THE HYDROLYTIC ENZYME—LIPASE.

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The first definite information concerning the existence in oleaginous seeds of an enzyme having the power of inducing the hydrolysis of oils, is due to Green[1]. The presence of such a substance had been suggested many years previously by Pelouze[2], and again, some years later, by Schützenberger[3], but it was Green who first gave conclusive proof of such an enzyme, by obtaining from the germinated castor-oil bean an extract capable of hydrolyzing oils. He likewise found that the non-germinated seed, if treated with dilute acetic acid for a time, gave an extract which was also lipolytic. His results are, for the most part, mostly qualitative, yet none the less conclusive that a hydrolytic enzyme was present in the extract.

About the same time that Green published his results, Sigmund[4] showed that when the resting rape, castor, poppy, hemp, pumpkin, maize and flax seeds are finely divided and treated with water or glycerol, an extract is obtained which, on the addition of alcohol, produces a precipitate. Sigmund found that this precipitate had hydrolytic power. There must, of necessity, be some doubt as to the quantitative value of Sigmund's experiments because he used no means of inhibiting bacterial growth.

This latter objection is not, however, applicable to the experiments of Sigmund which he published the following year.[5] In this paper, Sigmund gives results obtained with seeds in both a resting as well as a germinated condition. The resting seeds were ground with chloroform water and after twenty-four hours, the amount of acid present determined by titration. A determination of the amount of acid was made in a similar mixture as soon as prepared, the difference between the two results representing the acid formed by the lipolytic action of the lipase in the seed on the oil present, acting over a period of twenty-four hours. While the increase in the fatty acids was not marked, yet it was sufficient to warrant the conclusion that lipase was present in the seeds studied. Sigmund germinated rape, poppy and hemp seeds for two days and then dried

[1] *Proc. Roy. Soc.*, **48**, 370 (1890).

[2] *Compt. Rend.*, **40**, 605 (1855).

[3] "On Fermentation," 1876, International Scientific Series, p. 295.

[4] *Monatsh.*, **11**, 272 (1890).

[5] *Sitz. d. Kais. Akad. Wiss.* (Wien), **100**, 328 (1891).

them at a temperature of 35°. For his quantitative results, he used these dried seeds in the same fashion as the non-germinated ones, and found that the hydrolysis brought about by the germinated seeds is more intense than that developed by the resting ones. These results of Sigmund substantiate the suggestion made by Green with respect to the castor-oil bean, namely, that the lipase in resting seeds is present essentially as a zymogen, which gives rise to the enzyme during germination.

In 1898, Lumia[1] published some experimental results on the presence of lipase in germinated seeds, using for this purpose the castor-oil bean and the pumpkin seed (*Cucurbita pepo*). In the case of the castor-oil bean, the results were essentially the same as those obtained by Green, except that the intensity factor was more pronounced, for the lipase exerted its influence for a much longer period. At the expiration of six days, the increase in the fatty acids was determined, a total of 1.095 grams of acid (calculated as ricinoleic acid) being produced from 6 cc. of castor oil. From the results obtained with a similarly prepared extract of the germinated pumpkin seed, Lumia was not prepared to say whether the extract was capable of hydrolyzing oils or not, the increase in the fatty acids, even after seven days, being too trifling to warrant the conclusion that lipase was present. A few experiments were also made by Lumia with the resting seed of the cocoanut palm (*Cocos nucifera*) but only a very slight lipolytic effect was noted.

It is to Connstein, Hoyer and Wartenberg[2] that we owe most of our present knowledge of vegetable lipase and its activities. Their attention was confined to the resting castor-oil bean on which both Green and Sigmund had worked, and they found that under certain conditions, which they studied in detail, the zymogen in the resting seed could be converted into the active enzyme, and that, if sufficient time be given to the lipase in which to exercise its lipolytic action, the intensity of the hydrolysis is most marked, in some cases upward of 90 per cent. of the oil which had been worked into an emulsion with the seed being converted into free acid and glycerol. Among the many important results which were the outcome of this research, are two that deserve especial mention: first, that just as intense hydrolysis may be obtained with the resting seed as with the germinated; second, that Green's statement that free acid

[1] *Staz. sperim. agrar. ital.*, **31**, 397 (1898).

