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Acid–base buffering in organ preservation solutions as a function of temperature: new parameters for comparing buffer capacity and efficiency☆

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Abstract

Control of acidity and preventing intracellular acidosis are recognized as critical properties of an effective organ preservation solution. Buffer capacity and efficiency are therefore important for comparing the relative merits of preservation fluids for optimum hypothermic storage, but these parameters are not available for the variety of organ preservation solutions of interest in transplantation today. Moreover, buffer capacity is dependent upon both concentration and pH such that buffer capacity is not easily predicted for a complex solution containing multiple buffer species. Using standard electrometric methods to measure acid dissociation constants, this study was undertaken to determine the maximum and relative buffer capacities of a variety of new and commonly used hypothermic preservation solutions as a function of temperature. The reference data provided by these measurements show that comparative buffer capacity and efficiency vary widely between the commonly used solutions. Moreover, the fluids containing zwitterionic sulfonic acid buffers such as Hepes possess superior buffering for α-stat pH regulation in the region of physiological importance.

Keywords: Acid dissociation constants (pK); Acid–base control; Buffer capacity; Hypothermia; pH buffers; Organ preservation

Since the early developments of synthetic solutions in the 1960s for modern day organ preservation, it has been generally accepted that "intracellular-type" solutions provide the best cytoprotection during hypothermic storage. The principal design elements of "intracellular-type" flush solutions have been to adjust the ionic balance (notably of the monovalent cations) and to raise the osmolality by including an impermeant solute to balance the intracellular osmotic pressure responsible for water uptake. Attention to the biophysical properties of intracellular-type flush solutions, to restrict passive diffusional processes, has unquestionably led to the development of techniques that have provided the basis of clinical organ preservation during the past 30 years. With due consideration for the effects of ischemia, hypoxia, hypothermia, and reperfusion injury on cells, coupled with the proven efficacy of various existing organ preservation solutions, a

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Table 1
Composition of solutions

Components (mM)	PBS	KB	DMEM	EC	BMPS[a]	UW[a]	HTS/M[a]	Unisol[a] (UHK)	Celsior[a]	HTK[a]
Ionic										
Na^+	165.3	144.1	143.6	10.0	100.0	30.0	100.0	62.5	100.0	15.0
K^+	1.1	5.9	5.4	115.0	25.0	125.0	42.5	70.0	15.0	10.0
Ca^{2+}	—	1.3	1.8	—	0.5	—	0.05	0.05	0.26	—
Mg^{2+}	—	1.2	0.8	—	5.0	5.0	5.0	15.0	13.0	4.0
Cl^-	154.1	126.4	125.4	15.0	1.0	—	17.1	30.1	41.5	50.0
SO_4	—	1.2	0.8	—	—	5.0	—	—	1.2	—
pH Buffers										
$H_2PO_4^-$	1.1	1.2	1.02	15.0	25.0	25.0	10.0	2.5	—	—
HPO_4^{2-}	5.6	—	—	42.5	—	—	—	—	—	—
HCO_3^-	—	25.0	26.2	10.0	—	—	5.0	5.0	—	—
Hepes	—	—	—	—	10.0	—	25.0	35.0	—	—
Histidine	—	—	—	—	—	—	—	—	30.0	198.0
Total buffer	6.7	26.2	27.22	67.5	35.0	25.0	40.0	42.5	30.0	198.0
Impermeants										
Lactobionate	—	—	—	—	—	100.0	100.0	30.0	80.0	—
Raffinose	—	—	—	—	—	30.0	—	—	—	—
Sucrose	—	—	—	—	—	—	20.0	25.0	—	—
Mannitol	—	—	—	—	30.0	—	20.2	25.0	60.0	30.0
Glucose	—	11.0	5.5	194.0	10.0	—	5.0	5.0	—	—
Gluconate	—	—	—	—	85.0	—	—	70.0	—	—
Ribose	—	—	—	—	0.5	—	—	—	—	—
Colloids										
HES	—	—	—	—	5%	5%	—	—	—	—
Dextran 40	—	—	—	—	—	—	6%	6%	—	—

PBS, phosphate-buffered saline [Mediatech]; KB, Krebs–Henseleit bicarbonate buffer [39]; DMEM, Dulbecco's modified Eagle's medium [Sigma]; EC, EuroCollins [9,10,26]; BMPS, Belzer machine perfusion solution [Trans-Med and Organ Recovery Systems-KPSI] [4,31]; UW, University of Wisconsin solution [Viaspan/DuPont] [5]; HTS/M, Hypothermosol/maintenance [41]; Unisol (UHK) [Organ Recovery Systems] [42,48]; Celsior [Sangstat] [10], and HTK, Bretschneider [Custodiol] [9,10].

[a] These solutions contain various additional pharmacological components as specified in the literature references cited but these details are omitted from this table for clarity. Nevertheless, the complete formulations were used for the determination of pK_a in this study. Solutions used in this study were either obtained from commercial sources or prepared in our laboratory in accordance with the published formulations.

general consensus of the most important characteristics in the design of hypothermic storage solutions has emerged [5]. Control of acidity and preventing intracellular acidosis are recognized as critical properties of an effective organ preservation solution.

Among the principal events of the ischemic cascade, elevation of the concentration of protons, i.e., increasing acidity, is regarded as a contributory central event in the process of cellular injury ensuing from O_2 deprivation and energy depletion. Moreover, reduced temperatures are known to influence pH regulation, which is another important homeostatic mechanism for cell survival. It has frequently been reported that hydrogen ion concentration increases in a variety of mammalian cells during hypothermic storage such that tissue pH has been recorded to fall to 6.5–6.8 within a few hours of cold exposure [7,12]. Acidity is widely recognized as a hazard for cells, with the accumulation of protons contributing to a variety of deleterious processes including metabolic block of glycolysis, and structural damage. Destabilization of lysozomes releasing harmful proteases and catalysis of oxidative stress by mobilization of free heavy metals have been implicated as mechanisms of cellular tissue injury during acidosis. Control of pH and proton buffering capacity is therefore an important consideration in comparing the respective merits of hypothermic preservation solutions. Nevertheless, this parameter has rarely been published for the increasing variety of organ preservation solutions that have been formulated in recent years. Moreover, these solutions often comprise a cocktail of ingredients that usually include more than one chemical species with the ability to buffer protons. Since the components of a solution will interact to generate an overall effect, it is imperative that the buffer capacity of the solution as a whole is determined rather than reliance upon the reported buffer capacity of the principle buffer component. Moreover, buffer capacity is dependent upon both concentration and pH such that buffer capacity is not easily predicted for a complex solution. This study was therefore undertaken to determine the maximum and relative buffer capacities of a variety of new and commonly used hypothermic preservation solutions as a function of temperature. These data are necessary for evaluation of the relative merits of cold storage solutions in terms of their abilities to combat acidosis during low temperature preservation applications.

