Calcium and carbonate metabolism in the frog (Rana temporaria) during respiratory acidosis

K. SIMKISS
Department of Physiology and Biochemistry, The University, Reading, England

Two types of suggestions have been made about the functions of these enlarged organs in the amphibia. The first of these may be called the anatomical theories. They have been reviewed elsewhere (14) and as they are only poorly supported by experimental evidence they will be only briefly mentioned. They variously suggest that the sacs protect the spinal ganglia, act as reservoirs for endolymph displaced from the labyrinth, or transmit sound waves to the sensory regions of the inner ear. All these theories correlate the functions of the sacs with their morphological peculiarities. The second type of theory, however, considers that the large deposits of calcium carbonate provide the main physiological functions of these organs. Thus it has been suggested that the calcareous deposits could be withdrawn to assist in bone repair (9) or to provide calcium for bone formation at the time of the metamorphosis of the tadpole (2, 3, 6). Experimental evidence has recently been obtained to support both these hypotheses but a third possible function has attracted little attention. It has been shown that the calcareous deposits of the sacs dissolve and can no longer be seen radiographically if frogs are exposed to environments containing large amounts of carbon dioxide (16). This theory would appear to imply, therefore, that the calcium carbonate in the endolymphatic sacs can act as a buffer when the carbon dioxide concentration in the frog is increased experimentally:

$$\text{CaCO}_3 + \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{Ca}^{++} + 2\text{HCO}_3^-$$  (1)

Respiratory acidosis might also cause bone dissolution:

$$\text{Ca}_3\text{(PO}_4\text{)}_2\text{(OH)}_2 + 8\text{CO}_2 + 8\text{H}_2\text{O} \rightleftharpoons 10\text{Ca}^{++} + 6\text{HPO}_4^{-2} + 8\text{HCO}_3^- + 2\text{H}_2\text{O}$$  (2)

but since bone is normally continually being resorbed and reformed by cellular activity and since the blood is already in a supersaturated state for this mineral it is unlikely that such a simple relationship would be maintained.

It was therefore decided to treat frogs with carbon dioxide to try to obtain evidence pertinent to the following four questions: 1) Does respiratory acidosis in the frog produce an increase in the calcium excretion which would indicate the dissolving of mineral deposits? 2) Is
bicarbonate retained in the blood to increase its buffering ability? 3) Is the blood pH brought back to its normal pH value by these processes? 4) Is there any evidence which would indicate the normal relationship of the blood to the calcareous deposits of the endolymphatic sacs?

MATERIALS AND METHODS

The experiments were performed on the common English frog Rana temporaria. The animals were kept in running tap water for several weeks prior to their use and no attempt was made to feed them. Frogs will normally remain in a healthy state for many months without food and it was important in these experiments to starve the animals in order to avoid fecal contamination of the urine.

The frogs were kept in the laboratory in plastic funnels half-filled with glass beads upon which the animals could sit. The funnels were sealed at the bottom with clipped rubber tubes so that they could be filled with 50 ml of water. In the first experiments the frogs were placed in deionized water but this was later replaced with a 2 mM solution of sodium chloride so as not to upset the osmoregulation of the animals. This refinement had no effect on any of the results obtained. The water in the funnels half covered the frogs and could be drained off by releasing the rubber tubing. An inverted funnel prevented the animals from escaping and provided an inlet for an air line. The frogs were normally studied in these chambers for 1-2 weeks after which they were transferred to a plastic container and put into respiratory acidosis. The plastic container was subdivided to hold eight animals all of which breathed the same gas mixtures although they were individually housed in 50 ml of water which could again be withdrawn daily.

The water drained off from each frog was analyzed for calcium by means of an ethylenediaminetetraacetic acid titration using murexide as an indicator and with the end point determined photoelectrically (6). Proteins were removed from the solution by treating it with an equal volume of 20% trichloroacetic acid after which phosphate was determined colorimetrically by using an ammonium vanadomolybdate complex as previously described (6). Over 100 frogs were studied for periods of up to 2 weeks in order to determine their normal loss of calcium and phosphate into the water. The analyses were then continued while the animals were in respiratory acidosis.

