ON THE MECHANISM OF PERIODIC MOVEMENTS OF VARIATION

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(With Tab. XVIII).

CONTENTS.

CHAPTER I.

General introduction.

§ 1. *Movements of plants.*

Insufficient account of the very divergent points of view in distinguishing several types of movement gave rise to a serious confusion in the nomenclature in the field of plant movements.

If we restrict this discussion to movements of members of plants, in the first place a distinction can be made between *growth-movements,* in which growth is concerned, and *movements of variation,* due to changes in turgor.

A second distinction can be made between *autonomous* and *aitiogenous* (=paratonic) *movements.* In the autonomous movements it is not possible to connect them causally with any agent outside the plant, while in the paratonic movements this

is the case. The autonomous movements are due to a mechanism that is entirely determined by the structure of the plant; in plants showing paratonic movements, such a structure can react only with a preformed response to an agent outside the plant. In this latter case, where the agent causes a reaction-type, which is completely determined by the structure of the plant, one speaks of a *nastic movement.* Besides these are those plants, of which the structure is such, that the direction and the type of movement entirely is determined by the agent; this is the case with the *tropic movements.*

Whenever a movement is replicated consecutively in the same manner, whether under the influence of an inducing (periodic) agent or not, one speaks of *periodic movements;* besides, of course, there are purely *aperiodic movements.*

If one would like to summarize the above mentioned points of view in one system of classification of plant movements, it would be rather indifferent which distinction should be chosen for the main division. A usual system is that which for instance is reproduced in MEIRION THOMAS' "Plant Physiology"; it is as follows:

Movements shown by fixed members of fixed plants.

1. Autonomous movements (movements induced by internal stimuli).
   a. Growth movements.
   b. Movements of variation.
2. Paratonic movements (movements induced by external stimuli).
   a. Growth movements.
      i. Tropic movements.
      ii. Nastic movements.
   b. Movements of variation.

Hitherto the word *nasty* has been used for several kinds of movements of a divergent type, as for instance was the case with nyctinasty. For reasons of this kind I propose the following classification of the movements of members of plants, based on the arguments mentioned below. (see next page).

Instead of movements of variation one may also speak of "turgor reactions", as repeatedly has been done by several authors; this word, however, suggests that changes in turgor are not concerned in the case of growth movements, and this certainly does not hold true.

kind of movement	the movement induced by:	former names	direction of the movement determined by:	former names	growth or variation	examples:
endonomous	internal agent	autonomous	plant	nastic	growth	nutation, rotation, growth itself
					variation	part of nyctinastic movements
nastic	external agent	paratonic	plant		growth	photonasty thermonasty
					variation	seismonasty (*Dionaea*)
tropic	external agent		agent	tropic		phototropism, geotropism, etc.

When parts of plants show movements of variation these movements by the structure are restricted to a defined plane. This is not the case in tropisms and perhaps this is the reason why tropisms are not allied with movements of variation. If an organ, liable to tropisms and to movements of variation both, is affected by an external agent, first a tropic (growth) reaction occurs until the agent acts in the plane of the movements of variation.

§ 2. *The nature of the movements of Phaseolus.*

The sleeping movements of *Phaseolus, Canavalia* and other *Leguminosae* are movements of variation. Of course, the pulvini, which exclusively are the moving organs, show their movements of variation long before they are full-grown, but in that first period the mechanism of these movements may be considered superimposed upon the growth process.

In *Phaseolus* two types of movements of variation are clearly prominent:

a. Movements with *short latency.*

b. Movements with *long latency.*

BÜNNING (1936) has made a similar distinction. He adds that the movements sub a. especially are sensitive to the light of

shorter wavelengths, the pulvini relaxing during the sinking of the leaf, while those sub b. are sensitive to the red part of the spectrum, the pulvini remaining turgescent during the movement.

The light-turgor-reactions (movements of variation) with a short latency are called by MAR. BRAUNER (1933) "phototropic", if the light falls in the plane of the movement, and "photonastic", if the reaction is the result of a symmetrical light stimulus.

Both types of movements, sub a. and sub b., occur under the influence of light; these are nastic movements. Moreover, a mechanism in the plant regulates the movements in constant darkness; the convincing experiments of KLEINHOONTE (1929) have shown that the plants continue their movements in constant darkness (see also the second half of this paragraph). Even if the movements in the dark should be regarded as an after-effect of the preceding light stimulus it cannot be overlooked, that the period of the dark movements is independent of the period of the light stimuli which regulated the movement before. Therefore the movements in constant darkness may be called *endonomous movements;* the period of their rhythm is given by the structure of the plant.

Often the term "autonomous" has been used to characterize the dark movements of *Phaseolus* and *Canavalia.* However, this word suggests metaphysics and further autonomy in the strict sense is inconceivable in plants.

For these reasons I prefer the word "endonomous" to characterize the properties of this kind of movements.

Probably in the plant there are many distinct kinds of processes that may lead to endonomous, rhythmic movements. The periods of such movements vary in different objects from about 1 minute to many hours. As examples of plants with very short or short endonomous periods may be given:

Desmodium gyrans, period $\frac{1}{2}$ - 1 minute,
Trifolium pratense, period $1\frac{1}{2}$ - 4 hours,
Oxalis Acetosella, period $\frac{3}{4}$ - 2 hours.

Their periods are longer at low temperature and in aging (KABSCH 1861, PFEFFER 1875).

Also in *Phaseolus* or *Canavalia* one may find such endonomous, short periodic movements. Examples may be found in PFEFFER (1915, p. 93, fig. 28B) and in KLEINHOONTE (1929, fig. 37 and p. 67, fig. 33). These concern plants in which the long periodic

movements have been suppressed. One of the graphs of KLEINHOONTE is reproduced in fig. 1.

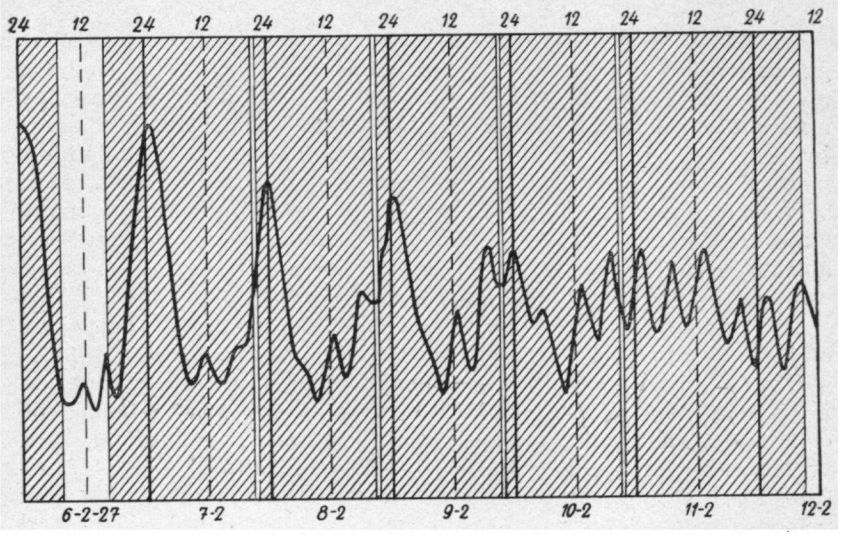


Fig. 1. Movements with short endonomic period in a graph of KLEINHOONTE (1929, fig. 33).

The endonomous periodic movements in the dark have caused the great confusion in literature and gave rise to the question at issue: are the movements endonomous or are they aitiogenous?

I will shortly give a historical review of the conceptions on this question[1]):

SACHS in 1857 came to the conclusion that "autonomous movements regulated by light" are involved; at least he seemed mostly inclined to this conclusion, though in 1863 he also mentioned an after-effect of the light influence in total darkness. PFEFFER in 1875 gave this classification:

1. Autonome oder spontane Bewegungen.
2. Receptionsbewegungen oder paratonische Bewegungen.
   a. die einfache Receptionsbewegung und deren Nachwirkung.
   d. die täglichen periodischen Bewegungen.

The sleeping movements were described as a result of the after-effects (not called autonomous) and continual rhythmic stimuli. In 1915 PFEFFER, apart from the autonomous spontaneous

[1]) In this historical survey I still use the word "autonomous" when the authors have done it.

movements, distinguished also autonomous after-effects. Sleeping movements he too then called: "autonomous movements regulated by light".

The experiments by STOPPEL (1932), BROUWER (1926) and others gave rise to doubt as to the autonomous character of the sleeping movements. They supposed the movements to be determined by external influences. BROUWER thought to make things clearer by introducing an, unfortunately not further to be defined, factor X.

KLEINHOONTE (1929) and BÜNNING (1932), at the hand of many experiments, brought the phenomenon again under the definition already given by SACHS (1857) and PFEFFER (1915): "autonomous movements regulated by light".

The endonomous processes determine the length of the period of the movements at constant external conditions.

If these conditions are not constant, the length of the period is only influenced by them to a certain extent. For instance, the endonomous rhythm depends on the rhythm of light and dark variation within the boundaries 6 — 6 — 6 — and 24 — 24 — 24 — (the figures indicate the length of subsequent light and dark periods in hours). Shorter alternations than 6 — 6 — 6 — and longer ones than 24 — 24 — 24 — were not followed by the endonomous processes; in these cases the normal 12 — 12 — 12 — rhythm of the movements was resumed again (KLEINHOONTE 1929).

BÜNNING (1931) has given another convincing proof of the endonomous character of the variation movements. He determined the length of the period at different temperatures. His results were that at a temperature of 15° C. the length of the period was about 30 hours and at 35° C. it was about 20 hours; intermediate temperatures gave intermediate periods.

Resuming it may be stated that the sleeping movements of the investigated plants are based upon *an endonomous system which, within certain limits, can be influenced by external stimuli,* e.g., light, temperature.

§ 3. *Statement of the problem.*

I have hitherto exclusively dealt with experiments and problems concerning the character of the movements, without mentioning the attempts which have been made to elucidate the internal mechanism of the processes resulting in moving of the leaves.

Several questions have engaged the investigators on this subject since SACHS. These questions develop from each other by logical reasoning, and that is the reason why I have treated

them subsequently in separate paragraphs of chapter IV. The order chosen by me does not only give a logic survey but also reflects more or less the chronological sequence in which the questions have been treated. Moreover, the study of the various processes gives a good idea of the difficulties to be met with when studying the mechanism of the endonomous system.

In chapter IV in this way the available data on the subsequent questions will be linked up and combined. This led to investigations on starch and amylase and on several questions concerning the starch-sugar metabolism in the plant.

Properly speaking, hitherto all data on the mechanism of the sleeping movements were descriptive; none of them gave rise to a theory on *the real problem of the sleeping movements: the endonomous periodicity in constant darkness.* It is this problem which continuously engaged me; it meant that I had to look for a system in the plant, which in itself could account for *periodic changes in volume.* It will be superfluous to say that these changes in volume were supposed to correlate directly with changes in sugar concentration of the cell sap. From this only a little step brought me to a possible role of starch and of amylase and so the outline of the investigations was traced.

The results and the conclusions given in the following pages are not exhaustive but also may be valuable by stimulating to detailed investigations on the complicated processes involved.

CHAPTER II.

The material; the motile organ and its anatomy.

§ 1. *The plants.*

In the course of time mainly two species showing sleeping movements have been chosen as an object for the study of nyctinasty, i.e., *Phaseolus multiflorus* L. and *Canavalia ensiformis* DC. Both plants show the movements equally well, perhaps *Canavalia* more regularly (that means, less disturbed by external influences) than *Phaseolus.* Especially in the winter season *Phaseolus* is more vigorous in growth and less liable to diseases. Although the sensitiveness to external agents is higher in *Phaseolus* than in *Canavalia,* they have in common the processes which result in the endonomous movements in constant darkness (see general introduction). Since many of my experiments had to be done in the winter season, I preferred *Phaseolus* to *Canavalia* (for even artificial day-lengthening by means of neon light could not

rule out the adverse influence of the season).

The first pair of leaves of *Phaseolus* shows the movements most clearly. By continually cutting off the main shoot, the first leaves develop to a large size and consequently the pulvini at the base of these grow favourably for a detailed study. The same pulvini (between petiole and lamina) have served as an object for the experiments of nearly all the earlier investigators.

Throughout the year the plants were cultivated in the greenhouse. In the winter the night period was reduced to 7 hours by means of neon light. The age at which the plants were used varied from about 3 weeks until two months after sowing. Besides the main shoot also the developing axillary buds must be continually cut off.

§ 2. *The anatomical structure of the pulvinus* (fig. 2) may be characterized by the following features:

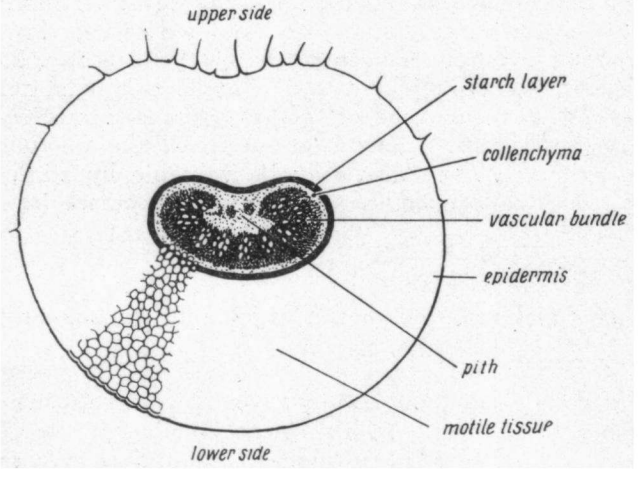


Fig. 2. Cross-section through the pulvinus.

Macroscopically the motile organ shows a dorsiventral structure, the surface of the lower side being cylindrical, that of the upper one is grooved longitudinally, so that a reniform transverse section results. The same reniform section is shown by the central vascular bundles, that are fused to one central cylinder in the pulvinus, in contrast to the several, peripherically arranged, strands in the petiole.

The microscopic structure is as follows: the narrow pith in

the centre consists of merely collenchyma cells. They are surrounded by the vascular strands, cylindrically arranged, except at the upper side, where the collenchyma of the pith is connected with the collenchyma that surrounds the vascular cylinder. Round the collenchyma sheath lies the starch layer, which since long ago drew the attention by its local storage of starch, exclusively found in the one or two layers of cells, which constitute the most central part of the motile tissue. The remaining motile tissue consists of parenchyma cells, almost isodiametric (somewhat stretched in a radial direction). Intercellular spaces cannot be found in this tissue, except in the more central part, where two or three layers of cells show air-filled intercellular spaces. This was already stated by SACHS (1857). The epidermis closely adjoins the motile tissue, it is unicellular in thickness. Its cells are smaller than those of the motile tissue, stomata occur very scarcely. The outer walls of the epidermal cells are somewhat thickened, the cuticule showing a peculiar, wavy striped, pattern. The surface is covered with hairs, that of the upper side rather densily, that of the lower side sparsely. Active hydathods occur, also more on the upper side than on the lower.

Microscopic reactions: only the walls of the vessels are lignified (phloroglucin-HCl). All other cell membranes consist of cellulose: with iodine in sulphuric acid they stain deeply blue, with iodine in zinc chloride violet. Boiling in Sudan-glycerin merely stains the cuticula (red). Starch is present in the starch layer in considerable amounts; whether it is present in the other cells of the motile tissue is difficult to state (perhaps in minute grains).

I will especially point again to the "accordeon" structure of the motile tissue (ZIMMERMANN 1929). Such an accordeonlike system promotes the changes in volume of the cells in axial direction.

§ 3. *The magnitude of the changes in volume of the motile cells.*

Measurements of the actual changes in volume of the motile cells have already been applied in a calculation given in a previous paper, in collaboration with J. B. THOMAS (1938). At that place we anticipated upon the argumentation which would be given in the present paper and which follows below.

Some shadowgraphs were made on photographic paper of the same pulvinus in a high and in a low position (corresponding to the extreme day- and night positions of the leaf). See fig. 3. Of course no exact measurements of the changes in volume of

each cell apart could be made, but measuring the changes in volume of the whole pulvinus gives an average value for the volume changes of all cells together.

It can be measured from the photographs that the increase in length of the upper side during a sinking of the leaves amounts to about 50% of the original length, while at the same time the lower side shows a decrease of about the same value. Assuming

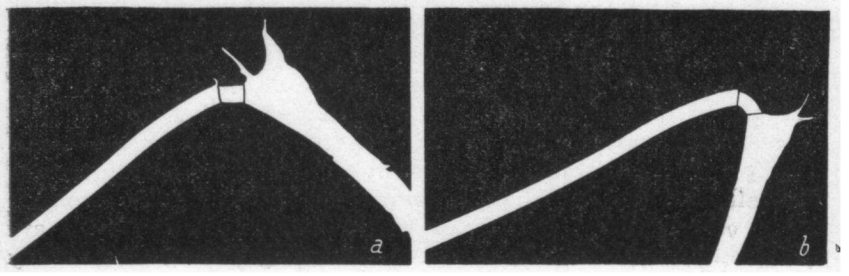


Fig. 3. Shadowgraphs of a pulvinus, a) in an uplifted position, b) in a drooped position.

that the changes in volume are simply linearly proportional to the changes in length (according to the "accordeon" structure and neglecting the volume changes in transverse directions), it may be said that the average changes in volume of the cells amount at least 1,5 $\times$.

This value has been adopted in the calculation in the previous paper l.c. and it will be used again in the last paragraph of chapter VII.

CHAPTER III.

Argumentation of the course of the research.

In chapters IV to VII of this paper I shall deal with the subjects that, more or less in chronological order, have engaged the attention of previous investigators. Where it proved to be necessary, I added some supplementary experiments of my own, while the material described in chapters V, VI and VII is new and led to the hypothesis discussed in chapter VIII.

The data, hitherto gathered by several authors in studying sleeping movements of plants, mainly have a phenomenological character. They form the assay to which the explanation of the mechanism of the movements always should be tested.

When I had the opportunity to state personally, by anatomical investigation, to which extent the starch layer in the pulvini is filled up with starch, it was very tempting to think of a possible relation between the storage of this starch and the processes underlying the movement. The simpliest relation, viz. a daily-periodic quantitative alteration in starch content, soon proved not to be observable. However, the starch might have the function of a source of energy and for that reason I attempted to determine the relation between the presence of starch and the occurrence of movements. This once done, as a logical consequence, I continued the investigation in the direction of influencing the starch content of the plant, hoping so to influence the movements. — All the experiments concerning starch are described in chapter V. —

Once conceiving a possible function of starch, it is a matter of course that one has to include amylases into the investigation, in order to connect changes in turgor, by way of alterations in the osmotic value of the vacuole, with the eventual role of a starch-sugar inversion. Now the most plausible assumption is that the quantity of amylase(s) in both halves of the pulvinus would alter reversibly, with the same rhythm as the daily-periodic movements. Accurate measurements of the amylase quantities in the upper and lower half of the pulvinus, in the uplifted as well as in the drooped position of the leaves, led to the conclusion that such differences, though they were perhaps demonstrated, are inadequate to account for the periodic changes of the osmotic values. However, it might be possible too that the total amount of amylase, present in the cells, is constant in its quantity, yet its activity being regulated by influences inside the cells. — Measurements of amylase activities are reported in chapter VI. —

In view of the endonomous periodic character of the processes investigated, it seemed attractive to examine changes in the concentration of the cell sap, due to changes in volume, and, in connection with the sensitiveness of enzymes especially to hydrogen-ion concentrations, it was obvious to focuss particular attention on the influence of pH on the amylase activity. — See chapter VII. —

It follows from the ever greater complexity of the processes involved that the conception given on the mechanism of the movements gradually gets an increasingly hypothetical character. The elements, however, hitherto are in agreement with the facts.