[2] *Ber.*, **35**, 3988 (1902).

hindered the hydrolysis is incorrect, but, on the contrary, it is necessary in order to obtain maximum hydrolysis.

Lipase has also been found in the jequirity seed (*Abrus precatorius*) by Braun and Behrend[1] but the conditions under which they worked were such that the seed showed no marked hydrolytic power.

Hoyer,[2] continuing the work of Connstein, Hoyer and Wartenberg, found it impossible to prepare a solution having notable hydrolytic power, from the resting castor-oil bean. Various attempts were made to isolate or concentrate the enzyme by mechanical means, but in this, Hoyer was likewise unsuccessful.

Fokin[3] has studied seeds of Russian origin to determine whether any of them had notable fat-splitting properties like the castor-oil bean. His examination included more than sixty different seeds from thirty different families, and he found but two that produced intense hydrolytic action. The seeds in question are the celandine (*Chelidonium majus*) and toadflax (*Linaria vulgaris*). Quite a number of seeds showed slight hydrolytic power (no experimental details are given with respect to these seeds), but none except these two were marked. Fokin's method of operation was akin to that of Connstein, Hoyer and Wartenberg.

Fokin has also made an extended study of the question of the decomposition of fats by enzymes[4] restricting himself, however, to a study of the lipase of the castor-oil bean. He has considerably extended our knowledge of the lipase of this bean and of the conditions under which it may operate to best advantage. It is to be noted that Fokin, like Hoyer, was unable to obtain a solution of the lipase which had any notable lipolytic power, such as is possessed by the bean itself.

Where Hoyer failed in working out a method of concentrating the enzyme in the castor-oil bean by mechanical means, Nicloux[5] has succeeded. Nicloux has shown how the cytoplasm of beans may be readily isolated mechanically, and proves that the hydrolytic power lies in the cytoplasm alone.

Walker and Bourne[6] also failed in an attempt to separate the lipase from the castor-oil bean and obtain a concentrated solution of

[1] *Ber.*, **36**, 1142, 1900 (1903).
[2] *Ibid.*, **37**, 1436 (1904).
[3] *Chem. Rev. u. Fett. u. Harz. Ind.*, **11**, 30, 48, 69 (1904).
[4] *Ibid.*, **11**, 91, 118, 139, 167, 193, 224, 244.
[5] *Compt. Rend.*, **138**, 1112, 1288, 1352 (1904); **139**, 143 (1904).
[6] *Tech. Quart.*, **17**, 284 (1904).

it. They found that germination lessens the intensity of the hydrolytic power of the bean.

Urbain, Saugon and Feige,[1] in a report of some experiments on the saponification of copra by means of the cytoplasm of the castor-oil bean, have brought out one point of especial interest, namely, that contrary to the statement of Connstein, Hoyer and Wartenberg they find that all glycerides are saponified with equal facility by the lipase of this bean. C., H., and W. found that the intensity of the hydrolysis of glycerides of fatty acids of low molecular weight was less than of those of high molecular weight, hence they assumed that all glycerides were not hydrolyzed with equal ease. The reason for this does not lie in the fact that the glycerides of fatty acids of low molecular weight are less easily saponified, but that one of the products of the hydrolysis, namely, the acid, as soon as it reaches a certain concentration, inhibits or destroys the activity of the lipase. This point is brought out very clearly with butyric acid, the authors showing that when the emulsion contains 10 per cent. of this acid, the hydrolysis is altogether stopped.

