J. Physiol. (1980), **303**, 535–547
With 5 text-figures
Printed in Great Britain

A COMPARATIVE STUDY OF THE KINETICS OF THE BOHR EFFECT IN VERTEBRATES

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(*Received 8 March 1979*)

SUMMARY

1. The kinetics of the Bohr effect and the role of carbonic anhydrase were studied in a series of representative vertebrates using a continuous flow rapid reaction apparatus.

2. The rates of the Bohr effect in vertebrates are very similar, and differences among classes are manifestations of the ambient temperature.

3. Complete carbonic anhydrase inhibition causes a fifteen to fortyfold reduction in the rate of the Bohr effect, sufficient to abolish its occurrence within capillary transit.

4. There is a twenty-three-fold (duck) to 360-fold (man) excess of carbonic anhydrase activity in vertebrate red cells for the normal generation of the Bohr effect.

5. When carbonic anhydrase is inhibited and CO_2 hydration becomes rate limiting, the stoichiometry of the Bohr effect ($\Delta \log p_{O_2}/\Delta pH$) is revealed in the ratio of the rates of proton formation in red cells to O_2 release from haemoglobin.

INTRODUCTION

The Bohr effect is the heterotropic interaction of hydrogen ions, carbon dioxide and oxygen upon their binding with haemoglobin. Since the discovery by Bohr, Hasselbalch & Krogh (1904), an enormous amount of work has been directed toward equilibrium measurements of the process in a myriad of animals (Hilpert, Fleischmann, Kempe & Bartels, 1963; Riggs, 1979). The protonation of certain oxylabile amino acids in haemoglobin causes a conformational change in the shape of the molecule, yielding a decrease in the affinity of the haem subunit for O_2. This increase in the P_{50} with acidosis (or right shift in the O_2 dissociation curve) and decrease with alkalosis enhances the exchange of O_2 and CO_2 in the tissue and gill or lung capillaries. In those animals displaying a large Bohr effect it may play a considerable role in O_2 exchange (Riggs, 1960; Bartels, 1973).

The process must proceed to completion rapidly in the short time course of

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0022-3751/80/8090-0084 $07.50

capillary transit to be of physiological value. The over-all rate of protonation plus O_2 release in simple solution:

$$H^+ + HbO_2 \rightleftarrows HbH^+ + O_2 \uparrow \quad (1)$$

is very rapid, having a half-time in the order of 10 msec (Roughton, 1964). However in blood, the generation of the Bohr effect must also include the diffusion of CO_2 and O_2 through the red cell membrane and cytoplasm and the intracellular hydration–dehydration reactions of CO_2. The kinetics in intact human red cells have been studied by Craw, Constantine, Morello & Forster (1963) and Forster & Steen (1968). They showed the process has a half-time of 120 msec, a rate sufficient to yield equilibrium in 0·75–1 sec, the average capillary transit time. They found inhibition of carbonic anhydrase results in a marked reduction in the rate. This corresponds well to a model in which the protonation of haemoglobin is dependent upon the hydration of CO_2, thus linking the functions of the two major proteins in the red cell. If the formation of H^+ from CO_2 determines the rate of the Bohr effect, then inhibition of carbonic anhydrase may have important chemical, physiological and clinical implications.

As part of our investigations of the role of carbonic anhydrase in respiration, we studied the rate of the Bohr effect with and without carbonic anhydrase in five representative vertebrate species: spiny dogfish (*Squalus acanthias*), goosefish (*Lophius americanus*), bullfrog (*Rana catesbeiana*), white Pekin duck (*Anas platyrynchos*) and man. We sought to answer these questions: (1) what is the quantitative relation between carbonic anhydrase inhibition and the Bohr effect rate? (2) do vertebrate haemoglobins show different kinetic Bohr effects? (3) is there a relation between the magnitude of the Bohr effect and the carbonic anhydrase activity in the blood of vertebrates?

METHODS

These experiments were performed with a modified Hartridge–Roughton (1923) rapid reaction apparatus (Foster & Steen, 1968). The object is to cause an abrupt change in the acid-base composition of the extracellular fluid and measure the resultant changes in dissolved O_2. A 1% suspension of red cells in Ringer solution of known pH, p_{CO_2} and p_{O_2} is mixed equally with the same Ringer solution of different pH or p_{CO_2} but equal p_{O_2}. The appropriate Ringer solutions are those following Forster & Steen (1968) for man and Prosser (1973) for the other vertebrates. The mixture travels at a known speed down a thin observation tube to a modified Clark electrode. The resultant change in p_{O_2} at different times is directly displayed on a Chemical Microsensor Model 1201 (Transidyne General Co.), calibrated directly by gas mixtures of known O_2 tension. The electrode response was the same for gases and solutions of equal p_{O_2}. O_2 concentrations (mM) were calculated from appropriate solubility coefficients. From these measurements, the time course and rate of the Bohr effect were calculated.

The blood from humans was drawn from an antecubital vein. Dogfish and goosefish blood was taken from a dorsal vein. Duck blood was collected from a catheter in a wing vein. Frog blood was obtained by ventricular puncture in the pithed animal. All blood was taken into heparinized syringes, placed in ice and used within 12 hr. Each point in the time course of the Bohr effect is the average of at least five measurements.

