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Facilitation by Carbonic Anhydrase of Carbon Dioxide Transport

T. Enns

The enzyme carbonic anhydrase greatly increases the rate of conversion of carbon dioxide to bicarbonate ions and the rate of the reverse reaction. In aqueous solution this rapid interchange of carbon dioxide between the form of dissolved gas and the form of bicarbonate ions may be utilized to enhance carbon dioxide transport, the limit of enhancement being the transport of bicarbonate ions. As many biological media contain more than 20 times as many bicarbonate ions as dissolved CO_2 molecules, the enhanced transport can thus become the major CO_2 transport system.

It can also be demonstrated that carbonic anhydrase alone, in the absence of bicarbonate ions, enhances CO_2 transport. While the function of this type of facilitated transport in biological systems has not been established, it may exist in cell membranes.

One can describe three types of transport systems in which carbonic anhydrase facilitates CO_2 transport: (i) flow transport, such as blood flow in pulmonary capillaries, in which the enzyme makes dissolved bicarbonate available for rapid conversion to CO_2 and effectively increases the amount of transported CO_2 by the amount of bicarbonate in the blood, and increases the amount of eliminated CO_2 by the bicarbonate *A-V* difference; (ii) diffusion transport, such as occurs in extravascular space, in which the enzyme mobilizes bicarbonate diffusion for CO_2 transport; and (iii) transport of CO_2 by the enzyme alone. The role of the enzyme in the first type of transport system was demonstrated by tracer experiments in the lungs of intact dogs (*1*). The second and third types of transport facilitation are demonstrated by data that I now present.

Diffusion of CO_2 and Bicarbonate Ions in Aqueous Solutions

The reversible reaction of CO_2 with water to form bicarbonate ions may be expressed in the following form:

$$CO_2 + H_2O \underset{H_2CO_3}{\overset{\text{Carbonic anhydrase}}{\rightleftharpoons}} HCO_3^- + H^+$$

The direct reaction of CO_2 with water to give bicarbonate ion in the presence of the enzyme has been suggested by Gibbons and Edsall (*2*) and others. The relation between dissolved CO_2 and bicarbonate concentrations at equilibrium is given by the simplified equation

$$pH = 6.1 + \log [HCO_3^-]/[CO_2] \quad (1)$$

It follows that in a solution of *p*H greater than 6.1 there is more CO_2 in the form of bicarbonate ions than in the form of dissolved CO_2 at equilibrium: for example, human blood plasma at *p*H 7.4 contains 20 times as many bicarbonate ions as CO_2 molecules.

Diffusion of dissolved CO_2 and bicarbonate ions may be described in terms of the following model. Consider a cylinder of unstirred solution of fixed area and thickness, subject to a difference in CO_2 tension between the flat end faces. Dissolved CO_2 diffuses

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from the face of high CO_2 tension to that of low CO_2 tension at a rate proportional to the tension difference. If conversion between CO_2 and bicarbonate is very rapid relative to the rate of diffusion, a difference in concentration of bicarbonate ions, corresponding to the CO_2-tension difference, will be established, and bicarbonate-ion diffusion will supplement CO_2 diffusion; the increase will be proportional to the bicarbonate-ion:CO_2 ratio of Eq. 1. In solutions in which there are no electrical fields, the transport by bicarbonate ions should be in approximately this same ratio to the transport of CO_2 molecules; thus, if the *p*H of lung tissue is 7.4, CO_2 diffusion from capillaries to alveoli may be increased a maximum of 20-fold (*3*).

