Plant Carbonic Anhydrase. Hydrase Activity and Its Reversible Inhibition†

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ABSTRACT: The hydrase activity of spinach carbonic anhydrase for CO_2 is inhibited by azide and acetazolamide under the conditions of stopped-flow experiments. At pH 6.82 the dissociation constants K_i for enzyme–azide and enzyme–acetazolamide are 3.3×10^{-5} and 2×10^{-4} M, respectively, The mode of inhibition is characterized as noncompetitive and the dissociation constants for the respective enzyme–inhibitor complexes are pH dependent. Each of these inhibition constants is in turn controlled by the ionization constant of the inhibitor as well as that of a catalytic group in the enzyme. Maximum inhibition occurs when the enzyme is in its acidic form and the inhibitor in its monoanionic form. The inhibitors bind in a 1:1 ratio with each independent site in the enzyme, a behavior which is identical with that found for CO_2 binding (Y. Pocker and J. S. Y. Ng (1973a), *Biochemistry 12,* 5127; Y. Pocker and J. S. Y. Ng (1973b), *Proc. Int. Congr. Biochem., 9th,* 65). Further investigation of the buffer effects on the catalytic constant, k_2 ($\equiv k_{cat}$), of the enzyme catalyzed CO_2 hydration reveals a pK_a of around 8.1 for the ionization group in the enzyme at zero buffer concentration. This pK_a is shifted to lower values with increasing buffer concentration. However, in the presence of inhibitors the enzyme-catalyzed hydration of CO_2 reveals a pK_a value in the vicinity of 8.4. A mechanism that allows for the above data is proposed.

Plant carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) is currently enjoying a renaissance which is affecting the research and thinking of plant physiologists. The resurgence of interest in this enzyme has gained significant thrust from its discovery in the leaves of all higher plants often in the same region as the primary enzymes of CO_2 metabolism (Everson and Slack, 1968; Everson, 1970; Poincelot, 1972; Black, 1973). The enzyme from higher plants exists in at least two electrophoretically separable types. One type, found principally in monocotyledon species, has a suggested molecular weight of 40,000 (Atkins *et al.*, 1972). The other, isolated from dicotyledon species, is an hexameric enzyme of molecular weight 180,000 containing six tightly bound zinc ions (Tobin, 1968, 1970; Kisiel and Graf, 1972; Pocker and Ng, 1973a,b).

The plant enzyme powerfully and reversibly catalyzes a reaction whose four components are intimately associated with photosynthesis (eq 1), but the physiological role of the

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \quad (1)$$

enzyme still remains to be elucidated. On the one hand, Everson (1970) reports that carbonic anhydrase is required for full photosynthetic activity, while on the other, Randall and Bouma (1973) indicate that a very low level of enzyme activity is sufficient for maximum photosynthesis. If the enzyme plays no role in facilitating the supply of CO_2 to the sites of carboxylation within the chloroplast, why is photosynthesis inhibited by acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) (Everson, 1970), a specific inhibitor of carbonic anhydrase?

While studies of the inhibitory action of acetazolamide on the erythrocyte enzyme are extensive (Kernohan 1966*a,b;* Pocker and Stone, 1968; Lindskog and Thorslund, 1968), little is known about its effect on the plant enzyme. Consequently, we deemed it important not only to characterize further the interaction between the spinach enzyme and its physiological substrate, carbon dioxide, but also to study in detail the kinetics of hydrase inhibition by both acetazolamide and azide. In contrast to the difficulties encountered by Tobin (1970) with the parsley enzyme, we were able to inhibit the spinach enzyme under both Wilbur-Anderson (1948) and stopped-flow conditions. A full account of our kinetic studies is presented here.

Experimental Section

p-Nitrophenol (Eastman) was purified by sublimation under vacuum. Imidazole was recrystallized three times from benzene. 1,2-Dimethylimidazole (Aldrich) was further purified by vacuum distillation. Acetazolamide (Lederle Laboratories), tested to be analytically pure, was used without further purification. All other chemicals were analytical grade reagents and were used without further purification.