Two types of experiments were performed. In type I, the rate of the Bohr effect was measured when the extracellular pH of the cell suspension was instantly changed by an equal addition of acidified Ringer solution containing 22 mM-lactic acid. These experiments were performed on dogfish and human blood. A dose response to the carbonic anhydrase inhibitor methazolamide (Neptazane, Lederle) was studied. Chemical and pharmacological properties of this drug have been described (Maren, Haywood, Chapman & Zimmerman, 1977). Dogfish red cell sus-

pensions and lactic acid Ringer solution were equilibrated with 98% N_2 and 2% O_2 at 16 °C. the human solutions were equilibrated with 95% N_2 and 5% O_2 at 37 °C. Relevant CO_2 concentrations were those generated by the simple stoichiometry of lactic acid added to bicarbonate solutions.

In type II experiments, the rate of the Bohr effect was measured when the extracellular p_{CO_2} was instantly changed. Dogfish (16 °C), goosefish (16 °C), frog (16 °C) and duck (41 °C) red cell suspensions were equilibrated with 98% N_2: 2% O_2 and the respective Ringer solution with 78% N_2: 2% O_2: 20% CO_2. In the human experiments, the red cell suspension at 37 °C was gassed with 95% N_2: 5% O_2 and the Ringer solution with 75% N_2: 5% O_2: 20% CO_2. A full dose response to methazolamide was examined in human blood and the effect of 10^{-3} M-drug in the other species. Relevant CO_2 concentrations (mM) were determined from the p_{CO_2} (calculated by the percentage CO_2 in the equilibrating gas at ambient barometric pressure and temperature) and the appropriate solubility coefficients. In the type II experiments with human blood, pH, p_{CO_2} and p_{CO_2} of the reacting solutions and final mixture were measured on an Instrumentation Laboratories (Lexington, Mass.) blood gas analyser calibrated with standard pH solutions and known gas tensions. Table 1 shows these values. In all experiments, drug was added to both red blood cell suspensions and Ringer solution and one half hour was allowed for equilibration with drug and gas mixtures.

RESULTS

Events occurring during measurement of kinetics of the Bohr effect are shown in Fig. 1. In type I experiments, lactic acid is added to the extracellular solution, which instantly reacts with $NaHCO_3$. H_2CO_3 then rapidly dissociates at the uncatalysed rate to CO_2 and water, CO_2 diffuses into the red cell where it is catalytically hydrated to yield HCO_3^- and protons for reaction with haemoglobin. We only consider the diffusion of CO_2 into the red cell in our calculations and not that of H_2CO_3, since the latter species is not present for an appreciable time. The rate of H_2CO_3 dehydration is so great that a concentration of less than 20 μM exists 22 msec (37 °C) or 115 msec (16 °C) after mixing (Fig. 1). In type I experiments, lactic acid (22 mM) in Ringer solution added to the red cell suspension in 8 mM-HCO_3^- (dogfish) or 25·6 mM-HCO_3^- (man), resulted in different CO_2 concentrations and pH at half-time. This is shown in Fig. 1. In type II experiments, CO_2 is added directly to the extracellular solution. All reactions and diffusion times occur with half-times less than 10 msec except the uncatalysed CO_2 hydration and carbonic acid dehydration (Forster, 1964). These reactions are greatly accelerated by carbonic anhydrase. We show the enzyme catalysing the reaction $CO_2 + OH^- \rightleftharpoons HCO_3^-$ which is the generally accepted mechanism for all pH values. At neutral pH, the uncatalysed reaction proceeds by the hydration of CO_2, but the product is still HCO_3^-.

The results of a dose–response study to carbonic anhydrase inhibition in a type I experiment with dogfish blood are shown in Fig. 2. Ninety-two per cent of the normal Bohr effect occurs by 6 sec, with a half-time of 1·4 sec. At the plateau of the dose response to methazolamide, the effect is only 16% complete, having a calculated half-time of 40 sec. This is approximately a thirtyfold reduction in the rate. Fig. 3 shows the results of the same type I experiment in human blood. The normal process is 98% complete at 1·5 sec, with a half-time of 0·32 sec. With total carbonic anhydrase inhibition, the effect is only 22% complete at 1·5 sec, having a calculated half-time of 3·7 sec. The reduction in the rate is twelvefold.

The results of type II experiments are shown in Fig. 4, in which the normal Bohr

effect and that at 10^{-3} M-methazolamide were examined in five representative vertebrates. The time courses are similar for human and duck and for goosefish, dogfish and frog. For the warm-blooded species, the half-time of the Bohr effect is 120 msec in man and 130 msec in the duck. In contrast the half-times for the three cold-blooded species range from 220 to 340 msec.

