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ACCUMULATION OF BICARBONATE IN INTACT CHLOROPLASTS FOLLOWING A pH GRADIENT

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SUMMARY

With silicone layer filtering centrifugation the uptake of radioactively labelled bicarbonate into isolated spinach chloroplasts was followed. This uptake was shown to have the following properties:

(a) It is so rapid that the kinetics of uptake usually cannot be resolved.

(b) Bicarbonate is accumulated in the stroma. The factor between the internal and external concentrations increases greatly when the pH of the medium is lowered from pH 8.5 to pH 7.0.

(c) The accumulation factor is independent of the concentration in the medium for a long concentration range.

(d) The accumulation of bicarbonate is increased when the chloroplasts are illuminated. This increase is abolished by the addition of uncoupler.

(e) Diamox, an inhibitor of carbonic anhydrase, inhibits the rate of bicarbonate uptake.

The activity of carbonic anhydrase was assayed in isolated chloroplasts and in leaf homogenates. In agreement with earlier reports the main activity was found to be located in the chloroplasts. This activity is latent; it can be only assayed if the chloroplasts are osmotically shocked.

From these results the following conclusions have been drawn:

(a) The inner membrane is impermeable to protons. Light-driven proton transport into the thylakoid space causes an alkalisation of the stroma.

(b) The uptake of bicarbonate proceeds *via* diffusion of CO_2 across the inner membrane. There are no indications for a specific transport of bicarbonate.

(c) The CO_2 concentration in the chloroplasts may be equal to the CO_2 concentration in the external space. The distribution of bicarbonate between the two compartments is inversely proportional to the distribution of protons.

A possible involvement of carbonic anhydrase and the bicarbonate pool in the stroma in increasing the CO_2 affinity of CO_2 fixation is discussed.

INTRODUCTION

The chloroplast envelope consists of two membranes, the outer and the inner. Of these the outer membrane has been shown to be freely permeable to metabolites

Abbreviation: DMO, dimethyloxazolidinedione.

of low molecular weight. The functional border between the metabolic compartment of the cytoplasm and the chloroplast (stroma) is the inner membrane[1].

It is the main function of chloroplasts to provide the cell with substrates, generated by photosynthesis. In spinach chloroplasts, 3-phosphoglycerate and dihydroxyacetone phosphate were shown to be the main products released[2]. This involves a transfer of CO_2, H_2O and inorganic phosphate across the inner membrane from the cytoplasm into the stroma, and of O_2, 3-phosphoglycerate and dihydroxyacetone phosphate in the other direction. A specific carrier catalyzing a counter-exchange of inorganic phosphate, 3-phosphoglycerate and dihydroxyacetone phosphate has been described by us recently[3,4]. In the present report we have investigated the problem of how the inorganic carbon is taken up. Until now, no direct measurements on this subject have been reported.

METHODS

(a) *Chloroplasts*

Spinach chloroplasts with intact envelope were prepared as described by Heldt and Sauer[1]. The integrity of the chloroplasts was checked by phase contrast microscopy[5]. Furthermore, as a quantitative measure of intactness the ferricyanide-dependent O_2 evolution was employed according to Heber and Santarius[6]. With this criterion, 80–90 % of the chloroplasts of a usual preparation appeared to be intact. Chlorophyll was assayed as described by Whatley and Arnon[7].

(b) *Incubation and filtering centrifugation*

The filtering centrifugation[8] was carried out with a Beckman Microfuge centrifuge using 0.4-ml polyethylene tubes (W. Sarstedt, Nümbrecht–Rommelsdorf). The tube contained 20 μl 2.5 M NaOH at the bottom, above this was a layer of 70 μl silicone oil (AR 100:AR 150 = 3:1, Wacker Chemie, München). On top of this 300 μl chloroplast suspension were added. If not stated otherwise, all the incubations were carried out at 4 °C in a medium containing 0.33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES) (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. The incubation was started by addition of radioactively-labelled $NaHCO_3$ (Amersham, England, specific activity according to the experiment from 20 to 0.5 Ci/mole) in a volume of 10 μl and was terminated by centrifugation. The centrifuge reaches its maximal velocity in less than 1 s. The separation of the chloroplasts from the medium occurs in less than 2 s after starting the centrifuge. This time can be extrapolated from the kinetics of uptake of phosphoglycerate into the sucrose-impermeable space of chloroplasts[4]. Immediately after centrifugation 20 μl 2.5 M NaOH were added to the supernatant in order to avoid diffusion of CO_2 through the silicone layer into the sediment fraction. The effectiveness of this addition is demonstrated in a control experiment of filtering centrifugation. In this experiment the radioactivity of [^{14}C]bicarbonate (0.5 mM) in the upper layer was 540000 dpm. When chloroplasts (20 μg chlorophyll per sample) had been added to the upper layer, and the sample was centrifuged, the radioactivity in the sediment fraction was 11300 dpm. When chloroplasts had been omitted, the radioactivity in the sediment fraction after centrifugation was only

