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A Contribution to the Study of the Synthesis of the Reserve Proteins in Ripening Pea Seeds

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The synthesis of the high-molecular reserve proteins in seeds during the ripening process has not been investigated with modern physical methods. The concentrations of various low-molecular nitrogenous substances, in particular amino acids and asparagine, have been determined, however, in the seeds and in other parts of plants during different stages of the ripening process. Emmerling [1–3] studied for 20 years the protein synthesis in ripening seeds of the broad bean, *Vicia faba major*. He showed that nitrogenous products are transported from different parts of the plant to the seed pod where they are stored before they are transported to the ripening seeds. Vassilieff [4] and Pfenninger [5] made the same observation. Emmerling stated that the seed proteins are synthesized from organic nitrogenous compounds (amides and amino acids) synthesized in the leaves. Schulze and Winterstein [6] found that the seed pods from the pea contain much asparagine and little arginine, while the seeds contain very little asparagine and large amounts of arginine. By analyzing the ripening pea seeds at 4 different stages they were able to show that the amount of protein nitrogen increases and the amount of non-protein nitrogen decreases during the ripening process. Most published values of protein concentration in seeds are not very reliable, however, because the determinations were carried out by precipitation of "total protein", in most cases by metals. Such methods precipitate all substances of a protein nature. Thus it is not possible to observe any relative changes of concentration between the different proteins.

In a previous paper [7] dealing with the germination process in pea seeds, the present author has shown that pea seeds contain a few well-defined protein fractions. A fractionating method was worked out by which two globulins, vicilin and legumin, and an inhomogenous albumin fraction could be determined

quantitatively. The amounts of low-molecular nitrogenous substances were determined by difference. This method has been used in the present study of the synthesis of the high-molecular reserve proteins in ripening pea seeds.

A. Treatment of the seed material

In the first experiments, seeds of the field variety of pea "Torsdagsärt II" were harvested in 1950 at different stages of the ripening process. The seeds were separated from the seed pods and were left to dry at room temperature. After about 1 month they were placed in water over night and then macerated in a Waring blendor for 6 minutes. After cooling to $-16°$ C, the product was dried *in vacuo* over $CaSO_4$. Table 1 gives some data on the seed material.

When the unripe seeds are dried in air, most of their water content is released. At stage I, 82 % of the original weight evaporated (in the form of water), and at stage VII 55 %.

B. Determinations of the nitrogen distribution in extracts of unripe, air-dried seeds

The lyophilized material from air-dried, unripe seeds, harvested in 1950, was extracted with 0.2 *M* NaCl, pH 7, as described in a previous paper [7]. At different stages of the ripening process the contents of extractable N, protein N, low-molecular N, albumin N and globulin N were determined. The results of these measurements are given in Table 2 and Figs. 1 and 2. At least five experiments were carried out at each stage of ripening. The values in Table 2

Table 1. Weight of 100 air-dried seeds, harvested in 1950, and nitrogen content of the lyophilized seed material at different stages of ripening.

Stage	Date of harvest	Weight per 100 seeds g	Nitrogen content (Kjeldahl) %
I	11.7	2.97	4.65
II	15.7	3.45	4.62
III	19.7	8.95	4.10
IV	23.7	14.60	4.19
V	27.7	19.00	3.86
VI	31.7	21.59	3.67
VII	4.8	21.47	3.55

Table 2. Nitrogen distribution in NaCl extracts from pea seeds at different stages of the ripening process. In all experiments, 1.50 g of dried seed material were extracted.

Stage	Total N in 1.50 g dried material, mg	Extractable N mg	Nondialysable N (protein N) mg	Dialysable N mg	Globulin N mg	Albumin N mg
I	69.7	52.4	8.2	44.2	2.6	3.5
II	69.3	52.8	13.6	39.2	8.2	3.6
III	61.5	44.0	21.1	22.9	15.1	3.5
IV	62.8	46.6	26.7	19.9	21.1	5.2
V	55.0	40.8	25.6	15.7	18.1	5.0
VI	57.9	38.0	22.5	15.5	17.2	3.7
VII	53.2	42.0	27.0	15.0	20.5	6.2

are average values from these determinations. The errors in the determinations were about the same as in the investigations on the germination process [7], except for stage I, where bigger errors were obtained depending on the very low protein content of the seeds at this stage of ripening. From Table 2 and Fig. 2 it can be seen that all values obtained for seeds from stage V are too low. This fact can probably be explained by assuming that something happened to

