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SOME ASPECTS OF THE EVOLUTION OF VERTEBRATE ACID-BASE REGULATION‡

This paper will consider two aspects of the evolutionary development of acid-base metabolism in the vertebrates:

1. The development of the H_2CO_3-HCO_3^- buffer system.
2. The relationship between pH and temperature.

Recent work from a number of laboratories provides data that can serve as a basis for such an analysis.

H_2CO_3-HCO_3^- BUFFER SYSTEM

Origin

The H_2CO_3-HCO_3^- system in vertebrates did not evolve directly from the original forms of life. Organic evolution probably dates back some 10^9 years.[1] Although there may have been small amounts of CO_2 present in the atmosphere and CO_2 was definitely present in the earth mantle, it is generally accepted that metabolic pathways were anaerobic in nature. This conclusion is based on several lines of evidence. The primitive atmosphere contained no free oxygen and any free oxygen formed from the photolysis or electrolysis of water could not have accumulated in the reducing atmosphere present at that time. If adequate concentrations of molecular O_2 in the atmosphere only arose from the metabolic activity of living forms, the original forms must have developed anaerobically.[1] Anaerobic metabolic processes in all existing organisms are very similar while oxidative processes are more variable; therefore, anaerobic metabolism would appear to be more primitive.[2] Biosynthetic processes requiring O_2 commonly have preliminary steps that are anaerobic. The biosynthesis of a number of important biological substances that are O_2 requiring in relatively higher forms are carried out by entirely anaerobic mechanisms in

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relatively simple forms.[3] It may, therefore, be concluded that O_2 dependent energy metabolism was absent in the most primitive forms.

Anaerobic energy metabolism does not involve substantial CO_2 production and in the absence of significant metabolic CO_2 generation, internal concentrations of CO_2 must be negligible. In the absence of substantial internal concentrations of CO_2, H_2CO_3 and HCO_3^- cannot be present in substantial concentrations. These two CO_2 containing compounds, although possibly present in the body fluids of the most primitive forms presumably did not represent a major buffer system. It appears that the H_2CO_3-HCO_3^- system as found in vertebrates did not evolve from the most primitive forms of life.

The evolution of forms capable of producing significant amounts of molecular O_2 by means of photosynthesis must have played the major role in the ultimate development of vertebrate CO_2 metabolism. It is not possible to estimate in a meaningful way the time of this occurrence. The development of photosynthesis involved the convergence of three more or less independent processes: CO_2 fixation, light energy utilization, and molecular oxygen production.[4] Of these three processes, the generation of molecular O_2 was the most decisive with respect to CO_2 metabolism. It may be visualized that the O_2 evolving mechanisms resulted in a progressive increase in O_2 tensions leading to the higher plants and to the widespread development of oxidative evolution. The evolution of animal organisms capable of using O_2 for various metabolic processes must have occurred subsequent to the above developments.

Among the major metabolic O_2 dependent pathways that developed was energy transduction by oxidation of substrate to CO_2 and H_2O. As a result, intracellular CO_2 generation became a quantitatively significant process and the H_2CO_3-HCO_3^- buffer system became a quantitatively important factor in biological systems.

Primitive vertebrates

Vertebrates probably appeared some 600×10^6 years ago.[5] These animals were aquatic, and conducted gas exchange in water.

Quantitative analysis of respiratory gas exchange in water-breathing vertebrates suggests that CO_2 tensions in the body fluids of these primitive vertebrates were quite low, being less than 5 torr.[6] The analysis may be developed as follows:

Given an organism conducting gas exchange in water:

In the steady state,

$$\dot{V}_{CO_2} = \dot{V}\alpha CO_2 \, (P_{E_{CO_2}} - P_{I_{CO_2}}) \qquad \text{(Eq. 1)}$$

$$\text{and } \dot{V}_{O_2} = \dot{V}\,\alpha O_2\,(P_{I_{O_2}} - P_{\bar{E}_{O_2}}) \qquad \text{(Eq. 2)}$$

where $\dot{V}_{CO_2}$ = CO_2 production ml/min.

$\dot{V}_{O_2}$ = O_2 consumption ml/min.

$\dot{V}$ = Minute volume of ventilation L/min.

αCO_2 = solubility coefficient of CO_2 (mM CO_2/mmHg pCO_2)

αO_2 = solubility coefficient of O_2 (mM O_2/mmHgpO_2)

$P_{\bar{E}_{CO_2}}$ = partial pressure of CO_2 in mean expired fluid

$P_{\bar{E}_{O_2}}$ = partial pressure of O_2 in mean expired fluid

$P_{I_{CO_2}}$ = partial pressure of CO_2 in inspired fluid

$P_{I_{O_2}}$ = partial pressure of O_2 in inspired fluid

Dividing equation (1) by equation (2):

$$\text{Respiratory exchange ratio (R)} = \frac{\alpha CO_2}{\alpha O_2} \times \frac{P_{\bar{E}_{CO_2}} - P_{I_{CO_2}}}{P_{I_{O_2}} - P_{\bar{E}_{O_2}}} \qquad \text{(Eq. 3)}$$

Solving for $P_{\bar{E}_{CO_2}}$ (assuming that $P_{I_{CO_2}} = 0$),

$$P_{\bar{E}_{CO_2}} = R \times \frac{\alpha O_2}{\alpha CO_2}\,(P_{I_{O_2}} - P_{\bar{E}_{O_2}}) \qquad \text{(Eq. 4)}$$

The following assumptions appear reasonable:

1. Oxidative phosphorylation was probably the major metabolic pathway for energy transduction so that R would have ranged between 0.7 and 1.0.

2. The ratio of O_2 solubility to CO_2 solubility, $\frac{\alpha O_2}{\alpha CO_2}$, would range between 1/20-1/40 (let us say 1/30) depending on the temperature and ionic strength of seawater at that time.

3. The oxygen tension of ambient seawater was generally in equilibrium with the tension of O_2 of the atmosphere and would have been 150 torr or less since the concentration of O_2 in the atmosphere has probably increased from that time to the present.

4. The CO_2 tension of ambient seawater was generally in equilibrium with the CO_2 tension of the atmosphere and was negligibly low in value.

Substituting appropriate values in equation (4) the following expression is derived:

$$P_{E_{CO_2}} = \frac{150\text{-}P_{\bar{E}_{O_2}}}{30} \qquad \text{(Eq. 5)}$$

Even if $P_{\bar{E}_{O_2}}$ equaled zero, $P_{\bar{E}_{CO_2}}$ would be only 5 torr. Substantial values of $P_{\bar{E}_{O_2}}$ would require even lower values of $P_{\bar{E}_{CO_2}}$. Since gas tensions in expired fluid are generally closely related to gas tensions in the plasma of post-respiratory exchange vessels ("arterial"), the provision of adequate O_2 tensions for cellular O_2 consuming processes implied the existence of low CO_2 tensions in plasma.

