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Doctoral thesis

Sources of error in interpretation of blood gas analysis: Current acid-base models of plasma

Zdroje chyb při interpretaci analýzy krevních plynů:
Současné acidobazické modely plazmy

Fonti di errore nell'interpretazione dell'emogasanalisi:
Attuali modelli acido-base del plasma

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Declaration

I declare that I am the sole author of the submitted thesis and that I properly referenced all resources and literature used. I declare that neither the submitted thesis nor its part has been used in a different final thesis.

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Key words

acid-base equilibrium, blood-gas analysis, plasma, buffers

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I would like to open my thesis with a quote by John W. Severinghaus (Astrup and Severinghaus 1986):

'I work on the hyperbolic system, by which K=ET. E is the effort I invest (in kilowatts),
T is time before the deadline in hours, and K seems to have a value near unity.'

The privilege of sharing this behavior pattern with one of the greatest minds in acid-base research and an expert on its history was something that provided me with relief while finishing this thesis.

In Prague, 31 March 2023

Martin Krbec

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List of abbreviations

CO_2	carbon dioxide
pCO_2	partial pressure of CO_2
O_2	oxygen
pO_2	partial pressure of O_2
BE(X)	base excess in fluid X (B = blood, P = plasma, Ecf = extracellular fluid)
SID	strong ion difference
β	buffer power
β(X)	buffer power of fluid X or chemical species X
$β(X)_{N(Y)}$	β(X) normalized for concentration of chemical species Y
K_a	dissociation constant of weak nonvolatile acids (if not clearly stated otherwise, e.g., K_a of lactate)
pK_a	negative decadic logarithm of K_a (if not clearly stated otherwise, e.g., pK_a of lactate)
HA	weak nonvolatile acid
$[HA]$, $[A^-]$	concentration of associated and dissociated form of HA
Atot	total concentration of weak nonvolatile acid
$Atot_{N(X)}$	Atot normalized for concentration of chemical species X
TP	total protein
Alb	albumin
$[Prot^-]$, $[Alb^-]$	charge displayed by proteins or albumin
A/G ratio	albumin to globulin ratio
Pi	inorganic phosphate
$[Phos^-]$	charge displayed by phosphates
r	Pearson correlation coefficient
R^2	coefficient of determination
RMSE	root-mean-square error
ICU	intensive care unit
ECMO	extracorporeal membrane oxygenation
SOFA	Sequential Organ Failure Assessment Score
APACHE II	Acute Physiology and Chronic Health Evaluation II
SAPS II	Simplified Acute Physiology Score II

Abstract

Introduction. Evaluation of acid-base status is an essential tool used by clinicians to guide therapy in a wide range of conditions. Of all biological fluids, in which the acid-base equilibrium has been studied, isolated plasma is the best described one. For its evaluation, two main approaches have been developed: One is the traditional bicarbonate-centered school based the work of Henderson, Hasselbalch, van Slyke, and Siggaard-Andersen. The other is the quantitative physicochemical approach invented by Stewart and further developed by Staempfli and Constable or Figge and Fencl. Each of the mentioned models uses a set of constants for characterizing the behavior of non-carbonic buffers (proteins and phosphates) in plasma, the value of which is crucial for precise calculation of derived parameters. The values of the constants used by the Stewart model, i.e., the total weak nonvolatile acid concentration (Atot) and its dissociation constant (pK_a), have only been experimentally determined once in a group of 8 healthy volunteers. Our aim was to experimentally determine the value of these constants in healthy volunteers and, for the first time ever, in two populations of critically ill patients. We hypothesized that buffer properties of weak nonvolatile acids are altered by critical illness, a fact that would manifest as a difference in pK_a or the conversion factor between Atot and the measured concentration of main non-carbonic buffers (albumin or total protein). On top of the described analysis, which is based on the Stewart and Staempfli-Constable model of plasma, we aimed to obtain estimates of the constants that characterize non-carbonic buffers in the classical and Figge-Fencl model of plasma.

Methods. Blood was taken from healthy volunteers (N=30), patients after an elective major abdominal surgery (N=27), and patients with sepsis (N=30) at general intensive care units of two medical centers in Czechia and Italy. Using a CO_2 tonometer, the partial pressure of carbon dioxide (pCO_2) in isolated plasma was manipulated in the range of 2 to 16 kPa (15 to 120 mmHg) while pH, pCO_2, and electrolyte concentration were repeatedly measured using a standard blood-gas analyzer. The experimental data were used by a nonlinear regression algorithm together with the equations that characterize the mentioned plasma models to determine the constants that characterize the buffer action of non-carbonic buffers in each model. These were then then compared among the study groups and with their previously published estimates.

Results. For the Stewart model of plasma, the values determined in healthy volunteers were as follows: Atot = 24.3 ± 1.9 mmol/L (equivalent of 0.510 ± 0.035 mmol/g of albumin or 0.340 ± 0.024 mmol/g of total protein) and pK_a = 6.63 ± 0.09. This model turned out to be prone to the confounding effect of unmeasured anions and, hence, inappropriate for postoperative and septic patients. Using the Staempfli-Constable model, which only considers the pH-dependent charge of plasma buffers and in

which the estimation of pK_a and Atot is not affected by unmeasured anions, the values obtained in healthy volunteers were as follows: Atot = 16.1 ± 3.4 mmol/ L (equivalent of 0.338 ± 0.073 mmol/g of albumin or 0.225 ± 0.049 mmol/g of total protein) and pK_a = 7.55 ± 0.17. Atot was significantly reduced in both patient populations (postoperative: 9.5 ± 4.4 mmol/L, septic: 11.9 ± 4.7 mmol/L), while pK_a was not different from healthy controls (postoperative: 7.48 ± 0.25, septic: 7.44 ± 0.36). Atot normalized for total protein concentration did not differ between healthy volunteers and either patient population. Results obtained with other mentioned approaches are also provided.

Conclusions. Using the Stewart model, the values of Atot and pK_a obtained in healthy volunteers matched their only previous experimentally derived estimates (Atot = 23.3 mmol/L, pK_a = 6.64). The analysis performed in healthy volunteers according to the Staempfli-Constable model validated the previously published value of Atot (17.2 mmol/L) but showed a difference of 0.45 in pK_a (previous estimate: 7.1). This is explained by a simultaneous difference in the amount of fixed charge displayed by non-carbonic buffers, so that, in the pH region of interest, our titration curve practically overlaps with the curve constructed using the previously published parameters. The absence of differences between the study groups in both Atot normalized to total protein concentration and pK_a (determined according to the Staempfli-Constable model) indicates that neither critical illness *per se* nor the presence of sepsis alters the structure of non-carbonic buffers to an extent that would measurably affect their buffer action.

Abstract (Czech)

Úvod. Hodnocení stavu acidobazické rovnováhy je základním nástrojem využívaným lékaři k vedení terapie u celé řady stavů. Ze všech biologických tekutin, v nichž byla acidobazická rovnováha zkoumána, je nejlépe popsaná izolovaná plazma. K jejímu hodnocení byly vyvinuty dva hlavní přístupy: Jedním z nich je tradiční bikarbonátová škola, na jejímiž vzniku se podíleli Henderson, Hasselbalch, van Slyke a Siggaard-Andersen. Druhým je kvantitativní fyzikálně-chemický přístup vyvinutý Stewartem, který dále rozvíjeli Staempfli a Constable nebo Figge a Fencl. Každý z uvedených modelů využívá soubor konstant, které charakterizují chování nebikarbonátových pufrů (proteinů a fosfátů) v plazmě a jejichž hodnota je rozhodující pro přesný výpočet odvozených parametrů. Hodnoty konstant využívaných Stewartovým modelem, tj. celková koncentrace slabé netěkavé kyseliny (Atot) a její disociační konstanta (pK_a), byly experimentálně stanoveny pouze jednou, a to u skupiny 8 zdravých dobrovolníků. Naším cílem bylo znovu experimentálně stanovit hodnoty těchto konstant u zdravých dobrovolníků a, vůbec poprvé, u dvou skupin kriticky nemocných pacientů. Naší hypotézou bylo, že pufrační vlastnosti slabých netěkavých kyselin se vlivem kritického stavu mohou měnit, což by se projevilo jako rozdíl v pK_a nebo v převodním faktoru mezi Atot a měřenou koncentrací hlavních nebikarbonátových pufrů (tj. albuminu nebo celkové bílkoviny). Nad rámec této analýzy, založené na Stewartově a Staempfliho-Constablově modelu plazmy, jsme hodlali určit hodnoty konstant, které charakterizují nebikarbonátové pufry v klasickém a Figgeho-Fenclově modelu plazmy.

Metody. Krev zdravých dobrovolníků (N=30), pacientů po elektivní rozsáhlé nitrobřišní operaci (N=27) a pacientů se sepsí (N=30) byla odebrána na všeobecných jednotkách intenzivní péče dvou lékařských center v Česku a Itálii. V izolované plazmě jsme pomocí CO_2 tonometru měnili parciální tlak oxidu uhličitého (pCO_2) v rozmezí 2 až 16 kPa (15 až 120 mmHg) za současného opakovaného měření pH, pCO_2 a koncentrace elektrolytů pomocí standardního analyzátoru krevních plynů. Experimentálně získaná data byla využita nelineárním regresním algoritmem spolu s rovnicemi charakterizující každý z uvedených modelů plazmy k určení konstant, které charakterizují pufrační aktivitu nebikarbonátových pufrů v každém modelu. Ty pak byly porovnány mezi studovanými skupinami a s jejich dříve publikovanými hodnotami.

Výsledky. Hodnoty stanovené u zdravých dobrovolníků pro Stewartův model byly následující: Atot = 24,3 ± 1,9 mmol/l (což odpovídá 0,510 ± 0,035 mmol/g albuminu nebo 0,340 ± 0,024 mmol/g celkové bílkoviny) a pK_a = 6,63 ± 0,09. Tento model se ukázal být náchylným na vliv neměřitelných aniontů, a tím pádem nevhodný ve skupině pooperačních a septických pacientů. Při použití Staempfliho-Constablova modelu, který zohledňuje pouze pH-dependentní náboj plazmatických pufrů a v němž není stanovení pK_a ani Atot ovlivněno přítomností neměřitelných aniontů, byly hodnoty

získané u zdravých dobrovolníků následující: Atot = 16,1 ± 3,4 mmol/l (což odpovídá 0,338 ± 0,073 mmol/g albuminu nebo 0,225 ± 0,049 mmol/g celkového proteinu) a pKa = 7,55 ± 0,17. Atot byla významně snížena u obou skupin pacientů (pooperační: 9,5 ± 4,4 mmol/l, septičtí: 11,9 ± 4,7 mmol/l), zatímco pK_a se nelišila od zdravých kontrol (pooperační: 7,48 ± 0,25, septičtí: 7,44 ± 0,36). Atot normalizovaná na koncentraci celkové bílkoviny se nelišila mezi zdravými dobrovolníky a žádnou z pacientských skupin. Výsledky získané pomocí ostatních zmíněných přístupů jsou také popsány.

Závěr. Při použití Stewartova modelu se hodnoty Atot a pK_a u zdravých dobrovolníků shodovaly s jejich jedinými předchozími experimentálně stanovenými hodnotami (Atot = 23,3 mmol/l, pK_a = 6,64). Analýza provedená u zdravých dobrovolníků podle Staempfliho-Constablova modelu validovala dříve publikovanou hodnotu Atot (17,2 mmol/l), ale ukázala rozdíl 0,45 v pK_a (předchozí hodnota: 7,1). To je vysvětleno současně přítomným rozdílem v množství fixního náboje na nebikarbonátových pufrech, což má za důsledek, že se v oblasti zkoumaného pH naše titrační křivka prakticky překrývá s křivkou sestrojenou podle dříve publikovaných parametrů. Absence rozdílů mezi studovanými skupinami v Atot normalizované na koncentraci celkové bílkoviny i v pK_a (určených dle Staempfliho-Constablova modelu) ukazuje, že ani kritické onemocnění jako takové, ani přítomnost sepse nemění strukturu nebikarbonátových pufrů do takové míry, aby byl měřitelně ovlivněn jejich pufrační účinek.

Abstract (Italian)

Introduzione. La valutazione dell'equilibrio acido-base è uno strumento essenziale, utilizzato dai medici per guidare la terapia in un'ampia gamma di condizioni cliniche. Tra i vari fluidi biologici di cui è stato studiato l'equilibrio acido-base, il plasma isolato è quello meglio descritto. Per la sua valutazione sono stati sviluppati due approcci principali: uno è quello della scuola tradizionale, incentrato sul bicarbonato e basato sul lavoro di Henderson, Hasselbalch, van Slyke e Siggaard-Andersen; l'altro è l'approccio fisico-chimico quantitativo, inventato da Stewart e ulteriormente sviluppato da Staempfli e Constable o da Figge e Fencl. Ciascuno dei modelli citati utilizza una serie di costanti per caratterizzare il comportamento dei tamponi non carbonici nel plasma (proteine e fosfati), il cui valore è fondamentale per poter effettuare un calcolo preciso dei parametri derivati. I valori delle costanti utilizzate dal modello di Stewart, cioè la concentrazione totale di acidi deboli non volatili (Atot) e la loro costante di dissociazione (pK_a), derivano da un unico studio sperimentale eseguito su un gruppo di 8 volontari sani. Il nostro obiettivo era quello di determinare sperimentalmente il valore di queste costanti in volontari sani e, per la prima volta, in due popolazioni di pazienti critici. Abbiamo ipotizzato che le proprietà tampone degli acidi deboli non volatili siano alterate dalla malattia critica; ciò si manifesterebbe come una differenza nella pKa o nel fattore di conversione tra gli Atot e la concentrazione misurata dei principali tamponi non carbonici (albumina o proteine totali). Oltre all'analisi descritta, che si basa sul modello plasmatico di Stewart e Staempfli-Constable, abbiamo cercato di ottenere stime delle costanti che caratterizzano i tamponi non carbonici nel modello plasmatico classico di Figge-Fencl.

Metodi. Un prelievo ematico è stato eseguito in un gruppo di volontari sani (N=30), in pazienti sottoposti elettivamente a chirurgia addominale maggiore (N=27) e in pazienti con sepsi (N=30) ricoverati presso le Unità di Terapia Intensiva Generale di due ospedali, situati rispettivamente in Repubblica Ceca e Italia. Tramite un tonometro a CO_2, la pressione parziale di anidride carbonica (pCO_2) del plasma isolato è stata manipolata in un intervallo compreso tra 2 e 16 kPa (15 e 120 mmHg). Il pH, la pCO_2 e le concentrazioni degli elettroliti sono stati misurati ripetutamente con un emogasanalizzatore standard. I dati sperimentali, insieme alle equazioni che caratterizzano i modelli plasmatici citati, sono stati analizzati tramite un algoritmo di regressione non lineare per determinare in ciascun modello le costanti caratterizzanti l'azione dei tamponi non carbonici plasmatici. Le costanti così ottenute sono state poi confrontate sia tra i gruppi di studio, sia con le stime precedentemente pubblicate.

Risultati. Applicando il modello di Stewart al plasma isolato, i valori determinati nei volontari sani sono stati i seguenti: Atot = 24,3 ± 1,9 mmol/L (equivalente a 0,510 ± 0,035 mmol/g di albumina o a 0,340 ±

0,024 mmol/g di proteine totali) e pK_a = 6,63 ± 0,09. Questo modello si è rivelato suscettibile all'effetto confondente esercitato dalla presenza di eventuali anioni non misurati ed è, quindi, inappropriato per la valutazione di pazienti post-operati e settici. Utilizzando il modello di Staempfli-Constable, che considera solo la carica pH-dipendente dei tamponi plasmatici e in cui la stima di pK_a e Atot non è influenzata dagli anioni non misurati, i valori ottenuti nei volontari sani sono stati i seguenti: Atot = 16,1 ± 3,4 mmol/L (equivalente a 0,338 ± 0,073 mmol/g di albumina o 0,225 ± 0,049 mmol/g di proteine totali) e pK_a = 7,55 ± 0,17. Gli Atot erano significativamente ridotti in entrambe le popolazioni di pazienti (post-operati: 9,5 ± 4,4 mmol/L; settici: 11,9 ± 4,7 mmol/L), mentre la pKa non differiva dai controlli sani (post-operati: 7,48 ± 0,25; settici: 7,44 ± 0,36). Gli Atot normalizzati per la concentrazione di proteine totali non differivano tra i volontari sani e i due gruppi di pazienti. Vengono inoltre mostrati anche i risultati ottenuti con gli altri approcci sopracitati.

Conclusioni. Utilizzando il modello di Stewart, i valori di Atot e pKa ottenuti nei volontari sani corrispondevano alle uniche stime note derivate sperimentalmente in precedenza (Atot = 23,3 mmol/L, pK_a = 6,64). L'analisi eseguita su volontari sani secondo il modello di Staempfli-Constable ha convalidato il valore di Atot precedentemente pubblicato (17,2 mmol/L), ma ha mostrato una differenza di pKa di 0,45 (stima precedente: 7,1). Ciò si spiega con una contemporanea differenza nella quantità di cariche fisse mostrate dai tamponi non carbonici, così che, nell'intervallo di pH considerato, la nostra curva di titolazione è praticamente sovrapponibile a quella costruita utilizzando i parametri precedentemente pubblicati. L'assenza di differenze tra i gruppi di studio sia per gli Atot normalizzati per la concentrazione totale di proteine, sia per la pKa (determinata secondo il modello di Staempfli Constable) indica che né la malattia critica in sé, né la presenza di sepsi alterano la struttura dei tamponi non carbonici in misura tale da influire in modo misurabile sulla loro azione tampone.

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1 Introduction

Blood gas analysis is a medical test that measures the amount of oxygen and carbon dioxide in the blood, as well as the pH and other parameters related to the acid-base balance.

The first observations of acid or alkaline reactions of body fluids (blood, gastric juice, or pancreatic juice) date back to the 18th century, but very little was known about their true chemical nature. Three milestones in our understanding of acid-base chemistry and physiology were the formulation of the Arrhenius theory of acids and bases in 1884, invention of electrometric measurement of the hydrogen ion activity by Wilhelm Ostwald and Walther H. Nernst in 1890, and introduction of the concept of hydrogen ion exponent (pH) by Søren P. L. Sørensen in 1909. At the same time, the importance of bicarbonate for acid-base stability of body fluids was recognized. The equation for dissociation carbonic acid, derived by Lawrence J. Henderson in 1908, was transformed to comply with the pH notation by Karl A. Hasselbalch in 1917. Hasselbalch was also the first to perform valid pH measurements of blood with a modified hydrogen electrode. However, this procedure was troublesome – the solution had to first be fully deoxygenated and saturated with hydrogen. Colorimetric methods were used as an alternative, but they lacked accuracy. Therefore, routine measurement of blood pH was not possible until the 1950s when the glass electrode, developed gradually since the beginning of the 19th century, became widely available (Severinghaus and Astrup 1985; Astrup and Severinghaus 1986).

In 1669, Robert Boyle demonstrated for the first time that blood exposed to vacuum releases dissolved gases. A hundred years later, Humphry Davy identified that these gases contain oxygen and carbon dioxide. Many investigators followed with qualitative as well as quantitative analyzes of blood gases. Initially, the only method to determine pCO_2 was chemical detection of CO_2 in small bubbles of gas equilibrated with the blood samples. After formulation of the Henderson-Hasselbalch equation, pCO_2 could calculated from pH (measured by platinum hydrogen electrode or colorimetrically) and total CO_2 released from acidified blood inside the van Slyke apparatus. When the pH electrode arrived, Paul Astrup invented the equilibration method for pCO_2 determination: pH was measured in an anaerobically drawn blood sample and after equilibration with two gases of known CO_2 tensions. The linear relationship between pH and log(pCO_2) was then used to determine pCO_2 in the original sample. A true revolution came with the construction of a pCO_2 electrode by Richard Stow and John W. Severinghaus in the 1950s and 1960s. Thanks to its precision and easy operation, the pCO_2 electrode soon replaced the equilibration technique in the clinical setting. (Astrup and Severinghaus 1986; Severinghaus and Astrup 1986a).

The measurement of pO_2 followed a path similar to that described for pH and pCO_2. The first analyzes used a small bubble of gas equilibrated with the blood sample. Another method determined pO_2 from measured blood oxygen saturation and the known oxygen–hemoglobin dissociation curve. Obviously, this method was imprecise due to factors that affect the dissociation curve and was completely useless for plasma or fully saturated blood. Electrochemical measurement of pO_2 using a platinum electrode, which was carried out successfully in protein-free solutions by the already mentioned lab of Walther H. Nernst, was not suitable for plasma or blood because the electrode was rapidly 'poisoned' by denatured protein. A solution to this problem was polarography with a dropping-mercury electrode invented by Jaroslav Heyrovský at Charles University in 1920s. The problem of protein accumulation was eliminated by the continuously renovated surface of mercury drops. The bubble equilibration method and the dropping-mercury electrode were the only reliable methods of estimating pO_2 in plasma and blood until Leland C. Clark, the author of the first blood bubble oxygenator, constructed a dedicated pO_2 electrode. He needed a reliable method for determination of pO_2 to characterize the function of his device. After experimenting with bare platinum electrodes, he came up with the idea of coating the electrode in material permeable to oxygen that would prevent the electrode from being 'poisoned'. The first working prototype of a pO2 electrode with a platinum core and polyethylene coating was constructed in 1954 and was an immediate success (Astrup and Severinghaus 1986; Severinghaus and Astrup 1986b; Severinghaus 2002; Clark et al. 1953).

Following the invention of dedicated electrodes for measuring pH, pO_2, and pCO_2, it was only a matter of time until a device combining all three electrodes would be constructed. The first device of this kind was assembled by John W. Severinghaus and A. Freeman Bradley in 1958 (Severinghaus and Astrup 1986b). In the 1970s, commercial blood gas analyzers were introduced and allowed the measurement of blood gases on a routine basis (Astrup and Severinghaus 1986). Since then, blood gas analysis has become an essential tool in acute care settings, such as intensive care units, emergency departments, and operating rooms. Modern analyzers use sophisticated technology to measure not only blood gases, but also a range of other parameters, such as electrolyte, lactate, glucose, and hemoglobin levels. This information is used to diagnose a wide range of medical conditions and guide therapy in the critically ill.

1.1 Models of acid-base equilibrium in plasma

Plasma, the acellular component of blood, is a single compartment fluid that consists of water, electrolytes, proteins, and various forms of carbonic acid including bicarbonate. In this chapter various approaches to the description of acid-base properties of plasma are introduced. These models use mathematical expressions to describe the role of the above-mentioned plasma constituents. Interestingly, little discussion takes place about the behavior of water, ions, phosphates, or bicarbonate. Thus, the debate about different acid-base models of plasma essentially narrows down to how they handle the acid-base interaction of plasma proteins.