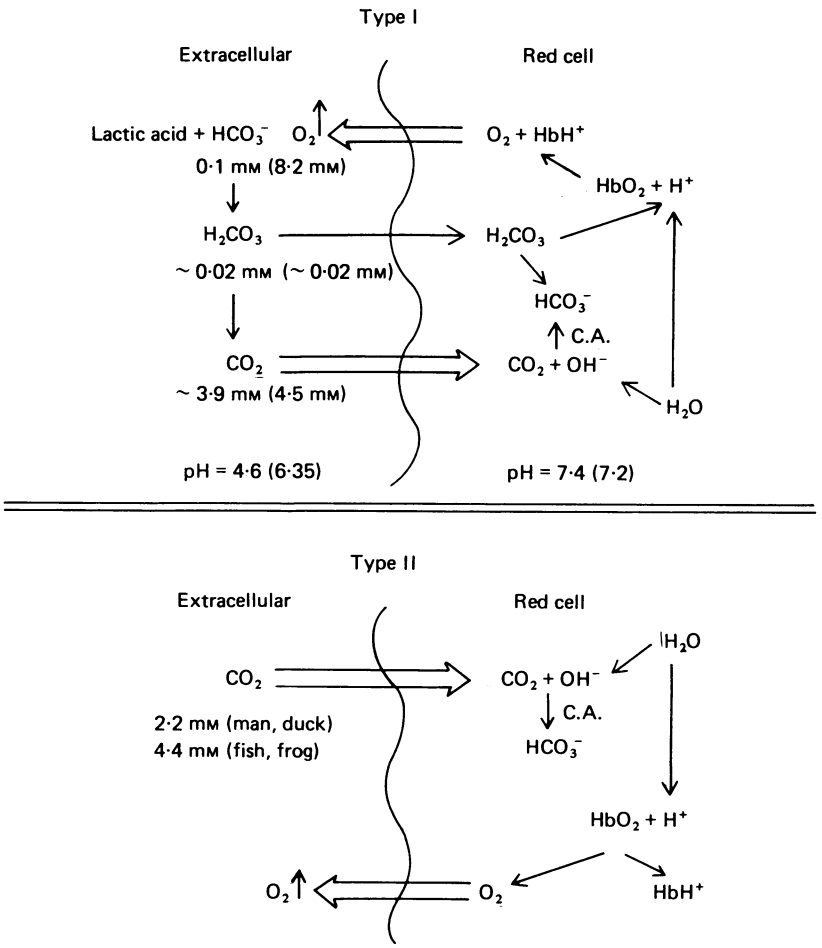

Fig. 1. Events in the measurement of the Bohr effect. Type I: concentrations shown are those for shark and for man (parentheses) 115 msec (shark) and 22 msec after mixing; these are the half-times for dehydration at 16 and 37 °C respectively. Type II: concentrations shown are those of CO_2 gas at the several temperatures used. C.A., carbonic anhydrase.

The dose response to methazolamide for human blood is presented in Fig. 5 for a type II experiment. Table 1 gives the measured compositions of the reacting solutions and the mixtures at equilibrium in these particular experiments and the postulated intra- and extracellular concentrations immediately after mixing before any CO_2 has reacted. There is a modest effect at 10^{-5} M with a peak effect a 3×10^{-4} M, which does not change when the drug concentration is increased threefold.

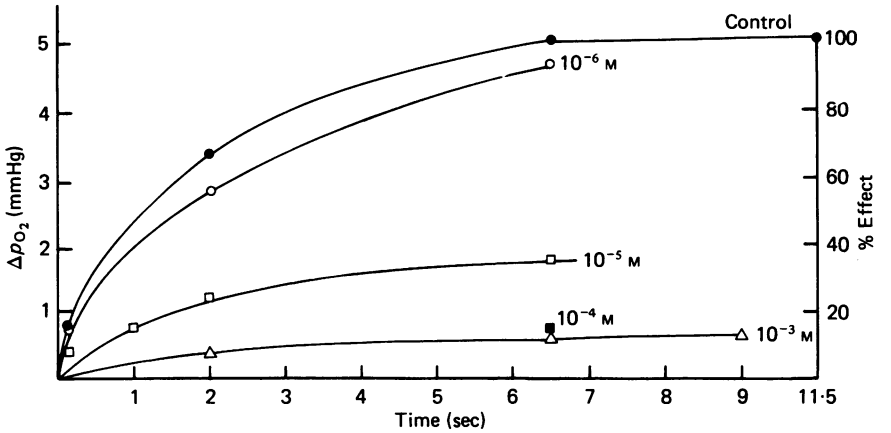

Fig. 2. The rate of O_2 release from red cells of dogfish following acidification (type I experiment), and its reduction by carbonic anhydrase inhibition. Concentrations are of methazolamide.

