MOLECULAR PHARMACOLOGY, 6, 430–440

Kinetics of Carbonic Anhydrase in Whole Red Cells as Measured by Transfer of Carbon Dioxide and Ammonia

THOMAS H. MAREN AND CHRISTINE E. WILEY

Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, Florida 32601

(Received April 13, 1970)

SUMMARY

The activity and inhibition of carbonic anhydrase (EC 4.2.1.1.) were studied in intact red blood cells by measuring the hemolytic rates induced when the cells were suspended in a solution of NH_4Cl and $NaHCO_3$. The inward diffusion of CO_2 and its subsequent hydration to HCO_3^- within the cell are matched by the inward diffusion of NH_3 and formation of NH_4^+. The new ionic pair attracts water, leading to swelling and hemolysis of the red cells. The rate-limiting step in the over-all process is the conversion of CO_2 to HCO_3^-. In the native cell, the calculated hemolysis time is about 0.1 sec; our observed hemolysis time is 20 sec. When the enzyme is totally inhibited by 100 μM ethoxzolamide, the hemolysis time is increased to 50 min. This corresponds to the time in which the uncatalyzed hydration of CO_2 generates an increase in ionic strength of about 60% in the cell. This intact erythrocyte system differs from the usual carbonic anhydrase assay in solution, in the important point that the catalytic hydration rate greatly exceeds the noncatalytic. This reflects the very high (approximately 0.1 mM) enzyme concentration in the red cell, analogous to that in certain secretory sites. Under these conditions, we have determined the K_I values of drugs at 99.99% inhibition, and have compared such values with the more conventional I_{50} data obtained from dilute enzyme in solution.

INTRODUCTION

Enzyme reactions are usually studied *in vitro* in dilute systems, in which their cellular milieu is destroyed. Extrapolation to systems *in vivo*, in both rate and inhibition kinetic studies, entails the assumptions that enzyme activity is proportional to concentration over perhaps a 10^4-fold range, and that the composition and geometry of the cell do not alter enzyme-substrate or enzyme-inhibitor relations. In our earlier work, the concentration of carbonic anhydrase (EC 4.2.1.1.) in cell lysates *in vitro* was at most 10^{-8} M; in the cell water of erythrocytes *in vivo* the concentration is about 10^{-4} M. Nevertheless, the results of kinetic studies *in vitro* have been applied to a variety of physiological situations (for review, see ref. 1).

An opportunity to obtain kinetic measurements on intact, living cells was afforded by the work of Jacobs and Stewart (2) on the role of carbonic anhydrase in certain ionic exchanges in the erythrocyte. Figure 1 gives a quantitative interpretation of the system of Jacobs and Stewart, based on both their results and our own. In this ionic milieu the red cells swell and then hemolyze in about

These studies were supported by National Institutes of Health Grants NB 01297 and GM AI 16934. A preliminary report was given before the American Society for Pharmacology and Experimental Therapeutics [*Fed. Proc.* 25, 320 (1966)].

20 sec, as NH_3 enters the cell by diffusion and is converted to NH_4^+ ions in accord with the pH equilibrium (1). The process requires new anions within the cell, which may be either HCO_3^- or Cl^-. Since Jacobs and Stewart (2) found that sulfanilamide, as a carbonic anhydrase inhibitor, reduced the rate of hemolysis, it appears that conversion of CO_2 to HCO_3^- within the cell is a rate-limiting event in the process. It is assumed that, as the NH_4^+ is formed, HCO_3^- is formed as the counter-ion and is then exchanged, wholly or in part, for external Cl^-. When a certain concentration of NH_4Cl or NH_4HCO_3 is attained (about 87 mmoles/liter of original cell water; see Table 3 and discussion), hemolysis occurs.

In the present experiments we have used the powerful heterocyclic sulfonamide inhibitors of carbonic anhydrase to abolish enzyme activity entirely, and thus have determined the uncatalyzed rate of anion accumulation in red cells. From this value we have estimated the true catalytic rate in the red cells and studied inhibition kinetics with various drugs. The studies on drug effects were of special interest, for the system has the unusual characteristic of permitting accurate estimations of fractional inhibition in the range above 0.999.

ION	AMBIENT SOLUTION mM	CONCENTRATION in ERYTHROCYTES INSTANTLY mM	CONCENTRATION in ERYTHROCYTES at HEMOLYSIS mM
NH_4^+	123	0	87 [a]
NH_3	2	2	2
CL^-	125	110	197 or less [b]
HCO_3^-	24.2	12	12 or more [b]
CO_2	0.8	0.8	0.8
Na^+	25	132	132
K^+	0	10	10
Hb^-	0	20	20

FIG. 1. *Ionic composition in test system*

The ambient solution was 125 mM NH_4Cl and 25 mM $NaHCO_3$. See the text under methods for calculation of acid-base equilibria. To 6 ml of this solution, 0.05–0.1 ml of dog erythrocytes was added. At the time of addition the cells had the composition shown under "instantly." The concentrations shown "at hemolysis" are those that would obtain without a volume change, i.e. without influx of water.

