In Vivo Environmental Temperature and the In Vitro Pattern of Luminal Acidification in Turtle Bladders

Evidence for HCO₃⁻ Ion Reabsorption

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ABSTRACT In this study, it is shown how to transfer tared aliquots of (HCO₃⁻ + CO₂)-containing luminal fluids directly into the mercury-sealed chamber of a modified Van Slyke apparatus and how to obtain direct as well as indirect manometric determinations of dissolved CO₂ ([CO₂]₀) in each aliquot of such fluids. It is next shown that the pattern of in vitro luminal acidification in an isolated turtle bladder sac depends upon the prior in vivo ambient temperature to which the donor turtle had become adapted. Under in vivo conditions, the food intake, physical activity, and acid excretion of 32°C-adapted turtles are greater than those of 21°C or 26°C-adapted turtles. Under in vitro conditions of incubating isolated bladder sacs (from 21, 26, and 32°C turtles) in (HCO₃⁻ + CO₂)-containing Ringer media at a single temperature (21°C), the patterns of luminal acidification are as follows: (a) The rate of depletion of luminal [HCO₃⁻] is greatest in bladders from the 32°C-adapted turtles. (b) Concomitant decreases in luminal [CO₂]₀, [HCO₃⁻], and pH (the 'CO₂-decreasing pattern' of luminal acidification) develop in all bladders from 32°C turtles, in half of those from 26°C turtles, but in less than one-fifth of those from 21°C-adapted turtles; and (c) a CO₂-increasing pattern of luminal acidification is found in most of the bladders from 21°C-adapted turtles. A postulated bicarbonate ion-reabsorbing pump is consistent with all of these patterns of luminal acidification.

INTRODUCTION

Background

In principle, a time-dependent decrease or increase in the luminal concentration of dissolved CO₂ ([CO₂]₀) should be the most critical parameter for determining whether bicarbonate reabsorption or proton secretion is the mechanism responsible...
for the observed acidification of (HCO₃ + CO₂)-containing luminal fluids in isolated turtle bladder sacs (Brodsky and Schilb, 1974). This claim would be undeniable, if one were able to estimate and correct for any concomitant diffusion of CO₂ from nonacidifying and acidifying cells into the enclosed luminal fluid or from the enclosed luminal fluid into these cells.

Experimentally however, it has been difficult to verify either one or both of these predictions, because the definitive findings from one laboratory differed from those of another, even though the in vitro bathing conditions imposed on the isolated bladders were similar. For example, Schilb and Brodsky (1966, 1972) and Schilb (1978) found a CO₂-decreasing pattern of luminal acidification, which could be attributed to the action of a bicarbonate-reabsorbing pump, even without the knowledge of how much CO₂ had diffused from the surrounding epithelial cells into the luminal fluid during the period of its acidification. But subsequently, Green et al. (1970) and Schwartz et al. (1974), using different methodology in similarly incubated turtle bladder sacs, found a CO₂-increasing pattern of luminal acidification, which these authors attributed to the action of a proton-secreting pump.

Present Objectives

The purpose of the present study was to determine whether the aforementioned discrepancy was due to an artifact or to some overlooked physiological factor that induces the generation of a CO₂-decreasing or a CO₂-increasing pattern of luminal acidification in the in vitro as well as in the in situ urinary bladder. To reach this objective, the present study was divided into two parts. In the first part, the development of a new methodological system enabled us to obtain doubly verified, calibrated determinations of [CO₂] in single samples of standard solutions, which had been preequilibrated with gas mixtures of known pCO₂. In the second part, experiments were designed to determine whether the prior in vivo temperature (to which turtles adapt) can be correlated with any characteristic of the in vitro luminal acidification process generated by the subsequently isolated bladders from such thermally adapted turtles.

We have elected to use the in vivo temperature to which turtles become adapted as a probable physiological determinant of certain characteristics of the in vitro luminal acidification generated by the isolated urinary bladders obtained from such turtles. This was because of the well-established neuroregulatory effects of temperature on oxygen consumption, acid base balance and other functions in several ectothermic animals including the turtle. These phenomena, extensively reviewed by White and Somero (1982), will be discussed later in relation to some of our present observations.

On the basis of results to be shown, it can be said that there were no artifacts in the previous estimates of luminal [CO₂] in our early experiments (Schilb and Brodsky, 1966, 1972) or in those of others (Green et al., 1970; Schwartz et al., 1974); and that the ambient temperature to which turtles adapt is a major determinant of the rate and consequent direction of change in luminal [CO₂] during the in vitro process of luminal acidification generated by the isolated urinary bladders obtained from such adapted turtles.
METHODS

Analytical Procedures

CO₂ Diffusion from an Aqueous into a CO₂-free Oily Phase

Rationale. In previous reports from this laboratory (Schilb and Brodsky, 1966, 1972), the concentration of dissolved CO₂ ([CO₂]₇) was estimated in (CO₂ + HCO₃⁻)-containing luminal fluids, which had been removed from the interior of closed turtle bladder sacs under a layer of CO₂-free oil and transferred into the chamber of a Van Slyke (VS) apparatus. Since this procedure of fluid collection and transfer required 1-2 min, dissolved CO₂ could have diffused out of the collected fluid into the overlying layer of oil and, consequently, the determined values of [CO₂]₇ in the bladder fluids might have been spuriously low.

It was therefore decided to determine empirically how much CO₂ is lost (per unit of time) from unstirred and stirred, CO₂-containing solutions kept under stagnant layers of paraffin oil, initially devoid of CO₂. The measured quantity of CO₂ lost from such fluids would provide the required direct evidence for discarding or retaining our previously reported data on the decreasing levels of [CO₂]₇ in acidified luminal fluids from closed turtle bladder sacs (Schilb and Brodsky, 1966, 1972).

Procedures. Two gravimetrically prepared solutions were used in these tests: (a) 5.0 mM NaHCO₃ plus 95 mM NaCl, and (b) 0.03 mM NaHCO₃ plus 100 mM NaCl. A glass pH electrode was kept immersed in 3 ml of either one of these solutions within a 9.0-ml test tube (inner diameter, 2.7 cm) that contained a magnetic stirring rod. The pH of that solution was continuously monitored before, during, and after its equilibration with a water-saturated gas mixture (5% CO₂ + 95% O₂). Equilibration was operationally defined as having been reached when the pH reached a constant level. At that time, gas flow into the solution was stopped, the solution was overlaid with an equal volume (3.0 ml) of CO₂-free paraffin oil, magnetic stirring of the underlying solution (not of the oil layer) was initiated, and the pH was continuously monitored for the ensuing 30-60 min.

Regardless of the results from these experiments, it was decided to develop entirely new and independent methods for the collection and analyses of (CO₂ + HCO₃⁻)-containing fluids from closed turtle bladder sacs or from any other reservoir, as is outlined in what follows.

Rationale for a New and Independent Approach

In order that no dissolved gas could possibly diffuse out of luminal fluids removed from closed turtle bladder sacs and that the analytical determination of [CO₂]₇ in such fluids could be verified, the following requirements were set. (a) The luminal fluid in such a sac (connected directly to the chamber of a VS apparatus) was to be collected and transferred to that chamber under a column of mercury. (b) The size of any aliquot delivered into the VS chamber was to be determined without pipetting or other handling; and (c) the [CO₂]₇ of that fluid should be determined by a direct analytical technique. (d) Finally, the directly determined value of [CO₂]₇ should match the indirectly determined value obtained by the classical method of Van Slyke. To satisfy these requirements, it was first necessary to modify the VS apparatus.

Modification of the VS Apparatus

A VS apparatus was modified by (Fig. 1): elongating the side arm port of stopcock S-1 to provide a direct connection between the manometric chamber and the mucosal interior of a bladder sac, which is secured to the terminal opening of the side arm; and by elongating the vertical exit port of stopcock S-2 into the form of a spigot. With these modifications, solu-
tions were transferred from an external container or bladder lumen (via the side arm) under mercury directly into the chamber (or in the reverse direction) without the loss of dissolved gases and without pipetting. The volume of any sample (0.5 to 1.0 ml) delivered into the chamber (via stopcock S-1) displaced an equal volume of mercury out of the closed system (via stopcock S-2) into a tared flask. The weight and specific gravity of the displaced mercury provided a precise measure of the aliquot volume within the chamber. Then degassed water was added so that the volume of aliquot plus water in the VS chamber was always 6.0 ml. These and other transfers of solution (above or below mercury) were achieved by appropriate manipulations of the stopcocks and leveling bulb, as described by Peters and Van Slyke (1932).

**GENERAL SET-UP SHOWING POSITIONS OF LEVELING BULB FOR:**

a. INCUBATION OF BLADDER and

b. DELIVERY AND ANALYSIS OF MUCOSAL FLUID

**FIGURE 1.** The modified Van Slyke manometric apparatus. See text for description and use of the added components.

**Directly Determined Values of \([CO_2]_f\)**

In \(HCO_3\) solutions devoid of non-\(HCO_3\) buffers. A direct manometric determination of \([CO_2]_f\) in any \(CO_2\)-containing solution (with no non-\(HCO_3\) buffers) can be obtained by adding a single step to those of the conventional VS method for determining total \(CO_2\) content. Before acidifying the \((HCO_3 + CO_2)\)-containing solution in the VS chamber, the dissolved \(CO_2\) (and \(O_2\)) in this solution are first evacuated into a fixed volume of supernatant gas phase. The pressure \((P_f)\) generated by these evacuated gases is proportional to the concentrations of
dissolved CO\textsubscript{2} and O\textsubscript{2} in the preevacuated solution; i.e.,

\[ P_1 = a[CO_2]_f + b[O_2]_f + pH_2O, \]

where the coefficients, \( a \) and \( b \) are derived from the Henry's law constant for the equilibrium distribution of each gas between the known volumes of solution and gas phase, and from Avagadro's law on the pressure exerted per gram mole of any gas which occupies a known volume at a given temperature, while \( pH_2O \) denotes the partial pressure of water vapor within the VS chamber.

Once \( P_1 \) is obtained, the supernatant gases are redissolved and the solution is acidified so that all of the HCO\textsubscript{3} is converted to CO\textsubscript{2}. The dissolved CO\textsubscript{2} and O\textsubscript{2} in the acidified sample are evacuated into the same supernatant gas volume, and the pressure (\( P_2 \)) generated therein is now proportional to the concentrations of dissolved CO\textsubscript{2}, HCO\textsubscript{3}, and O\textsubscript{2} in the preevacuated sample, or

\[ P_2 = a[CO_2] + a[CO_2]_{bic} + b[O_2] + pH_2O, \]

where the subscript 'bic' denotes the CO\textsubscript{2} that is derived from all of the bicarbonate ions in the original, preanalyzed solution.

