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Carbonic Anhydrase in Marine Algae[1]

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Abstract. **An electrometric system for determination of carbonic anhydrase activity was constructed. Enzyme activity was assayed in homogenates of marine macroscopic *Chlorophyta, Rhodophyta,* and *Phaeophyta.* Plants surveyed included *Ulva expansa* (Setchell) Setchell and Gardner, *Codium fragile* (Suringar) Hariot, *Enteromorpha* sp., *Chaetomorpha torta* (Farlow) McClatchie (*Chlorophyta*); *Laurencia papillosa* (Greville), *Plocamium coccineum* var. *pacificum* (Kylin) Dawson, *Pterocladia capillacea* (Gmelin) Bornet and Thuret, *Gigartina armata* J. Agardh (*Rhodophyta*); *Eisenia arborea* Areschoug and *Macrocystis pyrifera* (*Linnaeus*) C. A. Agardh (*Phaeophyta*). Activity was present in all algae; in the *Phaeophyta* this could be demonstrated only after dialysis. *p*-Chloromercuriphenylsulfonic acid (10^{-4} M) decreased activity in 1 species, *Plocamium;* this inhibition could be almost completely overcome with the addition of 10^{-3} M dithiothreitol. In 2 green and 2 red algae assayed for sensitivity to acetazolamide (Diamox), inhibition was complete at 10^{-4} M concentration of inhibitor. Dithiothreitol at a concentration of 10^{-3} M did not enhance activity in any of the homogenates, and was not necessary for enzyme expression.**

Carbonic anhydrase catalyzes the reaction: $H_2O + CO_2 \rightleftharpoons H^+ + HCO_3^-$. This reaction also takes place in the absence of the enzyme but apparently not at a rate fast enough for the normal metabolism of cells and organisms (10). No survey has yet been made on the presence or absence and distribution of carbonic anhydrase in marine macroscopic algae.[2] Its activity has been reported in 9 bacteria (22), microscopic algae (15, 21), higher plants (8, 24), invertebrates (18), and vertebrates (17), and probably is of fundamental importance wherever CO_2 is transported or exchanged.

Very little work has been done on the plant enzyme, but there are indications that the enzyme in plants is distinct from that found in animal tissues and red cells, although it is far from being clear to what extent this is so. Plant carbonic anhydrase is thought to be only weakly affected, if at all, by sulfonamides (5, 9) although the literature shows some variability (1, 11, 13, 19, 24). It has been found by some workers that free sulfhydryl groups are necessary for expression of the plant enzyme or for its enhancement (1, 23) but variability in this regard has also been reported (15, 24).

With this as a background, it was decided to initiate a study of representatives of the 3 major divisions of macroscopic algae in the sea—the *Chlorophyta, Rhodophyta,* and *Phaeophyta.* The first part of the study was the construction of an apparatus capable of measuring carbonic anhydrase activity in tissue homogenates. The most sensitive method was found to be an electrometric one whereby one measures enzyme activity in the hydration reaction by following continuously the change in hydrogen ion concentration: $H_2O + CO_2 \xrightarrow{\text{enzyme}} H^+ + HCO_3^-$. After this work was completed, a survey of the enzyme activity in the marine algae was begun. In addition, the importance of sulfhydryl groups in expression of activity was studied by addition of dithiothreitol, a sulfhydryl stabilizing agent (3), and the monosodium salt of *p*-chloromercuriphenylsulfonic acid, a sulfhydryl inhibitor. Also, sensitivity to a sulfonamide, acetazolamide (Diamox), was checked with 4 species.

Materials and Methods

The species of algae were collected at various intertidal and subtidal regions (to a depth of 20 feet) in the La Jolla area. Care was taken to select only those plants free from epiphytic growth. In the laboratory the plants were washed with a cold 3.5 % NaCl solution, blotted to absorb excess water, and weighed out in approximately 100 g quantities. This was done either immediately after collecting or within 18 hr of this time; running sea water aquaria were used as holding tanks. The homogenizing solution was 0.004 M NaH_2PO_4-Na_2HPO_4, of pH 7.0. The extractions were done in a semi-micro monel metal container on a Waring Blendor base; the homogenate

[1] Supported by Grant HE-10222 from the United States Public Health Service.