Methods

The effect of temperature on the "practical," or "apparent" dissociation constants, as previously defined [37,47], and buffer strength of the variety of biological buffers and preservation solutions listed in Table 1 was determined by standard electrometric titration methods as described below. Table 1 summarizes the comparative formulations of the solutions selected in this study.

Acid–base end-point titrations were carried out using a Radiometer TitraLab90 system (Hach Company, Loveland, CO) that, for high efficiency, included a titration manager (TIM900) and a high precision autoburette (ABU901 (5 ml)). In conjunction with these, a glass combined electrode (pHC4400, Radiometer) and glass thermometer (T201, Radiometer) were used for accurate, simultaneous reading of sample pH and temperature. Prior to the experiment, the pH electrode was calibrated in certified IUPAC solutions (Radiometer), using a two-point calibration method (pH 7.00 and pH 4.01 at 22 °C). This calibration was applied for the entire set of experiments using the TIM900 titration manager, which allowed for temperature compensations when the sample temperature differed from the calibration temperature. For complete remote control of the titration manager, TimTalk 1.2, windows based software (Radiometer) was used. For each solution triplicate titrations were carried out in a water-jacketed glass titration vessel with temperature controlled by circulating water cooled and pumped from a refrigerated bath (Fisher ISOTEMP-1028S; set at $T \pm 0.1$ °C).

As illustrated in Fig. 1, potentiometric titration curves were generated for each solution by back-titration of 5 ml of solution with standardized acid (0.1 N HCl; Fisher, Cat. # SA54-1), after adjustment to a pH value in the range of 12–13 by adding 0.5 ml of 1 N NaOH (Fisher, Cat. #SS266-1) to the sample volume. Titration curves were generated at 20 °, 15 °, 10 °, or 5 °C using a 0.5 ml maximum burette increment and a burette speed of 2 ml/min, until the stop-point of pH 2.0 was reached.

Data analysis and results

Using the conventional electrometric methods described above, practical acid dissociation constants (pK'_a) were derived from the titration curves by calculating the pH at half equivalence as

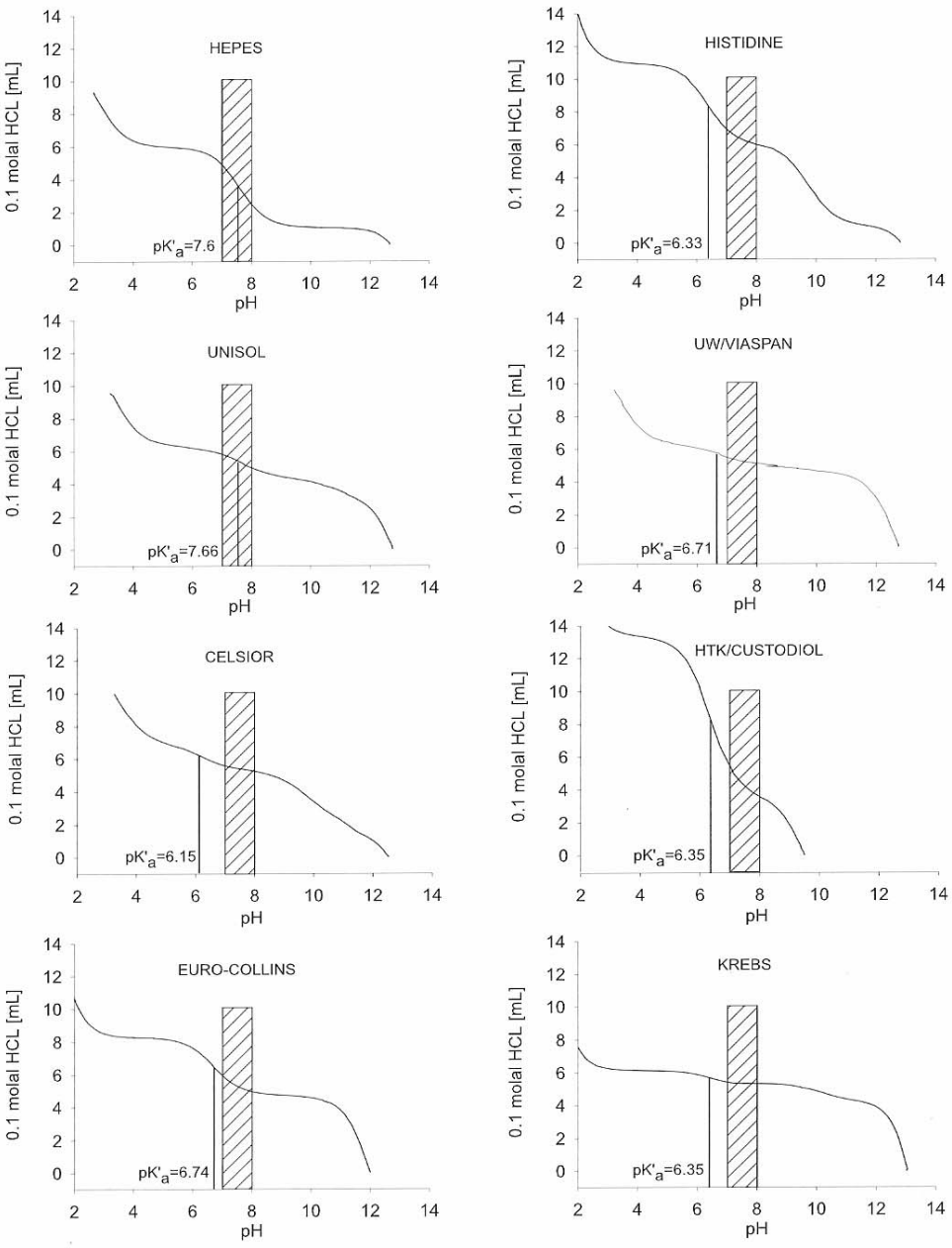

Fig. 1. Representative titration curves for selected preservation solutions at 5 °C. The shaded area demarcates the interaction of the titration curve with the pH range of physiological importance. The point of maximum buffering, as determined by the dissociation constant, pK'_a, is shown as a vertical line in relationship to this critical pH range. The comparative curves illustrate that only Hepes and solutions containing this aminosulfonic acid buffer provide maximum buffering in the pH range of physiological importance.

described elsewhere [37,38,47]. In the present study this was accomplished mathematically by analysis of that portion of the titration curve flanked by the end points. Acid dissociation constants are conventionally denoted as pK_a values and, as in the past, a pK'_a notation was adopted in

this study. This is the accepted convention to reflect that for most practical purposes at low ionic strengths, the Henderson–Hasselbalch equation relating pH to the ionization constant (K_a) and the thermodynamic activities of the acid–base species can be expressed in terms of ionic concentrations [37]. Moreover, the specific objective of this study was to derive averaged pK'_a values from the titration curves of complex multi-component organ preservation solutions as a means of comparing their relative buffer capacities.