Accurate models of plasma acid-base equilibrium are vital for accurate and reliable interpretation of blood-gas analysis. First, they serve as a basis on which the more complex models (i.e., of blood or whole-body) can be developed. Second, they allow for electroneutrality calculations that may unveil hidden components circulating in plasma (Chapter 1.3).

1.1.1 Classical model of plasma

In this thesis, the term classical model refers to the bicarbonate-centered approach of acid-base evaluation that was developed by Ole Siggaard-Andersen and several other Danish scientists around the 1960s. Their work relies heavily on the experiments by Henderson, Hasselbalch, and van Slyke, which are, too, introduced in this chapter.

According to the classical approach, two types of derangements exist: respiratory, characterized by alteration of pCO_2, and metabolic, for which the choice of a suitable quantifier has been long and eagerly discussed.

1.1.1.1 Henderson and Hasselbalch: The dissociation of weak acids

Lawrence J. Henderson updated the already-known law of mass action for the dissociation of weak acids in aqueous solutions to account for mixtures of an acid and its salt (Henderson 1908a; 1908b):

$$[H^+] = K \times \frac{[HA]}{[A^-]} \qquad \textit{Eq. 1}$$

where [HA] is the concentration of the associated form, $[A^-]$ is the concentration of the dissociated form, and K is a constant. Henderson also noted the ability of such mixtures to maintain stable $[H^+]$ even when a significant amount of acid is added and concluded that the most important acid/salt pairs for maintaining the neutral reaction in human blood are proteins, carbonic acid/bicarbonate, and phosphates. After introduction of the pH scale, Karl A. Hasselbalch transformed the equation that governs the dissociation of weak acids into a logarithmic form:

$$pH = pK + log\frac{[A^-]}{[HA]} \qquad \textit{Eq. 2}$$

which can be, in the case of carbonic acid/bicarbonate, rewritten as (Hasselbalch 1917):

$$pH = pK_1' + log\frac{[HCO_3^-]}{S \times pCO_2} \qquad \textit{Eq. 3}$$

where S is the CO_2 solubility coefficient in water. Eq. 3, the Henderson-Hasselbalch equation has since than occupied a central position the evaluation of acid-base status according to the classical approach.

1.1.1.2 Van Slyke: Quantifying the effect of buffers

Buffers are molecules that contain a titratable site which provides the ability to bind and release H^+ in response to changes in pH. A buffer is characterized by its concentration and the acidic dissociation constant (pK_a). The interaction between buffer dissociation and pH follows Eq. 2. For nonvolatile buffers, the law of mass conservation also applies:

$$[HA] + [A^-] = const. \qquad \textit{Eq. 4}$$

It is important to realize, that the relationship between the dissociation of titratable sites and pH, as described by Eq. 2, is reciprocal. To illustrate this fact, consider a solution of a weak monoprotic acid in water. It is clear that when the pH of such solution is manipulated by an addition of a strong acid or base, the ratio between [HA] and $[A^-]$ changes. At the same time, the presence of the weak acid affects how much the pH of the solution changes upon the addition of the strong acid or base: Under certain conditions, the pH shift may be significantly lower than if no weak acid were present. This concept is called buffering and the ability of a solution to minimize pH excursions can be described quantitatively by its buffer capacity – β (Van Slyke 1922):

$$\beta = \frac{dB}{dpH} = \frac{-dA}{dpH} \qquad \textit{Eq. 5}$$

where B and A represent the amount of a strong base or acid added. A titration curve of a weak monoprotic acid is shown in Figure 1-1 (left panel) and the corresponding β in Figure 1-1 (right panel) It is apparent that, for ordinary acids, the buffer action is limited to a pH range in the proximity of pK_a. Outside of this range, molecules of the acid carry a fixed charge and can be treated as strong ions. Despite its significance, the width of this range is not universally accepted. It can be assumed with only a negligible error that β = 0, if pH and pK_a differ by more than ±3 (Fencl et al. 2000), but a lower threshold, such as 1.5, is also used (Constable 1997).

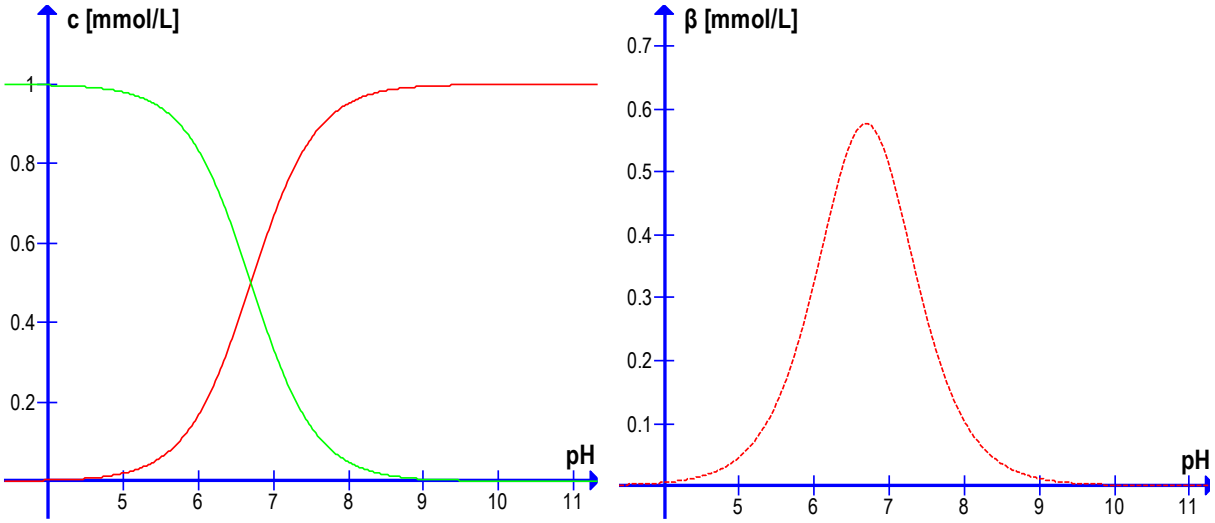

Figure 1-1. Titration of a weak monoprotic acid (pK_a = 6.7, Atot = 1 mmol/L) by a strong acid or base. ***Left panel:*** *The change in concertation (c) of the associated ([HA], green) and dissociated ($[A^-]$, red) form of a weak acid in response to pH.* ***Right panel:*** *The corresponding buffer capacity (β).*

Within the buffer range of a weak acid, almost all H^+ that are introduced during the addition of a strong acid are immediately bound by the dissociated form of the weak acid $[A^-]$. This leads to reduction of $[A^-]$ that is numerically equivalent to the amount of the strong acid or base added:

$$dB = -dA = d[A^-] = -d[HA] \qquad \textit{Eq. 6}$$

The buffer power of an individual weak acid (HA) can, therefore, be expressed as:

$$\beta(HA) = \frac{d[A^-]}{dpH} = -\frac{d[HA]}{dpH} \qquad \textit{Eq. 7}$$

It is also apparent from Figure 1-1 that β of a weak acid is a function of pH: Maximum β is reached when pH = pK_a, which is present when [HA] = $[A^-]$. In solutions containing multiple weak acids or proteins with several titratable sites, buffering is more complex: When pH of the solution is close to pK_a of several titratable sites, they all take part in the overall buffer action. The contribution of each site depends on its concentration and the difference between its pK_a and pH.

An important property of complex buffer solutions is that the total buffer capacity of a solution (S) at any pH can be obtained as a simple sum of the buffer effects of all individual titratable sites (TS), obtained from Eq. 7, at the given pH (Van Slyke 1922):

$$\beta(S) = \beta(TS_1 \subset S) + \beta(TS_2 \subset S) + \beta(TS_3 \subset S) + \cdots \qquad \textit{Eq. 8}$$

Plasma proteins are complex macromolecules that contain many functional sites. In the classical model of plasma, the buffer power of proteins is approximated by a constant (Siggaard-Andersen 1974). The

rationale for such an approximation derives from the fact that proteins possess several titratable sites. If pK_a of these sites are different, equally distributed, and separated by less than 1 unit, then as the buffering effect of one site fades away, another one starts to act. This may create a region of constant buffer power (Van Slyke 1922), as illustrated in Figure 1-2 and expressed by the following equation:

$$\frac{d[Pr^-]}{dpH} = const. \qquad \textit{Eq. 9}$$

It follows that in this range of constant buffer power, the negative charge carried by plasma proteins, $[Pr^-]$, can be expressed as a linear function of pH:

$$[Pr^-] = k_1 \times pH + k_2 \qquad \textit{Eq. 10}$$

These theoretical considerations were confirmed by experimental data for hemoglobin (Hastings et al. 1924; Van Slyke et al. 1922) and for equine serum proteins (Van Slyke et al. 1928). In the last article, the authors developed the following formulae for the estimation of the charge carried by equine albumin and globulins:

$$[Alb^-]\ (mEq/l) = 0.123 \times Albumin\ (g/l) \times (pH - 5.16) \qquad \textit{Eq. 11}$$

$$[Glob^-]\ (mEq/l) = 0.076 \times Globulins\ (g/l) \times (pH - 4.89) \qquad \textit{Eq. 12}$$

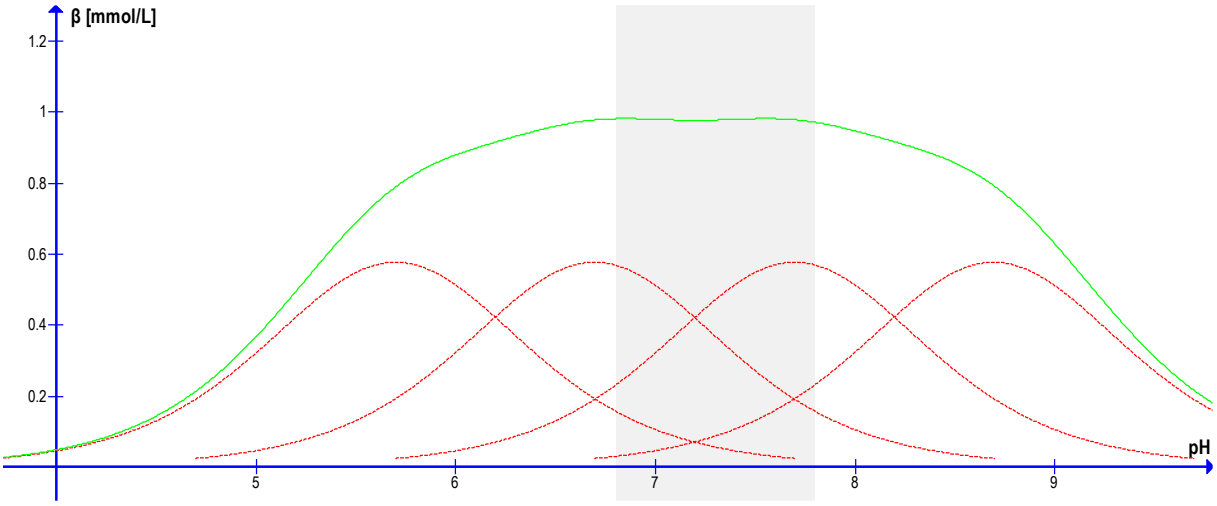

Figure 1-2. The buffer power of a macromolecule with four titratable sites. Red curves: the buffer power of each titratable site (pKa = 5.7, 6.7, 7.7, and 8.7). Green curve: the overall buffer power, obtained as a sum of the four individual curves. Note that due to the summation of buffer action of several titratable sites, the overall buffer power may be approximated by a constant very well in the pH range of interest (grey area). Adapted from Van Slyke, 1922.

In Eq. 11 and Eq. 12, a factor of 6.35, applied in the original source, was used to convert protein nitrogen mass to protein mass. For the normal albumin to globulin ratio in human plasma, they derived a formula that, despite being based on equine serum proteins, became widely used:

$$[Pr^-] = 0.104\ mmol/g \times TP\ (g/l) \times (pH - 5.08) \qquad \textit{Eq. 13}$$

where TP stands for total protein. According to this equation, the charge of plasma proteins under normal conditions (TP = 70 g/L, pH = 7.4) is 17.1 mEq/L. Differentiation of Eq. 13 with respect to pH provides the buffer power of plasma proteins:

$$\beta(TP) = \frac{d[Pr^-]}{dpH} = 0.104\ mmol/g \times TP\ (g/l) \qquad \textit{Eq. 14}$$

The titration curve according to Eq. 13 is presented in Figure 1-4, Chapter 1.2. The coefficient 0.104 is, in fact, the buffer power of plasma proteins normalized for TP concentration:

$$\beta(TP)_{N(TP)} = \frac{\beta(TP)}{TP} = 0.104\ mmol/g \qquad \textit{Eq. 15}$$

The buffer power of phosphate, another non-carbonic buffer of plasma, can be calculated from its concentration, pK_2, and pH (Siggaard-Andersen et al. 1977):

$$\beta(Pi) = 2.303 \times Pi \times \frac{10^{-pK_2} \times [H^+]}{(10^{-pK_2} + [H^+])^2} \qquad \textit{Eq. 16}$$

which with pK_2 of 6.8 provides β(Pi) of 0.44 mmol/L under normal conditions (Pi = 1.2 mmol/L, pH = 7.4). According to Eq. 8, the overall buffer power of non-carbonic buffers in plasma under normal conditions is:

$$\begin{aligned} \beta(NC) = {} & \beta(TP) + \beta(Pi) = 0.104\ mmol/g \times 70\ g/L + 0.44\ mmol/L \\ & = 7.7\ mmol/L \end{aligned} \qquad \textit{Eq. 17}$$

Due to the minor contribution of phosphate, β(NC) is often normalized for TP concentration, providing $\beta(NC)_{N(TP)}$:

$$\beta(NC)_{N(TP)} = \frac{\beta(NC)}{TP} = \frac{7.7\ mmol/L}{70\ g/L} = 0.110\ mmol/g \qquad \textit{Eq. 18}$$

1.1.1.3 Peculiarities of the bicarbonate buffer system in vivo

The pK_1' of carbonic acid in plasma is 6.105 (CLSI 2009). With the interval of ~1.3 between pK_1' and pH encountered in human plasma, one would expect the buffer ability of the bicarbonate buffer system, β(Bic), to be severely limited (compare Figure 1-1, right panel). Under normal conditions, an equivalent

of Eq. 16 would provide β(Bic) of 2.7 mmol/L, which is substantially less than β(NC) or β(TP). Despite that, bicarbonate is the most powerful buffer of human plasma in vivo. Its advantage stems from the fact that, in vivo, the carbonic acid species are not limited by the law of mass conservation (Eq. 4), as a stable pCO_2 is maintained by the respiratory system. This property, unique to the open bicarbonate buffer system, increases β(Bic) to overwhelming 56.6 mmol/L under normal conditions in vivo (Van Slyke 1922; Siggaard-Andersen et al. 1977).

1.1.1.4 The respiratory component – pCO_2

Partial pressure of carbon dioxide (pCO_2) has been used to quantify the respiratory component of acid-base equilibrium ever since respiration became known to alter acid-base balance and has remained unchallenged to date. This is true across all approaches to acid-base equilibration mentioned in this thesis and even for respiratory derangements of the acid-base equilibrium in blood or at the whole-body level.

1.1.1.5 The metabolic component – Siggaard-Andersen's Base excess

Since the introduction of acid-base analysis, pioneers of the classical approach have searched for a suitable parameter to describe the metabolic component of acid-base disorders. Initially, $[HCO_3^-]$ was seen as a surrogate for metabolic acid-base status (Van Slyke and Cullen 1917). A strong limitation of this simplistic view is that $[HCO_3^-]$ is altered by both metabolic and respiratory derangements.[1] Although the effect of respiration on $[HCO_3^-]$ is less pronounced, a more universal tool was needed. A possible candidate was the standard bicarbonate, i.e., bicarbonate concentration in plasma of blood equilibrated to pCO_2 of 40 mmHg (Jørgensen and Astrup 1957). However, another parameter, the Base Excess (BE), became widely used (Siggaard-Andersen 1974). BE is defined as the amount of strong acid that is needed to restore the pH of 7.4 after normalization of pCO_2 has been achieved. In current blood-gas analyzers, however, neither CO_2 equilibration nor titration by a strong base takes place and the value of BE is calculated solely from pH and pCO_2 according to the following equation (Siggaard-Andersen 1977):

$$BE(P) = [HCO_3^-]_{act} - 24.4 + \beta(NC) \times (pH - 7.4) \qquad \textit{Eq. 19}$$

[1] When a polio epidemic hit Copenhagen in 1952, some clinicians initially assumed that the victims were dying from a metabolic alkalosis of mysterious origin. This is not surprising as the only routinely available acid-base measurement at that time was 'bicarbonate' (i.e., total CO_2 released in the van Slyke apparatus). This was disputed by Bjørn Ibsen and Poul Astrup who argued that CO_2 accumulation was a more likely cause. Their suspicion was quickly confirmed by finding extremely low pH in the blood of polio patients. This observation not only saved hundreds of lives, but also paved the way for routine measurement of pH in clinical practice. (Astrup and Severinghaus 1986).

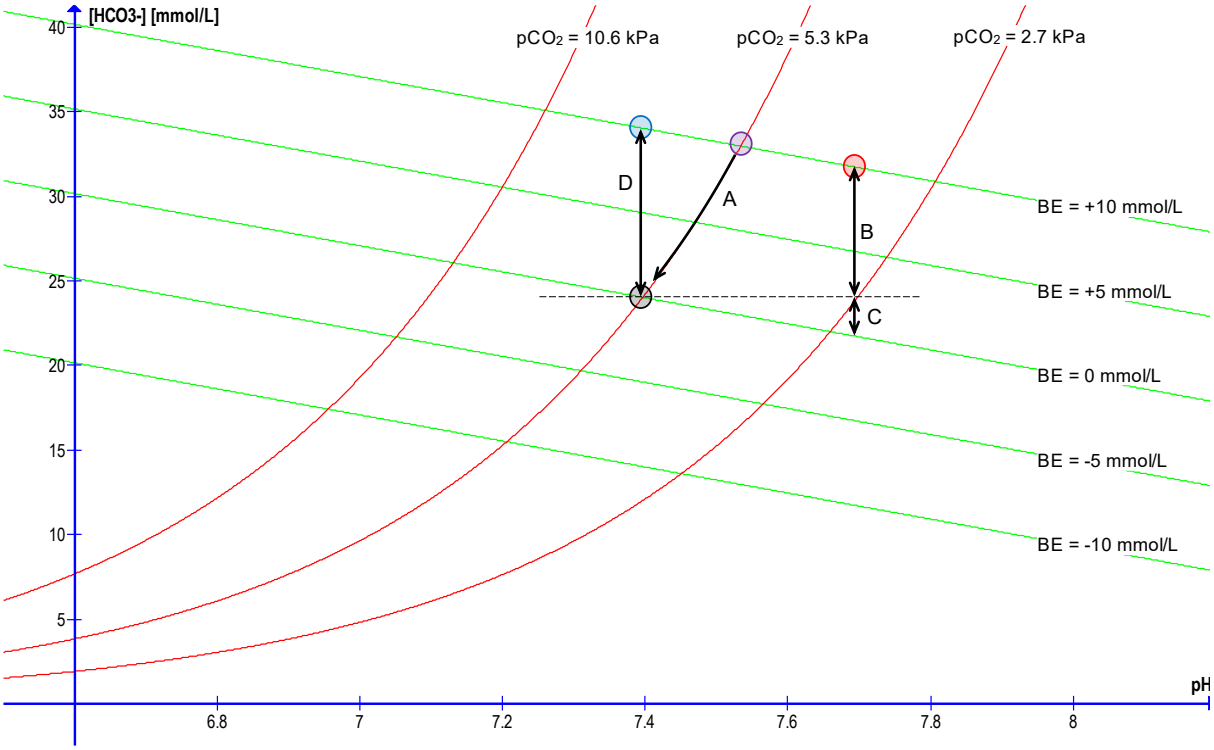

Figure 1-3. Davenport diagram with visualization of the relationship between pH, pCO_2, $[HCO_3^-]$ and BE(P) in plasma and three interpretations of BE(P) calculation. For details see text.

The logic behind this equation is best explained using the classic Davenport $[HCO_3^-]$/pH diagram (Davenport 1974) depicted in Figure 1-3. This diagram contains two kinds of isopleths: green descending lines that represent CO_2 titration at fixed BE(P) and red ascending curves that represent strong acid titration at fixed pCO_2. Let us consider a plasma sample of unknown metabolic acid base status with pH of 7.7, pCO_2 of 3.53 kPa, and $[HCO_3^-]$ of 32 mmol/L (red circle). The classical definition of BE says that first normal pCO_2 must be achieved (purple circle) and then a strong acid or base is added until the pH reaches 7.4 (arrow A). Although simple to understand, this concept is not useful for calculation of BE(P) nor for its experimental determination because the addition of a strong acid or base to a test tube, which is a closed system, alters pCO_2 (i.e., diverts titration from the red line). One useful way to interpret Eq. 19 is that it provides the net change of buffer base concentration, i.e., how much $[HCO_3^-]$ deviates from 24.4 mmol/L (arrow B, approx. 8 mmol/L) plus how much the net charge of other buffers deviates from the normal value it has at pH of 7.4 (arrow C, approx. 2 mEq/L). Another way of interpretation is the following: Imagine manipulating pCO_2 of the plasma sample in such a way that its pH reaches 7.4 (blue circle). The negative charge of plasma proteins is now exactly the same as it is under normal conditions (pCO_2=40 mmHg, BE=0, pH=7.4 – black circle), which means that any deviation of $[HCO_3^-]$ from its reference value of 24.4 mmol/L can only be explained by excess (or deficit) of a strong base (arrow D, 10 mmol/L). Both numerical interpretations are equal (B + C = D) and both require a variable that characterizes H^+ transfer between the buffer anions. This variable is the already

introduced non-carbonic buffer power of plasma, β(NC), which describes the net capacity of buffers other than bicarbonate. In the graph, β(NC) is represented by the slope of the green lines. Ideally, β(NC) would be calculated from TP concentration (Eq. 18), but this quantity is not measured by blood-gas analyzers and the default value of 7.7 mmol/L is usually used instead (CLSI 2009). Importantly, the value of β(NC) can be determined experimentally by CO_2 titration of plasma (Siggaard-Andersen et al. 1977).

The validity of Eq. 19 is limited to plasma. In the case of whole blood, a more complex calculation is needed (CLSI 2009).

1.1.2 Stewart model of plasma

Stewart developed a concept of acid-base physiology that differs from the classical one in many important aspects (Stewart 1981; 1983; 1978). In this theory, the acid-base status of any biological fluid is determined exclusively by three independent variables (see below), while H^+, pH, and HCO_3^- have become mere bystanders that passively adapt to set conditions. Such a huge conceptual shift in perceiving the importance the traditionally appraised parameters has been criticized (Kurtz et al. 2008; Siggaard-Andersen and Fogh-Andersen 1995) and the debate still continues (Seifter 2014: correspondence).