[2] Enzyme activity has been looked for previously in the brown alga, *Fucus serratus,* (2,20) but with negative results. As this work was completed Ikemori and Nishida (11) reported activity in a *Chlorophyte, Ulva pertusa.*

consisted of 100 g of algal tissue (wet wt) to 100 ml of buffer solution. The tissue and buffer were added together slowly over a period of 2 min to insure maximum breakage of the tough cell walls, and the resulting mixture was passed through 4 layers of cheesecloth. In most instances the plants were first cut into small pieces before being mixed with the buffer. This procedure took place in a 5° cold room. The resulting homogenate was then centrifuged at 12,100*g* for 20 min in a refrigerated centrifuge. Higher speeds and longer times were attempted but these did not influence subsequent enzyme activity.

The electrometric system used for measuring enzyme activity is a modification of a more complex apparatus built previously by Davis (4). Beckman glass and calomel electrodes were connected to a Cary model 31 vibrating reed electrometer, and this served as the pH meter. The electrometer in turn was connected to a Bausch and Lomb 10 mV VOM 5 chart recorder. This system registers changes in E.M.F. and the graph is therefore a millivolt scale. Hydrogen ion concentrations were determined by calibrating with standard buffers. Previous to this, a manometric device based on the familiar boat method (12) was tested with several algal extracts and with purified beef red blood cell enzyme, but the system did not give sufficiently precise results. The reaction measured is a dehydration whereby CO_2 is evolved after $NaHCO_3$ is mixed with the homogenate. Major disadvantages of this method are that the enzyme may be partially denatured due to the vigorous shaking of the reaction vessel, the rate measurement is strongly dependent upon rate of CO_2 removal from solution, and carbonic anhydrase activity may be modified by the strength of buffer employed (0.1–0.2 M) (6).

The reaction vessels were 20 ml pyrex beakers. They were chilled in a 10° refrigerator before use, as were all solutions and other glassware. Before every test 5 ml of the homogenate plus 0.5 ml of buffer, dithiothreitol (DTT)[3] (10^{-3} M final conc.; dissolved in the buffer) or *p*-chloromercuriphenylsulfonic acid (PCMPS)[4] (10^{-4} M final conc.; dissolved in the buffer), were injected by syringes into the beakers. The DTT and PCMPS were incubated with the homogenates for 30 min periods. Five min before the run, the beaker with solution would be set in a 10° cold bath and the pH electrodes lowered into it. Stirring was started at this time with a Teflon-coated magnetic stirring bar (8.0 mm) (slowing turning over) controlled by a device lying below the water level. The stirring was found necessary to condition and stabilize the electrodes since the recording was on occasion erratic for the first 1 or 2 min even with all parts carefully grounded. At the end of the 5 min period, 3 ml of a 1.693×10^{-3} mole/l. CO_2 solution was injected by syringe into the beaker. After a slight inflection of the recording needle caused by disturbance of the electrical field near the electrodes, the pH of the solution begins to fall—this drop is gradual for the controls, which contain just buffer, but steeper in the algal homogenates. Even though a smoother tracing could be obtained by turning the stirrer off after injection, it was not felt that reaction rates could be correctly measured for 1 to 2 min (4 min for the blanks) in unstirred solutions, the average time for the pH to drop to its lowest point. Chart recorder speed was 1 inch per min. Representative runs for *Plocamium* and its blank (buffer only) are shown in Fig. 1 and 2. The scale in these figures is the same; the EMF drop in the blank is sometimes slightly greater than in the homogenates because the buffering capacity of the latter is greater.