I am fully aware that in fact I have not exhaustively investigated the several parts of the problem. The system soon

proved to be so complicated that this could not be avoided. It would have been much easier to lose myself in a detailed study of a well defined inferior part of the process than to survey the system as a whole, and yet the latter was my intention. Certainly I may have overlooked vital processes which also play a more or less important part besides the phenomena described in the next pages.

CHAPTER IV.

Problems of and views on the mechanism. Historical review.

§ 1. *Turgor.*

Although, at present, it is beyond doubt that the changes in volume of the motile cells are due to changes in turgor of these cells, it is interesting that once this subject has been discussed in literature. Since in 1857 SACHS had mentioned that it is the turgor of the cells that changes periodically without thinking of another possibility, HOFMEISTER in 1862 suggested that the phenomenon of imbibition of the cell walls ("Zellhäuten") was much more likely the base of the changes in volume. HOFMEISTER was influenced by the data and the theories published in 1861 by GRAHAM on the properties of colloidal and crystalloidal substances. The imbibition of the cell walls would be followed by water absorbtion by the cell sap. The hydrating and de-hydrating power of the colloidal system, conditioned by all kinds of factors, would offer a suitable means to explain the rapid changes in volume of the motile cells.

In 1875 PFEFFER could not decide which of these two views should be preferred, but he felt that some data were in favour of the opinion of SACHS. In 1909 LEPESCHKIN published experiments, which served to demonstrate that the mechanical properties of the cell wall did not alter during the movement. He measured the flexibility [1]) of the pulvini, after plasmolysis, before and after light and dark periods. The flexibility did not prove to alter and therefore LEPESCHKIN concluded that only changes of the turgor occur during the movement. Since that time the attention entirely has been focussed upon changes in

[1]) To this purpose he used the method of BRÜCKE: the angle between lamina and petiole is compared in a normal and in an inverse position of the plant; the weight of the lamina causes a bending of the pulvinus proportionate to its flexibility.

the turgescence of the cells, and never since has been spoken of imbibition or such. Yet it may be useful to consider the possibility that, to a slight extent, changes in the water content of the cell walls may play a part in the moving effect.

Another question is the co-ordination of the turgor changes in the upper- and in the lower half of the pulvinus during the movements. In 1857 it has been stated by SACHS that the changes in turgor take place in an opposite sense in the antagonistic halves. He supported his statement by comparing the flexibility (measured in the same way as by LEPESCHKIN) of the pulvinus in a drooped and in an uplifted position of the leaf. PFEFFER (1875) has endorsed SACHS' view by experiments with parts of pulvini. He cut away the upper- or the lower half of the organ and registered the movements of the remaining halves in constant darkness. I have reproduced his results in fig. 4, for they

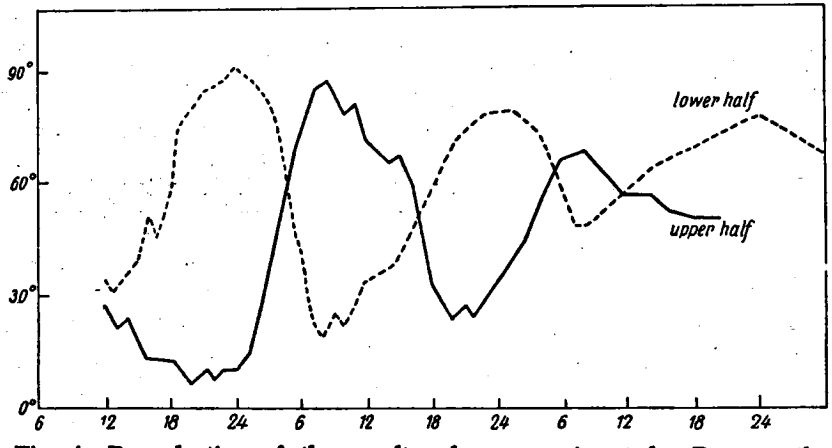


Fig. 4. Reproduction of the results of an experiment by PFEFFER; the movement of pulvini of which the upper or the lower half had been removed.

convincingly prove the correctness of SACHS' original observation. It was clearly distinguished by PFEFFER that his statement only holds true for the daily periodic movements (and for the autonomic movements) and not for the movements due to a singular reception with after-effect (see the scheme by PFEFFER, given on p. 763). The latter he attributes to changes in turgor, acting in the same sense but with a different velocity.

Although this part of the problem seemed to be solved, at

about the beginning of this century the question on the co-ordination of the turgor changes in the pulvinus arose anew. JOST (1898) and SCHWENDENER (1887) undermined the results and the conclusions of PFEFFER. During several years SCHWENDENER worked with *Phaseolus* (and *Oxalis*); all his observations were made in the greenhouse without any precautions regarding constancy of temperature, humidity, etc.; the cloudiness influenced his results, as can be seen from his protocols. So it is not amazing when he remarks: "An widersprechenden Reaktionen fehlte es zwar auch bei dieser Versuchsreihe nicht!" Still, he came to a conclusion contrary to that of PFEFFER: the rapid reactions upon light stimuli ("Einfache Receptionsbewegung" of PFEFFER) were ascribed to an opposite change of the turgor in both halves of the pulvinus.

PANTANELLI (1901), in experiments with *Robinia Pseudacacia* and *Porliera hygrometrica,* confirmed the results of PFEFFER. JOST has critisized this work; he called the investigation superfluous. — PANTANELLI (1901), in a reply to JOST, held the view that the investigations of JOST and SCHWENDENER were more likely superfluous than his own, since he worked with unknown plants and the others used a material that had already extensively and seriously been investigated by PFEFFER.

WIEDERSHEIM (a student of PFEFFER), in 1904 attempted to show that the controversy between PFEFFER and PANTANELLI and JOST and SCHWENDENER was due to a different way of operating (halving) the pulvini. He held the depth of the cutting responsible for the result: PFEFFER should have cut up to the vascular cylinder, SCHWENDENER should have halved the vascular cylinder too. His experiments did not convincingly prove that this view is correct. — LEPESCHKIN (1909), however, has investigated the same subject, and he confirmed the results and the conclusions of WIEDERSHEIM.

In spite of all the supplementary investigations, we conclude that still PFEFFER was right when he discriminated between two types of movements and two types of co-ordination of the changes in turgor in both pulvinar halves.

Recapitulating:

a) Rapid movements, induced by an external stimulus (light, temperature) — changes of turgor in the upper- and in the lower pulvinar half in the same sense but at different rates.

b) Daily periodic movements, "slow movements", — changes of turgor in both pulvinar halves in an opposite sense.

In 1926 BROUWER once more investigated this subject. He

stated that separated upper halves (lower half removed) did not show movements, while the separated lower halves moved in a daily periodic rhythm. He cut the pulvini so deep that the white vascular cylinder just became visible. I do not see how his differing results can be explained.

§ 2. *The osmotic value of the cell content.*

Many times it has been attempted to determine the osmotic value of the cell content and often the results were contradictory. This must be ascribed to a deficient distinction between the two types of movements, the rapid and the slow ones. HILBURG in 1881 attempted to state whether changes in the concentration of the cell content after a short period of illumination of the plant could be measured. This failed.

KERSTAN (1909) applied the plasmolytic method (DE VRIES); he found a difference in the "saltpetre value" of the upper- and the lower pulvinar half, in a day- and in a night position. He did not account for the changes in volume that had occurred in the meanwhile.

LEPESCHKIN (1909) combined the data of KERSTAN with the magnitude of the changes in volume as indicated in 1875 by PFEFFER (40%) and concluded that no changes in the osmotic values of the turgescent cells had been proved by KERSTAN. He made no experiments of his own.

ZIMMERMANN (1929) estimated the osmotic value of cells when just turgid, of both pulvinar halves, during the daily periodic movements. He found striking differences. The variations of the osmotic value appeared to occur antagonistically in both halves and parallel to the changes in volume. When the decrease in volume of cells just turgid was taken into account (according to URSPRUNG, 1930), the differences still remained. WEIDLICH (1930), using also the formulae of URSPRUNG, came to the same results. Unpublished results of experiments by Miss VAANDRAGER (Utrecht 1931) confirmed this too.

Since LEPESCHKIN (1934) and BÜNNING (1936) have critisized the results and the conclusions of ZIMMERMANN and WEIDLICH, I decided to examine once more the osmotic conditions in the upper and the lower pulvinar halves during the daily periodic movements.

The osmotic value and the suction pressure of the cells of a tissue can be measured in various ways. Always it has been considered necessary to reduce the experimental data, with the

aid of more or less complicated formulae (URSPRUNG 1930, WEIDLICH 1930, LEPESCHKIN 1933, 1934), to the actual volume of the cells in the tissue.

Now, in studying the movements of variation, the most important thing to know is, whether and how the suction pressure of the tissue as a whole changes relative to the movements. I used a somewhat simplified method, inasmuch as I compared the volume (proportionate to the length) of a certain strip of pulvinar tissue in water and in a hypertonic solution of glucose. The 1 mol. glucose solution caused plasmolysis; in this state the length of the tissue strip was minimal. The length of the strips in water, as compared to the minimal length in glucose solution, was taken as a measure for the suction pressure of the tissue.

The strips were first placed in water, then in the glucose solution; controls showed that first placing in glucose and then in water gave almost the same values. The shortening in glucose solution as compared to the length in water was expressed in percentage of the length in the glucose solution. The strips were longitudinal (radial) sections of the pulvinar motile tissue of the upper and of the lower half. To facilitate the measuring, the outline of the strips, in water as well as in the sugar solution, was traced on paper. An example of this procedure is given in fig. 5.

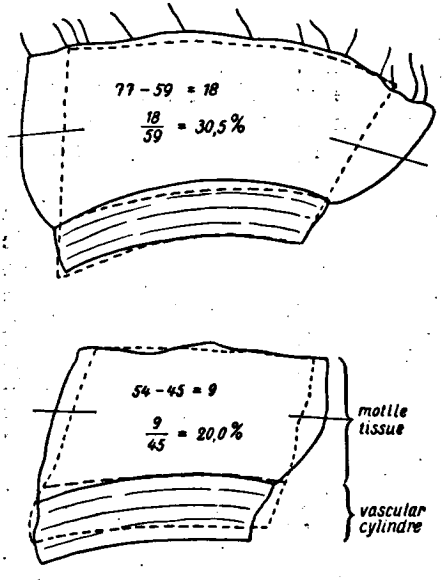


Fig. 5. Two examples of the measurement of the shortening of strips, one of an upper half (with hairs) and one of a lower half.
——— outline in water
- - - outline in glucose solution.

Sometimes an increase of length in a transverse direction was noticed to be combined with a decrease of length in the longtudinal direction, when the strips were placed in the glucose solution. This may support the existence of an "accordeon" structure of the motile parenchyma (ZIMMERMANN 1929, MAR. BRAUNER 1933).

Examples:

object	*shortening*	*broadening*
1. upper half	23,8%	9,0%
2. lower half	26,8%	5,6%
3. lower half	30,0%	14,5%

With the aid of tangential strips I first have determined the shortening of more or less peripherically situated parts of the pulvinar tissue. The results of these experiments are given in table 1.

TABLE 1.

objects	shortening in % of the length in glucose			
	epidermis	parenchyma	parench. near vasc. cylinder	vascular cylinder
E 1. upper half	11,3%	10,8%	6,7%	—
lower half	2,3%	6,5%	1,5%	—
E 2. upper half	19,5%	19,7%	11,9%	0%
lower half	12,5%	15,7%	14,7%	0%
E 3. upper half	11,8%	12,7%	7,5%	0%
upper half	9,5%	24,3%	7,7%	0%
lower half	12,7%	27,8%	13,2%	0%
lower half	5,8%	30,4%	17,0%	0%

To ascertain that endosmosis or exosmosis might not give false results, I arranged special experiments. In these series I placed the strips first in paraffin oil (P), to trace the original outline. Then in two series I placed them first in water (W), then in glucose solution (S), in two other series first in glucose solution (S), then in water (W). In each of the media the outline of the strips was traced. Changes in length were expressed in percentages of the length in paraffin oil. The results are given in the following table.

(sequence)	upper half		lower half	
	W	S	W	S
I. P—W—S	+ 33,2	+ 0,7	+ 26,7	— 1,9
II. P—S—W	+ 33,3	— 7,2	+ 31,5	— 9,1
III. P—W—S	+ 29,1	+ 5,7	+ 22,7	+ 4,1
IV. P—S—W	+ 30,2	— 5,4	+ 25,2	— 4,3

(each figure is an average of 3—5 measurements).

Sometimes even a slight lengthening in sugar occurred, showing that the osmotic value may exceed that of 1 mol glucose.

A comparison of the series P—W—S and P—S—W shows that

the relative length of the strips in water shows differences between the upper and the lower halves, while the values of the S-column scarcely differ in both halves. Therefore the length of the strips in water as compared to that in glucose solution (and expressed in percentage of the length in glucose) may be used for comparing the suction pressure (and so the osmotic values) of the tissue as a whole. No actual values of the osmotic pressures or suction pressures have been calculated, since I only intended to investigate whether differences between both halves, in various positions of the leaf, could be detected.

The results of the experiments, carried out to this purpose, have been summarized in table 2. The percentages given in this table are averages of 3 to 8 measurements each. For each percentage I have calculated the mean error from the formula $m = \pm \sqrt{\frac{\Sigma a^2}{n\ (n\text{-}1)}}$; in the table I have mentioned the mean error of the difference between the values for the two antagonistic halves of one pulvinus calculated (from the formula $m_v = \pm \sqrt{m_1^2 + m_2^2}$, ($m_1$ and m_2 being the mean errors of the two values in question).

TABLE 2. Comparison of the osmotic values in both pulvinar halves.

I. *Leaves in an uplifted position (day).*

series	object	number of measure-ments	shortening	difference	3 × m_v
G	upper half	5	18,8%	10,1	10,26
	lower half	5	28,9%		
O	upper half	8	26,6%	11,5	6,24
	lower half	6	37,5%		
H	upper half	6	17,8%	5,5	6,99
	lower half	7	23,3%		

II. *Leaves in a drooped position (night).*

J	upper half	7	29,4%	12,9	10,26
	lower half	5	16,5%		
L	upper half	4	27,8%	9,4	12,9
	lower half	4	18,4%		
M	upper half	5	34,5%	19,9	6,54
	lower half	3	14,6%		
N	upper half	7	25,0%	9,5	8,32
	lower half	6	15,5%		

III. For leaves in an intermediate position all differences found between upper and lower halves were far within the limits of error.

The positions of the leaves were valued from the angle between lamina and petiole; no registering of the movements took place, since too many plants were involved in the experiments. Therefore not all results of the series with strips of uplifted leaves exceeded the limits of error.

The results of these experiments are in full agreement with the results of the former investigators. They all lead to the conclusion, that *the osmotic value of the upper half is minimal in an uplifted position of the leaf, while that of the lower half is maximal in that case; when the leaf is in a drooped position, the situation is reverse.*

It may be emphasized again that this only bears upon the slow, daily periodic movements.

§ 3. *Permeability.*

It may be useful, but it is adventurous, to express the course of a certain vital phenomenon in terms of mathematics. For, when the formulae are erroneous, the conclusions drawn from them are false.

LEPESCHKIN in 1933 and 1934 has published papers on the osmotic processes in cells (cells apart and in a tissue) and on the mechanism of the periodic movements of variation. He has given a set of formulae, expressing the relation between turgor pressure, suction pressure and water withdrawing forces of a cell in a tissue. He also has taken into account the permeability, but particularly that part of his calculations has led him to somewhat strange conclusions on the influence of changes in the permeability of cell membranes on the turgor pressure of the cells.

Before critisizing the work of LEPESCHKIN I will first define some symbols used by me and partly also used by him:

T — *turgor pressure,* the pressure exerted on the cell wall by the cell content, straining elastically the wall.

V — the *volume* of the cell.

W — the *quantity of molecules of water* in the cell.

O — the *quantity of molecules of osmotically active substances* in the cell.

P_w — the *partial hydrostatic pressure of the water* in the solutions outside the cell.

P_o — the *partial hydrostatic pressure of the solved substances* in the solutions outside the cell.

p_w — the *permeability* for *water* of the cell membrane.

p_o — the *permeability* for *osmotically active substances* of the cell membrane.

a — the *total area of the surface of the membrane* (assuming the inner- and the outer surface to be equal).

Permeability (p) is defined: the ratio of the number of molecules passing a membrane to the number of molecules colliding against it.

Always is: $T = K \frac{W + O}{V}$

(K = a constant, dependent on properties of the cell wall and of the tissue and on conditional factors).

Now LEPESCHKIN is right in deriving his formulae until the point where he introduces the permeability into the problem. Once assuming that the membrane is permeable to osmotically active substances no final turgor pressure can be expected in a cell placed in water.

LEPESCHKIN (1933) expresses the relation between the suction pressure (S_l) of a solution and its osmotic pressure (P) in the equation: $S_l = P\ (1-\mu)$, where μ represents the "permeability-factor" for the solved substance. Considering the permeability for water too, he gives the equation: $S_l = P\ (1-\mu)\ (1-\frac{\sigma}{\mu})$, where σ represents the "permeability-factor" for water. Now these formulae cannot be used for describing the effect of a change of μ or σ, since P and μ or P and σ are not varying independently. This has not been mentioned by LEPESCHKIN. Differential calculus should have been applied in this case! The problem is rather difficult to solve and falls beyond the scope of this paper, I shall not treat it here.

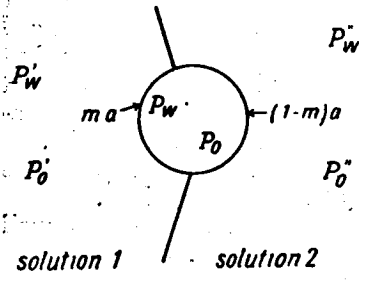


Fig. 6. See text.

I prefer to consider the case of a cel in a system (a tissue), where a part of its surface is in contact with a solution 1 and the other part with a solution 2 [1]). Several assumptions must be made before a final turgor value of the cell can be expected. The most important assumption is that constantly the loss of osmotically active substances (caused by permeating) by the cell is supplied by a mechanism in the cell (viz. photosynthesis or

[1]) The symbols regarding solution 1 are marked "that regarding solution 2 are marked"; the symbols regarding the cell content have no indices.

activity of enzymes). In this case O is kept constant. I assume further that $P''_w > K.\frac{W}{V} > P'_w$ and $K.\frac{O}{V} > P''_o > P'_o$ (see fig. 6), and that a part m of the cell surface is in contact with solution 1, and that P'_o, P'_w, P''_o and P''_w are constant (viz. continuously refreshed solution). For the equilibrium to be established, we now may write that in a certain period the numbers of molecules entering and leaving the cell must be equal:

$$ma.P'_w.p_w.t + (1-m)a.P''_w.p_w.t + ma.P'_o\,p_o.t + (1-m)a.P''_o.p_o.t$$
$$= a.P_w.p_w.t + a.P_o\,p_o t. = a.K.\frac{W}{V}.p_w.t + a.K\frac{O}{V}.p_o\,t$$

(see p. 778). This gives:

$$V = K.\frac{W.\frac{p_w}{p_o} + O}{\frac{p_w}{p_o}\left\{(1-m).P''_w + m.P'_w\right\} + (1-m).P''_o + m.P'_o}$$

It is clearly expressed by this formula how (when equilibrium has been reached) the volume of the cell V depends on O and on P'_w, P''_w, P'_o, P''_o and m. How the value of the ratio $\frac{p_w}{p_o}$ influences the value of V is entirely dependent on the mutual proportion of P_w and P_o in the solutions 1 and 2 (i.e. the osmotic values of the solutions surrounding the cell).

