TABLE I

Ouabain Binding in Slices of Dogfish Rectal Gland

Ouabain Concentration	Potassium mM	Control	Theophylline 2.5 mM + Dibutyryl cyclic AMP 0.5 mM	(theo + cAMP)-control
10^{-3}M	5	23.54 ± 6.09 (3)	26.04 ± 10.31 (3)	2.26 ± 1.27 (3) p < 0.05
	40	23.63 ± 6.11 (2)	22.10 ± 10.03 (2)	1.53 ± 3.92 (2)
	5-40	0.19 ± 2.48 (2)	-0.36 ± 3.18 (2)	-0.55 ± 5.66 (2)
10^{-4}M	5	30.51 ± 7.48 (3)	23.79 ± 8.27 (3)	-6.72 ± 2.49 (3) p <0.025
10^{-6}M	5	3.37 ± 1.87 (6)	4.00 ± 1.91 (6)	0.63 ± 0.62 (6) p <0.05
	40	0.54 ± 0.33 (6)	1.21 ± 0.36 (6)	0.67 ± 0.62 (6) p <0.025
	5-40	2.83 ± 1.87 (6)	2.80 ± 1.91 (6)	-0.04 ± 0.62 (6)
10^{-8}M	5	0.047 ± 0.026 (6)	0.076 ± 0.045 (6)	0.027 ± 0.016 (6) p <0.005
	40	0.013 ± 0.008 (3)	0.034 ± 0.022 (3)	0.021 ± 0.017 (3)
	5-40	0.036 ± 0.027 (6)	0.041 ± 0.041 (6)	0.006 ± 0.016 (6)
10^{-9}M	5	0.0057 ± 0.w019 (5)	0.0095 ± 0.0045 (5)	0.0038 ± 0.0025 (5) p <0.01
	40	0.0019 ± 0.0004 (2)	0.0053 ± 0.0017 (2)	0.0034 ± 0.0018 (2)
	5-40	0.0039 ± 0.0019 (5)	0.0042 ± 0.0039 (5)	0.0004 ± 0.0024 (5)

Values are in units of nanomoles per gWW, mean ± SD. Only paired t test were calculated and only for the (theo + cAMP)-control column. Only significant values for p are recorded. Incubation was for 120 min at 15°C in shark Ringer's solution.

be considered non-specific, with the reservation that concentrations of K^+ higher than 40 mM were not used in these experiments.

At lower ouabain concentrations of 10^{-6}, 10^{-8} and 10^{-9}M, ouabain binding was consistently increased by cAMP stimulation. The increase was apparent, however, in slices incubated with 40 mM as well as 5 mM of K^+ (Table 1), suggesting that non-specific as well as specific binding of ouabain was enhanced. No significant changes were noted in the difference between binding in the presence of 40 mM and 5 mM K^+. It is not possible, therefore, to interpret these data as establishing an activation of specific Na-K-ATPase ouabain-binding sites in the stimulated slice. Further experiments will be necessary in the perfused gland and with slices to delineate the nature of the increase in ouabain binding produced when ion transport is stimulated by cAMP in rectal gland cells.

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TELEOST ERYTHROCYTE CARBONIC ANHYDRASE: ISOLATION AND PARTIAL CHARACTERIZATION

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Red cell carbonic anhydrase has been studied and well characterized in a number of species. In addition to physical methods the enzyme can be categorized on the basis of kinetic properties and inhibition by sulfonamides and certain anions. This information has made possible a description of the phylogeny of carbonic anhydrase (Maren and Rittmaster, Bull. MDIBL. 17:35, 1977) and further suggests that the archetypal vertebrate enzyme (as in elasmobranchs) was of low activity, with the first high activity form appearing in teleosts. The kinetics and in-

hibition parameters of red cell carbonic anhydrase have been studied in several teleost species; all have a high activity enzyme akin to the high activity C type enzyme in mammals. (Maren and Friedland, Bull MDIBL, 1978). Girard and Istin (Biochem. Biophys. Acta 381:221, 1975) report that euryhaline eel (Anguilla anguilla) red cell has high and low activity isoenzymes. Their finding that these isozymes have K_M and k_{cat} that are more than an order of magnitude greater than all other vertebrate carbonic anhydrases is quite surprising and questionable.

