XXVI. STUDIES ON THE NATURE OF THE PROCESS OF GERMINATION.

A NEW METHOD FOR THE DETERMINATION OF PROTEINS BY MEANS OF ADSORPTION APPLIED TO THE DECOMPOSITION OF PROTEINS IN GERMINATING PEA SEEDS.

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EARLIER publications dealing with the question of decomposition of proteins in germinating seeds, have not given adequate consideration to proteolytic hydrolysis accompanying the extraction process.

Osborne has pointed out that a short extraction at a normal temperature, even if incomplete, yields more satisfactory results than prolonged extraction with heat because in the latter case it is impossible to avoid changes resulting from fermentation.

It is for this reason that the figures given in earlier publications relating to the proportion of protein N to amino N require to be reconsidered. As we are dealing only with proteins which are soluble in neutral solutions of sodium chloride (globulins and albumins) it will be best not to attempt a complete extraction but to determine the proportion of protein nitrogen to amino nitrogen in equivalent parts of the maceration liquid.

It will be later shown that it may be possible to obtain approximately absolute figures.

The usual methods for the determination of proteins were also considered to be unsatisfactory.

The method most often applied in Agricultural Chemistry, *i.e.* the determination of the total nitrogen content and multiplication by a certain factor, is of course useless in this case, in which proteins as well as non-proteins (amino acids, etc.) have to be taken into account.

Precipitation with phosphotungstic acid [Abderhalden and Pettibone, 1912] cannot furnish exact data for the degree of protein decomposition. This precipitation process is accompanied by the precipitation of other more or less complicated protein derivatives together with the protein itself.

Similarly the "Stutzer" method [Rosenthaler] (precipitation with copper hydroxide) does not yield accurate results.

Osborne's method [1924] of fractional "salting-out" is, apart from its intrinsic difficulty, quite useless for our purpose, because it fails completely as a quantitative method. (The method was in fact never worked out for this purpose.)

With a view to separating the proteins from other nitrogenous substances, we have tried to adsorb them by suitable adsorption media and afterwards to eluate¹ them.

Adsorption with aluminium hydroxide gel was first tried. As this substance, except in the presence of special reagents which had to be avoided, is easily soluble in excess of alkali, the less soluble iron hydroxide gel was chosen. It was proved that relatively small quantities of iron hydroxide gel were quite sufficient to adsorb quantitatively all the proteins. The filtrates from this adsorption did not react in the least to Spiegler's or Esbach's reagent, *i.e.* they were absolutely free of proteins.

By the use of weak alkalis it is possible to eluate the proteins almost quantitatively.

An experiment with kaolin (the purified kaolin of May and Baker was used) failed, at first, completely; better results were secured only after slight acidification, *i.e.* acidification of the maceration liquor in slight excess of the optimum for the coagulation of the proteins.

A maceration liquor showed, for example, a p_H of 4.8 (determined with platinum coated electrodes by Luther's method), and was adsorbed only to a slight extent at that concentration. Only when the p_H had been brought to the value of 3.8 by acidification was the adsorption brought practically to completion. The filtrates showed only a weak protein reaction with Spiegler's reagent, after standing for a long time. From the kaolin adsorbate it was possible to eluate the proteins almost quantitatively.

The following figures indicate comparative results with iron hydroxide gel and with kaolin.

100 cc. of maceration liquor contained:

	Fe(OH) ₃	Kaolin
Total N before adsorption	0.451	0.451
Non-protein N after adsorption	0.038	0.099
Protein N	0.413	0.352
First elution (2 % NaOH) N	0.280	0.288
Second elution (4 % NaOH) N	0.093	0.034
Third elution (6 % NaOH) N	0.020	0.009
Elution (1 + 2 + 3) N	0.393	0.331

It is evident that it is possible to eluate the protein N almost quantitatively. The higher content of non-protein N in the filtrate from the kaolin adsorption is easily explained, because as we have already mentioned the first adsorption by kaolin is by no means absolutely quantitative.

Previous experiments [Abderhalden and Fodor, 1919] and our own experience show that no amino acids are adsorbed when using such reagents.