It cannot be read from the formulae whether an initial change of the turgor, if a change of $\frac{p_w}{p_o}$ might cause such, finally results in a different value for V, since the formulae represent the statics of the processes, i.e. the equilibrium. The factors in the formulae are not independent of each other and therefore an alteration of one factor affects the value of others and the result cannot be predicted.

For these reasons I cannot see how LEPESCHKIN (1935) and BÜNNING (1936), from their formulae (or even without describing the phenomena in formulae), could conclude that changes in permeability (for water and solved substances) of the cell membranes would result in a definite change of the volume or of the turgor value.

CHAPTER V.

The relation between the presence of starch and the occurrence of movements.

§ 1. *Introduction.*

A study of the anatomy of the pulvinus especially called my attention to the great quantity of starch that normally fills up the starch layer. Since it seemed probable that this starch might play a fundamental part in affecting or in supporting the endonomous processes on which the movements are based, I tried to get some data on this storage of starch under various circumstances.

In the petiole as well as in the pulvinus the starch is stored mainly in the starch layer (surrounding the vascular bundles), in granula of rather large dimensions. In the more peripherical cells of the pulvinar tissue starch will be probably present, but in scarcely demonstrable grains (very small ones) or perhaps in a solved form.

The starch layer consists of two or three layers of parenchyma cells, adjoining closely the collenchyma that surrounds the central vascular bundle in the pulvinus. Generally on the upper side the starch storage is markedly less than on the lower side.

§ 2. *The starch content during a 24-hours period.*

To examine the starch content of the moving pulvinus during a period of 24 hours, every hour of a series of plants one pulvinus was isolated and fixed (in alcohol 96%); afterwards these pulvini were tested on starch content. At the same time of all plants the angle between petiole and pulvinus was measured (also every hour), while temperature and air humidity were continually noted too.

The estimation of the starch content (here and in other experiments) was made by staining the starch with iodine (in potassium iodide) in a hand-made transverse section of the pulvinus. Of course minor differences in starch content could not be noticed in this way.

The following scale was used:

1. very much starch (more than normal)
2. much starch (normal)
3. little starch (less than normal)
4. very little starch (some grains)
5. no starch

This standard only refers to the starch present in the starch layer. When starch occurs in a considerable amount elsewhere

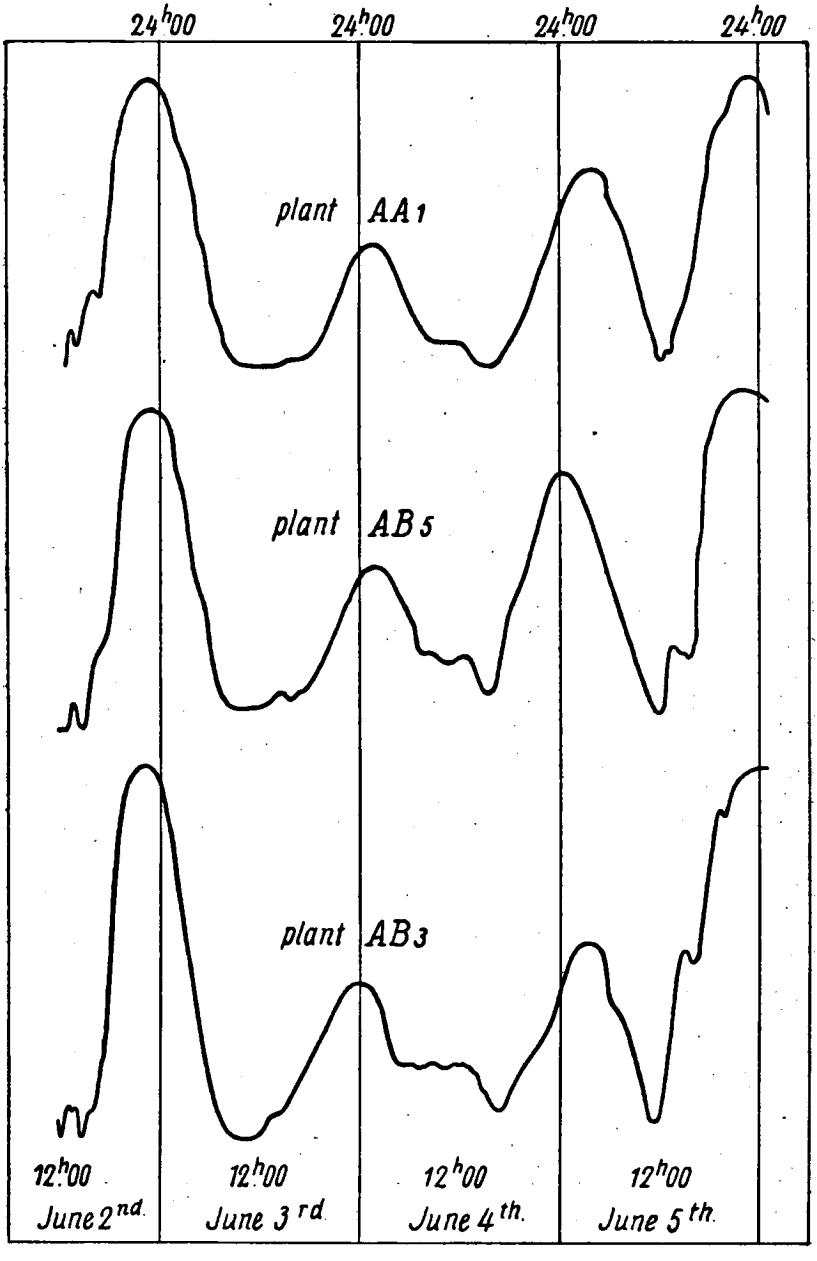


Fig. 7. The result of a registration of the movements of three plants, AA 1, AB 5 and AB 3, placed side by side in the greenhouse.

in the tissue it is especially mentioned.

For the 24-hours experiment a number of plants were chosen grown from the same sowings and under equal conditions of light, temperature and air humidity, all having a normal and healthy appearance. During the experiments the plants were left in their normal habitat (i.e., in the greenhouse, under natural day and night succession). With the experimental method just described *a variation in the starch content of the pulvinar starch layer during 24 subsequent hours could not be detected.* Throughout the 24 hours the starch layer was filled up with starch to the same extent.

§ 3. *Plants in constant darkness.*

By earlier investigators (e.g. KLEINHOONTE) it was noticed repeatedly that the sleeping movements still continue in the usual 12-12-12-hours rhythm for one or two weeks in constant darkness until they finally stop. Now it might be of importance to control the behaviour of the starch during such a dark period.

An ever felt objection to this kind of experiments is that one cannot control the movement of the leaves after having done a starch estimation, for to this purpose the pulvinus is cut off, fixed and microscopically tested. In consequence, conclusions must be drawn from experiments with more plants, grown under the same conditions. This is permissible as is demonstrated by fig. 7, which shows the result of a registration of the movements of three plants, placed side by side in the greenhouse. The dull and rainy days, 3rd and 4th of June, have had a corresponding effect in all three plants (AA 1, AB 3, and AB 5, of two sowings).

In a dark room, with temperature about 23° C. (not accurately constant), the movements of the same three plants were registered from June 11th until June 25th. The results of the last part of this registration are given in the graph of fig. 8. This figure shows, that the plants AA 1 and AB 5 after one week had fairly well stopped their movements, while those of AB 3 still continued and even had not stopped after 14 days [1]). At that moment the starch content of the pulvini of this series was estimated and proved to be of

AA 1 — no starch
AB 3 — little starch
AB 5 — very little starch.

[1]) Thus, in spite of the similar behaviour shown before, in the dark these plants displayed an individual character; the latter is due to internal conditions, while the parallelism before was induced by external stimuli.

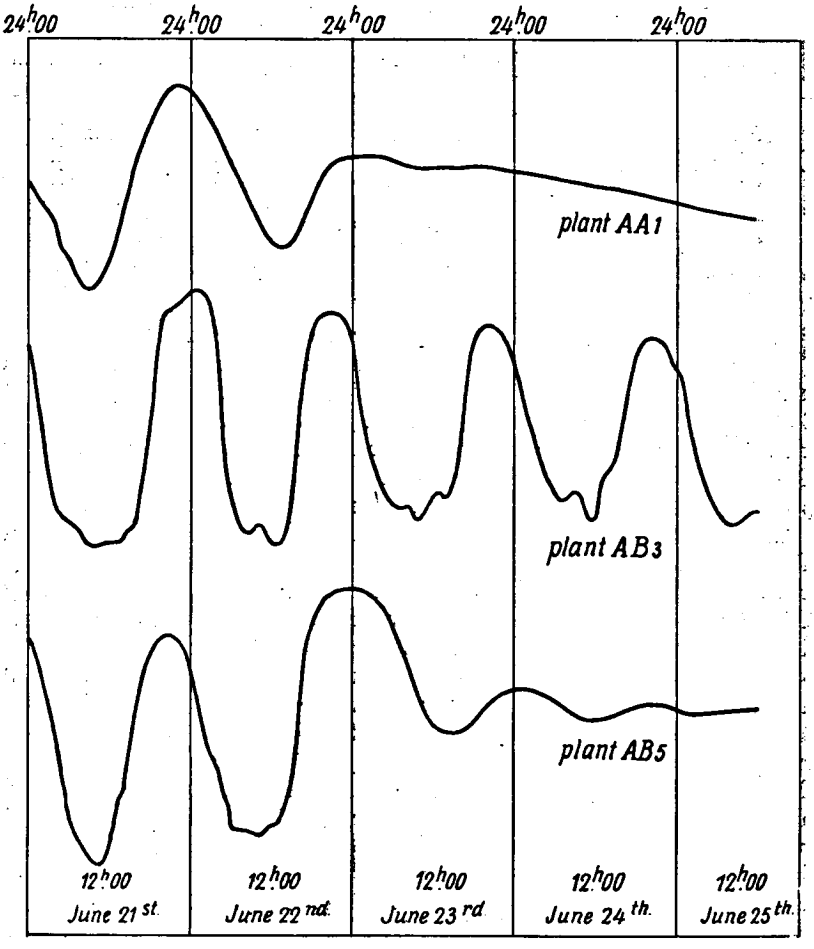


Fig. 8. The last part of the registration of the movements of the plants AA 1, AB 5 and AB 3 in constant darkness.

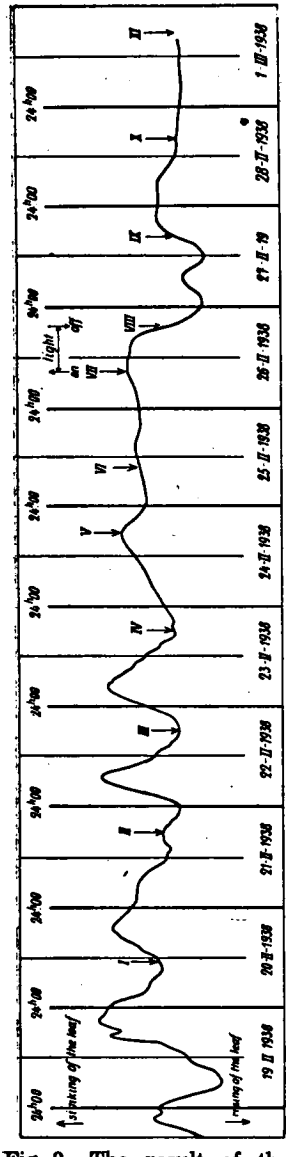
Of course these preliminary indications ought to be controlled in more objects. The following table gives some more observations on this subject:

TABLE 3. Relation between movement and starch content.

Plants	length of period in dark room	state of movement	starch content
AD 34	Oct. 19th—29th	no movement	no starch
AD 35	Oct. 19th—29th	no movement	no starch
AD 36	Oct. 19th—29th	no movement	no starch
AD 2	Oct. 23rd—29th	still moving with smaller amplitude	little starch
AD 20	Oct. 30th—Nov. 9th		little starch
AD 21	Oct. 30th—Nov. 9th	practically stopped	very little starch

These data were completed by an experiment in Februar 1938: A great number of plants of the same sowings (CY) and grown under equal conditions, were placed in a dark room (temp. about 20° C.) at the same time. Each day one pulvinus was cut off and fixed to be examined on starch. The movements of one of the pulvini were registered during the whole experiment, this pulvinus being fixed itself at the end.

The movements of the registered pulvinus continued until about Februar 25th, with some irregularities perhaps caused by the insufficient constancy of the temperature in the room. During this first period the starch content gradually decreased, as demonstrated by the microphotographs of the starch layer (the starch stained deeply blue with iodine). See the graph of fig. 9 and Tab. XVIII, A. On Februar 26th, from 9h30 until 20h00, the plants were illuminated by a bulb of 500 W. half a meter over the leaves (with a water bath between plant and bulb to eliminate thermic rays). The reaction to the illumination of the registered plant was a lifting of the leaf. After some oscillations an arrest was reached again somewhere near March 1st. I especially call the attention to the fluctuations of the starch quantity during the last part of the experiment. At the end of the light period not only the starch layer had been filled up with starch, but also the parenchyma cells of the motile tissue were unusually full of starch, though in grains considerably smaller than those in the starch layer. As to how this phenomenon is connected with the illumination and as to how (c.q. whether) movement is linked up with the high starch content, is not clear. The last photograph shows that after all *with the final arrest of the*



movement the starch has disappeared from the starch layer.

§ 4. *Plants in constant light.*

Several investigators, a.o. PFEFFER (1915), STOPPEL (1932), CREMER (1923), have mentioned that the plants stop their sleeping movements in constant light. That this does not hold true has convincingly been demonstrated by BROUWER (1926) and by KLEINHOONTE (1932).

I decided to examine the progress of the starch content during a period of constant illumination of the plants. To this purpose three plants were placed in a dark room and illuminated by a bulb of 200 decalumen, 50 cm over the leaves. The movements of one of these leaves (AF 9a) were registered from Januar 9th until 15th (see fig. 10), while in that period the starch content of one pulvinus each day was estimated. The results were that no fluctuation of the starch content could be noticed.

It may be concluded that *during this period of constant light the starch content did not increase nor decrease,* nor did any starch appear outside of the starch layer.

§ 5. *Conclusions.*

The nature of the problem prevents that absolute certainty can be gained as to the direct relation between the starch storage and the pulvinar motility, since for studying the changes in starch content during a long period it is necessary to draw more than one plant into the investigation. Therefore all results must be regarded as averages of several values.

None the less it may be stated that

Fig. 9. The result of the registration of the movements of one among several plants placed in a dark room. For further explanation see text. — This figure should be compared with Tab. XVIII, A.

without doubt starch plays an important part as a storage of energy for the processes that result in movement.

As long as starch is present movements can occur, though I want to emphasize that this only holds true for a single type of processes, all concerned with starch metabolism. Apart from

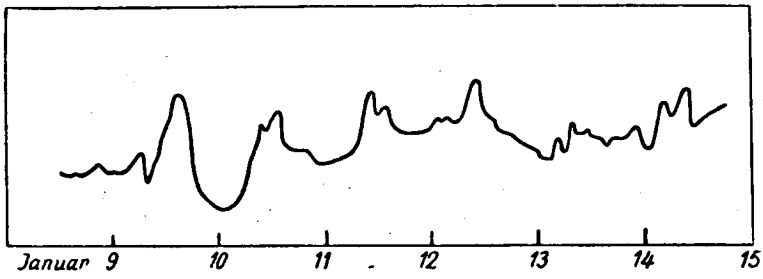


Fig. 10. Result of the registration of the movements of the plant AF. during a period of constant light.

these, it is conceivable that, e.g., changes in permeability may cause alterations of volume which have nothing to do with the starch metabolism. I cannot, however, visualize how such volume changes could be reversible.

CHAPTER VI.

Amylase.

§ 1. *Introduction.*

As already indicated on p. 769 the possible part played by starch in the mechanism of the sleeping movements drew the attention to enzymatic actions inside the cells of the motile tissue. In connection with starch, amylase(s) [1]) should be the most important enzymes. The methods, described by VAN KLINKENBERG (1931), for demonstrating the activity and the compounds of diastasis (malt-amylases), with slight alterations, proved to be very suitable for a qualitative proof of the occurrence of amylase in the pulvinus. A more careful examination of the amylase activity in different parts of a transverse section through the pulvinus enabled me to localize roughly the amylase. The next problem was to measure as accurately as possible the exact quantity of amylase present in both halves, the upper

[1]) I further use the term "amylase" for all amylolytic enzymes, eventually present in the pulvinar tissue.

and the lower, of the pulvinus. To this purpose I made myself familiar with the method described by LINDERSTRØM-LANG, for quantitative estimation of reducing sugars by means of a micro-titration. Once acquainted with this micro-method, I needed a good deal of time for finding the best way to extract the amylase from the object. Not until these things were settled, I could continue my attempts to establish whether a difference in amylase quantity between both pulvinar halves could be demonstrated or not.

§ 2. *Qualitative proof of the presence of amylase.*

The method first used by WIJSMAN (1889) and recently by VAN KLINKENBERG (1931) to study the properties of the amylase system of malt, seemed to me a suitable means to examine whether amylases could be detected in the pulvinar tissue of *Phaseolus multiflorus L.*

In this method a gelatin-starch plate is used to demonstrate the action of the amylase. The plate is made by casting a gelatin-starch solution (8% of gelatin, ¼% of soluble starch) in a Petri dish. The enzyme is allowed to hydrolyse the starch of the substrate for a long time (1—3 days) at low temperature in a refrigerator. — VAN KLINKENBERG has usually worked with enzyme solutions, only a few times he placed parts of a plant tissue (from barley kernels) on the gelatin-starch substrate. — The action of amylases is determined by staining the plates by a dilute iodine solution.

I have applied the same method for examining the amylase content of the pulvinar tissue. I placed some transverse slices of the pulvinus and of the petiole on a gelatin-starch substrate; after 24-hours incubation in the refrigerator, the plate was stained with iodine. The tissue proved to contain a clearly demonstrable quantity of amylase. The influence of alcohol and ether was then tested; alcohol inactivated the enzyme, particularly when heated up to 70° C. Ether did not inactivate at all, even a slight activation was noticeable, but the latter might be attributed to a better diffusing of the enzyme from a dead tissue than from a living one. A difference in amylase content between the upper and the lower half of the pulvinus could not be detected. The best period for diffusing of the enzyme from the tissue appeared to be about 20 hours (at 0° C.).

No further data being obtainable with this crude method, I have tried to apply the same principle in a more detailed investigation, which will be described in the next paragraph.

