Biochimica et Biophysica Acta, 1143 (1993) 29–37

BBABIO 43821

Effect of buffers and osmolality on anion uniport across the mitochondrial inner membrane

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(Received 8 July 1992)
(Revised manuscript received 27 October 1992)

Key words: Mitochondrion; Mitochondrial inner membrane; Anion channel; Zwitterionic buffer; Matrix volume

The effects of buffers and osmolality of the suspending medium on the pH-dependent anion uniport across the inner membrane of isolated rat liver mitochondria have been studied using the light scattering technique to measure passive osmotic swelling. In contrast to some other transport processes the rates of entry of chloride and other anions via the anion-conducting channel decreased steeply with increasing solute concentration. This effect appears to be a result of increased osmolality or decreased matrix volume rather than inhibition by the anion since it was also produced by increasing the osmolality by addition of non-penetrant solutes. The effects of some pH buffers on the mitochondrial anion-conducting channel were also investigated. Some zwitterionic buffers had little effect other than that produced by increasing osmolality but Tricine, Popso and Caps produced marked additional inhibition of anion uniport and several other zwitterionic buffers were also inhibitory. The correlation between increased anion conductivity and increased matrix volume supports the proposal that this channel functions in regulation of the volume of the mitochondrial matrix.

Introduction

There is now a substantial body of evidence for the existence of channels in the mitochondrial inner membrane not only from investigations using the technique of passive osmotic swelling [1–3] but also by direct observation of ion conduction using the technique of patch clamping micro-electrodes [4–8]. Nevertheless, the physiological functions and regulation of these channels remain unknown.

In the light of reports [9–14] that some of the commonly used zwitterionic buffers have inhibitory or blocking actions on ion channels, including a thorough study of the effects of several different buffers on the epithelial chloride channel by Hanrahan and Tabcharani [15], we commenced investigation of the effects of these buffers on the pH dependent anion-conducting channel in the mitochondrial inner membrane. However investigation of the inhibitory effects of buffers has the intrinsic and unavoidable complication that varying the concentration of the buffer in the required range, from 0 to > 20 mM, produces significant changes in the composition, ionic strength and overall osmotic pressure or osmolality of the medium. Compensation by varying the concentration of another component, either the penetrant solute or an additional non-penetrant component, the so-called spectator ion or solute does not eliminate this problem. Varying the penetrant concentration may itself alter the rate and adding a spectator solute introduces yet more uncertainties since it also may have concentration-dependent effects on channel or carrier activity. For example, Knauf and Mann observed inhibition of Cl^- exchange across the human erythrocyte membrane by high chloride concentration [16] and proposed an inhibitory chloride site.

As well as inhibitory effects of buffers or permeant ions, channels can be sensitive to osmolality, membrane stretch or cell volume. For example; stretch-activated channels, in rat dorsal root ganglion [17], in chicken skeletal muscle [18], in snail heart [19] and the pressure-sensitive channel reported by Martinac et al. [20] in spheroplasts of *Escherichia coli*.

Brierley et al. [21] found that increasing the concentration of external solutes decreased the ionic perme-

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Abbreviations: FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone. See Table I for abbreviations for zwitterionic buffers.

ability of beef heart mitochondria and Azzone's group [22,23] reported inhibitory effects of increased external solute concentration on ion transport in liver mitochondria. In contrast, Garlid and Beavis [24] found that the permeability coefficient for a non-ionic solutes such as erythritol or malonamide remained constant over a wide range of osmolalities indicating that neither the surface area of the inner membrane nor the transporter characteristics altered in response to osmotic pressure or matrix volume. However the same authors have suggested [2] that the anion-conducting channel in the mitochondrial inner membrane functions in the control of the volume of the mitochondrial matrix.

In this paper we present an empirical study of the effect of osmolality and a wide range of zwitterionic buffers on the anionic conducting channel of the mitochondrial inner membrane in order to provide information for investigation of this channel and other volume-dependent properties of mitochondria.

We report not only that certain buffers have a significant inhibitory action on anion uniport via the pH-dependent anion channel in the mitochondrial inner membrane but also that this channel is sensitive to medium osmolality.

Experimental procedures

Materials

Rotenone, antimycin A, Hepes, Tris, Tricine and the other zwitterionic pH buffers used in this investigation were obtained from Sigma, FCCP and ammonium isethionate from Aldrich, ammonium chloride, ammonium thiocyanate, ammonium nitrate from Merck, sodium acetate from Baker and ethanesulfonic acid from Fluka. All chemicals used in these experiments were obtained as highest purity grade available. Ammonium ethanesulfonate was prepared by neutralising ethanesulfonic acid with ammonium hydroxide.

pK_a values for buffers at 30°C were calculated from published data [25,26].

Preparation of mitochondria

Liver mitochondria from adult Wistar rats were isolated by differential centrifugation as previously described [27]. The mitochondria were finally re-suspended to 50 mg of protein/ml in 0.25 M sucrose containing 5 mM Hepes with pH adjusted to 7.5. The mitochondrial suspension was stored on ice with continuous stirring in the air to maintain high and constant anionic conductivity [28,29]. The mitochondria used in these experiments had a respiratory control ratio of not less than 4 and an ADP/O ratio of 1.5–2.0 (with succinate as the substrate) measured using a Clark-type oxygen electrode.