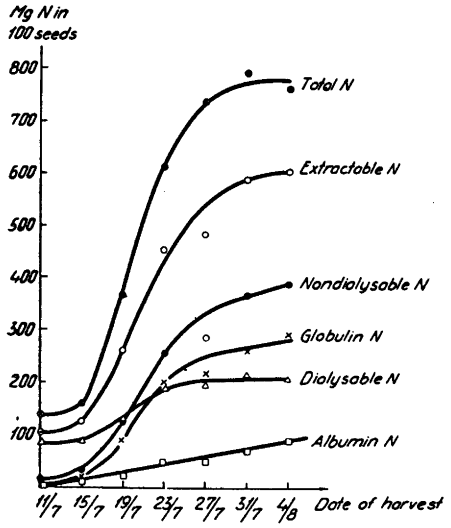

Fig. 1. Nitrogen distribution in pea seeds at different stages of the ripening process.

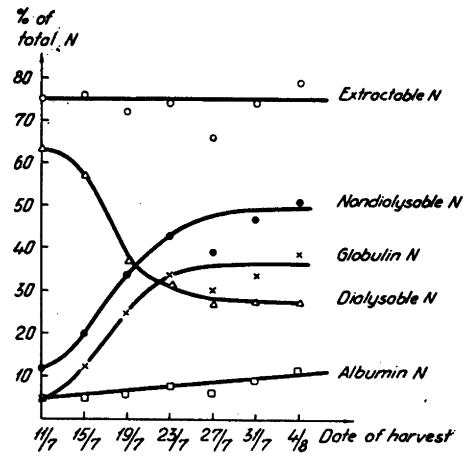

Fig. 2. Specific N as a percentage of the total N in developing pea seeds.

these seeds during the drying process. Five different extractions were carried out on this fraction at different times, and all determinations agree within $\pm$ 6 %. Thus the low values cannot depend on errors in the extraction method used.

C. Ultracentrifuge experiments on extracts from air-dried seeds

Extracts which had been dialyzed against the extraction buffer were analyzed in the ultracentrifuge, as described in a previous paper [7]. The ultracentrifuge diagrams for unripe seeds were very similar to those obtained for ripe seeds. The results are shown in Table 3. The results of some experiments on the garden pea variety are also found in this table [8]. The different stages of ripening for the two varieties are based on seed weight. Stage VII refer in both cases to ripe seeds. As can be seen from Table 3, the unripe seeds have a higher $\frac{\text{vicilin}}{\text{legumin}}$ ratio than the ripe seeds. For the peas of the field variety, the same ratio, 1.5, has been obtained for ripe seeds from three different years.

Table 3. The ratio $\frac{vicilin}{legumin}$ *in extracts from unripe pea seeds as determined from ultracentrifuge diagrams.*

Stage	Field variety $\frac{\text{vicilin}}{\text{legumin}}$	Average	Stage	Garden variety $\frac{\text{vicilin}}{\text{legumin}}$	Average
II	2.54 2.63 2.61 2.91	2.7	III	3.42 3.26 3.62	3.4
III	2.90 2.46 2.96	2.8	IV	2.70 2.70 3.13	2.8
VII	1.40 1.52 1.54	1.5	VII	1.30 1.19 1.28 1.15	1.2

D. Ultracentrifuge investigations on extracts from unripe seeds preserved by freezing

The experiments described above were carried out on seeds which had been dried in air after they had been harvested. It is very probable that some changes in the nitrogen distribution occur during this drying process, however. The main problem is whether or not any protein, especially well-defined high-molecular protein components, is synthesized, after harvesting, from low-molecular nitrogen constituents of the undeveloped seed.

In order to get some information on this point, seeds of the field variety of pea "Torsdagsärt II" were harvested in 1951 at different stages of the ripening process. Immediately after harvesting the seeds were separated from the seed pods, some seeds being preserved by cooling to — 20° C and some being allowed to dry at room temperature. After about two months both fractions were macerated and dried (lyophilized) as described above. A homogeneous green powder was obtained in both cases. Table 4 gives some data on the seed material used. Extractions were carried out with 0.2 M NaCl, pH 7, and after dialysis of the extracts against the extraction buffer the solutions were analyzed in the ultracentrifuge. The results of these investigations are given in Figs. 3 and 4.

