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Evidence for Mediated HCO_3^- Transport in Isolated Pea Mesophyll Protoplasts[1]

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ABSTRACT

The kinetics of ^{14}C fixation, and inorganic C (C_{inorg}) accumulation, have been followed in isolated pea mesophyll protoplasts. $NaH^{14}CO_3$ was supplied to the protoplasts in media the pH of which was varied between 7 and 8.

When $^{14}CO_2$ fixation was plotted against the calculated concentration of free CO_2 in the media, the apparent K_m for CO_2 was observed to rise as external pH increased. The V_{max} did not alter significantly. Similarly, when C_{inorg} uptake, either in the light or in the dark, was plotted against external CO_2 concentration the slope of the curves was steeper at higher external pH.

Investigation of the time course of uptake showed that internal C_{inorg} concentration rose throughout the experimental period, and that in the light it surpassed the external C_{inorg} concentration after about 3 minutes. Irradiation of protoplasts previously taking up $^{14}C_{inorg}$ in the dark brought about a sharp increase in the rate of $^{14}C_{inorg}$ accumulation which was sustained for at least 20 minutes.

Estimates of internal pH based on the distribution of labeled 5,5-dimethyloxazoladine-2,4-dione (DMO) between protoplast and medium suggested that internal pH altered relatively little with change in external pH. The values for internal pH as calculated from C_{inorg} distribution were always higher than those calculated from DMO distribution, *i.e.* the internal C_{inorg} concentration was higher than would be predicted on the assumption of passive distribution in accordance with pH.

Addition of carbonic anhydrase to the external solution was without effect either on rate of $^{14}CO_2$ fixation or C_{inorg} accumulation.

Various possible interpretations of the results are considered. It is concluded that the most reasonable explanation, consistent with all the data, is that HCO_3^- ions can cross the protoplast membranes, and that their passage is mediated by a transfer mechanism.

It has often been assumed that only free CO_2, and not HCO_3^-, is the C_{inorg}[2] species transferred to the carboxylating site in C_3 plants (*e.g.* 9, 28). The ability of aqueous angiosperms to utilize HCO_3^- from the medium (3, 8, 18) has been attributed by Raven (21) to a secondary adaptation to submerged conditions. Relevant experimental data, however, either for or against this view are scarce. Jones and Osmond (15) and Ullrich-Eberius *et al.* (27) studied the dependence of photosynthetic O_2 evolution by leaf slices on the external C_{inorg} concentration at various pH values. They concluded that CO_2 is in fact the only species transferred. Poincelot (20) and Champigny and Bismuth (6) have deduced that spinach chloroplast envelopes are capable of transferring HCO_3^- at a rate which is highly significant for the provision of C_{inorg} to the chloroplast stroma. This work has been challenged by Heldt (13) who maintains that C_{inorg} uptake proceeds via diffusion of CO_2 across the chloroplast envelope.

The work to be reported here has been carried out with isolated pea mesophyll protoplasts. The isolated protoplast system has the following advantages:

Where the object of investigation is a membrane process, it is desirable that the membrane should be directly exposed to the solute species in question, without intervening compartments such as the cell walls and intercellular spaces.

The bicarbonate transfer mechanism, if it exists, may be sited in the plasmalemma. Investigation of isolated chloroplasts, therefore, would not reveal it (see 28) but it should be detectable in protoplasts.

A suspension of mesophyll protoplasts is a relatively uniform population, and allows precise control of factors at the membrane surface such as pH, concentration of solutes, etc.

In the present investigation we have attempted to collect data which could contribute to a decision as to whether HCO_3^- can cross the protoplast membranes as an anion; if so, whether it crosses by diffusion or with the aid of a transport mechanism; and whether HCO_3^- transport capacity contributes toward limiting the rate of photosynthesis.

MATERIALS AND METHODS

The experimental plant was *Pisum sativum* L. var. "Dan," and conditions of growth and the procedure for isolating protoplasts were as described in earlier papers (9, 10). Protoplasts were suspended in a medium made up of 0.55 M mannitol, 1 mM KNO_3, 0.2 mM KH_2PO_4, 0.5 mM $CaCl_2$, 3 mM $MgSO_4$ (pH 5.8).