Respiratory acidosis was induced by making the frogs breathe various concentrations of carbon dioxide. In the early experiments the frogs were exposed to carbon dioxide and air mixtures of various proportions prepared by adjusting the gas flow through calibrated Rotameter tubes. The exact composition of the gases prepared in this way varied slightly from day to day and for more exact work the animals were supplied from cylinders of compressed gases. Various mixtures of from 5 to 20% carbon dioxide in oxygen were given in this form at a rate of about 250 ml/min. Care was taken to equilibrate the water to be put into the respiratory chambers with these gases before they were introduced since the frog has a large exchange of respiratory gases across the skin.

The frogs which were used in these experiments normally weighed from 25 to 35 g and had an estimated total blood volume of only 1-2 ml. Only about 0.5-1.0 ml of this blood can be withdrawn under normal circumstances so that frogs have to be killed in order to get a blood sample and only one complete set of pH, Pco₂, and bicarbonate analyses can be obtained for each animal. All readings unless otherwise stated were therefore performed in quadruplicate and untreated frogs were always killed at the same time and used as controls. These precautions are necessary as there is considerable variation

---

**FIG. 1.** Endolymphatic sac of a newly metamorphosed frog. Sac is only drawn on the right-hand side of the animal and the vertebral column have been omitted from that region. (Modified after Whiteside (18).)

**FIG. 2.** Standard curves used for determining values of base excess (mEq/liter). Normal value of frog blood with pH 7. and Pco₂ 18 mm Hg was used to plot the line labeled 0.
both between individual frogs and between batches of frogs. Fourteen different experiments were performed and blood was analyzed from 105 hypercapnic frogs. Blood samples were collected in heparinized capillary tubes from a cardiac puncture after the animals had been given a severe blow on the head. Three blood samples are necessary for the analyses used and these were all obtained within 1–2 min of the animal being removed from the respiratory chamber. If the animal had been subjected to a high Pco2 the blood samples were taken from the frog while it was retained in a plastic bag containing the gas mixture to which it had been acclimatized. The frogs were decapitated immediately after the blood samples were obtained.

The first sample removed from the frog was used to obtain a pH reading for the blood by using a Radiometer microelectrode system at 25 C. The other two blood samples were equilibrated with analyzed samples of 4 and 8% CO2–O2 gas mixtures using the Astrup microtonometer after which their pH was read. The relationship of log Pco2 and pH is linear so that it is possible to derive both the pH and the Pco2 content of the frogs blood from these readings using the methods of Siggaard-Andersen (12). It is then possible to calculate bicarbonate values from this data by means of the Henderson-Hasselbalch equation

\[ \text{pH} = pK_1 + \log \frac{\text{HCO}_3^-}{\text{aCO}_2} \]

using a value of 6.15 for \( pK_1 \) at pH 7.66 and 25 C (11) and 0.041 for \( \alpha \) at 25 C (10).

From this it is also possible to calculate carbonate values by using the log form of the law of mass action

\[ \text{Pco}_3 = \text{Pco}_2 \cdot \text{pH} + pK_2 \]

and taking pK2 as 10.22 which is the value for mammalian blood (5).

In order to demonstrate changes in blood bicarbonate which are independent of the blood carbon dioxide tension, values of “base excess” have been used in plotting the results. Base excess is defined as “the titratable base on titration to normal pH at normal Pco2 and normal temperature” (12) and it is a measure of nonrespiratory changes in the base content of the blood.

Base-excess calibration curves for frogs' blood were constructed by pooling the blood obtained from 30 frogs and titrating 0.2- or 0.4-ml samples with 0.2 M sodium bicarbonate or hydrochloric acid. These blood samples were then equilibrated with analyzed samples of 4 and 8% CO2–O2 gas mixtures after which their pH values were read. Curves were constructed by plotting the values of pH against titer for both sets of equilibrated blood (13). The average value of 44 samples of normal frogs' blood taken at various times of the year was pH 7.660 at Pco2 18 mm Hg and this was therefore taken as a standard value from which base-excess curves were derived as shown in Fig. 2.