The independent parameters, according to Stewart, are pCO_2, Strong Ion Difference (SID), and total concentration of weak nonvolatile acids (Atot). pCO_2 determines the effect of carbonic acid species and obeys the known Henderson-Hasselbalch equation (Eq. 3). The SID is the net charge attributable to all strong (i.e., fully dissociated) electrolytes:

$$SID = [Na^+] + [K^+] + [Ca^{2+}] + [Mg^{2+}] - [Cl^-] - [Lac^-] \qquad \textit{Eq. 20}$$

where the terms in square brackets denote concentration expressed in mEq/L. This value is obtained by multiplying the molar concentration of each ion by its valence. Finally, Atot characterizes the contribution of weak nonvolatile acids (i.e., non-carbonic buffers: proteins and phosphate). Their combined effect is modeled using a hypothetical weak monoprotic acid, HA, the behavior of which is described by two equations, one for dissociation equilibrium and one for mass conservation:

$$K_a = \frac{[H^+] \times [A^-]}{[HA]} \qquad \textit{Eq. 21}$$

$$A_{tot} = [A^-] + [HA] \qquad \textit{Eq. 22}$$

Not surprisingly, these equations are identical to Eq. 1 and Eq. 4 of the classical approach. Combining Eq. 21 with Eq. 22 and transforming them logarithmically, we obtain:

$$[A^-] = A_{tot} \times \frac{1}{1 + 10^{pK_a - pH}} \qquad \textit{Eq. 23}$$

Comparison of Eq. 23 with Eq. 13 of the classical approach unveils the contrast in handling of proteins: A titration curve of HA in the Stewart model is S-shaped. It spans between 0 and Atot and has an inflection point at pH = pK_a (Figure 1-4). Atot is often expressed as a product of a constant and albumin (or TP) concentration. An interesting similarity between the classical and Stewart approach is that, despite the conceptual difference, a knowledge of two constants and albumin (or TP) concentration is needed to predict the charge of proteins from actual pH.

The Stewart approach, often described as physicochemical, emphasizes the quantitative relationships between the acid-base parameters. All charged species present in plasma are represented in the equation of electroneutrality:

$$SID - [HCO_3^-] - [CO_3^{2-}] - [A^-] + [H^+] - [OH^-] = 0 \qquad \textit{Eq. 24}$$

from which the quantitatively negligible parameters can be omitted without affecting its precision, yielding the so-called simplified electroneutrality equation (Constable 1997):

$$SID - [HCO_3^-] - [A^-] = 0 \qquad \textit{Eq. 25}$$

Combining Eq. 3 for $[HCO_3^-]$, Eq. 23 for $[A^-]$, and Eq. 25 with any given set of the independent parameters (pCO_2, Atot, and SID) leads to a high-order polynomial equation with a unique solution for pH and all other dependable variables. A notable drawback of the Stewart approach is that it cannot, in its native form, handle multi-compartment environments (e.g., whole blood).

1.1.2.1 Values of K_a and Atot

Stewart did not make any attempt to experimentally determine K_a or Atot in human plasma, but he frequently used Atot of 19 (Stewart 1978; 1983) or 20 mmol/L (Stewart 1981) and K_a of 4×10^{-7} (pK_a = 6.40, Stewart, 1978), 2×10^{-7} (pK_a = 6.70, Stewart, 1981) or 3×10^{-7} (pK_a = 6.52, Stewart, 1983). Stewart provided no theoretical background for the preferred K_a and Atot values. One possible explanation is that the K_a of 2×10^{-7} mmol/L was chosen to closely match the K_a value of imidazole corrected for the temperature of 37 °C, which is 1.9×10^{-7} (Constable and Stämpfli 2009). Another possibility is that Stewart chose such values of K_a and Atot that, closely follow the experimental data of protein titration by Van Slyke (Eq. 13; Figure 1-4, top panels). However, no proof of either intention exists. In turn, the

arbitrarily chosen value of K_a and the lack of a conversion factor between Atot and any measurable plasma quantity (e.g., TP or albumin and phosphate concentration) were considered major drawbacks (Constable 1997; 2001).

The titration curve of plasma weak nonvolatile acids constructed with one set of Stewart's preferred parameters (Atot = 20 mmol/L, $K_a = 2 \times 10^{-7}$) is presented in Figure 1-4 (top right panel) along with the corresponding buffer power (bottom right panel). With these parameters, the net negative charge displayed by plasma weak nonvolatile acids (i.e., proteins and phosphates) at the pH of 7.4 is 17.5 mEq/L.

1.1.2.2 Early use of the Stewart approach

The Stewart model has been used to describe pathophysiological processes in a number of conditions such as salt-loading (Anderson and Jennings 1988b; 1988a) or intensive exercise (Forster et al. 1990b; 1990a; Kowalchuk et al. 1988a; 1988b; Lindinger et al. 1987; 1992; Weinstein et al. 1991). Most of the mentioned studies focused on the changes in the SID and examined the contribution of weak nonvolatile acids only briefly, which is consistent with a rather small variation in Atot during exercise and electrolyte disturbances. Moreover, majority of them did use the Stewart approach to evaluate the experimental data but did not attempt to validate it by comparing the measured values of dependent variables (e.g., pH) with the predictions derived from the Stewart equations. Studies for which these statements do not apply or that are otherwise noteworthy are further discussed in the next paragraphs.

Some of the mentioned studies (Lindinger et al. 1992; e.g., Weinstein et al. 1991) seem to be affected by a methodological error affecting Atot calculation. Atot was calculated as a product of TP and a factor of 0.243 or 0.246 mmol/g, which were derived from Eq. 13 (Van Slyke et al. 1928), solved for the pH of 7.4, or from experimental data of manipulation with protein concentration in blood *in vitro* (Rossing et al. 1986, Table 5). There appear to be several problems with this conversion: First, the equation by van Slyke only refers to plasma proteins, but there are other non-carbonic buffers in plasma (mainly phosphates) that also contribute to Atot by at least 2.4 mmol/L. Second, the product of TP and the conversion factors provides the actual charge displayed by either proteins (van Slyke *et al.*) or all nonvolatile weak acids (Rossing *et al.*), while Atot refers the maximum charge they may possibly exhibit if they dissociate completely (Constable 2001). Finally, it was suggested that Eq. 13 overestimates the net protein charge and much lower estimates (12.6 and 12.0 mEq/L) were published by other authors (Figge et al. 1992; Van Leeuwen 1964). It was speculated that the reason that led to the overestimation of $[Pr^-]$ by Eq. 13 was contamination of van Slyke's pure globulin solutions with albumin (Figge et al. 1991; Watson 1999). The same issue of problematic distinguishing between $[A^-]$ and Atot also affected

the study by Anderson and Jennings, where the correctly obtained Atot (calculated from experimental data using Stewart's Eq. 25 and Eq. 23) was directly compared with $[Pr^-]$ derived from Eq. 13 (Anderson and Jennings 1988a).

The fact that the studies criticized in the previous paragraph for improper handling of Atot provided results that were not strikingly wrong may attributed be to the ability of the two errors (the overestimation of $[Pr^-]$ and the confusion between $[A^-]$ and Atot) to partly compensate for each other as they alter the obtained Atot in opposite directions. Yet, a more general explanation exists. The values of K_a and Atot in the Stewart model must be treated as mutually dependent: For a relatively narrow pH range, even grossly inappropriate values of Atot and K_a can produce a good fit with experimental data, if their deviations combine in such a way that Eq. 23 provides a reasonable $[A^-]$. Note that several different values of K_a were used at that time (Chapter 1.1.2.1) and the choice was at discretion of each author.

The study by Kowalchuk *et al.,* whose principal aim was to describe intracellular, arterial, and venous acid-base equilibria during and after intensive exercise (Kowalchuk et al. 1988b), was also the first to asses validity of the Stewart approach. In this study, the $[H^+]$ calculated from SID, pCO_2, and Atot by Stewart's equations was compared with the measured values, finding only small differences. The study by Weinstein *et. al.*, in which acid-base evaluation was performed before and after maximum treadmill exercise (Weinstein et al. 1991), is noteworthy not only for the aim to validate the Stewart approach in a similar way as Kowalchuk *et al.* but also because it is the only experimental study ever co-authored by P. Stewart. In this study, the good agreement of the predicted $[H^+]$ with experimental data was interpreted as an 'unequivocal experimental validation of Stewart's quantitative approach to acid-base status analysis'. While I agree that both studies confirmed the validity of the Stewart approach in general, they did not specifically validate the K_a and Atot values used because of the narrow range of $[H^+]$ explored (approximately 40 to 100 nmol/L, pH 7.0 to 7.4) and the related concerns expressed in the previous paragraph.

1.1.2.3 Experimental determination of pK_a and Atot

Experimental determination of pK_a and Atot was performed in a number of species, e.g., horse (Constable 1997; Stämpfli et al. 1999), cat (McCullough and Constable 2003), dog (Constable and Stämpfli 2005), calves (Constable et al. 2005), and pigeon (Stämpfli et al. 2006). The first step toward the determination of K_a in humans was made in a retrospective analysis of four studies in which strong acid titration and CO_2 tonometry of plasma were performed (Constable 2001). This study, however, had several limitations, the most striking one being that almost half of all data points (42/94) originated from plasma of only two individuals. Furthermore, two of the analyzed studies did not report the SID,

a crucial factor for the algorithm that estimates K_a. In these cases, the SID had to be obtained by an approximate calculation from the reported Base excess.

To date, only one prospective experimental study to determine Atot and K_a in human plasma has been performed (Staempfli and Constable 2003). They repeatedly manipulated pCO_2 in plasma samples from eight healthy volunteers, measuring pCO_2, pH, and the concentration of the main electrolytes at each level. These values were used as input for a nonlinear regression algorithm along with the Stewart equations to solve simultaneously for Atot and K_a. Four different methods of SID calculation were applied and each of them produced a different set of Atot and K_a values. This implies that the estimates of K_a and Atot are very sensitive to the SID used, which was also shown theoretically (Matoušek 2013, pages 96-98 and 109-111). The values obtained with $[SID_6]_{constant}$ (i.e., using the mean $[Na^+]$, $[K^+]$, $[Ca^{2+}]$, $[Mg^{2+}]$, $[Cl^-]$, and $[Lac^-]$ of each subject) were $K_a = 2.27 \pm 0.66 \times 10^{-7}$ and Atot = 23.3 ± 1.5 mmol/L, which corresponded to 0.521 ± 0.026 mmol/g of albumin or 0.308 ± 0.016 mmol/g of TP (Staempfli and Constable 2003, table 5).

This study of Staempfli and Constable was limited by a small sample size of eight subjects and the inclusion of healthy volunteers only. Critically ill patients in intensive care often have a reduced concentration of albumin (Vincent et al. 2002; Caironi et al. 2014), altered A/G ratio, and a variable concentration of phosphate. It follows that their Atot should be altered accordingly. More importantly, plasma buffers of certain critically ill patients (e.g., patients with sepsis) may also be altered qualitatively (see Chapter 1.5), which may affect their K_a and/or the conversion factor between albumin or TP and Atot.

1.1.3 Staempfli-Constable model of plasma

Experimental evidence suggests that part of the charge carried by plasma proteins is fixed (i.e., does not depend on plasma pH). This can be inferred from a detailed characterization of titratable sites on the albumin molecule – pK_a of many sites is so far away from the pH range compatible with life that they cannot contribute to the buffer action (Figge et al. 1991; 1992).

A possible remedy for this fact was explored in the same study that was described in the previous chapter (Staempfli and Constable 2003) by employing a novel approach to the handling of weak nonvolatile acids, attributing them with a small amount of fixed charge. To obtain the parameters needed to describe the new model, the measured concentrations of strong electrolytes were disregarded, and an appropriate value of the SID ($SID_{estimated}$) was determined by the nonlinear regression algorithm together with Atot and K_a. The values of Atot = 17.2 ± 3.5 mmol/L (equivalent of 0.378 ± 0.078 mmol/g of albumin or 0.224 ± 0.044 mmol/g of TP), $K_a = 0.8 \pm 0.6 \times 10^{-7}$ ($pK_a = 7.10$), and

$SID_{estimated}$ = 37.1 ± 4.1 mEq/L were obtained. Importantly, the $SID_{estimated}$ was lower than the SID calculated from the directly measured concentration of strong ions by up to 8.9 mEq/L (depending on the SID calculation used), which means that during the simultaneous determination of Atot, K_a, and $SID_{estimated}$, part of the negative charge carried by plasma proteins was incorporated into the $SID_{estimated}$ partition.

Compared to the truly measured SID, described in the previous chapter, the modification with $SID_{estimated}$ led to higher R^2 and was deemed more accurate. Note that the K_a and Atot values, obtained this way, refer only to the pH-dependent portion of protein and phosphate charge. For the remaining part, dedicated formulae were developed (Table 1-1). Apart from the distinctive handling of weak nonvolatile acids, the Staempfli-Constable model of plasma follows the general rules of the original Stewart model.

Charged moiety	Type of charge	Calculation	Approx. value under normal conditions
Proteins	fixed	0.090 mEq/g of albumin or 0.052 mEq/g of TP	3.8 mEq/L
	pH-dependent	Eq. 23 for Atot and K_a see text	11.5 mEq/L
Phosphate	fixed	1 mEq/mmol of Pi	1.2 mEq/L

Table 1-1. pH-dependent and pH-independent charge of weak nonvolatile acids in plasma according to the Staempfli-Constable model.

The work of Staempfli and Constable was further developed by Lloyd, who incorporated the described principles into an automatic diagnostic tool (Lloyd 2004; Lloyd and Freebairn 2006).

Some limitations of the study by Staempfli and Constable such as inclusion of healthy volunteers only were already described in Chapter 1.1.2.3. However, a new problem arises with the inclusion of $SID_{estimated}$ among the parameters that are derived by the nonlinear regression algorithm. The pH range explored is relatively narrow (~1 unit) and the exchange of [HCO_3^-] for [A-] in response to CO_2 manipulation is relatively small (~7 mEq/L in healthy subjects) and is further decreased in patients with hypoproteinemia or hypoalbuminemia. Under such conditions, the model may become overparameterized, unstable, and fail to provide reliable estimates of the required variables.

1.1.4 Figge-Fencl model of plasma

Based on the work of Stewart and thanks to the advances made in protein structure analysis, a model that treats phosphate and all titratable sites of albumin individually (i.e., with a unique dissociation constant) was developed (Figge et al. 1991; 1992). The dissociation constants were determined from

either acid-base titration or nuclear magnetic resonance spectrometry. The validity of the model was verified by checking its predictions against measured data from variously manipulated samples of human serum and several artificial solutions of human albumin. This complex mathematical model that employs a total of 23 different dissociation constants can be, in the physiological pH range, simplified to a linear form that follows the general form of the electroneutrality equation (Eq. 25):

$$[XA^-] = SID - [HCO_3^-] - Alb \times (0.123 \times pH - 0.631) \\ -Pi \times (0.309 \times pH - 0.469) \qquad \textit{Eq. 26}$$

where $[XA^-]$ represents the possibly present unmeasured anions and is close to zero in healthy subjects.

The linear form of the albumin term resembles Eq. 10 and Eq. 13 of the classical approach (Chapter 1.1.1). The constant 0.123 mmol/g (later referred to as F1), therefore, represents the buffer power of albumin normalized for its concentration – $\beta(Alb)_{N(Alb)}$. Notice the small difference from $\beta(NC)_{N(Alb)}$ of the classical approach, which comprises the buffer capacity of all non-carbonic buffers, including other proteins and phosphate. In the Figge-Fencl model, the contribution of phosphate is covered by a separate term and the contribution of proteins other than albumin is claimed to be negligible. Interpreting the coefficient 0.631 is difficult. It may, however, be modified as follows:

$$[Alb^-] = Alb \times (0.123 \times pH - 0.631) = Alb \times [0.123 \times (pH - 7.4) + 0.279] \qquad \textit{Eq. 27}$$

After this modification, the coefficient 0.279 mEq/g (later referred to as F2) allows calculation of the charge carried by albumin at pH of 7.4 while the unchanged 'buffer' factor (F1) still explains how this charge varies with pH.

According to Eq. 26, the net negative charge carried by albumin under normal conditions is 11.7 mEq/L and the net negative charge carried by all weak nonvolatile acids (i.e., albumin and phosphate) is 13.9 mEq/L. The titration curve of albumin and phosphate according to Eq. 26 is presented in Figure 1-4.

Besides the clinical usefulness of the Figge-Fencl model for detection of acid-base abnormalities (Kaplan and Kellum 2004; 2008; Murray et al. 2007; Dondorp et al. 2004; Durward et al. 2005), it was probably the first derivative of the Stewart model (albeit considerably modified), in which the necessary constants were not a mere guess or assumption but rather derived from rigorous measurements.

In 1999 Watson presented a model that also treats phosphate individually but, in comparison to the full-scale Figge-Fencl model, simplifies the albumin part (Watson 1999). The charge of albumin is divided into pH-dependent positive portion, $[AH^+]$, attributed to 16 histidine residues present on each

molecule and characterized by a single association constant, and a fixed negative portion, [A^-_{fix}], representing the net effect of all remaining proton binding sites. The model was verified by finding only minor discrepancies between the predictions and experimental data of Figge, Rossing, and Fencl in the pH range of 6.8 to 7.8. The net charge carried by weak nonvolatile acids under normal conditions estimated by this model is as a sum of [A^-_{fix}] = -13.3 mEq/L, [AH^+] = 1.9 mEq/L, and [P^-] = -2.1 mEq/L and equals -13.5 mEq/L.

In 2012 an updated model, referred to as Figge-Fencl model 3.0, was constructed (Figge et al. 2018; Figge 2023). It incorporated new findings on the titratable sites of the albumin molecule in order to provide accurate predictions over a wider pH range. However, the updated version can still be, in the pH range of clinical interest, reliably replaced by the linear Eq. 26.

1.2 Comparison of the models of acid-base equilibrium in plasma

The pH-independent buffer power of proteins, which results from a linear titration curve, is included in the classical model (Chapter 1.1.1) and the Figge-Fencl model (Chapter 1.1.4) of plasma. On the other hand, the Stewart model (Chapter 1.1.2) and the Staempfli-Constable model (Chapter 1.1.3) employ a sigmoid titration curve, which corresponds to a pH-dependent buffer power of proteins and weak nonvolatile acids in general (Figure 1-4).

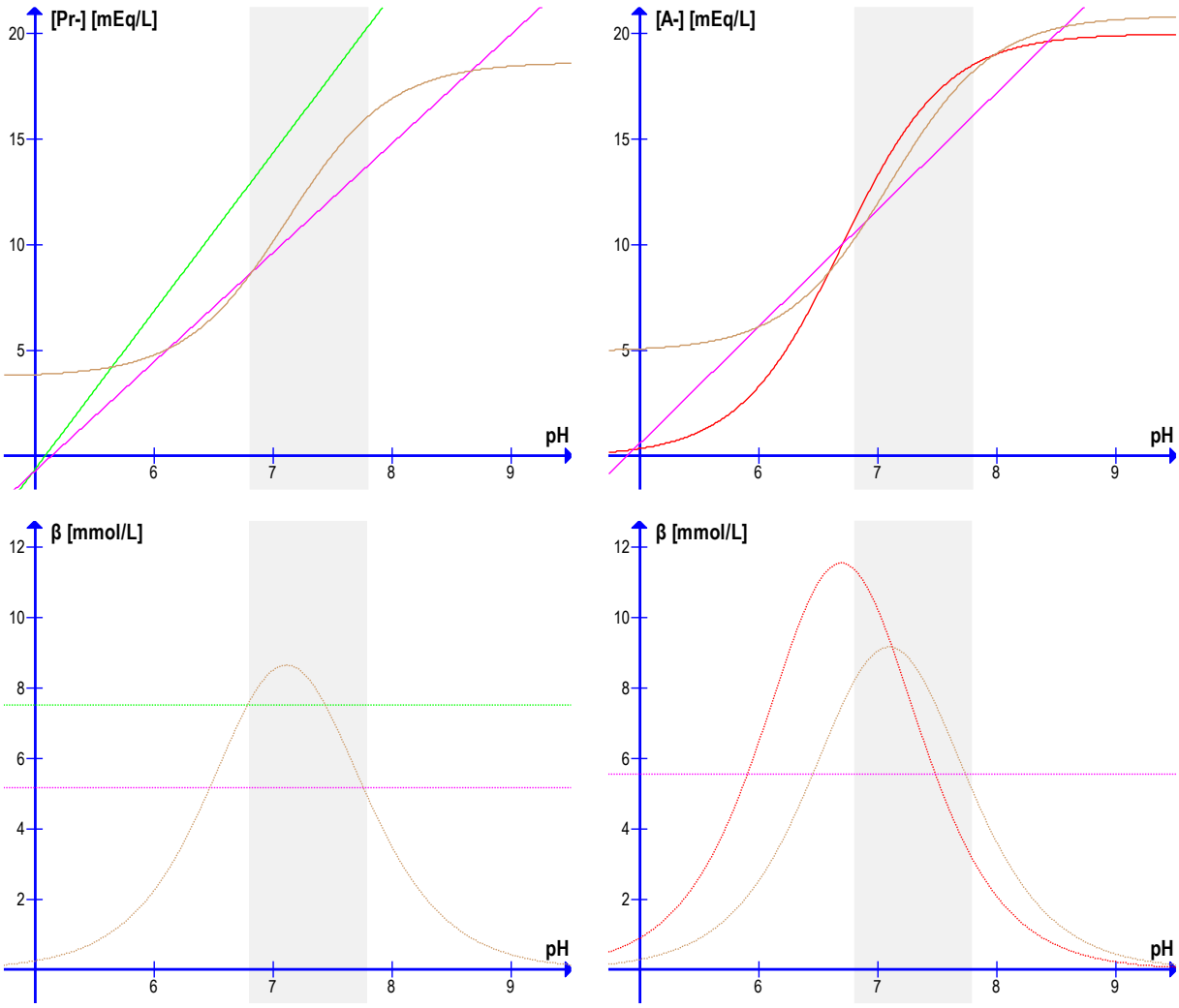

Figure 1-4. Titration curves and buffer powers of plasma proteins or all weak nonvolatile acids under normal conditions. ***Top left panel:*** *Dissociation curve of plasma proteins according to classical (green, Eq. 13, TP=70 g/L), Staempfli-Constable (brown, Eq. 23, coefficients from Table 1-1), and Figge-Fencl approach (pink, Eq. 26, albumin = 42 g/L).* ***Top right panel:*** *Dissociation curve of all weak nonvolatile acids in plasma according to Stewart (red, Eq. 23, Atot = 20 mmol/L and pK_a = 6.7), Staempfli-Constable (brown, Eq. 23, coefficients from Table 1-1), and Figge-Fencl approach (pink, Eq. 26, albumin = 42 g/L and Pi = 1.2 mmol/L).* ***Bottom panels:*** *The buffer power of plasma proteins (left) or all weak nonvolatile acids in plasma (right) obtained as the first derivative of the dissociation curves. The same source of equations and color coding is used. In all graphs, the pH range that may be encountered in human plasma (6.8 – 7.8) is marked grey. Note that the classical approach only works with the charge of proteins and is, therefore, not represented in the right-hand graphs while the Stewart model does not distinguish between the charge carried by proteins or phosphate and is, therefore, not represented in the left-hand graphs.*

Proponents of the classical theory did realize that linearity of the titration curve of plasma proteins is just an approximation. Van Slyke stated that: '*There is no reason to assume that the curves representing the relationship of base bound*[2] *by albumin or globulin to the pH of their solutions should be linear. They happen, over the limited range of physiological pH, to approximate straight lines with sufficient closeness to enable us to represent the results by linear formula with errors no greater than those of the experimental measurements.*' (Van Slyke et al. 1928). Indeed, Siggaard-Andersen later described that the buffer power of plasma proteins is a function of pH and has a local maximum at pH of approximately 7.1 (Siggaard-Andersen 1974) or 7.3 (Siggaard-Andersen et al. 1977). These data are shown in Figure 1-5, left panel. Interestingly, these data closely resemble the behavior of weak nonvolatile acids in plasma as described using the physicochemical approach (Figure 1-5, right panel) and implies that pK_a of weak nonvolatile acids is located in this region (Constable 2001). Siggaard-Andersen was aware of the rising popularity of the Stewart approach and heavily criticized its basic principles (Siggaard-Andersen and Fogh-Andersen 1995), but did not comment on this interesting similarity.