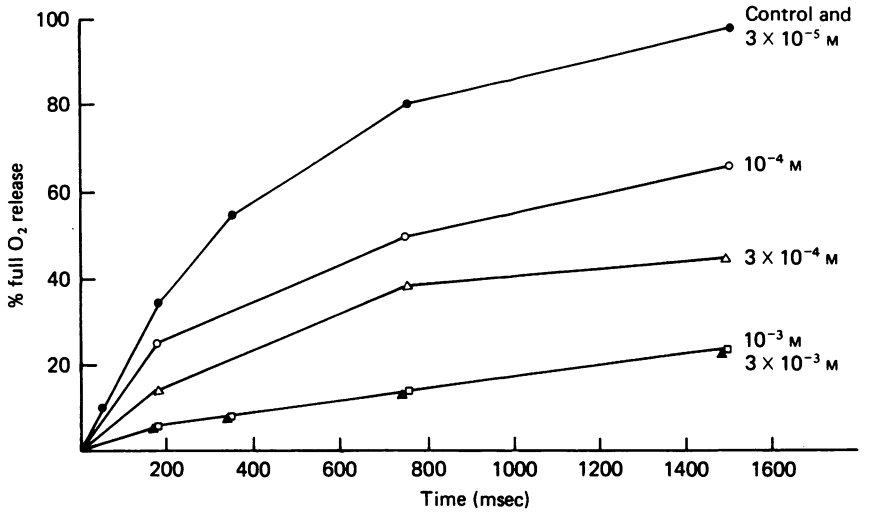

Fig. 3. The rate of O_2 release from human red cells following acidification (type I experiment), and reduction by carbonic anhydrase inhibition. Concentrations are those of methazolamide.

The release of oxygen from haemoglobin in these experiments depends upon the intracellular formation of protons from CO_2. Table 2 compares the rates of O_2 release with proton formation from the type II experiments of Fig. 4. Values for rates of O_2 release per unit volume red cells are calculated directly from Fig. 4 by the rise in p_{O_2} of the total suspension. Calculations of the proton generation rates (columns 2 and 4) are based on the uncatalysed reactions and the kinetic properties of the species' carbonic anhydrase, its intracellular concentration and inhibition by methazolamide and chloride. These data are given in Table 3 and the calculations made as follows.

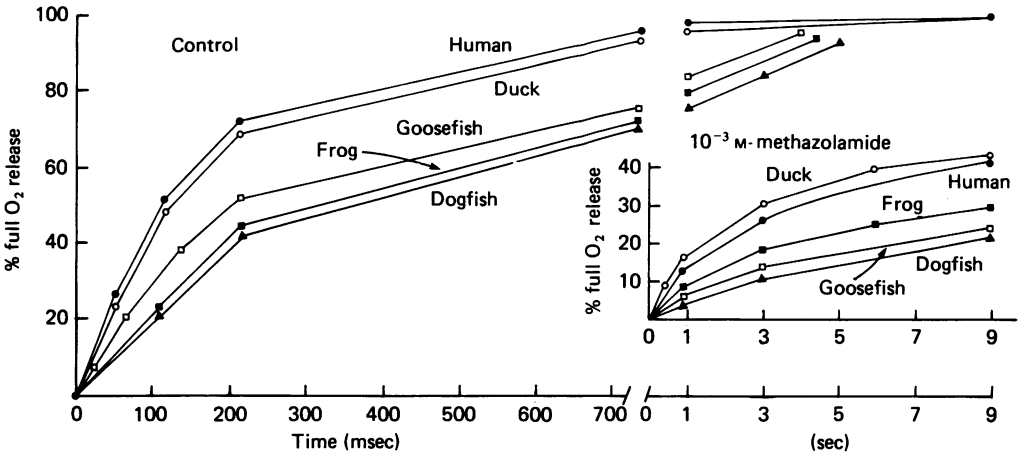

Fig. 4. Rates of Bohr effect in representative vertebrates, and effect of complete inhibition of carbonic anhydrase (inset). Type II experiments; CO_2 added at time zero.

TABLE 1. Composition of reacting solutions and mixtures in the type II human experiments

	p_{CO_2} (mmHg)	$[CO_2]$ (mM)	pH	$[HCO_3^-]$ (mM)	p_{O_2} (mmHg)
Buffer solution* extracellular	145	4·40	6·84	25·6	38
Red cell solution* extracellular	8	0·24	7·89	15·0	38
At initial mixture					
extracellular†	76	2·3	7·11	20·3	38
intracellular†	76	2·3	—	—	38
At equilibrium* mixture	50	1·52	7·25	21·5	55

* These are measured values as described in the text (n = 3).
† These are predicted values before any CO_2 reactions have occurred.

TABLE 2. Rates and half-times for the Bohr effect in five vertebrates compared with chemistry of H^+ generation (CO_2 hydration)

	Bohr effect factor	Control (mM sec^{-1} (half-time in sec))		10^{-3}M-methazolamide (mM sec^{-1} (half-time in sec))	
		H^+ generation	O_2 release	H^+ generation	O_2 release
Frog	−0·25	498	0·55	0·064	0·053
		(0·003)	(0·240)	(53)	(10·5)
Dogfish	−0·34	182	5·0	0·09	0·07
		(0·006)	(0·340)	(53)	(4·2)
Goosefish	—	380	18	0·06	0·17
		(0·004)	(0·220)	(53)	(7·2)
Duck	−0·53	150	13·0	0·21	0·19
		(0·017)	(0·130)	(8·7)	(11)
Man	−0·48	2850	16·5	0·20	0·38
		(0·0004)	(0·120)	(8·7)	(4·2)

Frog, dogfish, and goosefish blood was studied at 16 °C, duck at 41 °C and man at 37 °C. Rates for the Bohr effect are listed without parentheses and the corresponding half-times in parentheses. The values in column four are the sum of the calculated uncatalysed and residual catalysed rates. The uncatalysed rates are frog (0·057), dogfish (0·057), goosefish (0·057), duck (0·210) and man (0·160). See text for calculation of these rates.