Accumulation of acid-stable and acid-labile ^{14}C during incubation of the protoplasts with $^{14}C_{inorg}$ was followed by means of a filtering centrifugation technique similar to that described earlier (17, 28). Aliquots of 20 μl protoplast suspension were transferred to 400 μl plastic tubes already containing the following layers (from top to bottom): 180 μl suspension medium buffered to the required pH with a Mes-Hepes mixture, each 25 mM, which had been degassed under vacuum and flushed with N_2; 70 μl of a 4:1 mixture of 550 (50) and 510 (50) silicone oils (Dow-Corning); and 20 μl 3 M NaOH. The tubes were preincubated for 60 to 120 s in the light (45 nE cm^{-2} s^{-1}, 400–700 nm) before addition of $NaH^{14}CO_3$. After varying periods incubation was terminated by centrifugation of the protoplasts (microfuge B, Beckman Instruments) through the silicone oil into the NaOH, which trapped all inorganic C. Fifteen μl of 3 M NaOH were then added to the supernatant to avoid diffusion of CO_2 into the pellet. It was checked (in experiments where the protoplasts were omitted) that CO_2 diffusion from the supernatant through the silicone layer was negligible. The intactness of the protoplasts after centrifugation

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[2] Abbreviations: C_{inorg}, inorganic C; Evans Blue, 6,6'-[(3,3'-dimethyl-4,4'-biphenylene)bis(azo)]-bis(4-amino-5-hydroxy-1,3-naphthalenedisulfonic acid)tetrasodium salt; DMO, 5,5-dimethyloxazoladine-2,4-dione.

through silicone oil was examined by staining with Evans Blue (16). When protoplasts were centrifuged through a silicone oil layer into 1 M mannitol and subsequently treated with Evans Blue over 95% of the protoplasts excluded the dye, indicating that they were intact.

In certain longer term experiments where the effect of transition from dark to light, or DMO distribution between protoplast and medium, was examined, the protoplasts were incubated in tubes held in a glass water bath at 27 C, laterally illuminated. At intervals 200 μl aliquots were transferred to microfuge tubes and centrifuged through silicone oil as described above.

The microfuge tubes were next frozen in liquid N_2 and the tip containing the pellet was excised and placed in a closed vial containing 600 μl 0.1 N NaOH. Two samples of 200 μl each were withdrawn; one was transferred to an equal volume of 0.1 N NaOH while the other was transferred to an equal volume of 0.5 N HCl and left at room temperature for 30 min, during which it was frequently flushed with CO_2 to remove acid-labile ^{14}C. Radioactivity in both samples was measured in a Packard Tri-Carb 3385 liquid scintillation spectrophotometer, quenching being corrected for by an automatic external source ratio.

Rates of ^{14}C fixation were related to the Chl content of the pellet measured on parallel aliquots where the protoplasts were centrifuged into 1 M mannitol instead of NaOH and subsequently extracted with 80% acetone.

To estimate the volume of the cells in each aliquot, a sample of the cell suspension was incubated simultaneously with [^{14}C]mannitol (0.1 μCi) and tritiated H_2O (2 μCi) for 10 s under conditions identical to those of the $NaH^{14}CO_3$ or DMO incubations described above. "Water space" and "mannitol space" were given by the ratio between the dpm of each isotope in the pellet and the corresponding dpm in 10 μl of supernatant. The mannitol-impermeable space (*i.e.* THO space—mannitol space) was regarded as a measure of intracellular volume. Intracellular concentrations were calculated after correcting the counts in the pellet attributable to the solute under consideration for the amount of solute present in the extracellular volume (*i.e.* the "mannitol space"). One μl mannitol-impermeable space had a Chl content of about 6.5 μg.

Intracellular pH was estimated by applying the Henderson-Hasselbach equation

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

to the observed distribution of weak acids between protoplast volume and medium; where pK_a is the pK of the weak acid and $[A^-]$ and [HA] are the concentrations of dissociated and undissociated acid, respectively. [HA] was first calculated for the known conditions of the medium and then, on the assumption the [HA] is in equilibrium across the membrane, internal pH was calculated from the observed total HA + A^- concentration in the protoplasts.

Chemicals. Tritiated H_2O (5 mCi/ml) was obtained from the Nuclear Research Centre, Israel.

