined on a minimum of three samples of each extract. The results were calculated by reference to the original volume of the extractant rather than to the volume of solution after extraction (Danielsson, 1951). This is not strictly accurate, but the error resulting from changes in volume upon the dissolution of the seed material is minimal. On the other hand, when the results are referred to the volume of solution measured after centrifuging or filtration of the seed material, the nitrogen in the solution retained by the precipitate (amounting to several millilitres) is excluded from analysis.

Since the quantitative determination of proteins, especially in the presence of polypeptides, presents great difficulties, several precipitation methods were checked against known quantities of different pea-protein fractions, whose ultracentrifuge patterns had been previously determined in order to verify their 'purity'. The globulins and albumins were prepared according to the method of Danielsson (1949*b*). Peptides U were prepared essentially according to the technique of Borsook, Deasy, Haagen-Smit, Keighly & Lowy (1949), after the extracts had previously been freed from legumin by isoelectric precipitation, and from vicilin and albumin by heat coagulation; this peptide fraction comprised the low-molecular albumin component (probably the proteose of earlier workers) and some smaller peptides. As can be seen from Table 2, no clear-cut separations could be obtained except with tungstic acid, which precipitated both proteins and peptides completely. The latter reagent was further tested with low-molecular peptides (Table 3) and on whole extracts; a comparison between these results and results obtained by dialysis is given in Table 4.

The incomplete precipitation of proteins by Stutzer's reagent confirms earlier observations (Hart & Bentley, 1915; Hiller & Van Slyke, 1922). This reagent is one most extensively used in plant analyses, and it should be noted that the results, although too low in terms of absolute values, might still be valid on a comparative basis but are in need of revision.

The precipitating efficiency of trichloroacetic acid (TCA) is dependent on the concentration of the original solution,

Table 2. *Comparison of various protein precipitants*

All solutions were allowed to stand for 2 hr. before centrifuging off the precipitate. U and R refer to preparations from unripe and ripe peas respectively.

Material	Concn. (mg. of N/ml.)	Reagent: 2·5% TCA	5% TCA	10% TCA	Cupric hydroxide†	Tungstic acid‡
		(N as % of total)				
Peptides U	0·113	68	—	—	—	100
Albumin R	0·157	80	88	95	—	100
Albumin U	0·105	78	76	82	—	100
Globulin	—	100	—	—	—	100
Peptides U	0·153	—	—	—	89	—
Albumin R	0·320	—	—	—	94	—
Albumin U	0·243	—	—	—	83	—
Peptides U*	0·133	23	—	73	—	—
Albumin R*	0·133	50	—	65	—	—

* The material was precipitated from 'standard buffer', instead of from distilled water.
† 1·5 ml. of 0·1 N-NaOH was added to 1 ml. of sample solution, followed by 1·5 ml. of 0·11 N-$CuSO_4$ (Blish, 1922).
‡ 1 ml. of solution was precipitated with 1 ml. of 10% sodium tungstate and 1 ml. of 0·1 N-H_2SO_4 (Hiller & Van Slyke, 1922).

Table 3. *Precipitation of asparagine and low-molecular peptides with tungstic acid*

Samples (2 ml.) of the extract were precipitated with 1 ml. of 10% sodium tungstate solution and 0·5 ml. of 6 N-H_2SO_4.

Material	Precipitation time	Concn. of N in soln. (mg. %)	N precipitated (%)
Asparagine	30 min.	17·8	37
	24 hr.	17·8	38
Glycyltyrosine	30 min.	9·75	15
	30 min.	9·75	15
	24 hr.	9·75	19
	24 hr.	9·75	17
Diglycylglycine	30 min.	19·8	27
	30 min.	19·8	27
	24 hr.	19·8	28
	24 hr.	19·8	28

Table 4. *Comparison of non-protein nitrogen values obtained by dialysis and by tungstic acid precipitation of extracts of whole peas*

Peas	Dialysis	Tungstic acid: 30 min. (N as % dry wt.)	16 hr.
U	1·23	1·22	0·97
		1·26	0·96
		1·24	—
R	0·63	0·74	0·65
		0·72	0·62
Stage V	3·00	2·57	—
		2·42	—
Stage VI	2·60	1·90	—
		1·72	—

especially for the intermediate range of small proteins and large peptides (e.g. peptides U), and also on specific factors other than molecular size.

