acid from the soil. All of the humic acid fractions obtained in our fractionation scheme (*4*), with the exception of fractions 1A and 1B which are clay-humic acid complexes, can be methylated by this technique.

In our work with diazomethane (*7*) we dissolved the humic acid fractions in pyrrolidone. We have found, however, that in most instances *N*,*N*-dimethylformamide (DMF) may be substituted for pyrrolidone as solvent for the humic acid fractions. This solvent has the advantage of being easier to remove than pyrrolidone after the reaction. The methylation can then be carried out as described in (*7*) with the following modifications: (i) 0.25 percent (weight-volume) solutions of each humic acid fraction are prepared in redistilled DMF which has been dried over molecular sieve 5A; (ii) after methylation, ether and diazomethane are driven out of the solution by heating in a water bath; the material is completely dried under vacuum, and the dried product is redissolved in DMF (0.25 percent).

Methyl sulfinyl carbanion was prepared as outlined by Corey and Chaykovsky (*9*), and 1 ml of the resulting solution was added to 20 ml of the humic acid solution that had been methylated with diazomethane. An equal amount (1 ml) of methyl iodide that had been distilled over molecular sieve 5A was added, and the mixture was allowed to stand overnight.

At the end of the reaction period 2 ml of redistilled HCl solution (approximately 20 percent HCl) and 20 ml of water were added to the reaction mixture. This solution was then extracted three times with 20-ml aliquots of distilled methylene chloride. After three extractions only a very faint color was observed in the water layer, an indication that essentially all of the methylated humic acid had been extracted into the methylene chloride. The methylene chloride aliquots were combined and exhaustively extracted with water to remove the DMF and other reagents. Any free iodine produced may be removed by washing with a sodium thiosulfate or potassium iodide solution. The methylene chloride solution was then evaporated to dryness in a hot water bath. The product was redissolved in methylene chloride and remethylated with diazomethane in order to methylate any acidic groups resulting from the hydrolysis of esters by the acidification step. The final product was evaporated to dryness in a hot-water bath.

The humic acid derivatives obtained by this procedure have been fractionated by gel permeation chromatography and high-pressure liquid chromatography. Our preliminary examination of these products indicates that this methylation method is a useful approach to the elucidation of the chemical structure of humic acids.

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References and Notes

1. M. Schnitzer and S. U. Khan, *Humic Substances in the Environment* (Dekker, New York, 1972); M. M. Kononova, *Soil Organic Matter* (Pergamon, Oxford, 1966).
2. M. Schnitzer, in *Environmental Biogeochemistry*, J. O. Nriaga, Ed. (Ann Arbor Science, Ann Arbor, Mich., 1976), vol. 1, pp. 89–107.
3. R. L. Wershaw, D. J. Pinckney, S. E. Booker, *J. Res. U.S. Geol. Surv.* **5**, 565 (1977).
4. R. L. Wershaw and D. J. Pinckney, *ibid.* **1**, 361 (1973).
5. Sephadex is a registered trademark of Pharmacia Fine Chemicals, Inc. Use of the term in this report is for descriptive purposes only and does not constitute an endorsement of the product by the U.S. Geological Survey.
6. G. C. Briggs and G. J. Lawson, *Fuel* **49**, 39 (1970); M. Schnitzer, M. I. Ortiz de Serra, K. Ivarson, *Soil Sci. Soc. Am. Proc.* **37**, 229 (1973).
7. R. L. Wershaw, D. J. Pinckney, S. E. Booker, *J. Res. U.S. Geol. Survey.* **3**, 123 (1975).
8. S. Hakomori, *J. Biochem. (Tokyo)* **55**, 205 (1964); H. E. Conrad, *Methods Carbohydr. Chem.* **6**, 261 (1972).
9. E. J. Corey and M. Chaykovsky, *J. Am. Chem. Soc.* **87**, 1345 (1965).
10. We thank Dr. Mayer B. Goren of the National Jewish Hospital and Drs. Wilfred E. Pereira and Thomas R. Steinheimer of the U.S. Geological Survey for many helpful discussions and suggestions.