It is now generally assumed that during the germination of oleaginous seeds, a lipolytic enzyme is formed which hydrolyzes the oil present as a preliminary step in the utilization of this reserve food-stuff for the nourishment of the growing embryo. While this assumption may be correct, yet it rests on altogether too slender experimental grounds to warrant its acceptance as a principle applicable to all oleaginous seeds. The work of Green, Sigmund Lumia, Connstein, Hoyer and Wartenberg, as well as of Walker and Bourne, relates to but five germinated seeds. Of one of these five, namely, the castor-oil bean, C., H., and W. state that the germinated seed is no more active than the resting seed, while Walker and Bourne say that germination lessens its activity. However this may be, it is apparent that in the case of this bean, germination is unnecessary to demonstrate the presence of lipase and that it is capable of producing intense hydrolysis. With the germinated pumpkin seed, Lumia obtained entirely negative results. There remain then but the three seeds, on which Sigmund experimented, which may be used as a basis for the acceptance of the principle that lipase is produced during germination. It was necessary for Sigmund to show that under the same set of conditions the non-

[1] *Bull. Soc. Chim.* (3), **31**, 1194 (1904).

germinated seeds were less active; this he has done and the point appears to be proven.

Yet, since our knowledge of the lipase of the castor-oil bean has been so much extended, and we know the conditions most favorable for its activities, it is desirable to try other resting and germinated seeds under conditions which we now know are favorable for lipase to exercise its specific functions. Then if we obtain marked divergence in the intensity factor of the hydrolysis, it points still more clearly to the formation of lipase during germination. It has long been known that during germination, oleaginous seeds increase markedly in their acid content. If lipase is produced during germination, it then becomes evident what the function of this lipase is.

In the experimental part of this paper, we have studied four seeds in a resting state and two of them in a germinated condition. The resting flax, peanut, croton and almond seeds are not hydrolytic, or only so to a very slight extent. Neither is the zymogen of these seeds converted into an active enzyme by means which activate the castor-oil bean, celandine and toadflax. The experiments were carried over a sufficient period of time so that the time factor, so important in the case of the castor-oil bean, could be made use of, if such were possible. Failure to activate by these means, points conclusively to the fact that the zymogen of these seeds differs from those of the three seeds mentioned above.

Comparison of results obtained with the germinated flax and peanut, point unquestionably to the production of lipase during germination. A prolongation of the period over which the lipase could induce hydrolysis, showed that the hydrolysis augmented day by day, as would be expected, were lipase present. That hydrolysis was not due to bacterial action, was assured by the use of chloral, which Connstein, Hoyer and Wartenberg showed to be quite efficient for this kind of experimentation.

We have been able to prepare quite active solutions of lipase. In spite of numerous attempts, this has not been accomplished by those who have worked with the castor-oil bean. It is probable that not only the zymogen, but also the lipase of the bean differs from the lipase of the germinated flax and peanut.

The active solutions obtained from both the flax and peanut, produce a precipitate when treated with alcohol. The lipase, in the case of the peanut, is precipitated (doubtless along with much albuminoid matter), for the precipitate thus obtained shows marked

lipolytic power. Solutions of the flax lipase produce a precipitate when treated with alcohol, but this precipitate shows no hydrolytic activity. It is therefore evident that the lipase of the peanut differs from that of the flax.

EXPERIMENTAL WORK.

RESTING SEEDS.

The following seeds were used in this part of the investigation: 1, peanut (*Arachis hypogaea*); 2, flax (*Linum usitatissimum*); 3, almond (*Prunus amygdalus*, var. *dulcis*); 4, croton (*Croton tiglium*).

At the outset of this investigation, it was necessary to determine what substance could be most expeditiously used in order to inhibit bacterial growth in the emulsions. A number of trials were made with toluene, but this was found useless for this type of work, although seemingly satisfactory in Buchner's zymase investigations[1]. Chloroform and chloral were investigated, and while the former was satisfactory in the majority of trials made, yet in a few, the emulsions were not prevented from souring. Chloral proved satisfactory in all cases and it was consequently adopted. We owe to Connstein, Hoyer and Wartenberg's work the suggestion as to the use of chloral.

The general method of procedure was as follows: The resting seeds were thoroughly ground until a mass of more or less buttery consistency was obtained.[2] This mass was then thoroughly mixed with olive oil; water (or dilute acid of known strength) added until a perfect emulsion was obtained, the whole emulsion being made as thoroughly homogeneous as possible. The chloral was incorporated with the water before addition. The exact amount of each ingredient was determined as added. In a few of the experiments, the ground seed was extracted with ether before use.

