STUDIES ON THE CRASSULACEAN ACID METABOLISM

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STUDIES ON THE CRASSULACEAN ACID METABOLISM

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CHAPTER I

INTRODUCTION

§ 1. OLDER VIEWS

In the green, succulent parts of many plants organic acid is formed during the night and broken down in the day-time. This diurnal acid variation, called the Crassulacean acid metabolism, is an old subject-

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matter of research into plant physiology. Day-time deacidification was discovered by HEYNE (1815), but the accompanying variations in gas intake and output were already found by DE SAUSSURE (1804). An intensive study in Germany, France and Holland during the second half of the nineteenth century resulted in the following picture of the phenomenon.

A considerable diurnal acid variation was found to occur generally in species of the *Crassulaceae*, *Cactaceae*, *Euphorbiaceae*, *Bromeliaceae* and *Aloineae*. Plants belonging to the families *Mesembryanthemaceae* and *Orchidaceae* and to the genera *Pinus*, *Polypodium*, *Ilex*, *Rhododendron*, *Clivia*, *Tradescantia* and *Sanseviera* showed but a slight diurnal change in acidity. The variation has never been demonstrated in thin-leaved plants, nor in chlorophyll-free parts of succulents, such as flowers, fruits, roots and etiolated organs (WARBURG, 1886). The latter finding suggests that the acid metabolism is connected with the carbohydrate metabolism of succulent plants. A positive correlation was found between the nightly acidification and the assimilation in the preceeding day-time (KRAUS, 1885; AUBERT, 1892*a*). Moreover, it was demonstrated that plants, kept in a carbon dioxide-free atmosphere during the day, could still produce starch (KRAUS, 1885) and oxygen (AUBERT, 1892b) whilst deacidifying. Kraus and Aubert suggested that in the day-time organic acid is oxidized into carbon dioxide, which is, in turn, used in photosynthesis. However, already LIEBIG (1846) submitted that the organic acids in plants are not merely built up from carbon dioxide in order to produce this compound again at a later point of time, but that they are intermediates in the pathway from carbon dioxide to carbohydrates: "Die organischen Säuren . . . sind die Uebergangsglieder der Kohlensäure in Zucker" (LIEBIG, 1865). According to this view, carbohydrates are oxidized into organic acid during the night, while in the day-time light reduces acid again into carbohydrates; the organic acid was called "Crassulacean malic acid" or "isomalic acid", since its salts differed from malates in some respects.

Acidification and deacidification were considered to be the resultants of various reactions, the rates of which depended on external conditions (DE VRIES, 1884, 1885). Under constant conditions of not too long a duration, the amount of titrable acid tended to become constant too; changes in temperature, light conditions or composition of the surrounding atmosphere resulted in acidification or deacidification. Light and higher temperatures stimulated acid breakdown or retarded acid formation, whereas darkness, lower temperatures and increased carbon dioxide tensions (*e.g.* 25 %) brought about the reverse. Acidification could be correlated with a net carbon dioxide intake, deacidification—in the dark, at a higher temperature—with carbon dioxide output (WARBURG, 1886). The respiratory and assimilatory quotients were found to differ from unit (DE SAUSSURE, 1804). In various plant species of the genus *Sedum*, both the diurnal changes

in RQ and AQ and the diurnal acid variation appeared to depend on the measure of succulence (AUBERT, 1892*a*).

About the significance of this acid metabolism in relation to succulence and milieu conditions, views were conflicting. AUBERT (1892*a*) suggested that the high acid concentration prevents transpiration. KRAUS (1885) and DE VRIES (1885) considered it to be an adaptation to dry calcareous soils by plants, that can neutralize the lime with their acids. LIEBIG (1846, 1865), MAYER (1875) and WARBURG (1886) regarded the organic acids as byproducts of carbohydrate metabolism, partly oxidized in dissimilation and possibly serving as a carbon source in assimilation, substituting for carbon dioxide, because of the limited gas exchange of succulent leaves.

The chemical mechanism of the Crassulacean acid metabolism remained obscure at that time.

§ 2. CHEMICAL ASPECTS

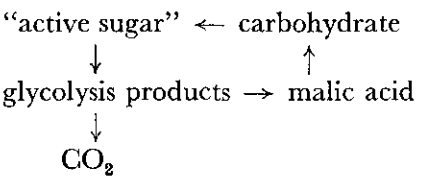
After a long pause, the attack on the subject was resumed with renewed interest, since advanced techniques allowed for detailed analyses of the compounds involved, and pathways of carbohydrate metabolism have become cleared up. PUCHER (1942) found that "isomalic acid". consisted of a mixture of organic acids, the greater part of which was isocitric, about one third was malic, some per cent were citric, while traces of other organic acids could be detected. Together, the organic acids of the Crassulacean plant *Bryophyllum calycinum* formed about a quarter of the total dry weight. PUCHER *et al.* (1947*a*, *b*, *c*, *d*, 1948*a*, *b*, 1949) and VICKERY (1952*a*) demonstrated that the diurnal variation appeared, indeed, mainly in the malic acid fraction.

Recent investigations confirm the relationships between Crassulacean acid metabolism and carbohydrate metabolism. The close connection between acid formation and starch breakdown, and conversely, became clear from the correlation coefficients found: .91 for starch and total acids, and even .97 for starch and malic acid (VICKERY, 1952*a*). The overall-reaction, however, is not yet established, since the proportion of the changes in starch and acid contents, calculated for as glucose units and malic acid respectively, varied considerably: from —.54 to —1.52 in the experiments of WOOD (1952) and from —.7 to +3.6 in those of VICKERY (1952*a*), dependent on the period of the day, for which the figures were computed. Moreover, other organic acids and carbohydrates showed a diurnal variation too, to some extent, whilst the composition of about a quarter of the organic matter of *Bryophyllum calycinum* leaves is still unknown and might contain more participants in the metabolic systems concerned (PUCHER *et al.*, 1947*a*; VICKERY, 1952*a*). Therefore, detailed balance equations have not been given at present (THIMANN and BONNER, 1950; WOOD, 1952; VICKERY, 1952*b*; THOMAS and RANSON, 1954; MOYSE, 1955).

The elucidation of pathways of carbohydrate metabolism allowed for explanations as to the mechanism of the conversion of carbohydrate into acid. WETZEL and RUHLAND (1932), WOLF (1932) and BENNET–CLARK (1933) suggested the formation of malic acid from some C_3-glycolysis compound.

The view of the Leipzig school (Wetzel, Ruhland and Wolf) was that in the dark, respiration would be narcotized by the increasing carbon dioxide tension in the succulent leaves. This would give rise to fermentation until accumulating acetaldehyde would, in turn, inhibit the carboxylase activity. Under these circumstances, pyruvic acid would be converted via diketoadipic acid and succinic acid into malic acid. On illumination, carbon dioxide was taken away, the acetaldehyde concentration reduced and carboxylase reactivation would cause deacidification.

BENNET–CLARK (1933) demonstrated that the maximum aldehyde concentration in succulent leaves was only about one tenth of that, required for a 50 % inhibition of the carboxylase activity. Moreover, diketoadipic acid was never found in plant tissues. According to Bennet–Clark, glycolysis would lead partly to a resynthesis of carbohydrate, in which latter pathway malic acid would be an intermediate:



The energy for carbohydrate resynthesis was presumed to originate from the oxidation of part of the acid or some other compound: oxidative anabolism. The active sugar could be a hexose or a heptose, *e.g.* sedoheptulose, which occurs in considerable amounts in Crassulacean plants. Malic acid would accumulate because the velocity of its formation would exceed that of its further conversion. Light deacidification was thought to be caused by the high temperatures prevailing in illuminated succulent leaves, which have an only limited transpiration. No opinion as to the mechanism for the conversion of glycolysis products into malic acid was given.

With the discovery of the heterotrophic carbon dioxide assimilation in propionic acid bacteria by WERKMAN and WOOD (1942), such a mechanism was at hand. In this Wood–Werkman reaction, pyruvic acid is carboxylated into oxaloacetic acid, which can be hydrogenated into malic acid by reduced diphosphopyridine nucleotide (DPNH), catalyzed by malic acid dehydrogenase:

$$\underset{\text{pyruvic acid}}{CH_3.CO.COOH} + CO_2 \rightleftharpoons \underset{\text{oxaloacetic acid}}{COOH.CH_2.CO.COOH}$$

$$COOH.CH_2.CO.COOH + DPNH + H^+ \rightleftharpoons$$
$$\rightleftharpoons \underset{\text{malic acid}}{COOH.CH_2.CHOH.COOH} + DPN^+$$

BONNER and BONNER (1948), THOMAS (1949) and WOLF (1949) suggested that this system could count for the Crassulacean acid metabolism. Later on, however, further biochemical investigations led to the discovery of other enzymes in higher plants, catalyzing the carboxylation of glycolysis intermediates into oxaloacetic or malic acid (VENNESLAND, 1949; CONN, VENNESLAND and KRAEMER, 1949; VENNESLAND, GOLLUB and SPECK, 1949; BANDURSKI and GREINER, 1953; TCHENN and VENNESLAND, 1954, 1955; BANDURSKY, 1955).

Three of these enzymes are now known.

The *malic enzyme* carboxylates pyruvic acid into malic acid with the aid of reduced triphosphopyridine nucleotide (TPNH), according to OCHOA, MEHLER and KORNBERG (1948):

$$CH_3.CO.COOH + CO_2 + TPNH + H^+ \rightleftharpoons \rightleftharpoons COOH.CH_2.CHOH.COOH + TPN^+$$

The *oxaloacetate carboxylase* combines phospho-enolpyruvic acid and carbon dioxide to oxaloacetic acid under phosphorylation of adenosine or inosine nucleotides (UTTER and KURAHASHI, 1954):

$$CH_2{:}COPO_3H_2.COOH + CO_2 + \frac{ADP}{IDP} \rightleftharpoons \rightleftharpoons COOH.CH_2.CO.COOH + \frac{ATP}{ITP}$$

These two enzymes catalyze reversible reactions.

The third enzyme, *phospho-enolpyruvate carboxylase*, converts phospho-enolpyruvic acid and carbon dioxide irreversibly into oxaloacetic acid (BANDURSKI, 1955):

$$CH_2{:}COPO_3H_2.COOH + CO_2 + H_2O \rightarrow \rightarrow COOH.CH_2.CO.COOH + H_3PO_4$$

The presence of these three enzymes and of malic acid dehydrogenase in extracts from succulent plants has been recently demonstrated (WALKER, 1956; SALTMAN, KUNITAKE, SPOLTER and STITT, 1956; COLES and WAYGOOD, 1957) and, therefore, various mechanisms for dark acidification are possible at present. These possibilities will be discussed in Chapter IV.

Carboxylating enzymes play, indeed, a role both in acidification and in deacidification. Strong indications in favour of a heterotrophic carbon dioxide assimilation in succulent plants have been obtained by BONNER and BONNER (1948) and by Thomas and co-workers (THOMAS, 1949; THOMAS and BEEVERS, 1949; THOMAS, 1951*a*; THOMAS and RANSON, 1954). In experiments with *Bryophyllum calycinum* plants, acidification and deacidification depended on the carbon dioxide tension, illumination and temperature, and proved to be closely connected with carbon dioxide intake and output respectively. The authors submitted that it is the carbon dioxide tension at a

carboxylating enzyme surface, which decides whether acid production or consumption prevails.

That in dark acidification carbon dioxide is, indeed, incorporated into malic acid, is convincingly demonstrated by the use of $C^{14}O_2$ (THURLOW and BONNER, 1948; VARNER and BURRELL, 1950; STUTZ and BURRIS, 1951; THOMAS and RANSON, 1954; SALTMAN, LYNCH and STITT, 1955). In experiments of short duration, up to 95 % of the radioactive isotope taken in, could be traced in malic acid (THOMAS and RANSON, 1954). Since the isotope hardly spreads among the other organic acids, there is no indication of an actively operating tricarboxylic acid cycle (STUTZ and BURRIS, 1951). VARNER and BURRELL (1950), feeding $C^{14}O_2$-enriched carbon dioxide to *Bryophyllum calycinum* plants during 2.5 hours in the dark at 10° C, found 92 % of the radioactivity of malic acid back in the carboxyl groups (cf. § 17, TABLE II). Under these conditions, only .1 % of the total radioactivity taken in could be traced in starch. If plants, thus enriched with radioactive malic acid, were allowed to deacidify in the light during .5 hour without $C^{14}O_2$ in the atmosphere, 3.7 % of the total activity was detected in the starch fraction, *viz.* 2 % in the C_3- and C_4-atoms of the glucose unit and 1.7 % in the other four carbon atoms. This unequal distribution of radioactivity suggests a conversion of malic acid into starch by way of a β-decarboxylation into a glycolysis intermediate, possibly next to a starch synthesis of carbon dioxide from respired malic acid. HAÏDRI (1955) stated that when *Bryophyllum calycinum* leaves were infiltrated with pyruvic acid-2-C^{14} or malic acid-2-C^{14}, after 3 hours of illumination 43–46 % of the starch radioactivity was traced back in the C_2- and C_5-atoms of the glucose unit. This confirms the view, that starch is, at least partly, formed by decarboxylation of malic acid and a reversal of the glycolysis pathway.

The conversion of malic acid into starch is not restricted to succulent plants, since Haïdri obtained similar results with tobacco and oat leaves.

§ 3. INFLUENCES OF EXTERNAL FACTORS

Whereas the interconversion of starch and malic acid is not typical for the Crassulacean acid metabolism, and the enzymes required have been detected throughout the plant kingdom, it is the diurnal variation in acid content which characterizes the metabolism of succulent plants. The problem, therefore, focuses on the question as to how the relative activities of the acid producing and consuming enzyme systems are varied in such a way, that a diurnal acid variation results.

This variation in enzyme activities does not depend on an internal factor. Although NUERNBERGK (1955) demonstrated a diurnal change in carbon dioxide intake of continuously illuminated succulent plants, GREGORY, SPEAR and THIMANN (1954) could not detect such an endonomous rhythm. Moreover, the amount of titrable acid becomes

constant under constant conditions (WARBURG, 1886; THOMAS and RANSON, 1954; VICKERY, 1956). It are external factors which govern the diurnal acid variation: temperature, illumination and atmosphere composition; the first two factors vary periodically.

A direct effect of *temperature variation* on the acid metabolism results from different Q_{10}-values of the various acidifying and deacidifying reactions. The current opinion is that the stimulation of deacidification and the inhibition of acidification by enhancement of temperature, is the result of a shift in the proportion of acid breakdown to acid formation, the former being stimulated to a larger extent than the latter.

A direct effect of *variation in illumination conditions* on the Crassulacean acid metabolism has not been demonstrated up to now. LASHER and BONNER (1955) stated in a short note, that red light caused a stronger deacidification in leaves of an unmentioned *Bryophyllum* species than blue light did. Since this should not agree with the general action spectrum of photosynthesis, they concluded that light might influence the Crassulacean acid metabolism directly. The authors do not report anything about the light intensities used and about the anthocyans, mostly present in considerable amounts in *Bryophyllum* leaves, which could be responsible for a strong non-effective absorption of blue light. Further detailed information is necessary before conclusions can be drawn.

The experiments of SOMERS (1951) on *Kalanchoe daigremontana* leaf discs showed, that the influence of light on the acid metabolism is not brought about by a change in temperature as a side-effect of illumination. The leaf discs, previously kept in the dark at 11°, 21° and 31° C respectively, were transferred to 21° C in the light or in darkness. Somers observed that deacidification and acidification depended on the illumination: irrespective of the preceeding temperature, the acid content of the illuminated discs became lower than that of those kept in continuous darkness.

In short, only little is known about direct influences of temperature and light on the Crassulacean acid metabolism. However, these factors might affect this metabolism indirectly as well.

First, if upon a change in temperature or illumination conditions *the permeability of the tonoplast* were altered and, by that, the accessibility of acids, accumulated in the vacuole, to enzymes in the protoplast, the rates of acid breakdown and formation would probably change. Similar changes might be produced by alterations in permeability of other protoplast membranes. Little is known about the effects of light and temperature on permeability. Most of the statements on the subject are questionable, since, until rather recently, it was not generally realized, that transport of solutes and metabolism are closely interrelated and that, therefore, changes in transport rates may be the result of an altered metabolism, rather than of a change in permeability (KRAMER, 1955; COLLANDER, 1957). Active absorption and transport at the cost of dissimilatory energy is well known (LUNDEGARDH, 1955; KRAMER, 1955), whilst VAN LOOKEREN CAMPAGNE (1957) demonstrated a light-dependent chloride absorption of *Vallisneria* leaves in a carbon dioxide-free milieu, utilising the excitation energy of chlorophyll. On the other hand, VIRGIN (1951) found an increase in permeability of *Helodea* leaf cells, caused by a lowered viscosity of the protoplasm, upon illumination; whilst BRAUNER (1956) argues changes in permeability by the release of photo-electrons from illuminated membranes.

The other explanation is that both temperature and light indirectly affect the diurnal acid variation by influencing *the carbon dioxide and oxygen tensions in the intercellular spaces of the succulent leaf.* Temperature rules the turnover capacity of dissimilatory and assimilatory enzyme systems that produce or remove carbon dioxide and oxygen. One may expect, that in the dark at a higher temperature more carbon dioxide will accumulate and less oxygen will be left in the intercellular spaces than at a lower temperature, at which respiration is less intensive; in sufficiently strong light, the reverse is to be expected. Moreover, temperature influences the solubility of carbon dioxide and oxygen in the protoplasm and may, by this means, affect the acid metabolism.

At first sight, the effect of temperature and illumination by way of the internal gas composition may look doubtful, since the composition of the outer atmosphere remains constant. One must realize, however, that there need not be a direct relationship between the tensions of a gas compound outside and inside the succulent leaf. The unfavourable surface/volume ratio of these leaves and the stomatal resistance against gas exchange may well lead to an intercellular gas composition, which is determined more by the metabolic activities of the cells than by the composition of the outer atmosphere.

As was mentioned before, the influence of *the carbon dioxide tension of the outer atmosphere* on the acid metabolism is frequently observed (WARBURG, 1886; THOMAS and BEEVERS, 1949; THOMAS and RANSON, 1954; MOYSE, 1955). It has invariably been found, that enrichment of the atmosphere with carbon dioxide stimulated dark acidification and inhibited light deacidification. THOMAS and BEEVERS (1949) even report acid production in *B. calycinum* leaves in the light at 10 % CO_2.

The effect of *the outer oxygen tension* on the Crassulacean acid metabolism is less clear. GUSTAFSON (1925) and MOYSE (1955) were unable to show any influence of the oxygen tension on dark acidification. Other authors (WARBURG, 1886; AUBERT, 1892*a*; WOLF, 1932) conclude that oxygen stimulates dark acidification; anaerobically, however, acid is still produced in the dark. According to WARBURG (1886), light deacidification little depends on the presence of oxygen, but, in the dark, deacidification is inhibited under anaerobic conditions.

If one wishes to study the relationships between some gaseous compound and the cell metabolism of a succulent tissue, one can work best with pieces of this tissue, of which one knows that the gaseous composition in the intercellular spaces is about equal, at least proportional, to that of the surrounding atmosphere.