27 September 1977; revised 15 November 1977

Inhibition of Carbonic Anhydrase by Anions in the Carbon Dioxide–Bicarbonate System

Abstract. *Chloride and iodide ions exhibit noncompetitive inhibition of carbon dioxide hydration catalyzed by bovine carbonic anhydrase and competitive inhibition of bicarbonate dehydration. Consideration of the net velocity at equilibrium leads to a satisfactory resolution of the dilemma concerning the different concentrations of such anions required to halve the initial reaction rate for each substrate.*

A controversy has arisen in connection with the anionic inhibition of carbonic anhydrase with CO_2 and HCO_3^- as substrates (*1*–*3*). Maren *et al.* (*1*) reported that the values of I_{50} (the concentration of an inhibitor that produces 50 percent of the original activity) in HCO_3^- dehydration were about three times those in CO_2 hydration. Koenig and Brown (*2*) argued from equilibrium considerations that these two sets of I_{50} values should agree and questioned the experimental procedure of Maren *et al.*, especially with regard to the large variation in *p*H accompanying their colorimetric measurements. Maren (*3*) replied that the anions exhibited noncompetitive inhibition of the enzymatic hydration of CO_2 and competitive inhibition of the HCO_3^- dehydration, and that under these conditions the Haldane relation need not have been violated by the different I_{50} values obtained by Maren *et al.*

We thought it desirable to determine independently the nature of the anionic inhibition of carbonic anhydrase, and also to provide a clear-cut explanation of why the addition of inhibitory anions to an equilibrium mixture in the presence of enzyme does not shift the position of equilibrium, if the anions reduce the initial reaction rates from the two substrates to different extents, as Maren *et al.* (*1*) reported. Consequently, we examined the effect of added anions on initial reaction rates in the interconversion of CO_2 and HCO_3^- catalyzed by bovine carbonic anhydrase, using a stopped-flow technique in which the *p*H variation during the monitoring of initial rates can be minimized.

The experimental procedure was similar to that described by Khalifah (*4*), except that experimentally obtained buffer factors were used instead of calculated ones. *N*-Methylimidazole (0.02*M*) and *p*-nitrophenol ($4 \times 10^{-5}M$) were used as a buffer-indicator system. Ionic strength was maintained at 0.1 with Na_2SO_4. Anions were added as their potassium salts. In HCO_3^- dehydration, a calculated small amount of H_2SO_4 was added to the buffer solution with a microsyringe to obtain the correct *p*H on mixing, considering the H_2CO_3-HCO_3^--CO_3^{2-} equilibrium in substrate solutions. The pK_a values of the H_2CO_3-HCO_3^- and HCO_3^--CO_3^{2-} transitions were taken as 3.6 and 10.3, respectively (*5*). Substrate concentrations were 0.0034 to 0.017*M* for CO_2 hydration and 0.01 to 0.05*M* for HCO_3^- dehydration. The concentration of enzyme was $7.8 \times 10^{-8}M$, measured spectrophotometrically and corrected by using ethoxzolamide (*6*).

The initial portion of the reaction was traced by using the Durrum-Gibson stopped-flow apparatus, which was interfaced with the PDP-8L digital computer. The initial *p*H was 7.00, and the maximum *p*H variation during the observation period was less than 0.1. From the

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observed absorbance change per unit time and the buffer factor (reciprocal absorbance change with a change in the H^+ concentration), one can calculate the reaction rate. The enzymatic rates V_{enz} were plotted against $V_{enz}/[S]$, where [S] is the substrate concentration, to yield V_{max} and K_m in the Michaelis-Menten expression.

As shown in Fig. 1a, with CO_2 as substrate, both Cl^- and I^- acted as noncompetitive inhibitors, with inhibition constants K_i of 0.094 and 0.0075*M*, respectively. Figure 1b indicates that the inhibition by these anions in HCO_3^- dehydration should be considered competitive to the first approximation (*7*). In CO_2 hydration, 7.5 m*M* I^- would reduce the activity by 50 percent, whereas, depending on the substrate concentration, about 15 m*M* would be required to reduce the activity by 50 percent in HCO_3^- dehydration in the substrate concentration range usually employed.

Our results can be explained as follows. For the enzymatic reaction described by Eq. 1 (where E stands for enzyme, S is CO_2, and P is HCO_3^-) the initial reaction rate with respect to each substrate can be expressed by Eq. 2. At equilibrium, the net velocity (Eq. 3), expressed in terms of the respective parameters for initial reaction rates, should be zero, leading to the Haldane relation (*5*, p. 135; *8*) in Eq. 4.