$NaH^{14}CO_3$ and [^{14}C]mannitol were obtained from the Radiochemical Centre, Amersham, England.

[^{14}C]DMO was obtained from New England Nuclear. Unlabeled DMO was obtained from Sigma.

RESULTS

Dependence of CO_2 Fixation on Free CO_2 and Total C_{inorg} Concentration in the Medium. This relationship has been investigated in a series of experiments where the pH of the medium was varied between 7 and 8. Figure 1a shows the curves relating rate of CO_2 fixation to total external C_{inorg} concentration in one such experiment where the pH values investigated were 7.1 and 7.7. The curve for pH 7.1 lies to the left of that for pH 7.7 up to a C_{inorg} concentration of approximately 0.55 mM, where the two

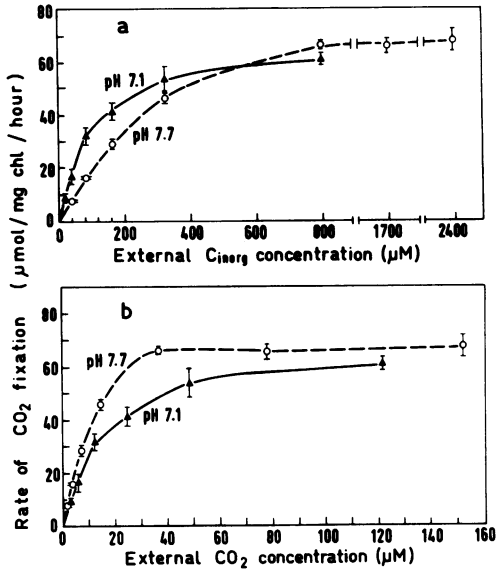

FIG. 1. Rate of CO_2 fixation by pea mesophyll protoplasts as a function (a) of total C_{inorg} concentration or (b) of free CO_2 concentration as calculated using $pK_a = 6.37$. Protoplasts were preincubated for 30 s in the light before addition of $NaH^{14}CO_3$. Incubation period 75 s; suspension medium buffered with 50 mM Mes + 50 mM Hepes; 25 C. Error bars indicate ± SD from plotted mean of triplicates.

curves cross. In Figure 1b, where the rate of CO_2 fixation is plotted against the calculated concentration of free CO_2 in the media, the curves for the two pH values do not coincide, but now the opposite picture is obtained—that for pH 7.7 lies to the left of that for pH 7.1. The apparent affinity for CO_2 is higher at the former pH. Plotting S/V against S (where S = substrate concentration and V = velocity) shows a relationship reasonably close to a straight line, justifying the use of Michaelis-Menten kinetics to arrive at an approximate characterization of the curves. In 10 experiments of the type exemplified in Figure 1, a and b, the K_m for CO_2 at the lower pH was always higher than that at the higher pH. According to the sign test (23) the probability of obtaining these results by chance is 0.0019 and the difference in K_m (CO_2) is thus highly significant. The V_{max} at the two pH values, however, did not differ significantly.

At least four possible explanations suggest themselves to account for the results reported above. The three set out below appear to be the most probable and have been addressed experimentally in this investigation:

(a) Not only CO_2 is penetrating the membrane; HCO_3^- is penetrating as well.

(b) Internal pH is varying with external pH, and is affecting the affinity of the carboxylating system (in particular RuBP carboxylase) for CO_2 (22).

(c) The rate of equilibration between CO_2 and HCO_3^- is pH-dependent, and external pH is thus affecting the rate of supply of CO_2 in the medium.

Possible Entry of HCO_3^- Ions. Figure 2 shows an experiment where the kinetics of C_{inorg} uptake were investigated both in the light and in the dark. To minimize the effect of C fixation on the kinetics of uptake, the experimental period was limited to 15 s, so that the proportion of fixed to free C was relatively small (about 40% in the light and less than 10% in the dark). The relationship between the rate of total C_{inorg} uptake and total external C_{inorg} concentration (Fig. 2a) is seen to be curvilinear and pH-dependent. If the data are replotted, this time against external CO_2 concentration (Fig. 2b), the two curves do not coincide, as would be expected if only CO_2 were taken up. However, the position of the curves relative to each other is now reversed—that for pH 8 lies to the