Carbonic anhydrase from spinach was isolated and purified as described previously (Pocker and Ng, 1973b). To eliminate relative error, a single purified batch of enzyme was used in this study. Apparatus, procedures, kinetic techniques, and calculations have also been reported earlier (Pocker and Ng, 1973b).

The enzyme was preequilibrated for at least 15 min with the inhibitors in buffer before mixing with the CO_2 solutions in 1:1 ratio in the stopped-flow spectrophotometer. The rate of hydration was monitored at 420 nm when *p*-nitrophenol was used as indicator and at 578 nm when *m*-Cresol Purple was used instead. The slight change in pH due to proton released in the hydration reaction causes the change in the color of the indicator, which is related to the actual hydration rate by a buffer factor. A detailed account of the theory and the methods of computation were reported

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TABLE I: The Influence of Buffer Concentrations on k_2 of CO_2 Hydration Catalyzed by Spinach Carbonic Anhydrase.

Buffer[a]	pH	$[Buffer]_{Total} \times 10^2$ (M)	$K_m \times 10^3$ (M)	$k_2 \times 10^{-5}$[b] (sec^{-1})
Phosphate	6.14	7.0	27.5	0.46
	6.14	6.0	25.8	0.58
	6.14	5.0	19.7	0.53
	6.14	4.0	15.7	0.51
	6.58	7.0	18.2	1.03
	6.58	6.0	16.0	0.93
	6.58	5.0	14.1	0.87
	6.58	4.0	11.2	0.80
	6.58	3.0	10.6	0.76
	7.50	5.0	6.7	2.60
	7.50	4.0	4.0	1.86
	7.50	3.0	4.4	1.58
	7.50	2.0	3.4	1.23
1,2-Dimethyl-imidazole	8.00	5.0	6.5	6.90
	8.00	4.0	5.6	6.15
	8.00	3.0	2.1	4.81
	8.00	2.0	3.8	3.96
	8.64	5.0	1.5	11.50
	8.64	3.0	1.2	8.10
	8.64	2.0	2.0	6.89

[a] All buffer solutions contained 5×10^{-2} M NaCl and 5×10^{-4} M EDTA for stabilization of the enzyme. Ionic strength was kept at 0.15 for the phosphate buffers and 0.075 for the 1,2-dimethylimidazole buffers with Na_2SO_4. [b] Lineweaver and Burk plots were used to evaluate all k_2 values.

in a previous paper (Pocker and Ng, 1973b). The pseudo-first-order rate constants were executed by a CDC-6400 digital computer using a Fortran IV computer program of a least-squares method written by Dr. N. Watamori in our laboratories. Since there is as yet no evidence concerning the actual number of active sites per molecule of enzyme, we have expressed the enzymatic velocity in moles of hydrogen ion released per mole of enzyme, mol wt 180,000. Consequently, the constant k_2 ($\equiv k_{cat}$) in sec^{-1} refers to turnover number of the hexamer. Unless otherwise stated, all K_i values were obtained from plots of V_0/V_i *vs.* [I] as advocated by Dixon (1953). At least two different concentrations of substrate and six concentrations of inhibitors were used for each K_i determination. Each kinetic run was repeated four times and the averaged values were taken. Control runs without enzyme were performed and the nonenzymic rates were subtracted from the enzymic rates. To reduce possible error caused by enzyme denaturation during storage, V_0, the enzyme catalyzed hydration rate in the absence of inhibitor was always determined along with the inhibited rates. All buffer systems, except where specifically mentioned, were maintained at an ionic strength of 0.15 by adding an appropriate amount of sodium chloride. In studying the effect of pH on K_i, imidazole was used as buffer in the pH region 6.8–7.5 and 1,2-dimethylimidazole was used in the pH range 7.55–9.38. The total buffer concentration was kept at 25 mM and hydrochloric acid was added to adjust to the desired pH.