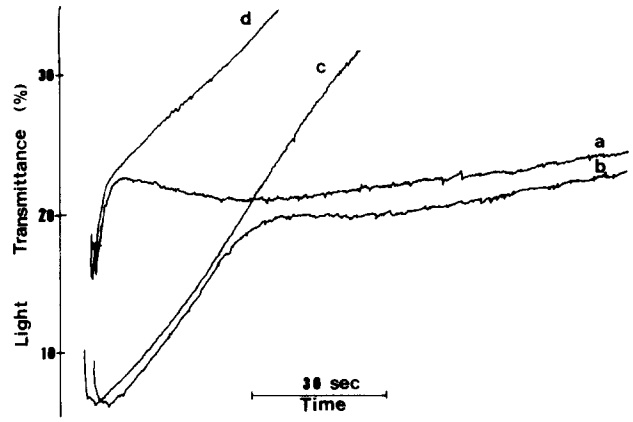

Fig. 1. Recordings of light scattering showing mitochondrial swelling in media containing NH_4-Hepes, NH_4Cl and $NH_4Cl + NH_4$-Hepes. Swelling was started by injecting the mitochondrial suspension, 1.5 mg protein, into the following media: (a) 20 mM NH_4-Hepes; (b) 20 mM NH_4-Hepes in 100 mM NH_4Cl; (c) 117 mM NH_4Cl; (d) 17 mM NH_4Cl. Media (a) and (d) had the same osmolality, 36 mmol/kg and media (b) and (c) were 243 mmol/kg. Rotenone, antimycin A and FCCP were added to each medium prior to the mitochondria at concentrations given in the experimental section, except that FCCP was not added in experiment (a).

Assay

Mitochondrial anion uniport was assayed using the technique of passive osmotic swelling by recording the light scattering changes of the mitochondrial suspensions which were stirred continuously in a cylindrical glass cuvette thermostatted at 30°C. A loose fitting plunger touching the surface of the liquid prevented vortex formation and allowed rapid stirring; under these conditions mixing time was less than 0.2 s. For each assay, 1.5 mg of mitochondrial protein suspension was added to a 4.0 ml of buffered or buffer-free medium (of known osmolality) containing 0.6 μM rotenone and 0.5 μM antimycin A to prevent endogenous respiration plus 7.5 μM FCCP to induce permeability to H^+. In control experiments in NH_4Cl and the other permeant anion media in the absence of FCCP the rate of light scattering change was zero or extremely slow (after the initial rapid adjustment to medium osmolality) showing that this technique measures swelling produced by anion uniport.

Adjustment of the mitochondria to changed osmolality is very rapid, see Fig. 1, and rates of swelling due to solute entry were estimated from the tangent to the initial region of the curve following this rapid adjustment to medium osmolality.

All solutions were adjusted to pH 8.0 with ammonium hydroxide, HCl or the selected buffer, as appropriate. Although the assay is at pH 8.0 which is outside the normal buffering range of NH_4^+, pK_a 9.15, the high concentration of NH_4^+ (around 100 mM) produces effective buffering in the absence of added buffers.

The osmolalities of the assay media were determined using a Wescor 5500 Vapor Pressure Osmometer.

Presentation of light scattering data

Mitochondria behave as near perfect osmometers in response to external osmolality changes and undergo large-amplitude swelling when there is net entry of solute into the matrix space since this is accompanied by influx of water [30]. The technique of recording mitochondrial light scattering to estimate changes in matrix volume has been used in many studies on solute entry [1,2,21,31,32] and the relationship between matrix volume and reciprocal absorbance has been put on a sound basis by Tedeschi and Harris [33] and Garlid's group [24,34]. As previously described, over a limited but useful range, % transmittance is a good approximation to reciprocal absorbance [35]. Since this technique measures entry of water rather than entry of solute, conversion to rates of solute entry requires correction for the inverse relationship between water entry per mol of solute and osmolality of the medium. In this paper the term 'relative rate' refers to the rate of solute entry and is given by the following expression.

$$\text{Relative rate } \% = \frac{\Delta\%T_{\text{Exp}} \times \text{Osmolality}_{\text{Exp}} \times 100}{\Delta\%T_{\text{Std}} \times \text{Osmolality}_{\text{Std}}} \quad (1)$$

Where $\Delta\%T$ is the rate of change of % transmittance per min. Subscript Exp refers the parameters to a particular set of conditions and Std to the standard conditions. Unless otherwise specified the standard conditions are 100 mM NH_4Cl at pH 8.0 with no buffer present, osmolality 207 mM.

There are, however, perturbations in the relationship between osmolality and light scattering which are correlated with irreversible structural changes at large matrix volumes [31,32,36]. This difficulty can be circumvented by using pre-swollen mitochondria or low osmolality media but these methods, which irreversibly alter the mitochondrial structure, preclude investigation of the effects of high osmolality and therefore low matrix volume on mitochondria in near-natural condition. In an alternative approach Garlid and Beavis [24] applied differing correction factors, obtained from equilibrium data on the relationship between light scattering and osmolality, to different regions of the swelling time-course curves in order to correct for the perturbations produced by the structural changes. However, we found that the perturbation in the response to low osmolality was time dependent (see results section) and in the light of this and the empirical nature of light scattering estimations we have presented the relative rates as defined above without further mathematical manipulation.