The empirical mathematical expression (correlation coefficient $R > 0.99$), used to describe pH changes between the experimentally determined end points regardless of solution nature and temperature, is given by

$$y = f(x) = y_0 + a/(1 + \exp(-(x - x_0)/b)), \qquad (1)$$

where y_0, a, b, and x_0 are constants (with values depending upon solution and temperature), x and y coordinates designated the pH and titrant volume (ml), respectively. The inflexion point (defined at the x coordinate where the second derivative is null and changes signs) coincides with the pK'_a value. Therefore, solving $f(x)'' = 0$ yields $x = x_0 = pK'_a$. The corresponding titrant volume is calculated as the function's value in that point, $f(x_0) = y_0 + a/2$ (ml). This analysis was applied to the triplicate titration curves derived for each solution at each of the four temperatures, the resulting mean pK'_a values (±SEM) are reported in Table 2. The variation of pK'_a with temperature is shown graphically in Fig. 2a, for the range of solutions tested and Table 2 lists the temperature coefficients calculated as the slope of the line between 20 and 5 °C.

The effectiveness of a buffer, or the buffer capacity, used to characterize the change in pH with the addition of a known amount of acid or base, is defined in a conventional way, as the increment in the acid–base volume dV (in millimol/L) needed to change pH by one unit (originated by VanSlyke in 1922 [49])

$$\beta_{conv} = dV/dpH. \qquad (2)$$

This value, which is the reciprocal of the slope of the pH-neutralization curve, is calculated directly from the sigmoid titration curve, using Eq. (1), with pH (x variable in the equation) receiving the values of $pK'_a \pm 1$, which are known to be in the central part of the curve where any buffer solution is most effective and where the changes in pH are small. The calculated values of β_{conv} are

Table 2
Average pK'_a values, theoretical and conventional buffer capacity (slykes) and temperature coefficient for the potential perfusion solutions

Solutions	β^t_{max}	20 °C		15 °C		10 °C		5 °C		$\Delta pK'_a$ °C
		pK'_a	β_{conv}	pK'_a	β_{conv}	pK'_a	β_{conv}	pK'_a	β_{conv}	
Hepes[a]	57.57	7.44 ± 0.01	38.8	7.47 ± 0.00	39.0	7.54 ± 0.00	39.0	7.60 ± 0.02	38.0	−0.0106
Histidine[a]	57.57	6.06 ± 0.00	40.9	6.12 ± 0.00	40.4	6.23 ± 0.02	40.5	6.33 ± 0.01	40.1	−0.0180
PBS	3.85	6.72 ± 0.00	2.7	6.73 ± 0.00	2.8	6.75 ± 0.01	2.7	6.79 ± 0.03	2.7	−0.0046
Krebs	15.08	6.33 ± 0.01	7.4	6.37 ± 0.01	7.4	6.35 ± 0.02	7.2	6.35 ± 0.00	7.2	−0.0013
DMEM	15.66	6.21 ± 0.00	15.6	6.21 ± 0.01	15.3	6.16 ± 0.04	14.3	6.12 ± 0.01	13.2	0.0060
EuroCollins	38.86	6.72 ± 0.03	27.7	6.72 ± 0.00	27.9	6.72 ± 0.01	27.4	6.74 ± 0.01	27.7	−0.0013
UW/Viaspan	14.39	6.36 ± 0.07	10.1	6.50 ± 0.09	10.0	6.40 ± 0.15	10.1	6.71 ± 0.09	10.1	−0.0233
Belzer/MRS	20.15	6.92 ± 0.00	12.2	6.92 ± 0.01	12.9	6.95 ± 0.02	13.0	6.99 ± 0.01	12.7	−0.0046
HTK/Custodiol	113.99	6.14 ± 0.00	78.8	6.21 ± 0.00	79.6	6.28 ± 0.00	79.1	6.35 ± 0.00	78.9	−0.0140
Celsior	17.27	5.96 ± 0.01	14.0	6.03 ± 0.04	13.6	6.13 ± 0.02	13.2	6.15 ± 0.02	13.4	−0.0126
HTS/M	23.03	7.27 ± 0.01	13.3	7.44 ± 0.02	12.9	7.51 ± 0.01	12.8	7.62 ± 0.02	13.2	−0.0233
Unisol/UHK	24.46	7.47 ± 0.00	14.3	7.55 ± 0.00	14.3	7.60 ± 0.01	13.9	7.66 ± 0.01	14.0	−0.0126

[a] Solutions of the pure buffer compounds, Hepes and Histidine were prepared at a concentration of 100 mM and used as baseline reference solutions.

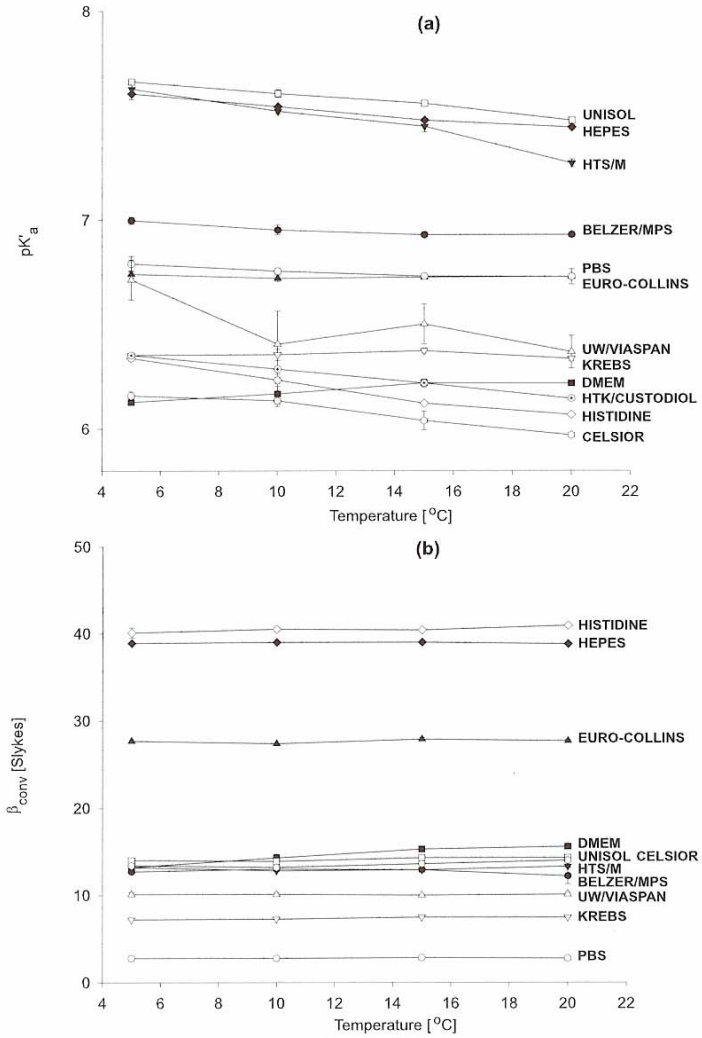

Fig. 2. Variation of dissociation constants (a), and conventional buffer capacities (b), with temperature. The values were calculated directly from the empirical titration curve equations derived for each solution. Buffer capacity, $\beta_{conv} = dV/dpH$. Strategies to combat acidosis by employing preservation solutions containing buffers that will maintain a high pH under hypothermic conditions are best accomplished using solutions with high pK'_as. (a) Shows clearly that only solutions containing the aminosulfonic acid buffer, Hepes, have pK'_a-values higher than 7 over the entire temperature range; all other solutions are spread in the range of 6–7.

listed in Table 2 for the entire set of solutions (SEM for the buffer capacity was less than 8%). Subsequently, the average temperature coefficients have been obtained assuming a linear relationship of pK'_a with temperature, between 20 and 5 °C, as $\Delta pK'_a/°C = (pK'_{a_{20}} - pK'_{a_5})/(20 - 5)$.