103 dpm. When the sample was stored at 4 °C for 7 days, the radioactivity in the sediment fraction was found to be 92 dpm. These data clearly indicate that there was no significant diffusion of CO_2 a cross the silicone layer.

The separation of the sediment from the supernatant fraction and the counting of radioactivity were carried out as described earlier[1]. For assay of fixed carbon 50 μl of the supernatant or 100 μl of the sediment fraction were added to 500 μl of 0.2 M HCl and heated 15 min at 90 °C.

In each experiment the chloroplast spaces accessible to tritiated water (^{3}HHO) and for [^{14}C]sucrose were assayed as described before[1]. The sucrose-permeable space comprises the medium which has been carried through the silicone layer and the space between the outer and the inner membrane. This space is unspecifically permeable to all substrates[1]. The amount of substrate present in the sucrose-permeable space is calculated from the substrate concentration in the medium and subtracted from the amount of substrate found in the sedimented chloroplasts. The substrate concentration in the chloroplasts is related to the sucrose-impermeable ^{3}HHO space.

Illumination was carried out with a tungsten–halogen light source, provided with a RG 630 cut off filter (Schott, Mainz) and a Calflex-C heat filter (Balzers, Lechtenstein). The light intensity was 50000 ergs·s^{-1}·cm^{-2}. The illumination was begun 3 min before adding bicarbonate to the chloroplasts. The centrifugation was carried out in the dark. With the low temperatures employed, illumination during centrifugation had no significant effect on the results. The data shown in this report are all mean values from triplicate experiments, which have been carried out simultaneously.

(c) *Assay of carbonic anhydrase activity*[9]

10 g spinach leaves were homogenized 5 times or 10 times 15 s with 15-s intervals in a medium containing 5 mM $MgCl_2$ and 10 mM sodium pyrophosphate (pH 7.9) with a Bühler (Tübingen) homogenizer. During the procedure the vessel was cooled with ice. 0.1 ml of the homogenate was added to a mixture of 5.0 ml 0.1 M sodium barbital, pH 8.2 and 5.0 ml H_2O saturated with CO_2 at 0 °C. The velocity of the change of pH between pH 8.2 and 8.0 was measured with a glass electrode and recorded. For the uncatalyzed reaction a blank was subtracted. The scale was calibrated by addition of known quantities of HCl. With intact chloroplasts the assay was carried out in the presence of 0.33 M sorbitol. Chloroplasts were osmotically shocked by adding 1 vol. of chloroplasts to 9 vol. of distilled water.

RESULTS AND DISCUSSION

(1) *Uptake of bicarbonate*

Radioactively labelled bicarbonate was added to an illuminated suspension of spinach chloroplasts. At the times indicated the incubation was terminated by centrifuging the chloroplasts through a layer of silicone oil into a NaOH solution. The uptake of bicarbonate was calculated from the total radioactivity in this sedimented fraction (Fig. 1A). For the assay of fixed carbon an aliquot of the sedimented fraction was treated with HCl to remove CO_2 and bicarbonate. The amount of CO_2 + bicarbonate within the chloroplasts is calculated from the difference between the total uptake of bicarbonate and the fixed carbon. Furthermore the fixed carbon

