CCXXIII. STUDIES ON THE DISTRIBUTION OF ENZYMES IN DORMANT AND GERMINATING WHEAT SEEDS.

I. DIPEPTIDASE AND PROTEASE.

II. LIPASE.

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I. DIPEPTIDASE AND PROTEASE.

THE enzymes of wheat, especially amylases, have been the subjects of studies for many years, but, since most of the work has been done on flour or ground whole seeds, very little knowledge of the distribution in the seed has been obtained. Such knowledge is of great interest especially in relation to germination, because our information on the *in vivo* functioning and interrelations of intracellular enzymes, in general, is extremely meagre. The enzymic micro-methods originated by Linderstrøm-Lang and Holter [1934] conveniently permit, for the first time, a study of the distribution and activity of enzymes in relation to structure.

The present paper reports the distribution of dipeptidase (tested on alanyl-glycine) and protease (tested on edestin) in five easily-dissected parts of the wheat seed, both dormant and germinating. These parts are (1) the hull, including epidermal, parenchymatous and aleurone cells; (2) the endosperm, which stores starch and protein; (3) the scutellum, a yellow sheath of cells between the embryo and the rest of the seed; and the embryo, cut in a rather arbitrary manner into (4) cotyledon and (5) radicles. The importance of the scutellum as a site of enzyme formation and activity at the time of germination is, for the first time, emphasised with proteolytic enzymes.

The chief work on enzymes in germinating wheat has been done in Russia [see especially Bach *et al.*, 1927]. An increase in the content of amylase, catalase, peroxydase and protease on germination has been found. It is unfortunate that such an extensive investigation should have been done without regard to optimum p_H and on dried, ground, extracted seeds. The great irregularities observed are probably related to the fact that drying has a varying effect on the enzymes. The physiological factors involved in the germination of grasses have been systematically reviewed by Lehmann and Aichele [1931]. It should also be noted that many years ago the scutellum was suggested as an important functional part of grass seeds. Experimental evidence, however, has been lacking, except in some old, partly discredited, investigations on carbohydrases.

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EXPERIMENTAL.

Preparation of the enzymes. The wheat used was Manitoba hard red spring wheat kindly supplied by Saltsjöqvarn, Stockholm. The dormant seeds were dried *in vacuo* over H_2SO_4 for 24 hours. In germinated seeds the enzymic activity was determined directly on fresh tissue, but simultaneous moisture determinations were made, drying in the above manner. All results have been expressed in activity per mg. dry weight. Dissected tissue was kept moist in small test-tubes, containing moist cotton in the caps. Weighing on the micro-balance was done in a small, covered container, and the weighed tissue was put into a test-tube containing about 8 μ l. of 60 % glycerol.

Dissection was carried out under a tripod lens. The epicarp over the embryo was removed, and then insertion and gentle prying with a blunt scalpel under the embryo caused it to come out quite cleanly. The embryo was at once cut in two along an arbitrary line which may be considered as the boundary between the cotyledon and the part containing the three radicles. A whole piece after weighing was used for each test. The embryo of more than 36 hours' germination was too large to use in such a manner. Since the work of Linderstrøm-Lang and Holter [1932] on barley rootlets had indicated a localisation of the peptidase activity, it was considered inadvisable to try cutting up either the cotyledon or the radicles; therefore, no determination was made when they became too large to use in the above manner. Pieces of hull were cut off and cleaned from attendant, whitish, starch cells. A lateral section was made and pieces of endosperm prepared. To obtain the scutellum it was found most convenient to cut the seed along the same axis as the groove, after removing the embryo. The yellowish layer was then easily seen in each half and was removed free of hull and endosperm.

Enzymic activity was determined exactly as described by Linderstrøm-Lang and Holter, using a burette of the type in which the mercury directly touches the solution. The procedure involves the direct titration of amino-groups by acid, in the presence of acetone, using naphthyl red as an indicator; the principle was explained by Linderstrøm-Lang [1928].

RESULTS.