The present investigation is concerned neither with an analysis of possible influences of permeability changes, nor with the effects of temperature and of oxygen tension on the acid metabolism. Since carbon dioxide is a common metabolite of both acid and carbohydrate metabolism, it might occupy a key position in the Crassulacean acid metabolism. It is examined, therefore, firstly, whether the composition

of the intercellular gas actually varies considerably, when daily illumination and nightly darkness alternate. For this purpose, it is investigated in Chapter II, whether the gas exchange with the outer atmosphere is small in proportion to the metabolic gas exchange.

In Chapter III, the effects of external factors on the acid metabolism are studied, whereby attention is focused on the question, whether illumination directly affects the acid metabolism or acts by means of the carbon dioxide tension only.

The results of these experiments are analyzed on a biochemical basis in Chapter IV, whilst in Chapter V the significance of the Crassulacean acid metabolism for the succulent plant is discussed.

CHAPTER II

THE GAS EXCHANGE OF EXCISED LEAVES AND OF LEAF SECTIONS

§ 4. Introductory remarks

In this Chapter, the extent to which the gas exchange of succulent leaves is hampered, is investigated. The starting-point for the experiments was the consideration, that cells of leaf sections will be in a far more close contact with the outer atmosphere than cells of intact or excised leaves. If, under various conditions as to gas composition, temperature, *etc.*, the respiration rates of excised leaves were found to differ from those of leaf sections, these differences could possibly be explained in terms of availability of oxygen. So a comparison of excised leaf respiration to section respiration should lead to an insight into the measure of gas exchange of succulent leaves and, from that, into the amplitude of the diurnal variation in intercellular gas composition.

The object in all experiments was *Bryophyllum tubiflorum* Harv.. This Crassulacean species has phyllodes of an almost cylindrical shape, 5 to 10 cm long, which form a very handsome material for preparing homogeneous samples of cross-sections. Since phyllodes are leaves, physiologically speaking, and morphologically not essential different from laminae (Goebel, 1923; Troll, 1939), they will, henceforth, be called leaves, for the sake of convenience.

In the course of these investigations, marked differences between respiration rates of excised leaves and of leaf sections came to light, which differences could be reduced to a limitation of excised leaf respiration. Closer analysis of this limitation showed the importance of stomatal aperture for leaf respiration. It turned out, that this stomatal aperture determines the measure of gas exchange and, by that, of leaf respiration rate.

§ 5. Plant material and methods

Bryophyllum tubiflorum plants were propagated vegetatively from bulbils, originally from one single plant. They were grown in a green-

house, in which in winter an additional artificial illumination during 14 hours per day was given. At first, fluorescent tube-lamps (Philips TL 40 W) were used as a light source, which were replaced afterwards by 100 W incandescant lamps. In the experiments, just full-grown leaves from ½ to 1 year old plants were used; these leaves were found to have the largest metabolic activity.

Excised leaves were obtained by cutting them with a razor blade; they were immediately placed with the cut into .1 ml of a .02 M KCl-solution. Leaf sections were cut with a microtome, with which cross-sections from .1 to 2 mm thick could be prepared, using 11 leaves at a time; tops and bases were discarded to enhance the homogeneity of the samples. The sections, mostly .5 or 1 mm thick, were washed three times in about 100 ml of a .02 M KCl-solution and superficially dried with filter paper after each washing.

The determination of respiration rates was carried out by means of the Warburg manometric technique, using flasks of about 20 ml with a central well and a side arm (see UMBREIT, BURRIS and STAUFFER, 1948).

The oxygen intake was measured in the dark by the direct method. Excised leaves were placed in the central well of the flasks, in .2 ml of a .02 M KCl-solution, the outer compartment containing 2 ml of a 10 % NaOH-solution or of a CO_2-buffer (see below). Sections, usually 40, 1 mm thick, or 80, .5 mm thick, were examined, mostly floating in 2 ml of a .02 M KCl-solution in the outer compartment, with .2 ml of a 10 % NaOH-solution or .4 ml of a CO_2-buffer (see below) in the central well. The respiration rates of excised leaves were converted into μmol O_2 per 500 mg of fresh weight, those of sections into μmol O_2 per 500 mg or per flask. Respiration in an atmosphere, enriched with CO_2, was studied, using CO_2-buffers after Pardee (PARDEE, 1949; KREBS, 1951; BURK, SCHADE, HUNTER and WARBURG, 1951). In the experiments with leaf sections, .4 ml of buffer in the central well were absorbed by 2 pieces of filter paper, 4 × 2 cm², one folded fanwise, the other one surrounding the former cylindrically; the brim of the central well was greased. The thermobarometer flasks contained the same amount of buffer. After 30 min. of equilibration, the readings were started. The CO_2-buffers after Pardee are composed of the weak base diethanolamine, which can be brought into equilibrium with carbon dioxide, resulting in a practically constant CO_2-tension above the liquid, up to 3 %. The buffer solutions were prepared from the following prescription.

Warm diethanolamine (B.D.H., techn. qual.) in a water bath of 40° C; mix 60 ml with 40 ml of distilled water, add 2 g of norit and shake. Filter through Delta no. 378 filter paper. In 20 ml of the colourless filtrate, 6.0 g K_2CO_3 and 30 mg of thiourea are dissolved, which takes about half an hour. While continuously shaking, add slowly 4.4 ml 6 N HCl and make up the solution to 30 ml with distilled water. After one night at 35° C, this solution is in equilibrium with 1 % CO_2 in the gas phase (buffer I). Buffer II, in equilibrium with ½ % CO_2 at 35° C, is made in the same way, using 3.0 g instead of 6.0 g K_2CO_3.

To compare respiration rates of leaves with those of sections, a correction had to be made for the cut cells of the sections. A microscopical examination showed that 1 mm-sections were built up of about 8 layers of intact cells; ½ mm-sections of about 3 such layers. With each cutting of the microtome blade two cell-layers are destroyed, their contents are washed away and their metabolism falls out. This means, that an entire leaf contains about 10 cell-layers per mm, which is 25 % more than 1 mm-sections. Therefore, an adjustment of 25 %

was applied to section respiration, when compared with leaf respiration. That this adjustment is correct ,was demonstrated when the respiration rates of 40 sections, 1 mm thick, were compared with those of 80 sections, .5 mm thick, the former being 1.25 times as high, whilst the theoretical ratio is 1.3. Obviously, respiration is restricted to the undamaged cells only.

One might consider whether another adjustment is required, *viz.* for the weight of the liquid, penetrated into the sections. This adjustment is not important, however, as was shown in the following way. Sections, $\frac{1}{2}$ mm and 1 mm thick, were cut alternately from the same leaves, washed and dried as usual, and weighed. The average weight of 20 sections, 1 mm thick, was 156.6 mg, that of 40 sections, $\frac{1}{2}$ mm thick, 159.7 mg. Since the difference in weight of 2 % was not significant, no adjustment was required.

The porometer experiments were made with the use of the horizontal porometer as designed by HAMORAK and LUBYNSKYJ (1930), and modified by HARTSUYKER (1935). The excised leaf was placed gas-tight on a rubber tube, which was connected with the porometer (Fig. 1). The gas was sucked into the leaf through the stomata and

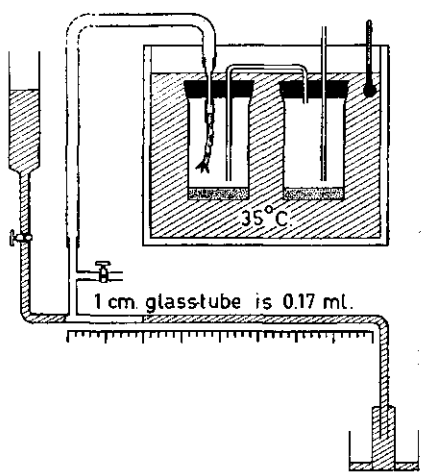


Fig. 1.

flowed, via the intercellular spaces and through the cut, into the porometer, with a constant negative pressure of about 7 cm of water. The volume of the gas was determined every 3 minutes. The leaf was placed in a 150 ml jar over 5 ml of a NaOH-solution, a CO_2-buffer or a glycerol-water mixture. The CO_2- and O_2-tensions, the humidity and the pressure in the jar were kept constant by means of a second jar, in which the same conditions prevailed and which was in connection with a gas tank or with the open air. The two jars were kept in a constant temperature water bath in the dark.

§ 6. The respiration of leaf sections

Sections floated in watery solutions to prevent their drying-out. This introduced a number of milieu-factors, the influences of which on respiration had to be estimated, before a comparison with leaf respiration could be made. The most favourable solution turned out to be a .02 M KCl-solution at a $P_H = 7$. It made no difference to the rate of oxygen intake, whether the sections floated in the liquid with gas-filled intercellular spaces or were submersed by evacuation *in vacuo*. Added substances penetrated very rapidly into the sections, irrespective again of whether the intercellular spaces were filled with gas or with liquid. The optimum respiration rate was measured between 30° and 35° C. After 30 hours at 35° C, no measurable disturbance in the determination of section respiration by interference from microbial gas exchanges could be detected: the oxygen intake in Warburg flasks, in which from zero up to 500 I.U. of penicillin were added to the medium liquid of the sections, was equal in all flasks. No special precaution against bacterial infection, therefore, was taken.

After these preliminary experiments, it was investigated, what factor limited the respiration rate of leaf sections. For that purpose, the influence of the oxygen tension in the atmosphere and of additions of substrates was examined: Experiments 1 and 2.

Exp. 1. *Respiration of leaf sections at various oxygen tensions.* Outer well: 40 sections, 1mm, in 2 ml of a .02 M KCl-solution. – Central well: .2 ml 10 % NaOH-solution.– Gas phases: mixtures of N_2 and O_2 from cylinders. – 37° C . – Dark.
Duple and triple determinations.

time (min.)	oxygen intake per flask in μl.		
	100 % O_2	20 % O_2	5 % O_2
60	52	51	56
120	92	94	101
180	129	131	134

Exp. 2. *Respiration of fresh leaf sections with and without various substrates.* Outer well: 40 sections, 1 mm, in 2 ml of solution at $P_H = 7.0$: 2 ml of a .12 M KCl-solution or 1 ml of a .04 M KCl-solution + 1 ml of a .20 M substrate solution. – Central well: .2 ml of a 10 % NaOH-solution. – Gas phase: air. – 37° C. – Dark.
K-pyruvate was recrystallized twice.
Duple and triple determinations.

substrate	none	glucose	pyruvate	malate
time (min.)	oxygen intake per flask in μl.			
60	77	69	73	70
120	140	127	129	126
180	186	174	176	175

Experiment 1 shows that the oxygen supply of the sections was amply sufficient, even in 5 % of oxygen. In a similar experiment it was found, that the oxygen intake with 1 % of oxygen in the atmosphere was retarded to 45 % of the maximum rate.

The amount of substrate, present in fresh leaf sections, limited the respiration rate no more than the oxygen supply did (Exp. 2). The endogenous respiration could not be enhanced by additions; on the contrary, additions of glucose, pyruvate or malate invariably slightly delayed the oxygen intake (it may be added here, that acetate strongly inhibited respiration: after 2 hours, no more oxygen was absorbed. A similar effect was found by LATIES (1949) using barley roots). That the compounds, added as a substrate, can, indeed, be used as such, is demonstrated in Experiment 3.

Exp. 3. *The effect of the addition of substrates on the respiration of starving leaf sections.* Outer well: 40 sections, 1 mm, in 2 ml solution. – Central well: .2 ml of a 10 % NaOH-solution. – Gas phase: 95 % N_2 + 5 % O_2. – 35° C. – Dark.

After 24 hrs, .5 ml of the .02 M KCl-solution was replaced by .5 ml of a .2 M glucose- or Na-malate-solution.

Single determinations.

time (hours)	oxygen intake per flask in μl.	
	.02 M KCl	.02 M KCl
0—1	46	45
0—2	88	84
0—3	120	119
21—22	29	26
21—23	59	49
	.015 M KCl + + .05 M glucose	.015 M KCl + + .05 M malate
24.30—25.30	40	37
24.30—26.30	79	72
24.30—27.30	115	111

This experiment shows, that after about 24 hours at 35° C in the dark, the oxygen intake of sections was slowed down. Glucose, malate and pyruvate, too, are now able to enhance the respiration rate of these starved sections and to restore it almost completely.

The conclusion, drawn from these experiments, is that the respiratory enzyme system in fresh leaf sections, being limited neither by oxygen deficiency nor by a lack of substrates, works at full capacity.

§ 7. COMPARISON BETWEEN RESPIRATION RATES OF EXCISED LEAVES AND THOSE OF LEAF SECTIONS

In a number of experiments, respiration rates of excised leaves and of leaf sections from the same whorls were compared with one another

under equal conditions. In these experiments, two important differences in the respiration rates of leaves and of sections came to light.

First, the oxygen intake of leaves was smaller than that of sections. For example, in an atmosphere of air without carbon dioxide, the respiration rate of excised leaves was, on an average, only half of that of sections, varying from 39 % to 80 % in the single tests. The explanation, that this difference is due to oxygen deficiency in the succulent leaves was, however, not definitely proved by experiments with leaves in air and in pure oxygen: in the latter, the oxygen intake was, on an average, 15 % larger than in air, but the increase varied from —8 % to 60 % in the single tests.

The second difference was that, whereas section respiration is independent of the presence of carbon dioxide in the milieu, the oxygen intake of excised leaves is considerably increased by the addition of .5 % or 1 % CO_2 to the atmosphere. Experiments 4 and 5, and Table I illustrate this difference.

Exp. 4. *Respiration rates of leaf sections in atmospheres with and without carbon dioxide.* Outer well: 35 sections, 1 mm, in 2.0 or 1.8 ml of a .02 M KCl-solution. – Central well: .2 ml of a 10 % NaOH-solution or .4 ml of a CO_2-buffer I. – Gas phase: air. – 35° C. – Dark.
Duple determinations.

time (min.)	oxygen intake per flask in μl.	
	— CO_2	1 % CO_2
60	51	53
120	93	93
180	132	133

Exp. 5. *Respiration rates of excised leaves in atmospheres with and without carbon dioxide.* Central well: 1 excised leaf in .1 ml of a .02 M KCl-solution. – Outer well: 2 ml of a 10 % NaOH-solution or a CO_2-buffer I or II. – Gas phase: air. – 35° C. – Dark.
Duple and triple determinations.

time (min.)	oxygen intake per 500 mg in μl.		
	— CO_2	.5 % CO_2	1 % CO_2
60	31	40	45
120	58	82	91
180	85	130	138

In 23 experiments on leaf respiration, the oxygen intake in a 1 % CO_2-containing atmosphere was, on an average, 150 % ($\pm$ 7) of that in a CO_2-free milieu.

TABLE I.

The effects of the oxygen and carbon dioxide tensions on leaf and section respiration rates. Mean values of all the experiments on the comparison of leaf with section respiration. Oxygen intake calculated on a basis of 500 mg fresh weight (with correction for the cut cells of the sections), in relation to the respiration rate of sections in carbon dioxide-free air = 1.

gas composition (%)			relative respiration rate	
O_2	CO_2	N_2	excised leaves	sections
20	0	80	.5	1.00
100	0	0	.6	.98
20	1	79	.8	1.01
99	1	0	1.0	—

When looking for an explanation of the effect of carbon dioxide on leaf respiration, it was observed that excised leaves lost more water in a CO_2-containing atmosphere than in one free of CO_2; the difference may rise to 10 mg in 4 hours. This led to the idea, that stomata were opened wider in a carbon dioxide containing environment. The hypothesis was put forward, that carbon dioxide has no direct influence on the respiration of excised leaves, but affects stomatal aperture, which determines the oxygen supply of the leaves. This supposition is supported by the finding that both in air and in oxygen, carbon dioxide enhances the respiration rate of leaves (Table I). Conclusive proof could be obtained by measurement of stomatal aperture in atmospheres of various composition. Since the weighing of leaves before and after experiments in air and in oxygen showed, that less water had evaporated in oxygen than in air, probably oxygen, too, had an influence on stomatal aperture. Therefore, in the next section, experiments on stomatal aperture in atmospheres with various tensions of carbon dioxide and of oxygen, will be discussed.

§ 8. EXPERIMENTS ON STOMATAL APERTURE

Three different methods were tried out to measure the extent of the stomatal aperture. The experiments were all performed under the same conditions as indicated in the previous section: the influence of various gas mixtures was studied at 35° C in the dark.

First, Lloyd's fixation method (see e.g. CRAFTS, CURRIER and STOCKING, 1949), by means of which SAÏD and TOLBA (1948) examined stomata of *Kalanchoe*, was applied. By this method only practically closed stomata could be demonstrated under all circumstances. It is well known that this method is unsuitable for many plants. If the stomata react very quickly, or if the epidermis does not very easily become detached from the parenchymatous tissue, the same stomatal aperture will invariably be found (HARTSUYKER, 1935; HEATH, 1950).

Apart from this method, two others were used: determination of the amount of transpiration and porometer measurements. Trans-

spiration was determined by weighing samples of plucked leaves at the beginning and at the end of periods, during which they had been kept in various gas mixtures at constant relative humidity and temperature in the dark.

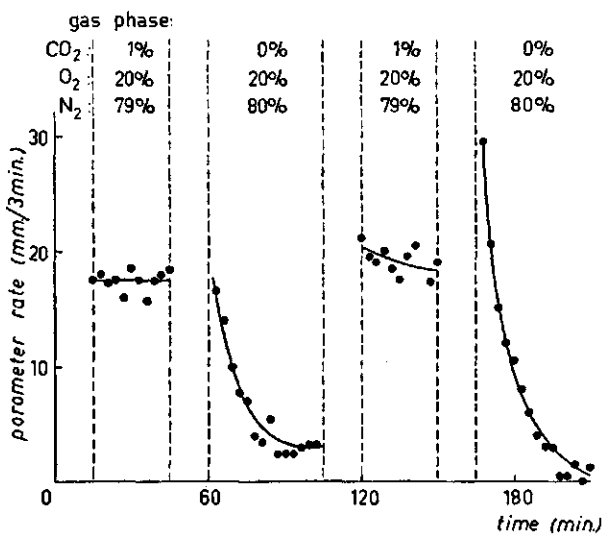
Examples of transpiration experiments are the Experiments 6 and 7, whilst the Experiments 8 and 9 present two of the porometer tests.

Expts. 6 and 7. *The effects of carbon dioxide and oxygen tensions on the transpiration of plucked leaves.* Samples of 4 leaves. – Duration: 18 hrs. – 35° C. – Dark. Quadruple determinations.

