The Breakdown of the High Molecular Reserve Proteins of Peas during Germination

CARL ERIK DANIELSSON

Institutes of Biochemistry and Physical Chemistry, Uppsala, Sweden

The protein breakdown in different seeds during germination has been studied for about 150 years. The most fundamental experiments were done by Pfeffer, Schulze and Prianischnikow. For a review of their work and other references Chibnall's monograph¹ on this subject is recommended, where also a large number of references can be found.

In most experiments described by earlier authors the most important question concerned itself with the amino acids formed in the young plant. The amino acids have been identified and their concentrations have been determined at different stages during germination. These old determinations are probably inaccurate. Many experiments have been performed in order to determine the large amounts of asparagine, and in certain cases glutamine, which are accumulated in the shoots during germination. These amides probably store all nitrogen which can not be used immediately by the plant. The leguminosae plants have a very high protein content, and have therefore been investigated most. These experiments have given many important contributions to the understanding and knowledge of the nitrogen metabolism in the plants.

The present study of the protein breakdown has been undertaken from another point of view. Instead of studying the breakdown products of the reserve proteins in the seed, the amounts of unaltered proteins have been determined at different stages during the germination. This has been possible with the help of the new methods for the study of high molecular substances which have been worked out during the last decades *i.e.* ultracentrifugation and electrophoresis.

Earlier investigations have shown that seeds of peas, *Pisum sativum*, contain two well-defined globulins, vicilin and legumin. Osborne² was the

first one to prepare these two globulins. The methods used by Osborne, however, do not give complete separation of the two globulins. By using simple precipitation methods, the present author succeeded in separating the two globulins completely from each other. The two resulting proteins were homogeneous in the ultracentrifuge and electrophoresis ^{3,4}. The molecular weight for vicilin was determined to be 186 000 with an iso-electric point at pH 5.5. For legumin the corresponding values were 331 000 and pH 4.8. Vicilin and legumin are also different in their chemical composition. Osborne and Campbell ⁵ showed that legumin contains considerably more sulfur than vicilin, and the present author has shown that the tryptophan content of legumin is about four times higher than that of vicilin. A third high-molecular protein fraction can be obtained from seeds of peas. This fraction is still very poorly defined, but it is called the albumin fraction in this paper because it is soluble in water. Vicilin and legumin are insoluble in water but soluble in dilute salt solutions. If a solution containing albumin, vicilin and legumin is dialysed against water, the two globulins are precipitated and the albumin remains in solution. The albumin fraction is, however, not homogeneous. Ultracentrifugation has shown that it contains at least two components. The albumin fraction is called legumelin by Osborne and Campbell ⁵.

The experiments described in this paper were performed in order to determine at which stage during the germination and at which speed the breakdown of the reserve proteins occurred.

EXPERIMENTAL

A. The treatment of the seed material before extraction

For the present experiments ripe, air-dried seeds from yellow peas, field variety "Torsdagsärt II" from the Royal Agricultural College in Uppsala were used. The seeds were placed in water overnight in order to swell and were then allowed to germinate in Petri dishes between wet filter papers. After 5 days, when the roots were about 3—4 centimeters long, the seeds were placed in a nutrient solution on a metal gauze in such a way that the roots were dipping into the solution but the seeds themselves were above the surface. The nutrient solution had the following composition: 1 g · Ca(NO₃)₂, 1 g · KNO₃, 1 g · MgSO₄ · 7 H₂O, 1 g · KH₂PO₄, 0.5 g · KCl and 5 drops 5 % FeCl₃ to 7 l H₂O. This nutrient solution was used in order to get germination conditions as normal as possible. It is very difficult to grow peas under sterile conditions, but the experiment

Fig. 1. The lengths in cm of the roots and the shoots at different stages of the germination. Each value in the figure is the average of 25 determinations.

was discarded if moulds could be seen on the seeds. The time of germination was counted from the day when the seeds were first placed in water to swell. Prianischnikow started to count one day later when the seeds had been placed between wet filter papers⁶. After different time intervals the germination was interrupted, the material was washed with water and finely ground with distilled water in a Waring blender for 6 minutes. The product obtained was frozen down to -16°C and dried in vacuum over CaSO_4 in the frozen state. The dry material consisted of a fine powder. Kjeldahl nitrogen determinations on each preparation showed that the material was rather homogeneous. The water content of each preparation was determined by heating to 105°C for 20 hours. The results of the nitrogen determinations are shown in Table 1. Fig. 1 shows the length of the roots and the shoots at different stages of germination.

