NOTES ON METABOLIC CHANGES IN THE GERMINATION OF SEEDS

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Statement of the Problem.

For the study of physiological processes seeds are a favorite object. This choice is not an arbitrary one, for the latency of the various processes in dormant seeds represents a convenient base to which the various processes, which accompany the first stages of germination, may be referred.

The most evident physiological phenomenon during germination is *respiration*, which expression is used here in the broadest sense and may be defined as the *clastic* (as opposed to the hydrolytic) changes, both aerobic and anaerobic in reserve foods or (and) assimilates (K l u y v e r 79).

In the earliest stages of germination the substrate consists, of course, entirely of reserve food.

As the last link in the chain of clastic breakdown is concomittant, at least in higher plants, with the intake of oxygen, one of the most obvious criteria for the estimation of metabolism should be the rate of oxygen-intake. Inasmuch a CO_2 production might also take place under reduced oxygen-pressures, and might even continue in an environment deprived of oxygen, oxygen-intake as a function of oxygen-pressure might yield valuable informations.

The first part of these experiments are, therefore, concerned with the oxygen-intake at various oxygen-pressures.

As the peculiarities observed may be partly due to the substrate, both oily and starchy seeds were used in these experiments (Chapter I).

The behaviour of mustard-seed *(Brassica nigra Koch)* showed many interesting features and this seed was, therefore, used in most of our further experiments.

In the first place the behaviour of one of the enzymes which are supposed to play a part in the process of respiration, *catalase*, was investigated and its activity determined at various stages of germination. The characteristic features of this reaction were compared with various mathematical expressions representing supposed chemical mechanisms (Chapter II).

Inasmuch as catalase may be considered as a mild de-oxygenating agent, the activity of the antagonistic principle, *dehydrogenase*, in the broadest sense, was tested by means of qualitative tests with methylene-blue. The latter tests were applied to both oily and starchy seeds in various stages of germination (Chapter III).

In the classical reserve foods, carbohydrates, fats and proteins, the latter substance is used sparingly and apparently only in

starvation („protein-economy"). In this paper the protein-metabolism is not considered, although it is realized that „common opinion" is a dangerous guide. In normal conditions and in the early stages of germination reported upon in this paper, it is assumed that the protein-breakdown plays a subordinate, and possibly negligeable, role in respiration (vide Maquenne 94, Kostytschew 81, Stiles 136).

Changes in carbohydrates and oils were considered more closely. Carbohydrate metabolism, although it presents many obscure points, is known to consist of a hydrolytic and a clastic stage. The clastic stage is made up of a series of hydrogen transferences (anaerobic phase) and terminates, at least in higher plants, with the acceptance of atmospheric oxygen. The process in higher plants seems to be comparable in many respects to that in yeasts and bacteria, inasmuch as it involves a hydrolytic change into monoses, which are changed into phosphoric esters before the clastic process may be initiated. In the clastic processes, reducing substances (e.g. acetaldehyde) are formed in higher plants as well as in microbes. The general scheme of this clastic breakdown is now almost universally accepted (Kluyver 79).

No general guiding schemes exist for fat metabolism. Mothes (104) states, not without reason; „Der Fettstoffwechsel der Pflanzen ist ein stark vernachlässigtes Gebiet".

Since the early work of Hellriegel (62) and Sachs (122) it is generally conceded that during germination the reserve fat is changed into carbohydrates, which subsequently serve as respiratory substrate. This point, however, has not been proved beyond doubt. Equally unsettled is the course of the fat dissimilation. A study of fat metabolism, therefore, involves carbohydrates as well and in Chapter IV mustard-seed has been tested, after a *preliminary microchemical study*, on the *presence of fermentable sugars* in subsequent germination stages.

The same germination stages were used for the *study of changes in the content and the composition of the oil.* Special care was taken to choose characteristics as might be important in relation to oxygen-intake (Chapter V).

Higher plants are so complicated that conclusive results can hardly be expected.

Still the influence of the substrate is unmistakable and future experiments as to the further characterisation of the substrate seem highly desirable *(Conclusions).*

CHAPTER I.

Oxygen-intake of Oily and Starchy Seeds.

§ 1. *Introductory Remarks.*

The following investigation was prompted by the work of Dolk and van Slogteren (25) on the respiration of flower bulbs and by the results obtained by de Boer (10) on the respiration of Phycomyces Blakesleeanus. The former authors found that the oxygen-intake of hyacinth-bulbs, measured as a function of oxygen-tension, remained constant as the air contained from 21—14% O_2, while at lower tensions the oxygen consumption decreased steadily. Phycomyces proved to be able to consume oxygen of low tension when cultivated on a oil-medium, while on a starchy medium this behaviour could not be observed.

The aim of our work has been to investigate oxygen consumption of germinating oily and starchy seed as a function of oxygen-tension.

§ 2. *Review of the Literature.*

The literature on this subject is scanty. Already de Saussure (123) mentions that most seeds do not show decrease in the rate of oxygen-intake when brought at ½ of the normal pressure (± 10% O_2).

Godlewski (44) used an apparatus which may be considered a forerunner of a similar one used by Dolk (25). He measured volumetrically the oxygen-intake by determination of the rise of mercury in a calibrated tube connected with the respiration-vessel, while the CO_2 secreted by the seeds was absorbed by a KOH-solution. The seeds of *Raphanus, Cannabis, Linum* and *Medicago* were found to have a decreased oxygen-intake when the O_2-pressure was less than 10% of the atmospheric pressure. Six days old plants of *Raphanus* had a decreased oxygen-intake when the O_2-pressure was less than 20% of the atmospheric pressure. Buds of the *poppy* were able to absorb all the oxygen available.

Elaborate work was done by Stich (134) on the seeds of *Brassica, Helianthus, Pisum* and *Triticum.* This author found that only at tensions as low as 3—4% O_2 the oxygen-intake diminished, while no difference in the behaviour of oily and starchy seeds could be observed. Inasmuch as his respiration-vessel was quite large (800 cc) and, moreover, the oxygen was determined by macromethods, two rather large sources of error were introduced. Puriewitsch (119) later substantiated Stich's conclusions.

Shoup (128) found that the oxygen-deficiency of luminous

bacteria became noticeable only at 3% O_2, while Warburg and Kubowitz (145), measuring the oxygen-consumption of an unclassified bacterium, found a decrease of 50% at as low a tension as 2×10^{-5} mm Hg at 10° C.

As appears from the above most authors found a diminution of oxygen-intake at very low oxygen-tensions. Only Dolk and van Slogteren come to a different conclusion.

§ 3. *Material and Methods.* [1])

The oxygen-intake was determined volumetrically in a modified Krogh-respirometer, constructed by Mr. H. J. Kersten (fig. 1).

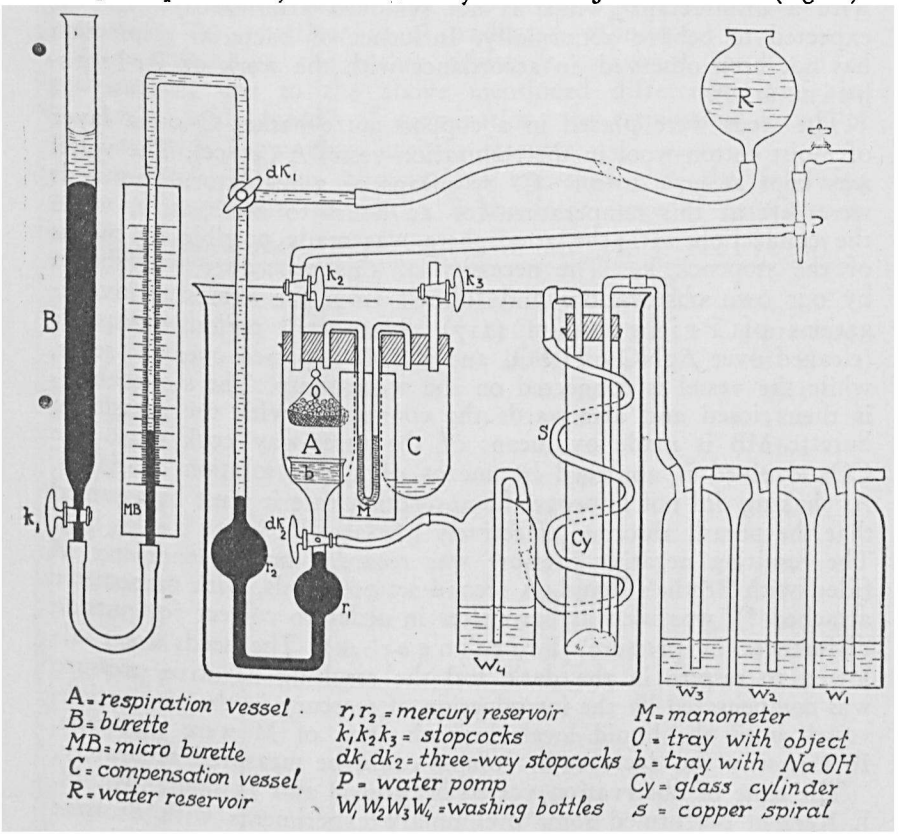


Fig. 1.

[1]) I am much indebted to Mr. A. J. Stuivenberg, master mechanic of the Botanical Laboratory, for the construction of the apparatus and for his valuable assistance and aid in its manipulation.

This instrument is essentially a reduced edition of the instrument used by Dolk. The seeds were placed on moist filter paper in Petri dishes in the dark at 30° C. and brought to a definite stage of germination. Fungous infection was checked by means of dilute Bromine-water, with which the seeds were shaken for some time after which they were washed in tapwater. Preliminary experiments showed that this treatment did not affect germination in any respect.

The experiments were not carried out under sterile conditions because of very great difficulties in technique, while seeds treated with a disinfectant (which is not removed afterwards) might be expected to behave abnormally. Influence of bacterial respiration has not been observed, in accordance with the work of Pringsheim (116).

The seeds were placed in a copper gauze basket O on a layer of moist cotton-wool in the respiration-vessel A (45 cc). The vessel was kept at 30° ± 0.1° C. by means of a regulator. The seeds were left at this temperature for 12 hours to acclimatize, while the connection with the atmosphere was made possible by means of the stopcock k_2. The necessity of this procedure was shown by our own observations and follows from the extensive investigations of Pringsheim (117). After this period moist air (cleaned over $AgNO_3$, H_2SO_4 and KOH) is passed over the seeds, while the vessel is connected on the waterpump. The stopcock k_2 is then closed and afterwards the connection with the measuring burette MB is made by means of the three-way cock dk_2. The CO_2 evolved is absorbed by means of a 5% solution of KOH. As shaking did not improve the absorption rate it must be assumed that the potash absorbed effectively (see also Pringsheim l.c.). The resulting negative pressure was recorded on a manometer M filled with Brodie's liquid. A second set of vessels, with manometer attached *), was used as barometer in order to correct for outside differences in pressure (Frietinger 41). The seeds were allowed to respire in the dark and the resulting negative pressure was compensated by the introduction of mercury in the respiration-vessel, until the liquid levels in both arms of M were the same. In this way the decrease in volume could be measured accurately.

The time of observation varied between 8 and 12 hours. Mr. H. J. Kersten performed some preliminary experiments with *Brassica rapa*, while we worked with *Brassica nigra*, *Linum usitatissimum* (oily seeds) and *Triticum spelta*, *Fagopyrum esculentum* (starchy seeds).

*) not shown in the drawing.

The number of seeds used varied with their size. The experiments were carried out with seeds in two stages of germination. In the first stage the seeds were placed in the vessel when the young root had just emerged; on the second stage the roots were 1—3 cm long. The root length does not take into account the differences of development of mono- and dicotyledonous seeds, e.g. in Triticum the lateral roots replace the main root at an early stage of germination. Oxygen-intake as a function of oxygen-pressure was determined in both stages. Killed seeds did not show an appreciable effect. The vessel was calibrated by weighing with water. The volume of basket, seeds and KOH-vessel was determined by measuring the overflow in a graduated cylinder.

Notwithstanding the rather arbitrary choice of the stage of germination, due to the above mentioned differences, but also to the specific „speed" of germination, the respiration curves of various seeds show characteristic features.

The results of our observations are shown in the following tables. A represents the early, B the later stage of germination.

1. *Brassica Rapa* (observations of Mr. H. J. Kersten)

A		B	
cc O_2 absorbed in ½ hour	O_2-pressure in %	cc O_2 absorbed in ¼ hour	O_2-pressure in %
0.410	18.2	0.450	19.5
0.490	16.9	0.420	18.0
0.455	15.5	0.440	16.4
0.380	14.1	0.440	14.8
0.455	13.0	0.460	13.2
0.405	11.8	0.410	11.8
0.500	10.0	0.435	10.3
0.450	8.9	0.395	8.8
0.355	8.0	0.365	7.6
0.400	6.6	0.330	6.4
0.455	5.0	0.275	5.4
0.405	3.9	0.230	4.6
0.375	3.0	0.210	3.8
0.375	1.6	0.175	3.2
0.385	0.7	0.160	2.7
0.150	0.0	0.150	2.2
0.000	0.0	0.100	1.8
		0.100	1.5
		0.060	1.2
		0.040	1.0

2. *Brassica nigra*

A		B	
cc O_2 absorbed in ½ hour	O_2-pressure in %	cc O_2 absorbed in ½ hour	O_2-pressure in %
0.229	20.2	0.439	19.8
0.216	19.5	0.412	18.6
0.250	18.6	0.441	17.3
0.223	17.8	0.413	16.1
0.204	17.0	0.371	14.9
0.199	16.3	0.325	13.9
0.197	15.5	0.355	12.8
0.196	14.7	0.309	11.7
0.185	14.0	0.362	10.5
0.177	13.3	0.277	9.5
0.185	12.5	0.284	8.5
0.205	11.6	0.245	7.6
0.195	10.8	0.262	6.6
0.234	9.8	0.222	5.7
0.216	8.8	0.272	4.7
0.230	7.8	0.198	3.9
0.203	6.8	0.255	2.9
0.189	5.9	0.129	2.3
0.175	5.1	0.176	1.6
0.202	4.1	0.144	1.0
0.255	2.8		
0.148	2.0		
0.144	1.2		
0.128	0.6		

3. *Linum usitatissimum*

A		B	
cc O_2 absorbed in ½ hour	O_2-pressure in %	cc O_2 absorbed in ½ hour	O_2-pressure in %
0.608	18.9	0.206	20.2
0.496	17.1	0.170	19.6
0.659	14.8	0.214	18.8
0.435	13.3	0.185	18.2
0.536	11.4	0.214	17.4
0.464	9.7	0.194	16.7
0.375	8.4	0.200	16.0
0.382	7.1	0.196	15.3
0.376	5.8	0.217	14.5
0.175	5.1	0.195	13.8
0.206	4.4	0.218	13.0
0.223	3.3	0.188	12.3
0.225	2.8	0.197	11.5
0.055	2.6	0.209	10.8
0.131	2.2	0.225	10.0
0.089	1.9	0.224	9.1
		0.189	8.5
		0.196	7.8
		0.204	7.0
		0.204	6.3
		0.195	5.6
		0.194	4.9
		0.174	4.3
		0.134	3.8
		0.158	3.2
		0.175	2.6

4. *Triticum Spelta*

B		A	
cc O_2 absorbed in ½ hour	O_2-pressure in %	cc O_2 absorbed in ½ hour	O_2-pressure in %
0.275	20.0	0.321	19.7
0.262	19.0	0.309	18.5
0.232	18.2	0.240	17.6
0.262	17.2	0.297	16.4
0.252	16.3	0.347	15.1
0.232	15.5	0.239	14.1
0.235	14.6	0.309	12.9
0.208	13.8	0.288	11.8
0.246	12.9	0.272	10.7
0.254	12.0	0.269	9.6
0.179	11.4	0.290	8.5
0.147	10.8	0.227	7.6
0.190	10.1	0.215	6.8
0.082	9.8	0.227	5.9
0.106	9.4	0.214	5.0
0.155	8.9	0.214	4.2
0.066	8.6	0.159	3.6
0.00	8.6	0.176	2.9
0.129	8.2	0.180	2.2
0.009	8.1	0.227	1.3
		0.077	1.0

5. *Fagopyrum esculentum*

A		B	
cc O_2 absorbed in ½ hour	O_2-pressure in %	cc O_2 absorbed in ½ hour	O_2-pressure in %
0.222	20.2	0.257	20.0
0.212	19.4	0.321	18.8
0.192	18.7	0.320	17.6
0.248	17.8	0.318	16.4
0.217	17.0	0.341	15.1
0.188	16.3	0.370	13.7
0.178	15.6	0.309	12.5
0.183	15.0	0.378	11.0
0.197	14.2	0.332	9.8
0.221	13.4	0.354	8.4
0.190	12.7	0.326	7.2
0.180	12.1	0.317	6.0
0.204	11.3	0.319	4.8
0.168	10.7	0.279	3.7
0.187	10.0	0.273	2.7
0.235	9.1	0.238	1.8
0.203	8.4	0.213	1.0
0.224	7.6	0.177	0.3
0.180	6.9	0.105	0.0
0.224	6.1		
0.195	5.4		
0.205	4.6		
0.247	3.4		
0.235	2.5		
0.219	2.0		
0.165	1.4		
0.145	0.9		

In the figures 2—6 the ordinates represent the amount of O_2 absorbed per unit time, while the abscissae represent the oxygen-pressure. The full line gives the respiration characteristic of stage A, while the broken line represents the germination in the later stage B.