As can be seen from Fig. 3, the well-defined globulin components appear rather suddenly in the middle of the ripening process. In the present case these components appear between stage III and stage IV. The amounts of high-molecular substances, as determined from the ultracentrifuge diagrams, were about the same for stage I—III, but for stage IV this value increased by 125 %. In earlier stages traces of well-defined globulin components can be

Table 4. Wet and dry weight of 100 seeds, harvested in 1951, after drying at room temperature.

Stage	Date of harvest	Weight per 100 seeds (g.)		Moisture
		Wet	Dry	%
I	14.7	—	—	85
II	18.7	15.1	2.8	82
III	21.7	19.3	3.6	82
IV	25.7	23.7	5.3	78
V	28.7	31.2	8.6	72
VI	1.8	34.4	10.8	69
VII	4.8	39.8	13.9	65
VIII	8.8	43.2	18.9	56

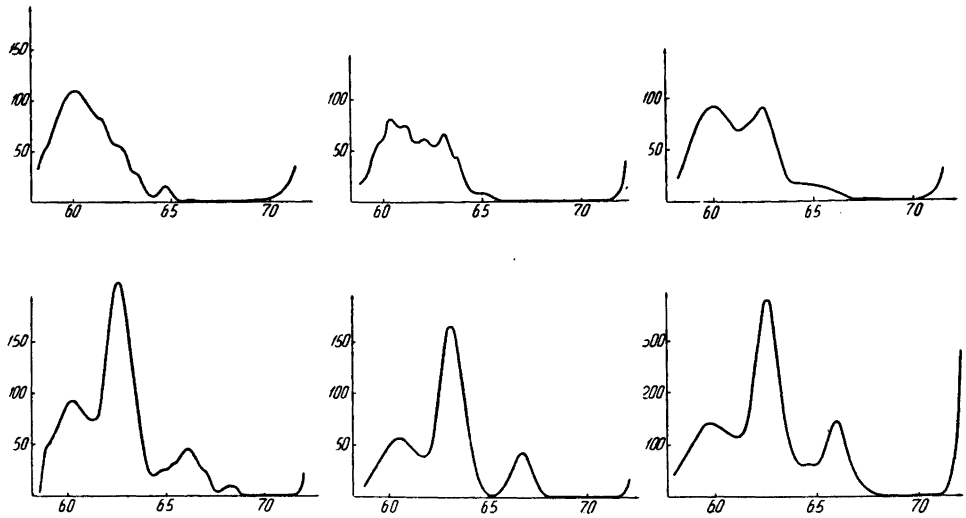

Fig. 3. Ultracentrifuge diagrams from dialyzed extracts of unripe pea seeds preserved by freezing. The abscissae represent distance from the axis of of rotation (cm.), the ordinates represent the scale-line displacement (μ). The exposures were all taken 35 minutes after full speed. Scale distance 120 mm. Centrifugal field 260,000 × gravity.

Top left: stage I.
Top centre: stage II.
Top right: stage III.
Bottom left: stage IV.
Bottom centre: stage V.
Bottom right: stage VI.

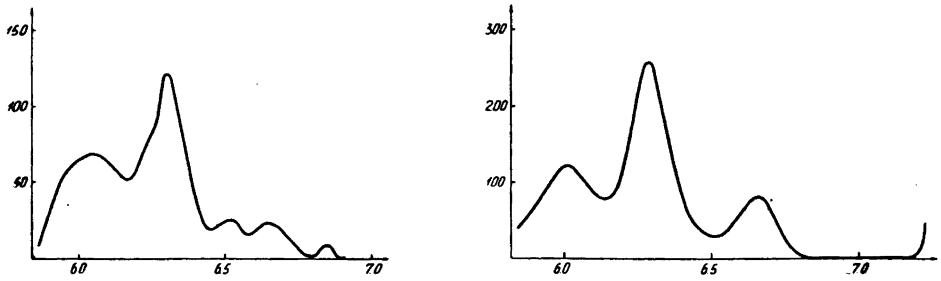

Fig. 4. Ultracentrifuge diagrams for unripe pea seeds which had been dried at room temperature. The experimental conditions were the same as those given in the caption of Fig. 3.

Left: stage I. Right: stage II.

Table 5. The ratios $\frac{vicilin}{legumin}$ *and* $\frac{albumin}{globulin}$ *in extracts from pea seeds, preserved by freezing. The values were obtained from ultracentrifuge diagrams.*

Stage	$\frac{\text{vicilin}}{\text{legumin}}$	$\frac{\text{albumin}}{\text{globulin}}$
II	7.7	6.4
III	5.1	4.8
IV	3.7	2.9
V	3.9	2.7
VI	2.3	2.2
VII	2.5	2.0
VIII	1.7	2.4

Table 6. Content of amino nitrogen in water extracts of lyophilized pea seeds, harvested in 1951.