Results

In view of the impossibility of independent variation of osmolality and solute concentrations we have investigated the effects of: (i) varying the penetrant concentration at zero or low (constant) buffer concentration, (ii) adding spectator solutes such as sucrose, (iii) varying the buffer concentration at fixed penetrant concentration, (iv) varying the buffer concentration at different osmolalities of the permeant solute.

We first report the kinetics of light scattering changes produced by passive osmotic swelling of mitochondria in media containing both impermeable and permeable solutes.

Fig. 1 shows recordings of the light scattering changes of mitochondrial swelling in media which contained either NH_4Cl or NH_4-Hepes or both. Mitochondria were added to a solution of 20 mM Hepes with no FCCP added, recording (a), initially the light scattering decreased very rapidly as the mitochondria swell in the low osmolality medium, then the light scattering increased as the mitochondria apparently adjust to an overshoot, following that the light scattering decreased at a very slow steady rate indicating a slow rate of Hepes entry. Identical effects were observed in the presence of 7.5 μM FCCP (result not shown). When 20 mM Hepes was incorporated into NH_4Cl medium containing FCCP, recording (b), the initial rate was similar to the rate in buffer-free NH_4Cl medium, recording (c). However, the swelling stopped almost completely when the mitochondria attained osmotic equilibrium with the Hepes in the medium, compare with recording (a), whereas in the buffer-free NH_4Cl assay mitochondria swell continuously without ever achieving osmotic equilibrium. The rate of swelling was rapid when the mitochondrial suspension was added to the hypotonic buffer-free NH_4Cl medium (17 mM NH_4Cl has the same osmolality as 20 mM NH_4-Hepes), recording (d): there was firstly a phase of very rapid adjustment to the osmolality of the medium, secondly a phase of fast swelling and thirdly a faster steady rate. During the second and third phases NH_3, H^+ and Cl^- enter the matrix, the less rapid rate in the second phase corresponds with the increase in light scattering seen in the response to overshoot in 20 mM NH_4-Hepes, recording (a).

These recordings show some important features. Firstly, the almost vertical downward start of the curve is the increase in light-scattering as the mitochondria are injected and mix in the media and is followed by changes in mitochondrial light-scattering. The steepness of both downward and initial upward parts of the curves, together with the rapid reversal of direction at this point, see records (a) and (b) shows that mixing is very fast, complete within about 0.2 s. Furthermore, the rate of water entry in hypotonic media is very fast with a $t_{1/2}$ of less than 1 s.

Secondly, the extent of the light scattering change is markedly limited by the osmotic balance provided by the impermeant solute, Hepes, at 20 mM. This limita-

tion of the extent of swelling may lead to an underestimate of the rate of swelling in media of higher non-permeant osmolality.

Thirdly, in the response to low osmolality in either NH_4Cl or NH_4-Hepes there appears to be an element of overshoot and subsequent adjustment. This is obvious in the Hepes curve and is visible but less clear in the recording of swelling in 17 mM NH_4Cl + FCCP since it is superimposed on the steep slope of swelling produced by NH_4Cl entry. This apparent overshoot and reversal occur at light-scattering levels similar to those at which the structural perturbations occur in the equilibrium response of mitochondrial light scattering to osmolality and may therefore indicate a time-dependence ($t_{1/2}$ for the reversal of the overshoot being approx. 12 s) of these structural changes. An alternative explanation is that alkalinisation of the matrix by entry of NH_3 activates the K^+/H^+ antiporter [37–39] and that the reversal is due to relatively slow exit of matrix K^+ ions. In either case this time-dependent reversal cannot be corrected by factors obtained from equilibrium relationships between light scattering and medium osmolality.

Fig. 2 shows traces of light scattering records of mitochondrial swelling when 25 mM NH_4-Tricine was present in the medium: this concentration being chosen to have the same osmolality as 20 mM NH_4-Hepes. The initial very rapid rate of adjustment to medium osmolality was similar in 25 mM NH_4-Tricine, recording 2(a), to that in 20 mM Hepes. The rate of Tricine entry shown by the third phase was slow but faster than the rate of Hepes entry but, as with Hepes, FCCP had no detectable effect on mitochondrial swelling in NH_4-Tricine medium (result not shown). However, the effect of Tricine in 100 mM NH_4Cl medium, recording