The following method for calculating relative values of enzyme activity was used. The total EMF change was read. Then the time at which one-third of this change was attained was noted as t_1. Similarly, the time at which two-thirds of the change was attained was noted as t_2. The interval between these 2 times, in min, varies as the reciprocal of the enzyme activity. Because the logarithmic plot of $\triangle$ EMF (EMF minus final EMF) is a straight line over its central third (Fig. 3), the above procedure was chosen. Relative enzyme activities are obtained as $\frac{\log \triangle \mathrm{EMF}t_1 - \log \triangle \mathrm{EMF}t_2}{t_2 - t_1}$. The blank value is subtracted from this and the final result expressed as net enzyme activity per mg of protein:

$$\left[\frac{\log \triangle \mathrm{EMF}_{t1} - \log \triangle \mathrm{EMF}_{t2}}{t_2 - t_1}\ \text{enzyme} - \frac{\log \triangle \mathrm{EMF}_{t1} - \log \triangle \mathrm{EMF}_{t2}}{t_2 - t_1}\ \text{blank}\right] \div \text{mg of protein.}$$

It may be seen from the development in the appendix that any 2 points on the curve might have been used to obtain a time interval characteristic of the enzyme activity, provided that they be the same values of the function of pH for every preparation and blank.

Protein determinations were made by the method of Lowry *et al.* (16). Precision of these measurements was checked by the Biuret method (14) for some species of algae. Absorbance was measured on a Beckman DU spectrophotometer with a Gilford photometer.

The CO_2 solution was made as follows. Pure CO_2 was bubbled into approximately 80 ml of deionized water contained in a 125 ml serum bottle, for about 10 min. The bottle was then plugged with a serum bottle stopper and clamped in an inverted

[3] Calbiochem, Los Angeles, California.
[4] Sigma Chemical Company, St. Louis, Missouri.