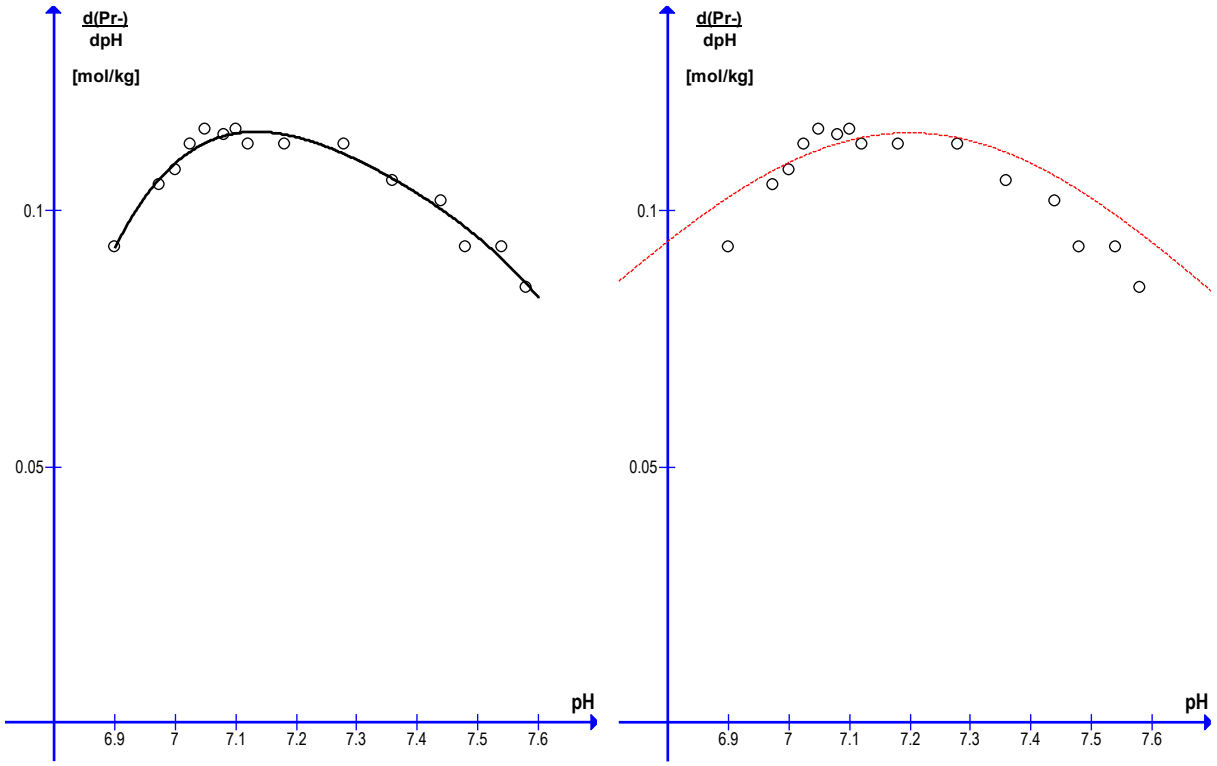

Figure 1-5. The relationship between the buffer power of plasma proteins and pH. ***Left panel:*** *Experimental data (adapted from Siggaard-Andersen, 1974, page 44). The buffer power is expressed per kg of plasma protein.* ***Right panel:*** *The same data with a curve representing buffer power of a weak monoprotic acid as defined by Stewart. Its concentration and pK_a were chosen to match the experimental data of Siggaard-Andersen as closely as possible.*

[2] In current terminology *negative charge carried* would be used instead.

Stewart was, too, aware that his treatment of plasma proteins is only approximate. He believed, however, that it does not introduce any significant error and hence did not attempt to provide a more precise description of the weak nonvolatile acid dissociation (Stewart 1981). One of the reasons why Stewart did not go with a linear titration curve could be the fact that he often explored extreme ranges of pH and SID (e.g., SID = 1000 mEq/L with pH ≈ 9 or SID = 0.001 mEq/L with pH ≈ 4). Figure 1-4 reveals that a linear approximation is not suitable for such a wide pH range. Conversely, the best known successor to the original Stewart model, the Figge-Fencl model, treats the albumin titration curve as linear within the physiological pH range (Figge et al. 1991; 1992).

In summary, the advocates of the classical theory, which is characterized by constant β(NC), have speculated that the titration curve of plasma proteins is not completely linear and that β(NC) is, therefore, not perfectly constant. On the other hand, followers of the Stewart theory introduced a model in which protein dissociation is, in the physiological pH range, very accurately represented by a linear function of pH.

For practical purposes that are limited by the narrow pH range compatible with life, the difference between the concepts of treatment of protein dissociation may be small. In turn, all of the above-mentioned approaches may describe the acid-base behavior of plasma with acceptable accuracy, provided that they use reasonable estimates of their respective constants. This was confirmed in a study (Anstey 2005) that mathematically compared three of the mentioned strong ion models (Stewart, Figge-Fencl, and Watson). The curves predicted by each model under normal conditions and in states with an altered SID and/or weak acid content were plotted in the standard pH-log(pCO_2) diagram. Differences between the obtained curves were not statistically significant. However, derangements in phosphate concentration were not studied and neither were the predictions tested against any measured values.

1.3 Unmeasured anions and their detection

Acid-base perturbations are termed acidosis or alkalosis based on the direction of pH shift they favor and are described as respiratory or metabolic depending on the nature of the acid or base that causes them (Barrett et al. 2012). Metabolic acidoses develop due to the accumulation of nonvolatile organic or inorganic acid in the extracellular space. This has two simultaneous effects on the electrolyte composition. First, H^+ from the acid combines with HCO_3^- forming carbonic acid[3] that is eventually eliminated by the lungs. Second, the anionic moiety of the accumulated acid takes the place vacated by [HCO_3^-] (Emmett and Narins 1977). Only some of these accumulated anions, such as Cl^- or lactate, are routinely measured, while a whole bunch of other species, collectively known as unmeasured anions, are not. Despite that, they are extremely important for clinical practice as their accumulation accompanies several life-threatening yet treatable conditions listed in Table 1-2 (Emmett and Szerlip 2022).

Type of metabolic acidosis	Accumulated anion
Ketoacidosis (diabetic, alcoholic or starvation)	aceto-acetate and β-hydroxybutyrate
Acidosis in renal failure	Inorganic acids (phosphates, sulphates)
Acetylsalicylic acid overdose	salicylates
Paracetamol overdose	pyroglutamate
Methanol poisoning	formiate
Ethanol poisoning	acetate
Ethylene glycol poisoning	glycolate and oxalate
D-lactic acidosis	D-lactate

Table 1-2. List of metabolic acidoses due to accumulation of unmeasured anions (adapted from Emmett and Szerlip, 2022).

Detection of unmeasured anions represents a diagnostic challenge. As they are not routinely measured, signs of their presence are indirect. Two strategies of such detection have been developed. The first is based on the principle of electroneutrality, which dictates that in all fluids, the sum of positive charges must be equal to the sum of the negative ones. If the difference between the two

[3] In fact, a majority (but not all) of the added H^+ combines with HCO_3^- – a smaller part (ranging from tenths to several mmol/L) combines with other buffers present, e.g., phosphate and proteins, and an even smaller portion (in the order of nmol/L) remains dissociated. Consequently, the decrease in [HCO_3^-] is smaller than the amount of acid added. Therefore, standard bicarbonate is not as good a parameter of metabolic acid base status as the Base excess, which expresses the reduction in Buffer base, i.e., the reduction in [HCO_3^-] plus the reduction in [A^-] of other buffers (Siggaard-Andersen 1974; Langer et al. 2022).

sums is higher than what can be explained by naturally occurring unmeasured anions, presence of some of the species listed in Table 1-2 is suspected. Such calculations are the Anion gap, Corrected anion gap, and Strong ion gap. The other approach is represented by various modifications of Base excess partitioning.

1.3.1 Anion gap and Corrected anion gap

The oldest tool for the detection of unmeasured species is the Anion gap (AG). Its calculation is simple:

$$AG = ([Na^+] + [K^+]) - ([Cl^-] + [HCO_3^-]) \qquad \textit{Eq. 28}$$

As $[K^+]$ is fairly stable in human plasma, even a simpler equation is sometimes used:

$$AG = [Na^+] - ([Cl^-] + [HCO_3^-]) \qquad \textit{Eq. 29}$$

The normal value of AG calculated according to Eq. 18 is around 12 (8 to 16) mEq/l. (Emmett and Narins 1977).

Since the beginning of its use, AG has been known to have several limitations. The normal value of AG is, in fact, the net charge carried by charged species not incorporated in the equation. These are Ca^{2+} and Mg^{2+} on the cationic side, and phosphate and proteins on the anionic side. Fluctuations of $[Ca^{2+}]$ and $[Mg^{2+}]$ are rather small and can be measured if abnormal values are suspected. The concentration of phosphate can vary but its concentration is, too, routinely measured. The most problematic part is represented by plasma proteins, whose concentration may vary substantially, especially in the critically ill (Iberti et al. 1990). This led to the development of a formula that corrects the calculated AG for the abnormalities in plasma protein concentration, producing the Corrected anion gap AG_{corr} (Figge et al. 1998):

$$AG_{corr} = AG + 0.25 \times ([normal\ albumin\ (g/l\ )] - [observed\ albumin\ (g/l)]) \qquad \textit{Eq. 30}$$

As this equation corrects AG for abnormal albumin levels, its reference range remains the same as that for AG. It should be noted that for the development of AG_{corr} the acid-base characteristics of albumin were taken from the Figge-Fencl model (Figge et al. 1991; 1992) in which the authors also provided their own tool for unmeasured anion detection, the Strong ion gap.

1.3.2 Strong ion gap – a gap calculation derived from the Stewart model

The idea of comparing the SID obtained by direct measurement of electrolytes with the SID that satisfies Stewart's equations for the measured pH to estimate whether any unmeasured anions are

present comes from Jones. He also noted that such calculation is, in fact, similar to AG, with the advantage that [A^-] is treated as a variable rather than a constant (Jones 1990).

Figge and Fencl modified the original Stewart approach by treating all titratable sites of albumin individually. The resulting titration curve of albumin was almost linear in the physiological pH range, which allowed them to replace the Stewart equations for HA dissociation by two simple linear equations for albumin and phosphate (Chapter 1.1.4). They then introduced a calculation for detection of unmeasured anions, later named the Strong Ion Gap (SIG). Its structure resembles the AG, which it outperforms by incorporating all measurable strong ions and, more importantly, the charge carried by albumin and phosphate, both of which are dependent on the total concentration of the respective species and pH (Kellum et al. 1995b; Figge et al. 1992).

1.3.3 Computer models of acid-base homeostasis

At least two complex physiological models of whole body acid-base equilibrium, that are capable of estimating the amount of unmeasured anions present, have been described (Wolf and Deland 2011; Anstey 2010a; 2010b) and are developed to date (Wolf 2023). Both have shown good performance in detecting unmeasured anions in a single in-vivo study (Morgan et al. 2017) but have never become widely used in clinical practice. This is probably due to the complexity of the calculations used, which cannot be performed without obtaining the original computer program.

1.3.4 Simplified methods

The calculation of the SIG was often considered too difficult to use in everyday practice, which led many authors to develop simplified versions. In general, their authors propose to simplify calculation of the charge carried by weak nonvolatile acids or to limit the number of strong ions that are taken into account. A suggestion to calculate [P^-] and [Alb^-] in a pH-independent manner (Fencl et al. 2000) may serve as an example of the former simplification. The latter approach is represented by the Bicarbonate gap, which, in addition to the pH-independent calculation of [P^-] and [Alb^-], also ignores [Ca^{2+}] and [Mg^{2+}] (Agrafiotis et al. 2018).

Another simplified method of unmeasured anion detection is the Base excess partitioning. This name covers a group of several distinct calculations that, nevertheless, rely on the same principle: First, the composition of plasma (i.e., the concentration of main electrolytes and albumin) is used to calculate the expected Base excess (BE). This value is then compared with the BE calculated by a blood-gas analyzer using the van Slyke equation (i.e., from pH and pCO_2). If the Base excess reported by the analyzer is significantly lower (or more negative) than the expected value, an unknown acidifying substance must be present (Gilfix et al. 1993; Balasubramanyan et al. 1999; Boyle and Lawrence 2003;

Story et al. 2004). Accuracy of this concept is negatively affected by several assumptions. Their detailed analysis is, unfortunately, beyond the scope of this thesis.

In this chapter on unmeasured anions, I have shown why they are clinically relevant and how their detection is interconnected with the evaluation of acid-base status. Individual calculations were introduced in order to show that, with the exception of Anion gap, they need to quantify the charge carried by plasma proteins. Correspondingly, poor performance of the Anion gap in hypoalbuminemia is considered its main drawback (Figge et al. 1998). In conclusion, an accurate estimation of unmeasured anions can only be done if the acid-base characteristics of plasma proteins are well studied.

1.4 Unmeasured anions in sepsis

It was recognized as early as 1963 that organic acids other than lactate may contribute to the high anion gap metabolic acidosis in certain patients (Waters et al. 1963). A handful of such conditions were described, and the accumulated anion was readily identified (Table 1-2). However, the application of electroneutrality-based calculations to critically ill patients, in whom such conditions were unlikely, has consistently indicated the presence of an unmeasured non-lactate anion using AG (Forni et al. 2005; Rudkin et al. 2022), corrected AG (Mecher et al. 1991; Moviat et al. 2003), or SIG (Moviat et al. 2008; Noritomi et al. 2009; Mallat et al. 2012; Moviat et al. 2003). The studies by Mecher *et al.* and Mallat *et al.* targeted specifically patients with severe sepsis and septic shock as defined by Sepsis-1 (ACCP/SCCM Consensus Conference Committee 1992) or Sepsis-2 (Levy et al. 2003).

Animal models of sepsis have shown that unmeasured anions contribute to the overall metabolic acidosis (Rackow et al. 1990) and that the liver may be the site of acid production (Kellum et al. 1995a). In humans with lactic acidosis and acidosis of unknown origin, several intermediate metabolites associated with the Krebs cycle were found by enzymatic assays in quantities that could explain 3 or even 5 mEq/L of AG (Forni et al. 2005). In another human study, Moviat *et al.* used advanced methods such as ion-exchange column chromatography, reverse-phase high-performance liquid chromatography, and gas chromatography/mass spectrometry to identify several organic anionic substances that were significantly elevated in high SIG metabolic acidosis (in both septic and non-septic patients), yet their aggregate effect could only explain 0.6 mEq/L or 7.9 % of the observed SIG elevation (Moviat et al. 2008).

It has been speculated that acute phase proteins may be responsible for at least part of the observed SIG (Kellum 2003), but no correlation was found between C-reactive protein or fibrinogen concentration and SIG (Kneidinger et al. 2007).

With the above-mentioned contradictory results and controversies, the nature of unmeasured anions in sepsis remains unclear.

1.5 Potential effect of sepsis on non-carbonic buffers

Sepsis has been strongly linked to inflammatory and oxidative stress (Duran-Bedolla et al. 2014) which leads to oxidation of plasma proteins, including albumin (Bonifazi et al. 2021). Furthermore, commercial human albumin solutions, which contain a higher percentage of oxidized albumin forms than plasma of healthy subjects (Bar-Or et al. 2005), are often used for fluid resuscitation in patients with sepsis or septic shock as suggested by recent Surviving Sepsis Campaign International Guidelines (Evans et al. 2021). Oxidation of albumin causes changes in its conformation and has been shown to alter some of its crucial biological properties (Kawakami et al. 2006).

Computer optimalization of the parameters that describe the acid-base action of albumin in the full-scale Figge-Fencl model, performed in order to accurately match experimental data, suggests that not all proton-binding sites of the albumin molecule are titratable. Some may be buried within interior of the protein (Figge et al. 1991; 1992). It is, therefore, possible that changes in the conformation of albumin molecule may alter the number of titratable sites that are exposed to the surrounding environment or modify their pK_a by allosteric effects. While this speculation shares some similarity with the unconfirmed hypothesis about the effect of acute phase proteins by Kellum (Chapter 1.4), it is certainly more plausible, because albumin concentration is always several times higher than that of all acute phase proteins. Indeed, Langer *et al.* has described a significant reduction in $\beta(NC)_{N(Alb)}$ in septic patients accompanied by a different amount of specific albumin proteoforms identified by two-dimensional electrophoretic separation of plasma samples (Langer et al. 2021).

2 Aims and hypotheses

Our objective was to reproduce the work of Staempfli and Constable and determine the value of K_a and A_{tot} in healthy volunteers, increasing the sample size to 30 subjects. Moreover, we aimed to repeat the same procedure in two distinct groups of patients receiving intensive care: 30 patients with sepsis and 30 patients after elective major abdominal surgery.

Pursuant to the first objective, CO_2 titration of plasma samples from a total of 90 subjects (healthy and critically ill) was to be performed. Using this data, we planned to study the interaction between plasma non-carbonic buffers and pH from different perspectives and analyze the possible differences between the studied groups.

We hypothesized that buffer properties of plasma weak acids may be altered during critical illness due to the reasons described in Chapter 1.5.

3 Methods

This prospective observational case-control study in human subjects was conducted simultaneously in two centers – Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy and FNKV University Hospital, Prague, Czechia. The study was carried out in accordance with the Declaration of Helsinki and was approved by the ethics committee at both centers. The study was registered in an independent database (Clinicaltrials.gov: NCT03966664). Written informed consent was obtained from all participants. For patients with impaired decision making, an independent physician evaluated the associated risks and allowed enrollment only if these were assessed as negligible. On recovery, a consent with data analysis from these patients was obtained.

3.1 Study subjects

Three study groups were defined as follows: healthy volunteers, patients admitted to the ICU after major elective surgery, and patients with sepsis or septic shock admitted to the ICU. The inclusion and exclusion criteria are summarized in Table 3-1. Sepsis-3 definition (Singer et al. 2016) was used.

	Healthy volunteers	Postoperative patients	Septic patients
Inclusion criteria	Age > 18 years Informed consent No known significant health problems	Age >18 years Informed consent Planned ICU admission after elective surgery	Age > 18 years Informed or deferred informed consent Diagnosis of Sepsis
Exclusion criteria	Pregnancy	Pregnancy Bilirubin >4 mg/dL Minor or major thalassemia Transfusion of more than 4 units of RBC and/or 1 L of plasma during the 24 hours prior to enrollment Liver cirrhosis Onco-hematological diseases Diagnosis of sepsis	Pregnancy Bilirubin > 4 mg/dL Minor or major thalassemia Transfusion of more than 4 units of RBC and/or 1 L of plasma during the 24 hours prior to enrollment

Table 3-1. Study A, study groups, inclusion and exclusion criteria.

3.2 Sample size determination

The primary endpoint of the study was the difference in pK_a of isolated plasma measured in healthy volunteers and septic patients as defined by the Stewart model of plasma (Chapter 3.6.2). Secondary endpoint was the average difference in pK_a of isolated plasma measured in septic and non-septic patients.

The sample size for the primary endpoint of the study has been calculated using the software SigmaPlot 11.2 (Systat Software Inc., San Jose, CA, USA) using a paired t-test and using the difference in K_a of isolated plasma between critically ill patients with sepsis and heathy controls as outcome parameter. Based on previous studies (Staempfli and Constable 2003; Langer et al. 2015), we considered a difference in K_a of 1.9×10^{-7} as clinically relevant and estimated a standard deviation of 2.2×10^{-7}.

After defining the following parameters: minimum detectable difference in means = 1.9×10^{-7}, expected standard deviation of residuals = 2.2×10^{-7}, desired power = 0.90, and alpha error = 0.05, the estimated sample size was n = 30 for each group. Given the lack of preliminary data about non-septic patients, we decided to enroll 30 non-septic patients to perform adequate comparisons.

3.3 Enrollment, baseline variables

Each participating center was supposed to enroll 15 subjects per group. The enrolment of patients was not consecutive but was based on availability of staff and technical resources. At enrollment, the following data were collected for both patient groups: anthropometric data, medical history, vital functions, critical illness severity scores [SOFA (Vincent et al. 1996), APACHE II (Knaus, William et al. 1985) and SAPS II (Le Gall 1993)], ongoing therapy (including type and amount of intravenous fluids administered), and laboratory results. In septic patients, the presumed sepsis origin and time from diagnosis were recorded. In healthy volunteers, we collected anthropometric data, medical history, and laboratory results only.

