and Evans, 1980, op. cit.), but contrast with our recent findings with the perfused S. acanthias head where both afferent pressures and preferential shunting of perfusate into the dorsal aorta are controlled by beta receptors (Evans and Claiborne, 1981, op. cit.). The fact that epinephrine addition resulted in an initial increase in branchial vascular resistance (which is increased after beta-blockade) suggests that alpha-adrenergic receptors are involved in the pressure responses to epinephrine, but definitive statements await experiments testing the effects of pheontolamine on this response.

In summary our data indicate that the head of Mustelus canis pups can be readily perfused for relatively long periods and used to examine various factors in the control of elasmobranch branchial hemodynamics. Importantly, they, like the perfused S. acanthias pup head, also represent a potential resource for investigating mechanisms of solute transport across the elasmobranch branchial epithelium (Evans et al., this volume). This research was supported by NSF PCM 81-04046.

THE DISSOCIATION OF CO_2 HYDRATION AND RENAL ACID SECRETION IN THE DOGFISH, SQUALUS ACANTHIAS

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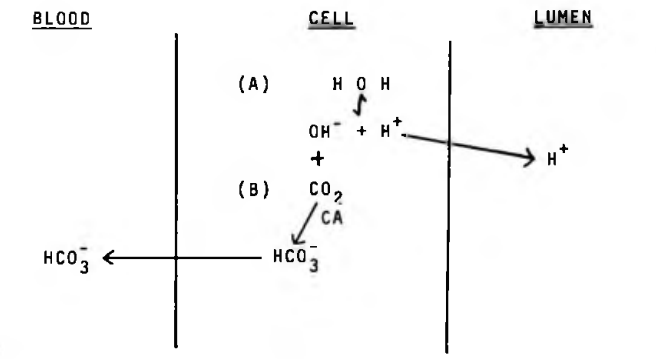
Since the discovery of renal hydrogen ion secretion and its reduction by carbonic anhydrase inhibition (Pitts and Alexander, Am. J. Physiol. 144:239, 1945), the enzyme, CO_2 and H^+ secretion have been inextricably linked. However, recent physiological work and reconsideration of the chemical reactions involved suggest that this linkage may be dissociated. The primary event in acid serretion is the formation of protons from the protolysis of water (an inexhaustible source) with translocation of H^+ toward one cell border and OH^- oppositely directed (Maren, Can. J. Physiol. Pharmacol. 52:1041, 1974). The proton is extruded into the tubular lumen and the hydroxyl ion moves into the blood (Figure 1, Step A). Our questions are: 1) how is this accomplished, and 2) is noncatalyzed or carbonic anhydrase catalyzed buffering of hydroxyl ion by CO_2 an absolute requirement for renal hydrogen ion secretion (Figure 1, Step B).

FIGURE 1

Since the discovery of renal H^+ secretion, (Pitts, 1945), the process has been linked with CO_2 hydration and carbonic anhydrase. In terms of what we now know of the chemistry of these processes, the scheme would be:



Marine fish are well suited chemically for such a study since they lack renal carbonic anhydrase, and their low body temperature and low pCO_2 result in a very slow uncatalyzed reaction. Marine fish are also well suited physiologically for such a study, since they reabsorb all filtered HCO_3^- and excrete acid at rates independent either of HCO_3^- loads or (since there is no renal enzyme) the administration of carbonic anhydrase inhibitors (Hodler et al., Am. J. Physiol. 183:155, 1955). Table 1 shows acid-base equilibria in plasma and urine of S. acanthias, both in the normal and alkalotic states.

In the present experiments we repeat the basic work of Pitts, but in S. acanthias, presenting a large buffer load to the kidney and measuring maximal rates of H^+ secretion. These rates will be compared to those of the uncatalyzed reactions of CO_2 with water, which yield H^+ ions. If the physiological rates are notably higher than the chemical, a process for H^+ secretion independent of CO_2 must be invoked.