It may, therefore, be concluded that primitive vertebrates being water-breathing animals and faced with an obligatory requirement for O_2 possessed plasma CO_2 tensions less than 5 torr.

The concentrations of HCO_3^- in extracellular fluid must likewise have been low, say less than 10 mEq/L. This stems from the fact that only a relatively narrow range of extracellular pH is compatible with vertebrate survival. Since the equilibrium ratio of HCO_3^- to pCO_2 is fixed by pH, a vertebrate with a low pCO_2 must likewise possess a relatively low plasma HCO_3^- concentration.

Since extracellular fluid is electrically neutral, the existence of a low plasma HCO_3^- concentration must have been balanced by a relatively high plasma concentration of Cl^- or other similar non-HCO_3^- anions.

Finally, it might be anticipated that the properties of buffer systems present in blood and body fluids of the primitive vertebrates would be especially suitable for buffering H_2CO_3 during circumstances of elevated CO_2 tensions. The nature of these buffers will be discussed below.

The early terrestrial vertebrates

Vertebrates left the water for land during the Devonian period some 500×10^6 years ago.[9] The development of gas exchange in air rather than in a liquid medium must have been one of the most important consequences of this transition. It now became possible to supply adequate amounts of O_2 with a lower ventilatory volume for the following reasons: In a gaseous medium with R=1, equation (5) becomes:

$$P_{\bar{E}_{CO_2}} = P_{I_{O_2}} - P_{\bar{E}_{O_2}} \qquad \text{(Eq. 6)}$$

It is unlikely that gas exchange in air involving simple diffusion as the mode of gas exchange could be associated with values of $P_{\bar{E}_{O_2}}$ greater than 110 to 120 torr. Under these circumstances, $P_{\bar{E}_{CO_2}}$ values would be sub-

stantially greater than 10 torr. It would, therefore, seem that the transition from water to air breathing was associated with a change from relatively low to relatively high CO_2 tensions in body fluids.

As indicated above, a high plasma pCO_2 must have been accompanied by relatively high HCO_3^- and relatively low Cl^- (non HCO_3^- anions) concentrations in plasma. Thus, the transition from an aquatic to a terrestrial habitat must have been accompanied by substantial changes in the gaseous and ionic composition of extracellular fluid.

In terms of patterns of buffering with respect to H_2CO_3 during the transition from water to air there are two *a priori* possibilities:

1. That the same pattern of buffers was maintained during the transition. This possibility might require the development of additional mechanisms for dealing with the augmented levels of body CO_2 associated with aerial gas exchange.

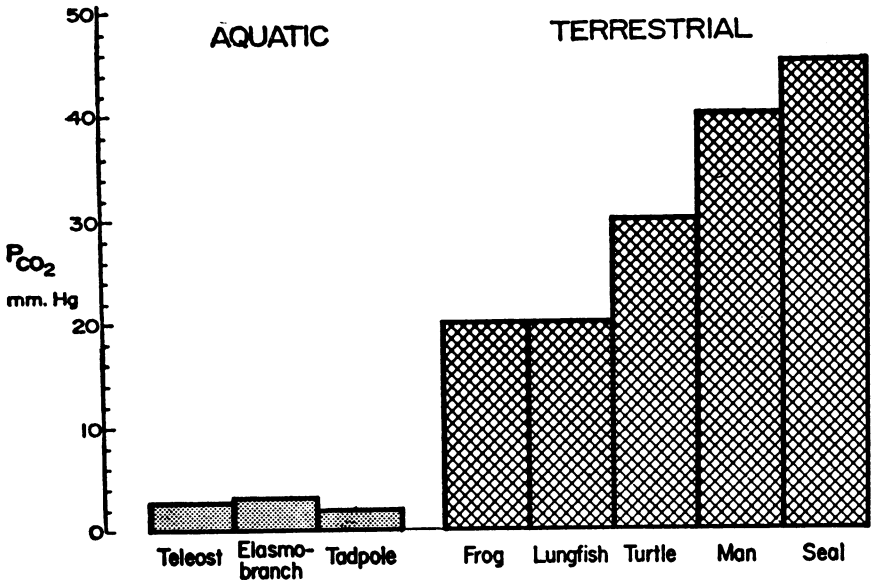
2. That a new pattern of body buffers evolved to meet the new levels of CO_2.

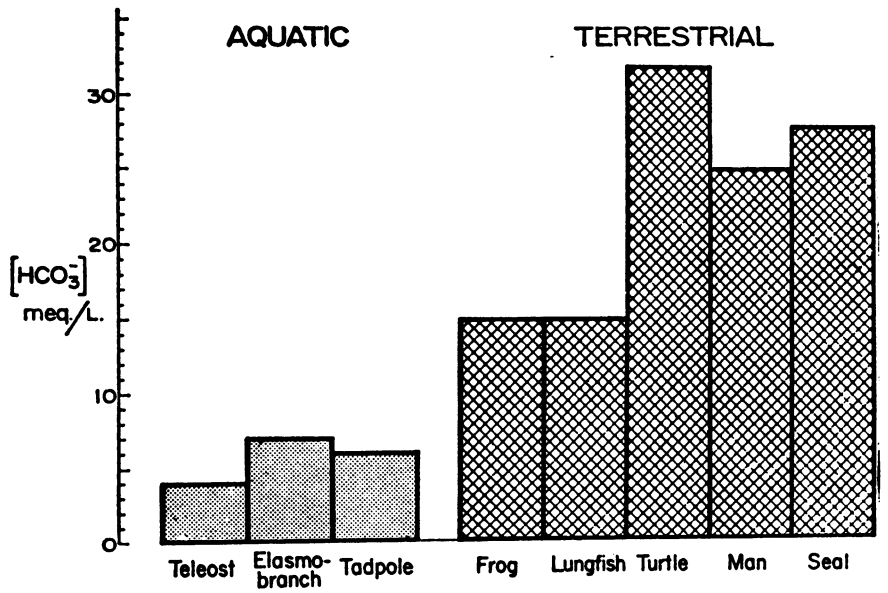
It will become apparent that the first possibility is the more likely one (see below).

