EVALUATION OF STEWART'S MODEL FOR ACID-BASE BALANCE IN PATIENTS WITH DIABETIC KETOACIDOSIS

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DECLARATION

I declare that this Research Report is entirely my own work, except where otherwise indicated in the text or references. This work has not been submitted for any degree or examination at any other university or institution.

This study has been approved by the Committee for Research on Human Subjects of the University of the Witwatersrand.

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J. E. Paiker Date

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I am indebted to my husband Dr. David Rubin for introducing me to Stewart's model and encouraging me to undertake this project many years ago, when the model was almost unknown in the medical literature. I am also grateful that in view of his knowledge in this field, he agreed to act as a co-supervisor for this project at my departments request. As he has subsequently become my spouse, he has formally requested the postgraduate committee to excuse him from involvement in any aspect of the examination process.

I am also indebted to Dr Rowe for being the other co-supervisor of this project. Dr Rowe's encouragement, as well as her experience and knowledge of research methodology proved to be invaluable in the execution of this endeavour.

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ABSTRACT

The importance of the regulation of pH in body fluids is well established, and measurements in this regard are made routinely in a number of clinical situations.

In order to fully appreciate the nature of an acid-base disorder, a precise understanding of the determinants of hydrogen ion concentration is required.

Unfortunately, current physiological teaching tends to be over simplified, and does not incorporate fundamental physico-chemical aspects of aqueous solutions, which are essential for a thorough understanding of the subject.

This deficiency was addressed by Stewart who analysed complex aqueous solutions and proposed a model based on sound chemical theory and a number of simplifying assumptions.

The model accounts for the simultaneous influences of the important variables affecting hydrogen ion concentration, rather than using a single equation (Henderson Hasselbalch for CO_2) to explain acid base equilibria.

Stewart's model differentiates between **dependant** variables such as hydrogen, hydroxyl and bicarbonate ions, and **independent** (controllable) variables namely the partial pressure of carbon dioxide pCO_2, the concentration of net ionic charge of strong electrolytes known as the strong ion difference SID, and total weak acids A_{tot}. The model goes on to predict the relationships between all these variables such that any dependant variable can be calculated uniquely for measured or postulated values of the three independent variables.

To establish whether this model could account for all factors influencing acid-base changes in a clinical setting it was decided to study patients with Diabetic Ketoacidosis, as the biochemistry of this disorder is well known. Arterial and venous blood was collected from twenty patients with the diagnosis of Diabetic Ketoacidosis as part of their routine management. Hydrogen ion concentrations and the concentrations of the independent variables were measured, and the results of measured hydrogen concentrations and predicted hydrogen ion concentrations based on Stewart's model using measured independent variables were compared.

Extreme discrepancies were found between the measured hydrogen ion concentrations and those calculated using Stewart's model in this patient group. As the model is able to accurately predict hydrogen ion concentrations from simple solutions, the reasons for the discrepancies in complex biological solutions remain uncertain. The two major areas which may account for these changes are accuracy of measurement and protein chemistry. In conclusion, the sound chemical and thermodynamic basis of Stewart's model makes it likely to be correct in principle, however further research is essential to elucidate the specific causes of discrepancy between theory and measurement in the clinical setting.

TABLE OF CONTENTS

INTRODUCTION AND LITERATURE REVIEW

Acid-base balance in physiological solutions has been studied for many decades, and while a number of controversies still exist regarding exact mechanisms of regulation, there is general consensus on most aspects of the problem in the orthodox physiological and medical literature. In fact, much of the theory currently promulgated in acid-base literature was developed in the 1920's and has undergone relatively little change since then.

Unfortunately, all is not well with the conventional view on the subject, and the late 1970's saw the emergence of a new theory produced by P.A. Stewart, a biophysicist at Brown University [1].

Stewart recognized that the problem of acid-base balance must be analysed in terms of modern thermodynamic theory of aqueous solutions, and subsequently proceeded to develop a model which accounts for the theoretical inconsistencies of the currently held views [2].

About one and a half decades have passed since Stewart published his model, and, while there has been a gradual increase in the number of citations of Stewart's work, there is still no broad recognition of its validity and applicability. The reasons for this are numerous, however the most likely are:

1) Stewart's model is essentially quantitative, and the biological and medical sciences have until recently been more descriptive, resulting in very few researchers who are able to assess the model.

2) There is a very vocal core of researchers whose work would be invalidated or at least require substantial modification if Stewart's view were to prevail.

3) Experimental validation of Stewart's model presents a number of intimidating obstacles in terms of the precision and accuracy required in measuring the determining analytes.

Notwithstanding the above, Stewart's approach to acid-base balance in physiological solutions represents the most elegant and theoretically consistent view of the subject to date, and a small but consistent group of researchers have supported his view.

Summary of the Conventional View of Acid-Base Physiology

The traditional view holds that in the course of normal metabolism approximately 20 000 - 25 000mmol of carbonic acid (H_2CO_3) is generated but due to the equilibrium $H_2CO_3 \leftrightharpoons H_2O + CO_2$ this acid component is exhaled via the lungs as carbon dioxide. A further 50-100 mmol of H^+ is produced daily as non-volatile acids as a result of catabolism of proteins and the incomplete oxidation of carbohydrates and lipids. These non-volatile acids are normally excreted via the kidneys. The kidneys are thought to play a further role in acid -base homeostasis by means of the excretion of bicarbonate.

The maintenance of pH within a narrow range is ascribed to buffering. Two mechanisms are thought to play a major role in this regard. The first is metabolic reactions which preserve homeostasis by changing their rates in response to acid-base disturbances and in so doing

either consume or produce protons or its equivalents namely OH^- and HCO_3^-. The second mechanism is due to proton pumping or the transport of H^+ or its equivalents across membranes and in so doing allowing cytosolic pH to be maintained at a much higher level than plasma.

For many years it has been proposed that the understanding of the Henderson-Hasselbach reaction is central to the understanding of acid -base physiology. This equation relates the pH of the buffer to the concentration of the buffer acid and the buffer base.

$$pH = pK + \log\frac{acid}{base}$$

As the bicarbonate buffer system is thought to play a major role in maintaining pH, the above equation can be written as follows.

$$pH = 6.1 + \log\frac{[HCO_3^-]}{pCO_2 \times 0.03}$$

where the denominator of the log term represents the concentration of dissolved carbon dioxide written as the partial pressure of carbon dioxide multiplied by its solubility coefficient in blood.

As the proton pump in the distal renal tubular cells is only able to maintain a gradient of 1:1000 across the cell membrane it was proposed that urinary buffers, ammonia which is

produced from glutamine in the distal renal tubular cells and phosphate, bind hydrogen ions which allow excretion of H^+ ions to proceed.

The presumed reactions in the renal tubules relating to ammonium and phosphate are:

$$NH_3 + H^+ \leftrightharpoons NH_4^+$$

$$HPO_4^- + H^+ \leftrightharpoons H_2PO_4$$

More recently it was thought that these theories regarding the buffer mechanisms were erroneous as it was found that the glutaminase reaction occurs mainly in the proximal tubule and that the pK of the reaction $NH_3 + H^+ \leftrightharpoons NH_4^+$ is 9.03 at physiological pH in which case ammonium is produced which does not allow for the excretion of hydrogen ions.

Halperin and Jungas [3,4,5] proposed the following explanation for the excretion of hydrogen ions by the renal tubules taking the above mechanisms into account:
The metabolism of glutamine in the proximal renal tubule yields two molecules of ammonium and two molecules of bicarbonate by the formation of oxo-glutarate via the gluconeogenic and tricarboxylic acid pathways. Bicarbonate is subsequently returned to the blood while the ammonium is lost in the urine, resulting in a nett loss of hydrogen ions.

Atkinson and Bourke[6,7] proposed an alternative view which states that the liver is thought to be the main organ responsible for acid -base regulation. The mechanisms by which the liver fulfills this function is thought to be: 1. via amino acid catabolism with the consequent

production of bicarbonate and ammonium ions and 2. the consumption of bicarbonate during the ureagenesis cycle.