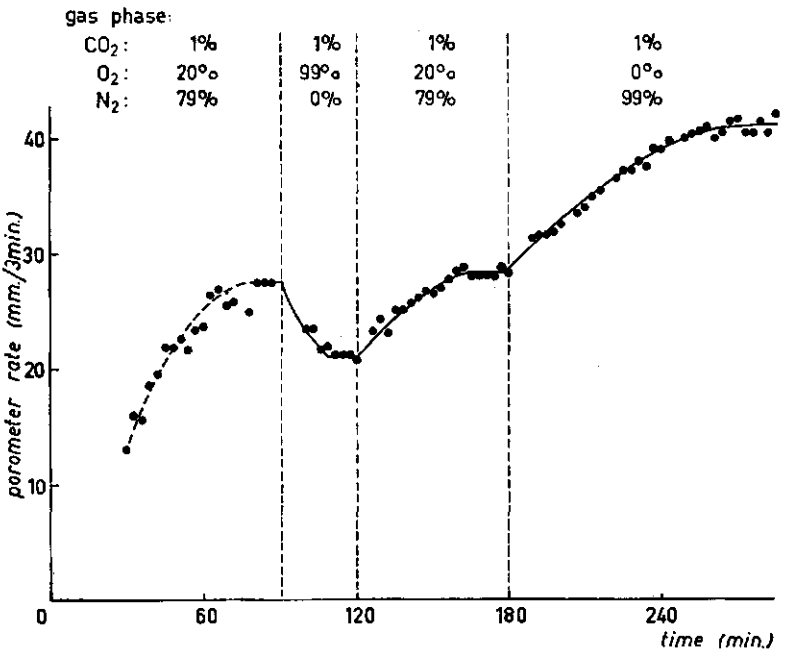
The decrease of weight by transpiration is given in % of the initial weight.

		Experiment 6: effect of CO_2			Experiment 7: effect of O_2			
composition gas mixture in %	O_2	20	20	20	0	5	20	99
	CO_2	0	1	5	1	1	1	1
	N_2	80	79	75	99	94	79	0
decrease of weight, in %		10	19	12	31	20	16	13

Expts. 8 and 9. *Porometer experiments.* Technical data, see § 5.



Exp. 8. The influence of the CO_2-tension.



Exp. 9. The influence of the O_2-tension.

The two methods produced corresponding results. Carbon dioxide, at a tension of 1 %, had an opening effect on stomata, as compared with 0 % and 5 %. On the other hand, a closing effect of oxygen is obvious: from 100 % to 0 %, stomatal aperture was progressively widened, whilst the stomata opened fully only under anaerobic conditions.

Experiment 9 shows an experimental difficulty. Stomata were often closed at the beginning of the porometer experiment. This occurred in atmospheres both with and without carbon dioxide. Probably it was the result of the mechanical shock of the cutting and the attachment to the apparatus; about 60 to 90 minutes after the setting up the experiments proper could be started.

From further investigations it was concluded, that stomata of *Bryophyllum tubiflorum* leaves are not very sensitive to stimuli other than CO_2, O_2 and mechanical ones. An illumination of 20×10^3 ergs/cm^2.sec during 1 hour, in air with 1 % CO_2, had no effect on the aperture. Only at a low relative humidity of the gas mixture stomata closed. There was hardly any reaction to changes in temperature; at lower temperatures stomata tended to open a little wider.

§ 9. Discussion and conclusions

The stomata of *Bryophyllum tubiflorum* phyllodes are equally distributed, about 30 per mm^2, which, according to Stålfelt (1956*a*), is

few. These stomata are normally built, the guard cells being 40 μ long and containing chloroplasts. They are level with and surrounded by 3 to 5 subsidiary cells, which is a characteristic of the Crassulaceae (FRITSCH and SALISBURY, 1938). A pore with a maximum width of 10–12 μ separates a respiratory cavity of about .01 μl from the surrounding air.

These stomata behave in a rather uncommon manner, since they are mainly sensitive to the concentrations of carbon dioxide and oxygen in the atmosphere. Both transpiration and porometer measurements show, that stomata are opened wider in 1 % CO_2 than in a carbon dioxide-free atmosphere, and that an increase of the oxygen tension causes the stomatal aperture to narrow.

Being indirect, these methods give mean values of the behaviour of a great many stomata, although the two methods are not equally exact. Using transpiration rates of leaf samples as a measure of stomatal aperture—which may be done in experiments of not too long a duration and under controlled conditions—, the figures obtained are correct only to some per cent, because of the variability of response of the individual leaves. Moreover, when interpreting results of transpiration experiments, one has to assume, that it is the composition of the external atmosphere, rather than that of the internal one, to which the stomata react.

The porometer experiments, on the other hand, give the precise reactions of the whole body of stomata of one single leaf to changes in the composition of gas, surrounding and sucked through the leaf. The extent of these reactions differed in the various experiments, on account again of the individual variability of the leaves, but the effects of changes in carbon dioxide and oxygen tensions were invariable distinct and reproducable. If the gas movement varies under influence of different circumstances and the leaf remains turgescent, porometer readings give reliable information about changes in stomatal aperture, since it is inconceivable how the resistance in the intercellular spaces could then vary considerably (VAN SLOGTEREN, 1917; HARTSUYKER, 1935). Moreover, the intercellular spaces in *Bryophyllum tubiflorum* leaves are large, taking up about one fifth of the total leaf volume and running throughout the leaf. It is highly improbable, therefore, that their resistance limits the porometer rate.

The influence of carbon dioxide on stomatal aperture of *Bryophyllum* leaves seems to be in contradiction to the rather commonly accepted view, that carbon dioxide causes closure (LINSBAUER, 1917; HEATH and RUSSELL, 1954; STÅLFELT, 1956*b*). However, SCHELLENBERG (1896) and PAETZ (1930) reported that the stomatal aperture of several plant species narrowed when carbon dioxide was absorbed from the air by KOH-solutions. SCARTH (1932) and FREUDENBERGER (1941) found opening of stomata in higher CO_2-concentrations, which could be toxic. Since I dispose of data on stomatal reaction at only three carbon dioxide concentrations, and the relationships between the carbon dioxide tension and stomatal behaviour appear to be complex, I feel not justified in making a stand in this controversy.

The very obvious closing effect of oxygen on stomatal aperture is quite compatible with the theory of WILLIAMS (1954), who suggested that it is the closing movement of stomata, rather than the opening one, which is the active, energy consuming part of stomatal movement. SCARTH, WHYTE and BROWN (1933) reported that stomata of *Zebrina pendula* (?) open at low oxygen tensions, under water and in oil. GREGORY and ARMSTRONG (1936) found a wide opening of *Pelargonium*

stomata in a hydrogen atmosphere in the light, and an incapability of closure in subsequent darkness, unless the hydrogen was replaced by air. The authors gave no further interpretation of these findings. According to FREUDENBERGER (1941), stomata of *Canna* leaves close at first in nitrogen in the dark, but afterwards open fully; this too might be caused by oxygen deficiency. Recently, HEATH and ORCHARD (1956) tried to test Williams's hypothesis indirectly, by investigating the stomatal response to closure treatments under aerobic and anaerobic conditions. They carried out porometer experiments on illuminated wheat leaf stomata. The authors were unable to demonstrate an opening effect of oxygen deficiency. The interpretation of the results is hindered by the facts, that for the comparison of stomatal behaviour in air and in nitrogen the initial values differ, and that the variability in the single tests was considerable. Obviously, illuminated wheat stomata are rather insensitive to changes in oxygen tension and, therefore, no felicitous subject for an investigation into oxygen influences. This insensibility itself does not disprove the view, that closure is the energy-consuming part of stomatal movement, since, especially in the light, chlorophyll-containing cells are able to gain energy from other than respiratory sources (ARNON, 1956).

Respiration of leaf sections is not limited through lack of oxygen or of substrates. It is concluded that the endogenous respiration of leaf sections is determined exclusively by the turnover capacity of the respiratory enzyme system in the intact cells.

The respiration rate of excised leaves differs in two aspects from that of comparable leaf sections: it is lower than the latter and it can be stimulated by the addition of carbon dioxide (in concentrations of .5–1 %) and, possibly, by an increase of the oxygen tension. The respiration rate of sections is affected neither by oxygen tensions above 5 %, nor by carbon dioxide.

Carbon dioxide influences the acid metabolism (see Chapter III), but it must be concluded from the absence of any effect on section respiration, that the CO_2-influence on the acid metabolism is of no consequence to the rate of respiration. The accelerating effect of carbon dioxide on leaf respiration can be fully explained from the results of the investigation into stomatal behaviour. In 1 % CO_2, the stomata are opened much wider than in a carbon dioxide-free atmosphere, so that the oxygen entry is facilitated. This means that the lower rate of respiration in leaves is due to oxygen deficiency and depends on stomatal aperture.

If this view is correct, then the variation in the extent of reaction of stomatal aperture to CO_2 and O_2 should be reflected in a similar variation of the leaf respiration rate at different CO_2- and O_2-tensions. This was found to be the case: whereas section respiration showed only little variability, that of leaf respiration was quite considerable.

According to the opinion, given above, a raised oxygen tension in the atmosphere should enhance leaf respiration. This increase in

oxygen intake was hardly found, since the average increase of 15 % was not significant. The closing effect of oxygen on stomatal aperture, however, counts for the failure to demonstrate an evident stimulation of respiration at higher oxygen concentrations.

Even in pure oxygen, leaf respiration proved, on an average, to be only 60 % of that of sections; section respiration in 1 % O_2 was still 45 % of the maximum rate. It is quite obvious from these findings, that in leaves a very steep gradient must exist between the 100 % O_2 surrounding the leaf, and the oxygen tension in the intercellular spaces; the latter can come to only a few per cent! Consequently, the gas exchange between the intercellular spaces and the outer atmosphere must be extremely poor.

The respiration rate of leaves in 99 % O_2 + 1 % CO_2 was generally about as high as that of comparable sections. This means, that the oxygen tension in the intercellular spaces was no longer limiting, because of the abundance of oxygen outside the leaf, together with the opening effect of carbon dioxide on stomatal aperture.

The experiments were performed at the optimum temperature, 35° C. At a moderate temperature, it is true, respiration will be less intensive—*e.g.* at 15° C about 25 % of that at 35° C, according to Q_{10}-calculations from preliminary experiments—, so the inequality between metabolic gas exchange and that with the outer atmosphere will be lessened. Assimilation, however, readily surpasses dissimilation in intensity and, therefore, will have an even more vigorous influence on the composition of the intercellular gas in the light, than respiration so plainly has in darkness. The alternation of these two metabolic processes, opposite to one another as to their gas exchanges, must cause an intensive diurnal variation in the intercellular gas composition.

It was not attempted to obtain direct information about this variation by determination of gas samples from the intercellular spaces, taken in the morning and in the evening. The frequently used method of gas extraction *in vacuo* (MAGNESS, 1920) is convincingly rejected by GORTER and NADORT (1941), who demonstrated that by this treatment large amounts of carbon dioxide are extracted, which could not possibly have been present in the original intercellular gas. SHAFER (1938) found that carbon dioxide, physically dissolved in the cell sap or even loosely chemically bound, is extracted *in vacuo*. Especially in the case of succulents, with their large content of organic acids and active carboxylases, this might be a considerable source of errors. DEVAUX (1891) developed a much milder technique, leading to more reliable results, but applicable only to massive tissues such as large tubers and fruits.

That the composition of the intercellular gas in succulent leaves differs from that of the outer atmosphere, was submitted by KAKESITA (1930) to explain the observation, that *Bryophyllum calycinum* plants formed bulbils on the leaves both after a treatment at 35° C and after

being kept in a hydrogen atmosphere; the common factor in the two cases was considered to be oxygen deficiency.

Although no direct data on the subject can be presented, it is obvious from the limited respiration rates of leaves—even in pure oxygen—, as compared to those of sections, that the gas exchange between the intercellular spaces and the outer atmosphere is, indeed, very poor, much too small to satisfy metabolic requirements. The inevitable consequence is the occurrence of a probably very considerable diurnal variation in the composition of the gas in the intercellular spaces of *Bryophyllum tubiflorum* leaves.

CHAPTER III

THE INFLUENCES OF EXTERNAL FACTORS ON THE ACID METABOLISM

The characteristic feature of the Crassulacean acid metabolism is the diurnal variation in acid content, caused by external factors (Chapter I). External factors, especially illumination, affect the gas composition of the intercellular spaces in the succulent leaf; they induce a diurnal change in the carbon dioxide and oxygen tensions (Chapter II). In this Chapter, it will be investigated as to how far the influence of illumination conditions on the Crassulacean acid metabolism depends on changes in the carbon dioxide tension in the intercellular spaces.

§ 10. Methods

A. **Apparatus**

The plant material used was similar to that used in Chapter II. In most experiments, the Warburg apparatus was used again, which permitted of experiments of long duration under controlled conditions and measuring of the gas exchange. The flasks were painted white, except for the bottom, through which light from sodium lamps (Philips 140 SO) could enter. The light intensity was measured with a waterproof photocell as designed by Lange (1940), gauged at 589 mμ. By using suitable filters, the light could be adjusted to any value up to 37×10^3 ergs/cm^2.sec. Cooling with tap water slightly overcompensated the heat supply from the lamps. Most experiments were performed at 20.0° C. The CO_2-buffer I (see § 5) is in equilibrium with $\frac{1}{4}$ % CO_2 in the atmosphere at 20° C. If flasks had to be kept in the dark, whilst others were illuminated, they were wrapped in black paper and tin-foil.

B. **Fixation and extraction of acids**

There are quite a variety of procedures to extract organic acids from plant tissues and to separate and determine them. The excellent

reviews by WOLF (1955) and RANSON (1955) give detailed accounts of these subjects.

The ether extraction from dried, acidified material after PUCHER, VICKERY and WAKEMAN (1934, 1941) was unsuitable for our purposes, as it took too much time and was not easily convertible to a microscale. Moreover, one may doubt whether isocitric lactone is formed during drying at 50° C. A safer way to fix plant material is deep-freezing (ISHERWOOD, 1946). For the fixation and extraction of the acids, the following procedure was followed.

Transfer 1 leaf or 40 leaf sections, 1 mm thick, quickly into a thin-walled glass tube, 80 × 7 mm, and freeze during 10 minutes in acetone, cooled with solid CO_2, in a Dewar vessel. Store the tubes at —10° C in the dark.

For the extraction, grind up the plant material of 1 tube in a mortar with 300 mg of quartz sand and allow to thaw out for some minutes. Transfer the slurry quantitatively into centrifuge tubes, 100 × 18 mm, make up to 3 ml with distilled water and precipitate at 1700 G during 5 minutes. Collect the clear supernatant, whirl up the precipitate in distilled water at 3 ml and centrifuge again. After 4 extractions with distilled water, make up the gathered supernatant liquids to 25 ml in a test tube, 15 × 3 cm; this water extract contains over 99 % of the water soluble salts and acids, originally present in the plant material.

C. **Titration**

In most experiments, the total amount of free titrable acid was determined. For this purpose, 6 drops of 1 % phenolphthalein in 70 % ethanol were added to the extract, and CO_2-free nitrogen was bubbled through from 5 minutes before the titration began, to remove dissolved carbon dioxide. In the nitrogen stream the solution was titrated with .01 N NaOH-solution from a Pellet microburette, the capillar outlet-tip being underneath the liquid surface. The titration was stopped when the entire solution was just slightly pink; at that P_H, 8.9–9.0, over 99 % of the acids are neutralised. To facilitate the perception of the colour change, the test tube had been placed in a porcelain dish. The accuracy of the titration was 10 μl, corresponding to .1 μeq.

D. **Total acid determination**

When, in some experiments, the total acid content had to be determined, a cation exchange resin was used (Imac C12, Handelsmij. Activit, Amsterdam; this resin is a monofunctional, sulfonated, cross-linked polystyrene resin). The resin, 70–140 mesh, was made up into a column, 18 × 1 cm, and generated in the H^+-form with 6 % HCl, which afterwards was washed away with distilled water, until the eluate gave no more opalescence with a $AgNO_3$-solution.

The collected supernatant liquids, obtained with the extraction (see B), were, instead of being made up to 25 ml, passed through

the column and eluted with distilled water until 50 ml of eluate were gathered; the elution rate was adjusted to .5 ml/min. From experiments, in which Na-malate was added to sections before deep-freezing, it was concluded that the recovery was 99–100 %.

E. **Isolation of the organic acids**

a. Purification

In order to examine what organic acids are present and which of them participate in the diurnal variation, the organic acids were separated and determined by means of paper chromatography. It turned out that the plant sap had to be purified to prevent tail formation of the spots. For this purification the silicagel method, as recommanded by ISHERWOOD (1946), was used. Celite, although taking less time and fewer chemicals (STUTZ and BURRIS, 1951), was found to absorb varying amounts of organic acids and was, therefore, discarded. The purification was carried out as follows.

Prepare silicagel according to ISHERWOOD (1946) and dry it thoroughly over P_2O_5. Mash 4 g of it over a 140 mesh sieve and mix it with 50 ml of the elution liquid, which is chloroform/butanol, v/v = 50/50, equilibrated against .2 N sulfuric acid. Pour the slurry into a glass tube of 2 cm diameter on a perforated porcelain disc, covered with a filter paper disc, to form a column, about 5 cm high.

Extract 4 g of leaf (fresh weight) according to the prescription given in B, using 2 g of quartz sand. Adjust 5 ml of the 25 ml of water extract to $P_H = 2.0$ with concentrated sulfuric acid and mix it thoroughly with so much of dry silicagel, that the latter remains superficially dry. Make this silicagel up to a homogeneous slurry with the elution liquid and transfer the slurry quantitatively upon the column made before. Allow the column, in our experiments about 16 cm high, to run just dry and subsequently add more elution fluid, until 400 ml of filtrate is gathered. This filtrate contains the organic acids, whereas inorganic acids, sugars, *etc.* are held back. Add drops of concentrated ammonia to the filtrate and shake firmly, until so much ammonia is added, that a drop of the mixture colours red with a drop of a 1 % phenolphthalein solution in 70 % ethanol. The organic acids are now dissociated and after separation of the liquid layers in a funnel, the aqueous one is collected. Wash the non-polar layer 5 times more thoroughly with each time about 2 ml of distilled water and evaporate the combined aqueous liquids *in vacuo* at room temperature. Dissolve the dry ammonium salts in 1 ml of water; the concentration, about 3 μeq of salts per 10 μl, is suitable for paper chromatography.

b. Separation and determination

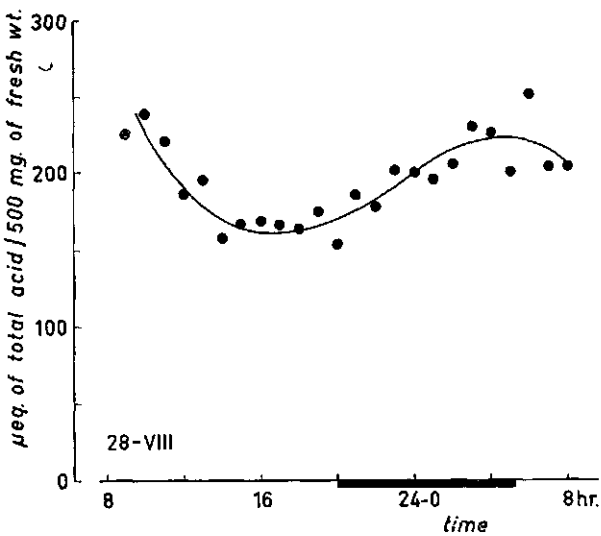
Descending paper chromatography was performed in a glass tank, using a paraffined polyvinyl trough in a stainless steel frame. Strips of Whatman no. 1 filter paper, 45 × 19 cm, were washed before use, first with 2 N acetic acid, next with distilled water and finally with the solvent. The lower margin of the strips was notched to allow the solvent to flow off without disturbing the chromatogram. As a solvent, *n*-propanol/NH_4OH (25 %)/H_2O = 3/1/1, was used (ISHERWOOD and HANES, 1953). The propanol was refluxed with NaOH-pellets and next distilled at 96–97° C.