The advantage of the method described above is that it is possible to use a known amount of the substance for each extraction experiment, and the homogeneity and water content of the material is also known. Before this method was worked out the material was extracted without previous drying and the same number of seeds or plants were used for each experiment. This method, which has been used by many authors on this subject is, however, of too low accuracy for investigations of this kind.

Table 1. Determinations of nitrogen content in dried preparations of peas, germinated for different times. The values are average values from three determinations with a maximum deviation from the mean of $\pm 2\%$.

Time of germination days	Nitrogen content of the dry weight %
1	4.43
2	4.13
3	4.41
4	4.29
5	4.31
6	4.43
7	4.52
8	4.61
9	4.51
10	4.82
12	5.65
15	6.32
19	6.55

B. Determination of the best extraction conditions

In order to determine the best extraction conditions, some experiments with different extraction solutions and different extraction times were done. In all experiments the temperature was $+4^{\circ}\text{C}$ which is convenient inasmuch as it reduces the effect of any proteolytic enzymes. The risk of bacterial growth is also lower at this temperature. Previous experiments with the pure globulins have shown that the extraction should be done at a pH value close to 7 because legumin is insoluble in the pH range 4—6³.

Two (2.0) grams of macerated, frozen and dried seeds, germinated for one day, were extracted with 100 ml of different extraction solutions at $+4^{\circ}\text{C}$ with stirring. At different time intervals 3 ml were withdrawn with a pipette, centrifuged at 3 500 r.p.m. (radius 5 cm) for 5 minutes. The nitrogen content of this solution was then determined according to Kjeldahl. The results are shown in Table 2.

From Table 2 it can be seen that 0.2 *M* NaCl, buffered to pH 7.0 with 0.03 Na_2HPO_4 and 0.02 NaH_2PO_4 is as effective as a buffer with much higher ionic strength. This buffer solution will be referred to as "standard buffer" in this paper and was used in all experiments here described. From Table 2 it can be seen that water is almost as effective as standard buffer in the first stage of the extraction, but after prolonged extraction the nitrogen content of the

Table 2. Determination of the best extraction conditions of nitrogen compounds from seeds of peas at pH 7. Temperature + 4° C. 2.0 g dried seed material and 100 ml extraction solution were used for each extraction experiment.

Extraction solution	Nitrogen content in extract after different extraction times mg N/ml				
	10 min.	60 min.	3 hours	9 hours	24 hours
H ₂ O	0.636	0.615	0.580	0.539	0.537
0.2 M NaCl	0.654	0.662	0.686	0.690	0.703
0.03 M Na ₂ HPO ₄					
0.02 M NaH ₂ PO ₄					
1 M NaCl	0.647	0.662	0.688	0.691	0.703
0.06 M Na ₂ HPO ₄					
0.04 M Na NaH ₂ PO ₄					

water extract decreases. According to definition globulins should not be soluble in water. The fact that the globulins can be extracted with water may be explained by assuming protective colloids in the seeds. If a water extract from pea seeds is dialysed against water for about one hour or is stored for a few days, the globulins are precipitated. This effect can be seen from the values in Table 2.

C. Preparation methods used

One and a half grams (1.50 g) of the dried material were weighed into an Erlenmeyer flask of 100 ml and one drop of toluene was added. The extraction was carried out for 20 hours with 20 ml standard buffer at + 4° C with stirring. The extraction was interrupted and after centrifugation the precipitate in the centrifuge tube was again extracted for 1 hour with another 10 ml standard buffer. After centrifugation the two extracts were mixed and filtered through a glass filter (Jena G 2) and the volume was measured. 1.0 ml was taken off for nitrogen determination. In the final calculations corrections were made for all volumes taken off for nitrogen determinations and ultracentrifugations. The extract was then dialyzed in a thin cellophan bag against 1 000 ml of standard buffer. The dialysis was continued for 48 hours and the standard buffer outside the bag was changed every twelve hours. After the dialysis was finished the volume was measured and 1.0 ml was taken off for nitrogen determination and 2.0 ml for ultracentrifugation. The remaining solution was then dialysed for 24 hours against running tap water (+ 8° C), immediately followed by dialysis against distilled water for 48 hours at + 4° C. By this procedure a dense precipitate of globulins was obtained, which was centrifuged down. The volume of the supernatant solution was measured, and nitrogen determination was carried out on 3.0 ml of the solution. The precipitate was dissolved in standard buffer, the volume measured and the nitrogen content of 1.0 ml of the solution determined. The fractionation scheme is shown below.