Ureagenesis would therefore be inhibited in the presence of metabolic acidosis and stimulated in the presence of a metabolic alkalosis thereby maintaining physiological pH balance.

From the above, it is evident that current physiological acid-base theory is inadequate to account for the complexities of biological solutions.

REVIEW OF STEWART'S MODEL

With the greater availability of computers in the 1970's, it became possible and practical to deal with a large class of mathematical and physical problems, which had previously proved too complex for ready manipulation.

It was primarily this factor, combined with his difficulty with the rationale behind the conventional approach to physiological acid-base balance, that prompted Peter A. Stewart, a Biophysicist at Brown University, to re-examine the subject of acid-base balance, and to challenge the conventional viewpoint. His work led to a novel approach to the subject which is based on modern chemical and thermodynamic principles of solution chemistry [1,2].

In order to understand Stewart's model, it is first necessary to review a few basic aspects of aqueous solutions which Stewart used as his starting point:

Consider first a hypothetical beaker of absolutely pure water. The aqueous solution contained therein must consist primarily of molecules of H_2O as well as some H^+ and some OH^- ions. Clearly, the origin of the H^+ and OH^- ions is due to the dissociation of water molecules. This is in fact the only reaction that occurs in pure water, and it must be recognised that this reaction will proceed to form hydrogen and hydroxyl ions which in turn recombine to form water molecules at a rate dependant on their concentrations, such that a state of dynamic equilibrium is reached where the rate of forward reaction equals the rate of the reverse reaction, and the concentrations of all three species remain constant even though both reactions continue.

$$H_2O \leftrightharpoons H^+ + OH^-$$

The point at which this equilibrium occurs is, in the case of pure water, only dependent on the temperature, and so the ratio of product to reactant at any given temperature is a constant which will be called K_w.

$$K_w = \frac{[H^+][OH^-]}{[H_2O]}$$

It should be mentioned, as pointed out by Stewart, that in accordance with thermodynamic theory, dissolved species should be described in terms of their thermodynamic activities rather than their concentrations. However, in dilute solutions, which body fluids can reasonably be considered to be, there is very little numerical difference in dealing with concentrations as compared to activities, and for this reason, the more familiar concentration units of measurement will be used throughout. It must be borne in mind however, that for a more exact treatment of the subject, activities will have to be retained [8].

Now the central question to be answered is: what determines the hydrogen ion concentration of this pure water sample at a given temperature?

The first step in answering this question is to make a simplifying assumption regarding the

water dissociation equation.

The value of K_w, while temperature dependent, is always a very small number, implying that the concentrations of H^+ and OH^- ions are about one thousand million times less than the concentration of undissociated water molecules. Changes in temperature will cause a change in the value of K_w and consequently in the concentration of H^+ (and OH^-), however even temperature changes that cause the hydrogen ion concentration to vary 10 fold or even 100 fold for that matter, will hardly change the water concentration from its value which at 37^oC is about 55.3M.

For this reason, the water concentration can be considered to be constant with negligible loss in numerical precision. This means that the equation can be simplified by multiplying the essentially constant concentration of water by K_w to give a new temperature dependent constant called K_w', resulting in the following:

$$K_w' = [H^+][OH^-]$$

This equation is still not sufficient to determine the hydrogen ion concentration for a known K_w', ie. a graph of hydrogen ion versus hydroxyl ion concentration will produce a hyperbola which implies an infinite number of possible values for H^+ with correspondingly infinite values for OH^-.

Clearly, a second equation is required to solve for two variables. This is provided by the

absolute requirement for electrical neutrality. The solution has no net charge, and thus the concentration of H^+ and OH^- ions must be identical yielding the second equation ie.

$[H^+] = [OH^-]$ or $[H^+] - [OH^-] = 0$

Clearly, the intersection of the straight line graph produced by the above equation and the hyperbola produced by the hyperbolic equation yields the solution for hydrogen ion concentration and hydroxyl ion concentration for this pure water sample. Algebraically, this can be solved as:

$$[H^+] = [OH^-] = \sqrt{K_w'}$$

Note that hydrogen and hydroxyl ion concentrations for pure water are always equal, making this a neutral solution at all times. The only remaining requirement for determining actual concentrations, is to know the numerical value of K_w' at the temperature under consideration. At a temperature of $25°C$, the value of K_w' is 1.01×10^{-14}. This implies a hydrogen and hydroxyl ion concentration of about 10^{-7}, or a pH of 7.

It is thus clear that at a different temperature (with a different K_w'), the pH of pure water would no longer be 7 even though the solution would still be neutral.

In order to make this pure water solution a little more like a body fluids, one must introduce strong ions (strong electrolytes). Strong ions are defined as ions which are always in an almost completely dissociated state in aqueous solution. Examples in physiology include sodium, potassium and chloride ions. For example, there is almost no $NaCl$ in solutions but only

dissociated Na^+ and Cl^-.

The importance of strong ions in terms of acid base behaviour is that they add new terms to the electrical neutrality equation. As an example, one could imagine an aqueous solution containing only sodium and chloride (not necessarily in the same concentrations). This again requires two equations to describe this solution, namely, the equation as before in the pure water case $K_w' = [H^+][OH^-]$, and a new electrical neutrality equation incorporating all the ions ie:

$$[Na^+]-[Cl^-]+[H^+]-[OH^-] = 0$$

Once again, this electrical neutrality equation implies that the total anion concentration is equal to the total cation concentration, and real solutions may require that other ions such as potassium are considered.

For convenience, one may group the difference between the concentrations of strong cations and strong anions into a single term called the strong ion difference abbreviated $[SID]$. ie. In this example let $[SID] = [Na^+]-[Cl^-]$

The two equations describing this system then become:

$$[SID]+[H^+]-[OH^-] = 0$$

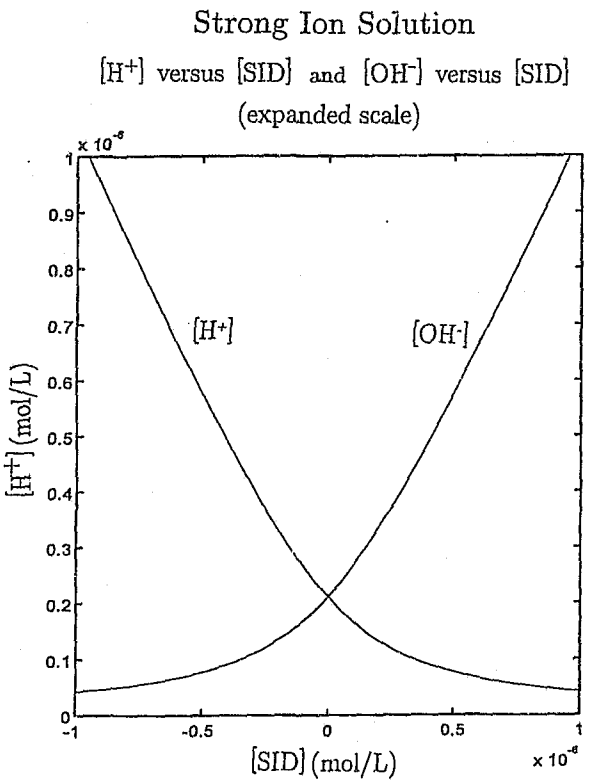
$$K_w' = [H^+][OH^-]$$

The algebraic solution of the two simultaneous equations is a quadratic equation which can be solved explicitly for $[H^+]$ and $[OH^-]$ as follows:

$$[H^+] = \sqrt{K_w' + ([SID]/2)^2} - ([SID]/2)$$

$$[OH^-] = \sqrt{K_w' + ([SID]/2)^2} + ([SID]/2)$$

A graphical representation shown below [1,12] (in this case at an arbitrary temperature of 44°C) of these equations shows that when strong cation and strong anion concentrations are equal, the solution is again neutral and identical to the pure water case (except for a small effect of osmolality on K_w' which can be ignored with very little effect numerically). When the $[SID]$ becomes positive, the OH^- concentration rises sharply while the H^+ concentration tends towards zero. The opposite is true for negative $[SID]$ values, where the H^+ concentration rises sharply and the OH^- concentration tends towards zero. The sodium and chloride ions can be added to the solution as sodium hydroxide and hydrochloric acid respectively.