In the experiments I describe, diffusion of $C^{14}O_2$ tracer through aqueous solutions described by this model was determined. The transport system had the following characteristics: (i) it could be regarded as a steady-state system because accumulation of tracer on the side of low concentration remained very small; (ii) all but two measurements were made under conditions of diffusion equilibrium with respect to all components except the tracer. Thus all concentration gradients are eliminated except those of the tracer, which describes diffusion from one face of the solution to the other while an equal unlabeled flux transports in the opposite direction. Under these circumstances it may be expected that dissolved $C^{14}O_2$ flux and $HC^{14}O_3^-$ flux will follow Fick's law, their diffusion coefficients having approximately the inverse ratio of the square roots of their molecular weights, 1.18. This transport involves no net flux of cations.

Determination of CO_2 Transport by Bicarbonate Ion Diffusion

The enhancement of CO_2 diffusion by bicarbonate diffusion was studied by measuring transfer of CO_2 gas through an aqueous solution supported in a Millipore filter; except for two (to be described presently), the measurements were made in terms of $C^{14}O_2$ tracer transport from gas phase on one side of the solution to gas phase on the other side. There was no movement of total CO_2, because CO_2 tension was maintained equal on both sides of the aqueous solution and chosen so that the dissolved-CO_2 concentration was in equilibrium with the bicarbonate-ion concentration in accordance with Eq. 1. The solutions were prepared with phosphate buffer to obtain the desired *p*H.

The enzyme in these experiments was bovine red-cell carbonic anhydrase (grade B, 1000 units per milligram; *4*); it was used without further purification. The preparations may contain several forms of the enzyme, having different reaction rates; such different forms have been reported for human red-cell carbonic anhydrase (*2*).

The apparatus is shown in Fig. 1. The Millipore grade-HA filter containing the solution had an area of 1.27 square centimeters and a thickness of 0.015 centimeters. The pore size was nominally 0.45 microns; the void space, 80 percent. Total gas pressure was ambient room pressure; the gas was a mixture of air and CO_2. The gas mixture was equilibrated with the solution for 15 minutes before injection of tracer $C^{14}O_2$ through the port in the upper chamber.

The radiation counters were proportional gas-flow counters, with windows 3.1 centimeters in diameter and 0.9 milligram per square centimeter in thickness; counter output was read from decade scalers and counting-rate meters. The counting rate was essentially proportional to $C^{14}O_2$ concentration in the gas chambers; the contributions from Millipore filter and upper chamber, to the radioactivity measured in the lower chamber, were negligible. Because the gas-chamber volumes were equal, the counting rates were also proportional to total $C^{14}O_2$ in each chamber.

The transport data are expressed as fractions of the difference, between radioactivity in the upper chamber and radioactivity in the lower chamber, transferred to the lower chamber per minute. If R_1 is the counting rate of the upper counter in counts per minute, R_2 is the counting rate of the lower counter in counts per minute, ΔR_2 is the change in counting rate in the lower counter in Δt minutes, and T is the transport rate in fraction per minute of net $C^{14}O_2$ in the upper chamber,

$$T = (\Delta R_2/\Delta t) \times 1/(R_1 - R_2)$$

when R_1 and R_2 are measured at the midpoint of the time interval Δt. In all experiments the calculated rates were based on measurements in which the counting rate of the lower counter did not exceed 3 percent of the counting rate of the upper counter.

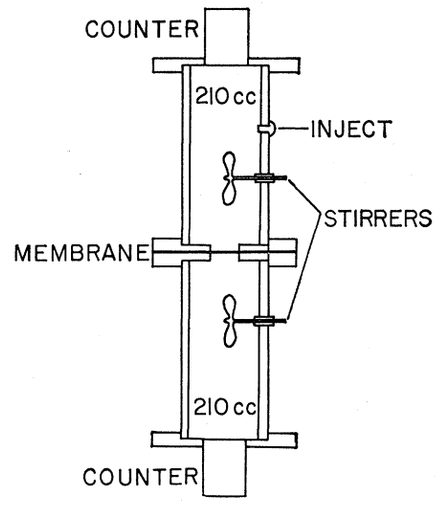

Fig. 1. Apparatus for measuring CO_2 transport. Membrane is aqueous solution in Millipore filter.