§ 3. *The localization of amylase in the pulvinus.*

The experiments, described in § 2, only generally demonstrated the presence of amylase in the pulvinus (as well as in the petiole). Once familiar with the method of enzyme testing after VAN KLINKENBERG, I adapted the method to a more detailed examination of the localities in the plant (in petiole and in pulvinus), where the enzyme is present in the greatest quantity, or at least where it is most active. It needs scarcely to be said, that it would be difficult to discriminate whether one measures the quantity or the activity of amylase in the plant.

To apply the method of VAN KLINKENBERG for a minute examination, I casted the gelatin-starch solution (see foregoing §) in a thin layer on a slide. The edges of the glass were coated with paraffin, so that by floating it on a water surface, to get an exact horizontal position of the slide, the gelatin layer became as homogenous in thickness as possible. After solidifying of the gelatin the slides prepared in this way were dried completely for several hours and an "enzymographic plate" was obtained that, after "development" in iodine solution, fairly well reproduced differences in amylase activity of various parts of plant tissue brought into contact with it.

Yet there is one point which easily can spoil the results and give a false idea on the distribution of the amylase in the tissue. An important condition for a good result is that the surface of the tested tissue must be entirely in good contact with the enzymographic plate, for if this is not the case the results, of course, are misleading. The good contact between plant and plate can be judged from the mark of the tissue that should be visible on the plate.

Another, less serious, difficulty is the irregularity in staining intensity, that inevitably occurs when a iodine solution of too high a concentration is used. The concentration should be low enough to permit a slow "development", which can be stopped at any moment desired.

After the development the "picture" of the amylase activity may be copied on photographic paper and in some cases even an enlargement can be made. [1])

Not too great an accuracy may be expected from this method, since, during the cutting of the pulvinus (or the petiole), the blade unavoidably spreads some of the cellsap (with or without amylase) over the surface.

[1]) No grain limits the magnification, only the sharpness does!

Many experiments were made, of which one is reproduced (Tab. XVIII, B).

Results: It must be concluded from the experiments that the greatest quantity of amylase is situated near to the vascular bundles or in some cases in the vascular bundles itself. Of course one is inclined to think of the starch layer as a source of amylase, but it must be doubted whether this is always the case. Moreover, it is generally known in literature that the amylase content of a tissue may vary rather rapidly, as will be amply discussed on p. 804.

Conclusion:

The motile tissue contains the highest quantity of amylase in the more central parts, while in some cases the vascular bundle itself (except the collenchyma sheath) *is very rich of amylase.*

How these facts are to be explained still remains uncertain, since we do not know where starch in a solved form is present (if such ever be the case). It is, however, clear that, once starch and amylase both present in the highest quantity in the same region of the tissue, slight activating or paralysing influences already may bring about considerable alterations in the sugar content of the tissue.

§ 4. *Micro-method for the estimation of reducing sugars and amylase quantities.* [1])

The quantity of reducing sugars in a solution is estimated by means of the following chemical method. The sugar, e.g., glucose, is oxidized stoechiometrically to gluconic acid by adding freshly prepared iodine solution of a suitable alcalinity:

$C_6H_{12}O_6 + J_2 + 3\ NaOH \longrightarrow C_6H_{11}O_7Na + 2\ NaJ + 2\ H_2O.$

The excess of iodine is determined by titration with thiosulphate in acid solution.

To secure that glucose and maltose are oxidized and saccharose, fructose and starch are not, the iodine reaction occurs in a carbonate-bicarbonate buffer of pH 10,5. (5 vol. 0,4 N Na_2CO_3 + 1 vol 0,4 N HCl). — Since iodine reacts with a number of substances, all the material used must be cleaned with scrupulous care. Also the sublimation of the iodine should be prevented.

With due precautions these conditions are realized in the micro-method described in detail by LINDERSTRØM-LANG and

[1]) In the §§ 4, 5 and 6 of this chapter the figures represent the quantities of reducing sugars, formed by hydrolysis, expressed in mm^3 thiosulphate; the italicized figures represent the values obtained after reducing to the same weight of tissue.

HOLTER (1933). I will shortly describe this method again.

From the solution of which the sugar content must be estimated, a certain exact quantity (15 mm^3) is pipetted with a half-automatic micro-pipette into a micro test tube. These tubes are made from Jena glass (they are about 25 mm high, outer diameter 6 mm) and completely coated with a thin (fully transparent) paraffin layer. As to the preparing and the cleaning of these tubes I refer to LINDERSTRØM-LANG l.c.. About 50 mm^3 of a carbonate buffer of pH 10,5 is added to the sample taken with the half-automatic pipette, in order to establish the conditions required for the oxidizing by iodine. Next 11,9 mm^3 of a 0,15 N iodine solution in potassium iodide is added to the mixture with the automatic pipette (for a description of the automatic and the half-automatic pipettes I refer to the paper by the inventors of these useful instruments). Iodine sinks to the bottom and sublimation is prevented. To check it entirely, immediately afterwards a ring of 50 mm^3 1,2 N sulfuric acid is laid above the level of the mixture with a micro-pipette. By capillary forces the ring sticks to the wall of the micro-tube. In the same way another ring of liquid (consisting of $\pm$ 30 mm^3 0,3% starch solution) is placed above the sulfuric acid, in order to serve as an indicator at the titration. Then the tube is allowed to stay for 30 minutes. In this period part of the free iodine is bound, dependent on the quantity of reducing sugar present in the mixture; when the 30 minutes are over [1]), the two liquid rings in the tube are added to the reaction mixture by centrifuging the tube. The sulfuric acid acidifies the mixture (while producing CO_2) and then the micro-burette allows a vary careful addition of the N/20 thiosulphate. During the titration the mixture is continuously stirred with the aid of a periodic electromagnetic interruptor and minute beads of iron melted in glass. To estimate the amount of sugar, the result of the sugar titration is compared with a check experiment, in which exactly the same volumes of solutions are used, yet water instead of the sugar containing solution. In this way the amount of sugar is expressed in mm^3 N/20 thiosulphate. How many mg sugar correspond to 1 mm^3 thiosulphate completely depends on the actual concentration of the solutions used, i.e., the iodine- and the thiosulphate solution. Generally the concentrations slowly alter in the course of time, even during one experiment if this lasts for some hours. Therefore it can be indicated only approximately how much

[1]) Experiments of LINDERSTØM-LANG and HOLTER have shown that this time is sufficient.

sugar corresponds to 1 mm³ N/20 thiosulphate; for maltose this quantity is about $4{,}3 \times 10^{-3}$ mg. The accuracy of the method is about 0,06 mm³ N/20 thiosulphate or about $2{,}5 \times 10^{-4}$ mg maltose.

In the experiments of the following paragraphs the amounts of maltose always are given in mm³ N/20 thiosulphate, since only the results of one experiment can be mutually compared. Moreover, it could not always be avoided to break micro-pipettes. Now and then a narrow capillary tip of a pipette was broken and it is difficult to make a new micro-pipette of exactly the same volume. This is also a reason why only the results of no more than one experiment can be compared.

All the figures representing amounts of maltose estimated *are means of at least two parallel titrations.* The accuracy of the method can be judged from the graphs, in which almost none of the estimated points falls beyond the smoothly drawn curves.

To use the above described method for the estimation of amylase quantities, it is necessary that the amylase acts on a solution of pure starch to form maltose (I used "Kahlbaum" soluble starch p.a.). For the sake of comparison the samples shall be treated in exactly the same way; all quantities of solutions shall be the same and so the time of hydrolysis and of sugar oxidation and, of course, the temperature must be kept as constant as possible all the time.

The amylase is extracted from the tissue (for details see § 5), and consequently *the amount of amylase depends on the quantity of tissue extracted.* In comparing the amylase contents of two pieces of plant tissue it is therefore necessary to reduce the quantities of amylase estimated to the same weight of tissue. Yet in this reduction is a difficulty, due to the nature of the hydrolysing process. The general shape of the curve, representing the course of the process of hydrolysis — forming sugars through the action of amylase out of starch —, is reproduced in figure 11 (Exp. 01). It can be seen that the curve of the

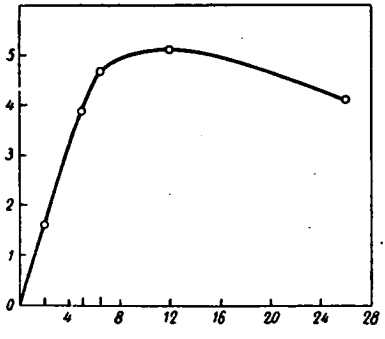


Fig. 11. The general shape of the starch hydrolysis curve.
ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

sugar production starts almost linear, then bends slowly to a certain limit, the level of which is determined by the quantity of starch available for hydrolysis. One might also say: the quantity of amylase determines the rate of sugar-forming until the quantity of available starch becomes the limiting factor in the process. In most of the graphs on the next pages one easily recognizes the theoretical shape of the hydrolysis curve. This shape is the result of the quantity of maltose formed per time unit, whilst this quantity continuously depends on the quantity of the available starch-amylase complex (SE). During the first part of the process of hydrolysis the concentration of the starch is very high relative to that of the amylase and the quantity of SE formed per time unit will be constant. The first part of the curve is a straight line. As soon as the starch quantity has decreased below a certain limit, besides the amount of amylase also the amount of starch will affect the rate of the process $S + E \rightleftharpoons SE$; the reaction-velocity slowly decreases until the amount of enzyme has become high relative to that of the starch and then, at the end of the reaction, the curve will be almost a straight line again. In several cases I have clearly noticed such a theoretical course of the hydrolysis curve. — If hydrolysis lasts for a very long time the amount of sugars produced decreases again; it is not with certainty known to what kind of processes this is due.

If the reduced values lie in the sphere of influence where the available starch quantity is limiting factor, a reduction of the

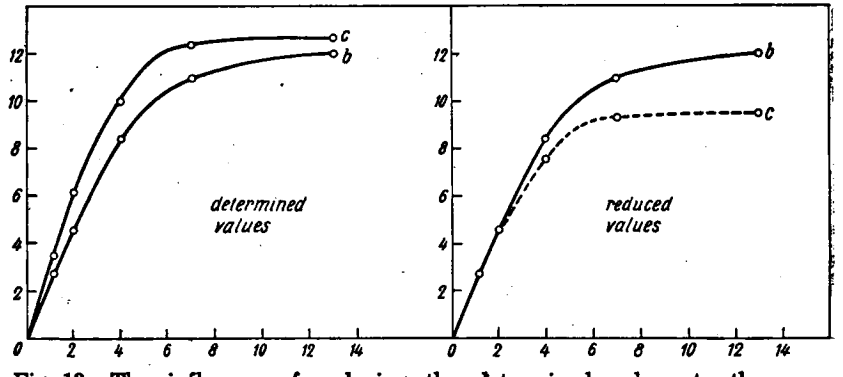


Fig. 12. The influence of reducing the determined values to the same weight of tissue on the shape of the curve.
ordinate: sugar in mm³ thio sulphate,
abscissa: time in hours.

values to the same weight of tissue will give a false idea of the amylase activity. This is demonstrated by the experiment 02, in which two pulvini were cut into thin slices; these were mixed and divided into two unequal parts, each of which was weighed and extracted in the usual way.

Exp. 02. Extraction at 0° C. for 24 hours.
2 pulvini sliced and divided in:
b - 11,7 mg tissue, extracted in 170,8 mm³ buffer pH 5,9.
c - 15,6 mg tissue, extracted in 170,8 mm³ buffer pH 5,9.
results hydrolysis in table 4 and in fig. 12.

TABLE 4. Reduction to the same weight of tissue.

time of hydrolysis	b		c	
1h10	2,67	*2,67*	3,57	*2,67*
2 h	4,55	*4,55*	6,13	*4,59*
4 h	8,40	*8,40*	9,98	*7,49*
7 h	10,95	*10,95*	12,35	*9,26*
13 h	12,00	*12,00*	12,60	*9,45*

It always has been a puzzle how to express the quantity or the activity of enzymes. Generally one is accustomed to compare the quantities of sugar formed by hydrolysis in a definite time. It will be evident from the last experiment that the application of this procedure may lead to completely false values on the relative amylase activities, when not the greatest precaution is taken. This will be demonstrated in the next experiment:

Exp. 03. Extraction at 0° C. for 24 hours.
2 pulvini extracted in 88,4 mm³ buffer pH 5,9.
a_1 — undiluted extract — amylase content x
a_2 — diluted: 3 aq. dest.: 1 a_1 — amylase content 1/4 x
a_3 — diluted: 3 aq. dest.: 1 a_2 — amylase content 1/16 x.

TABLE 5. Appreciation of amylase activity.

ratio:		samples: a1 a2 a3
actual ratio		16 : 4 : 1
read from the graph after:	hydrolysing 1 hour	16 : 4 : 1
	hydrolysing 2 hours	12,5 : 4 : 1
	hydrolysing 4 hours	7 : 3,7 : 1

In the table 5 I have compiled the ratios of amylase activity as can be read from fig. 13, considering several periods of hydrolysis.

It appears clearly from this experiment that *for comparing amylase quantities it might be the best to compare the slope of the hydrolysis curve in its initial part,* viz. expressed in the tangent of the angle curve/abcissa.

In many of the experiments described in the next paragraphs it will be seen that the hydrolysis curve at the hour 0 does not

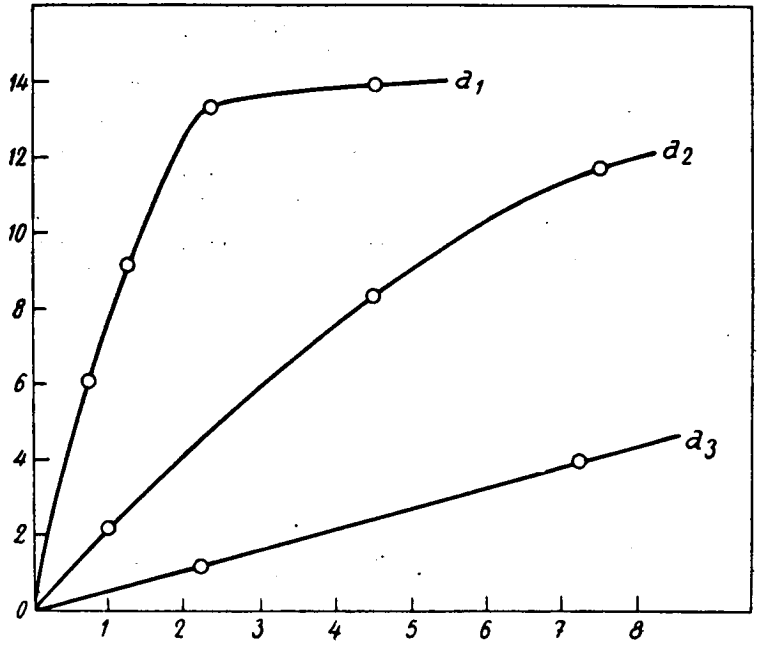


Fig. 13. Appreciation of amylase activity. See text.
ordinate: sugar in mm³ thiosulphate,
abscissa: time in hours.

start at zero. This is due to an initial sugar content of the extract or of the substrate (starch solution), in most cases of the extract. To eliminate these errors one should always try to estimate two points of the first straight part of the curve.

For a full application of the procedures explained above may be referred to the experiments 68 and 69, p. 809.

§ 5 *Methods of extraction and hydrolysis* [1]).

Since the quantity of amylase extracted from the tissue is estimated by the quantity of reducing sugars, formed by hydrolysis of starch, it is necessary to keep in mind that the quantity of sugars determined is not a measure for the amylase quantity without comment.

The quantity of reducing sugars, formed from starch by hydrolysis through the action of amylase, depends on:

I. the quantity of amylase,

II. the conditions under which hydrolysis takes place.

I. The quantity of amylase on its turn depends on the conditions under which the extraction of the tissue happens, viz.:

a. the liquid in which the amylase is extracted,
b. the way of fixing the tissue,
c. the way of crushing the tissue,
d. the temperature,
e. the time of extraction,
(f. the activation by various substances).

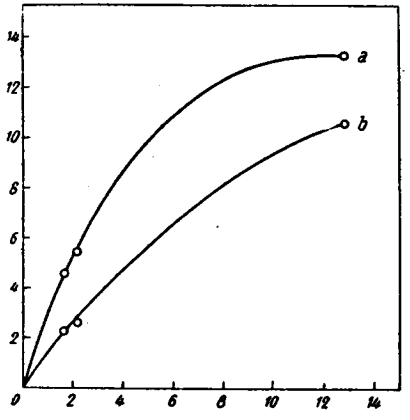


Fig. 14.

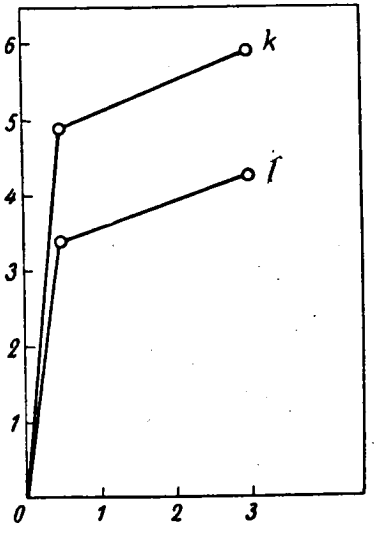


Fig. 15.

Extraction in different liquids. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

[1]) See note on p. 789.

Of all these factors I had to determine the effect on the sugar quantities finally measured.

a. As liquids to extract amylase from the plants I compared distilled water and buffer solutions of various composition.

Exp. 11 gives the result of a comparison between the extraction in aq. dest. and in a phosphate buffer of pH 5,9.

Extraction at 0° C. for 27 hours.

a — 25,3 mg tissue in 170,8 mm³ *buffer pH 5,9*

b — 19,7 mg tissue in 170,8 mm³ *aq. dest.*

results in table 6 and in fig. **14.**

TABLE 6. Influence of extraction liquid.

time of hydrolysis	a		b	
1h40	5,79	*4,58*	2,14	*2,17*
2h10	6,85	*5,42*	2,55	*2,59*
12h50	13,32	—	10,27	*10,60*

Exp. 12 shows that extraction in buffer pH 5,9 gives a higher yield than that in buffer pH 10,5;

Extraction at 35° C. for 6 hours.

k — 16,9 mg tissue in 170,8 mm³ *buffer pH 5,9*

l — 23,7 mg tissue in 170,8 mm³ *buffer pH 10,5.*

results hydrolysis in table 7 and in fig. **15.**

TABLE 7. Influence of extraction liquid.

time of hydrolysis	k		m	
30 min.	4,14	*4,89*	3,98	*3,36*
180 min.	4,98	*5,90*	5,05	*4,26*

b. In order to stop as abruptly as possible the metabolic processes of the tissue, I looked for a method of fixing the tissue before or perhaps during the extraction. Preliminary experience had already tought me, that ether doesnot affect the amylase, while alcohol does.

