secretin (75 U/ml) and vasoactive intestinal peptide (1.6×10^{-6} M) were investigated by the in vitro isolated organ perfusion technique. Neither secretin nor glucagon had any effect over the concentration range studied. Vasoactive intestinal peptide (VIP) had a marked stimulatory effect on active chloride transport producing a greater than 500% increase in transport compared to control perfusions (230 ± 51.3 to 1222 ± 304 μEq/hr/gWW, $p < 0.01$, n=6).

Additional studies were carried out to determine whether this concentration of VIP produced a concomitant increase in intracellular cyclic AMP concentration. VIP produced a doubling of intracellular cyclic AMP compared to controls (15.4 to 29.8 pmoles cAMP/mg protein) and was increased further by theophylline (1 mM) (27.7 to 94.8 pmoles cAMP/mg protein). Thus this hormone increases active chloride transport and intracellular cyclic AMP accumulation and is synergistic with theophylline. Additional experiments were carried out with the intact fish to establish the presence of the hormone and define the factors which regulate its release. These results are not available at the present time.

In summary, active chloride transport in the rectal gland of the spiny dogfish is tightly coupled to the intracellular level of cyclic AMP. Cyclic AMP concentration is regulated in part by the activity of the degradative enzyme, cyclic nucleotide phosphodiesterase which is inhibited by theophylline. In addition there appears to be a feedback mechanism which either alters the activity of the diesterase or inhibits adenylate cyclase activity resulting in a fall of the intracellular level of the nucleotide. Furthermore our studies indicate that extracellular sodium concentration modifies the cyclic AMP response to theophylline. Our studies with VIP demonstrate that this hormone activates the gland in vitro resulting in an increase in cyclic AMP production and active chloride transport. Further studies are required to establish whether this hormone plays an important regulatory role in the intact fish.

AN INTRODUCTION TO COMPARATIVE STUDIES OF THE RATE OF THE BOHR EFFECT

Erik R. Swenson and Thomas H. Maren, Department of Pharmacology, University of Florida, College of Medicine, Gainesville, Florida

The Bohr effect describes the protonation of HbO_2, which elicits a conformational change in the protein, leading to release of O_2. It yields a "shift to the right" in the oxygen dissociation curve, meaning that pO_2 increases with acidosis, at any level of oxygen saturation. The rate of the reaction $H^+ + HbO_2 \rightarrow HHb + O_2$ is extremely rapid, half-time being of the order of 10 milliseconds. The rate of the Bohr shift is thus determined by the rate of formation or appearance of H^+ within the red cell.

Our interest in this subject was effected by the finding of R. E. Forster and J. B. Steen (J. Physiol., 196:541, 1968) that carbonic anhydrase inhibition reduced the rate of the CO_2-mediated Bohr shift in human red cells by about thirty-fold. This corresponds well to a model in which the protonation of hemoglobin is linked to the hydration of CO_2 in tissue capillaries. Thus the formation of the two chief proteins (hemoglobin and carbonic anhydrase) in red cells is linked.

We have begun a study of the Bohr shift, with the following questions in mind: (1) What are the quantitative relations between inhibition of carbonic anhydrase and rate of the Bohr shift? (2) Are there physiological and clinical sequelae to marked reduction in rate of the Bohr shift? (3) Does the magnitude of the Bohr shift in vertebrate and invertebrate species have any relation to carbonic anhydrase activity of the blood?

The question of the Bohr effect in *S. acanthias* (which includes the pacific dogfish referred to as *S. suckleyi*) had not been settled (Riggs, Fish Physiology, Vol. IV, p. 209, 1970). However, both Manwell (Arch. Biochem. Biophys., 101:504, 1963) and Kent et al. (Bull. Mt. Desert Island Biol. Lab., 14:51, 1974) report Δ log pO_2/Δ pH of about 0.34 in this species.