[a] Concentration in red cell water (if there were no volume change) to match formation of HCO_3^- in 50 min (the uncatalyzed time) from the rate constant of 2.2 min^{-1} and (CO_2) of 0.8 mM. See the text under discussion.

[b] Concentration if all HCO_3^- formed were exchanged for Cl^-. If none were exchanged, Cl^- would be 110 mM, and HCO_3^-, 99 mM.

METHODS

The standard electrolyte solution selected for most tests (see the first section under RESULTS) was a mixture of 5 ml of 150 mM NH_4Cl and 1 ml of 150 mM $NaHCO_3$. The pH of this solution was 7.65, and its ionic composition was HCO_3^-, 24.2 mM; CO_2, 0.8 mM; NH_4^+, 123 mM; and NH_3, 2 mM. We used an apparent pK_a for HCO_3^-/CO_2 of 6.17 (reviewed in ref. 1) and a pK_a for NH_3/NH_4^+ of 9.25 (3). These and other constants used are for 25°. Experiments were done at room temperature. Figure 1 shows chemical details of the system.

For red cells of the elasmobranch *Squalus acanthias*, the same system was used, except that the NH_4Cl concentration was raised to 500 mM, which yielded a solution essentially isosmotic with the blood. These experiments were done at the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine.

The reaction system contained either 0.05 ml of mammalian blood or 0.2 ml of elasmobranch blood. The approximate ratio of the enzyme concentration in mammalian to elasmobranch blood is 35:1 (1). Table 5 lists the drugs used and their lipid solubility and rate of diffusion into red cells. Their chemistry is fully reviewed in reference 1.

In some cases (method A) drug was added to the electrolyte (outside) solution. The highest concentration was 100 μM, which had an insignificant effect on the osmotic strength. The reaction was started by the addition of blood. Only very lipid-soluble drugs were diffused from the electrolyte medium to red cells rapidly enough to ensure the attainment of equilibrium well before the 20-sec interval that comprised the control period (i.e., without drug) required for hemolysis. The concentrations of rela-

tively lipid-insoluble drugs in the cell increased as the hemolytic reaction proceeded.

For this reason, method B was used, in which the drug, in sodium phosphate buffer, pH 7.1, was incubated with an equal volume of red cells for 30 min, after which 0.05 ml of this mixture was added to the 6 ml of electrolyte solution. This procedure allowed both diffusional and chemical equilibrium (with enzyme) to be attained within the buffer–red cell mixture. The attainment of a new equilibrium between the drug concentrations in the red cell and in the solution was still slow, however, except for lipid-soluble drugs. Therefore, the actual concentration of drug within the red cell at any one time was difficult to estimate, and decreased as the reaction proceeded. For very lipid-soluble drugs, equilibrium was complete well before the 20-sec control hemolysis time. Thus, the value of K_I could be established only for the lipid-soluble drugs, and depended on closely corresponding experimental results from methods A and B. Only in this circumstance could we be sure that the added calculated or measured concentration of drug in the outside solution (designated I_f) was in equilibrium with enzyme within the cell.

The reaction was carried out in test tubes, and hemolysis was gauged visually by clearing of the solution to a degree that permitted illuminated newsprint to be clearly legible through the tubes. This end point was reproducible within about 10%, and had the advantage of being applicable to marine biological laboratory conditions. Since the range of time covered was 20–3000 sec, small errors in determining the end point were of little consequence.

Drugs. All drugs were supplied by the Organic Chemical Division of Lederle Laboratories (courtesy of Dr. Selby Davis), except for ethoxzolamide, which was supplied by Upjohn. Their properties have been described fully (1) (see also Table 5).

Calculations. The following symbols, constants, and calculations were used (1).

V_{unc} = uncatalyzed velocity of the reaction $CO_2 \rightarrow H_2CO_3$

V_{cat} = observed velocity of the reaction in the presence of carbonic anhydrase

V_{obs} = observed velocity of partially inhibited reactions

V_E = theoretical velocity of the enzymatic reaction

$[E]_0$ = total molar concentration of carbonic anhydrase in red cells. In human red cells this is 150 μM, taking into account both B (125 μM) and C (25 μM) forms. In the dog, the value is 36 μM, and only one form is found with certainty (1)

$[E_f]$ = free or active concentration of carbonic anhydrase

$[I_0]$ = total concentration of drug

$[EI]$ = concentration of drug-enzyme complex

K_I = dissociation constant of $[EI]$:

$$K_I = \frac{[E_0 - EI]\,[I_0 - EI]}{EI} \qquad (1)$$

I_f = free drug:

$$I_f = I_0 - EI \qquad (2)$$

i = fractional inhibition:

$$i = \frac{EI}{E_0} \qquad (3)$$

Substituting Eqs. 2 and 3 into Eq. 1 and rearranging yields

$$i = \frac{I_f}{I_f + K_I} \qquad (4)$$

When $I_0 > E_0$ or EI, $I_f \cong I_0$, and

$$i = \frac{I_0}{I_0 + K_I} \quad \text{or} \quad K_I = \frac{I_0}{i} - I_0 \qquad (5)$$

The important term i will be considered in terms of rate, as a theoretical base, and then in terms of time, to conform with the actual observations. We define a term a, denoting fractional activity, such that $i = 1 - a$.

$$a = \frac{E_f}{E_0} = \frac{E_0 - EI}{EI} \qquad (6)$$

$$a = \frac{V_{obs} - V_{unc}}{V_E} \qquad (7)$$

Notice that the uncatalyzed rate is subtracted from the observed rate to yield the

true inhibited rate. Fractional inhibition is then

$$i = 1 - \frac{V_{obs} - V_{unc}}{V_E} \quad (8)$$

If $V_{unc} \ll V_{obs}$, as is true for enzymatic and partially inhibited reactions faster than about 1000 sec in this system, V_{unc} may be neglected in these expressions.