Finally, strong alkali 5.0 M KOH is anaerobically delivered into the VS chamber. This produces a sink for CO\textsubscript{2} in the aqueous phase (i.e., \( CO_2 + OH^- \rightarrow HCO_3^- \)), which in turn reduces the pCO\textsubscript{2} of the gas phase. Consequently, the total gas pressure in the chamber decreases to \( P_3 \), which is proportional to the partial pressures of O\textsubscript{2} and water vapor above the solution, or

\[ P_3 = b[O_2] + pH_2O. \]

Therefore, the directly measured pressure difference, \( P_1 - P_3 \) (or \( P_{13} \)) is equal to the partial pressure of CO\textsubscript{2} in the gas phase (pCO\textsubscript{2}(g)) and proportional to [CO\textsubscript{2}]\textsubscript{f} in the preevacuated solution, or

\[ \Delta P_{13} = pCO_2(g) = a[CO_2]_f. \]

**Limitation of direct method.** Using the direct method in the presence of a nonbicarbonate buffer in a (CO\textsubscript{2} + HCO\textsubscript{3})-containing solution, we obtained a spuriously high value for [CO\textsubscript{2}]\textsubscript{f} because of the following events. (a) During the step of evacuating dissolved CO\textsubscript{2} from the unacidified solution into the supernatant gas phase in the VS chamber, the pH of the underlying solution must increase. (b) This in turn induces an increase in the degree of dissociation of the protonated member of the non-HCO\textsubscript{3} buffer pair (e.g., the phosphate buffer) with the consequent production of additional protons. (c) The newly formed protons then titrate the HCO\textsubscript{3} ions to produce an additional quantity of dissolved CO\textsubscript{2} over and above that originally present in the solution. In formal terms:

\[ \Delta P_{13} = pCO_2(g) = a[CO_2]_f + a[CO_2]_{bic}, \]

where [CO\textsubscript{2}]\textsubscript{bic} is the additional CO\textsubscript{2} formed by the titration of HCO\textsubscript{3} with protons dissociated from the non-HCO\textsubscript{3} buffers. Consequently,

\[ pCO_2(g) > a[CO_2]_f, \]

which means that the direct method is valid, only if the quantity of non-HCO\textsubscript{3} buffer is vanishingly low relative to that of HCO\textsubscript{3} in any (HCO\textsubscript{3} + CO\textsubscript{2})-containing solution.
Indirectly Determined $[\text{CO}_2]$ with a Directly Estimated $pK'_a$

The level of $[\text{CO}_2]$ in phosphate-containing bicarbonate solutions was estimated from: (a) the manometrically measured, total $\text{CO}_2$ content ($[\text{CO}_2]_t$) and (b) the electrometrically measured pH in one aliquot; together with (c) an operationally determined $pK'_a$ (for the $\text{HCO}_3:\text{CO}_2$ system) in a second aliquot of the same solution.

The first aliquot. Having been transferred from the external container to the chamber, the solution sample was raised into the oil-filled cup unit where its pH was determined within 1 min, after which an aliquot was quantitatively transferred into the chamber. Once in the chamber, the solution was evacuated and the first gas pressure ($P_1$) was measured. Next the solution was acidified (1.0 ml, 0.6 N $\text{H}_2\text{SO}_4$) so that all of the $\text{HCO}_3^-$ was converted to $\text{CO}_2$, and reevacuated to obtain the second gas pressure ($P_2$). Finally, the acidified solution was alkalized (1.0 ml, 4.0 N $\text{NaOH}$) so that all of the $\text{CO}_2$ was converted to $\text{HCO}_3^-$, and the resulting gas pressure ($P_3$) was measured.

The measured difference, $P_2 - P_3$ ($\Delta P_{23}$, derived from Eqs. 2 and 3, is directly proportional to the total $\text{CO}_2$ content ($[\text{CO}_2]_t$) in the original solution, i.e.,

$$\Delta P_{23} = a[\text{CO}_2]_t + a[\text{CO}_3]_{bic} = a[\text{CO}_2]_t,$$

which is the manometrically determined parameter of the classical VS method, where $[\text{CO}_3]_{bic} = [\text{HCO}_3^-]$ in the original solution (Peters and Van Slyke, 1932).

Using the values of pH and $[\text{CO}_2]_t$, one can estimate $[\text{CO}_2]_f$ from the equation,

$$[\text{CO}_2]_f = \frac{[\text{CO}_2]_t}{1 + \text{antilog} (\text{pH} - pK'_a)^{-1}},$$

provided that the $pK'_a$ of the $\text{HCO}_3^-:\text{CO}_2$ system in that particular solution is known from an independent determination. Such a $pK'_a$ determination was next carried out on the second aliquot of the original solution.

The second aliquot. It was necessary that the $[\text{CO}_2]_t$ in this second aliquot be set at a known level to determine the $pK'_a$. Accordingly, the second aliquot of the aforementioned luminal fluid was transferred into the cup unit of the VS apparatus (Fig. 1), where it was bubbled and equilibrated with a gas of known $p\text{CO}_2$. Once equilibrated, the pH of this second aliquot ($pH_2$) was determined. The solution was then quantitatively delivered into the VS chamber, where another value of dissolved $\text{CO}_2$ concentration ($[\text{CO}_2]_{bic}$) and another value for the total $\text{CO}_2$ content ($[\text{CO}_2]_t$) were determined.

From these determined values, the $pK'_a$ of the $\text{HCO}_3^-:\text{CO}_2$ system in the original solution is defined as,

$$pK'_a = pH_2 - \log \left( \frac{[\text{CO}_2]_{bic} - [\text{CO}_3]_{bic}}{[\text{CO}_2]_{bic}} \right),$$

Characterization of the $pK'_a$. To obtain an independent check on the validity of this $pK'_a$ determination, the procedures described for the second aliquot were carried out on a standard, $\text{CO}_2$-equilibrated, $\text{HCO}_3^-$-containing solution at five different temperatures between 20 and 40°C. Like any equilibrium constant, the determined $pK'_a$ was found to be a linear inverse function of temperature (Fig. 2), and to be greater (by 0.07 pH units) than the thermodynamically defined $pK'_a$ of Harned and Bonner (1945) over the entire temperature range. The distinction between a thermodynamically defined dissociation constant ($pK'_a$) and an operationally defined dissociation constant ($pK'_a$), along with the advantage of the latter, has been discussed rigorously by Bates (1954). In essence, $pK'_a$ is derived from the molality (not from the activity) of hydrogen ions in solution, and this molality is calculated from electrical poten-
Acidification-induced Decrease in pCO₂

On the other hand, pK' is derived from the activity of hydrogen ions in solution, as measured with the conventional glass electrode. It would be fundamentally incorrect to substitute a value of pK' into Eq. 8, if the pH had been measured with a glass electrode. Consequently, a value for pK' operationally defined for the presently used solution, is the only correct parameter to use (in Eq. 8) for obtaining a valid estimate of [CO₂] in that solution.

Estimation of non-HCO₃ buffer concentration. The value for ([CO₂] + [CO₂]_{luc}), determined from the direct method, minus that for [CO₂]_{luc} determined from the indirect method, is equal to [CO₂]_{luc}, which in turn is a measure of the non-HCO₃ buffer content in the original solution. When [CO₂]_{luc} = 0, there are no non-HCO₃ buffers in the solution; and when [CO₂]_{luc} > 0, its magnitude depends upon the total concentration and the pK of the non-HCO₃ buffer relative to the corresponding parameters of the HCO₃:CO₂ buffer in the original solution.

Therefore, [CO₂]_{luc} was used to determine whether or not significant quantities of a non-HCO₃ buffer (phosphate or other) could diffuse into a fluid (initially devoid of non-HCO₃ buffer) during a 4–6-h exposure of such a fluid to the luminal surface of an acidifying turtle bladder.

Solutions

Solution A (devoid of non-HCO₃ buffer). This solution, consisting of 5 mM NaHCO₃ and 95 mM NaCl, was equilibrated with water-saturated gas mixtures of 6–7% CO₂ in O₂ at 25°C.

Solution B (containing a non-HCO₃ buffer). This solution consisted of 75 mM NaCl, 20 mM NaHCO₃, 4 mM KCl, 0.8 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 2.0 mM CaCl₂, and 10 mM glucose; and was equilibrated with the same gas mixture, after which the average concentration of dissolved CO₂ was 2.5 mM and the pH was 7.12.

Solution C. This solution contained sulfuric acid, 0.05 M and when it was equilibrated with the same water-saturated gas mixture, the concentration of dissolved CO₂ was used as an analytical standard for the precision of the measured concentrations of dissolved CO₂ in solutions A or B, which had been equilibrated with the same gas mixture. It was found that when different gas mixtures were used, the concentration of dissolved CO₂ differed in accordance with physical laws.
Physiological Procedures

General Procedures

In this part of the present study, each freshly excised bladder sac (from a *Pseudemys scripta* turtle) was ligated around the terminal end of the mercury-filled glass capillary tube, which was connected to the chamber of a modified VS manometric apparatus (Fig. 1). This allowed for transfers of luminal fluids under mercury from the bladder to the manometric chamber and vice versa without the loss of dissolved CO$_2$. The ligated bladder was then surrounded by 1 liter of a HCO$_3$-containing Ringer solution (solution B), in which the concentration of dissolved CO$_2$ was kept constant during the preincubation and incubation periods by continuous bubbling with a gas mixture of known pCO$_2$.

The preincubation period was initiated by transferring 12 ml of a luminal solution (containing 5 mM HCO$_3$ and equilibrated with the same gas as that used for the serosal solution) from the chamber of the manometric apparatus to the lumen of the bladder and back again into the manometric chamber from which it was discarded. After repeating this rinsing cycle three times, another aliquot (12 ml) of the same luminal solution was introduced into the bladder, after which 6 ml were removed, while 6 ml remained in the lumen, and the lower end of the mercury column in the capillary tube was flush with the neck of the bladder, thus eliminating any dead space of luminal fluid (See Fig. 1). Under these conditions, the closed bladder sac, filled with luminal fluid, was allowed to preincubate for 1 h in the CO$_2$-equilibrated serosal fluid. At this time, the period of preincubation was terminated by the removal of the luminal solution.

The incubation period was initiated by introducing 12 ml of fresh luminal solution into the preincubated bladder sac. After a few seconds, ~6 ml of this solution were removed from the bladder and discarded. Then three consecutive 0.7-ml aliquots of the luminal fluid were anaerobically transferred to the manometric chamber and analyzed separately for pH, [CO$_2$], and total CO$_2$ content ([CO$_2$]) as described above. These determinations (the M$_1$ sample) were used to define the initial preacidified state of the luminal fluid within the bladder sac.

The filled bladder sac was then allowed to incubate for 4–6 h, during which time the luminal acidification process progressed. This period was terminated after transferring three consecutive 0.7-ml aliquots of the acidified luminal fluid to the manometric chamber for analyses of pH, [CO$_2$], and [CO$_2$]; these determinations (the M$_2$ sample) were used to define the final acidified state of the luminal fluid within the bladder sac.