Stage	Amino nitrogen, g equivalents/ml × 10^5	
	Seeds preserved by freezing	Air-dried seeds
I	9.35	10.58
II	7.16	7.56
III	6.95	6.66
IV	6.00	5.04
V	5.27	3.91
VI	3.73	3.75
VII	3.66	3.41
VIII	3.69	3.52

detected in the diagrams. Fig. 4, however, shows that if the seeds are allowed to dry at room temperature, rather well-defined components are found even at stage I. Table 5 shows the ratios between the different components.

E. Determinations of amino nitrogen

0.1 g lyophilized seed material was extracted with 2 ml H_2O for 24 hours. After centrifugation amino nitrogen was determined by acetone titration according to the Linderstrøm-Lang method [9]. Each titration was carried out with 0.1 ml extract and 1 ml acetone. The results are given in Table 6. Exactly the same experiment was also carried out on pea seeds which had been preserved at $-20°$ C for seven months. The new values were identical with those in Table 6.

DISCUSSION

Table 1 shows that the nitrogen content of the seeds, calculated as percentage of dry weight of the lyophilized material decreases almost linearly during the ripening process, probably depending on the fact that the in-flow of non-nitrogenous substances increases as ripening proceeds. Fig. 1 shows the increase in concentration of the different nitrogenous substances in 100 seeds during the development of the seeds. This figure gives a clear picture of the conditions during the ripening process in a single plant which delivers the nitrogenous substances synthesized in the leaves to a fixed number of seeds.

The absolute amount of all nitrogenous substances increases during the ripening process according to Fig. 1. Of special interest is the fact that the high-molecular nitrogenous substances are formed at an early stage of seed development, *e.g.*, 2/3 of the final amount of globulin is synthesized during the first half of the period of ripening. It is also significant that the amount of albumin increases slowly at a constant rate.

As was mentioned above, the experiments were carried out on seeds which had been harvested in two different years, 1950 and 1951, and the seed material had been treated in different ways. However, a comparison can be made between the two crops if all results obtained are expressed in terms of seed weight. From the seeds harvested in 1950, well-defined globulin components were obtained in the ultracentrifuge even in the first stages of ripening. It can be seen from Fig. 4 that when the seeds are allowed to dry in air at room temperature well-defined globulin components are found even in very small seeds. When the seeds are preserved by freezing, no such components can be detected in these early stages. (Fig. 3) Thus the ripening process continues in seeds which have been separated from the mother plant, and those nitrogenous substances which are delivered to the very small seed from its mother plant have the proper composition to form the well-defined globulin components.

Fig. 2 gives some information about different categories of nitrogen in ripening pea seeds, if the fact that the ripening process has been going on after harvesting is taken into consideration. This figure shows specific N as a percentage of the total N in the seeds. Extractable N is practically constant during the whole of the period studied. Protein N and globulin N increase mainly during the first part of the ripening process. Albumin N increases slowly at a constant rate all the time. Low-molecular N decreases at the same rate as protein N increases. The curves of formation for protein N, globulin N and albumin N during the ripening process are very similar to those obtained for the breakdown of these fractions during germination [7]. The most interesting fact is that the albumin fraction is synthesized and broken down quite independently of the globulin fraction. Together with the finding that the chemical composition of these two high-molecular protein fractions are different [10], this result gives strong evidence that the globulins and albumins are two quite different protein fractions.

The ultracentrifuge experiments which were carried out on unripe seeds harvested in 1951 showed (Fig. 3) that well-defined globulin components could be isolated at stage III—IV, corresponding to stage II for the seeds harvested in 1950. Thus according to Figs. 1 and 2, synthesis of well-defined globulin components begins at the same time as the content of protein N and globulin N begins to increase.

Determinations of the ratio $\frac{\text{vicilin}}{\text{legumin}}$ from ultracentrifuge diagrams show that this ratio decreases during the ripening process. The differences are so large that even the rather considerable errors involved in the methods used cannot have any effect on this result. The changes in the ratio $\frac{\text{vicilin}}{\text{legumin}}$ during the ripening process can be seen most clearly from the experiments on the pea seeds preserved by freezing. Vicilin is formed before legumin during the first part of the ripening process, and the two globulins are formed at different rates as ripening progresses. This fact can be clearly understood if the values from Table 3 are combined with those from Table 2. The absolute amounts of both vicilin and legumin increase during the ripening process, which fact can also be seen directly from the ultracentrifuge diagrams. The alternative that vicilin is synthesized first and than transformed to legumin is thus ruled out. During the germination process, however, the two globulins are broken down at the same rate [7].