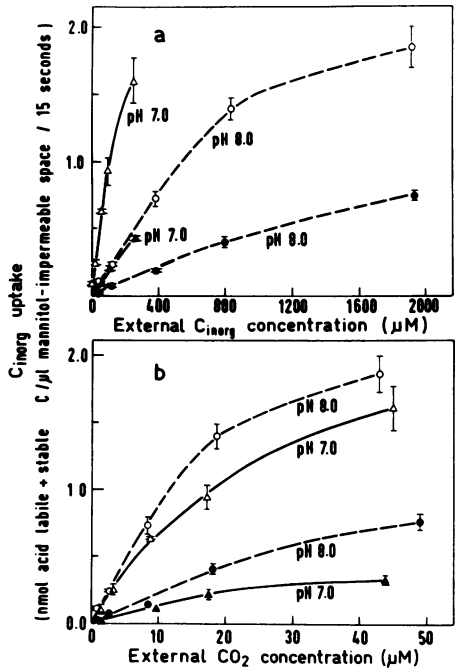

FIG. 2. Dependence of C_{inorg} uptake (nmol) by pea mesophyll protoplasts on total external C_{inorg} concentration (a) or CO_2 concentration (b) at two pH values in the light (○, △) and in the dark (●, ▲). Protoplasts were preincubated in the light for 45 s before addition of $NaH^{14}CO_3$. In the case of dark treatments, illumination ceased 10 s before addition of bicarbonate. Incubation period 15 s; buffer 25 mM Mes + 25 mM Hepes; 25 C. Error bars indicate ± SD from plotted mean of triplicates.

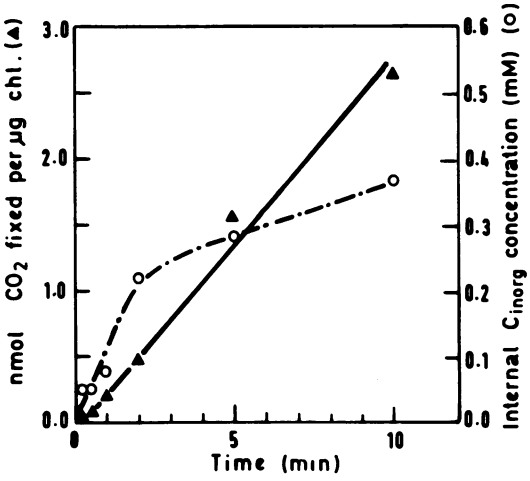

FIG. 3. Time course for C_{inorg} accumulation (right-hand scale) and CO_2 fixation (left-hand scale) by pea mesophyll protoplasts supplied with 0.24 mM $NaH^{14}CO_3$ at pH 7.45 in the light. Preincubation period 30 s in the light.

left of that for pH 7. Note that this result is obtained in the dark as well as in the light.

The progress of uptake with time is shown in Figure 3. The curves show:

The internal C_{inorg} concentration rises with time throughout the experimental period (10 min) though the rate of uptake tends to decrease.

After approximately 3 min the internal C_{inorg} concentration reaches and subsequently surpasses the external total C_{inorg} concentration. The latter effect was more pronounced when the external concentration was relatively low, *i.e.* up to about 0.6 mM.

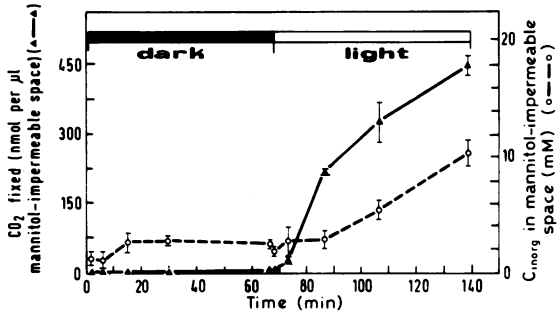

FIG. 4. Effect of transition from dark to light on CO_2 fixation (left-hand scale) and C_{inorg} accumulation (right-hand scale) by pea mesophyll protoplasts supplied with 5 mM $NaH^{14}CO_3$. Medium buffered at pH 7.5 with 50 mM Hepes. Error bars indicate ± SD from plotted means of triplicates.