Stewart points out that it is not the addition of the weak hydroxyl and hydrogen ions that changes the resulting concentrations, but rather the strong ions that are added at the same time as these weak ions namely sodium and chloride ions in this example that determine the hydrogen ion concentration. This indicates again that hydrogen and hydroxyl ions are dependent on the strong ions (independent variables) in solution, and their addition to solutions cannot in itself bring about a change in their resulting concentrations. (It should be noted that ionic species cannot be introduced on their own without either removing an equal number of like charges or adding an equal number of opposite charges such as in the case of *NaOH* or *HCl*).

The confusion is compounded by the apparent rise in H^+ concentration by x mol per unit volume when adding x mol of hydrogen ions in the form of *HCl*, and the equivalent effect on

OH^- by adding $NaOH$, however this apparent one to one correspondence of hydrogen and hydroxyl ions by the addition of hydrochloric acid and sodium hydroxide respectively is erroneous. Examination of the quadratic equation for hydrogen ion concentration shows that the response of the H^+ concentration becomes very linear against $[SID]$ when $[SID]$ is very negative but this correspondence is lost as $[SID]$ approaches to zero. An equivalent effect for OH^- at negative $[SID]$ values can be seen by examining the quadratic equation for hydroxyl ions.

In order to simulate plasma or other body fluids more closely, it is necessary to introduce weak acids into the system, traditionally classed amongst the so called buffers. Weak acids are distinguished by their partial dissociation in aqueous solution, in contrast to strong acids such as HCl which dissociate fully to yield strong ions. Weak acid dissociation can be represented by the following chemical reaction:

$$HA \leftrightharpoons H^+ + A^-$$

This can be represented quantitatively as:

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

where K_a represents the dissociation constant for the weak acid under consideration, and is largely temperature dependent.

The most numerically important weak acids in biological solutions are the proteins, while weak acids such as phosphates are usually present in very small concentrations and can usually be ignored with very little loss in precision. If one considers the thousands of proteins present in blood plasma, the problem at first sight appears daunting because a different value for K_a must be found for each protein. Stewart proposed that in the physiological range of concentrations, the plasma proteins have K_a values which are sufficiently close to each other, that a single lumped representative value for K_a can be assumed without much loss in numerical precision. Even though this procedure may not be strictly valid, it remains a valuable concept in explaining the model, and additional proteins can always be taken into account as and when required [9,10,11].

The effect of adding this hypothetical single weak acid in the form of a total protein value is to introduce an additional anion into the electrical neutrality equation, namely A^-, and due to the new anion, the electrical neutrality equation becomes:

$$[SID]-[A^-]+[H^+]-[OH^-] = 0$$

A second mathematical equation results from the physical requirement that the total amount of weak acid A_{tot} added to the solution must remain constant even though some of the molecules will be in dissociated form as H^+ and A^-, and some will be undissociated HA. This equation of conservation of total weak acid is:

$$[HA]+[A^-] = [A_{tot}]$$

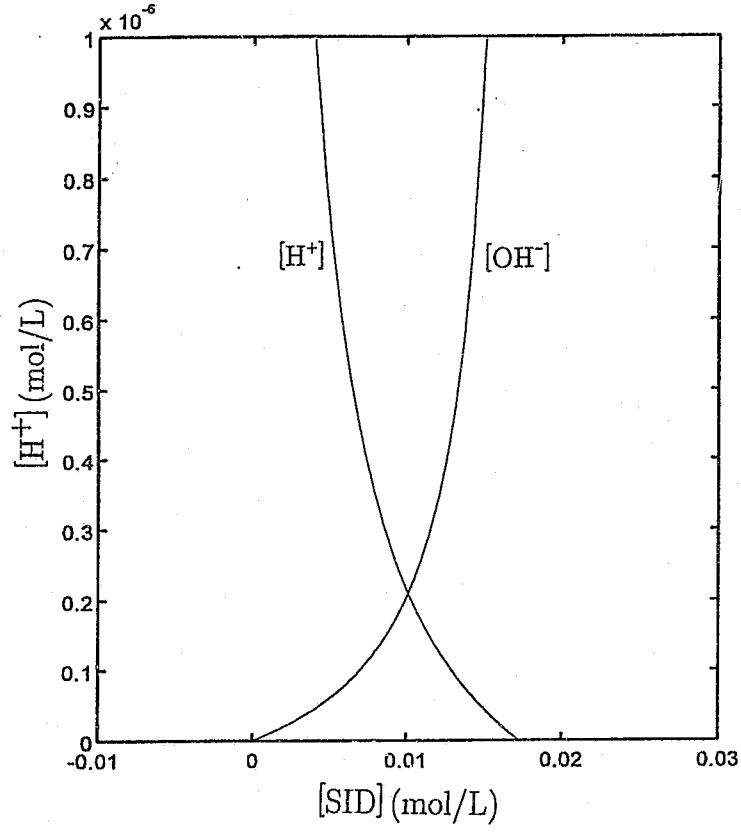
When the three new equations resulting from weak acid in solution are considered together with the additional requirement for water dissociation namely $K_w' = [H^+][OH^-]$, and solved simultaneously, the result is a third order polynomial in $[H^+]$

$$[H^+]^3+([K_a]+[SID])[H^+]^2+([K_a]([SID]-A_{tot})-K_w')[H^+]-K_aK_w' = 0$$

The effect of adding a weak acid is to shift the neutrality point to a positive non-zero $[SID]$ value, ie. The $[SID]$ value at which H^+ and OH^- concentrations are equal now occurs at a positive $[SID]$ value rather than an $[SID]$ of zero as was the case for pure water and aqueous solutions with strong ions only. In addition to this, the rate of change of $[H^+]$ and $[OH^-]$ with respect to $[SID]$ is greater than in the pure water and strong ion case for the range under consideration in physiology. This can be seen by inspecting the gradients of the plots of the resulting equations and comparing them to the strong ion case. This is surprising when one considers that buffers are traditionally said to decrease this rate of change. Stewart's model shows that this only happens in specific regions of the $[SID]$ range which are usually not of interest in physiology.

Strong Ion – Weak Acid Solution

$[H^+]$ versus [SID] and $[OH^-]$ versus [SID]



At this point it should be noted that weak bases can be analysed in an equivalent way, however they are usually not present in physiological solutions in appreciable quantities and can thus usually be ignored. One example of an exception to this is the presence of the ammonia/ammonium ion equilibrium in urine in appreciable quantities.

One further solute is needed to realistically simulate biological fluids in general and plasma specifically, namely carbon dioxide. The concentration of CO_2 is determined by the rate of metabolism and the rate of elimination by the lungs. CO_2 concentrations are usually expressed

in terms of an equivalent interfacial gas phase partial pressure which would maintain the dissolved CO_2 concentration in question at equilibrium. Typically, in healthy human arterial plasma, CO_2 is maintained at a partial pressure of about 40mmHg.

There are five chemical reactions that occur in aqueous solution due to the presence of dissolved CO_2, and these are:

$$CO_2(aq) \leftrightharpoons CO_2(g)$$

$$CO_2 + H_2O \overset{CA}{\leftrightharpoons} H_2CO_3 \leftrightharpoons H^+ + HCO_3^-$$

$$CO_2(aq) + OH^- \leftrightharpoons HCO_3^-$$

$$HCO_3^- \leftrightharpoons H^+ + CO_3^{2-}$$

It should be noted that the carbonic anhydrase catalised reaction (abbreviated CA) is normally a very slow reaction in comparison to the others, and this catalyst has the effect of increasing the reaction rate accordingly, and in so doing, results in rapid equilibration of the system which would otherwise not occur.

It should also be noted that some CO_2 reacts with groups on protein molecules, however this reaction, forming carbamino compounds is usually numerically insignificant and will be ignored in this analysis.

One of the features of CO_2 reaction in water is the formation of the carbonate ion (CO_3^{2-})

which is just as theoretically relevant as bicarbonate but numerically many orders of magnitude smaller, and is frequently ignored in physiological literature. It should also be noted that bicarbonate and carbonate ions are dependent variables in the same way as hydrogen and hydroxyl ions.