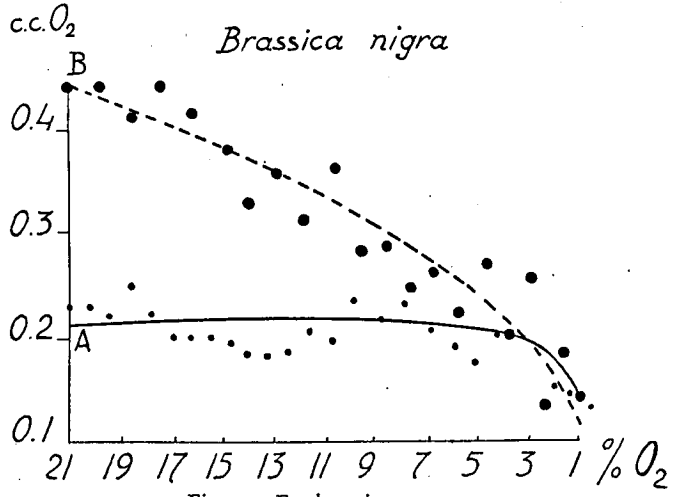


Fig. 2. Explanation see text.

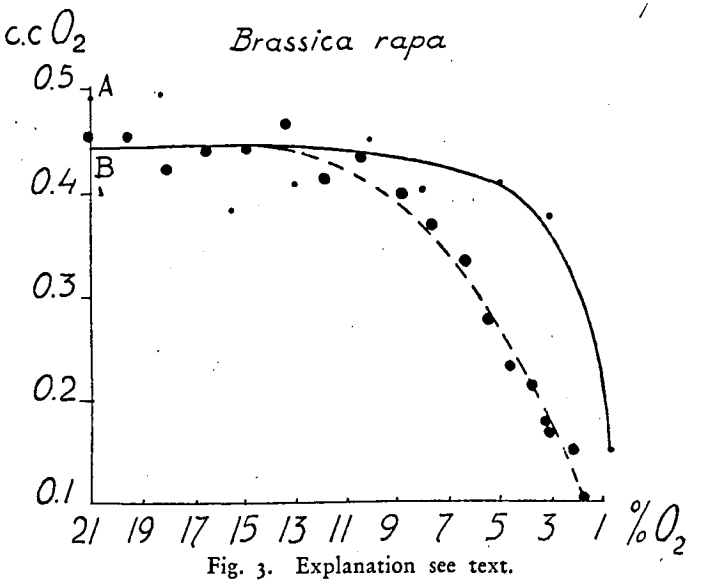


Fig. 3. Explanation see text.

§ 4. *Discussion.*

It appears from the figures that:

1. Both species of Brassica (fig. 2, 3) possess the ability, at an early stage of germination, to absorb oxygen at a normal rate at low oxygen-pressures (3% O_2). At lower O_2-tension the oxygen-intake decreases gradually and finally stops. At a later stage of germination respiration is materially retarded at much higher oxygen-tensions (for Brassica Rapa at 13% O_2, for Brassica nigra at 17% O_2).

2. Linseed shows, in the first stage of germination, a retarded

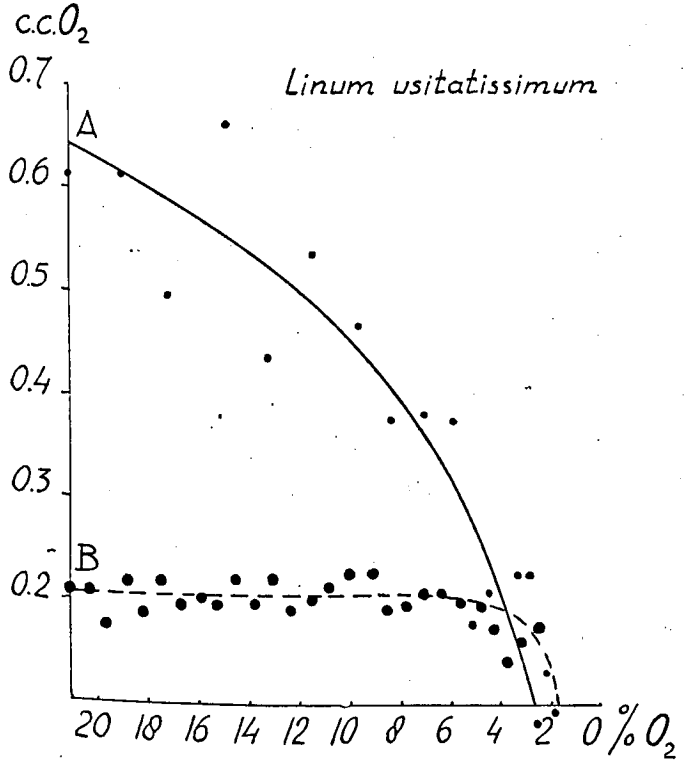


Fig. 4. Explanation see text.

oxygen-intake almost immediately, while in a later stage it continues to respire normally down to 3% O_2 (fig. 4).

3. Spelt shows at the first stage a decrease at 15% O_2, while in the fully germinated seeds the oxygen-intake decreases steadily

with diminishing pressure, to stop entirely between 9 and 7% O_2 (fig. 5).

4. Buckwheat behaves, at early stages of germination, much like Brassica; decrease in O_2-intake became manifest at low oxygen-pressure (3% O_2), while at the second stage an increase followed by a decrease (at 11% O_2) in oxygen-intake could be observed (fig. 6).

Volatile by-products might influence the results. In the duration of the experiment, however, alcohol was not formed, the seeds were free from one another and they were kept in a moist, not in a soaked condition (Stiles 135, Pringsheim 118). At higher O_2-pressures, anaerobiosis may probably not occur. It is still possible, however, that inconvenient by-products may occur at lower O_2-pressures. But in this case the curve would have a much steeper slope even to an excess-pressure in the respiration-vessel, a fact which has not previously been stated.

Due to the fact that earlier literature usually does not mention the stage of germination and that careful work has been done, thus far, almost exclusively with lower plants (de Boer, Shoup, Warburg) it is impossible to find adequate analogies. Of the older work, that of Godlewski shows the greatest similarity to our experiments. Phycomyces (de Boer) cultivated on a "oily" medium seems to behave like Brassica in stage A, while the same fungus, grown on "starchy" medium, behaves like Brassica in stage B. Dolk's results are directly comparable with ours. The oxygen-intake of hyacinth-bulbs shows the same characteristic dependence of pressure as the later germination stages of Brassica and the early stages of Triticum and Linum. Less sensitive to oxygen are stage A of Brassica and Fagopyrum and stage B of Linum, while the spelt is more sensitive to oxygen-deficiency. Fagopyrum in stage B is a case apart.

Several authors (Stiles and Leach 136, Pringsheim 117, Frietinger 41, Stälfelt 131 and others) mention the structure of the seed-coat as influencing the oxygen-intake. A heavy seed-coat, especially a mucilaginous layer, might particularly hamper it. Linseed, with its mucilaginous epidermis (Karsten 73) might be a case of such a protected seed, where in the early stages of germination the oxygen-intake is only possible at high outside pressure, while at a later stage of germination no such barrier will exist.

The seed of Brassica nigra, which also has a mucilaginous outer layer (Tschirch 142), shows, however, an entirely different respiratory behaviour. Moreover the seeds of Fagopyrum

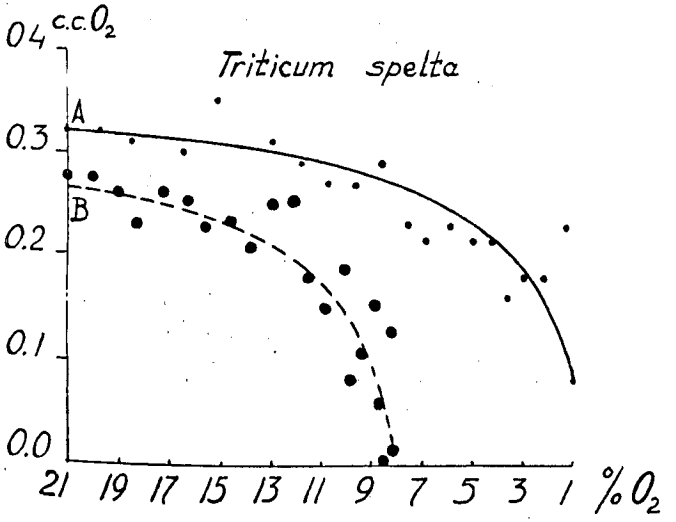


Fig. 5. Explanation see text.

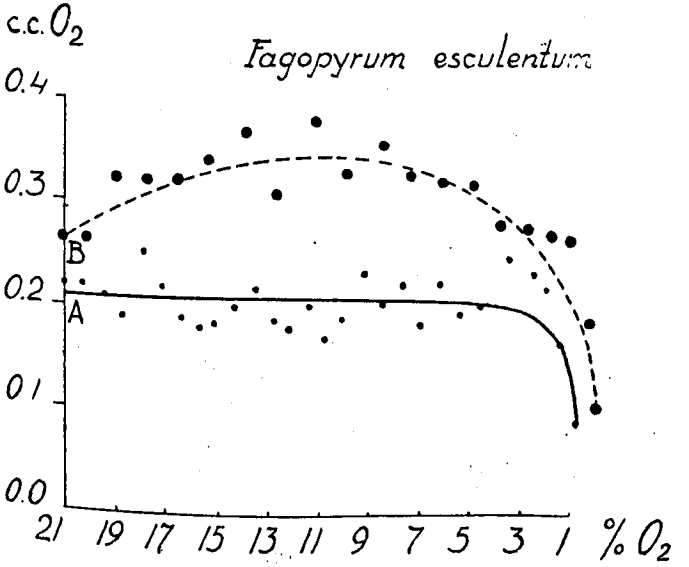


Fig. 6. Explanation see text.

were used with the testa and showed low sensitivity, while the spelt, which has a thin-walled outer layer, appeared to be highly sensitive to diminution in O_2-pressure.

While the influence of oxygen-diffusion cannot be denied, other factors will exert their influence on the oxygen-intake, for instance the chemical nature of the reserve foods. In Chapter IV it is shown that Brassica does not contain carbohydrates in the first germination stages. Oil might be, therefore, the cause of the remarkable respiratory behaviour, an assumption also made by de Boer for Phycomyces.

In stage B the oil might be changed in composition and this change in composition might account for the increase in sensitivity for diminished pressure during germination.

The data obtained on linseed seem to be in contradiction with this assumption. It is possible, however, that in the first stages of germination the testa appears in this case as a limiting factor, while only in the second stage the oil will exert its influence. The data of Triticum agree with those of de Boer with Phycomyces on starchy media.

Wheat-germs, however, contain 10—12% of oil (Wehmer 149). The absence of linolic and linoleic acid in the wheat-oil (which compounds are present in both Brassica and Linum) might account for its apparent inactivity.

The behaviour of the buckwheat, which contains much starch (67%) and very little oil (1.5%), remains unaccountable.

A third possible explanation of the experiments follows from the behaviour of the seeds in relation to methylene-blue as H_2-acceptor, as described in Chapter III.

CHAPTER II.

Catalase Activity at various Stages of Germination.

§ 1. *Review of the Literature.*

Catalase is one of the best studied enzymes because of its almost universal distribution in living cells, which distribution warrants the assumption that this enzyme might play an important role in physiological processes. In order to prove this point, however, it is necessary to study the nature and mode of action of this enzyme as well as the factors influencing the process and the methods of its quantitative determination.

The opinions on all these questions are extremely divergent. This is chiefly due to the fact that some look upon the enzyme

as a chemical compound, while others look upon the accelerated decomposition of H_2O_2 as caused by the colloidal nature of the enzyme.

The specificity of the enzyme was shown in 1901 by Loew (89), who gave the word catalase to the principle properly to catalyse the reaction: $2\ H_2O_2 \rightarrowtail 2\ H_2O + O_2$. According to Loew there are 2 catalases; insoluble α- and soluble β-catalase.

Already in 1899 Bredig (11) has drawn a parallel between the action of enzymes and his Pt-sols. He coined the term "inorganic ferments" for his colloidal metal-solutions. He states e.g. that both enzymes (Loew's catalase as well as the inorganic ferments) may be poisoned by H_2S and HCN. Bredig accounts, moreover, for the monomolecular nature of the reaction by two chain-reactions:

1. $H_2O_2 \rightarrowtail H_2O + O$.
2. $O + O \rightarrowtail O_2$, of which the reaction-velocity of the second is infinitely large. The similarity between inorganic and organic ferment is accounted for by him by their colloidal state. He called the attention to the great influence of dispersion and adsorption on the decomposition of the peroxide.

According to Issajew (69, 70), yeast-catalase causes, below a certain critical peroxide-concentration and at low temperature, a strictly monomolecular decomposition of H_2O_2. The velocity-constant symbates with catalase-concentration, but is not proportional to the latter. This proportionality, however, was found by Senter (126).

Engler and Herzog (30) account for the mechanism of the decomposition of H_2O_2 as follows:

$$\text{Catalase} + H_2O_2 \rightarrowtail [\text{catalase}]\begin{matrix}-O-OH\\ -H\end{matrix}$$

$$2\left([\text{catalase}]\begin{matrix}-O-OH\\ -H\end{matrix}\right) \rightarrowtail 2\ (\text{catalase} + H_2O + O_2)$$

An adsorption precedes here the decomposition.

Appleman (3) found evidence of the existence of α- and β-catalase in potatoes. Crocker (20) makes the same distinction for various grains, peach- and Amaranthus seeds.

Michaëlis and Pechstein (98) stress the ampholytic nature of the enzyme, the activity of which depends upon both the anions as well as upon the undissociated enzyme-molecules.

Baas Becking and Hampton (4) find a monomolecular reaction for the catalase from the grown tips of hemp. Higher peroxide-concentrations seem to destroy part of the enzymatic activity; deviations of the monomolecular equation occur here.

α - and β -catalase might represent different degrees of dispersion of the same enzyme. There existed proportionality between the velocity-constant and the catalase-concentration, results similar to those found by Euler (34) for Boletus scaber.

Wieland (152) accounts for the decomposition by dehydrogenation as follows:

1. HO—OH ⇝ O—O + 2H
2. HO—OH + 2H ⇝ $2H_2O$

He also assumes, that the K of the second reaction is infinitely large and that, therefore, the monomolecular reaction is apparently satisfied.

Willstätter (154) introduced his well-known concept of the composite nature of enzymes; a colloidal "carrier" with an active group fixed upon this "carrier" in a not further defined way. The "carrier" causes the stability and the activity of the enzyme, while the active group accounts for the specificity.

Morgulis (102), using liver-preparations, found the proportion substrate:enzyme dictating the order of the reaction (uni-, 1.5— to bimolecular). The peroxide seems to oxidize the enzyme. This may be counteracted by experimenting with highly dilute H_2O_2 and at low temperature (0° C.).

Hennichs (63) succeeded for the first time to free to a high degree liver-catalase from admixtures. He points to the fact that its characteristic activity "in vitro" is different from that "in vivo", subsequently that during the preparation changes have taken place.

Within the frame of his general theory, Euler (32) assumes a dissociable compound enzyme-substrate and that accordingly, the decomposition is determined by the concentration and the dissociation-constant of this compound. Further particulars are given by Haldane (56).

Battelli and Stern (5) assume the presence of two antagonistic principles, anticatalase and philocatalase, which hypotheses were further tested by Stephan (133). In view of the colloidal nature of the enzymes there seems, however, to be slight probability that all these principles should exist.

Charmandarian (15) changed the degree of dispersion of malt-catalase by temperature and changes in chemical environment. He found concomittant changes in the reaction-velocity.

Leggatt (85) concludes from his experiments with various seeds on the existence of α -thermolabile and β -thermostabile catalase. As he worked, however, with unpurified extracts, the presence or absence of protective colloids might account for his findings.

Zeile (155) found as the active group of the catalase a porphyrin-iron compound both in animal and in vegetable extracts.

As a divergent opinion we mention the work of Haber and Willstätter (53). They look upon the decomposition of H_2O_2 as a dehydrogenation in a chain-reaction, in which OH and H_2O serve as chains. The initial reaction is:

Catalase + H_2O_2 ⇝ Desoxycatalase + H_2O

The master-reactions are:

1. $H_2O + H_2O_2 \rightsquigarrow O + H_2O + OH$
2. $OH + H_2O_2 \rightsquigarrow H_2O + O_2H$

By reoxidation, for instance through intracellular iron-compounds, the desoxycatalase could be regenerated. Many objections might be given against this mode of reaction (Zeile 156 and others).