The resulting emulsion was then divided among a number of Erlenmeyer flasks (50 cc.), the weights of which had previously been accurately determined. By reweighing the flasks and their contents, we could then readily calculate the amount of the various constituents. These flasks were stoppered and placed in an incubator and kept at a constant temperature of 30°. The acid value of one of the samples was always determined at once, the others at varying intervals of twenty-four to forty-eight hours, in order to determine the progress of the hydrolysis.

[1] "Die Zymasegärung," p. 176.

[2] In all these cases, the seeds (except the flaxseed) were freed from their outer shell before using.

EXPERIMENT 1.

3.8 grams of the peanut butter, previously extracted with ether; 37.4 grams of oil; 10.2 grams of water; 0.4 gram chloral.

At once.	One day.	Two days.	Four days.	Six days.
3.5	5.5	3.7	3.9	4.5[1]

This shows very clearly that no hydrolytic enzyme exists as such in the resting peanut. The retrogression in the acid value following, in this case, the first day, has been noted in a number of cases. We are as yet unable to explain this retrogression. One explanation is possible, but we hardly think probable, namely, that our emulsions were not homogeneous. Great care was used in all cases to obtain perfectly homogeneous emulsions by prolonged trituration. Experiments 3 and 4, for example, give some idea of how the acid value increased very slowly from one determination to another without any retrogression or, in fact, any very *marked* increase, yet what increase there was, was fairly regular, pointing to the fact that the emulsions were homogeneous.

EXPERIMENT 2.

4.1 grams of peanut (same as in Expt. 1); 32.5 grams of oil; 12.1 grams of 0.4 per cent. sulphuric acid;[2] 0.35 gram chloral.

At once.	One day.	Three days.	Four days.	Five days.	Seven days.
4.1	5.6	4.8	6.2	7.3	7.5

The presence of mineral acid has not the power of activating the zymogen of the resting peanut. The most that can be said is that in the presence of mineral acids, the hydrolysis is but feeble.

This same experiment was repeated with peanuts that had not been extracted with ether, and, in the presence of small amounts of mineral acids, the results were of the same order as those just given, showing that the inactivity did not lie in any cause produced by the ether extraction.

EXPERIMENT 3.

7.5 grams of flaxseed; 32.4 grams of oil; 15 grams of water; 20.0 grams of 0.4 per cent. sulphuric acid; 0.35 gram of chloral.

At once.	One day.	Three days.	Five days.	Seven days.
2.9	4.3	7.1	8.0	9.8

[1] The acid value in all cases, unless otherwise stated, is based on the per cent. of oil in the sample, and represents the number of milligrams of potassium hydroxide necessary to neutralize the acids in 1 gram of the oil. All due corrections were made when mineral acids were present.

[2] Slightly weaker than $N/_{10}$ H_2SO_4. Connstein, Hoyer and Wartenberg found that the most intense action was produced by acid varying between $N/_{10}$ and $N/_8$.

As in the case of the peanut (Expt. 2) the flaxseed shows only a slight hydrolytic power. That even this slight hydrolytic power is not in any way due to the presence of the mineral acid, is shown by the following experiment, in which the addition of the mineral acid was omitted.

EXPERIMENT 4.

6.5 grams of flaxseed; 26.7 grams of oil; 31.4 grams of water; 0.36 gram of chloral.

At once.	One day.	Two days.	Four days.	Six days.
4.0	6.3	6.3	8.2	9.0

The higher acid value shown on immediate titration, is due to the fact that it was a different sample of oil from that used in the preceding experiment. As in Expt. 3, the results show that the flaxseed has a slight hydrolytic power, but by no means pronounced. Another experiment in which the total amount of mineral acid (calculated in per cent. of the emulsion) was only one-third that of Expt. 3, showed results comparable with that of Expts. 3 and 4.

EXPERIMENT 5.

7.8 grams of croton seeds; 40.8 grams of oil; 15.0 grams of 0.4 per cent. sulphuric acid; 0.43 gram of chloral.

At once.	One day.	Three days.	Five days.	Seven days.
7.1	8.3	9.8	10.6	11.2

EXPERIMENT 6.