Tungstic acid produces complete precipitation of proteins and any intermediary products, but, according to Hiller & Van Slyke (1922), it does not precipitate amino acids; however, it cannot be used for separating either proteins and peptides from amino acids or proteins from amino acids and peptides, since it precipitates small peptides only partially (Table 3). When only small amounts of low-molecular diffusible peptides or asparagine or both are present, as they are in extracts of seeds U and R, the amount of non-protein nitrogen as determined by a rapid (30 min.) tungstic acid precipitation agrees well with the amount determined by dialysis (Table 4). If, however, a large quantity of peptides or asparagine or both is present (Table 8), the values for non-protein nitrogen as determined by tungstic acid will be low. Conversely, if protein is estimated by the determination of Kjeldahl nitrogen on the residues from tungstic acid extraction (Petrie & Wood, 1938), these values will be much too high because they incorporate not only all the unextracted nitrogen (10–30% of the total) but also a considerable percentage of the peptides present.

It is clear from the above that fractionations by precipitating agents depend upon many factors in addition to size. Moreover, since precipitates dissociate according to mass action, rigorously standardized conditions, including concentrations, are needed to yield meaningful results. Dialysis, on the other hand, offers a means of separation which depends more directly upon molecular size and is relatively less sensitive to changes in the compositions of the solutions used.

Accordingly, a simple procedure based upon dialysis was adopted in the present investigation. The extract (5 ml.) was placed in a thin cellophan bag (Visking casing) tied so as to have a compressed bubble of air above the liquid, and was dialysed against 300 ml. of 'standard buffer' (or other, depending upon the extract) for about 42 hr. No appreciable difference was noticed in the results if the extracts were dialysed for twice that time. After dialysis, the bags were opened and samples were taken for nitrogen determinations. Total Kjeldahl nitrogen of the dialysed solution was taken as protein nitrogen. The results were referred back to the original volume since the change in volume when dialysis is performed in a tightly closed bag, against the same buffer, is negligible.

Since non-protein nitrogen was taken as the difference between total nitrogen and protein nitrogen, a further

check on the procedure was made by determining nitrogen on samples of the diffusate as well as of the dialysed solution. The values for non-protein nitrogen obtained by the direct method were 0·69% for ripe peas and 1·18% for unripe ones, whereas those obtained by difference were 0·60% and 1·24% respectively. The values for protein nitrogen obtained by dialysis were independent of concentration, as can be seen from Table 5.

Amino nitrogen was determined by the method of Van Slyke, with a reaction time of 20 min. For determinations of amide nitrogen the samples were hydrolysed in approximately 6 N-HCl (1 part of extract to 1 part of conc. HCl) for exactly 1 hr. at 95°. After hydrolysis, the sample was diluted, transferred to a Kjeldahl distillation flask, made alkaline with 30% NaOH, steam-distilled, and titrated in the usual manner. The method was tested with pure asparagine and compared with Kjeldahl nitrogen of the same solution. Total amino and amide nitrogen values were obtained from determinations on whole extracts, 'protein' amino and amide nitrogens from determinations on the dialysed solution. Two samples of each solution were analysed.

The values for 'protein' amino nitrogen show a certain inconsistency which suggests some abnormal reactivity toward the Van Slyke reagent, and therefore may well not represent true protein amino groups. The 'protein' amide nitrogen, on the other hand, is probably part of the protein, since it remains relatively constant in terms of percentage of total protein (6·5–8·4%).

Table 5. *Nitrogen distribution in extracts from ripe peas as a function of the extractant and concentration of the starting material*

Results are given in % of dry material.

