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The Relation between Photosynthesis, Respiration, and Crassulacean Acid Metabolism in Leaf Slices of *Aloe arborescens* Mill.[1]

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ABSTRACT

Leaves and leaf slices from *Aloe arborescens* Mill. were used to study the interrelations between Crassulacean acid metabolism, photosynthesis, and respiration. Oxygen exchange of leaf slices was measured polarographically. It was found that the photosynthetic utilization of stored malic acid resulted in a net evolution of oxygen. This oxygen production, and the decrease in acid content of the leaf tissue, were completely inhibited by amytal, although the rate of respiratory oxygen uptake was hardly affected by the presence of this inhibitor of mitochondrial electron transport. Other poisons of respiration (cyanide) and of the tricarboxylic acid cycle (trifluoroacetate, 2-diethyl malonate) also were effective in preventing acid-dependent oxygen evolution. It is concluded that the mobilization of stored acids during light-dependent deacidification of the leaves depends on the operation of the tricarboxylic acid cycle and of the electron transport of the mitochondria.

A comparison of enzyme activities in extracts from *Aloe* leaves and from other plants and studies of leaf anatomy and chloroplast morphology revealed typical characteristics of C_3-, as well as C_4-, plants in *Aloe*.

Extensive research, mainly between 1940 and 1960, has provided much of the presently available information about the biochemical mechanism underlying the so-called CAM[3] (for reviews see refs. 3, 27, 28, 41). The nightly acidification of the leaves results primarily from an enzymatic carboxylation of PEP with atmospheric CO_2 which yields oxalacetate and, through subsequent reduction, malate (37). In the light, stored acids are decarboxylated again, and the liberated CO_2 is photosynthetically converted into carbohydrates. This mechanism constitutes an adaptation to an arid environment, since it permits a closure of the stomata during the hot daytime by making CO_2 available for photosynthesis from the endogenous acid pools (24).

Many striking resemblances exist between the mechanism of CO_2 assimilation in CAM and that in the C_4-dicarboxylic acid pathway of CO_2 fixation which is characterized by the appearance of labeled dicarboxylic acids as first stable products of the photosynthetic assimilation of $^{14}CO_2$. Many data and concepts have recently been published on the physiological differences which separate C_4-plants from plants with the "classical" Calvin-Benson pathway of CO_2 fixation (C_3-plants). Investigations on CAM, however, have been few. This situation is reflected in Hatch and Slack's (13) review on the mechanisms of CO_2 assimilation.

The photosynthetic use of CO_2 from stored malate in chlorophyllous tissue with CAM should be reflected in a light-dependent net evolution of oxygen even in the absence of external CO_2. It was the aim of this work to devise a rapid and reproducible method for the measurement of O_2 exchange in such tissue under various conditions. This approach was hoped to permit an analysis of the physiological conditions regulating the storage and photosynthetic utilization of CO_2 in plants with CAM.

The use of intact leaves was not desirable because their gas exchange is influenced by the activity of the stomata and by the diffusion of gases through the bulky tissue. Sectioning of the leaf would lessen, or eliminate, such problems and facilitate the entry of added inhibitors and other substances. Bruinsma (6) had observed, however, that CAM was adversely affected when *Bryophyllum* leaves were sliced. This appeared to have been due to actions of leached tannins and other phenolic compounds. Therefore, we had to find a plant which was relatively free of such potentially inhibitory compounds. This criterium eliminated nearly all plants which had been used by other investigators in the past, *e.g.*, from the genera *Bryophyllum*, *Sedum*, and *Kalanchoe*. Leaves from *Aloe arborescens* Mill. (Liliaceae), however, had a very low content of tannin bodies and yielded slices with high metabolic activity.

When *Aloe* leaf slices were suspended in a buffered solution of mannitol, their O_2 exchange could be measured easily with a Clark type oxygen electrode. By use of metabolic inhibitors we were able to show that the rapid mobilization of stored malate in the light required unimpaired mitochondrial activity. However, the number of functional respiratory sites appeared more important than the over-all rate of respiration in the cells.

MATERIALS AND METHODS

Maintenance of the Experimental Plants. Our *Aloe* plants were cut as branches from the same parent plant (courtesy of

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[3] Abbreviations: CAM: Crassulacean acid metabolism; PEP: phosphoenolpyruvate; APO: photosynthetic oxygen evolution from the utilization of stored acids; CPO: photosynthetic oxygen evolution dependent on exogenous CO_2; HPMS: hydroxypyridine-methane sulfonic acid.

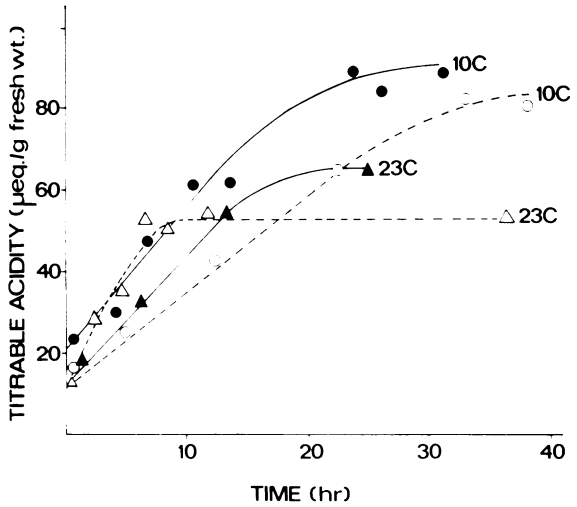

FIG. 1. Dark acidification of intact and sliced leaves from *Aloe* at two temperatures. Intact leaves (●, ▲) were kept in air, and transverse slices (○, △) were kept in standard buffer, which was bubbled vigorously with air.