With this extraction method only 70—80 % of the total nitrogen in the seeds is extracted. If larger volumes are used the yield is higher but the solutions are then too dilute for investigation in the ultracentrifuge and the losses

by dialysis against water are larger because small amounts of vicilin remains in the supernatant even after prolonged dialysis. The nitrogen remaining in the extracted material is probably bound in the cells in such a way that it can not diffuse out through the cell walls during the extraction. This nitrogen could possibly be extracted by Miller and Chibnalls ether-water method⁷ but previous experiments on the purified globulins have shown that these proteins are denatured by ether. Thus the ether extraction is not suitable in this special case. The preparation method used here seems to give very little denaturation of the proteins. The experiments described have been performed in order to study the breakdown of the globulins, and it has been impossible to isolate any products of globular character from the leaves, stems and roots.

In the following discussion the numbering of the fractionation scheme will be used. At no. 8 an extract with a maximal amount of extractable nitrogen will be obtained. After the dialysis at no. 9 all low-molecular nitrogen has dialysed out through the bag, and at no. 10 the sum of the globulin N and albumin N is obtained. This nitrogen can be called protein nitrogen, even if the expression is not exactly correct but the error is very small. In this paper the nitrogen determined at no. 10 is called non-dialysable N. The amount of dialysable nitrogen (low-molecular N) is obtained by taking the difference between the determinations at no. 8 and no. 10. The ultracentrifugation at no. 10 gives the relative amounts of albumin, vicilin and legumin according to what will be said below. The dialysis at no. 11 separates the albumins from the globulins. The amount of albumin N is determined at no. 13 and the globulin N at no. 12.

The following example may be given:

One and a half grams (1.50 g) of the dried material, germinated for 4 days, were extracted according to the fractionating scheme above. At no. 8 the volume was 24.0 ml, and the nitrogen determinations gave the value 1.00 mg N/0.5 ml. Thus 48.0 mg N had been extracted. After dialysis against standard buffer according to no. 9, at no. 10, 23.0 ml solution containing 0.697 mg N/0.5 ml were obtained. Thus, after correcting for the solution taken off for nitrogen determination at no. 9, 33.5 mg non-dialysable nitrogen had been obtained. After dialysis against water according to no. 11 and dissolving of the precipitate at no. 12 the following values were obtained. The precipitate was dissolved in 34.0 ml and this solution contained 0.586 mg N per 1.0 ml. Thus 19.9 mg globulin N could be found. After correction for the volumes which had been taken off (1 ml at no. 8

and 3 ml at no. 10) $\frac{19.9 \times 24}{20} = 23.9$ mg globulin N are obtained. The volume of the

supernatant solution at no. 13 was 28.0 ml, since the volume increases during the dialysis to the saltfree state. It contained 0.657 mg N/3.0 ml. Thus 6.1 mg albumin N were found, which value after correction increases to 7.4 mg albumin N.

Fig. 2. Ultracentrifugation diagrams of NaCl-extracts of pH 7 from seeds of peas, germinated for 1 day (left), 9 days (centre) and 15 days (right). The extracts had been dialyzed for 48 hours against the extraction buffer. The exposures are taken 45 minutes after full speed at 65 000 r.p.m. ($300\,000 \times g$). The same scale distance (80 mm) has been used. The areas have been measured in the way indicated on the diagram to the left. In the diagrams the abscissae represent the distance from the centre of rotation (cm), the ordinates represent the scale-line displacement (Z in μ).

The ultracentrifugation * at no. 10 showed that the solution, 24.0 ml, contained 0.15 % (g/100 ml) albumin, 0.34 % vicilin and 0.24 % legumin. Thus 36.0 mg albumin, 81.6 mg vicilin and 57.6 mg legumin were found in the solution. By dividing these values with the factor 6.25, the values above correspond to 5.8 mg albumin-N, 13.0 mg vicilin-N and 9.2 mg legumin-N, or totally 22.2 mg globulin-N. If these values are compared with those obtained from the nitrogen determinations, it is clear that the ultracentrifugation method gives lower values. The sedimentation diagrams are shown in Fig. 2.