Initial rates of uncatalysed intracellular proton generation are calculated from the equation

$$\dot{V}_{uncat} = (k_{CO_2})\,[CO_2] - (k_{HCO_3^-})\,[HCO_3^-], \quad (2)$$

where the k terms are first order rate constants for the appropriate pH, temperature and ionic strength (Edsall, 1969); these constants are not materially affected by the presence of haemoglobin (Roughton, 1964). The right-hand term cannot be computed

TABLE 3. Carbonic anhydrase hydration kinetics and inhibition constants

	k_{cat} (sec^{-1})	K_m (mM)	$[E]_0$ (mM)	Chloride K_I (M)	R.B.C. Cl^- (mM)	Methazolamide K_I (M)
Frog	4×10^{5a}	10^a	1×10^{-2b}	$2{\cdot}7 \times 10^{-1b}$	70	4×10^{-8b}
Dogfish	$2{\cdot}5 \times 10^{4c}$	5^c	$2{\cdot}4 \times 10^{-2c}$	$1{\cdot}2 \times 10^{-1c}$	130	2×10^{-7c}
Goosefish	10^{6c}	10^c	10×10^{-3c}	$1{\cdot}7 \times 10^{-1c}$	52	2×10^{-9c}
Duck	$3{\cdot}8 \times 10^{5d}$	10^d	$4{\cdot}4 \times 10^{-2d}$	$1{\cdot}4 \times 10^{-2b}$	80	$2{\cdot}7 \times 10^{-8d}$
Man[i]					80	
HCA-B	$4{\cdot}7 \times 10^{4e}$	4^e	$1{\cdot}25 \times 10^{-1f}$	6×10^{-3g}		$1{\cdot}7 \times 10^{-8h}$
HCA-C	9×10^{5e}	9^e	$2{\cdot}5 \times 10^{-2f}$	2×10^{-1g}		$1{\cdot}6 \times 10^{-8h}$

For cold-blooded species, at 16°, for duck 41°, for man 25°

a Bundy & Cheng (1976)

b Unpublished data, T.H. Maren & E. R. Swenson.

c Maren, Friedland & Rittmaster (1980).

d Bernstein & Schraer (1972). Data on chicken, except K_I, which are our values for duck.

e Khalifah (1971). Current work in our laboratory shows that the difference between values at 25 and 37 °C are very small. These values were obtained at 0·2 ionic strength.

f Magid (1970).

g Maren, Rayburn & Liddell (1976).

h Maren *et al.* (1977). There is no difference between these inhibition constants and that obtained on whole blood. For methazolamide and chloride, the inhibition constant is not affected by length of incubation or order of addition among enzyme, substrate or drug.

i HCA-B = human carbonic anhydrase, HCA-C = human carbonic anhydrase C.

directly because it is difficult to know the exact pH and bicarbonate concentration in the red cell. However the solution is at equilibrium before mixing ($\dot{V}_{uncat} = 0$), so the intracellular rate, $(k_{HCO_2^-})\,[HCO_3^-]$ must equal the rates of extracellular HCO_3^- hydration and CO_2 hydration. Thus we know the initial value for the right-hand term of eqn. (2), and we only need the new, experimentally raised carbon dioxide concentration at mixing ($[CO_2]_{exp}$) to solve eqn. (2). The extent of disequilibrium is given by

$$\frac{(k_{CO_2})[CO_2]_{exp} - (k_{HCO_3^-})[HCO_3^-]}{((k_{CO_2})\,[CO_2]_{exp}}. \quad (3)$$

Initial rates of unopposed proton generation (Table 2, column 2) in the normal are computed from the equation

$$\dot{V}_{cat} = \frac{k_{cat}[E]_0[CO_2]_{exp}}{K_m + [CO_2]_{exp}}, \quad (4)$$

where k_{cat} and K_m are hydration constants for the enzyme and $[E]_0$ is the intracellular enzyme concentration (Table 3). CO_2 concentrations are as shown in Fig. 1. Correction for the small degree of HCO_3^- dehydration is applied by multiplying $\dot{V}_{cat}$ by the

extent of disequilibrium. We assume that initial rates obtain through the course of our measurements for the following reasons. CO_2 tension is instantly raised tenfold at mixing within the red cell, while rapid equilibration of $[H^+]$ and $[HCO_3^-]$ with the external solution is prevented by resistance of the red cell membrane to rapid shifts of these ions (Forster, 1964). Extracellular CO_2 is not depleted because the concentration of red cells is very dilute and CO_2 hydration outside the cells proceeds at the slow uncatalysed rate.