Plasma CO_2 tensions and HCO_3^- concentrations in existing vertebrates

Existing vertebrates might be expected to, and do demonstrate a pattern of plasma CO_2 tensions and HCO_3^- concentrations that is in agreement with the theoretical considerations outlined above. As shown in Figure 1A and Figure 1B, there is a bimodal distribution of CO_2 tensions and HCO_3^- concentrations.[8] Water-breathing forms generally have plasma CO_2 tensions less than 5 torr and HCO_3^- concentrations less than 10 mEq/L whereas air-breathing forms generally have CO_2 tensions greater than 10 torr and HCO_3^- concentrations greater than 20 mEq/L. In this respect, the Amphibia represent a key group both because some members are aquatic and others are air breathers, and also because there are forms that during larval life are aquatic and during adult life are terrestrial. It may be noted from Figure 1A that the aquatic tadpole has a pCO_2 less than 5 torr while the pCO_2 of the frog is approximately 20 torr.

This does not imply that every aquatic vertebrate (individual or species) has a low CO_2 tension and every terrestrial vertebrate (individual or species) has a high CO_2 tension. Aquatic vertebrates living in water with high ambient CO_2 tensions would obviously have high body fluid CO_2 tensions. Terrestrial species under unusual conditions of temperature or with unusual mechanisms for ventilatory regulation might conceivably have CO_2 tensions less than 5 torr. Any apparent exceptions must reflect the operation of the physical-chemical mechanisms involved in vertebrate gas ex-





FIGS. 1A and 1B. CO_2 tensions and $[HCO_3^-]$ in some presently existing vertebrates.

change as influenced by environment or regulation. Although there may be exceptions to the bimodal distribution of CO_2 tensions among vertebrates, the remarkable fact is that exceptions are relatively rare!

Vertebrate blood CO_2 titration (dissociation) curves

CO_2 titration curves define the changes in total CO_2, HCO_3^- or H^+ concentration that occur as a result of alterations of pCO_2. In acute studies, these changes occur as a result of buffering of H_2CO_3 in body fluids. Such curves have been obtained *in vitro* and *in vivo* on whole blood over the entire vertebrate range of CO_2 tensions and are generally represented graphically by plotting total CO_2 or $[HCO_3^-]$ as a function of pCO_2.

As indicated in Figure 2 the form of these curves is quantitatively similar among all vertebrates, consisting of an initial portion (pCO_2, 0 torr to approximately 10 torr) with a relatively steep slope followed by a portion with a less steep slope (pCO_2's of 10 to 150 torr).[10-13] It may be concluded that the general pattern of blood and body buffering of H_2CO_3 is similar among aquatic and air-breathing vertebrates.

The shape of CO_2 titration curves is determined by the nature and amount of buffers available in the body fluids. Therefore, the similarity of shape implies that the buffer composition of body fluids is fundamentally similar among all vertebrates.

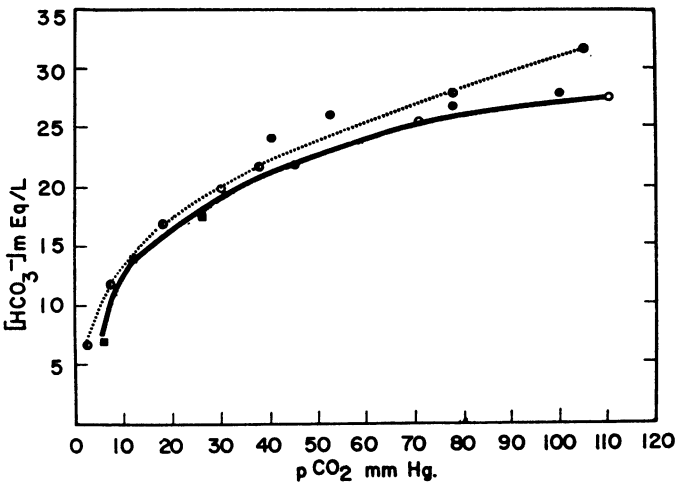


FIG. 2. The general shape of vertebrate blood CO_2 titration curves. The solid black line represents *in vivo* data with the squares representing data obtained in the elasmobranch,[10] the open circles, data obtained in man[11] and the solid circles, data obtained in the dog.[13] Although the curve appears to be continuous, this probably represents coincidence since the data have not been corrected for differences in temperature. The dotted curve represents data obtained *in vitro* in human (mammalian) blood.[13]

It is useful to consider the chemical species giving rise to the observed pattern of vertebrate CO_2 titration curves.

As long ago as 1907, L. J. Henderson showed that the titration of H_2CO_3 by buffers with a pKa value near 7 would give rise to a hyperbolic relationship between pCO_2 and $[HCO_3^-]$ of the general type characteristic of *in vitro* titration curves of mammalian blood.[14]

Edsall and Wyman developed these relationships extensively both on a theoretical and on an experimental basis.[15] Applying the requirement of electro-neutrality to equilibrium systems, they derived the following equations to define the relationship between pCO_2 and H^+ or HCO_3^- as a function of the equilibrium constant of any buffer system, $B + H^+ \rightleftharpoons BH^+$ with $K'_B = \dfrac{[B]\,[H^+]}{[BH^+]}$

$$pCO_2 = \frac{\dfrac{C \cdot [H^+]}{K'_B + [H^+]} + [Na^+] - [Cl^-]}{\dfrac{\alpha CO_2 \cdot K'_{H_2CO_3}}{[H^+]}} \qquad \text{(Eq. 7)}$$

$$pCO_2 = \frac{1}{\alpha CO_2} \cdot \frac{K'_B}{K'_{H_2CO_3}} \cdot \frac{[HCO_3^-]\,[HCO_3^- - D]}{C - HCO_3^- + D] \qquad \text{(Eq. 8)}}$$

where C is the total concentration of buffer, $[B] + [BH^+]$

Na^+ represents the concentration of all "fixed" cations

Cl^- represents the concentration of all "fixed" anions

D represents $Na^+ - Cl^-$.

The contribution of the terms $[H^+]$, $[OH^-]$, and $2\,[CO_3^=]$ in the equation describing electroneutrality are considered negligible at $4<pH<8$ and have been discarded.

These workers showed that the typical characteristics of mammalian *in vitro* CO_2 titration curves were reproduced by buffer solutions with a pK between 7 and 8 at a concentration similar to concentrations of such buffer groups present in hemoglobin. Indeed, using a simple solution consisting of 0.02 M $NaHCO_3$, 0.025 M 4-methyl-imidazole and 0.025 M 4-methyl-imidazole hydrochloride (pK'=7.4) a relationship between pCO_2 and HCO_3^- was demonstrated that closely parallels mammalian *in vitro* titration curves (Fig. 3).