The value of i could be calculated directly from measurements of the reaction periods. With time denoted by T and using the same subscripts as above, Eq. 8 becomes

$$i = 1 - \frac{T_E \cdot (T_{unc} - T_{obs})}{T_{obs} \cdot T_{unc}} \quad (9)$$

When $T_{unc} \gg T_{obs}$, the latter term may be dropped from the numerator. It will be shown that $T_E = 0.1$ sec. The simplified equation then reduces to

$$i = 1 - \frac{0.1 \text{ sec}}{T_{obs}} \quad (10)$$

RESULTS

Effect of CO_2 and HCO_3^- Concentrations on Rate of Hemolysis

Development of "standard experiment." Tables 1 and 2 show the relationship between CO_2 equilibria in the ambient solution and the rate and time of hemolysis, respectively. The hemolysis times denoted as "catalyzed" in Table 2 refer to those observed in the absence of inhibitor, and correspond to rates designated V_{cat}. The times denoted as "uncatalyzed" refer to those seen in the presence of appropriate inhibitors, which are shown in the next section to have abolished enzyme activity completely. These hemolysis times correspond to rates designated by V_{unc}. The data suggest that both the catalytic (normal) and uncatalytic (inhibited) rates of red cell hemolysis depend closely upon the CO_2 concentration in the solution. Table 2 shows that these rates change about 2-fold with a 2-fold change in CO_2 concentration, under circumstances in which the HCO_3^- and H^+ concentrations change 8-fold. This confirms the idea that the driving force of the system is the conversion of CO_2 to HCO_3^- within the cell (2) (Fig. 1).

The dependence of the uncatalyzed rate

TABLE 1

Hemolysis rates of human red cells (0.05 ml of blood) in mixtures of NH_4Cl and $NaHCO_3$ (150 mM)

NH_4Cl	$NaHCO_3$	pH	CO_2[a]	V_{cat}
ml added			*mM*	*$sec^{-1} \times 100$*
5	0	5.70	0.1[b]	0.28
5	0.05	7.00	0.35	0.37
5	0.1	7.18	0.51	0.43
5	0.2	7.34	0.76	0.80
5	0.3	7.42	0.95	1.0
5	1.0	7.65	1.6	3.3

[a] Calculated from final equilibrium of mixture, not from measured pH.

[b] Source of CO_2 in this case was the red cells.

TABLE 2

Hemolysis times of normal and carbonic anhydrase-inhibited canine red cells (0.05 ml of blood), in mixtures of NH_4Cl and $NaHCO_3$ (150 mM)

NH_4Cl	$NaHCO_3$	pH	HCO_3^-	CO_2	Times for hemolysis	
					Catalyzed	Uncatalyzed[a]
ml added			*mM*	*mM*	*sec*	
5.75	0.25	7.15	5.75	0.50	30	3360
5.50	0.50	7.40	11.9	0.60	23	2520
5.0	1.0	7.63	24.2	0.80	17	2000
4.5	1.5	7.80	36.6	0.90	19	2000
4.0	2.0	7.90	49.2	0.80	20	2000

[a] In the presence of 10^{-4} M CL 13,580.

on CO_2 concentration is anticipated from the simple first-order relation

$$V_{unc} = k_1[S] \quad (11)$$

where V_{unc} is the velocity of the uncatalyzed reaction, k_1 is the rate constant, and $[S]$ is the CO_2 concentration. Similarly, the observed catalytic rate, V_{cat}, is dependent on $[S]$, since the K_m is about 10 mM (reviewed in ref. 1), and thus $K_m > [S]$. The relationship is

$$V_{cat} = \frac{V_{max} \cdot S}{K_m + S} \quad (12)$$

in which S in the denominator may be neglected.

Table 3 shows that the observed catalytic rate increases when the volume of red cells

TABLE 3

Effect of volume of dog red cells on observed hemolysis time in mixture of 5 ml of NH_4Cl (150 mM) and 1 ml of $NaHCO_3$ (150 mM)

Cells	Times for hemolysis	
	Catalyzed	Uncatalyzed[a]
ml	*sec*	
0.025	11	1680
0.050	17	1920
0.10	26	
0.15	30	
0.20	33	1920

[a] In the presence of 10^{-4} M CL 13,580.

in the system decreases. The probable explanation for this is that sensing of the catalytic rate by clearing of the solution is easier in the more dilute system. In the much slower, nonenzymatic reaction, however, the rate is independent of the volume of cells: here rapid sensing is not involved. Theoretically, the catalytic as well as the noncatalytic rate should be independent of the volume of red cells, since ideally we are studying events as they may be imagined in a single cell.