TABLE 4. Comparison of proton formation and O_2 release in the Bohr effect

Human type 1 experiments

	Fractional carbonic anhydrase activity $a = 1-i$	Initial proton generation (mM sec^{-1})	$t\frac{1}{2}$ proton generation (sec)	Initial O_2 release (mM sec^{-1})	$t\frac{1}{2}$ O_2 release (sec)
Control	1	5410	0·0005	3·9	0·3
Methazolamide					
10^{-5} M	$1{\cdot}6 \times 10^{-3}$	—	—	—	—
3×10^{-5} M	$5{\cdot}5 \times 10^{-4}$	2·96*	1·0	3·9	0·3
10^{-4} M	$1{\cdot}6 \times 10^{-4}$	1·21*	2·4	1·71	0·74
3×10^{-4} M	$5{\cdot}5 \times 10^{-5}$	0·64*	4·6	1·13	1·7
10^{-3} M	$1{\cdot}6 \times 10^{-5}$	0·43*	6·9	0·52	3·8
3×10^{-3} M	$5{\cdot}5 \times 10^{-6}$	0·37*	8·1	0·52	3·8

* Value includes contribution of uncatalysed rate (0·34 mM sec^{-1}).

The data of column 2, Table 2 include a correction for inhibition of carbonic anhydrase by Cl^-; the appropriate concentrations (free drug = I_f) and K_I are given in Table 3. Fractional inhibition (i) is calculated as

$$i = \frac{I_f}{I_f + K_I}. \tag{5}$$

This is the general expression for non-competitive inhibition, but applies approximately to the present case, (since $[Co_2] < K_m$) even though anion inhibition of hydration is of 'mixed' type (Maren & Couto, 1979). The numbers of column 2, Table 2 are the values of eqn. (4) × residual activity ($a = 1-i$) in the presence of red-cell chloride. Proton generation rates following methazolamide include both the uncatalysed rate and residual catalytic component. This component is the normal rate (column 2, Table 2) multiplied by the degree of residual activity (column 2, Table 4) following methazolamide. Inhibition by methazolamide is also calculated according to the simplifying assumptions of eqn. 5. The precise mechanism of sulphonamide inhibition is not yet known.

Tables 4 and 5 show calculated data from the experiments of Figs. 3 and 5. One can employ an alternative approach to calculating net catalysed CO_2 hydration rates in the human experiments by use of the more rigorous kinetic equation (Segel, 1977), in which primes denote dehydration constants:

$$V_{cat} = \frac{[(k_{cat})[E]_0[CO_2]_{exp}/K_m] - [(k'_{cat})[E]_0[HCO_3^-]/K'_m]}{1 + ([CO_2]_{exp}/K_m) + ([HCO_3^-]/K'_m)}. \tag{6}$$

This equation requires a knowledge of the intracellular HCO_3^- concentration, which we take as 12·8 mM. The constants are k'_{cat} (HCA – B) = 7400 sec^{-1}, k'_{cat} (HCA – C) = 183,000 sec^{-1}, K'_m (HCA – C) = 40 mM, K'_m(HCA – B) = 18 mM (Maren & Couto, 1979 and current work). The net catalysed CO_2 hydration (including chloride

TABLE 5. Comparison of proton formation and oxygen release in the Bohr effect

Human type II experiments

	Initial proton generation (mM sec^{-1})	$t\frac{1}{2}$ proton generation (sec)	Initial O_2 release (mM sec^{-1})	$t\frac{1}{2}O_2$ release (sec)	% Bohr effect in 1 sec
Control	2850	0·0005	16·5	0·12	99·7
Methazolamide					
10^{-5} M	4·72*	0·29	7·6	0·22	96%
3×10^{-5} M	1·67*	0·83	3·5	0·54	74%
10^{-4} M	0·62*	2·2	1·3	1·3	42%
3×10^{-4} M	0·31*	4·5	0·57	2·9	21%
10^{-3} M	0·20*	6·9	0·38	4·2	15%

* Value includes contribution of uncatalysed rate (0·16 mM sec^{-1}).

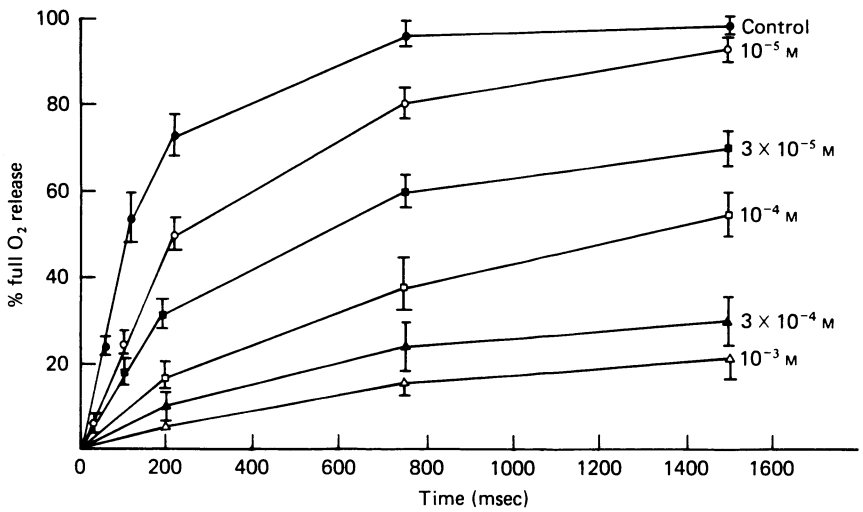

Fig. 5. Rate of Bohr effect in human red cells, and effect of carbonic anhydrase inhibition by methazolamide (type II experiment; CO_2 at time zero).