Waldschmidt-Leitz (146) stresses the importance of the active group as a limiting factor in the catalase-action, whilst Sumner (137) points to the proteinaceous nature of the enzyme.

Albers (1) looks upon the enzyme-substrate compound as a real chemical compound, which is able to fix 2 molecules of H_2O_2. The decomposition follows the scheme:

$$\text{Catalase} + H_2O_2 \rightleftharpoons \text{Catalase}\begin{pmatrix}HO—OH\\HO—OH\end{pmatrix} \rightsquigarrow \text{Catalase}\begin{pmatrix}HO—OH\\ \searrow \quad \swarrow \\HO—OH\end{pmatrix}$$

$$\rightsquigarrow \text{Catalase} + O_2 + 2H_2O$$

This scheme shows great affinity to that of Wieland. Albers assumes the presence of another non-reactive compound [enzyme. $(H_2O_2)_6$] which is formed with excess H_2O_2.

Frankenburger (38), like Höber (66), looks upon enzymes as microheterogeneous systems, which contain active groups (i.e. iron-porphyrin) at definite loci of the enzyme-complex, which chemically activate the substrate. A brief summary of his hypothesis follows.

1. Adsorption of H_2O_2 on enzyme-particles.
2. Shift of the adsorbed molecules to the active groups.
3. The active group causes the loosening of the valence-bonds in HO..O..H, respectively split in H_2O and O.
4. Fixation of H_2O_2 and O on the „carrier" by secondary valences; the iron-porphyrin is free to act upon another molecule of H_2O_2. H_2O and O_2, the latter originated by two atoms of O, are liberated from the „carrier".

One of the processes will be limiting factor. If reaction 3 be limiting, the reaction will appear to be monomolecular.

Frankenburger stresses the difference between models of and natural catalase. The latter show greater activity as well as pronounced specificity. Catalase only decomposes H_2O_2, models are able to decompose other peroxides. The difference in activity is shown in the following table:

1 mol. Fe $^{++}$ (or Fe $^{+++}$) decomposes in aqueous solution 10^{-5} mol. H_2O_2.

1 mol. hemin or mesohemin under the same conditions 10^{-2} mol. H_2O_2.

1 mol. (?) catalase under the same conditions 10^5 mol. H_2O_2.

Frankenburger's views form a synthesis between the colloidal and chemical theories of catalase-action.

In the latest paper that came to our attention, Zeile (156) states that catalase consists of a comparatively small „carrier" in dissociation-equilibrium with a colloidal protective system.

No consensus of opinion exists as to the function of the catalase. The chief difficulty seems to be that we do not possess a valid index of its strength. The only rational index would be the velocity-constant (Hampton 58, Baas Becking 4, Höber 66, Euler 33), in which the kinetics of the process is taken into account. No reliable index of enzyme-activity can be obtained when only the amount of O_2 liberated in a certain period of time is stated (e.g. Appleman 3, Choate 17, Crocker 20, Nemec and Duchon 108, 109, Haut 60, Leggatt 85, Marks 95, Pope 115). The same objection holds for the authors who give the period of time, necessary to liberate a certain amount of O_2 (e.g. Heinicke 61). They are useful, however, when strictly standardized, as comparative measures as, for instance, in the work of Leggatt (85) and Heinicke (61).

Many authors, apparently, have not realised the influence of the environment. Graçanin (46) used no buffers, his preparations were dried at 40° C.; Nemec and Duchon (108, 109), using no buffers either, did not state temperatures and used a large excess of H_2O_2, etc.

The available data seem to indicate that catalase-action should be related to respiration. Both respiration and catalase-action are decreased by H_2S and HCN (Bredig 11, v. Herk and Badenhuizen 64).

According to Loew (89), „activated" H_2 is accepted by at-

mospheric oxygen and H_2O_2 is formed, a strong protoplasmic poison, which has to be decomposed immediately by catalase.

Wieland (152) is of the same opinion; catalase is a „Hilfsferment" in respiration.

Zeile (156) looked upon catalase as a principle, protecting the „Atmungsferment" of Warburg, the latter forming H_2O_2, which is decomposed by the former.

It should, moreover, exercise a regulatory action in cells, which are poor in peroxidase.

French (39) has shown that Chlorella decomposes H_2O_2 below 20° C. according to a monomolecular reaction. He also assumes a correlation between respiration and catalase-action.

This correlation is shown by v. Herk and Badenhuizen (64) in an entirely different way. Sections of Sauromatum, placed in a purified enzyme-preparation of the same plant, showed an increased O_2-intake in a Warburg-apparatus. They express no opinion as to the causes of this phenomenon.

In the quest for a reliable index of germinating power of seeds the relation of seed-vitality and catalase-activity has been frequently investigated (Appleman 3, Crocker 20, Nemec and Duchon 108, 109, Graçanin 46, Leggatt 86, Haut 60, Knecht 77, Stephan 133).

It is generally accepted that such a correlation exists.

Fodor, Frankenthal and Biletzky (37) claimed to have demonstrated antagonism between dehydrogenases and catalase with maize. As a concomittant increase of dehydrogenases and catalase-activity often appears, their hypothesis is far from being proven.

As for the influence of environmental factors, plant-catalase appears to be most sensitive.

For the influence of temperature two antagonistic effects have been observed.

1. Reversible influence on reaction-velocity.
2. Irreversible damaging of the enzyme (Issajew 69, Morgulis 103, Zeile 156). As the damage is usually greater than the acceleration, it is advisable to work at temperatures below 20° C.

The activity decreases with storage (Crocker 20, Charmandarian 15, Euler 34).

The stability decreases with dilution (Euler 33).

These 2 factors are chiefly active in unpurified extracts.

The influence of pH is universally accepted. The activity is markedly decreased in acids (Sörensen 130, Baas Becking

4, v. Herk and Badenhuizen 64). The optimum is situated between 6.5—9 (Zeile 156), the solutions are usually kept at 7.0.

Peroxide-concentration has a pronounced effect on the activity of the enzyme and may lead in high concentrations to partial inactivation (Baas Becking 4, Morgulis 102). The monomolecular reaction in this case does not suffice. Consequently, the relation between velocity-constant and enzyme-concentration will be variable. Euler (34) fixes the maximal strength of H_2O_2 at 0.01 n.

§ 2. *Review of the Methods.*

There are 3 methods to determine catalase-activity.

A. The original method consists of titration of the undecomposed H_2O_2 in acid solution of $KMnO_4$. This method is still generally used. Objections against it are;

1. Latency time (Baas Becking 4); the oxygen-development does not start immediately after the addition of the peroxide.
2. In unpurified extracts the end-point of the titration is uncertain.

B. An acid solution of KI is added to the undecomposed H_2O_2, the iodine liberated is titrated with $Na_2S_2O_3$. The objections to this method are similar as those mentioned under A.

C. Gasometric methods, in which the volume of the liberated O_2 is measured. According to Euler (34), this procedure does not yield as accurate a result as the other two methods. The gasometric methods, however, have the great advantage, that the color of the extract does not influence the result.

Certain precautions, however, are indicated. To prevent supersaturation of the solution of H_2O_2, care should be taken not to use too much liquid and to use a reaction-vessel with a broad bottom, while the apparatus has to be shaken continuously. Violent shaking might decrease the activity, however (Stephan 133, Leggatt 85, Heinicke 61).

An extension of this method is the autographic method, which has the advantage that the entire reaction is demonstrated „ad oculos". This is, moreover, the only way, in which the latency-time may be determined. Autographic experiments before 1920 are summarized by Baas Becking (4). Leggatt (85) has used this method in his preliminary experiments. For his final series he used titration with $KMnO_4$ without giving reasons for this proceeding. Heinicke (61) elaborated the method for 12 simultaneous determinations. The autographic method is used in

this paper to determine catalase-activity of germinated and ungerminated mustard-seed.

§ 3. *Own Investigations.*

Leggatt (85) has stipulated the necessity of standardized proceedings.

In all our experiments the germination took place in the following way.

A definite weight of seed was soaked in tapwater in a test-tube. After swelling for 5 minutes, the seeds were placed quantitatively in Petri dishes on moist filter paper and germinated in a thermostate at 30° C. for 24 and 48 hours. Germination was irregular. After 24 hours a few roots broke through, after 48 hours some cotyledons are visible. Technical difficulties (size of the seed and low germination-index, vide Chapter 5) prevented the selection of equal stages of germination.

The weight of the air-dry seed was taken as a basis for all computations, as it appeared that this weight did not change when the seeds were kept in a sealed bottle.

The change per seed in germination follows from these numbers, because of the smallness of the seed.

As from the literature-survey the lability of the enzyme appeared, the work was done with unpurified extracts.

20 grammes ungerminated ground or germinated seed were rubbed for ½ hour in a mortar with 2 grammes of $CaCO_3$ and 78 grammes distilled water. This extract was shaken for 1 hour in an Erlenmeyer at a temperature of 17.5° C. Presumably the catalase will go into solution, while the lime will neutralize the noxious acids.

The possibility remains that the activity of the enzyme changes during these manipulations (Euler 33). On the other hand time should be allowed for the peptisation or the solution of the enzyme. Charmandarian (15), moreover, found that the activity did not decrease after one hour.

In order to be able to sample the liquid, it had to be centrifuged for 10 minutes at 3000 revolutions/minute.

The yellow, opaque extract of pH 7 remained at constant activity during the experiments (4 to 5 hours). The next day, however, the extract proved to be completely inactive.

The description of the apparatus will be found in the publication of Baas Becking (4). The oxygen developed in the vessel, will cause a change in the level of a mercury column, which change is registered by means of a kymografion.

The experiments were carried out in a constant temperature room at 17.5° C.

To prevent the ejection of the stopper through the pressure developed, the vessel was securely clamped. The peroxide was placed in a small container suspended from the stopper by means of a platinum-wire. By carefully turning the vessel, the container dropped from its hook and the peroxide was quantitatively mixed with the liquid.

Registration starts at this moment, while the vessel was rotating regularly throughout the experiment.

The volume of the vessel was calculated by the determination of the excursion of the recording needle with a known amount of liquid containing a known amount of H_2O_2 decomposed by $KMnO_4$ in H_2SO_4. The curve obtained is shown in fig. 7. The abscissae give the time in minutes, the ordinates the quantity of H_2O_2 decomposed.

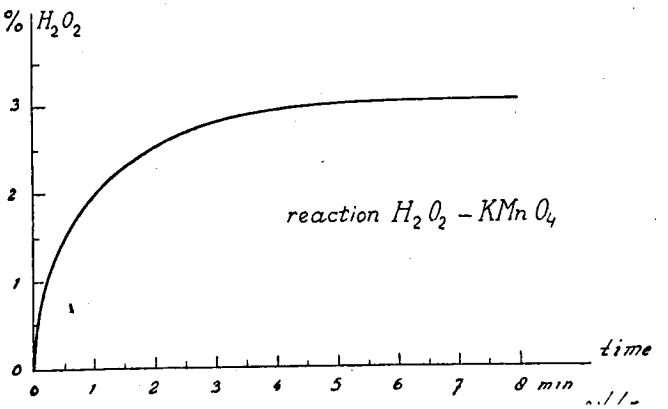


Fig. 7. Explanation see text.

If the area of the junction manometer-reaction-vessel be a cm^2; the maximum excursion of the needle in cm. h; x the number of grammol. O_2 already present in the reaction-vessel; Δ x grammol. O_2 liberated from H_2O_2; v the volume of the reaction vessel; Δ v its increase in volume; furthermore R the gas-constant, T the absolute temperature, p pressure in cm Hg, Δ p increase in pressure by oxygen-development, we obtain, neglecting the amount of O_2 present in the liquid:

$$\begin{array}{l} (p + \Delta p)(v + \Delta v) = RT(x + \Delta x) \\ p\,v \qquad\qquad\quad = RT\,x \end{array}$$

$$p\,\Delta v + v\,\Delta p + \Delta p\,\Delta v = RT\,\Delta x$$

$$\Delta v = a\,h \qquad \Delta p = 2\,h.$$

$$v = \frac{RT\,\Delta x}{2\,h} - a\,(\tfrac{1}{2}\,p + h).$$

$v = 110$ cc.

If the volume of the reaction-vessel is known, a control on total decomposition of H_2O_2 may be obtained by means of

$$\Delta x = \frac{(a\,p + 2\,v)\,h + 2\,a\,h^2}{RT}$$

When we take as average p 77 cm Hg and when we work at a temperature $T = 273 + 17.5°$ C., we obtain

$$\%\ H_2O_2\ decomposed = 0.9\ h$$

If the volume of the liquid be 10 cc more (second experimental series), we obtain

$$\%\ H_2O_2\ decomposed = 0.82\ h$$

Preliminary experiments were performed with 1 cc 3% H_2O_2. Lower concentrations were unsuitable as the excursion of the needle became too small.

The enzyme was not destroyed by H_2O_2; renewed addition of peroxide yielded O_2-development.

As a stock solution 30% H_2O_2 purissimum Merck was used, which fulfilled all the conditions imposed by the Ned. Pharm. (107). Fresh dilutions were prepared every day and the dilutions controlled by means of titration by $KMnO_4$.

The experiments were carried out with 24 and 48 hours germinated seed with the following solutions;

1. 5 cc extract + 5 cc phosphate buffer pH 7 + 1 cc 3% H_2O_2.

2. 10 cc extract + 10 cc phosphate buffer pH 7 + 1 cc 3% H_2O_2.

The complete decomposition of the H_2O_2 is shown in the following table, where the titer of the H_2O_2 is compared with the calculated value from the curves.

	Ungerminated seed		24 hours germinated seed		48 hours germinated seed	
	5 cc extract	10 cc extract	5 cc extract	10 cc extract	5 cc extract	10 cc extract
H_2O_2 value in % according to curve	3.09	2.31	2.74	2.58	2.66	2.58
H_2O_2 value in % according to titration	3.09	2.30	2.68	2.68	2.70	2.70

The accuracy varies from 100—95%. The discrepancies may be accounted for by sampling errors and measuring errors in the graphical record.

The latency-time of 10—30 seconds was always observed, a relation between this time and quantity of enzyme, as supposed by Baas Becking (4), could not be found.

Figures 8, 9 show the records obtained. The abscissae of the graphical records are shortened and the ordinates lengthened in these figures.

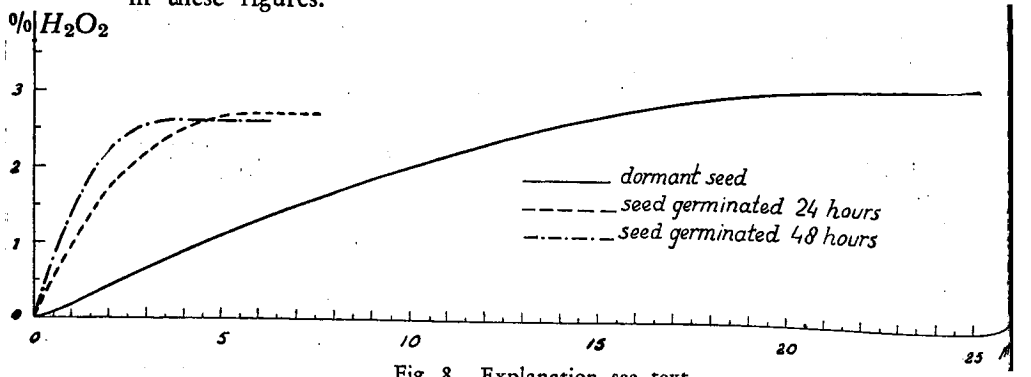


Fig. 8. Explanation see text.

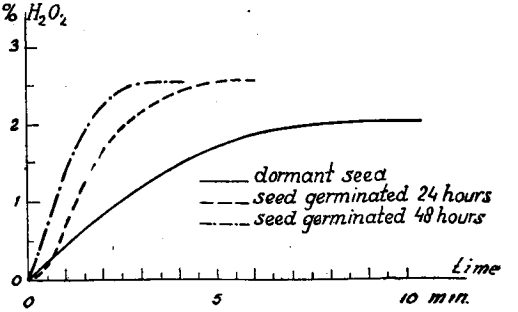


Fig. 9. Explanation see text.

As the construction of the asymptote is inaccurate, the halftime, i.e. the reaction-time measured from the end of the latency-time until the moment that one half of the 3% H_2O_2 is decomposed, is given in the following table.

	Ungerminated seed		24 hours germinated seed		48 hours germinated seed	
	5 cc extract	10 cc extract	5 cc extract	10 cc extract	5 cc extract	10 cc extract
Half-time in seconds	418	225	110	100	63	64

From the table it appears that only in the ungerminated seed the reaction-time seems to be inversely proportional to the quantity of enzyme, while in the 24- and the 48 hour-seeds no marked effect could be observed.

