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FORMULATION AND SOME BIOLOGICAL USES OF A BUFFER MIXTURE WHOSE BUFFERING CAPACITY IS RELATIVELY INDEPENDENT OF pH IN THE RANGE pH 4–9

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A mixture is described which has a buffering capacity which is essentially independent of pH in the range pH 4.0–9.0. It is shown how this buffer mixture may be used to determine the force–flux relationship of proton transfer between two aqueous phases separated by a phospholipid bilayer in vesicular systems and so demonstrate that this relationship is linear over a wide range of $\Delta\tilde{\mu}_{H^+}$. The buffer mixture can, furthermore, be employed to determine the volume enclosed within a vesicular preparation.

Key words: proton transfer; pH-independent buffering capacity; internal volume; force–flux relationship.

INTRODUCTION

The chemiosmotic theory of biological energy transduction (e.g. [1–6]) has aroused great interest in the role and nature of proton gradients in biochemical energy coupling. There are consequently several experimental situations in which it would be desirable to have available a buffer whose proton-buffering capacity is essentially independent of pH over a wide range of pH values. Yet inspection of the literature reveals that whilst buffer mixtures have been described, such as those of Britton and Robinson, McIlvaine or Sørensen [7], which exhibit strong buffering power over various pH ranges, none has thus far been reported whose buffering capacity is essentially independent of pH over a wide span of pH values of physiological interest. It is the purpose of this communication to describe the formulation and properties of such a buffer mixture and to illustrate its utility in the study of proton transfer processes in multi-compartment systems. It is further shown that the buffer may be exploited to determine the volume enclosed by a vesicular preparation.

MATERIALS AND METHODS

Determination of pH

All pH measurements were made using an Orion Model 901 Microprocessor Ionalyser (MSE Scientific Instruments, Crawley, Sussex, U.K.) with a Russell pH electrode (impedance less than 20 MΩ) and an Orion double-junction reference electrode, in a reaction vessel thermostatted at 25°C. This pH meter was accurate to ±0.001 pH units. The output from the meter was directed both in analogue form to a potentiometric chart recorder (Servoscribe Model 1s, Smiths Industries, Wembley, Middlesex, U.K.) and in binary-coded decimal form, twice per second, to a SWTPC MP68 Microcomputer. The microcomputer, its interface to the Ionalyser and a version of an interpreted BASIC called BASICION were supplied by M. James, Research Resources Ltd, 40 Stonehills, Welwyn Garden City, Hertfordshire, U.K., from whom further details may be obtained. All programs were written by the authors in BASICION. Output from the Ionalyser was stored on a mini-floppy disc for subsequent retrieval and data analysis.

Preparation of phospholipid vesicles

Soybean lecithin (480 mg) and sodium cholate (20 mg) in 10 ml of a tenfold dilution of stock KM3 buffer (Table 1) were sonicated for 5 min at room temperature using an MSE Sonicator operating at high power (150 W) and an amplitude, peak-to-peak, of 5 μ. The final temperature was within the range 30—40°C. The dispersed phospholipid/cholate mixture was dialysed for a total of 30 h at 37°C against 3 × 500-ml vols. of the tenfold-diluted KM3 buffer fortified with 2.5 mM $MgCl_2$. The resultant vesicular preparation was used without further purification.

TABLE 1

Composition of the stock solution of Buffer KM3

The stock solution (pH ≈ 3.5) was adjusted to the desired pH with HCl or KOH.

Substance	Molarity	g/l stock solution
Malonic acid	40 mM	4.164
DL-Malic acid	75 mM	10.097
Dipotassium oxalate	80 mM	13.296
Tripotassium citrate	25 mM	7.66
Maleic acid	75 mM	8.768
Disodium-β-glycerophosphate	25 mM	7.65
Dipotassium hydrogen phosphate	100 mM	17.43
N-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonate	40 mM	9.53
Triethanolamine hydrochloride	25 mM	4.64
Tris(hydroxymethyl)methylglycine	75 mM	13.94
Glycylglycine	100 mM	13.21
2-Amino,2-methyl-propanediol	25 mM	2.628
Sodium metaborate	80 mM	11.029
2-Amino,2-methyl-propanol	75 mM	9.42

Chemicals

All chemicals were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. or B.D.H. Chemicals, Poole, Dorset, U.K. and were of the highest quality available. Water was double-distilled in an all-glass apparatus.