Table 4 shows that red cells of the elasmobranch behave in the same general way as mammalian erythrocytes. The longer hemolysis times of inhibited elasmobranch erythrocytes may reasonably be ascribed to their greater osmolarity, which would necessitate a greater accumulation of ions to cause hemolysis. The fact that the 35-fold lower concentration of carbonic anhydrase in the elasmobranch cell is not reflected in the observed catalytic rate further suggests that the concentration of enzyme is not a limiting event in this system, and will be discussed in a later section.

From the data of Tables 1–3, the standard system as described in METHODS was selected for further work. In this system (125 mM NH_4Cl, 25 mM $NaHCO_3$, and 0.05 ml of blood), the pH and CO_2 equilibria were close to those found *in vivo*.

Complete Inhibition of Enzymatic Reaction

Quantitative aspects of enzyme-drug interaction using ethoxzolamide and methazolamide.

TABLE 4

Hemolysis times of normal and carbonic anhydrase-inhibited red cells from squalus acanthias

NH_4Cl (0.5 M)	$NaHCO_3$ (0.15 M)	CO_2	Times for hemolysis		
			Catalyzed		Uncatalyzed[b] 0.1–0.2 ml
			0.1 ml[a]	0.2 ml	
ml	*ml*	*mM*	*sec*		
5	1	1.16	38	65	>4800

[a] These are the volumes of red cells used.

[b] In the presence of 10^{-4} M CL 13,580.

Figure 2 shows an experiment carried out in the standard system, using dog cells with concentrations of ethoxzolamide added to the aqueous solution (method A) from 0.02 to 100 μM. The critical point is that the three highest concentrations yielded essentially the same rate, which now can be taken as that of the uncatalyzed reaction. The suitability of ethoxzolamide for this determination was dictated by its very high activity against the enzyme (1), and decisively by its high lipid solubility, which permitted very rapid entrance into the red cell. Holder and Hayes (4) found that the rate constant for entry into dog red cells is about 0.7 sec^{-1}, so that near equilibrium between drug in solution and red cell is achieved within about 5 sec. Since the observed catalytic rate is 20–30 sec (Table 1), diffusion is not rate-limiting in the system. Thus a smooth curve is generated, over the range of observed inhibited times for hemolysis, from 40 sec to 50 min.

The uncatalyzed (fully inhibited) hemolysis time of 50 min makes it possible to estimate the true catalytic time, since Roughton (5) and others (reviewed in ref. 1) have calculated that carbonic anhydrase in red cells increases the rate of CO_2 hydration about 30,000-fold. Thus the enzymatic reaction should be complete in 0.1 sec, 200 times faster than that observed. The difference is assumed to be due to our slowness in sensing the hemolytic process.

The calculation of i from Eq. 9 or 10 does not involve V_{cat}, the observed catalytic time, which is about 20 sec. Thus the "sensing error," or delay, is not involved in the estimation of fractional inhibition,

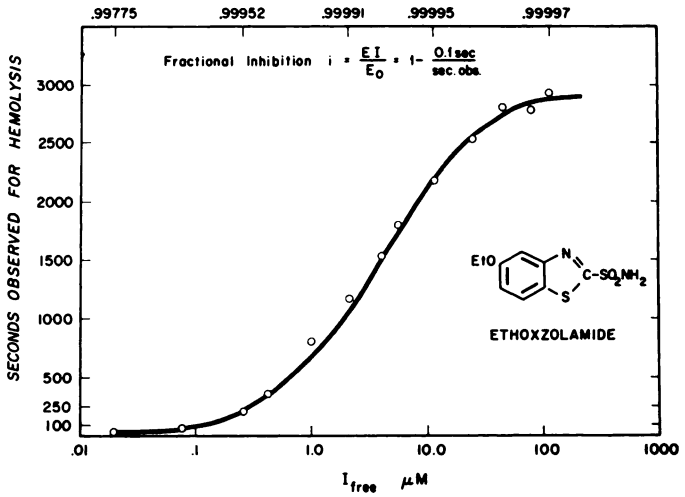

FIG. 2. *Effect of carbonic anhydrase inhibition by ethoxzolamide on osmotic hemolysis of dog red cells* Method A, drug not incubated with red cells.

which is calculated from times much closer to those of the nonenzymatic reaction. The latter are so slow that the observed time is the true time of the ionic transfer reactions described in Fig. 1. In other words, the physical limitations on detection determine the rate observed experimentally in the range of V_E and V_{cat}, but in the range of V_{uncat} the rate-limiting event resides in the chemistry of the reaction itself.