Knowing that the change in pH is minimal near the solution pK'_a, a different approach is considered in defining the buffer capacity. This is based upon the fact that in the immediate vicinity of the pK'_a value (where the assumption of local linearity of pH–titrant volume curve is justified) the change in pH is minimal for a known amount of acid added to solution, which based on Eq. (2), yields the maximum value, β_{max}, for buffer capacity. Moreover, the first derivative of $f(x)$

(Eq. (1)) with respect to x, at a point represents the angular coefficient of the geometrical tangent to the graph (curve) of $f(x)$ at that point. Therefore, for $\mathrm{pH}_{1/2} = x_0 = \mathrm{p}K'_a$, the first derivative $f'(x_0) = |a/(4b)|$ is the angular coefficient or the slope of the tangent to the curve at that location. The absolute value is taken for physical meaning considerations. Also, the slope of a line delimited by two points, $P_1(x_1, y_1)$ and $P_2(x_2, y_2)$, is given by $(y_2 - y_1)/(x_2 - x_1)$, as shown in Fig. 3. If the linearity assumption on the pH–titrant volume curve is implied, the tangent and the "linear" portion of the curve (in the vicinity of $\mathrm{p}K'_a$) are overlapped, and their slope coincides. Consequently, $|a/(4b)| = |(y_2 - y_1)/(x_2 - x_1)|$, in this case $P(x_1, y_1)$ and $P(x_2, y_2)$ define the "limits of linearity" of the titration curve, in the vicinity of $\mathrm{p}K'_a$. Summarizing this in one expression gives:

$$\mathrm{d}V/\mathrm{dpH} = \beta_{\max} = |a/(4b)| = |(y_2 - y_1)/(x_2 - x_1)|, \quad (3)$$

where again $\mathrm{d}V$ is expressed in millimol/L yielding buffer capacity in "slykes." Fig. 2b shows the conventional buffer capacity change with temperature. The principle purpose of this study is to compare the effective buffer capacity of some new and commonly used hypothermic preservation solutions as a function of temperature based upon determination of the practical acid dissociation constants. To aid in these comparisons, two new quantitative indices are proposed to compare the relative buffer efficiency of preservation solutions

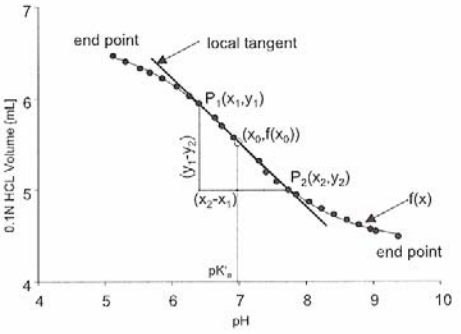

Fig. 3. Graphical illustration of the dissociation constant coordinates on a representative titration curve (Hepes) and the empirical derivation of the maximum buffer capacity. Maximum buffer capacity (slope of the tangent to the titration curve), $\mathrm{d}V/\mathrm{dpH} = \beta_{\max} = |(y_2 - y_1)/(x_2 - x_1)|$.

as a function of temperature and environmental pH. First efficiency, η, is defined as the proportion of the solution buffer strength used under given ambient pH conditions relative to the maximum available buffer strength, obtained at pH values close to solution's $\mathrm{p}K'_a$. Hence, efficiency is the ratio of the solution capacity to its maximum capacity. When the conventional buffer capacity is used, the efficiency is defined as the conventional efficiency

$$\eta_{\mathrm{conv}} = \beta_{\mathrm{conv}}/\beta_{\max} (< 1). \quad (4)$$

Second, as seen in Table 2, in this study the values of the $\mathrm{p}K'_a$ span the range of 5.96–7.66. Of practical interest are those solutions with a $\mathrm{p}K'_a$ close to the physiological value of 7.4, which corresponds to the pH of extracellular fluid. Therefore, a different buffer capacity, identified as physiological buffer capacity β_{phys}, and defined as the ratio of acid volume increment to change the pH with ± 0.4 units in the region of the 7.4 value, is introduced. This is designed specifically to evaluate relative buffer capacities in the range of 7.0–7.8, which is known to be the region of maximum importance for control of pH for biological homeostasis. Qualitatively, this represents the effective buffer capacity of a given solution in the vicinity of physiological pH and provides a useful index for comparing the relative buffering abilities of complete solutions as a function of temperature (see Fig. 4a) in the range of critical importance for controlling acidosis during the ischemic and preservation interval. Here again, Eq. (1) is used, with pH values of 7.4 ± 0.4 falling inside the definition domain of $f(x)$ (in between the two end points). The solution efficiency in the physiological pH range is given by $\eta_{\mathrm{phys}} = \beta_{\mathrm{phys}}/\beta_{\max} (< 1)$ (see Fig. 4b). Table 3 summarizes the characteristics of the solutions in terms of buffer capacity and efficiency for the considered temperature range. Relative buffer capacity as a function of pH is also critical for considering the relative merits of different solutions for acid–base control in the vicinity of physiological pH. Fig. 5 illustrates the change in buffer capacity as a function of pH for some of the solutions analyzed in this study and Table 4 lists the peak buffer value and its corresponding pH value for the complete list of solutions. These data serve to illustrate that solutions containing the ampholyte buffer Hepes show excellent buffering in the critical pH region of pH 7.4–7.8 and, in accordance with its design specification, Unisol offers superior buffering as discussed more fully below.