The pH, [CO$_2$], and [CO$_2$] of the serosal fluid were determined in 3 aliquots obtained from that fluid at the beginning and again at the end of the incubation period.

Temperature Control Procedures

In vitro. All of the bladder sacs used in this set of experiments had been removed from turtles adapted to a single in vivo temperature, 21°C. After their removal, each sac and the surrounding serosal bath were maintained at a selected in vitro temperature (21, 26, or 32°C) by thermostating of the entire laboratory room and all of its contents (solutions, manometric apparatus, etc.). The objective of these experiments was to evaluate any changes in the rate of luminal HCO$_3$ depletion and in the CO$_2$ changing pattern of the in vitro acidification process that might be induced by imposed variations in the in vitro temperature.

In vivo. Groups of donor turtles were maintained for 2–4 wk in a metal tank fitted with a readily removable plastic cover. The tank water, renewed twice daily, was thermostatted at 21, 26, or 32°C, depending on which experimental group was being thermally adapted. The turtles remained submerged, except for brief periods when they breathed by raising their heads over the water's surface.

The time required for the in vivo thermal adaptation of the turtle was determined as fol-
Acidification-induced Decrease in pCO₂

After being kept for two or more weeks at a temperature of 21°C, the period of thermal adaptation of the turtle was started by imposing a step increase in temperature of the aqueous environment from 21–32°C. On each consecutive day after this change, the pH levels in bladder urine, cardiac blood, and coelomic fluid were determined in one of the turtles in the 32°C environment before the removal of its bladder for the in vitro incubation at 21°C. The period of adaptation was operationally defined as the number of days required for steady state levels of pH in the aforementioned fluids to be reached.

Diet

Raw fish, in amounts of 200–250 g per turtle, were offered to and ingested by the turtles at weekly intervals. This interval was adopted to provide the time required for digestion and absorption of the food; the onset and dissipation of the postprandial period, during which alkali is secreted into the urine; and for the onset and maintenance of the postabsorptive period, during which acid end products of the metabolized protein are excreted into the urine. The maximal time required for passing through the first two of these in vivo metabolic states was defined by the time required for a bolus of fish bones to arrive at the ileocecal valve. Bones were found in the neighborhood of the ileocecal valve within 3 d of a single fish feeding.

Bathing Fluids

Luminal solution. The initial composition of the luminal incubation fluid (in millimolar) was 95 NaCl and 5 NaHCO₃; and the final pH was 6.44–6.69, depending on the pCO₂ of the CO₂:O₂ gas mixture used in any given experiment.

Serosal solution. The composition of the serosal fluid (in millimolar) was: 75.5 NaCl, 4.0 KCl, 0.8 NaH₄PO₄, 0.5 NaH₂PO₄, 2.0 CaCl₂, 10.0 glucose, and the final pH was 6.99–7.22, again depending on the pCO₂ of the gas mixture.

Analytical Techniques

Both the direct and indirect methods, described previously in the Methods section, were used to determine the [CO₂]ᵢ in every luminal fluid sampled. In principle, the value of the directly determined [CO₂]ᵢ should be equal to that of the indirectly determined [CO₂]ᵢ whenever there is no nonbicarbonate buffer in the fluid being analyzed; this was the initial state of the luminal fluids (See Table I for verification). But, also in principle, the directly determined value of [CO₂]ᵢ would, of necessity, be spuriously high in the presence of a nonbicarbonate buffer; this was indeed the case for the serosal fluids, in which the concentration of inorganic phosphate is 1.3 mM (See Tables II and III for verification). Because of the presence of inorganic phosphate, the level of [CO₂]ᵢ in the serosal fluid was estimated from the determined values of pH and [CO₂]ᵢ, using an independently determined pKᵢ value on a separate aliquot of the same sample, as described previously as the indirect method.

RESULTS

Validity of Old and New Methods for Determining the Concentration of Dissolved CO₂ in Solutions

Were Previously Reported Levels of Luminal [CO₂] Spuriously Low?

Strategy. In the first part of this study, experiments were designed to challenge the technical validity of previously determined decreases in the [CO₂]ᵢ levels of luminal fluids, which had been acidified during an incubation within isolated turtle blad-
der sacs. After the acidified luminal fluids had been removed from these sacs, they were kept under CO₂-free oil for 1–2 min before carrying out the definitive determinations of CO₂ content and pH (Schilb and Brodsky, 1966, 1972).

Therefore, it is necessary to determine how much of these decreases could have been due to diffusional losses of CO₂ from the collected luminal fluids into the contiguous CO₂-free oil layers, and how much could have been due to the effects of a postulated bicarbonate-reabsorbing mechanism on the precollected luminal fluids during their acidification within the bladder sacs. Recognizing that the diffusional component of these CO₂ losses can be evaluated most precisely in the absence of the physiological component, we adopted the following protocol. Iso-capnically equilibrated solutions (with bicarbonate concentrations similar to those before [5.0 mM] and after [0.03 mM] acidification of previously studied luminal fluids) were initially overlaid with equal volumes of CO₂-free oil. The resulting two-phase system was then incubated for periods of 1–2 h, during which time the levels of pH and, concomitantly, those of [CO₂]ᵢ in the aqueous were continuously monitored.

**Raw Data: Time-dependent Changes in pH and [CO₂]ᵢ**

*In the unstirred system.* When the oil-covered bicarbonate solutions (5.0 or 0.03 mM) were allowed to remain stagnant, the levels of pH and, consequently, those of [CO₂]ᵢ in the aqueous phase remained constant, not only for the first 1–2 min, but for the subsequent 4 h.

This finding alone could well be sufficient to show that no detectable quantity of dissolved CO₂ had been lost from the luminal fluids that had been kept under oil for 1–2 min during our earlier studies on the luminal acidification processes in isolated turtle bladder sacs (Schilb and Brodsky, 1966, 1972). However, there might have been some overlooked perturbation of the interface formation when the acidified luminal fluids contacted the supernatant oil layer in those studies. It was therefore decided to determine the effect(s) of continuously stirring the aqueous phase while the oil phase was allowed to remain stagnant.

*In the stirred system.* During the first 5–10 min of incubation under the condition of aqueous phase stirring the levels of pH and [CO₂]ᵢ in the aqueous solution remained essentially constant, which indicates a near-zero rate of CO₂ diffusion from the aqueous to the oil phase. However, after the ensuing 60 min of this incubation, the pH in each of two oil-covered aqueous solutions (0.03 and 5.0 mM bicarbonate) had increased by 0.06 pH units: from 4.47 to 4.53 pH units in the 0.03-mM solution; and from 6.70 to 5.76 pH units in the 5.0 mM solution. Concomitantly, the level of [CO₂]ᵢ, which was initially 1.67 mM in each of these isocapnically-equilibrated solutions, had decreased by 0.20 mM in the 0.03-mM solution, and by essentially the same amount (0.23 mM) in the 5.0-mM bicarbonate solution.

In summary, the unchanging levels of pH and [CO₂]ᵢ during the initial 1–5 min of incubating these (HCO₃⁻ + CO₂)ₐ-containing solutions (with or without stirring) provides a qualitative validation of the previously reported CO₂-decreasing pattern of luminal acidification in the turtle bladder (Schilb and Brodsky, 1966, 1972). A semi-quantitative validation of these older data can also be obtained by calculating the minute rate of CO₂ diffusion from the measured amount of dissolved CO₂ that had
disappeared from each of the aqueous, CO$_2$-containing bicarbonate solutions after a 1-h period of incubation under oil. These values are presented in what follows.

**Derived Data: Parameters of CO$_2$ Diffusion from Aqueous-to-Oil Phase**

Because of physical laws that govern the rate of solute diffusion in a closed, two-solvent phase system, the initial 1–2-min rates and quantities of CO$_2$ diffusion out of any aqueous phase (standard solutions or acidified luminal fluids) must be finite, and in the presently analyzed system, small enough to produce no more than an insignificant error in the determined values of pH and [CO$_2$]$_f$ in the aqueous phase. These finite quantities are as follows.

(a) The quantity of dissolved CO$_2$ lost by diffusion out of the oil-covered (HCO$_3$ + CO$_2$)-containing standards during the first 1–2 min of the incubation period (11–22 nmol of each 3-ml aliquot) amounted to no more than 0.2–0.4% of the total initial quantity of dissolved CO$_2$ in that 3-ml aliquot. This creates a real, but nonetheless quantitatively insignificant error in the determined value of [CO$_2$]$_f$.

(b) It is also worth noting that this diffusional loss of 11–22 nmol is no more than 0.26–0.52% of that lost if the aqueous-to-oil diffusion had been allowed to reach an equilibrium state, in which the concentration of CO$_2$ in the oil phase can be as much as fourfold higher than that remaining in the aqueous phase (Washburn, 1928; Lange, 1939).

This oil/water distribution of CO$_2$, a time-independent equilibrium parameter, depends on the solubility of CO$_2$ in oil relative to that of CO$_2$ in water and is in no way related to the time-dependent (off-equilibrium) diffusion process. It is therefore fundamentally erroneous to claim (Al-Awqati, 1978) that a high oil/water distribution coefficient is the cause of the previously determined CO$_2$-decreasing pattern of luminal acidification reported by Schilb and Brodsky (1966, 1972). Such a claim would require that a diffusion equilibrium state develops within 1–2 min of the time that these acidified fluids had been kept under CO$_2$-free oil, a requirement clearly excluded by the present observations.

(c) It follows from these evaluations, that the oil/water interface must be the major barrier to diffusion of CO$_2$ from the presently analyzed standard solutions (or from the previously analyzed luminal fluids) into a contiguous layer of oil. In this connection, the apparent CO$_2$ diffusivity of the oil/water interface in the present experiments can be estimated from the following parameters.

Expressed in units conventionally used in physical systems, the rate of aqueous-to-oil diffusion of dissolved CO$_2$ ($J$) normal to an interfacial area ($A$) of 2.3 cm$^2$, amounted to $1.83 \times 10^{-10}$ mol/s per cm$^2$. This diffusion had occurred along an assumed interfacial path length of $3 \times 10^{-5}$ cm and down a concentration difference ($C$) of $1.67 \times 10^{-6}$ mol/cm$^3$. Substituting these values into Fick’s first equation ($J = -DA\Delta C/\Delta X$) yields a value for CO$_2$ diffusivity ($D$) of $1.43 \times 10^{-7}$ cm$^2$/s, which is <1/100th of the value for CO$_2$ ($2 \times 10^{-5}$ cm$^2$/s) in aqueous solutions.

In summary, the raw and derived data from this set of experiments are sufficient to rule out pitfalls that might have been associated with the placement of luminal fluids under CO$_2$-free oil for 1–2 min in the earlier studies (Schilb and Brodsky, 1966, 1972). Nevertheless, we developed new and independent methods for obtaining a more precise evaluation of [CO$_2$]$_f$ than that obtained with the oil collection
method. What follows is the experimental verification of the new methodological system in standard solutions of known composition.