Extractant	Starting material/100 ml. of extractant (g.)	Total N	Protein N	Non-protein N	Amino N
'Standard buffer' (20 min.)	6·17	3·30	2·82	0·48	0·34
	6·27	3·34	2·97	0·37	0·34
	6·30	3·32	2·57	0·75	—
	6·30	3·65	2·65	1·00	—
	6·05	3·58	2·88	0·70	—
	6·05	3·22	2·93	0·29	—
	6·05	3·22	2·94	0·28	—
	4·05	3·60	2·93	0·67	—
	4·15	3·45	2·87	0·58	—
	2·02	3·21	2·67	0·54	—
	2·05	3·32	2·86	0·46	—
	2·01	3·27	2·63	0·64	—
	1·00	3·30	2·93	0·37	—
	1·02	3·35	3·01	0·34	—
	Average	3·36	2·83	0·53	0·34
'Standard buffer' (16 hr.)	6·40	3·68	2·83	0·85	—
	6·22	3·46	2·85	0·61	—
	1·09	3·62	3·21	0·41	—
	Average	3·59	2·96	0·63	0·34
0·1% KOH	2·11	3·97	3·45	0·51	—
	1·03	3·82	3·46	0·47	—
	Average	3·90	3·45	0·49	—

Table 6. *Distribution of protein in extracts*

Component ...	Legumin		Vicilin		Albumins	
	R	U	R	U	R	U
			(% of total protein)			
	9	19	59	64	32	17
	10	15	55	68	35	17
	23	17	43	61	34	27
	20	15	47	49	33	36
	21	24	52	52	27	24
	23	24	50	53	27	23
	26	24	48	54	26	22
	24	28	41	56	35	16
	25	17	48	59	27	24
Average	20±1·6*	21±1·4	49±1·3	57±1·6	31±1·0	23±1·4

* Standard error.

The determination of peptide by difference is open to objection on the ground that it includes any undetermined soluble nitrogenous compounds which might be present. The concentration of these products, however, is so small that no serious error can be introduced. The spread of results in this fraction would be expected to be large, since it reflects the accumulation of errors made in the other determinations. The average of a number of determinations, however, is useful to indicate the trend which this fraction follows.

The presence of peptides has been demonstrated more directly in several ways. In one experiment, the diffusate of a 'standard buffer' extract was concentrated and treated with 10% TCA. A small amount of precipitate was obtained, which gave one immobile and several mobile ninhydrin-positive spots on a paper chromatogram, with propanol–butanol–0·1 N-HCl (2:1:1) as the solvent. In another experiment an aqueous extract was electrodialysed, the precipitated protein removed by centrifuging and the three compartments were chromatographed separately. In all three, a large number of spots unaccountable for by amino acids were obtained.

The concentration of the different protein components in the extracts were determined by measuring the corresponding areas in the ultracentrifuge diagrams, according to Danielsson (1951). This method is not very exact, as can be seen from the data given in Table 6, and requires a large number of determinations for accurate results; since such were not critical for the purposes of the present investigation, ultracentrifuging was carried out four or five times for each stage of development.

RESULTS

The distribution of total and amino nitrogen in whole peas is given in Table 7. The nitrogen distribution in the extracts is given in Table 8. Fig. 2 represents a comparison between extracted and unextracted total and amino nitrogens respectively. The curves are sufficiently parallel to justify accepting the results obtained with extracts as representative of the whole seeds.

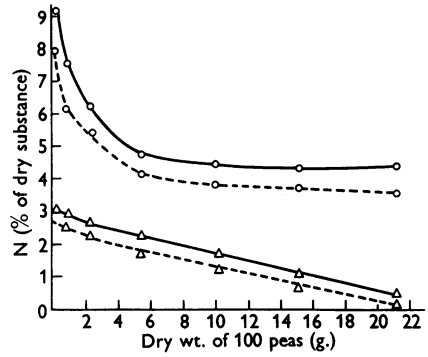

Fig. 2. Comparison of extracted and total nitrogen. The solid lines denote nitrogen determined directly on the dry substance; the broken lines, on extracts; ○, Kjeldahl N; △, amino N.

