about 0.25 mm. By fusion of some of the tubules at various levels of the gland (notably at 0.25, 0.6, 1.1 and 1.4 mm from the periphery) the number of tubules at the central canal is reduced four fold. Although we can distinguish 4 orders of tubules (3 fusions) for some tubules, others may have fused only once or twice so that there is an admixture of tubules with large or small lumens even at the central canal where the average diameter of the lumen is about 50 μm.

We have found about 512 tubules at the periphery of the gland and about 64 surrounding the central canal so that there are on the average 8 peripheral tubules for each central (primary) one. The length (and diameter) of the gland varies with the individual fish but assuming a gland 3.5 cm long with peripheral tubules spaced 35 μm apart we would find 512,000 peripheral tubules and 64,000 primary tubules entering the excretory ducts. Not all secretory tubules branch at the same level nor for the same number of times. From measurements of lumen diameters we conclude that the total lumenal volume has increased at least 4.5 times en route to the central canal and that there is little difference in this volume in fixed preparations taken from theophylline stimulated and ouabain inhibited glands.

Blood sinusoids - There is very little connective tissue between the secretory tubules and the sinusoids of the parenchyma and only very few collagenous strands extend from the capsule. Beneath the capsule the thin walled capsular arteries give rise usually directly to sinusoids of about 10 μm diameter but occasionally to subcapsular cisterns about 150 μm in diameter from which many small sinusoids arise. Sections across the tubules at the periphery show the tubules closely packed with blood sinusoids between them in such a way that the bases of about half the tubule cells abut on other tubules and about half on sinusoids. This close packing extends about 0.4 mm from the periphery at which level one finds some large (fused) tubules intermixed. At 1.5 mm from the periphery the tubules are about 95% surrounded by sinusoidal walls. From 1.8 mm to the central canal large secretory tubules predominate and adjacent tubules are only intermittently in contact with other tubules; the surrounding sinusoidal space being about 35 μm across. The sinusoidal endothelium is extremely thin, usually 0.2 μm thick, but varying from 0.03 μm to 0.5 μm except where the nuclei are present (2-3 μm). Despite this thinness we have found no fenestrae.

KINETIC PROPERTIES OF RED CELL CARBONIC ANHYDRASE IN *S. acanthias* AND *L. americanus*, IN RELATION TO THE VERTEBRATE PHYLOGENY OF THE ENZYME

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Primate red cells contain two isoenzymes of carbonic anhydrase, with very similar active sites and tertiary structure, but only 60% homology in amino-acid sequence. The enzyme designated C is some 20-fold more active than B, although the latter is about 5 times more abundant, being (at 4 g/liter RBC) the second most concentrated protein in red cells. Analysis of enzyme patterns in other mammals, as well as in birds and amphibia, suggest that a high activity enzyme, akin to C, is always present in red cells. Most mammals have both types, although certain ones (dolphin, sheep, ox, cat) have only C. All submammals thus far examined have but a single enzyme, and kinetic analysis in the frog and chicken, for example, show it to be akin to C (Bundy, Comp. Biochem. Physiol. 57B:1, 1977). In human populations, C is far more stable in concentration and iso-electric pattern, while B is variable and occasionally absent (Kendall and Tashian, Science 197:471, 1977). No function has yet been found for B; the very powerful catalytic activity of C provides a generous reserve for respiratory events, including exercise to exhaustion (Maren and Swenson, Federation Proc. 1978).

These and other considerations have suggested that C may be the archtypal enzyme, with gene duplication occurring some 10^8 years ago (Tashian and Carter, in Advances in Human Genetics, Vol. 7, Plenum Press, 1976). Only one study has been made on the kinetics of fish carbonic anhydrase, that of Maynard and Coleman

(J. Biol. Chem. 246:4455, 1971), who gave the amino acid composition and chemical and physical properties of the single red cell enzyme from the bull and tiger sharks. In certain respects these were quite different from mammalian carbonic anhydrases, notably in having 100 additional amino-acid residues, and much higher cystine content. From limited stopped flow measurements, they suggested that the shark enzyme is of the high activity type, akin to mammalian C.

