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Ammoniuretic Activity of Dog Plasma When Tested on the Urinary Bladder of *Bufo marinus* (40859)

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Abstract. Hemibladders from the toad *Bufo marinus* were used in all experiments. A protein free preparation of plasma was made from dogs in metabolic acidosis, respiratory acidosis, and metabolic alkalosis. These plasma preparations were tested for ammoniuretic activity by placing them on the serosal side of the urinary hemibladders of *Bufo marinus* mounted *in vitro*. Both acidotic plasma preparations caused increased ammonia excretion into the mucosal media as compared to control hemibladders. Metabolic alkalotic plasma preparations did not produce significant ammoniuretic activity. Using paired hemibladders, the effect of metabolic acidotic plasma preparations showed increased ammoniuretic activity when compared to metabolic alkalotic plasma preparations ($P < 0.05$).

Frazier and Vanatta (1) reported that the urinary bladder of *Bufo marinus* excreted ammonia. Frazier (2) reported a 3-fold increase in ammonia excretion by the urinary bladder in response to a NH_4Cl induced acidosis. In mammals it is known that ammonia secretion can increase 10-fold in response to metabolic acidosis. Frazier and Vanatta (3) also reported that various preparations of both toad plasma and dog plasma were capable of increasing ammonia excretion in isolated urinary bladders obtained from toads in normal acid–base balance.

The mechanism by which either mammal or toad increases ammonia excretion in acidosis is not clear. These studies report evidence for the existence of an ammoniuretic substance in acidotic dog plasma. The observed effect is on the isolated toad urinary bladder.

Methods. The basic experimental procedure was to make preparations of dog plasma and assay these for their ammoniuretic activity on toad bladders in an *in vitro* preparation. *Bufo marinus* of either sex were used in all experiments. The toads were collected near Baranquilla, Colombia and supplied by Charles P. Chase of Miami, Florida. Recently, it became impossible to obtain toads from Colombia. Toads collected from Panama have been obtained from the same supplier. Although no experiments on Panamanian toads are included in this report, in other experiments we have found no difference between Colombian and Panamanian *Bufo marinus* with regard to ammonia excretion (4). Toads were kept in our laboratory in deionized water without food for 1–30 days before use. These were considered to be in normal acid–base balance.

Toads were pithed and the urinary bladder removed. Hemibladders were mounted between lucite chambers with a cross sectional area of 3.09 cm^2. Each chamber contained 2.0 ml of the indicated fluid, and were sealed together with an O-ring. After mounting the bladder, both serosal and mucosal solutions were placed on the bladder and allowed to equilibrate for 15–30 min before the beginning of the excretion period. The mucosal chamber was then drained, washed once with fresh mucosal solution, and then 2 ml of mucosal Ringer solution was placed in the chamber at time zero. After 120 min the mucosal solution was collected and analyzed for ammonia content.

The control serosal solution contained 10 m*M* sodium phosphate buffer, pH 6.97, and 1% v/v ethanol. In addition, the serosal solution contained, in m*M:* NaCl, 105.0; KCl, 3.0; and $CaCl_2$, 0.9. The mucosal solution contained 0.6 m*M* sodium phosphate buffer, pH 6.44. This solution also contained in m*M:* NaCl, 117.0; KCl, 3.0, and $CaCl_2$, 0.9. One hemibladder from each

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toad was bathed with the control serosal solution, and the paired hemibladder was bathed with the appropriate plasma preparation. Humidified 100% O_2 was bubbled into the mucosal medium throughout the experiment.

The 120-min excretory period was terminated by draining the chambers. Then the bladders were cut from the chambers and blotted on filter paper, and wet weight was determined. Bladders were then dried at 100° and dry weight determined. The water content of each bladder was calculated, and all bladders showed similar percentage water content.

Ammonia was determined by the Berthelot reaction as described by Chaney and Marbach (5). The indolphenol dyestuff formed in this reaction was measured with a Bausch and Lomb Spectronic 20 spectrophotometer. Nine replicate analyses of a standard 40 μM NH_4Cl solution gave an average value of 40.01 μM with a standard deviation of individual values of 0.58 μM, which is 1.45%. Nine replicate analyses of a 10 μM solution averaged 10.29 μM, with a standard deviation of individual values of 0.67 μM, which is 6.7%. In the experiments reported, most analyses were within the range of 10–60 μM, with a few analyses slightly below 10 μM.