Spots of purified plant extracts and of known solutions of organic acids were brought up from a 12.4 μl capillar; the distance between the spots was 2.5 cm. After equilibration over night, the solvent flowed

over during 5 hours. The strips were dried in an air flow at room temperature, sprayed lightly with ammoniacal silver nitrate (PARTRIDGE, 1946) and dried at 105° C. After about 15 minutes the spots showed up on a darkened background; they could be determined by their relative rates of movement and by the spot colour, hydroxy acids showing a more or less intensive brown to violet ring around the white centre. The spots were immediately cut out and weighed. Because, within certain limits, spot area and weight are proportional to the logarithm of the quantity of acid (BRYANT and OVERELL, 1953), the amount of acid could be found by relating the weight of its spot to those of known quantities of the same acid on the same strip. The accuracy of this estimation was 5–10 % for 2–.5 μeq of acid per spot.

§ 11. THE DIURNAL ACID VARIATION

The commonly used plant species in studies on the Crassulacean acid metabolism is *Bryophyllum calycinum;* sometimes *B. daigremontanum* is used. In illuminated leaves of the former species, PUCHER *et al.* (1947*c*) found the malic acid content to decrease from 17 to 7 g/kg of fresh weight; isocitric acid varied hardly and irregularly, citric acid seemed to follow the malic acid changes but slightly, whilst a trace of oxalic acid changed hardly significantly. Among the other plant species, tested for the occurrence of a diurnal acid variation, *Bryophyllum tubiflorum*, used in the present investigations, is not met with. It had to be verified, therefore, whether the acid metabolism in *B. tubiflorum* leaves behaves similarly to that of other succulent plant species.



Exp. 10. *The diurnal variation in total acid content of leaves of a greenhouse plant.* Light intensities up to 44 × 10^3 ergs/cm².sec, darkness from 8 p.m. till 5 a.m. Temperatures from 15.6 to 27.6° C. Determinations of single leaves.

In Experiment 10, lasting 24 hours, each hour a leaf was gathered from a plant in the greenhouse and its total acid content determined. It turned out that in the day-time about one third of the total acid content disappeared gradually, and was restored gradually again at night. The amplitude of the variation, about 9.4 g of acid per kg of fresh weight (the acid calculated for as malic acid), agrees with the above-mentioned findings of Pucher *et al.*.

Part of the acid is in the form of salt. That this salt fraction does not participate in the diurnal variation, can be concluded from Experiment 11.

Exp. 11. *The diurnal variation in total and free acid content.* Samples of 9 leaves, gathered from a greenhouse plant at 5 p.m. and 9 a.m.. Duplo titrations before and after passing the water extracts over a cation exchange resin (see §10, D).

	μeq of acid per 500 mg of fresh wt		
	5 p.m.	9 a.m.	acidification
total acid . . .	130	173	43
free acid . . .	9	53	44
salt	121	120	—1

In the evening, the free-acid content was quite low. Over night, free acids were produced, whereas the salt fraction hardly varied. Since the change in salt content comes within the measure of accuracy of the acid determination, one is justified in determining the amount of free acid as a measure for the acid variation, in stead of the more time-devouring method for total-acid determination.

It was investigated whether, next to organic acids, a considerable inorganic-acid fraction occurs. Total-acid determinations showed that removal of the inorganic anions by means of the silicagel method (see § 10, E, *a*), did not reduce measurably the acidity of watery leaf extracts. The inorganic-acid fraction may, therefore, be neglected.

Paper chromatographic methods (see § 10, E, *b*) were used to trace what organic acids are present in *B. tubiflorum* leaves, and which of them exhibit a diurnal variation. Experiment 12 shows that the diurnal acid variation mainly concerns malic acid; isocitric and citric acids, which are present in considerable quantities, do not vary in the over all balance.

Exp. 12. *The diurnal variation in the organic-acid content of leaves.* Samples of 9 leaves, gathered from a greenhouse plant at 5 p.m. and 9 a.m.. Leaf extracts purified over silicagel (see § 10, E, a). Qualitative and quantitative analyses with paper chromatography (see § 10, E, b). Duple determinations.

	μeq of acid / 500 mg of fresh wt	
	5 p.m.	9 a.m.
malic acid	trace	72
isocitric + + citric acids . .	175	172
other acids . . .	trace	trace

We may conclude from the foregoing, that the main features of the acid metabolism in *Bryophyllum tubiflorum* phyllodes coincide with the general pattern of the Crassulacean acid metabolism: a diurnal variation occurs in the free malic acid content, the large amounts of isocitric and citric salts being inert.

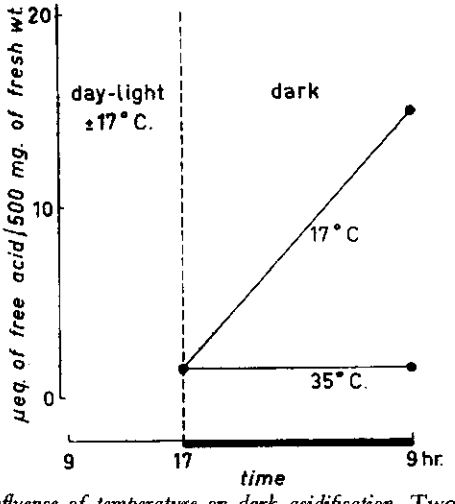
§ 12. Influences of external factors on the acid metabolism in leaves

We will now consider what factors affect the acid variation. It was repeatedly observed (*e.g.* Kraus, 1885; Aubert, 1892*a*; Gustafson, 1925), that *weather conditions* in the day-time have an effect on deacidification during that day, as well as on acidification in the subsequent night. A bright day was found to induce a larger amplitude in the acid variation than a dull day. An illustration of this phenomenon, as shown by *B. tubiflorum* leaves, is provided by Experiment 13.

Exp. 13. *The influence of weather conditions on the diurnal acid variation.* Samples of 2 leaves, gathered from a greenhouse plant, at 3 subsequent days at 9 a.m. and 5 p.m.. Duple determinations of the total amount of acid.

date	weather	μeq of total acid / 500 mg of fresh wt			
		9 a.m.	5 p.m.	nightly acidification	daily deacidification
3 XI 53	sunny	195	155		40
4 XI 53	rainy	216	191	61	25
5 XI 53	overcast	200	167	9	33

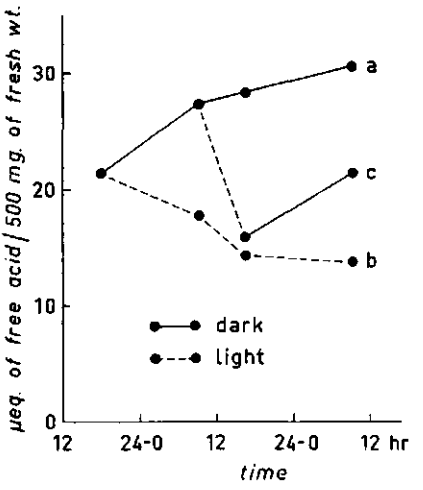
Since the relative humidity in the greenhouse was rather constant, the two main weather factors, which could influence the diurnal acid variation, were temperature and illumination.



Exp. 14. *The influence of temperature on dark acidification.* Two plants, commonly pretreated in the greenhouse until 5 p.m., were next kept in the dark at different temperatures. Duple determinations of samples of two leaves.

Experiments on the influence of *temperature* showed that, in accordance with the data in the literature, higher temperatures led to lower acid contents; they stimulated light deacidification and inhibited, or even reversed, dark acidification (Experiment 14).

The influence of *illumination conditions* was studied in experiments on excised leaves, placed in Warburg flasks, in which temperature could be kept constant throughout the experiment. Nrs. 15 and 16 are two of these experiments.



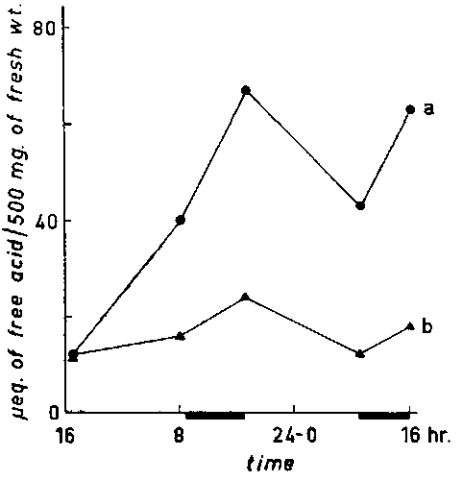
Exp. 15. *The influence of illumination conditions on the acid metabolism of excised leaves.* Freshly-excised leaves, with the cut in a .02 M KCl-solution. – Air + 1/4 % CO_2. – 20.0° C. – Duration: 40 hrs. – Intensity sodium light: 37×10^3 ergs/cm².sec. Single and duple determinations of samples of 3 leaves.

a: continuously darkened; b: continuously illuminated; c: illuminated from 9 a.m. till 5 p.m. only.

Experiment 15 shows that, at a constant temperature, the acid content of leaves increased during prolonged darkness and decreased in continuous light, without showing a diurnal rhythm. An alternation of acid formation and breakdown only occurred if light and dark periods alternated with one another.

That the diurnal acid variation is at least predominantly exogenous, is demonstrated by Experiment 16. At a constant temperature, excised leaves were illuminated during the night and kept in the dark in the day-time. As a result of this treatment, the acid metabolism reversed and followed the new rhythm within 24 hours.

It is concluded that, if temperature is kept constant, the acid variation largely depends on the illumination conditions.



Exp. 16. *Reversal of the diurnal acid variation of excised leaves by reversal of the illumination period.* Freshly-excised leaves, with the cut in a .02 M KCl-solution. – 20.0° C. – Duration: 47½ hrs. – Intensity sodium light: 22 × 10^3 ergs/cm².sec. Light: 5 p.m. till 9 a.m.; dark: 9 a.m. till 5 p.m. and 9 a.m. till 4p. m.. Duple determinations of samples of 3 leaves.
a: air + 5 % CO_2; b: air without CO_2.

§ 13. Influences of illumination and carbon dioxide tension on the acid metabolism in leaf sections

In alternately illuminated and darkened succulent leaves, a considerable diurnal fluctuation in the intercellular gas composition, notably in the carbon dioxide tension, must be expected (see Chapter II). This diurnal change in CO_2-content might govern the acid variation either by participation of carbon dioxide in acid production and consumption in an equilibrium or cycle reaction, or because a continuous acid breakdown is overcompensated by acid formation if the CO_2-tension surpasses a certain level.

If one wishes to examine, whether light affects the Crassulacean acid metabolism in this way, one has to perform two types of experiments. Cells must be exposed, on the one hand, to various illumination conditions at a constant CO_2-tension, and, on the other hand, to various concentrations of CO_2 under constant illumination conditions. Since it is demonstrated in Chapter II, that sections of *Bryophyllum tubiflorum* leaves have a good gas exchange with the outer atmosphere, they form a promising material, conformable to requirements.

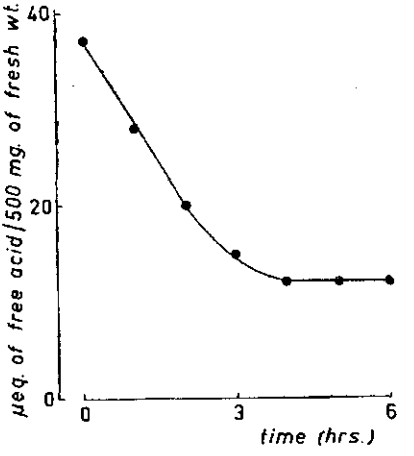
A. Experiments with non-adapted leaf sections; the adaption

The interpretation of experiments on the effects of illumination and carbon dioxide tension on the acid metabolism of fresh-cut leaf sections, is complicated by interference from the adaptation of the acid metabolism to the conditions, under which the leaf cells are kept

during the experiment. At the beginning of the experiment, an accidental amount of acid is present, brought about by the coincidence of conditions in the greenhouse, from which the material is taken. When leaves are cut into sections and these are brought under constant experimental conditions, the amount of acid changes until a level is reached, adapted to the prevailing conditions.

This adaptation of the amount of acid to the experimental conditions can be obtained in four ways: one can start with acid-rich or deacidified plant material and adapt either to light or to darkness. Since adaptation of deacidified sections to light has not been carried out, three ways are left to discuss.

1. Sections, cut in the morning, and, therefore, containing a large amount of free acid, can be adapted to illumination and a low carbon dioxide tension within a few hours, as follows from Experiment 17. Under these conditions, sections rapidly deacidify and a low, constant acid level is reached in about 4 hours.

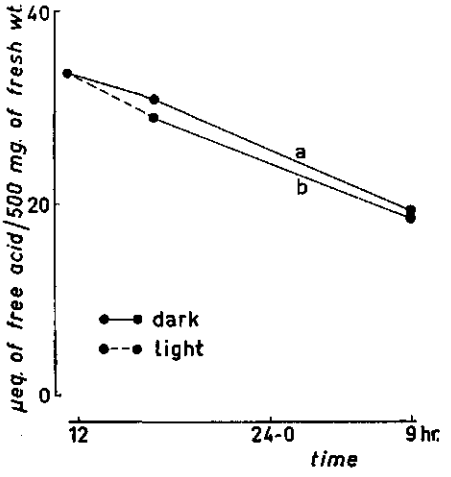


Exp. 17. *Adaptation of acid-rich sections to illumination at a low carbon dioxide tension.* Samples of 30 sections, 1 mm, cut in the morning, floating in a .02 M KCl-solution. – Laboratory air. – 20.0° C. – Intensity sodium light: 37×10^3 ergs/cm².sec. Duple and single determinations.

2. If sections, cut in the morning, are adapted either to light and a higher CO_2-tension, *e.g.* 5 %, or to darkness—at any CO_2-concentration—, a rather slow deacidification sets in. In the dark, at 20° C, the final acid level of adapted sections is lower than that of comparable excised leaves. If illuminated sections are exposed to 5 % CO_2, the rapid, light-induced deacidification, which occurs at low CO_2-concentrations, is completely suppressed, and the acid metabolism is indistinguishable from that in the dark: Experiment 18. At the end of this experiment, adaptation was still incomplete.

3. Sections, cut in the evening from deacidified leaves, which are adapted to darkness, reach a constant acid level after about 20 hours.

The amount of acid depends on the CO_2-tension, temperature and thickness of the sections. In 1-mm-sections, dark acidification never equalled that of comparable excised leaves under the same conditions (Experiment 19).



Exp. 18. *Adaptation of acid-rich sections to illumination and a high carbon dioxide tension, and to darkness.* Samples of 40 sections, 1 mm, floating in a .02 M KCl-solution. – Air + 5 % CO_2. – 20.0° C. Duple determinations.
a: continuous darkness;
b: illuminated from 11.20 a.m. till 4.30 p.m. (sodium light, 37 × 10^3 ergs/cm². sec), next darkness.

Exp. 19. *The adaptation of deacidified sections and leaves to darkness.* Plant material gathered from a greenhouse plant in the afternoon. – Samples of 30 sections (1 mm), floating in a .02 M KCl-solution, and of 2 leaves, placed with the cut in this solution. – Laboratory air. – Dark. – 20.0° C.

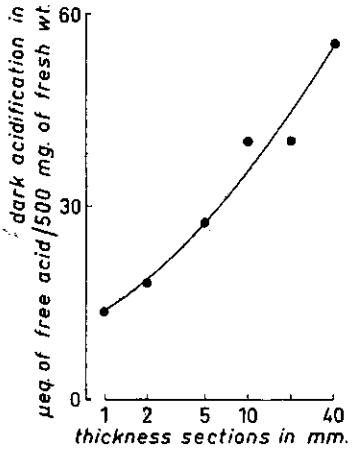
Duple determinations.

	μeq of free acid/500 mg of fresh wt	
	4.30 p.m.	9.30 a.m.
sections	4.4	9.6
excised leaves . .	4.4	20.9

No external factor could be found responsible for this reduced dark acidification of sections, as compared to that of leaves. Imitating the intercellular gas composition of darkened leaves by an up to 20 % CO_2-enrichment and an O_2-tension, lowered down to zero, of the atmosphere above the sections, enhanced dark acidification somewhat, but by no means sufficiently at any CO_2/O_2 ratio tried. No unfavourable factors, such as ion composition, P_H, osmotic value, nor any inhibiting substance could be traced in the solution, in which the sections floated, whilst no essential compound diffused from the sections into the liquid or the atmosphere. Nor was there any factor either found necessary to be transported along the longitudinal axis of the leaf. Both in cut pieces of leaf and in leaves, pinched lightly with a pair of tweezers, dark acidification was decreased for a part, larger than that

accounted for by the number of visibly damaged cells. There was, however, no evidence that acids from injured cells hindered the acid production in intact ones, nor that the sudden entry of the outer atmosphere into the leaf had any influence on the acid metabolism.

Experiment 20 shows the relation between the thickness of sections, cut in the evening from deacidified leaf material and allowed to acidify during the night in darkness at a high CO_2-tension, on the one

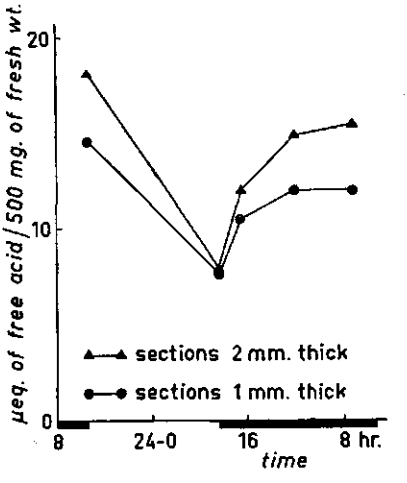


Exp. 20. *The relation between acidification and the logarithm of the thickness of sections, during dark adaptation.* Sections, cut from a greenhouse plant at 5 p.m., floating in a .02 M KCl-solution. – Air + 5 % CO_2. – Dark. – 20.0° C. – Duration: $16^1/_2$ hrs. At 5 p.m., 4.5 µeq of free acid/500 mg of fresh wt was present. Duple determinations of the following samples: 40 sections (1 mm), or 20 sections (2 mm), or 8 sections (5 mm), or 4 sections (10 mm), or 2 sections (20 mm), or 1 section (40 mm).

hand, and the constant acid level, reached in the morning, on the other hand. Sections, 40 mm thick, contained as much as comparable excised leaves, but the thinner the sections were, the smaller the measure of acidification.