inhibition calculated from eqn. (5)) is 2030 mM sec^{-1}, a value ~ 70% of that given in Table 5, column 1, row 1. We believe that these values are equivalent, given the limits of present methodology. Carbonic anhydrase activity at each drug level is given in Table 4 and applies also to Table 5. As described above, proton generation rates are calculated from known kinetic data, and O_2 release from the experimental curve. The results clearly show a quantitative relation between proton generation and O_2 release when carbonic anhydrase is sufficiently inhibited. The stoichiometry of the Bohr effect in vertebrates ranges between 1:1 to 1:3, depending on the nature of the experiment. This will be analysed in the Discussion.

DISCUSSION

The kinetics of the Bohr effect appear remarkably similar in the blood of these vertebrates. The half-time in duck and human is approximately 120 msec, while in the goosefish, dogfish and frog it ranges from 220 to 340 msec. Forster & Steen (1968) showed that the human Bohr effect at 37 °C has a half-time of 120 msec and at 23 °C of 190–310 msec, values only slightly different from those of the cold-blooded species at 16 °C.

The classical Bohr effect has been shown to be the result of both H^+ binding and CO_2 binding as carbamate (Roughton, 1970). Hlastala & Woodson (1975) showed that the contribution of each is critically dependent on the degree of Hb saturation, base excess and 2,3-diphosphoglycerate levels. When haemoglobin saturation is 70% and 2,3-diphosphoglycerate is present in normal concentrations (our experimental conditions) the contribution of direct CO_2 binding is less than 10% of the total Bohr effect. Our results confirm this. If there had been a large contribution by carbamate formation this would have been apparent during carbonic anhydrase inhibition, since the enzyme plays no role in this process. However neither we nor Forster & Steen (1968) saw a rapid appearance of O_2 followed by a slower second phase of O_2 release when the enzyme was inhibited.

The data of Table 2 permit an analysis of the basic chemistry of the Bohr effect. Normally the potential for H^+ formation (column 2) greatly exceeds the measured O_2 release (column 3). It must be noted that calculations of H^+ generation in the normal yield the potential for H^+ formation and are not true rates *in vivo*. This calculation provides a measure of the enzyme excess, that is the relation between $\dot{V}_{cat}$ and $\dot{V}_{obs}$ In these experiments, $\dot{V}_{obs}$ (the true rate of H^+ generation) is measured only indirectly by the rate of O_2 release. The Tables show that $\dot{V}_{cat}/\dot{V}_{obs}$ (enzyme excess) is 23–360 depending on the species. There appears to be no direct quantitative relation between the magnitude of a species Bohr effect and the activity of its red-cell carbonic anhydrase.

Total inhibition of carbonic anhydrase may be defined as the observed plateau of a dose–response study, or when the fully inhibited observed rate is equal to the calculated uncatalysed rate (Maren, 1977). Our data fulfil both of these criteria. When carbonic anhydrase is totally inhibited by 10^{-3} M-methazolamide, the Bohr effect rate is markedly reduced to a plateau level (Figs. 2 and 3) and the calculated uncatalysed rate of H^+ generation nearly matches O_2 release (Table 2). The Bohr effect factor ($\Delta \log p_{O_2}/\Delta pH$) varies from $-0{\cdot}10$ to $-2{\cdot}1$ in vertebrates (Hilpert *et al.* 1963; Riggs, 1979). Wyman (1964) provided the mathematical treatment which equates the classic Bohr effect factor with the number of protons bound to haemoglobin per O_2 molecule released. Our data comparing this range of vertebrates suggest within a twofold range the quantitative relation between proton formation and O_2 release.

The Bohr effect factor for human haemoglobin is $-0{\cdot}5$ (Hilpert *et al.* 1963), meaning that the binding of one proton is accompanied by the release of two O_2 molecules. The dose–response relations of methazolamide to the human Bohr effect in Tables 4 and 5 provide further quantification of the basic chemistry. In the type I experiments (Table 4) the H^+/O_2 ratio is about $-0{\cdot}67$, which is slightly high since there is some finite delay in CO_2 formation from H_2CO_3, generated in the extracellular

solution (Fig. 1). In the type II experiments (Table 5), this artifact is absent and the true factor, $-0{\cdot}5$, is very nearly observed. We emphasize this stoichiometric relation between the rates of proton formation and O_2 release when inhibition of carbonic anhydrase decreases the rate of CO_2 hydration to less than that of the normal Bohr effect.

These data suggest that when CO_2 hydration is rate limiting, the buffering of protons by haemoglobin is solely by those amino acids involved in the Bohr effect. Whether this phenomenon of specific Bohr amino-group buffering occurs during normal capillary transit is unknown, but our results add further to the concept of binding and interaction between haemoglobin and carbonic anhydrase (Silverman, Tu & Wynns, 1978). The rate and half-time of the normal Bohr effect ($t_{\frac{1}{2}} = 0{\cdot}12$ sec) suggest that it is the bicarbonate-chloride exchange process ($t_{\frac{1}{2}} = 0{\cdot}09$ sec) (Chow, Crandall & Forster, 1976) which is the rate limiting step when carbonic anhydrase is present.