¹ We are adopting the terminology of Willstätter.

The method of determination is briefly as follows: 100 g. of finely-ground seed-flour is macerated for one hour with 500 cc. of a solution of 10 % sodium chloride at the temperature of the room.

The liquor is then filtered through a battery of folded filter papers and toluene and thymol are added for preservation. In this maceration liquor the nitrogen content is determined by Kjeldahl's process (micro-method of Abderhalden-Fodor).

100 cc. of the maceration liquor is mixed with a corresponding quantity of iron hydroxide gel, completely neutralised and well shaken for half-an-hour. (The iron hydroxide gel was prepared by precipitation of a solution of FeCl_3 with the equivalent amount of sodium carbonate and removal of excess CO_2 by boiling.) The resultant precipitate is filtered and thoroughly washed. The filtrate must be absolutely free from proteins; if this is not the case the quantity of iron hydroxide used has been insufficient. The non-protein nitrogen in the filtrate is then determined. To recover the proteins, the residuum is eluted for 2 hours at first with 2 % NaOH, subsequently with 4 % NaOH and finally with 6 % NaOH. About 300 cc. of alkaline liquor are used for both elution and washing on each occasion. Finally by acidifying up to the optimum of coagulation, the proportion of protein which can be precipitated and the proportion non-precipitable can be determined. Protein which cannot be precipitated by acid can be dialysed and isolated. The difference between the amount of total N in the maceration liquor and the adsorption filtrate indicates the content of protein nitrogen. Lipins are present in such small quantities that they can be ignored.

To obtain approximately absolute figures, it is only necessary to determine the proportion of nitrogen in the flour and the nitrogen content in the maceration liquor and to multiply the protein nitrogen content found in the maceration liquid with the corresponding factor.

Thus we found a total nitrogen content of 0.451 % in the maceration liquid, whereas it contained 0.413 % of protein nitrogen. The nitrogen of the seed-flour used amounts to 3.976 %.

It was therefore necessary to multiply the figure obtained by the factor $\frac{3.976}{0.451} = 8.82$. From this, by multiplying the amount of protein nitrogen by 5.7 (the usual factor for conversion of the nitrogen of Leguminosae into protein) [Grafe, 1914], the protein content is found to amount to 20.76 %.

The alternative method of complete extraction cannot be recommended, because as we have already mentioned, proteolytic hydrolysis is liable to take place under such circumstances.

In the examination of germinated seeds smaller quantities of elution reagent and a shorter period of reaction have been used although we did not recover all the proteins in this way.

This new method of protein determination in plant extracts possesses in our opinion the following advantages:

(1) The proportion of protein nitrogen to non-protein nitrogen is quantitatively determined.

(2) The proteins are purified and can easily be recovered.

(3) Proteolytic hydrolysis is avoided.

(4) The method has the advantage of great rapidity.

EXPERIMENTAL.

The peas, "Prince Albert," supplied by the firm Vilmorin, which contain 3.976 % of nitrogen (air dried after removal of the skin) were used. They were sown in germination boxes and Tel-Aviv dune-sand was used as substratum.

The following summary explains the plan of our experiment:

	Root	Stalk
Experiment Light 1 (after 3 days)	3-4 cm.	—
Experiment Light 2 (after 11 days)	Strongly developed	Ca. 5 cm. Cotyledons green
Experiment Dark 1 (after 3 days)	3-4 cm.	—
Experiment Dark 2 (after 11 days)	Strongly developed	Ca. 8 cm. Cotyledons pale

As we had to work on the problem of chemical processes in the seed itself, the latter had to be completely separated from skins, roots and stalks. The following figures indicate the content of protein N and non-protein N.

In 100 parts of the maceration liquor:

	Not germinated	Light 1	Light 2	Dark 1	Dark 2
Total N	0.451	0.560	0.648	0.526	0.671
Non-protein N	0.038	0.058	0.143	0.065	0.139
Protein N	0.413	0.502	0.505	0.461	0.532

From these figures the proportion of protein N to non-protein N can easily be calculated.

