Biochem. J. (1978) **176**, 983–991
Printed in Great Britain

Anion/Calcium Ion Ratios and Proton Production in Some Mitochondrial Calcium Ion Uptakes

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(*Received* 23 *June* 1978)

The uptake of Ca^{2+} by liver mitochondria, when phosphate movement is inhibited, occurs when CO_2 is present and not in its absence. Uptake of Ca^{2+} to form $CaCO_3$ yields $2H^+/Ca^{2+}$. Heart mitochondria, when phosphate movement is inhibited, will take up Ca^{2+} with the exact equivalent of hydroxybutyrate, lactate or acetate. By providing a carrier for Cl^- with tributyltin, a stoicheiometric uptake of Cl^- with the Ca^{2+} takes place. The uptakes appear to occur without significant pH change; there appears to be no CO_2-dependent uptake into heart mitochondria. Oxygenation of anaerobic heart mitochondria, in the presence of an inhibitor of phosphate movement and of generation of phosphate from internal ATP, does not yield significant change of external acidity in relation to the amount of O_2 added. Use of Bromothymol Blue as an indicator of the distribution of a weak acid anion confirms that the transient nature of the response of the dye distribution to Ca^{2+} is connected with movement of endogenous phosphate. Bromothymol Blue accumulated in response to Ca^{2+} is discharged when entry of the Ca^{2+} (in the presence of mersalyl) is mediated with nigericin. It is concluded that Ca^{2+} uptakes will occur alternatively with the equivalent of anions or in exchange for endogenous K^+ and that proton production is connected with the changes of ionization of phosphate (unless phosphate movement is inhibited) and in liver mitochondria with the hydration of CO_2.

When mitochondria take up Ca^{2+} with expenditure of energy there is usually an acidification of the medium as well as an uptake of anions (Chance, 1965; Lehninger, 1970). The best-known example is the uptake of Ca^{2+} with P_i, which occurs in the ratio $1.67 Ca^{2+}$ ions/P_i molecule in intact mitochondria (Rossi & Lehninger, 1963) and 1.5 Ca^{2+} ions/P_i molecule in inner-membrane vesicles (Pedersen & Coty, 1972). It was suggested originally (Brierley *et al.*, 1964; Carafoli *et al.*, 1964) that the acidification of the medium was attributable to the formation of $Ca_3(PO_4)_2$, so that, according to the pH of the medium (Harris, 1973), the charge on the phosphate, whether endogenous or added, changed from −1.6 to −3 electronic charges. This change would release about 1.4 H^+ ions/P_i molecule or about 0.9 H^+ ion/Ca^{2+} ion.

This simple explanation of the origin of the acidity was questioned later, because (1) there was no evidence for formation of crystalline $Ca_3(PO_4)_2$, (2) without added phosphate about 1 H^+ ion appears per Ca^{2+} ion taken up to a limit of attainable uptake between 50 and 80nmol/mg of protein, (3) with added permeant acids, such as acetic, lactic, propionic and β-hydroxybutyric, there is still some acid formation (Rasmussen *et al.*, 1965), and (4) when inhibitors of phosphate movement are added to liver mitochondria there is an increase in the ratio H^+/bivalent cation (for Mn^{2+}, see Pozzan *et al.*, 1976; for Ca^{2+} ion, see Brand *et al.*, 1976*b*). In the latter paper the authors stress the importance of movement of endogenous phosphate in determining the ratio H^+ ion/Ca^{2+} ion observed in the presence of other permeant acids. If we accept the ubiquity of endogenous phosphate, which is well documented (Brand *et al.*, 1976*b*; Hoek *et al.*, 1971), then its movement with Ca^{2+} ion can explain point (2) above, and the ionization change can be the source of the acidity mentioned in point (3). Point (1) can be met by supposing that the $Ca_3(PO_4)_2$ remains in protein-associated amorphous form. Point (4) can now be met (Fig. 2 of the present paper) by invoking the additional generation of $2H^+$ ions for each Ca^{2+} ion accumulated as $CaCO_3$, by using dissolved CO_2 as the source of carbonate. Although the latter process was shown to occur by Elder & Lehninger (1973), the importance of the proton yield was not underlined. Thus inhibition of phosphate movement, which yields about 1 H^+ ion/Ca^{2+} ion, can leave the hydration of CO_2 with its yield of 2 H^+ ions/Ca^{2+} ion as a more prolific source of protons.