The dose response to carbonic anhydrase inhibition differs for the type I and II experiments in human blood. This is explained as follows. In type I (Table 3), 10^{-4} M-methazolamide results in a 2·5-fold reduction in the Bohr effect rate, while the same concentration in type II (Table 4) shows an eleven fold decrease. The initial CO_2 concentration at the start of the reaction in type I experiments is 4·6 mM and in type II it is 2·3 mM. Since the red cells in each experiment are pre-equilibrated with the same gas tensions their respective intracellular $[H^+]$ and $[HCO_3^-]$ must be equal prior to mixing. Entry of CO_2 into the cell without corresponding instantaneous shifts in $[H^+]$ and $[HCO_3^-]$ results in a disequilibrium or CO_2 excess. In type I experiments this initial disequilibrium favours CO_2 hydration over HCO_3^- dehydration twentyfold and in type II experiments tenfold. The greater the disequilibrium the faster the reaction (catalysed and uncatalysed). Thus the level of enzyme inhibition must be higher to produce equivalent reductions in the Bohr-effect rate.

The general principle that the CO_2 gradient is a determinant of the degree of inhibition necessary to yield an effect has been shown in other systems (Maren, 1977). An important physiological corollary is that inhibition of the physiological effect ($\dot{V}_{obs}$) can be diminished or overcome by increasing this substrate. For example, in the lung partial inhibition of carbonic anhydrase can be countered by tissue CO_2 retention and hyperventilation. These responses widen CO_2 gradients between blood and lung, gill or tissue and magnify the activity of residual uninhibited enzyme to maintain normal CO_2 exchange (Swenson & Maren, 1978).

The present data show that partial inhibition of the Bohr effect is apparent at 10^{-5} M-methazolamide in the type II experiments (row 2, Table 5) but would have little physiological significance (column 6, Table 5). The physiological Bohr effect (defined as the amount occurring in capillary transit) is reduced to 30% by about 2×10^{-4} M-methazolamide. In these experiments (type I) the p_{CO_2} difference was 70 mm Hg, a steep gradient only observed in conditions of maximal metabolic or ventilatory stress. We plan further experiments in which this gradient is greatly reduced to simulate conditions *in vivo*. The protocol of type II experiments will be used with the p_{CO_2} gradient reduced to 7 mm Hg. The initial normal (full catalytic rate of H^+ formation) would then be 334 mM sec^{-1}, sufficient to generate a Bohr effect of equivalent magnitude to that in Table 4, 16·5 mM sec^{-1}. We anticipate that the dose response to methazolamide would show apparent inhibition at 10^{-6} M,

without physiological significance; initial rate of O_2 release would be two-thirds normal, but will allow 96 % of the physiological Bohr effect. At 10^{-5} M, the physiological Bohr effect would be reduced to 30 %. Such concentrations of unbound methazolamide are generally achieved in man with the therapeutic use of this drug, without any overt toxicity (Maren *et al.* 1977).

Despite considerable chemical and biochemical research into the Bohr effect, little has been done to quantify the role of this process physiologically. Two very recent papers provide stimulating evidence for a major function of the Bohr effect in c.s.f. formation and cerebral respiration. Jankowska & Grieb (1979) have demonstrated that during hypoxia c.s.f. p_{O_2} is higher than arterial p_{O_2}. This is attributed to the acidification of choroid plexus capillary blood by the metabolic activity of this tissue in the generation of a bicarbonate rich nascent c.s.f. (Maren, 1972). A c.s.f. – arterial p_{O_2} difference is not seen in normoxia, since the slope of the oxyhaemoglobin dissociation curve is relatively flat at high p_{O_2} values. Laux & Raichle (1978) measured cerebral blood flow and oxygen consumption before and after complete carbonic anhydrase inhibition (acetazolamide 30 mg/kg I.V.) and showed a 30 % increase in blood flow and a 30 % decrease in cerebral O_2 uptake. These authors estimate by statistical analysis that less than 50 % of the blood-flow increase could be attributed to an elevated p_{CO_2} secondary to carbonic anhydrase inhibition. The major increase in cerebral perfusion was postulated to be a consequence of tissue hypoxia, subsequent to interference of O_2 unloading in the brain capillaries.

We do not know the consequence of loss of the Bohr effect at submaximal levels of carbonic anhydrase inhibition as used clinically and analysed in this work. In the face of a diminished Bohr effect the body may compensate by increasing perfusion, dilating and recruiting capillaries, widening the p_{O_2} and p_{CO_2} gradients, increasing red blood corpuscle 2,3-diphosphoglycerate or shifting to a greater degree of anaerobic metabolism. Whether a reduced Bohr effect does impose any physiological disadvantage, especially in conditions of cardiovascular or pulmonary insufficiency remains for further investigation.

This work was supported by NIH Grant GM 16934. We wish to thank the Pharmaceutical Manufacturers Association, Inc. for the award of a Medical Student Fellowship in Clinical Pharmacology to Erik R. Swenson.

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