Table 7. *Distribution of total and of amino nitrogen in fresh and dry peas at different stages of maturity*

Stage	N as % wet wt.		N as % dry wt.		N as mg./100 peas		Moisture (%)
	Total	Amino	Total	Amino	Total	Amino	
I	1·41	0·46	9·24	3·03	18·5	6·1	84·7
II	1·16	0·44	7·60	2·85	70·7	26·5	84·7
III	0·96	0·39	6·29	2·54	140·9	56·9	84·7
IV	0·92	0·42	4·74	2·14	256·0	115·6	80·6
V	0·99	0·38	4·45	1·73	452·6	175·9	77·8
VI	1·30	0·32	4·38	1·06	661·4	160·1	70·2
VII	4·16	0·39	4·46	0·42	936·2	96·6	6·7
U	1·35	0·14	4·93	0·54	550	61	73·8
R	4·32	0·36	4·72	0·39	1160	97	8·5

Table 8. *Nitrogen distribution in extracts of peas in different stages of maturity*

Percentages were corrected for moisture content of the lyophilized material.

Stage	Total N	Protein N	Non-protein N			
			Total	Amino	Amide	Peptide*
	(% dry wt.)					
I	8·17	1·54	6·63	2·65	0·6	3·4
II	6·25	1·48	4·87	2·52	0·7	1·6
III	5·39	1·02	4·37	2·21	0·7	1·4
IV	4·17	0·77	3·40	1·75	0·5	1·2
V	3·93	0·79	3·14	1·27	0·5	1·4
VI	3·79	1·36	2·43	0·71	0·2	1·6
VII	3·56	2·95	0·61	0·12	0·1	0·4

* Obtained by difference.

In order to assure a clear picture of the development of the seeds, results are given, not only as percentage of dry substance, but also as percentage of original (wet) material, and as mg. of nitrogen/100 seeds. It is important to know the absolute amounts of nitrogenous materials during the development of the individual seed, in order to bring out the manner in which the incoming nitrogen is used for synthesis, and to follow possible interconversions of nitrogenous fractions.

It can be seen from Table 7 that, whereas the concentration of amino nitrogen in the fresh seed (calculated as percentage of wet weight) remains quite constant throughout the entire period of development, the concentration of total nitrogen decreases at first and later increases markedly. This rapid increase begins at stage V and corresponds to the onset of desiccation, as may be seen from the last column of Table 7. From Table 8 it can be seen that on a dry-weight basis the concentrations of all components, with the exception of protein, decrease as the seed ripens. More eloquent, however, is the absolute distribution of the different nitrogen fractions, as represented by Fig. 3. It can be seen that there is a uniform influx of nitrogen into the seed which, at first, is distributed more or less uniformly among the fractions. At stage V amino and amide nitrogen begin to decrease, while peptide and protein nitrogen continue to increase. At stage VI, peptide nitrogen also begins to decline, and only protein continues the sharp increase. At the same time there is a sharp increase in the 'protein' amino and amide nitrogen, as shown in Table 9.

The distribution of the protein components in the extract is given in Table 10. The figures represent averages of at least four runs on different extracts. Fig. 4 shows characteristic ultracentrifuge diagrams of extracts, stages IV–VII. Because of the preponderance of albumin in the earlier stages, no satisfactory diagrams could be obtained; convection could not be eliminated and, further,

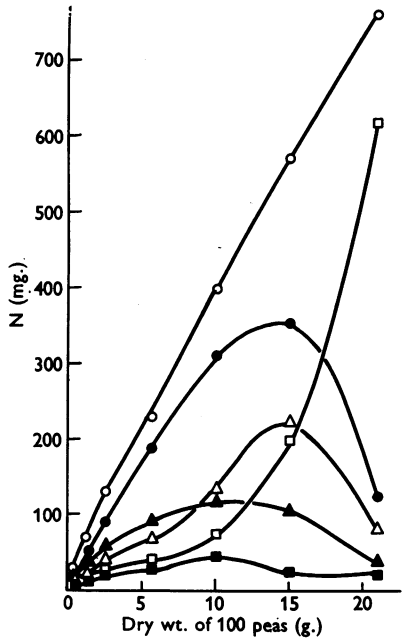

Fig. 3. Absolute nitrogen distribution in extracts from peas throughout the maturation process. The experimental points denote the different developmental stages (I–VII) used for the analyses: ○, total N; □, protein N; ●, total non-protein N; ▲, amino N; △, peptide N; ■, amide N.