The following experiments were made to control these effects:

Exp. 21. Extraction at 0° C. for 4 hours.

a — 7,9 mg tissue from 10^h30 — 14^h30 in 170,8 mm³ buffer pH 5,9.

b — 7,7 mg tissue from 10^h30 — 12^h00 in 44,2 mm³ *ether* and from 12^h00 — 14^h30 in 170,8 mm³ buffer pH 5,9.

results hydrolysis in table 8 and in fig. **16.**

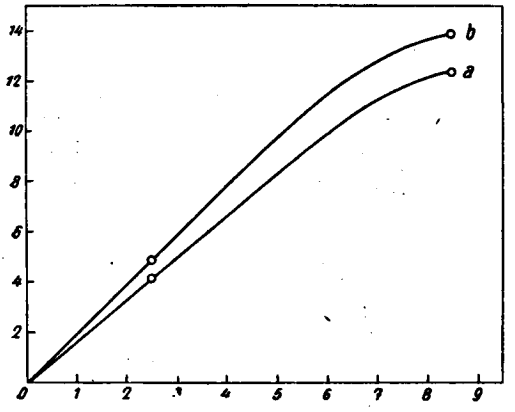


Fig. 16. The influence of ether in extracting. (b — ether).
ordinate: sugar in mm³ thiosulphate,
abscissa: time in hours.

TABLE 8. Influence of ether.

time of hydrolysis	a		b	
2h30	3,29	*4,12*	3,72	*4,83*
8h30	9,64	*12,20*	10,76	*13,94*
21h00	12,98	—	13,42	—

Exp. 22. Extraction at 0° C. for 6½ hours, of which first 1 hour in fixative.
a — 10,1 mg tissue in 44,2 mm³ *alcohol 96%*, then in 170,8 mm³ buffer pH 5,9
b — 10,4 mg tissue in 44,2 mm³ *ether*, then in 170,8 mm³ buffer pH 5,9
results hydrolysis in table 9 and in fig. 17.

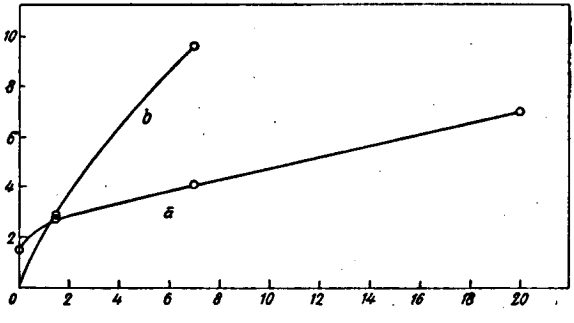


Fig. 17. The influence of alcohol and ether. See text.
ordinate: sugar in mm³ thiosulphate,
abscissa: time in hours.

c. The common way in which I prepared the pulvini before extracting them, was to cut them into thin slices. I compared

TABLE 9. Influence of alcohol and ether.

time of hydrolysis	a		b	
0h00	1,53	*1,52*	0,43	*0,41*
1h30	2,78	*2,75*	3,03	*2,89*
7h00	4,15	*4,10*	10,02	*9,63*
20h00	7,08	*7,02*	13,72	—

this way of extraction to a crushing of the sliced tissue in the extraction-vessel (with a glass-stamper). Experiments 31 and 32 show the results of this comparison:

Exp. 31. Extraction at 35° C. for 2½ hours.
a — 40,0 mg tissue in 170,8 mm³ buffer pH 5,9, *in slices.*
b — 35,9 mg tissue in 170,8 mm³ buffer pH 5,9, *crushed.*
results hydrolysis in table 10 and in fig. 18.

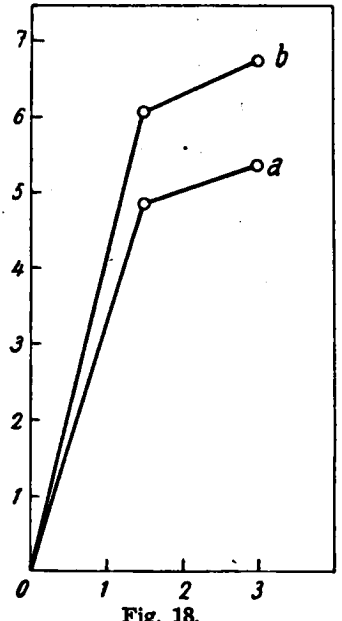


Fig. 18.

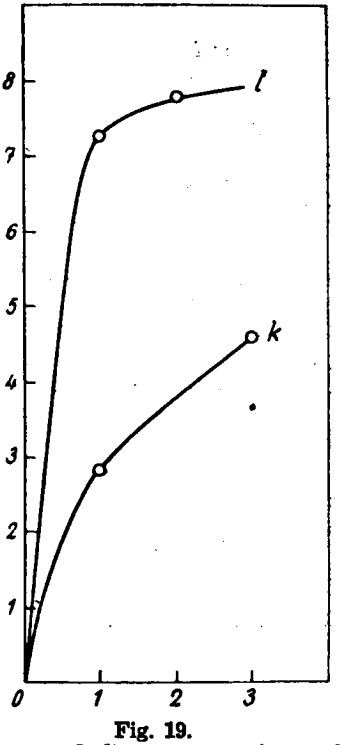


Fig. 19.

The influence of crushing the tissue. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

TABLE 10. Influence of crushing the tissue.

time of hydrolysis	a		b	
1h30	4,86	*4,86*	5,45	*6,07*
3h00	5,35	*5,35*	6,04	*6,73*

Exp. 32. Extraction at 0° C. for 16 hours.
k — 28,2 mg tissue in 170,8 mm³ buffer pH 5,9, *in slices.*
l — 17,8 mg tissue in 170,8 mm³ buffer pH 5,9, *crushed.*
results hydrolysis in table 11 and in fig. 19.

TABLE 11. Influence of crushing the tissue.

time of hydrolysis	k		l	
1 h	3,30	*2,92*	5,17	*7,25*
2 h	—	—	5,54	*7,78*
3 h	5,18	*4,59*	—	—

d, e. The influence of the temperature on the quantity of active amylase, extracted from the pulvinus, often was investigated in combination with the effect of the time of extraction.

Exp. 41. Extraction for 12½ hours.
a — 1 pulvinus in 170,8 mm³ aq. dest., *at 35° C.*
b — 1 pulvinus in 170,8 mm³ aq. dest., *at room-temp. (18° C.)*
results hydrolysis in table 12 and in fig. 20.

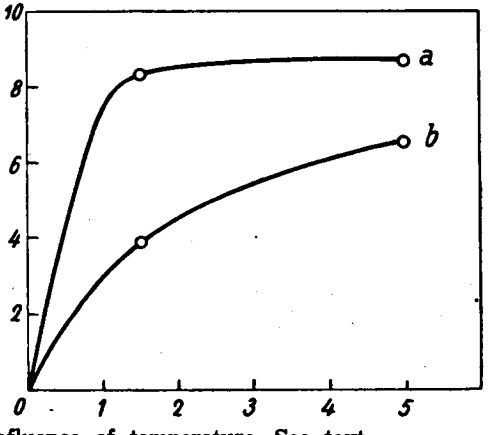


Fig. 20. The influence of temperature. See text.
Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

TABLE 12. Influence of temperature.

time of hydrolysis	a	b
1h30	8,35	3,94
5h00	8,69	6,37

The experiments 31 and 32 show that the same order of values is reached with the extraction at 35° C. and that of 0° C., though of course the extraction times are not the same.

Exp. 42. 2 pulvini in 341,6 mm³ buffer pH 5,9, at roomtemp. The amylase content is estimated after 6, 8 and 11½ hours of extraction; hydrolysis for 1½ hours.

results hydrolysis in table 13 and in fig. 21.

TABLE 13. Influence of extraction time.

time of hydrolysis	time of extraction:		
	6	8	11½ hours
1h30	1,60	2,12	3,00

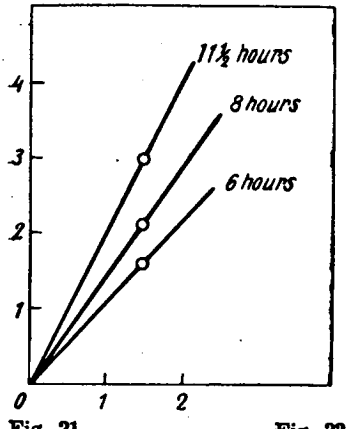


Fig. 21.

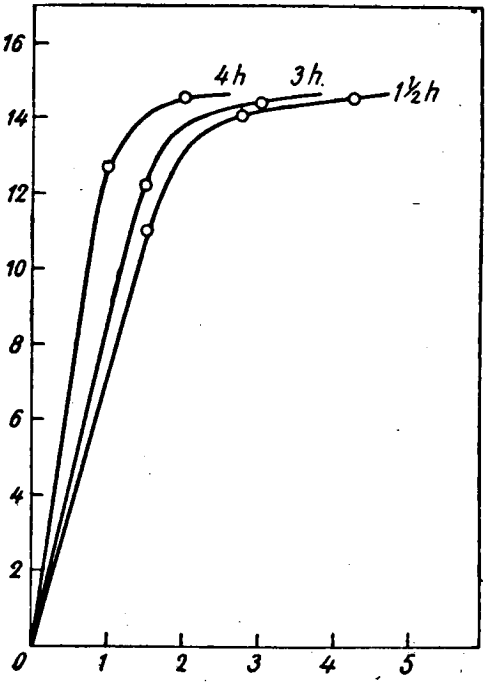


Fig. 22.

The influence of the time of extraction. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

Exp. 43. Extraction at 0° C.
31,1 mg tissue in 170,8 mm³ buffer pH 5,9.
Extraction-time 1½, 3 and 4 hours.
Results hydrolysis in table 14 and in fig. 22.

TABLE 14. Influence of extraction time.

	time of extraction:		
	1½	3	4 hours
time of hydrolysis	(1h30) 11,05 (2h45) 14,13 (4h15) 14,55	(1h30) 12,25 (3h00) 14,40	(1h00) 12,73 (2h00) 14,58

The amylase quantity after these three extraction times may be compared from the curves representing the rate of hydrolysis.
Exp. 44. Extraction at 35° C.
a — 18,5 mg tissue in 170,8 mm³ buffer pH 10,5.
b — 23,7 mg tissue in 170,8 mm³ buffer pH 10,5.
a — extracted for 2½ hours, b — for 6 hours.
Results hydrolysis in table 15 and in fig. 23.

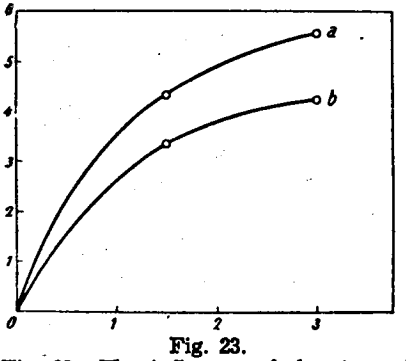


Fig. 23.

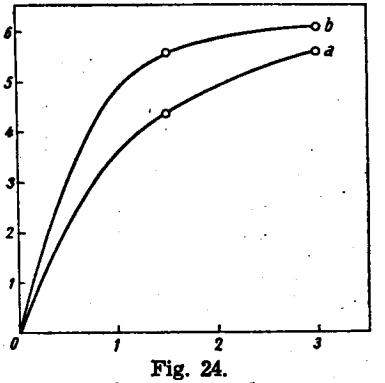


Fig. 24.

Fig. 23. The influence of the time of extraction. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.
Fig. 24. The influence of papayotin. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

TABLE 15. Influence of extraction time.

time of hydrolysis	a		b	
1½ h	4,01	4,33	3,98	3,36
3 h	5,17	5,58	5,05	4,26

The injurious effect of the longer extraction time probably must be due to the high temperature during the extraction.

f. In one experiment only I examined the activating influence of *papayotin;* since this aspect of the enzymatic reaction was of no interest to me, I did not continue my experiments in this direction. Later on I never have used the stimulating effect (that will be shown below) of the papayotin.

Exp. 51. Extraction at 35° C. for 2½ hours.
a — 18,5 mg tissue in 170,8 mm³ buffer pH 10,5.
b — 19,9 mg tissue in 170,8 mm³ buffer pH 10,5, + papayotin.
Results hydrolysis in table 16 and in fig. 24.

TABLE 16. Influence of papayotin.

time of hydrolysis	a		b	
1½ h	4,01	4,33	5,52	5,55
3 h	5,17	5,58	6,00	6,03

II. Conditions during hydrolysis.

The temperature determines the rate of starch hydrolysis. For completeness' sake this may be demonstrated by the results of the experiment 55, which follow below:

Exp. 55. Extraction at 0° C. for 16 hours.
22,1 mg tissue in 170,8 mm³ buffer pH 5,9.
Results hydrolysis in table 17 and in fig. 25.

TABLE 17. Influence of temperature on hydrolysis.

temp.	time of hydrolysis:				
	¾	1¾	3¾	6½	11 hours
35° C.	5,07	5,26	5,69	5,69	—
0° C.	2,27	3,29	4,54	5,05	5,30

Another factor of importance in hydrolysis is the hydrogen-ion concentration. This subject will be dealt with in detail in the next chapter.

§ 6. *Amylase content of upper- and lower half of the pulvinus in various positions of the leaf.* [1])

The original assumption, leading to the extensive study of

[1]) See note on p. 789.

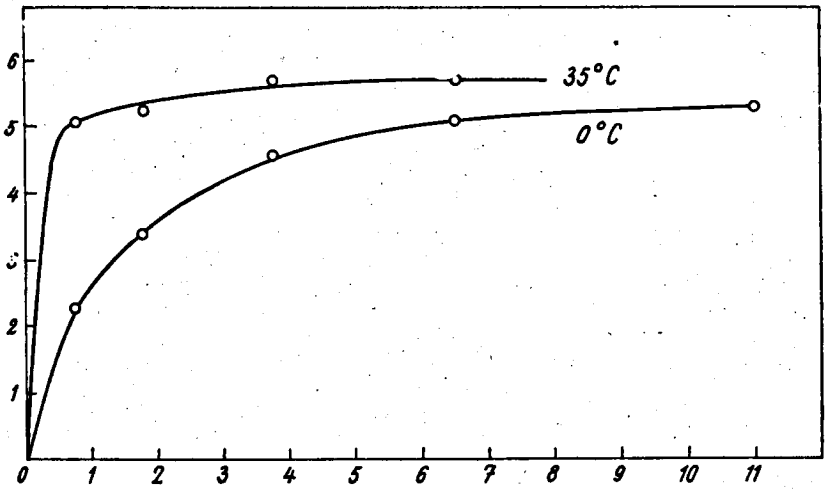


Fig. 25. The influence of temperature on hydrolysis. See text.
ordinate: sugar in mm³ thiosulphate,
anscissa: time in hours.

amylase-extraction methods, was that the mechanism of movements of variation possibly might be simply explained, if the amylase content of both halves of the pulvinus would prove to be unequal in various positions of the leaf.

For this reason I arranged a series of experiments to examine whether such a difference between both halves could be detected.

The dependency of the activity of the pulvinar amylases on several environmental influences (e.g. temperature, hydrogen-ion concentration) urged me to do the experiments in various ways.

Exp. 61.

a — 15,1 mg upper half in 170,8 mm³ buffer pH 5,9
b — 16,1 mg lower half in 170,8 mm³ buffer pH 5,9
k — 16,9 mg upper half in 170,8 mm³ buffer pH 5,9
m — 18,9 mg lower half in 170,8 mm³ buffer pH 5,9
Beginning of the extraction: a and b — 0h30.
(extr. for 6 hours) k and m — 14h00.
Results hydrolysis in table 18 and in fig. 26.

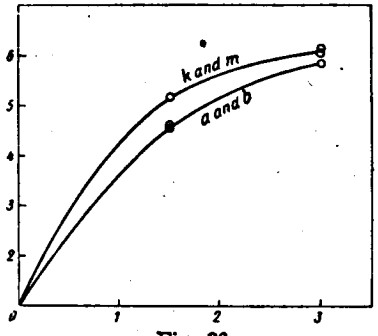


Fig. 26.

Amylase content of upper and lower half. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

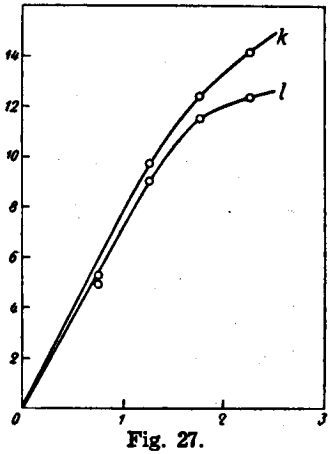


Fig. 27.

TABLE 18. Amylase content of upper and lower half.

time of hydrolysis	a	b	k	l
1½ h	3,20 3,60	3,35 3,54	4,14 4,17	4,63 4,17
3 h	4,32 4,86	4,60 4,85	4,98 5,01	5,72 5,14

There is a striking conformity in this experiment between the amylase content of the upper and of the lower half, both at 0^h30 and at 14^h00; the sum of the amounts of amylase of both halves seems to differ to a certain extent at different times. This, however, can be attributed to the fact, that the pulvini at 0^h30 and at 14^h00 were from two different plants and it is a well-known fact, that two individuals need not contain the same amount of active amylase[1]).

In this connection it may be emphasized that this divergency too is another reason (besides that already mentioned in § 4 of this chapter) why the figures, representing the estimated

[1]) I refer, for instance, to a remark of OPARIN (1934): "Wir sehen demnach, dasz die Aktivität irgendeines Ferment (z.B. der Amylase oder Invertase) keinen für eine bestimmte Zelle charakteristischen, konstanten Wert vorstellt. Die Fermentaktivität erfährt im Gegenteil under dem Einflusz innerer und äuszerer Faktoren weitgehende, rasch ablaufende, reversible Veränderungen".

quantity of reducing sugars, cannot be mutually compared in more than in one experiment only.

Exp. 62. Extraction at 0° C. for 5 hours, beginning 7^h00.

k — 11,8 mg *upper* half of 2 pulvini in 170,8 mm³ buffer pH 5,9.

l — 13,2 mg *lower* half of 2 pulvini in 170,8 mm³ buffer pH 5,9.

Results hydrolysis in table 19 and in fig. 27.

TABLE 19. Amylase content of upper and lower half.

time of hydrolysis	k		l	
¾ h	4,86	*4,94*	5,76	*5,23*
1¼ h	9,52	*9,67*	9,93	*9,02*
1¾ h	12,20	*12,40*	12,65	*11,50*
2¼ h	13,85	*14,18*	13,60	*12,35*

In this experiment the reduction of the figures to the same weight of tissue leads to a divergency in the curves (see graph), which, however, is not essential at all. Only in this case I have calculated and plotted the reduced values completely, to demonstrate the conformity of these two divergent curves to that of exp. 02 (§ 4).

Exp. 63. Extraction at 0° C. for 5 hours, beginning 10^h15.

a — 12,5 mg *upper* half in 170,8 mm³ buffer pH 5,9

b — 12,8 mg *lower* half in 170,8 mm³ buffer pH 5,9.

Results hydrolysis in table 20 and in fig. 28.

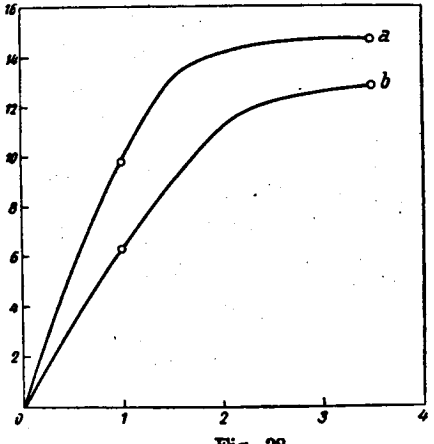


Fig. 28.