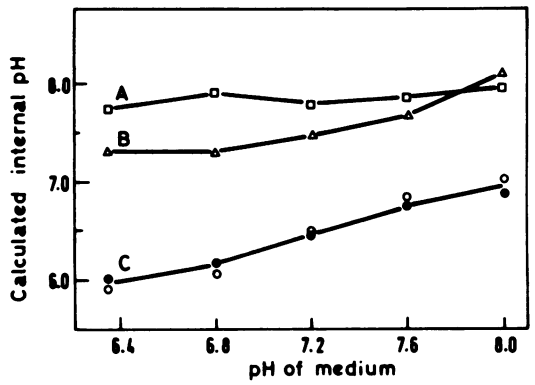

FIG. 5. Internal pH in pea mesophyll protoplasts as affected by external pH. Curves A and B calculated from C_{inorg} distribution between protoplasts and medium, Curve C from DMO distribution. Curve A, $^{14}C_{inorg}$ concentration in medium ranging from 33 μM at pH 6.4 to 710 μM at pH 8, CO_2 concentration constant, DMO concentration 20 μM. Curve B, $^{14}C_{inorg}$ concentration 500 μM, DMO concentration 20 μM. Curve C, C_{inorg} concentration 500 μM, [^{14}C]DMO concentration 20 μM. Buffer Mes-Hepes mixture, each 25 mM. Pretreatment in buffer + unlabeled substance for 2 min. Incubation with [^{14}C]DMO was for 2 min (●) or 7 min (○). Incubation with $NaH^{14}CO_3$ was for 2 min.

A very slight lag following the introduction of $^{14}CO_2$ is detectable in the curve for fixed C. This lag was frequently, though not always, present.

Figure 4 shows the effect of light on the rate of accumulation of total C_{inorg}. Experiments of this type were relatively long-term, the protoplasts being supplied with labeled C_{inorg} for 30 to 60 min before irradiation. During the preliminary dark period the level of C_{inorg} accumulation was relatively low. Irradiation produced a sharp increase in accumulation rate as indicated by the change of slope seen in Figure 4 (in the case of the experiment shown in Figure 4 after an initial lag, possibly related to the very high rate of CO_2 fixation at this period which may have depleted an internal pool). A rate of C_{inorg} accumulation well above the dark rate was maintained for at least 20 min. In the dark the internal C_{inorg} level did not reach the external; in the light, however, it rose above it. Parallel experiments were carried out with DMO (see next section below) to examine whether the C_{inorg} uptake curve was reflecting a pH change in the protoplasts. No appreciable change in overall pH could be observed after dark to light transition.

Effect of Rise in External pH on the Δ pH Between Medium and Protoplast. We have attempted, as have many other workers (*e.g.* 7, 14) to estimate internal pH in protoplasts by following the distribution of the weak acid DMO between the protoplast and the medium. We recognize, however, that information gained in

this way relates only to the overall DMO distribution inside the protoplast, and does not give precise information as to the pH in any one of its several compartments. However, because the pK for DMO is close to that for H_2CO_3, passive distribution of H_2CO_3 should follow that of DMO through all subcellular compartments. We have taken the pK of DMO over our temperature range as 6.36 (1).

An experiment in which the DMO distribution was determined at a number of external pH values is shown in Figure 5 (curve C). A certain degree of dependence of internal pH on external pH is seen. In seven experiments of this type, overall internal pH rose from 0.3 to 0.6 units per rise of 1 pH unit in the medium.

We have also calculated the internal pH on the basis of C_{inorg} distribution. The assumptions here are that the CO_2 penetrates the membranes so quickly that diffusion equilibrium between the cell interior and exterior is rapidly set up (4); and that the difference in HCO_3^- concentration between the cell interior and exterior is a function of Δ pH according to the Henderson-Hasselbach equation. Figure 5 compares the values for internal pH as calculated according to DMO distribution and C_{inorg} distribution, respectively. In order that the treatments should be comparable, C_{inorg} and DMO were supplied simultaneously to all samples. In the case of curve B the $^{14}C_{inorg}$ concentratioin was kept constant over the pH range while for curve A $^{14}C_{inorg}$ concentration was varied so that $^{14}CO_2$ concentration remained constant; in both cases DMO was provided at the same concentration as in the labeled DMO treatment. Figure 5 shows that the estimates of pH based on DMO distribution are lower than those based on C_{inorg}. More C_{inorg} is detected in the cells than would be predicted on the basis of the pH indicated by DMO, were the distribution of C_{inorg} governed by the assumptions set out above. Two min incubation with DMO gave virtually the same results as 7 min incubation (Fig. 5). The lower pH estimates based on DMO distribution are, therefore, not likely to be due to the fact that DMO has not yet equilibrated across the membrane.