**TABLE 1. Loss of calcium and phosphate ions from frogs (R. temporaria) under various treatments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Observations</th>
<th>Calcium</th>
<th>Phosphate</th>
<th>Ca/P04</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st day in deionized water</td>
<td>131</td>
<td>8.42±4.85*</td>
<td>13.00±8.69†</td>
<td>0.65</td>
</tr>
<tr>
<td>4th day in deionized water</td>
<td>118</td>
<td>2.20±2.21‡</td>
<td>8.87±6.50§</td>
<td>0.25</td>
</tr>
<tr>
<td>3 days before acidosis</td>
<td>81</td>
<td>1.77±2.01§</td>
<td>9.65±7.07§</td>
<td>0.18</td>
</tr>
<tr>
<td>Day before acidosis (control)</td>
<td>76</td>
<td>1.53±1.68</td>
<td>9.92±7.11</td>
<td>0.15</td>
</tr>
<tr>
<td>Increase in Ca and P04 over control values</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st day in 5% CO2 95% O2</td>
<td>34</td>
<td>4.89±3.30*</td>
<td>2.60±6.47‡</td>
<td>1.08</td>
</tr>
<tr>
<td>1st day in 6% CO2 94% O2</td>
<td>0</td>
<td>7.28±2.32*</td>
<td>0.46±6.36§</td>
<td>15.89</td>
</tr>
<tr>
<td>1st day in 10% CO2 90% O2</td>
<td>22</td>
<td>10.12±3.54*</td>
<td>6.75±6.99*</td>
<td>1.50</td>
</tr>
<tr>
<td>1st day in 20% CO2 80% O2</td>
<td>12</td>
<td>13.72±2.95*</td>
<td>8.45±6.40†</td>
<td>1.62</td>
</tr>
</tbody>
</table>

All values are given in pmoles/24 hr. Values are means ±sd. Significance of all values evaluated by the Student t test in comparison with the values obtained on the day before acidosis was induced. * \( P < 0.1% \). † \( P < 1% \). ‡ \( P < 5% \). § Not significant.
The quantity of calcium lost from a frog increases enormously when the animals are exposed to atmospheres containing carbon dioxide. The magnitude of the increase is related to the partial pressure of carbon dioxide and in all cases the increase over control values is highly significant. There is also an increase in the quantity of phosphate lost from the frogs but this is more variable and not always significantly different from control values except in the more severe experiments (Table 1).

The loss of calcium from the frog exposed to high carbon dioxide gas mixtures shows a characteristic pattern of responses with time. The initial rapid rise lasts for only a short time and then progressively falls off so that after about 5 days it is almost back to its original value (Fig. 4). The quantity of phosphate lost from the acidotic animal is more variable than the loss of calcium and the only trend is a tendency towards increasing amounts. In some experiments, e.g., in Fig. 4A there is no increase in phosphate loss.

When frogs are returned to air after 3 days in an elevated carbon dioxide environment, calcium excretion falls to control values or lower. If the frogs are then returned to the same CO₂-Ø₂ mixture as before, the increase in calcium excretion is much less than it was during the initial carbon dioxide exposure (Fig. 5).

The results of the analyses of the blood of treated frogs can be expressed as base excess values or as bicarbonate concentrations. The base excess values were determined graphically by measuring the displacement of the log Pco₂/pH lines and comparing them with the standardization lines shown in Fig. 2. Bicarbonate values were calculated from the Henderson-Hasselbalch equation. The two methods agree closely in their estimation of changes in blood base (Table 2) but the base excess values are simpler to follow since they only measure the nonrespiratory component and they are derived directly from the log Pco₂/pH plots. The results are therefore shown graphically in this form in which each point represents the mean of four animals analyzed simultaneously. Fourteen different experiments have been performed but only the crude data are given in this form in two examples (Figs. 6 and 7).