Results

We reported earlier on the purification and characterization of carbonic anhydrase from spinach leaves. This hexameric enzyme of molecular weight 180,000 is shown to contain six zinc ions and 12 sulfhydryl groups exposed upon the dissociation in 6 M guanidine hydrochloride. The spinach enzyme powerfully catalyzes the hydration of CO_2. The catalytic constant k_2 ($\equiv k_{cat}$) exhibits a simple sigmoidal behavior as a function of pH while K_m values extrapolated to zero buffer concentration are more nearly pH independent. An ionizing group of $pK_a = 7.7$ in the presence of buffers governs the activity of the enzyme and a very large

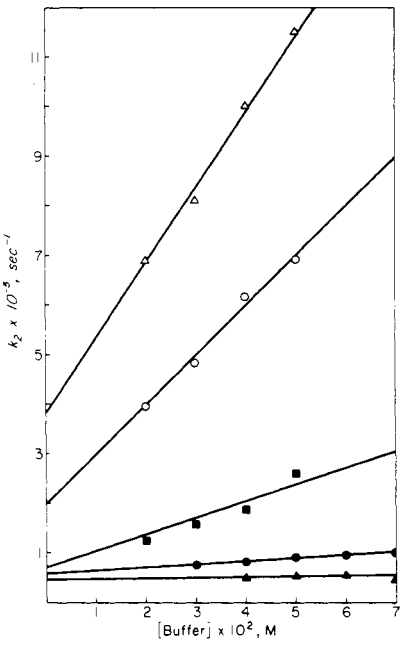

FIGURE 1: Effects of buffer concentration on k_2 at different values of pH. Values of k_2 in sec^{-1} in phosphate buffers at (▲) pH 6.14, (●) pH 6.57, and (■) pH 7.55; and in 1,2-dimethylimidazole buffers at (○) pH 8.00, and (△) pH 8.64. Each k_2 value was determined by a Lineweaver–Burk plot with six different $[CO_2]_0$. All the values of enzyme catalyzed and uncatalyzed rates in this study were the average value of at least four runs. Ionic strength of all solutions was maintained at 0.15 with Na_2SO_4; 5×10^{-2} M NaCl and 5×10^{-4} M EDTA were added to each solution to stabilize the enzyme. $[E]_0 = 3 \times 10^{-8}$ M.

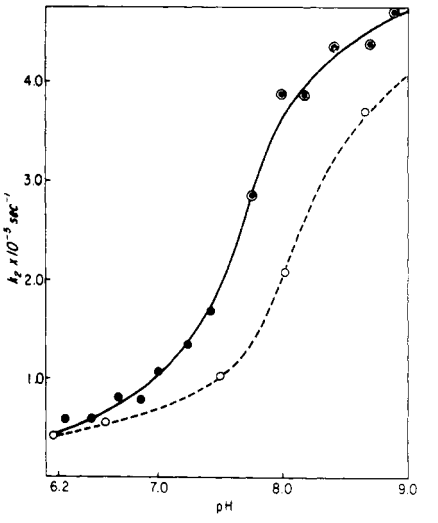

FIGURE 2: The turnover number k_2 in sec^{-1} for the spinach carbonic anhydrase catalyzed hydration of CO_2 as a function of pH at 25.0° and ionic strength of 0.075 maintained with NaCl. Total buffer concentration, 1.5×10^{-2} M: (●) phosphate; (⊙) 1,2-dimethylimidazole. Broken line passes through the extrapolated values of k_2 at zero buffer concentration (○).

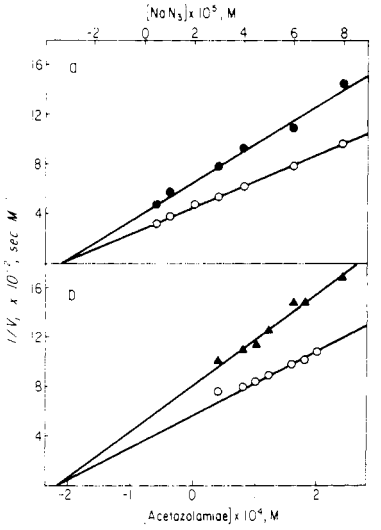

FIGURE 3: Dixon plots of $1/V_i$ against inhibitor concentration. Effects of (a) sodium azide and (b) acetazolamide on the CO_2 hydrase activity of spinach carbonic anhydrase. Rates were performed at 25.0° in 0.02 M phosphate, 9×10^{-3} M NaCl, 9×10^{-5} M EDTA, and 5×10^{-5} M *p*-nitrophenol (pH 6.82). $[CO_2]_0$ = 16 mM (○); 6.5 mM (●); 8.5 mM (▲). Each point represents the mean value of at least four kinetic runs with $[E]_0 = 3.12 \times 10^{-8}$ M.