The relation between dark acidification and thickness of sections was studied more closely in experiments, of which nr. 21 is a typical example. In this experiment, sections of 1 mm and 2 mm thick, cut in the evening from deacidified leaves, were allowed to acidify over night in the dark, at $\frac{1}{4}$ % CO_2. The next morning, different acid contents were found. The nearly adapted sections (the adaptation time, 14 hrs, was rather short), were illuminated at $\frac{1}{4}$ % CO_2 during 24 hours, deacidified and the same low acid level was reached in both types of sections. Probably, the remainder of titrable acid bears no direct relation to malic acid metabolism and is an indifferent rest. During the subsequent dark period, at $\frac{1}{4}$ % CO_2, samples were taken which showed, that both the rate and the measure of dark acidification in the thicker sections were larger than those in the thinner sections. These differences can not be ascribed to different carbon dioxide tensions within the two types of sections, since at 5 % CO_2, which

highly saturates dark acidification (see below), a similar difference in final acid content occurred: at 9.30 a.m., 20.6 μeq of free acid per 500 mg fresh weight was present in 1-mm-sections, whereas in the 2-mm-sections 25.3 μeq was found. It rather looks as though more cells are inactivated in the sections, than can be accounted for by the



Exp. 21. *The relation between thickness and dark acidification of adapted sections.* Samples of 20 sections (2 mm) and of 40 sections (1 mm), floating in a .02 M KCl-solution. Adaptation during 14 hrs in the dark, in air + $^1/_4$ % CO_2, at 20.0° C. Illuminated from 12 noon (sodium light, 9 × 10^3 ergs/cm^2.sec) till 10.30 a.m., next dark again. Total duration: 67 hrs. Single and duple determinations.

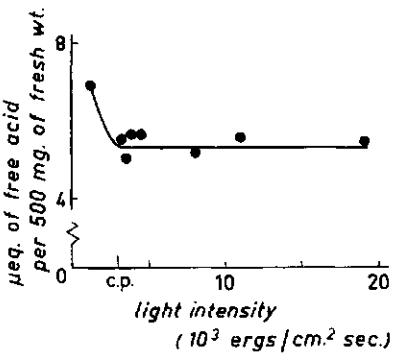
cut ones. I am inclined to believe, that through the mechanical act of the cutting the acid metabolism is damaged in cells, which still respire normally and appear undamaged under the microscope.

Experiment 21 further provides a demonstration of sections, attaining a constant acid level, whilst adapting themselves to changed conditions, in this case darkness.

B. **Experiments with adapted leaf sections**

The acid level of adapted leaf sections was investigated in darkness and at different illumination conditions, *viz.* in strong light and at the intensity of the compensation point. The latter intensity, at which respiratory and assimilatory O_2-exchanges balance, was from manometric determinations found to be 2.7 × 10^3 ergs/cm^2.sec. As Experiment 22 illustrates, the light intensity itself has no influence on the height of the final acid level, provided that the intensity surpasses that of the compensation point.

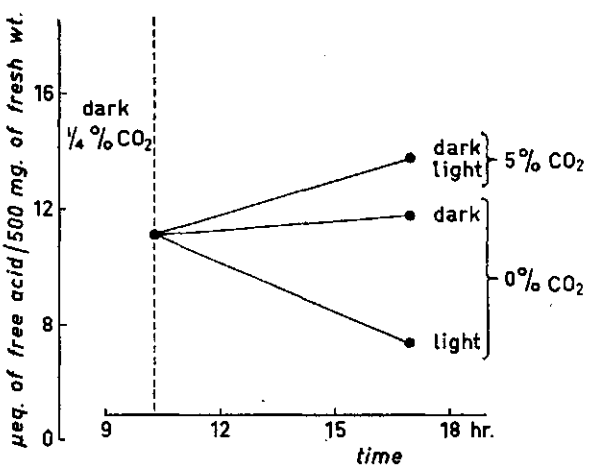
The relation between the carbon dioxide tension and the illumination conditions will be considered from the results of three typical experiments.



Exp. 22. *The influence of the light intensity on the acid level.* Samples of 30 sections (1 mm), in 2 ml of a .02 M KCl-solution. – Air without CO_2. – 20.0° C. Single and duple determinations after 44 hrs of illumination (sodium light). c.p. = compensation point.

In Experiment 23, the influence of strong illumination at a low and a high carbon dioxide tension was investigated. After adaptation over night in the dark, at ¼ % CO_2, four different conditions were applied: a low and a high CO_2-tension, each in the light and in darkness. In continuous darkness, the replacement of ¼ % CO_2 by 0 % CO_2 caused no measurable change in the acid content; in 5 % CO_2, however, the acid level rose: CO_2 stimulates dark acidification.

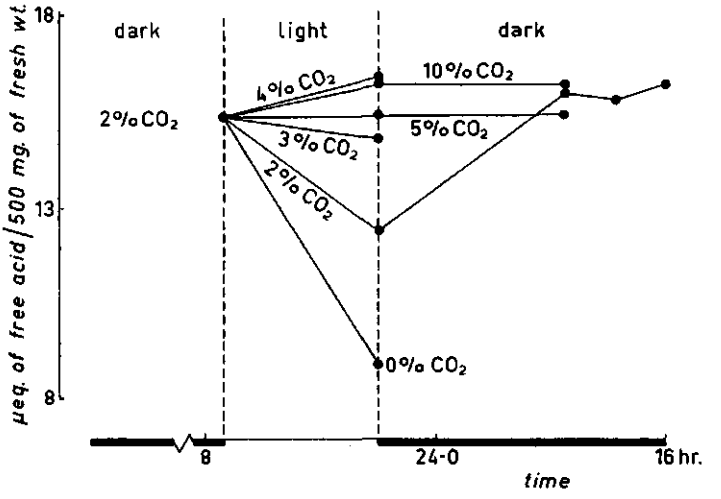
Upon illumination, the acid content of the sections in 0 % CO_2 fell considerably; this light deacidification, on the other hand, did not occur in 5 % CO_2. As in the case with adapting sections, at a high Co_2-tension, it is entirely immaterial whether sections are illuminated



Exp. 23. *The influence of strong illumination at a low and a high carbon dioxide tension.* Samples of 40 sections, 1 mm, in 2 ml of a .02 M KCl-solution. – 20.0° C. Adaptation during 14 hrs in air + ¼ % CO_2 in the dark. At 10.15 a.m.: air without CO_2, light or darkness, air + 5 % CO_2, light or darkness. Light intensity: 8.7 × 10^3 ergs/cm².sec. Total duration: 21 hrs. Triple determinations.

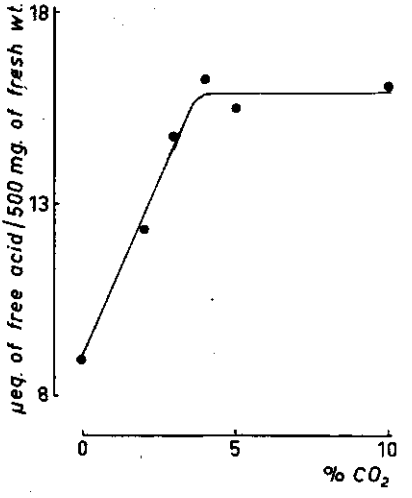
or not. Light only affects the acid content if the carbon dioxide tension is low. This will be further analysed in the next experiment.

The influence of light-dark alternations at various carbon dioxide tensions was studied in Experiment 24. After adaptation in the dark at 2 % CO_2, samples were exposed to various CO_2-concentrations in



Exp. 24a. *The influence of light-dark alternations at various carbon dioxide tensions.* Samples of 40 sections, 1 mm, in 2 ml. of a .02 M KCl-solution. – 20.0° C.

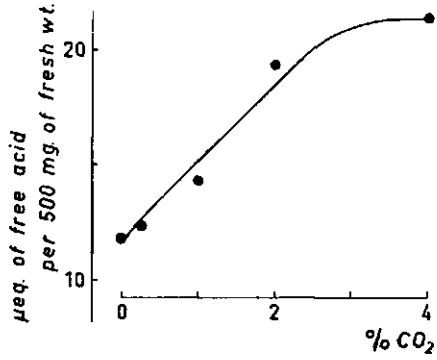
Adaptation during 65 hrs in the dark, in air + 2 % CO_2, refreshed twice. At 10 a.m., atmosphere replaced by air + 0, 2, 3, 4, 5 or 10 % CO_2; illumination: 8.7 × 10^3 ergs/cm².sec until 8 p.m.. Samples in air + 2, 5 and 10 % CO_2 next in darkness till 4 p.m., atmospheres repeatedly refreshed. Total duration: 95½ hrs. Single determinations.



Exp. 24b. *The relation between carbon dioxide tension and acid content at strong illumination.* Acid values at the end of the illumination period.

the light. In accordance with Experiment 23, deacidification set in at the lower CO_2-tensions only. At these lower tensions a linear relationship existed between the height of the adapted acid levels and the CO_2-concentration. From about 4 % up to 10 %, carbon dioxide no longer limited the acid content, whilst light no longer influenced the acid metabolism. At the prevailing temperature, the optimum acid level appeared to have been reached.

Whereas, in strong light, about 4 % CO_2 was needed to saturate the acid metabolism, in the dark, this saturation was reached at 2 % CO_2 already, as follows from the acid level after the adaptation and from the acidification at 2 % CO_2 in the dark period, next to illumination. During this dark period, the optimum acid level was either maintained (at 5 % and 10 % CO_2) or reached again (at 2 % CO_2). The strict reversibility of the acid level in sections at 2 % CO_2 clearly demonstrates the dependence of its height on the external conditions: re-establishment of the original conditions resulted again in the original amount of acid being restored.



Exp. 25. *The relation between carbon dioxide tension and acid content on illumination at compensation point intensity.* Samples of 40 sections, 1 mm, in 2 ml of a .02 M KCl-solution. – 20.0° C. – 2.7 × 10^3 ergs/cm².sec. throughout the experiment.
Adaptation during 20 hrs in air + $^1/_4$ % CO_2. Next, atmosphere replaced by air + 0, $^1/_4$, 1, 2 or 4 % CO_2. Single and duple determinations after another 27 hrs.

Experiment 25 investigates into the influence of the carbon dioxide tension on the effect of illumination at compensation point intensity. Since, at strong illumination, the acid metabolism is saturated at about 4 % CO_2, but, in the dark, at 2 % CO_2 already, it was interesting to examine, at which tension CO_2 would become saturating, if respiration and photosynthesis balance. According to the expectation, this concentration was found to be reached at about 3 % CO_2.

§ 14. Discussion

An investigation into the general features of the acid metabolism in *Bryophyllum tubiflorum* phyllodes, described in § 11, demonstrates that the typical diurnal acid variation occurs in this plant species. This

plant can, therefore, be used as a subject of investigations into the Crassulacean acid metabolism.

It is the intention of the present paper to analyse some of the factors on which the diurnal acid variation depends.

As was discussed in § 3 already, some *endonomous factor* is not known to occur. In the present experiments, on the contrary, under constant conditions an acid level was obtained, which remained constant for many hours. The acid variation, at least in excised leaves, depends on external factors only, *viz.* on temperature, illumination and atmospheric composition.

In the literature, a consensus of opinion prevails on the influence of *temperature*. The view of DE VRIES (1884) is that the proportion of simultaneously occurring acid production and consumption is determined by temperature, as a result of the greater accelerating effect which a rise in temperature has on the rate of acid breakdown rather than on the rate of acid formation. With this view, my few experiments on the subject are in accordance.

For technical reasons, *viz.* in order to ensure a constant temperature throughout the course of the experiments, the effect of *illumination* was studied, using excised leaves. Excised leaves are often used in experiments on the Crassulacean acid metabolism and appear to give reliable results. According to PUCHER *et al.* (1947*d*), excised *B. calycinum* leaves hardly differ from those, still attached to plants, as regards the course of their metabolism. In the present experiments, excised leaves, placed with the cut in a .02 M KCl-solution, lived on without showing any abnormality. If a diurnal illumination rhythm was given, they exhibited a diurnal acid variation for at least five days.

From these experiments, performed at a strictly constant temperature, light (589 mμ, from sodium lamps) proved to be a very important factor indeed. In continuous light or darkness, the acid content gradually shifted towards a constant level; it was the alternation of dark and light periods which caused the acid level to fluctuate (Exp. 15).

The diurnal acid variation in succulents can thus be described by the influences of temperature and light: in the dark and relatively cool night, acidification occurs, alternated by deacidification during the light and warmer day-time.

In § 13 the influence of illumination was analysed more in detail to see, whether the alternation of dark and light periods directly affects the acid metabolism, or acts by way of the changes in intercellular carbon dioxide content, which will occur simultaneously as a result of these alternations.

The data in the literature on the effects of carbon dioxide and oxygen on the Crassulacean acid metabolism were discussed in § 3. They were all concerned with experiments on leaves. The interpretation of these experiments suffers from the difficulty that, in view of the results of Chapter II, the composition of the atmosphere outside the leaf may differ considerably from that in the intercellular spaces,

with which the metabolizing cells are actually concerned. Moreover, since both carbon dioxide and oxygen affect stomatal aperture, as was demonstrated in § 8, one has no certainty at all, that a change of these gases in the outer atmosphere will lead towards a corresponding change of the intercellular gas composition.

It was tried in the present investigations, to overcome these difficulties by using leaf sections in stead of excised leaves, in order to separate the influences of illumination and carbon dioxide tension. It still leaves, however, the difficulty, that the concentration of CO_2 within the cells remains obscure, but a ready gas exchange with the outer atmosphere is greatly facilitated.

Freshly cut sections contain any amount of acid, as a result of the influences of the accidental conditions in the greenhouse. Adaptation to the experimental conditions appeared an improvement, permitting of quite appropriate responses of the acid metabolism to changes in these conditions. This expectation was, indeed, confirmed, but another complication arose in these experiments, since the acid level of adapted sections always turned out to be lower than that of comparable excised leaves, depending of the thickness of the sections. This phenomenon could neither be attributed to the number of visibly damaged cells, nor to a difference in internal carbon dioxide concentrations. To justify the use of this adapted section material, it is stated, that this material has distinct advantages over excised leaves as regards their gas exchange; that the results, obtained with non-adapted sections were essentially conformable to those from adapted ones, differing only by interference from the adaptation trend; and, finally, that at low carbon dioxide tensions, by alternation of dark and light periods, a balanced diurnal acid variation was brought about again, proportional to that of leaves and maintained for at least five days.

The height of the constant acid level of adapted sections depends on temperature and on the conditions of illumination and carbon dioxide tension. If temperature is kept constant and one of the other factors is changed, there is a response in the acid content, which is fully reversed as soon as the original conditions are re-established (Exp. 24). Whether there is a response to illumination or not, depends on the carbon dioxide tension. If this tension is over 4 %, light deacidification is completely absent. That this is not due to a narcotic influence of carbon dioxide on the acid metabolism, is evident from the fact, that both acid production and consumption can proceed in 5 % CO_2 (*e.g.* Experiments 18 and 20). Below 4 % CO_2, the height of the acid level decreases proportionally to the CO_2-concentration (Experiment 24). At the light intensity, at which assimilatory and dissimilatory oxygen exchanges balance, 3 % CO_2 saturates the acid metabolism, whilst, in the dark, the optimum acid level is reached at 2 % CO_2 already.

No fruitful discussion of these facts is possible, unless it is known, how carbon dioxide and light can influence the acid metabolism. This will be dealt with in the next Chapter, in which the biochemical

pathways of the diurnal acid variation will be considered and the above mentioned relationships discussed.

CHAPTER IV

BIOCHEMICAL ANALYSIS OF THE CRASSULACEAN ACID METABOLISM

§ 15. Conditions

The characteristic feature of the Crassulacean acid metabolism, *viz.* the diurnal variation in acid content, is not restricted to Crassulacean plants only, but occurs in succulents, belonging to 12 plant families throughout the plant kingdom (Warburg, 1886; Bennet-Clark, 1949, Thomas and Ranson, 1954). It is far more probable that, under influence of succulence, the relative activities of generally occurring enzymes have been changed, than that in all the separate cases of succulence some peculiar enzyme system would have evolved to induce the diurnal acid variation. Up to now, no enzyme has been detected in succulent plants, which failed to occur in non-succulent tissue. Much of our general knowledge of cell metabolism will, therefore, be applicable to succulent-plant metabolism as well. For these reasons, it is tried to produce a picture of the diurnal acid variation on basis of this general knowledge of plant biochemistry.

Generally, organic acids occupy a central position in cell metabolism, since they are intermediates of carbohydrate, protein and fat metabolism. Data from literature, discussed in the Chapters I and III, however, quite convincingly demonstrate that, in the case of Crassulacean acid metabolism, malic acid is the main acid involved in the diurnal variation, and that this acid variation is closely connected with carbohydrate metabolism. The later retracted statement, that the acid variation might be related with protein metabolism (Pucher *et al.*, 1947*c*, *d*, 1948*b*), can be left out of consideration.

Malic acid metabolism and related pathways of carbohydrate metabolism are combined in Fig. 2. Data about photosynthesis are from Calvin (1955), those about glycolysis from Stumpf (1954) and those about the pentose phosphate pathway from Horecker and Mehler (1955) and from Axelrod and Beevers (1956). We have to face the question, which of the reactions, present in the scheme, are actually found to occur in succulent leaves. These are only few, since enzyme isolation from these leaves is precarious: rupture of cells immediately causes destruction of enzymes by acids from the vacuoles.

PATHWAYS of CARBOHYDRATE and MALIC ACID METABOLISM

Legend:

⇝ photosynthesis pathway

⇢ pentose phosphate pathway

⟹ glycolysis pathway

TCA-cycle: tricarboxylic acid cycle

① phospho-enolpyruvate carboxylase

② oxaloacetate carboxylase

③ malic enzyme

④ malic acid dehydrogenase

⑤ carboxydismutase

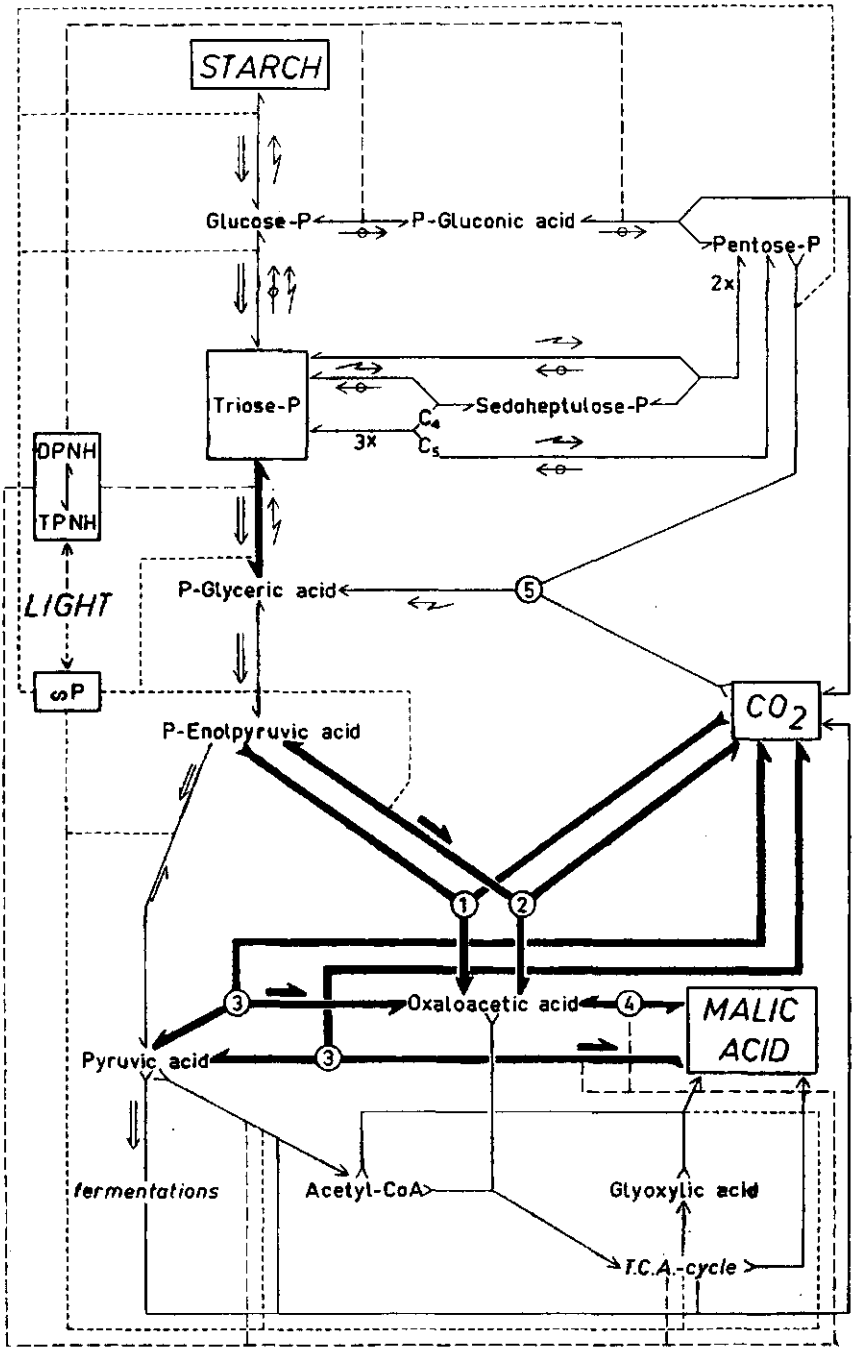

STARCH
Glucose-P
P-Gluconic acid
Pentose-P
2x
Triose-P
Sedoheptulose-P
C4
C5
3x
OPNH
TPNH
LIGHT
P-Glyceric acid
5
ωP
CO2
P-Enolpyruvic acid
1
2
3
Oxaloacetic acid
4
MALIC ACID
Pyruvic acid
3
fermentations
Acetyl-CoA
Glyoxylic acid
T.C.A.-cycle

Fig. 2.