Knowledge of the true catalytic time permits an analysis of inhibition kinetics, according to the arithmetic model given in the *Calculations* section above. Figure 2 shows the fractional inhibition (i) throughout the experiment. We have the interesting circumstance that very high degrees of i may be recognized with accuracy. Unlike our system *in vitro* (6), the time of the uncatalyzed reaction is much slower than the catalytic or properly chosen inhibited reactions. This arises from the large amount of enzyme in the intact cell (on the order of 10^{-4} M), in contrast to that in the system *in vitro* (on the order of 10^{-9} M). The kinetics in this system is therefore allied to that *in vivo*, where similar enzyme concentrations exist, and where similarly high degrees of fractional inhibition have been studied (7).

From the fractional inhibition value of i = 0.9999, the K_I may be calculated with the use of Eq. 5. I_0, the concentration of drug in aqueous solution, is approximately equal to I_f, as demonstrated below. This value, from Fig. 2, at i = 0.9999, is 2 μM, yielding $K_I = 2 \times 10^{-10}$ M. This may be compared with the results obtained in the dilute system *in vitro*, in which E_0 was 10^{-4} times that in the intact red cell and the K_I for ethoxzolamide was variously reported as 15×10^{-10} M (8) and 3×10^{-10} M (9). It has been noted that K_I values calculated in this way are not accurate for the very active drugs, because I_{50} and E_0 are numerically very close (6, 7).

Ethoxzolamide was also evaluated (method B) by preliminary incubation of drug with red cells prior to addition of the cells to the electrolyte solution. A curve similar to that of Fig. 2 was obtained. Using the 1000-sec time, which should theoretically yield i = 0.9999, we obtained a value for I_f of 3 μM, which yielded a K_I of 3×10^{-10} M, in close agreement with the findings in Fig. 2. It is thus clear that equilibrium of drug between cellular enzyme and solution can be attained rapidly from either side.

A second lipid-soluble, highly active drug against carbonic anhydrase, 2-*o*-chlorophenylthiadiazole-5-sulfonamide (CL 13,580), was used with results very similar to those found with ethoxzolamide (Table 5). The agreement between the K_I values for intact cells and hemolysates was particularly good.

Figure 3 shows an experiment (method A, dog cells) with methazolamide, a less active drug, with a fairly rapid diffusion rate into red cells. According to the data of Holder and Hayes (4), the rate constant for entry is about 0.06 sec^{-1}, whence the half-time to reach equilibrium is about 10 sec. While this appears long in relation to the observed catalyzed time (20 sec in this experiment), the data show the same time plateau of about 50 min, as is evident in Fig. 2 for ethoxzolamide. Furthermore, when methazolamide was incubated with dog red cells according to method B, a curve quantitatively similar to that of Fig. 3 was generated. Thus equilibrium is achieved within a time permitting quantification, as for ethoxzolamide; conceivably diffusion is more rapid in this system than in that of Holder and Hayes (4).

The K_I of methazolamide was calculated from the 1000-sec (i = 0.9999) point of Fig. 3, which yields $I_0 = I_f$ = 160 μM. From these values, K_I is 1.6 × 10^{-8} M; in the dilute system *in vitro* it was 3.5 × 10^{-8} M (8) or 6.5 × 10^{-8} M (9).

Several additional points are relevant to the quantitative interpretation of these experiments. One concerns the effect of NH_4^+ on the enzyme itself. When enzyme activity *in vitro* was measured by the standard method in this laboratory (6) but in the presence of 125 mM NH_4Cl and 25 mM $NaHCO_3$, the catalytic rate was decreased about 50%. In the presence of these electrolyte concentrations, however, the inhibitory activity of the drugs was not altered. When red cells were exposed to 125 mM NH_4Cl for 5 min and then centrifuged, diluted, and analyzed in the usual way for enzyme activity, the catalytic rate was normal. It thus appears that the artificial environment of the red cell used in the present study does not substantially alter its carbonic anhydrase activity. The maximal correction factor that might be applied is a decrease in the theoretical catalyzed rate by half, making the time constant 0.2 sec (see calculations above). However, the necessity for this correction is not certain, and in any case it would not seriously alter our calculations.

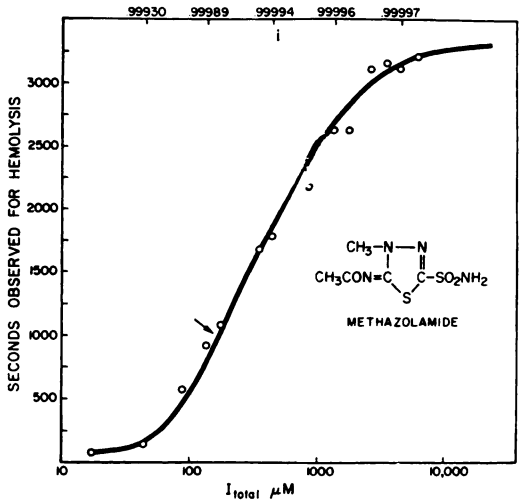

FIG. 3. *Effect of carbonic anhydrase inhibition by methazolamide on osmotic hemolysis of dog red cells*

Method A, drug not incubated with red cells.