The mathematical descriptions of the above chemical reactions relating to CO_2 are as follows:

$$[CO_2(d)] = S_{CO_2} \cdot pCO_2$$

$$[H_2CO_3] = S_{H_2CO_3} \cdot pCO_2$$

$$[CO_2(d)] \cdot [OH^-] = K_1 \cdot [HCO_3^-]$$

$$[H^+] \cdot [HCO_3^-] = K_2 \cdot [H_2CO_3]$$

$$[H^+] \cdot [CO_3^{2-}] = \frac{K_3}{2} \cdot [HCO_3^-]$$

The division of the constant by 2 has been introduced [12] to correct for the fact that carbonate (as well as other concentrations) is expressed in mmol/L whereas Stewart used mEq/l which resulted in carbonate ion having twice the numerical value due to it being a divalent cation.

It should be noted that by combining variables, the first four equations can be simplified to one equation ie.

$$[H^+]\cdot[HCO_3^-] = K_A\cdot S_{CO_2}\cdot pCO_2$$

where is must be noted that K_A is distinct from K_a , and that the product $K_A\cdot S_{CO_2}$ will be represented by K_c to retain consistency with Stewart[12].

The addition of carbon dioxide therefore requires two additional equations to describe the solution mathematically, where the constant K_c in the second equation is just the appropriate algebraic combination of constants from the four equations giving rise to it.

The second equation is clearly the non-logarithmic form of the Henderson Hasselbach equation:

$$pH = pK_A + \log_{10}\frac{[HCO_3^-]}{S_{CO_2}\cdot pCO_2}$$

The carbon dioxide in solution adds two new anions to the electrical neutrality equation, namely the bicarbonate ion and the carbonate ion.

$$[H^+]+[SID]-[HCO_3^-]-[A^-]-2[CO_3^{2-}]-[OH^-] = 0$$

Note that this again differs from Stewart's original formulation in that carbonate ion concentration is multiplied by 2 due to the fact that it is a divalent cation and is expressed in mmol/L whereas Stewart worked in mEq/L which accounted for the doubling due to charge [12].

Finally, there are six equations which describe the acid-base status of a solution namely:

$$K_w' = [H^+][OH^-]$$

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

$$[H^+]\cdot[HCO_3^-] = K_c \cdot pCO_2$$

$$[H^+]\cdot[CO_3^{2-}] = \frac{K_3}{2}\cdot[HCO_3^-]$$

$$[HA]+[A^-] = [A_{tot}]$$

$$[SID]-[A^-]+[H^+]-[OH^-]-[HCO_3^-]-2[CO_3^{2-}] = 0$$

Each of the six equations represents a physical requirement, and all these requirements must be met simultaneously. Solving the six equations simultaneously leads to the following fourth order polynomial in $[H^+]$:

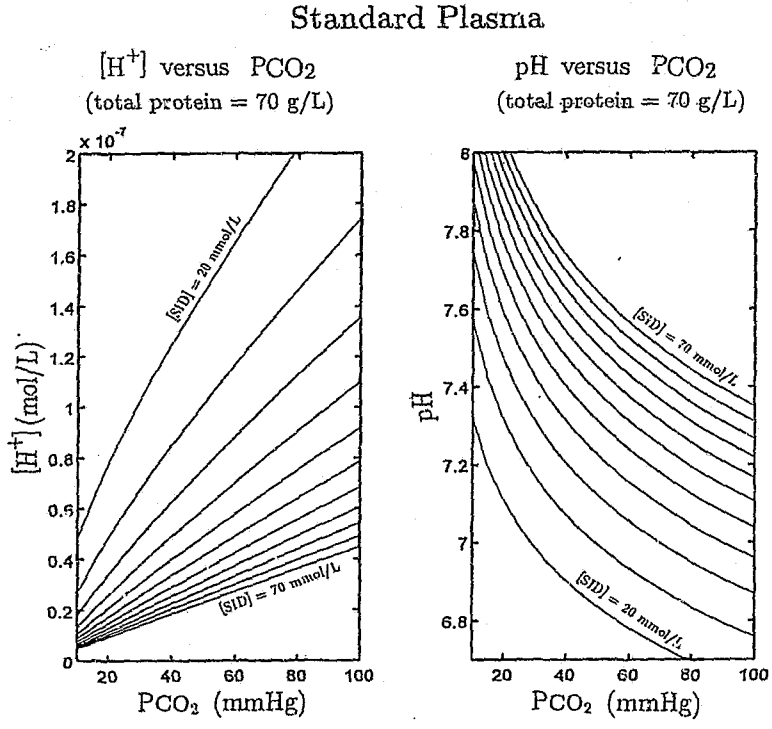
$$\begin{aligned}&[H^+]^4+(K_a+[SID])[H^+]^3\\&\quad+(K_a([SID]-A_{tot})-(K_c\cdot pCO_2+K_w'))[H^+]^2\\&\quad\quad-(K_a(K_c\cdot pCO_2+K_w')+K_3\cdot K_c\cdot pCO_2)[H^+]\\&\quad\quad\quad-K_a\cdot K_3\cdot K_c\cdot pCO_2 = 0\end{aligned}$$

This then is in essence Stewart's model.

This equation can be solved easily with a digital computer to produce values for hydrogen ion concentration (or pH), as a function of the independent variables of A_{tot}, pCO_2 and $[SID]$ [13].

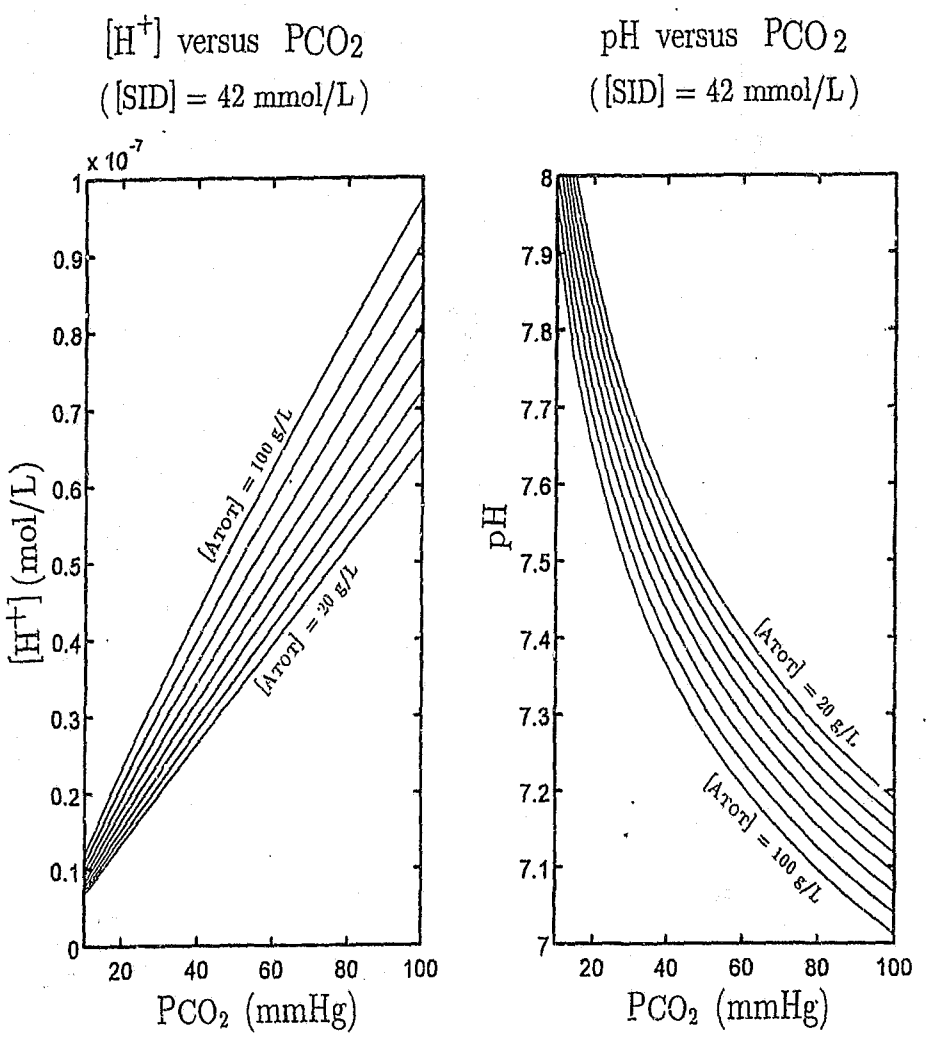
$[H^+]$ is just one of the dependent variables which can be solved for, the others being bicarbonate ions, carbonate ions, hydroxyl ions, A^- ions and HA [13].