TABLE 1

Showing the failure of a high HCO_3^- load to influence HCO_3^- reabsorption or acid excretion in a marine fish (Squalus acanthias). (n = 4)

	Urine				Plasma		
	pH	Flow, kg^{-1}	H^+	HCO_3^-	pH	HCO_3^-	CO_2
0 - 2 hrs	5.7	0.9 ml/hr	30 mM	0.1 mM	7.46	4.4 mM	0.19 mM
	Infuse	30 m moles	$NaHCO_3$	at 2 - 4 hrs			
4 - 5 hrs	5.8	1.3	35	0.9	8.19	30	0.24

In terms of mammalian physiology, an extraordinary result:

1. Increasing filtered load of HCO_3^- 7 - fold does not affect HCO_3^- excretion.
2. Titratable acid does not change in the face of greatly increased HCO_3^- reabsorption, suggesting that these are independent events.

METHODS--Male sharks weighing approximately 2 kg were caught by net in Frenchman Bay and kept in live cars until used. During the experiment they were placed in small boxes with free flowing seawater ($T = 15^\circ - 16^\circ C$). The dorsal aorta and the urinary papilla were cannulated with PE-90 polyethylene tubing and in earlier experiments the animals were restrained in a ventral side-up position throughout the study. However, in later experiments after catheter placement fish were restrained in a normal position by use of two soft encircling sponge rings that fit snugly into the box. The latter technique allowed the fish limited movement of his head and body and resulted in little or no struggling. Despite the obvious preference of method we found no difference in gas exchange or hemodynamics in the fish over the usual time course of an experiment. Blood pressure was monitored continuously and arterial blood samples were taken anaerobically for measurement of pO_2, pCO_2, pH, total plasma CO_2, plasma phosphate, imidazole and inulin. Urine was collected for measurement of flow rate, pH, total CO_2, inulin, phosphate and titratable acid. Arterial pO_2 and pH were measured on a blood gas analyzer (Instrumentation Laboratories, model 203, Watertown, Mass.). Titratable acid was measured by titration of 1 ml of urine to pH 7.8 with 0.1 N NaOH. Plasma and urine total CO_2 were measured manometrically using a Kopp-Natelson Microgasometer. Plasma and urine phosphates were measured using the method of Fiske and Subba Row (J. Biol. Chem. 66:375, 1925). For measurements of GFR, 2 μCi of ^{14}C-inulin were injected several hours before the start of the infusion. Plasma and urinary imidazole were measured by careful titration of plasma and urine over the range of 7.1 - 8.1 with 0.1 N NaOH or 0.1 N HCl. A value of each animal's normal buffering over this range was determined before infusion of imidazole and was subtracted from the total plasma or urinary titratable buffer to yield the imidazole concentration. The method was validated by determinations (< 5% error) of known amounts of imidazole added to plasma or urine to generate concentrations > 3 mM.

We tested a number of buffers for their ability to increase renal titratable acid secretion. The basic design of the experiment included a two-hour period of control baseline measurements followed by a two-hour infusion of buffer (30 ml/hr) with repeat measurement of all pertinent variables. The compositions of the differing buffers in dogfish Ringers at pH = 7.7 were 120 mM for phosphate, phenol red and dimethadione (DMO) and 225 mM for imidazole and 200 mM for piperazine-N,N'-bis[2-ethane sulfonic acid] (PIPES). In several experiments with imidazole infusion the effects of Darstine, an inhibitor of organic base secretion and methazolamide, a carbonic anhydrase inhibitor, were examined. The rate of buffer infusion was of some concern and we present only those data of animals whose blood pressure remained stable (> 30 cm H_2O) and whose arterial oxygenation never fell below 90 mm Hg.