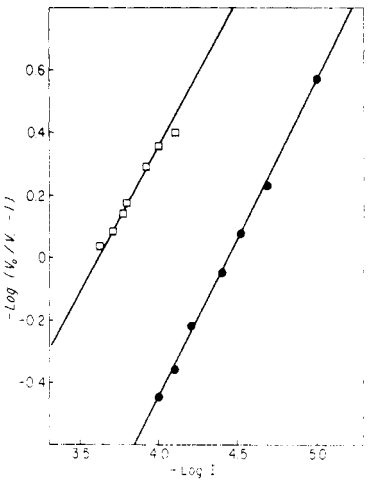

FIGURE 5: Plot of $-\log[(V_0/V_i) - 1]$ *vs.* $-\log$ [I]: (□) acetazolamide; (●) sodium azide. $[CO_2]_0$ = 16 mM. $[E]_0 = 3.12 \times 10^{-8}$ M. The slopes are 0.99 and 0.93 for azide and acetazolamide, respectively.

increase in activity is observed when this group ionizes to its basic form. However, these kinetic parameters are shown to be buffer sensitive (Table I). At low pH, values of K_m are highly sensitive to the concentration of the acidic component of the buffer whereas, at high pH, k_2 is affected significantly by the basic component of the buffer. Extrapolating to zero buffer concentration (Figure 1), the pH profile of k_2 is shifted to higher values of pH (Figure 2) resulting in an increase of 0.4 unit in the pK_a value of the ionizable group. In other words, at zero buffer concentration, the pK_a of the catalytic group is 8.1.

In our stopped-flow spectrophotometric studies both azide and acetazolamide are found to inhibit the hydrase activity of spinach carbonic anhydrase. However, the concentration of acetazolamide needed is about 10^4 higher than that required for the same degree of inhibition with the erythrocyte enzyme. In the case of azide, the concentration needed is roughly the same for both enzymes. The effects of these inhibitors on the plant enzyme are shown as Dixon plots in Figure 3. Thus, in contrast to its remarkably powerful inhibitory effect on the erythrocyte enzyme, acetazolamide is, in fact, a weaker inhibitor than azide with respect to the plant enzyme. Both the Dixon plots (Figure 3) and Lineweaver–Burk plots (Figure 4) characterize these inhibitions as noncompetitive. The binding of these inhibitors to the enzyme causes a change in V_m but has no effect on K_m. At pH 6.82, the dissociation constants, K_i, for enzyme-azide and enzyme–acetazolamide complexes are found to be 3.3×10^{-5} and 2×10^{-4} M, respectively. The number of inhibitor molecules binding to the enzyme can be determined by the method of Johnson *et al.* (1942) (Figure 5). The slopes of the respective lines indicate a 1:1 stoichiometry between active site and inhibitor.

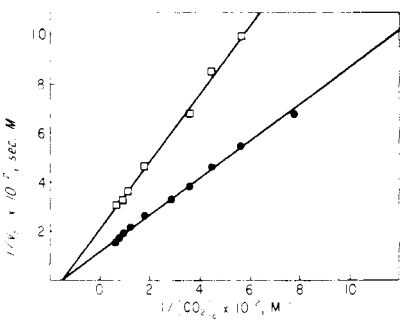

FIGURE 4: Lineweaver–Burk plots of CO_2 hydration rates catalyzed by spinach carbonic anhydrase in the (●) absence and (□) presence of 3.5×10^{-5} M sodium azide. Conditions are given in the legend to Figure 3.

The dependence of K_i on pH is shown in Table II for both inhibitors. In the case of azide (Figure 6) values of K_i increase with increasing pH and are controlled by an ionizing group having a pK_a of 8.4. In other words the anion-sensitive site in the enzyme bears a group which has a pK_a in the vicinity of 8.4 and its transformation to the basic form reduces the inhibitory power of the azide ion. The extent of acetazolamide inhibition is controlled by two ionizing groups having pK_a values of 7.2 and 8.4 (Figure 7). The former represents the ionization of the SO_2NH_2 group in acetazolamide with a pK_a value of 7.2 whereas the latter coincides with that observed for azide inhibition. The maximum inhibition occurs when the enzyme is in its acidic form. In other words, the acetazolamide anion, as well as azide anion, binds preferentially to the protonated form of the enzyme which predominates at low pH.