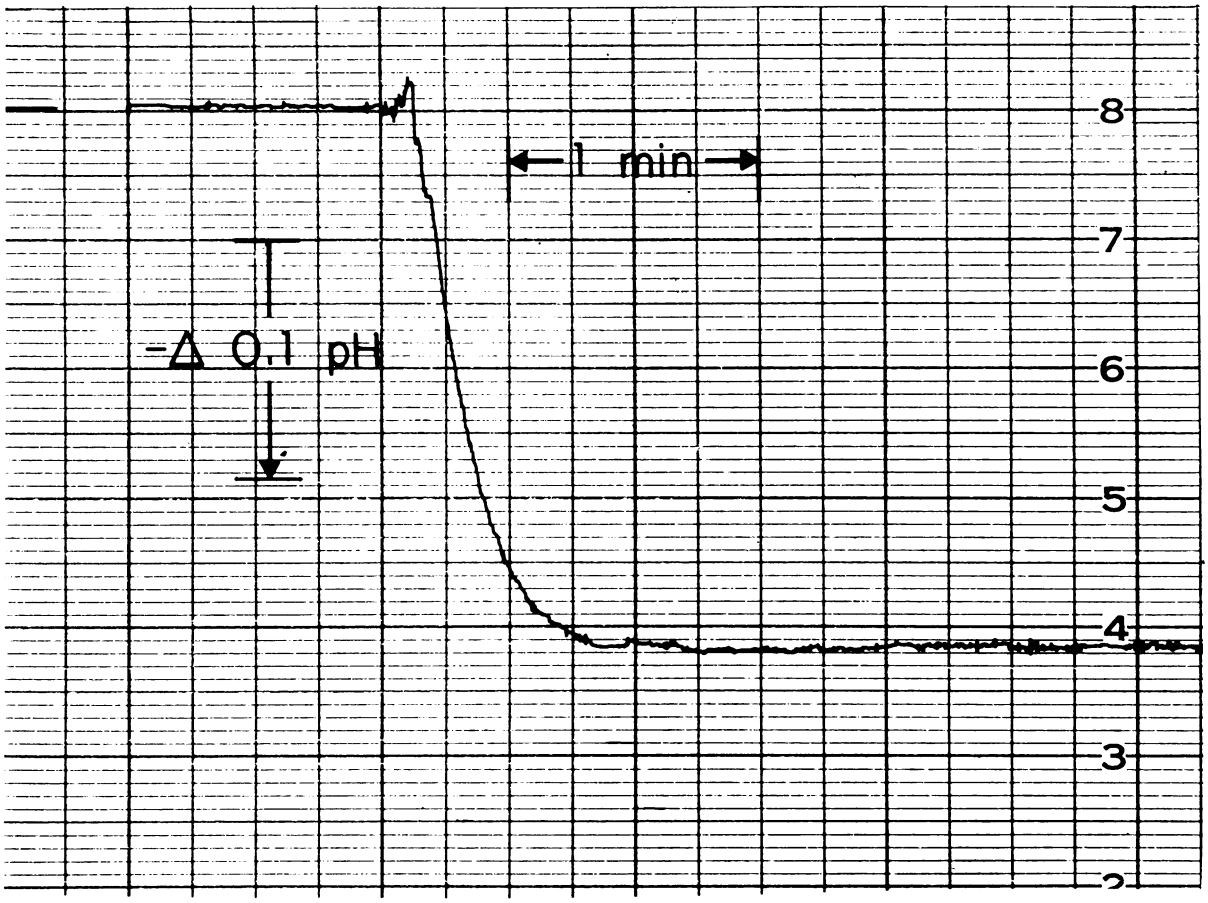

FIG. 1. Change in pH after injection of CO_2 substrate. 5 ml of *Plocamium*-0.004 M NaH_2PO_4-Na_2HPO_4 homogenate + 0.5 ml 0.004 M NaH_2PO_4–Na_2HPO_4.

Table I. *Carbonic Anhydrase Activity*

	Species	Month collected	Enzyme activity[1,2] 4×10^{-3} M Buffer	10^{-3} M DTT	10^{-4} M PCMPS	10^{-4} M Acetazolamide
A.	*Chlorophyta*					
	Ulva expansa (Setchell) Setchell and Gardner	November	0.061	0.057	0.057	0
	Codium fragile (Suringar) Hariot	August	0.303	0.285	0.290	0
	" " (boiled)		0	...	...	...
	Enteromorpha sp.	August	0.152	0.152	0.146	...
	Chaetomorpha torta (Farlow) McClatchie	November	0.113	0.118	0.107	...
B.	*Rhodophyta*					
	Laurencia papillosa (Greville)	May	0.151	0.136	0.139	0
	Plocamium coccineum var. *pacificum* (Kylin) Dawson	September	0.294	0.290	0.100[4]	0
	" " (boiled)		0	...	...	...
	Pterocladia capillacea (Gmelin) Bornet and Thuret	September	0.210	0.216	0.205	...
	Gigartina armata J. Agardh	October	0.183	0.193	0.197	...
C.	*Phaeophyta*					
	Eisenia arborea Areschoug	November	0.110	0.116	0.110	...
	" " (boiled)		0	...	...	...
	Macrocystis pyrifera (*Linnaeus*) C. A. Agardh	October	0.004	0.004	0.004	...
D.	Spinach (*Spinacea oleracia*) homogenate		9.720	...	...	...
E.	Bovine erythrocyte carbonic anhydrase (purified)[3]		664.200	...	...	...

[1] See text for explanation of index of enzyme activity.
[2] To 5.0 ml of algal homogenate was added 0.5 ml of buffer, DTT, PCMPS, or acetazolamide.
[3] Calbiochem. Los Angeles, California.
[4] Activity in homogenate with both 10^{-3} M DTT and 10^{-4} M PCMPS was 0.272.