Table 1. Amino acid composition of some representative vertebrates

	Wolffish	Tiger Shark[1]	Toad[2]	Turtle[3]		Chicken[4]	Human[5]	
							B	C
LYS	23	29	14	19	11	15	18	24
HIS	6	14	14	14	14	13	11	12
ARG	43	13	6	7	10	9	7	7
ASP	19	40	24	29	27	24	31	29
GLU	18	40	20	21	29	26	22	25
GLY	33	32	18	20	21	21	16	22
ALA	22	23	12	13	14	19	19	13
VAL	9	13	11	14	13	18	17	17
LEU	7	34	21	24	21	17	20	25
ILE	5	16	6	13	11	10	10	9
PRO	ND.*	21	17	N.D.	N.D.	18	18	17
SER	8	31	17	24	20	20	30	19
THR	9	13	10	14	13	10	14	13
CYS	ND	18	3	1	4	7	1	1
MET	2	3	1	0	2	3	2	1
PHE	1	18	8	9	11	10	11	12
TYR	1	9	8	8	8	8	8	8
TRP	ND	7	7	7	6	7	6	7
Total residues	~206	374	193	~237	235	255	261	261
activity	high	low	high	low	high	high	low	high

[1]Maynard and Coleman, J. Biol. Chem., 246:4455, 1971.

[2]Scott and Skipski, Comp. Biochem. Physiol. 63B:429, 1979.

[3]Hall and Schraer, Comp. Biochem. Physiol. 63B:561, 1979.

[4]Bernstein and Schraer, J. Biol. Chem., 247:1306, 1972.

[5]Nyman and Lindskog, Biochem. Biophys. Acta, 85:141, 1964.

*Not determined.

This note reports the first attempt at purification and amino acid analysis of a teleost erythrocyte carbonic anhydrase. In addition I compare my findings with those in elasmobranchs and other vertebrates with particular attention to the problem of isoenzymes in these species.

The purification of teleost red cell carbonic anhydrase was attempted with affinity gel chromatography using CM Biogel-A (Bio Rad) coupled to para-aminoethylbenzene-sulfonamide (a carbonic anhydrase inhibitor) through carbodiimide (Osborne and Tashian, Analytical Biochem., 64:297, 1975). Hemolysates from wolffish (Anarichas lupus) were added to the affinity gel, which then washed free of hemoglobin and other red cell constituents with large volumes of buffer solution. Carbonic anhydrase was eluted with the same buffer solution containing KCNO, a strong anionic inhibitor. The protein concentration of the eluate was determined spectrophotometrically at 280 mμ using bovine serum albumin standard. The eluate was dialyzed against large volumes of distilled water before performing activity and amino acid analyses. Carbonic anhydrase activities of the eluate and hemolysates from wolffish, flounder (Pseudopleuronectes americanus) and goosefish (Lophius americanus) were measured using the micro-method for CO_2-hydration at 0-1°C with barbital buffer and bromothymol blue indicator (Maren, J. Pharmacol. Exp. Therap. 130:26, 1960). Determinations of the intracellular red cell carbonic anhydrase concentrations in these species and the dogfish (Squalus acanthias) were made by two independent methods. The first entails the titration of hemolysate enzymic activity with the powerful inhibitor benzolamide (Maren et al., J. Pharmacol. Exp. Therap. 130:389, 1960). The second measures sulfonamide binding in intact erythrocytes using the tightly bound highly lipid soluble inhibitor ethoxzolamide (Maren et al., Biochem Pharmacol., 6:218, 1961). Amino acid analysis was performed according to the method of Hirs, Stein and Moore (J. Biol. Chem. 211:941, 1954).

Table 1 shows the amino acid composition of wolffish erythrocyte carbonic anhydrase and also those of a representative species of elasmobranch, amphibia, reptile, bird and mammal reported in the literature (see references in Table 1). The low activity shark enzyme has a greater number of amino acids than any other species as well as a high cysteine content. It was not possible to determine directly the cysteine content of wolffish enzyme since the acid hydrolysis usually degrades a large fraction of the cysteine. The wolffish enzyme appears to be more akin to the higher vertebrate enzymes on the basis of total amino acid residues per molecule. It differs considerably in the content of arginine, leucine, serine, phenylanine and tyrosine. The isolation procedure resulted in a loss of enzyme activity, requiring that activity be measured in fresh hemolysates. The K_i for benzolamide is 2.4×10^{-9}M and titration with this inhibitor yielded the result that 1 enzyme unit in the assay system is 6×10^{-10}M. These values compare closely to those for the proven high activity goosefish (Maren and Rittmaster, vide supra) red cell enzymes.