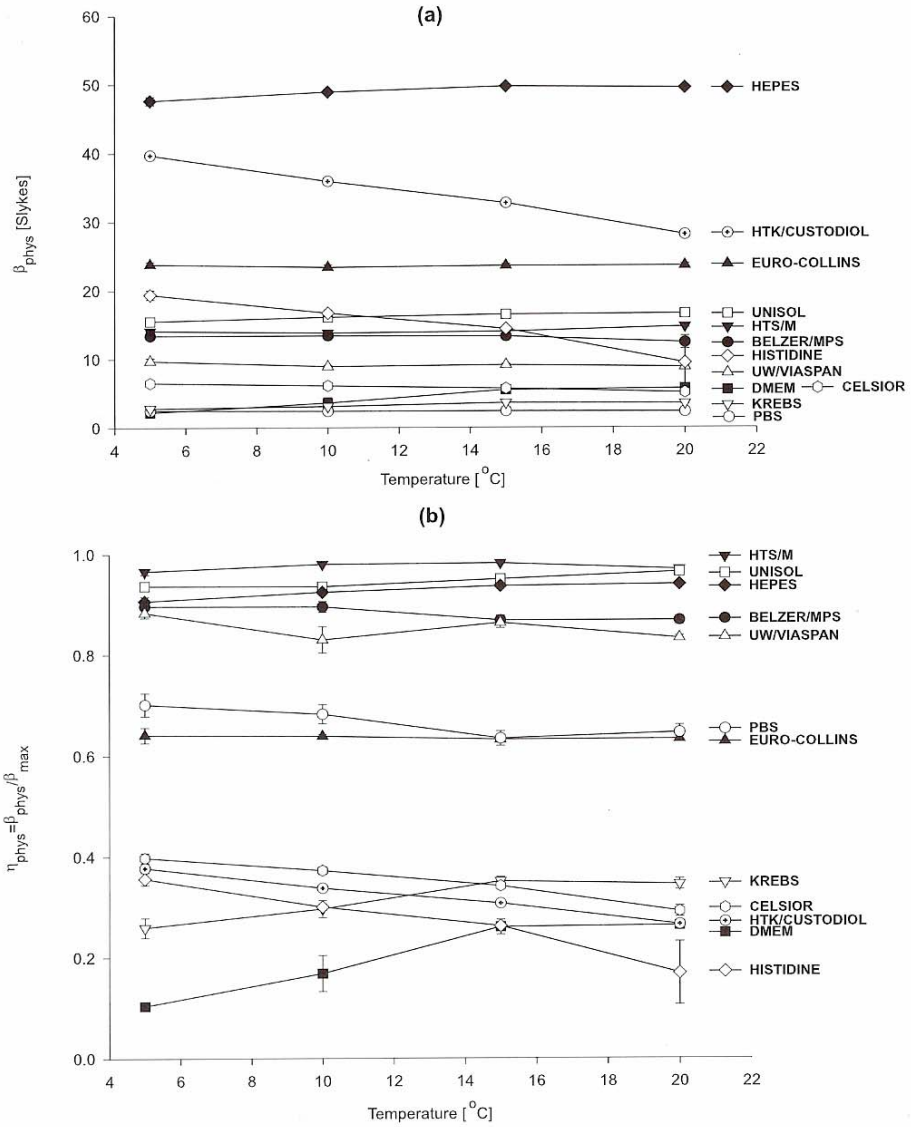

Fig. 4. Variation of solutions buffering capacity (a) and efficiency (b), with temperature in the physiological range of pH 7.0–7.8. Physiological buffer capacity, $\beta_{phys} = dV/(dpH)_{7\text{-}7.8}$. The preservation solutions showing the highest buffer efficiency (>0.9) in the physiological range ($\eta_{phys} = \beta_{phys}/\beta_{max}$) are those containing the aminosulfonic acid buffer Hepes. PBS and EuroCollins have intermediate efficiencies in the range of 0.6–0.7, while all other solutions had low efficiencies (<0.4).

Discussion

A great deal is known about the effects of cold on cells since cooling has proved to be the foundation of nearly all effective methods of protecting and preserving cells and tissues for applications such as transplantation. Transplantation science calls for effective methods of preservation since it is unavoidable that donor cells, tissues, and organs are required to withstand a period of *ischemia* and *hypoxia* as part of any transplantation procedure when the blood supply is temporarily interrupted.

Table 3
Maximum and physiological buffer capacity (slykes) and efficiency for the potential perfusion solutions

Solutions	20 °C				15 °C				10 °C				5 °C			
	β_{phys}	β_{max}	η_{conv}	η_{phys}	β_{phys}	β_{max}	η_{conv}	η_{phys}	β_{phys}	β_{max}	η_{conv}	η_{phys}	β_{phys}	β_{max}	η_{conv}	η_{phys}
Hepes	49.5	52.7	0.736	0.940	49.7	53.0	0.735	0.937	48.9	52.8	0.737	0.925	47. 6	52.5	0.741	0.907
Histidine	9.4	56.2	0.727	0.168	14.4	55.1	0.732	0.261	16.7	55.7	0.728	0.299	19.4	54.4	0.736	0.356
PBS	2.3	3.6	0.754	0.646	2.4	3.7	0.744	0.634	2.4	3.6	0.768	0.688	2.5	3.6	0.764	0.702
Krebs	3.5	10.2	0.731	0.345	3.6	10.4	0.720	0.351	3.1	10.6	0.690	0.296	2.8	10.9	0.663	0.259
DMEM	5.7	21.7	0.718	0.263	5.5	21.4	0.715	0.260	3.6	21.8	0.655	0.168	2.2	21.8	0.604	0.103
EuroCollins	23.6	37.2	0.745	0.633	23.6	37.4	0.745	0.632	23.4	36.5	0.753	0.639	23.8	37.2	0.744	0.641
UW/Viaspan	8.8	10.6	0.945	0.833	9.11	10.6	0.943	0.864	8.9	10.7	0.937	0.830	9.7	11.0	0.920	0.884
Belzer/MPS	12.4	14.3	0.849	0.869	13.0	15.2	0.849	0.869	13.4	14.9	0.871	0.896	13.4	14.9	0.853	0.897
HTK/Custodiol	28.1	106.1	0.743	0.264	32.7	106.7	0.745	0.306	35.9	106.4	0.743	0.337	39.7	106.3	0.742	0.373
Celsior	5.0	17.4	0.803	0.291	5.7	16.8	0.813	0.341	6.1	16.4	0.806	0.372	6.5	16.5	0.812	0.397
HTS/M	14.7	15.2	0.877	0.970	14.0	14.2	0.906	0.982	13.8	14.1	0.911	0.980	14.1	14.6	0.904	0.966
Unisol/UHK	16.6	17.2	0.835	0.965	16.5	17.4	0.820	0.950	16.1	17.2	0.812	0.936	15.5	16.5	0.846	0.937

The basis of this hypothermic protection is that cooling can help to combat the deleterious effects of ischemia, but the consequences of cooling are not exclusively beneficial such that hypothermic storage is a compromise between the benefits and detriments of cooling [40]. Perturbation of pH regulation during both ischemia and hypothermia is recognized to be a significant problem during organ preservation for transplantation. The role of preservation fluids in combatting acid–base changes is of great importance but comparative details of buffer capacities and efficiencies, as a function of temperature, are not available for all of the principal solutions used for organ preservation. Moreover, studies are often published describing undesirable pH changes during organ preservation with various solutions without clear explanations of the underlying mechanisms. For example, Ahmed et al. recently reported a marked difference between UW solution and phosphate-buffered sucrose solutions (PBSL) for controlling detrimental shifts in pH during hypothermic liver preservation. Their study showed that PBSL flush solutions were associated with alkalinization of the perfusion solution while UW was associated with acidification, but the authors concede that the reason for this phenomenon are unknown at this stage [1]. Such studies serve to illustrate the importance and need for detailed knowledge of the acid–base chemistry of the preservation fluids that our work seeks to provide.