Further, the pH indicated by the distribution of C_{inorg} is seen to depend on C_{inorg} concentration. Where the latter was higher (curve B) a lower pH is calculated. This again indicates that the relation between C_{inorg} uptake and C_{inorg} concentration is nonlinear, and may suggest there is a saturable component contributing to C_{inorg} uptake (*e.g.* a carrier) and at higher concentrations uptake consequently falls below that predicted for diffusion. (However, an alternative explanation might be that higher internal concentrations cause acidification).

Promotion of Rate at Which Equilibrium Reached Between CO_2 and HCO_3^-. The effect of adding carbonic anhydrase to the external solution was examined both when the pH of the latter was 7 and when it was 8. The final concentration of the added enzyme was 0.5 mg/ml. No effect was observable either on the rate of $^{14}CO_2$ fixation nor on total C_{inorg} detectable in the protoplasts when the latter was followed over 2 min (data not shown).

DISCUSSION

As we have pointed out previously, although it is believed that in microalgae (2) and in some aquatic angiosperms a transport mechanism operates for the uptake of HCO_3^- ions, it is commonly assumed that in higher land plants virtually only free CO_2 penetrates to the carboxylation site, and that this occurs by diffusion. The present investigation has provided indications that bicarbonate may be taken up in the form of the anion by isolated pea mesophyll protoplasts. The main indications are provided by the following findings:

(a) If CO_2 were in fact the sole penetrating species, the curve for ^{14}C fixation as a function of external C_{inorg} concentration at pH 7 would be expected to differ from that at pH 8, owing to the different ratio of CO_2:HCO_3^- for a given C_{inorg} concentration at the two pH values: but the curves would be expected to coincide when plotted against external CO_2 concentration. This however was not the case. A statistically significant difference in apparent affinity for external CO_2 was observed at the two pH values. On the other hand the V_{max} values were not appreciably different at the two pH values. Servaites and Ogren (22) have reported similar observations for isolated soybean mesophyll cells.

(b) Similarly, for a given external CO_2 concentration, uptake of C_{inorg} was higher at pH 8 than at pH 7 (Fig. 2). This effect was expressed after 15 s or less of incubation, and was shown both in the light and in the dark. In the latter case fixed ^{14}C formed a relatively small part of the total internal ^{14}C pool.

In algae, increased apparent photosynthetic affinity for CO_2 with increasing pH has been regarded as evidence for the utilization of bicarbonate ions from the medium (17, 21). Servaites and Ogren (22) attributed the increased apparent affinity which they observed in their soybean cell system to increased affinity of RuBP carboxylase for CO_2. That a change in internal pH of sufficient magnitude to bring about the required change in the K_m (CO_2) of RuBP carboxylase (22) can wholly explain our own findings seems unlikely for the following considerations. First, our observations suggest that overall internal pH rose relatively slightly with rising external pH, and other investigators using a variety of techniques have reported that the internal change is even smaller (25). Two membranes and the buffering zone of cytoplasm separate the external medium from the chloroplast stroma where RuBP carboxylase is sited and the results suggest that stromal pH would not be very sensitive to change in extracellular pH. Secondly, stromal pH is not likely to rise continuously with time under constant external conditions. Yet, C_{inorg} uptake as observed in this investigation was protracted (Figs. 3 and 4). This continued uptake over many minutes contrasts with the observations of Werdan and Heldt (28) on isolated chloroplasts, whose results (in experiments carried out at 4–9 C) indicate that C_{inorg} uptake occurred with a $t_{1/2}$ of approximately 10 s. These workers are of the opinion that CO_2 is in diffusion equilibrium across the membrane, and that C_{inorg} accumulation within the chloroplast is accounted for by the Δ pH between the medium and the stroma, the latter being the more alkaline in the light. However, one would expect alkalization of the stroma to be complete within a few seconds of illumination or at most within a minute or two (11, 12) and our experiments showed continued uptake in the light over a substantially longer period (Fig. 4).