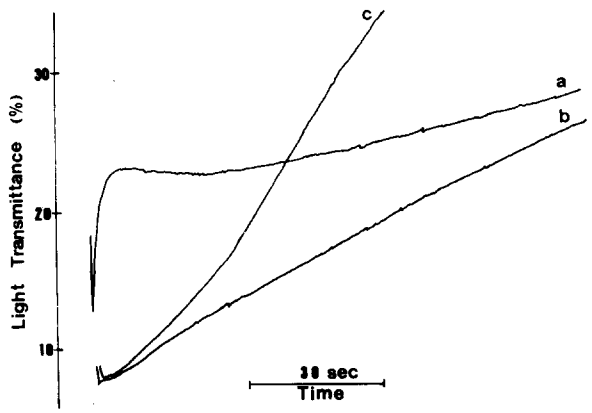

Fig. 2. Recordings of light scattering showing mitochondrial swelling in media containing NH_4-Tricine, NH_4Cl and NH_4Cl + NH_4-Tricine. Swelling was started by injecting the mitochondrial suspension, 1.5 mg of protein, into the following media: (a) 25 mM NH_4-Tricine; (b) 25 mM NH_4-Tricine in 100 mM NH_4Cl; (c)119 mM NH_4Cl. Medium (a) was of osmolality 40 mmol/kg and media (b) and (c) 247 mmol/kg. All media contained rotenone, antimycin A and FCCP added prior to the mitochondria.

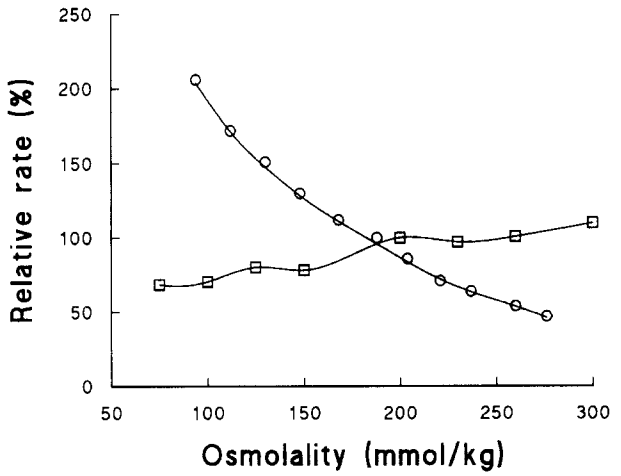

Fig. 3. Effect of osmolality on the rate of solute entry in NH_4Cl and ribose media. Solute uptake was initiated as described in Fig. 1 and the experimental section by injecting the mitochondrial suspension, 1.5 mg protein, into media of differing concentrations of solute. ○, NH_4Cl at pH 8.0 containing FCCP, rotenone and antimycin A; rates are expressed relative to that in the standard medium 100 mM NH_4Cl. □, ribose at pH 8.0 containing 5 mM K-Hepes, rotenone and antimycin A, rates are expressed relative to that in 200 mM ribose.

Fig. 2(b), is different from the effect of Hepes, the swelling rate is markedly inhibited and settles to a slow rate which appears to be the sum of a slow (inhibited) chloride entry plus the slow Tricine entry. The time-dependent second phase is seen clearly in recordings 2(a), 1(a) and 1(b); it is detectable in 1(d) and is presumed to be present in recording 2(b) but masked because the rate of swelling decelerates with a similar time constant.

The effect of increasing osmolality of NH_4Cl on the rate chloride uniport is shown in Fig. 3. The rate of light scattering change was much faster when mitochondria were added to solutions of lower ammonium chloride concentration (lower osmolality) and the rate decreased progressively as the ammonium chloride concentration was increased (higher osmolality). Even after correcting for the osmolality, using Eqn. 1, in order to compare rates of solute entry rather than water entry, a substantial decrease, about 50%, in the rate of solute entry rate was observed after increasing the ammonium chloride concentration in the medium from 100 mM to 130 mM. When Cl^- was replaced by ethanesulphonate or isethionate, Fig. 4, there was no significant difference in the effect of medium osmolality on the relative rate of solute entry when rates were expressed relative to the rate in 100 mM medium of the same salt although the rates of swelling in the ammonium isethionate and ethanesulphonate media at 100 mM were 15% and 30%, respectively, of the rate in ammonium chloride at the same concentration. The effects of osmolality on the swelling rates were much less when NH_4Cl was replaced by NH_4SCN, sodium

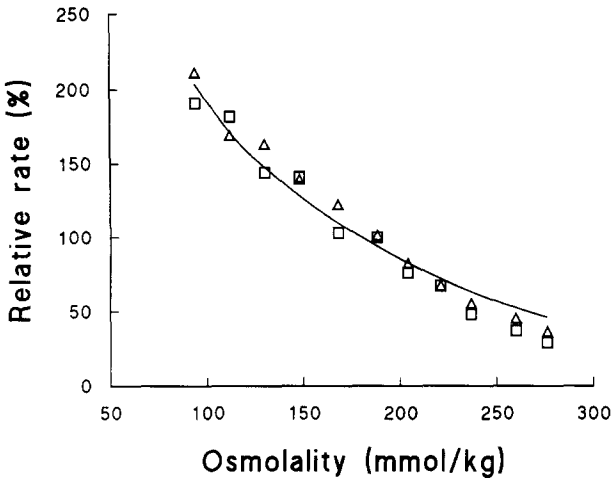

Fig. 4. Effect of osmolality on the rate of solute entry in ammonium isethionate and ethansulphonate media. Mitochondrial suspension, 1.5 mg protein, was added into media of differing concentrations of ammonium isethionate, △, and ammonium ethanesulphonate, □, at pH 8.0 containing FCCP, rotenone and antimycin A as described in the experimental section. The salt concentrations were varied over the range 50 to 150 mM. For each medium the rates are expressed relative to that under the standard conditions, 100 mM of the same salt. The curve is that fitted to the NH_4Cl data in Fig. 3 shown without the data points in this figure.