RESULTS

Formulation of the buffer, and pH-dependence of its buffering capacity

Though the test ingredients of the buffer mixture were first selected on theoretical grounds, ionic strength effects rendered it impossible to optimise the mixture, with respect to the pH-independence of its buffering powers, by recourse solely to calculations based on concentration and pK_a values. Further improvement was achieved by semi-empirical adjustments of the concentrations of the components, leading eventually to the mixture, designated KM3, described in Table 1. This stock solution was hereafter diluted tenfold for all experiments. The buffering capacity of the tenfold-diluted KM3 mixture over the range of pH values of greatest interest to biochemists is shown in Fig. 1. Though the buffering capacity is not entirely independent of pH over the range pH 4—9, the maximum variation about the median buffering capacity is only 12%.

Determination of force—flux relations for H^+ transfer across liposomes

Mitchell and Moyle [8] demonstrated that, in contrast to what one might perhaps have expected, topologically closed biological membrane systems are

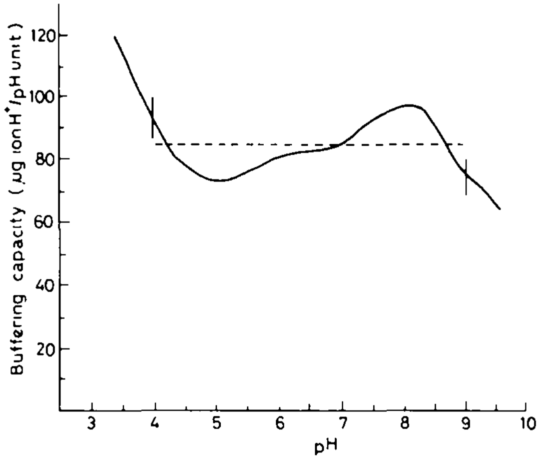

Fig. 1. Effect of pH on buffering capacity of Buffer KM3. A tenfold-diluted sample of Buffer KM3 (Table 1) was adjusted to pH 9.8 with KOH. Microlitre volumes of 10 M HCl were added to a 6 ml volume of sample such that the pH excursion was approximately 0.06 pH units, and the buffering capacity was calculated from the relation $B = -\delta H^+/\delta pH$.

rather impermeable to protons. Thus, when a weakly buffered suspension of mitochondria was challenged with a pulse of acid sufficient to change the pH measured with a macroscopic glass electrode by approximately 0.05—0.1 pH units, the response of the pH electrode could be subdivided into two parts: a rapid acidification of the medium occurring faster than the response time of the pH electrode, ($t_{1/2}$ = ca. 1 s), followed by a slower ($t_{1/2}$ = 70—90 s) and smaller alkalinisation. These phenomena were correctly ascribed to the titration of two kinetically distinct compartments separated by a relatively proton-impermeable osmotic barrier (the M phase). It was initially assumed, and subsequently demonstrated [8], that the rate of proton transfer across the M phase was linearly related to the concentration gradient of protons $\vec{\Delta}$pH across the barrier M phase for $\vec{\Delta}$pH values less than 0.1 pH units. The buffering capacities of the outer (rapidly-titrating) and inner (more slowly titrating) aqueous phases are given [8] by the equations

$$B_O = -\Delta H^+/\Delta pH_0^\alpha \qquad (1)$$

$$B_T = -\Delta H^+/\Delta pH_0^\omega \qquad (2)$$

$$B_I = B_T - B_O \qquad (3)$$

where ΔH^+ is the quantity of H^+ added to the system, B_O, B_T and B_I refer to the buffering capacities of the outer, total (inner plus outer) and inner phases, respectively, and the meaning of ΔpH_0^α and ΔpH_0^ω is given in Fig. 2. In view of the present interest in the force—flux relationships of protonmotive systems (e.g. [9—13]), we decided to determine the relationship between the rate of H^+ transfer and pH gradient across the M phase of such systems when $\vec{\Delta}$pH is much greater than 0.1 The method relies upon the ability to calculate the internal pH of such vesicular systems using the knowledge that the buffering capacity of the inner phase is essentially independent of pH. The terminology used for describing this type of experiment is given in Fig. 2, where changes in a given phase are prescribed by the operator Δ, whilst differences between phases are denoted by $\vec{\Delta}$ (cf. [8]). If the starting