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P \quad (1)$$

$$\left.\begin{aligned} V^S_{enz} &= V^S_{max}[S]/(K_m{}^S + [S]) \\ V^P_{enz} &= V^P_{max}[P]/(K_m{}^P + [P]) \end{aligned}\right\} \quad (2)$$

$$V_{net} = \frac{V^S_{max}[S]/K_m{}^S - V^P_{max}[P]/K_m{}^P}{1 + [S]/K_m{}^S + [P]/K_m{}^P} \quad (3)$$

$$V^S_{max}[S]_{eq}/K_m{}^S = V^P_{max}[P]_{eq}/K_m{}^P \quad (4)$$

A careful inspection of Eqs. 2 and 4 shows that V^S_{enz} and V^P_{enz} are generally different at the equilibrium concentrations of the two substrates except for the case when $K_m{}^S/[S]_{eq}$ is equal to $K_m{}^P/[P]_{eq}$. For bovine carbonic anhydrase this occurs only at *p*H ~ 6.5 (*9*). To maintain the equilibrium when an inhibitor is added to the CO_2(S)-HCO_3^-(P) system, assuming competitive inhibition for the dehydration and noncompetitive inhibition for the hydration, it must hold that

$$\frac{V^S_{max}/(1 + C_i/K_i{}^S)}{K_m{}^S}[S]_{eq} = \frac{V^P_{max}}{K_m{}^P(1 + C_i/K_i{}^P)}[P]_{eq} \quad (5)$$

where C_i is the concentration of inhibitor. Thus, $K_i{}^S$ must be equal to $K_i{}^P$ from the consideration of equilibrium, and I_{50} is equal to K_i for the noncompetitive inhibition with CO_2 as a substrate and to $(1 + [P]/K_m{}^P)K_i$ for the competitive inhibition with HCO_3^- as a substrate. In the latter case I_{50} depends on the substrate concentration and agrees with that for noncompetitive inhibition only at infinitely low concentrations of P.

Table 1 shows V_{max}/K_m values obtained from our experiments. The results indicate that a concentration ratio $[CO_2]/[HCO_3^-]$ of about 1/5 will satisfy the Eqs. 4 and 5, and be at the equilibrium value at every inhibitor concentration. A ratio $[CO_2]_{eq}/[HCO_3^-]_{eq}$ of 1/5 at *p*H 7.0 implies a pK_a of about 6.3 for the dissociation equilibrium between these two substrates, which is in good agreement with the value in the literature (*5*).

From Eqs. 2 and 6, using initial rate measurements

$$V_i{}^S = \frac{V^S_{max}/(1 + C_i/K_i{}^S)}{K_m{}^S + [S]}[S] \qquad V_i{}^P = \frac{V^P_{max}}{K_m{}^P(1 + C_i/K_i{}^P) + [P]}[P] \quad (6)$$

one expects different extents of rate decrease with the addition of inhibitors, which is actually seen in Fig. 1. The equilibrium position, however, is not affected by the addition of anions, since Eq. 4 or 5 holds in each case; that is, the Haldane relation is not violated by these observations.

In conclusion, although K_i must agree for both reactions, I_{50} may be different for the two substrates, depending on the inhibition mechanism, without violating the requirement of equilibrium between the two substrates. In the CO_2-HCO_3^- interconversion catalyzed by bovine carbonic anhydrase, Cl^- and I^- were found to be noncompetitive inhibitors of CO_2 hydration and competitive inhibitors of HCO_3^- dehydration. In order to reconcile the observations related to anion inhibition of carbonic anhydrase in the CO_2-HCO_3^- system, one has to realize that in enzyme catalysis the two initial reaction rates (that is, for the hydration of CO_2 and the dehydration of HCO_3^-) are generally different with equilibrium concentrations of the two substrates,

Table 1. Effect of anions on V_{max}/K_m for the initial reactions in the CO_2-HCO_3^- interconversion catalyzed by bovine carbonic anhydrase at *p*H 7.00 and 25°C. Inhibitors were premixed with the enzyme-buffer solution. The concentration of enzyme was 7.8×10^{-8}*M*.

Reaction	V_{max}/K_m (sec^{-1})				First-order rate constant* (sec^{-1})
	No inhibitor	KI (*M*)		KCl (*M*)	
		0.015	0.030	0.030	
CO_2 hydration	3.55	1.16	0.74	2.66	0.034
HCO_3^- dehydration†	0.63	0.24	0.15	0.53	0.0065
$(V_{max}/K_m)^{CO_2}/(V_{max}/K_m)^{HCO_3^-}$	5.6	4.8	4.9	5.0	5.2‡

*First-order rate constant in the absence of enzyme and inhibitors. †Corrected for the reverse reaction due to the presence of CO_2 in HCO_3^- solutions ‡Ratio of the two first-order rate constants (0.034/0.0065) in the absence of enzyme.

