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Titration of Mitochondrial Buffer by Accumulated Anions

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Summary. Acidimetric titration of intact rat liver mitochondria discloses a buffer power of about 45 mEquiv per g between pH 7 and 8, the value rises to 60 mEquiv per g per pH unit after lysis using Triton X-100. The existence and properties of this buffer system have been related to mitochondrial anion accumulation. The uptake of permeant anions by mitochondria occurs to a charge-dependent extent and they are in electrochemical equilibrium with each other and the protons as in a Donnan system. Adding permeant anion causes the intramitochondrial anion content to rise towards a saturation level, the inside to outside concentration ratio falls and concomitantly the transmembrane proton gradient diminishes, making the interior less alkaline. The falling internal pH is associated with protonation of the internal buffer, thus providing a second method for measuring the buffer power, a method which also tests the arguments used in the calculations. The titration curve is constructed by relating the internal pH (deduced from the permeant anion ratio) to the total internal anion equivalents which in turn determines the ionization state of the buffer because the sum of the internal anion equivalents, including the buffer anion, equals the equivalents of internal cation. The buffer power so measured agrees with the acidimetric method applied to lysed mitochondria. The disparity between the acidimetric data from lysed and unlysed mitochondria follows theoretical predictions.

Mitochondrial anion accumulation is often attributed to the operation of a series of transmembrane exchanges between different species, starting with that of a phosphate for a hydroxyl (Chappell & Crofts, 1966). According to one hypothesis, the latter ion is supposed (Mitchell, 1971) to be generated internally from water following an energized extrusion of protons. However, when respiratory inhibitors and oligomycin are present, cutting off the energy supply, anion accumulation still occurs. This must reflect an ionization change of an internal buffer which had previously been 'charged up' by the active transport of protons. It is the internal buffer which is studied in this paper. An alternative view of metabolite anion accumulation is that they are in transmembrane electrochemical equilibrium and they are

Table 1. Anion contents of fresh twice-washed rat liver mitochondria

Anion	Range of contents (mmole/g protein)	Equivalents (mEquiv/g protein)
Phosphate	3–5	6–10
Citrate	1–4	3–12
Malate	1–3	2–6
Pyruvate	0.2–1	0.2–1
Phosphoenolpyruvate	0.5–2.5	1.5–7.5
Oxoglutarate	1–2	2–4
Glutamate	1.4–4	1.4–4
Asparate	1.3–7	1.3–7
Hydroxybutyrate	0.4	0.4
Glutamine	0.8	—
Glutathione	2.7	5.4
Serine	3.0	—
Hydroxyproline	1.5	—
Cysteine	1.0	2
Taurine	0.9	1.8
Glycine	1.0	—
Alanine	1.3	—
α-Glycerol phosphate	2–4	3–6

The substrate anion values are collected from many experiments in which initial contents of anions were assayed. The amino acids were analyzed using an auto-analyzer.

The total mEquiv/g are 67 in the unlikely case of all acids being present at their maximum amount.

accumulated because of active cation transport into the mitochondria. After the addition of the inhibitors there remains a store of internal cations (K^+, Ca^{2+}) associated with an internal buffer anion denoted in the following by X^-. The quantity of X^- is diminished as the internal pH is lowered because it is protonated to XH; the electrostatic balance between cations and anions can then be maintained as more permeant anions enter.

It is generally accepted that the accumulation of K^+ and Ca^{2+} is energy-dependent, and we propose that the cation store should be regarded as being associated with the maintenance of that chemically negligible charge surplus required to make the interior positive. As in the classical Gibbs-Donnan system the internal positive potential is explained if anion entry is associated with simultaneous neutralization of the buffer anions X^- by protons derived from outside.