3.4 Sample collection and handling

In patients, blood samples were taken preferably from a central venous line. If it had not been placed or if the sample could not be easily drawn, an arterial line or other suitable catheter was used instead. In healthy volunteers, the blood was taken from a peripheral vein on the upper extremity. The total amount of blood collected was around 30 mL. One collection tube with clot activator (4-8 mL) and one tube with ethylenediaminetetraacetic acid (EDTA, 3-4 mL) were sent to certified in-hospital laboratory for biochemistry examination (magnesium, phosphate, total protein, and albumin concentration and serum protein electrophoresis) and complete blood count. Another 16 mL of blood collected in lithium heparin tubes ware used for CO_2 tonometry of plasma, 3-4 mL blood collected in tubes with EDTA was used for frozen plasma storage, and 1mL of blood was collected in a syringe with dry electrolyte-balanced heparin for baseline blood gas analysis and point-of-care ketone (beta-hydroxybutyrate) measurement. Where applicable, plasma was obtained by centrifugation at 4 °C at 3000 rpm for 12 minutes.

3.5 CO_2 tonometry of plasma and blood-gas analyses

The tonometry of plasma was performed immediately after collecting and centrifuging the sample. Using an EQUILibrator CO_2 tonometer (RNA Medical, Devens, MA, USA) and custom gas mixtures (0%, 2%, 12% and 20% CO_2 in air), we manipulated pCO_2 in the range of 2 - 16 kPa (15 - 120 mmHg) at 37 °C and measured pH, pCO_2 and other variables using ABL90 FLEX PLUS blood gas analyzer (Radiometer, Copenhagen, Denmark), collecting at least 20 datapoints for each sample.

3.6 Endpoint variables, calculations, and statistical analysis

Every model of plasma is characterized by its own set of parameters, determination of which requires specific handling of the directly measured parameters. These transformations are described in the next chapters, which are ordered in a way that reflects their logical rather than chronological development, starting with the classical model of plasma, advancing to the Stewart model and its two derivatives.

Across all acid-base models of plasma and blood, the SID was calculated according to Eq. 24 and the concentration of bicarbonate was calculated according to Henderson-Hasselbalch equation (Eq. 3) with the solubility coefficient S = 0.0307 mmol/L/mmHg and apparent dissociation constant pK_1'=6.105 in plasma and 6.095 in whole blood (CLSI 2009). As the two centers use a different system of units, a conversion 1 kPa = 7.500638 mmHg was used (Radiometer 2018) to comply with the transformations performed automatically within the blood-gas analyzer.

In the baseline characteristics of our subjects, we specifically analyzed the relationship between albumin, non-albumin protein and TP concentration as this is important for the subsequent analyses in which we used linear regression to determine whether TP or albumin better predicts the measured characteristics of non-carbonic buffers.

The data were handled in Microsoft Excel 365, statistical tests were performed with GraphPad Prism 8, if not stated otherwise. p value of 0.05 was considered statistically significant.

3.6.1 Classical model of plasma

In this model, the main variable of interest is the mean non-carbonic buffer power of plasma, β(NC), in each group, defined by Eq. 9. The law od electroneutrality dictates that during manipulation of pCO_2 any change of the charge carried by plasma non-carbonic buffers $[A^-]$ is mirrored by a change in $[HCO_3^-]$ of the same magnitude:

$$\beta(NC) = \frac{d[A^-]}{dpH} = -\frac{d[HCO_3^-]}{dpH} \qquad \textit{Eq. 31}$$

As explained in Chapter 1.1.1, β(NC) is considered constant in the physiological pH range. In each plasma sample, β(NC) was, therefore, determined as negative of the slope of the linear regression line between pH and $[HCO_3^-]$ during CO_2 equilibration.

To explore the relationship between β(NC) and the concentration of main plasma buffers, we normalized β(NC) in each plasma sample for the concentration of albumin and TP [$\beta(NC)_{N(Alb)}$ and $\beta(NC)_{N(TP)}$, respectively]:

$$\beta(NC)_{N(Alb)} = \frac{\beta(NC)}{[Alb]} \quad \textit{Eq. 32}$$

$$\beta(NC)_{N(TP)} = \frac{\beta(NC)}{[TP]} \quad \textit{Eq. 33}$$

Mean values of $\beta(NC)_{N(Alb)}$ and $\beta(NC)_{N(TP)}$ were than compared between groups.

Finally, we aimed to determine which parameter better predicts the value of β(NC). In pooled data from all three groups, we performed a linear regression of albumin concentration vs. β(NC) and a linear regression of TP vs. β(NC). Superiority of the methods was assessed according to R^2 and root mean square error (RMSE) of each regression.

3.6.2 Stewart model of plasma

The SID was calculated according to Eq. 20. The concentration of all necessary electrolytes was obtained from the blood gas analyzer during CO_2 tonometry, except for $[Mg^{2+}]$, which was measured along with Pi, TP and albumin in a certified hospital laboratory. The SID fluctuates throughout the tonometry of plasma because of the accumulation random analytical errors (further amplified by the additive nature of the SID calculation), systematic analytical errors caused by interference on the ion selective electrodes, and ion-protein binding (Maas et al. 1985; Burnett et al. 2000; Meyerhoff and Opdycke 1986; Fogh-Andersen et al. 1993). Staempfli and Constable, in their study on K_a and Atot, noticed a dependency of $[Na^+]$ and $[Ca^{2+}]$ on pH and speculated that the most likely explanation was salt-type binding of Na^+ and Ca^{2+} to plasma proteins. To account for this effect, they worked not only with the average SID obtained from all measurements within one subject, but also repeated the estimation of K_a and Atot using the actual SID of each individual measurement (Staempfli and Constable 2003). If the speculation of Staempfli and Constable is correct, the magnitude of Na^+ and Ca^{2+} fluctuation should correlate with albumin and/or TP concentration. Presence of such correlation could not be verified by Staempfli and Constable as they only enrolled healthy volunteers with normal albumin and TP concentration.

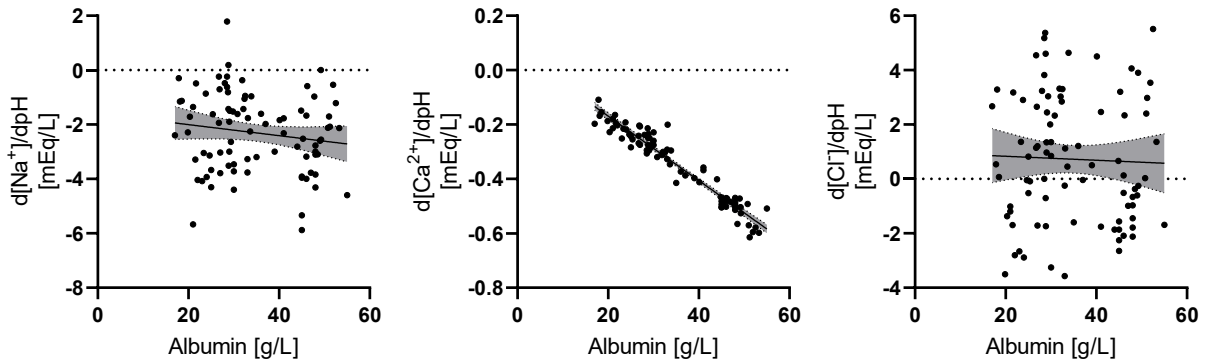

Figure 3-1. The relationship between magnitude of [Na^+], [Ca^{2+}], and [Cl^-] variation, expressed as d[electrolyte]/dpH, during CO_2 equilibration, and albumin concentration.

We performed a preliminary analysis analogous to the one described by Staempfli and Constable during which all electrolyte concentrations measured by ion-selective electrodes were regressed against pH. We observed a relevant pH-dependent change in [Na^+] (mean d[Na^+]/dpH -2.29 ± 1.45 mmol/L per pH unit), [Ca^{2+}] (mean d[Ca^{2+}]/dpH -0.35 ± 0.13 mmol/L per pH unit), and [Cl^-] (mean d[Cl^-]/dpH 0.75 ± 2.36 mmol/L per pH unit). Individual d[electrolyte]/dpH values obtained in each plasma sample were then plotted against albumin concentration (Figure 3-1). We found a strong negative correlation ($r = -0.96$, $p < 0.0001$) between d[Ca^{2+}]/dpH and albumin concentration while no such relationship was found for Na^+ and Cl^-. This shows that ion protein binding of measurable magnitude only happens in the case of Ca^{2+} and that this phenomenon contributes little, if at all, to the variation of [Na^+] and [Cl^-] during CO_2 equilibration of plasma. The most probable explanation of the pH dependency of [Na^+] and [Cl^-] is, in our opinion, the interference of ion selective electrodes with H^+ or HCO_3^-. As the magnitude of pH-dependent variation of [Ca^{2+}] is little compared to [Na^+] and [Cl^-], we decided to disregard the actual SID and to only use the average SID (SID_{avg}) of each plasma sample for the subsequent analyses. Such handling of the SID minimizes the accumulation of random and systematic (i.e., pH related) analytical errors but neglects the effect of Ca^{2+}–protein binding.

For calculation of the parameters of the Stewart model of plasma, Eq. 25, the simplified strong ion electroneutrality equation, was combined with Eq. 3 for [HCO_3^-], and Eq. 23 for [A^-] to provide the following equation:

$$SID_{avg} - 0.0307 \times pCO_2 \times 10^{pH-6.105} - \frac{Atot}{1 + 10^{pK_a - pH}} = 0 \qquad \textit{Eq. 34}$$

which was used by a nonlinear regression algorithm along with the experimental data to solve simultaneously for pK_a and Atot in each plasma sample. Mean pK_a and Atot were compared between groups.

Total concentration of weak nonvolatile acids (Atot) is usually expressed as a function of albumin or TP concentration. We, therefore, defined parameters $Atot_{N(Alb)}$ and $Atot_{N(TP)}$, which refer to Atot normalized for albumin and TP concentration:

$$Atot_{N(Alb)} = \frac{A_{tot}}{[Alb]} \quad \textit{Eq. 35}$$

$$Atot_{N(TP)} = \frac{A_{tot}}{[TP]} \quad \textit{Eq. 36}$$

Mean values of $Atot_{N(Alb)}$ or $Atot_{N(TP)}$ were calculated and compared between groups.

Finally, we tried to determine which parameter better predicts the value of Atot. In pooled data from all three groups, we performed a linear regression of albumin concentration vs. Atot and a linear regression of TP vs. Atot. Superiority of albumin or TP in predicting Atot was assessed according to R^2 and root mean square error (RMSE) of these regressions.

3.6.3 Staempfli-Constable model of plasma

To derive parameters of the Staempfli-Constable model of plasma, the measured values of strong electrolytes were disregarded, and the nonlinear regression algorithm was used estimate the SID (SID_{est}) along with Atot and pK_a. The simplified strong ion electroneutrality equation (Eq. 25), was combined with Eq. 3 for $[HCO_3^-]$, and Eq. 23 for $[A^-]$ to provide the following equation:

$$SID_{est} - 0.0307 \times pCO_2 \times 10^{pH-6.105} - \frac{Atot}{1 + 10^{pK_a - pH}} = 0 \quad \textit{Eq. 37}$$

which was used by a nonlinear regression algorithm along with the experimental data to solve simultaneously for SID_{est}, Atot, and pK_a in each plasma sample. Then, the difference between the measured and estimated SID (ΔSID) was calculated for each plasma sample:

$$\Delta SID = SID_{avg} - SID_{est} \quad \textit{Eq. 38}$$

Mean values of the estimated parameters (SID_{est}, ΔSID, pK_a, and Atot) were then compared between the studied groups.

The relationship between Atot and albumin or TP concentration was analyzed identically as in the case of Stewart model (Chapter 3.6.2), i.e., using the normalized values ($Atot_{N(Alb)}$ and $Atot_{N(TP)}$) and linear regression of pooled Atot values vs. albumin and TP.

3.6.4 Figge-Fencl model of plasma

The simplified equation of the Figge-Fencl model for the physiological pH range (Eq. 26) uses a linear term for phosphate charge. While this is appropriate for routine calculations, as the induced error is negligible (Figure 3-2), we rather expressed phosphate charge in a more precise manner:

$$[Phos^{-}] = Pi \times \left(1 + \frac{1}{1 + 10^{pK_2' - pH}}\right) \qquad \textit{Eq. 39}$$

with pK_2' of 6.66 (Sendroy and Hastings 1927). The other two dissociation constants of phosphoric acid are more than 4 units away from the explored pH range and may, thus, be ignored.

We used SID_{avg} due to the same reasons as described in Chapter 3.6.2. In the study where the Figge-Fencl approach was first introduced, the SID was lowered by 1.5 mEq/L to include the charge of sulfates present in serum filtrands (Figge et al. 1991). Sulphate concentration was not measured in our subjects, but we have decided to include the same factor in order to obtain comparable results. To derive the parameters of the Figge-Fencl model, we set $[XA^-] = 0$ in Eq. 26, knowing that this assumption may confound the result in the patient populations. $[HCO_3^-]$ and $[Phos^-]$ were calculated with Eq. 3 and Eq. 39 and the more intuitive expression for the titration curve of albumin (as shown in Eq. 27) was used. The two parameters that characterize protein titration curve in this model were labeled F1 and F2:

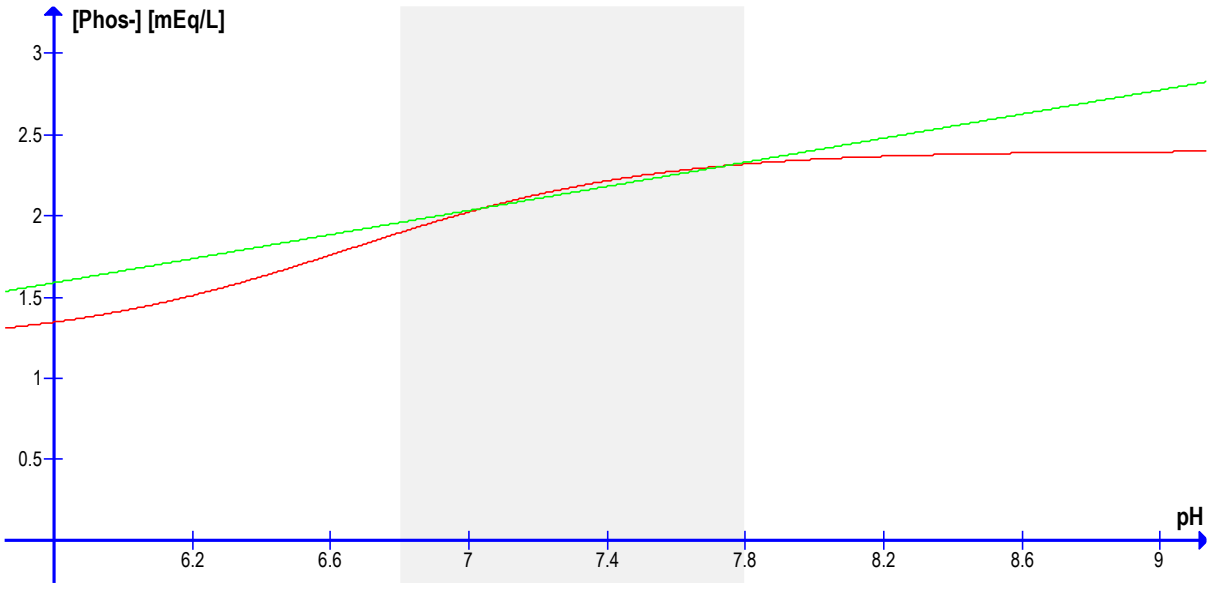

Figure 3-2. The dissociation curve of phosphates. ***Red:*** *the accurate sigmoid dissociation curve (Eq. 39).* ***Green:*** *the approximate linear dissociation used in the Figge-Fencl model (Eq. 26). In both cases, Pi of 1.2 mmol/L was used. The pH range of clinical interest is marked grey.*

$$SID - 1.5 - 0.0307 \times pCO_2 \times 10^{pH-6.105} - [F1 \times (pH - 7.4) + F2] - Pi \times \left(1 + \frac{1}{1 + 10^{6.66-pH}}\right) = 0 \quad \textit{Eq. 40}$$

Finally, the obtained parameters (F1 and F2) were normalized for albumin ($F1_{N(Alb)}$ and $F2_{N(Alb)}$) or TP concentration ($F1_{N(TP)}$ and $F2_{N(TP)}$) and the mean values were compared between groups.

4 Results

Recruitment of subjects was started in March 2019. The study was interrupted between August 2020 and June 2021 because of the outbreak of COVID-19 pandemic. Collection of samples was terminated in October 2021 after enrolling a total of 30 volunteers, 27 postoperative, and 30 septic patients. Reaching the prespecified number of 90 subjects was not possible due to lack of human resources in one of the participating centers.

In all 87 subjects the sample collection and subsequent analyses were performed as described in the Methods with the following deviations: Baseline blood gas analysis was not performed in 2 controls and 3 postoperative patients, complete blood count was not obtained in 3 controls, serum protein electrophoresis was not performed in 2 postoperative patients, and albumin concentration was not measured in 1 postoperative patient. Except for albumin concentration, the lack of these data only affected the baseline characteristics as these data are not needed for determination of parameters that characterize buffers in various acid-base models. Albumin, on the other hand, is needed for calculation of the parameters normalized for buffer concentration [e.g., $\beta(NC)_{N(Alb)}$, $Atot_{N(Alb)}$]. The normalized parameters were, therefore, determined for a reduced number of subjects (N = 86).

Baseline characteristics of the enrolled subjects are summarized in Table 4-1. The three populations did not differ in sex or age. Usually, patients were sampled on the first day after admission to the ICU, but later sampling was common in Septic patients, especially if they were admitted due to a different diagnosis and developed sepsis during their ICU stay. Septic patients performed worse in SOFA as well as other disease severity scoring systems which corresponded to a higher need for organ support and translated into a trend towards higher mortality. There was a significant difference in total fluid intake between Postoperative and Septic patients, but no differences in fluid management were found when the length of ICU stay was considered.

In the septic patient cohort, the most common source of infection was respiratory tract (N=15, 50 %), followed by abdominal (N=7, 23 %) and genitourinary tract (N=3, 10 %). In 5 patients (17 %) the source of infection was different or unknown. No significant difference was found between the study centers in the percentage of septic patients who received exogenous albumin [Prague: N=5 (29 %); Milan: N=8 (62 %); Fisher's exact test: p = 0.14] or in the dose of albumin administered [Prague: 0 (0– 20) g; Milan: 20 (0–50) g; Mann-Whitney test: p = 0.08].

		Control (N = 30)	Postoperative (N = 27)	Septic (N = 30)	p value
N		30	27	30	
Site	N (%) in Prague	14 (46.7)	17 (63.0)	17 (56.7)	0.46
Sex	N (%) of female	14 (46.7)	9 (33.3)	12 (40.0)	0.59
Age	years	54 ± 15	61 ± 16	56 ± 18	0.31
Time since ICU admission	days	-	1 (1–2)	1 (1–3)	<0.05
Time since sepsis diagnosis	days	-	-	2 (1–3)	-
SOFA		-	4.4 ± 1.4	9.7 ± 2.5	<0.0001
APACHE II		-	6.0 ± 2.8	16.4 ± 6.8	<0.0001
SAPS II		-	14.5 ± 6.5	39.2 ± 13.0	<0.0001
Fluids administered	L	-	7.3 (2.2–10)	9.5 (3.7–15)	<0.05
Fluids administered per day	L/day	-	4.1 ± 3.4	3.8 ± 1.9	0.68
Fluid balance	L	-	4.0 (0.1–6.3)	5.1 (2.0–9.1)	0.20
Fluid balance per day	L/day	-	2.0 ± 2.1	2.2 ± 1.9	0.73
Albumin administered	g	-	0 (0–0)	0 (0–23)	<0.001
Mechanical ventilation	N (%)	-	1 (3.7)	26 (86.7)	<0.0001
ECMO	N (%)	-	0 (0)	5 (16.7)	0.05
Vasopressors	N (%)	-	7 (25.9)	26 (86.7)	<0.0001
ICU mortality	N (%)	-	1 (3.7)	7 (23.3)	0.05
Hemoglobin	g/L	144 ± 10	109 ± 17*	95 ± 13*	<0.0001
Total protein	g/L	72 ± 5	50 ± 6*	47 ± 7*	<0.0001
Albumin	g/L	48 ± 3	32 ± 4*§	24 ± 4*	<0.0001
Non-albumin protein	g/L	24 ± 3	18 ± 3*	22 ± 6	<0.0001
A/G ratio		1.64 ± 0.20	1.48 ± 0.26	1.09 ± 0.34*	<0.0001
Phosphate	mmol/L	1.09 ± 0.13	0.97 ± 0.28	1.22 ± 0.56	<0.05
pH		7.38 ± 0.03	7.40 ± 0.04	7.35 ± 0.09	<0.05
pCO_2	kPa	6.5 ± 0.8	6.0 ± 0.6	5.8 ± 1.2*	<0.01
$[HCO_3^-]$	mmol/L	28.6 ± 2.1	27.5 ± 3.3	24.5 ± 6.8*	<0.01
BE(Ecf)	mmol/L	3.4 ± 1.9	2.6 ± 3.8	-1.1 ± 7.9*	<0.0001
SID	mEq/L	43.7 ± 2.4	39.4 ± 2.8*	39.1 ± 6.2*	<0.0001
SIG	mEq/L	-0.1 ± 1.1	0.9 ± 2.0	5.7 ± 2.8*	<0.0001

Table 4-1. Study A, baseline characteristics. Data are presented as mean ± SD, median (1Q–3Q), or N (%) as appropriate. When all three groups were compared the p values refer to the result of Chi-squared test or one-way ANOVA, as appropriate. For the latter, multiple comparisons were performed and an asterisk (*) denotes a significant difference between the marked value and Controls. For comparisons between the two patient populations, the p values refer to the result of student's t-test, Mann-Whitney test, or Fischer's exact test, as appropriate. §For the marked result N = 26.

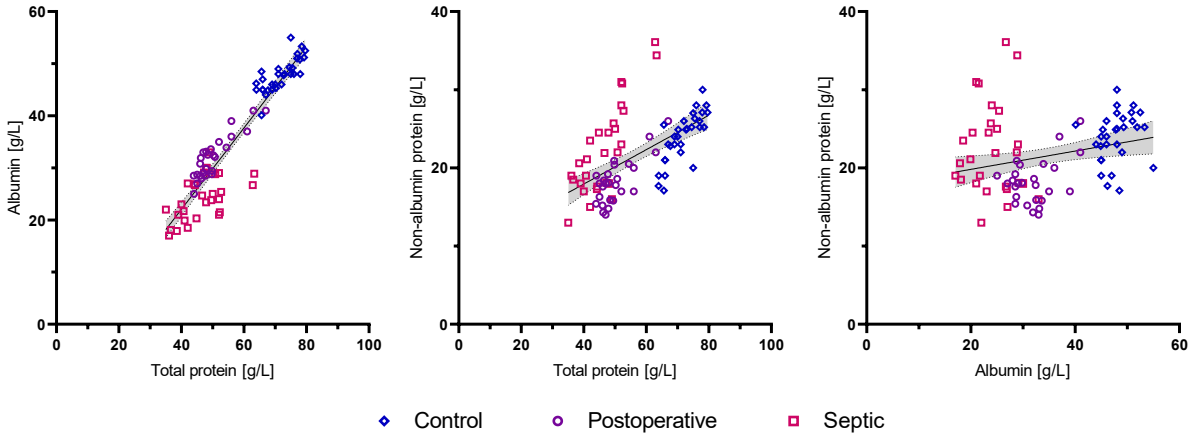

Figure 4-1. Mutual relationship between serum TP, albumin, and non-albumin protein concentration.