![Fig. 4. Loss of calcium and phosphate ions from frogs in air and when exposed to A: 5% CO₂-95% O₂ (4 frogs); and B: 10% CO₂-90% O₂ (4 frogs). Response to 5% CO₂-95% O₂ has been selected as an example which shows an increase in calcium loss with little effect upon phosphate excretion.](#)

![Fig. 5. Mean values for the loss of calcium from 4 frogs in 2 mmoles sodium chloride in air and during two treatments with 5% CO₂-95% O₂. Abscissa in days.](#)
TABLE 2. Comparison of the changes in blood bicarbonate levels of frogs in acidosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Base Excess, mEq/liter</th>
<th>Bicarbonate, mEq/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% CO₂ 95% O₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 2 hr</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>After 24 hr</td>
<td>-4</td>
<td>25</td>
</tr>
<tr>
<td>After 48 hr</td>
<td>+9</td>
<td>40</td>
</tr>
<tr>
<td>Total change</td>
<td>+9</td>
<td>+8</td>
</tr>
<tr>
<td>10% CO₂ 90% O₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 2 hr</td>
<td>-2</td>
<td>34</td>
</tr>
<tr>
<td>After 72 hr</td>
<td>+10</td>
<td>46</td>
</tr>
<tr>
<td>After 120 hr</td>
<td>+20</td>
<td>54</td>
</tr>
<tr>
<td>Total change</td>
<td>+22</td>
<td>+20</td>
</tr>
</tbody>
</table>

Base excess values derived graphically from Fig. 2 and bicarbonate calculated from the Henderson-Hasselbalch equation. Average data from 32 frogs.

Samples of blood taken from frogs within 2 hr of their being exposed to carbon dioxide showed that they rapidly equilibrated with the experimental gas mixtures. Thus the pH of the blood fell rapidly when the animals were put into respiratory acidosis. In all cases, however, there was a rapid compensation and the pH of the blood had started to rise within 24 hr (Fig. 6). The exact timing and extent of these compensatory responses depended upon the degree of acidosis imposed (Figs. 6 and 7) but in all cases they had the following characteristics: First, the compensation was fast and virtually complete within from 2 days (for 5% carbon dioxide) to 5 days (for 20% carbon dioxide). Secondly, the compensation stopped before the pH of the blood had returned to its original value. Thirdly, the slowing down and cessation of the changes in blood pH correlated with the decline in the quantity of calcium lost from the frog.

The plasma calcium level of six control frogs was 2.5 ± 0.3 mEq/liter. This fell during the first 2 days of acute acidosis but then returned to its original value (Table 3).

The hemoglobin content of the blood of 14 frogs averaged 8.8 ± 2.3 g/100 ml.

DISCUSSION

The excretion of calcium by *R. esculenta* in distilled water was studied by Krogh (4) who found that the quantity lost via the urine declined from 3.2 to 1.0 μmoles/kg per hr during a period of 10 days. There was a fairly constant loss of 2.8 μmoles/kg per hr from the skin. It is therefore apparent that the skin can be an important avenue for the loss of calcium ions from the frog and in the present experiments the cutaneous and renal losses of calcium have been measured together. The average calcium loss from 76 specimens of *R. temporaria* after they had come to a steady state in deionized water was about 1.53 μmoles per frog per 24 hr, or about 2 μmoles/kg per hr, which is a similar value to that found by Krogh.

These steady-state values constitute the control observations necessary to determine whether calcium carbonate deposits in the endolymphatic sacs contribute to buffering respiratory acidosis in the frog. If these deposits were so used then the amounts of both calcium and bicarbonate ions would increase in respiratory acidosis.