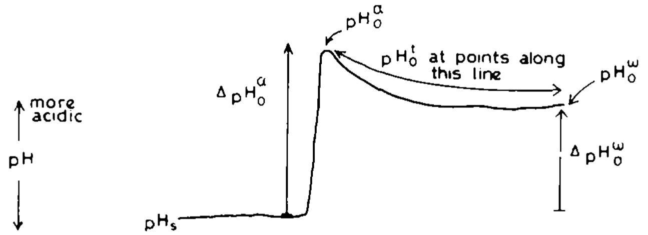

Fig. 2. Terminology used in describing an acid-pulse experiment. The figure shows the different phases of an acid-pulse experiment as described in the text, and diagrammatically, the direction of the pH changes encountered in such an experiment.

pH is pH_S and the initial and final pH excursions following the acid pulse are ΔpH_O^α and ΔpH_O^ω (Fig. 2), then knowing the quantity of H^+ added, we may calculate the internal and external buffering capacities from Eqns. 1—3. If these quantities are known to be independent of pH, we may then calculate the internal pH over the time of the decay of the acid pulse across the M phase. The boundary conditions are that at $t = 0$, $pH_I = pH_S$ and $pH_O = pH_O^\alpha$, and that at $t = \infty$, $pH_I = pH_O$. Thus, at any point on the decay curve we may obtain the number of protons that have moved across the membrane from the relationship

$$\delta H^+ = (pH_O^\alpha - pH_O^t)/B_O \tag{4}$$

Since these protons must appear inside the vesicles we may thus obtain the intravesicular pH from the equation

$$pH_I = pH_S + (pH_O^t - pH_O^\alpha) \times (B_I/B_O) \tag{5}$$

$\vec{\Delta}pH$ at any point on the curve is obviously given by the relationship $\vec{\Delta}pH = pH_I - pH_O$ whilst the rate of H^+ transfer may be obtained from the slope of a plot of $-\ln(pH_O^\omega - pH_O^t)$ versus time. Thus we may determine the pH gradient and force—flux relationship at any point on the decay curve using measurements of the external pH alone.

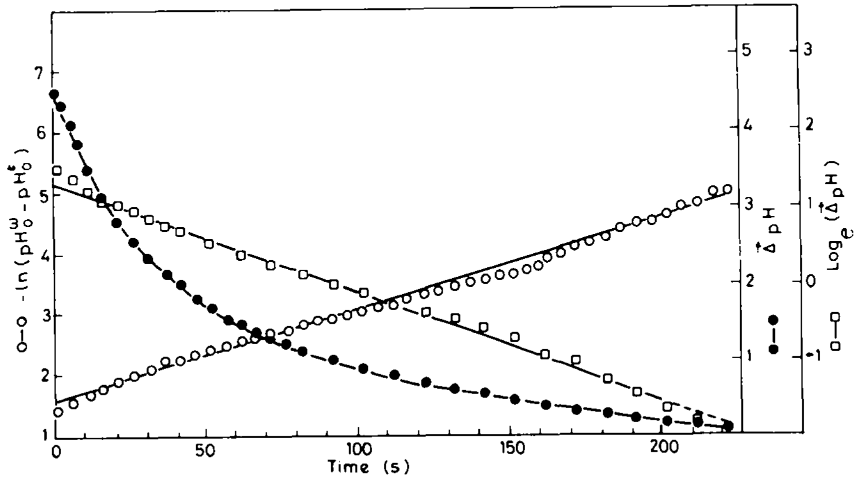

Fig. 3. Secondary plot of various parameters of an acid-pulse experiment performed using the KM3 Buffer. The reaction mixture contained 6 ml of a suspension of phospholipid vesicles (20 mg phospholipid/ml) and 128 μg carbonic anhydrase in tenfold-diluted KM3 Buffer (pH = 8.712) prepared as described in Materials and Methods. At $t = -2$ s, 42 μl of 10 M HCl were added to bring the pH to 4.16, and the subsequent decay of pH_O was followed as described in Materials and Methods. Data storage and the calculation of $\vec{\Delta}pH$ was performed, as described in the text, by the microcomputer.