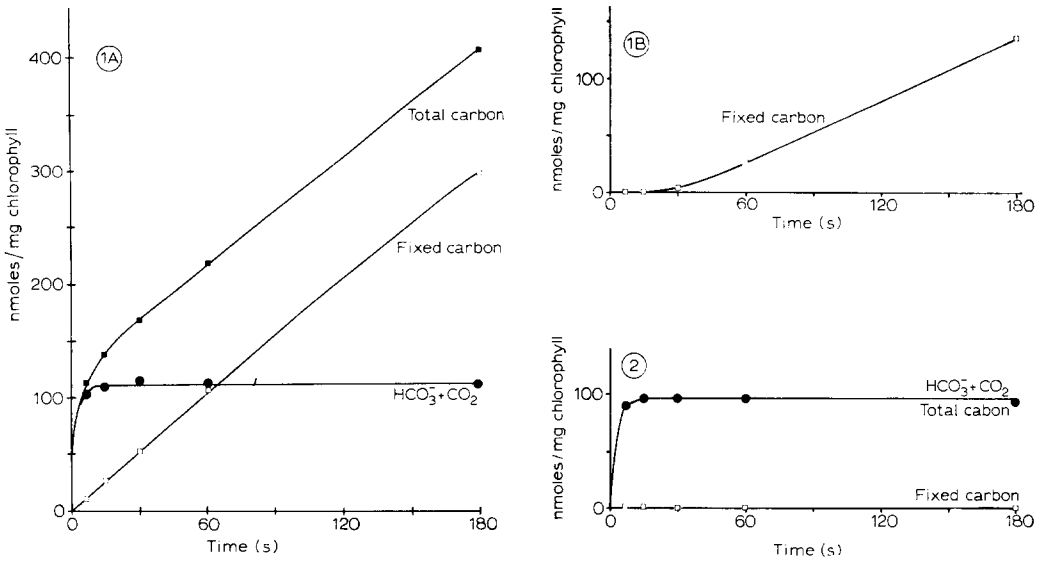

Fig. 1. (A) Bicarbonate uptake and accumulation of fixed carbon in the sucrose-impermeable ^{3}HHO space of chloroplasts in the light. Temp. 9 °C, bicarbonate in the medium 0.5 mM, 28 μg chlorophyll per ml, sucrose-impermeable ^{3}HHO space 50.1 μl/mg chlorophyll. (B) Release of fixed carbon into the medium of a chloroplast suspension in the light. For conditions, see (A).

Fig. 2. Bicarbonate uptake in the sucrose-impermeable ^{3}HHO space of chloroplasts in the dark. See legend Fig. 1A.

appearing in the medium was measured (Fig. 1B). Within the time scale of these figures, the CO_2 fixation did not reach linearity with time. The rate of CO_2 fixation was evaluated from the fixed carbon measured in the chloroplasts (569 nmoles/mg chlorophyll) and in the medium (368 nmoles/mg chlorophyll) after 5 min (between 3 and 5 min). It was 15.1 μmoles CO_2 fixed per mg chlorophyll per h (9 °C).

The same experiment has been carried out in the dark (Fig. 2). There is no CO_2 fixation, the total uptake of radioactive carbon representing CO_2 + bicarbonate in the chloroplasts. In both experiments the CO_2 + bicarbonate are taken up so rapidly that it is impossible to resolve the kinetics of uptake.

From the amount of CO_2 + bicarbonate taken up, and the size of the sucrose-impermeable water space, the concentration within the chloroplasts can be calculated. Since in intact chloroplasts the thylakoid space is only a small part of the sucrose-impermeable ^{3}HHO space, the concentrations evaluated here may be regarded as concentrations in the stroma (H. W. Heldt, K. Werdan and G. Geller, unpublished). The concentrations of CO_2 + bicarbonate in the chloroplasts (2.20 mM in the light, 1.90 mM in the dark) are about 4 times higher than in the medium. In this experiment, illumination causes a 15 % stimulation of the uptake (see also Fig. 4 and Table IV).

The accumulation of CO_2 + bicarbonate is strongly dependent on the pH in the medium (Fig. 3). At pH 7.0 the concentration in the chloroplasts is about 19 times higher than in the medium, at pH 8.5 it is only 1.5 times higher. This accumulation factor is independent of the bicarbonate concentration for a relatively long concentration range (Fig. 4). It shows that the bicarbonate concentration in the chloroplast can be very high. In extreme cases (spinach harvested in winter)

linearity of uptake was observed up to a concentration of 300 mM bicarbonate in the chloroplasts (K. Werdan and H. W. Heldt, unpublished results). Again the uptake of CO_2 + bicarbonate is enhanced by illumination, as shown in the experiment in Fig. 4. This stimulation decreases with higher bicarbonate concentrations.