Total ammonia appearing in the mucosal media was calculated and expressed as "units" defined as (nmoles NH_4^+) (100 mg wet wt of bladder)$^{-1}$(min)$^{-1}$. A set of data from a single pair of experiments are shown in Table I.

Heparinized plasma was obtained from mongrel dogs of either sex. Three dogs were placed in metabolic acidosis by administering 20 g of NH_4Cl in six divided doses over a 48-hr period. The efficacy of this treatment in producing an acidosis was confirmed by determining the [HCO_3^-] of plasma from one animal at the time of bleeding. The concentration was 9.5 meq/liter. Plasma from two dogs was used in Experiment 1, and from the third dog in Experiment 4.

Two dogs were placed in metabolic alkalosis by administering 20 g of $NaHCO_3$ in six divided doses over a 48-hr period. The existence of an alkalosis was confirmed by determining the [HCO_3^-] of plasma obtained from one animal. The concentration was 39.5 meq/liter. Plasma from one dog was used in Experiment 3 and from the other in Experiment 4.

Respiratory acidosis was produced in one dog by placing it in an environment of 5% CO_2 for 48 hr. The percentage CO_2 in the air in the cage was checked by analysis on four occasions during this period. The bicarbonate concentration of the plasma of this animal was 22 meq/liter before the onset of the acidosis, and 28 meq/liter at the time of bleeding. This change in bicarbonate concentration is evidence that the dog's kid-

TABLE I. RESULTS OF A SINGLE PAIR OF HEMIBLADDERS FROM THE SERIES COMPARING EFFECTS OF PLASMA FROM DOG IN METABOLIC ACIDOSIS WITH EFFECTS OF DOG IN METABOLIC ALKALOSIS

	Metabolic alkalosis plasma preparation		Metabolic acidosis plasma preparation	
	Serosal	Mucosal	Serosal	Mucosal
[NH_4^+] before μM	44.8	0	44.8	0
[NH_4^+] after μM	84.3	9.9	35.3	11.8
Total net NH_4^+ excretion per bladder in 120 min (nmole)	19.8		23.6	
Wet weight of hemibladder	10.28		9.11	
Dry weight of hemibladder	2.73		2.42	
Net NH_4^+ excretion (nmole·[100 mg bladder]$^{-1}$·[min]$^{-1}$)	1.61[a]		2.16	

[a] NH_4^+ excretion was determined by measuring the NH_4^+ concentration in the mucosal bath and normalizing this concentration for weight and time as follows: (9.9 nmole/ml) (2 ml)·100/10.28 mg·1/120 min = 1.61 nmole·(100 mg bladder)$^{-1}$·min^{-1}.

neys had compensated for the respiratory acidosis. This plasma was used in Experiment 2.

All blood drawn for making the plasma preparations was obtained under nembutal anesthesia, following which each dog was killed by an overdose of anesthesia.

Plasma from the above animals was prepared by adding 4 vol of acetone to 1 vol of plasma, which precipitated the proteins. The mixture was filtered, and the filtrate evaporated under reduced pressure with a maximum temperature of 40°. When the acetone was removed, 0.5 *N* HCl was added to pH 2.0 and the evaporation continued to dryness.

The final volume for reconstituting the solution was calculated based on:

$$(155 \text{ meq/liter})\ (V) = (120 \text{ meq/liter})\ (W),$$

where V = initial volume of plasma, and W = volume of water to be added for reconstituting.

The purpose of this is to reduce the concentration of total salts from that of mammalian body fluids to that of toads.

A volume of ethanol equal to 0.01 *W* was added to the residue, and then a volume of water, *W*. Also, assuming 2.0 m*M* phosphate was present in the initial plasma, the amount of phosphate necessary to make the final phosphate concentration 10 m*M* was added in the form of Na_2HPO_4. The pH of the resulting mixture was adjusted to 6.97 by adding 0.5 *N* NaOH or 0.05 *N* NaOH. Such preparations were free of proteins when tested by the Heller test as described by Todd and Sanford (6). In the test the solution is layered on top of concentrated nitric acid.