Fig. 3 shows the results of a typical acid pulse experiment of the type described above, in which $\Delta \mathrm{pH}_0^{\alpha}$ (and thus, of course, $\vec{\Delta}\mathrm{pH}$) was made to exceed 4 units. From many experiments of this type, the following general conclusions could be drawn (cf. Fig. 3):

(a) For values of $\vec{\Delta}\mathrm{pH}$ between 0.1 and 4.5 the rate of decay of H^+ across the M phase of the phospholipid vesicles is accurately described by a single exponential equation; in other words, the relationship between $\Delta\tilde{\mu}_{H^+}$ and the rate of H^+ translocation was linear even at values of the transmembrane electrochemical proton gradient far removed (>250 mV) from equilibrium.

(b) Under conditions of a pH-independent buffering capacity of the two phases, $\vec{\Delta}\mathrm{pH}$ also decayed in an exponential fashion.

(c) The lack of dependence on pH of the rate of passage of H^+ across the vesicular M phase indicated that no significant transmembrane H^+ transfer was effected by the penetration across the membrane of any of the buffer components in its uncharged form.

(d) Under the present conditions the osmotic volume enclosed by the vesicle preparation may be accurately obtained from the relationship

$$\text{Internal volume fraction (in ml per ml reaction mixture)} = \frac{\text{Inner volume}}{\text{Total volume}}$$

$$= \frac{\Delta \mathrm{pH}_0^{\alpha} - \Delta \mathrm{pH}_0^{\omega}}{\Delta \mathrm{pH}_0^{\alpha}} \qquad (6)$$

Under conditions in which the buffering capacity of the system per se, relative to that of the added buffer, is not insignificant, the enclosed volume fraction is equal to the ratio of the slopes of plots of the measured B_{I} and B_{T} versus the added buffer concentration.

DISCUSSION

In a number of recent studies (e.g. [14,15]) of the protonic coupling mechanism of electron transport phosphorylation, it has been assumed that the force—flux relationship between $\Delta\tilde{\mu}_{H^+}$ and the rate of transmembrane H^+ transfer is a linear one even at rather high values of $\Delta\tilde{\mu}_{H^+}$. The present analysis shows that this assumption may be regarded as correct, provided that the coupling protons are osmotically active, a view which is not universally held [5,9—11,13,16]. The present system should be of value in testing the view that the proton channel of the F_0F_1-ATPase has rather special current—voltage properties as regards its protonophoric activity (e.g. [17—19]). Finally, we would mention that the present buffer system has proved useful as a supporting electrolyte in polarographic studies [20]; it may also find utility in isoelectric focussing systems of the type described by Prestidge and Hearn [21].

SIMPLIFIED DESCRIPTION OF THE METHOD AND ITS APPLICATIONS

The multicomponent buffer mixture whose formulation is described in this paper demonstrates a buffering capacity which is relatively independent of pH in the range pH 4—9. It has been employed to demonstrate that the force—flux relationship of proton transfer between two aqueous phases separated by a phospholipid bilayer in vesicular systems is linear over a wide range of $\Delta\tilde{\mu}_{H^+}$. It has further been shown how this buffer mixture can be exploited to determine the volume enclosed within such a vesicular preparation. The buffer mixture has proved useful as a supporting electrolyte in polarographic studies and is also likely to prove most valuable in isoelectric focussing procedures and a variety of biochemical assays.

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Following earlier work (Hellingwerf, K.J. (1979) Thesis, University of Amsterdam), Arents, van Dekken, Hellingwerf and Westerhoff (Westerhoff, H.V., personal communication) have also employed a buffer mixture of the present type, which possesses a buffering capacity independent of pH in the range pH 4—6, to demonstrate that electroneutral proton flux across bacteriorhodopsin-containing liposomes depends linearly upon the pH gradient, in line with a previous assumption (Westerhoff, H.V., Scholte, B.J. and Hellingwerf, K.J. (1979) Biochim. Biophys. Acta 547, 544—560).