9.5 grams of almonds; 28.2 grams of oil; 19.5 grams of 0.4 per cent. sulphuric acid; 0.42 gram of chloral.

At once.	Two days.	Four days.	Five days.
6.3	6.4	7.3	10.0

Experiments 5 and 6 show that the croton and almond seeds, like those of the peanut and flax, contain a zymogen which is but slightly, if at all, activated by mineral acids.

GERMINATED SEEDS.

The peanut and flaxseed were the only seeds used in these experiments. In germinating the peanut, they were first soaked in water for twenty-four hours and then planted in white pine sawdust which was kept moist. After germination for about a week at 30°, the hypocotyledonary portion was separated and rejected. The remainder of the seed was freed from external moisture, thoroughly

pulped, and in this condition used as in the experiments with the non-germinated seeds[1].

Of a large number of experiments carried out with the germinated peanut, only two need be given.

EXPERIMENT 7.

19.4 grams of germinated peanut; 33.1 grams of oil; 8.9 grams of 0.4 per cent. sulphuric acid; 0.35 gram of chloral.

At once.	One day.	Three days.	Four days.	Six days.
6.4	10.5	13.0	14.8	29.3

This experiment shows very clearly the formation of lipase during germination. The hydrolysis steadily increased up to the fourth day, and during the next forty-eight hours, the acid value increased to about twice the value obtained on the fourth day. This marked increase is akin to the "Sprung" of Connstein, Hoyer and Wartenberg, but in degree, is not so intense.

EXPERIMENT 8.

Another sample of germinated peanut, gave the following results.

9.4 grams of germinated peanut; 30.6 grams of oil; 10.0 grams of 0.1 per cent. sulphuric acid; 0.31 gram of chloral.

At once.	One day.	Two days.	Four days.	Six days.
4.9	8.9	11.3	17.0	34.8

These results are quite similar to those of Expt. 7, except that the total amount of mineral acid was considerably reduced, without any marked effect on the general trend of the results. In this experiment, the total amount of germinated peanut used was (based on its per cent. of the emulsion) a little over half that of the previous experiment.

When we compare the results of the last two experiments with those obtained with the resting peanut, it at once becomes evident that lipase is produced during the process of germination.

A few experiments were made to concentrate the lipase of the germinated peanut. The germinated seeds were very thoroughly ground with clean quartz sand and a very small amount of water.

[1] In calculating the acid value, based on the oil in the sample, the results are necessarily slightly in error due to the free fatty acids developed during germination. This error is, however, constant throughout any experiment. The same is true of the results in Experiments 3, 4, 5 and 6, due to small amounts of free fatty acids in the non-germinated seeds.

This grinding with sand was done to break up the cells of the germinated seed. The resulting mass was then subjected to a pressure of about 250 atmospheres, the press liquor obtained being of a grayish color and slightly turbid. This press liquor was poured into absolute alcohol and the precipitate thus obtained was filtered, washed thoroughly with ether and then dried *in vacuo*. The final product was grayish in color but became somewhat darker on standing.[1]

The following experiments show that the lipase had been extracted from the germinated seed and that the precipitated product was very active.

EXPERIMENT 9.

3.0 grams of the precipitate; 30.7 grams of oil; 12.3 grams of water; 0.41 gram of chloral.

At once.	Two days.	Three days.	Five days.	Seven days.
8.9	30.9	37.4	45.7	53.2

EXPERIMENT 10.

2.7 grams of precipitate; 32.5 grams of oil; 10.0 grams of 0.4 per cent. sulphuric acid; 0.41 gram of chloral.

At once.	One day.	Two days.	Four days.	Six days.
7.1	25.2	39.4	50.9	60.3

In Expts. 9 and 10, the precipitated product used formed respectively but 6.5 and 5.9 per cent. of the total emulsion; *i. e.*, the hydrolysis was brought about by a comparatively small amount of the precipitated product, showing clearly that the lipase had been concentrated.

A number of experiments were also carried out with the press liquor itself, but it is unnecessary to give the figures obtained. We found that gum acacia was necessary in these experiments to obtain a permanent emulsion. All the experiments with the press liquor showed that this liquor contained lipase, and that it was quite active in hydrolyzing oils.