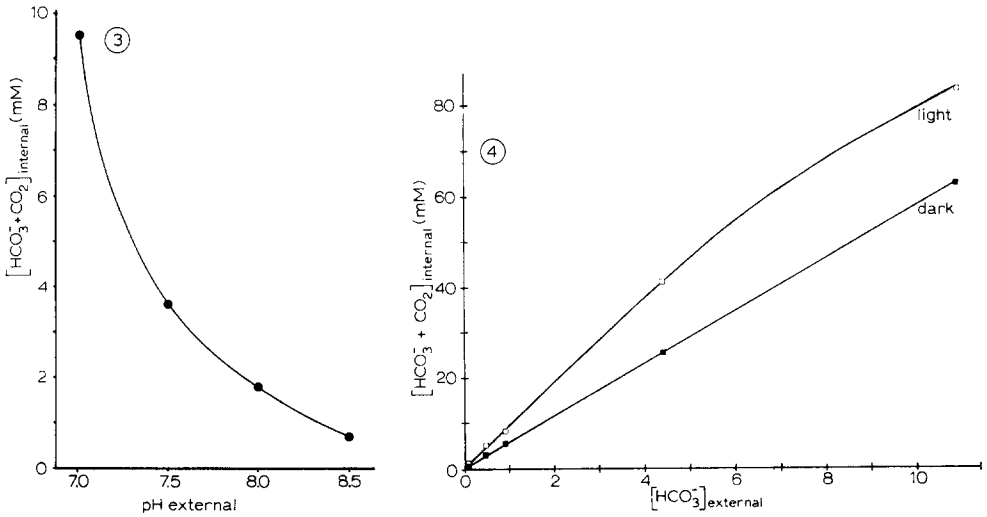

Fig. 3. Bicarbonate uptake into the sucrose-impermeable ^{3}HHO space of chloroplasts depending on the pH in the medium. Bicarbonate in the medium 0.5 mM, 17 μg chlorophyll per ml, incubation time 3 min, illumination.

Fig. 4. Bicarbonate uptake into the sucrose-impermeable ^{3}HHO space of chloroplasts depending on the bicarbonate concentration in the medium. 92 μg chlorophyll per ml, incubation time 3 min.

The question of whether transport of bicarbonate may be accompanied by counter transport of another anion was investigated. We tested a number of anionic compounds, which are known to be present in the chloroplasts and are readily transported across the inner membrane, *e.g.* inorganic phosphate, 3-phosphoglycerate, aspartate, glutamate and malate[2,3,10–12]. The chloroplasts were preloaded with one of these compounds, and washed afterwards[3]. As shown earlier, inorganic phosphate and 3-phosphoglycerate are liberated by the addition of inorganic phosphate; and aspartate, glutamate and malate are liberated by the addition of malate[3]. However, none of these compounds were released on addition of bicarbonate (Table I). From this it is concluded that there is no counter exchange of bicarbonate with one of these anions.

(2) *Carbonic anhydrase in chloroplasts*

The dehydration of carbonic acid and the hydration of CO_2 are relatively slow processes[13]. As shown in Table II a high activity of carbonic anhydrase, catalyzing these reactions, is present in spinach chloroplasts. Similar results have been reported earlier[14–16]. For the assay of carbonic anhydrase in spinach leaves the material was homogenized in a hypotonic medium (see Methods). In this way, the chloroplasts were all disrupted. In another experiment (not shown here) boiling of the homogenate destroyed 99% of the carbonic anhydrase activity, thus showing

TABLE I

RELEASE OF RADIOACTIVELY LABELLED INORGANIC PHOSPHATE, 3-PHOSPHOGLYCERATE, MALATE, GLUTAMATE AND ASPARTATE FROM THE CHLOROPLASTS ON ADDITION OF BICARBONATE

The chloroplasts were incubated for 2 min at 4 °C with radioactively labelled anions (1 mM) and washed afterwards. The back exchange was started by the addition of unlabelled anions, and was terminated by filtering centrifugation. The amount of substances released was calculated from the radioactivity in the supernatant.