A series of transport measurements were made at equal bicarbonate-ion concentrations of 0.025*M*, but with

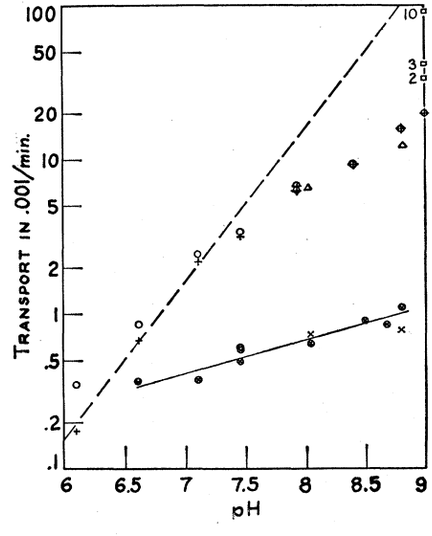

Fig. 2. Carbon dioxide transport through solution containing 0.025*M* $NaHCO_3$. Symbols: hollow circles, total transport with carbonic anhydrase at 1 milligram per milliliter; crosses, same values minus CO_2-diffusion transport; triangles, total transport with carbonic anhydrase at 1 milligram per milliliter and no CO_2 in the lower chamber; squares, increased concentration of carbonic anhydrase (numbers represent concentrations of carbonic anhydrase in milligrams per milliliter); circled x's, buffer solution only; x's, 1 milligram of carbonic anhydrase per milliliter plus acetazoleamide at 0.01 milligram per milliliter at *p*H 8 or at 10 milligrams per milliliter at *p*H 8.8.

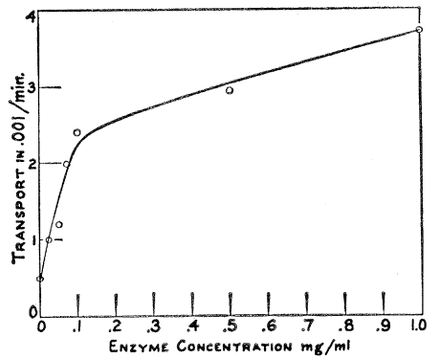

Fig. 3. Carbon dioxide transport through solution containing 0.025*M* $NaHCO_3$ at *p*H 7.5, at varying concentrations of carbonic anhydrase.

increasing *p*H and correspondingly decreasing CO_2-gas tension. Figure 2 gives the data obtained with carbonic anhydrase at 1 milligram per milliliter and without carbonic anhydrase. As a further check on the effect of the enzyme, two measurements were made with enzyme solutions to which the inhibitor acetazoleamide (*5*) was added.

The CO_2 transport due to bicarbonate ions alone may be deduced from these data; this is the total transport in the enzyme solution, minus the transport due to diffusion of dissolved CO_2. For this purpose it was assumed that at low *p*H all bicarbonate ions transported CO_2, and that the contribution of dissolved CO_2 was proportional to the relative concentration of dissolved CO_2 as calculated from Eq. 1. The crosses in Fig. 2 represent the calculated bicarbonate-ion transport; the broken line, indicating the transport expected if all bicarbonate-ion diffusion were available for CO_2 transport, is extrapolated from the values at low *p*H.

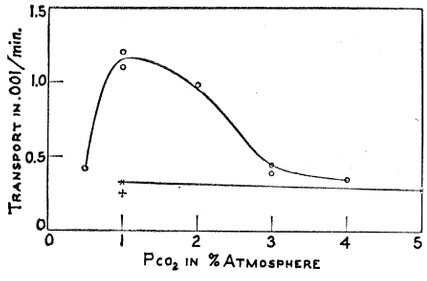

Fig. 4. Carbon dioxide transport at varying CO_2 pressures in the gas phase. Symbols: circles, 100 milligrams of carbonic anhydrase per milliliter at *p*H 5.2; x's, buffer at *p*H 5.2; cross, 100 milligrams of carbonic anhydrase per milliliter at *p*H 5.2 plus 10 milligrams of acetazoleamide per milliliter.