The existence and the properties of the internal buffer are worthy of consideration. When the sum of the concentrations of internal identified anions is made (*see* Table 1) its maximum is seen to be little more than

half the equivalents of internal K^+ and Ca^{2+} (about 120 mEquiv/g protein), to say nothing of the Mg^{2+} which behaves as if it is tightly bound in an energy-independent way. Some anionic charge must reside on the protein to make up the deficiency. A parallel case exists in the erythrocyte whose hemoglobin contributes some 30 mEquiv of anionic charge per ml cells at pH 7.4 (Harris & Maizels, 1952). In the mitochondrion, the charge balance can be written:

$$K^+ + 2\,Ca^{2+} = \text{Equivalents small anions} + X^-. \quad (1)$$

Even if there are fixed or impermeant negative charges (X^-) inside, anion accumulation can still occur passively provided prior expenditure of energy has charged up the interior with cations to effectively produce an impermeant positive net charge inside. Anion accumulation can then occur passively to reach electrochemical equilibrium even after further energy supply is cut off. It has been shown, however, that during storage there is a slow loss of cations (Harris, 1968*a*) and also of the ability to accumulate anions (Harris, 1968*a*, *b*). Although loss of Ca^{2+} after energy deprivation is rapid (Thomas, Manger & Harris, 1969), that of K^+ is slow, unless the membrane has been made highly permeable with an ionophore. If an uncoupler is added to the suspension, anions are no longer accumulated (Harris, 1968*b*).

The applicability of Eq. (1) insofar as there are parallel upward or downward changes of content of cations and mobile anions has been demonstrated. Ca^{2+} uptake is accompanied by gain of anions (Kimmich & Rasmussen, 1968; Harris & Berent, 1969); massive K^+ uptake induced by an ionophore can carry in nearly its equivalent of certain anions (Harris, 1968*a*). Discharge of K^+ is accompanied by loss of anions (Harris & van Dam, 1968).

Turning to the interrelation between the content of permeant anions and X^- there are experimental results showing that more anion is accumulated as the external pH is lowered (Palmieri, Quagliariello & Klingenberg, 1970). In our interpretation, this effect can be accounted for by the greater protonation of X^- at lower pH. The only interrelation so far not explicitly examined under conditions of constant cation content and pH is that between the content of metabolite anions and X^-. As metabolite anion is accumulated a change of X^- to XH must occur so that, in effect, the internal buffer is progressively titrated. A source of intramitochondrial protons is evidently required but direct membrane permeability to them is not essential as will be seen when phosphate distribution is considered.

In the Donnan system, the conditions for electrochemical equilibrium of the respective anions (A^-, B^{2-}, C^{3-}) lead to the relation between the

inside and external concentrations

$$[A^-]_i/[A^-]_e=[B^{2-}]_i/[B^{2-}]_e^{\frac{1}{2}}=([C^{3-}]_i/[C^{3-}]_e)^{\frac{1}{3}}\equiv r. \quad (2)$$

These relations applied to the phosphate ions bearing charges of -1 and -2 lead to the proton ratio $[H^+]_e/[H^+]_i\equiv r$. This follows on substituting for the respective anionic concentrations in the dissociation equations for inside and for outside and taking the ratio of proton concentrations. Accordingly, in the absence of membrane permeability to protons a cycling of phosphate will lead to adjustment of the proton ratio to the same value as it would have in a passive or Donnan system. The transmembrane pH difference can be written as

$$\Delta\,\mathrm{pH}=\log r=\frac{1}{n}\log\frac{[A^{n-}]_i}{[A^{n-}]_e}. \quad (3)$$

The ratio between the internal and external concentrations of any chosen *permeant* anion (i.e., one which can freely move to its electrochemical equilibrium distribution) can be used to find ΔpH and hence pH_i (*cf.* Addanki, Cahill & Sotos, 1968). The total content, in equivalents, of metabolite can be substituted in the electrostatic equivalence Eq. (4) to obtain X^- and so the dependence of X^- on pH_i is found:

$$\text{Equivalents of internal cation} - \text{equivalents of metabolite anion} = X^-. \quad (4)$$

In practice, it is most convenient to plot pH_i or Δ pH, for a fixed pH_e against the total content of metabolite anions in equivalents. The curve so obtained relates the difference, cations $-X^-$, to pH_i. While it is likely that the buffering by the internal protein will remain fairly constant from one preparation to another, one may expect displacements between the curves when the cation contents of the preparations are unequal.