Experiments were done at 16°C on blood of *S. acanthias*, using the method of Forster and Steen (vide supra). A 5% suspension of red cells in dogfish Ringers was mixed in a rapid flow apparatus with similar Ringer's solution to which 20 mM lactic acid had been added. The rise in pO_2 was measured with a modified Clark electrode. pO_2 was displayed directly on the Chemical Microsensor Model 1201 (Transidyne General Co.). Both solutions were gassed with 2% O_2 and equilibrated with various concentrations of the carbonic anhydrase inhibitor, methazolamide. This drug has a dissociation constant (K_I) of 2×10^{-8} M against carbonic anhydrase of red cells of this species when assayed at 16°C in barbital buffer (J. Pharmacol. Exp. Ther., 130:26, 1960) in the hydration reaction. The carbonic anhydrase concentration in red cells of *S. acanthias* is 10^{-7} M.

The reaction studied is shown in Figure 1. When the Ringers-lactic acid solution is mixed with the red cell suspension containing 8 mM $NaHCO_3$, the extracellular concentrations shown are

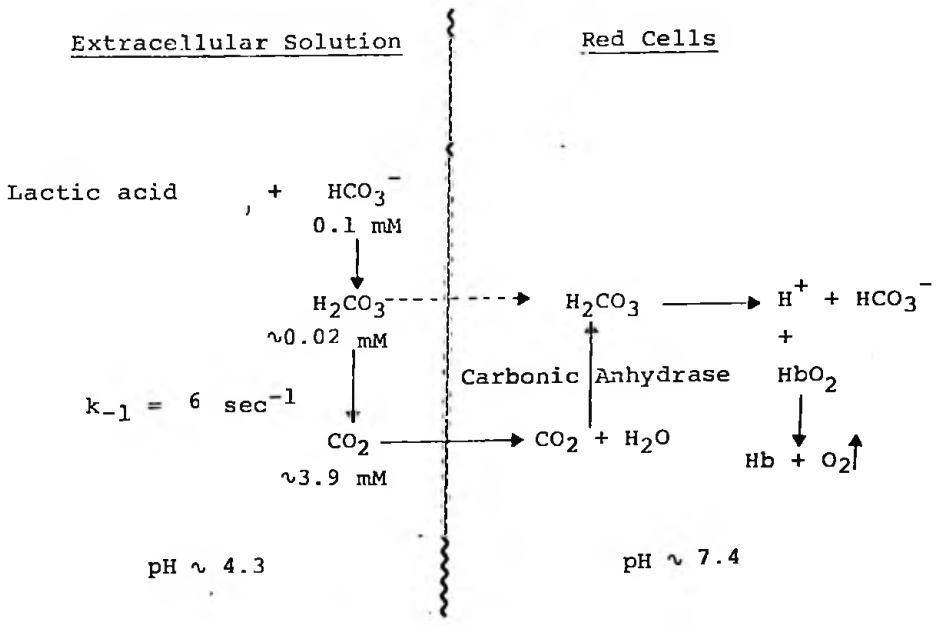

Figure 1. Events in experiments to measure rate of Bohr effect in *S. acanthias*. The concentrations shown are estimations of these at 115 milliseconds after mixing, which is the half-time of the dehydration of carbonic acid.

generated in about 115 milliseconds, the half-time for the dehydration of H_2CO_3. These numbers are 50% of the equilibrium values between H_2CO_3 and CO_2; the equilibrium ratio is 1:400. It should be noted that because this ratio favors CO_2 so strongly, the concentration of CO_2 even at 10% of equilibrium (which is achieved in about 16 milliseconds), is almost that of equilibrium. Therefore, the concentration of CO_2 in the extracellular solution - nearly 4 mM - is virtually

constant throughout the reaction. Lactic acid does not penetrate the red cell under these circumstances; H_2CO_3 is probably much less permeable than CO_2, and owing to the rapidity of its dehydration is not present in appreciable concentration for any length of time. Acidification of the red cell thus depends upon diffusion in and hydration of CO_2, as shown on the right of Figure 1.