Mrs. Grace Denius, Melbourne, Fla.). They were rooted and grown in regularly watered and fertilized soil in a greenhouse. Other plants were kept under similar conditions and have been described elsewhere (31). In our original survey, and for comparative experiments, we have used many species of the Crassulacean family, most of which were purchased from Ne-Smith's Nursery, Tallahassee, Fla.

Selection, Conditioning, and Preparation of the Leaf Material. All experimental leaves were mature, at least 8 inches long, and free from visible damage or disease. "Deacidified" leaves, excised at the end of a light period, were used as soon as possible. In the time interval between arrival in the laboratory and the start of the experiment, such leaves were kept under a bank of fluorescent lamps. "Acidified" leaves were harvested early in the morning and then subjected to a dark treatment of 10 C for about 24 hr. Such additional dark treatment yielded more satisfactory and reproducible acidities of the leaf tissue because the temperature of Florida summer nights was usually too high for optimal acidification to occur.

Slicing was performed with sharp razor blades either vertical to the leaf axis ("transverse slices," about 2 mm thick) or parallel to it ("parallel slices," about 3 × 3 mm). The latter could be freed from the nonchlorophyllous parenchyma cells simply by gentle scraping. The slices were placed in a phosphate-buffered (40 mM, pH 6.8) solution of 0.7 M mannitol ("standard buffer") and used at random for the experiments.

Oxygen Exchange Measurements. Measurements of oxygen production and consumption were performed in a water-jacketed, clear plastic vessel (volume 3.7 ml) into which a Yellow Springs Instruments oxygen sensor had been incorporated. The signal was displayed on a Sargent Model SRG recorder. Before being placed into this apparatus, the medium with the leaf slices was evacuated for 30 sec using a laboratory aspirator. This procedure removed gases from the intercellular spaces and also aided in the penetration of inhibitors into the tissue. The vessel was filled to the top with the suspension medium and covered with a tightly fitting lid. Additions could be made from a syringe through a sealable hole in the lid.

Incorporation of $^{14}CO_2$ and Its Determination. Uptake of $^{14}CO_2$ by leaf slices occurred in stoppered Erlenmeyer flasks from "standard buffer" supplemented with $NaH^{14}CO_3$ at pH 6.8. More than 90% of the radioactivity incorporated in the dark could be extracted from small leaf sections at 70 C with 20% (v/v) ethanol in the course of 30 min. Aliquots of such extracts were dried on planchets, and the radioactivity was determined with a model C-110 B Nuclear Chicago gas flow counter.

Extraction Procedures and Enzyme Analyses. The extraction and titration of organic acids was performed by the procedure of Bruinsma (6) and the determination of glycolate by that of Zelitch (44). Since we did not succeed in separating the various organelles of *Aloe* cells by the method of Tolbert *et al.* (35), our extracts for enzyme analyses were prepared directly from the green portions of deacidified *Aloe* leaves and from other leaves at 4 C under N_2 with a VirTis homogenizer at high speed. The medium contained 0.1 M tris-HCl buffer, pH 8.3, 10 mM $MgCl_2$, 10 mM EDTA, 10 mM 2-mercaptoethanol, and occasionally 1 to 2% polyvinyl pyrrolidone as a protective agent (5). Any further treatment of the extract, *e.g.*, centrifugations and fractionations on Sephadex G-25 columns, were done with only minor modifications according to published methods for assays of the various enzymes. The following procedures were adopted: carboxydismutase (E.C. 4.1.1.39) and PEP carboxylase (E.C. 4.1.1.31), Slack and Hatch (33); pyruvate-P_i-dikinase, Andrews and Hatch (1); malic enzyme (E.C. 1.1.1.40), Ochoa (25); carbonic anhydrase (E.C. 4.2.1.1.), Chen *et al.* (8); glycolate oxidase (E.C. 1.1.3.1.), Salin and Homann (30); glyoxylate reductase, *i.e.*, hydroxypyruvate dehydrogenase (E.C. 1.1.1.26), Tolbert *et al.* (35, 36).

Chlorophyll was extracted from leaf slices with methanol at 60 C or at room temperature with 80% acetone from aliquots of leaf extracts. The absorbance was measured with a Zeiss PMQ II Spectrophotometer for calculation of the chlorophyll content (19, 31).

Illumination. Light from 1000-w projection lamps was passed through round-bottom flasks filled with running water and red plastic filters. White or blue light was usually avoided because under such conditions undesirable photooxidation reactions tended to occur between added substances and blue absorbing pigments which had leached from the leaf tissue.

The light intensities were determined with an Instruments Speciality Co. spectroradiometer.

Microscopy. For electron microscopy with a Hitachi 111 C instrument, leaf slices were fixed with glutaraldehyde according to Sabatini *et al.* (29), dehydrated in 100% ethanol, postfixed with propylene oxide, and embedded in araldite. Silver sections obtained with a Porter-Blum microtome were stained with uranyl acetate and, subsequently, with Reynold's lead citrate (32).

Tannin bodies were identified light-microscopically according to Bate-Smith (2).