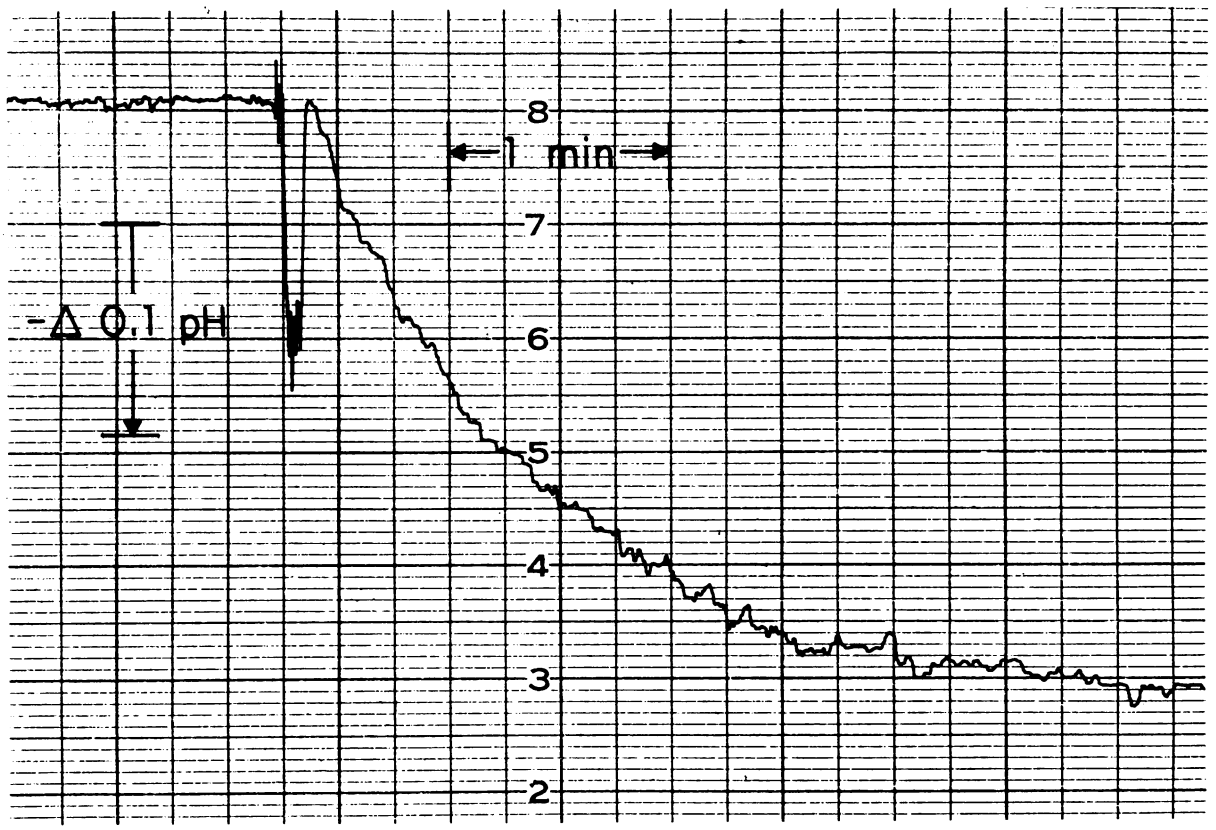

FIG. 2. Change in pH after injection of CO_2 substrate. Blank. 5.5 ml 0.004 M NaH_2PO_4–Na_2HPO_4.

position. Then approximately 15 cc of pure CO_2 was injected into this solution through the stopper to create some pressure inside the bottle so that later withdrawal of water by syringe would not reduce the pressure on this water, which could extract some CO_2 out of solution. Next, a 100 cc glass syringe was fitted with a short piece (3 inch) of rubber tubing which was clamped shut, and filled with de-ionized water. A Teflon-coated magnetic stirring bar (8 mm) was placed in this water. The syringe plunger and clamp were adjusted so that the solution was freed of air bubbles and a final volume of 95 cc of water was left in the syringe. Then a 5 cc syringe was used to extract 5 cc of CO_2-saturated water from the serum bottle, and this water was injected into the 100 cc syringe through the clamped piece of rubber tubing. Mixing of the CO_2 into the large volume was accomplished by rotation of the stirring bar for a few min. To extract the 3 cc of CO_2 solution for the experiment, the needle of a 5 cc syringe was inserted through the tubing and the barrel of the larger cylinder was forced down, pushing water into the smaller syringe.