As can be seen from Fig. 2, three main peaks are obtained, albumin, vicilin and legumin. The albumin peak is divided into two peaks after about 100 minutes at 65 000 r.p.m. The sedimentation constants of these peaks are about 1 S and 4 S. In some diagrams a new component, sedimenting still faster than legumin, can be seen. Its sedimentation constant is about 19 S. It is an association product of legumin. When a solution containing purified legumin is heated to 90°C large amounts of this component are formed. In some cases legumin has been quantitatively transformed into this new component. Solutions containing pure legumin in high concentrations also show this high-molecular component in the ultracentrifuge.

For information about the ultracentrifugation method see Svedberg and Pedersen's monograph on the subject⁸.

D. Results from the nitrogen determinations

The values obtained from the nitrogen determinations are shown in Table 3 and Fig. 3.

Table 3. Nitrogen distribution in NaCl-extracts from seeds of peas, germinated for different times. In all experiments 1.50 g dried material has been extracted.

Days of germination	Tot. N in 1.50 g dried material mg	Extractable N mg	% of total N	Nondialys- able N (protein N) mg	% of total N	Dialysable N mg	% of total N	Globulin N mg	% of total N	Albumin N mg	% of total N
1.	64.6	49.8		37.2		12.6		26.0		6.4	
		48.1		37.5		10.7		26.2		7.1	
		45.3		36.1		9.2		28.4		8.5	
		52.4		39.7		12.7		28.5		8.6	
		47.5		37.3		10.2		26.5		8.2	
		Av: 48.6	75	37.6	58	11.1	17	27.1	42	7.8	12
2.	55.6	46.8		38.8		8.0		26.4		7.3	
		44.2		35.7		8.5		25.8		8.4	
		44.7		40.3		4.4		25.2		10.7	
		45.6		34.5		11.1		25.9		8.1	
		45.2		36.6		8.6		25.1		10.7	
		Av: 45.3	82	37.2	67	8.1	15	25.7	46	9.0	16
3.	61.2	44.9		36.5		8.4		26.2		7.2	
		44.2		36.2		8.0		24.7		8.5	
		Av: 44.6	73	36.4	59	8.2	13	25.5	42	7.9	13
4.	59.8	48.0		33.5		14.8		23.9		7.4	
		49.7		33.0		16.7		26.0		6.5	
		Av: 48.9	82	33.3	56	15.8	26	25.0	42	7.0	12
5.	58.8	48.4		31.9		16.5		22.6		7.8	
		48.8		29.6		19.2		22.4		7.0	
		41.4		29.3		12.1		21.5		7.8	
		49.6		33.7		15.9		25.6		7.4	
		46.6		34.2		12.4		24.6		7.9	
		Av: 47.0	80	31.7	54	15.2	26	23.3	40	7.6	13
6.	61.0	45.2		28.0		17.2		24.8		7.1	
		45.5		30.9		14.6		22.4		7.5	
		46.7		31.9		14.8		21.4		5.4	
		48.9		32.4		16.5		22.4		6.7	
		Av: 46.6	76	30.8	50	15.8	26	22.8	37	6.7	11

Table 3 (cont.)