Validity of methods and practical issues

The purpose of this study was to determine the practical acid dissociation constants and comparative buffer capacities of a range of new and commonly used organ preservation solutions. For this we chose to use conventional electrometric methods to carry out potentiometric acid–base titrations. Such measurements with a standardized glass/calomel electrode endow the measurements with as much thermodynamic significance as possible and are superior to indirect methods of measuring hydrogen ion activity such as magnetic resonance spectroscopy or pH indicators [38].

To generate complete titration curves showing the end points depicted in Fig. 1, it was necessary to carry out back-titrations in a conventional way by adjusting the initial pH of the respective solutions with standard alkali to commence titration from pH > 12. In some instances this resulted in a visible precipitation that resulted in "noise" on the titration curve. This was particularly prevalent

Table 4
Peak buffer values (slykes) and the corresponding pH values

Solution	Peak buffer values[a]	pH[b]
Hepes	52.4	7.62
Histidine	54.4/54.6	9.60/6.47
PBS	3.6	6.90
Krebs	11.6/10.4	10.08/6.56
DMEM	22.6/20.46	0.35/6.24
EuroCollins	40.3	6.64
UW/Viaspan	17.8	6.81
Belzer/MPS	16.4	6.91
HTK/Custodiol	107.0	6.41
Celsior	29.2/17.2	9.78/6.31
HTS/M	15.2	7.69
Unisol/UHK	20.0	7.70

[a] Derived from the incremental changes in the experimental titration curves.
[b] Value corresponding to the peak buffer value.

for the UW-Viaspan solution as seen in the typical curve depicted in Fig. 1. In all cases the precipitate re-dissolved spontaneously as the pH was lowered during titration. This phenomenon is consistent with one or more products of the solutions having a pH-sensitive solubility product.

Importance of pH control during cold ischemia

Under physiological conditions (37 °C) cells actively regulate pH within narrow limits with intracellular pH being maintained at a lower value (pH 7.0 ± 0.3) [33] than the extracellular fluid (pH 7.4). The structure/function relationships of biomacromolecules, especially proteins, are governed by their tertiary and quaternary structures which, in turn, rely upon the maintenance of charged moieties within the molecule. Perturbation of pH homeostasis in conjunction with the altered ionic environment during hypothermia can serve to markedly alter the structure/activity relationships of molecules such as enzymes that rely on electrochemical neutrality to maintain their net charged state, relative to the neutral point of water [28]. In aqueous solutions such as the intracellular fluid, the concentrations of protons (H^+) and hydroxyl ions (OH^-) are determined by the ionization constant of water (pK_w) which increases as temperature decreases (see Fig. 6). Intracellular electrochemical neutrality (equal concentrates of H^+ and OH^-) is therefore maintained at reduced temperatures only if pH rises in concert with pK_w to maintain a constant OH^-/H^+ ratio (i.e., the quantity of protons needed for neutrality falls as temperature decreases). It is important to understand that a given pH value is not a measure of electrochemical neutrality unless it is related to a specific temperature because neutrality is also dependent upon the concentration of hydroxyl ions. Hence, it is only at 37 °C that an extracellular pH of 7.4 yields a "neutral" intracellular pH that is optimal for physiological function. In other words, if pH does not rise as temperature is lowered there will be a relative excess of protons despite an apparently "normal" pH value. The disturbance of intracellular neutrality by the accumulation of H^+ during hypo-

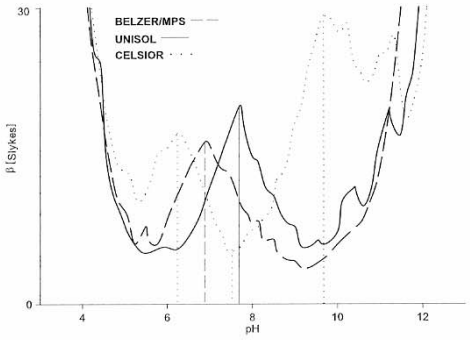

Fig. 5. Change of buffer capacity as a function of pH at 5 °C for three representative solutions (Belzer-MPS; Unisol (UHK), and Celsior). Celsior has two peaks corresponding with the two pK'_a values of its main buffer component, histidine. The trough representing the lowest point for buffer capacity on the Celsior curve is located at pH 7.5, which is also seen to correspond with the point of peak buffer capacity for Hepes-buffered Unisol. The data illustrate that solutions containing the ampholyte buffer Hepes show excellent buffering in the critical pH region of 7.4–7.8.

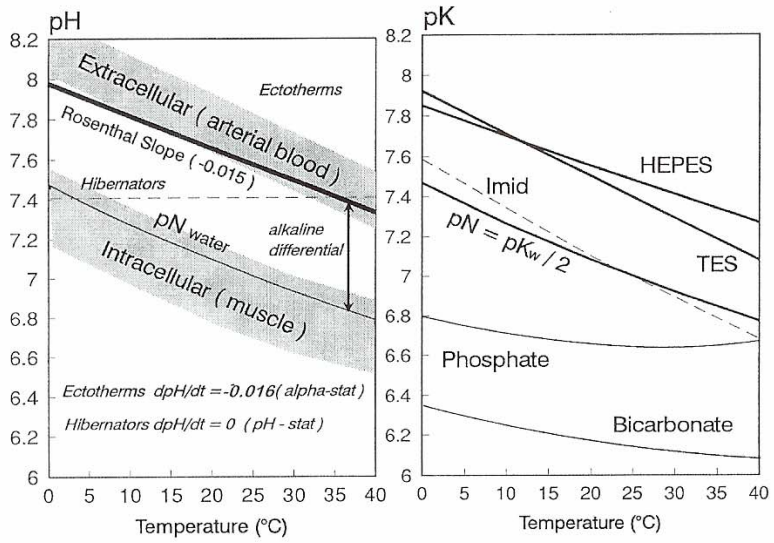

Fig. 6. The relationships between pH and pK as a function of temperature relative to acid–base control in biological systems during hypothermia. Shaded areas show the range of pH values reported for both the blood and intracellular fluid of ectotherms (cold-blooded animals) as a function of body temperature. The blood of warm-blooded mammals including human falls within this range when cooled without gas exchange. The pH change of blood during cooling parallels that of the neutral point of water (pN) and the temperature coefficient is given by the Rosenthal slope [27]. In contrast hibernators do not maintain a constant degree of alkalinity between the extracellular and intracellular compartments, but instead maintain a constant pH of 7.4 (pH-stat vs. α-stat regulation). In contrast to the physiological buffers, phosphate and bicarbonate, that do not retain their relative buffer capacity during cooling, the imidazole (Imid) group of histidine and synthetic buffers such as Hepes and TES are effective buffers over the entire temperature range due to the fact that their temperature coefficients (dpK/dt) parallel that of water (pK_w = dissociation constant of water) (adapted and extended from various sources including [24,25]).

thermic ischemia not only has a profound effect upon macromolecular structure and poisons the active sites of enzymes, but also causes metabolites to lose their charged state such that they are able to diffuse down concentration gradients as non-ionized lipophilic molecules. The depletion of important metabolites for the regeneration of high-energy phosphates during reperfusion is therefore exacerbated by relative acidity. The Donnan equilibrium responsible for maintenance of transcellular ion gradients and cellular water content is also dependent upon electrochemical neutrality within the cell.