RESULTS

Some Physiological Characteristics of *Aloe* Leaf Slices. Considering the possible experimental problems mentioned in the introduction, it was important to ascertain that *Aloe* leaf slices displayed all aspects of CAM of intact leaves without significant decrease in magnitude. Figure 1 compares the time course of acidification in intact leaves and in leaf slices from *Aloe*. At 10 C, the rate of acidification in slices was about half that observed for intact leaves, but the final acid content was the same. The acidification at 23 C, however, was faster after the leaves had been sliced. Quite obviously, the rate of CO_2 fixation by intact leaves at the higher temperature had been limited by the rate of diffusion of CO_2 into the tissue. In agreement with observations of other authors (3, 4, 34, 37), the level of acidification remained lower at the higher temperature. When, after acidification at 10 C, leaf slices were transferred to higher temperatures, the acidity began to decrease markedly,

and this deacidification was greatly accelerated by light (Fig. 2).

Since our findings corroborated the earlier results of other authors with intact Crassulacean leaves, we were encouraged to use leaf slices from *Aloe* for further investigations.

Oxygen Consumption and Evolution by Leaf Slices from *Aloe*. Oxygen uptake by slices from *Aloe* leaves in magnetically stirred standard buffer at 25 C did not depend on the state of acidification. Bruinsma (6) had found that O_2 uptake by intact *Bryophyllum* leaves was enhanced by increasing the external CO_2 concentration, possibly as the result of CO_2-dependent changes in the stomatal aperture. Accordingly, it should be absent in thin leaf slices. As expected, respiratory O_2 exchange in leaf slices from *Aloe* did not vary with the amount of added bicarbonate in standard buffer up to at least 1 mM.

Oxygen consumption by these slices was, however, strongly dependent on the O_2 concentration of the medium below 50% air saturation. Such a strong dependence of respiration on the external O_2 concentration is well known for many types of bulky plant organs. This phenomenon has generally been ascribed to diffusion problems, although data obtained with tissue pieces of varying size were found to be inconsistent with such an explanation (10, 11, 18).

In leaf slices from *Aloe*, the respiration rate depended linearly on the logarithm of the oxygen tension between 2 and 14% O_2. This is essentially what Moyse (22) and Moyse and Jolchine (23) found for intact *Bryophyllum* leaves, but the situation in their particular case was somewhat more complex. Ducet and Rosenberg (10) were unable to account for the logarithmic dependence by any simple mechanism. From Arrhenius plots of the temperature dependence of respiration in *Aloe* slices between 16 and 30 C, we calculated activation energies between 8 and 10 kcal. Such values are too high for a diffusion-limited process and are indeed higher than those obtained by other investigators from their data on the respiration of bulky plant tissues (*cf.* ref. 11).

An often discussed supposition invokes the involvement of more than one terminal oxidase system as an explanation for the observed effects of increasing oxygen concentrations on the respiration rate (10, 11, 42). One might expect that these oxidases differ in their sensitivity to cyanide. Accordingly, respiration in *Aloe* leaf slices, which is partially resistant to cyanide, ought to be inhibited by this poison to a different degree depending on the oxygen concentration. However, within the range of experimental error, we did not observe such a varying sensitivity to cyanide.

In the light, and in the absence of added bicarbonate, deacidified leaf slices did not show any significant net exchange of O_2, while there was a net production of oxygen from fully acidified slices (Fig. 3). Assuming a respiratory quotient of unity, and a slow diffusion of CO_2 out of the tissue, these results indicate a complete photosynthetic refixation of all respiratory CO_2 in deacidified slices. Net O_2 evolution was possible only when stored acids provided an additional source for CO_2. When more than 0.1 mM bicarbonate was added, a net oxygen evolution appeared also with deacidified slices which saturated at about 1 mM bicarbonate. Maximal rates of oxygen evolution in the presence of bicarbonate were identical for acidified and deacidified slices (Fig. 3). This finding did not come as a surprise, since light-dependent deacidification is known to be inhibited by external CO_2 (6).

The intimate relationship between oxygen evolution in illuminated acidified slices and the loss of accumulated acids was expressed by a linear dependence of the maximal net rate of oxygen production on the amount of free acids in the leaf tissue (Fig. 4). Furthermore, an addition of the photosynthetic inhibitor DCMU to the medium stopped the light-induced de-

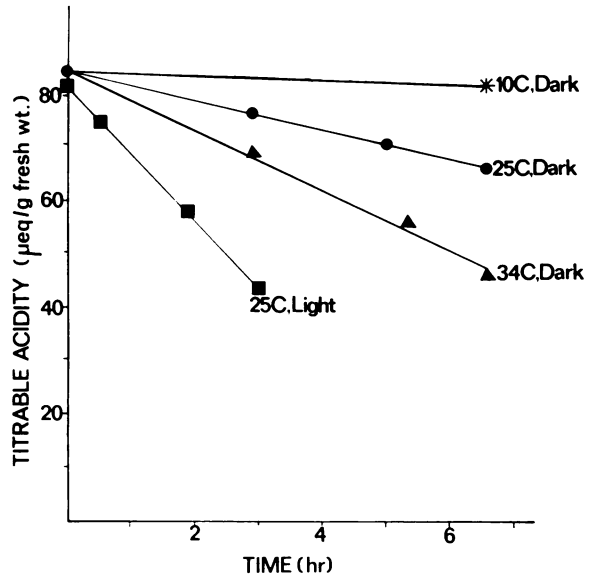

FIG. 2. Deacidification of leaf slices. Acidified leaves were sliced, and the slices were incubated in standard buffer. Illumination with $5.5 \cdot 10^4$ erg·sec^{-1}·cm^{-2} red light.