7.	63.0	43.3		28.3		15.0		20.6		6.1	
		51.8		29.7		22.1		22.2		7.3	
		47.6		31.0		16.6		20.1		6.8	
		44.6		28.0		16.6		19.2		7.0	
		39.9		26.3		13.6		19.5		7.0	
		Av: 45.4	72	28.7	46	16.8	27	20.3	32	6.8	11
8.	63.2	44.9		18.8		16.1		13.4		5.4	
		44.1		20.5		23.6		13.4		5.6	
		46.6		22.1		24.5		12.7		6.7	
		47.2		23.7		23.5		15.1		8.0	
		Av: 45.7	72	21.3	34	21.9	35	13.7	22	6.4	10
9.	60.8	44.7		20.9		23.8		13.9		5.8	
		53.6		24.3		29.3		13.8		6.0	
		Av: 49.2	81	22.6	37	26.6	44	13.9	23	5.9	10
10.	65.7	46.3		20.3		26.0		10.9		6.7	
		41.8		18.4		23.4		9.2		6.4	
		Av: 44.1	67	19.4	30	24.7	38	10.1	15	6.6	10
12.	75.5	41.0		15.3		25.7		9.4		4.8	
		38.0		12.8		25.2		7.4		4.9	
		49.2		15.7		33.5		7.2		6.7	
		Av: 42.7	57	14.6	19	28.1	37	8.0	11	5.5	7
15.	86.6	24.2		6.2		18.0		2.5		2.5	
		36.3		8.3		28.0		2.9		3.4	
		Av: 30.3	35	7.3	8	23.0	27	2.7	3	3.0	3
19.	87.4	35.7		8.8		26.9		3.8		3.2	
		34.6		7.9		26.7		3.2		2.1	
		Av: 35.2	40	8.4	10	26.8	31	3.5	4	2.7	3

It can be seen from Table 3 that the amount of extractable N varies strongly in the different preparations. The amount of non-dialysable N has been determined with a maximum deviation from the average of $\pm 14\%$. The deviation in the determinations of globulin N is lower than $\pm 10\%$ with one exception. For the preparation germinated for 12 days the deviation is $\pm 17\%$. The albumin N has been determined with a maximum deviation of $\pm 20\%$ from the average. The determinations are more difficult to carry out and the accuracy is lower the longer the time of germination. This depends mainly on two things, first the protein concentration decreases and second the extracts contain a great deal of breakdown products which together with the

Fig. 3. Nitrogen distribution in peas at different stages of germination.

chlorophyll and other products formed during the germination lower the accuracy of the determinations. As can be seen from Table 3 only 70—80 % of the total nitrogen in the seeds can be extracted. If less nitrogen is extracted the extracts contain relatively more of low molecular products. This is shown by the following experiment.

One and a half grams of the dried material, germinated for three days, were extracted with 25 ml standard buffer without subsequent washing of the precipitate according to nos. 4—6 in the fractionating scheme. By this method only 60—65 % of the total nitrogen can be extracted. Three experiments were carried out. The ratios $\frac{\text{Dialysable N}}{\text{Non-dialysable N}}$ obtained were 0.58, 0.49 and 0.48 respectively when 64, 63 and 60 % of the total nitrogen were extracted. From Table 3 this ratio after three days is 0.22, when 73 % of total nitrogen was extracted.

Thus the low-molecular nitrogen is more easily extracted than the high-molecular nitrogen, and it is important to extract as much nitrogen as possible in order to get a true picture of the nitrogen distribution in the seeds.

E. Results from the ultracentrifuge experiments

As was mentioned in connection with the preparation method, determinations of concentrations from sedimentation diagrams give lower values than

those obtained with other methods, *e. g.* determination of the nitrogen content and refractive index of the solution. In some cases the values obtained from the sedimentation diagrams are only half of those obtained from the nitrogen determination. This depends on the fact that relatively large amounts of low-molecular material are present and part of these substances do not sediment in the ultracentrifuge. Quantitatively the values from the centrifuge diagrams can not be directly compared with those obtained with other methods. The sedimentation diagrams, however, can give an idea of the relative amounts of the different protein components. It is, however, important to remember that the concentration obtained from centrifugation diagrams of the faster sedimenting component always is too low. This effect has been discussed by several authors⁹⁻¹¹ but it is of little importance at concentrations lower than one percent. In our case the solutions contain three components, two of which, vicilin and legumin, are always in about the same relative concentrations. When determining the relative amounts of vicilin and legumin from the ultracentrifuge diagrams the error caused by the effect mentioned above should be of about the same magnitude all the time.

In order to compare the relative concentrations of vicilin and legumin, ultracentrifuge diagrams taken 45 minutes after full speed at 65 000 r.p.m. were used. The areas were measured in the way indicated in Fig. 2. The errors in these determinations are large and increase with germination of the seeds. The longer the germination has been going on the more non-sedimenting products are present in the solutions in comparison with the amounts of vicilin and legumin. This fact can be clearly seen from Fig. 2. The ratio vicilin/legumin was determined after 1, 2, 3, 5, 6, 7, 9 and 12 days of germination. In all cases the average values of this ratio from the different determinations were between 1.1 and 1.5. After what has been said about the errors in these determinations, it is impossible from these measurements to detect any definite differences in the breakdown velocities of the two globulins. According to these experiments vicilin and legumin are broken down with the same speed during the germination. The absolute amounts of albumin, vicilin and legumin determined from sedimentation diagrams gave breakdown curves of the same form as the curves obtained from nitrogen determinations.