The most important question in these investigations is whether or not any high-molecular intermediate products can be observed during the synthesis of the well-defined reserve proteins. There are many conceivable possibilities with regard to protein synthesis. For this discussion we assume that proteins are built up by condensation of amino acids [11] and not by polymerization of some simple unit, *e.g.*, formaldehyde and ammonia [12]. Wood and Petrie [13] found a correlation between amino acid and protein nitrogen and from their experiments they concluded that protein is formed by direct condensation of amino acids. Steward and Preston [14], however, who worked with metabolizing potato discs, thought that the amino acids are first deaminated. Simple amino acids may gradually condense to a long peptide chain which then forms a globular protein by coiling. A second possibility is that many amino acid molecules are arranged in the appropriate way and then condense very rapidly, all at the same time, to a peptide chain, large enough for the formation of one protein molecule. The third possibility is that some rather high-molecular peptide chains are first synthesized, which react with each other through a few active groups and form the protein molecule. It is very difficult to throw any light upon the mechanism of these reactions but some information about intermediate products may be obtained from Fig. 3. During stage I of the ripening process, no well-defined globulin components can be seen in the ultracentrifuge diagrams. The diagrams from stage II and III show very small amounts of the pure components, but the diagrams from stage IV show well-defined components in considerable quantity, and the diagrams are very similar to those obtained from the ripe seeds. The sudden change in the

amounts of high-molecular substances, as determined from the ultracentrifuge diagrams, indicates that the formation of the well-defined components is rather rapid, contrary to what happens during the germination process [7]. It is, however, very difficult to settle definitily from the ultracentrifuge diagrams whether any high-molecular nitrogenous substances serve as intermediate products in the protein synthesis. So a new method had to be used.

Figs. 3 and 4 show clearly that the ripening process is going on in the seeds which have been separated from the mother plant and left to dry at room temperature. The seeds from stage I contained no globulin components at all when the ripening process had been broken by freezing, but when the seeds had been dried at room temperature, all high-molecular nitrogenous substances were transformed to well-defined components. Thus chemical analysis of the two fractions from stage I can give some information about the synthesis of the reserve proteins. In our case amino nitrogen was determined by acetone titration according to the Linderstrøm-Lang method [9]. As can be seen from Table 6, amino nitrogen decreases at a constant rate during the first half of the ripening process until a constant value of the amino nitrogen content is obtained. All experiments indicate rather small differences in the amounts of extractable amino nitrogen compared with the total change during the ripening process between the fractions preserved by freezing and those dried in air. The air-dried fractions contained less amino nitrogen than the frozen fractions as was to be expected. At stage I the amount of amino nitrogen is three times higher than the value for stage VIII. Some conclusions can be drawn from these results. Firstly, the small differences in amino nitrogen between fractions containing no well-defined components and fractions from which well-defined components could be isolated indicate that high-molecular intermediate products are probably involved in protein synthesis, because the formation of large molecules (M = 186 000 and 330 000) from rather large intermediate molecules should give only small changes in amino nitrogen. The fact that amino nitrogen decreases linearly with respect to time also indicates that the intermediate products in the synthesis of proteins are of a high-molecular nature, because the ultracentrifuge diagrams show that the globulin components are formed rather rapidly.

Of course, much work remains to be done in this field. Unripe seeds may be more convenient for the study of protein synthesis than other materials which have been used, such as regenerating liver, because the protein systems in the seeds are rather simple, and large amounts of material can be obtained at different stages of protein synthesis. Furthermore the substances under investigation are all the time in a closed system, which is advantageous in many respects.

SUMMARY

1. Studies have been made of the nitrogen distribution in developing pea seeds.

2. Globulin N increases rapidly during the first part of the ripening process. Albumin N increases at a constant rate. Globulins and albumins are synthesized independently.

3. Vicilin and legumin are synthesized at different rates. The concentration ratio $\frac{\text{vicilin}}{\text{legumin}}$ is diminished as ripening proceeds.

4. Protein synthesis proceeds in unripe seeds which have been harvested. This synthesis is arrested by cooling to $-$ 20° C.

5. The possibility of high-molecular intermediate products in the synthesis of the reserve proteins is discussed.

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