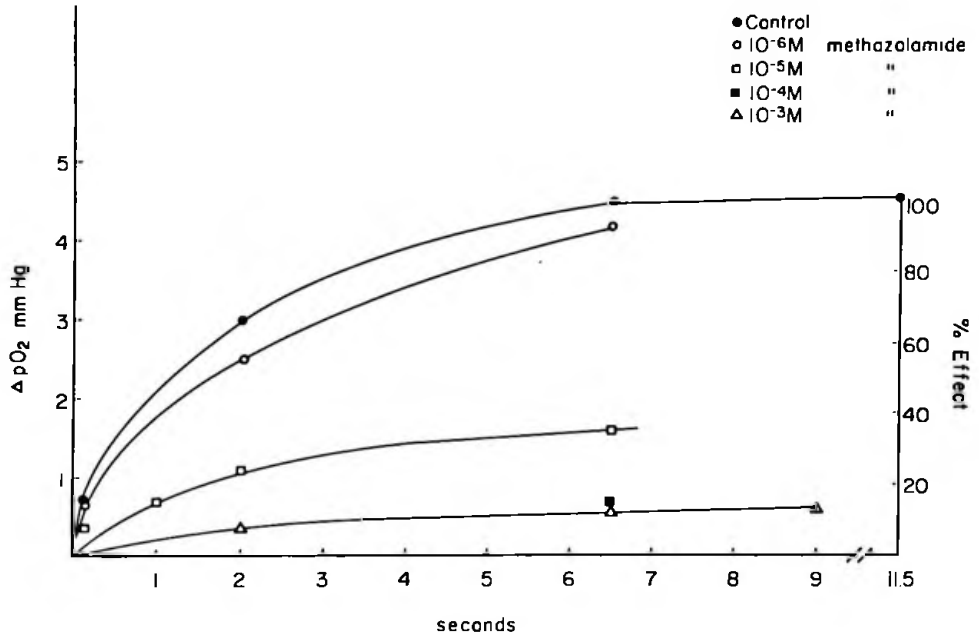

Figure 2. Time course of O_2 release from red cells of *S. acanthias*. At 0 time a cell suspension at pH 7.8 was mixed with dogfish Ringers-lactic acid solution (pH 3.7). Methazolamide was added to each solution at least 30 minutes before the experiment.

Figure 2 shows the data. The full increment of pO_2 following acidification, set as 100% on the ordinate, is 4.5 mm Hg. In control blood this is reached in 4-6 seconds. When carbonic anhydrase is completely inhibited (note identity of data when 10^{-3} and 10^{-4} M methazolamide are used) only a small fraction of this oxyten increment (about 15%) is evolved in 9 seconds.

Table 1 shows rate constants (k) and half-times ($T_{\frac{1}{2}}$) taken from control and completely inhibited (i.e., uncatalyzed) curves of Figure 2. We also show the chemical rate constants for the uncatalyzed (k_1) and catalyzed (k_{enz}) hydration of CO_2. From these chemical constants and the calculated CO_2 concentration (Figure 1) we estimate the H^+ production in fish red cells, and compare these to the rates of O_2 release. At 50% O_2 saturation, one mole of O_2 is generated from HbO_2 by about 0.5 moles H^+ in a variety of mammals (Hilper et al., Am. J. Physiol., 205:337, 1963). Roughly this appears true for elasmobranch species, but, as noted above, details are at present controversial. H^+ production is given by $k_1(CO_2)$ = 47 μM sec^{-1} and $k_{enz}(CO_2)$ = 15 mM sec^{-1}. The corresponding rates of O_2 release (from Figure 2) are about 0.4 μM sec^{-1} for the uncatalyzed reaction and 29 μM sec^{-1} for the catalyzed. Thus both the uncatalyzed and

TABLE 1

RATE CONSTANTS AND HALF-TIME FOR BOHR EFFECT,
COMPARED WITH CHEMISTRY OF CO_2 HYDRATION

	k sec^{-1}		$T_{1/2}$ sec	
	Uncat	Normal = cat	Uncat	Normal = cat
Dogfish (16°) Bohr*	0.02	1.34	35	0.52
Chemical†	0.012	3.8	57	.18
Man (37°) Bohr* (Forster & Steen)	0.07	2.2	10	0.31
Chemical†	0.14	2100	5	3×10^{-4}

*From Figure 2 above, and Table 3 of Forster and Steen

†Uncat constants, k_1 are from literature, see Magid and Turbeck, BBA 165: 515 (1968). Catalytic constants are

$$k_{enz} = \frac{k_{cat} \cdot E}{CO_2 + K_m}$$

k_{cat} and K_m are given by Khalifah, and adjusted for temperature. (J. Biol. Chem. 246: 2561, 1971). E is 10^{-7} M for fish red cells and 250 x 10^{-7} M for Enzyme C in man. CO_2 is taken as 3.9 mM for fish and 10 mM for human experiments.