Materials and Methods

The rat liver mitochondria were prepared by homogenizing in 0.25 M mannitol or sucrose containing 1 mM ethyleneglycol-*bis*-(2-aminoethyl)-tetracetic acid (EGTA), pH 7; the cell debris was centrifuged down by taking the speed up to 5,000 rpm (10-cm rotor) and then switching off. The mitochondria were recovered by centrifuging for 64,000 g · min, washed twice in 0.3 M sucrose containing 5 mM KCl and 1 mM EGTA. Finally the pellet was taken up in 0.25 M mannitol or sucrose. In the course of the work it was noted that using mannitol led to lower respiratory rates and in subsequent preparations sucrose was used as in the earlier work quoted. The Ca^{2+} content of the mitochondria was 4 to 7 μmole/g protein.

The anion distributions were measured using the silicone layer technique described before (Harris & van Dam, 1968; Harris & Berent, 1970). The incubation was either carried out with a ^{14}C-labeled compound in the presence of a suitable inhibitor to stop

its oxidation or the anion contents were determined directly by means of specific enzyme assays made on samples taken during the course of metabolism. The inorganic phosphate was measured as described before (Harris, 1970).

The titration of the mitochondrial protein was performed on an automatic titrimeter (Radiometer TTT1).

Results

The measurements of mitochondrial anion contents with different external anion concentrations are summarized in Table 2. The ratio between the internal content (corrected for the amount carried by contaminating external medium, as measured by the sucrose accessible space) and the applied concentration was used to calculate the ΔpH from Eq. (3). It was assumed that the internal anions were distributed in 1-μliter matrix water per mg protein (Harris & van Dam, 1968). This value may be at the high end of the range since the water inaccessible to sucrose was often only

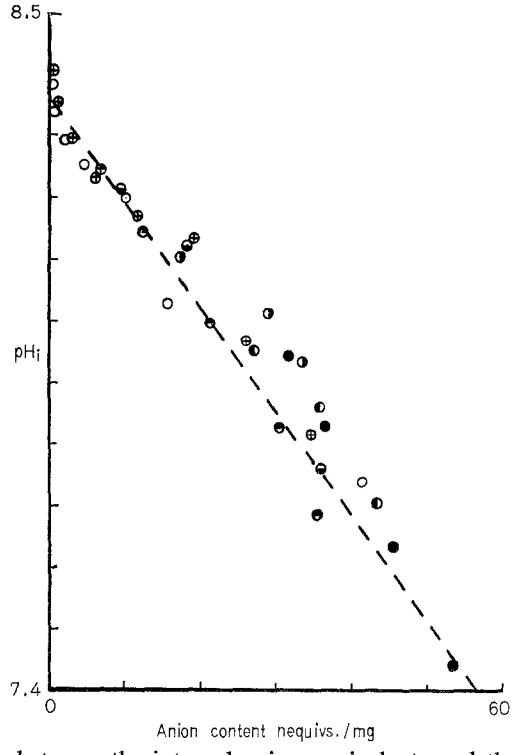

Fig. 1. The relation between the internal anion equivalents and the internal pH found by adding $\Delta pH \equiv \frac{1}{n} \log \frac{A_i^{n-}}{A_e^{n-}}$ to the external pH. The differently marked points refer to the experiments listed in Table 2. ●, 1; ◓, 2; ◒, 3; ⊕, 4; ◐, 5; ◑, 6; ○, 7. As more internal anions are present the inside/outside concentration ratio and the ΔpH falls, so the pH$_i$ moves from alkalinity toward acidity. This is associated with protonation of the internal buffers