Statistical analysis of each series was done using the one-tailed Student *t* test as reported by Fisher (7). The mean difference ± SEM is given. The one-tailed test was appropriate since ammoniuretic activity was present only if the experimental hemibladder excreted more ammonia than the control hemibladder. Statistical analysis on Experiment 2, Table II, was done by the one-tailed Wilcoxon's paired sample test.

Results. Experiments 1, 2, and 3. Experiment 1 consisted of 19 paired hemibladders in which a plasma preparation from a metabolic acidotic dog was placed on the serosal surface of the experimental hemibladder, and 10 m*M* PO_4 Ringer solution on the serosal surface of the control hemibladder. Table II shows the results of these experiments. It is obvious that an extract of the plasma from a dog in metabolic acidosis will stimulate NH_4^+ excretion in the toad urinary bladder ($P < 0.025$). In addition, Table II also shows the results of Experiment 2 which was obtained when a preparation of plasma from a dog in respiratory acidosis was tested against control Ringer solution. Again, there was significant stimulation of NH_4^+ excretion in the bladder by this extract of plasma from a dog in respiratory acidosis ($P < 0.05$).

In contrast, in Experiment 3 consisting of a series of 10 paired hemibladders in which the plasma preparation was from a dog in metabolic alkalosis, the average ammonia excretion of the experimental hemibladders was 3.13 units compared with the average of the control hemibladders of 2.87 units.

TABLE II. THE EFFECT OF PLASMA EXTRACTS ON NH_4^+ EXCRETION IN THE TOAD URINARY BLADDER

Expt No.	State of dog from which plasma was obtained	*N*	Average NH_4^+ excretion in nmoles (100 mg bladder)$^{-1}$ (min)$^{-1}$		Mean difference ± SEM	*P* value
			Experimental	Control		
1	Metabolic acidosis	19	2.88	2.31	0.56 ± 0.26	<0.025[a]
2	Respiratory acidosis	10	1.99	1.57	0.42 ± 0.29	<0.05[b]
3	Metabolic alkalosis	10	3.13	2.87	0.26 ± 0.43	>0.25[c]

[a] *P* value determined by the one-tailed Student's *t* test.
[b] *P* value determined by one-tailed Wilcoxon's paired sample test.
[c] *P* value with either one-tailed Student's *t* test or Wilcoxon's paired sample test.

The mean difference was 0.26 ± 0.43 units ($P > 0.25$). The ammoniuretic activity was not present in plasma when the dog was in metabolic alkalosis.

Experiment 4. Since plasma preparations from an alkalotic dog gave a positive, but not statistically significant different result in comparison to a control Ringer solution, it was necessary to directly compare the effects of the two plasma preparations. A plasma preparation from another acidotic dog was placed on the serosal side of hemibladder A, and a plasma preparation from the alkalotic dog was placed on the serosal side of hemibladder B. The mean ammonia excretion of 10 hemibladders A was 3.06 units and of 10 hemibladders B was 1.98 units with a mean difference of 1.08 ± 0.50 units ($P < 0.05$).

In these experiments the slight ammonia concentration in the preparations was matched at the beginning of the experiment. At the end of the experiment the serosal ammonia concentration of the plasma preparation from the acidotic dogs averaged 88.6 ± 11.8 μM, and from the alkalotic dogs averaged 85.3 ± 1.7 μM. In five of the pairs the final concentration was higher in the serosal media from the acidotic dogs, and in four of the pairs the final concentration was higher in the plasma preparation from the alkalotic dog. In one pair, the serosal concentrations were the same on both hemibladders at the end of the experimental period. As can be seen in Table I, an increased excretion of ammonia can take place in a hemibladder in both absolute amounts, and when corrected for bladder weight, even though it has a lower concentration gradient across it than its pair.

Discussion. We have collected plasma from dogs in either metabolic acidosis, respiratory acidosis, or metabolic alkalosis. The plasma proteins were precipitated and the osmolarity adjusted to that of amphibian plasma (≅237 mOsm/liter). We have shown that a substance with ammoniuretic activity is present in the plasma of dogs in metabolic acidosis and respiratory acidosis. No ammoniuretic activity was found in the plasma of a dog in metabolic alkalosis. A related finding is reported by Alleyne and Roobal (8). They found a factor in plasma of acutely acidotic rats which increased ammonia formation in kidney slices of normal rats.