The influence of grinding on the ungerminated seed was investigated by the use of a corn mill [1]), which yielded particles of about 450 μ. When 5 cc extract were prepared from this flour in the usual way the reaction-time proved to be very large (> 30 minutes). Calculation of these very flat curves is exceedingly inaccurate. With 10 cc extract the halftime proved to be 345 seconds. Not all H_2O_2 was decomposed; added 2.9%, decomposed 2.7% H_2O_2. *Fine grinding has a deteriorating effect upon the activity of the catalase* (Euler 33, Leggatt 85).

The kinetics of the reaction were considered more in detail. The following equations were made use of;

1. Monomolecular reaction *); $K = \frac{1}{t} \ln \frac{a}{a-x}$
2. Bimolecular reaction *); $K = \frac{1}{t} \frac{x}{a(a-x)}$
3. Trimolecular reaction *); $K = \frac{1}{t} \frac{x(2a-x)}{2a^2(a-x)^2}$
4. 1.5 - molecular reaction **); $K = \frac{\sqrt{a} - \sqrt{a-x}}{t \sqrt{a \cdot (a-x)}}$
5. Schütz' formula ***); $K = \frac{x}{\sqrt{t}}$

[1]) I am much indebted to Prof. Dr. G. v. Iterson Jr. for the use of the instrument.

*) Nerst (110), p. 596.
**) Herzog cited in Oppenheimer (113) II, p. 963.
***) Schütz (125).

6. Autocatalytic reaction ****); $K = \frac{1}{2t} \log \frac{a+x}{a-x}$

In regard to the autocatalytic reaction as mentioned by Höber, it should be mentioned that it differs materially from the autocatalytic reaction given by several other authors (Robertson, Pearl and others). Their reaction reads;

$$x = \frac{a}{1+ce^{-mt}}$$

derived from the differential equation;

$$\frac{dx}{dt} = b\,x\,(a-x)$$

while the reaction mentioned in Höber is derived from the differential equation;

$$\frac{dx}{dt} = k_1\left(1 + k_2\,\frac{x}{a}\right)(a-x)$$

illustrating a quite different mechanism. As our curves did not show a pronounced point of inflection, the equation as given by Pearl has not been used.

The results were as follows;

A. Ungerminated seed. No equation seems to fit.

B. 24 and 48 hours germinated seed. Constant K could be obtained with equation 6. The magnitude of K is, apparently, a function of the H_2O_2-concentration.

	24 hours germinated seed		48 hours germinated seed	
	5 cc extract	10 cc extract	5 cc extract	10 cc extract
$K \times 10^5$	248 286 261 262 274 286 262 271 287	302 305 319 300	429 484 427 420 454 433 468 528 521	503 555 499 484 480
Average $K \times 10^5$	272	306	463	506
Probable error of the mean	8.8	5.1	26.4	18.1

****) Höber (66), p. 322.

For 5 cc extract; K (48 hour seed) : K (24 hour seed) = 1.7
For 10 cc extract; K (48 hour seed) : K (24 hour seed) = 1.65

§ 4. *Discussion* *).

A. Suppose that the deviations in the curve for ungerminated seed are caused by a time-factor and suppose, moreover, that the reaction-velocity is proportional to the concentration of the undecomposed substrate, we get

$$\frac{dx}{dt} = k\,(a - x)\,f\,(t)$$

$$\frac{dx}{a-x} = k\,f\,(t)\,dt$$

Integrated; $\ln \frac{a}{a-x} = K\,F\,(t)$, in which $\frac{d\,F\,(t)}{dt} = f\,(t)$

This may be tested graphically by plotting $\log \frac{a}{a-x}$ against time and by determining the tangents at various points of this curve.

The constancy of K may be tested at various observation-times. In this way the following table was obtained:

t in seconds	427	598	668	789	915	1490
f (t)	1.33	1.37	1.68	1.57	1.47	0.55

This may be accounted for by the surmise that the degree of dispersion of the enzyme increases at the start of the reaction in such way that the decomposition of H_2O_2 is increasingly furthered (increasing f (t)), but that later the H_2O_2 starts to inactivate the enzyme (decreasing f (t)).

In the ungerminated seed the catalase is present, apparently, in such a labile condition, that no further conclusions in regard to its activity may be drawn from our experiments.

B. 24 and 48 hours germinated seeds.

Inasmuch as the K for the monomolecular reaction increases steadily, the H_2O_2 cannot have poisoned the enzyme.

The „autocatalysis" may be accounted for as follows;

Suppose reaction-velocity is proportional to the concentration of the undecomposed substrate (a-x) and further proportional to the active enzyme surface c_1. Suppose further that this active surface is inactivated proportional to the H_2O_2-concentration, we get;

*) I am much indebted to Mr. E. F. Drion for the following considerations.

$\frac{d\,x}{d} = K\ (a - x)$ (act. surface c_1)

Inactive surface $= c\ (a - x)$

Total active surface at the start $= c_1$, $c_1 > c\,a$

$\frac{d\,x}{d\,t} = K\ (a - x)\ [c_1 - c\ (a - x)\ ]$

$\frac{d\,x}{d\,t} = K_1\ (a - x)\ (b + x) \qquad b = \frac{c}{c_1} - a$

Integrated $K_1\ t = l\,n\ \frac{b + x}{a - x} \times \frac{a}{b}$

When b=a, we obtain the „autocatalytic" equation. In that case c_1=2ac, that means that at the start of the reaction one half of the enzyme is inactivated. This could be considered as a case of *negative „autocatalysis"*. The difference of K for 5 and 10 cc extract could be accounted for by a change in b. The appearance of the „autocatalytic" reaction must be accounted for by the fact that it is a logarithmic function.

Inasmuch as kinetic studies of catalase action are scarce (Issajew 69, 70, Baas Becking 4, Euler 33), similar phenomena are, as far as we know, not recorded in the literature. Comparisons with other investigations, therefore, are difficult. Euler (33) mentioned increased catalase-activity during the germination of barley and based his conclusions on comparison of average monomolecular constants. These constants were averages of a number which markedly decreased during the reaction. No quantitative conclusions are warranted on such an irregular foundation.

From our experiments, it does not appear evident that the amount of catalase increases or decreases during the first stages of germination, because in the different stages the processes are of a different order. Examination of K for 5 cc extract and 10 cc extract seems to indicate that catalase-activity is increased from 24 to 48 hours.

Comparison with the ungerminated seed is impossible. Comparison with the half-time numbers, however, would induce some to conclude continuous increase of activity.

The theory of Frankenburger (38) might account for the „autocatalytic" line, because at higher H_2O_2-concentrations the displacement of the adsorbed peroxide to the active groups might be retarded.

No correlation of enzyme-activity and the respiratory phenomena, described in Chapter I, could be observed.

CHAPTER III.

Experiments with Methylene-blue.

§ 1. *Review of the Literature.*

The reduction of methylene-blue to leucomethylene-blue under the influence of yeast- or bacterial dehydrogenases has been studied extensively, while higher plants have been used occasionnaly and only very recently.

After the conclusion of our experiments, the work of Grande (47), Mazza (97) and Zeller (157) concerning fatty acid dehydrogenases, came to our attention.

Mazza stated an increase of oxygen-intake, when higher fatty acids were added to a suspension of *Bacterium coli* in a Warburg apparatus. Because no CO_2 had been developed (how this has been verified, is not indicated in this paper), he concluded that, in the first instance, dehydrogenation had taken place. After this dehydrogenation, the fatty acids must have been further oxidized by the bacteria. No increase of oxygen-intake could be observed after heating the bacterial suspension at 100° C. After the addition of toluene to the suspension, only in the beginning an increasing oxygen-intake could be stated. M. supposes in this case that only dehydrogenation, no further oxidation of the fatty acids had taken place. Chodat and Junquera (18) mentioned an experiment with *Endomyces Chodati,* which was able to reduce methylene-blue without addition of a H_2donator, when brought in Thunberg tubes. H_2donator would be glutathione. When, however, one considers the fact, that Endomyces contains much fat, it is not impossible, that higher fatty acids have been H_2donators too. The same remark may be made with regard to the experiments of Wertheimer (151), who stated that baker's yeast could reduce methylene-blue without addition of a H_2donator.

Fodor and Frankenthal (36) observed the presence of dehydrogenases for plant acids and purin derivatives in seeds of barley, wheat and oats. The watery extract of the 3 grains, brought in Thunberg tubes, could only reduce methylene-blue without addition of a H_2 acceptor, at pH > 8.

Grande (47) found dehydrogenases for palmitic and stearic acid in the seeds of e. g. *Agrostemma, Papaver* and *Amaranthus.* His experiments were made in Thunberg tubes, pH amounted to 7—7.5, the color agent used was 1-naphtol-2-Nasulfonate-indo-2.6. dichlorphenole. Because all these seeds contain fats (Wehmer 149), the presence of fatty acid dehydrogenases is comprehensible.

Zeller (157) stated that in extracts of *Cucurbita pepo*, a reduction of methylene-blue occurred without an addition of a H_2donator, when brought in Thunberg tubes. The decoloration-time is shortened considerably when salts of palmitic, stearic and oleic acid are added; the dehydrogenases observed are fatty acid dehydrogenases. Which dehydrogenation products are formed could not be detected, in no case linolic acid, because the addition of the salt of this acid to the extracts did not have any influence on the decoloration-time.

The purpose of our experiments, was not so much to demonstrate the presence of fatty acid dehydrogenases, but the presence of the system: H_2donator-dehydrogenase-H_2acceptor.

§ 2. *Methods used and Experiments.*

The same simple experimental scheme as indicated by Wertheimer for yeast was used here.

A certain number of seeds or a definite weight of ground or rubbed seeds is brought into a test tube. 10 cc. tapwater, 0.5 cc. phosphate buffer (pH 6.6) and 2 drops of methylene-blue solution (1-20.000) are added, the whole mixture covered up with a layer of toluene. The beginning of a reduction of the methylene-blue is visible after 24 hours as a colorless zone, in the lower end of the tube, above and round the seeds. Always, just as well in the cases when no reduction had taken place, methylene-blue is absorbed by the plant tissues. The shade of the liquid might, at most, become a trifle clearer. Especially the root tips, never the hypocotyledon, would take up methylene-blue.

That a real reduction of the methylene-blue had taken place, was proved by very carefully bringing H_2O_2 in the colorless zone with a pipette. A light blue color was than produced. Considering the fact that the reaction leucomethylene-methylene-blue is a reversible one (Reid 121), one must not wonder about the observation that never the original deep blue color could be brought back by oxidation with H_2O_2. At the same time, this oxidation proved that the decoloration of the liquid was not due to the absorption of the methylene-blue by the plant tissues. A tube filled with 0.5 cc. buffer, 10 cc. tapwater, 2 drops of methylene-blue and a layer toluene was used as a blank.

The following scheme indicates some of the experiments performed.

— no reduction	± feeble reduction
+ reduction	+ + strong reduction

	Brassica	Linum	Triticum	Fagopyrum
A Dormant seed	— After 30 hours ±	—	—	—
B Ground dormant seed	+	—	—	—
C Ground dormant seed, boiled ½ hr. in tap-water	—			
D Germinated seeds, only root tip visible	+	—	—	—
E Germinated seeds, roots visible, cotyledons still invisible	+	+		
F Full developed plants	—	—		

Some experiments were also made with an ethereal extract of germinated and latent seeds, emulsified or non-emulsified, compared with almond-oil emulsion and with the extracted seed.

G. Mustard-seed oil with 10 cc. tapwater, 0.5 cc buffer and 2 drops of methylene-blue; no reduction. Emulsion of mustard-seed oil with gum arabic; reduction. Emulsion of almond oil with gum arabic; no reduction. Seed remainders, not wholly free from oil; strong reduction.

H. Emulsion with gum arabic of the ethereal extract from well developed plants of Brassica; no reduction. Rubbed extracted seed; no reduction.

§ 3. *Discussion.*

These experiments prove that in the given environment, a system H_2donator-dehydrogenase-H_2acceptor is present in seeds of *Linum*

and *Brassica*, not in the seeds of *Triticum* and *Fagopyrum* (A-E). The system is sensitive to increase of temperature (C). It is already present in *Brassica* in the ungerminated seed (B), in *Linum* it appears later on. That no or only a very feeble reduction could be observed in the intact seeds of *Brassica*, may be explained by the small permeability of the seed-coat for methylene-blue.

Linum shows only the system already indicated, when the seed has been germinated for some time.

Both *Brassica* and *Linum* seem to have lost the system when the seeds are wholly germinated (F, H). The necessity for a great dispersion of the oil is shown by experiment G. This observation is in accord with the remark of Netolitzky (111), that oil must be present in a colloidal, dispersoid state in the cytoplasm of the plant. The experiment with almond oil proves that not all oils may induce methylene-blue reduction.

It is remarkable that, apparently, the dehydrogenases are not hampered by the ether. Besides, they seem to be present in the oil as in the seed remainders as well.

In connection herewith, a publication of Traxl (141) must be mentioned. He investigated the influence of the addition of oil emulsions on the respiration of hen's erythrocytes. It appeared that oil emulsions hampered or stimulated the respiration. The stimulation of the respiration was caused by a „thermolabile substance", present in the oil, that induced an increase of the iodine number by chain-reactions. It seems not to be improbable, that this „thermolabile substance" was a dehydrogenase.

It is not impossible, that the dehydrogenases, found in *Brassica* and *Linum*, dehydrogenate fatty acids (vide Grande and Zeller!). Supposing this to be the case, the remarkable conduct of *Brassica* with regard to oxygen-intake could, partially at least, be explained by the hypothesis that after one day's germination, fatty acid dehydrogenases are chiefly predominant. At the same time an unknown H_2acceptor, taking up the activated H_2, when little or no oxygen is available, must be present in the seed. In normal circumstances, oxygen is the H_2acceptor, in abnormal circumstances (i.e. at low oxygen-tension), it is the unknown substance.

At the second day, the influence of the dehydrogenases is decreasing, changes of the reserve substances have taken place, so that the physiological processes are retarded at a higher oxygen-tension than at the first day.

That the system is still present is demonstrated by experiment E, only later on it disappears completely (F).

In no case, absence of H_2donators, i.e. fatty acids, may be considered as the microchemical experiments (vide Chapter IV) show their presence even in fully developed plants.

Linum, which already contains carbohydrates in the latent seed in contrast with *Brassica* (Chapter IV), would use these carbohydrates primarily when germinating; after that, the oil is used (Ermakoff 31). Therefore, the fatty acid dehydrogenases are appearing later on. Which of the three components, H_2donator, dehydrogenase, H_2acceptor, are lacking in *Fagopyrum* and *Triticum*, was not further examinated.

The remarkable conduct of *Fagopyrum* in regard to the oxygen-intake may probably be found in the different composition of its reserve as compared with *Triticum*.

In any case, it appears from the experiments described above, that dehydrogenases play an important part in germination.

CHAPTER IV.

Microchemical Investigations and Quantitative Sugar Analyses.

§ 1. *Microchemical Experiments.*

As a preliminary some microchemical tests were made on the dormant seed and on etiolated plants, when the cotyledons were just visible.

For a detailed microscopical description of *Brassica nigra* we refer to the studies of Hartwich and Vuillemin (59). Following the lines of Tunmann (143) tests were made on oil, protein, reducing and higher carbohydrates.

Oil.

In the dormant seed the oil drops are distinctly visible in the cotyledons; the same is the case in the cotyledons of the young plants. With Sudan III in both cases a red color of the drops can be affirmed. In the plants the color was stronger in the outer than in the inner cell layers. In the radicle no coloring could be observed.

With a mixture of one part of 50% NaOH-solution and one part of 25% NH_3, soap crystals were formed. These were pleochroitic and feebly negative in the dormant seed, and further a little smaller than in the cotyledons of the plants. When plants were taken, whose cotyledons were fully developed, the soap crystals were formed very slowly. The soap crystal could never be observed in the hypocotyledons and in the radicle.

Protein.

In the dormant seed and in the young plants distinctly visible round idioblasts, the myrosin cells, could be observed, especially in the cotyledons. These cells were colored dark brown by IKI and dark red by Millon's reagent. With Millon's reagent, IKI and the xanthoproteine reaction, in both stages protein proved to be present.

Reducing Carbohydrates.

With Fehling's solution neither in the seed nor in the plants a visible reduction could be observed.

Higher Carbohydrates.

With IKI there appeared nowhere in the seed a blue color, caused by starch.

In the etiolated plants the middle lamellae of the cells of the hypocotyledon, especially in the outer cell layers, were colored greenish blue. The same occurred in the tops of the root hairs. According to the experiments of Ziegenspeck (158), this is caused by the presence of a higher carbohydrate, viz. amyloid.

In the cotyledons starch was nowhere visible, neither in the stomata. In the hypocotyledon a starch sheath, filled with small starch grains, could be observed. The starch grains were lying together either on one or on the other side of the cell or they were lying scattered throughout the whole cell. As soon as the plants were exposed to the light for some hours, the stomata of the hypocotyledon and the cotyledons were filled with small starch grains.

Discussion.

The presence of reducing sugars cannot be proved; only a trifling quantity of starch and some amyloid made their appearance.