It appears likely that the basis for the similarity of vertebrate CO_2 titration curves is the presence of appropriate concentrations of buffers in blood and body fluid with pK values ~7. In addition to organic and inorganic phosphate, buffers with a pK of 7 are essentially limited to proteins containing imidazole residues and free αamino groups. This point is

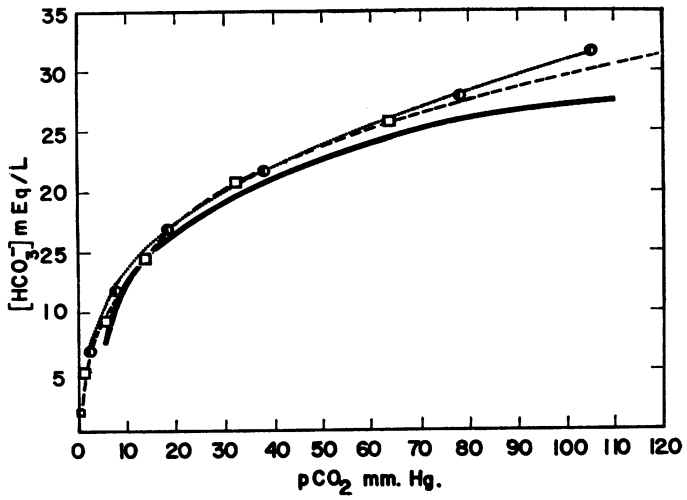


FIG. 3. Comparison of an *in vivo* vertebrate blood CO_2 titration curve (solid line), *in vitro* mammalian CO_2 titration curve (dotted line) and the CO_2 titration curve obtained on a solution containing 0.025 M methyl imidazole (pK = 7.4) and 0.025 M methyl imidazole hydrochloride (interrupted line).

illustrated in the data shown in Table 1 taken from data of Tanford, *et al.*,[16] by Edsall and Wyman.[15] Intrinsic pK values for bovine serum albumin are compared with these values in other typical proteins. The pK values distribute themselves into three discrete groups: low pK values <5 related

TABLE 1. IONIZABLE GROUPS IN BOVINE SERUM ALBUMIN AND THEIR INTRINSIC pK VALUES*

Group	*Amino acid analysis (No./molecule)*	*Albumin (pk_{int})*	*Other proteins** (pk_{int})*
α-Carboxyl	1	(3.75)	3.6
Carboxyl (Asp and Glu)	101	3.95	4.3- 4.7
Imidazole (His)	17	7.0	6.4- 7.0
α-Amino	1	7.8	7.4- 7.9
ε-Amino (Lys)	57	9.8	10.1-10.6
Phenolic (Tyr)	18	10.35	8.5-10.9
Guanidine (Arg)	22	>12	11.9-13.3

* Assumed molecular wt. of albumin, 65,000.

** The other proteins for which comparison data are given here include insulin, β-lactoglobulin, ovalbumin, lysozyme, and ribonuclease.

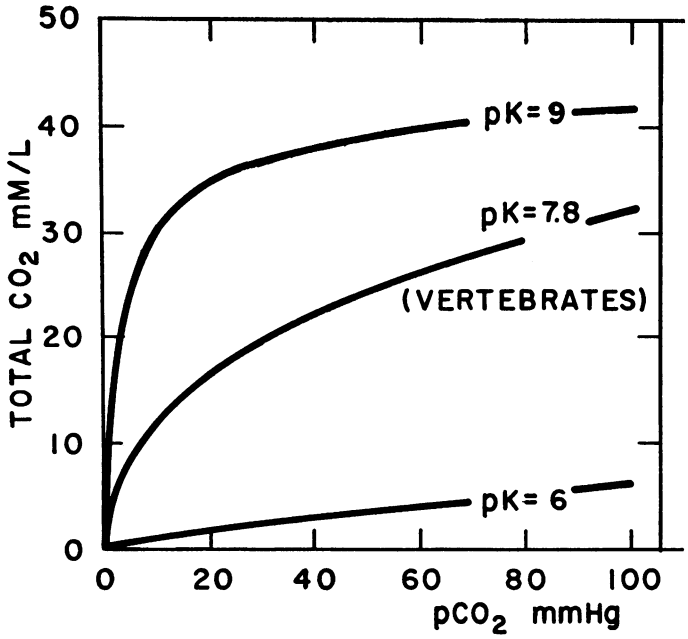


FIG. 4. Comparison of calculated CO_2 titration in a simple solution with buffer pK'$_a$ of 9.0, 7.8 and 6.0 respectively.

to ionization of α carboxyl and carboxyl groups; pK values of approximately 7-8 related to imidazole (histidyl) and αamino groups; and high pK values >8.5 related to ϵ-amino, phenolic, and guanidyl groups.

It is possible to construct the CO_2 titration curves that result from buffers with various pK values. Figure 4 shows calculated CO_2 titration curves taken from Edsall and Wyman in 0.06 M buffers with pK values of 6, 7.8, and 9. We may consider the physiological consequences of each of these curves.

Considering the lowermost curve (pK = 6.0) it may be noted that the curve, although essentially linear, has a small slope which is largely produced by an increase in dissolved gaseous CO_2. This relationship would involve several profound disadvantages. The animal would be acidotic at almost any pCO_2 value. There would be essentially no generation of HCO_3^- in response to conditions associated with increased CO_2 tensions without high concentrations of buffer with such low pKa values. Apart from the question of survival at these low pH values, the transport and excretion of metabolically generated CO_2 would pose great problems.

This may be illustrated by the following example. Consider the excretion of metabolically generated CO_2 in man.

$$\dot{V}_{CO_2} = C.O.\ (C_{\bar{v}_{CO_2}} - Ca_{CO_2}) \qquad \text{(Eq. 9)}$$

where $\dot{V}_{CO_2}$ = CO_2 production: mM/min.

C.O. = cardiac output: L/min.

$C_{\bar{v}_{CO_2}}$ = total CO_2 concentration of mixed venous blood: mM/L

$C_{a_{CO_2}}$ = total CO_2 concentration of arterial blood: mM/L

Under basal circumstances $\dot{V}_{CO_2} = 10$ mM/min.

If mixed venous pCO_2 is 47 torr and arterial pCO_2 is 40 torr, the $\bar{v}$-a ΔCO_2 concentration would be 0.4 mM/L. Therefore, basal cardiac output would be $\frac{10}{0.4} = 25$ L/min, a value five times as high as normal basal cardiac output. In order to improve blood CO_2 transport, one would require either very low arterial pCO_2 values (which implies inordinately high alveolar ventilation) or very high mixed venous pCO_2 (which implies inordinately high tissue pCO_2 values).[17] Under conditions of exercise with increased CO_2 production, the problem posed would be correspondingly magnified.