In our preparation of the plasma, HCl was added in sufficient quantity to convert all the bicarbonate to carbonic acid and then the preparation was evaporated to dryness under reduced pressure. Such procedures prevent the reformation of significant quantities of bicarbonate when the phosphate is added. If H_2CO_3 were not removed, the reaction

$$H_2CO_3 + Na_2HPO_4 \rightarrow NaHCO_3 + NaH_2PO_4$$

would take place. The above procedure and the subsequent adjustment of pH eliminate H^+, HCO_3^-, and CO_2 as the ammoniuretic substance. Analysis of plasma preparations were done to confirm that there was no detectable bicarbonate.

It is reasonable to exclude glutamine as the substance stimulating ammonia excretion. Pitts (9) reports that there is no evidence that plasma glutamine concentration changes in response to an acidosis. Therefore, in the experiments comparing the plasma of metabolic alkalosis and acidosis, the plasma preparations bathing hemibladders A and B can be assumed to have similar glutamine concentrations. The excretion of ammonia by A was increased. Furthermore, in experiment from Table II using plasma from a dog in metabolic alkalosis, the hemibladder with the plasma preparation and therefore with glutamine did not show significant increases in ammonia excretion in comparison with the bladder with no exogenous glutamine.

It is known that ammonia salts given by mouth are rapidly converted to urea (10) so that ammonia blood levels do not rise in response to ammonia chloride administration. Therefore, the metabolic acidosis plasma could not have produced an ammonia gradient across the bladder which increased ammonia excretion.

The primary purpose of this paper is to report the data which indicate the presence of the ammoniuretic substance in dog plasma during different states of acid–base balance. A related question is "What is the source of the ammonia excreted?" Some

speculation is in order with regard to this quesiton. We have shown that the toad bladder excretes ammonia *in vitro* without adding either exogenous precursors or ammonia. The ammonia is produced from precursors in the bladder. In other experiments (4) we have found that the rate of hydrolysis of glutamine in Ringer solution was increased when bladders were added to the media. This suggests that glutaminase is present in the toad urinary bladder. However, before the glutamine–glutaminase system can be implicated in this ammonia excretory tissue, additional studies with more stringent criteria must be done.

The average control ammonia excretion in the metabolic alkalosis series is high. One must then consider the following premise. Could the metabolic alkalosis plasma contain an amminouretic substance, but because the control ammonia excretion was near a limit, further stimulation of the bladders could not elicit a higher rate of ammonia excretion. This premise does not seem likely. First, it appears that the rate of excretion can go higher than the value of 3.13 units reported. In our laboratory we have previously reported (4) a series of 53 paired hemibladders in which the control value averaged 3.30 units of ammonia excretion, and the experimental hemibladders, stimulated by a urine extract, excreted 4.60 units. The mean difference was 1.30 ± 0.40 ($P < 0.005$). Thus the value of 3.13 does not seem to be near a limit of maximum excretion.

The portion of the premise which suggests that the plasma contains an ammoniuretic substance is disproven by the series in which the effects of preparations from plasma of acidotic dogs is compared with the effects of preparations from alkalotic dogs. The hemibladders treated with plasma from the alkalotic animals excreted an average of 1.98 units, which is in the range of the control values of Experiments 1 and 2 reported in Table II.

In these experiments and other experiments there has been quite a bit of variation in control values for ammonia excretion. By using the technique of paired hemibladders, the effect of this variation on comparisons between control and experimental procedures is minimized. The exact source of these variations has not been determined. Since seasonal variation explains many phenomena like this in amphibia, one could speculate that this is the probable cause of the variation in the control values.

Melton *et al.* (4) report a similar ammoniuretic activity of a crude extract of human urine when tested on toad urinary bladder. This activity is present in urine obtained from individuals in metabolic acidosis. They were unable to find activity in extracts of urine obtained from the same individuals when metabolic alkalosis was produced by ingestion of $NaHCO_3$.

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