As amyloid is a cellulose derivative (vide Ziegenspeck), this can be ignored as a respiration substrate.

It is equally clear that, when the cotyledons are fully developed, they still retain some of their reserve matter, the oil. Therefore, in the first stages of germination a trace of the oil can be transformed into higher carbohydrates, a possible transition to glucose or other reducing sugars could not be observed.

This microchemical description differs greatly from those made with other oily seeds.

Sachs (122) observed in the ricinus-bean a decrease of the oil, occurring parallel with a considerable increase of starch. Von Ohlen (112), who made an extensive microchemical study

of the germination of the soy-bean, records besides the oil, always reducing sugars and starch, however, in changing quantities. It is as well to recall that with these seeds in the ungerminated stage oil as well as carbohydrates were found. This was not the case with Brassica nigra. A quantitative study of the sugars in the dormant and in the germinating seed must complete these preliminary experiments.

§ 2. *Quantitative Sugar Analyses.*

Quantitative sugar analyses belong to the most difficult problems of biochemistry. Ruhland (120) indicates different obstacles, which can be met.

For, apart from the question that there must be a rational basis, to which all values must be reduced (vide Chapter II), attention must be paid to the manner, in which the plant extract is made and how it is rid of impurities, and also to the method, by which one finally determines the carbohydrates.

Firstly, if one wishes to duplicate the tests, the seeds must be germinated according to a wholly standardized method. This point Zeller (157), whose observations we can fully agree with, stresses strongly. Therefore, in these experiments the same operating modus was always followed.

Germination took place as described in Chapter II.

To prevent any qualitative or quantitative changes in the composition of the carbohydrates, occurring through drying or through storage for any time, the germinated seed was analysed immediately. For the sugar analysis of the dry seed this was ground and a certain part of it directly analysed.

With regard to the extraction of the sugars from the seed, the procedure followed was the same as that described by Tollenaar (139).

Where in the first place it was wished to determine the quantity of sugars, which directly, as glucose, or indirectly by inversion by enzymes, as starch and maltose, could serve as respiration substrates, takadiastase was immediately added to the watery extract. Takadiastase was taken, not hydrolysing by HCl, because it was necessary to be sure that no substances of the cell wall might be corroded. Davis and Daish (23) proved this corrosion possibility to be true for hydrolysing with HCl.

The method of extraction is lengthy; the possibility that the carbohydrates fall to pieces is not absolutely excluded. As much as possible, the disintegration of the carbohydrates was checked

by keeping the extracts between the different treatments at a low temperature.

A half year after ending our sugar analyses, Zeller's publication (157) arrived. He avoids the intricate extraction by extreme grinding of the substance to be analysed. Besides, he does not free the extract of impurities, while always determining the sugar content as the difference of reduction before and after fermentation, so that the reducing not fermentated substance cannot cause any mistakes. There was no time to determine whether with mustard-seed, with its great quantity of proteins and oils and minimal quantities of sugars, this method would be possible. In any case, the filtration of the extract is a time-consuming work and a possible cause of failure.

That the sugars were entirely extracted, was proved by the microscopical examination of the seed remaining, in which no starch could be detected, and by the extraction of the seed residue with water, which extract did not reduce Fehling's solution.

Like Lehmann (87), iron-free coal was used as a cleansing medium. Basic lead acetate was not taken for reason of its toxicity, because after having purified the solution, it was the intention to ferment it. To wash the sugars wholly out of the coal, great quantities of hot water should be used, although, according to Kruyt (82), the absorption of sugars by coal is not great. The extract was before the purification brownish yellow, afterwards practically colorless, but slightly opalescent. An extraction of the coal with water did not reduce Fehling's solution, so no sugars were left in the coal.

Furthermore an inversion of the solution with H_2SO_4 was necessary, while maltose and saccharose might be present. When neutralising the acid liquid by $Ba(OH)_2$, not only $BaSO_4$, but also the last residue of colloid substances was precipitated. The total reduction of the wholly clear liquid should then be determined.

Sugars can be determined polarimetrically, biochemically, colorimetrically, gravimetrically and titrimetrically. The last 3 methods are based on the property of sugars to oxidise easily. For an extensive description of the different quantitative methods of sugar analysis reference is made to the article by Lehmann (87) and to the handbooks concerning carbohydrates, such as those of v. d. Haar (52) and Tollens (140).

Whilst there is a great possibility of the quantity of sugars being small (vide microchemical investigations!), a micro-method should be chosen. The polarimetric methods should, therefore, be avoided.

The exclusively biochemical methods, as worked out by Kluyver (78), where certain yeasts ferment certain sugars, are not applicable to our purpose, because it was not known, with which sugars these experiments had to deal.

Colorimetric methods were not possible either, because sometimes the extract showed a light yellow color.

In regard to the titrimetric methods, which were employed to determine not the quantity of the sugars, but that of the organic substances (vide for instance Malhotra 93), these were not used as being not sufficiently specific and exact.

In the preliminary experiments 3 methods were tested, i.e. that of Fehling-Lehmann-Schoorl, as described in v. d. Haar (52), that of Maclean, as used by de Wilde (153) and that of Hagedorn-Jensen, as applied by Lehmann (87). Our first experiments already indicated that, given the great dilution, with which should be worked, the first method falls below the limit of the required accuracy, so that this could not be used further.

Duggan and Scott (27), who compared different micromethods for sugar analysis, came to the conclusion that the Hagedorn-Jensen method (55) is the most suitable. Furthermore, the solution does not contain copper salts, which can be precipitated by the impurities. Objections against it are the great dilution of the thiosulphate solution, the quick deterioration of the solutions, which, therefore, always must be made fresh. The Maclean method is not recorded, although this, according to de Wilde, is sufficiently exact and the technique is less complicated than that of Hagedorn-Jensen.

Weinbach and Calvin (150) controlled the reduction value of physiologically important sugars. They found that the Hagedorn-Jensen method and the latest modification of the Shaffer-Hartmann method (127) showed no remarkable difference with regard to the reduction value, expressed in glucose units. A great objection is the time required for making up a special reduction table. Furthermore, it is necessary to have a notion, which sugars are present, a thing we simply did not know.

Hulme and Narain (68) indicate that a mixture of different sugars exactly shows the sum of reduction of the composing sugars by using the Hagedorn-Jensen method.

Eventually the Hagedorn-Jensen method was chosen, because it showed the lowest reduction values, i.e. most of the reducing non-carbohydrates are then excluded. According to the Hagedorn-Jensen method, sugars are oxidised by $K_3Fe(CN)_6$. The remaining $K_3Fe(CN)_6$ after the reduction is quantitatively converted

by KI in $K_4Fe(CN)_6$, because the latter is precipitated as an insoluble compound by adding Zn SO_4.

$$2\ K_3Fe(CN)_6 + 2\ KI \rightleftarrows 2\ K_4Fe(CN)_6 + 2\ I$$
$$2\ K_4Fe(CN)_6 + ZnSO_4 \rightarrow K_2\ Zn_3\ [Fe(CN)_6]_2 + 3\ K_2SO_4$$

When titrating, the prescriptions, as given by Lehmann, were accurately followed.

The reduction, which occurred after fermentation, proved that fermentation after the first titration was not superfluous. Kerstan (76), too, pointed to the great residual reduction after fermentation, which frequently occurs. The divergent results of several authors (Malhotra 93, Schroeder and Horn 124, Tollenaar 139, Gäumann 43 and others) with regard to the transformation of carbohydrates in plants and seeds, are mainly due to the fact that they did not determine the residual reduction. For the same reason, earlier investigations concerning sugar contents, e.g. those of Detmer (24), Maquenne (94), Iwanow (71), are not reliable.

A 10% yeast suspension (koningsgist of the Gist- en Spiritusfabrieken at Delft) gave such a high selfreduction that 1 cc of it was much too much. We did not succeed until 1 cc of a 0.5% yeast suspension had been used. An increase of reduction after fermenting above the limits of accuracy could not be stated. Decomposition of the glucosid sinigrin, which, moreover, is present in very small quantities (vide Chapter 5), had not occurred then.

Whether the yeast in the extract kept its vital properties was determined by spreading a drop of the fermented liquid on malt agar. Yeast colonies appeared abundantly within 24 hours.

Before the determination of the reduction after fermentation, centrifuging of the yeast cells was necessary, because, otherwise, exact duplicates could not be obtained.

The total amount of carbohydrates was determined as follows. 2 grammes of ground or germinated rubbed seed were boiled for half an hour with 75 cc tapwater. After cooling the mixture, 50 mgr takadiastase and approximately 10 drops of toluene were added, the whole liquid being left at 38° C. for 24 hours. After heating for half an hour and cooling for the same time, the liquid was then filtered through a folded filter. The filtrate was kept in an icebox. The rest with filter was boiled for half an hour with 50 cc tapwater and was cooled afterwards; toluene was added and the whole left for 12 hours in the icebox. This was

filtered off in the first filtrate, the rest was boiled with filter and 50 cc tapwater for half an hour. After cooling for half an hour, this extract was added to the combined filtrates, to which the wash water of the latest used filter was added. To this extract a knife-point quantity of coal was added and the liquid heated gently for half an hour, by means of which the coal could circulate throughout the solution and some proteins were precipitated. After filtering off the coal on a Buchner filter and washing out with boiling water, the filtrate was filled up to 500 cc. To 50 cc of this solution so much H_2SO_4 was added that the concentration of the acid was 3.75%. After that, the liquid was hydrolysed for 3 hours with reflux. After neutralising by $Ba(OH)_2$ and filtering off, the filtrate was filled up to 100 cc. In 5 cc of this hydrolysate the reducing value is determined with $K_3Fe(CN)_6$ solution. The reducing power, too, was determined of 5 cc of an extract, made in exactly the same way as the seed extract, of 50 mgr diastase in 500 cc H_2O. This value was taken into account as blank, when determining the reduction before and after fermentation. 10 cc of the neutralised hydrolysate with 1 cc 0.5% yeast suspension in closed reagent tubes were fermented at 37° C. during 4 hours and shaken every quarter of an hour. Four hours fermenting was sufficient, because preliminary tests proved that after 3 hours the residual reduction did not change any more. After centrifuging, 5 cc of the clear liquid was pipetted off, from which the reduction was determined. The extract of the takadiastase was not fermented, because no fermentable sugars were present in indicative quantities. The extracts, in which the sugar analysis was made, were at most one week old. If through unforeseen circumstances the extraction had required more time than that, no sugar analysis was made.

The following are the average data, taken from determinations obtained from different extracts.

1. Ungerminated seed.	
In 5 cc extract present before fermentation	0.082 mgr glucose
In 5 cc extract present after fermentation	0.072 mgr glucose
Carbohydrates present in 5 cc extract	0.010 mgr glucose
2. 24 hours germinated seed.	
In 5 cc extract present before fermentation	0.038 mgr glucose
In 5 cc extract present after fermentation	0.016 mgr glucose
Carbohydrates present in 5 cc extract	0.022 mgr glucose

3. 48 hours germinated seed.

In 5 cc extract present before fermentation	0.030 mgr glucose
In 5 cc extract present after fermentation	0.039 mgr glucose
Carbohydrates present in 5 cc extract	—0.009 mgr glucose

The negative value of the carbohydrate content of the seed germinated in 48 hours fell below the limit of accuracy. One can say rightly that the carbohydrate content in this case is not determinable chemically. The same can be said of the carbohydrate content in the dormant seed.

One could say that the carbohydrate content of the seed germinating during 24 hours is 0.2%, expressed in % glucose. With regard to the complicated determination method, however, it is safer to conclude that qualitatively a minimum content of carbohydrates can be present.

The quantitative analyses of the sugars agree perfectly with the microchemical tests. In no one of the 3 stages studied was a quantity of sugar present that was worth mentioning: therefore, in no case oil is converted into stabile sugars, when mustard-seed begins to germinate.

The possibility that the oil was converted into transitory labile glucose, but immediately afterwards transformed to CO_2 and H_2O, still remains.

It is clear that after this quite negative sugar analysis there is no question of differentiating between different sugars.

CHAPTER V.

Chemical and Physical Changes of Oil during Germination.

§ 1. *Review of the Literature.*

Since the experiments of Hellriegel (62), Sachs (122) and Detmer (24), it is tacitly assumed in biochemistry that during germination of oily seeds oil has been converted into sugar, before respiration or synthesis of new cells may take place. Most authors only tested, therefore, how much oil disappeared and what quantity of sugar appeared from it.

A survey of these investigations is given by Detmer (24) for the older publications, by Miller up to 1910 and 1912

(99, 100), by Matthes (96) and Bürckle (14) until 1927 and 1929. Furthermore, the papers of Iwanow (71), Cynberg (21), Ermakoff (31), Malhotra (92, 93), Daggs (22), Murlin (105, 106) and Zeller (157) must be mentioned.

All authors stated a decrease of oil and an increase of carbohydrates. Only a few authors (Godlewski 44, Pringsheim (117) suggested that oil may be used directly as a respiration substrate.

One of the greatest objections to be made to most experiments (those of Zeller excepted) is that in regard to most carbohydrate determinations, such analytical errors (e.g. no determination of residual reduction!) have been committed that only qualitative, but no quantitative conclusions are warranted.

In any case, it is very likely that part of the oil, at least, is converted into carbohydrates.

Only Cynberg (21) could not find any carbohydrates, neither in the dormant seed nor in the germinating seed. She worked on *Brassica raphanus,* of which one may expect almost the same chemical composition as *Brassica nigra.* In the latter no carbohydrates could be found either (Chapter IV)!

These investigations are markedly different from those made by Zeller (157) with *Cucurbita pepo,* which seed already contains carbohydrates in the latent stage. Therefore, it is not at all improbable that in the first days of germination Brassica uses its oil in a different way as Cucurbita.

Further objections may be made to the fact that most authors studied the changes occurring during germination at long intervals of time, Detmer (24), for instance, after 4, 7 and 10 days, Iwanow (71) after 4, 8 and 12 days, Miller (99, 100) after 3 or 4 days of germination.

An exception to this objection must be made for the investigations of Matthes (96), Malhotra (92), Murlin (106), Ermakoff (31) and Zeller (157), which authors state the changes in the seed during germination in short intervals of time.

While an extensive literature exists on the change of the oil into carbohydrates, it is very scarce on the changes of composition of the oil itself.

The oldest observations to be found were those of v. Fürth (42), who found an unchanged iodine number of the oil of 4 weeks-germinated linseed and of 9 days-germinated ricinus-bean. The germination, however, took place in the light!

On the other hand, Miller (99, 100) noted a decrease of

the iodine number and an increase of the acid number for seeds of *Helianthus annuus*. The germination, however, took place in the light and the temperature during germination was not kept constant.

In regard to the iodine number, Iwanow (71) confirmed Miller's observations for linseed, hemp-, *Brassica Napus* and poppy seed. For the same objects, he stated a decrease of the hexabromide[1]) number and of the esterfication number [2]). Germination took place in the dark, the temperature was about 25° C.

The observations of Matthes (96) are unreliable, because the germination of the sunflower-seed took place in the light and not at constant temperature. Besides, duplicates from the same extraction, not from different extractions, were made. On the whole, Matthes stated a fairly great oscillation of his constants (refraction, iodine, acid, Polenske[3]) and Reichert-Meiszl number[4]).

In contrast with v. Fürth, Ermakoff (31) examined the first stages of germination of linseed; he observed constancy of iodine number. That, with constancy of iodine number, still a great change of the oil components is possible is shown by the experiments of Bauer (6) in regard to the changes of oil during the ripening of sunflower-seed. A great change of the amount of linolic, linoleic and oleic acid was stated in that case. Ermakoff found a decrease, afterwards an increase of the acid number, the refraction remaining constant.

Zeller (157) determined only the change of the total fatty acid content; he found a strong decrease, running parallel with an increase of carbohydrates.

To gain insight into the change of composition of fats, one may examine the behaviour of lower plants on a substrate containing fats or fatty acids.

One of the oldest sets of observations so far, those of van Tieghem (138), showed the normal development of a yeast species, of different *Mucor* species and especially of *Penicillium glaucum*, on a fat substrate. The moulds were able to cause an increase of the acid number of the fat, which indicates that the fat is decomposed extracellularly in glycerol and fatty acids.

Stärkle (132) indicated for *Aspergillus* a decomposition

[1]) denotes the amount of linolic acid present.

[2]) denotes the average molecular weight of the fatty acids.

[3]) indicates the amount of less volatile acids.

[4]) indicates the amount of more volatile acids.

of fatty acids with less than 14 C-atoms to ketones, if NH_3 is present, according to the reaction of Dakin:

$$R.CH_2.CH_2.COONH_4 \xrightarrow{+O} R.CHOH.CH_3. \xrightarrow{-2H} R.CO.CH_3$$

Den Dooren de Jong (26) investigated different bacteria (especially *Pseudomonas* and *Mycobacterium)* attacking oleic acid. They were unable, however, to do so with stearic and palmitic acid.