Table 2. Data for relating anion contents to the pH calculated from the ratio holding between anion concentration inside/outside[a]

Exp.	Pyruvate	Malate	Citrate	Phosphate	OG[b]	Malonate	Acetate	Sum	Donnan ratio r	log r $= \Delta$pH	pH_i
1. Citrate additions to medium. Extl pH 7.2	1.0	1.4	10.5	14.6	3.8			31.3	5.5[c]	0.74[c]	7.94
	0.8	0.8	18.0	13.0	3.8			36.4	4.2[c]	0.625[c]	7.82
	0.5	0.35	29.7	11.0	3.8			45.3	2.7[c]	0.43[c]	7.63
	0.3	0.2	39.3	10.1	3.8			53.7	1.65[c]	0.218[c]	7.42
2. Phosphate additions to medium. Extl pH 7.2				12.0					8.7	0.94	8.14
				21.1					5.1	0.71	7.91
				30.0					4.2	0.62	7.82
				35.5					3.05	0.485	7.685
3. Malonate additions to medium. Extl pH 7.2						9.2			6.42	0.807	8.07
						17.5			5.03	0.70	7.90
						28.0			4.32	0.63	7.83
						36.2			3.67	0.565	7.76
4. Acetate (A) additions to medium. Extl pH 7.4							0.84		10.3	1.01	8.41
							2.07		8.9	0.95	8.35
							3.45		7.75	0.89	8.29
							7.35		6.7	0.82	8.22
							11.60		5.8	0.765	8.16
(B)							6.42		6.90	0.84	8.24
							25.60		4.13	0.56	7.96
5. Citrate additions in presence of added malate. Extl pH 7.3		9.2	17.6					26.8	4.58	0.66	7.96
		5.8	30.0					35.8	3.66	0.56	7.86
		5.4	37.8					43.2	2.70	0.40	7.70
6. Phosphate additions to medium. Extl pH 7.2		2.5	3.3	12.1				17.9	8.2	0.91	8.11
		2.0	3.3	23.0				28.3	6.5	0.81	8.01
7. Pyruvate (A) additions to medium. Extl pH 7.4	0.80								9.5	0.98	8.38
	3.0								7.8	0.89	8.29
	4.45								7.0	0.85	8.25
	10.0								6.4	0.80	8.20
(B)	1.06								8.85	0.94	8.34
	15.6								4.2	0.65	8.05
	41.6								2.2	0.34	7.94

[a] The concentrations inside are deduced from the contents, corrected for the carry down in the volume of fluid accessible to sucrose, assuming a matrix water space of 1 ml/g protein (Harris & van Dam, 1968). Anions measured in mEquiv/g.

[b] OG: oxoglutarate.

[c] Calculated from citrate.