The effect of drying the germinated seeds before or after dissection was to increase the apparent enzymic activity, as shown in Table I. The activity determined on fresh tissue, but expressed on the basis of dry weight, was consistently

Table I. *Dipeptidase activity in different parts of germinated wheat seeds, determined on fresh and on dried material, showing maximum and minimum means obtained in different series, and thus errors resulting from drying.*

	μ l. N/20 HCl per mg.			
	Fresh tissue		Dried tissue	
	12 hours	24 hours	12 hours	24 hours
Hull	5.4 $\pm$ 0.7	5.4 $\pm$ 0.2	42.0 21.1	24.9 11.5
Endosperm	3.2 $\pm$ 1.0	3.6 $\pm$ 0.3	0.3 2.6	7.0 0.7

smaller. Furthermore, the variation on drying was great. While it is possible to get the proper results by this procedure, the variations between the means of different series were large. Owing to these results it was decided to work with

fresh tissue. It seems possible that the effect of drying is to liberate the enzymes from zymogens which are supposed to exist in the cell, and that this liberation is irregular owing to somewhat varying internal conditions.

p_H-activity curves. In common with most dipeptidases the optimum *p_H* of that in wheat was found to be about 7.5. About the same curve was obtained for each part of the wheat. The protease, similarly to many plant proteases, has an optimum about *p_H* 5.0, which is also the approximate isoelectric point of the substrate used, edestin. Table II shows the values found.

Table II. *Showing the activity of wheat dipeptidase and protease in relation to p_H.*

Activity is expressed as $\mu\text{l. of } N/20 \text{ HCl}$.													
p_{H}	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Protease	1.8	4.2	9.4	11.9	12.9	6.5	2.0	0.5	0	0	0	0	0
Dipeptidase	0	0	0	0	0	1.5	4.0	9.5	24.0	26.0	24.0	16.0	5.0

Time of extraction with glycerol. Whilst the best time varies a little with different parts of the seed and especially between dried and fresh tissues a standard time of 2 hours was adopted after various experiments. Table III shows typical results.

Table III. *Activity of dipeptidase in whole dormant wheat embryo, after extracting in glycerol for different lengths of time.*

Activity in $\mu\text{l. } N/20 \text{ HCl per mg.}$					
Time of extraction (min.)	20	40	60	120	240
Activity	6.0	8.0	9.0	11.0	11.3

Time of digestion with substrate. The time of 2 hours, at 40°, as used by Linderstrøm-Lang, was adopted. The digestion-time is always somewhat arbitrary, depending partly on considerations of initial velocity or equilibrium, and partly on the amount of hydrolysis required to give sufficient accuracy in the determination. Table IV shows the type of results obtained with the two enzymes.

Table IV. *The relation between the amount of hydrolysis of alanylglycine and of edestin by wheat embryos and the digestion time.*

$\mu\text{l. } N/20 \text{ HCl per mg.}$						
Time in hours	0.5	1	2	3	4	7
Dipeptidase	4.0	7.0	9.8	11.1	11.2	11.8
Protease	2.9	5.0	8.0	11.0	11.0	11.6

The relation between enzyme concentration and activity, as shown for both enzymes by using finely-cut pieces of dry cotyledon and varying the weight from 0.04 to 1.00 mg. was found to be linear. This fact justifies the conditions prevailing in this work, in which slightly different weights of tissue were used in each tube, and the titrations were corrected to a certain dry weight of tissue (1 mg.) for comparison.

Accuracy of the results. When the working conditions for the enzymes had been established the study of the relative activity in different parts of the seed was begun. Experiments with the five parts for any time of germination were made simultaneously, each being done in 6 to 8 tubes, and the whole series was repeated. The resulting averages are shown in Table V, Figs. 1 and 2. The

probable errors of the means are given in the Table, because these are rather higher than expected. The errors involved depend not only on the method of determining activity, but more especially on variations from one seed to another.

It should be noted that in every case of determining the activity in hull and endosperm at least one-third of the tubes showed no enzymic activity at all.