The difference between the transport rate attributed to bicarbonate-ion diffusion (crosses in Fig. 2) and the maximum possible values shown by the broken lines results from an insufficient concentration of enzyme at *p*H values above 7.5. Increase in the enzyme concentration at *p*H 9 to 2, 3, and 10 milligrams per milliliter increased the transport rate (squares in Fig. 2).

Data on the relation between transport rate and enzyme concentration at *p*H 7.5, bicarbonate-ion concentration of 0.025*M*, and 3 percent CO_2 in the gas phase (Fig. 3) may be compared with those of Fig. 2 by considering the bicarbonate ion–transport fraction of maximum bicarbonate-ion transport as a function of enzyme concentration and CO_2-gas pressure; this value decreases when either enzyme concentration or CO_2 pressure decreases, indicating that it depends on the reaction of enzyme and gaseous CO_2 at the surface of the membrane.

The data that I present show unidirectional transport of labeled CO_2 although there is no net transport of CO_2, but transport rates are almost equal when there is no CO_2 in the lower chamber. Figure 2 shows two such measurements demonstrating net CO_2 transport at *p*H 8 and 8.9.

Measurement of CO_2 Transport by Carbonic Anhydrase Preparation

Since the reaction of carbonic anhydrase with CO_2 implies the formation of an intermediate compound in which CO_2 is bound to the enzyme, such an intermediate should act as a carrier of CO_2, perhaps in the manner in which hemoglobin is a carrier of oxygen (*6*). This transport was measured in solutions at *p*H 5.2 containing carbonic anhydrase at 100 milligrams per milliliter. According to Eq. 1 the solutions contained 8 times as much dissolved CO_2 as bicarbonate ions, so any major increase in transport had to be attributed to the enzyme preparation. Addition of acetazoleamide at 10 milligrams per cubic centimeter stopped the enhancement of the transport.

The bovine carbonic anhydrase that I have described was used for the measurements shown in Fig. 4; each point was obtained with a freshly prepared solution to minimize deactivation of the enzyme. The gas mixtures were of air and CO_2.

Red-Cell Ghosts Facilitated CO_2 Transport

The transport apparatus was used to test carbonic anhydrase activity in washed red-cell ghosts from fresh rabbit blood. The ghosts were deposited on the upper side of a Millipore filter saturated with phosphate buffer at *p*H 7.5. The transport rate was 2.3 times that obtained with buffer alone. This arrangement, of enzyme-containing particles on one side of the solution, does not permit quantitative evaluation of these measurements, but the enhanced transport was demonstrated repeatedly. Filtrate from the lysed cell solution also facilitated CO_2 transport. One may conclude that a fraction of the enzyme either was part of the cell ghosts or adhered closely to them.

Discussion

The role of carbonic anhydrase in facilitating diffusion of CO_2 in the presence of bicarbonate has been demonstrated. This mechanism must be operative wherever the enzyme exists in animal or plant aqueous solutions; it accounts for the greater part of diffusion-CO_2 transport when (i) the *p*H exceeds 6.1, (ii) the enzyme concentration is sufficient, and (iii) there is no electric field in the solution.

The possibility of enzyme activity in red-cell ghosts was examined. Location of the enzyme on the outer surface of red cells would make conversion of plasma bicarbonate ions to CO_2 more rapid than if the enzyme were confined to the red-cell interior. The latter location of the enzyme would make it necessary for plasma bicarbonate ions to diffuse into the red cells—a relatively slow process—before the enzyme could convert them to CO_2. Experiments with anesthetized dogs had demonstrated (*1*) that the enzyme in the pulmonary capillaries produces complete exchange between bicarbonate ions and CO_2 in a time interval no greater than that required for blood to flow from the heart to the lungs; these findings certainly are compatible with location of enzyme activity at or near the exterior of the red cells.