Table 9. *Distribution of protein and free amino and amide nitrogen in pea extracts*

Stage	Per cent of total amino N: 'Protein' amino N	Per cent of total amino N: Free amino N	Per cent of total amide N: 'Protein' amide N	Per cent of total amide N: Free amide N
I	0	100	14	86
II	0	99	11	89
III	5	95	10	90
IV	6	94	10	90
V	8	92	11	89
VI	11	89	43	57
VII	54	46	83	17

Table 10. *Distribution of legumin, vicilin and albumin in extracts of peas in different stages of development*

Stage	Total protein % of dry substance	Per cent of total protein: Legumin	Vicilin	Albumin
III	6·4	—	—	100
IV	4·8	(7)	11	83
V	4·9	9	29	60
VI	8·5	16	46	38
VII	18·4	37	46	17

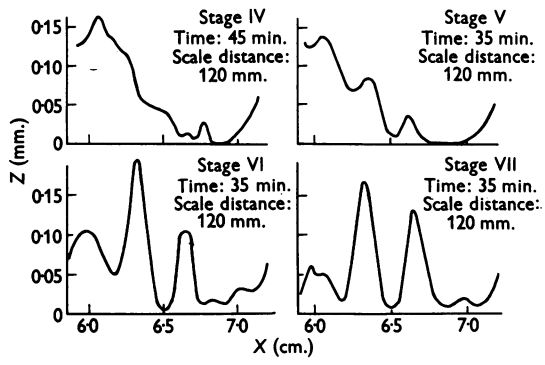

Fig. 4. Characteristic ultracentrifuge diagrams of extracts from peas at different stages of development. Co-ordinates as in Fig. 1.

sporadic peaks, probably due to association products, were observed. In stage III the bulk of the material is found in the albumin peak (which is not well differentiated), and neither vicilin nor legumin can be detected. There are, however, high-molecular components (S_{20} 18–20) present, which are believed to be association products of legumin (Danielsson, 1951).

In stage IV the vicilin peak is discernible for the first time. The peak ahead of vicilin is probably not legumin, although its appearance is persistent. Its sedimentation coefficient is in the neighbourhood of 16, but this in itself is not of great significance; owing to the small concentration of these components, the sedimentation coefficients are unreliable. More significant evidence against the peak being due to legumin is the low ratio of vicilin to legumin at this stage, as compared with subsequent ones. In stage V, vicilin and legumin are clearly present. It is difficult to determine the concentration, however, since the vicilin is poorly differentiated from the large albumin peak.

Analyses of peas U and R indicate that there is an increase rather than a decrease of albumin in the last stage of ripening (Table 6); furthermore, this increase was localized entirely in the lower molecular component (S_{20} approx. 1) of the albumin fraction and was coupled with a corresponding decrease in the peptide fraction (Table 11). The apparent discrepancy between this observation and the results obtained in the second series of analyses is easily explained by the fact that peas U were much closer to maturity than peas of stage VI, as can be seen from the values for protein and amino nitrogen.

DISCUSSION

A survey of the foregoing data permits the drawing in some detail of a picture of the fate of the nitrogenous compounds entering the pea seed throughout the process of its growth and ripening. One striking feature, which is brought out in Table 8, is that after the initial stages the amount of nitrogen entering the seed remains proportional to the amount of total dry matter during its whole development. However, at a certain stage, corresponding to the onset of water loss, the seed develops a tendency towards increased condensation, as reflected in the falling off of the values for the fractions representing non-protein nitrogen.

The changes in the three main nitrogenous fractions, amino nitrogen, peptide nitrogen and protein nitrogen (Fig. 3), are clearly suggestive of curves representing consecutive reactions. The phase of high 'condensing capacity' is marked at the beginning by a progressive increase in the peptide nitrogen at the expense of the amino nitrogen, and later by a striking increase in the protein nitrogen at the expense of the peptide nitrogen.