Table 2. Intracellular erythrocyte carbonic anhydrase concentrations

	Sulfonamide Titration of hemolysate activity (μM)*	Red Cell Sulfonamide Binding ǂ (μM)
Wolffish	6	----
Goosefish	5	72
Flounder	13	105
Dogfish	24	20
Man	28	180

*Using benzolamide. Method of Maren et al., J. Pharmacol. Exp. Therap., 130:389, 1960).

ǂUsing Ethoxzolamide. Method of Maren et al, Biochem. Pharmacol., 6:218, 1961). Results in this method are independent of the sulfonamide used.

The discovery of carbonic anhydrase isoenzymes in the blood of many mammals was made in the early 1960s; however, only recently has there been any evidence that lower vertebrates may have red cell isozymes. Hall and Schraer (Comp. Biochem. Physiol., 63B:561, 1979) showed that in turtle and Girard and Istin (vide supra) in the

feel that there are high and low activity erythrocyte carbonic anhydrases. Certain tissues in the dogfish (which lacks a high activity red cell enzyme) have a high activity carbonic anhydrase, (Maren and Friedland, vide supra). The isolation procedure employed here would not readily separate isoenzymes and the possibility of isoenzymes in teleost red cells was explored in a different manner.

In cells that contain only one isoenzyme it is possible to calculate the intracellular enzyme concentration from an Easson-Stedman analysis (Maren et al., J. Pharmacol. Exp. Therap., 130:389, 1960). The actual molar concentration of the enzyme is obtained by direct titration with benzolamide or another powerful carbonic anhydrase inhibitor. However, should low and high activity forms co-exist, this method will underestimate the true intracellular enzyme concentration because a low activity isozyme contributes little to overall activity while physically binding the inhibitor. Measurement of the total binding of a carbonic anhydrase inhibitor in intact cells provides a direct determination of the intracellular enzyme concentration, independent of the presence or absence of isoenzymes. This method offers no information about absolute or relative activities of the enzymes. By employing both methods, one ascertains the presence of high and low activity enzymes, if the two values are divergent. The results for several teleosts, an elasmobranch and man are shown in Table 2. The wolffish is rarely caught in the waters off Mount Desert Island and I was not able to do erythrocyte ethoxzolamide binding in this fish. Therefore I studied two other teleosts and the pattern is quite clear. The data on ethoxzolamide binding indicates that these teleosts have 8-14 times more enzyme than direct titration shows. One sees the same discrepancy in human red cells, (see references in Table 2), that are known to have low and high activity isozymes. The red cell concentration of the high activity enzyme C is approximately 25 μM, whilst for the low activity enzyme B it is 125-150 μM. The value for direct titration with benzolamide in human blood gives 27 μM, almost identical to the concentration of enzyme C, and the value from red cell ethoxzolamide binding gives the sum of enzymes B and C. The same situation would probably obtain in the turtle erythrocyte, whose low activity enzyme is 8-10 times more concentrated (Hall and Schraer, vide supra). This probably is the case in the wolffish since recovery of enzyme as determined by protein content yields four times as much enzyme as would be calculated from direct titration data of Table 2. In contrast the shark red cell is known to have only a low activity enzyme (Maynard and Coleman, J. Biol. Chem., 246:4455, 1971) and the equivalent results of the two methods (Table 2) support this conclusion.

Further work will be necessary to characterize these isoenzymes in teleost erythrocytes. However the preliminary data strongly suggests that red cell high and low activity isozymes of carbonic anhydrase have existed in the lower vertebrates dating back to the evolution of the teleosts.

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DOGFISH (Squalus acanthias) RENAL AMMONIA RESPONSE TO AN ACID LOAD

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Although acid-base balance and its regulation has been well studied in mammalian systems, little is known concerning the control of acid-base balance in fish. It is generally assumed that ion-exchange mechanisms operating in the gill maintain the normal pH of the body fluids, but definitive evidence for this hypothesis is lacking. The role of the kidney has received relatively little attention. Recently the rainbow trout has been shown to completely compensate for an acid load by renal H^+ excretion (Wood and Caldwell, J. Exp. Zool., 205(2):301-307, 1978).

In mammals, the ability of the kidney to eliminate H^+ and anions as urinary NH_4^+ salt provides one of the major mechanisms for disposing of an acid load. The purpose of the present study was to examine the renal ammonia