The evidence indicates that this is, in fact, the case for the average total calcium loss from 34 frogs increases three- to fourfold when the animals are put into respiratory acidosis with 5% carbon dioxide. The base excess of the blood increases by about 8–9 mEq/liter for the animals referred to in Table 2, raising the blood pH from about 7.25 to 7.40 in the first 48 hr of acidosis. The average increase in the calcium loss from the animals during the same period is 10.4 μmoles and the phosphate excretion increases by 2.9 μmoles. These results are therefore in keeping with the suggestion that in the frog increases in carbonic acid are, at least partially, buffered by dissolving calcareous deposits. There are, however, a number of other possible explanations. In this study, the in vivo responses to respiratory acidosis include not only buffering by blood but also other extracellular and intracellular buffering as well as renal compensations (7). In this regard it is likely that some bicarbonate ions arise from sources other than the calcium carbonate deposits, perhaps as described by equation 2 due to bone dissolution. With bone dissolution phosphate ions would also be liberated. An increased excretion of phosphate is, however, a typical response of many animals to acidosis and it is generally thought to be at least partly derived from the soft tissues (7). In the present experiments phosphate excretion increased during acidosis but the results are very variable and not always significant (e.g., Fig. 4, A and B) except in the more severe forms of acidosis. In these extreme cases, however, the increased excretion of
TABLE 3. Plasma calcium levels of control frogs and frogs exposed for various periods of time to 10% CO₂-90% O₂

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of observations</th>
<th>Ca, mEq/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls in air</td>
<td>6</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>24-hr acidosis</td>
<td>4</td>
<td>1.9±0.3*</td>
</tr>
<tr>
<td>48-hr acidosis</td>
<td>4</td>
<td>1.9±0.2*</td>
</tr>
<tr>
<td>72-hr acidosis</td>
<td>6</td>
<td>2.7±0.4†</td>
</tr>
</tbody>
</table>

Values are means ± sd. Significance of differences from controls evaluated by a Student t test. * P < 0.05. † Not significant.

![Graph](http://ajplegacy.physiology.org/)

Calcium and phosphate ions is highly significant and occurs in the ratio of 1.62:1 which is close to the ratio found in bone (Table 1). This may be purely coincidental but it obviously needs further investigation.

It will be apparent therefore that although there is circumstantial evidence to suggest that the calcium carbonate deposits of the frogs’ inner ear are dissolved during acidosis the suggestion relies a great deal upon the observed increase in the excretion of calcium. For a 30-g frog in 5% carbon dioxide this amounts to 10.4 μmoles in 48 hr. The phosphate excretion is 2.9 μmoles over the same period and if it is assumed that this has all been derived from bone it accounts for 4.8 μmoles of calcium. This leaves 5.6 μmoles calcium as being possibly of carbonate origin i.e., as a source of 11.2 μEq of bicarbonate. The observed increase in plasma bicarbonate is 8 mEq/liter. According to the analyses of Prosser and Weinstein (8) the plasma volume is about 7% and the remaining extracellular fluids about 20% of the body weight of a frog. This gives a plasma volume of 1-2 ml and a remaining extracellular volume of 5-6 ml. Thus the observed increase of 8 μEq/ml of base excess probably represents a net gain of about 50-60 μEq bicarbonate. On these assumptions, therefore, the calcareous deposits are responsible for about 20% of the total increase in bicarbonate. This estimate ignores the possibility of an increased renal loss of bicarbonate which was found by Yoshimura et al. (19) in their short-term study of respiratory acidosis in the frog, R. limnocharis. In most animals respiratory acidosis rapidly leads to a decrease in urinary bicarbonate and further work needs to be done before it can be accepted that the frog is exceptional in this respect.

These calculations indicate that the schemes described by equations 1 and 2 are in keeping with the experimentally observed results and that the mineral deposits of the frog could account for a significant fraction of the buffering of the animal exposed to carbonic acid. This leaves unexplained, however, both the normal decline in calcium loss with sustained acidosis (Fig. 4) and the incomplete compensation for blood pH which actually occurs (Fig. 6). There is no obvious reason from equation 1 why the bicarbonate level should not continue to rise until the pH of the blood is restored to normal. It might be better, therefore, if equation 1 were rewritten in the form:

\[
\text{CaCO}_3 \text{(solid)} \rightarrow \text{Ca}^{++} + \text{CO}_3^{-} + \text{H}^{+} + \text{CO}_2^{-}
\]

Equation 4 emphasizes that carbonate ions in solution react with added hydrogen ions to form bicarbonate until or unless calcium ion increases so much that the solubility product of calcium and carbonate ions is exceeded.

