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Effects of phosphate, HEPES, N_2O and CO on the kinetics of human erythrocyte carbonic anhydrases B and C

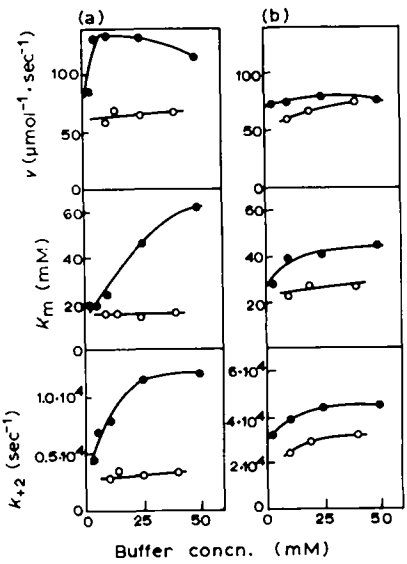
From data obtained by measuring the infrared difference spectrum of CO_2-equilibrated bovine carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) in solutions, RIEPE AND WANG[1] concluded that CO_2 is loosely bound to a hydrophobic surface of the active site of the enzyme. N_2O was found to compete with CO_2 in binding to this site with approximately the same affinity. According to these findings, N_2O can be expected to act as an inhibitor of carbonic anhydrase. One object of the present study has been to investigate the expected inhibitory effect of N_2O on human erythrocyte carbonic anhydrase form B and form C. In preparation to this task we have studied the effect of phosphate which has been widely used as a buffer constituent in studies of the kinetics of carbonic anhydrase.

We prepared electrophoretically pure preparations of carbonic anhydrase B and C by fractionation of human red blood cells using the chromatographic procedure described by ARMSTRONG *et al.*[2]. The rate measurements were performed by means of the pH-stat method[3] in which the rates of the dehydration or hydration reactions are registered as the amount of acid (or base) which must be added per unit time to the reaction medium in order to maintain a constant pH. During the dehydration experiments a CO_2-free gas is driven through a solution of $NaHCO_3$ whereby the reaction product, CO_2, is expelled as rapidly as it is formed. In this study pure N_2, N_2O or CO were used as expellants. The course of the dehydration reaction was registered until approximately 80% of the HCO_3^- initially present had been converted to CO_2. The Michaelis–Menten parameters, K_m and v_{max}, were calculated from the data obtained from each dehydration experiment (procedure II, ref. 3). The rate constant, k_{+2}, was calculated as v_{max} divided by the molar concentration of the enzyme. In the hydration experiments, the initial rate of the reaction (v) was determined using a gas mixture containing a known concentration of CO_2.

We investigated the dehydration kinetics of carbonic anhydrase B and C in HEPES[4] (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) and in phosphate buffers. Marked differences in the behavior of the two enzymes are apparent (Figs. 1a and b). Phosphate acts as an activator of carbonic anhydrase B, while HEPES is without effect on this enzyme. At a buffer concentration of 25 mM, the initial rate v, K_m and k_{+2} are, respectively, 2.1, 3.1, and 3.8 times higher in phosphate than in HEPES. The effect of phosphate on carbonic anhydrase B resembles the effect of SO_4^{2-} on carbonic anhydrase from the midgut epithelium of *Hyalophora cecropia*[5]. The phosphate effect on v for carbonic anhydrase C is negligible, although K_m and v_{max} are both affected to some degree.

The dehydration kinetics of carbonic anhydrase B in phosphate and in HEPES were investigated between pH 6.8–8.0 (Fig. 2). While K_m appears to be almost independent of pH in phosphate buffers (*cf.* ref. 3), a linear relation between log K_m and pH with slope + 0.5 are found in HEPES buffers. The data in Fig. 2 suggest that the effect of phosphate decreases with increasing pH.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.