The second point concerns the binding of drug to enzyme in the presence of the artificial ionic solution. In a typical experiment, 0.27 μmole of ethoxzolamide was dissolved in 50 ml of the standard solution of NH_4Cl–$NaHCO_3$ (I_0 = 5.5 μM), to which 0.4 ml of dog red cells was added. After 2 min the cells were centrifuged. They contained 48 μM drug (0.02 μmole), while the outside solution (I_f) contained 5.2 μM. The concentration in red cells was approximately that found in earlier experiments in which the entry of drug from plasma was studied (10), and it agreed roughly with the concentration of carbonic anhydrase in the cells (6). This experiment also shows that drug can be recovered quantitatively from the system. Finally, it is clear that the amount of drug bound to the very small volume of red cells was small relative to the amount added, so that $I_0 \cong I_f$. Similar findings were obtained with methazolamide and CL 13,580.

Experiments of the type of Figs. 2 and 3 were also carried out with human red cells. Ethoxzolamide and CL 13,580, when tested by either method A or method B, gave a maximal time of 2049 sec, with a mean of about 1800 sec, in a total of eight tests. The K_I values were of the same order observed

for the dog erythrocytes, and are summarized in Table 5.

In the elasmobranch, using the system outlined in Table 4, inhibition with CL 13,580 yielded no hemolysis in 80 min. Thus the uncatalyzed reaction time, like the catalyzed, was considerably longer than that for the mammal, probably because of the much higher osmolarity of the dogfish red cell.

Behavior of other sulfonamides in the system. In view of the early experiments with sulfanilamide, the wide use of acetazolamide, and our interest in the pharmacology of benzolamide, it appeared worthwile to summarize experiments with these drugs (Table 5). None of these three agents could be used for the quantitative analysis of total inhibition described above, for a variety of reasons. Sulfanilamide is too weak an inhibitor for full inhibition; the other drugs diffused too slowly for equilibration with enzyme in the short time (<20 sec) before the beginning of hemolysis. Table 5 shows that the half-time for diffusion of acetazolamide and benzolamide was 2–3 min, far too long for these drugs to have any effect when added to the outside solution. Accordingly, they were inactive when used in method A. Conversely, when acetazolamide and sulfanilamide were tested by method B, they seemed more active than in the standard test *in vitro*, because more drug than had been calculated (on the basis of final diffusional equilibrium between cells and aqueous solution) was present in the red cells during the first few minutes of the experiment. Sulfanilamide yielded a K_I far too low when tested by method B, but showed reasonable agreement with the K_I value obtained *in vitro* by method A, suggesting that here diffusion was nearly adequate. The rate of diffusion of sulfanilamide into red cells is considerably faster than that for acetazolamide and benzolamide, although slower than for the other three drugs of Table 5. Jacobs and

TABLE 5

K_I of sulfonamides against carbonic anhydrase, related to some physical properties in intact red cells and solution

Drug	Ether/ buffer partitions[a]	$t_{1/2}$, buffer to red cells[b]	K_I				
			Dog cell lysates[c]	Intact cell[d]			
				Dog		Human	
				Method A	Method B	Method A	Method B
		min	*μM*	*μM*		*μM*	
Ethoxzolamide	140	0.017	1.5	0.2	0.3	0.4	
CL 13,580[e]	79	0.1	0.5	0.4	0.3	2	0.6
Methazolamide	0.62	0.2	35	10	10	39	
Sulfanilamide	0.15	0.5	9100	2310 (500 sec)	212 (100 sec)	5895 (253 sec)	146 (70 sec)
Acetazolamide	0.14	2	40		0.2–2	N[f]	1–5
CL 11,366[g] (benzolamide	0.001	3	2.5			N[h]	

[a] From reference 11.

[b] From reference 4, except for CL 13,580, which was determined later by the same methods.

[c] From reference 8. $K_I = I_{50} - \frac{1}{2} E_0$, where $E_0 = 5 \times 10^{-9}$ M.

[d] From the concentration ($I_{99.99}$) at which the hemolysis time is 1000 sec, whence $i = 0.9999$, except for sulfanilamide, for which $i \cong 0.999$ and the times are noted in parentheses.

[e] 2-*o*-Chlorphenylthiadiazole-5-sulfonamide.

[f] The reaction time was 100 sec when the concentration was 5 mM in the outside solution. N denotes essential inactivity in the test.

[g] 2-Benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide.

[h] The reaction time was 48 sec when the concentration was 70 μM in the outside solution. N denotes essential inactivity in the test.

Stewart (2) also observed that the effect of sulfanilamide was somewhat limited by diffusion. The K_I for sulfanilamide was calculated from the 100–500-sec point (representing less inhibition than for the other drugs), because full inhibition (more than 99.99%) could not be reached at maximum solubility.

Thus it is clear that only rapidly diffusing drugs can yield quantitative information, and that agreement between methods A and B is the experimental criterion.