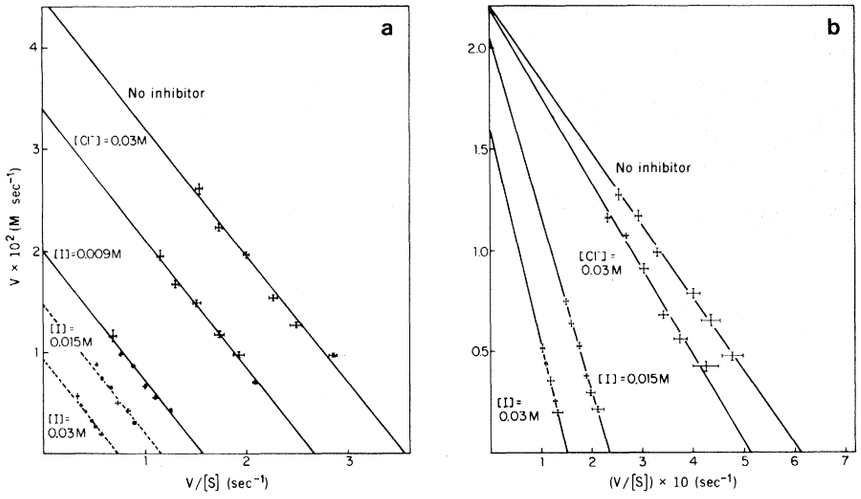

Fig. 1. Plot of V_{enz} against $V_{enz}/[S]$ for (a) CO_2 hydration and (b) HCO_3^- dehydration. The solid lines are based on a least-squares calculation. The dotted lines were drawn by using the average slope of the three other lines in (a).

and they can be further different in the presence of inhibitors, depending on the inhibition mechanism. The only requirement for the attainment of equilibrium is that the Haldane relation, as expressed by Eqs. 4 and 5, be obeyed.

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References and Notes

1. T. H. Maren, C. S. Rayburn, N. E. Liddell, *Science* **191**, 469 (1976).
2. S. H. Koenig and R. D. Brown III, *ibid.* **194**, 745 (1976).
3. T. H. Maren, *ibid.*, p. 746.
4. R. G. Khalifah, *J. Biol. Chem.* **246**, 2561 (1971).
5. H. Gutfreund, *Enzymes: Physical Principles* (Wiley-Interscience, London, 1972), p. 48.
6. Y. Pocker and M. W. Beug, *Biochemistry* **11**, 698 (1972).
7. Although small differences were noted in V_{max} values for different anion concentrations (Fig. 1b), the variation in slope with the change in anion concentration was indicative of competitive inhibition in this system.
8. J. B. S. Handane, *Enzymes* (Longmans, Green, London, 1930).
9. The K_m values for both CO_2 hydration and HCO_3^- dehydration were found to be nearly independent of *p*H in the *p*H range where activity showed a major change. Although K_m was dependent on the buffer concentration, the ratio $K_m^{CO_2}/K_m^{HCO_3^-}$ did not vary significantly with buffer concentration (Y. Pocker and N. Tanaka, in preparation).
10. We thank S. Sarkanen for valuable discussions and D. Moore for his diligent assistance in all aspects of the computer work. Supported by grants from the National Institutes of Health and the National Science Foundation.

25 March 1977; revised 17 October 1977

Transovarial Transmission of Japanese Encephalitis Virus by Mosquitoes

Abstract. *Female* Aedes albopictus *and* Aedes togoi *mosquitoes infected with Japanese encephalitis virus either by intrathoracic inoculation or by ingestion of a virus-sucrose-erythrocyte mixture transmitted the virus to a small percentage of their* F_1 *progeny. Adult* F_1 *female* Aedes albopictus *thus infected transmitted the virus in turn to newly hatched chickens by feeding on them.*

In terms of human morbidity and mortality, Japanese encephalitis (JE) is by far the most important of the mosquito-borne virus encephalitides. Japanese encephalitis virus is found throughout much of eastern Asia, extending from the southeastern Soviet Union in the north to Indonesia in the south and as far as India to the west. Human disease caused by the virus occurs throughout this vast area but is more frequent in the northern part of the range (for instance, there were 2429 deaths in South Korea in 1949). Because of the higher incidence of human disease, the most extensive epidemiologic studies of JE virus have been carried out in northern temperate areas, where mosquitoes are not active in the winter. The virus reappears in the same locality year after year, and consequently considerable effort has been devoted to determining how the virus survives when its vectors are inactive.