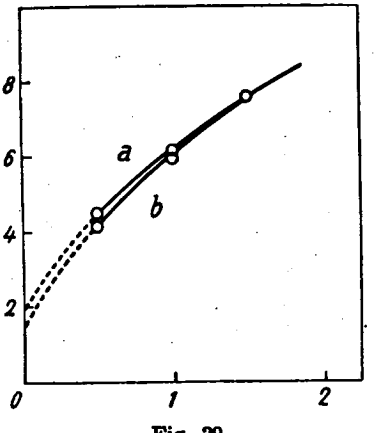


Fig. 29.

Amylase content of upper and lower half. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

TABLE 20. Amylase content of upper and lower half.

time of hydrolysis	a		b	
1 h	9,79	*9,79*	6,45	*6,30*
3½ h	14,70	*14,70*	12,85	*12,80*

Exp. 64. Extraction at 0° C. for 5 hours, beginning 8^h00.
a — 10,8 mg *upper* half in 170,8 mm³ buffer pH 5,9.
b — 10,6 mg *lower* half in 170,8 mm³ buffer pH 5,9.
Results hydrolysis in table 21 and in fig. 29.

TABLE 21. Amylase content of upper and lower half.

time of hydrolysis	a		b	
½ h	4,47	*4,47*	4,05	*4,13*
1 h	6,13	*6,13*	5,85	*5,96*
1½ h	7,60	*7,60*	7,45	*7,59*

In this experiment the initial sugar content of the extract seems to have been rather high (see § 4).

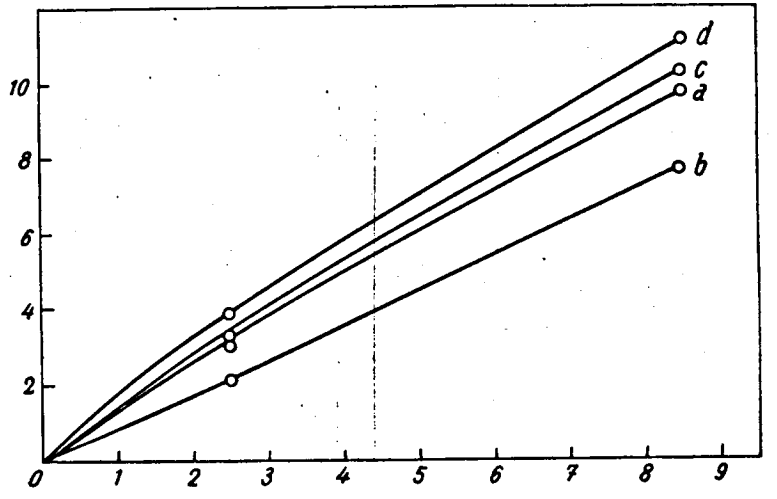


Fig. 30. Amylase content of upper and lower half. See text.
Ordinate: sugar in mm³ thiosulphate,
abscissa: time in hours.

Exp. 65. Extraction at 0° C. for 4 hours, beginning 10^h30.
a — 7,9 mg *upper* half in 170,8 mm³ buffer pH 5,9.
b — 8,8 mg *lower* half in 170,8 mm³ buffer pH 5,9.
c — 7,1 mg *upper* half in 170,8 mm³ buffer pH 5,9.
d — 7,7 mg *lower* half in 170,8 mm³ buffer pH 5,9.
Results hydrolysis in table 22 and in fig. 30.

TABLE 22. Amylase content of upper and lower half.

time of hydrolysis	a	b	c	d
2½ h	3,29 *3,33*	2,16 *2,07*	2,75 *3,10*	3,72 *3,87*
8½ h	9,64 *9,76*	8,46 *7,69*	9,02 *10,27*	10,76 *11,18*
21 h	12,98	12,71	13,12	13,42

The apparent difference in amylase content between both pulvinar halves, at least partly, might be the effect of a difference in initial sugar content of the extract or of the starch solution used for hydrolysis.

Exp. 66. Extraction at 0° C. for 6½ hours, beginning 8^h30.
a — 8,8 mg *upper* half in 170,8 mm³ buffer pH 5,9.
b — 10,4 mg *lower* half in 170,8 mm³ buffer pH 5,9.
Results hydrolysis in table 23 and in fig. 31.

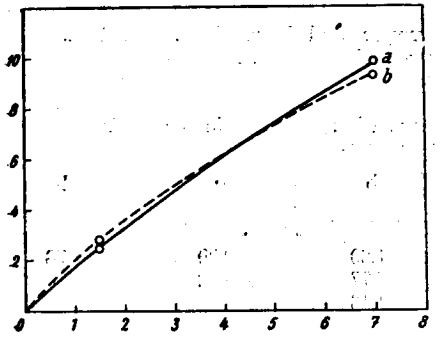


Fig. 31. Amylase content of upper and lower half. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

TABLE 23. Amylase content of upper and lower half.

time of hydrolysis	a		b	
1½ h	2,31	*2,49*	3,03	*2,77*
7 h	9,17	*9,90*	10,02	*9,32*
20 h	13,79	—	13,72	—

Exp. 67. Extraction at 0° C. for 6 hours, beginning 8^h15.
a — 12,6 mg *upper* half in 170,8 mm^3 buffer pH 5,9.
b — 12,4 mg *lower* half in 170,8 mm^3 buffer pH 5,9.
c — 10,3 mg *upper* half in 170,8 mm^3 buffer pH 5,9.
d — 10,7 mg *lower* half in 170,8 mm^3 buffer pH 5,9.
Results hydrolysis in table 24 and in fig. 32.

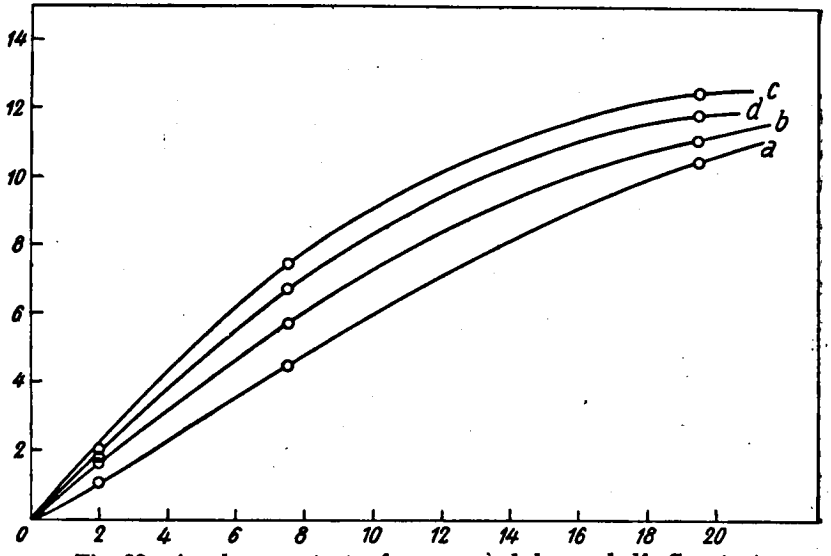


Fig. 32. Amylase content of upper and lower half. See text.
Ordinate: sugar in mm^3 thiosulphate,
abscissa: time in hours.

TABLE 24. Amylase content of upper and lower half.

time of hydrolysis	a	b	c	d
2 h	1,04	1,60	2,00	1,78
$7^1/_2$ h	4,54	5,77	7,42	6,76
$19^1/_2$ h	10,53	11,16	12,48	11,85

(The figures have not been reduced to the same tissue weight, because the differences in weight between the two halves of one pulvinus are minimal).

In two experiments the amylase content of upper and lower halves was measured during 24 hours (every 6 hours an extraction was started). The extraction happened at 0° C. for 2 hours in 44,2 mm^3 ether, then for 22 hours in 170,8 mm^3 buffer pH 5,9.

Exp. 68.

at 6h00	a — 17,1 mg *upper* half b — 16,3 mg *lower* half	each sample from two pulvini.
at 12h00	c — 13,0 mg *upper* half d — 15,9 mg *lower* half	
at 18h00	e — 20,4 mg *upper* half f — 24,2 mg *lower* half	
at 24h00	g — 25,0 mg *upper* half h — 21,3 mg *lower* half	

The sugar formed by hydrolysis was estimated in each pair of extracts after 1, 3, 5, 9¼ and 34 hours, to be sure that the part of the curves used for comparison still is below the bending of the hydrolysis curve. Only the curves of one pair of extracts (e and f) are given as an example of this procedure (fig. 33).

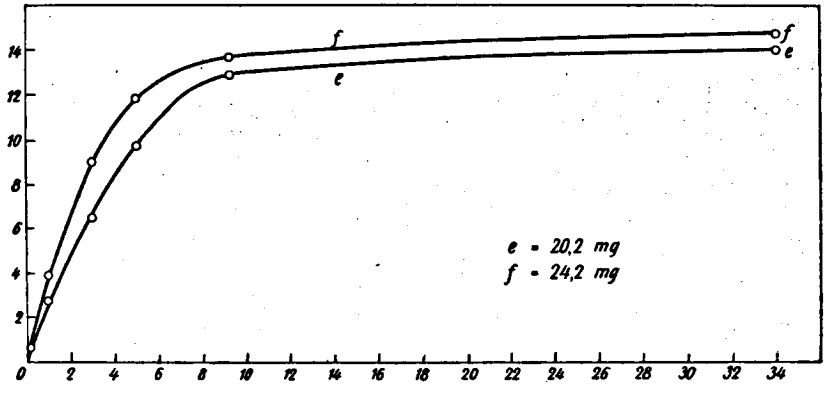


Fig. 33. For explanation see text.
Ordinate: sugar in mm³ thiosulphate,
abscissa: time in hours.

Results of hydrolysis (after 1 and 3 hours, and all reduced to the same weight of 17,1 mg) in table 25 and in fig. 34.

To give an easy survey, I represented the amylase activity by

TABLE 25. Amylase content during 24 hours.

time of hydrolysis	a	b	c	d	e	f	g	h
1 h	1,80	2,18	2,17	2,74	2,36	2,70	2,59	2,73
3 h	4,90	5,77	5,31	6,69	5,52	6,36	5,73	6,14

the tangent of the angle between the hydrolysis curve and the abscissa in the graphs. In this way a single figure expresses the differences in amylase activity between the upper and the lower half of the pulvini. At the same time eventual errors by differ-

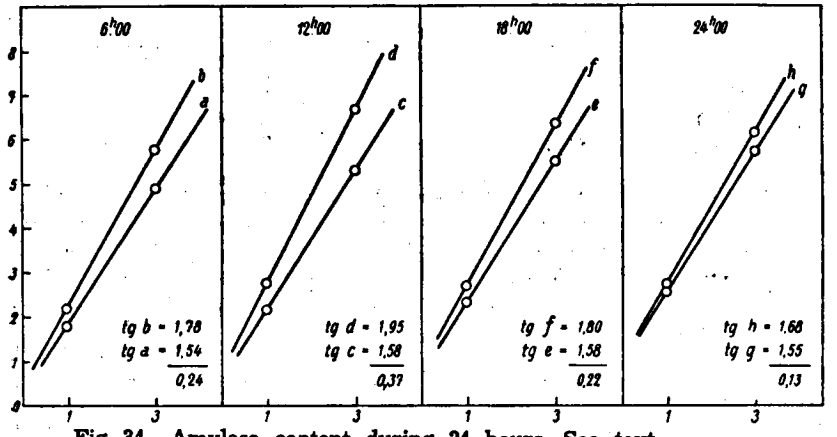


Fig. 34. Amylase content during 24 hours. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

ences in the initial sugar content of the extracts are eliminated. The slope of the amylase-activity curve is a better standard for the amylase activity than e.g. the quantity of sugars formed by hydrolysis in a certain period.

At all four hours chosen the lower half appears to have a higher amylase content than the upper half, although the differences were small. For the values see fig. 34. The values calculated for the upper halves (expressed in the tangents described above) prove to be strikingly equal in all four cases.

The same procedure was repeated in

Exp. 69.

at 0h00	a — 11,1 mg *upper* half b — 13,2 mg *lower* half	each sample from two pulvini.
at 6h00	c — 25,6 mg *upper* half d — 19,7 mg *lower* half	
at 12h00	e — 15,8 mg *upper* half f — 15,9 mg *lower* half	
at 18h00	g — 18,3 mg *upper* half h — 16,9 mg *lower* half	

Results of hydrolysis (all reduced to the same tissue weight of 15,7 mg) in table 26 and in fig. 35.

TABLE 26. Amylase content during 24 hours.

time of hydrolysis	a	b	c	d	e	f	g	h
1 h	3,08	2,42	2,07	2,73	2,34	2,65	2,95	3,72
2 h	5,52	4,40	3,52	4,81	4,03	4,39	5,12	6,60

The values for the amylase activity and for the differences in amylase activities between the upper and lower halves can be read from fig. 35.

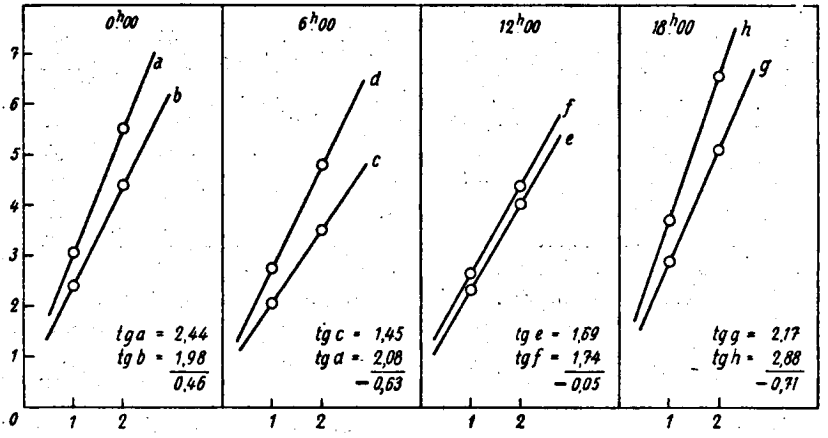


Fig. 35. Amylase content during 24 hours. See text. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

§ 7. *Discussion and conclusions.*

Summarizing the results of the experiments in this chapter, I have shown that amylase is present in the pulvini of *Phaseolus*, further where it occurs and how the method of extraction affects the finally measured quantity of reducing sugars, formed by hydrolysis from soluble starch.

For extracting, buffer solutions of a certain pH proved to yield a higher amylase content of the extract than distilled water. I, therefore, further always used a buffer solution (of pH 5,9) as extraction liquid.

It has been stated by OPARIN and RISKINA (1932) that extractions in buffer (they used a buffer solution of pH 8,0 after Mc. ILVAINE) show a much higher amylase content than extractions in water. They suppose, that in the first case the total quantity of enzyme (also the inactive part) is extracted from the tissue, while in the latter only the active enzyme compound(s) enters into the extraction solution. They remark further, that for this reason the daily changes in amylase activity of leaves would not be observable when extraction happens in a buffer, while extracting in water would reveal differences.

I found this information at a time, when all my attempts to state a difference between the amylase contents of both pulvinar halves had already been made. Besides, it still remains open to doubt whether the changes in amylase activity under the influence of hydrogen-ion concentration in the tissue (the importance of which will be described in chapter VII and VIII) ever might be reflected in the amylase content of extracts, independently of the extraction liquids used.

The reason why I used ether to start the extraction in most of the experiments, was to kill the tissue without destroying the amylase from the tissue.

Crushing the tissue instead of only slicing it yielded a much higher demonstrable quantity of amylase. Yet I have never used this method in estimations where a high degree of accuracy was desired, because it proved impossible not to alter the total quantity of tissue already weighed, in removing from the liquid the glass-stamper used for crushing.

A perusal of the results of the experiments on the influence of the temperature and the time of extraction on the amylase content of the extract will show that the higher the temperature and the longer the time of extraction, the higher the amylase content. Still I hesitated to extract at a high temperature and for a long time, because it is uncertain whether one does not partially destroy the enzymes (though raising their activity).

It is known from literature that most of the enzymes are gradually losing their activity, when extracted from the living tissue, while also many enzymes are known to be very sensitive to too high or too low temperatures. Therefore I have applied several temperatures and different times of extraction, when trying to find a difference in amylase quantity between the upper and the lower half of the pulvinus.

These latter attempts have not been successful. In some cases a difference was found but reverse results were so frequent,

that no sufficient proof is present, to maintain the possibility of differences in amylase quantity between both halves as a probable means for explaining the mechanism of the movements.

CHAPTER VII.

Hydrogen-ion concentration.

§ 1. *H-ion concentration and amylase activity.*

It is a well-known fact that the activity of enzymes is highly dependent on the concentration of certain ions, among which H-ions play the most important part.

In literature the influence of H-ion concentration on amylase activity has repeatedly been investigated. An extensive review is given bij OPPENHEIM (1936). Almost all investigators have described an optimum-curve. The subject has thoroughly been investigated by VAN KLINKENBERG (1932), who estimated the pH optimum-curves for both α- and β-amylase (from malt). Of all types of amylases, hitherto examined on their activity in different H-ion concentrations, the pH optima lay between pH 4 and pH 6. In some cases the range of optimal activity is wide, in other cases it is only a minor part of the investigated H-ion concentration traject.

In view of the dependency of the amylase activity on pH, I decided to determine the pH activity curve of the pulvinar amylase, in order to see whether these data somehow might reveal a relation with the phenomena of reversible volume changes. Of course I expected to find a pH optimum-curve with one optimum at about pH 5, and so I wondered, when finding a two-peaked curve, or, if one prefers to say so, a curve with a very broad optimum (from pH 4 to pH 7) and a sinking near pH 5,8 — 6,0 (see fig. 36).

Experiments.

In 9 experiments I examined the activity of the pulvinar amylase extract in media of various hydrogen-ion concentrations. The results of two of the experiments are reproduced (fig. 36 and fig. 37).

The hydrogen-ion concentrations were obtained in using buffer solutions after Mc ILVAINE; the buffering compounds are 0,1 mol citric acid and 0,2 mol Na_2HPO_4. The pH's obtained with the aid of these buffer solutions cover a range from about pH 2 to pH 8. The values of the pH were controlled

colorimetrically or, in some cases, by means of a chinhydrone-electrode.

The pulvini were extracted first in ether and then in distilled water. No buffer was used here, to prevent shifting of the pH of the solutions in which hydrolysis had to occur. Since rather large quantities of extract were required in these experiments, in some cases I made the extract from a mixture of petiolar and pulvinar tissue; no deviations as compared with plain pulvinar extracts were noticed.

The extracts were filtered through a glass-filter, in order to avoid inaccurate readings by slight contaminations in the solutions. — In the experiments described below all quantities used are exactly mentioned.

Exp. 71.

Extracted 1 g petiolar and pulvinar tissue (cut into thin slices) in 5 cm³ aq. dest. — temp. 35° C.