catalyzed chemical rates greatly exceed the respective rates of O_2 release. It was surprising to find that the chemical uncatalyzed rate slightly exceeded the normal (catalyzed) rate of O_2 release; at first this seems paradoxical to the very large effect of carbonic anhydrase inhibition. But these facts all point in the same direction: the chemical potential of the hydration reaction is not evident in the rates of O_2 release. Reasons include diffusion time for CO_2 and O_2, back (dehydration) reaction within the cell, and lag in recording. The great dependence on carbonic anhydrase suggests that in the final path $CO_2 + H_2O \xrightarrow{C.A.} H_2CO_3 \rightarrow H^+$, the CO_2 gradient for the reaction is small.

Calculations of the data of Forster and Steen from human red cells show the same relations. The chemical uncatalyzed rate K_1S exceeds the normal measured rate of O_2 release; yet there was a

very large decrease in O_2 release rate following acetazolamide. A further point of interest in comparing fish and human red cells is that the Bohr rate constant in fish is about 1/2 that in man, despite the 600-fold difference in k_{enz} (Table 1). This shows, as in all other systems studied, that carbonic anhydrase is not rate limiting or regulatory.

Since 10^{-4} M methazolamide reduces the rate of the Bohr shift so greatly that it cannot mediate the release of O_2 in the capillaries, we may ask whether this question has been tested in vivo. This concentration of methazolamide (or acetazolamide, which has the same potency) is readily achieved in vivo following doses of 20-50 mg/kg in *S. acanthias* (Comp. Biochem. Physiol., 5:201, 1962; Amer. J. Physiol., 222:885, 1972). No overt toxicity has been observed; it remains for further investigation in this and other species to find whether limitation of the Bohr effect does impose any physiological disadvantage.

Supported by NIH Grant GM 16934 and a Medical Student Research Fellowship (to E.R.S.) from the Pharmaceutical Manufacturers Foundation.

ACUTE EXPERIMENTS ON THE EURYHALINITY OF *Fundulus heteroclitus* (Linn)

C. E. Wilde, Jr., Department of Zoology, University of Rhode Island, Kingston, R.I.

The euryhalinity of *Fundulus heteroclitus* (Linn) does not appear to have been studied under crisis conditions, nor has the appearance of this well known condition in the adult fish been studied in the fry. Protected by its chorion, the developing embryo can be reared in all dilutions of sea water from full strength to distilled. Under these varying circumstances there are variations in the rate of incorporation of amino acid into proteins (Crawford, R. B., Heinemann, M.-H., and Wilde, Jr., C. E., Bull. MDIBL, 8:1968) but there appear to be no differences in overall morphogenesis. This latter statement requires further study in detail.

Fry are euryhaline from the moment of hatching. I report here two experiments in which intense osmotic stress was placed upon *Fundulus* fry for a period of hours by rapidly changing their ambient medium every ten minutes. They were kept under constant observation.

Seven *Fundulus* fry were removed from their growth medium, millipore filtered 50% sea water, and placed in millipore filtered distilled water. Seven fry were similarly placed in full strength millipore filtered sea water.

Every 10 minutes the fry were removed from their present medium and placed in fresh medium of opposite type, that is, if the fry were in sea water they were placed in fresh water or vice versa.

Ten cycles of change were carried out. The ambient temperature was 21°C and the media were equilibrated to that temperature. During the exchange maneuver care was taken to transfer a minimum of medium with the fry. The exchanges were carried out in the tips of a series of Pasteur pipettes. The fry were approximately 2 mm in length and the dishes of sufficient size so that the medium was essentially infinite in relation to the mass of the fry.

The fish were observed continuously for behavioral change, morbidity and mortality.

There were no apparent physiological or behavioral changes to be observed. The fry remained vigorous continuously and there was no morbidity or mortality. At the end of the experiment the fry were placed in full strength sea water for further observation and experiment.

The results led to a second experiment of similar type with the same fry except that the stress lay between distilled water and double strength sea water (kindly prepared and donated by