RESULTS--The effect of PO_4 infusion is seen in Table 2. The rate of phosphate and titratable acid excretion rises four-fold following ten-fold elevation of plasma phosphate. Further elevations of plasma phosphate could not be tolerated. Note the very high U/P ratio for phosphate in the normal, which actually declines during phosphate infusion. Results with phenol red and DMO were equally disappointing; maximal titratable acid output did not exceed 100 μEq/hr·kg at maximum tolerated doses.

We then turned to organic bases which buffer at pH 5-8. Table 3 shows the data with imidazole (pK = 7.1) and

TABLE 2

Augmentation of Renal Titratable Acid Excretion by Phosphate

	URINE					PLASMA
	pH	Flow	Titratable Acid		PO_4	PO_4
CONTROL		(ml/hr·kg)	(meq/L)	(μeq/hr·kg)	(mM)	(mM)
(n = 5)	5.7 ± 0.1	1.1 ± 0.2	33 ± 4	36 ± 7	45 ± 5	1.4 ± 0.2
PHOSPHATE INFUSION						
(n = 5)	5.6 ± 0.1	1.8 ± 0.3	77 ± 6	140 ± 10	108 ± 10	11 ± 1.0

n = number of experiments, values include standard error of the mean.

TABLE 3

Augmentation of Renal Titratable Acid Excretion By Imidazole

	URINE				PLASMA
	pH	Flow	Titratable Acid		Imidazole
CONTROL		(ml/hr·kg)	meq/L	μeq/hr·kg	mM
(n = 5)	5.72 ± 0.03	1.0 ± 0.1	28 ± 3	33 ± 4	--
IMIDAZOLE INFUSION					
(n = 5)	5.81 ± 0.04	2.6 ± 1.0	149 ± 15	390 ± 30	6.3 ± 1.0
IMIDAZOLE INFUSION + DARSTINE					
(n = 1)	5.80	2.3	162	365	5.2
PIPES INFUSION					
(n = 2)	5.7	2.0	130	260	--

n = number of experiments.
± = standard error of the mean.

PIPES (pK = 6.8). The augmentation of titratable acid excretion was twelve-fold with imidazole and eight-fold with PIPES. In order to rule out active cationic base secretion (i.e., the transport of acidic imidazole) which would contribute to urinary titratable acid independent of direct proton secretion by the tubule, we gave Darstine (mepiperphenidol), an inhibitor or organic base secretion (Torretti et al., J. Clin. Invest. 41:783, 1962). At doses (see Table 2) known to block completely this mechanism in mammalian kidney (but against far lower concentrations of organic base) the results are no different from controls. Assuming that titratable acid equals urinary imidazole, calculations from Table 3 show a U/P ratio of 24 for imidazole (pK = 7.1). This is very close to the theoretical value of 25 for non-ionic diffusion when urine pH = 5.7 and plasma pH = 7.5.

Table 4 shows more complete data from a single experiment which also includes the effect of methazolamide, a carbonic anhydrase inhibitor, on maximal acid secretion. In this experiment the U/P ratio for imidazole is $120 \div 6.3 = 19$, somewhat less than the predicted 25 for the process of nonionic diffusion. However, when we correct for the ionized fraction in plasma (30%) and the filtered acid imidazole in urine, the U/P ratio is 25.5, very close to the theoretical value. The small decrease in titratable acid secretion seen with methazolamide (also without effect in two other experiments, not shown) is due to the declining plasma level of imidazole and need not be ascribed to any inhibition of enzyme activity.