Results

Carbonic anhydrase activity was present in all 10 algae examined (table I). Activity could only be demonstrated in the brown algae, *Eisenia* and *Macrocystis*, after dialysis overnight against 2 ten volume dilutions of the 0.004 M phosphate buffer. Initial interference was perhaps due to the abundant amount of mucilage present in these plants. *Codium*, a green alga, also appeared to have a mucilaginous homogenate but this did not appear to interfere with enzyme expression. Homogenates of 3 plants, *Codium*, *Plocamium*, and *Eisenia* were boiled for 10 min and this destroyed all activity.

Dithiothreitol at a concentration of 10^{-3} M did not enhance activity in any of the algal homogenates after 30 min incubation. One plant, *Chaetomorpha*, was homogenized also in buffer plus DTT, but the activity expressed was the same as that where the stabilizer was added later to the buffer homogenate. Only 1 plant, *Plocamium*, was affected by the PCMPS, activity being decreased by 66 % at 10^{-4} M. Only 7.5 % inhibition occurred when the homogenate was incubated with 10^{-4} M PCMPS and 10^{-3} M DTT. It is doubtful that the inhibition shown by both DTT and PCMPS in *Laurencia* is significant. The changes produced by these compounds in other plants were about 5 % and cannot be considered significant. Four species (2 red and 2 green), *Laurencia*, *Plocamium*, *Ulva*, and *Codium* were tested for their susceptibility to inhibition by acetazolamide. Enzyme activity of all 4 plants was completely inhibited at 10^{-4} M concentration.

The graph showing the effect of protein concen-

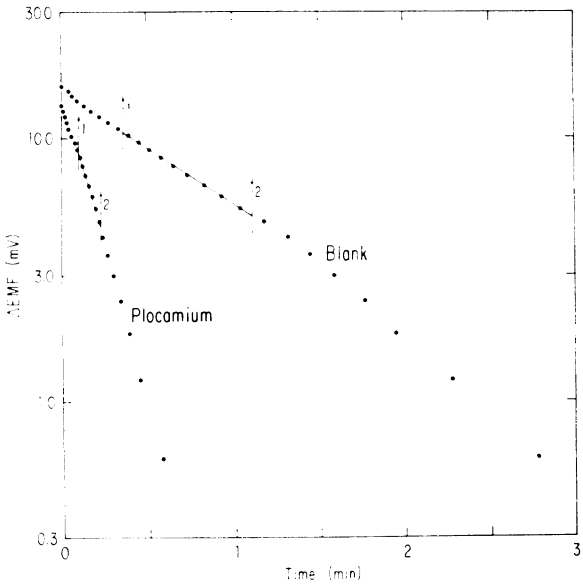

FIG. 3. Logarithmic plot of Fig. 1 and 2.

tration (from diluted homogenates) on the rate of log EMF drop for 3 species (Fig. 4) demonstrates that the relative enzyme activities listed in table I are a function of tissue-buffer ratios at time of homogenization and will change with a change in these ratios. Protein concentrations in undiluted homogenates (approx. 5 mg/ml) yield a rate of log △ EMF drop not much greater than the highest figure shown on the graph, and with some species (*e.g. Codium*) this rate starts to decrease before this concentration is reached. The species in Fig. 4 were collected in a different month (November) and at a different location than their counterparts in table I.