DISCUSSION

The results of these experiments will be discussed in terms of (a) quantification of the hydration of CO_2 to HCO_3^- within the red cell as it applies to the phenomenon reported by Jacobs and Stewart (2), (b) use of the system of Jacobs and Stewart for determining the kinetics of inhibition of carbonic anhydrase at the levels (more than 99%) important *in vivo*, and (c) relevant earlier work.

a. In the system described we have achieved complete inhibition of carbonic anhydrase activity, so that known rate constants for the uncatalyzed hydration of CO_2 can be applied. From this we can determine the extent of intracellular HCO_3^- and NH_4^+ formation. As noted in the introduction, and implicit in the model of Jacobs and Stewart, HCO_3^- is formed in the cell from CO_2, but only because a new cation (NH_4^+) is also available as a counter-ion. According to Jacobs and Stewart, HCO_3^- does not necessarily accumulate in the cell, but may be exchanged for external Cl^-. In the model shown in Fig. 1, the accumulated anion is denoted as Cl^-, with the representation that less Cl^- and more HCO_3^- could be present. This distinction is not important in the present context, since the hydration of CO_2 to form HCO_3^- is the initial process and is the one controlled by the enzyme. The sum of newly present HCO_3^- and Cl^- in the cell is equivalent to the amount of HCO_3^- formed, and is also equivalent to the newly formed NH_4^+ within the cell.

It is simple to calculate the amount of new HCO_3^- formed under conditions of total enzyme inhibition. The rate constant for the uncatalyzed hydration of CO_2 at 25° is 2.2 min^{-1} (12), and the concentration of CO_2 in the system is 0.8 mM. Thus the rate of hydration (Eq. 11) is

$$\begin{aligned} V_{unc} &= k_1 (CO_2) \\ &= 2.2 \text{ min}^{-1} \cdot 0.8 \text{ mM} \\ &= 1.73 \text{ mM min}^{-1} \end{aligned}$$

Hemolysis occurs in about 50 min, at which time 87 mM HCO_3^- has been formed, "trapping" 87 mM NH_4^+. Some (or all) of the HCO_3^- will be exchanged for Cl^-; in any case, the cell (at constant volume) will increase its osmolarity by 174 mOsM, or 58%. Actually, of course, this osmotic force causes water to enter, swelling and hemolyzing the cell. These forces appear roughly analogous to those generated by adding red cells to hypotonic (0.4%) NaCl solutions.

In the catalyzed reaction in normal, uninhibited red cells, the same osmolar forces are presumably at work, but are generated much more rapidly. In terms of the end point used here, 20 sec are required. But Jacobs and Stewart (2) have demonstrated that swelling begins within 1 sec after the cells have been suspended with NH_4^+, HCO_3^-, and Cl^-. We have calculated that the true catalytic rate is much greater than the actual swelling and hemolysis rates observed by outselves or recorded by Jacobs and Stewart (2). This will be discussed in the following paragraphs.

b. From the data of Figs. 2 and 3, showing the time course for the uncatalyzed reaction (approximately 50 min), we obtain the catalytic time, since the magnification of rate by carbonic anhydrase in the red cell is well known from the work of Roughton (5). This value is about 30,000, and thus the enzymatic reaction time is 0.1 sec. This is due to the very high turnover number of carbonic anhydrase and its high concentration in red cells (1), which places the catalytic interconversion $CO_2 \rightleftharpoons HCO_3^-$ in the time range of inorganic reactions.

Table 6 shows that 50% inhibition of the enzyme would be totally unrecognized in this system; it would occur in 0.2 sec, which is 100 times faster than the observed normal uninhibited rate of hemolysis. The factors that make this observed rate relatively slow have not been identified, but must include

TABLE 6

Model relations between carbonic anhydrase activity and observed hemolysis rates in dog red cells suspended in NH_4Cl-$NaHCO_3$

Reaction	Time	Velocity	E_f	EI	i
	sec	*M/min*	*μM*	*μM*	
Calculated Enzymatic					
Normal	0.1	60[a]	36	0	0
50% inhibited	0.2	30	18	18	0.5
Observed enzymatic					
Normal	20	0.3[b]	0.18	35.82	0.995
Partially inhibited[c]	100	0.06	0.04	35.96	0.9990
	1000	0.006	0.004	35.996	0.9999
Observed uncatalyzed =	3000	0.002[d]	0.001	~36	0.99997
totally inhibited			≅0		≅1

[a] V_E

[b] $V_{cat.}$

[c] Drug effects begin here.

[d] $V_{uncat.}$

diffusion rates, resistance of the membrane to swelling, and our inability to determine accurately the end point of hemolysis. The relationship between the calculated and observed enzymatic rates in this system is the same as that observed *in vitro*, the former being 10^2–10^3 times as fast as the latter (1). Clearly, enzymatic catalysis is not rate-limiting in the present model or *in vivo*, unless it is inhibited more than 99%.

Because of the very high concentration and activity of the enzyme, an enormous range of possible reaction rates occurs, from the observed, which appears to utilize only 0.5% of available enzyme, to the totally inhibited, which involves less than 0.003% of the enzyme. In the usual cell-free dilute enzyme system, particularly at 37°, the uncatalyzed rate is appreciable, and makes determination of catalytic and inhibited rates susceptible to considerable method error. Determination of $i > 0.5$ is particularly difficult, and of $i > 0.8$, virtually impossible. The present experiments enabled us to study a system *in vitro* with three unusual and important properties: intact cells are used, enzyme concentration is high, and fractional inhibition in the range of 0.999 may be estimated with accuracy. This system provides all the properties of carbonic anhydrase activity *in vivo*, in which enzyme and inhibition kinetics can be approached.