DISCUSSION--The remarkable constancy of urinary pH at about 5.7 in marine fish is well known (W. W. Smith, J. Cell. Comp. Physiol. 14:59, 1939). The necessity for an acid urine relates to the fact that the kidney is responsible for excretion of magnesium whose salts precipitate above pH 6.0. Experiments by Pitts (J. Cell and Comp. Physiol. 4:389, 1934), W. W. Smith (J. Cell. Comp. Physiol. 14:59, 1939) and Hadler et al (Am. J. Physiol. 183:155, 1955) taken as a whole document that hydrogen ion secretion varies in proportion to the excretion of phosphate and other urinary buffers to insure a fixed acid pH. Kempton (J. Morph. 73:247, 1943) showed that prompt tubular acidification occurs very early in the proximal tubule and is maintained throughout the length of the nephron. The quantitative capacity for H^+ secretion in the dogfish was studied by J.J. Cohen (J. Cell. Comp. Physiol. 53:705, 1959) using infusions of trimethylamine (TMA), a weak base with pK of 9.8. Assuming no active secretion of cationic TMA but only passive diffusion of the nonionized amine, he showed maximal acidification rates of approximately

TABLE 4

REPRESENTATIVE EXPERIMENT OF IMIDAZOLE INFUSION AND EFFECT OF A CARBONIC ANHYDRASE INHIBITOR

(Fish Wt = 1.9 kg)

	URINE						PLASMA†
	Flow	GFR	pH	Titratable Acid	Phosphate	Imidazole*	Imidazole
	(ml/hr·kg)			(uEq/hr·kg)	(umol/hr·kg)		(mM)
CONTROL (-2 to 0 hr)	1.0	2.2	5.70	50	60	10	0
IMIDAZOLE* INFUSION (2-3 hr)	5.2	9.5	5.80	676	52	624	6.3
30 mg/kg METHAZOLAMIDE (3-4 hr)	5.0	8.7	5.76	590	55	535	5.3

*Imidazole measured as the difference between titratable acid and phosphate. In this experiment the Imidazole was infused from 0-2 hrs, and baseline measurements made at 2-3 hrs; then methazolamide given and measurements made at 3-4 hrs.

†Plasma values were unchanged through the experiment as follows: Phosphate 1.1 mM; pH 7.5; pCO_2 5-6 mm Hg; HCO_3^- 6-7 mM.

350 μEq/hr·kg, just as in our experiments. At these rates a rise was noted in the urine pH to 6.2, suggesting a possible limit to this secretory mechanism.

Our results with phosphate confirm those of Smith (1939), Hodler et al (1955) and Wolbach (Am. J. Physiol. 219:886, 1970). The failure to reach maximal acid secretory rates with phosphate is due to its toxicity at high serum levels and its near maximum active secretion in the basal state. With phosphate infusion the percentage of PO_4 secreted to total excreted falls from 90% to less than 50%, the remainder representing filtered phosphate. Thus phosphate is not a suitable means of studying variation in acid excretion in the fish. Our data with imidazole are comparable to those of Cohen (1959) and are consistent with the passive diffusion of unionized base and subsequent ionic trapping. However, we did not see a marked or statistically significant increase in urinary pH at these high rates. These rates of acid secretion, 0.4 millimoles per hr per kg fish, are not necessarily maximal. Greater quantities of imidazole could not be used because of toxicity.

The question of the role of CO_2 in this process can now be addressed. Since carbonic anhydrase is absent, the important reactions are:

$$H_2O + CO_2 \underset{k_1}{\longrightarrow} H_2CO_3 \longrightarrow H^+ + HCO_3^- \qquad (1)$$

and

$$H_2O \longrightarrow H^+ + OH^- \qquad (2)$$

$$CO_2 + OH^- \underset{k_2}{\longrightarrow} HCO_3^- \qquad (3)$$

If H^+ output is dependent on reaction (1), then its rate is:

$$V = k_1 \cdot [CO_2] = 12 \text{ mM/hr}$$

where $k_1 = 1 \text{ min}^{-1}$ at T = 16°C and $CO_2 = 0.2$ mM ($pCO_2 = 4.5$ mm Hg). The kidney weight in these animals is ~5 g/kg and making the very liberal assumption that the appropriate reaction volume (i.e., those cells involved in H^+ secretion) is half this then;

$$V = 30 \text{ } \mu \text{ moles/hr} \cdot \text{kg fish.}$$

This figure supposes no back reaction and thus overestimates the true velocity. If the gradient for CO_2 from cell to tubule is similar to the A-V gradient of 15%, then a more realistic value is ~ 5 μ moles/kg · hr. It is clear that

such a value can account for neither basal nor stimulated acid output. Furthermore, the failure of this reaction adds further to the concept that protolysis of water (reaction 2) must be the primary reaction in the generation of protons and not CO_2 hydration and carbonic acid formation.