Anion added	*Radioactive substance released (nmoles/mg chlorophyll, 20 s, 4 °C)*				
	Inorganic phosphate	*3-Phospho-glycerate*	*Malate*	*Glutamate*	*Aspartate*
Bicarbonate	0.1	0.1	0.5	0.1	0.8
3-Phosphoglycerate	47.3	15.5	0.1	0.4	0.5
Malate	0.2	0.2	46.4	13.1	25.0

TABLE II

CARBONIC ANHYDRASE ACTIVITY IN SPINACH LEAVES

For details see Methods.

	Activity (μmoles/min per mg chlorophyll, 0 °C)
Leaf homogenate A, 75 s homogenized	3580
Leaf homogenate B, 150 s homogenized	3540
Supernatant from homogenate A	3690
Intact chloroplasts	165
Intact chloroplasts, treated with an osmotic shock before assay	2980

that the carbonic anhydrase activity is due to an enzyme. The activity in the homogenate is not changed when the time of homogenisation is doubled. This indicates that the homogenisation is complete and that there are no losses of enzyme activity during the homogenisation. Furthermore, it was found that the activity of carbonic anhydrase in the homogenate did not decrease during the time of the experiment. The total activity is found in the supernatant of the homogenate, which shows that all the enzyme is soluble.

85 % of the total carbonic anhydrase activity in the leaf, as related to the chlorophyll content, is found in isolated chloroplasts. From phase contrast microscopy it was estimated that about 80 % of the chloroplasts in this preparation were structurally intact. Most of the 20 % broken chloroplasts, which still retain chlorophyll, will have lost the soluble enzyme when the chloroplast preparation was washed. Therefore, the actual activity of this enzyme related to chlorophyll content in the chloroplast will be higher than measured. It has also been observed by Everson and Slack[14] that in spinach a large proportion of the carbonic anhydrase activity is located in the chloroplasts, whereas it has been concluded by Poincelot[16] that only 63 % of the enzyme occurs in the chloroplasts. All these data show that at least most of this enzyme is located in the chloroplasts.

The carbonic anhydrase activity in intact spinach chloroplasts is latent, with the exception of a very low activity, which could be attributed to some chloroplasts which may have been broken in the suspension. In order to assay carbonic anhydrase the chloroplasts are treated with an osmotic shock. For the assay CO_2-saturated medium is added and the formation of protons followed using a pH electrode.

$$CO_2 + H_2O \xrightarrow{\text{Carbonic anhydrase}} HCO_3^- + H^+$$

The latency of this enzyme implies that the inner membrane surrounding the chloroplast is either impermeable to the CO_2 required, or to the protons formed. On the one hand CO_2 is known to be lipid soluble and to be able to diffuse rapidly through artificial membranes[17], on the other hand there is evidence that the inner membrane is impermeable to protons (see next paragraph). From these data it is safe to conclude that the products of the reaction, protons and bicarbonate, cannot leave the stroma space. It has been proposed that carbonic anhydrase is present both on the surface and inside spinach chloroplasts[15]. From our data there seems to be no evidence for this conclusion. Functionally the carbonic anhydrase appears to be located in the stroma, and not to be accessible to protons and bicarbonate from the external space.

(3) *How does the inorganic carbon get into the chloroplast?*

The uptake of inorganic carbon (CO_2 and bicarbonate) could involve a transfer of bicarbonate or of CO_2. The latter, as mentioned before, has been shown to diffuse through artificial phospholipid bilayers with a velocity similar to diffusion through water[17]. Since membranes are principally impermeable to ions, a direct transport of bicarbonate would require a special mechanism, a carrier for instance. If there were to be transport of bicarbonate, either a cation would have to be transported in the same direction or another anion in the opposite direction, for the sake of electroneutrality. If the cation is a proton, or the anion a hydroxyl ion, which comes to the same thing, the bicarbonate transport would involve the transfer of a proton. From the data shown here, a decision between these different possibilities can be made.