Three replicate runs were made for each type of solution, and the precision of this system was estimated by dividing the average deviation of the 3 slopes (from t_1 to t_2) by the mean slope. This deviation was 5.41 %. Measurements of protein amounts in the crude homogenates by the Lowry and the

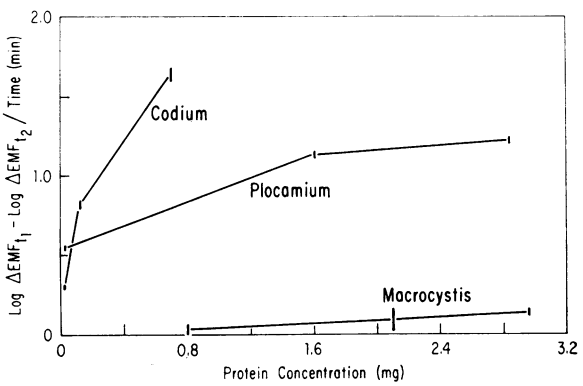

FIG. 4. Slope of log EMF *versus* protein concentration.

Biuret methods yielded some variability. Biuret/Lowry ratios for the same volume of homogenate were as follows: *Codium*, 4.0; *Plocamium*, 1.3; *Macrocystis* (dialyzed), 1.1; and spinach, 0.7. The Biuret method appears to produce higher estimates in *Codium* due to the large amount of suspended material, and in *Plocamium* as a result of the presence of the dominant accessory pigment, phycoerythrin, a biliprotein with an absorption maximum at approximately 550 mμ. For comparative purposes, activity measurements were made also on a spinach homogenate and purified bovine erythrocyte enzyme, and these values are listed (table I).

Discussion

At present the function of carbonic anhydrase in plants is not understood. Related to this is the fact that it is still to be shown whether the primary acceptor of carbon dioxide in the chloroplasts, ribulose 1,5 diphosphate carboxylase, receives the substrate as CO_2 or as HCO_3^-. One possible function of carbonic anhydrase is to greatly increase transport of carbon dioxide across membranes (7). In solutions at physiological pH, carbon dioxide is mostly in the form of bicarbonate which diffuses almost as fast as dissolved CO_2, and hence accounts for most of the carbon dioxide transport. However, in a membrane, CO_2 gas diffuses about 100 times faster than ions. Hence, if the enzyme is present, it converts the bicarbonate to CO_2 gas and greatly facili-

APPENDIX

The following calculations of enzyme kinetics yield a function of hydrogen ion concentration which is equal to the product of enzyme activity and time.

$$CO_2 + H_2O + \text{enzyme} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{enzyme-substrate complex} \underset{k_3}{\overset{k_3}{\rightleftharpoons}} \text{enzyme} + HCO_3^- + H^+$$

Let $s = [CO_2]$, $E' =$ free enzyme conc., $Es =$ enzyme-substrate complex conc., and $p = [HCO_3^-]$

(1) $\frac{ds}{dt} = -k_1 \cdot s \cdot E' + k_2 \cdot Es$

(2) Es is small $\therefore ds = -dp$, $\frac{dEs}{dt} \approx 0$, $s_o = s + p$

(3) $\frac{dEs}{dt} = -(k_2 + k_3)Es + k_1 \cdot s \cdot E' + k_4 \cdot p \cdot [H^+] \cdot E' = 0$

(4) $\frac{dp}{dt} = -k_4 \cdot p \cdot [H^+] \cdot E' + k_3 \cdot Es$

From (3) $Es = \frac{k_1 \cdot s \cdot E' + k_4 \cdot p \cdot [H^+] \cdot E'}{k_2 + k_3}$

(5) Then $\frac{dp}{dt} = \frac{E' \cdot k_1 \cdot k_3}{k_2 + k_3}\left(\frac{-k_2 \cdot k_4}{k_1 \cdot k_3} \cdot p \cdot [H^+] + s_o - p\right)$

Let NaH_2PO_4 conc. before dissociation = A
Let Na_2HPO_4 conc. before dissociation = A
$[Na^+] = 3A$

$[Na^+] = [H_2PO_4^-] + 2[HPO_4^{--}] + [HCO_3^-]$ since $[H^+]$ is negligible.