AXELROD and BANDURSKI (1953) reported the presence of the glycolysis enzyme phosphoglyceryl kinase in *Bryophyllum* species. In water extracts from *B. calycinum*, which had previously been incubated with infiltrated 1 % ammonia, COLES and WAYGOOD (1957) found malic enzyme, malic acid dehydrogenase and oxaloacetate carboxylase. The occurrence of phospho-enolpyruvate carboxylase and malic acid dehydrogenase in cell-free *Bryophyllum* extracts was independently demonstrated by WALKER (1956) and SALTMAN, KUNITAKE, SPOLTER and STITT (1956). The reactions, catalyzed by the enzymes mentioned, are indicated in Figure 2 by heavy lines.

SEIFTER (1954) demonstrated coenzyme A in extracts of two *Bryophyllum* species. After $C^{14}O_2$-feeding to *Sedum spectabile*, TOLBERT and ZILL (1954) could isolate radioactive fructose, glucose, sucrose and sedoheptulose; the last-mentioned sugar is a well-known compound of Crassulacean plants (BENNET–CLARK, 1933).

From these data in literature we see, that enzymes and intermediates of all main pathways, present in Figure 2, are, indeed, found to occur in plants, showing the Crassulacean acid metabolism. In the application of the scheme to the explanation of this metabolism, one has to be prepared for pitfalls. The scheme shows biochemical possibilities and probabilities of interconversions between carbohydrates and acids. Not each datum, however, has to play a role, indeed, in the Crassulacean acid metabolism; only part of the biochemical possibilities may be physiologically realised because of absence or spatial separation of enzymes. Whereas biochemical probabilities, *e.g.* as regards the position of reaction equilibria, are only of limited value in discussing physiological trends, since reaction products, even if accumulating in the cell, might be rapidly removed from the site of reaction.

§ 16. ACID CONSUMPTION IN THE DARK

Malic acid breakdown can proceed by the activities of malic enzyme and of oxaloacetate carboxylase.

A. Oxydative decarboxylation by malic enzyme leads to pyruvic acid.

B. In the other case, malic acid has first to be dehydrogenated by malic acid dehydrogenase into oxaloacetic acid, which can then be decarboxylated into phospho-enolpyruvic acid. This decarboxylation requires an energy-rich phosphate bond. Phospho-enolpyruvic acid can again be converted in two ways.

B.1. The compound can be dephosphorylated by enolase into pyruvic acid, in which case an energy-rich phosphate bond is released; thus, a conversion of malic acid into pyruvic acid, both by means of malic enzyme and by oxaloacetate carboxylase activity, does not require any energy supply.

Pyruvic acid will be completely oxidized in the tricarboxylic acid cycle or, under conditions of oxygen deficiency, possibly be fermented. According to KAKESITA (1930) and THOMAS and RANSON (1954), there are indications of ethanol production in succulent leaves. VISHNIAC, HORECKER and OCHOA (1957) state, however, that, generally speaking, adult photosynthetic tissues lost their fermentative capacity,

probably because of disappearance of carboxylase during the growing-up. WARBURG (1886) reports that dark deacidification is completely inhibited under anaerobic conditions.

B.2. On the other hand, phospho-enolpyruvic acid can be converted to the carbohydrate level. Since, however, this conversion would require another high-energy bond in addition to reduced phosphopyridine nucleotides, and since one may not expect that, in the dark, these cofactors are available in abundance, a synthesis of carbohydrate from malic acid is improbable. In fact, this conversion is rarely found in the dark: only PUCHER *et al.* (1947*d*) and VICKERY (1952*a*, 1954*b*) reported a slight increase in starch content in some out of a number of experiments, in which excised leaves of *B. calycinum* deacidified in prolonged darkness at about 23° C. VARNER and BURRELL (1950) found only .5 % of the total activity of $C^{14}O_2$, absorbed by *B. calycinum* leaves in the dark during 2 hours, to be incorporated in the combined carbohydrate fractions.

When comparing the various possibilities of malic acid consumption in the dark, and considering the experimental evidence on the subject, it is highly probable that dark deacidification mainly proceeds by decarboxylation and subsequent respiration.

§ 17. ACID PRODUCTION IN THE DARK

The main sources of acid production in the dark are starch and sedoheptulose (BENNET-CLARK, 1933; PUCHER *et al.*, 1947*c*). Any malic acid production from these carbohydrates must proceed via phospho-enolpyruvic acid. The conversion into this compound is either oxidative or carboxylative.

A.1. *Oxidative production of phospho-enolpyruvic acid* from starch can proceed either by glycolysis or by combined steps from the pentose-phosphate pathway and glycolysis, *viz.* by oxidation of glucose-phosphate via phospho-gluconic acid into pentose-phosphate, conversion of this intermediate into triose-phosphate and subsequent oxidation in glycolysis. By this latter pathway, sedoheptulose, too, can enter acid-producing enzyme systems.

A.2. *Carboxylative production of phospho-enolpyruvic acid* occurs when ribulose-1,5-diphosphate combines with carbon dioxide in the reaction, catalysed by carboxydismutase, to produce phospho-glyceric acid. Pentose-phosphates can be formed from starch and from sedoheptulose by enzymes of the photosynthetic and pentose-phosphate cycles. Because of the large pools of starch and sedoheptulose, reactions of these cycles can proceed without the cycles having to be closed. The formation of the pentose-diphosphate requires an energy-rich bond, but the same holds for fructose-diphosphate production in glycolysis. The carboxylative cleavage of ribulose-diphosphate into phospho-glyceric acid easily proceeds in the dark (QUAYLE, FULLER, BENSON and CALVIN, 1954; RACKER, 1955; ARONOFF, 1957).

After the discussion of the conversion of phospho-enolpyruvic acid into malic acid, evidence will be presented for the occurrence of this

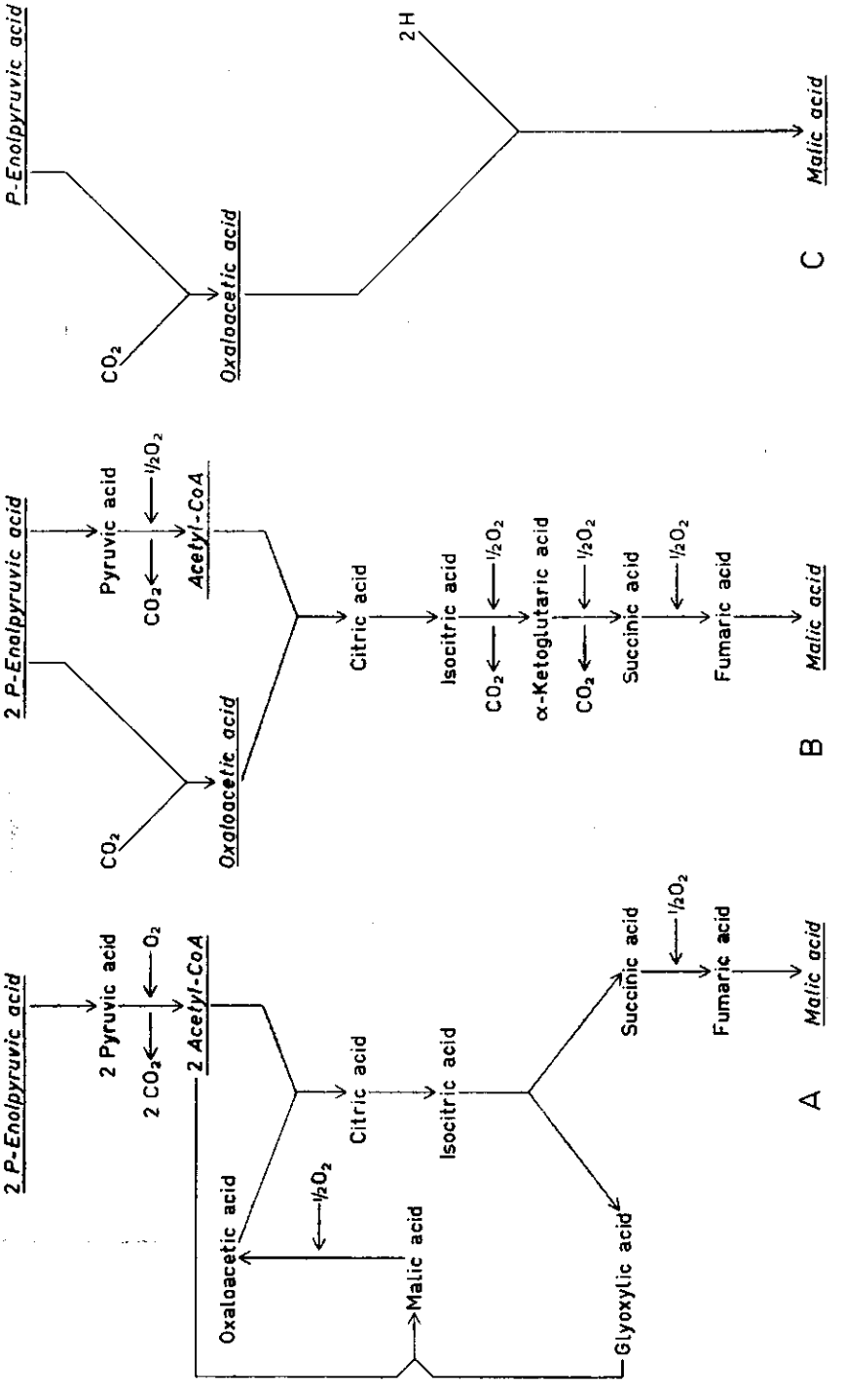

2 P-Enolpyruvic acid
2 Pyruvic acid
O2
2 CO2
2 Acetyl-CoA
Oxaloacetic acid
½O2
Citric acid
Isocitric acid
Malic acid
Glyoxylic acid
Succinic acid
½O2
Fumaric acid
Malic acid
A
2 P-Enolpyruvic acid
Pyruvic acid
½O2
CO2
Acetyl-CoA
CO2
Oxaloacetic acid
Citric acid
Isocitric acid
½O2
CO2
α-Ketoglutaric acid
½O2
CO2
Succinic acid
½O2
Fumaric acid
Malic acid
B
P-Enolpyruvic acid
2H
CO2
Oxaloacetic acid
Malic acid
C

Fig. 3.

carboxylative formation of phospho-enolpyruvic acid in the dark in the Crassulacean acid metabolism.

From phospho-enolpyruvic acid, malic acid can be formed either by oxidative or by reductive transformations, the former either with or without carboxylation.

B.1.a. *Oxidative synthesis of malic acid without carboxylation* (Fig. 3A). By means of pyruvic acid and acetyl coenzyme A, phospho-enolpyruvic acid can enter the tricarboxylic acid cycle. From this cycle itself, malic acid cannot accumulate, since it is consumed at the same rate at which it is formed. Enzymes of the cycle, however, might contribute towards malic acid production.

Recently, KREBS (1958) suggested that the activity of isocitritase, the enzyme which cleaves isocitric acid into succinic and glyoxylic acids (SAZ and HILLARY, 1956), opens the possibility of oxidative synthesis. Glyoxylic acid can combine with acetyl coenzyme A to form malic acid, which via oxaloacetic acid and another acetyl coenzyme A gives isocitric acid again. Succinic acid would then be left for *e.g.* malic acid synthesis.

B.1.b. *Oxidative synthesis of malic acid with carboxylation* (Fig. 3B). The other way of oxidative malic acid accumulation could be, that part of the phospho-enolpyruvic acid is converted into acetyl coenzyme A, whilst the remainder is carboxylated into oxaloacetic acid. From these two intermediates malic acid can be formed by the sequence of reactions of the tricarboxylic acid cycle.

WOLF (1949, 1951, 1953) and SOMERS (1951) suggest that in the Crassulacean acid metabolism malic acid accumulates through the activities of enzymes of the tricarboxylic acid cycle. There is, however, convincing experimental evidence against any oxidative malic acid production.

Acidification does not strictly depend on aerobic conditions. WARBURG (1886), GUSTAFSON (1925), WOLF (1932) and MOYSE (1955) reported dark acidification of succulent leaves under anaerobic conditions, though mostly the rates were more or less decreased. In one of Moyse's experiments, respiration was limited by oxygen deficiency at all oxygen tensions up to 100 % O_2, whereas dark acidification had the same rate at all oxygen tensions down to 1 % O_2. Conclusive evidence against any oxidative pathway is that acidification is not related to the liberation of carbon dioxide. The overall reaction of oxidative malic acid production from carbohydrates is:

$$C_6H_{12}O_6 + 3O_2 = C_4H_6O_5 + 2CO_2 + 3H_2O.$$

On the contrary, acid production has invariably been found to be intimately connected with carbon dioxide incorporation. This is proved both by correlation tests and by isotope experiments (see Chapter I).

B.2. *Reductive synthesis of malic acid* (Fig. 3C).

In this way, malic acid can be formed by reductive carboxylation

of either phospho-enolpyruvic acid or pyruvic acid. For biochemical reasons, carboxylation of phospho-enolpyruvic acid into oxaloacetic acid and subsequent hydrogenation by malic acid dehydrogenase is by far the more probable way. Malic enzyme and oxaloacetate carboxylase catalyse reversible reactions, it is true, but the equilibrium positions of these reactions normally greatly favour decarboxylation. For instance, HARARY, KOREY and OCHOA (1953) stated that in a malic enzyme preparation, at 5 % CO_2, in equilibrium the malic acid: pyruvic acid ratio was only 3.5 %.

Phospho-enolpyruvate carboxylase, on the contrary, appears hitherto to fixate carbon dioxide irreversibly into oxaloacetic acid, which in turn can readily be hydrogenated into malic acid by malic acid dehydrogenase.

This carboxylation of phospho-enolpyruvic acid requires the incorporation of one molecule of carbon dioxide per malic acid molecule formed. THOMAS and RANSON (1954) report that the CO_2-intake: acid production ratio surpasses unity in dark acidification. The lower the temperature, the higher the acid accumulation was and the larger, too, the proportional excess of carbon dioxide incorporation. This can be explained by the carboxylative production of phospho-enolpyruvic acid, discussed in A.2. The stronger the acid production, the more this process will participate.

Further evidence in favour of carboxylative phospho-enolpyruvic acid formation is obtained from isotope experiments with $C^{14}O_2$. If phospho-enolpyruvic acid is formed by carboxylation, not only the β-carboxyl group of malic acid, derived from the carboxylation of phospho-enolpyruvic acid into oxaloacetic acid, will be labelled, but the α-carboxyl group must expected to become labelled too. This α-carboxyl group of malic acid comes from the carboxyl group of phospho-glyceric acid, half of which will be labelled by carboxydismutase activity. Therefore, if all malic acid should be formed from phospho-enolpyruvic acid, which was produced carboxylatively, then in the presence of $C^{14}O_2$, the α-carboxyl group should contain about half the amount of radioactivity of the β-carboxyl group. This expectation is confirmed by data, given by VARNER and BURRELL (1950) on the labelling of malic acid, formed in the dark in an atmosphere, enriched with carbon dioxide, containing $C^{14}O_2$: TABLE II.

TABLE II

The distribution of labelling in malic acid, formed in dark acidification (after VARNER & BURRELL, 1950).

B. calycinum leaves were exposed to an atmosphere with 5 % CO_2 containing $C^{14}O_2$, during 2.5 hrs at 10° C in the dark. – Acids extracted with ether and separated by a silicagel column.

Degradation products of malic acid	$-CH_2.CHOH-$	α-COOH–	β-COOH
Labelling, in %	8	38	54

Since exchange reactions of the carboxyl groups by way of fumaric acid can be neglected, in view of the results of STUTZ and BURRIS (1951) (see § 2), the distribution of labelling found by Varner and Burrell confirms the predominantly carboxylative formation of phospho-enolpyruvic acid. Additional evidence against oxidative phospho-enolpyruvic acid production rises from the observations, mentioned in B.1., that acidification still proceeds under anaerobic conditions.

Summarizing it is suggested, that in dark acidification malic acid is formed from starch and sedoheptulose by a mainly carboxylative production of phospho-enolpyruvic acid, which in turn is carboxylated by phospho-enolpyruvate carboxylase into oxaloacetic acid; this compound is reduced by malic acid dehydrogenase into malic acid.

§ 18. ACID CONSUMPTION IN THE LIGHT

In the light, photosynthesis occurs, since energy-rich phosphate bonds and reduced phosphopyridine nucleotides are produced, necessary for the conversion of phospho-glyceric acid into triose-phosphate. If the competition for phospho-glyceric acid, as a substrate, between the enzymes involved in carbohydrate synthesis, on the one hand, and the acidifying enzyme system, on the other hand, is unilateral in favour of the former, and, if, moreover, the supply of phospho-glyceric acid from carboxydismutase activity were to fell short, then malic acid production will be lowered or even stopped. Since its consumption by respiration may continue, a net deacidification will occur. The light deacidification, which occurs at low carbon dioxide tensions can, however, not been explained by a continued malic acid respiration only, as can be calculated from respiration rates, given in Chapter II. Some other deacidifying system must, therefore, exist.