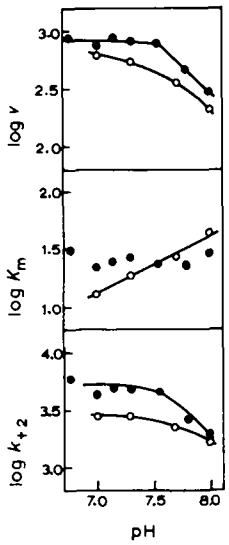


Fig. 1. Effects of phosphate and HEPES on (a)carbonic anhydrase B and (b) carbonic anhydrase C. The rate of the dehydration reaction was measured at 2° in phosphate (●—●) and in HEPES (○—○) at pH 7.30. v (μmol $l^{-1} \cdot sec^{-1}$) is the initial rate of the reaction at the substrate concn. $[HCO_3^-]$ = 25 mM. (The preparation of carbonic anhydrase C used in these experiments had an unusually low specific activity (*cf.* values for k_{+2} in Table I and ref. 3)).

Fig. 2. Effect of pH on carbonic anhydrase B in phosphate and in HEPES buffers. The rate of the dehydration reaction was measured at 2° in 10 mM phosphate (●—●) and in 10 mM HEPES buffers (○—○). Ordinate: log v (v in μmol $l^{-1} \cdot sec^{-1}$), log K_m (K_m in mM) and log k_{+2} (k_{+2} in sec^{-1}). v is the initial rate of the reaction at $[HCO_3^-]$ = 25 mM.

The results from our study of the effect of N_2O on the dehydration kinetics of carbonic anhydrase B and C in HEPES buffers are shown in Table I. We also investigated the effect of CO which previously has been suspected to be a carbonic anhydrase inhibitor[6]. N_2O and CO had no demonstrable effect on carbonic anhydrase

TABLE I

EFFECTS OF N_2O AND CO ON CARBONIC ANHYDRASE B AND C

The rate of the dehydration reaction was measured at 2° and pH 7.30 in 10 mM HEPES buffer. The results are mean values of n experiments. v is the initial rate of the reaction at $[HCO_3^-]$ = = 25 mM.

	Gas (*mM*)		*n*	*v* (*μmol l⁻¹·sec⁻¹*)	K_m (*mM*)	$k_{+2} \cdot 10^{-3}$ (*sec⁻¹*)	*S.E.* (%)
Human carbonic anhydrase B	N_2	(1.0)	6	61.1	16.0	3.02	6–14
	N_2O	(58.0)	6	55.7	17.8	2.87	6–9
	CO	(1.5)	4	55.5	17.5	2.84	5–6
Human carbonic anhydrase C	N_2	(1.0)	12	81.4	22.3	80.6	4–8
	N_2O	(58.0)	7	75.9	29.7	86.9	4–6
	CO	(1.5)	7	83.5	22.9	83.8	7–9

B. Neither did CO have a measurable effect on carbonic anhydrase C. A small but significant effect of N_2O is noted on K_m ($P < 0.001$) and v_{max} ($0.01 < P < 0.05$) for carbonic anhydrase C. The decrease of the initial rate of the reaction in the presence of N_2O in a concentration of 58 mM was less than 10%. Results similar to those shown in Table I were obtained in 10 mM phosphate buffers.

The initial rate of the hydration reaction was measured in 10 mM phosphate buffers containing 1.6 mM CO_2 in the absence and presence of N_2O (N_2O concn., 34 mM). No significant differences in the activity of either carbonic anhydrase B or C were found. The measurements were performed at pH 7.30 and 2°.

We conclude that an inhibitory effect of N_2O on erythrocyte carbonic anhydrase is either small or nonexistent. A similar conclusion has been reached by Dr. R. KHALIFAH of Havard University (personal communication), who also investigated the kinetics of bovine erythrocyte carbonic anhydrase. Thus a distinct discrepancy exists between the results referred to above, obtained by RIEPE AND WANG[1] and the kinetic data according to which no binding of N_2O to the active centre in carbonic anhydrase can be demonstrated.

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