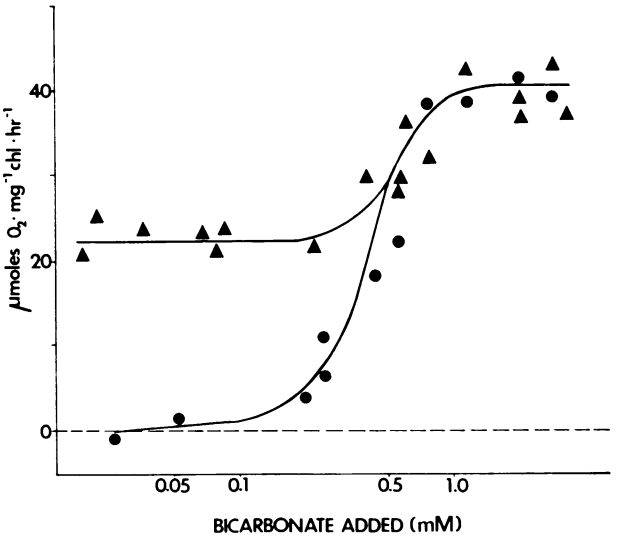

FIG. 3. Oxygen exchange of slices from *Aloe* leaves as function of the added amount of bicarbonate under photosynthesis saturating light intensity (5.5 erg·sec^{-1}·cm^{-2} red light). Standard buffer, 25 C. Transverse slices from acidified (▲) or deacidified (●) leaves.

acidification, and reduced the oxygen exchange to that of dark respiration (not shown).

Acidification proceeds best in the cold, whereas deacidification is favored at higher temperatures (3, 4, 34, 37). The question arose, therefore, whether net APO could be increased significantly by raising the temperature of the medium. In the experiment of Figure 5, any net production of oxygen required temperatures of at least 20 C. At 15 C, nonphotosynthetic fixation of respiratory CO_2, and its escape from the tissue to the outside, appeared to compete successfully with photosynthesis since the overall exchange of O_2 remained below the compensation level.

Relation between Photosynthetic Oxygen Evolution and Respiration in Acidified Leaf Slices from *Aloe*. After acidification of *Aloe* leaves in $^{14}CO_2$-enriched air, more than 80% of the

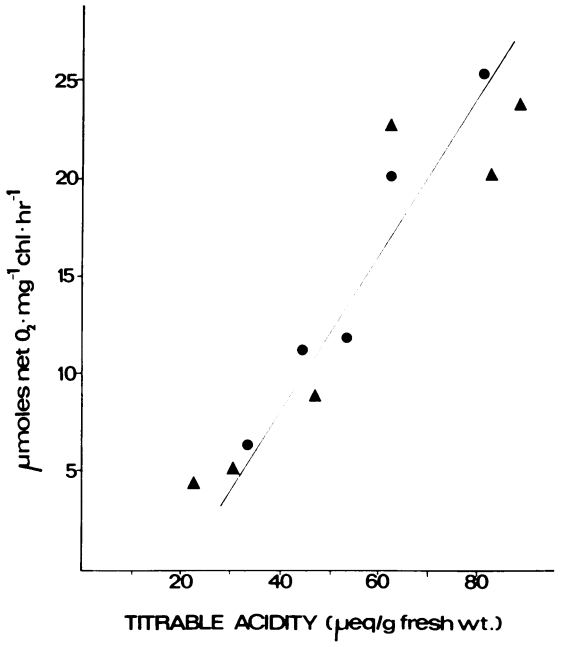

FIG. 4. Dependence of photosynthetic oxygen production from *Aloe* leaf slices on their acid content. Deacidified leaves were placed in the dark at 10 C under air. During the course of acidification, slices were taken and their acidity and maximal ability to evolve oxygen in the light were determined. Oxygen measurements in the absence of $NaHCO_3$ at 25 C in standard buffer equilibrated with 10% O_2 in N_2; $6 \cdot 10^4$ erg·sec^{-1}·cm^{-2} red light. Data from two leaves.

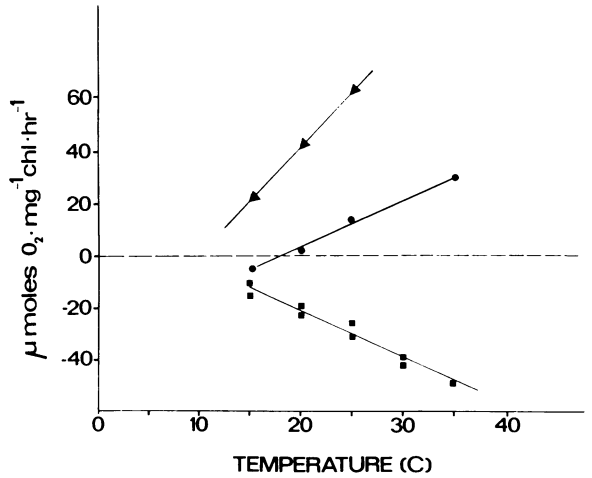

FIG. 5. Temperature dependence of respiration and photosynthesis in acidified transverse slices from *Aloe* leaves. Standard buffer, equilibrated with 10 to 12% O_2 in N_2; $5.5 \cdot 10^4$ erg·sec^{-1}·cm^{-2} red light; ■: respiration (dark); ●: photosynthetic oxygen evolution in absence of added $NaHCO_3$; ▲: same, after addition of 1.5 mM $NaHCO_3$.

incorporated radioactivity was found in chromatographically identified malate. In the light, the stored malate is mobilized and then oxidatively decarboxylated to pyruvate (3, 6, 28) which could be broken down further to CO_2 and water via the respiratory pathway. A simple calculation shows that the refixation of CO_2 released in a complete combustion of malate can result in a net oxygen evolution of maximally 40% of the respiratory oxygen uptake. This presupposes that the degradation of pyruvate is responsible for all respiratory activity during light-dependent deacidification and that the glyoxylate cycle does not operate in *Aloe* leaves. Neither we nor others (*cf.* ref. 28) have found any evidence for the presence of the latter in plants with CAM. In our studies, we quite often encountered a net oxygen evolution in the absence of added CO_2 which was higher than two-fifths of the respiration rate in the dark. Assuming that respiration was not significantly increased during photosynthesis, we have to postulate that pyruvate was partly or totally utilized in synthetic reactions as was proposed earlier by Haidri (12). Milburn *et al.* (21) came to the same conclusion after an analysis of the respiratory gas exchange of *Bryophyllum* leaves.