100 parts of N contain:

	Not germinated	Light 1	Light 2	Dark 1	Dark 2
Protein N	91.57	89.64	77.93	87.62	79.28
Non-protein N	8.43	10.36	22.07	12.38	20.72

This summary shows us that there is a steadily increasing dissociation which has about the same value for normal and for etiolated germinated seeds for equal intervals of time. Previous literature has generally given a figure of 89 : 11 for the proportion protein N : amino N [Adolf Mayer].

This somewhat high content can probably be explained by the fact that proteolytic hydrolysis had occurred during the extraction process.

The following figures of Schulze and Flechsigs [1885] for germinated seeds can serve for comparison (the peas were germinated on saw-dust in the dark and the figures relate to the whole plant).

N in % of total N:

	Not germinated	First stage
Protein N	86.44	38.33
Amino N	11.62	30.34
N soluble in ethyl acetate	1.94	11.33

The higher content of amino acids in the first stage may be chiefly ascribed to the fact that all these analyses relate to the whole plant. It is well known,

e.g., that the formation of asparagine is chiefly located in the axial portions of the plant.

Schulze [1878] found in 11-day old lupins in the axial portions 31.81 % asparagine in the cotyledons, only 7.62 % in the dry substance. Priaschnikow obtained similar results. It is confirmed that with regard to the axial portions, the N of the asparagine forms a much higher percentage of the total N than in the cotyledons.

Our experiments showed that not all the protein derivatives are immediately utilised for regeneration, but that a high percentage remains in the seed. Later experiments will enable us to decide whether after the dissociation of protein there is also a qualitative difference between the amino acids immediately used for regeneration and those which at first remain in the seed.

Osborne separated the proteins contained in peas by means of fractional "salting out" in different concentrations. We preferred to distinguish between protein fractions, which can be precipitated and those which cannot be precipitated by acid.

We therefore determined on the first two elutions the proportion between these two fractions. For this purpose we carefully acidified to the optimum of coagulation (*i.e.* methyl red showed a weak reaction), and obtained the following results, expressed as % of protein N in the first two elutions:

	Not germinated	Light 1	Light 2	Dark 1	Dark 2
Protein precipitable by acid	31.17	44.02	47.14	47.08	57.75
Protein non-precipitable by acid	68.83	55.98	52.86	52.92	42.25

These figures are in no way quantitative, as the proportion was only determined in two elutions, in addition to which, as already mentioned, complete recovery of proteins was not carried out, because of the danger of proteolytic changes. It may be mentioned for the characterisation of these proteins precipitated by acid that *they are quantitatively dispersed in alcohol of 70-80 %*.

We hope to make further investigations of the alcohol sols of the plant globulins.

As it has been suspected that these proteins contain phosphoric fractions [Osborne and Strauss, 1915] this question was also examined. According to our experiments iron hydroxide gel is not suited for this purpose as it liberates the phosphoric acid on its surface.

We therefore left the protein of our maceration liquor to be adsorbed by kaolin and examined it after removal of all the proteins to see whether a difference could be observed in the content of phosphoric acid before and after the adsorption.

	Non-germinated	% P Light 1	Dark 1
Maceration liquor before adsorption	0.07	0.06	0.05
Maceration liquor after adsorption	0.07	0.06	0.04

Hence it follows that there are no phosphoric fractions either in the ungerminated or in the germinated seeds.

SUMMARY.

(1) The method of adsorption with iron hydroxide gel is very well suited for the determination of protein and the derivatives of the protein decomposition process. It enables us to obtain the proteins in a pure state and to characterise them.

(2) The content of non-protein N in germinating seeds increases almost equally during the process of germination, whether the germination takes place in dark or in light.

(3) Only a part of the derivatives obtained at the cost of the proteins is immediately used for regeneration.

(4) In non-germinating seeds there is a proportion of 30 : 70 of protein precipitable by acid to protein not precipitable by acid; this proportion changes in germinating seeds where we find almost equivalent quantities of the two kinds of protein. (At the same time there is a loss of total protein N.)

(5) The protein precipitated by acid is dispersed by alcohol of 70–80 %.

(6) Neither in non-germinating seeds nor during germination are there any phosphoproteins.

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