Of cardinal interest was to find whether drug activity in this high range of inhibition would conform to the prediction from work with cell lysates, in which the concentration of enzyme interacting with drug was about 10^{-4} times lower than the concentration in the intact cell. For three of the drugs for which diffusion was not found to be rate-limiting, the K_I calculated from $i = 0.9999$ in intact cells (see Table 6 for enzymatic equilibra) was close to that calculated from $i = 0.50$ in solution (Table 5). This validates our earlier work, in which fractional inhibition exceeding 0.99 was calculated for observations *in vivo*, based on K_I values for the drugs derived from the I_{50} data obtained in solution (7). Attempts have also been made to show that cellular constituents do not seriously alter the K_I (8); this also appears to be confirmed.

c. Keilin and Mann (13) were the first to measure carbonic anhydrase within red cells. They converted hemoglobin to methemoglobin, and used the colorimetric shift of this pigment with pH to follow hydration or dehydration of CO_2 within the cell. Wistrand and Bååthe (14) used this method to study inhibitors and drew conclusions similar to ours; i.e., when the catalyzed reaction rate was reduced by 50%, enzyme activity was inhibited by 98.6% with one method of calculation and by 99.95% with another. The

contributions of both human red cell enzymes B and C were taken into account. Erythrocytes from various species and from a human neonate were also studied. An advantage of the method of Wistrand and Bååthe is that drug and enzyme could be equilibrated (60 min was the longest time necessary) before the reaction, after which the reaction was carried out within 2 min. Under these conditions, unlike ours, complete inhibition was measurable for the ionized compounds CL 11,366 and acetazolamide; about 10^{-4} M and 10^{-3} M concentrations of drug were required, respectively. A disadvantage of their method is the small spread between the catalytic (30-sec) and noncatalytic (120-sec) reaction times, making the measurement of i inaccurate in the 0.99 range.

Constantine, Craw, and Forster (15) studied CO_2 uptake by whole red cells in a rapid-flow system. The uncatalyzed reaction rate was magnified 7000-fold in this system, but 1 mM acetazolamide (in full equilibrium with enzyme) inhibited the catalyzed reaction rate only by about 90%. This result, taken in the context of our data and those of Wistrand and Bååthe (14), suggests that there exist other intracellular receptors than carbonic anhydrase for CO_2, and indicates the extent of their participation, about 10%.

Kernohan and Roughton (16) used a rapid thermal measurement for CO_2 hydration in concentrated red cell lysates and intact cells. They found that enzyme activity was equal in these two situations, showing that catalytic activity was not lowered in concentrated solutions or in the cellular milieu.

The present data thus accord with previous work in this and other laboratories, for both dilute and concentrated enzyme solutions. The principal advances are the study of the Jacobs and Stewart reaction in the complete absence of active carbonic anhydrase, and the quantification of sulfonamide activity against enzyme when inhibition exceeds 99%.

ACKNOWLEDGMENT

We thank Dr. Betty Vogh for her careful and helpful reading of the manuscript.

REFERENCES

1. T. H. Maren, *Physiol. Rev.* **47**, 595 (1967).
2. M. H. Jacobs and D. R. Stewart, *J. Gen. Physiol.* **25**, 539 (1942).
3. R. G. Bates and G. D. Pinching, *J. Amer. Chem. Soc.* **72**, 1394 (1950).
4. L. B. Holder and S. L. Hayes, *Mol. Pharmacol.* **1**, 266 (1965).
5. F. J. W. Roughton, *Harvey Lect.* **39**, 96 (1943).
6. T. H. Maren, A. L. Parcell and N. M. Malik, *J. Pharmacol. Exp. Ther.* **130**, 389 (1960).
7. T. H. Maren, *J. Pharmacol. Exp. Ther.* **139**, 140 (1963).
8. T. H. Maren and C. E. Wiley, *J. Med. Chem.* **11**, 228 (1968).
9. T. H. Maren, *J. Pharmacol. Exp. Ther.* **139**, 129 (1963).
10. T. H. Maren, B. Robinson, R. F. Palmer and M. E. Griffiths, *Biochem. Pharmacol.* **6**, 21, (1961).
11. P. J. Wistrand, J. A. Rawls and T. H. Maren, *Acta Pharmacol. Toxicol.* **17**, 337 (1960).
12. B. H. Gibbons and J. T. Edsall, *J. Biol. Chem.* **238**, 3501 (1963).
13. D. Keilin and T. Mann, *Nature* **148**, 493 (1941).
14. P. J. Wistrand and P. Bååthe, *Acta Pharmacol. Toxicol.* **26**, 145 (1968).
15. H. P. Constantine, M. R. Craw and R. E. Forster, *Amer. J. Physiol.* **208**, 801 (1965).
16. J. C. Kernohan and F. J. W. Roughton, *J. Physiol. (London)* **186**, 138P (1966).