For a counter exchange to occur, a very high concentration of exchangeable anions within the chloroplasts would be required, since there is a linear relationship observed between the concentration of CO_2 + bicarbonate inside and outside the chloroplasts up to rather high concentrations (Fig. 4). In the experiments of Table I none of the anions tested (which are present in the chloroplasts in considerable amounts) appeared to be involved in a counter exchange with bicarbonate. For these reasons, and also in view of the very high velocity of $CO_2 + HCO^-_3$ uptake (Figs 1 and 2) a specific counter exchange of bicarbonate with another anion may be regarded as being most unlikely. On the assumption that the permeability properties of the inner membrane are the same for both directions of transport, cotransport of bicarbonate together with a proton can be also excluded on the grounds of the latency of carbonic anhydrase activity within the chloroplasts.

It appears from the data of Fig. 3 that the distribution of bicarbonate follows a pH gradient. This implies that the inner membrane separating the stroma from the external space is impermeable to protons. Impermeability of the inner membrane to protons has been also concluded by Heber and Krause[18]. From these findings

it seems to be a plausible suggestion that the transfer of inorganic carbon into the chloroplasts proceeds by a diffusion of CO_2 across the inner membrane:

$$HCO_3^- + H^+ \rightleftharpoons H_2O + CO_2 \overset{\text{External}}{\underset{\text{Inner membrane}}{\rightleftharpoons\!\!\!\!\rightleftharpoons}} CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad \text{(Internal)}$$

In the system we are dealing with, bicarbonate was added to a chloroplast suspension. It is feasible that the CO_2, which is in equilibrium with the external bicarbonate (partly due to some carbonic anhydrase activity, probably arising from chloroplasts which have been broken in the suspension), diffuses across the inner membrane and is hydrated in the stroma by the carbonic anhydrase located there. In this way, CO_2 diffusion facilitates an indirect cotransport of bicarbonate and a proton across the inner membrane.

There have been speculations about this rather obvious possibility before[19]. The diffusion of CO_2 across the inner membrane might be so rapid that there are equal CO_2 concentrations on either side of the membrane. In this case, the logarithm of the ratio of the internal (int) and external (ext) bicarbonate concentrations should be equal to the ΔpH across the inner membrane (Eqn 3).

$$\frac{[H^+]\cdot[HCO_3^-]}{CO_2} = K \tag{1}$$

$$[H^+]_{int}\cdot[HCO_3^-]_{int} = [H^+]_{ext}\cdot[HCO_3^-]_{ext} \tag{2}$$

$$\log\frac{[HCO_3^-]_{int}}{[HCO_3^-]_{ext}} = pH_{int} - pH_{ext} \tag{3}$$

The validity of this mechanism can be tested by plotting the logarithm of the ratio $[HCO_3^-]_{int}/[HCO_3^-]_{ext}$ against the ΔpH across the inner membrane (Eqn 3). For this it is necessary to know the internal pH. In the experiment shown in Fig. 5 the external pH was varied and the internal pH was assayed from the distribution of [^{14}C]dimethyloxazolidinedione (DMO)[20]. For the different values of external pH the CO_2 concentrations were calculated according to Eqn 1, and were subtracted from the sums of HCO^-_3 and CO_2 in both compartments, yielding the corresponding bicarbonate concentrations. The experimental points are very close to the theoretical linear function with a slope of 1. Thus our experimental data seem to agree rather well with the above mechanism.

(4) *The pH of the stroma*

Previously the pH in the thylakoids had been evaluated from the distribution of DMO[21] and of methylamines[22,23]. It appears from the results of Fig. 5 that the pH in the stroma can be calculated either from the distribution of bicarbonate or of DMO. With both compounds essentially the same results are obtained, demonstrating the validity of the method. The pH values for the stroma in the dark calculated in this way show a large variability depending on the conditions of growth and of the experiment. Values from pH 6.8–8.8 have been observed (H. W. Heldt, K. Werdan and G. Geller, unpublished).

When chloroplasts are illuminated, the accumulation of bicarbonate is en-

hanced (Table III, see also Fig. 4), indicating an alkalisation of the stroma, which might be due to light-driven proton transport across the thylakoid membrane[24]. Using DMO and methylamine, we have shown recently that this light-induced alkalisation of the stroma is indeed accompanied by an acidification of the thylakoid space[20]. A detailed report on these pH changes in intact chloroplasts is in preparation.