There is substantial evidence that light deacidification is brought about by an oxidative decarboxylation of malic acid into phospho-enolpyruvic acid, which is next transformed into phospho-glyceric acid and used up in carbohydrate synthesis. This is, in principle, the reversed pathway of acid production. The evidence comes from isotope experiments by VARNER and BURRELL (1950) and HAÏDRI (1955). These authors allowed succulent leaves to convert C^{14}-labelled malic acid in the light; degradation of the glucose units of the starch formed, showed a distribution of C^{14}, corresponding to a conversion via phospho-glyceric acid (see § 2).

The oxidative decarboxylation of malic acid into phospho-enolpyruvic acid cannot be performed by exactly the same pathway, which produces malic acid in the dark, since phospho-enolpyruvate carboxylase, which is active in acidification, appears to catalyse an irreversible reaction. Two other ways may account for light deacidification.

A. Malic enzyme converts malic acid into pyruvic acid, which is then phosphorylated.

B. Malic acid dehydrogenase oxidizes malic acid into oxaloacetic acid, which can be decarboxylated either by malic enzyme into pyruvic acid or by oxaloacetate carboxylase into phospho-enolpyruvic acid.

All these reactions require an energy-rich phosphate bond.

One may question, whether pyruvic acid will be phosphorylated into phospho-enolpyruvic acid by enolase. Reversibility of this reaction is established *in vitro* (LARDY and ZIEGLER, 1945). Isotope experiments showed that, in animal tissues, the production of phospho-enolpyruvic acid from pyruvic acid proceeds via the enzymes of the tricarboxylic acid cycle and oxaloacetate carboxylase, rather than by enolase (TOPPER, *et al.*, 1949; LORBER *et al.*, 1950). Since, however, energy-rich phosphate bonds may expected to be available in illuminated photosynthetic tissue, one cannot exclude participation of malic enzyme and enolase in light deacidification.

The dehydrogenating activities of malic acid dehydrogenase and malic enzyme obviously are not seriously hindered by deficiency of oxidized phosphopyridine nucleotides, as might be expected in the light. Nothing is known about spatial separation of metabolic pathways in the Crassulacean acid metabolism; it would be of great interest to know, whether malic acid can be produced and consumed by isolated chloroplasts.

For the same reason, our ignorance hitherto prevents us from deciding, whether respiration proceeds at the same rate in the light as it does in the dark. CALVIN and MASSINI (1952) suggested an interruption of the tricarboxylic acid cycle in the light; BROWN (1953), however, found that the intake of labelled oxygen by *Chlorella* suspensions was not hindered by illumination.

§ 19. THE INFLUENCES OF ILLUMINATION AND THE CARBON DIOXIDE TENSION

We now come to discuss the main results of the experiments, described in § 13, B, *viz.* the observation that carbon dioxide enrichment of the atmosphere can prevent light deacidification and enables the acid level to be maintained at the same height as in darkness. We will consider a possible influence of carbon dioxide on A: acid consumption by respiration (dark deacidification); B: the conversions of phospho-enolpyruvic acid into oxaloacetic acid and *vice versa;* and C: the synthesis of phospho-glyceric acid.

A. An effect of the carbon dioxide tension on the rate of acid consumption by respiration has to be considered, since respiration is preceeded by malic acid decarboxylation. If an increase of the CO_2-tension would decrease the rate of decarboxylation, respiratory acid consumption would diminish. That carbon dioxide, however, has no such an influence, follows from the comparison between respiration rates of sections in atmospheres without and with 1 % CO_2 (Experiment 4): the presence of carbon dioxide fails to decrease the oxygen

intake. Moreover, biochemical evidence conflicts with the view, that the decarboxylating activities of malic enzyme and oxaloacetate carboxylase would show any marked decrease, if a few per cent of carbon dioxide were present (see § 17, B.2.).

B. The same applies to the decarboxylation reactions in light deacidification, since there, too, oxaloacetate carboxylase and, possibly, malic enzyme participate.

No more than the rates of decarboxylations of malic and oxaloacetic acids into pyruvic and phospho-enolpyruvic acids, can the rate of carboxylation of phospho-enolpyruvic acid into oxaloacetic acid be expected to depend on the carbon dioxide concentration. The enzyme involved, phospho-enolpyruvate carboxylase, has an extremely high affinity to carbon dioxide: TCHEN and VENNESLAND (1955) were unable to show a limiting effect of carbon dioxide deficiency in enzyme preparations, freed as much as possible from CO_2. The CO_2-concentrations, at which, at the various illumination conditions, light deacidification was just prevented, must have been far over the concentration, necessary to saturate phospho-enolpyruvate carboxylase.

C. If, at a low carbon dioxide concentration and strong illumination, the rate of photosynthesis is limited by an insufficient supply of CO_2, this means that the enzymes, converting phosphoglyceric acid into carbohydrates, are only partly active. In this situation, malic acid is converted into phospho-glyceric acid; this light deacidification makes up the deficiency and gives the carbohydrate-synthesizing enzymes full employment.

At a slightly higher CO_2-tension, more phospho-glyceric acid is formed by carboxydismutase activity; light deacidification is, therefore, required to a smaller extent only and less acid disappears. Thus a proportional relation between carbon dioxide tension and increase of acid content will occur, until so much CO_2 is available, that photosynthesis becomes saturated, i.e. carboxydismutase produces phospho-glyceric acid at such a rate, that it fully provides for the wants of the carbohydrates-synthesizing enzymes. If this is the case, no more light deacidification can occur.

At CO_2-tensions above the saturation point of photosynthesis, carboxydismutase can produce phospho-glyceric acid at a still faster rate, and any excess, not used up in photosynthesis, can be transformed into malic acid along the same pathway as in the dark. The proportional relation between CO_2-tension and the increase of the acid content will be continued until the CO_2-tension becomes so high, that the acidifying enzyme system, too, is saturated by phospho-glyceric acid supply. Unless the turnover capacity of carboxydismutase is limited at these CO_2-concentrations, it is immaterial whether illumination occurs or not.

This picture of a phospho-glyceric acid production, limited by the carbon dioxide tension, fully accounts for the results of the experiments, described in § 13, B.

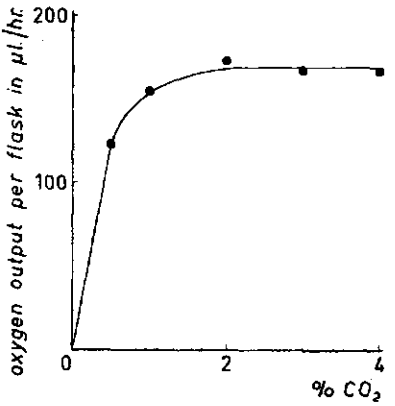
At strong illumination, a linear relationship exists between the acid

content and the carbon dioxide tension, up to about 4 % CO_2 (Experiment 24). At these lower concentrations, carboxydismutase activity is unable to saturate both photosynthesis and acidification. At higher concentrations, these processes are, indeed, both saturated by the supply of phospho-glyceric acid. The final acid level reached is exactly as high as in the dark, since *a*: the lock from phospho-glyceric acid to carbohydrates is fully occupied and, therefore, closed for products of deacidification, as it is in the dark, when no energy-rich phosphate bonds and reduced phosphopyridine nucleotides are available; *b*: acidification occurs at the same rate as in the dark, since it is not limited by deficiency of phospho-glyceric acid; and *c*: deacidification by respiration apparently proceeds at the same rate as in the dark.

In fully adapted sections, the rate at which malic acid is formed (*b*) must exactly equal that of its disappearance by respiration (*c*) since, otherwise, a constant acid level would be impossible. In the present conception, it is included that the height of the acid level, finally reached under constant conditions, is a measure for the rates of acid production and consumption. The mechanism of the coupling of these rates is, however, unknown.

At a low illumination intensity, e.g. at the compensation point, photosynthesis is saturated at a lower carbon dioxide concentration already, and phospho-glyceric acid will correspondingly become available for acidification at lower CO_2-concentrations. In accordance with this view, the acidifying enzymes proved to be saturated at about 3 % in stead of about 4 % CO_2 (Experiment 25).

In the dark, when no phospho-glyceric acid is used up in carbohydrate synthesis, all phospho-glyceric acid, produced by the carboxydismutase reaction, is available for acidification. Carboxydismutase is



Exp. 26. *The relation between the rate of photosynthesis and the carbon dioxide tension.* Outer well: 40 sections, 1 mm, in 10 ml or in .5 ml of .05 M KCl-solution. – Gas phases: air with various CO_2-tensions. – Illumination: 25 × 10^3 ergs/cm².sec (sodium light). – 20.0° C.

Single and duple determinations after the indirect Warburg method.

then able to saturate the acidifying enzyme system at 2 % CO_2 already (Experiment 24).

The main condition for this picture is, that the turnover rate of carboxydismutase largely depends on the carbon dioxide tension, at least up to 4 % CO_2. This is, indeed, the case, as was demonstrated by MAYAUDON, BENSON and CALVIN (1957). The authors state, that a highly purified carboxydismutase preparation from *Tetragonia* leaves had a much higher affinity to ribulose-diphosphate than to carbon dioxide; at 20° C and a $P_H = 6.8$, a concentration of 6×10^{-3} M HCO_3^-, corresponding with 5 % CO_2 in the atmosphere, was still insufficient to saturate the enzyme.

A second condition is that photosynthesis, at strong illumination at 20° C, becomes saturated at about 2 % CO_2. Experiment 26 shows that this was, indeed, the case with *B. tubiflorum* leaf sections under the prevailing conditions.

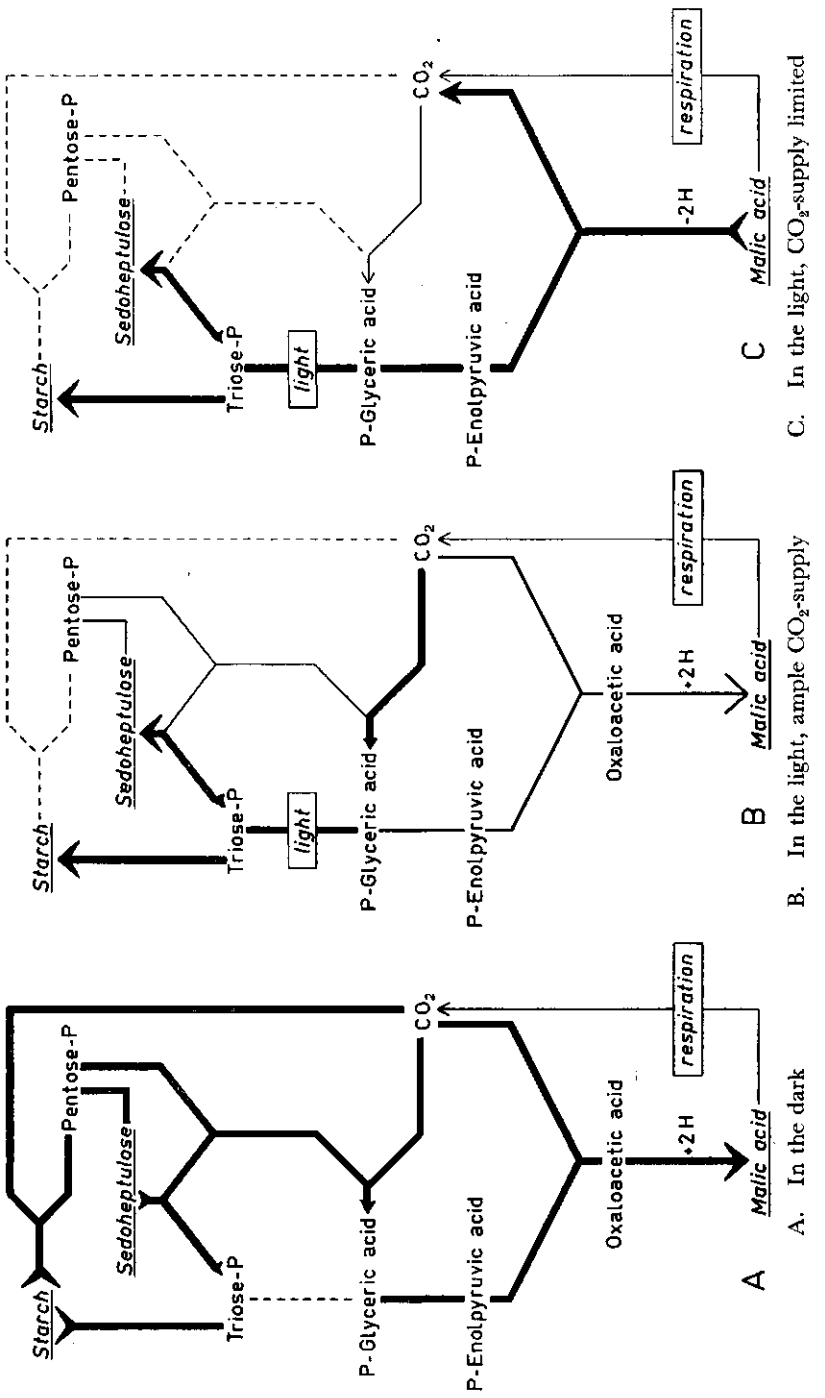
CHAPTER V

THE PHYSIOLOGY OF THE CRASSULACEAN ACID METABOLISM

§ 20. THE SIGNIFICANCE OF THE DIURNAL ACID VARIATION

As a result of the discussion in the previous Chapter, the course of the acid metabolism in succulent leaves under natural conditions, can be described in the following manner.

At night (Fig. 4A), carbohydrates disappear. They are partly respired in the form of CO_2, partly converted into malic acid. The latter conversion proceeds by a mainly carboxylative breakdown of starch and sedoheptulose into phospho-enolpyruvic acid, which is carboxylated by phospho-enolpyruvate carboxylase and subsequently hydrogenated by malic acid dehydrogenase into malic acid. The malic acid formed is partly respired again, after decarboxylation. Whether a net acidification occurs, depends on the ratio between the activity of the carboxylating enzymes, on the one hand, and the respiration rate, on the other hand. At higher temperatures, malic acid respiration appears to be about as high as acid formation, and only a slight acidification, or even a deacidification, occurs. Mostly, however, nights will be relatively cool and acidification sets in as a result of a carboxylative acid production surpassing the oxidative acid consumption; probably, the latter has a larger Q_{10}-value. Possibly the accumulation of carbon dioxide and the impoverishment in oxygen content, which occur in darkened succulent leaves (Chapter II), play a role, too, in the attainment of an overweight of carboxylative over oxidative metabolic pathways. Anyhow, it is this preponderance of carboxylating rates over respiratory rates, which is the *conditio sine qua non* for the diurnal acid variation. The only difference in enzymatic apparatus



A. In the dark

B. In the light, ample CO_2-supply

C. In the light, CO_2-supply limited

Fig. 4.
THE COURSE OF THE DIURNAL ACID VARIATION

of succulent and non-succulent leaves may well lie in a relatively larger amount of phospho-enolpyruvate carboxylase in the former.

This carboxylative acid synthesis enables the succulent leaf to exhibit a considerable metabolic activity with a minimum necessity for gas exchange. The oxygen uptake can be low and respiratory carbon dioxide is retained. Carbon dioxide can even be absorbed from the outer atmosphere, during the night, and used up in acid synthesis. This phenomenon, leading to negative RQ-values, was discovered by De Saussure, using *Opuntia* leaves, as early as 1804; the occurrence of this "De Saussure effect" has been repeatedly confirmed since. It permits of an uninterrupted carbon dioxide uptake, night and day, by leaves which have, in order to reduce their transpiration, a limited gas exchange.

In the day-time, temperature rises, which leads to deacidification, probably again because acid respiration has a larger Q_{10}-value than acid synthesis. This deacidification by respiration, however, cannot account for the total deacidification and its high rate during the day.

In the light, photosynthesis sets in and consumes phospho-glyceric acid rapidly in carbohydrate synthesis. In so far and as long as the carbon dioxide tension in the intercellular spaces of the leaf is high enough, carboxydismutase produces phospho-glyceric acid at a rate, sufficient to supply both photosynthesis and acidification (Fig. 4, B). A simultaneous acidification and carbohydrate synthesis in succulent leaves was observed by VICKERY (1952*a*) in the afternoon, when the latter process was considerably reduced for some unknown reason.

If the carbon dioxide tension is, however, too low to allow of a sufficient turnover rate through carboxydismutase, as is expected to occur soon in illuminated succulent leaves, acidification will be slowed down, stopped and even reversed (Fig. 4, C): whereas phospho-enolpyruvate carboxylase is no longer active through substrate deficiency, decarboxylating enzymes convert malic acid into phospho-enolpyruvic acid, which is hydrated into phospho-glyceric acid and consumed in carbohydrate synthesis: light deacidification.

By this way, two molecules of malic acid are required for the synthesis of one molecule of glucose. If the carbon dioxide from the decarboxylation and from acid respiration is used up completely in phospho-glyceric acid production, deacidification during the day proceeds according to the formula:

$$3C_4H_6O_5 + 3H_2O \rightarrow 2C_6H_{12}O_6 + 3O_2,$$

leading to an acid consumption: carbohydrate production ratio = 1.5. Experimental values in literature differ from 1.1 to 1.8, with one observation of .6 by PUCHER *et al.* (1947*d*), suggesting that, generally, carbon dioxide uptake from the outer atmosphere plays a minor role only in carbohydrate synthesis in succulents, the main carbon source being malic acid. Since any conversion of malic acid into carbohydrates goes via the reductive phosphorylation from phospho-glyceric acid into phospho-glyceric aldehyde, the light-dependent

production of carbohydrates from malic acid is rightly named photosynthesis.

If sufficient carbon dioxide is available, this is preferably consumed in photosynthesis, but as soon as the carbon dioxide supply falls short, photosynthesis shifts towards the other substrate: malic acid. This allows succulents to photosynthesize in the day-time, if necessary, with closed stomata.

In so far as this malic acid is respired first in the form of carbon dioxide, no essentially new feature enters the picture. Isotope experiments showed, however, that another pathway exists, indeed, in which the radioactive label, attached to one of the malic acid carbon atoms, is not distributed at random in the glucose units of starch (sedoheptulose has not been investigated in this respect), but according to the direct conversion via phospho-enolpyruvic and phosphoglyceric acids. This direct conversion occurs in non-succulent plants, too (HAÏDRI, 1955), but to a much smaller extent, since, in non-succulent tissue, malic acid is not accumulated. The proportion between starch formation from malic acid via phospho-enolpyruvic and phospho-glyceric acids, on the one hand, to that by way of respiratory carbon dioxide, on the other hand, cannot be computed with certainty from the experimental data, presented by VARNER and BURRELL (1950) and HAÏDRI (1955); one can approximate that this ratio is probably between .5 and 1.0.

A frequently applied adaptation of plant life to resist drying-up in arid regions, is the development of succulence. Thus, by reduction of leaf area, mostly combined with a decreased number of stomata per surface unit, transpiration is restrained. As a result, however, gas exchange, necessary for a vivid metabolic activity, is curtailed too. By relative enlargement of the activities of carboxylating enzymes, especially of phospho-enolpyruvate carboxylase, succulents are able to absorb continuously carbon dioxide and to have a nightly production of malic acid, which not only can give rise to respiratory carbon dioxide for photosynthetic purposes, but also replaces carbon dioxide as a photosynthetic substrate. In this manner, an active metabolism is maintained, night and day, allowing for the fixation of energy and carbon with a minimum need for gas exchange.