Discussion

Similar to the erythrocyte enzyme, plant carbonic anhydrase is inhibited by both azide and acetazolamide. These inhibitors form 1:1 complexes with each independent site in the enzyme, and their mode of inhibition can be characterized as noncompetitive with regard to CO_2 hydration. The extent of inhibition is controlled by the ionization of the active group in the enzyme and that of the inhibitor, if the inhibitor ionizes within the range of pH studied. In view of the fact that both K_m and k_2 are buffer and anion sensitive, we suggest that the pK_a of 8.4 corresponds to the ionization of the activity-linked group in the plant enzyme having a pK_a of 8.1 at zero buffer concentration. Such an increase in pK_a is understandable if the inhibitor anions form H bonds with the zinc coordinated water or with any other BH^+ residue located in the active site of the enzyme. Clearly the proton in the EH^+I^- complex will be released less readily in comparison to that of the free (uninhibited) en-

TABLE II: Inhibition Constants of Azide and Acetazolamide as Functions of pH

Buffer[a]	pH	$K_i \times 10^5$ [b] (M) (Azide)	pH	$K_i \times 10^4$ (M) (Acetazolamide)
Imidazole	6.83	3.3	6.80	2.0
	7.16	4.7	7.16	1.5
	7.55	3.8	7.55	1.1
1,2-Dimethyl-	7.72	4.8	7.55	1.1
imidazole	7.98	4.7	7.70	1.2
	8.18	5.4	8.17	1.4
	8.57	6.9	8.74	2.5
	8.72	9.4	9.06	3.9
	9.15	11.5		
	9.38	18.3		

[a] Total buffer concentration in all solutions was 25 mM. HCl was used to adjust the pH. Ionic strength was maintained at 0.075 with NaCl. The enzyme was further stabilized by 5×10^{-4} M EDTA. [b] Values of K_i were determined by Dixon plots as described in the experimental section. $[E]_0 = 3.12 \times 10^{-8}$ M.

zyme, EH^+ (Laidler and Bunting, 1973). It is interesting to note that such anionic effects are also reflected in pH–rate plots as reported by Kernohan (1965) for bovine carbonic anhydrase. Thus, in the presence of the noninhibitory anions, sulfate and phosphate, he estimated the pK_a to be 6.35 whereas in the presence of 0.08 M Cl^-, the pK_a is shifted to 6.9. This type of behavior is not limited to the CO_2 hydration but has also been observed in the esterase reaction (Thorslund and Lindskog, 1967; Pocker and Stone, 1967). Coleman (1967) has pointed out that the inflection point of the esterase pH–rate profiles of erythrocyte carbonic anhydrase from various species and isozymes differ almost by 1 pH unit as a function of species and isozyme. However, at present it is not yet clear whether one general catalytic mechanism is operating in the erythrocyte as well as the plant enzyme.

One striking difference between the plant enzyme and the erythrocyte enzyme is in the inhibition by acetazolamide. An excellent inhibitor for the erythrocyte enzyme ($K_i \simeq 10^{-8}$ M, Kernohan, 1966; Pocker and Meany, 1965, 1967; Pocker and Stone, 1967; Lindskog and Thorslund, 1968), acetazolamide, is only a moderate inhibitor for the plant enzyme ($K_i > 10^{-5}$ M). It is possible that the entrance to the active center is much smaller in the plant enzyme than in the erythrocyte enzyme or that since the plant enzyme is hexameric, there may be more steric hindrance around the active site, thus restricting the entry of the inhibitor molecule. It is also likely that there are fewer van der Waals interactions and hydrogen bondings between the plant enzyme and the inhibitor molecule. With HCA-C, Bergsten *et al.* (1972) had also demonstrated that besides the direct coordination of the sulfonamide group to the zinc ion following the displacement of the zinc-bound water molecule, the inhibitor molecule seems to have other additional interactions with the enzyme. Due to the bulk of this inhibitor, all the solvent molecules are displaced from the active site cavity. The rest of the inhibitor molecule with the heterocyclic ring points out from the active center. The large size of the active site cavity in HCA-C contributes to its astounding catalytic versatility. Since the plant enzyme is not only much less susceptible to inhibition by the specific but rather sizable sulfonamide molecules, like acetazolamide, but also shows little catalytic versatility toward the larger substrates, one is led to believe that the active crevice of this enzyme is much smaller than its counterpart in erythrocytes.