acetate or NH_4NO_3, as shown in Fig. 5 (note the expanded vertical axis in this figure). The complexity of the effects in NH_4NO_3 and NH_4SCN media reflects the multiplicity of processes occurring. Both NO_3^- and SCN^- not only enter via the anion conducting channel but also by simple diffusion through the membrane [1,40], the latter mode being rapid in the case of SCN^-. The rate of entry by diffusion is proportional to the concentration gradient and hence increases with increasing anion concentration and it would appear, Fig. 5, that this increase in diffusion rate balances the inhibitory effect on entry via the channel at high NO_3^- concentrations and outweighs it at high SCN^- concentrations.

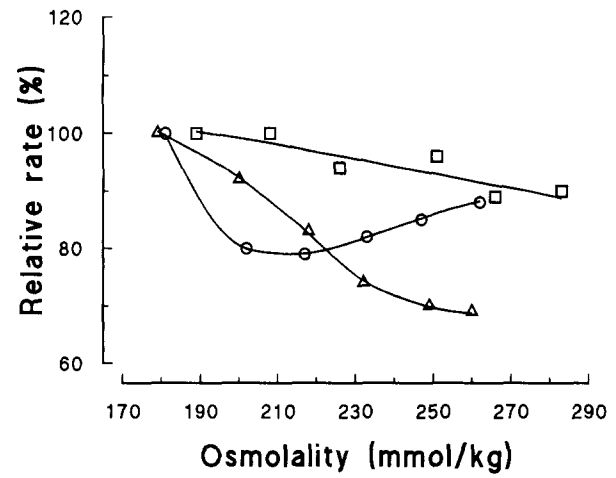

Fig. 5. Effect of osmolality changes on the rate of mitochondrial swelling in various media: △, NH_4NO_3; ○, NH_4SCN; □, sodium acetate. Assays were as described in Fig. 3 except that no FCCP was added to the sodium acetate medium. Concentration of each medium varies from 100 mM to 150 mM. The rates for each medium are presented relative to their respective standard assays at 100 mM of the same salt.

The effect of increasing osmolality on ribose entry, Fig. 3, is markedly different from the effect on chloride, isethionate or ethansulphonate uniport as the rate of ribose entry increases with increasing ribose concentration. This observation is similar to that of Garlid and Beavis [21] who found that the rate constant for erythritol entry was unaffected by osmolality when allowance was made for structural changes.

Thus, there is an effect of high salt concentration or osmolality on anion entry via the pH-dependent inner membrane anion-conducting channel which is not observed with Na^+ ions which enter via carrier-mediated Na^+/H^+ antiport, with entry of the non-ionic solute ribose or with entry of SCN^- or NO_3^- by diffusion. The identical effects of increasing osmolality in ammonium chloride, isethionate and ethanesulphonate suggest that osmolality rather than binding of the anion to an inhibitory site is the factor which produces the decrease in rate of anion entry since the different anions would be expected to have differing affinities for an inhibitory site. To distinguish between the effects of osmolality and anion concentration we investigated the effect of increasing the osmolality by adding non-permeant solutes to the NH_4Cl medium. As shown in Fig. 6, increasing concentrations of sucrose (non-ionic), ammonium gluconate (non-permeant anion) or Tris chloride (non-permeant cation/non-ionic plus chloride) all decreased the rate of chloride uniport when added to 100 mM NH_4Cl media. Increasing the osmolality by increasing the NH_4Cl concentration concomitantly increases the driving force for Cl^- entry. Since even simple channels exhibit saturation effects, Stein [41], the question arises as to whether the channel is operating at near saturating capacity, in which case the rate of chloride entry should be independent of Cl^- concentration or well below the pseudo K_m in which case the rate should be proportional to Cl^- concentration. In the latter case slower rates would be expected in media of the same osmolality in which Cl^- has been partially replaced by other solutes. As shown in Fig. 6, the rates in such media are lower than the corresponding rates in NH_4Cl. However, when the rate in NH_4Cl is corrected for the increased Cl^- concentration by assuming that the rate is first order in Cl concentration, using Eqn. 2,

$$\text{Corrected rate } \% = \text{relative rate } \% \times \frac{[Cl^-]_{Std}}{[Cl^-]_{Exp}} \quad (2)$$

it is found that the curve for ammonium gluconate is similar to the corrected NH_4Cl curve. The lower rates

seen when the osmolality is increased with Tris-HCl, which produces a smaller increase in Cl^- concentration than that produced by adding NH_4Cl, and sucrose suggest either an effect of the NH_3 gradient or an inhibition by polyols, as reported by Lehninger [42]. When these rates are compared with the rates in NH_4Cl corrected for both Cl- and NH_3 (or NH_4^+) concentrations using Eqn. 3

$$\text{Doubly corrected rate \%} = \text{relative rate \%} \times \frac{[Cl^-]_{Std} \times [NH_4^+]_{Std}}{[Cl^-]_{Exp} \times [NH_4^+]_{Exp}} \quad (3)$$