Considering the uppermost curve (pK = 9), it may be noted that the initial portion ($pCO_2 < 5$ torr) has a sharp ascending slope, but that over the remainder of the curve there is progressively less change in total CO_2 concentration. It is clear that substantial variations in CO_2 content of the solution only occur at pCO_2 values < 5 torr. The pH of such solutions would be 8.5 to 9. If such pH values were tolerable, adequate CO_2 transport and excretion might be feasible over the limited range of low pCO_2 values. However, under conditions resulting in an increase in ambient pCO_2, even aquatic forms would be faced with extreme difficulties in the transport and excretion of metabolically generated CO_2. Air-breathing forms would require inordinately high alveolar ventilation or inordinately high cardiac output (or both) to provide adequate CO_2 transport and excretion.

The middle curve (pK = 7.8), which closely resembles vertebrate CO_2 titration curves, provides both for adequate buffering of H_2CO_3 and for CO_2 transport and excretion over the entire vertebrate pCO_2 range without unduly elevated cardiac output or alveolar ventilation.

This analysis indicates that the original aquatic vertebrates would not have been able to survive without an adequate concentration of buffers of pK of at least 7. Moreover if available buffers had a pK substantially greater than 8, then aquatic animals could only have survived with very

low pCO_2 values and the transition to air-breathing forms would have been virtually impossible.*

Indeed, it may be speculated that the development of oxidative phosphorylation of carbon-containing compounds with its attendant CO_2 production and H_2CO_3 generation ($pK'_a = 6.1$) required the presence of buffers with pK values in the range of 7-8. Since these buffers would produce pH values of about this magnitude in solutions containing them, the basis for the presently existing pH values of vertebrate extracellular fluid may have been established.

The relationship between pCO_2 and HCO_3^- in any animal should be predictable given the values of $\frac{1}{\alpha CO_2}$, $\frac{K_B}{K_{H_2CO_3}}$, the total concentration of buffer, and the difference in concentration between fixed cations and anions (Eq. 8). Since temperature has an important influence on the values of some of these parameters, it is pertinent to consider the effect of temperature change on CO_2 titration curves. The value of $\frac{1}{\alpha CO_2}$ and $\frac{K_B}{K_{H_2CO_3}}$ are primarily influenced by changes in temperature. αCO_2 falls with a rising temperature and assuming that imidazole represents the major blood buffer, $\frac{K_B}{K_{H_2CO_3}}$ increases with increasing temperature. As a result, the amount of HCO_3^- generated at any pCO_2 decreases with increasing temperature. Figure 5 shows the calculated effect of changing temperature on the CO_2 titration curve obtained on 0.06 M imidazole solution from 0° to 40°C. in a system where all HCO_3^- is derived from buffering of H_2CO_3. It may be noted that the general shape of the curve is unaffected by temperature; that as temperature increases, less HCO_3^- is generated at any pCO_2 and that the difference in HCO_3^- concentration as a function of temperature increases with increasing pCO_2s. It appears that as homothermic vertebrates with relatively high body temperatures evolved, the simple chemical

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* A very large increase in concentration of buffer compounds with pK 6.0 might accomplish CO_2 transport; however, this would involve major changes in osmotic relations as well as in protein structure.

The elegant unitary nature of these relationships is demonstrated by the fact that CO_2 transport in tissues and in respiratory exchange units not only depends on the shape of the CO_2 titration curve but also is facilitated by the isohydric shift; that is, it depends on the fact that oxygenated hemoglobin is a stronger acid than reduced hemoglobin. In the lung, the shift in pH as hemoglobin becomes oxygenated provides H^+ which combines with red cell HCO_3^-: $H^+ + HCO_3^- \rightleftarrows H_2CO_3 \rightleftarrows CO_2 + H_2O$. This provides CO_2 for diffusion from the animal to the ambient environment. It is generally accepted that the isohydric shift is related to changes in the ionization of histidyl residues. Thus, the same amino acid accounts for both the favorable shape of the CO_2 titration curve and for providing adequate CO_2 for respiratory excretion.

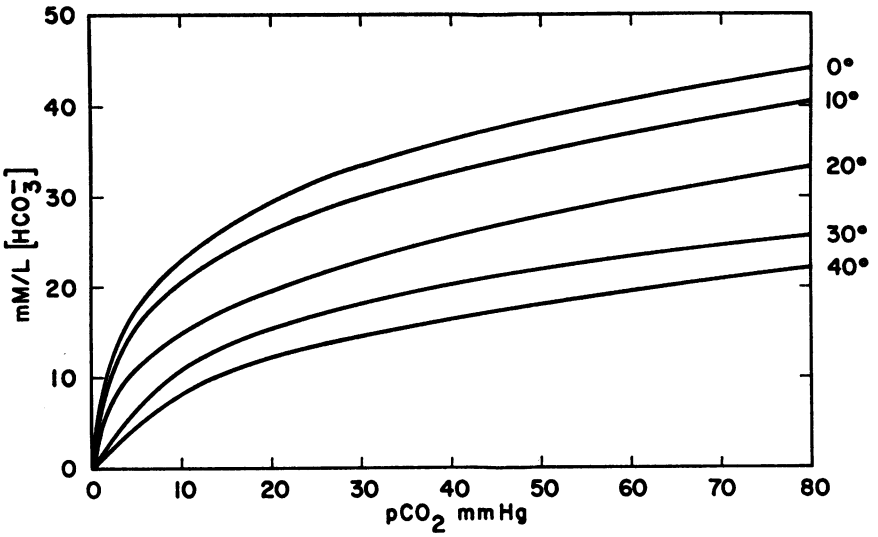

FIG. 5. The effect of changes in temperature from 0°C to 40°C on calculated CO_2 titration curves found in an 0.06 M 4-methyl imidazole solution in which the HCO_3^- present results from buffering of H_2CO_3.

buffering of H_2CO_3 became relatively less efficient than was true in cold blooded animals and gave rise to the requirement for additional regulatory mechanisms involving the relationship between H_2CO_3 and HCO_3^- in body fluids.

Consideration of the quantitative aspect of HCO_3^- generation from CO_2 in living forms suggests that any CO_2 titration curve can be separated into two components. One component is, of course, the HCO_3^- generated by buffering of protons within the organism. The other component is the addition (or subtraction) of HCO_3^- produced by regulatory mechanisms or metabolic changes. This separation can be quantitatively accomplished by the Edsall-Wyman analysis. For example, the calculated changes produced in the CO_2 titration curve on a solution of 0.06 M 4-Me imidazole by the initial addition or removal of $NaHCO_3$ are shown in Figure 6. It may be seen that such changes do not fundamentally alter the shape of the curve but change the concentration of HCO_3^- found at any pCO_2. Regulatory changes involving HCO_3^- are usually produced by renal mechanisms. Renal regulatory mechanisms which serve to augment simple buffering of H_2CO_3 may well have become of great importance as vertebrates became air breathers and as relatively high body temperatures became an important feature of vertebrate existence.