Extraction-time: ether 30 minutes
aq. dest. 150 minutes

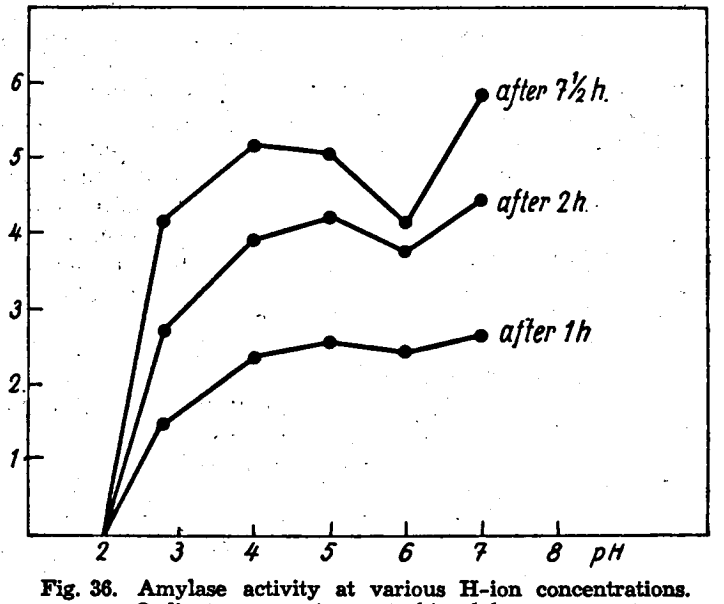


Fig. 36. Amylase activity at various H-ion concentrations. Ordinate: sugar in mm³ thiosulphate, abscissa: time in hours.

Quantities used in hydrolysis:
76,2 mm³ extract
170,8 mm³ buffer + 1% soluble starch.

results: (see fig. 36)

TABLE 27. Amylase activity at various pH [1]).

time of hydrolysis	pH: 2,0	2.8	4.0	5.0	6.0	7.0
1 h	0	1,45	2,35	2,55	2,45	2,65
2 h	0	2,70	3,90	4,20	3,75	4,45
7½ h	0	4,15	5,15	5,05	4,15	5,85

Exp. 72.

Extracted 265 mg pulvinar tissue in 1.30 cm³ aq. dest. temp. 35° C.
Extraction-time: ether 30 minutes, aq. dest. 150 minutes.
Quantities used in hydrolysis:
58,6 mm³ extract
126,4 mm³ buffer + 1% soluble starch.

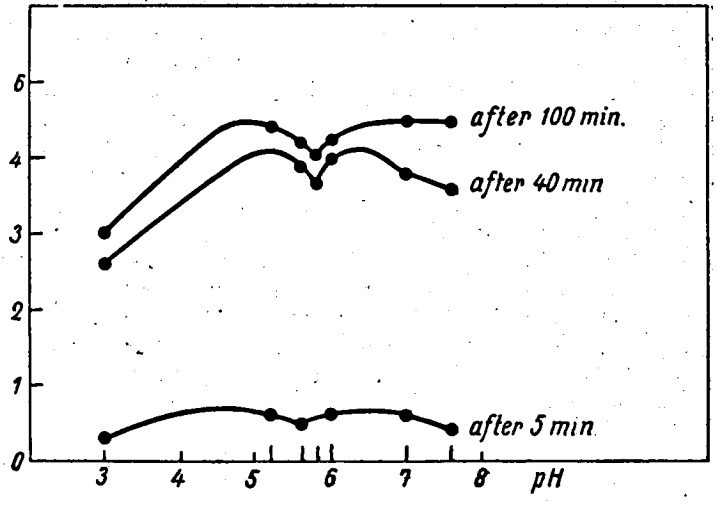


Fig. 37. Amylase activity at various H-ion concentrations. Ordinate: sugar in mm³ thiosulphate, abscissa: H-ion concentration in pH.

[1]) In tables 27, 28 and 29 the figures represent the quantities of reducing sugars, formed by hydrolysis, expressed in mm³ thiosulphate.

results: (see fig. 37)

TABLE 28. Amylase activity at various pH.

time of hydrolysis	pH: 3.0	5.2	5.6	5.8	6.0	7.0	7.6
5 min.	0.30	0.60	0.50	0.60	0.60	0.60	0.40
40 min.	2.60	—	3.90	3.70	4.00	3.80	3.60
100 min.	3.00	4.40	4.20	4.05	4.20	4.50	4.50

Since I did not investigate whether α- or β-amylase was present in the pulvinar extract [1]), I am fully aware that one may argue that the presence of both amylases may acount for the two-peaked curve. I believe that this cannot be the case, since VAN KLINKENBERG (1931) has shown that, though the curve of β-amylase has its optimum at pH 4,5—5,2, the optimum of the α-amylase curve (pH 5.8—6.0) just falls in the sinking of teh pH-activity curve of the pulvinar amylase (see figs 36 and 37).

A much more serious objection might arise from the method used in estimating the sugars formed by hydrolysis. This method allows the estimation of the quantities of "reducing sugars" and therefore, besides maltose, its cleavage product (glucose) might also be determined. Now the enzyme maltase, which splits up the maltose in twice as much glucose, acts optimally at a pH of about 7-8. Thus, the two-peaked curve might be caused by the action of maltase, partly hydrolysing the maltose formed by the amylase from the starch. One molecule maltose (with one reducing bond) forms two molecules glucose (each with one reducing bond). At pH 5,5—6,0, where the activity of the amylase decreases, that of the maltase is increasing. If the pulvinar extract contains maltase, this fact would neutralize the effect of the decrease in amylase activity.

To control this posibility, I have examined the extract on its maltase content. Using extremely pure maltose ("KAHLBAUM" p.a.) as a substrate, no maltase appeared to be present in the extract. One of three experiments is reproduced here.

Exp. 73.

Extracted 100 mg pulvinar tissue in 500 mm³ aq. dest. — temp. 35° C.

Extraction-time: ether 30 minutes
aq. dest. 150 minutes

[1]) I was interested only in the activity of the extract as such at various H-ion concentrations. Whether this activity has to be ascribed to one or more different amylases is a subject for a special detailed enzymatic study.

Quantities used in hydrolysis:
58,6 mm³ extract
126,4 mm³ buffer + 1% maltose (or 1% starch)

TABLE 29. Maltase not present in the extract.

time of hydrolysis	pH: 4.6	7.0	10.5	
0 min.	6,95	7,40	6,40	substrate: Maltose
60 min.	6,93	7,40	6,68	
180 min.	6,90	7,70	7,15	
0 min.		0,00		substrate: Starch
90 min.		7,67		

Though, as I have already mentioned, generally only one single pH-optimum has been described for amylases in literature, I have found some indications as to the occurrence of two-peaked pH curves. ORRÚ (1930) described a pH optimum at 5,2—5,4 and a second, though less prominent one, at 6,4—6,6. GIRI and SREENIVASAN (1937) reported on the amylase system of the rice-kernel that the pH-optimum of β-amylase lays at 4,5—5,1 and that of α-amylase at 6,4—7,1. BOIS et NADEAU (1936) estimated the "neutral points" of amylase and found that these lay at pH 4,6—4,9 and at 6,5—6,7.

It thus appears that the phenomenon reported by me stands not alone. It would be useless to try to explain the two-peaked curve of the amylase activity; to this purpose it would be necessary to investigate in detail which types of amylase are present in the extract and how their activity is at various hydrogen-ion concentrations.

An explanation, however, is at yet not of special interest to me; the important thing is that *the pulvinar amylase is less active at pH* 5,8—6.0 *than at both higher and lower hydrogen-ion concentrations.*

Still there is one thing to be considered: the amylase content of the tissue must be much higher than that measured in the extracts, since in extracting one dilutes to a high degree the original amylase concentration. It seems probable, that the extract is at least about 5× diluted as compared to the tissue. [1])

[1]) Viz. in exp.'s 71, 72 and 73 a certain weight of tissue is extracted in 5 × as much water!

This being the case, also the amylase activity of the tissue at any pH must be about 5× higher, and it will be clear that *the shape of the amylase activity curve,* given in fig. 36, *is only a faint reflection of the activities of the actual amylase concentration in the tissue.*

To realize what this means, we want to compare for instance the amylase activity at pH 5,8 with that at pH 6.5.

The same difference in the amounts of produced sugar, plotted in figs. 36 and 37, would have been obtained in at least 1/5 of the time at a concentration of amylase as present in the tissue. Further it also may be probable that the differences would be much more pronounced.

§ 2. *H-ion concentration of the tissue.*

It cannot be denied that, up to now, it has not been possible to measure the pH of the cell sap in a tissue. Yet it seemed necessary to me to try to get some information on the pH in the pulvinar tissue. Direct measurements, of course, were out of question.

One of the great difficulties met with, is the small quantity of juice of which the H-ion concentration is to be determined.

Since I was once accustomed to the use of micro test tubes and micro-pipettes, I applied these tools for a colorimetrical method of comparing H-ion concentrations. To this purpose I made a colour standard for several pH-indicators, viz. methylred, bromthymolblue and bromcresolpurple. For preparing the scale I used buffer solutions of citric acid and Na_2HPO_4 (Mc ILVAINE;

TABLE 30. (for explanation of the characters see text).

		indicator:		
No.	pH	methylred	bromthymolblue	bromcresolpurple
I	4,70	red		
II	5,08			yellow
III	5,29		yellow	
IV	5,83			← aq. dest.
V	6,28		← c	← d
VI	6,66	a	← b	← e
VII	6,87		← tap-	
VIII	7,28	yellow	blue water	violet
IX	7,72			

already mentioned on p. 816). For each indicator a series of tubes was filled with 126,4 mm^3 buffer solution and 9,4 mm^3 indicator solution, the pH of the buffers being controlled ahead with a chinhydrone-electrode. The series used for the three types of indicators are given in full in table 30.

Also some tests are given in that table. Most of the observations were made with bromcresolpurple; the results are marked with symbols which mean:

a. pulvinar tissue, pressed out in tap water, changed the colour from purely yellow (tap water) to the blue side.

b. pulvinar tissue, cut into slices and extracted in tap water, changed the colour of tap water to the yellow side (between VI and VII).

c. pulvinar tissue, the same as that of b, now crushed by means of a glass-stamper. The colour shifted still more to yellow, now being between V and VI.

In the experiments with bromcresolpurple, distilled water was used to extract the tissue. Distilled water itself started with a nearly yellow colour as soon as water and indicator had been brought together; however, its colour changed rather rapidly to completely violet. Now, in observating the colour changes brought about by the tissue in aqua destillata, it stroke me that the colour of the latter did not change at all for many hours, while this is the case with plain distilled water.

d and e represent the colour given by the juice of the pulvinar tissue (the pulvinus first being cut up into thin slices). In some cases the colours obtained were between IV and V (d), but in most of the experiments the pH of the extracted juice was between 6,28 and 6,66.

It has been attempted to determine too whether a difference in pH of the upper- and the lower half might be detected, but I have never succeeded in showing such a difference; probably it will not be possible to detect any differences in H-ion concentration between both sides by means of this crude method.

Conclusions: Generally the value of pH in the extracted cell sap was found at about pH 6,5. — Since in extracting, the electrolyte solutions of the cell sap have considerably been diluted, the actual value of the pH of the cell sap in the tissue

must be less than 6,5. It depends on the buffering capacity of the cell sap how much the actual value has changed by diluting. Therefore we may conclude, that *the pH of the cell sap probably lies between* 5,5 *and* 6,5 (assuming a maximal dilution of 1 : 10 in extracting).

No differences in pH of the upper- and lower half of the pulvinus could be demonstrated with this method.

§ 3. *Shifting of H-ion concentration recorded by measurements of potential differences.*

Since more than a year J. B. THOMAS is engaged with measurements of P.D.'s in several plants. When he proposed me to proceed to measurements of the P.D. of the petiole and the pulvinus of *Phaseolus multiflorus*, I have eagerly accepted that proposal, since no means should be neglected which perhaps might throw new light on the mechanism of the movements.

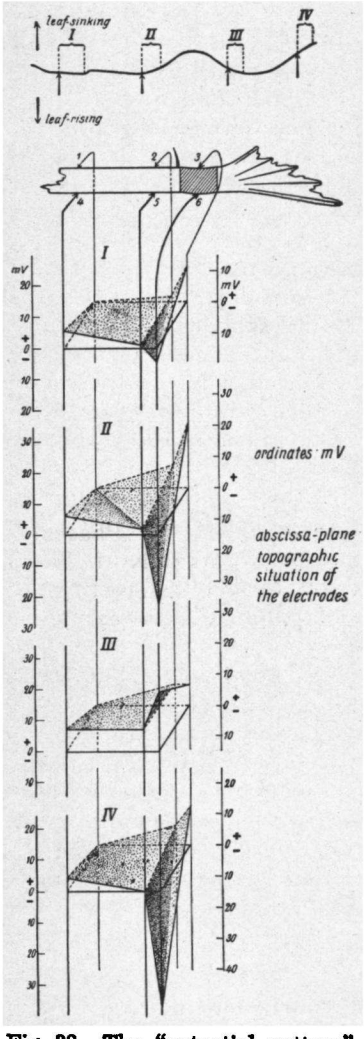
So we started to investigate whether a relation could be found between the movements of the leaves of *Phaseolus multiflorus* and the P.D. variations in the motile pulvini. When such a relation had been stated, it seemed interesting to examine to which extent the P.D. variations could be explained as concentration effects.

The method of measuring potential differences was described in a previous paper (DE GROOT and THOMAS 1938). I will shortly report the experiments which are of special interest for an explanation of the motile mechanism.

In 11 experiments we measured the P.D. in petiole and pulvinus four times a day. At the same time the movement of the leaf was registered. The distribution of the potential in the petiole appeared not to be related with the movement, while just in the pulvinus such a relation exists. The results of one experiment are reproduced in fig. 38.

In all experiments a shifting of the "potential level", synchronous with the movement, was found in the pulvinus. The results of this series of experiments show that a constant relation exists between the value of the P.D. across the pulvinus and the state of movement; besides, the potential level in the petiole remained practically constant during each experiment.

Subsequently we measured continuously (from minute to



minute) the shifting of the P.D. in the pulvinus, synchronously with the movement. The results of one of several experiments have been reproduced in fig. 4 of our previous paper. It was evident that the variations of the movement are preceded by those of the P.D. of the pulvinus. The space of time between both reactions amounts to 20—30 minutes. The lower side of the pulvinus showed more intensive changes in its potential than the upper one. The potential levels of both sides in general change in an opposite direction.

A discussion of the results yielded data of special interest in relation to the periodic movements. We have calculated the potential differences to be expected when a leaf moves from a high to a low position, if one considers the membranes in the plant impermeable for anions and assuming that the changes in volume and in concentration of the cell sap amount 3/2 in the upper half and 2/3 in the lower half (for argumentation see § 3 of chapter II). Substituting these values in the formula of NERNST for diffusion potentials, we found:

Fig. 38. The "potential pattern" in petiole and pulvinus, represented in a 3-dimensional graph. The horizontal plane 1-2-3-6-5-4-shows the topographic situation of points measured on the median section through petiole and pulvinus: see situation at the top of the graphs. The potential value of 1 each time was chosen as the level of the abscissa plane; the potential of each point is referred to 1. The upper side of the "potential plane" is dotted, its under side shaded.

$$E = \frac{RT}{nF} \frac{l_c - l_a}{l_c + l_a} \ln \frac{c'}{c} = \frac{RT}{F} . 1 . \ln \frac{3}{2} \left(\frac{2}{3}\right)$$

This gave E = + 10,45 mV for the upper half and E = — 10,45 mV for the lower half; the difference between the P.D. in a high and in a low position of the leaf should be 20,90 mV across the pulvinus. As an average of all the experiments we found that the difference of the values at a sinking and a rising state of the leaf amounts to + 24,85 mV (theor. + 20,90 mV) across the pulvinus, while for the upper and the lower side apart (compared to the constant potential level in the petiole) the corresponding values are resp. + 14,21 mV (theor. + 10,45 mV) and — 10,03 mV (theor. — 10,45 mV). This rather close agreement made us assume that we were right when considering the membranes practically impermeable for anions, while it also seems correct to assume the value for n (in the formula) = 1. Since among the univalent cations the speed of the H-ions is of a paramount rate, it is plausible that the influence of other cations, even if they were polyvalent, is only of secondary importance.

In this connection it is important too to know which kind of electric effects were really measured by means of the gelatin-$ZnSO_4$-electrodes applied to the plants. Some experiments with models, to be reported by J. B. THOMAS in a next paper, pointed in the direction that we were exclusively measuring the effect of the shifting of H-ion concentrations.

These results and conclusions considerably will support the theory, discussed in the next chapter, which attributes a probable function to alterations in the hydrogen-ion concentration in the movements. However, no definite method is available to verify the measured values of the pH-changes and to ascertain which changes actually occur.

CHAPTER VIII.

Theory, discussion and conclusions.

§ 1. *A Theory on the mechanism of the nyctinastic movement.*

In the preceding chapters I have made an attempt to analyse some of the complicated processes involved in the motile system of the pulvini of *Phaseolus multiflorus* (and allied species). A tentative synthesis will be given now.

The data presented in the preceding pages allow to conceive

a hypothesis on the role of the subjects studied. The periodic movements in the dark can be explained as follows [1]).

Continuously sugars are removed from the tissue by the respiration and by diffusion to places of lower concentration. The sugar content of the cells is maintained by the action of amylase, which converts starch into sugar. If the removal by respiration and diffusion exceeds the supply by hydrolysis the volume will decrease, if the supply exceeds the removal the volume will increase, because the sugar content chiefly determines the osmotic value of the cells. However, changes in volume must be accompanied by changes in the concentration of the electrolytes solved in the cell sap. It is highly probable that the ionic equilibria in the cell sap will be disturbed when a volume change occurs. A new equilibrium will be established and, for instance, the hydrogen-ion concentration will have got another value. This possibility has been proved by the measurements of P.D.'s (chapter VII). The pH of the cell sap was found to be about 6.0 and it is exactly in this region that the amylase activity in a peculiar way depends on the H-ion concentration. As a matter of course these facts have led me to the assumption that the interaction between amylase activity and H-ion concentration forms a system that in itself may account for the periodic changes in volume.

Similar thoughts on the influence of ionic equilibria on enzyme activity I have found in a paper of St. J. VON PRZYLECKI (1935). He says: "Angenommen, dasz die Amylase ein amphoterer Stoff ist, so könnten wir uns die Aktivität folgenderweise vorstellen: Um die maximale Amylasewirkung zu erreichen ist 1. ein bestimmtes Verhältnis zwischen dissoziierten Anionen/Kationen Gruppen, 2. vielleicht auch ihre Menge (d.i. die Entfernung von gleichnamig und entgegen geladenen Gruppen u.s.w.) nötig. Die Grösze des Verhältnisses übt vielleicht einen Einflusz auf die Affinität, besonders aber auf die Stabilität des Komplexes E-S aus". In the same paper he remarks: "Ein charakteristisches Merkmal der lebenden Materie bildet ihre Fähigkeit zu Autoregulationen". — "Die Regulierung der chemischen Prozesse im Zellverbande ist innigst mit der Regulation der Enzymreaktionen verbunden".

The system may be described with the aid of the scheme in fig. 39. A closed chain of reactions consists of the following components: the amylase activity varies with the pH of the cell sap,

[1]) For the sake of simplicity I have purposely confined the considerations to the sugar-starch metabolism in the pulvinar tissue.