Kluyver (79) found, that sulfate reducing bacteria were able to oxidise organic substances simultaneously, for instance stearic and palmitic acid;

1) $C_{15}H_{31}\,COOH + 30\,H_2O \rightarrow 16\,CO_2 + 92\,H$
2) $H_2SO_4 + 8\,H \rightarrow H_2S + 4\,H_2O$

The reactions indicated are the result of a series of primary reactions, consisting of hydrogenations and dehydrogenations.

Especially German authors (vide the review given by Küssner 83) indicate that the fat is decomposed extracellularly by the moulds.

Flieg (35) observed for *Aspergillus niger* a conversion to transitory starch after saponification of the fat.

Küssner investigated the changes in fats caused by the action of *Phycomyces nitens, Aspergillus niger* and *Penicillium glaucum* by determination of several oil constants. He made, however, several serious mistakes in his analytical methods. For instance, after the mould had been growing on a mixture of aqueous nutrient medium and fat, the fat was not dried, but was analysed as such. Further, after the first superficial division of fat and liquid, the dried mould was extracted with petroleum ether. The weight of the evaporation-residue is stated as pure fat; examination for other substances present were not made. Küssner determined the iodine number, hydroxyl [1]), esterfication, Polenske, Reichert-Meiszl and acid number. He observed an increase of the acid number, a decrease of the esterfication number, oscillation of the hydroxyl number, the iodine number and the fat content, the fats being triolein, tripalmitin or sesame oil. Glycerol always disappeared immediately.

Another possibility to gain insight into the mechanism of fat decomposition is to determine certain intermediate products, whose existence is expected, by an „Abfangverfahren". Pirschle (114)

[1]) indicates the amount of hydroxyl groups.

showed acetaldehyde as a possible intermediate product of fat decomposition by this method.

By infiltration of the seeds with certain substances or by addition of them to the autolysed extract of the seeds of *Cucurbita pepo*, Zeller tried to cause a shift of the equilibrium: fat ⇄ sugar. An increase of starch content was found, indeed, by addition of sorbic acid, aceto-acetic acid and acetaldehyde, for instance.

With regard to the several theories about fat decomposition, these have to be accepted with much reserve. Kostytschew [2]) (81) rightly says: „Der Vorgang der Zuckerbildung aus Fett ist zur Zeit noch nicht recht begreiflich".

For lower organisms Buchanan (12) indicated oxidation to ketone-acid, afterwards decomposition to an acid, containing 2 C-atoms less, as follows:

$R.\ CH_2.\ CH_2.\ CH_2.\ COOH + O \rightarrow R.\ CH_2.\ CHOH.\ CH_2.\ COOH + O \rightarrow R.\ CH_2.\ CO.\ CH_2.\ COOH + H_2O \rightarrow R.\ CH_2.\ COOH + CH_3.\ COOH$, etc.

Küssner (83) gave the following scheme:

$R.\ CH_2.\ CH_2.\ R' + O \rightarrow R.\ CHOH.\ CH_2.\ R' - H_2O \rightarrow$

$R.\ CH = CH.\ R' + O \rightarrow R.\ CHOH.\ CHOH.\ R' + O \rightarrow$

$R.\ COOH + R'.\ COOH$

Iwanow (71) supposed the acids of the linolic type to be decomposed easily, saturated acids being more inactive.

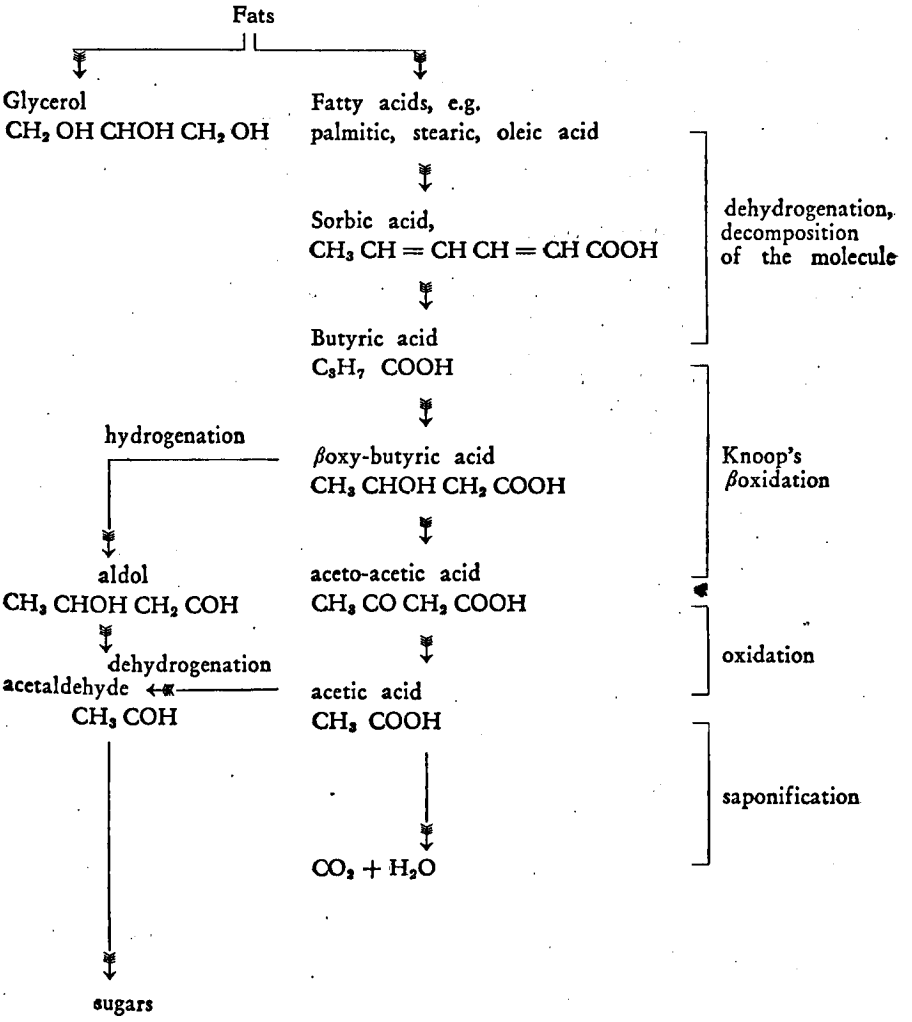
Matthes (96) gave the following hypothesis: The oil is saponified gradually via di- and monoglycerides to free acids and glycerol. Alternately, glycerides with saturated or unsaturated acids are attacked. Glycerol disappears immediately. Fatty acids are subsequently decomposed into lower fatty acids with C-chains of different length. From these acids, oxy-acids and oxy-aldehydes arise, which are converted into carbohydrates.

Zeller (157) supposed the decomposition to take place as follows: See scheme next page.

The oxidation following the scheme of Knoop (80) would take place in lower fatty acids, in contrast to the theory current in animal physiology. Zeller does not stress the fact that in his scheme the possibility also occurs that fatty acid, via acetic acid, is burnt directly to CO_2 and H_2O.

Summarizing one may say that fats certainly have both a respiratory and a storage function in plants. At first, a decomposition

[2]) Pflanzenphysiologie, 1, p. 398.



to glycerol and fatty acid may take place. Glycerol as „Dreikohlenstoffkörper" is used up immediately (K o s t y t s c h e w). Further oxidation of the fatty acids now occurs, either by dehydrogenation or by formation of hydroxyl groups. In both cases decomposition to lower fatty acids takes place, which acids may be changed to carbohydrates, to CO_2 or other substances.

§ 2. *Methods used.*

1*a*. Germination of the seeds, vide Chapter II.

1*b*. Extraction of the seeds. During 5 minutes the germinated seeds are brought to 80° or 90° C., by which action the enzymatic processes are stopped. To prevent possible decomposition or conversion of the oil by higher temperature, the seeds were dried at 40° C. at most. The seeds were rubbed in a mortar and brought in an extraction-thimble quantitatively.

If the latent seed had to be examined, the seed was ground and a certain weight of it brought in the extraction-thimble quantitatively. Always 10 grammes of air-dry seed were taken.

The seed was extracted with ether in a Soxhlet apparatus during 12 hours at least. In the seed no remainders of the oil could be detected microchemically.

Often, petroleum ether is mentioned as extraction liquid (Grün 49), because it should yield more fat. Murlin (106) and Twisselmann (144) showed, however, that the difference between the 2 extraction liquids is negligible, that, furthermore, petroleum ether extracts yield less oxidation products than ethereal extracts. And the latter are the very products of importance for these experiments!

Rightly, Chibnall and Channon (16) say that the ethereal extract only may be called „oil" or „fat", if the amount of the unsaponifiable matter is not large. In our experiments this is the case, so, henceforth, the ethereal extract is called „oil". Besides esters of fatty acids and glycerol, caritinoids, phosphatids, steroles and other substances are extracted.

After evaporation of the ether at 60°—80° C., the remainder, i.e. the „oil", is dried over concentrated H_2SO_4 in presence of strips of paraffin, the latter to withdraw the last traces of ether from the oil.

To check the decomposing influence of light, air and water upon the oil, this was always worked up as quickly as possible and was kept for 14 days at most.

II. *Qualitative Experiments.*

a. Phosphate reaction to prove the presence of phosphatids as indicated in the „Ned. Pharm." (107), at Lecithine, p. 278. The oil is saponified with KOH; after acidification the liquid is filtered off and the filtrate evaporated. After incineration the remainder is dissolved in concentrated HNO_3; a yellow precipitate with ammoniummolybdate indicates the presence of a phosphate, i.e. a phosphatid.

b. Reaction of Liebermann (88) for Steroles.

The oil is dissolved in anhydrous acetic acid. After cooling, a red, afterwards a blue color on addition of a few drops of concentrated H_2SO_4, indicates the presence of steroles.

c. Modified Test of Kreis, according to Lea (84), for Epihydrinaldehyde.

A mixture of 1 gramme of oil, 2 cc benzene and 1 cc concentrated HCl is shaken for 1 minute. Then 1 cc 0.1% solution of phloroglucin in peroxide-free ether is shaken again for 1 minute. By centrifuging the aqueous layer is separated, which has, if epihydrinaldehyde is present, a red rose color. The amount of epihydrinaldehyde may be determined semi-quantitatively by comparison of the color obtained with $KMnO_4$ solutions (0.2 gr $KMnO_4$ in 1000 cc water and diluting in four subsequent steps each time to 0.4 of its strength).

III. *Physical Constants.*

As for the chemical methods to be mentioned yet, duplicate numbers are obtained from 2 different extractions.

a. Specific gravity was determined in a pycnometer with ground stopper. The s.g. was calculated at 15°/4° C.

b. Refraction was determined in the refractometer of Abbe, not only at 25° C., but also the change of the refraction from 15°—50° C. was determined.

c. Viscosity.

For this determination the viscosimeter of Ostwald was used. With regard to the precautions taken, reference is made to the paper of Bungenberg de Jong (13). The relative value $\frac{\text{time of flow of oil}}{\text{time of flow of distilled water}}$ at 30° C. was calculated. Calculation of absolute viscosity was superfluous, because only the relative changes of the oil viscosity were of importance to us.

IV. *Chemical Methods.*

a. Dry weight was determined by drying until constant weight at 103—105° C. of 1 gramme of air-dry seed or of 1 gramme of seed, germinated for 24 or 48 hours.

b. Acid Number, i.e. the number of mgr KOH necessary for the neutralisation of free acid, present in 1 gramme of oil. The determination as described in the „Warenwet" (147) was used. 5 gr oil are dissolved in 30 cc of a neutral mixture of alcohol and ether and titrated with 0.1 n alcali and phenolphtalein as indicator.

c. Unsaponifiable Matter is determined following the description found in the „Analysemethoden voor den handel in vetten, oliën en oliezaden" (2). About 5 gr. oil are boiled in a reflux with 50 cc alcoholic alcali in a waterbath. After quantitative transfer with 30 cc distilled water to a separatory funnel, the liquid is shaken with 40 cc petroleum ether. When the layers have been separated, the aqueous layer is brought into another separatory funnel, the ethereal solution being deposited in a flask, previously weighed. Two or three subsequent times, the aqueous liquid is shaken, as indicated above, with 25—30 cc petroleum ether. From the assembled ethereal extracts the petroleum ether is distilled off and the remainder, dried at 80° C., is weighed as „unsaponifiable matter".

This constant is important, because the „unsaponifiable matter" may be considered often as the accumulation of the end-products of metabolism (vide Jordan and Chibnall 72).

d. Amount of Allylisocyanate, which indicates, how much glucoside is present by determination of its aglucon allylisocyanate. The method used is taken from the „Commentaar" IV, p. 112 (45). 5 gr powdered seed or seed germinated for 24 or 48 hours and rubbed in a mortar, are brought in a Erlenmeyer with 100 cc distilled water. After closing, the Erlenmeyer is kept for 2 hours at room-temperature, the flask being swung at intervals. The enzyme myrosin has converted the glucoside sinigrin into allylisocyanate, glucose and $KHSO_4$.

$$\underset{\text{sinigrin}}{C_3H_5.NC.(OSO_2OK.)S.C_6H_{11}O_5} + H_2O \xrightarrow{\text{myrosin}} \underset{\text{allylisocyanate}}{C_3H_5.NCS} + C_6H_{12}O_6 + KHSO_4$$

After addition of 90% alcohol, by which the enzymatic reaction is stopped, the liquid is heated slowly until boiling, care being taken that not too much foaming occurs. Then, heating strongly, the allylisocyanate is distilled over in a measuring flask of 100 cc, which contains already 10 cc 10% NH_4OH. An excess of $AgNO_3$, i.c. 25 cc 0.1 n $AgNO_3$-solution, is added, when about 50 cc of the liquid have been distilled off. To precipitate the Ag_2S completely the flask is kept for 12 hours, then filled up to 100 cc. The following reaction has taken place:

$$\underset{\text{allylisocyanate}}{C_3H_5.NCS} + 3\,NH_3 + 2\,AgNO_3 \rightarrow \underset{\text{allylcyanamid}}{C_3H_5.NH.CN} + Ag_2S + 2\,NH_4NO_3$$

After filtering off, 6 cc concentrated HNO_3 and 1 cc satu-

rated solution of iron-alum is added to 50 cc of the filtrate and the excess $AgNO_3$ titrated back with KCNS (= a cc). The procentual amount of a allylisocyanate is then: 2 × (12.5-a) × 0.099%.

f. Oxygen-absorbing Capacity of the oil is determined according to Grün, 1, p. 284 (49). A drop of oil is brought on each of 2 tared glass plates, surface 10 × 10 cm, and divided carefully upon the whole surface. By keeping the plates sloping for half an hour, the superfluous oil is removed. After weighing the plates with the oil they are brought under a darkened bell jar, while air is led through during the course of the experiment.

So the action of light upon the oxygen-intake of the oil is excluded. The plates were weighed every day until an increase of weight, subsequent to the preliminary decrease, occurs. The maximum increase of weight was noted. In spite of all precautions taken duplicates were not at all satisfactory, so only a qualitative value may be attached to this experiment.

f. Iodine Number, i.e. the amount of I_2 in grammes, taken up by 100 grammes of oil, which is a measure of the amount of non-conjugated double bonds present in the oil. The method Moszler-Winkler, as indicated by the „Ned. Pharm." (107), under Olea pinguia, p. 313, was used. This method is simple and convenient. For iodine numbers, higher than 200, too low values may be obtained. But this was of no influence on our experiments, because such high values were never obtained. The determination runs as follows. In a flask with ground stopper of 300 cc, 150—200 mgr oil are weighed, dissolved in 10 cc CCl_4 and mixed with 25 cc 0.2 n $KBrO_3$, 5 cc KBr (30 = 100) and 7 cc diluted HCl. The flask is closed immediately, shaken and kept, for one hour at least, in the dark, while swinging round repeatedly. As quickly as possibly, 10 cc KI (166 gr KI/L) are added, the excess I_2 is titrated with 0.1 n $Na_2S_2O_3$.

If all precautions are taken, the iodine number is reliable to a tenth of one percent.

Substitutions, not addition of I_2, is possible, when hydroxyl groups are present;

$$R.CHOH + HI \rightarrow R.CHI + H_2O$$

Unsaponifiable matter may also take up iodine (steroles with hydroxyl groups!).

The properties of isomeric oleic acids were studied by Eckert and Halla (28). They stated that the double bond is easily shifted by addition and subsequent splitting off of the added substance. For instance, the reaction with HI is:

$$R.\,CH_2.\,CH = CH.\,R_1 \xrightarrow{+HI} R.\,CH_2.\,CHI.\,CH_2.\,R_1 \xrightarrow{-HI} R.\,CH = CH.\,CH_2.\,R_1$$

Pure 9—10 oleic acid (with the double bond between the 9th and the 10th C-atom from the carboxyl group) may add the theoretical 89.4% I_2 indeed, 2—3 oleic acid, however, only 9.05%, 3—4 oleic acid 16.27%, 4—5 oleic acid 26.96% I_2.