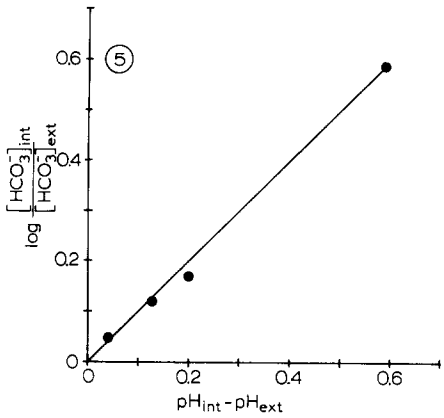

Fig. 5. The logarithm of bicarbonate distribution between the sucrose-impermeable ^{3}HHO space and the medium depending on the ΔpH between these two spaces. Temp. 8 °C, medium: 3.3 mM bicarbonate, 1.0 mM DMO, 80 μg chlorophyll per ml, sucrose-impermeable ^{3}HHO space 25 μl/mg chlorophyll. For the calculation of pH from DMO distribution see ref. 20. The measurement of pH and of bicarbonate distribution was carried out in parallel samples containing either [^{14}C]-bicarbonate + unlabelled DMO or unlabelled bicarbonate + [^{14}C]DMO. The specific activity of DMO (N.E.N. chemicals) was 0.3 Ci/mole. The samples were illuminated.

TABLE III

CALCULATION OF THE pH IN THE STROMA FROM THE BICARBONATE UPTAKE INTO THE SUCROSE-IMPERMEABLE ^{3}HHO SPACE OF CHLOROPLASTS

Bicarbonate in the medium 3.3 mM, temp. 4 °C, incubation time 3 min, 57 μg chlorophyll per ml.

Conditions of incubation	*HCO_3^- uptake (mM)*	*Internal pH*
Dark	2.08	7.40
Light	6.31	7.88
Light + carbonylcyanide *m*-chlorophenylhydrazone (1 μM)	1.68	7.31

The effect of light on the pH in the stroma is abolished by low concentrations of uncouplers, *e.g.* carbonylcyanide *m*-chlorophenylhydrazone, which are known to cause the decay of the light-dependent proton gradient across the thylakoid membrane[25]. This is further support for our conclusion that the increase of the pH in the stroma on illumination is due to light-driven proton transport into the thylakoids.

(5) *The effect of Diamox*

Diamox, an inhibitor of carbonic anhydrase (see ref. 26) inhibits the uptake of bicarbonate measured after 7 s (Table IV). The final level of bicarbonate uptake, as measured in this experiment after 120 s, is not influenced. Apparently it is the

TABLE IV

INHIBITION OF THE BICARBONATE UPTAKE INTO THE SUCROSE-IMPERMEABLE ^3HHO SPACE OF CHLOROPLASTS BY DIAMOX

Bicarbonate in the medium 0.1 mM, 32 μg chlorophyll per ml.

Time (*s*)	*Diamox* (*mM*)	HCO_3^- *uptake* (*mM*)	*Inhibition* (%)
7	—	0.31	
	0.5	0.12	61
120	—	0.60	
	0.5	0.62	0

rate of the uptake which is decreased by the inhibitor. This seems to be due to an inhibition of the hydration of CO_2 in the stroma. For the dehydration of bicarbonate in the medium there is actually no carbonic anhydrase required in our experiments because diluted chloroplast suspensions have been used. The amount of bicarbonate taken up by the chloroplasts is only 10 % of the CO_2 which is in equilibrium with the bicarbonate in the medium. However, Diamox did not always inhibit CO_2 accumulation in our experiments. As the carbonic anhydrase in the stroma is very active, it may be sometimes difficult to obtain an inhibition strong enough to be visualized.

With 1 mM Diamox, CO_2 fixation of intact chloroplasts using bicarbonate was shown to be inhibited by 50 %[19]. From this result it had been concluded that carbonic anhydrase is involved in the overall pathway of CO_2 fixation. Unfortunately 1 mM Diamox does not only inhibit carbonic anhydrase, but it has also been shown to inhibit Photosystem II and therefore also the photoreduction of 3-phosphoglycerate[27], which is part of the overall process of CO_2 fixation. Independently we have also observed this inhibition of 3-phosphoglycerate reduction in intact chloroplasts on the addition of Diamox. These findings raise the possibility that the inhibition of CO_2 fixation by Diamox does not involve carbonic anhydrase activity. For this reason, Diamox seems to be a rather unreliable tool for elucidating the role of carbonic anhydrase in CO_2 fixation.