Since malate is believed to be stored in the vacuoles (3, 28), its mobilization might occur at the expense of energy derived from respiration. We checked, therefore, whether APO, like respiration, was influenced by the oxygen tension. In Figure 6A, gross O_2 evolution *versus* light intensity has been plotted for acidified slices at three oxygen tensions. The efficiencies and the saturation levels of APO seem to be different. However, when the light intensity dependence of the actual O_2 exchange (Fig. 6B) was analyzed, it became apparent that net APO did not change with the oxygen tension. It was also seen that the compensating light intensity was independent of the original rate of respiratory O_2 uptake. Light intensity curves of normal CPO revealed the same phenomena (not shown). Such results would be expected if, at the compensation point, sufficient photosynthetic O_2 accumulates in the cells to permit maximal rates of respiration regardless of the external oxygen concentration.

Whether peroxisomal respiration ("photorespiration") contributed significantly to the respiratory processes in the light has to remain an open question because of the experimental

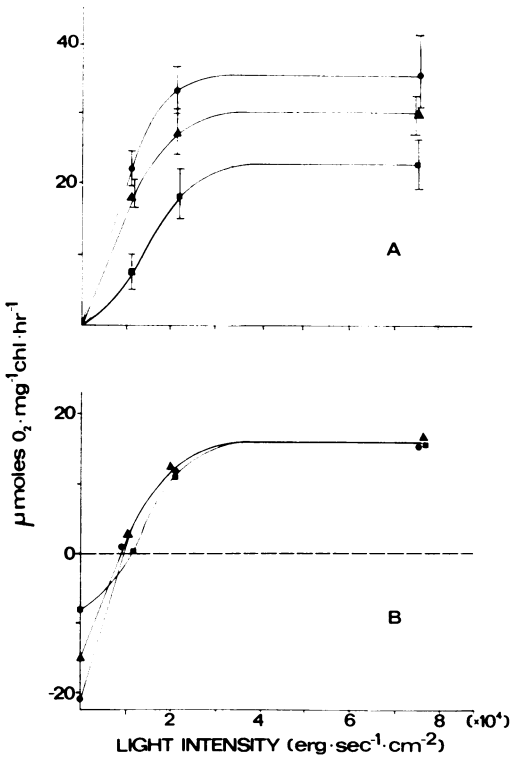

FIG. 6. Oxygen exchange of acidified leaf slices from *Aloe* at three oxygen tensions. Transverse slices in standard buffer at 25 C equilibrated with 3% (■), 7% (▲), and 10% (●) O_2 in N_2. A, Oxygen evolution corrected for respiration in the dark; absolute variations of values from 5 experiments as indicated. B, Actual oxygen exchange; only mean values plotted.

difficulties arising from the slow exchange of O_2 and CO_2 by *Aloe* leaf tissue. HPMS, a widely used inhibitor of glycolate oxidase (43), also could not be used because it strongly inhibited photosynthetic O_2 evolution (*cf.* ref. 26). In addition, we noted that highly active peroxidases in *Aloe* leaf extracts catalyzed a rapid autoxidation of any added HPMS.

A dependence of APO on mitochondrial respiration was indicated by the nearly identical response of these two processes to additions of the respiratory poison cyanide (Figs. 7 and 8). CPO, on the other hand, was much more resistant. In fact, when low concentrations of the inhibitor were added to de-

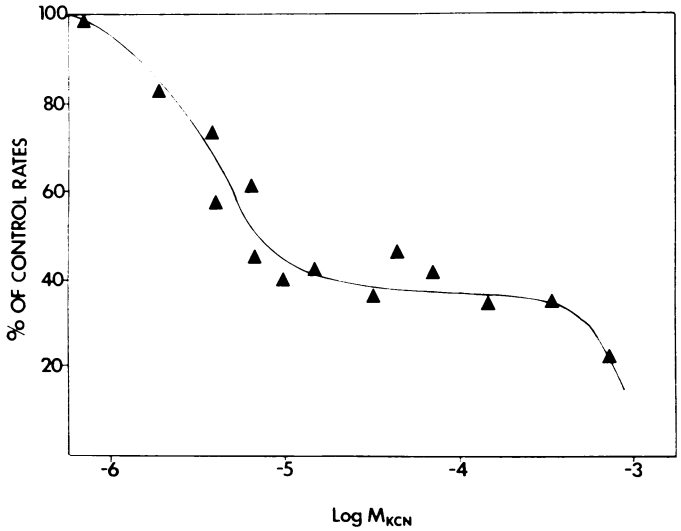

FIG. 7. Inhibition by KCN of respiration in leaf slices from *Aloe*. Transverse slices in standard buffer, equilibrated with 10% O_2 in N_2, at 25 C. An addition of 1 mM bicarbonate did not affect the response.