A graph of $[H^+]$ versus pCO_2 plotted from the above equation for a variety of values of $[SID]$ while A_{tot} remains fixed throughout shows quantitatively the very definite increase in $[H^+]$ as pCO_2 rises (respiratory acidosis) for any given $[SID]$ value, while the $[H^+]$ also rises rapidly at a fixed pCO_2 for dropping $[SID]$ values, a form a metabolic acidosis.



A similar plot of $[H^+]$ versus pCO_2 for a variety of A_{tot} values shows how rising weak acids (mainly plasma proteins) produce a form of metabolic acidosis.

Standard Plasma

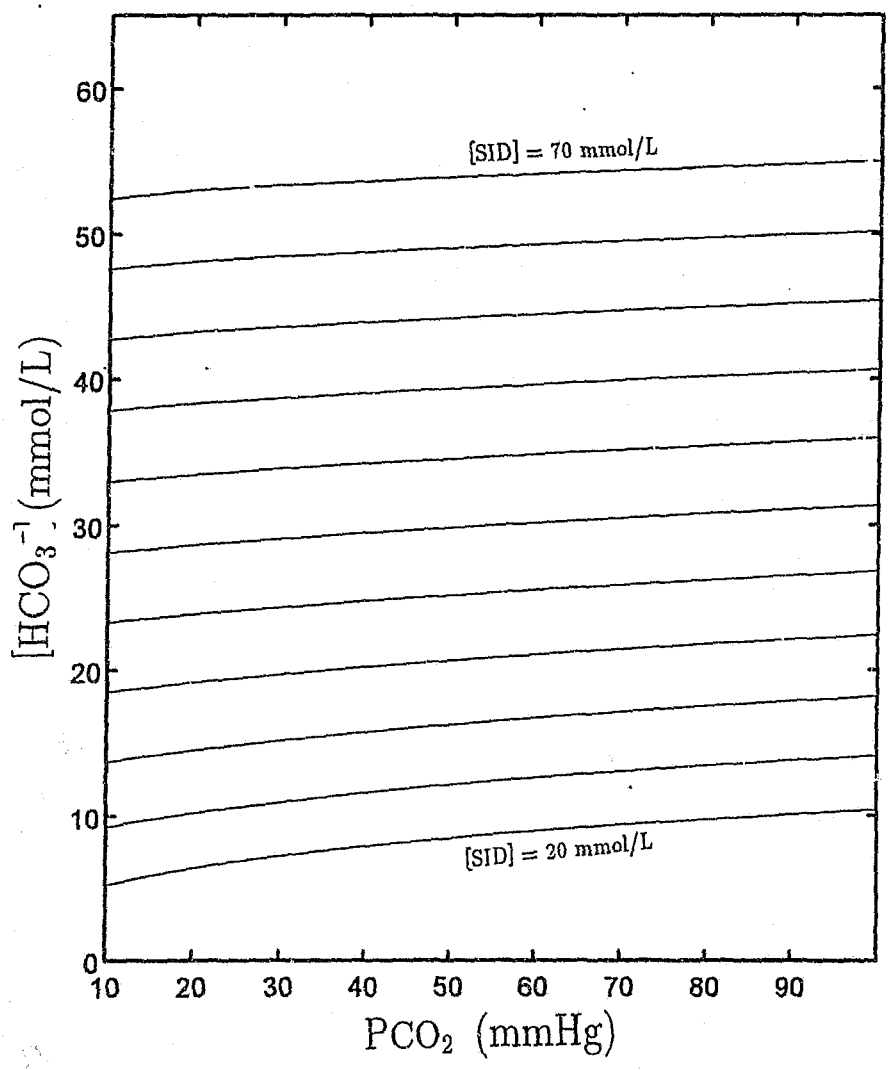


It is also instructive to plot bicarbonate values against pCO_2 for a variety of [*SID*] values, and it is clear that an increase in pCO_2 has only a slight effect on $[H^+]$ while a metabolic effect such as a change in [*SID*] has a relatively dramatic effect on bicarbonate concentrations.

Standard Plasma

$[HCO_3^-]$ versus PCO_2

(total protein = 70 g/L)



One of the striking features of Stewart's model is the fact that with the appropriate values for the constants, and by using physiologically correct values for the independent variable, the values for $[H^+]$ and other dependent variables produced by the equation are within the expected range.

In summary, Stewart's model identifies three independent variables ie. pCO_2, A_{tot} and $[SID]$ which determine all the other variables including bicarbonate and hydrogen ion concentration.

It is therefore evident that one cannot postulate a pumping in or out of hydrogen ions or bicarbonate ions from the plasma compartment as a mechanism to control the final pH value.

There have been a number of reports relating to the obvious advantages of Stewart's model, as well as highlighting some of the controversies, while other authors have been severely critical of this work [14,15,16].

Other authors have utilised Stewart's model to analyse various biological solutions [17,18,19,20,21,22], and there has been a reasonably successful verification of the model in normal subjects [23].

AIMS AND OBJECTIVES

The objective of this study was to evaluate whether Stewart's model of acid-base regulation, which has been shown to be correct in simple solutions, could be applied to a complex clinical condition namely diabetic ketoacidosis. In addition, the objective of the project was to highlight any discrepancies between the model's predictions and clinical measurements in this common pathological condition, and to try to elucidate obvious sources for any observed discrepancies.

Diabetic ketoacidosis was chosen to evaluate the behaviour of the model for two reasons:

1) This condition constitutes a group of patients who require urgent venous and arterial blood sampling for clinical management making it convenient to obtain blood samples.

2) Diabetic ketoacidosis is a well understood clinical entity with well characterised biochemical changes which could thus be analysed with relative simplicity.

SUBJECTS

Arterial and venous blood samples were collected from twenty patients with the diagnosis of diabetic ketoacidosis. All samples were obtained as part of the routine management of these patients, at the discretion of the managing doctor. Only patients who were treated with insulin, fluids and electrolyte replacements were included in the study. Patients known to be on any other medication were excluded.

Methods

Blood for arterial blood gas analysis was collected in commercially prepared heparinised syringes (Instrumentation Laboratory). Arterial blood gas analysis was performed immediately following collection, by means of ion selective electrodes (ISE) at 37 degrees centigrade on the IL 1306 blood gas analyser (ILEX). The blood gas analyser was calibrated and three levels of controls were run before each specimen was analysed.

Venous blood for electrolytes, ketones and protein analyses was collected in normal clotted tubes while blood for lactate and glucose analyses was collected in fluoride tubes. Blood was centrifuged at 3500 rpm for 10 minutes. Aliquots of blood were immediately analysed and used for clinical management while duplicates were stored at -70^0 C and analysed as a batch at a later time to exclude inter-batch variation, for the purposes of the project. Electrolytes (ISE), total proteins (Biuret) and albumin (Bromocresol-green) were analysed on the Hitachi 747 autoanalyser (Boehringer Mannheim). The 747 autoanalyser was calibrated and controls (PPU and PNU, Boehringer Mannheim) at two levels were used to determine the accuracy of analysis. Precision of analysis was determined using Monotrol control material at two separate levels. Lactate was measured on the TDX (lactate dehydrogenase, Abbott Diagnostics), three different levels of control material (Abbott) was used to determine the accuracy of the test prior to analysing the specimens. Ketones were analysed on the Cobas mira using the Randox Rambut kit. Randox control material at two different levels was used to determine the accuracy of the aceto- acetate kit. As no quality control material for Beta-hydroxybutyrate was commercially available, control material was made up adding a known quantity of Betahydroxybutyrate (Sigma Chemicals) to laboratory grade water.