So, while considering the value of the iodine number, this complication has to be taken into account. In oils, extracted from latent oily seeds, mostly 9—10 oleic acid occurs. But what happens to it during germination is absolutely unknown.

In the literature, no agreement exists in regard to the composition of the fatty acids of mustard-seed oil. Wehmer (149) mentioned from older publications:

5.6% stearic, arachic and behenic acid ($C_{17}H_{35}.COOH$, $C_{19}H_{39}.COOH$, $C_{21}H_{43}.COOH$);
78% oleic acid ($CH_3.(CH_2)_7.CH = (CH.(CH_2)_7.COOH$);
4.5% linolic acid ($CH_3.(CH_2)_4.CH = CH.CH_2.CH = CH.(CH_2)_7.COOH$);
6.5% linoleic acid ($CH_3.CH_2.CH = CH.CH_2.CH = CH.CH_2.CH = CH.(CH_2)_7.COOH$).

Grün (50), however, indicated for English mustard-seed (iodine number 104, unsaponifiable matter 3.3%);
traces of stearic and arachic acid;
2% palmitic acid ($C_{15}H_{31}.COOH$);
2% lignoceric acid ($C_{23}H_{47}.COOH$);
24.5% oleic acid;
50% erucic acid ($CH_3.(CH_2)_7.CH = CH.(CH_2)_{11}.COOH$);
19.5% linolic acid;
2% linoleic acid.

The large amount of unsaturated fatty acids in the oil is striking in any case. But just for these unsaturated acids the discrepany between the data given by different authors is the greatest.

The presence of dehydrogenases (vide Chapter III) could make it possible that more double bonds are produced during germination. Therefore, it would be of great importance to determine quantitatively the changes of the amount of the unsaturated fatty acids in the different stages mentioned. Different methods are available, but none of them seems reliable.

Kaufmann (74) stated that $(CNS)_2$ may be added to the double bond in oleic acid, but in linolic acid only to one of the 2 double bonds, this in contrast to the iodine number,

where I_2 is added to all double bonds available. From the difference between iodine and thiocyanate number, the amount of oleic and linolic acid may be determined in a mixture of both. Also for compounds with 3 double bonds, such as *β*elaeostearic acid, $(CNS)_2$ could be added only to one double bond. Kaufmann (75), however, has worked with chemically pure compounds, seldom with mixtures.

Groszfeld (48) stressed the fact that especially mixtures of erucic, oleic, linolic and linoleic acid, are separated with difficulty. And mustard-seed oil happens to be just such a mixture!

Hilditch (65), while asserting that with his methods a quantitative separation of fatty acids is possible, declares, however, that this is only possible with simple mixtures and only for the acids present in excess.

v. Loon (90) found that stereo-isomers of unsaturated fatty acids could give different thiocyanate numbers. The possibility that such isomers are present in the mustard-seed oil is not at all excluded.

Watermann (148) refers to the fact that the thiocyanate number has not been controlled for all unsaturated fatty acids present in oils, that the influence of small impurities in oil, such as coloring agents, on this number is still unknown.

Another method to separate the different unsaturated fatty acids quantitatively is by addition of Br_2, producing bromides with different solubilities in ether. So linolic acid gives a soluble tetrabromide, linoleic acid an insoluble hexabromide.

v. Loon (91), however, showed this method not to be quantitative for oil of the seed of *Cheiranthus Cheiri.* At most, he could get an approximate value for linoleic acid. The solubility of the tetrabromide, however, is so strongly influenced by the presence of other bromides that no quantitive determination of linolic acid is possible.

Eckstein (29) added known quantities of linolic and linoleic acid to butter; he recovered at most 22% of the linolic acid and 50% of the linoleic acid!

Bengis and Anderson (8), following the bromide method, found too low values for linolic acid in the oil of the coffee-bean.

Explicitly, Snider and Bloor (129) also indicate that a quantitative separation of the unsaturated fatty acids by their bromides is insufficient. Different isomers, for instance, produce bromides with a different solubility in ether. With the method of Kaufmann they also found discrepancies.

The difficulties arising in the separation and the quantitative determination of the saturated fatty acids seem even greater than those for the unsaturated fatty acids (vide Grün 49 and Groszfeld 48). These acids will not be considered, because they only constitute a very small fraction of the mustard-seed oil.

So, for the time being, a quantitative determination of the fatty acids in the different stages of germination had to be abandoned.

§ 3. *Results of the Experiments.*

The phosphate reaction was always positive, the same was the case with the Liebermann reaction. So phosphatids and steroles are present in the oil during the three stages of germination studied.

An attempt to find intermediate products of the decomposition of the oil was made with the Kreis test. For in rancid oil the following decomposition may take place:

$$CH_3.(CH_2)_7.CH = \underset{9.\ 10.\ \text{oleic acid}}{CH.(CH_2)_7.COOH} \rightarrow$$

$$CH_3.(CH_2)_{14}.CH = \underset{2-3\ \text{oleic acid}}{CH.COOH} \xrightarrow{+O_2 - H_2O}$$

$$CH_3.(CH_2)_{12}.CH = CH.CH{-}CH.COOH \xrightarrow{O_2}$$
$$\backslash O /$$

$$COH.CH{-}CH.COOH \xrightarrow{-CO_2}$$
$$\backslash O /$$

$$CH_2.CH.COH$$
$$\backslash O /$$

epihydrinaldehyde

Epihydrinaldehyde produces the red rose color of the aqueous solution. This reaction, however, proved to be always negative in our experiments. So it is probable, that the decomposition of the oil during germination seems to follow another course.

Oil constants were always calculated for 1 or 100 grammes of oil. In analogy with the so-called „Einkeimlingszahl" of Matthes and Bürckle, three constants, i.e. iodine and acid number and unsaponifiable matter were also reduced to the amount of oil, present in 1 gramme of seed. As indicated already in Chapter II, in 1 gramme of seed approximately the same number of seeds is present, so our constants „per 1 gramme of seed" are comparable

with the „Einkeimlingszahl". So an insight is gained how the composition of the oil per seed is changing during germination. For instance, the acid number being 2.25 and the oil content 28.25%, the acid number per 1 gramme of seed is 0.2825 × 2.25 = 0.6356.

A great difficulty, which arises, when experimenting with *Brassica nigra*, is its low germination value. When germinated on filter paper in Petri dishes at 30° C. in a thermostate, a value of 40% was found after one week of germination. Therefore, the values obtained of 24 or 48 hours germinating seeds are always too low.

To compensate for this factor somewhat, all values were calculated for 100% germination. One must have in mind, however, that, where germination is very irregular, the germination value after 24 and 48 hours still will be lower than 40%, so the real values of 100% germination will be higher than those mentioned. The constants per 1 gramme of seed are calculated for 100% germination. The data found and calculated are given in the following tables.

Refraction of the oil from 15—50° C.

Temperature in degrees centigrade	*Dormant seed*	*Seed germinated for 24 hours*	*Seed germinated for 48 hours*
15	1.4762	1.4764	1.4758
20	1.4742	1.4749	1.4739
25	1.4725	1.4729	1.4718
30	1.4704	1.4710	1.4700
35	1.4689	1.4692	1.4682
40	1.4670	1.4672	1.4664
45	1.4651	1.4656	1.4647
50	1.4633	1.4638	1.4629

1. Dormant seed. 2. Seed germinated for 24 hours. 3. Seed germinated for 48 hours.

	Color	*Odor*
1.	Yellowish green	Oily
2.	Faintly yellowish green	As mustard
3.	Yellow	As mustard

	Data found	*100% germination*	*Data per 1 gramme of seed*
Oil content in %			
1.	28.25	28.25	
2.	25.23	20.70	
3.	21.96	12.52	
Specific gravity			
1.	0.9187	0.9187	
2.	0.9154	0.9105	
3.	0.9142	0.9075	
Refraction at 25° C.			
1.	1.4724	1.4724	
2.	1.4730	1.4739	
3.	1.4717	1.4707	
Viscosity			
1.	57.7	57.7	
2.	57.0	56.6	
3.	66.4	79.5	
Dry weight in grammes			
1.	0.9123	0.9123	
2.	0.8770	0.8241	
3.	0.8784	0.8276	
Oil-free dry weight in grammes			
1.	0.6298	0.6298	
2.	0.6247	0.6171	
3.	0.6588	0.7024	

	Data found	*100% germination*	*Data per 1 gramme of seed*
Acid number			
1.	2.25	2.25	0.6356
2.	2.17	2.05	0.4244
3.	2.94	3.98	0.4983
Unsaponifiable matter			
1.	2.38	2.38	0.6724
2.	2.42	2.48	0.5134
3.	4.38	7.41	0.9277
Amount of allylisocyanate in %			
1.	0.92	0.92	
2.	0.69	0.35	
3.	0.68	0.32	
Oxygen absorbing capacity in %			
1.	1.30		
2.	0.45		
3.	0.15		
Iodine number			
1.	108.3	108.3	30.59
2.	112.5	118.3	24.59
3.	113.1	120.3	15.06

§ 4. *Discussion.*

The refraction from 15°—50° C. is decreasing gradually in all stages of germination, nothing could be concluded from it.

Rightly, Boekenoogen (9) stated that the greatest caution has to be taken, when conclusions from the determined constants are derived, plant oils being a mixture of many glycerides. Therefore, it seems to be very premature to derive such far-reaching conclusions from chemical constants as done by Matthes and Bürckle.

Moreover, the value these authors put on their „Einkeimlings-

zahlen" may be much too great. Decrease of iodine number, for instance, may mean one of two things. Either (and only this is mentioned by the authors) the double bonds may have become saturated and more saturated compounds are produced. But secondly, it may mean also the disappearance of compounds with double bonds, being broken down completely to CO_2 and H_2O, while so many unsaturated acids are left or newly formed that the iodine number of the oil may be increasing.

Further, the authors overlooked the complications that are innate in the determination of the iodine number, as already indicated.

After the determination of several chemical and physical constants, at most one may conclude, which process is „taking the lead". Subtle, but maybe important changes, remain concealed.

In regard to the constants of the dormant seed, the following values were mentioned in the literature:

Oil content; Wehmer (149) 29—33%, Huber and v. d. Wielen (67) 25.7—33.7%, Benessayag (7) 25%.
Specific Gravity; Grün (50) $15°/15°$ 0.9143—0.9230, Huber and v. d. Wielen $15°/15°$ 0.919—0.923.
Refraction; Grün, at 40° C. 1.4655, at 20° C. 1.4739, Huber and v. d. Wielen at 22° 1.4712—1.4731.
Acid number; Grün 1.4—12.0.
Unsaponifiable matter; Grün more than 1%.
Amount of allylisocyanate; Huber and v. d. Wielen 0.63—1.23%, Ned. Pharm. 0.7% required at least, Benassayag 0.9—1.1%.
Iodine numbers; Grün 65—107, Huber and v. d. Wielen 113.5—120.

All constants, found by us, are within the given limits.

In regard to the changes of the constants during germination all authors mention a decrease of oil content. The only exception found is made by Ermakoff (31), who indicates that linseed after 18 hours germination did not show a noticeable decrease in oil content. This would confirm our hypothesis, given in Chapter III, that the oil of linseed is not attacked immediately, a possibility also mentioned by Ermakoff.

Only the observations of Hafenrichter (54), who worked with 2 varieties of soy-beans, those of Matthes (96) on sunflower-seed, those of Ermakoff on linseed and those of Zeller (157) on seeds of Cucurbita pepo are comparable with ours, because those authors studies the first stages of germination by the deter-

mination of several oil constants. Still, precautions must be taken, when comparisons are made. For an indication of the time, in which germination of a specific seed stopped, does not at all guarantee the same stage of germination, the „speed" of development being characteristic for each species (vide Chapter I). It is probable, moreover, that the composition of the reserve stuff greatly influences this „speed" qualitatively as well as quantitatively.

In regard to the research of Matthes, only the first 4 of his 10 stages of germination will be mentioned in this paper. See table p. 60.

Differences of our constants with those mentioned are the following. Refraction; Matthes only found decreasing values, in this paper the values increase and subsequently decrease. Oil-free dry weight; Matthes found decreasing values, in this research the values increase after a small decrease. Iodine number; Ermakoff and Matthes found practically constant values, in this paper an increase is found. Dry weight; Zeller, calculating the mean error, concludes that dry weight of *Cucurbita pepo* does not decrease during germination. For the other investigations always a more or less decrease of dry weight, comparable to ours, was observed.

For the estimation of the found values, we refer to the information given by Grün (49).

In general, when plotting the found values against time, three types of curves are obtained.

1. Curves, in which the changes are gradual, i.e. a straight line, either ascending or descending, is obtained. To this group belong iodine number per 1 gramme of seed, oil content (fig. 10), specific gravity.

2. Curves, where the change from latent to 24 hours germination is small, from 24 till 48 hours germination, the change being great. To this group belong; refraction (fig. 11), viscosity (fig. 11), oil-free dry weight, acid number, unsaponifiable matter, the latter per 1 gramme of seed included.

3. The opposite of 2. To this group belong; dry weight, amount of allylisocyanate (fig. 12), oxygen-absorbing capacity, iodine number (fig. 12).

A possible explanation of these phenomena could be the following.

1. Because the sugars are absent (Chapter IV), only oil has been used in germination of *Brassica nigra*. The only conclusion one can make from the numbers, obtained for specific gravity

	Hafenrichter	Matthes	Ermakoff	Zeller
	72 hours germinated. On dry weight of dormant seed	1) dormant seed 2) 75 hours germinated 3) 103 hours germinated 4) 125 hours germinated	1) dormant seed 2) 17 hours germinated 3) 70 hours germinated	1) 3 days germinated 2) 4 days germinated 3) 5 days germinated
Dry weight	Decrease 4.48% 1.85%	Einkeimlingszahl 1) 55 mgr 2) 47 mgr 3) 47 mgr 4) 46 mgr	Of 10 grs seed 1) 9.5200 gr. 2) 9.3070 gr. 3) 9.0506 gr.	Einkeimlingszahl 1) 0.308 ± 0.041 gr. 2) 0.308 ± 0.058 gr. 3) 0.331 ± 0.060 gr.
Refraction		n_{25} 1) 1.4728 2) 1.4726 3) 1.4724 4) 1.4729	n_{20} 1) 1.4727 2) 1.4726 3) 1.4727	
Oil-free dry weight		Einkeimlingszahl 1) 27.3 mgr 2) 27.7 mgr 3) 24.1 mgr 4) 24.9 mgr		
Acid number		1) 0.4 2) 0.25 3) 0.3 4) 0.8 Einkeimlingszahl $\times 10^3$ 1) 11.1 2) 5.8 3) 6.9 4) 16.9	1) 0.71 2) 0.37 3) 9.00	
Iodine number		1) 130.9 2) 131.2 3) 131.9 4) 133.2 Einkeimlingszahl $\times 10^3$ 1) 36.3 2) 30.6 3) 30.2 4) 28.1	1) 172.7 2) 172.5 3) 171.6	

(while keeping in mind that a decrease may be caused by many opposite influences) is, in connection with the other constants, that probably a decomposition of the glycerides has taken place. In that case, the fatty acids present are decomposed chiefly after 24 hours germination (vide acid number per 1 gramme of seed). Decrease of iodine number per 1 gramme of seed indicates disappearance of double bonds. The mechanism of this process,

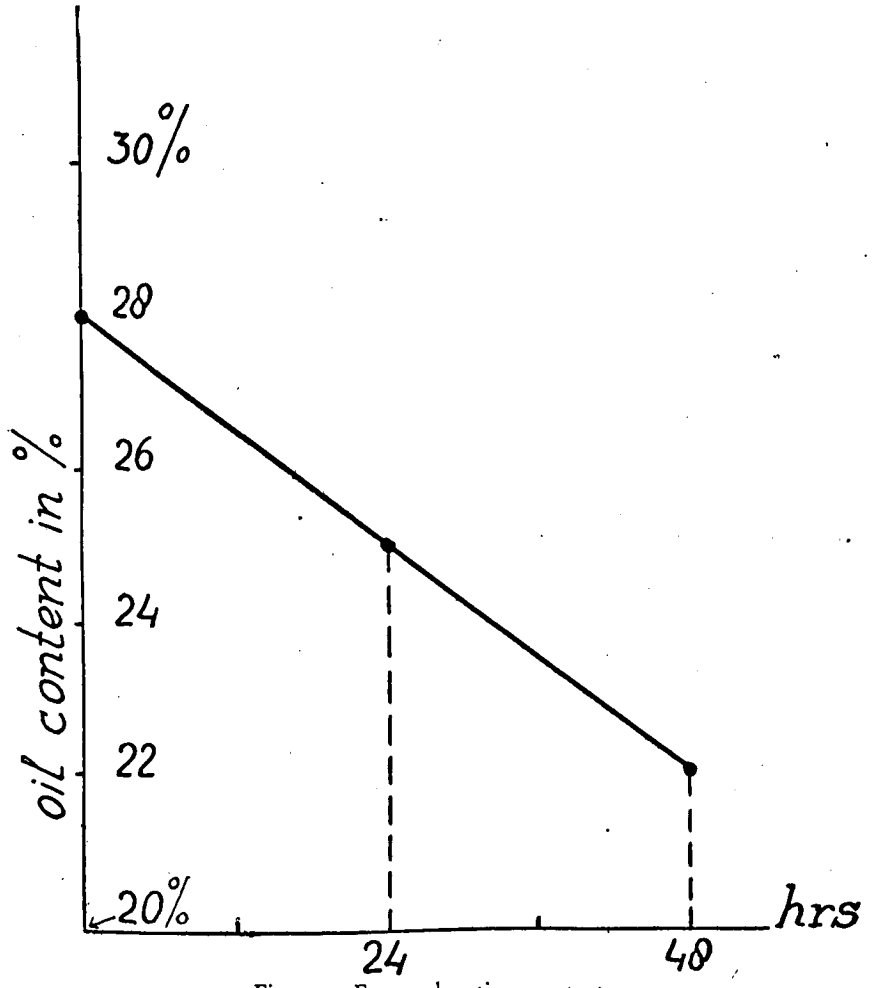


Fig. 10. For explanation see text.