it is found, Fig. 6, that the doubly corrected NH_4Cl rates are similar to the rates with Tris-HCl but greater than the rates in sucrose. It might be inferred from this that sucrose but not Tris is inhibitory. However, Tris has a very similar effect in ammonium isethionate medium, inset to Fig. 6, but, because the rates in isethionate are very much slower than in Cl^-, any changes in the rate of ammonia entry should have much less effect on the overall rate in isethionate medium. From this we infer that the rates in both chloride and isethionate media are limited by the rate of anion entry and therefore no correction should be made for ammonia concentration. On this basis comparison of the curve for Tris-HCl with the NH_4Cl curve corrected using Eqn. 2 indicates that Tris has an inhibitory effect.

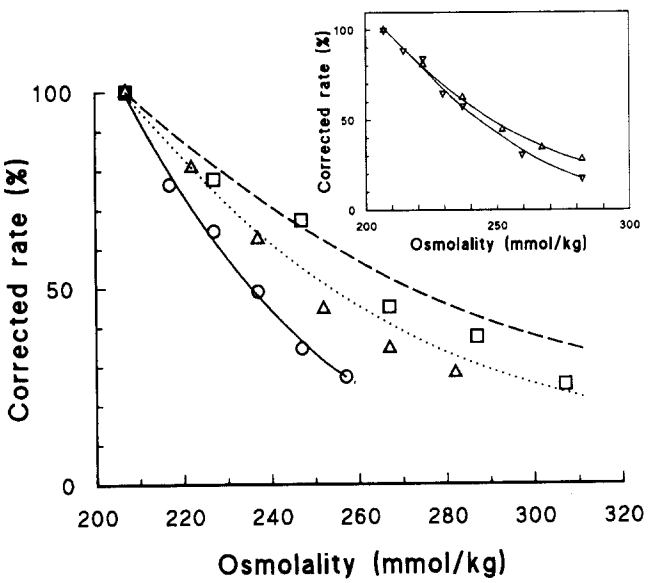

Fig. 6. Effect of some non-permeant solutes on the rate of chloride entry. Main figure: assays were conducted as in Fig. 3 with non-permeant solutes added to 100 mM NH_4Cl medium. ○, sucrose; △, Tris-Cl; □, NH_4-gluconate. The dashed line (— — —) represents the rates for NH_4Cl medium corrected for chloride concentration using Eqn. 2 and the dotted line (· · · · · ·) the same data corrected for both chloride and ammonia concentration using Eqn. 3. Inset: reproduces the data for Tris-Cl added to NH_4Cl medium shown in the main figure, △, and shows also the effect of adding Tris-isethionate to 100 mM ammonium isethionate medium, ▽.

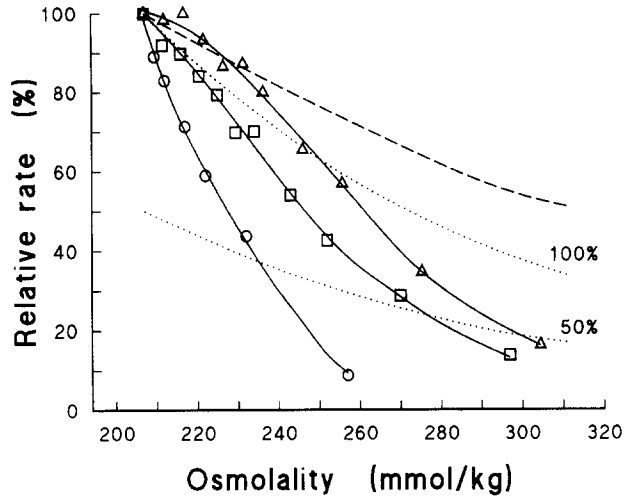

Fig. 7. Effect of some zwitterionic buffers on the rate of chloride entry. 1.5 mg of mitochondrial protein was added to 100 mM buffer-free NH_4Cl containing varying concentrations of: △, Pipes; □, Hepes; ○, Caps. Rates are measured relative to the standard assay in 100 mM buffer-free NH_4Cl. The broken line (— — —) shows the variation of the rate with osmolality in buffer-free NH_4Cl, symbols omitted as in Fig. 4; the dotted lines (· · · · · ·) represent 100% and 50%, as labelled, of these rates corrected for chloride concentration using Eqn. 2. The intersection of the line for 50% of the corrected rate in NH_4Cl with the curve for a buffer defines the I_{50} value corrected for the effect of osmolality and chloride concentration on the rate in NH_4Cl.

Overall, we conclude that inhibition of anion entry via the channel is a function of osmolality rather than anion concentration but some solutes have additional inhibitory activity.

The zwitterionic buffers are generally impermeant and investigation of their effects serves not only the practical purpose of screening them for inhibitory effects but also provides further tests of the role of osmolality and direct inhibition by solutes. Fig. 7 shows the inhibitory effect of some buffers in NH_4Cl medium with the curves for the rates in NH_4Cl corrected for chloride concentration and 50% of these rates shown for comparison. From results of experiments of this type, it appeared that many buffers have little effect on the rate of swelling other than that produced by increasing the osmolality of the medium but a few exhibit inhibitory effects superimposed on the osmolality effect. We have characterised the effects of these buffers by graphical interpolation to estimate the concentrations (I_{50}) at which the rate is inhibited to 50% of the corrected rate in buffer-free ammonium chloride of the same osmolality.