DATA ANALYSIS

The relationship between the measured hydrogen ion concentration and that calculated using Stewart's model, was analysed by means of a simple regression analysis. It is self evident that perfect agreement would constitute a 45 degree regression line of identity.

In addition to this, techniques of error analysis were used, whereby expected random error was calculated for over a wide range of measurements based on the known variability established from repeated measurements on control material. The actual percentage errors of the calculated results with respect to measured hydrogen ion concentrations were determined and compared.

RESULTS

The strong electrolyte concentration data obtained from the 20 serum samples expressed in *mmol/L* were as follows:

Table 1 *Analytes determining [SID] (mmol/L)*

Na^+	K^+	Cl^-	*Lactate*	*Aceto-acetate*	*β-OH butyrate*	**[SID]**
133.0	5.0	91.0	2.3	3.0	8.2	33.5
130.0	5.8	87.0	7.5	2.0	10.9	28.4
137.0	4.9	103.0	1.8	3.0	7.9	26.2
144.0	4.4	105.0	3.6	2.8	12.6	24.4
145.0	3.7	110.0	2.3	1.6	9.3	25.4
135.0	5.3	97.0	1.7	2.0	7.9	31.8
131.0	5.0	90.0	4.7	3.0	10.4	28.0
117.0	4.8	75.0	2.8	4.2	9.5	30.3
138.0	4.1	94.0	5.5	2.7	7.3	32.5
139.0	3.0	119.0	2.1	0.6	0.7	19.6
162.0	3.6	129.0	5.2	0.4	8.2	22.8
137.0	3.1	112.0	2.7	1.1	5.8	18.5
126.0	7.5	85.0	5.9	4.7	32.2	5.8
139.0	3.7	114.0	2.6	2.8	5.0	18.3
137.0	4.0	110.0	1.0	0.4	1.3	28.4
133.0	4.6	92.0	3.8	5.7	11.2	24.9
125.0	5.5	91.0	2.1	4.5	12.5	20.5
136.0	4.9	110.0	2.9	3.6	9.0	15.5
133.0	5.5	90.0	2.4	3.6	15.2	27.4
142.0	3.3	111.0	3.7	2.3	7.4	20.9
139.0	3.5	108.0	4.1	2.8	9.2	18.5

The calculated [*SID*] values based on the above table, as well as the other two independent

variables viz. pCO_2 and total protein A_{tot} are presented below:

Table 2 Independant variables

pCO_2 *mmHg*	A_{tot} *g/L*	[SID] *mmol/L*
12.2	57.0	33.5
24.0	67.0	28.4
14.8	63.0	26.2
27.4	46.0	24.4
11.7	82.0	25.4
22.8	79.0	31.8
16.1	74.0	28.0
29.3	59.0	30.3
27.2	76.0	32.5
25.9	49.0	19.6
31.0	78.0	22.8
19.4	57.0	18.5
12.5	71.0	5.8
21.4	58.0	18.3
23.5	58.0	28.4
12.2	80.0	24.9
12.4	83.0	20.5
12.6	65.0	15.5
23.9	84.0	27.4
12.8	57.0	20.9
13.2	73.0	18.5

From this data, predicted hydrogen ion concentrations were calculated using Stewart's model by utilising a BASIC computer program specifically written for this analysis and based on an

algorithm suggested by Stewart. The constants[1] used at 37 degrees C were: $K'_w=4.4\times10^{-14}$, $K_a=3\times10^{-7}$, $K_3=6\times10^{-11}$, $K_c=2.46\times10^{-11}$. The "measured" hydrogen ion concentrations were derived from measured pH values. The pH and both the measured and calculated hydrogen ion concentrations are shown below, as well as the actual and absolute percentage error, with the measured values being regarded as the "true" concentrations. (It should be remembered that even the measured values have small errors).

Table 3 Measured pH, measured $[H^+]$, calculated $[H^+]$

pH measured	$[H^+]$ *measured* nmol/L	$[H^+]$ *calculated* nmol/L	*Error*	*Absolute error (%)*
7.02	94.76	24.10	70.66	74.57
7.13	73.79	31.90	41.89	56.77
6.91	121.90	25.80	96.1	78.83
7.01	97.72	42.70	55.02	56.31
7.26	54.95	38.60	16.35	29.76
7.10	80.17	34.10	46.07	57.46
6.96	110.41	41.10	69.31	62.77
7.20	62.81	41.60	21.21	33.76
7.30	49.89	65.70	-15.81	31.69
7.32	48.19	93.00	-44.81	92.97
7.32	48.19	67.40	-19.21	39.85
6.89	128.82	688.00	-559.18	434.06
*7.91	12.30	75.90	-63.60	516.94
7.31	49.55	37.00	12.55	25.32
7.03	93.97	31.00	62.97	67.01
7.06	87.10	74.60	12.50	14.35
7.05	88.72	94.40	-5.68	6.41
7.20	62.66	58.50	4.16	6.64
7.21	61.38	37.40	23.98	39.06
7.17	67.14	77.30	-10.16	15.13

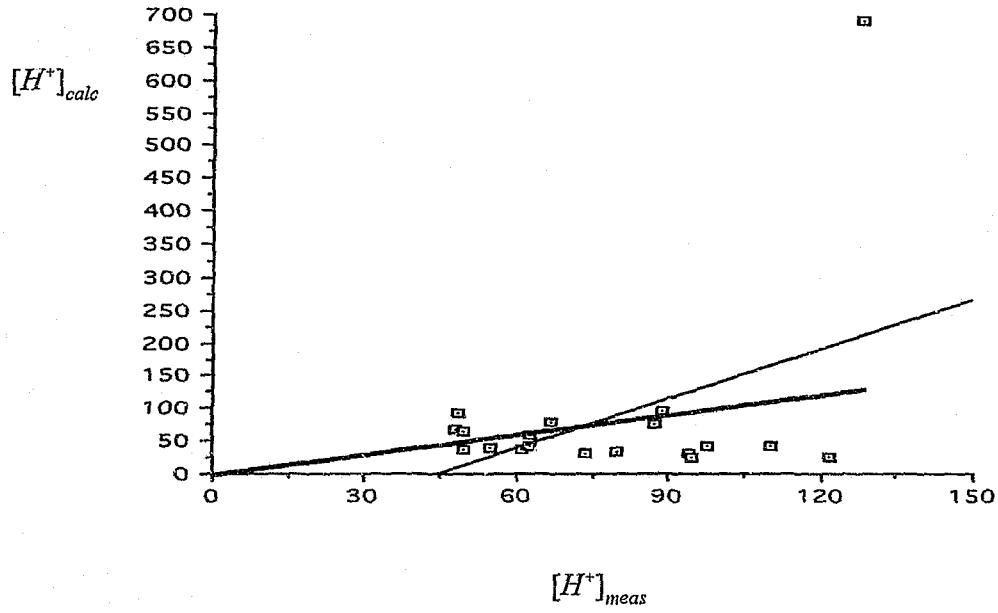
* The pH of 7.91 is almost impossible in diabetic ketoacidosis, and was probably a transposition error. This result was therefore not included in the analysis.

The best fit regression plot of the calculated versus the measured hydrogen ion concentrations is shown below, and it is clear that the line deviates enormously from the 45 degree identity line which would be expected for perfectly matching results. Only 19 of the 20 data points are shown, with the 20th point deviating to such an extent that it could not be included on a graph of this scale.

Calculated v.s. measured [H⁺]

Regression line: $[H^+]_{calc} = 2.55[H^+]_{meas} - 114.3$

(The heavy line represents the line of identity)



Below, is a table showing the estimated one standard deviation errors for each of the analytes based on repeated measurements of control samples at a number of different concentrations with the range. The names or labels given to the various control materials are shown, and when followed by the letters L, M or H, this indicates low, medium and high values respectively. The table shows means after a number of measurements of the same control, as well as standard deviation (σ), and % coefficient of variation (CV), which is identical to the percentage error from the mean of one standard deviation.