Table V. *Enzymic activity in different parts of the wheat seed during germination.*

Activity is expressed as $\mu\text{l. N/20 HCl}$ per 1 mg. dry weight; the probable errors of the means are given.

are given.		Condition of seed				
Part of seed	Dormant	Germinating				
		12 hours	24 hours	36 hours	48 hours	96 hours
		Dipeptidase activity.				
Hull	7.4 ± 0.7	5.4 ± 0.7	5.4 ± 0.2	8.6 ± 1.0	4.5 ± 0.6	—
Endosperm	2.6 ± 1.2	3.2 ± 1.0	3.6 ± 0.3	3.9 ± 1.1	2.6 ± 0.1	—
Scutellum	17.1 ± 1.0	39.0 ± 0.9	46.8 ± 5.6	69.3 ± 2.4	55.0 ± 1.0	6.3 ± 0.5
Cotyledon	11.3 ± 2.0	47.2 ± 3.8	37.3 ± 2.6	24.0 ± 1.0	17.8 ± 0.6	—
Radicles	20.7 ± 3.9	32.2 ± 4.3	29.3 ± 2.0	26.5 ± 1.5	—	—
Protease activity.						
Hull	12.9 ± 1.6	14.2 ± 0.7	13.7 ± 1.2	10.0 ± 1.3	14.7 ± 1.7	—
Endosperm	7.1 ± 1.0	4.6 ± 0.6	7.6 ± 1.1	3.6 ± 0.8	6.8 ± 0.4	—
Scutellum	17.8 ± 2.0	45.1 ± 2.6	61.0 ± 6.2	52.1 ± 3.2	41.3 ± 4.2	34.4 ± 2.2
Cotyledon	15.8 ± 1.3	31.2 ± 4.4	25.6 ± 1.0	17.8 ± 1.2	—	—
Radicles	11.8 ± 0.7	25.6 ± 1.4	22.1 ± 1.6	12.4 ± 0.8	—	—

Fig. 1. Dipeptidase activity of different parts of wheat seeds during germination.

Fig. 2. Protease activity of different parts of wheat seeds during germination.

The determination of protease activity by titrating amino-groups can be seriously criticised. Proteolysis probably begins with disaggregation of the protein molecule, a process ultimately producing polypeptides, which are hydrolysed by polypeptidase, giving titratable amino-groups. It is often possible to demonstrate the disappearance of precipitable protein before any change in titration has occurred. In the present work, however, some correlation was found, using a semi-quantitative turbidimetric procedure, involving the use of 20 % salicylsulphonic acid. Both procedures showed the same optimum p_{H} and the same relative activities for different times of germination.

DISCUSSION.

It can be observed from Table V and Figs. 1 and 2 (1) that the hull and endosperm contain a relatively small amount of enzyme and that the amount does not change in a regular and significant manner on germination; (2) that the amount of enzyme is greatly increased, after 12 hours of germination, in the scutellum and embryo, the increase being somewhat greater in the cotyledon than in the radicles; (3) that after 12 hours the enzyme content in the embryo decreases fairly rapidly, but that in the scutellum it continues to increase for a time, later (after 36 hours) clearly decreasing but still maintaining a fairly high level. Slight differences exist between the curves for dipeptidase and protease.

The sudden increase of enzymic activity in the first few hours of germination is a familiar phenomenon associated with the change of the embryo from a dormant to an active condition.

It is well known that in germination the starch and protein of the endosperm ultimately appear in the embryo, usually as sugars and amino-acids, there to be used for building the new materials required by the developing plant. It has generally been suggested that the starch and protein are hydrolysed in the endosperm and then transported to the embryo, and enzymes are commonly held to be responsible for these changes. While some theoretical discussions have taken place, *e.g.* as to whether the enzymes are in the hull and embryo and are secreted into the stored food, little real knowledge, so far as the wheat seed is concerned, has been obtained. It is usually considered that a high enzymic activity in a given tissue indicates a site of formation, either for secretion or for doing the enzymic work, or possibly both. These considerations have a bearing on the results of the present investigation.

Since both hull and endosperm sometimes showed no proteolytic activity, never showed much and showed no change on germination, it can be doubted if they are the active centres in the proteolytic changes of the wheat seed. Furthermore there was no indication that the scutellum secretes enzymes into the endosperm, since no increase occurred.

The decrease of activity in the embryo after 12 hours is rather surprising since requirements must be increasing, even admitting that the proteolytic enzymes may not be the most important. This decrease was roughly proportional to the increase in total weight of the embryo, but since the dry weight was nearly constant, it is perhaps only an accidental relationship.