(6) *On the physiological role of carbonic anhydrase and the bicarbonate pool*

The CO_2 concentration needed for half-maximal rate of CO_2 fixation is about two orders of magnitude higher when envelope-free chloroplasts or purified ribulose diphosphate carboxylase are employed instead of intact chloroplasts or whole plants (see ref. 28). It was shown that the affinity for CO_2 of the purified enzyme is enhanced by adding Mg^{2+} (refs 29 and 30). But even in the presence of high concentrations of Mg^{2+}, the affinity for CO_2 of a reconstituted system for CO_2 fixation using envelope-free chloroplasts is about 1 order of magnitude lower than for CO_2 fixation of intact chloroplasts[31]. It has been presumed that the CO_2 concentration at the site of CO_2 fixation may be elevated by active transport of CO_2 or bicarbonate[32,33]. It was further speculated that an accumulation of bicarbonate might be achieved by a pH gradient across the envelope[19]. The present publication shows this to be the fact. However, this does not solve the problem, since CO_2 and not bicarbonate was found to be the substrate for purified ribulose diphosphate carboxylase[34] and the CO_2 concentration in water, being in equilibrium with the CO_2 in the air, is not affected by the pH.

The following question arises: What is the function of the very high activity of carbonic anhydrase found in the chloroplast stroma? Since the transfer of inorganic carbon from the cytoplasm to the stroma occurs by diffusion of CO_2 and since CO_2 is the substrate for ribulose diphosphate carboxylase, one might conclude that the formation of bicarbonate catalyzed by carbonic anhydrase is not in the pathway of CO_2 fixation, but in a blind alley. Because of the relatively high pH in the stroma, the CO_2–bicarbonate equilibrium is shifted to bicarbonate. Therefore the bicarbonate pool in the chloroplasts will function as a CO_2 buffer. But this buffer has only a limited capacity. The CO_2 thus stored would only last for seconds if photosynthesis is proceeding at the maximal rate. As well as this function, it should be realized that carbonic anhydrase may play an essential role in the utilization of CO_2 present in low concentrations. This seems obvious from the comparison of carbonic anhydrase activity in different species and its dependence on growth conditions. Carbonic anhydrase was found to be absent in chloroplasts from plants with the C_4-dicarboxylic pathway[14]. The physiological role of the C_4-dicarboxylic pathway is understood to be to concentrate CO_2 for utilization by ribulose diphosphate carboxylase[35]. Furthermore, it has been shown that in some green algae carbonic anhydrase is absent when the algae are grown in 5 % CO_2, whereas the enzyme is present in algae grown with low CO_2 concentration[36]. These results led to the speculation that carbonic anhydrase may be physically associated with ribulose diphosphate carboxylase in order to increase the local availability of CO_2.

In view of our results, this hypothesis could be more clearly defined. It may be visualized that there are regions in the stroma proteins where protons can be localized. The carboxylation of ribulose diphosphate leads to the formation of two protons. At the site where these protons are formed, the bicarbonate equilibrium could be shifted to the formation of CO_2. The very high activity of carbonic anhydrase would enable a rapid response of CO_2 formation from bicarbonate following the generation of these protons. The ribulose diphosphate carboxylase may be arranged in such a way that the CO_2 liberated by carbonic anhydrase is bound to the enzyme before it diffuses from its microenvironment. In this way a local rise of CO_2 concentration may make possible CO_2 fixation by means of local acidification. It is likely that in the dense protein gel of the stroma the enzymes are not distributed at random, but organized in a certain structure. In the highly diluted enzyme solutions which are usually employed *in vitro*, it may be not possible to demonstrate such a cooperation of the two enzymes. Therefore, negative results in attempting to isolate an enzyme complex from ribulose diphosphate carboxylase and carbonican hydrase[36] may not disprove this hypothesis.

This mechanism implies that bicarbonate is actually the substrate utilized in CO_2 fixation of intact chloroplasts, due to the cooperation of the two enzymes. In this scheme, the proton gradient across the inner membrane which enables the accumulation of bicarbonate in the stroma would be responsible for the high CO_2 affinity of CO_2 fixation in intact chloroplasts.

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