In summary, the shape of blood CO_2 titration curves which determines effectiveness in CO_2 transport and excretion is largely determined by the

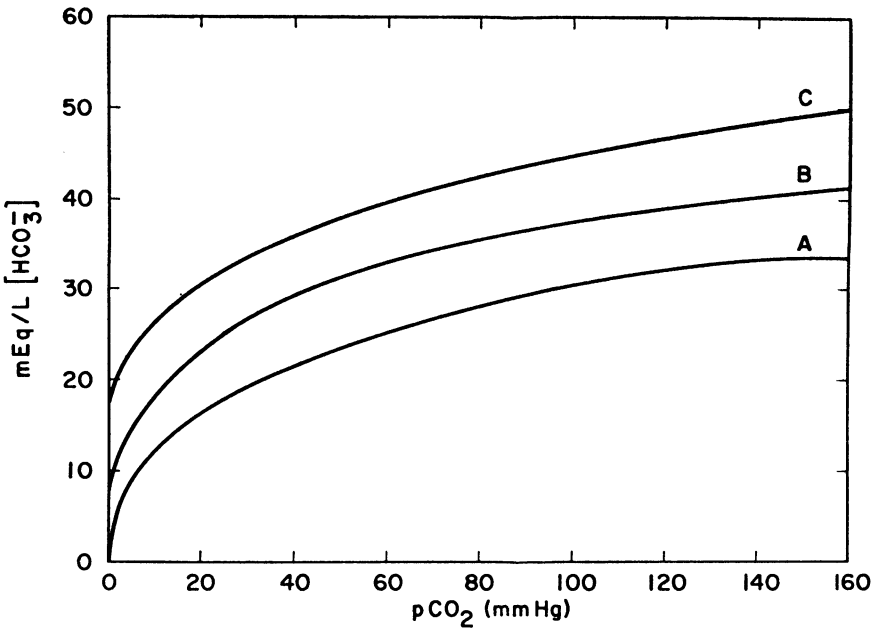

FIG. 6. The effect of adding or removing $NaHCO_3$ on calculated CO_2 titration curves in an 0.06 4-methyl imidazole solution. A. 10 mM/L HCO_3^- removed. B. No HCO_3^- added; all HCO_3^- related to buffering of H_2CO_3. C. 10 mM/L added. Such additions or losses of HCO_3^- would occur by regulatory processes in intact biological systems. $K_B = 10^{-7.4}$ M/L; $K_{H_2CO_3} = 10^{-6.1}$ M/L; $\alpha = 3 \times 10^{-2}$ mM/L/mm.

pK of blood buffer systems. The absolute values of HCO_3^- produced at equilibrium at any CO_2 tension depend upon a number of additional factors (temperature, metabolic regulation) none of which greatly affect the shape. The survival of vertebrates required adequate concentrations of buffers with pK values ~ 7 which are primarily found in proteins with imidazole residues and free α amino groups. The similarity of CO_2 titration curves in existing vertebrates suggests that this common pattern of buffer protein composition is an evolutionary heritage occurring early in vertebrate or even pre-vertebrate development.

TEMPERATURE—pH RELATIONSHIPS IN VERTEBRATES

Temperature is one of the most familiar and important physico-chemical variables. The evolution of vertebrates involved a change from poikilothermic animals whose body temperature varied with the ambient temperature to homothermic animals with a relatively constant and relatively high body temperature. As a consequence, the body temperature of existing vertebrates spans a range of approximately 0° to 42°C.

The relationship between temperature and pH is complex from the physical-chemical standpoint. The thermodynamic basis of pH measurement does not strictly permit the comparison of pH values obtained at different temperatures since,

$$E_T = E^\circ - \frac{RT}{F} \ln aH^+ \qquad \text{(Eq. 10)}$$

where E_T = measured potential produced by a given H^+ activity (aH^+) at temperature T
E° = ground state potential
R = Universal gas constant
T = absolute temperature
F = Faraday

Since E° must be arbitrarily defined at any temperature, the absolute value of E_T cannot be precisely determined.[18] However, as indicated above, biological systems do present the problem of interpretation of pH values obtained at various temperatures.

Early studies in this area involved *in vitro* measurements of blood pH as a function of temperature. It was demonstrated that for mammalian blood, a rise of 1°C. was associated with a fall in measured pH of 0.014 pH units.[19] A more recent study of the effect of changes in temperature in a poikilothermic vertebrate, the pond turtle (*P. Scripta elegans*) showed that the same relationship between temperature and pH $\frac{\Delta pH}{°C} = -0.014$ was not only true of blood whose temperature was manipulated "in vitro" but was likewise true of the blood of the animal as it existed "in vivo" at different temperatures.[20] These studies were extended by Rahn who noted that the same relationship held for a variety of poikilothermic vertebrates including fish, amphibia, and reptiles. Of particular note was that at a temperature of 37°C., the blood pH of these animals was approximately 7.4, a value similar to the pH of homothermic man.[21]

In attempting to define the meaning of these observations, Rahn noted that the relationship between pH and temperature in the various species studied could be described by a line whose slope was equal to that of the line defining the relationship between temperature and pN where pN = ½ pK_w and K_w is the dissociation constant of H_2O. Since pOH = pK_w − pH, a plot of pOH against temperature produced a line that was likewise parallel to the pN line (Fig. 7). On the basis of this analysis he suggested that biological regulatory mechanisms for pH operated to maintain a constant $\frac{H^+}{OH^-}$ ratio at various temperatures.[21]