Levels of $[\text{CO}_2]_f$ in (HCO$_3$ + CO$_2$)-containing Solutions

Estimated by the direct method in the absence of non-HCO$_3$ buffers. To establish the validity of the direct method for determining $[\text{CO}_2]_f$ in any solution devoid of non-HCO$_3$ buffers, a HCO$_3$-containing solution (A) and a strong (0.05 M) H$_2$SO$_4$ solution (B), were each equilibrated with the same water-saturated gas mixture (nominally, 7% CO$_2$, 93% O$_2$). Once a known volume of each solution was enclosed within the mercury-sealed chamber of the VS apparatus and evacuated (without any addition of acid), the partial pressure of CO$_2$ evolved into a known volume of the gas phase [$p$CO$_2$(g)] and was measured at first before and then after absorption of that CO$_2$ by overalkalinizing the solution phase (see Methods). If the $p$CO$_2$(g) generated from unit volume of the HCO$_3$ solution (A) were equal to that generated per unit volume of the H$_2$SO$_4$ solution (B), then and only then could one conclude that the direct method provides a valid measure of $[\text{CO}_2]_f$ in any CO$_2$-containing solution devoid of non-HCO$_3$ buffers.

| TABLE I |
| --- |
| Calibration of the Direct Method for Determination of $[\text{CO}_2]_f$ |

| Coequilibrated solutions | pH $\pm$ SEM | $[\text{HCO}_3]$ $\pm$ SEM | $[\text{CO}_2]_f$ $\pm$ SEM |
| --- | --- | --- | --- |
| A) NaHCO$_3$ + NaCl | 6.43 $\pm$ 0.56 | 4.51 $\pm$ 0.34 | 2.74 $\pm$ 0.057 |
| B) H$_2$SO$_4$ <1.0 | $\sim$0.0 | 2.75 $\pm$ 0.060 |

Values shown are means ($\pm$ SEM) for the pH, $[\text{HCO}_3]$, and $[\text{CO}_2]_f$ in a CO$_2$-equilibrated, (NaHCO$_3$ + NaCl)-containing solution (A) and in a CO$_2$-equilibrated H$_2$SO$_4$ solution (B) of equivalent ionic concentration. The mean ($\pm$ SEM) of the individual paired differences (n = 19) between $[\text{CO}_2]_f$ in solutions A and B was 0.0068 $\pm$ 0.015 mM ($P > 0.6$). Note that in going from one to another sample, $[\text{CO}_2]_f$ varied from 2.51 to 3.27 mM because four different CO$_2$:O$_2$ gas tanks were used and because the prevailing temperature varied from 21 to 27°C during different gas equilibration periods.

This requirement was confirmed experimentally in 19 paired samples of solutions A and B after each had been equilibrated with the same gas mixture (Table I). The value of $[\text{CO}_2]_f$ in each sample of solution A (the HCO$_3$ solution) was indistinguishable from that of $[\text{CO}_2]_f$ in the paired sample of solution B (the H$_2$SO$_4$ solution), which had been preequilibrated with the same gas mixture. Each individual pair of solutions had been preequilibrated with the same gas mixture, but four different gas tanks had been used in the 19 paired determinations. Nevertheless, averaged over the 19 pairs, the mean value of the individual paired differences was not significantly different from zero.

In summary, the direct manometric method is a precise as well as a valid way to measure the level of $[\text{CO}_2]_f$ in HCO$_3$-containing solutions devoid of non-HCO$_3$ buffers, or in H$_2$SO$_4$ solutions. Moreover, the measured levels of $[\text{CO}_2]_f$ present in Table I are in keeping with Henry's law for the solubility of gases in aqueous solutions of equivalent electrolyte concentration.
Estimated by the direct method in the presence of a non-HCO₃ buffer. When estimated by the direct method in five CO₂-equilibrated phosphate-containing bicarbonate solutions, the mean value for [CO₂]ₙ was significantly greater than that determined in five paired H₂SO₄ solutions that had been equilibrated with the same CO₂ gas mixture (Table II). The mean value of this excess, 0.2 mM, can be attributed to the quantity of protons released from a known quantity of H₂PO₄ when dissolved CO₂ is evacuated from the phosphate-containing (HCO₃ + CO₂)-containing solution into the supernatant gas phase within the mercury-sealed chamber of the VS apparatus (See Methods). Although calculable when the concentration and pKₐ of the nonbicarbonate buffer is known, it became necessary to use an extension of the classical indirect method for determining [CO₂]ₙ when these parameters are or might be unknown.

| Experiment No. | pH | [HCO₃] | [CO₂]ₙ | Δ[CO₂]ₙ | Estimated concentration non-HCO₃ buffer |
|---------------|----|--------|--------|---------|----------------------------------------|
|               |    | mM     | mM     | mM      | (d-h) (d-a)                            |
| 1             | 7.35 | 26.6 | 2.05 | 1.91 | 1.97 | 0.14 | 0.08 |
| 2             | 7.34 | 24.5 | 2.09 | 1.81 | 1.86 | 0.28 | 0.23 |
| 3             | 7.13 | 26.5 | 3.47 | 3.18 | 3.26 | 0.29 | 0.21 |
| 4             | 7.12 | 25.4 | 3.52 | 3.24 | 3.20 | 0.28 | 0.32 |
| 5             | 7.02 | 20.6 | 3.32 | 3.27 | 3.27 | 0.05 | 0.05 |

Effect of the presence of a specific concentration (1.3 mM) of a known non-HCO₃ buffer (P) on the value of [CO₂]ₙ, when determined by the direct method (d) as compared with that (in the same solution) by the indirect method (h). In addition to the pH and [HCO₃], values presented are those for [CO₂]ₙ in each serosal solution and in a paired sulfuric acid solution (a), which had been equilibrated with the same CO₂-containing gas mixture. Values under the heading Δ[CO₂]ₙ are those for the paired differences (d-h) or (d-a) (means ± SEM, 0.21 ± 0.04 and 0.18 ± 0.04; P < 0.01 and P < 0.02, respectively), which provide a measure of the non-HCO₃ buffer concentration. The mean ± SEM of the paired difference (h-a) was −0.03 ± 0.02 (P > 0.2), which demonstrates the validity of the indirectly determined value (h) of [CO₂]ₙ in non-HCO₃ buffer-containing solutions. It can be seen under the column, H₂SO₄, that two different gas mixtures were used in these experiments.

Estimated by the indirect method. As described in the Methods section, [CO₂]ₙ was estimated from the determined values of total CO₂ content ([CO₂]ₜ) and pH, along with an operationally determined value for the pKa, in each sample of luminal and serosal fluid. With this approach, the value obtained for [CO₂]ₙ in the CO₂-equilibrated phosphate-containing bicarbonate solutions was found to be indistinguishable from that determined in H₂SO₄ solutions which had been equilibrated with the same CO₂ gas mixtures (Table II).

In summary, the direct method provides a valid measure of [CO₂]ₙ in a HCO₃-containing or HCO₃-free (H₂SO₄) solution, provided that such a solution is devoid of non-HCO₃ buffers. Also, the indirect method provides a valid measure of [CO₂]ₙ independent of the presence or absence of nonbicarbonate buffers. The difference
between the directly and the indirectly determined [CO$_2$]$_f$ in a given HCO$_3$ solution is proportional to the quantity of non-HCO$_3$ buffer in that solution.

**Do Non-HCO$_3$ Buffers of Serosal or Cell Fluids Diffuse into Luminal Fluids Devoid of Such Buffers?**

If sufficient quantities of non-HCO$_3$ buffers (such as inorganic phosphate, lactate, or ammonia) were to diffuse from the serosal fluid and/or from cell fluid of an isolated turtle bladder sac into a (HCO$_3$ + CO$_2$)-containing luminal fluid (initially devoid of non-HCO$_3$ buffer), the directly measured luminal [CO$_2$]$_f$ would become spuriously high, but the indirectly determined level of [CO$_2$]$_f$ would be valid (Table II). On the other hand, if the quantity of non-HCO$_3$ buffer diffusing into the lumen were very small, the directly determined level of [CO$_2$]$_f$ would be indistinguishable from the indirectly determined level.

To test the validity of this prediction, CO$_2$-equilibrated bicarbonate solutions (initially devoid of non-HCO$_3$ buffers) were placed into the luminal compartments of closed turtle bladder sacs surrounded by (HCO$_3$ + CO$_2$)-containing Ringer solutions. After 3–5 h of incubation, the acidified solution was removed from the bladders, reequilibrated with the same gas mixture as that used before incubation, and analyzed for [CO$_2$]$_o$, [CO$_2$]$_i$, pK"a, and pH. It was found that the directly determined [CO$_2$]$_f$ was not significantly different from the indirectly determined [CO$_2$]$_f$ (Table III). Therefore, no significant quantity of non-HCO$_3$ buffer could have diffused into these solutions during their exposure to the luminal surface of the bladder. These findings, in harmony with those found previously using another method (Schilb and Brodsky, 1966), completed the methodological part of the present study.

**Physiological Determinants of [CO$_2$]$_f$ during Luminal Acidification**

**Lack of Effect of In Vitro Temperature**

After the removal of urinary bladder sacs from turtles that had been adapted to a single in vivo temperature (21°C), they were divided into three in vitro groups: one was incubated at 21°C; another, at 26°C; and a third at 32°C (Table IV). During these in vitro incubation periods, the luminal [CO$_2$]$_f$ increased while the pH and HCO$_3$ concentration decreased without exception in each bladder of all three groups (Table IV). There were three bladders in each of the three groups, so that the data shown are not amenable to any rigorous statistical analysis. Nevertheless, the mean values of the individual decreases in luminal [HCO$_3$] were statistically significant ($P < 0.02$) in each of the three groups. Although the mean changes in pH and [CO$_2$]$_f$ were not statistically significant, the direction of the individual changes was the same in all nine bladders, i.e., pH decreased while [CO$_2$]$_f$ increased. Evidently, a CO$_2$-increasing pattern of in vitro luminal acidification under these conditions is independent of the in vitro temperature.