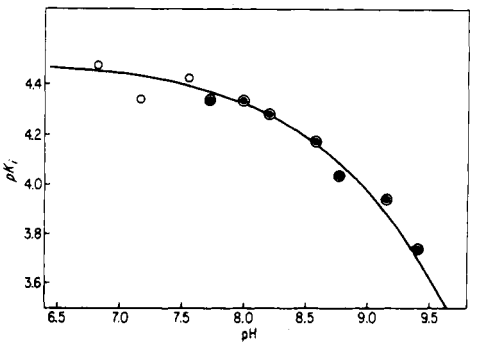

FIGURE 6: Influence of pH on the inhibition constants for sodium azide. Each K_i value was obtained from at least two plots of V_0/V_i *vs.* $[I]_0$ at different CO_2 concentrations and for each line, a minimum of five different concentrations of inhibitor were used. The noninhibited enzyme catalyzed rates were always determined along with the inhibited rates. Buffer components used were imidazole (○) and 1,2-dimethylimidazole (⊙) with concentrations of 0.025 M and ionic strength of 0.075 maintained by NaCl. $[E]_0 = 3.12 \times 10^{-8}$ M.

Mounting evidence from infrared spectroscopy (Riepe and Wang, 1968), nuclear magnetic resonance quadrupolar relaxation (Ward, 1969, 1970), X-ray (Kannan *et al.*, 1972), and visible spectroscopy (Lindskog, 1966) shows that in the erythrocyte enzyme the inhibitor anion is often coordinated directly to the metal ion at the active center (Lindskog and Coleman, 1973). A displacement of a water molecule presumably existing within the coordination sphere of the metal was observed by Fabry *et al.* (1970) upon the binding of sulfonamide and monovalent anions to the cobalt(II) enzyme. Whether such displacement also occurs in the plant enzyme awaits further investigation.

At least in the case of erythrocyte enzyme, the sulfonamides probably bind to the enzyme in the anionic state (King and Burgen 1970). X-Ray investigations strongly support the hypothesis that anions and sulfonamides are closely associated with the zinc ion. Other independent studies using kinetic (Pocker and Stone, 1968; Lindskog and Thorslund, 1968) and spectroscopic (Coleman, 1967) methods reveal that the sulfonamides compete with anionic inhibitors for the same, or nearby interacting, binding site.

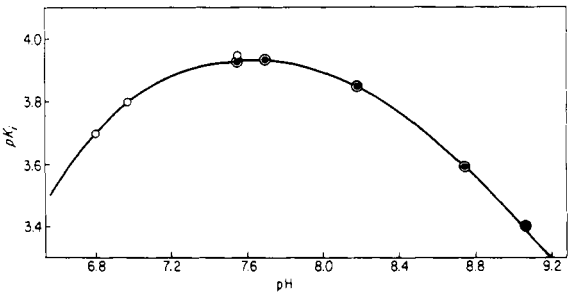

FIGURE 7: Acetazolamide inhibition of SCA-catalyzed hydration of CO_2 as a function of pH at 25.0°. $[E]_0 = 3.12 \times 10^{-8}$ M. Methods and conditions were given in the legend to Figure 6.

This type of binding may also occur in the plant enzyme, as discussed above.