DISCUSSION

From Table 3 some important conclusions can be drawn. The amount of extractable nitrogen shows, even if the errors in these determinations are large, a clear tendency to decrease as the germination is going on. According to Table 3 the new plant does not lose any nitrogen during the germination.

During the last stage of the germination it takes up nitrogen from the nutrient solution. Thus, when the total nitrogen in the plant increases while the extractable nitrogen decreases, the nitrogen in the developed plant must be present in a different form than in the seeds. The proteins of the leaves are synthesized when the new plant grows up and according to Chibnall¹² these proteins cannot be easily extracted without previous cytolysis. According to Table 3 the amount of extractable nitrogen begins to decrease after about 6 days of germination, or exactly at the time (Fig. 1) when the new plant begins to grow and green leaves are formed. Thus a clear dependence of the amount of extractable nitrogen on growing can be seen. The breakdown curve of the non-dialysable nitrogen, or protein nitrogen, shows a maximum rate of breakdown after 8—10 days of germination. A similar curve was obtained by Prianischnikow¹³ with a maximum breakdown after 9 days of germination. The globulin nitrogen, however, is broken down in a still more interesting way. During the first stage of the germination, about the first five days, globulin is broken down very slowly. After 5—6 days the breakdown of the globulins increases, and after 15 days practically no globulin remains. A comparison of this curve (Fig. 3) with Fig. 1 shows that the breakdown of the globulins is most intense at the time when the new plant is growing. Thus the conclusion can be drawn that the globulins, which amount to 70 % of the total protein content of the seeds, are used for the synthesis of the proteins in the new leaves.

The albumin nitrogen is slowly broken down with constant speed during the germination. The fact that albumin is broken down more slowly than globulin can be seen clearly from the ultracentrifuge diagrams in Fig. 2. The role of the albumin during germination is not clear. The author has two theories on this subject. Firstly albumin can be an intermediate product in the breakdown of the high-molecular globulins to amino acids. Secondly, and more probably, the albumin is broken down quite independently of the globulins, and the breakdown products from the albumin fraction serve other purposes than the breakdown products from the globulins.

As can be seen from Table 3, it is difficult to determine the amount of dialysable (low-molecular) nitrogen with satisfactory accuracy. It can be seen, however, that during the first 10 days of the germination the amount of low-molecular nitrogen increases at about the same rate as the breakdown of the globulins and the albumin, during which of course amino acids and other low-molecular nitrogen products are formed. After ten days the amount of low-molecular nitrogen decreases, because this nitrogen is probably being used by the plant for the synthesis of new proteins. From the results of these experiments, it is clear that the protein breakdown is most effective after 5—10 days'

germination. Thus, the proteolytic enzymes are most active at this stage. Very little is known about the proteolytic enzymes from seeds of plants belonging to the *Leguminosae*. The present author has isolated a protein fraction with proteolytic activity by extracting overnight macerated pea seeds, germinated for 8 days with water or 50 % glycerol. By dialysis of the extracts against water and precipitation of the supernatant solution from the dialysis with $(\text{NH}_4)_2\text{SO}_4$, the enzyme can be concentrated and purified. A still more active enzyme preparation can be obtained by extracting unripe seeds with water and subsequent dialysis of the extract, by which procedure the globulins are precipitated. The proteolytic activity follows the albumin fraction. These investigations will be described in a later paper.

SUMMARY

1. The breakdown of albumins and globulins in seeds of peas has been studied during germination.
2. From the ripe seeds, 70—80 % of total N can be extracted at pH 7 with 0.2 M NaCl. After ten days' germination less nitrogen can be extracted.
3. The breakdown curve of the globulins shows a pronounced maximum between 5—10 days' of germination which is the period when the new plant begins to form leaves. Vicilin and legumin are broken down with the same speed.
4. The breakdown of the albumins is slow and occurs at a constant rate.
5. A protein fraction with proteolytic activity has been isolated and purified. The enzyme follows the albumin fraction.

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