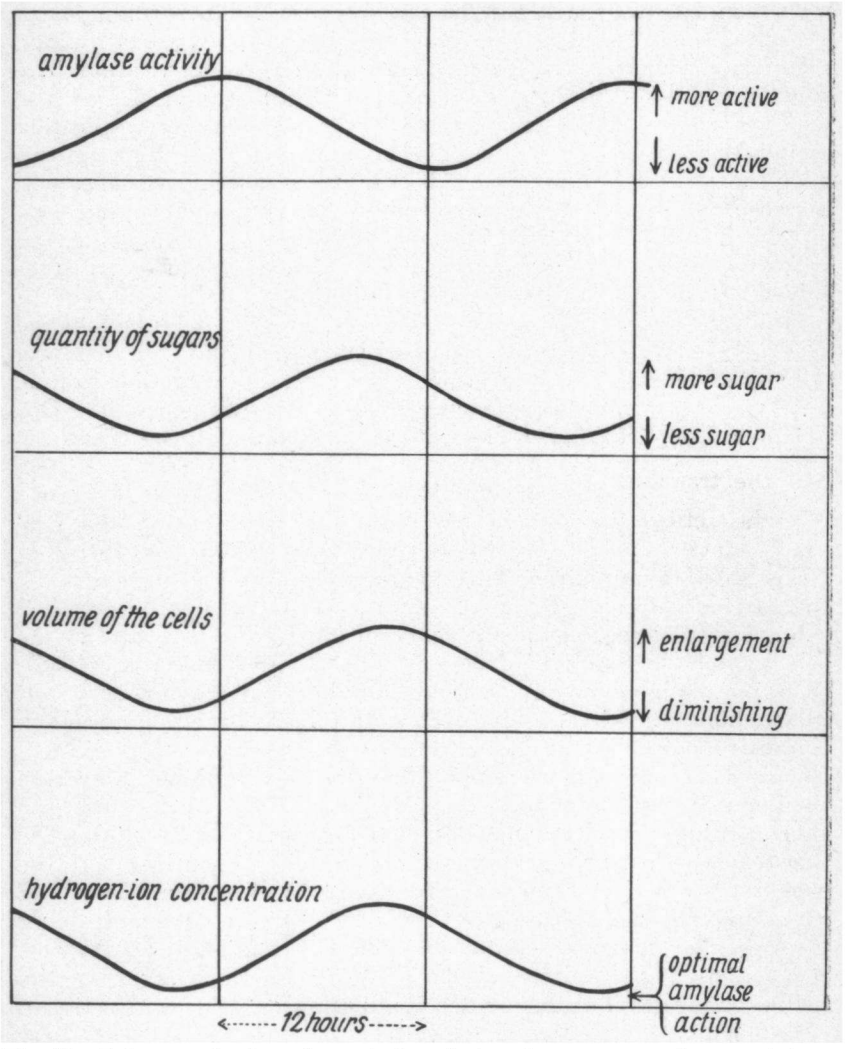


Fig. 39. Scheme of the theoretical relation between amylase activity, quantity of sugars, volume of the cells and H-ion concentrations. For reasoning see text.

the pH depends on the volume of the cell, the volume varies according to the osmotic value of the cell sap and this osmotic value is dependent on the amylase activity. One might also say: The pH is closely related to the water content of the cell, and the amylase activity is determined by the pH as well as it determines itself the water content and the volume of the cell.

I emphasize again that this system concerns merely the periodic movements in the dark. The theory, however, can account for all changes in turgor, also those in the light. For superimposed on the periodic (endonomous) dark processes, all kinds of "stimuli" may influence the various parts of the processes resulting in a certain turgor pressure. To realize how this might proceed, we have to consider how the three processes determining the sugar content (and the turgor) of the cells are dependent on various external and internal agents. The three processes involved are:

I. the respiration
II. the transport by diffusion } — removing sugars
III. the action of amylase — supplying sugars.

The intensity of these processes is dependent on various conditions:

I. The intensity of respiration is affected by a) the amount of available respirable substrate, b) the concentration of oxygen in the air, c) the water content, d) the concentration of carbon dioxide in the air, e) the acidity, f) the salts present, g) the temperature and h) the light (?).

II. As to diffusion of soluble material out of the cells, it must be remembered that

a) the direction of the diffusion is determined by the differences in concentration of the solutions involved,

b) the rate of the diffusion is determined by 1) external conditions, as light, temperature, etc. and 2) internal conditions, as acidity, concentration of electrolytes, etc., which all effect the state of the permeable membranes in the tissue.

III. The effect of the action of amylase is dependent on:

a) external conditions: temperature, light (?),

b) quantity of amylase,

c) quantity of available starch,

d) internal conditions: pH and all kinds of activating or paralyzing influences.

In the day light the processes are complicated by photosynthesis,

and, if I would be complete, I should have to mention all kinds of metabolic processes determining the physiological state of the cells in the pulvinar tissue.

In spite of the great uncertainty as to the actual part of each process in the resulting value of the turgor of a cell, some indications may be given to account for phenomena known in literature and connected with pulvinar movements.

For instance, it seems highly probable that the rapid changes in turgor, caused by the light, studied by Mar. BRAUNER (1933) and others, have nothing to do with amylase action. They can be attributed to an influence of the light on the diffusion system in the tissue. I do not say "on the permeability of the cell membrane", since I cannot imagine how an increase or a decrease of the permeability — at a certain concentration gradient — ever might account for a reversible change in the turgor of the cell. Of course changes in permeability may have something to do with the influence of light, but certainly they cannot merely account for the phenomena.

§ 2. *Discussion.*

In this paragraph I will discuss how the various processes, studied by former investigators and by myself, are related to the system described in the last §.

The endonomous periodic movements in constant darkness can be explained by the endonomous periodic changes in amylase activity in the cells of the motile tissue. No changes in the intensity of respiration or in the rate of diffusion need to be involved in the mechanism of the movements. This does not mean that, if they exist, the explanation given by me would fail. Besides the reversible system of the amylase action other reversible processes concerned in the sugar-starch metabolism may also exist; the result of their joint activity is reflected in the movements.

The results of the measurements of the osmotic values of the upper and the lower half of the pulvinar tissue have shown that in an uplifted position of the leaf the osmotic value of the cells in the lower half was higher than that of the cells in the upper half and reversely. This fact matches with the statements by ZIMMERMANN (1929) and others; all data indicate that changes in the osmotic value of the cells run parallel to changes in volume. The greater the volume, the higher the osmotic value of the cell sap. The objection of BÜNNING (1936), that this parallelism precludes the possibility that the volume changes are

caused by changes in the osmotic value, does not hold. If changes in volume may be induced by alterations of the sugar content of the cells, the effect (the volume decrease or -increase) will rapidly follow alterations of the osmotic values, since the time of latency only depends on the water permeability of the membranes. This latention lasts for so short a time (perhaps several minutes, perhaps half an hour), that it is practically impossible to state the sequence of processes suggested by BÜNNING.

When plants of *Phaseolus* are inverted, the movements continue, but their period is also rapidly inverted (within 2 or 3 hours). This phenomenon can be explained as follows: in the normal position the weight of the lamina tends to enlarge the volume of the cells in the upper half and to decrease that of the cells in the lower half. When the plant is inverted, the weight acts in an opposite way, so that the cell volume in the anatomical upper half is mechanically decreased and in the anatomical lower half is enlarged, compared with the former, normal position. Now the cell volumes that were decreased will increase (and vice versa), according to the same mechanism of starch-sugar conversion, which accounts for the movements in the normal position of the plant. It may be remembered that it is a general feature of the system, that the volumes which have been decreased tend to increase, while those which have been enlarged tend of decrease. I draw the attention to the fact, that the weight of the leaves (acting in downward direction) is no *conditio sine qua non* for the occurrence of movements, since with considerably reduced laminae the pulvini continue their movements. The fact, that the plants stop moving when fixed on the horizontal axis of a rotating clinostat indicates, that the continuously varying way in which the weight of the laminae influences the processes in the tissue, in that case, balances the changes in amylase activity in the cells. The movements on the clinostat have not been studied by registering them (technical difficulties prevented this); the occurrence of movements has been valued. Therefore it still remains possible, that slight movements (in daily periodic rhythm) have escaped the observation of the investigators. Perhaps a detailed study of the influence of gravity on the daily periodic movements might reveal new and important data for the knowledge of the motile mechanism.

SUESSENGUTH (1922) has discussed, among other subjects, the influence of wilting on the action of amylases in plants. His conclusion from a survey of literature is that wilting causes the

hydrolysing enzymes to grow active. This remarkable effect — for, while wilting withdraws water from the cell, by hydrolysis water is bound — has been explained in two ways. VON MOLISCH (1921) thinks of an increase of the enzyme concentration (besides that of all other solved substances), as a result of the loss of water. SUESSENGUTH has mentioned the possibility that adsorbtion interfaces are set free by the withdrawal of water, and so the substrate becomes accessible for the enzyme (or the enzyme for the substrate).

The explanation of VON MOLISCH might as well be applicable to the changes in water content during the movements of the motile pulvini. If the total amount of amylase were constant in both halves of a pulvinus, the concentration of the amylase would be altered with the cell volume. This change of the amylase concentration, by a decreased (or increased) production of osmotically active substances, would counteract the direction of alteration of the volume, just as supposed in the hydrogen-ion-amylase system described in the last §. However, the differences in amylase concentration should be measurable and this has not appeared to be possible (chapter VI).

LEPESCHKIN (1934) deduced from his formulae that the turgor pressure of a tissue depends on the concentration of the fluids outside the tissue, with which it is in contact. To illustrate this thesis (which generally of course, holds true), he points to the fact that, when the lamina of a leaf is totally cut off, the movements stop in a few days. According to LEPESCHKIN the lack of a transpiration stream through the vascular bundle causes, in this case, the concentration of the osmotically active substances outside the motile tissue gradually increase, until the turgor of the motile tissue has so much decreased that no movement can occur. — This conception does not match the facts, since the pulvini (with removed lamina) keep their rigidity and therefore their turgor has not decreased.

The phenomenon of the stopping of the movements after cutting off the lamina, can be much easier explained in the following way: the starch content of the starch layer in the pulvinus seems not only to be supplied by the photosynthesis of the pulvinar tissue itself but, for an important part, by that of the leaf. If the lamina has been cut off, the starch of the pulvinar starch layer will gradually be consumed in the motile mechanism and then the movements will stop. — In my experiments I have often used plants, of which the lamina had been cut off for the greatest part. These plants kept on moving in the normal day- and night

succession but, once placed in constant darkness, they stopped moving within a few days, since the reduced starch content of the starch layer cannot maintain the motile mechanism for a longer time.

I add the results of some experiments which support this view (table 31 and fig. 40).

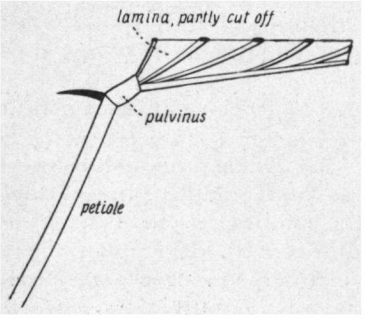


Fig. 40. A sketch illustrating the extent to which the lamina had been cut off in the experiments of table 31.

TABLE 31. Experiments with reduced lamina.

No. of leaf:	lamina cut off:	placed in the dark room:	fixed:	starch content:
AD 14a	Nov. 2nd	—	Nov. 8th	no starch
AD 14b	(normal, control)	—	Nov. 8th	much starch
AD 8a	Nov. 2nd	—	Nov. 14th	very little starch
AD 8b	(normal, control)	—	Nov. 14th	much starch
AD 9a	Nov. 2nd	—	Nov. 14th	no starch
AD 9b	(normal, control)	—	Nov. 14th	much starch
AD 7a	Nov. 2nd	Nov. 8th	Nov. 14th	no starch
AD 7b	(normal, control)	Nov. 8th	Nov. 14th	little less than normal

As to the movements of these leaves was noted:

AD 8a, AD 9a: still moving fairly well on Nov. 8th/9th, movements slowly decreasing from Nov. 8th to 14th.

AD 7a: moving weakly until Nov. 13th, then stopped.

§ 3. *Conclusions.*

Resuming all experimental and theoretical data given in the preceding chapters, we may conclude that the mechanism of the pulvinar movements in constant darkness can be explained by enzymatic processes in the tissue. Changes in the osmotic values

of the cell contents, and thus changes of the volumes, interact with these enzymatic actions.

No differences of amylase quantities at different stages of the movement could incontestably be observed. Some results pointed to differences, but these are so inconsistent that, in a theory on the mechanism, the possibility of differences in amylase quantities cannot yet be accounted for.

The alterations of the potential in the pulvinus found a satisfactory explanation in the shifting of ionic concentrations during the movement, and at the same time they strongly endorse the possible occurrence of changes in the hydrogen-ion concentrations in the motile cells.

The theory still is rather vague as far as the details of the process are concerned, but this was unavoidable since, hitherto, reliable data on the details of the motile mechanism are too scanty. No independent study in different fields of plant physiology ever will be able to reveal complicated processes such as underlie the movements of variation of the pulvini of *Phaseolus multiflorus* and allied species. Only the combination of as many aspects of the problem as possible may finally result in a detailed knowledge of all processes involved in the motile mechanism.

SUMMARY.

1. The mechanism of the pulvinar movements of *Phaseolus multiflorus* L. was studied in several ways.

2. A comparison of the osmotic values of the cell content in the upper and in the lower half of a pulvinus at various positions of the leaves, showed that in an uplifted position of the leaf the lower half has a higher suction power than the upper half, while in a drooped position of the leaf the upper half has a higher suction power. These results confirm those of ZIMMERMANN (1929) and WEIDLICH (1930).

3. The relation between the presence of starch in the starch layer of the pulvini and the occurrence of movements was investigated. The results of the experiments show that, as long as starch is present, movements may take place; if the starch has disappeared from the starch layer the movements stop. The starch probably acts as a source of energy for the processes resulting in movement.

4. The methods of extracting amylase from the tissue and of testing its activity were studied. — In some cases a difference in amylase activity of the upper and the lower half of the pulvinar

motile tissue could be detected. In other cases no differences at all were found. No conclusions as to a periodic change in the amylase activity or -quantities could be drawn from these experiments.

5. The amylase extracted from the pulvinar tissue showed a remarkable difference in activity at different hydrogen-ion concentrations. The pH of the pulvinar tissue appeared to lie between 5,5 and 6,5, and it is just in this region that a decrease in the amylase activity was demonstrated.

In 3 series of experiments, in collaboration with J. B. THOMAS, the potential differences on petiole and pulvinus were measured. A parallelism between movement and P.D. variations was demonstrated, which made it probable that mainly shifting of H-ion concentrations was measured.

6. A theory was given that enabled to account for all phenomena described by me and by earlier investigators. In short the hypothesis is this: the volume of the cells depends (in upper and lower antagonistic halves) on the osmotic value of the cell content (sugar); sugar is removed constantly by respiration and by diffusion, it is supplied from starch by the action of amylase; the amylase thus may influence the total amount of sugars in the cells, and at the same time its activity is regulated by the pH; it was made probable that fluctuations of the pH parallel to changes in volume occur.

7. In this way the principles of an endonomous automatism were suggested and a mechanism was described liable to all kinds of conditional (internal and external) factors.

I owe much to the kind interest and the encouragement of Prof. dr. V. J. KONINGSBERGER.

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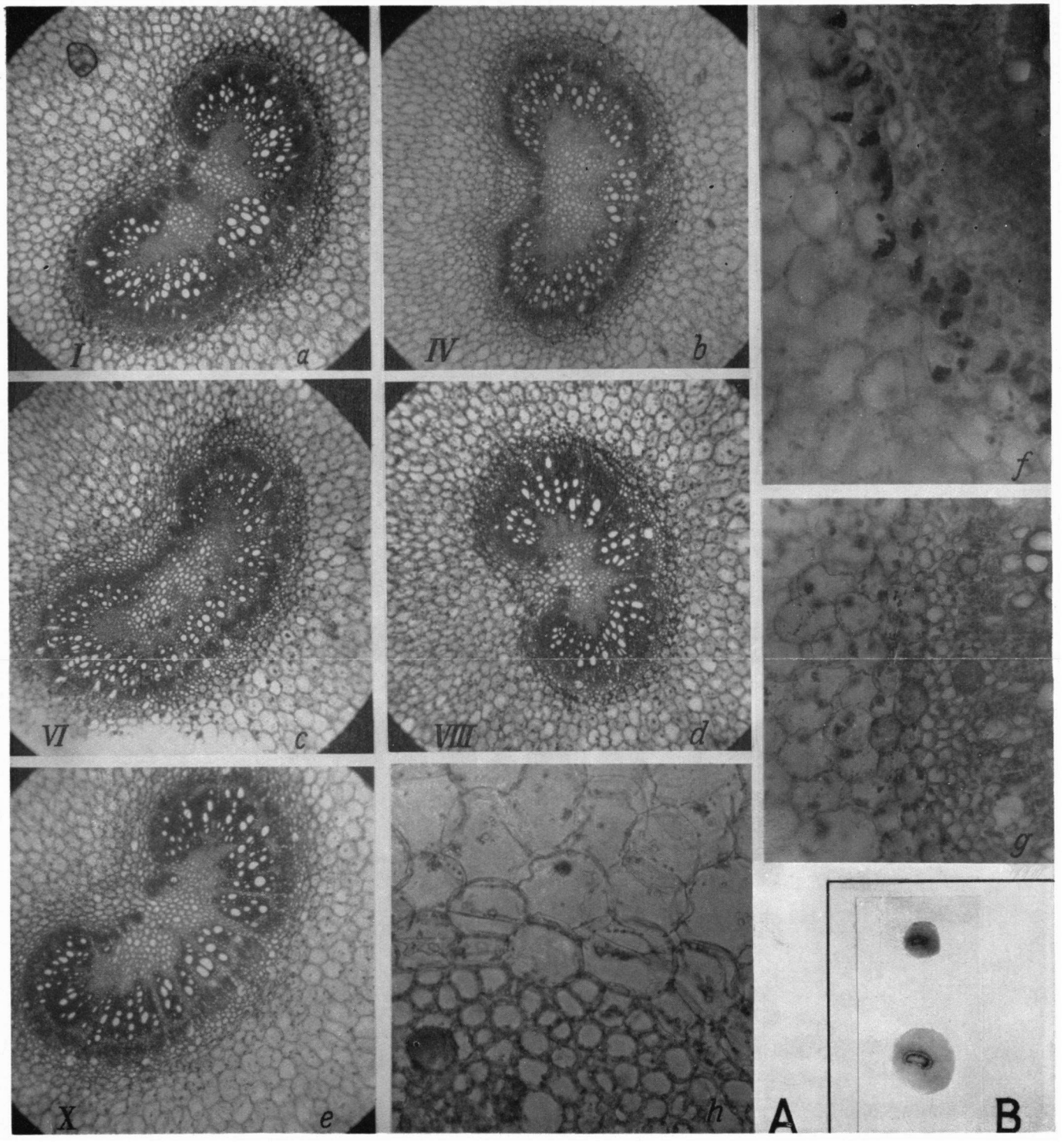

A. Micro-photographs of cross sections of the pulvini of the experiment of fig. 9 (See text p. 784). The Roman numerals refer to the time of fixation of the pulvinus, as indicated in fig. 9. The photographs f, g and h give a detailed picture of the starch layer at the resp. moments I, VI and XI of fig. 9.

B. One example of a print and one of an enlargement made from an "enzymographic plate" (see p. 789).

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For a full survey of recent and early literature I refer to KLEINHOONTE (1929) and BÜNNING (1936).