We wished to isolate and analyze the kinetic properties of shark and other fish carbonic anhydrases, as begun here in 1965 (Maren and Wiley, Bull. Mt. Desert Island Biol. Lab. 5(#2):25, 1965). Affinity gel chromatography was attempted, using CM Biogel A (Biorad) coupled to Prontosil (a potent carbonic anhydrase inhibitor) through carbodiimide (Osborne and Tashian, Analytical Biochem. 64:297, 1975). Hemolysates of dogfish red cells were added to the affinity gel, and the carbonic anhydrase eluted with a second inhibitor, methazolamide. The eluate was dialyzed against large volumes of water. However, in the procedure, enzyme activity was rapidly lost, so that yields were negligible. This recalled Maynard and Coleman's experience, in which specific activity of shark enzymes dropped notably during purification, and in the case of one species, the hammerhead, was completely inactivated.

Kinetic work was therefore carried out on hemolysates of red cells, using the micromethod for CO_2 hydration at 0-1°, with barbital buffer (4-10 mM) and bromthymol blue as indicator. The pH of the reaction is from 8.2 to 7.3 (Maren, J. Pharm. Expt. Therap. 130:26, 1960). In this system human red cells (with 125 µM B and 25 µM C) have 20,000-25,000 units of enzyme per ml. *Squalus acanthias* red cells contained 2000 units per ml. Titration of enzymic activity with the powerful inhibitor ethoxzolamide (Maren, J. Pharm. Expt. Therap. 130:389, 1960) yielded the relation that 1 enzyme unit in the reaction system (1.2 ml) is 10^{-8} M. Thus the concentration of enzyme in dogfish red cells is 2.4×10^{-5} M. The K_I for ethoxzolamide is 1×10^{-8} M.

Reciprocal plots of $1/CO_2$ vs. $1/H^+$ formation yielded K_m of 5 mM and $k_{cat} = V_{max}/E = 20{,}000\ sec^{-1}$. Table 1 compares these data to human B and C enzymes. It is clear that the *S. acanthias* carbonic anhydrase

TABLE 1

Properties of fish red cell carbonic anhydrase

	Dogfish	Goosefish	Human B	Human C
$k_{cat} = \frac{V_{max}}{E} \times 10^{-4}\ sec^{-1}$	2	50	3	23
K_m, mM	5	10	6	11
E, µmol/L red cells	24	10	125	25

At 1°C. Increase in temperature to 37° increases k_{cat} about 5-fold. Changes in K_m with temperature are not accurately known.

is of the low activity type. Calculation of k_{cat} from Table 3 of Maynard and Coleman (vide supra) for red cell carbonic anhydrase of bull and tiger shark yields about 30,000 sec^{-1} at 25°, in close agreement with the present work.

The low activity human B carbonic anhydrase has a high affinity for anions (Maren et al. Science 196: 469, 1976). These data are reproduced in Table 2, and compared with those for the dogfish enzyme. Except for Cl^-, the dogfish enzyme also shows high anion affinity; and this is the first example of a carbonic anhydrase showing susceptibility to F^-. This last finding conforms to the data of Maynard and Coleman (vide supra), in that there are many amino-acid variations in the shark enzyme, as well as striking differen

TABLE 2

Inhibition by anions and sulfonamides of fish red cell carbonic anhydrase. K_I inhibition of hydration of CO_2

	Dogfish	Goosefish	Human B	Human C
			10^{-3} M	
F^-	2	-	> 300	> 300
Cl^-	120	17	6	200
Br^-	20	-	4	200
I^-	1	0.03	0.3	26
ClO_4^-	0.15	-	3.6	1.3
CNS^-	0.3	-	0.2	0.6
CNO^-	0.01	0.005	7×10^{-4}	0.02
			10^{-7} M	
Acetazolamide	2	-	1	0.11
Methazolamide	1	0.1	0.07	0.06
Ethoxzolamide	0.1	0.01	0.01	∿ 0.002
Benzolamide	0.2	0.1	0.01	∿ 0.004
Sulfanilamide	900	-	800	40

Methods as described by Maren (J. Pharm. Expt. Therap. 130:29, 1960; and Science 191:469, 1976). In present experiments, barbital buffer (10 mM) was used, temperature was 1°C, and CO_2 concentration 70 mM.