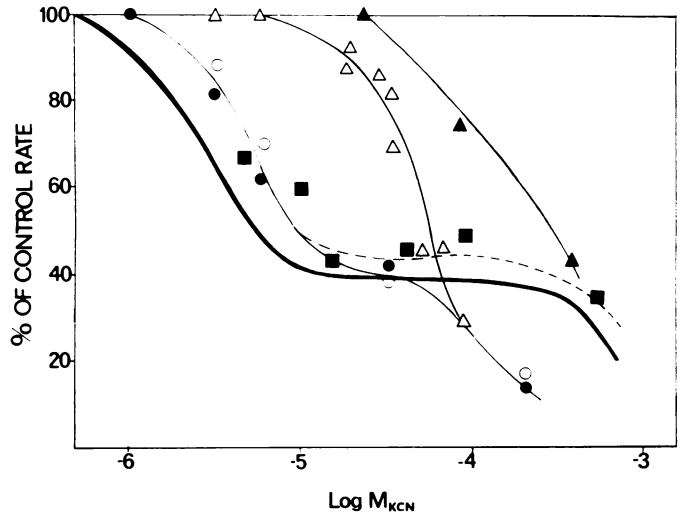

FIG. 8. Inhibition by KCN of photosynthesis, respiration, and acidification in leaf slices from *Aloe*. Conditions for oxygen measurements as for Figure 7. Photosynthetic oxygen evolution corrected for corresponding dark respiration rate. Heavy line: respiratory oxygen uptake (from Fig. 10); ○, ●: oxygen evolution from utilization of stored acids, no $NaHCO_3$ added; △, ▲: oxygen evolution in the presence of added $NaHCO_3$ (1.5 mM); ■: acidification of transverse leaf slices in standard buffer in the presence of labeled $NaHCO_3$, measured as relative amount of incorporated ^{14}C. Light intensities (red): $6 \cdot 10^4$ erg·sec^{-1}·cm^{-2} (open symbols; ○, △) or $2.2 \cdot 10^4$ erg·sec^{-1}·cm^{-2} (closed symbols; ●, ▲).

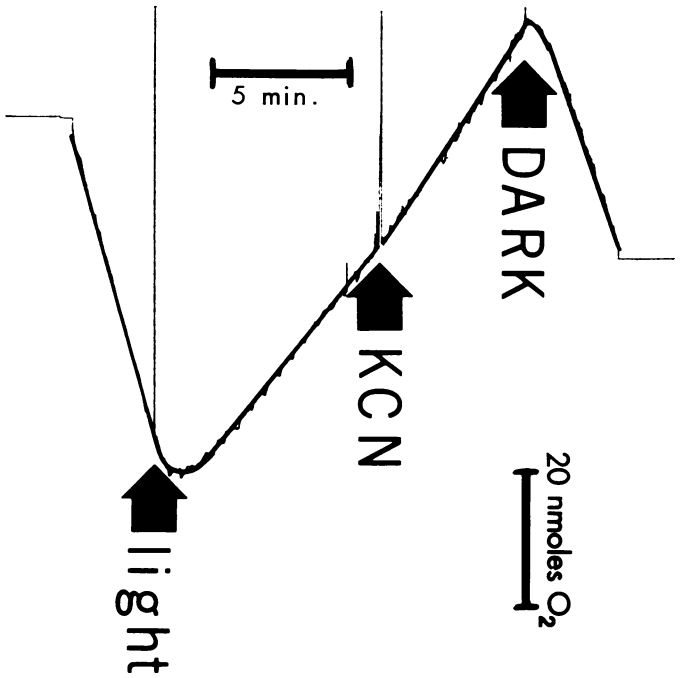

FIG. 9. Effect of an addition of 5 μM KCN on the photosynthetic oxygen exchange in transverse leaf slices from *Aloe*. Conditions as for ▲ in Figure 8. Note that this figure depicts an actual recording from the oxygen electrode system. Hence, the trace includes the response from the oxygen consumption by the electrode itself.

Table I. *Effect of Respiration Inhibitors on Photosynthetic and Respiratory Oxygen Exchange in Leaf Slices from Aloe*

Acidified parallel slices in standard buffer, pH 4.5, were equilibrated with 10% O_2 in N_2 at 25 C and in $5.5 \cdot 10^4$ erg·sec^{-1}·cm^{-2} red light. Six μmoles $NaHCO_3$ were added where indicated.

	No Inhibitor	10 mM Fluoro-acetate	10 mM Diethyl-malonate	1.5 mM Amytal
		μmoles O_2/mg chl·hr		
Respiration				
No inhibitor	−10	−11	−11	−10
+ Inhibitor		−6.5	−10.5	−11
Net oxygen evolution				
No inhibitor	+24	+22	+28	+24
+ Inhibitor		+4.5	+4.5	+2.0
+ Inhibitor + bicarbonate	+43	+27	+50	+42

acidified slices in the presence of bicarbonate, net photosynthetic O_2 production was enhanced (Fig. 9). This finding supported our assumption that mitochondrial respiration continued during photosynthesis.

Since cyanide is a rather unspecific metabolic poison, it was desirable to provide additional evidence for a coupling between APO and respiratory processes. The most clearcut results were obtained with amytal and rotenone, inhibitors of mitochondrial electron transport. Amytal had no effect on the respiratory oxygen exchange in the dark, but it completely inhibited net APO (Table I) and light-dependent deacidification (Table II). Rotenone acted in a similar way.