Table 4 Estimated analyte errors

Analyte	*Control*	*mean*	*σ*	*CV*
Sodium ion	**Na mon1**	**134.5**	**1.5**	**1.13**
(mmol/L)	**Na mon2**	**112.7**	**1.2**	**1.07**
	Na PNU	**116.1**	**2.9**	**2.51**
	Na PPU	**136.7**	**2.8**	**2.06**
Potassium ion	**K mon1**	**6.45**	**0.1**	**1.63**
(mmol/L)	**K mon2**	**4.35**	**0.05**	**1.26**
	K PNU	**4.5**	**0.12**	**2.57**
	K PPU	**6.19**	**0.15**	**2.37**
Chloride ion	**CL mon1**	**99.6**	**1.9**	**1.96**
(mmol/L)	**CL mon2**	**91**	**2**	**2.2**
	CL PNU	**88.3**	**2.1**	**2.33**
	CL PPU	**107.4**	**2.4**	**2.21**
Total Proteins	**TP mon1**	**74.7**	**2**	**2.63**
(g/L)	**TP mon2**	**43.3**	**1.2**	**2.79**
	TP PNU	**51.1**	**0.9**	**1.76**
	TP PPU	**48.6**	**1.6**	**3.3**
B-OH butyrate	**BHYD 1**	**302.8**	**8.5**	**2.8**
(µmol/L)	**BHYD 2**	**1148.6**	**34.3**	**3**
Aceto-acetate	**Acet L**	**111.56**	**5.72**	**5.13**
(µmol/L)	**Acet M**	**222.22**	**14.02**	**6.3**
	Acet H	**564.22**	**14.66**	**2.6**
Lactate	**Lact L**	**2.23**	**0.09**	**4.05**
(mmol/L)	**Lact M**	**5.11**	**0.09**	**1.76**
	Lact H	**10.25**	**0.17**	**1.66**
pCO_2	**pCO_2 L**	**21.86**	**0.52**	**2.37**
(mmHg)	**pCO_2 M**	**36.68**	**0.7**	**1.9**
	pCO_2 H	**61.71**	**1.32**	**2.14**
Hydrogen ion	**H L**	**24.81**	**0.23**	**0.93**
(nmol/L)	**H M**	**38.13**	**0.53**	**1.39**
	H H	**67.7**	**1.03**	**1.52**

The error analysis performed on Stewart's model is as follows:
Experimental data are always subject to some error, and interpretation of results must always take this factor into account.

Broadly speaking, there are two types of error that can occur in experimentation viz. random error and systematic error.

Random error refers to random fluctuation in the measurement about the "true" value. This "true" value can be estimated by the mean of multiple readings of the same data. One important characteristic of random error is that it has a Normal or Gaussian distribution about the mean value, and the distribution can be fully characterised by the mean and standard deviation. The presence of random error gives rise to the finite precision of a measurement, which can be improved by attention to the experimental technique and by averaging multiple readings.

Systematic error applies to a fixed or well characterised deviation of the measurement from the "true" value, and gives rise to the finite accuracy of a measurement. This can be corrected for by calibration against a standard measure.

The data measured in this project was calibrated by the use of standards to produce calibration curves which were internally generated in most of the instrumentation. It is therefore expected that systematic error will play a small role in the overall error.

More importantly, random error must be well characterised and accounted for.

Controls for each of the analytes were measured a number of times to establish mean and standard deviations, and in so doing, to characterise the error in the individual measurements.

For the remainder of this discussion, the error in any measurement will refer to one standard deviation generally expressed as a percentage of the mean.

In any calculation based on a mathematical model, the error in the input variables produce an error in the calculated variable, the magnitude of which depends on a formula derived from the original model.

The general form of this formula is:

$$\sigma_{[H^+]} = \sqrt{\left(\frac{\partial[H^+]}{\partial[SID]} \cdot \sigma_{SID}\right)^2 + \left(\frac{\partial[H^+]}{\partial[A_{Tot}]} \cdot \sigma_{A_{Tot}}\right)^2 + \left(\frac{\partial[H^+]}{\partial pCO_2} \cdot \sigma_{pCO_2}\right)^2}$$

where the sigmas represent the standard deviations resulting from random error for the various subscripted variables. As can be seen, the standard deviation of the calculated variable depends not only on the standard deviations of the input variables, but also on the partial derivatives of the calculated variable with respect to each of the input variables.

As Stewart's model is expressed in implicit form, the partial derivatives were computed by

implicit differentiation and then solved for in each case.

The solutions for the partial derivatives are as follows[12]:

$$\frac{\partial[H^+]}{\partial fCO_2} = \frac{{}_c[H^+]^2 + K_a K_c[H^+] + K_3 K_c[H^+] + K_a K_3 K}{\nabla}$$

$$\frac{\partial[H^+]}{[SID]} \quad \frac{-[H^+]^3 - K_a[H^+]^2}{\nabla}$$

$$\frac{\partial[H^+]}{[A_{Tot}]} \quad \frac{K_a[H^+]^2}{\nabla}$$

where:

$$\nabla = 4[H^+]^3 + 3K_a[H^+]^2 + 3[SID][H]^2 + 2K_a[SID][H^+] - 2K_a A_{Tot}[H^+] - 2K_c pCO_2[H^+] - 2K_w'[H^+] - K_a K_c pCO_2 - K_a K_w' - K_3 K_c pCO_2$$

The $[SID]$ itself is subject to error, however as it is simply computed by taking the sums and differences of the various electrolyte charge concentrations, the partial derivatives in the error formula are unity, and the error equation reduces to being square root of the sum of the squares of the *one standard deviation* values for each of the analytes.

A BASIC computer program was developed for the formulae developed above, and typical worst case *one standard deviation* errors were used, as shown in the table 4 for each of the independent analytes, namely $[SID]$, pCO_2, and A_{tot} the following *one standard deviation* errors for calculated hydrogen ion concentrations are shown below for a range of values for the independent variables. The one standard deviation worst case error for $[SID]$ was estimated at 12.5% using the formula described above utilising worst case values for the electrolytes from table 4. The other one standard deviation worst case errors based on table 4 were estimated at 3.3% for A_{tot} and 2.4% for pCO_2.

Table 5 Error analysis for selected values

[SID] *(mmol/L)*	A_{tot} *(g/L)*	pCO_2 *(mmHg)*	error $[H^+]$ *(%)*
20	45	20	21.6
40	75	40	19.6
60	95	60	18.23
20	95	40	23.3
40	75	60	18.6
60	45	20	15.2
20	75	60	20
40	95	40	22

As table 5 indicates, the error analysis produced estimates for *one standard deviation* errors

with respect to the "true" concentration of hydrogen ions varying between about 15% and 23%

Clearly the errors obtained from the analysis as presented in table 3 are much greater than those predicted by the random error analysis, with only 4 out of the 20 percentage errors falling within one standard deviation of the expected value.

DISCUSSION AND CONCLUSION

The very elegant model proposed by Stewart to explain acid-base physiology and the numerous factors involved in its control is a great stride forward into research of this most complex and important field.

From the data presented however it is clear that the percentage errors obtained from using Stewart's model to calculate hydrogen ion concentration are far in excess of those predicted based purely on an analysis of the random errors.

In order to begin to achieve an understanding of the discrepancies, a number of possibilities should be explored.

It has been mentioned that Stewart was well aware of some of the simplifying assumptions that he made in developing this model, and it is very possible that some of these factors could contribute greatly to the errors. However, in view of the relative thermodynamic rigour which Stewart brought to bear on his analysis, and his strict adherence to sound principles of physical chemistry, it is highly unlikely that the entire model is incorrect, but rather that if any changes are required, they are likely to relate to detail and non-linearities in coefficients.

Unaccounted for substances remain a strong possibility for the source of error, and can be divided into two categories.

1) Those substances which are well known but are considered to exist in low enough concentrations as to be numerically insignificant.

2) Substances such as medications and metabolic products which are not known to exist in the sera under examination or have not been measured.

The first group, ie. those substances regarded as being numerically insignificant, would include substances such as calcium, magnesium, phosphates and sulfates. In general, these substances would be found in ionic concentrations less than about 2 or 3 mmol/L, although in terms of ionic contribution this must be doubled to accommodate the effects of divalent cations and anions. It must also be borne in mind that phosphates are weak ions, and as such they do not contribute as dominantly as the other strong ions. Furthermore the positive and negative ions would at least to some extent cancel out their effect.