Concentrations of the main plasma and blood buffers (albumin, TP, and hemoglobin) were significantly reduced in both patient populations. Exploring the relationship between TP, albumin, and non-albumin protein (Figure 4-1) showed these variables are mutually corelated (TP vs. albumin: $r = 0.93$, $p < 0.0001$; TP vs. non-albumin protein: $r = 0.59$, $p < 0.0001$; albumin vs. non-albumin protein: $r = 0.26$, $p < 0.05$).

4.1 Classical model of plasma

The mean non-carbonic buffer power of plasma, β(NC), in each group is reported in in Table 4-2 and Figure 4-2 (top left panel). The values recorded in both Postoperative and Septic patients were significantly lower than in Controls. Mean non-carbonic buffer power of plasma normalized for albumin and TP concentration [$\beta(NC)_{N(Alb)}$ and $\beta(NC)_{N(TP)}$] are presented in Table 4-2 and Figure 4-2 (top middle and top right panel).

		Control (N = 30)	Postoperative (N = 27)	Septic (N = 30)	p value
β(NC)	mmol/L	8.1 ± 1.4	4.1 ± 1.9*	5.2 ± 1.8*	<0.0001
$\beta(NC)_{N(Alb)}$	mmol/g	0.169 ± 0.027	0.127 ± 0.060*§	0.218 ± 0.078*	<0.0001
$\beta(NC)_{N(TP)}$	mmol/g	0.113 ± 0.019	0.082 ± 0.039*	0.113 ± 0.036	<0.0001

*Table 4-2. Classical model of plasma. Results of linear regression using CO_2 equilibration data for determination of model parameters in the three populations. p value refers to one-way ANOVA. *The marked results differ significantly from Controls. §For the marked result N = 26.*

The relationship between individual β(NC) values and albumin or TP concentration is shown in Figure 4-2 (bottom panels). Predictors of β(NC) were determined using pooled data from all three groups. The linear regression of β(NC) vs. albumin yielded a slope of 0.164 (95%CI 0.153 to 0.176), Y intercept not different from zero, R^2 = 0.32, and RMSE = 1.96 mmol/L (Figure 4-2, bottom left panel). The linear regression of β(NC) vs. TP had a slope of 0.104 (95%CI 0.097 to 0.111), Y intercept not different from zero, R^2 = 0.39, and RMSE = 1.85 mmol/L (Figure 4-2, bottom right panel).

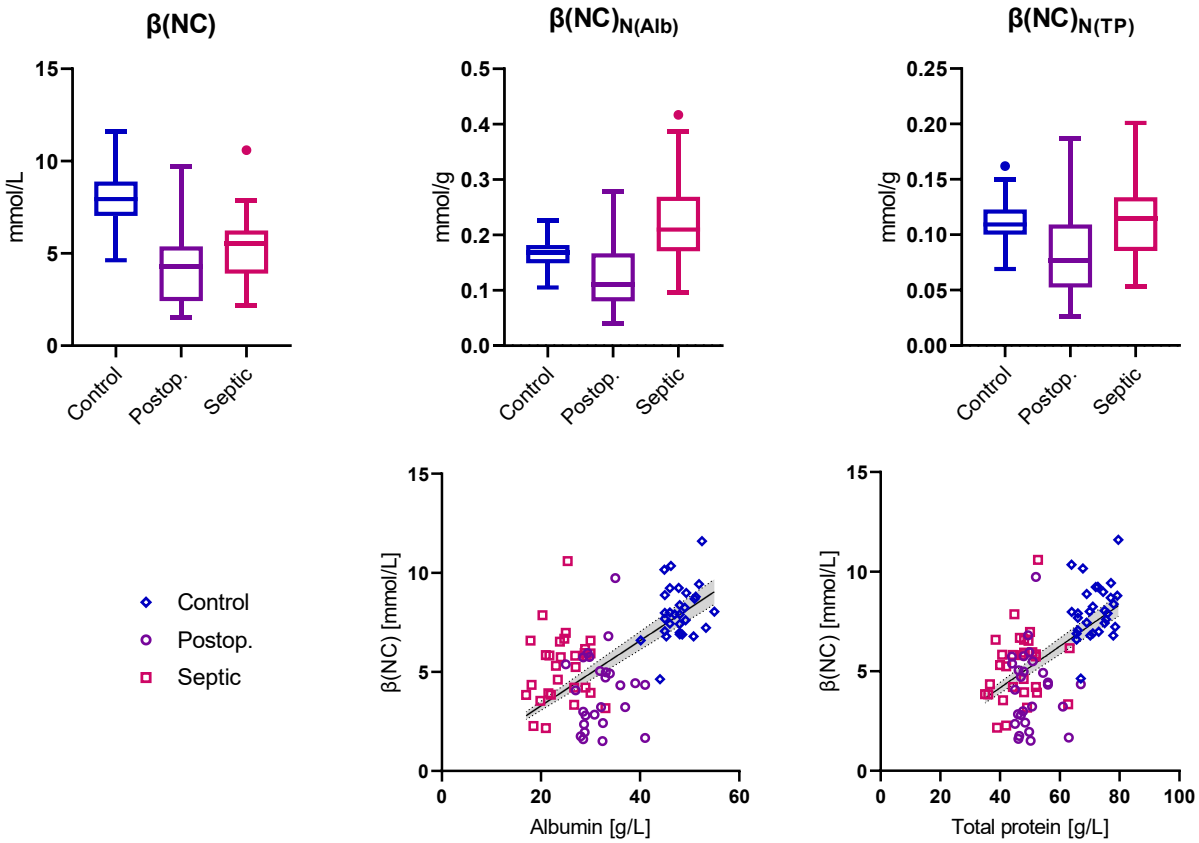

Figure 4-2. Classical model of plasma. ***Top panels:*** *boxplots of non-carbonic buffer power of plasma [β(NC)], non-carbonic buffer power of plasma normalized for albumin [$\beta(NC)_{N(Alb)}$], and non-carbonic buffer power of plasma normalized for TP [$\beta(NC)_{N(TP)}$]. Whiskers span form min to max. Outliers detected by Tukey's outlier detection method are shown as separate points.* ***Bottom panels:*** *individual β(NC) values and linear regression of all β(NC) values vs. albumin or TP with 95% confidence bands.*

4.2 Stewart model of plasma

The nonlinear regression algorithm provided a valid fit for all studied plasma samples.

The mean weak nonvolatile acid concentration (Atot) and its pK_a in each group are reported in in Table 4-3 and Figure 4-3 (first and second panels in the top row). The values recorded in both patient populations differed significantly from Controls. Mean Atot normalized for albumin and total protein [$Atot_{N(Alb)}$ and $Atot_{N(TP)}$] are presented in Table 4-3 and Figure 4-3 (third and fourth panels in the top row). The values recorded in both patient populations are, again, significantly different from Controls.

		Control (N = 30)	Postoperative (N = 27)	Septic (N = 30)	p value
pK_a		6.63 ± 0.09	6.32 ± 0.25*	6.33 ± 0.21*	<0.0001
Atot	mmol/L	24.3 ± 1.9	18.8 ± 2.0*	22.2 ± 3.9*	<0.0001
$Atot_{N(Alb)}$	mmol/g	0.510 ± 0.035	0.588 ± 0.078*§	0.924 ± 0.190*	<0.0001
$Atot_{N(TP)}$	mmol/g	0.340 ± 0.024	0.377 ± 0.046*	0.480 ± 0.079*	<0.0001

*Table 4-3. Stewart model of plasma. Results of nonlinear regression using CO_2 equilibration data for determination of model parameters in the three populations. p value refers to one-way ANOVA. *The marked results differ significantly from Controls. §For the marked result N = 26.*

The relationship between individual Atot values and albumin or total protein concentration is shown in Figure 4-3 (bottom panels). Predictors of Atot were determined using pooled data from all three groups. The linear regression of Atot vs. albumin yielded a slope of 0.146 (95%CI 0.081 to 0.212), Y intercept of 16.8 (95%CI 14.4 to 19.1), R^2 = 0.19, and RMSE = 3.22 mmol/L (Figure 4-3, bottom left panel). The linear regression of Atot vs. TP had a slope of 0.157 (95%CI 0.107 to 0.206), Y intercept of 13.1 (95%CI 10.2 to 15.9), R^2 = 0.31, and RMSE = 2.95 mmol/L (Figure 4-3, bottom right panel).

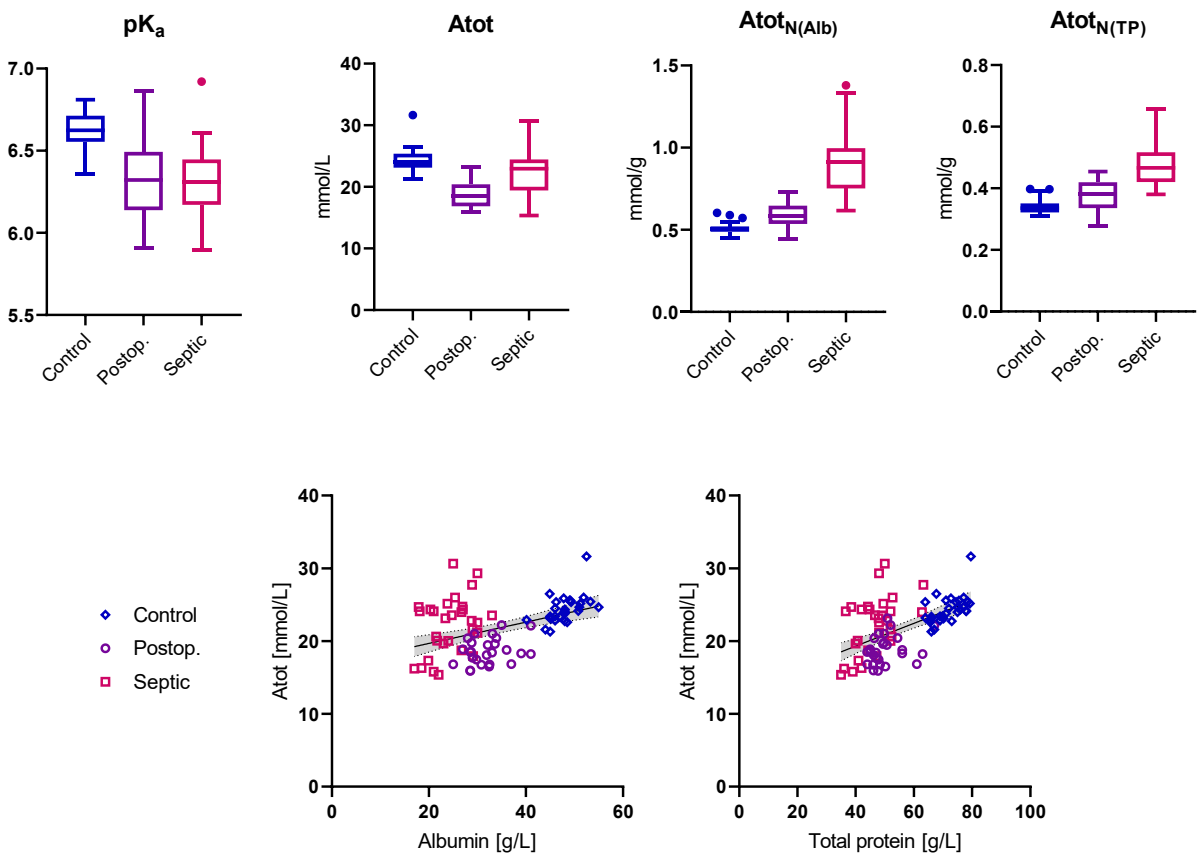

Figure 4-3. Stewart model of plasma. ***Top panels:*** *boxplots of the dissociation constant of weak nonvolatile acids (pK_a), total concentration of weak nonvolatile acids (Atot), total concentration of weak nonvolatile acids normalized for albumin [$Atot_{N(Alb)}$], and total concentration of weak nonvolatile acids normalized for TP [$Atot_{N(TP)}$]. Whiskers span form min to max. Outliers detected by Tukey's outlier detection method are shown as separate points.* ***Bottom panels:*** *individual Atot values and linear regression of all Atot values vs. albumin or TP with 95% confidence bands.*

4.3 Staempfli-Constable model of plasma

The nonlinear regression algorithm provided a valid fit in all Controls but yielded ambiguous parameters in 8 postoperative (29.6%) and 6 septic (20.0 %) patients. These results were not used for further analyses.

Mean SID_{est} differed significantly between Controls and both patient populations (Table 4-4 and Figure 4-4, first and second panels in the top row). The difference between the measured and estimated SID (ΔSID), on the other hand, did not differ between Controls and either patient population.

The mean weak nonvolatile acid concentration (Atot) and its pK_a in each group are reported in Table 4-4 and Figure 4-4 (first and second panels of the middle row). Atot was significantly reduced in the patient populations while pK_a did not differ between groups. Mean Atot normalized for albumin and total protein [$Atot_{N(Alb)}$ and $Atot_{N(TP)}$] are presented in Table 4-4 and Figure 4-4 (third and fourth panels of the middle row). $Atot_{N(Alb)}$ was significantly higher in Septic patients. $Atot_{N(TP)}$ did not differ between Controls and either patient population.

		Control (N = 30)	Postoperative (N = 19)	Septic (N = 24)	p value
SID_{est}	mEq/L	32.8 ± 2.0	28.9 ± 3.5*	26.0 ± 6.3*	<0.0001
ΔSID	mEq/L	14.0 ± 1.5	13.0 ± 3.2	15.8 ± 3.7	<0.01
pK_a		7.55 ± 0.17	7.48 ± 0.25	7.44 ± 0.36	n.s.
Atot	mmol/L	16.1 ± 3.4	9.5 ± 4.4*	11.9 ± 4.7*	<0.0001
$Atot_{N(Alb)}$	mmol/g	0.338 ± 0.073	0.292 ± 0.146[§]	0.490 ± 0.216*	<0.001
$Atot_{N(TP)}$	mmol/g	0.225 ± 0.049	0.192 ± 0.093	0.255 ± 0.098	<0.05

*Table 4-4. Staempfli-Constable model of plasma. Results of nonlinear regression using CO_2 equilibration data for determination of model parameters in the three populations. p value refers to one-way ANOVA. *The marked results differ significantly from Controls. [§]For the marked result N = 18.*

The relationship between the SID_{est} values and measured SID_{avg} is shown in Figure 4-4 (top right panel). The linear regression of SID_{est} vs. SID_{avg} yielded a slope of 0.84 (95%CI 0.70 to 0.97), Y intercept of -7.1 (95%CI -13.2 to -1.1), R^2 = 0.68, and RMSE = 0.91 mEq/L mmol/L The relationship between individual Atot values and albumin or total protein concentration is shown in Figure 4-4 (bottom panels). Predictors of Atot were determined using pooled data from all three groups. The linear regression of Atot vs. albumin yielded a slope of 0.197 (95%CI 0.098 to 0.297), Y intercept of 5.8 (95%CI 2.1 to 9.6), R^2 = 0.18, and RMSE = 4.49 mmol/L (Figure 4-4, bottom left panel). The linear regression of Atot vs. TP had a slope of 0.221 (95%CI 0.204 to 0.239), Y intercept not different from 0, R^2 = 0.20, and RMSE = 4.42 mmol/L (Figure 4-4, bottom right panel).

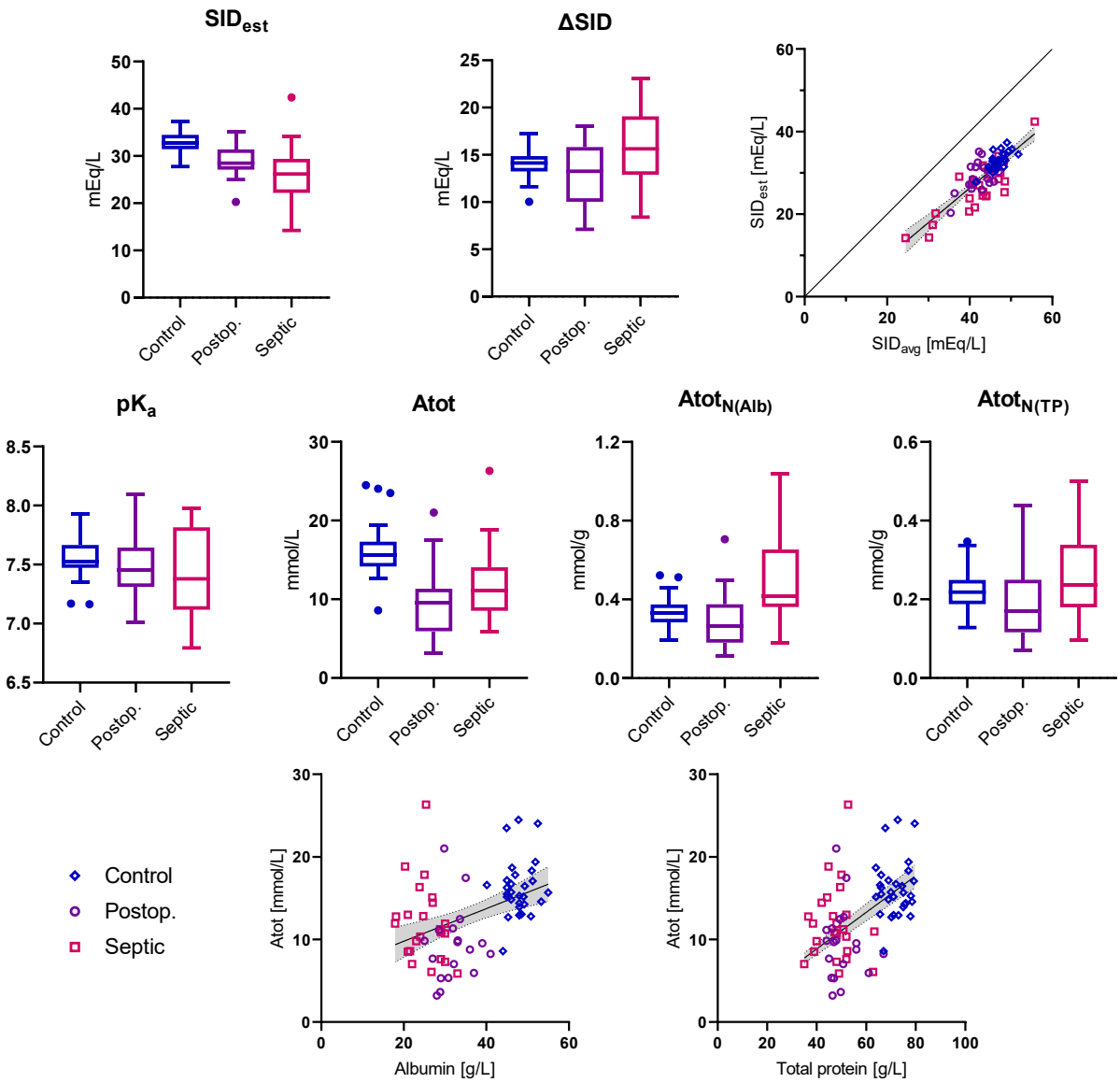

Figure 4-4. Staempfli-Constable model of plasma. ***Top panels:*** *boxplots of the estimated SID (SID_{est}) and the difference between the estimated and average measured SID (ΔSID) in each sample. The right graph shows individual SID_{est} values and linear regression of SID_{est} vs. SID_{avg} with 95% confidence bands and line of identity.* ***Middle panels:*** *boxplots of the dissociation constant of weak nonvolatile acids (pK_a), total concentration of weak nonvolatile acids (Atot), total concentration of weak nonvolatile acids normalized for albumin [$Atot_{N(Alb)}$], and total concentration of weak nonvolatile acids normalized for TP [$Atot_{N(TP)}$]. Whiskers span form min to max. Outliers detected by Tukey's outlier detection method are shown as separate points.* ***Bottom panels:*** *individual Atot values and linear regression of all Atot values vs. albumin or TP with 95% confidence bands.*

4.4 Figge-Fencl model of plasma

The mean estimates of F1 and F2 in each group are reported in in Table 4-5 and Figure 4-5 (left panels in the second and fourth row). The values of F1 recorded in both Postoperative and Septic patients were significantly lower than in Controls. Mean F1 and F2 normalized for albumin and TP concentration [$F1_{N(Alb)}$, $F1_{N(TP)}$, $F2_{N(Alb)}$, and $F2_{N(TP)}$] are presented in Table 4-5 and Figure 4-5 (middle and right panels in the second and fourth row).

		Control (N = 30)	Postoperative (N = 27)	Septic (N = 30)	p value
F1	mmol/L	7.7 ± 1.4	3.7 ± 1.9*	4.8 ± 1.7*	p < 0.0001
$F1_{N(Alb)}$	mmol/g	0.161 ± 0.027	0.116 ± 0.060*§	0.198 ± 0.076*	p < 0.0001
$F1_{N(TP)}$	mmol/g	0.107 ± 0.019	0.075 ± 0.039*	0.103 ± 0.035	p < 0.001
F2	mEq/L	17.1 ± 1.3	13.8 ± 1.8*	16.5 ± 3.2	p < 0.0001
$F2_{N(Alb)}$	mEq/g	0.358 ± 0.022	0.428 ± 0.064*§	0.684 ± 0.142*	P < 0.0001
$F2_{N(TP)}$	mEq/g	0.238 ± 0.015	0.275 ± 0.039*	0.356 ± 0.061*	p < 0.0001

*Table 4-5. Figge-Fencl model of plasma. Results of linear regression using CO_2 equilibration data for determination of model parameters in the three populations. p value refers to one-way ANOVA. *The marked results differ significantly from Controls. §For the marked result N = 26.*

The relationship between individual F1 and F2 values and albumin or TP concentration is shown in Figure 4-5 (panels in the second and fourth row). Predictors of F1 and F2 were determined using pooled data from all three groups. The linear regression of F1 vs. albumin yielded a slope of 0.154 (95%CI 0.143 to 0.166), Y intercept not different from zero, R^2 = 0.35, and RMSE = 1.91 mmol/L (Figure 4-5, left panel in the second row). The linear regression of F1 vs. TP had a slope of 0.098 (95%CI 0.091 to 0.105), Y intercept not different from zero, R^2 = 0.39, and RMSE = 1.85 mmol/L (Figure 4-5, right panel in the second row). The linear regression of F2 vs. albumin yielded a slope of 0.074 (95%CI 023 to 0.124), Y intercept 13.2 (95%CI 11.4 to 15.1), R^2 = 0.09, and RMSE = 2.49 mEq/L (Figure 4-5, left panel in the fourth row). The linear regression of F2 vs. TP had a slope of 0.090 (95%CI 0.050 to 0.130), Y intercept 10.8 (95%CI 8.5 to 13.1), R^2 = 0.19, and RMSE = 2.36 mEq/L (Figure 4-5, right panel in the fourth row).