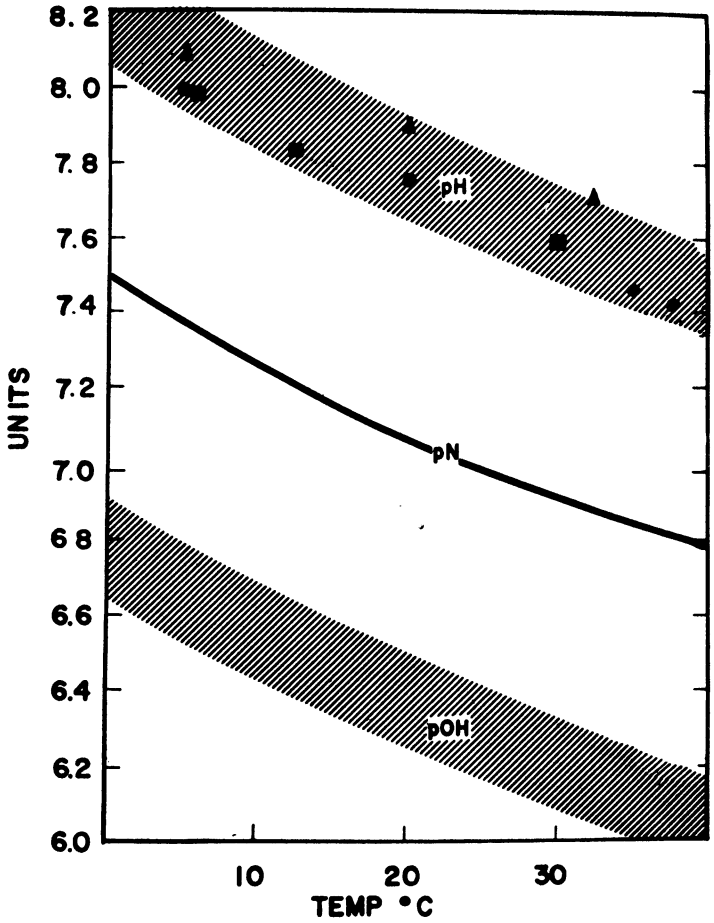

FIG. 7. Relationship between changes in blood pH and temperature in a number of vertebrate species obtained by Rahn.[23] Note the approximately parallel pH, pN and pOH lines.

Albery and Lloyd have pointed out that this temperature-pH relationship can be duplicated by a buffer system with a pK approximately one half that of water.[22] Their derivation is as follows:

From well-known thermodynamic considerations,

$$\frac{\partial \ln K_A}{\partial T} = \frac{\Delta H_A}{RT^2} \qquad \text{(Eq. 11)}$$

For water,

$$\frac{\partial \ln K_w}{\partial T} = \frac{\Delta H_w}{RT^2} \qquad \text{(Eq. 12)}$$

Since $K_w \cong 10^{-14}$ and $\Delta H_w \cong 14$ K cal/mole,

$$\frac{\ln 10^{-14}}{T} \cong \frac{14}{RT^2} \qquad \text{(Eq. 13)}$$

If we now consider a buffer with a K of approximately 10^{-7}, we see that its ΔH must be approximately 7.

Given the mathematical description of Rahn's data as:
log $[H^+]$ = ½ log K_w+ constant over the vertebrate temperature range,

$$\frac{\ln [H^+] \text{ blood}}{T} = \tfrac{1}{2} \frac{\ln K_w}{T} = \frac{\Delta H_w}{2RT^2} \qquad \text{(Eq. 14)}$$

$$\frac{\ln [H^+] \text{ blood}}{T} = \frac{\ln K_a}{T} = \frac{\Delta H_A}{RT^2} \qquad \text{(Eq. 15)}$$

Thus, if $\Delta H_A \cong \tfrac{1}{2} \Delta H_w$, $\Delta H_A = \tfrac{1}{2} \times 14$ Kcal/mole = 7 Kcal/mole, and pK_A must $\cong$ 7.

It therefore requires only the presence of a buffer system with a pK_A of 7 to give rise to the observed relationship. Since it has been shown above that proteins with a pK 7 are responsible for the vertebrate CO_2 titration curve, it seems reasonable that these proteins are likewise responsible for the observed relationship between temperature and pH in vertebrates.

To test this hypothesis, pH was measured as a function of temperature in a number of simple solutions. These included protein-free buffered fluid (urine); 5% Bovine Serum Albumin (BSA), and 5% BSA with 0.025 M $NaHCO_3$ equilibrated with 5% CO_2.[23]

The values found are superimposed on the data obtained by Rahn (Fig. 8). It may be seen that the protein-free fluid does not exhibit the relationship between pH and temperature found in vertebrate blood. However, a solution containing 5% BSA alone has properties qualitatively similar to those described by Rahn. Solutions containing 5% CO_2—.025 M $NaHCO_3$—5% BSA qualitatively and quantitatively reproduce the Rahn relationship between pH and temperature.

It should be pointed out that in homothermic vertebrates plasma pH may be regulated at a "constant" value in the face of changes in temperature. This is suggested by the finding that hibernating mammals maintain an arterial plasma pH of 7.4 (measured at body temperature) despite a sharp decrease in body temperature.[24] This evidence of additional regulatory mechanisms may be considered analogous to the ability of renal mechanisms in some vertebrates to modify the blood CO_2 titration curve.

The precise physiological function served by the temperature-pH relationship is not clear. Nor is it clear whether this relationship represents

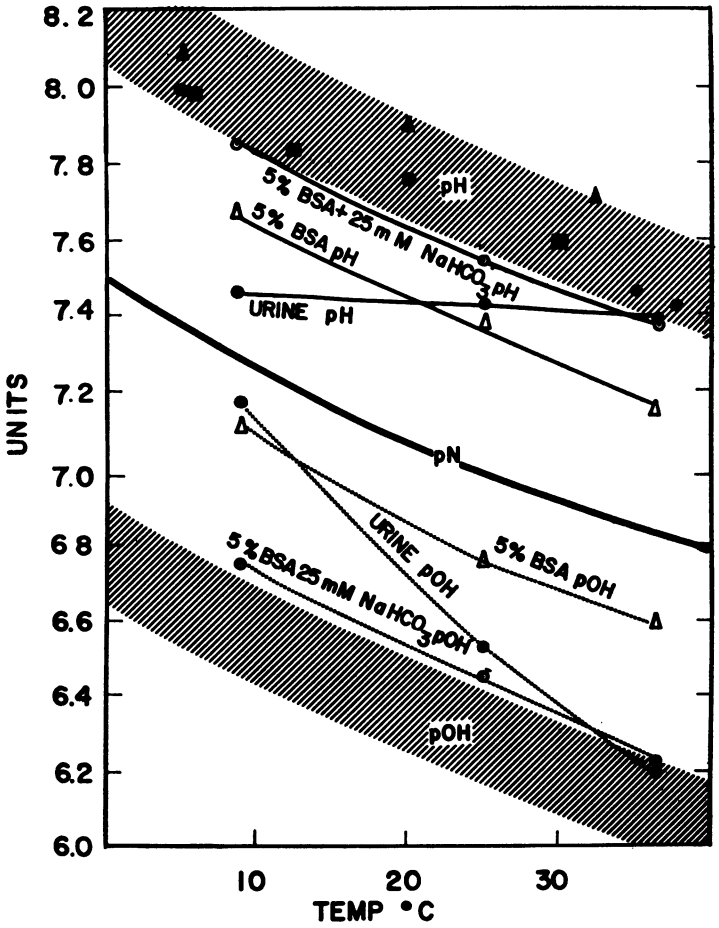

FIG. 8. The relationship between pH, pN and pOH in various solutions. Note that the 5% beef serum albumin (BSA) gives a qualitatively consistent relationship like that of vertebrate blood and that a solution with 5% BSA plus 0.025 M HCO_3^- gives a relationship indistinguishable from the *in vivo* pattern found in vertebrates.