In this connection, Schwartz et al. (1974), using a pCO$_2$ electrode (rather than a manometric apparatus) for estimating [CO$_2$]$_o$, also found a CO$_2$-increasing pattern of in vitro luminal acidification in bladders from turtles that had been adapted to room temperature. It can therefore be inferred that the measured increment in
TABLE III
Equality of Direct and Indirect Determinations of [CO\textsubscript{2}]\textsubscript{i} in Luminal Fluids after Incubation in Bladder Sacs

| Experiment No. | pH          | [HCO\textsubscript{3}] \textsubscript{i} | [CO\textsubscript{2}]\textsubscript{i} \textsubscript{(d)} | [CO\textsubscript{2}]\textsubscript{i} \textsubscript{(a)} | Estimated conc. of non-HCO\textsubscript{3} buffer |
|---------------|-------------|---------------------------------|----------------|----------------|----------------------------------|
|               | mM          | mM                             | mM            | mM             | (d-h)                            |
| 1             | 6.65        | 4.60                           | 1.82          | 1.80           | 1.73                             | 0.02                           | -0.09                           |
| 2             | 6.75        | 5.85                           | 1.79          | 1.81           | -                   | -0.02                           | -                                |
| 3             | 6.22        | 1.82                           | 1.88          | 1.88           | 1.83                             | 0                              | 0.05                            |
| 4             | —           | 1.54                           | 1.94          | 1.94           | 1.83                             | 0                              | 0.11                            |
| 5             | 5.04        | 0.13                           | 1.98          | 2.10           | 1.86                             | -0.12                          | 0.12                            |
| 6             | 6.26        | 3.10                           | —             | 2.70           | 2.63                             | —                              | —                               |
| 7             | 5.43        | 0.49                           | 2.96          | 2.98           | 2.99                             | -0.02                          | -0.03                           |
| 8             | 6.43        | 4.71                           | 2.90          | 2.99           | 2.90                             | -0.09                          | 0                               |
| 9             | 6.21        | 2.97                           | 3.19          | 3.19           | 3.27                             | 0                              | -0.08                           |
| 10            | 5.96        | 1.70                           | 3.24          | 3.20           | 3.27                             | 0.04                           | -0.05                           |

These data show that no detectable quantities of non-HCO\textsubscript{3} buffer appear in the CO\textsubscript{2}-equilibrated, (NaHCO\textsubscript{3} + NaCl)-containing luminal solutions that had been incubated in, and acidified by, 10 closed turtle bladder sacs. Analyses were carried out after each incubated luminal solution and a paired H\textsubscript{2}SO\textsubscript{4} solution had been equilibrated with the same gas mixture (four different mixtures were used). In addition to the luminal HCO\textsubscript{3} concentration and pH, values shown in the table include: the dissolved CO\textsubscript{2} concentration ([CO\textsubscript{2}]\textsubscript{i}), determined by the direct (d) and indirect (a) methods in the luminal solutions, and that determined in the paired H\textsubscript{2}SO\textsubscript{4} solution (a). The concentration of non-HCO\textsubscript{3} buffer is defined by the difference between the direct and indirectly determined levels of dissolved CO\textsubscript{2} (d-h), and between the level of CO\textsubscript{2} in H\textsubscript{2}SO\textsubscript{4} and that determined directly in the luminal fluid (d-a) (means ± SEM, -0.021 ± 0.019 and 0.007 ± 0.030; P > 0.2 and P > 0.8, respectively). The precision of the indirectly determined concentration of dissolved CO\textsubscript{2} is inversely related to the magnitude of its deviation from that in the paired, coequilibrated H\textsubscript{2}SO\textsubscript{4} solution; i.e., by the magnitude of the difference, d-h. The mean ± SEM for (h-a) is 0.045 ± 0.026 and its P > 0.2.

TABLE IV
Lack of Effect of In Vitro Incubation Temperature on a CO\textsubscript{2}-increasing Luminal Acidification Pattern in Bladders from 21°C-adapted Turtles

| Fluid Sample | Parameter | 21°C | 26°C | 32°C |
|--------------|-----------|------|------|------|
| Luminal      | pH        | 6.49 ± 0.03 | 6.53 ± 0.04 | 6.52 ± 0.04 |
|              | ΔpH       | -0.93 ± 0.50 | -0.78 ± 0.34 | -0.38 ± 0.15 |
|              | [HCO\textsubscript{3}] (mM) | 5.26 ± 0.31 | 5.16 ± 0.26 | 5.21 ± 0.07 |
|              | Δ[HCO\textsubscript{3}] (mM) | -4.29 ± 0.69 | -4.01 ± 0.66 | -2.70 ± 0.49 |
|              | [CO\textsubscript{2}]\textsubscript{i} (mM) | 2.91 ± 0.03 | 2.52 ± 0.03 | 2.05 ± 0.03 |
|              | Δ[CO\textsubscript{2}]\textsubscript{i} (mM) | +0.29 ± 0.15 | +0.11 ± 0.05 | +0.29 ± 0.03 |
| Serosal      | pH        | 7.16 ± 0.01 | 7.08 ± 0.01 | 7.04 ± 0.01 |
|              | [HCO\textsubscript{3}] (mM) | 24.3 ± 0.45 | 19.6 ± 1.02 | 17.8 ± 0.07 |
|              | [CO\textsubscript{2}]\textsubscript{i} (mM) | 2.92 ± 0.01 | 2.52 ± 0.01 | 2.14 ± 0.04 |

Mean values (± SEM) for the initial levels of luminal pH, HCO\textsubscript{3}, and [CO\textsubscript{2}]\textsubscript{i} in these levels after incubation of bladders from 21°C-adapted turtles at three different in vitro temperatures (n = 3 bladders for each group), and for the corresponding levels, which remained constant in the serosal fluid during the incubation period. The luminal changes are the means of the differences between the M\textsubscript{i} and M\textsubscript{a} samples.
luminal \([\text{CO}_2]_l\) is also independent of any valid analytical technique used for its determination.

**Effects of In Vivo Temperature on the Donor Turtles**

Turtles that had been adapted to a 21°C aqueous environment for 2 wk were transferred to and kept in a 32°C aqueous environment for the next 2–3 wk. During this time, the sequence of in vivo changes was as follows. For the first 2 d, there were no observable changes in the physical activity (swimming actions were slow and infrequent), there was little or no appetite for offered food, and little or no change in acidity of the bladder urine (which remained at or near pH, 7.0). Between the second and fifth day at 32°C, the physical activity and appetite increased progressively, with no change in acidity of the bladder urine. After six or more days at 32°C, the turtles swam with vigor, consumed raw fish offerings rapidly, while the acidity of the bladder urine increased (reaching pH, 6.0 or less). After 2 wk at 32°C, the physical activity and appetite of these turtles remained at high levels, while the mean value (± SEM) for the pH of bladder urine was 5.83 ± 0.22 (range, 4.89–6.81; n = 9).

The changes in 32°C-adapted turtles were found to be distinctly different from those found in 21°C-adapted turtles. The physical activity and appetite of 21°C turtles remained at low levels, while the mean value (± SEM) for the pH of bladder urine was 7.63 ± 0.23 units (range, 6.61–8.00; n = 6). The range of pH in the cardiac blood of the 21°C-adapted (7.40–7.80) was the same as that in the 32°C-adapted turtles.

Each of the adaptive responses of turtles kept at their behaviorally preferred temperature of 32°C for two or more weeks is in harmony with that expected from well documented findings of others (Boyer, 1965; Gatten, 1974; Dawson, 1976) who have studied thermoregulatory phenomena intensively in ectothermic reptiles. For example, during adaptation of such reptiles to a warmer environmental temperature, Myhre and Hammel (1969) and Simon (1974) have shown that thermosensitive neurons of the brain stem and spinal cord transmit behavior-modifying signals, which could account in part for the increased physical activity and appetite seen in the presently studied turtles in a 32°C environment. During similar periods of adaptation, it has been shown that the rate of total oxygen consumption increases (Jackson, 1977; Coulson and Hernandez, 1983), and the systemic acid-base balance is shifted toward an acidotic state in which the extracellular level of \([\text{CO}_2]_l\) is elevated, while that of \([\text{HCO}_3^-]_l\) remains constant (Jackson, 1971; Kinney et al., 1977; Reeves, 1977). These findings are thought to be reflected in the progressive increase of urinary acidity in the presently studied turtles adapted to a 32°C temperature.

In summary, the physical activity, the food intake, and the urinary acid excretion in 32°C-adapted turtles were greater than the corresponding characteristics in 21°C-adapted turtles. In the next set of experiments, it is shown how these different in vivo states modify the pattern of in vitro luminal acidification in isolated bladders under a single set of incubation conditions.

**Effects of In Vivo Temperature on the In Vitro Urinary Bladder**

After each of the bladders had been removed from turtles adapted to environmental temperatures of 21, 26, or 32°C, they were preincubated and incubated at room temperature using the luminal and serosal fluids described in the Methods.
Acidification-induced Decrease in pCO₂

Luminal pH and HCO₃⁻. During in vitro acidification periods, the mean values for the final decrements of luminal pH and HCO₃⁻ concentration in bladders from 32°C donor turtles were not significantly different from those in bladders from 26°C or 21°C turtles.

Rate of luminal HCO₃⁻ depletion. Since the time required for evoking this degree of in vitro luminal acidification in the 32°C group was shorter than that required in the other bladder groups, the average hourly rate of luminal bicarbonate depletion in the 32°C group was faster than that in bladders from turtles adapted to the lower temperatures. Mean values (± SEM) for the rates of luminal HCO₃⁻ depletion (in micromoles per hour) were 6.15 ± 0.04 in the 32°C bladder group, 3.36 ± 0.42 in the 26°C group, and 3.63 ± 0.42 in the 21°C group.

Changes in luminal [CO₂]f (Table V, Fig. 3). It was found that the luminal [CO₂]f increased significantly in over 80% of the bladders from 21°C-adapted turtles, but decreased significantly in all of the bladders from 32°C-adapted turtles. It was also found that the luminal [CO₂]f decreased significantly in two bladders from the 21°C-adapted turtles, as well as in five from the 26°C-adapted turtles; and that there was no detectable quantity of CO₂-absorbing or proton-absorbing buffers in any of the acidified luminal fluids, as shown by the measured equality between the directly and indirectly determined values of [CO₂]f (see the Results section, and Tables I and II).

Evidently, the greater the in vivo temperature of the donor turtle, the greater the magnitude of the in vitro decrement of luminal [CO₂]f during the period of luminal acidification in the isolated bladder taken from the turtle. This relationship is best visualized when the transmural gradients of [CO₂]f (developed during the course of luminal acidification) are depicted in relation to the prior in vivo temperature of the donor turtles in each of the three groups (Fig. 3). The directional orientation, as
well as the magnitude of the final transmural gradient of \([\text{CO}_2]_f\) in any single bladder group was significantly different from that in either one of the other groups.

Whereas the \(\text{CO}_2\)-increasing pattern is in harmony with the findings of Schwartz et al. (1974) and Green et al. (1970), the \(\text{CO}_2\)-decreasing pattern of acidification is in harmony with those of Schilb and Brodsky (1966, 1972). As far as we can now determine, both sets of data are technically valid, but differ because the previously maintained body temperatures or metabolic states of the donor turtles were not the same in both studies.

In summary, a single parameter of the luminal acidification process in these bladder sacs, the time-dependent directional change in luminal \([\text{CO}_2]_l\) (or the final transmural gradient of \([\text{CO}_2]_f\)), was found to be a uniformly reproducible and predictable consequence of the prior in vivo body temperature and metabolic of the donor turtle.