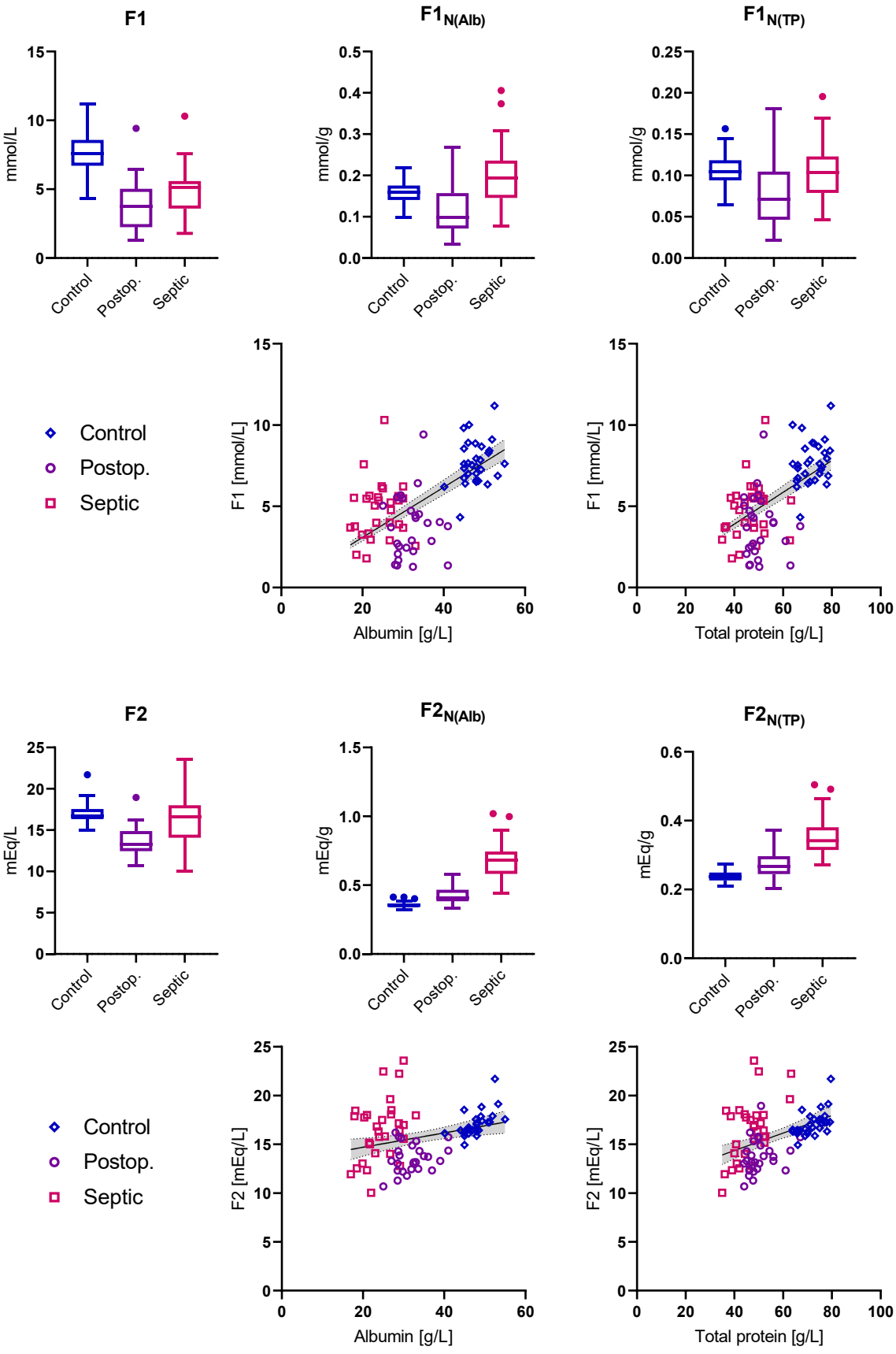

Figure 4-5. Figge-Fencl model of plasma. Boxplots of the estimated parameters. ***First and third row:*** *parameters F1 and F2 – raw and after normalization for albumin and TP concentration. Whiskers span form min to max. Outliers detected by Tukey's outlier detection method are shown as separate points.* ***Second and fourth row:*** *individual F1 and F2 values and linear regression of these values vs. albumin or TP with 95% confidence bands.*

5 Discussion

In this thesis, I describe a series of *in-vitro* CO_2 tonometry experiments with human plasma. With 87 participants representing three distinct populations (healthy volunteers, postoperative patients, and septic patients), this study qualifies as one of the largest of its kind ever performed. We successfully completed our main objective and obtained reliable estimates of pK_a and Atot according to the Stewart model (for healthy volunteers only, see below) and Staempfli-Constable model of plasma (for all three studied groups). Other approaches to the description of plasma acid-base equilibrium (classical and Figge-Fencl model) were also studied and the values of constants that characterize non-carbonic buffers in those models were determined.

In the following subchapters, I compare the results we obtained with each of the mentioned models with their previous theoretical and experimental estimates and discuss the differences we observed between the studied groups.

In the baseline data, a strong correlation between serum albumin and TP concentration was found. This is an expected result because the former is the principal constituent of the latter. Nevertheless, the strength of this correlation rises a concern that the two parameters are so closely related that deciding whether albumin or TP concentration shall be the preferred quantity for normalizing the parameters of plasma buffers may be difficult. The correlation between albumin and non-albumin protein concentration was also significant, yet weak.

5.1 Classical model of plasma

Not surprisingly, we found that the mean β(NC) was decreased in both patient groups, where the amount of non-carbonic buffers (assessed by albumin and total protein concentration) was also reduced. For comparison of the β(NC) we found in healthy volunteers with results of other authors see Table 5-1. Our value (8.1 ± 1.4 mmol/L) agrees well with the values repeatedly proposed by Siggaard-Andersen and with the currently recommended equation for the calculation of the Base excess but exceeds the values reported by some other authors. Notably, the study by Langer *et al*. also described a cohort of septic patients, in which the mean β(NC) was 0.5 mmol/L. This is markedly less than what we found in the current study (5.2 mmol/L). A possible cause of this discrepancy is discussed at the end of the next chapter.

Authors, year	β(NC) [mmol/L]	Note / method
Ellison, Straumfjord and Hummel, 1958	5.8	Strong acid titration of human plasma. The effect of bicarbonate buffer was subtracted.
Siggaard-Andersen, 1974	7.5	Derived from previously published data of strong acid and CO_2 titration of equine plasma (Van Slyke et al. 1928)
Davenport, 1974	5.4	No link to experimental data provided.
Siggaard-Andersen, 1977 CLSI, 2009	7.7	Obtained from the equation for BE(B) by setting Hb=0 g/L.
Langer et al., 2021	3.7	CO_2 titration of plasma.

Table 5-1. β(NC) of plasma in healthy subjects as described by other authors.

Opinions on whether albumin or TP concentration is a more suitable variable for evaluation of $\beta(NC)_N$ differ. Some authors claim that not only albumin but also other proteins (globulins) carry pH-dependent charge and, therefore, contribute to buffering (Van Slyke et al. 1928; Siggaard-Andersen and Fogh-Andersen 1995; Siggaard-Andersen et al. 1977; Constable and Stämpfli 2009) while others say that, at physiological pH, albumin is the only protein that carries appreciable amount of pH-dependent charge (Figge et al. 1991). Experimental data supporting both conclusions have certain limitations: Van Slyke *et al.* were limited by their contemporary equipment, worked with horse serum proteins and performed only a limited number of experiments. Figge *et al.* only used two kinds of samples: either artificial globulin-free solutions or serum filtrands with normal A/G ratio. An obstacle that hinders precise determination of how much various protein moieties contribute to the overall plasma β_{NC} is the collinearity between TP, albumin, and non-albumin protein concentration (Figure 4-1), especially in healthy volunteers.

Our data, which also included individuals with altered A/G ratio (Table 4-1, Figure 4-1), suggest that β(NC) can be accurately predicted from albumin or TP concentration alone (Y intercept of both

regressions did not differ from zero, Figure 4-2, bottom panels), yet TP turned out to be a slightly better predictor of β(NC) than albumin (R^2 0.39 vs. 0.32). Accordingly, when albumin concentration was used to normalize β(NC) in septic patients, unexpectedly high $\beta(NC)_{N(Alb)}$ was obtained (Figure 4-2, top middle panel). This oddity resolved with $\beta(NC)_{N(TP)}$ (Figure 4-2, top right panel). Such an observation seems to be appropriate from a physiological point of view: Our subjects had 13-36 g/L of protein other than albumin in plasma (Figure 4-1, right panel). Despite being very heterogenous, these proteins certainly contain at least some titratable sites in their structure that contribute to the overall buffer action. An unexpected finding is the significant reduction of $\beta(NC)_{N(TP)}$ in postoperative patients. This result needs to be interpreted with caution for two reasons: First, I am not aware of a pathophysiological mechanism by which critical illness, but not sepsis, should reduce $\beta(NC)_{N(TP)}$. Note that the septic cohort suffered from more severe organ dysfunction according to all three scoring systems used. If critical illness were to alter the buffer action of non-carbonic buffers, the septic cohort should be affected even more. Second, this hypothesis was not formulated *a priori*. With the number of statistical tests described in this thesis, false positive results may appear.

The mean $\beta(NC)_{N(TP)}$ obtained in Controls (0.113 ± 0.019 mmol/g) and the slope of the regression line of β(NC) vs. TP in all subjects (0.104 mmol/g, 95%CI: 0.097 to 0.111) agree very well with the value 0.110 mmol/g (Eq. 18) used in the classical model of plasma (Siggaard-Andersen 1974).

In a recent study (Langer et al. 2021), a significant reduction of $\beta(NC)_{N(Alb)}$ was reported in septic patients (Control: 0.08 vs. Septic: 0.01 mmol/g). Our results are not compatible with such an observation: Not only were our values of $\beta(NC)_{N(Alb)}$ much higher in both groups but an opposite trend was observed (Control: 0.167 vs. Septic: 0.218 mmol/g). This discrepancy is present despite very similar methodology and comparable populations. A possible explanation could be the use of an older blood-gas analyzer by Langer *et al*. Direct comparison of the two machines revealed a considerable disagreement in variation of $[HCO_3^-]$ in response to pCO_2 manipulation (own observation, unpublished data). Two features that render our current results as the more plausible option are their agreement with previously published values of β(NC) and $\beta(NC)_{N(TP)}$ of healthy subjects, and the fact that some of the septic subjects in the study of Langer *et al*. had negative values of β(NC) and $\beta(NC)_{N(Alb)}$, for which an explanation is hard to find.

5.2 Stewart model of plasma

Results of the parameters derived according to the Stewart model show that both pK_a and Atot was significantly lower in both patient populations than in healthy volunteers. An interesting observation is that Atot was higher in Septic than in Postoperative patients even though Septic patients had a lower concentration of both albumin and TP, the principal non-carbonic buffers in plasma. This is most apparent when Atot was normalized for albumin concentration but is also present after normalization for TP (Figure 4-3, top panels). Such results seem unlikely. Possible causes of that will be discussed in the next paragraphs.

Studying the relationship between Atot and pooled data from all three groups, we found that in both studied regressions (Atot vs. albumin and Atot vs. TP) the Y intercept differed significantly from zero (Figure 4-3, bottom panels). Therefore, none of the parameters was sufficient to predict Atot.

In the control group, the values of pK_a and Atot obtained (Atot = 24.3 ± 1.9 mmol/L, pK_a = 6.63 ± 0.09) perfectly matched their only previous experimental estimates (Staempfli and Constable, 2003, table 5: Atot = 23.3 ± 1.5 mmol/L, pK_a = 6.64). Similarly, the normalized Atot values of healthy volunteers ($Atot_{N(Alb)}$ = 0.510 ± 0.035, $Atot_{N(TP)}$ = 0.340 ± 0.024) corresponded well with the results of the same authors ($Atot_{N(Alb)}$ = 0.521 ± 0.026 mmol/g, $Atot_{N(TP)}$ = 0.308 ± 0.016 mmol/g). This is a notable result given the fact that the experiments were carried out almost 20 years apart and with completely different equipment.

It was already mentioned (Chapters 1.1.2.2 and 1.1.2.3) that more pairs of experimentally determined K_a and Atot may fit the same data and that the estimates are very sensitive to the SID used. Unmeasured anions, that are not included in SID_{avg} but whose presence affects the acid-base properties of plasma, could have altered results of postoperative and septic patients and, at the same time, confound the pooled data. Even healthy volunteers have some amount of unmeasured circulating anions such as sulphate and uric acid but inter-individual variability in their concentration is low. It is, therefore, reasonable to assume that they affected the estimates of pK_a and Atot in healthy volunteers by Staempfli and Constable to the same degree as ours.

I will now explore how presence of unmeasured anions could have affected our data. Strong anions are considered as fully dissociated and, as such, do not need to have a specific pK_a assigned. However, if they were treated as weak acids, their pK_a would be far below 7.4. (e.g., lactate with pK_a of 3.86). During the process of simultaneous pK_a and Atot determination by nonlinear regression, anions, which are not included in the SID, fall into the $[A^-]$ term of Eq. 25 (electroneutrality equation). Their charge, attributed erroneously to weak nonvolatile acids, leads to a false increase in Atot and drags the

hypothetical pK_a of weak nonvolatile acids towards the acid part of pH spectrum. Both these trends were, indeed, observed the patient cohorts. To further confirm the described behavior and to understand the magnitude of this effect I repeated pK_a and Atot estimation in healthy volunteers after subtracting 5 or 10 mmol/L from measured [Cl^-]. Such manipulation simulates presence of unmeasured anions by hiding part of the negative strong ion charge from the algorithm. The results, presented in Table 5-2, revealed that Atot rose by 4.8 and 9.7 mmol/L, which is 96– 97% of the simulated unmeasured anion concentration. In both patient groups, this effect would combine with the "true" reduction of Atot due to decreased albumin and TP concentration. Interestingly, even with a considerable amount of simulated unmeasured anions in the plasma samples, the effect on pK_a was much less pronounced than in the actual results of postoperative and septic patients (Table 5-2). This could be explained by the fact that unmeasured anions, if present, constituted a relatively greater portion of Atot in patients and, therefore, had greater influence on the estimated pK_a.

		Original data of Controls	[Cl-] decreased by 5 mmol/L	[Cl-] decreased by 10 mmol/L
pK_a		6.63 ± 0.09	6.50 ± 0.09	6.41 ± 0.09
Atot	mmol/L	24.3 ± 1.9	29.1 ± 1.9	34.0 ± 1.8
$Atot_{N(Alb)}$	mmol/g	0.510 ± 0.035	0.610 ± 0.037	0.712 ± 0.041
$Atot_{N(TP)}$	mmol/g	0.340 ± 0.024	0.407 ± 0.025	0.475 ± 0.028

Table 5-2. Stewart model of plasma. Estimation of pK_a and Atot in plasma samples of Controls after presence of unmeasured anions was simulated by decreasing measured [Cl-] by either 5 or 10 mmol/L.

In an study that recruited both healthy and diseased animals (Constable et al. 2005), Atot and K_a determined in healthy calves and these values were used to describe the acid-base status of diseased animals which included quantification of unmeasured anions. It is apparent from previous paragraphs that such approach would not be reliable if the illness were suspected to alter K_a or normalized Atot. By similar logic, our aim to determine normalized Atot and pK_a in critically ill patients was not successful because unmeasured anions could have been (and likely were) present. This is a vicious circle – estimation of unmeasured anions requires precise quantification of the charge carried by weak nonvolatile acids in plasma, yet precise determination of Atot and pK_a in this cohort is confounded by presence of unmeasured anions. There appear to be at least two remedies for this issue: The first is to a switch to the Staempfli-Constable model, which does not rely on knowledge of the actual SID. The second would be to design experiments in which plasma proteins are separated from plasma water and electrolytes and each component is analyzed separately. Such has methodology has already been used in acid-base research (Figge et al. 1991) but only healthy volunteers were included.

5.3 Staempfli-Constable model of plasma

The nonlinear regression algorithm failed to provide valid results in 14 (16.2%) plasma samples, all of which belonged to the two patient groups. We believe that this is caused by the reduced concentration of non-carbonic buffers in these groups (Table 4-1) which led to a smaller variation in $[HCO_3^-]$ during pCO_2 manipulation. To illustrate this fact, consider an extreme case where non-carbonic buffers are completely absent from the plasma sample: $[HCO_3^-]$ is equal to the SID and does not response to any variation of pCO_2 until either $[CO_3^{2-}]$ or $[H^+]$ or $[OH^-]$ becomes quantitatively relevant (Eq. 24). When variation of $[HCO_3^-]$ is little, random errors of measurement gain significance and confound the next steps of analysis. The fact that the same error did not affect the Stewart model, which used the same data as an input, relates to the necessity to estimate an additional unknown parameter (i.e., SID_{est}). The more parameters are unknown, the higher error is present in each estimate. This increases the standard deviation of the estimates and may prevent some plasma samples from converging with the model altogether.

The values of Atot = 17.2 ± 3.5 mmol/L ($Atot_{N(Alb)}$ = 0.378 ± 0.078 mmol/g, $Atot_{N(TP)}$ = 0.224 ± 0.044 mmol/g) and $K_a = 0.8 \pm 0.6 \times 10^{-7}$ (pK_a = 7.10) have been previously published (Staempfli and Constable 2003, table 6). Our estimates of Atot, $Atot_{N(Alb)}$, and $Atot_{N(TP)}$ in healthy volunteers agree well with these values but our pK_a is higher by 0.45. I will discuss this discrepancy later in this chapter. We, nevertheless, observed the same trend in both Atot and pK_a (reduction of the former and increase in the latter) as Staempfli and Constable when the estimated SID was used in place of the measured SID (i.e., when the Stewart model was replaced by the Staempfli-Constable model). In particular, the movement of pK_a towards the physiological pH region is a finding with important consequences that are, too, discussed later.

The algorithm we used for estimation of parameters according to the Staempfli-Constable should not be affected by an alteration of the SID. To confirm that, the same post-hoc analysis as described in the previous chapter was performed. Estimates of SID_{est}, pK_a and Atot remained unaffected by this manipulation and the only parameter that changed was ΔSID (Table 5-3). Although predictable, this

		Original data of Controls	[Cl-] decreased by 5 mmol/L	[Cl-] decreased by 10 mmol/L
SID_{est}	mEq/L	32.8 ± 2.0	32.8 ± 2.0	32.8 ± 2.0
ΔSID	mEq/L	14.0 ± 1.5	19.0 ± 1.5	24.0 ± 1.5
pK_a		7.55 ± 0.17	7.55 ± 0.17	7.55 ± 0.17
Atot	mmol/L	16.1 ± 3.4	16.1 ± 3.4	16.1 ± 3.4

Table 5-3. Staempfli-Constable model of plasma. Estimation of SID_{est}, ΔSID, pKa, and Atot in plasma samples of Controls after presence of unmeasured anions was simulated by decreasing measured [Cl-] by either 5 or 10 mmol/L.

table nicely illustrates the complete independence of pK_a and Atot estimates on the true SID of plasma sample.

It should be appreciated that the nonlinear regression algorithm, despite having been provided with pH and pCO_2 data only, yielded SID_{est} and Atot estimates that were strongly linked to the actually measured SID_{avg} and TP concentration (Figure 4-4, top right and bottom right panels). Note that SID_{est} contains the possibly present unmeasured anions while SID_{avg} does not. This discrepancy, the magnitude of which can be at most several mEq/L, was little compared to range of SID_{avg} present in our subjects (23 – 56 mEq/L) and, therefore, was not sufficient to hide this correlation. Nevertheless, it probably caused the slope of regression line to deviate significantly from one (Figure 4-4, top right panel).

Between-group comparison of the characteristics of non-carbonic buffers in plasma did not reveal a significant alteration in critical illness: Estimated pK_a did not differ between the studied groups and neither did $Atot_{N(TP)}$ differ between Controls and either patient population. Of note, TP was, again, found to be a better predictor of Atot than albumin.

The titration curve of weak nonvolatile acids used in both Stewart and Staempfli-Constable model can approximated as having 5 areas: When pH is close to pK_a, i.e., in the middle of the S-shaped curve, there is a roughly linear ascending part that spans over an area of approximately 1 pH unit. At both extremes of pH, the curve is also almost linear, yet horizonal. Finally, in the transition zones between the said areas the curve is considerably bent (Figure 5-1, red curve). In both models, the curve can be shifted in the left–right direction by altering pK_a. In the Staempfli-Constable model, further

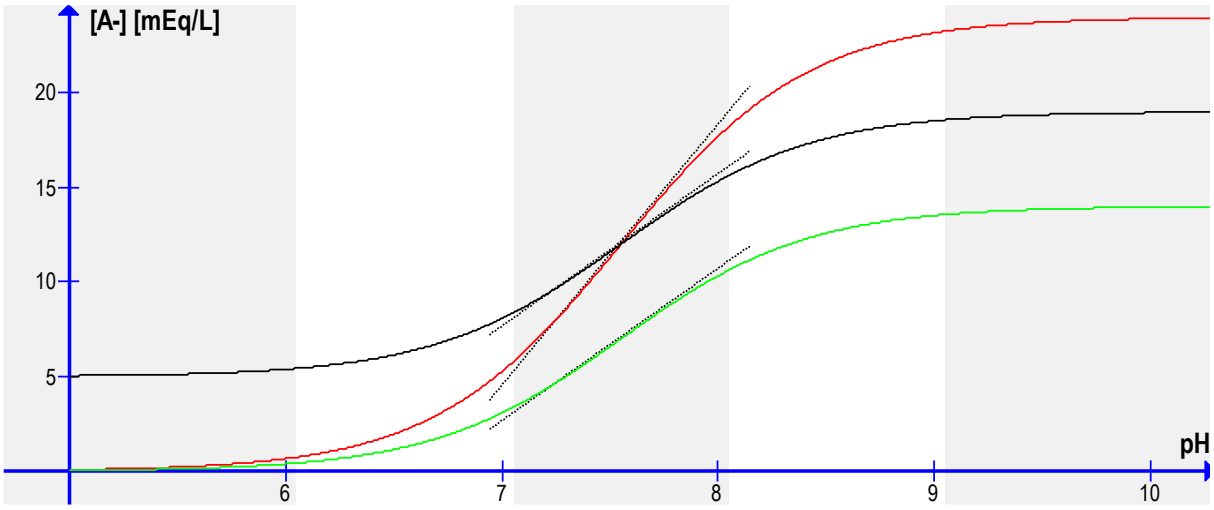

Figure 5-1. The sigmoid shape of weak nonvolatile acid titration curve in the Staempfli-Constable model of plasma. ***Red:*** *pK_a = 7.55, Atot = 24 mmol/L, and no fixed charge.* ***Green:*** *pK_a = 7.55, Atot = 14 mmol/L, and no fixed charge.* ***Black:*** *pK_a = 7.55, Atot = 14 mmol/L, and fixed negative charge of 5 mEq/L. The interrupted lines represent linear approximations at pH = pK_a. White and gray zones divide the titration curves into five segments as described in text.*

adjustments can be freely done: Atot can be modified to alter the slope of the curve in the pH region of interest (Figure 5-1, green curve). Such manipulation is limited in the Stewart model because it would alter the charge carried by weak nonvolatile acids and, thus, come into conflict with the equation of electroneutrality (Eq. 25). In the Staempfli-Constable model, however, one more parameter may be adjusted to compensate for the charge lost due to the reduction of Atot. By altering the amount of fixed charge attributed to weak nonvolatile acids, the whole curve can be moved up or down, (Figure 5-1, black curve). To summarize, if one only cares about the middle part of the S-shaped titration curve, the Staempfli-Constable model allows him to freely adjust its slope as well as its vertical and horizontal position. The fact that the mean estimated pK_a in all three studied groups (7.55, 7.48, and 7.44) fell into the physiological pH range can, therefore, be interpreted as an attempt of the nonlinear regression algorithm to fit the data with the most linear part of the S-shaped curve. Indeed, visual inspection of the pH/[HCO_3^-] relationship revealed that most of them follow an almost linear trace.