Further Werdan and Heldt's (28) hypothesis would not seem to be applicable to our results with protoplasts, inasmuch as Figure 5 showed that C_{inorg} accumulates within the latter to a greater extent than would be predicted on the basis of Δ pH between medium and cell as indicated by the DMO estimation of internal pH. The fact that different compartments of the protoplast may be at different pH values does not invalidate this line of reasoning, since the passive distribution of C_{inorg} should be close to that of DMO in all compartments. We have evidence (Volokita and Kaplan, in preparation) that the DMO distribution is likely to overestimate rather than underestimate internal pH.

The third possibility set out above to explain our findings, that external pH is affecting the rate of supply of CO_2 in the medium via an effect on the rate of equilibration between CO_2 and HCO_3^-, also seems to have low probability in view of the fact that addition of carbonic anhydrase to the medium was without effect either on $^{14}CO_2$ fixation rate or on the level of C_{inorg} detectable in the protoplasts.

There is a fourth possibility that might be considered. The passive permeability of the cell membranes for CO_2 might be altered by pH. This possibility, however, is purely speculative and has no basis, so far as we are aware, in the literature.

We conclude that the most reasonable explanation consistent with all our data is that HCO_3^- can penetrate the protoplast membrane. The difference between our results and those of Wer-

dan and Heldt (28) might indicate that the plasmalemma, but not the chloroplast inner membrane, can be penetrated by HCO_3^-; or alternatively that an HCO_3^- transfer mechanism does exist in the chloroplast inner membrane (*cf.* Poincelot 20) but does not function efficiently over the low temperature range of Werdan and Heldt's experiments.

Data which suggest that CO_2 is the only C_{inorg} species utilized in cotton leaf strips (15) and pea leaf strips (Kaplan, unpublished) may reflect the fact that CO_2 being a smaller and uncharged molecule diffuses much more rapidly than does HCO_3^- through the free space of the tissue, where fixed negative charges impede the progress of the anion: the uptake of the latter may therefore be masked.

If HCO_3^- in fact penetrates our protoplasts, as we have concluded it does, then it is highly likely that it can only do so by virtue of a transfer mechanism, since the passive driving force acting on this ion will be outwards from the cytoplasm as soon as the internal concentration has reached a value approximately one-tenth of the external concentration (*i.e.* the direction of the electrochemical potential gradient will be outwards, assuming a plasmalemma potential of −50mv [5]). Supporting evidence for the existence of an HCO_3^- transfer mechanism is provided by the following considerations:

The protracted uptake of HCO_3^- in the light cannot be explained satisfactorily by a continuous rise in internal pH (as already pointed out above); and probably not by continuous slow conversion of CO_2 to HCO_3^- within the cell, since the activity of carbonic anhydrase is known to be high in chloroplasts, the compartment where the pH is highest. The most likely explanation of the protracted uptake is continuous operation of a transfer mechanism.

In the light the internal C_{inorg} concentration reaches and surpasses the external. Accumulation ratios as high as 10 have been observed. Accumulation due to the activity of a transfer mechanism appears to be the most likely postulate, but this now raises questions as to the permeability barrier allowing intracellular accumulation of CO_2. Direct computation of the permeability of the plasmalemma to CO_2 has been attempted in the present investigation, and the figure arrived at is approximately 10^{-3} cm s^{-1} (taking into account the average diameter of protoplasts, osmotic volume, Δ $[CO_2]$ across the plasmalemma and initial CO_2 flux as measured in experiments such as in Fig. 3). This permeability coefficient is 10 to 100 times lower than that recently calculated for erythrocytes (24). Whether the existence of a permeability barrier for CO_2 is the explanation of the accumulation noted in these experiments, or whether some other process such as binding to cellular proteins including RuBP carboxylase is the explanation, must await clarification in further research.

Further investigation should also establish whether the presence of an HCO_3^- transfer mechanism can contribute towards resolving the discrepancy between the observed kinetic parameters of isolated RuBP carboxylase and those predicted on the basis of calculated CO_2 conductance to the carboxylation sites in intact leaves (26).

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