![Figure 3](image)

**DISCUSSION**

**General Aspects of Luminal Acidification**

**Mass Conservation of Luminal \(\text{CO}_2\)**

The presently demonstrated \(\text{CO}_2\)-changing acidification processes in \((\text{HCO}_3^- + \text{CO}_2)\)-containing luminal fluids can be analyzed rigorously, yet nonspecifically, within constraints of the mass conservation law for the turnover of intraluminal \(\text{CO}_2\). Accordingly, any time-dependent change in the luminal \(\text{CO}_2\) concentration, \(\Delta [\text{CO}_2]/\Delta t\), must be defined by the algebraic sum of two concomitantly occurring, time-dependent (rate) processes. These include: (a) the transepithelial diffusion rate \((J)\) of \(\text{CO}_2\) from the surrounding epithelial cells \((m)\) into the mucosal fluid \((m)\), which is defined as \(J(\text{CO}_2\text{diff, cm})\), or from the mucosal fluid into the surrounding cells, defined as \(J(\text{CO}_2\text{diff, mc})\); and (b) the dehydration of intraluminal \(\text{H}_2\text{CO}_3\), driven by a postulated secretion of protons into the \((\text{HCO}_3^- + \text{CO}_2)\)-containing mucosal fluid, defined as \(J(\text{CO}_2, \text{H}^+\text{pump})\), or, alternatively, the hydra-
tion of intraluminal CO$_2$ driven by a postulated reabsorption of bicarbonate ions from such a mucosal fluid, defined as $J$(CO$_2$, HCO$_3$ pump).

Experimentally, the unknown aspects of these rate processes include the magnitude and directional orientation of $J$(CO$_2$ diff) as well as the identity of the acidification mechanism in the function, $J$(CO$_2$ pump). Therefore the generalized equation for the mass conservation of intraluminal CO$_2$ must be cast in the form,

$$\Delta[CO_2]/\Delta t = J(CO_2 \text{ diff}) + J(CO_2 \text{ pump}).$$

The sign convention adopted for each parameter in Eq. 10 is the following. (a) $\Delta[CO_2]/\Delta t$ is positive whenever the luminal [CO$_2$]$_t$ increases, and negative when luminal [CO$_2$]$_t$ decreases. (b) $J$(CO$_2$ diff) is positive whenever the transapical diffusion of CO$_2$ is directed from the cells into the luminal fluid, and negative whenever it is directed from the luminal to the cellular fluid. (c) $J$(CO$_2$ pump) is positive for a postulated active secretion of protons into a HCO$_3$-rich luminal fluid, and negative for a postulated active reabsorption of bicarbonate ions from such a luminal fluid.

Under experimental conditions such as these that have been imposed on the turtle bladder sacs, one can determine the magnitude and direction of change in $\Delta[CO_2]/\Delta t$ after specified periods of active luminal acidification, as well as the rate of luminal acidification from the measured rate of luminal bicarbonate depletion, $\Delta[HCO_3]/\Delta t$. One cannot, however, identify the acid excretory mechanism from the value of $\Delta[HCO_3]/\Delta t$, which is the same for a bicarbonate-reabsorbing operation as it is for a proton-secreting operation. As long as the pumping function is active, the diffusion parameter $J$(CO$_2$ diff) is not measurable.

The cell-to-lumen diffusion of CO$_2$, $J$(CO$_2$ diff, cm), can be measured after the pumping mechanism has been turned off and the bladder sac filled with a CO$_2$-free luminal fluid, while the level of [CO$_2$]$_t$ in the serosal fluid is kept constant by continuous equilibration with a gas mixture of known pCO$_2$. However, there is no independent evidence from which one can conclude, along with others (Green et al., 1970; Schwartz et al., 1974) who have successfully carried out such experiments, that $J$(CO$_2$ diff, cm), after inactivating the acid-excreting pump mechanism, remains the same as that while the pumping mechanism is active. The minimal requirements for making such a comparison are a change (or lack of change) in the cell-to-lumen gradient of CO$_2$, which depends upon a change (or lack of change) in the cytosolic level of [CO$_2$]$_i$; both of these parameters depend on a corresponding change (or lack of change) in the rate of metabolic CO$_2$ production, not only in the acidifying cells, but also in the non-acidifying cells of the bladder epithelium.

**Conformity of Mass Conservation with Present Data**

Is bicarbonate reabsorption the single explanation for CO$_2$-decreasing patterns of luminal acidification? According to the mass conservation law for the turnover and balance of intraluminal CO$_2$ (Eq. 10), a postulated bicarbonate-reabsorbing pump, operating at a rate $J$(CO$_2$, HCO$_3$ pump) is consistent with the observed development of a CO$_2$-decreasing pattern of luminal acidification, such as that found in all bladders from 32°C-adapted turtles and in some from 26- and 21°C-adapted turtles. This is because $\Delta[CO_2]/\Delta t$, a negative quantity when luminal [CO$_2$]$_i$ decreases with time, requires that the algebraic sum of the pumping and diffusion rates be negative. Bicarbonate reabsorption is also a negative quantity because it contributes pri-
marily to decreasing the level of luminal \([\text{CO}_2]\), and secondarily to creating a trans-apical gradient of \([\text{CO}_2]\), which is oriented to drive a cell-to-lumen diffusion of \(\text{CO}_2\); i.e., \(J(\text{CO}_2 \text{ diff, cm})\). As long as the magnitude of a \(\text{HCO}_3\) pump exceeds that of \(J(\text{CO}_2 \text{ diff, cm})\), a positive quantity, a \(\text{CO}_2\)-decreasing pattern of luminal acidification, such as that observed in the present study, will inevitably develop.

To show uniqueness in the consistency of the postulated bicarbonate reabsorption with the observed \(\text{CO}_2\) decreasing pattern of luminal acidification, the inevitable consequences of a postulated proton-secreting pump should be inconsistent with the observed \(\text{CO}_2\)-decreasing patterns of luminal acidification such as those found in the present study. Such inconsistency arises from the following considerations. \(\Delta [\text{CO}_2]/\Delta t\), the observable parameter, is a negative quantity in Eq. 10. The postulated secretion of protons at any specified rate, \(J(\text{CO}_2, \text{ H}^+ \text{ pump})\), is a positive quantity, because it contributes primarily toward increasing the level of luminal \([\text{CO}_2]\), and secondarily toward creating a transapical \([\text{CO}_2]\) gradient, which is oriented to drive a lumen-to-cell diffusion of \(\text{CO}_2\). It is therefore required that the magnitude of \(J(\text{CO}_2 \text{ diff, mc})\) be greater than that of \(J(\text{CO}_2, \text{ H}^+ \text{ pump})\) in order to account for the observed negative value of \(\Delta [\text{CO}_2]/\Delta t\), which is the operationally defined parameter of the luminal \(\text{CO}_2\) decreasing phenomenon. However this condition,

\[
J(\text{CO}_2 \text{ diff, mc}) > J(\text{CO}_2, \text{ H}^+ \text{ pump}),
\]

is contradictory to the mass conservation law for the balance of intraluminal \(\text{CO}_2\). This is because the quantity of intraluminal \(\text{CO}_2\) generated by the secretion of a specified quantity of protons into an enclosed, \(\text{HCO}_3\)-containing luminal fluid must be greater than the maximal quantity of \(\text{CO}_2\) diffusing out of that luminal fluid into the surrounding epithelial cells.

In summary, a proton-secreting pump cannot possibly be responsible for generating or even for being concomitantly associated with an observed \(\text{CO}_2\)-decreasing pattern of luminal acidification, owing to its inconsistency with the mass conservation law under conditions that prevail during the development of the luminal \(\text{CO}_2\)-decreasing phenomenon. The alternative mechanism, that of a bicarbonate-reabsorbing pump, is therefore not only consistent, but uniquely consistent with the presently demonstrated \(\text{CO}_2\)-decreasing patterns of luminal acidification found in all 14 bladders from 32°C-adapted turtles as well as in six of 23 bladders from 21- and 26°C-adapted turtles.

*Why proton secretion is a valid, but not a unique explanation for \(\text{CO}_2\)-increasing acidification patterns.* Any observed, time-dependent increase in luminal \([\text{CO}_2]\) can be the direct consequence of a cell-to-lumen diffusion of \(\text{CO}_2\), as well as the direct consequence of a proton-secreting pump. However, one cannot rule out the possibility that a cell-to-lumen diffusion of \(\text{CO}_2\) can be associated with a concomitantly operating bicarbonate reabsorptive pump; if the rate of this diffusion were faster than that of the bicarbonate pumping process, a \(\text{CO}_2\)-increasing pattern of luminal acidification would develop.

The same cell-to-lumen diffusional process, \(J(\text{CO}_2 \text{ diff, cm})\) can also be associated with a concomitantly operating proton secretory pump, \(J(\text{CO}_2, \text{ H}^+ \text{ pump})\). Under this condition, the observed increment in luminal \([\text{CO}_2]\), would be derived in part
from the pump-driven conversion of luminal HCO$_3^-$ into CO$_2$, and in part from the diffusion of metabolic CO$_2$ out of the nonacidifying cells into the lumen.

However, the postulated proton-secreting operation $J(CO_2, H^+ \text{ pump})$, also a direct cause of the observed increase in luminal [CO$_2$]$_l$, is usually assumed to be associated with an oppositely oriented transapical CO$_2$ diffusion, i.e., $J(CO_2 \text{ diff, mc})$. This requires that the rate at which proton secretion generates CO$_2$ from intraluminal bicarbonate be sufficient to produce and maintain the luminal [CO$_2$]$_l$ at a level higher than that maintained in the nonacidifying cells, which account for 80% of all the epithelial cells lining the turtle bladder.

In summary, in keeping with the mass conservation law for the balance of intraluminal CO$_2$, a postulated bicarbonate-reabsorbing mechanism is consistent with the presently demonstrated CO$_2$-increasing patterns of luminal acidification in most of the bladders from 21- and 26°C-adapted turtles. Nevertheless, a postulated active secretion of protons is also consistent with the presently demonstrated CO$_2$-increasing patterns of luminal acidification. It follows that proton secretion is an explanation, but not the exclusive explanation for the demonstrated increases of luminal [CO$_2$]$_l$ in isolated turtle bladder sacs under the presently imposed conditions.

Can both the CO$_2$-decreasing and CO$_2$-increasing processes of luminal acidification be completely explained with a single type of acid-pumping mechanism or are two different types required for this purpose? The one-pump hypothesis. Because proton secretion cannot account for the CO$_2$-decreasing pattern of luminal acidification, bicarbonate reabsorption must be invoked as the single-pump mechanism in this hypothesis. Such an assumed, singly operating bicarbonate pump is uniquely consistent not only with the CO$_2$-decreasing pattern of luminal acidification, but also with the in vivo temperature dependence of the in vitro rate of acidification in the presently studied bladder sacs.