The trouble of finding pK_a (i.e., an inflection point) of an almost linear curve is evident and could explain the difference between our pK_a and the value published by Staempfli and Constable. Nevertheless, the difference in pK_a needs further discussion as the left-right shift of the curve by 0.5 pH unit changes considerably the charge carried in the pH region of interest. It was already mentioned that in the Stewart and Staempfli-Constable models of plasma, the estimated parameters cannot be evaluated in isolation. Figure 5-2 shows titration curves of weak nonvolatile acids in plasma of healthy volunteers constructed with mean ΔSID, pK_a, and Atot obtained in the current study and in the study by Staempfli

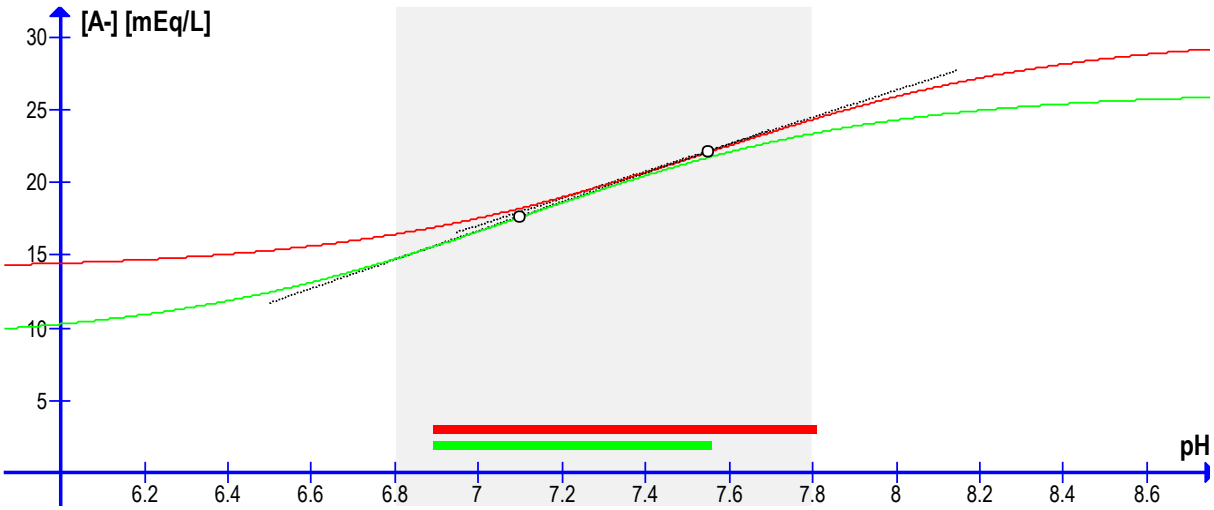

Figure 5-2. Titration curves of weak nonvolatile acid in healthy volunteers. ***Red curve:*** *our results (ΔSID = 14.0 mEq/L, pK_a = 7.55, Atot = 16.1 mmol/L).* ***Green curve:*** *results of Staempfli and Constable (ΔSID = 9.0 mEq/L, pK_a = 7.10, Atot = 17.2 mmol/L). For the latter, ΔSID was calculated according to Eq. 37 and individual values of the measured and estimated SID provided in the study. The gray zone represents pH range of interest. On each curve pK_a is marked by a white point and the dashed line represents a linear approximation in the region of pK_a ± 0.6. The lines of appropriate color at the bottom represent the pH range explored in each study.*

and Constable. The linear approximations of the titration curves in the proximity of pK_a practically overlap. This implies an excellent agreement of the results obtained in the two studies: In the pH range of interest, the difference in horizontal position (i.e., in pK_a) is fully compensated by an appropriate shift in vertical position (i.e., in ΔSID) and the resulting titration curves are almost identical. Furthermore, this result suggests that a straight line may be sufficient as well as more comprehensible for modeling the titration curve of weak nonvolatile acids in the physiological pH range.

If the middle part of the S-shaped titration curve is used to model protein dissociation using the Staempfli-Constable approach, Atot loses entirely its connection to the minimum, actual, or maximum charge of weak nonvolatile acid, because these variables are greatly influenced by the amount of fixed charge carried. In such case, Atot becomes essentially a measure of the slope of the titration curve in the proximity of pK_a, i.e., a measure of the buffer power. Derivation of Eq. 23 with respect to pH provides equation of the buffer power curve:

$$\beta = \frac{d[A^-]}{dpH} = Atot \times \frac{10^{pK_a - pH} \times ln\,10}{(1 + 10^{pK_a - pH})^2} \qquad \textit{Eq. 41}$$

which can be solved for its maximum value at $pH=pK_a$:

$$\beta_{max} = Atot \times \frac{ln\,(10)}{4} = Atot \times 0.5756 \qquad \textit{Eq. 42}$$

However, inspection of the buffer power curve (Figure 1-4, bottom right panel) reveals that the maximum buffer power at $pH=pK_a$ does not represent its mean value in any reasonably wide pH range. For a pH range of *q* units centered around the pK_a, the mean β can be calculated from Eq. 41 as:

$$\beta_{mean} = \frac{1}{q} \times \int_{pK_a - \frac{q}{2}}^{pK_a + \frac{q}{2}} Atot \times \frac{10^{pK_a - pH} \times ln\,10}{(1 + 10^{pK_a - pH})^2}\, dpH \qquad \textit{Eq. 43}$$

Solving Eq. 43 for a pH range of 1 unit (q = 1) provides:

$$\beta_{mean(1)} = Atot \times 0.5195 = \beta_{max} \times 0.9025 \qquad \textit{Eq. 44}$$

If the values of Atot in healthy volunteers obtained in this study (16.1 mmol/L) and by Staempfli and Constable (17.2 mmol/L) are transformed according to Eq. 44, they yield $\beta_{mean(1)}$ of 8.36 and 8.88 mmol/L, respectively. These results are comparable with the non-carbonic buffer power of plasma estimated either in this study (8.1 mmol/L, Table 4-2) or by other authors (Table 5-1).

Finally, I will discuss the variation of ΔSID between our studied groups. ΔSID comprises the fixed partition of protein and phosphate charge together with the charge of any unmeasured anions

present. Interestingly, ΔSID was not significantly different between Controls and either patient group, despite a significant reduction in albumin and TP concentration in the patient populations. This suggests that the relative contribution of ΔSID constituents differs between groups. In Controls, where excess of unmeasured anions is unlikely, most of ΔSID should be attributed to fixed protein and phosphate charge. By contrast, unmeasured anions must occupy a significant portion of ΔSID in the patient populations. An alternative could be that plasma proteins, modified by critical illness, exhibit more fixed negative charge. Such alteration, however, seems very unlikely without a simultaneous alteration in their buffer action, i.e., in either pK_a or $Atot_{N(TP)}$.

5.4 Figge-Fencl model of plasma

Evaluating the results of CO_2 tonometry of plasma of shall be straightforward as most of the interesting facts were already discussed in the previous chapters.

It should be emphasized that the Figge-Fencl model of plasma allows for the same freedom of titration curve adjustment as the linear part of the S-shaped curve in the Staempfli-Constable model (i.e, adjustment of both its slope and vertical position). This is, however, achieved using only two instead of three parameters (F1 and F2 vs. pK_a, Atot, and ΔSID). Such handling seems to be much more comprehensible while it, at the same time, eliminates the problem of non-converging samples and increases confidence of the estimated parameters.

The parameter F1 is by definition very close to β(NC). There was a difference of ~0.4 mmol/L between F1 and β(NC), attributable to the buffer action of phosphate (Eq. 16). Despite that, the similarity of the two parameters allows for applying the same conclusions that were drawn for β(NC) and $\beta(NC)_{N(TP)}$ to F1 and $F1_{N(TP)}$.

The overall buffer power of non-carbonic buffers suggested by Figge and Fencl for their model under normal conditions (Eq. 26: 0.123 mmol/g × 42 g/L + 0.309 mmol/mmol × 1.2 mmol/L = 5.5 mmol/L) agrees with some of the values listed in Table 5-1 (e.g., Ellison *et al.* and Davenport) but is considerably less than the value of 7.7 mmol/L, proposed by Siggaard-Andersen and used in the calculation of BE(P). Based on the experiments presented in this thesis [healthy volunteers: β(NC) = 8.1 mmol/L, F1 = 7.7 mmol/L], I conclude that the higher estimate is more appropriate.

Estimates of the F2 parameter can, similarly to ΔSID of the Staempfli-Constable approach, only be interpreted in the group of healthy volunteers, where the confounding effect of unmeasured anions is little. In the Figge-Fencl model, the normal charge of plasma proteins at pH of 7.4 is 11.7 mEq/L (Chapter 1.1.4) while our results indicate a value of 17.1 mEq/L. Our estimate is, therefore, considerably higher. On the other hand, it corresponds well with the estimate by Van Slyke (16.9 mEq/L, Eq. 13). This is yet another topic that deserves attention and should be addressed by future research.

5.5 Limitations

The most important limitation of the presented study is that unmeasured anions have very likely confounded estimation of some of the parameters that characterize non-carbonic buffers in plasma of postoperative and septic patients. Throughout the thesis, I pay special attention to disclose which parameters are affected by this problem: The confounding effect of unmeasured anions is limited to those parameters that determine the vertical position of the titration curve in the $[A^-]$/pH graph and does not apply to the parameters that affect its shape or slope. The rationale for this is that virtually all substances that might present as unmeasured anions have pK_a sufficiently distant from the physiological pH range to safely assume that they possess no buffer properties (Figge et al. 1992). However, if an unidentified substance with pK_a close to 7.4 were present in sufficient quantity, it would negatively affect all the parameters studied.

A limitation related to that described in previous article is that we can only comment with absolute certainty on the buffer action, and not the overall charge, of plasma non-carbonic buffers. Despite that, we consider these two faces of non-carbonic buffers as interlinked and conclude (Chapter 5.6), that a change of one in them is unlikely to happen without a measurable alteration of the other.

It is known that the course of sepsis, including mortality, is heavily influenced by many pathogen and host related factors such as age, underlying comorbidities, medications, and source of infection (Pieroni et al. 2022; Iskander et al. 2013; Singer et al. 2016). Hence, generalization of the results obtained in this septic cohorts to populations with a different patient mix must be done with caution. Similarly, postoperative patients cannot be readily compared with other patients without sepsis in the ICU, such as trauma, cardiac arrest, or stroke victims.

Of the common body fluids and compartments analyzed, isolated plasma is the simplest one. The complexity of buffer processes increases in blood (Krbec et al. 2022; Siggaard-Andersen 1974) and escalates further in the case of whole body *in-vivo* (Giebisch et al. 1955; Elkinton et al. 1955). Studying the mechanisms of buffer action in plasma cannot, therefore, provide a complete picture of how the human body mitigates disturbances of the acid-base equilibrium. On the other hand, knowledge of the rules that govern acid-base reactions in plasma is the gateway to understanding the principles that apply in more complex settings.

5.6 Clinical implications and future perspectives

Plasma is the fluid in which the law of electroneutrality is applied to perform electroneutrality-based calculations including the detection of unmeasured unions. Their estimation is of a great clinical importance, as it may be a sign of several life-threatening but treatable conditions. The simplest of the tools used, the Anon gap, is known to have poor sensitivity in patients with decreased albumin concentration. The more advanced tools, which are not affected by this limitation, require calculation of the charge displayed by plasma proteins. This is done from albumin or TP concentration with equations and constants unique for each model of acid-base equilibrium in plasma. If any of these constants is altered by critical illness or sepsis, the affected calculation may need to be corrected accordingly.

The results obtained in this study using the classical and Staempfli-Constable model of plasma indicated that TP corelates better than albumin with the variables that are supposed to vary proportionally with non-carbonic buffer content. In line, TP should be used to obtain the normalized values of these parameters. The non-carbonic buffer power normalized for TP concentration was not altered by sepsis. There was, however, a significant reduction in Postoperative patients. We interpret this finding with caution, because of the lack of pathophysiological explanation for such a phenomenon. Using the Staempfli-Constable model, no differences in the constants that characterize non-carbonic buffers (Atot normalized for TP, and pK_a) were found between Controls and either patient population.

The above findings imply that neither critical illness *per se* nor sepsis affects the buffer action of non-carbonic buffers to a measurable, clinically relevant degree. While the current study does not allow us to exclude that these conditions increase the amount of fixed charge carried by plasma proteins, we consider this possibility unlikely without at least a slight alteration in their buffer action.

Studies that definitively decide whether or not the net charge of plasma proteins change during sepsis or critical illness will need to separate the studied molecules from their aqueous environment, thus removing all possibly present unmeasured anions, by processes such as ultrafiltration or microdialysis.

6 Conclusions

We performed one of the greatest sets of CO_2 equilibration experiments with human plasma to date. By enrolling subjects from three distinct populations (healthy volunteers, postoperative patients, and septic patients), we were able to evaluate the effect of critical illness and, in particular, sepsis on the quantities that characterize plasma buffers. In this thesis, I thoroughly describe and compare the parameters obtained by analyzing the collected data according to several well-established models of acid-base equilibrium in plasma.

In the analyzes performed according to the classical model of plasma, β(NC) in healthy volunteers was 8.1 mmol/L, which corresponds well to the values that appear repeatedly in the literature. Within our study, β(NC) was significantly reduced in both patient populations. In septic patients, this could be explained by the reduced TP concentration so that β(NC) normalized for TP concentration remained constant. In postoperative patients, however, β(NC) was reduced beyond this effect. This finding, currently unexplainable, calls for further investigation.

The application of the Stewart model in healthy volunteers provided pK_a and Atot values that validated their only previous experimental estimates. In the patient populations, both parameters of the Stewart model turned out to be prone to the confounding effect of unmeasured anions and, hence, did not provide useful results for comparing characteristics of non-carbonic buffers.

The Staempfli-Constable model, the most complex approach used, did not converge in 16% of samples. In healthy volunteers, we validated the only previously published estimate of Atot, while pK_a differed by as much as 0.45. Subsequent analyzes revealed that this discrepancy in pK_a is fully explained by the simultaneous difference in the amount of fixed charge attributed to weak nonvolatile acids causing the titration curves of weak nonvolatile acid in healthy volunteers constructed with our and the previously published estimates of Atot and pK_a to overlap in the pH region of interest. Within our study, no difference in pK_a or Atot was found between Controls and either patient population. An important finding was that the estimated pK_a in all three study groups was within the explored pH rage and close to 7.4, which implies that the experimentally obtained titration data points were most accurately represented by the middle (i.e., the most linear) part of the S-shaped curve. In turn, a simpler, linear approach seems to be sufficient as well as more comprehensive for describing the buffer action of weak nonvolatile acids in plasma within the physiological pH range.

The differences between the studied groups analyzed according to the Figge-Fencl model provided similar results as the classical model, since the F1 parameter is very similar to β(NC). It was noted that the classical and Figge-Fencl model differ considerably in the buffer power they attribute to

non-carbonic buffers under normal conditions (classical: 7.7 vs. Figge-Fencl: 5.5 mmol/L). This difference is far from negligible and, too, deserves further attention. Our current experimental data favor the higher of the two mentioned values.

With respect to the principal aim of the current study, I conclude that we successfully reproduced the only study to date (Staempfli and Constable 2003), in which Atot and pK_a of weak nonvolatile acids in plasma were experimentally measured. We obtained reliable estimates of these parameters in healthy volunteers (for both the original Stewart and the modified Staempfli-Constable model), which agree extraordinarily well with the values provided in the mentioned study. It is likely that the presence of unmeasured anions confounded our estimates of pK_a and Atot obtained using the original Stewart model in both patient populations. In the Staempfli-Constable model, however, estimation of these two principal parameters is not affected by such a problem. The values of pK_a and $Atot_{N(TP)}$, obtained in this way, did not differ between the control group and either patient population. This implies that neither critical illness *per se* nor sepsis alters the structure of plasma proteins to such an extent that would measurably affect their macroscopic buffer action.

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Publication outcomes

Original research articles:

1. SCHMIDT, Matouš, Petr WALDAUF, Martin KRBEC and František DUŠKA, 2022. Perimortem dynamics of blood potassium concentration in patients dying in intensive care unit: A prospective nested cohort study. *Journal of Forensic Sciences* [online]. **67**(4), 1550–1556. ISSN 0022-1198. Available at: doi:10.1111/1556-4029.15054.
   Supplement n. 1, Clarivate 2021 Journal Impact Factor: 1.717

2. KRBEC, Martin, Petr WALDAUF, Francesco ZADEK, Serena BRUSATORI, Alberto ZANELLA, František DUŠKA and Thomas LANGER, 2022. Non-carbonic buffer power of whole blood is increased in experimental metabolic acidosis: An in-vitro study. *Frontiers in Physiology* [online]. **13**, 1009378. ISSN 1664-042X. Available at: doi:10.3389/fphys.2022.1009378
   Supplement n. 2. Clarivate 2021 Journal Impact Factor: 4.755

3. BRUSATORI, Serena*, Martin KRBEC*, Petr WALDAUF, Alberto ZANELLA, František DUŠKA and Thomas LANGER, 2023. Determination of Ka of isolated plasma of septic critically ill patients, healthy volunteers, and non-septic critically ill patients. Manuscript in preparation.
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Other publications:

1. KRBEC, Martin, Kateřina KOUDELKOVÁ and František DUŠKA, 2021. On acid-base effects of albumin. Letter to the editor. *Current Medical Research and Opinion* [online]. **37**(9), 1515–1516. ISSN 0300-7995. Available at: doi:10.1080/03007995.2021.1944074.
   Supplement n. 3. Clarivate 2021 Journal Impact Factor: 2.705

2. KRBEC, Martin and František DUŠKA, 2023. The buffer power of plasma proteins: experimental observations are consistent while the definitions are not. Letter to the editor. *Journal of Applied Physiology*. In press. ISSN 8750-7587. To be available at: doi:10.1152/japplphysiol.00039.2023.
   Supplement n. 4. Clarivate 2021 Journal Impact Factor: 3.881

Conference proceedings:

1. KRBEC, Martin, Petr WALDAUF, Paul W. G. ELBERS, Thomas LANGER and František DUŠKA. Mystery of Unidentified Anion in Sepsis Solved. Poster. XXVI. Congress of ČSARIM, Brno, 2-4 October 2019

2. KRBEC, Martin, Kateřina KOUDELKOVÁ and František DUŠKA. Albumin Is Not a Buffer in Human Plasma. E-poster. 33rd annual congress ESCIM LIVES, on-line event, 6-9 December 2020

3. KRBEC, Martin, Serena BRUSATORI, Francesco ZADEK, Alberto ZANELLA, František DUŠKA and Thomas LANGER. Non-carbonic buffer power of whole blood is increased in experimental metabolic acidosis: an in-vitro study. Poster. 33° Smart Meeting Anesthesia Resuscitation Intensive Care, Milan, 4-6 May 2022

4. BRUSATORI, Serena, Martin KRBEC, Francesco ZADEK, Alberto ZANELLA, František DUŠKA and Thomas LANGER. Experimental determination of Atot and pK_a of whole blood of healthy volunteers, patients with sepsis and postoperative patients: an in vitro study. Poster. 33° Smart Meeting Anesthesia Resuscitation Intensive Care, Milan, 4-6 May 2022

5. KRBEC, Martin, Petr WALDAUF, Francesco ZADEK, Serena BRUSATORI, Alberto ZANELLA, František DUŠKA and Thomas LANGER. Zvýšení nebikarbonátové pufrační kapacity krve během in-vitro navozené metabolické acidózy nevnáší měřitelnou chybu do výpočtu Base excess krve [The increase on non-carbonic buffer power in metabolic acidosis does not induce a measurable bias in the calculation of the Base excess of blood]. Poster. XXVIII. Congress of ČSARIM, Brno, 15-17 September 2022

6. BRUSATORI, Serena, Martin KRBEC, Francesco ZADEK, Alberto ZANELLA, František DUŠKA and Thomas LANGER. Experimental determination of Atot and pK_a of whole blood of healthy volunteers, patients with sepsis and post-operative patients: an in vitro study. Poster. 35th annual congress ESICM LIVES, Paris, 22-26 October 2022

7. KRBEC, Martin, Serena BRUSATORI, Alberto ZANELLA, František DUŠKA and Thomas LANGER. Experimental determination of pK_a and Atot of weak nonvolatile acids in plasma of healthy volunteers and its application in critically ill patients. Oral presentation. 19th International Medical Ph.D. Conference of Faculty of Medicine in Hradec Králové, Charles University, Hradec Králové 24-25 November 2022

Supplements

Supplement n. 1: Original article, Journal of Forensic Sciences

Supplement n. 2: Original article, Frontiers in Physiology

Supplement n. 3: Letter to the editor, Current Medical Research and Opinion

Supplement n. 4: Letter to the editor, Journal of Applied Physiology