Nonetheless, the possibility remains that one or more of these substances may exist in concentrations where the usual assumptions of insignificance no longer applies.

The second group of substances ie. the unknown group, would include medications and their metabolites, of which the staff are unaware, or even substance such as non-conventional or alternative medications. These substances may well exist in the se[illegible] f some patients and would be very difficult and in some cases impossible to identify and quantify without the benefit of a correct history and sophisticated methodologies.

The possible influence of plasma proteins on the model is perhaps the most difficult to

quantify, and may in fact be the very area in which the model needs modification.

Firstly, Stewart's assumption of a representative equilibrium constant for all plasma proteins may well be a serious oversimplification of reality. The constant may in fact prove to be highly non-linear due to a possible dependence of the equilibrium constant on hydrogen ion concentration itself, or perhaps even on other ionic species. This will only be determined by extensive experimentation.

Secondly, while some workers appear to have demonstrated that proteins may have complex effects [25], and that it is only albumin [10] which determines the acid-base effects of plasma proteins, this may in fact also be an oversimplification, and furthermore, even if this is correct, it would imply a requirement to first determine the albumin to globulin ratio and then apply a correction factor to the total protein values used in this study. Other workers have demonstrated that the equilibrium constant of protein depends in a rather complex way on the specific proteins structure and functional groups [10].

Thirdly, it is not at all impossible that factors as yet not considered may affect the behaviour of proteins. A possible example of this is the undetermined effect of glycation. It is well known that persistently high glucose levels encountered in diabetics produce glycation of various proteins. It is therefore not at all impossible that glycation may have an important effect on the equilibrium constants of plasma proteins, something which again will only be determined by further experimental research.

Finally, the important possibility of measurement errors needs to be considered as a cause for

discrepant results.

The first possibility for erroneous measurements is that of the partial pressure of carbon dioxide. While the random error for the controls used for carbon dioxide measurements are very acceptable, and would not contribute substantially to the error, it must be recognised that control samples were not available for the very low carbon dioxide levels encountered in keto-acidotic patients, and it may well be true that the error at this part of the range is unacceptably high. It should also be recognised, that in the absence of adequate standard and control material, not only is it impossible to characterize the random error, but also the possibility of systematic error due to non-linearity at the extremes of the range cannot be excluded.

The keto-acid β-hydroxy butyric acid measurements depended on controls and standards that were not well characterised at the levels under consideration. The standards and control materials for β-hydroxy butyric acid were available for levels far lower than those encountered in typical keto-acidotic sera, and for this reason, as in the case of carbon dioxide, it is not possible to draw definitive conclusions regarding systematic and random errors.

The aceto-acetate controls and standards were prepared in-house as commercially available assayed control material was unobtainable. Crystaline aceto-acetic acid was used to prepare control material. While this had the advantage of being able to achieve concentrations at any point in the anticipated range, the technique suffered from the disadvantage of not having an external assay system for calibration and control.

It is clear from the above discussion that there are numerous factors which have not been adequately accounted for, and that any one or more of these may produce gross errors in the predicted results.

Notwithstanding the above, Stewart's model provides an elegant explanation of the observed behaviour of hydrogen ions in physiological solutions In view of considerations of modern thermodynamic theory and physical chemistry, there can be little doubt that the model is correct in principle, and what remains is to reduce the simplifications to the point that the model accurately reflects conditions in physiological solutions.

It is thus clear that in order to validate the model and to possibly introduce modifications which correct for some of the oversimplifications, extensive and precise experimental work remains to be performed. However, it is doubtful if the routine chemical pathology laboratory can provide adequate analytical precision to achieve this goal.

Furthermore, even when the model is fully validated and adequately modified to reflect the complexity of biological solutions, it remains questionable as to whether the coarse precission and accuracy typically required for most medical decision making will be adequate to allow for the clinical use of the model. Nonetheless, Stewart's approach must surely be regarded as a high point in the understanding of an important physical and physiological process.

REFERENCES

1. Stewart PA. How to understand acid-base. Elsevier Press.1981; 1-181.

2. Stewart PA. Modern quantitative acid-base chemistry,. *Can. J.Physiol. Phamacol.*1983; **61**: 1444-1461.

3. Halperin ML. How much 'new' bicarbonate is formed in the distal nephron in the process of net acid excretion. *Kidney Int.* 1989;**35**: 1277-1281.

4. Halperin ML, Jungas RLL. Metabolic production and renal disposal of hydrogen ions. *Kidney Int.*1983; **24** :709-713.

5. Halperin ML, Jungas RLL, Cheema-Dhadi S, Brosman JT. Disposal of the daily acid load: an integrated function of the liver, lungs and kidney. *TIBS.* 1987;**12**:197-199.

6. Atkinson DE, Bourke E. The role of ureagenesis in pH homeostasis. .*TIBS.*1984;**9**:297-300.

7. Atkinson DE, Bourke E. Metabolic aspects of the regulation of systemic pH. *Am J Physiol.* 1987; **252**: F987-F956.

8. Robinson RA, Stokes RH. Electrolytic solutions. 1959 2nd ed. Butterworths Scientific Publications. London.

9. Rossing TH, Maffeo N, Fencl V. Acid base effects of altering plasma protein concentrations in human blood. *J.Appl Physiol.*1986; **61**:2260-2265.

10. Figge J, Mydosh T, Fencl V. The role of serum proteins in acid-base equilibria. *J Lab Clin Med* 1991; **117**: 453-467.

11. Figge J, Mydosh T, Fencl V. Serum proteins and acid-base equilibria: a follow-up. *J Lab Clin Med* 1992;**120**: 713-719.

12. D. M. Rubin - unpublished work.

13. Stewart PA. Independent and dependent variables of acid-base control. *Respir. Physiol.*1978; **33**:9-26.

14. Cameron JN. Acid-base homeostasis: past and present perspectives. *Physiological Zoology.* 1989; **62(4)**: 845-865.

15. Reeves RB. Commentary on the review article by Dr Peter Stewart. *Can.J.Physiol.Pharmacol.*1983;**61**:1442-1443.

16. Siggaard-Andersen O, Fogh-Andersen N. Base excess or buffer base (strong ion difference) as a measure of a non-respiratory acid-base disturbance. *Acta Anaesthesiol Scand.* 1995; **39** (sup 107):123-128.
17. Kowalchuk JM, Heigenhauser GJF, Lindinger MI, Sutton JR, Jones NL. Factors influencing hydrogen ion concentration in muscle after intense exercise. *J.Appl Physiol.*1988; **65**:2080-2089.

18. Kowalchuk JM, Heigenhauser GJF, Lindinger MI, Obminski G, Sutton JR, Jones NL. Role of lungs and inactive muscle in acid base control after maximal exercise. *J Appl Physiol.* 1988; **65**:2090-2096.

19. Lindinger MI, Heigenhauser GJF, Spriet HL. Effects of intense swimming and tetanic electrical stimulation on skeletal muscle ion and metabolites. *J.Appl. Physiol.*1987; **63**:2331-2339.

20. Stainsby WN, Eitzman PD. Role of CO2, O2 and acid in arteriovenous [H+] differences during muscle contractions. *J.Appl Physiol.*1988; **65(4)**:1803-1810.

21. Alfaro V, Peinado VI, Palacios L. Factors influencing acid-base status during acute severe hypothermia in unanesthetized rats. *Resp Physiol.* 1995: **100**: 139-149.

22. Jennings D. The physiochemistry of [H^+] and respiratory control: roles of pCO_2, strong ions and their hormonal regulators. *Can J Physiol Pharmacol.*1994; **72**: 1499-1512.

23. Weinstein Y, Magazanik A, Grodjinovsky, Inbar O, Dlin RA, Stewart PA. Re-examination of Stewarts quantitative analysis of acid-base status. *Med Sci Sports Exerc.* 1991; **23:** 1270-1275.

24. Taylor JR. An introduction to error analysis. The study of uncertainties in physical measurement.. Second edition. 1997. University Science Books.