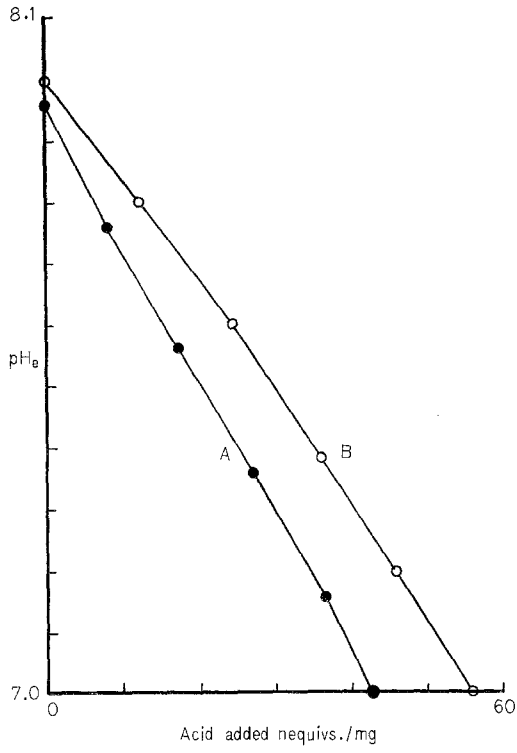

Fig. 2. Acidimetric titration curves for intact (*A*) and lysed (*B*) mitochondria. Triton (0.01 %) was added to *B*. The points were derived by taking the differences between the continuous curves obtained from the automatic titrimeter for the mitochondrial suspension and for medium alone. The addition of acid protonates the buffer. The lesser buffering obtained with intact mitochondria is explained (*see* Discussion) by movements of permeant anions which reduce the internal pH shift to a fraction of that in the medium

0.7 ml/g protein, in which case both the ΔpH and internal anion concentrations will be higher than the figures calculated in Table 2, the former by 0.15 unit and the latter by 1.4-fold. The ΔpH is plotted against the total content of measured anions in Fig. 1. The sets of points from a given experiment tend to fall on one of what would be a set of parallel lines because of minor differences in cation content as mentioned in the introduction, and differences in the matrix water. However, the data plotted, as well as other isolated points obtained from other experiments, support the concept that as anions accumulate there is a progressive fall in the ΔpH and hence in pH_i. The effective buffer value of X^- can be estimated from the slope as being about 60 mEquiv per pH unit per g protein (between pH 7.5 and 8.0; *see* dashed line, Fig. 1).

The acidimetric titration of mitochondrial protein was described earlier (Harris, Judah & Ahmed, 1966) which led to a value for buffering power of about 50 mEquiv per pH unit per g dry weight between pH 7 and 8. Since the dry weight is about 1.1 g per g biuret protein, the buffering power per mg protein is 55 mEquiv per pH unit. We have repeated the titration with and without Triton X-100 as a lytic agent to make the membrane permeable. Fig. 2 shows two typical curves. When the membranes have been made permeable, the buffering power between pH 7.5 and 8.0 is 61 mEquiv per pH unit per g protein. This value is close to the figure of Harris *et al.* (1966) and with the value obtained (here) from the anion titration.

Effect of Acidification of Ca^{2+} Content

Since we wish to discuss the consequence of acidifying the suspension medium, it is necessary to consider that a second factor responding to this change in conditions may exist; namely, the cation content. The importance of this in determining the anion content is seen in Eq. (1).

When the mitochondria contain a comparatively large amount of Ca^{2+}, the content is reduced as the medium is acidified (Table 3A, Lines 1 and 2). Only when the particles have a low initial Ca^{2+} content is the amount comparatively insensitive to pH. With the lower Ca^{2+} content obtained by energy depletion and resuspension to remove the released Ca^{2+}, the anion content increased in response to acidity (Table 3B).

Table 3. Effect of acidification on citrate content of rat liver mitochondria

A. With Ca^{2+} loss, citrate at 130 μM	Contents (μmole/g protein)	
	Citrate	Ca^{2+}
Initial pH 7.4	3.64	24.0
Adjusted to pH 6.6	3.47	3.5
Readjusted to pH 7.8	1.38	4.4

Loss of 41 μEquiv cation for ΔpH of 0.8 unit (7.4 to 6.6) compensates for diminished ionization of internal buffer. Buffer power = 51 μEquiv/pH per g.

B. Without Ca^{2+} loss (Acidified before citrate addition)	Extl conc (μM)	Contents (μmole/g protein)	
	Citrate	Citrate	Ca^{2+}
pH 7.4	48	1.44	12.0
pH 6.4	57	4.10	11.0

Discussion

Comparisons with a Model and with the Adsorption Isotherm

The results obtained with the penetrating anions show that the buffering power deduced from the internal anionic buffer is in quantitative agreement with the acidimetric titration data and allows one to estimate the total equivalents of internal anions at a given pH. A fair approximation to the system is obtained by assuming a cation content of 120 mEquiv/g and a buffer containing 150 mEquiv/g of ionizable groups ($X^- + XH$) and a pK of 7.8. This pK represents the weighted mean of a spread of ionization constants so does not allow identification of the buffer species. A better fit with the experiments could be obtained with a more detailed description of the buffer properties.