While it might clearly be misleading to press too far any analogy between a simple kinetic system and a complex plant organ, it is tempting to suggest that so far as these data bear on the problem of the mechanism of protein synthesis in pea seeds, the simplest interpretation would be in terms of a stepwise build-up of the proteins. It might be conceivable that the peptides are broken down to amino acids before utilization in the synthesis of the proteins; this is somewhat unlikely, however, since it would be necessary to assume the greatest degree of hydrolysis of peptides precisely at a time when the 'condensing capacity' of the seed is reaching its highest level, and when rapid loss of water (from 70·2 to 6·7 % moisture) would not seem to favour hydrolysis.

Curves typical of consecutive reactions, and very similar in character to those of Fig. 3 were obtained by Turba & Esser (1955) for the incorporation of ^{14}C into the different nitrogenous fractions of actively growing yeast cells. These authors isolated about 40 peptides, and in short-term experiments were able to demonstrate that the radioactive label was incorporated into peptides before it appeared in proteins.

Acceptance of the hypothesis of a stepwise build-up of the seed proteins would lead one to expect well-defined intermediates to fill the gap between the simple peptides and the globulins, but the question of the nature of these intermediates cannot at present be answered, although there are several points of evidence in favour of the formation of the globulins via albumin. The albumin fraction is extraordinarily heterogeneous and could thus be thought of as a pool of protein fragments varying greatly in size and amino acid composition.

In the beginning of seed formation, all the protein is in the form of albumin, the globulins appearing

Table 11. *Nitrogen distribution in pea extracts*

		Protein N (% dry wt.)					Non-protein N (% dry wt.)		
					Albumin				
Peas	Total N	Total	Legumin	Vicilin	*a*	*b*	Total	Amino	Peptide
U	3·96	2·72	0·54	1·55	0·29	0·34	1·24	0·46	0·78
R	3·36	2·83	0·57	1·39	0·55	0·32	0·53	0·34	0·19

only at stage IV. This would seem to indicate that a certain concentration of low-molecular protein has to be built up before synthesis of globulin can proceed. Once this level has been reached, globulins can be formed even in detached unripe seeds, which at first show only the albumin peak. As has been shown by Danielsson (1952) this appearance of globulin is associated with little or no change in amino nitrogen. This suggests that the combining fragments are of rather high molecular weight, which in turn means that they must appear in the ultracentrifuge diagrams located in the broad 'albumin' peak. The second point of evidence is that the final increase in protein just before desiccation is not distributed uniformly over all protein fractions, but is concentrated in the low-molecular albumin peak (Fig. 5, Table 11). Unfortunately, time has not yet permitted us to confirm this very interesting finding by analysing the albumin fractions of a different series of seeds, but the observation is, nevertheless, highly suggestive. It should be pointed out that the rapid increase of globulins as compared with a very gradual increase of albumins (Danielsson, 1952) does not argue against the latter being intermediates. On the contrary, since the globulins are largely insoluble in the seed, one would expect the equilibrium to be in their favour, the albumins being left at a more or less constant level.

Since the arguments against a stepwise synthesis of protein rely so heavily on the inability of many workers to demonstrate peptide intermediates or even to detect peptides in tissues in general, it might be appropriate to comment on this difficulty here.

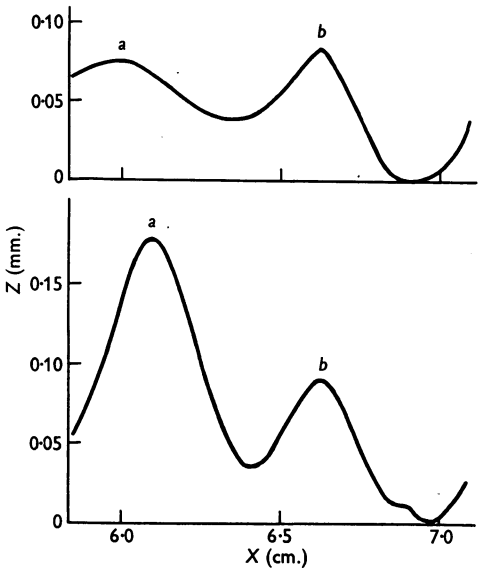

Fig. 5. Ultracentrifuge diagrams of the albumin fraction of peas, Upper, unripe peas (U); lower, ripe peas (R). Co-ordinates as in Fig. 1.