The outstanding feature of plant carbonic anhydrase is its hexameric nature with six tightly bound zinc ions. However, we found that it is not an allosteric enzyme within the limits of our experimental conditions and that a 1:1 complex is formed between each independent site and a substrate or inhibitor molecule. Although its affinity for CO_2 (K_m = 1.5 mM) is higher than that of the erythrocyte species (K_m = 12 mM for bovine B enzyme and 9 mM for human C enzyme) its turnover rate with $k_2 = 4.6 \times 10^5$ sec^{-1} at higher pH is only half of that of the erythrocyte species (10^6 sec^{-1} for bovine B and 1.4×10^6 sec^{-1} for human C enzyme). Evidently, the same general pathway may be operative in both the plant and the erythrocyte enzymes, but the nature of the participating groups and the detailed mechanisms may not be the same. In fact, their amino acid compositions are quite different, with the plant enzyme being additionally six times larger, indicating perhaps that a more complicated mechanism may be functioning here.

References

Atkins, C. A., Patterson, B. D., and Graham, D. (1972), *Plant Physiol. 50,* 214, 218.

Bergsten, P. C., Waara, I., Lovgren, S., Liljas, A., Kannan, K., and Bengtsson, U. (1972), *Proc. Alfred Benzon Symp., 4th, Oxygen Affinity Hemoglobin Red Cell Acid Base Status,* 363.

Black, C. C., Jr. (1973), *Annu. Rev. Plant Physiol. 24,* 253.

Coleman, J. E., (1967), *J. Biol. Chem. 242,* 5212.

Dixon, M. (1953), *Biochem. J. 55,* 161.

Everson, R. G. (1970), *Phytochemistry 9,* 25.

Everson, R. G., and Slack, C. R. (1968), *Phytochemistry 7,* 581.

Fabry, M. E., Koenig, S. H., and Schillinger, W. E. (1970), *J. Biol. Chem. 245,* 4256.

Johnson, F. H., Eyring, H., and Williams, R. W. (1942), *J. Cell. Comp. Physiol. 20,* 247.

Kannan, K., Liljas, A., Waara, I., Bergsten, P., Lovgren, S., Strandberg, B., Bengtsson, U., Carlbom, U., Fridborg, K., Jarup, L., and Petef, M. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36,* 221.

Kernohan, J. C. (1965), *Biochim. Biophys. Acta 96,* 304.

Kernohan, J. C. (1966a), *Biochim. Biophys. Acta 118,* 405.

Kernohan, J. C. (1966b), *Biochem. J. 98,* 31P.

King, R. W., and Burgen, A. S. V. (1970), *Biochim. Biophys. Acta 207,* 278.

Kisiel, W., and Graf, G. (1972), *Phytochemistry 11,* 113.

Laidler, K. J., and Bunting P. S. (1973), The Chemical Kinetics of Enzyme Action, 2nd ed. Oxford, Clarendon Press, p 160.

Lindskog, S. (1966), *Biochemistry 5,* 2641.

Lindskog, S., and Coleman, J. E. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2505.

Lindskog, S., and Thorslund, A. (1968), *Eur. J. Biochem. 3,* 453.

Pocker, Y., and Meany, J. E. (1965), *J. Amer. Chem. Soc. 87,* 1809.

Pocker, Y., and Meany, J. E. (1967), *Biochemistry 6,* 239.

Pocker, Y., and Ng, J. S. Y. (1973a), *Proc. Int. Congr. Biochem., 9th,* 65.

Pocker, Y., and Ng, J. S. Y. (1973b), *Biochemistry 12,* 5127.

Pocker, Y., and Stone, J. T. (1967), *Biochemistry 6,* 668.

Pocker, Y., and Stone, J. T. (1968), *Biochemistry 7,* 2936.

Poincelot, R. P. (1972), *Plant Physiol. 50,* 336.

Randall, P. J., and Bouma, D. (1973), *Plant Physiol. 52,* 229.

Riepe, M. E., and Wang, J. H. (1968), *J. Biol. Chem. 243,* 2779.

Thorslund, A., and Lindskog, S. (1967), *Eur. J. Biochem. 3,* 117.

Tobin, A. L. (1968), CO_2: Chemical, Biochemical and Physiological Aspects, NASA SP-188, Washington D. C., p 139.

Tobin, A. L. (1970), *J. Biol. Chem. 245,* 2656.

Ward, R. L. (1969), *Biochemistry 8,* 1879.

Ward, R. L. (1970), *Biochemistry 9,* 2447.

Wilbur, K. M., and Anderson, N. G. (1948), *J. Biol. Chem. 176,* 147.