Although not the direct cause of the CO$_2$-increasing pattern of luminal acidification, a postulated bicarbonate-reabsorbing process cannot be ruled out as the singly operating acidification mechanism during the development of a CO$_2$-increasing pattern of luminal acidification. This is because a rate of cell-to-lumen diffusion of CO$_2$, which exceeds that of the concomitant bicarbonate reabsorption, would produce an increasing pattern of luminal acidification. Such a situation might well be expected in view of the dominant proportion (80%) of nonacidifying epithelial cells in the turtle bladder.

The two-pump hypothesis. Despite the consistency of the one-pump (bicarbonate reabsorptive) hypothesis with all of the presently obtained data, the interesting alternative of a two-pump hypothesis has been suggested to us by a colleague. According to this hypothesis, a proton-secreting operation (and explicitly not a bicarbonate-reabsorbing operation) accounts for the demonstrated CO$_2$-increasing acidification process in most of the bladders from 21- and 26°C-adapted turtles. But during and after these same turtles become adapted to a 32°C environment, the proton secretory pump mechanism is progressively inactivated, while a bicarbonate-reabsorbing pump is progressively activated. As suggested, this hypothesis requires that any single acidifying cell of the bladder contain two qualitatively different acid excretory mechanisms, or that there are two different kinds of acid-excreting cells in any given bladder. The most compelling reasons for invoking this hypothesis are
those relating to the direct effects of each postulated pumping process on the luminal \([\text{CO}_2]_l\). Proton secretion cannot be excluded as a direct cause of the observed \(\text{CO}_2\)-increasing patterns of luminal acidification and bicarbonate reabsorption cannot be excluded as the direct cause of the observed \(\text{CO}_2\)-decreasing pattern of luminal acidification.

Such a two-pump hypothesis has no requirement for the undeniable possibility that either the cell-to-lumen diffusion of \(\text{CO}_2\) or the secretion of protons can be a direct cause for any observed increase in luminal \([\text{CO}_2]_l\). But, an observed \(\text{CO}_2\)-increasing pattern of luminal acidification can also be associated with (though not caused by) a bicarbonate-reabsorbing operation, the rate of which is less than the concomitant rate of the cell-to-lumen diffusion of \(\text{CO}_2\).

Therefore, the cell-to-lumen or the lumen-to-cell diffusion of \(\text{CO}_2\) must be included as part of the two-pump hypothesis. But \(J(\text{CO}_2 \text{ diff})\) is a nonmeasured quantity, and there is no independent evidence from which one can even infer, let alone demonstrate that any observed increment of luminal \([\text{CO}_2]_l\) is not the direct consequence of a cell-to-lumen diffusion of \(\text{CO}_2\). This uncertainty applies directly to the data of Green et al. (1970) and Schwartz et al. (1974), who found that the magnitude of a mucosal-to-serosal gradient of \(p\text{CO}_2\) was greater in turtle bladder sacs with high acidification rates than in those with little or no acidification. With the implicit assumption that the \(p\text{CO}_2\) of the cytosol in the acidifying cells remains constant in the face of increasing or decreasing acidification rates, one could conclude that proton secretion is the cause of any or all observed increases in luminal \(p\text{CO}_2\). However, as long as the cellular \(p\text{CO}_2\) and, consequently, the cell-to-lumen gradient of \(p\text{CO}_2\) remain unknown, one cannot conclude that proton secretion is the only direct cause of any observed increase in the mucosal-to-serosal gradient \(p\text{CO}_2\) (cf., Fig. 4 of Brodsky and Schilb, 1974).

Over and above this uncertainty, the two-pump hypothesis has to be supplemented with some ad hoc assumptions to account for the fact that the rate of luminal acidification is faster during \(\text{CO}_2\)-decreasing acidification periods (in 32°C-adapted turtles) than during \(\text{CO}_2\)-increasing acidification periods (in 21- or 26°C-adapted turtles). In accepting these assumptions, one might predict that the rate of proton secretion decreases with increasing ambient temperatures to which a given turtle can adapt. We are not aware of any biological process, the rate of which varies inversely with temperatures within a physiologically defined range.

Another ad hoc assumption of the two-pump hypothesis is that none of the observed increment in luminal \([\text{CO}_2]_l\) is derived from the cell-to-lumen diffusion of \(\text{CO}_2\) during the period of maximal proton secretion. If this could be shown, the nonexistence or nonfunctioning of a bicarbonate pump would be verified in bladders from 21- or 26°C-adapted turtles. But there is neither direct nor indirect evidence that shows that no \(\text{CO}_2\) from the nonacidifying cells diffuses into the lumen during the generation of a \(\text{CO}_2\)-increasing pattern of luminal acidification in the bladder sacs.

Conclusion. The one-pump (bicarbonate reabsorptive) hypothesis requires only a single assumption, based on the law of mass conservation of intraluminal \(\text{CO}_2\), to account for all of the present data. In contrast, the two-pump hypothesis requires at least three ad hoc assumptions (devoid of any experimental or theoretical support)
to account for all of the present data. Therefore in our opinion, it is logical to conclude (at least tentatively) that bicarbonate reabsorption is probably the single mechanism responsible for acidification of the urine in turtle bladders under all of the present conditions.

Specific Considerations: Experimental and Theoretical

Bicarbonate Reabsorption: A Frequently Rejected Hypothesis

The main reason for rejecting bicarbonate reabsorption as a mechanism of luminal acidification has been the consistency of proton secretion with what had been construed as critical data on acid excretion in the mammalian kidney and turtle urinary bladder. However, the single exception to what otherwise could be a generalized (or unified) theory of acid excretion is the presently validated, CO₂-decreasing pattern of acidification of HCO₃-containing luminal fluids in turtle bladder sacs (i.e., there were no significant losses of dissolved CO₂ from acidified luminal fluids into contiguous CO₂-free oil layers in our earlier studies [Schilb and Brodsky, 1966, 1972], as had been claimed by others [Green et al., 1970; Stienmetz 1974]).

In view of this single exception, it has become necessary to determine whether bicarbonate reabsorption had been rigorously excluded by the data upon which the hypothesis of proton secretion has been supported. A reexamination of these data shows that none constitutes unambiguous support for either proton secretion or bicarbonate reabsorption, that a specially contrived model for a bicarbonate-reabsorbing cell has been ruled out, but that neither the general concept nor an alternative model of a bicarbonate-reabsorbing cell has been ruled out.

Evidence used to rule out bicarbonate reabsorption. The observed excess of excreted titratable acid over concomitantly filtered CO₂ + H₂CO₃ in phosphate-loaded dogs (Pitts and Alexander, 1945) cannot exclude a bicarbonate-reabsorbing mechanism of urinary acid excretion unless one assumes that the apical membranes of the renal tubular cells block the cell-to-lumen diffusion but not the lumen-to-cell diffusion of CO₂ (Brodsky and Schilb, 1974).

The CO₂-increasing pattern of luminal acidification in turtle bladder sacs under conditions imposed by Schwartz et al. (1974) can also be ascribed to bicarbonate reabsorption as well as proton secretion. This is because it cannot be denied that the rate of metabolic CO₂ production changes when the acid excretory mechanism is inhibited, and because there is no evidence for the level or change in the level of intracytosolic [CO₂]c in either the acidifying or nonacidifying epithelial cells of this tissue (see above in the Discussion section).

The proposed distribution of metabolic CO₂, based on the mucosal-to-air evolution of CO₂ and the luminal acidification rate (in short-circuitur turtle bladders bathed by media devoid of exogenously added CO₂ and HCO₃) led Schwartz et al. (1974) to conclude that bicarbonate reabsorption was ruled out. This was because the calculated cell-to-mucosal flux of CO₂ would have to be over 3.5-fold greater than the calculated cell-to-serosal flux, and if so, the CO₂ permeability of the apical membrane would have to be 3.5-fold greater than that of the basal-lateral membrane. However, this cannot rule out bicarbonate reabsorption because the cell-to-lumen diffusion of CO₂ (as well as the cell-to-serosal diffusion) depends upon the
level of cytosolic \([\text{CO}_2]\), the magnitude of the \(\text{CO}_2\) concentration gradient, and the width of the unstirred fluid layers (most certainly, the width of that layer on the mucosal surface is about one-third of the layer bounding the basal-lateral membrane). Therefore, there is no requirement for the permeability of the apical membrane to be different from or to be the same as that of the basal-lateral membrane.

A bicarbonate pump (as well as a proton pump) could be responsible for generating the ATP-induced acidification process found in isolated membrane vesicles obtained from turtle bladder epithelial cells, even though these vesicles had been prepared and incubated in media devoid of exogenously added \(\text{HCO}_3\) and \(\text{CO}_2\) (Gluck et al., 1982; Youmans et al., 1983; Youmans and Brodsky, 1987). This is because all of the media used had been exposed to atmospheric air, the \(\text{pCO}_2\) of which is 0.3 mmHG at 25°C. Therefore, the dissolved \(\text{CO}_2\) in the extravesicular compartment of such media must have reached a level of 13 \(\mu\text{M}\) (in keeping with Henry's law), and the same level (13 \(\mu\text{M}\)) must have been reached in the intravesicular fluid (owing to the \(\text{CO}_2\) permeability of vesicular membranes). Furthermore, since these media were buffered at a pH of 7.4-7.6 (by the relative excess of a nonbicarbonate buffer), bicarbonate must have formed and reached levels of 197–312 \(\mu\text{M}\) in both the extra- and intravesicular fluids. In summary, the available data on the ATP-driven intravesicular acidification processes are as consistent with bicarbonate efflux as with proton influx.

**Theoretical considerations: the model cell used to rule out bicarbonate reabsorption.**

The concept of a bicarbonate reabsorbing process of urinary acidification has also been rejected, because a frequently invoked singularly contrived model (designated here as the type A model) fails to account for the findings related to the observed inhibition of carbonic anhydrase activity, not only in the turtle bladder but also in the mammalian nephron (Rector, 1976; DuBose et al., 1978, 1981; Lang et al., 1978; Lucci et al., 1980). We agree, for reasons other than those given by these investigators, that the type A model deserves to be rejected. Moreover, we suggest that this rejection is by no means tantamount to a valid exclusion of any alternative model or of the general hypothesis of bicarbonate reabsorption. In this connection, an alternative model (designated herein as type B) has been constructed (Durham et al., 1984).

**Questions relating to different models for luminal acidifying cells.** What exactly is wrong with the type A model for a bicarbonate reabsorbing cell, which has been categorically rejected every time that it has been proposed? On what grounds is the alternative type B model inherently valid and consequently more appropriate for experimental testing than the type A model? With respect to experimental challenges, how does the type B model compare with the classical, type C model (Pitts and Alexander, 1945). An answer to each of these questions is given in what follows.