In most experiments, enzyme and inhibitor are added to substrate (CO_2) and the reaction begun by addition of buffer (SEI method). In the alternative method, enzyme and inhibitor are mixed without substrate and then are added to the CO_2 solution, followed by addition of buffer (EI). The two methods usually agree: where EI gives lower inhibition constants (as for the more active sulfonamides against human C and goosefish enzyme) these numbers are used, based on the concentration of the drug when mixed with enzyme, prior to starting the reaction with substrate and buffer.

The values for anions against human enzymes are taken from Maren et al., vida supra, 1976, and done by the EI or SEI method at 25°C. There is little difference in K_I between the two temperatures.

in stability, compared to human B and C. Relation of F^- sensitivity to structure and activity of the shark enzyme remains a problem for the future. Imidazole (not shown) did not inhibit at 10 mM; this is a specific inhibitor of human B but not C (Khalifah J. Biol. Chem. 246:2561, 1971) by competing for CO_2 at a Zn co-ordination site (Kannan et al. FEBS Letters 73:115, 1977).

Table 2 shows the inhibition constants of five sulfonamides against the dogfish enzyme. In agreement with our previous work (Maren and Wiley, vide supra) all the drugs have somewhat less activity than against human C, by a factor of about 20. Susceptibility to sulfonamides of the dogfish enzyme is closer to that of human B.

Turning to the marine teleost, we examined red cells of the flounder *Pseudopleuronectes americanus*, and found very high carbonic anhydrase activity, although much less stability than in human or shark red cells. In the system described above, there were about 22,000 enzyme units/ml red cells, confirming (Maren Physiol. Rev. 47:667, 1967) that teleosts have some ten times as much carbonic anhydrase activity in red cells as the elasmobranchs. Parenthetically, it must be noted that the paper by Mashiter and Morgan (Comp.

Biochem. Physiol. 52A:713, 1975) purporting to show no carbonic anhydrase in red cells of the flounder *Platichthys flesus*, is based on an esterase, not a CO_2 assay.

In the goosefish, *Lophius americanus*, activity was the same as for flounder, 25,000 units/ml red cells. The enzyme was more stable, however, and permitted preliminary analysis of its specific activity and susceptibility to inhibition. Titration of activity with ethoxzolamide, as described above, yielded the surprising result that 1 enzyme unit in the system is 4×10^{-10} M. The carbonic anhydrase concentration in red cells is then 10^{-5} M. The K_I for ethoxzolamide is 1×10^{-9} M. Reciprocal plots of the activity of the goosefish enzyme show $k_{cat} = 5 \times 10^5$ per sec and $K_m = 10$ mM.

Table 2 shows that the sulfonamides are highly active as inhibitors of goosefish red cell carbonic anhydrase. Unexpectedly, we found great susceptibility to the anions, for iodide more than for human B. Since the Cl^- concentration of marine teleost red cells is about 50 mM, activity of the enzyme is lowered about fourfold by this environment.

The work to date suggests that the much higher activity of this enzyme in red cells of teleosts, compared to elasmobranchs, is not due to a higher concentration, but to the fact that teleosts have a "high activity" carbonic anhydrase, and elasmobranchs a "low activity" form. Shark and alligator (Wistrand and Whitis, P.S.E.B.M. 101:674, 1959) are the first examples in vertebrates in which the low activity form is the sole red cell carbonic anhydrase.

We suggest that a low activity type rather than the high or C type is the ancestral vertebrate enzyme. In Figure 1 we see a progression from low to high type in two of the classes (osteichthyes and amphibia), which represent an improvement and refinement of the enzyme in response to vertebrate evolution. The high activity or C type is well suited to mammalian life, having a higher k_{cat} and K_m than B (Table 1), and being more heat stable (Osborne and Tashian, Biochem. J. 141:219, 1974). With respect to mammals, a gene duplication appears to have occurred some 10^8 years ago (Tashian and Carter, vide supra), giving rise ultimately to the two enzymes now found. Our work and that of Maynard and Coleman shows quite clearly that although shark carbonic anhydrase is of low activity and relatively anion sensitive, it is very different from mammalian B, and Figure 1 shows no connection between them. In this light, we entertain the possibility that mammalian B is a relatively late evolutionary accident, with no physiological role.