One reason that peptides have not been found more frequently is undoubtedly that the deproteinization procedures commonly used remove the peptides together with the proteins. It has been seen, for example (Table 3), that tungstic acid precipitates even small peptides to a considerable extent. Moreover, as has been pointed out by Turba & Esser (1955) peptides have a great tendency to be adsorbed on to proteins and are thus precipitated with the latter even by reagents such as trichloroacetic acid, which normally would leave the peptides in the supernatant fluid. Many peptides also behave in an unconventional manner in extraction procedures. Deane & Truter (1955), for example, report that there are many peptides in suint that are soluble in 70–80 % alcohol, and therefore would be discarded with the amino acids fraction. Furthermore, these peptides, containing from 8 to 13 amino acids, cannot be distinguished from known amino acids in several two-dimensional chromatographic systems, and could only be revealed as peptides after hydrolysis and re-chromatography.

From the few examples given it appears that the problem of the natural occurrence of peptides in biological systems needs to be re-examined. The method described in the present paper might give valuable leads in the search for these compounds, although the quantitative data might be somewhat in error because of the indirect nature of the determinations. It should be remembered that if labile amides are present which give off the same nitrogen atom in both the amide and the Van Slyke analyses, the values for peptide nitrogen, obtained by difference, would be low. This error then would compensate some of the error in the opposite direction due to nitrogen of bases other than amino acids and to the non-amino nitrogen of the basic amino acids. The apparently high concentration of peptide nitrogen in the very young seeds (stage I) might, in part, be due to the high arginine content of such seeds (Schulze & Winterstein, 1910; E. W. Yemm, private communication); but since glutamine is also present in considerable amounts, the error, at least in part, would be corrected. Similar errors, however, cannot alter significantly the values in stages VI and VII, where the peptide nitrogen exceeds other soluble nitrogen (Table 8). Moreover, it is not so much individual values, as it is the whole trend of the peptide fraction (and especially the increase in peptide concentration in the peas of stages V and VI, and the subsequent decrease in stage VII) that is important for the conclusions drawn in the present paper, and that also would be important for the investigation in other systems of the role of peptides in protein synthesis.

SUMMARY

1. Extracts of *Pisum sativum* at seven different stages of development were analysed for total nitrogen, non-dialysable nitrogen, total and non-dialysable amino nitrogen and total and non-dialysable amide nitrogen; total non-protein nitrogen and peptide nitrogen were determined by difference.

2. The protein distribution in the extract was determined from ultracentrifuge diagrams.

3. In the early stages of development the incoming nitrogen was distributed about equally among the different fractions. With ensuing desiccation, however, there occurred first a sharp increase in peptide nitrogen and later a sharp increase in protein nitrogen at the expense of peptide nitrogen.

4. The data offer strong evidence in favour of peptide intermediates in the synthesis of the seed protein.

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Protein Synthesis in Ripening Pea Seeds

2. DEVELOPMENT OF EMBRYOS AND SEED COATS

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In seeds of the Leguminosae, the constituent parts do not all develop simultaneously. For example, it is known that the endosperm develops first, only to be later absorbed by the developing embryo (including the cotyledons). This means that while protein is being built up in some parts of the seed, it is being broken down in others, so that a study of the development of the whole seed gives only the net result of protein formation. A truer picture might be obtained by studying independently the development of different parts of the seed. As far as the author is aware, such a study has not previously been carried out.

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EXPERIMENTAL

The material consisted of peas of the same lot as those used in the preceding paper (Raacke, 1957). They were grouped into stages as previously described, but the groups represented here are not exactly the same as those of Part 1 of this investigation, although they roughly correspond. The embryos of peas belonging to stages I, II and III were too small to be separated, so that only embryos of peas of stages IV, V, VI and VII were used.

The seed coats of peas in stages IV, V and VI were removed by making a small incision in the coat, through which the embryo could be squeezed out. The ripe peas, stage VII, were allowed to soak overnight in water at 2° before removal of the coat.

The material designated as 'cotyledon IV' is a whole embryo of peas belonging to stage IV. From peas at stages V,