Table I summarises these I_{50} values with the corresponding osmolalities. The effect of high osmolality contribution by impermeant solutes was noted above in connection with the effect of Hepes, Fig. 1(a). When the osmolality contribution is over 50 mmol kg^{-1} the

limitation on extent of swelling prevents accurate measurement of additional inhibitory effects. The data presented in Table I show that Tricine is markedly inhibitory; Popso and Caps are also inhibitory; Tapso, Tes, Mopso, Hepes, Mes, Ches, Pipes, Taps, Mops, GlyGly, Epps, Tris and Heppso, form a series of decreasing inhibitory activity while Bicine and Bes have little or no inhibitory effect other than that produced by their non-permeant contribution to the medium osmolality. No correlations between inhibitory activity and the pK_a or structure of these buffers can be discerned. The most inhibitory group from Tricine to

TABLE I

Inhibitory action of buffers on chloride uniport

I_{50} values were estimated graphically from plots of buffer inhibition of chloride uniport in 100 mM NH_4Cl as shown and under the conditions in Fig. 7 and described in the text.

Buffer	I_{50} (mM)	ΔOsmol [a] (mmol/kg)	pK_a [b]	Chemical name
Tricine	17	26	7.9	*N*-tris(hydroxymethyl)methylglycine
Popso	24	39	7.8	piperazine-*N*,*N*'-bis(2-hydroxypropanesulphonic acid)
Caps	28	28	10.3	3-(cyclohexylamino)propanesulphonic acid
Tapso	32	55	7.6	3-(*N*-tris(hydroxymethyl)-methylamino)-2-hydroxypropanesulphonic acid
Tes	33	60	7.3	*N*-tris(hydroxymethyl)methylaminoethanesulphonic acid
Mopso	35	67	6.9	3-(*N*-morpholino)-2-hydroxypropanesulphonic acid
Hepes	37	67	7.5	*N*-2-hydroxyethylpiperazine-*N*'-ethanesulphonic acid
Mes	39	78	6.0	2-(*N*-morpholino)ethanesulphonic acid
Ches	46	48	9.4	2-(*N*-cyclohexylamino)-propanesulphonic acid
Pipes	47	91	6.7	piperazine-*N*,*N*'-bis(2-ethanesulphonic acid
Taps	49	63	8.4	*N*-tris(hydroxymethyl)methyl-3-aminopropanesulphonic acid
Mops	49	92	6.9	3-(*N*-morpholino)propanesulphonic acid
GlyGly	50	73	8.1	glycylglycine
Epps	(41%) [c]	75	8.0	*N*-2-hydroxyethylpiperazine-*N*'-propanesulphonic acid
Tris	(37%) [c]	75	8.0	*N*-tris(hydroxymethyl)-aminomethane
Heppso	(32%) [c]	81	7.8	*N*-hydroxyethylpiperazine-*N*'-2-hydroxypropanesulphonic acid
Bicine	(8%) [c]	69	8.2	*N*,*N*'-bis(2-hydroxyethyl)-glycine
Bes	(0%) [c]	95	7.1	*N*,*N*'-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid

[a] ΔOsmol is the increase in medium osmolality produced by the buffer at its I_{50} concentration in 100 mM NH_4Cl.
[b] pK_a values at 30°C were calculated from published data [22,23].
[c] Figures in parentheses are % inhibition at 50 mM buffer.

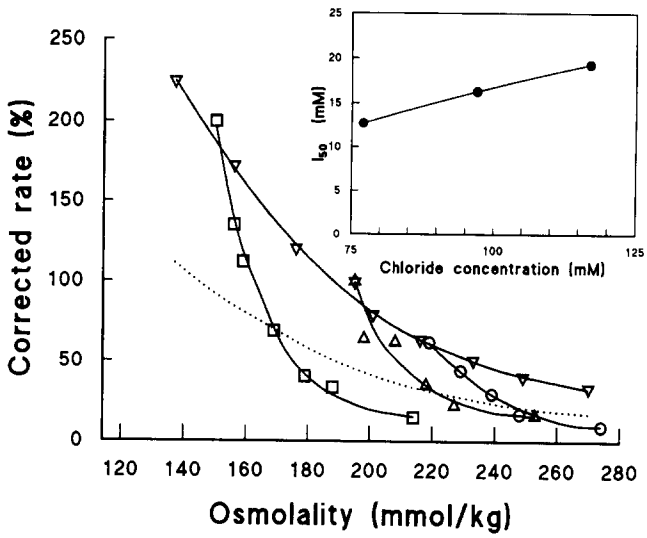

Fig. 8. Inhibition by Tricine at different concentrations of NH_4Cl. Mitochondrial suspension was added into media containing 5, 10, 20, 30 and 50 mM Tricine in: □, 80 mM NH_4Cl; △, 100 mM NH_4Cl and ○, 120 mM NH_4Cl. Rates are expressed relative to the standard assay in 100 mM buffer-free NH_4Cl and corrected for chloride concentration using Eqn. 2. ▽, rates in varying osmolalities of buffer-free NH_4Cl medium, the dotted line (· · · · · ·) represents 50% of these rates. I_{50} values for Tricine at different NH_4Cl concentrations were estimated as in Fig. 7 and plotted, •, against chloride concentration in the inset figure.