At the systemic level, a failure to understand that it is the OH^-/H^+ ratio that is the critical determinant of protein structure and enzyme function, rather than the pH value per se, has been responsible for much misunderstanding and controversy about acid–base management during hypothermia [20,32].

Acid–base regulation during hypothermia

It has been established in ectothermic (cold blooded animals) and in the blood of warm blooded animals cooled in a closed system that does not permit gas exchange, that pH rises in parallel with the neutral point of water (pN) during cooling in the range of 0–40 °C (the Rosenthal relationship, [27]). Fig. 6 shows that the rate of change of pH with temperature is −0.015 pH units/°C and is referred to as α-stat pH regulation in recognition of the fact that both intracellular pH and blood pH buffering is dominated by the degree of ionization (α) of the imidazole moieties of proteins (see [24,32]).

On the basis of both in vitro and in vivo experiments, it has been generally accepted that acid–base regulations in nearly all vertebrates are consistent with primary regulation of α-imidazole, resulting in a stable OH^-/H^+ ratio and the

observed change in blood pH with temperature (Fig. 6). Fig. 6 also shows that intracellular pH is close to neutrality and closely parallels the rise in pN whereas, in ectotherms and mammalian blood in vivo, body fluid pH is maintained higher, i.e., more alkaline, than intracellular pH. The purpose of maintaining an alkalotic extracellular milieu may be to provide the cell with a proton-sink for the acidic products of its metabolism. Although this pH management strategy is the most prevalent in the animal kingdom [51], an alternative process has evolved in hibernating mammals; their metabolism and metabolic function continue at a body temperature as low as 5 °C and they maintain arterial pH at 7.4 irrespective of systemic temperature (pH-stat regulation).

A detailed discussion of these pH-regulatory phenomena is beyond the scope of this communication but the implications for which strategy should be adopted for optimum protection during clinical hypothermia, or hypothermic preservation of isolated organs for transplantation, has been addressed in numerous publications over the past few decades (for example see [17,19,32,34,51]). Although these considerations have sometimes led to a controversial debate there are persuasive arguments with supporting evidence in favor of the α-stat strategy. Examples from studies on exquisitely sensitive ischemic tissues such as heart and brain are of particular interest. It has been shown in numerous studies that the electrical stability, contractility, and hemodynamics of the heart is better preserved during hypothermia when the α-stat scheme is adopted, as opposed to constraining pH to 7.4 [32,34,50]. Concerns for brain protection during hypothermic cardiopulmonary bypass have led to studies of the effect of pH and temperature on cerebral metabolism and cerebral blood flow with the latter being appropriately maintained during α-stat management [18]. In a direct comparison of the α-stat versus pH-stat management of dogs subjected to 60 min of cold (17 °C) ischemic circulatory arrest, it was demonstrated that the α-stat strategy resulted in better protection of ischemic tissues than in those animals whose pH was maintained at 7.4 throughout. Improved protection was manifested as a better cardiac output, twice the cerebral blood flow, lower peripheral resistance and a significantly better post ischemic ventricular performance on rewarming [3].

Strategies of pH management for optimum hypothermic preservation of isolated organs have not yet been studied extensively but if α-stat regulation proves to be generally beneficial to maintain a constant degree of alkalinity between the extracellular and intracellular compartments to preserve electrochemical neutrality during cooling, the question of appropriate pH buffers needs to be carefully considered. Fig. 6 illustrates that due to the temperature coefficients of their dissociation constants (dpK/dt) the intrinsic physiological buffers phosphate and bicarbonate do not retain their relative buffering capacity during cooling. In sharp contrast, the imidazole group of the amino acid histidine retains effective buffering over the entire temperature range because its dpK/dt is closely aligned with that of water. Histidine-based solutions have been reported to help maintain liver adenine nucleotides during cold ischemia [6]. In synthetic systems such as man-made organ preservation media, or hypothermic blood substitutes, other buffers are available with equivalent or greater temperature coefficients to that of the pK_w for water. For example, the aminosulfonic acid buffers introduced by Good et al. [16] have been shown to possess superior buffer capacity and temperature coefficients for applications involving hypothermia [11,16,47]. For example, the zwitterionic aminosulfonic acid compound Hepes[1] has found widespread in vitro use as a biocompatible buffer and the close match of its dpK_a/dt with that of water and imidazole is illustrated in Fig. 6. Moreover, the aminosulfonic acid buffers may be a better choice than imidazole/histidine for preservation solutions since imidazole has been criticized as being too reactive and unstable to be a satisfactory biological buffer [15]. Moreover, as shown unequivocally in this study, the ampholyte Hepes provides superior buffering capacity and efficiency compared with histidine for pH regulation in the critical range of pH 7.0–7.8. It has recently been reported that other aminosulfonic acid buffers such as Bicine and Tricine provided a higher buffer capacity and greater protection than histidine for hypothermic storage of livers [8]. Compared with Hepes however, Bicine and Tricine provide a lower buffer capacity in the physiological range due to their higher pK's as we have previously reported [47].

Control of acidosis in the cells of preserved organs will be impacted by the exchange of protons and buffer species between the intracellular and extracellular compartments. With respect to external buffering power and intracellular pH, Garlick et al. addressed the question of whether

[1] *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

protons produced within ischemic cells are transported to the extracellular space. Such an export process would slow down as extracellular pH decreased. They hypothesized that if the external pH was maintained by increasing external buffering, the proton export could continue longer thereby reducing the fall in pH_i. They provided some support for this hypothesis using hearts perfused with Krebs–Henseleit supplemented with Hepes [11]. Clearly, intracellular pH (pH_i) can be influenced by external buffers if there is exchange of intracellular buffer species such as phosphate and/or protons. Moreover, there is the possibility that external buffers can permeate into the intracellular space and thereby directly act as pH_i buffers. As far as we know, there have been very few specific studies to examine the permeation of buffer species into cells. However, relatively small organic compounds such as histidine (155 Da) and the aminosulfonic acid buffers (≈160–230 Da; e.g. Bicine = 163 Da and Hepes = 238 Da) might be expected to permeate during prolonged hypothermic exposure. There is recent evidence that much larger molecules such as the disaccharide trehalose (342 Da) can permeate into cells as a result of hypothermia-induced phase changes in the plasma membrane [2].