Previous work (Harris, 1968*a*, *c*) has shown that accumulation of permeant anions is strongly dependent on the charge of the ion. According to the present view, it is the charge which determines the concentration ratio at electrochemical equilibrium. Since the electrostatic term contributing to the electrochemical potential rises as the charge increases, it follows that multiply-charged anions will compete strongly with less-charged anions.

It is not a simple matter to compare the figures obtained from the conventional double reciprocal plots for uptake competition with theoretical values calculated for the model, because the competition exhibited depends on the total internal anion equivalents as well as on the external concentraitions of the ions involved. However, it can be shown that a dicarboxylate on will be from 5 to 10 times as effective a competitor as a monocarboxylate ion, while a tricarboxylate ion would be 30 to 80 times as effective as a monocarboxylate ion and 5 to 8 times as effective as a dicarboxylate ion. Just as the net movement of anions requires changes in the ionization of the internal buffer, so will the exchange of an anion for another bearing an unequal charge. This latter process has been observed to be enhanced by addition of uncoupler or phosphate (Robinson, Williams & Halperin, 1971) and can be explained by the uncoupler or phosphate (*cf.* Chappell & Crofts, 1966) serving as proton carriers thus facilitating the equilibration between X^- and XH.

Other Evidence for Internal Positivity

The existence of an intramitochondrial positive charge which is a function of the external anion concentration has been controversially supported by

Tupper and Tedeschi (1969) using microelectrodes. There is also inferential evidence from the fact that the cationic methylamine is less concentrated in the mitochondria than in the medium (Harris & Bassett, 1971).

The Effect of External pH on the Anion Distribution

Altering the external pH can change at least two factors affecting anion accumulation in opposite ways. As the suspension is acidified there is a loss of Ca^{2+} from the mitochondria to an extent which seems to depend on their initial content of Ca^{2+} (Table 3). This loss of cation decreases the permissible anionic content of the interior. On the other hand, with protonation of the internal buffer ($X^- + H^+ \rightarrow XH$), more metabolite anions can be accommodated to balance the internal cations electrostatically.

In their study of the effect of external pH on anions, Palmieri *et al.* (1970) state: "It is clear that the log of the anion distribution ratio is proportional to ΔpH for all the anions tested" but did not use this relation to obtain explicitly pH_i from the known pH_e. It is pH_i which determines the content of internal anions. In their discussion these authors also briefly mention that: "The saturation of anion uptake with increasing external concentrations can in principle be expected when the influx of the undissociated acid decreases the internal pH to a level where the internal buffering capacity becomes exhausted". We suggest that even if the anions enter as the charged species, the same relationships will hold, provided protons can also move to electrochemical equilibrium.

Some examples of the response of internal pH to a shift of external pH from 7.4 to 7.0 will be calculated. If a monobasic acid is present at 1 mM and is accumulated by 6.3 times (a typical experimental value) then the internal concentration is 6.3 mM, (A_i), the ΔpH is 0.81 [from Eq. (3)] and pH_i is 8.21 (fitting on the curve, Fig. 1). With the external pH lowered to 7.0, the internal pH must fall to such a value that the increased anion ratio A_i'/A_e corresponds to a $\Delta pH = \frac{1}{n} \log A_i'/A_e$ which when added to 7.0 gives a pH_i consistent with the new anion concentration A_i'. By trial it is found that with $A_i' = 14$ mM, the internal pH is $7.0 + 1.14 = 8.14$ which is on the curve (Fig. 1) for internal equivalents = 14. The internal pH has therefore fallen only 0.07 units in response to a shift of 0.4 units outside.