Caps includes buffers with and without hetero-cyclic rings and carboxylate as well as sulphonate compounds. Addition of an extra hydroxyl group can either increase or decrease the inhibitory action. Fig. 8 shows inhibition by Tricine in different concentrations of NH_4Cl. The I_{50} values for Tricine were estimated by the method shown in Fig. 7 and as shown in the inset to Fig. 8 the I_{50} values increased almost linearly with chloride concentration as would be expected for simple competitive inhibition, Eqn. 4.

$$I_{50} = K_i\left(1 + \frac{[S]}{K_m}\right) \quad (4)$$

(Where S represents chloride ion, K_m is a Michaelis type constant characterising the saturation kinetics for a simple channel and K_i is the inhibitor constant.)

Discussion

Our observations show, firstly, that the rate of mitochondrial swelling produced by entry of anions via the pH-dependent anion conducting channel is markedly affected by altering the osmolality of the medium: the rate decreases with increasing osmolality when this is increased either by increasing the concentration of the permeant anion or by adding an impermeant solute. This is in accord with previous reports of osmotic effects on ion transport [21–23] but defines the effect

in relation to a specific transport process. Secondly, some zwitterionic buffers have an additional inhibitory effect on this anion uniport. We have also confirmed previous reports that sucrose inhibits anion uniport across the mitochondrial inner membrane [42].

One possible mechanism for the effect of osmolality or matrix volume is that, as mentioned in the introduction with regard to other channels, changes in the membrane such as stretching or unfolding lead to higher conductivity or an increased number of accessible channels [17–20]. However, the major structural change in the mitochondrial membrane is associated with breakage of the outer membrane and as shown in the elegant study by Garlid's group [24,34] is not associated with changes in erythritol permeability. This structural change is observed as a perturbation in the curves relating light scattering to osmolality at osmotic equilibrium and a similar perturbation can be seen in the light scattering recordings of the kinetics of passive osmotic swelling. In the kinetics of swelling it can be seen that the rates before and after this perturbed region are similar indicating that no major change in ribose permeability or chloride uniport has occurred. Furthermore, as experiments conducted at osmolalities above and below the perturbed region of the light scattering response show similar effects of osmolality on the rate of solute entry both above and below the perturbed region these effects must be dependent on the matrix volume rather than the structural change associated with the perturbed region. Although it is not definitive, this lack of correlation between the major structural change and change in anion conductivity argues strongly against stretch sensitivity or channel accessibility being the mechanism by which this channel responds to osmolality.

An alternative explanation is that the pH-dependent anion-conducting channel is inhibited by a solute in the matrix, the concentration of which, and in consequence its inhibitory effect, increases as the matrix volume decreases in response to increased medium osmolality. As suggested by Garlid and Beavis [2] the inhibitory solute in the matrix may be Mg^{2+} ions as they have shown that this channel is inhibited by matrix Mg^{2+} [3]. However, the role of Mg^{2+} is uncertain as the same group, Beavis [3], have reported recently that under energised conditions the channel could open without depletion of matrix Mg^{2+} and was not inhibited by extramitochondrial Mg^{2+} ions.

We also present evidence for the inhibitory effects of some of the commonly used buffers on the mitochondrial anion-conducting channel. Some buffers have an inhibitory action in addition to the decrease in rate produced by increasing osmolality. Although no specific structural characteristics of the buffers can be correlated with inhibitory activity, the present observations and published observations of inhibition of other channels by zwitterionic buffers [9–14], emphasises the need to test the effects of these buffers whenever they are used in experiments on channels. In investigations in which the mitochondrial inner membrane pH-dependent anion-conducting channel may be involved Bicine and Bes appear to be the best choice for buffers; Tricine, Popso and Caps, which are markedly inhibitory, should not be used; the remainder of those tested, Tapso, Tes, Mopso, Hepes, Mes, Ches, Pipes, Taps, Mops, GlyGly, Epps, Tris and Heppso, form a series of decreasing inhibitory action and some may be usable at low concentrations.

The sensitivity of this channel to osmolality or matrix volume is of interest because the activity of several mitochondrial processes is modulated by the matrix volume and has been proposed as one mode of hormonal regulation of mitochondrial function [45]. Also, failure in the control of matrix volume may be involved in pathological effects such as those caused by ischaemia [46]. As the membrane potential of the mitochondrial membrane is negative on the matrix side and anion-conductivity is greater at higher matrix volume the pH-dependent anion-conducting channel would constitute a negative feedback system favouring exit of anions at large matrix volume and hence decrease of matrix osmolality, exit of water and decrease of matrix volume.

Acknowledgements

This research was supported by a grant from the National University of Singapore (RP890313). We thank Prof. C.H. Tan of the Department of Biochemistry for loan of the Wescor 5500 vapor pressure osmometer.

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