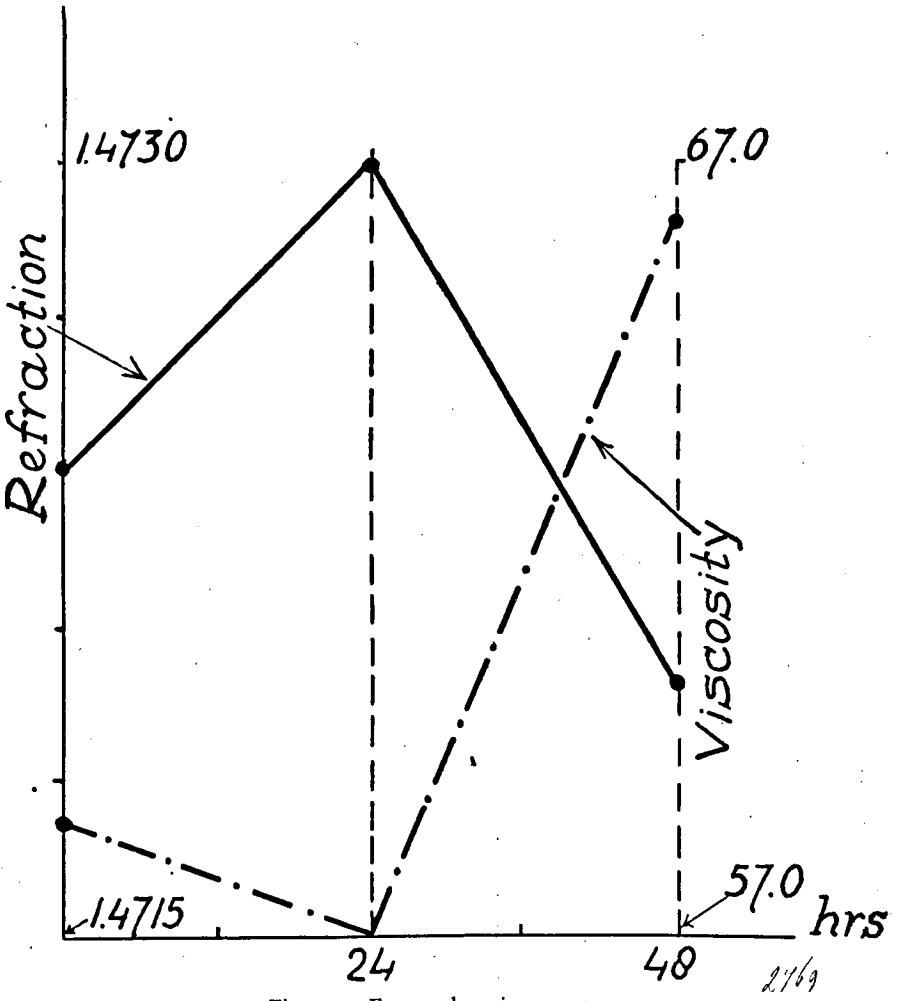


Fig. 11. For explanation see text.

whether by saturation or by decomposition of the fatty acids, must be left undecided until the discussion of the other values.

2. The increase, however small, of the refraction after 24 hours germination, makes it more probable that the oil becomes more unsaturated than in the latent seed, that dehydrogenation must have taken place. Other processes may be going on, too,

but the dehydrogenation process is the most obvious one. It is improbable that not more double bonds, but more hydroxyl groups are produced, because in that case the viscosity would have changed more. Greater possibility for the production of more hydroxyl groups is given after 48 hours germination in regard to the increase of the viscosity.

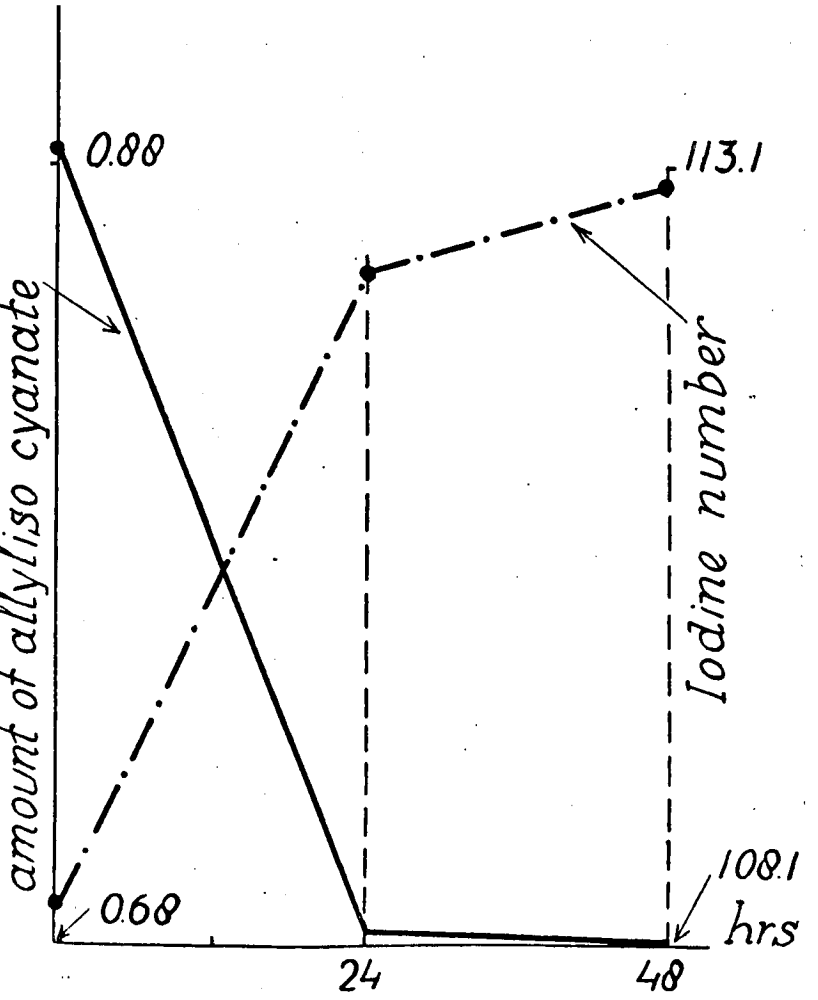


Fig. 12. For explanation see text.

Refraction, on the whole, is more influenced by an increase of double bonds than of hydroxyl groups. Therefore, there is no discrepancy between this supposition and the decrease of refraction after 48 hours germination. Further, the increase of the acid number as well as the increase of the unsaponifiable matter may also have a decreasing effect on the refraction. The unsaponifiable matter, probably, may also cause an increase of the viscosity.

Though reserve is essential, when drawing conclusions from the changes in viscosity, still one gets the impression that after 48 hours germination, the ethereal extract has lost more of its "fatty nature" than after 24 hours.

The strong increase of the oil-free dry weight after 48 hours germination would indicate the decomposition products of the oil to be changed to components of the young plant. So beside the use of the oil as respiration substrate, it would serve at the same time as synthetisizing material. Conform with this should be the increase of the unsaponifiable matter.

The increase of the acid number might indicate that the decomposition of the glycerides after 48 hours germination outweighs the disappearance of the free acid.

3. The rapid decrease of the amount of the allylisocyanate after 24 hours germination is evident, for Benassayag (7) mentions the decomposition of the glucoside by an increasing amount of water. Wehmer (149) mentions for young plants an amount of allylisocyanate of 0.17%. The role of the glucoside during germination is unknown. Observed by us (Chapter IV), also by Frey-Wissling (40), it remains a remarkable fact that the enzyme does not disappear during germination.

The acid number per 1 gramme of seed indicates clearly that after 24 hours germination acids have disappeared. After 48 hours this process is less pronounced, the decomposition of the glycerides is predominant.

The decrease of dry weight is so small that no conclusions may be drawn from it.

The increase of the iodine number after 24 hours germination is probably caused by the production of more double bonds (vide dehydrogenases and refraction). The simultaneous increase of iodine number and refraction show the improbability of a shift of the double bond in the direction of the carboxyl group.

Because O_2 is taken up chiefly by linolic and linoleic acid, it seems probable that the decrease of saturation is not caused by

the production of more linolic and linoleic acid (vide oxygen-absorbing capacity), but of other unknown acids. This is in conformity with the paper of Grande, who could not prove the presence of linolic or linoleic acid after the addition of dehydrogenases on oleic acid.

It is difficult to decide, if after 48 hours germination a shifting of the double bonds has taken place. The probability, however, is small, because otherwise the Kreis test should not have turned out to be negative. For, when it had been positive, 2—3 oleic acid must have been present.

The small increase of the iodine number may have been caused by the presence of more hydroxyl groups.

It was tried to increase the iodine number of the oil extracted from the dormant seed in the presence of air. This has succeeded with 2 methods.

1. *Spraying of the Oil* [1]).

Because the oil in the plant cell is present in small drops, it was tried to get the same condition experimentally by spraying the oil in very small drops (diameter about 1 μ) in air. The sprayed oil had a clearer color than the original. Immediately after the spraying, the iodine number of the sprayed and the original oil was determined, of the latter it is a little higher than mentioned before, because another batch of mustard-seed was used.

Iodine number before spraying 109.3.
Iodine number after spraying 119.0.
The sprayed oil gave a negative Kreis test.

2. *Emulsion.*

Another possibility to divide the oil to a high degree is the preparation of an emulsion. The intention was to make an emulsion as stable and as finely divided as possible.

Clayton (19) mentioned as an excellent emulgator 1% Na-oleate-solution. According to v. d. Minne (101) intermittent shaking may be a method for the preparation of an emulsion. When continuously shaken, the water is beaten into the oil drops; the oil cannot be divided more in the water.

A 20% emulsion was prepared with the oil from the dormant

[1]) I am much indebted to Prof. Dr. W. J. de Haas for the use of the facilities of his laboratory for the spraying of the oil.

seed and a 1% Na-oleate-solution by subsequent addition of small quantities of the Na-oleate-solution to the oil, shaking once or twice, waiting until the water-oil emulsion was settled, shaking anew, etc., until a stable emulsion was obtained. The mean diameter of the oil drops was 8 μ. An aliquot of the emulsion, about 50 grammes, was weighed twice in 2 wash bottles. In 2 wash bottles, moreover, 5 grammes of a mixture of 1 part ground seed and 1 part water was added to the emulsion. In 1 wash bottle a mixture of Na-oleate solution and distilled water was placed instead of oil. Purified, moist air (vide Chapter I) was led through all the 5 flasks during 12 hours. After that the emulsion was not yet broken, at most some „cream" gathered on top of the emulsion. It would be better, if the emulsion had been broken and the oil, thus obtained, had been used for the determination of the iodine number. This, however, was not possible, neither by centrifuging nor by addition of acids or salts. So the iodine number had to be determined in the emulsion itself. Because the effect of an emulsion upon the determination of the iodine number is as yet unknown, the oil before and after emulgating cannot be compared. The deviation obtained, because water had been evaporated, when air had been led through during 12 hours, has been reduced as much as possible by treating the blank Na-oleate-solution the same as the oil emulsions, as indicated above.

The iodine number was calculated on the oil present in the emulsion. The iodine number of the oil, present in the aqueous seed extract, was also taken into account. The values obtained were:

Iodine number of the oil without addition of the seed extract	95.5
Iodine number of the oil with addition of the seed extract ..	154.0

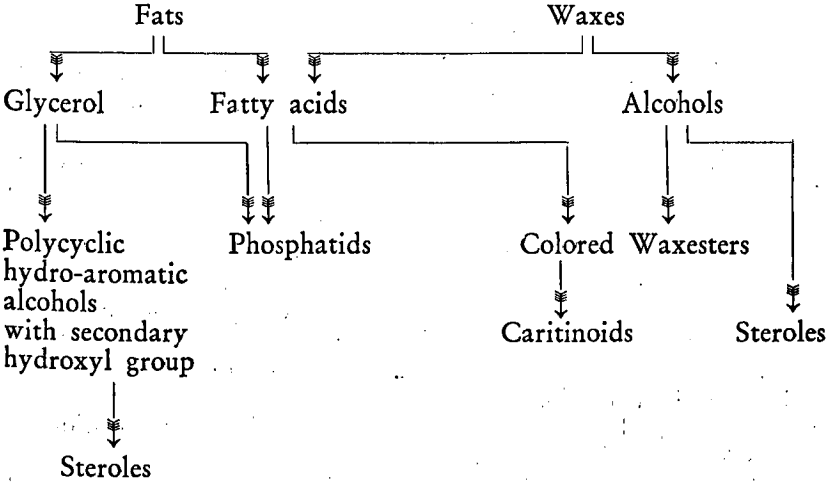
The increase of the iodine number is so great that, though no absolute reliance may be placed upon these numbers, the influence of the seed extract is unmistakable.

Where in Chapter III it was proved that in seed and oil of *Brassica nigra* dehydrogenases are present, the above experiments, spraying and emulsification, give an additional indication of the presence of dehydrogenases.

A comparison of the accepted theories concerning fatty acid decomposition with our own experiments increase the possibility of the dehydrogenation of the fatty acids and the production of hydroxyl groups as found in Zeller's scheme.

Besides, attention must be paid to the probability of synthesis of the decomposition products. Which possibilities one may expect in

the latter case, is indicated in Halden's scheme (57), reproduced here in a very abbreviated manner.



From this scheme it is obvious that phosphatids and steroles, important ingredients of the cell, may be produced by fatty acids, i.e. by fats and oils.

Concluding, it may be said that in *Brassica nigra* after 24 hours germination other processes are predominant than after 48 hours germination. After 24 hours germination the dehydrogenation processes are leading, fatty acids (possibly via sugars) serve as respiration substrate. On the second day the formation of hydroxyl groups should be predominating. At the same time the probability exists that fatty acids or derivatives are used for synthetisizing processes. It is not out of question that the latter are more sensitive to lower oxygen-pressures than the respiratory processes. This might give another explanation of the phenomena described in Chapter I.

In this place, I want to thank Prof. Dr. L. G. M. Baas Becking for his help and for his constructive criticism, which have been a constant source of inspiration to me.

SUMMARY.

1. The influence of decrease of oxygen-tension on the oxygen-intake of germinating starchy and oily seeds was determined gasometrically.

2. This influence appeared variable with the species investigated. *Brassica nigra* as well as *Brassica Rapa* were little affected by decreased oxygen-tension in the early stages of germination. This sensitivity increased in the later stages of germination.

3. Catalase-activity was determined in seeds of *Brassica nigra* in the latent, 24, 48 hour stages by an autographic method.

4. No velocity-constant could be determined from catalase-action in ungerminated seed, the catalase being present probably in a highly labile condition.

5. In 24 and 48 hour germinated seeds the decomposition of the peroxide followed the autocatalytic equation.

6. In *Linum usitatissimum* and *Brassica nigra* the presence of a system H_2donator-dehydrogenases-H_2acceptor could be demonstrated in certain germination stages.

7. A micro-method for the determination of fermentable sugars of *Brassica nigra* was elaborated.

8. At latent, 24, 48 hour germinated seeds, no measurable quantities of fermentable sugars could be found.

9. An ethereal extract was prepared of the same stages of germination of *Brassica nigra.* Several constants were determined from the „oil" prepared from this extract.

10. The constants showed a tendency to change appreciably up to 24 hour germination, while the change between 24 and 48 hour was less pronounced. The opposite phenomena also occur frequently.

11. Due to the simultaneous happening of a great many complicated processes, no far-reading conclusions may be drawn from the results obtained. At most, a certain trend in a definite direction may be observed.

12. It is supposed that after 24 hour germination, in *Brassica nigra* dehydrogenating processes change fatty acids, in which processes oxygen or another substance may serve as H_2acceptor. After 48 hour germination synthetic processes might act upon fatty acid derivatives. The activity of the dehydrogenases decreases, whereas the influence of the oxygen-tension increases.

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