With 0.2 mM dibasic acid in the medium, the internal concentration will typically be 6.5 mM (13 mEquiv/g) with a concentration ratio of 32.5, Donnan ratio 'r' 5.7, internal pH 8.155. Reducing pH_e to 7.0 will increase

'r' to 8.6, making pH_i 7.93 ($=7.0+\log 8.6$) and the internal concentration 14.8 mM ($8.6^2 \times 0.2$) or 29.6 mEquiv/g. The internal pH falls by about half the external change.

With 0.02 mM tribasic acid present in the medium, the internal concentration will typically be 4 mM (12 mEquiv/g) with a concentration ratio of 200, Donnan ratio 'r' 5.84, internal pH 8.166. Reducing the pH_e to 7.0 increases 'r' to 7.93 making pH_i 7.90 ($7.0+\log 7.93$) and the internal concentration 10 mM ($7.93^3 \times 0.02$) or 33 mEquiv/g. In this example internal pH falls by about 60% of the external shift. The internal changes obviously depend on how much anion is present but are always less than those made in the medium. For this reason, titration of intact mitochondria follows a curve corresponding to less buffering power than the mitochondrial contents possess (as shown experimentally in Fig. 2; also shown by Mitchell & Moyle, 1967).

Factors Affecting the Titration Results

If permeant endogenous anions are present in small amounts within the mitochondria, neglecting them results in the sum of the measured anions becoming only slightly less than the proper total. If, however, the endogenous permeant anions provide initially, say, 10 mEquiv acid groups per g protein, then as other anion(s) are added, the initial contents of endogenous anions fall (as r falls) so their contribution to the overall total is lessened. This means that the quantity of the added, measured anion, changes by more than does the true total and that data obtained from incomplete measurements will fall on a steeper curve than corresponds to changes of X^- alone.

Changing the cation content alters the co-ordinates but not the slope of the curve. The internal impermeant anions (e.g., oxoglutarate under some conditions) affect the curve in the same way because they do not distribute according to the Donnan relation, and lower the effective concentration of the cations. It will be noted that no entries for internal chloride or bicarbonate are included in Table 1. There is evidence that under physiological conditions of pH and in the absence of chelating agents, Cl^- is not a permeant anion (*see* e.g., Chappell & Crofts, 1966). If any internal chloride is present it will have the effect of diminishing the total of positive charges contributed by the cations. It is assumed that the preparation of the mitochondria removes bicarbonate, though if any did remain it would contribute to the buffer (X^-) as it is impermeant.

It may be properly questioned whether many of the anions conventionally found to act as substrates for isolated mitochondria, particularly when hypotonic media are used, do in fact freely pass the membrane when the particles are in the cytoplasm. Addition of colloids certainly impedes the utilization of many anions (Harris, Tate, Manger & Bangham, 1971) by rat liver mitochondria. This restriction does not impair the usefulness of the concept of the internal buffer but it does imply that we need more detailed knowledge of mitochondrial permeability under physiological conditions.

Binding of Phosphate to Ca^{2+}

The internal phosphate has been noted to accumulate rather more than corresponds to the accumulation of the weak acid dimethyloxazolidinedione (used to calculate internal pH) by Hoek, Lofrumento, Meyer and Tager (1971) who supposed that binding of the phosphate was responsible. Rossi and Lehninger (1963) showed that about 1.7 moles of Ca accompany each extra mole of internal phosphate during the energy-dependent uptake of these two ions. They supposed that hydroxyapatite was being formed. The matter has been more recently reviewed by Lehninger (1970). A procedure for measurement of free phosphate (not combined with Ca) should really be applied when it is desired to make valid comparisons between accumulations of phosphate and other anions. Similarly, sinks for other anions may well exist under certain conditions; for example, Robinson *et al.* (1971) found that adding extramitochondrial magnesium caused the citrate equilibrium distribution to alter, presumably on account of chelation.

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