Further work is indicated, particularly on the structure and kinetic properties of carbonic anhydrase in primitive animals in each of the classes, to test these ideas. Supported by NIH GM GM 16934.

FIGURE 1. VERTEBRATE PHYLOGENY OF CARBONIC ANHYDRASE IN RED CELLS

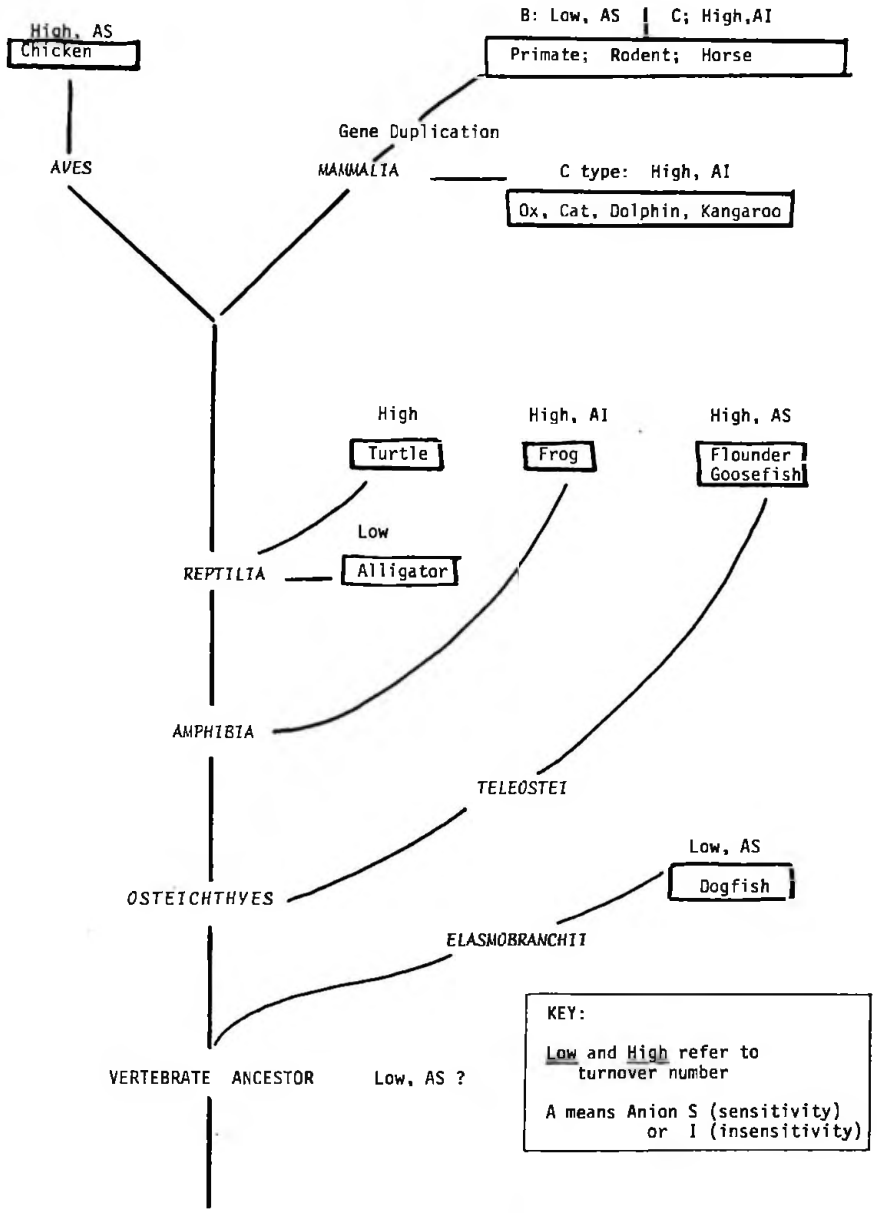

Figure 1. By High turnover number we mean about 3×10^4 sec^{-1} at 0°, and by Low, activity about 10% of this. By anion sensitivity we mean K_I of I^- of 10^{-3} M or less, and insensitivity, some 20 times greater. Except for dogfish enzyme, this also means K_I for Cl^- (in sensitive species) of about 10^{-2} M, and 20-fold higher when insensitive.