It is also interesting to consider the possibility that carbonic anhydrase may be located in mammalian red-cell membranes, and perhaps in other cell membranes, in such a manner as to fa-

cilitate CO_2 transport between cell interior and exterior by the process demonstrated in Fig. 4; such transport would be particularly effective at CO_2 pressures of about 0.01 atmosphere. Bicarbonate ions, in the absence of carbonic anhydrase, also enhance CO_2 transport. While this enhancement does not approach the transport rates made possible by the enzyme, it is still quite significant.

The presence of carbonic anhydrase in animal and plant tissues has been reported by many investigators. Almost unavoidably it will be decisive in determining rates of CO_2 transport wherever it is located; for this reason the enzyme can be expected to play an important role in metabolic processes ranging from pulmonary and kidney function in vertebrates to photosynthesis in plants.

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Ethical Issues in Research with Human Subjects

A rationale is formulated for a code of conduct in the recruitment of subjects for research.

Wolf Wolfensberger

A number of disciplines engaging in clinical practice with humans have been concerned with questions of ethics for some time, and their considerable experience provides a basis for the evolution of widely accepted codes of professional conduct. Ethics in research, however, is still rather virgin territory. What little there is in the way of codification is very inadequate. Cranberg (*1*) pointed out that the 1953 code of ethics of the American Psychological Association (*2*) was apparently the only one existent in 1963 that had been officially adopted by a scientific organization. Another code, applicable mainly to medical research with human subjects but not (as far as I know) officially embraced by any professional or scientific group, was promulgated at the Nuremberg war-crime trials (*3*). More recently, the World Medical Association (*4*) in 1964 passed a statement on human experimentation known as the Declaration of Helsinki. Britain's Medical Research Council, also in 1964, published a "Statement . . . intended to serve as a guide . . ." on "Responsibility in investigation on human subjects" (*5*). Other organizations also have taken steps toward codification of ethics in research but no code, statement, guide, or other set of widely adopted principles yet has the degree of clarity and adequacy that appears feasible and necessary to guide researchers through certain problem areas.

In the past, when lack or inadequacy of rules in research had not been perceived as a major problem, researchers muddled along in the belief or hope that procedures and conventions either in common use or approved by their peers were proper and ethical. At times it was even assumed that a novel procedure entailing unknown degrees of risk, or a procedure requiring definite risks, could be made respectable by having the experimenter share the risk with the subject, or by using volunteers. We have now arrived at a point in the evolution of science at which both scientists and the intelligent lay public consider universality of procedure, approval by peers, sharing of risks, and even use of volunteers questionable or even unacceptable and unethical in some research situations.

A number of recent events have thrust the question of ethics in research into sharp focus. The censuring by the regents of the University of the State of New York (*6*) of a respected hospital and of respected physicians will probably constitute a landmark in the development of codification of ethics in research. This decision censured research procedures that are believed by many scientists in the field to be rather conventional. Then there were the uproar over Project Camelot (*7*); the widespread concern with the psychological test movement, culminating in the recent congressional hearings (*8*); and even the battle over animal-research legislation, and the wave of recent publicity regarding instances of cruelty to research dogs. Indeed, the concern with research practices is probably only one expression of broader-current reexamination and formulation of the rights of the individual. Related expressions may be widespread interest in draft laws, civil rights, the rights of an accused, the right to privacy, compensation to victims of crime, and the concern expressed by both the public and various professional and scientific bodies about the need to update or establish professional codes of ethics in general (*9*).

The degree to which concern about conduct of research has grown among the intelligent lay public is of interest. Controversial news items about recent conduct of research were carried in many and diverse news media: The *Wall Street Journal* ran several articles on problems of ethics in research (*10*), and the *Saturday Review* (*11*) devoted 10 pages to the subject, covering some

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