This interpretation can be applied to the data in Fig. 6 by calculating the bicarbonate and carbonate concentrations before and during respiratory acidosis by the methods already described. The results of these calculations

![Graph](http://ajplegacy.physiology.org/)

...
are shown in Fig. 8. It is apparent that when the frog is placed in 5% carbon dioxide there is an immediate large rise in the bicarbonate level of the blood and an equally dramatic fall in the carbonate level. The reason for the fall in blood carbonate is apparent from equation 3 for if the buffering ability of the blood is poor, the ratio $\Delta \text{HCO}_3^-/\Delta \text{pH}$ is less than unity and the carbonate concentration decreases correspondingly. The main buffer of the blood is hemoglobin and this was found to be present at the relatively low concentration of 8.8 g/100 ml in the blood of R. temporaria. Thus, because of the poor buffering of the blood a rise in carbon dioxide tension leads to a fall in the carbonate ion concentration (15), causing more calcium carbonate to dissociate. This process continues until the solubility product is again obtained.

The calcium ion concentration of the blood is normally regulated at a constant level by calcitonin and the parathyroid hormone. During acidosis there is a tendency for hydrogen ions to displace calcium ions from the plasma proteins so that for a constant calcium ion concentration there is a fall in total plasma calcium. These changes appear to occur in the plasma calcium levels of the acidotic frog (Table 3) and can be interpreted as indicating the maintenance of a constant calcium ion concentration. Certainly there is no evidence for a rise in calcium ion activity. Thus any dissolution of calcium carbonate will continue until the carbonate concentration returns to its original value and this will only occur when the bicarbonate and pH values have been raised according to the scheme in equation 3. It can be seen from Fig. 8 that this has virtually occurred by the end of day 2 and that the increase in blood bicarbonate from day 0 to day 2 corresponds with the amount necessary to cause the shift in base excess in Fig. 6. This would account therefore for the large excretion of calcium during this period and for the incomplete return of blood pH to its original value.

If this interpretation of the changes in the acidotic frog is correct it should be possible to forecast what pH the blood will attain if a frog is subjected to any given carbon dioxide tension. The forecast can be made by first calculating the carbonate ion concentration for normal frogs' blood. From this it is possible to calculate by using equation 3 what bicarbonate concentration must exist at any given pH to give the original carbonate value. The carbon dioxide tension which corresponds to these bicarbonate and pH values can then be calculated from the Henderson-Hasselbalch equation. An "isocarbonate line" can then be plotted which relates the values of carbon dioxide tension and pH which will give a constant carbonate concentration. For comparison another line is calculated which shows the relationship between carbon dioxide tension and pH when the base excess value remains constant, i.e., a "no compensation line." Both curves are shown in Fig. 9 in relation to the pH of the frogs blood at any given carbon dioxide tension when a) the values were obtained after 2 hr in the gas (open circles) or b) the values were obtained after more than 2-5 days in the gas (filled circles). It is apparent that at first the values correspond to the no compensation curve but that after 2-5 days the values obtained are those which would be obtained if the compensation of the frog was limited by an isocarbonate relationship.

This interpretation implies therefore that there are deposits of calcium carbonate in equilibrium with the blood. This suggestion is in keeping with the rich capillary network and large mineral deposits associated with the endolymphatic sacs of frogs. It would also account for the initial large excretion of calcium from the acidotic frog, the rapid rise in base excess of the blood, and the fact that this compensation is incomplete. This same hypothesis would also explain why the endolymphatic deposits are resorbed from the sacs during bone formation (6) and why they increase in size during hypervitaminosis D (9) since variations in the concentration of plasma calcium ions should produce similar effects on the size of the endolymphatic deposits as variations in plasma carbonate concentrations.

It is a pleasure to acknowledge the assistance of Miss H. A. Roberts in these experiments.

REFERENCES


2. GUARDABASSI, A. Les sals de Ca du sac endolymphatique et les processus de calcification des os pendant la metamorphose.
42: 143-167, 1953.