SUMMARY

I

In the leaves of many succulent plants, organic acid is formed during the night and broken down in the day-time. This diurnal variation in acid content has been related to carbohydrate metabolism by various authors since the middle of the 19th century.

Modern research qualified the variation in acidity to changes in the malic acid fraction. The carbohydrate content, particularly that of starch and sedoheptulose, increases during the day and decreases at night. A strict correlation was found to occur between the two opposed variations in malic acid and in carbohydrate content.

Arguments both from biochemical and physiological research strongly plead in favour of malic acid production by way of β-carboxylation of some intermediate of

carbohydrate breakdown. Formation of carbohydrate from malic acid by the reversed way might occur.

The diurnal variation in acid content is caused by the external factors temperature and illumination. A change in the atmospheric composition, too, can effect the acid metabolism.

In the present paper it is submitted that temperature and illumination may influence the acid metabolism by means of the composition of the intercellular gas. The two factors affect the gas exchange of the leaf cells; if this gas exchange with the intercellular spaces surpasses that of the intercellular spaces with the outer atmosphere, a diurnal variation in intercellular gas composition will be caused by the diurnal alternation of assimilation and dissimilation. It is conceivable that this will happen in succulent leaves. Especially a variation in carbon dioxide content could be important, since this compound is known to be a metabolite in the acid variation.

It is the intention of the present paper to investigate whether illumination affects the Crassulacean acid metabolism by means of the carbon dioxide tension in the intercellular spaces, or in a more direct manner.

II

In order to determine whether a considerable diurnal variation in the gas composition of intercellular spaces can occur, indeed, in succulent leaves, the ability for gas exchanges with the outer atmosphere was studied on leaves—phyllodes, properly speaking—of the Crassulacean plant *Bryophyllum tubiflorum* Harv..

Respiration rates of excised leaves were compared with those of leaf sections, using the Warburg manometric method.

With leaf sections, the rate of gas exchange with the outer atmosphere allowed for an unrestricted respiration at the optimum temperature, about 35° C, at even 5 % of oxygen. The respiration rate could neither be enhanced by the addition of suitable substrates, nor could it be influenced by carbon dioxide (Expts. 1, 2, 3 and 4).

Excised leaves exhibited a smaller respiration rate than comparable sections under the same conditions. Leaf respiration could be accelerated by those changes in atmospheric composition, which caused stomatal aperture to widen (Expt. 5, TABLE I).

Transpiration measurements and porometer experiments demonstrated, that stomata of *B. tubiflorum* leaves are opened wider in 1 % of carbon dioxide than they are in 0 % or in 5 %. Oxygen, from zero concentration to 100 %, had a progressively greater closing effect on stomatal aperture (Expts. 6, 7, 8 and 9).

It is concluded that leaf respiration is limited by oxygen deficiency, even in a milieu of pure oxygen. The oxygen supply is controlled by stomatal aperture. Thus, the composition of the intercellular gas can differ greatly from that of the outer atmosphere. The diurnal alternation of light and darkness is expected, therefore, to cause a considerable diurnal variation in the intercellular gas composition by the alternating metabolic activities of the leaf cells.

III

The diurnal acid variation in *B. tubiflorum* phyllodes is comparable to that of plants, commonly used in this field of research: the amplitude is of the same order and the variation occurs in the free malic acid fraction (Expts. 10, 11 and 12).

Weather conditions in the day-time affect both deacidification during that day and acidification in the subsequent night (Expt. 13).

Rising temperature stimulates deacidification and retards acidification (Expt. 14).

At a constant temperature, a variation in the acid content can be brought about by light-dark alternations (Expts 15 and 16).

To study the effects of illumination and carbon dioxide tension separately and in any combination, leaf sections had to be used. These sections were previously kept under constant conditions as to temperature, illumination and atmospheric composition. Temperature was invariably kept at 20.0° C throughout the experiment. Under constant conditions, the acid content of sections becomes constant too. When sections had thus become adapted to their environmental conditions, illumination and/or carbon dioxide tension were changed and the response in the

acid content determined. The acid content of adapted sections, 1 mm thick, was always lower than that of comparable excised leaves under the same conditions. No conclusive explanation could be given for this phenomenon (Expts 17, 18, 19, 20 and 21).

In the dark, the acid content increases if the carbon dioxide tension rises between 0 % and 2 %; higher tensions do not further influence the acid level. The graph of the relation is a Blackman curve.

At the light intensity of the compensation point, the optimum acid level is reached at about 3 % of carbon dioxide, at strong illumination at about 4 %. Since this optimum level is equal both in the light and in the dark, at carbon dioxide tensions surpassing 4 %, it is immaterial for the acid level, whether the sections are illuminated or not (Expts 22, 23, 24 and 25).

IV

An attempt to elucidate the biochemical pathways of the Crassulacean acid metabolism starts from three conditions.

1. The main acid to be concerned is malic acid.
2. The variation in the malic acid content is related to carbohydrate metabolism.
3. Only enzymes, known to occur generally in the plant kingdom, play a role.

The following pathways are considered to be the most probable ones (Fig. 2):

1. *Dark deacidification*: oxidative decarboxylation of malic acid and subsequent respiration into carbon dioxide.
2. *Dark acidification:* carboxylative transformation of starch and sedoheptulose into phospho-glyceric acid, with the co-operation of the enzyme carboxydismutase; carboxylation of phospho-enolpyruvic acid, derived from phospho-glyceric acid, by the enzyme phospho-enolpyruvate carboxylase into oxaloacetic acid; reduction of this compound into malic acid (Fig. 4).
3. *Light deacidification:* oxidation and decarboxylation of malic acid into phospho-enolpyruvic acid; conversion of this compound into carbohydrates by reversal of the glycolysis pathway, with the aid of energy and reducing power from photolysis of water.

The results of the Expts. 23, 24 and 25 fit well in this picture.

The carbon dioxide tension is immaterial as to *dark deacidification*. It affects dark acidification and light deacidification.

In *dark acidification*, carbon dioxide, up to a tension of 2 %, is the limiting factor in the carboxylative production of phospho-glyceric acid from pentose-diphosphate by the enzyme carboxydismutase. At tensions over 2 %, sufficient carbon dioxide is available to saturate the acidifying enzyme system with phospho-glyceric acid.

Light deacidification occurs at carbon dioxide tensions up to 4 %. At these lower concentrations, the enzyme carboxydismutase falls short in supplying enough phospho-glyceric acid to saturate the carbohydrates synthesizing enzyme system. The gap is filled up by phospho-glyceric acid from malic acid: malic acid replaces carbon dioxide as a source in carbohydrate synthesis. If the carbon dioxide concentration rises between 0 % and 4 %, less and less malic acid is required. At low light intensities, *e.g.* at the compensation point, the carbohydrates synthesizing apparatus will be saturated sooner than at a strong illumination. If this apparatus is saturated with carboxylatively produced phospho-glyceric acid, and, in addition, part of this phospho-glyceric acid is available for the acidifying enzyme system, then the final acid level will be equal to that in the dark at that carbon dioxide tension. This is the case at the light intensity of the compensation point above about 3 %, in stronger light above about 4 % of carbon dioxide; at these concentrations, the horizontal parts of the Blackman curves are reached.

A literature datum shows, that *in vitro* carboxydismutase is still unsaturated at 5 % CO_2. Photosynthesis of leaf sections of *B. tubiflorum* is, under the usual experimental conditions and at strong illumination, not saturated untill a carbon dioxide tension of 2 % is applied.

V

During the relatively cool nights, succulent plants are able to produce malic acid out of carbohydrates, thanks to the preponderance of carboxylative enzyme

systems over oxidative ones. Possibly, the composition of the intercellular gas plays a role in this relationship.

In the day-time, at a normal composition of the outer atmosphere, succulent plants will use up their malic acid as a source for the energy-fixing synthesis of carbohydrates; partly, malic acid is directly converted into carbohydrates (Fig. 4).

Plants, resisting against desiccating climatic conditions by developing succulence, which curtails their ability for gas exchange, are able to maintain a lively metabolic activity by the diurnal variation in their acid content; this variation allows of a continuous fixation of carbon dioxide, night and day.

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SAMENVATTING

I

In bladen van vele vetplanten wordt 's nachts organisch zuur gevormd, dat overdag weer afgebroken wordt. Deze dagelijkse wisseling in het zuurgehalte werd reeds in de vorige eeuw door een aantal onderzoekers in verband gebracht met de koolhydraatstofwisseling.

Modern onderzoek heeft aangetoond, dat de schommeling in het zuurgehalte voornamelijk optreedt in het vrije appelzuur. Het koolhydraatgehalte, vooral dat van zetmeel en sedoheptulose, neemt overdag toe en 's nachts af, en tussen de tegengestelde variaties in de hoeveelheden appelzuur en koolhydraat bestaat een stricte correlatie.

Argumenten, ontleend zowel aan biochemisch als aan physiologisch onderzoek, pleiten voor de vorming van appelzuur door β-carboxylering van een tussenprodukt van de koolhydraatafbraak. Opbouw van koolhydraat uit appelzuur langs de omgekeerde weg is in principe mogelijk.

De dagelijkse schommeling in het zuurgehalte wordt veroorzaakt door uitwendige factoren: temperatuur en belichting. Verandering van de samenstelling van de atmosfeer kan eveneens het zuurmetabolisme beïnvloeden.

Ondersteld wordt, dat temperatuur en belichting via de samenstelling van het intercellulaire gas hun invloed op het zuurmetabolisme kunnen uitoefenen. Deze factoren beïnvloeden immers de stofwisseling, en dus de gasopname en -afgifte van de bladcellen. Indien de gaswisseling tussen de bladcellen en de intercellulaire ruimten groot is in verhouding tot die tussen de intercellulairen en de buitenatmosfeer, zal, door de dagelijkse afwisseling van assimilatie en dissimilatie, een dagelijkse schommeling optreden in de samenstelling van het intercellulaire gas. Dit wordt voor succulente bladen waarschijnlijk geacht. Vooral een variatie in het koolzuurgehalte kan belangrijk zijn, omdat koolzuur een rol speelt in het zuurmetabolisme.

Doel van het onderzoek is, na te gaan, in hoeverre belichting het zuurmetabolisme beïnvloedt via de intercellulaire koolzuurspanning, dan wel op een meer directe wijze.

II

Teneinde te onderzoeken, of bij succulente bladen inderdaad de samenstelling van het intercellulaire gas aan een aanzienlijke dagelijkse schommeling onderhevig is, is het vermogen tot gasuitwisseling nagegaan bij bladen (eigenlijk phylloden) van *Bryophyllum tubiflorum* Harv., een Crassulacee.

Met behulp van de manometrische methode volgens Warburg is de ademhalingssnelheid van afgesneden bladen vergeleken met die van bladcoupes.

Bij bladcoupes blijkt de uitwisselingssnelheid van intercellulair gas met de buitenatmosfeer voldoende, om bij optimale temperatuur, $\pm$ 35° C, zelfs in 5 % zuurstof nog een onbelemmerde endogene ademhaling toe te laten. Deze ademhaling is

niet te versnellen door toevoeging van geschikte substraten; koolzuur heeft op de ademhalingssnelheid geen invloed (Exp. 1, 2, 3 en 4).

Afgesneden bladen vertonen een lagere ademhalingssnelheid dan overeenkomstige coupes onder dezelfde omstandigheden. Deze bladademhaling is te versnellen door dezelfde wijzigingen in de buitenatmosfeer, welke ook de stomata wijder doen opengaan (Exp. 5, TABEL 1).

Transpiratieproeven en porometeronderzoek wijzen uit, dat de huidmondjes van *B. tubiflorum*-bladen in 1 % CO_2 wijder zijn geopend dan in 0 % of in 5 % CO_2. Voorts, dat zuurstof, van 0 % tot 100 %, een steeds toenemende sluitende invloed op de stomata heeft (Exp. 6, 7, 8 en 9).

Geconcludeerd wordt, dat de ademhaling van bladen belemmerd wordt door zuurstofgebrek, zelfs in een milieu van zuivere zuurstof, en dat de zuurstofvoorziening wordt beheerst door de openingstoestand van de stomata. De samenstelling van het intercellulaire gas blijkt zeer veel te kunnen verschillen van die der buitenatmosfeer. Bij de dagelijkse afwisseling van licht en donker is dus, door de daarmede gepaard gaande afwisseling in het metabolsime van de bladcellen, een aanzienlijke dagelijkse schommeling in de samenstelling van het intercellulaire gas te verwachten.

III

De phylloden van *B. tubiflorum* vertonen eenzelfde variatie in het zuurgehalte als bladen van andere vetplanten, welke voor het onderzoek naar de dagelijkse wisseling in het zuurgehalte gebruikt worden: deze wisseling heeft dezelfde amplitudo en speelt zich af in het vrije appelzuur (Exp. 10, 11 en 12).

De weersomstandigheden overdag beïnvloeden zowel de ontzuring gedurende die dag, als de verzuring tijdens de daarop volgende nacht (Exp. 13).

Temperatuursverhoging stimuleert de ontzuring en remt de verzuring (Exp. 14).

Bij constante temperatuur treedt een schommeling in het zuurgehalte op onder invloed van licht-donker wisselingen (Exp. 15 en 16).

Teneinde de invloeden van belichting en koolzuurspanning op het zuurmetabolisme afzonderlijk en in willekeurige combinaties te kunnen bestuderen, is het onderzoek voortgezet met bladcoupes. Deze coupes werden tevoren onder constante omstandigheden gebracht wat betreft temperatuur, belichting en atmosferische samenstelling. De temperatuur was steeds 20,0 °C. Onder constante omstandigheden bereiken de coupes een constant zuurgehalte, aan de omstandigheden aangepast. Wanneer de coupes aldus geadapteerd zijn, wordt de belichtingsconditie en/of de koolzuurspanning gewijzigd en de reacties in het zuurgehalte nagegaan. Het zuurgehalte van geadapteerde coupes van 1 mm dik, is steeds lager dan dat van vergelijkbare afgesneden bladen onder dezelfde omstandigheden. Een afdoende verklaring voor dit verschijnsel is niet gevonden (Exp. 17, 18, 19, 20 en 21).

In het donker neemt het zuurgehalte toe bij een stijging van de koolzuurspanning tussen 0 % en 2 %; hogere koolzuurspanningen beïnvloeden het zuurgehalte niet meer. De relatie heeft in grafische voorstelling de gedaante van een Blackmankromme.

Bij de lichtintensiteit van het compensatiepunt is het optimale zuurniveau bereikt bij ongeveer 3 % CO_2, in sterk licht bij omstreeks 4 % CO_2. Daar het optimale zuurniveau in licht en donker hetzelfde is, is er bij koolzuurspanningen boven 4 % geen invloed meer van belichting op het zuurgehalte (Exp. 22, 23, 24 en 25).

IV

Bij een poging, uit literatuurgegevens af te leiden, welke de biochemische wegen zijn, waarlangs het zuurmetabolisme van succulenten in licht en in donker verloopt, zijn drie voorwaarden gesteld.

1. Het bij de zuurwisseling in hoofdzaak betrokken zuur is appelzuur.
2. De variatie in het appelzuurgehalte hangt samen met de koolhydraatstofwisseling.
3. Uitsluitend enzymen, bekend uit de algemene biochemie van planten, spelen een rol.

De volgende wegen worden het waarschijnlijkst geacht (Fig. 2):

1. *Donkerontzuring:* decarboxylering van appelzuur en verbranding van het decarboxylatieprodukt tot koolzuur.
2. *Donkerverzuring:* carboxylatieve omzetting van zetmeel en sedoheptulose tot phosphoglycerinezuur, met behulp van het enzym carboxydismutase; carboxylering van het uit phosphoglycerinezuur verkregen phospho-enolpyrodruivenzuur, met behulp van het enzym phospho-enolpyruvaatcarboxylase, tot oxaalazijnzuur, en reductie hiervan tot appelzuur (Fig. 3).
3. *Lichtontzuring:* naast voortgaande ontzuring als in donker, een oxydatieve decarboxylering van appelzuur in phospho-enolpyrodruivenzuur, dat langs de omgekeerde glycolyseweg wordt opgebouwd tot koolhydraat, met behulp van energie en reducerend vermogen, afkomstig van de photolyse van water.

De resultaten van de Exp. 23, 24 en 25 passen in dit beeld.

De koolzuurspanning is niet van belang voor de *donkerontzuring*. Zij is van invloed bij de donkerverzuring en bij de lichtontzuring.

Bij de *donkerverzuring* is koolzuur, tot een spanning van 2 %, de beperkende factor bij de carboxylatieve vorming van phosphoglycerinezuur uit pentosediphosphaat door het enzym carboxydismutase. Boven 2 % is voldoende koolzuur aanwezig om het zuurproducerende enzymsysteem met phosphoglycerinezuur te verzadigen.

De *lichtontzuring* verloopt bij koolzuurspanningen tot 4%. Bij deze lagere concentraties kan het enzym carboxydismutase onvoldoende phosphoglycerinezuur vormen, om het koolhydraatsynthetiserend enzymsysteem te verzadigen. Voor zover deze carboxylatieve phosphoglycerinezuurproduktie te kort schiet, wordt uit het voorradige appelzuur phosphoglycerinezuur bijgeleverd: het appelzuur vervangt het koolzuur als bron voor de koolhydraatsynthese. Bij stijgende koolzuurspanning wordt steeds minder van het appelzuur verbruikt. Bij een lage lichtintensiteit, bij voorbeeld het compensatiepunt, zal het koolhydraatsynthetiserend apparaat eerder verzadigd zijn dan bij sterke belichting. Wanneer dit apparaat met carboxylatief gevormd phosphoglycerinezuur verzadigd is, en er bovendien nog van dit phosphoglycerinezuur beschikbaar is voor het zuurvormende enzymsysteem, dan zal het zuurgehalte hetzelfde zijn als in donker bij die koolzuurspanning. Dit is bij het compensatiepunt het geval boven ongeveer 3 %, in sterk licht boven omstreeks 4 % CO_2; bij deze concentraties wordt het horizontale gedeelte der Blackmankrommen bereikt.

Uit de literatuur volgt, dat carboxydismutase in vitro bij 5 % koolzuur nog onverzadigd is. De photosynthese van bladcoupes van *B. tubiflorum* wordt, onder de gebruikelijke omstandigheden en bij sterke belichting, eerst bij ongeveer 2 % koolzuur verzadigd.

V

In de relatief koele nachten kunnen vetplanten uit koolhydraten appelzuur opbouwen, dank zij het overwicht, dat carboxylerende enzymsystemen dan hebben op oxyderende. Mogelijk speelt hierbij ook de samenstelling van het intercellulaire gas een rol.

Overdag zullen, bij een normale samenstelling van de buitenlucht, vetplanten het appelzuur gebruiken als bron voor de lichtenergie vastleggende koolhydraatvorming; hierbij wordt het appelzuur ten dele rechtstreeks in koolhydraat omgezet (Fig. 4).

Planten, welke weerstand bieden aan uitdrogende klimaatsomstandigheden door de ontwikkeling van succulentie, kunnen, dank zij de dagelijkse wisseling in het zuurgehalte, ook bij beperkte mogelijkheid tot gasuitwisseling met de buitenatmosfeer, een levendig metabolisme handhaven, waarbij zij dag en nacht koolzuur kunnen vastleggen.