One possibility considered in early studies was transovarial transmission of the virus by mosquitoes, with overwintering of the agent in eggs, larvae, or imagoes, depending on the mosquito species involved. In fact, Japanese (*1*), Russian (*2*), and Chinese (*3*) workers reported the recovery of JE virus from adult mosquitoes reared from field-collected larvae and never exposed to JE virus in the laboratory. However, failure to confirm the initial findings for field-collected material and negative experimental results, both with JE (*4–6*) and with the related St. Louis encephalitis (*7*) and West Nile (*8*) viruses, led to the current consensus that transovarial transmission of JE virus does not occur (*9, 10*). Most of the initial positive results were obtained by "blind" passage in laboratory mice and were subsequently attributed to the use of mice already infected with either JE or a mouse encephalitis virus, or to infection of the mice in the course of the experiments by infected wild mosquitoes which gained access to the animal quarters.

Despite various hypotheses and considerable work since JE virus was first isolated in mice in 1935, there is no generally accepted explanation of how the virus survives the winter in the northern part of its range (*9*). The reports of transovarial transmission by mosquitoes of La Crosse virus (*11*), a bunyavirus, and Koutango virus (*12*), a flavivirus, prompted us to reexplore the possibility of transovarial transmission of JE virus by mosquitoes. We report here the demonstration of such transmission by *Aedes albopictus* and *Aedes togoi*.

Two hundred female *A. albopictus*, 3 to 6 days old, from a colony derived from mosquitoes collected on Oahu, Hawaii, were inoculated intrathoracically with JE virus isolated from *Culex annulus* mosquitoes captured in Taiwan, China. The *A. albopictus* had been caged with males and had access to a 10 percent sucrose solution, but not blood, before use. The virus was prepared from a suspension of male *A. albopictus* which had been infected by intrathoracic inoculation from the original *C. annulus* pool.

Six days after inoculation, the female *A. albopictus* were given an opportunity to feed on a normal laboratory mouse, and at least 90 percent did so. An oviposition cup was placed in the cage immediately afterward. Eggs were removed from the cage 4 days later, kept moist for one additional day, and then dried and stored at ambient temperature. The temperature in the room in which the parent mosquitoes and eggs were kept ranged from 25° to 31°C. Exposure to light varied since room lights were often left on at night.

Twenty-four days after removal from the cage, the eggs were immersed in water to hatch and the larvae reared on a diet of Tetramin fish food and Purina guinea-pig chow. The temperature of the room in which these larvae and later F_1 stages were kept was 29° ± 1°C with constant artificial light. Beginning 7 days after the eggs were first immersed and daily thereafter for nine additional days, all pupae were removed from the rearing pans and placed in one or more 4-liter cylindrical cardboard containers with fine nylon mesh covering one end. The F_1 adult mosquitoes were killed 2 to 17 days after emergence for virus assay. Before they were killed, some of the F_1 adult female mosquitoes were tested for ability to transmit JE virus by giving them an opportunity to feed on a single occasion between 9 and 17 days after emergence on newly hatched White Leghorn chicks. Chicks were tested for JE viremia 3 days after exposure to mosquitoes (by inoculation of Vero cell cultures) and for JE hemagglutination-inhibition (HI) antibody 20 days afterward.

When F_1 adult mosquitoes were killed, males and females from each container were pooled separately in groups of 1 to 120 for testing. Each pool was ground in a Ten Broeck tissue grinder in 1.5 to 2.0 ml of diluent (phosphate-buffered saline with 30 percent calf serum) and tested for the presence of JE virus by intrathoracic inoculation of *Toxorhynchites amboinensis* mosquitoes (*13*). The latter were examined by a direct fluorescent antibody procedure analogous to that described for dengue viruses (*14*).

Fourth-instar F_1 larvae were also removed from the rearing pans for virus assay. The larvae were pooled in groups of 100, washed in tap water, and killed by grinding in a tissue grinder with 2.0 ml of diluent. These pools were tested for JE

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