The next logical step was to test inhibitors of the tricarboxylic acid cycle. This approach was marred by unexpected difficulties. Malonate, for example, evidently did not enter the

Table II. *Inhibition of Deacidification by Amytal*

Each horizontal line represents one experiment. Parallel slices from acidified leaves were placed into standard buffer equilibrated with 10 to 15% O_2 in N_2 throughout the incubation period; temperature, 25 C; 1.5 mM amytal present where indicated; illumination, where applicable, with $7.5 \cdot 10^4$ erg·sec^{-1}·cm^{-2} red light.

Before Incubation	After Incubation (1 hr)			
	Light		Dark	
	− amytal	+ amytal	− amytal	+ amytal
	μequivalents acidity/mg fresh wt slice			
84	66	78	78	76
74	54	71	68	—[1]

[1] Sample lost.

Table III. *Activities of Important Enzymes of the C_3 and C_4 Pathways of Photosynthesis*

Enzyme extractions and determinations of their activities were as described in "Materials and Methods." The numbers give the activities in μmoles of substrate converted per mg chlorophyll per hr; only in the case of carbonic anhydrase was another type of unit used (8). Spinach, *Spinacia oleracea*, var. Bloomsdale; tobacco, *Nicotiana tobacum*, var. N.C. 95; corn, *Zea mays*, var. Sweet Corn, hybrid *Sorghum* var. Rio.

Enzyme	C_4-plants			C_3-plants	
	Aloe	Corn	Sorghum	Spinach	Tobacco
	units of activity				
Ribulose-diP carboxyiase	189	180	21[1]	515	210
PEP carboxylase	660	850	950[1]		27[2]
Glycolate oxidase	41			68	90
Glyoxylate reductase	120				310[2]
Malic enzyme	42	388			2
Pyruvate-P_i-dikinase	0	150			0
Carbonic anhydrase	2000	350		3800	

[1] From Slack and Hatch (33).

[2] From Salin and Homann (30).

leaf slices easily and gave inconclusive results. We finally settled for the use of the less efficient derivative diethyl malonate (39) at pH 4.5. Moreover, when we added acetate in our controls to the fluoroacetate experiments, we noticed that a combination of acetate and bicarbonate severely inhibited respiration and photosynthetic O_2 evolution. Propionate had the same effect. Presumably, the protonated acids diffused readily from the medium (pH 4.5) into the tissue thereby causing an intolerably large drop of the pH in the cells. On the basis of data obtained from various control experiments, we do not believe that such unspecific "acid effects" invalidated our data for diethyl malonate, but the results obtained with fluoroacetate have to be analyzed with caution. The data in Table I show, however, that APO was more sensitive to the inhibitors of the tricarboxylic acid cycle than was CPO. A similar dependence on this cycle appears to exist for the photosynthetic utilization of dicarboxylic acids in C_4-plants (15).

Inhibition of Acidification by Cyanide. The nightly accumulation of acids in CAM does not occur under anaerobic conditions (22, 23). Maximal rates in leaf slices from *Aloe* were sustained by less than 3% oxygen, in agreement with Moyse's (22) data on *Bryophyllum* leaves. We estimated that the accumulation of 1 μequivalent of acid required the simultaneous uptake of not more than 1 μmole O_2.

Since the acidification rate was saturated at such low rates of respiration, we expected it to be relatively insensitive to cyanide. Yet acidification responded to the presence of cyanide in the same manner as did respiration (Fig. 8). While interferences of even low concentrations of cyanide with essential reactions of the acidification process could not be ruled out, the data suggest that the acidification rate depended on the number of functional respiratory sites rather than on their turnover frequency.

Activities of Photosynthetic Enzymes in *Aloe* Leaf Extracts. In order to obtain some information about the relation of *Aloe* to typical C_3- and C_4-plants, we analyzed the activities of some chloroplastic and peroxisomal enzymes in leaf extracts from *Aloe* and from other types of plants (Table III). We are aware that even various precautions and control experiments could not make absolutely certain that no enzyme inactivations had occurred, particularly during extraction of *Aloe* leaves. For example, we believe that pyruvate-P_i-dikinase is not lacking in *Aloe* (*cf.* ref. 16). Moreover, the chlorophyll content per dry weight of chlorophyllous tissue of *Aloe* leaves is only about one-third of that of the other leaves listed in Table III. On a basis of tissue weight, therefore, the activities of enzymes isolated from *Aloe* leaves turn out to be quite low. However, since photosynthetic enzymes ought to be compared on a chlorophyll basis, the data in Table III suggest that *Aloe* is not a typical C_4-plant. The same conclusion can be reached from an analysis of the leaf anatomy and chloroplast morphology of *Aloe* (Fig. 10) and Crassulacean plants (14).

DISCUSSION

We have described in this paper the suitability of leaf slices from *Aloe arborescens* for studies of the oxygen exchange of tissue with CAM. The slices were permeable to inhibitors like cyanide and amytal, but apparently not to malonate at pH 4.8. We also were unable to substitute externally dissolved malate for the endogenous malate pool during photosynthesis in the absence of added bicarbonate. Moreover, the diffusion of gases into and out of the tissue remained a problem even after slicing the succulent leaves. This was evident from the strong dependence of respiration on the external O_2 concentration and from the observation that, in the light, all respiratory CO_2 appeared to be refixed photosynthetically.