Reactions 2 and 3 show acid secretion in terms of H_2O protolysis and hydroxylation of CO_2 as a means of buffering OH^-. For the latter reaction

$$V = k_2 \cdot [CO_2] \cdot [OH^-] = 29 \text{ mM/hr}$$

where $k_2 = 4000 \text{ sec}^{-1} \text{ M}^{-1}$ at 16°C, $CO_2 = 0.2 \mu M$ and OH^- is given a very high value of 10^{-5} M (pH = 9.0). This may be obtained in certain regions of the cell (i.e., the antiluminal border) but for purposes of argument we use it, realizing the average cell pH is much lower. Using the reaction volume of 2.5 ml (vide supra) then

$$V = 72 \mu \text{ moles/hr} \cdot \text{kg fish}$$

a value only 20% of the ovserved stimulated acid secretion.

Related experiments by Garvey and Maude (Am. J. Physiol. 240:F306, 19) in the isolated perfused rat kidney show a similar failure of the uncatalyzed CO_2 reactions to match acid secretory rates, when the kidney is perfused with $CO_2 HCO_3^-$ free Ringer's solutions and carbonic anhydrase is inhibited. Our experiments extend these observations in several salient ways; we studied acid secretion in an intact animal without renal carbonic anhydrase and attempted to augment the process.

The negative result with methazolamide suggests two important points. First, it rules out a small but heretofore undetectable amount of renal carbonic anhydrase whose activity might only be essential and manifested during such high rates of acid secretion. Secondly, red cell enzyme (inhibited by methazolamide) is not necessary for buffering of OH^- or other base generated by acid secretion.

Sanders et al (Am. J. Physiol., 225:1311, 1973) showed that acid secretion by the in vitro frog stomach is reduced but not abolished in the absence of exogenous CO_2. Their conditions were radically different and carbonic anhydrase was not inhibited. However, they found that the H^+ secretory rate could be returned to normal levels in CO_2 free conditions by the use of appropriate buffer concentration in the bathing media (HPO_4/H_2PO_4 = 25 mH and pH = 4.4). They attributed this to recycling of endogenous metabolic CO_2. Such an explanation could not explain the present results or conclusions, since we assume a constant concentration of CO_2 - as did Garvey and Maude (vide supra).

In conclusion, renal acid secretion appears to involve primary protolysis of H_2O, with H^+ and OH^- ion separation. This process need not be dependent upon a reaction with CO_2 or on renal or erythrocyte carbonic anhydrase. The mechanism for handling of hydroxyl ions remains unknown, but buffering by other intracellular acids (amino acids, phosphate) or direct Cl^-/OH^- exchange might be postulated. This work was supported by NIH grant HL-22258.

THE MECHANISM OF THE HYPOTONICITY-INDUCED K^+ ACCUMULATION IN SLICES OF THE RECTAL GLAND OF THE DOGFISH (SQUALUS ACANTHIAS)

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We have reported previously (Goldstein et al., Bull. MDIBL 21:3, 1981) that hypotonic (urea-free) media produced a) an efflux of urea from the slices; b) a modest increase in tissue H_2O; and c) a net influx of K^+ until the original electrochemical gradient has been reached. Several lines of evidence permitted us to exclude a direct coupling between urea efflux and K^+ influx. This report concerns the actual mechanism by which the efflux of an uncharged molecule brings about an influx of KCl into the cells.