**The Type A Model for a Bicarbonate-reabsorbing Cell**

As discussed in detail by its proposers (who were also its rejectors), the type A model was defined as a carbonic anhydrase-free luminal acidifying cell through which pumped bicarbonate ions flow from the apical region to the basal-lateral region of
the cytosolic fluid without interacting in any way with the available protons in that fluid. In the form presented and explicitly illustrated (Maren, 1956; Schwartz et al., 1974; Rector, 1976; DuBose, 1983), this model is seriously flawed because of the following considerations.

First of all, the assumed lack of interaction between bicarbonate ions (pumped into the cytosolic fluid) and the available protons in that fluid is contradicted by physico-chemical laws governing the association reaction between bicarbonate ions and protons (to form carbonic acid) in any aqueous solution, including the cytosol. Secondly, there is no requirement for any coupling of the continuously produced metabolic CO$_2$ with the available H$_2$CO$_3$ in the cytosolic fluid. Indeed, there is no explicit indication for the existence of the reaction CO$_2$ + H$_2$O $\rightarrow$ H$_2$CO$_3$ in the cytosol of this model.

For either of these reasons, the type A model could have been excluded on the basis of first principle alone, i.e., without the need for any experimental testing. But even if the model were corrected for these errors, it remains burdened with additional difficulties. It appears tautological (at least, in our opinion) to test for the validity of an assumed carbonic anhydrase-free model by carrying out experiments on a process such as urinary acid excretion, which is known to be sensitive to carbonic anhydrase inhibitors, and in tissues (kidney, turtle bladder) that are known to contain carbonic anhydrase activity. Moreover, neither the sensitivity nor the lack of sensitivity of any acidification process to carbonic anhydrase inhibitors is sufficient to identify that process as one of proton secretion or bicarbonate reabsorption, respectively.

**Common Characteristics of Models B and C**

*During a pump-off period.* Other than different pump mechanisms, the component parts of the plasma membranes and cytosol, as well as the metabolic activity in the type B model are the same as those in the type C model (Fig. 4). Therefore, if the pump mechanism were turned off, the distribution of metabolically produced CO$_2$ among the luminal, cellular, and serosal compartments of the type B model would be the same as that in the type C model. The time-dependent development of a steady state distribution of metabolic CO$_2$ can then be envisaged under conditions prevailing in a model bladder sac, filled initially with a relatively small volume of (HCO$_3$ + CO$_2$)-containing luminal fluid and surrounded by an infinitely large perfectly mixed volume of (HCO$_3$ + CO$_2$)-containing serosal fluid. Under these contrived conditions, the fate of metabolic CO$_2$, produced continuously at an assumed constant rate, is as follows. After reaching an unspecified intracellular level, CO$_2$ diffuses from the cell of origin into both the serosal and luminal fluids. During this time, the level of [CO$_2$]$_m$ in the infinitely large volume of serosal fluid remains constant, while that in the small volume of lumen fluid ([CO$_2$]$_m$) changes with time until the luminal level of [CO$_2$]$_m$ is nearly the same as the cellular level of [CO$_2$]$_c$. After this state is reached, practically all of the metabolically produced CO$_2$ will diffuse into the large volume of serosal fluid until the steady state profile of CO$_2$ concentration (from mucosa to cell to serosa) reaches the form,

$$[\text{CO}_2]_m = [\text{CO}_2]_c > [\text{CO}_2]_v.$$  \hspace{1cm} (12)
During a pump-on period. The turning of either pump mechanism leads to the following events in both models.

Across the apical membrane. The levels of pH and HCO₃⁻ decrease in the luminal fluid and increase in the apical region of the cytosol. The consequences of this initial change are that HCO₃⁻ flows out of the apical region toward the basal-lateral region of the cytosol, while H⁺ flows in the reverse direction across the cytosol.

Across the basal-lateral membrane. Part of the metabolic CO₂, along with all of the HCO₃⁻ arriving in the basal-lateral region of the cytosol diffuse into the infinitely large perfectly mixed volume of CO₂-equilibrated serosal fluid. The other part of metabolic CO₂ in the cytosolic region is hydrated as a consequence of the HCO₃⁻ depletion; this creates a source of H⁺ ions that flow out of this region toward the apical region (see Fig. 4).

**Figure 4.** (Upper panel) Pitts model (model C) for luminal acidification generated by a proton secretory pump in the apical membrane of a carbonic anhydrase-rich cell. (Lower panel) Current model (model B) for luminal acidification generated by a bicarbonate-reabsorptive pump in the apical membrane of a carbonic anhydrase-rich cell. The relative concentrations (sources and sinks) of each constituent (CO₂, H₂CO₃, H⁺, and HCO₃⁻) in the mucosal, cytosolic, and serosal compartments are designated by large and small lettering, respectively. In model C, the proton pump-induced sink for CO₂ (also a source of HCO₃⁻) is located in the apical region of the cytosol. In model B, the bicarbonate pump-induced sink for CO₂ (also a source of H⁺) is located in the apical boundary of the luminal fluid. Horizontal arrows and inequality signs denote the magnitude and direction of the transapical, transcytosolic, and transbasal lateral flow of each constituent. Vertical arrows denote the net direction of the CO₂-HCO₃ reactions in the luminal, cytosolic, and serosal fluids. Mucosal, indicates the relatively small volume of luminal fluid surrounded by the closed turtle bladder sac while Serosal, indicates the idealized large volume of CO₂-equilibrated serosal fluid in each model system. Details on the events induced by turning on each pump mechanism are discussed in the text.
Characteristics Unique to Model B (Fig. 4, Lower Panel) after Turning on the Bicarbonate Pump

**Luminal.** The activated pump induces an immediate reduction of \([HCO_3^-]_m\), which increases the degree of dissociation of \(H_2CO_3\), thereby decreasing the luminal levels of \([H_2CO_3]_m\) and \(pH_m\). The decrease in \([H_2CO_3]_m\) initiates an uncatalyzed hydration of \(CO_2\), thereby decreasing the luminal level of \([CO_2]_m\).

**Cytosolic (in the apical boundary region).** Concomitant with the luminal events, the pump induces an immediate increase of \([HCO_3^-]_c\), which increases the degree of association of pumped \(HCO_3^-\) with intracytosolic \(H^+\) ions. This in turn increases the cytosolic level of \([H_2CO_3]_c\), which initiates the carbonic anhydrase-catalyzed dehydration of \(H_2CO_3\), and thereby increases the cytosolic level of \([CO_2]_c\).

**Across the apical membrane.** The aforementioned changes in the luminal and cytosolic concentrations of \(H_2CO_3\) and \(CO_2\) form transapical concentration gradients, which induce passive diffusional flows of \(H_2CO_3\) and \(CO_2\) from the cytosol to the luminal fluid.

Characteristics Unique to Model B after Inhibition of Cytosolic Carbonic Anhydrase

In the apical boundary of cytosolic fluid. The level of \([H_2CO_3]_c\) increases and that of \([CO_2]_c\) decreases immediately after the cytosolic carbonic anhydrase is inhibited. This is because the pump mechanism (presumably not acted upon by carbonic anhydrase inhibitors) continues to transport bicarbonate against a progressively increasing transapical concentration gradient at a progressively decreasing rate.

**Across the apical membrane.** The immediate consequences of the aforementioned intracytosolic changes in \([H_2CO_3]_c\) and \([CO_2]_c\) are an accelerated cell-to-lumen diffusion of \(H_2CO_3\) and a retarded cell-to-lumen diffusion of \(CO_2\).

In the luminal fluid. The accelerated cell-to-lumen diffusion of \(H_2CO_3\) increases the luminal level of \([H_2CO_3]_m\) and consequently that of \([H^+]_m\). The retarded cell-to-lumen diffusion of \(CO_2\) is followed by a decrease in the luminal level of \([CO_2]_m\).

**Metabolic \(CO_2\).** Under these carbonic anhydrase–inhibited conditions, the continuously produced metabolic \(CO_2\) repletes the supply of cellular \(CO_2\), part of which diffuses at a reduced rate into the luminal fluid to yield sufficient bicarbonate ions for reabsorption, but at a much slower rate than that found in the absence of carbonic anhydrase inhibition.

Characteristics Unique to Model C (Fig. 4, Upper Panel); Events after Turning on the Proton Pump

**Intraluminal.** The entry of pumped protons into the luminal fluid decreases the levels of \(pH_m\) and \([HCO_3^-]_m\), and consequently increases that of \([H_2CO_3]_m\). The increase in \([H_2CO_3]_m\) triggers its uncatalyzed dehydration, which results in an increased luminal level of \([CO_2]_m\).

**Intracytosolic.** At the same time, the pumping of protons (out of the cytosol) increases the cytosolic levels of \(pH_c\) and \([HCO_3^-]_c\), thereby decreasing that of
This triggers a carbonic anhydrase-catalyzed hydration of CO₂, with a consequent decrease in the cytosolic level of \([\text{CO}_2]_c\).

Across the apical membrane. As a consequence of the aforementioned changes in CO₂, transapical concentration gradients develop, which drive diffusional flows of \(\text{H}_2\text{CO}_3\) and \(\text{CO}_2\) from the luminal into the cytosolic fluid.

**Characteristics Unique to Model C; Events after Inhibition of Cytosolic Carbonic Anhydrase**

**Intracytosolic.** The hydration of CO₂ is retarded (with little or no change in the metabolic production of CO₂). Consequently, the cytosolic level of \([\text{CO}_2]_c\) increases while that of \([\text{H}_2\text{CO}_3]_c\) decreases; this retards the rate at which protons are delivered into the pump mechanism.

**Intraluminal.** The level of \(\text{pH}_m\) decreases transiently, due to the continued, but retarded rate of proton secretion. Consequently, the luminal levels of \([\text{H}_2\text{CO}_3]_m\) and \([\text{CO}_2]_m\) continue to increase, but at slower rates than those prevailing in the presence of an active carbonic anhydrase element in the cytosol.

Across the apical membrane. The aforementioned changes reduce the magnitude of the lumen-to-cell concentration gradients. Consequently, the rates of lumen-to-cell diffusion of \(\text{H}_2\text{CO}_3\) and \(\text{CO}_2\) decrease, while proton secretion continues at a progressively decreasing rate.

**Summary**

Except for the presently verified, CO₂-decreasing pattern of luminal acidification, the type C model for a proton-secreting cell can be made to replicate all of the other observed characteristics of the luminal acidification process in turtle bladder sacs. But without exception, the type B model for a bicarbonate-reabsorbing cell can be made to replicate all of these observed characteristics. This analysis of specific models (B or C), together with the constraints imposed by the mass conservation law for the balance of intraluminal CO₂, makes it difficult to deny that bicarbonate reabsorption could be the dominant, if not the sole mechanism of luminal acidification in the turtle urinary bladder.

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