We have shown that the light-dependent deacidification in *Aloe* leaf slices was associated with a net evolution of O_2. Elevated temperatures favor the mobilization and decarboxylation of the malate pool (3, 4, 34, 37), but it is evident from Figure 2 that the deacidification in the light was not merely a temperature effect. Another driving force for the decarboxylation of malate may be the decrease in the CO_2 concentration of the cell when the photosynthetic apparatus is set into operation by light (27, 28, 34). The movement of malate from its site of storage to the site of decarboxylation might be controlled by the concentration gradient between these two loci. Our inhibitor data suggest, however, that the light-dependent deacidification depends on respiratory processes. The effectiveness of inhibitors of the tricarboxylic acid cycle and of mitochondrial electron transport supports the view that these systems are active in the light (20) and that they are involved in the conversion of stored malate into a photosynthetic substrate. On the basis of our gas exchange measurements, we have rejected the supposition that a complete combustion of malate to CO_2 and water via the tricarboxylic acid cycle constitutes an integral part of the deacidification process. Hence, the role of respiration may be the production of energy for a conversion

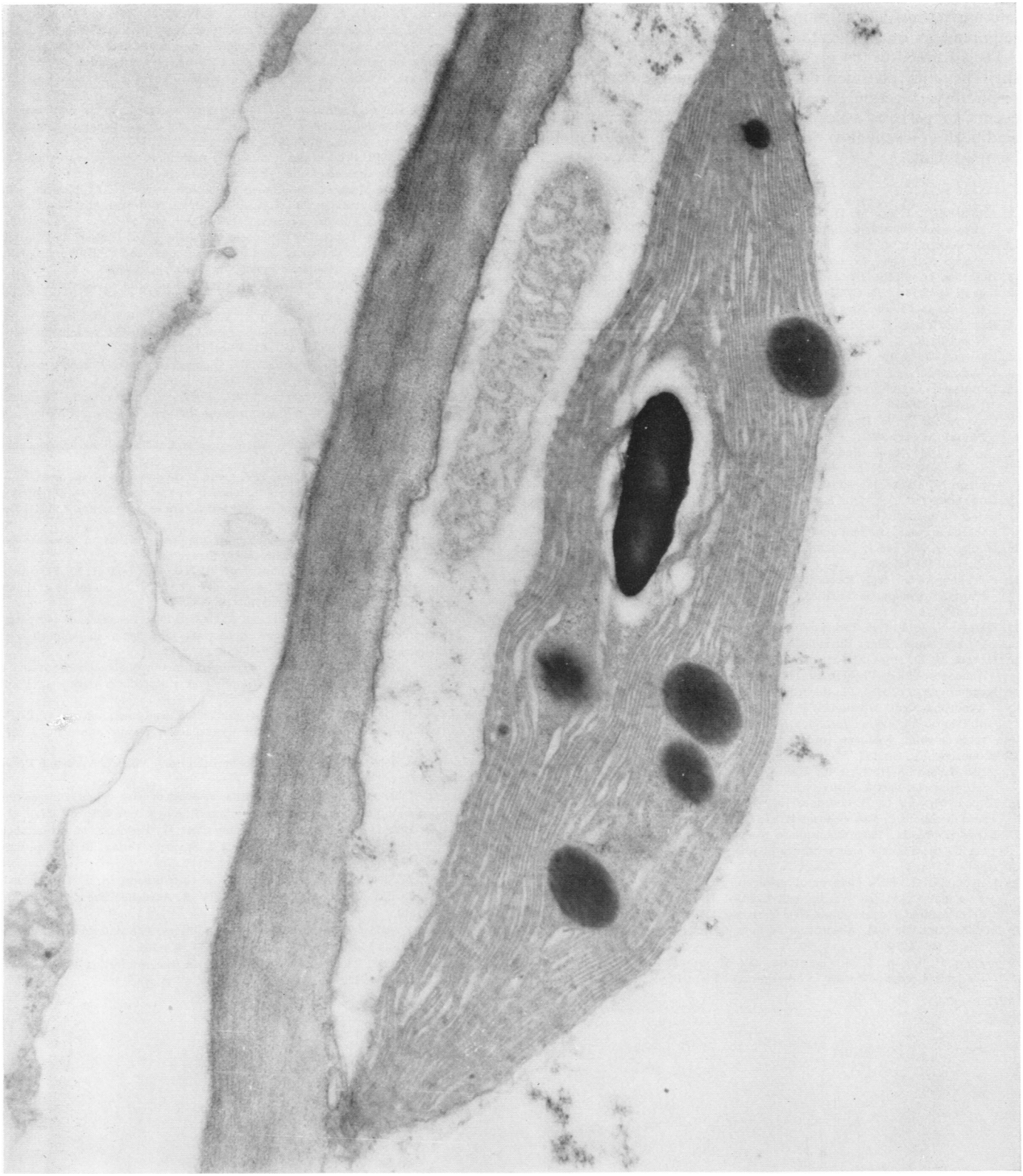

FIG. 10. Electron micrograph of an *Aloe* chloroplast, magnification 28,500. Note the absence of a peripheral reticulum (*cf.* ref. 9, 17). The dense thylakoid system was typical for our *Aloe* chloroplasts, but was not found in other plants with CAM.

of accumulated pyruvate into carbohydrates and perhaps also for the transport of malate out of the storage pool in the vacuole.

The accumulation of malate at its storage site during the nightly acidification process conceivably is an active process. The nearly identical sensitivity to cyanide of respiration and of the CO_2 assimilation in the dark may well be an expression of this fact. It remains to be explained, however, why the cyanide-

and amytal-resistant portions of respiration cannot satisfy the requirements of acidification and deacidification. Respiratory ATP is formed in the presence of both inhibitors (7, 40). We shall have to consider an additional involvement of other respiratory reactions, regulatory functions of respiratory events, or perhaps a dependence of the acidification and deacidification mechanism on processes in specific mitochondrial compartments.

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