Germinated Flaxseed.—The germinated flaxseed used in these experiments, was prepared as follows: The seed was placed between layers of moist filter-paper and kept at 30°, the papers being moistened from time to time. In twenty-four to forty-eight hours, the seed had germinated, in some cases, the hypocotyledonary portion being nearly a centimeter long. These seeds were then thoroughly macerated and the resulting paste used as in the preceding experi-

[1] This method is similar to the method employed by E. Buchner in extracting zymase from the yeast cell.

ments. While a large number of experiments under varying conditions and with varying amounts were carried out, yet it will be unnecessary to give more than one experiment in order to show that, as in the case of the peanut, lipase is produced during the germination of the flaxseed.

EXPERIMENT 11.

14.2 grams of germinated flaxseed; 23.3 grams of oil; 18.8 grams of water; 8.0 grams of 0.4 per cent. sulphuric acid; 0.36 gram of chloral.

At once.	One day.	Two days.	Four days.	Seven days.
6.7	24.5	34.7	52.5	74.4

If germinated flaxseed is ground with sand in order to disrupt the cells, and then treated with a small amount of 5 per cent. salt solution, the resulting mass being afterwards subjected to 250 atmospheres pressure, a press liquor is obtained which is grayish in color, turbid and viscous. When this liquor is used to induce hydrolysis, gum acacia is necessary in order to obtain a permanent emulsion. As was expected, this liquor was found capable of hydrolyzing oils.

A number of attempts were made to precipitate the lipase from this press liquor after the fashion used in precipitating the peanut press liquor. A bulky, stringy, slightly grayish precipitate was always obtained, which, on standing, became much darker in color. We found that when prepared in this way, the precipitate showed practically no hydrolytic power, the press liquor from which it had been obtained having been, however, active. In the production of an emulsion with this product, a large amount of water seemed to be necessary.

It was found impossible in this case to determine whether the lipase in the press liquor from the germinated flaxseed was water-soluble or not, for the press liquor was always so viscous as to render it impossible of filtration even after dilution, so that no satisfactory aqueous solution quite free from turbidity could be obtained to work with.

It is certain, however, that the active lipase in the turbid and viscous press liquor, if precipitated by the alcohol, is in such a form as to be no longer capable of inducing hydrolysis. In preparing the press liquor, varying methods were used; for example, in one case, the germinated seed was ground alone with sand and then pressed; in another, small amounts of water were used in addi-

tion to the sand; again, water, sand and Fuller's earth—but all of these gave inactive precipitates. Only one experiment need be given to show the type of results obtained.

EXPERIMENT 12.

7.0 grams of precipitate; 33.9 grams of oil; 29.9 grams of 0.4 per cent. sulphuric acid; 0.35 gram of chloral.

At once.	One day.	Three days.	Five days.	Seven days.
0.8	1.4	2.7	3.9	3.1

In conclusion, we wish to return to the question of the retrogression in the acid value, several examples of which occur in the experiments cited in this paper. We have met with this phenomenon a number of times, and the following experiment, which we have selected especially for this purpose, shows this admirably.

EXPERIMENT 13.

	At once.	Two days.	Four days.	Five days.	Six days.	Seven days.
Mineral acid present in terms of N H_2SO_4	0.41	0.33	0.37	0.38	0.48	0.38
Fatty acid in the oil in terms of N acid	0.21	0.17	0.19	0.19	0.24	0.19
Total acid	0.62	0.50	0.56	0.57	0.72	0.57
N KOH to neutralize the sample	0.45	0.49	0.45	0.49	0.69	1.38

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MECONIC ACID IN THE U. S. P. 1890 ASSAY OF OPIUM AND CERTAIN MECONATES.

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THE work outlined in the following pages has to do with the identification and properties of an impurity which usually contaminates, to a greater or less degree, the precipitated morphine which is obtained as the "form" to be weighed in the present (1890) U. S. P. method for the assay of opium. Incidentally, some of the salts of meconic acid with barium and calcium were prepared and examined. It should be stated at the outset that these salts seem to have a somewhat variable composition and offer