(as proposed by Rahn) active "defense" of H^+/OH^- ratios or whether it represents a physical-chemical "happening." However, it appears that the relationship depends ultimately on the presence of suitable proteins carrying groups with a pK of ~7. It thus appears likely that as in the case of the CO_2 titration curve, this common protein pattern developed early in vertebrate evolution (or in pre-vertebrate forms) and is responsible for the common relationship between temperature and pH found in blood of many existing vertebrates.

SUMMARY

The concept that pathways of energy transduction and protein structure are fundamental determinants of biological function is scarcely new. The present studies outline the specific operation of these factors with respect to some aspects of the evolution of acid-base regulation in vertebrates. The development of electron chain O_2 consumption resulted in substantial intracellular CO_2 generation. As a result the H_2CO_3-HCO_3^- system became important and the problem of CO_2 disposal developed. Early vertebrates were aquatic gas exchangers and, therefore, must have had low CO_2 tensions, low HCO_3^- concentrations and relatively high non HCO_3^- anion concentrations in extracellular fluid. The transition to a terrestrial life with attendant air breathing resulted in high CO_2 tensions, high HCO_3^- concentrations and lower non HCO_3^- anion concentrations in extracellular fluid. Two important aspects of acid-base metabolism, the CO_2 titration curve and the relationship between temperature and blood pH, appear to be similar in existing vertebrates. The similarity is based on appropriate concentrations of protein buffers with pK values ~7. It is suggested that this similarity is based on a common protein pattern that developed in the earliest vertebrates or even in earlier biological forms.

ACKNOWLEDGMENT

We are grateful to Sir Hans Krebs and Professor Hermann Rahn for advice and assistance with the development of the concepts presented in this paper.

REFERENCES

1. Florkin, M., Ed.: *Aspects of the Origin of Life.* International Series of Monographs of Pure and Applied Biology. Oxford, Pergamon Press, 1960.
2. Urey, H. C.: Primitive planetary atmospheres and the origin of life. In Reference 1, pp. 8-14.
3. Bloch, K.: Oxygen and biosynthetic patterns. *Fed. Proc.,* 1963, *21,* 1058-1063.
4. Calvin, M.: Evolution of enzymes and the photosynthetic apparatus. In Ref. #1, pp. 124-131.
5. Berrill, N. J.: The *Origin of Vertebrates.* Oxford, Clarendon Press, 1955.
6. The quantitative relationship between $P_{\bar{E}_{O_2}}$ and $P_{\bar{E}_{CO_2}}$ in presently existing aquatic forms was originally formulated by Rahn[7] and extended by this laboratory.[8]
7. Rahn, H.: Aquatic gas exchange: Theory. *Respiration Physiology,* 1966, *1,* 1-12.
8. Robin, E. D. and Murdaugh, H. V.: Quantitative aspects of vertebrate gas exchange. In, *Development of the Lung,* edited by A. V. S. de Reuck and R. Porter. New York, Little Brown & Co., 1967, pp. 85-98.
9. Smith, Homer, W.: *From Fish to Philosopher.* Summit, N. J., CIBA Pharmaceutical Production, Inc., 1959.
10. Cross, C. E., Packer, B. S., Linta, J. M., Murdaugh, H. V. Jr., and Robin, E. D.: H^+ buffering and excretion in response to acute hypercapnia in the dogfish *Squalus acanthias. Amer. J. Physiol.,* 1969, *216,* 440-452.
11. Brackett, N. C., Jr., Cohen, J. J., and Schwartz, W. B.: Carbon dioxide titration curve of normal man: Effect of increasing degrees of acute hypercapnia on acid-base equilibrium. *New Engl. J. Med.,* 1965, *272,* 6-12.
12. Cohen, J. J., Brackett, N. C., Jr., and Schwartz, W. D.: The nature of the carbon dioxide titration curve in the normal dog. *J. clin. Invest.,* 1964, *43,* 777-786.

13. Christiansen, J., Douglas, C. G., and Haldane, J. S.: The absorption and dissociation of carbon dioxide by human blood. *J. Physiol.*, 1917, *48*, 244-271.
14. Henderson, L. J.: *Blood: A Study in General Physiology.* New Haven, Yale University Press, 1928.
15. Edsall, J. T. and Wyman, J.: *Biophysical Chemistry: Vol. I.* New York, Academic Press, Inc., 1958, p. 562.
16. Tanford, C., Swanson, S. A., and Shore, W. S.: Hydrogen ion equilibrium of bovine serum albumin. *J. Amer. chem. Soc.*, 1955, *77*, 6416-6468.
17. Robin, E. D.: Intracellular CO_2 tension: Practice and theory. *Quart. J. Med. Col. Virginia*, 1967, *3*, 189-196.
18. Bates, R. C.: *Determination of pH. Theory and Practice.* New York, J. Wiley and Sons, Inc., 1964.
19. Rosenthal, T. B.: The effects of temperature on the pH of blood and plasma in vitro. *J. Biol. Chem.*, 1948, *173*, 25-30.
20. Robin, E. D.: Relationship between temperature and plasma pH and carbon dioxide tension in the turtle. *Nature*, 1962, *195*, 249-251.
21. Rahn, H.: Gas transport from the external environment to the cell. In, *Development of the Lung* (see Ref. 8), pp. 3-22.
22. Albery, W. J. and Lloyd, B. B.: Variation of chemical potential with temperature. In, *Development of the Lung* (see Ref. 8), pp. 30-33.
23. Unpublished observations of Bromberg, P. A., Murdaugh, H. V., Jr., and Robin, E. D.
24. Kent, K. M. and Pierce, E. C.: Acid-base characteristic of hibernating animals. *J. Appl. Physiol.*, 1967, *23*, 336-340.
25. Some readers may be interested in the following letter from an observer who somehow obtained a pre-publication copy of this manuscript:

March 20, 1969

Eugene D. Robin, M.D.
Department of Medicine
University of Pittsburgh
School of Medicine
Pittsburgh, Pennsylvania 15213

Dear Dr. Robin:

I read with considerable interest your speculation on the basis for the number 7 as the blood pH value for the vertebrate phylum.

It is My pleasure to inform you of the real reason for this designation. It actually resolved upon My choice of the bicarbonate buffer system for protoplasm and its inherent pK of 6.1. I was sort of hung up on this number six since it was the day on which I rested after making the world.

Transformation to 7 was an accident emerging from a later selection of a logarithmic system to the base 10.

I hope that this explanation will be helpful to you in your work.

Sincerely,
G.
God