$3A = 2A + [HPO_4^{--}] + p$

$[HPO_4^{--}] = A - p$; $[H_2PO_4^-] = 2A - [HPO_4^{--}] = A + p$

$\frac{[H^+][HPO_4^{--}]}{[H_2PO_4^-]} = K_f$

$\frac{[H^+](A - p)}{A + p} = K_f$

(6) $p = A\frac{[H^+] - K_f}{[H^+] + K_f}$

tates its passage out through the membrane. Conversely, if CO_2 is coming in through the membrane, the enzyme converts most of it to bicarbonate ions, increasing the diffusion rate in the solution by a corresponding amount.

The data reported here are not entirely in agreement with generalizations that have been made about carbonic anhydrase in plants. A sulfhydryl stabilizer, dithiothreitol, was not necessary to protect the plant enzyme, nor did it enhance activity. Inhibition of the −SH group by PCMPS occurred in 1 plant of the 10, *Plocamium*. The test with dithiothreitol can be interpreted as meaning that either there is no requirement for SH in the enzyme activity or that the enzyme remains in a sufficiently reduced state in the homogenate and is therefore protected. The inhibitor study shows that for *Plocamium*, the sulfhydryl group appears to be necessary for the enzyme activity. Also, a sulfonamide, acetazolamide, did cause an inhibition, which was complete, at 10^{-4} M in the 4 species examined.

$$(7)\quad \frac{dp}{dt} = \frac{2A \cdot K_f}{\left([H^+] + K_f\right)^2} \cdot \frac{d[H^+]}{dt}$$

From (5), (6), (7), K_m (Michaelis constant) $= \frac{k_2 + k_3}{k_1}$, and K (equilibrium constant) $= \frac{k_1 \cdot k_3}{k_2 \cdot k_4}$, it follows that:

$$(8)\quad \frac{d[H^+]}{dt} = \frac{E'}{2A \cdot K_f} \cdot \frac{k_3}{K_m} \left([H^+] + K_f\right) \left\{\left([H^+] + K_f\right) s_o - \frac{A}{K}\left([H^+] - K_f\right)\left(K + [H^+]\right)\right\}$$

Hence: (9)

$$\frac{d[H^+]}{\left([H^+] + K_f\right)\left\{-\frac{A}{K} \cdot [H^+]^2 + \left(s_o - A + \frac{A \cdot K_f}{K}\right) \cdot [H^+] + K_f \cdot s_o + K_f \cdot A\right\}} = \frac{E' \cdot k_3}{2A \cdot K_f \cdot K_m} \cdot dt$$

Because the denominator on the left side of the expression is cubic in $[H^+]$ and can be factored, (9) can be integrated and an exact solution obtained. This has the form

$$(10)\quad \alpha \cdot \ln\left([H^+] + K_f\right) + \beta \cdot \ln\left([H^+] + a\right) + \gamma \cdot \ln\left([H^+] + b\right) = \frac{k_3 \cdot E' \cdot t}{K_m}$$

Where α, β, γ, a and b are constants obtained from (9) and the initial value of $[H^+]$, and the constants s_o, A, K_f and K.

Equation (10) can be written as $\frac{E' \cdot k_3}{K_m} \cdot t = f\left([H^+]\right)$

Where $f\left([H^+]\right)$ is a function of $[H^+]$ only.

Emperically the greater part of $f\left([H^+]\right)$ varies as log (pH-pH_{final}). Therefore the logarithmic slope of the data obtained, $\frac{\Delta f\left([H^+]\right)}{\Delta t}$, was taken as a measure of $\frac{E' \cdot k_3}{K_m}$.

$\frac{E' \cdot k_3}{K_m}$ is here used as the enzyme activity.

Acknowledgments

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