It would seem that the really significant observation is the large and continued increase in activity of the enzymes of the scutellum. The importance of the scutellum has long been suggested by some old work on amylase [see Lehmann and Aichele, 1931] and can be inferred from the work on barley by Euler and Sjöman [1933], but its full place in relation to all parts of the seed is only now emphasised. It may be possible that the scutellum performs all the functions required by the changes known to occur in the seed at this time.

II. LIPASE.

Extraordinarily little work has been done on wheat lipases. It is, of course, generally thought that considerations of fat and fat hydrolysis in cereals are of minor importance. This conception has probably arisen from the low content of fat and the weak esterase activity, in comparison with *Ricinus*, for example.

Such a criterion is, however, not justified. It is probable that the fat in wheat, small though it is, and the lipase, weak though it is, can play a part in developing acidity as well as bad odours in stored flours. The present study can be of interest in this connection since it indicates the distribution of lipolytic activity in various parts of the seed.

From a physiological viewpoint the present study is of interest in demonstrating the changes of lipolytic activity on germination, though the significance of these changes is not obvious.

Apparently the only real study of wheat lipases is that by Sullivan and Howe [1933], working with ground wheat.

EXPERIMENTAL.

The wheat used, and the preparations made have been described in the preceding section. The method used was described by Glick [1934], employing methyl butyrate as substrate, glycine as buffer and involving an acid titration with the micro-apparatus of Linderstrøm-Lang. The method had to be modified owing to the weakness of the enzyme and the consequent small differences in titration. The modification consisted only in the use of $N/40$ HCl instead of $N/20$ for titration and doubling the quantity of indicator used. Even then the procedure was not wholly satisfactory, giving much less reproducible results than were obtained in the proteolytic studies.

An added difficulty was the rather high change in the titration value of the enzyme preparation on digestion without substrate. The correction was usually as large as the hydrolysis of substrate. This change may or may not be due to lipolytic hydrolysis of natural esters, since any change in the acidity of the tissue gives the same result, and there are various possibilities. The greatest corrections were found with hull and scutellum. A further difficulty is involved

Fig. 3. Lipase activity in different parts of wheat seeds during germination.

in trying to determine a p_H curve. The Glick method was of no value at a p_H lower than 7.5, partly because the initial titration is so low. In the present study therefore no optimum could be determined, a p_H of 8.2 being arbitrarily chosen in order to have suitable titration values. This is almost certainly not the optimum.

In spite of these difficulties some results were obtained which could be reproduced with fairly reassuring accuracy. These are presented in Fig. 3.

DISCUSSION.

(1) The very high esterase content of the scutellum, possibly associated with the high fat content of this part, is very striking, as is the sudden drop in activity on germination. (2) By far the greater part of the lipolytic activity of the embryo is in the cotyledon, and this increases on 12 hours' germination and then decreases sharply. (3) The endosperm contains a high amount of lipase, second only to, but much lower than, the scutellum, and this amount increases in the first 12 hours of germination, then gradually decreases. This is of interest in connection with the accidental use of sprouted wheat for flour-making. (4) The hull contains an appreciable amount of lipase, and no change occurs on germination.

SUMMARY.

1. A study has been made of the distribution of dipeptidase and protease in five parts of the dormant and germinating wheat seed, namely hull, endosperm, scutellum, cotyledon and radicle. Some conditions for the activity of the enzymes, relative to p_H , extraction, digestion time *etc.* have been determined.
2. The hull and endosperm sometimes showed no enzyme, and in any case only a very small amount which did not change significantly on germination.
3. The enzymes in the scutellum and embryo greatly increased in the first 12 hours of germination, after which they decreased in the embryo but continued to increase, for a time, in the scutellum.
4. The importance of the scutellum is thus emphasised, and the results are discussed in relation to popular conceptions concerning germination.
5. A micro-study has been made of the distribution of lipase activity in different parts of dormant and germinating wheat seeds. The results indicate high activity in the scutellum, decreasing on germination, whilst some parts show an increase in the first 12 hours and then a decrease.

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