Many of the reported studies purporting to examine changes in pH_i have relied upon NMR, but Lareau et al. have cautioned about the interpretation of intracellular pH changes based upon ^{32}P NMR spectroscopy because inorganic phosphate leaks from cells during prolonged hypothermic storage. For this reason it is not always possible to follow the time course of pH_i from the chemical shift of the Pi peak [19].

Comparative buffer capacities of organ preservation solutions

In light of the considerations outlined above, the present study was undertaken to evaluate the relative buffer capacities and efficiencies of some new and commonly used organ preservation media. While the acid dissociation constants (pK'_a) of many of the principal buffers used as components of organ storage media are documented, the specific acid–base buffering characteristics of the complex solutions, each containing different buffer compounds at different concentrations, has not been widely determined. Acid–base buffering is known to depend strongly upon buffer concentration and pH such that the relative efficacy of organ preservation solutions in this regard needs to be determined specifically in the pH range of critical interest for optimum cold storage. To this end, practical pK'_a values were determined for the individual solutions and titration curves were used to generate and compare buffer capacities and efficiencies within the pH range and at the temperatures of greatest interest for hypothermic organ preservation. The data highlight some important points that warrant particular discussion and emphasis.

The mean practical acid dissociation constants for all of the solutions analyzed in this study are available for comparison in Table 2 and their respective maximum and physiological buffer capacities are compared in Table 3. It was observed that for each solution the β_{max} value calculated in this analysis was in good agreement with the theoretical β^t_{max} listed in Table 2. Since β^t_{max} [42] was calculated using the total concentration of the principal buffer components as identified in Table 1, this verifies that the pH buffer characteristics of these multi-component preservation solutions is dictated by these principal buffer components.

Of the solutions evaluated in this study, HTK (Custodiol) has the highest buffer capacity due to the very high concentration of histidine (198 mM) in this solution. However, the buffering efficiency, η_{phys}, is relatively low (0.37) due to the fact that the pK'_a for this solution is 6.35 at 5 °C. Celsior, which contains 30 mM histidine as its only buffer component, has a relatively low buffer capacity ($\beta_{phys} = 6.5$ at 5 °C) and low efficiency ($\eta_{phys} = 0.4$). The buffering profile for Celsior depicted in Fig. 5 shows the two peaks corresponding with the two pK'_as for histidine. Moreover, the trough representing the lowest point for buffer capacity on the Celsior curve is located at pH 7.5, which is also seen to correspond with the point of peak buffer capacity for Unisol. These observations serve to emphasize that optimal buffering relies upon both the concentrations of the principal buffer components and their pK'_a values. For example, data for the pure reference buffers, Histidine and Hepes at the same concentration (100 mM), show that while their maximum buffer capacities are equivalent ($\beta_{max} = 54$ and 52.5, respectively), β_{phys} is markedly higher for Hepes (47.6) compared with 19.4 for histidine at 5 °C. Moreover, the buffering efficiency of Hepes, $\eta_{phys} = 0.9$ compared with $\eta_{phys} = 0.36$ for histidine. This difference is reflected in the effective buffering indices of preservation media containing Hepes as the principal buffer compared with those containing histidine. For example, Belzer's MPS, Hypothermosol (HTS/M), and Unisol have β_{phys} values of 13.4,

14.1, and 15.5, respectively at 5 °C, all with a buffer efficiency of 0.9 or greater. By comparison, with the exception of EuroCollins solution, all of the other buffer solutions had β_{phys} values of <10 slykes. It is noteworthy that physiological buffer media such as DMEM, Krebs' solution, and PBS have β_{phys} values <3 slykes at 5 °C and relatively low buffer efficiencies emphasizing that the composition of these solutions is markedly sub-optimal for applications at low temperatures. Moreover, the temperature coefficients for these physiological buffers are very low and do not match the Rosenthal slope of −0.015 units/°C deemed optimal for α-stat pH regulation as discussed above. In this context EuroCollins solution also has a temperature coefficient (−0.0013 pK units/°C) that is sub-optimal for α-stat regulation due to the fact that the principal buffer is phosphate. Parenthetically, the relatively high buffer capacity of EuroCollins of 23.8 slykes at 5 °C is due principally to the high concentration of phosphate (57.5 mM), but the buffer efficiency index is relatively low ($\eta_{phys} = 0.64$). In an earlier study comparing the relative merits of EuroCollins vs. Marshall's Hypertonic Citrate solution, Fuller et al. showed that EC had superior buffer capacity. This reflected the relative pK'_as of 7.2 for phosphate and 6.4 for citrate, respectively [13].

The other organ preservation solution that relies upon phosphate as its only effective buffer is UW (Viaspan), which is generally regarded as the "gold" standard for preservation of abdominal organs. Again the inferior role of phosphate as a suitable buffer under hypothermic conditions is reflected in the buffer parameters determined for UW solution. For example, the $pK'_a = 6.71$ at 5 °C with a corresponding buffer capacity, $\beta_{phys} < 10$ slykes at any temperature. These deficiencies have been rectified in the Belzer modification of UW for machine perfusion (Belzer's MPS) by addition of 10 mM Hepes. This raised the pK'_a to 7.0 at 5 °C, the β_{phys} buffer capacity to 13.4 and the buffer efficiency, η_{phys}, to 0.9.

It is obvious from these considerations that the physiological buffer capacity of hypothermic preservation solutions can be markedly elevated by increasing the concentration of the principal buffer. We have previously developed tissue preservation media containing 100 mM aminosulfonic acids such as Hepes and TES[2] yielding solutions with very high buffer capacities ($\beta_{phys} > 50$ slykes and $\eta_{phys} > 0.9$) [39,43,46]. An example is the CPTES solution developed principally as a corneal preservation solution containing 100 mM TES that has proved effective in a variety of low temperature applications [21,29,30,44–46]. The efficacy of improved buffering capacity using other aminosulfonic acid buffers such as Bicine and Tricine as additives to UW organ preservation solution has recently been demonstrated to improve the metabolic status of hypothermically stored livers. In Churchill and Kneteman's [8] study Bicine and Tricine provided a higher buffer capacity and greater protection than Histidine. Hepes has been documented to be highly effective in combatting the alterations in acid–base homeostasis of ischemic hearts [14,36]. We have demonstrated the effectiveness of a variety of these sulfonic acid buffers in cryobiological applications [39,43,46,47] and consideration of their use in vivo has recently been advocated [35,41].

It is concluded therefore that zwitterionic sulpfonic acid buffers, with pK'_a values in the region of 7.5 (of which Hepes is the most widely tested) offer the most ideal buffering contribution to hypothermic preservation solutions. This is due to their high buffer capacity, efficiency, and appropriate temperature coefficient for control of pH in the physiologically important range at reduced temperatures. These conclusions concur with the recommendation by Swan [35] that these synthetic buffers warrant consideration and further evaluation for low temperature applications in clinical medicine. This includes hypothermic clinical applications in addition to ex vivo organ preservation for transplantation.

[2] *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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