Brand *et al.* (1976*a*) found that the ratio between the uptakes of some permeant singly charged acid anions and Ca^{2+} ion by liver mitochondria became

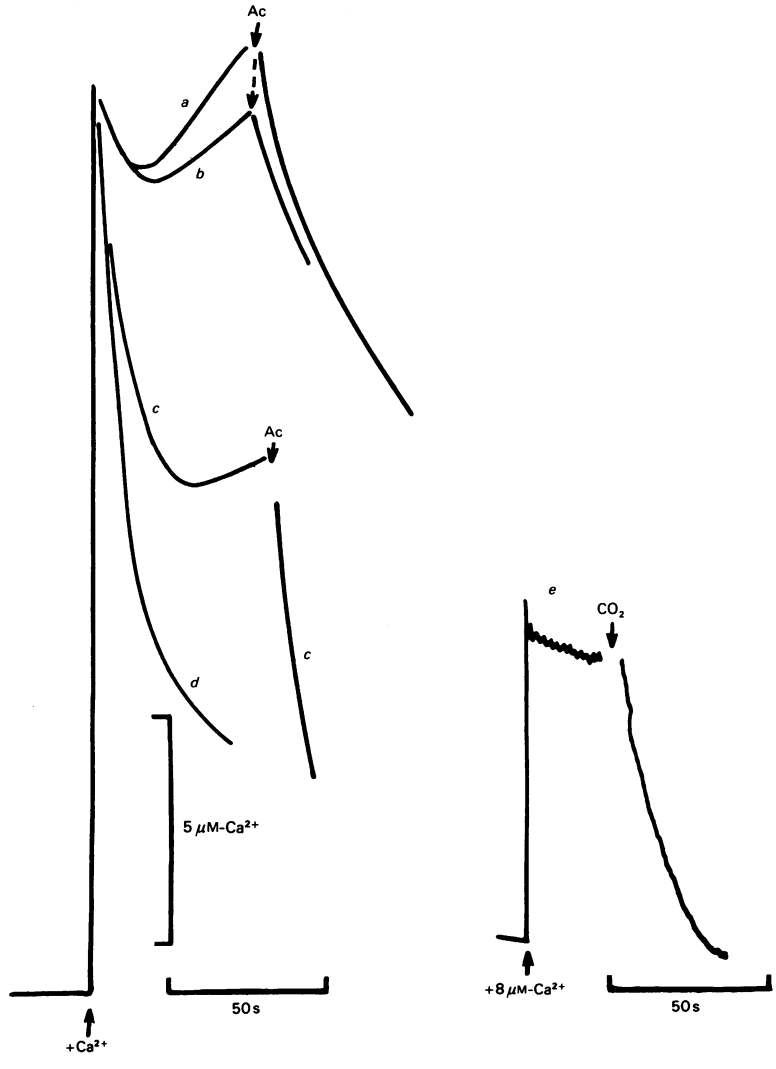

Fig. 1. *Effect of hydrated CO_2 on Ca^{2+} uptake by rat liver mitochondria*

The concentration of free Ca^{2+} ions in the mitochondrial suspension is indicated by the absorbance difference (685–675 nm) of the metallochrome dye Arsenazo III included in the medium. The suspension was supplemented with 20 μM-mersalyl to inhibit phosphate movement and with 2 μg of oligomycin/ml either to remove internal ATP or to prevent Ca^{2+}-ion-stimulated adenosine triphosphatase activity, since the latter would yield phosphate to react with the Ca^{2+} ions. Transfer of the added Ca^{2+} ions (20 μM for curves *a–d* and 8 μM for curve *e*) into the mitochondria is almost absent when either Diamox (20 μM) is added to inhibit carbonic anhydrase (curve *a*) or the medium is pre-gassed with pure O_2 to remove dissolved CO_2 (curves *b* and *e*). If the medium was used without pretreatment to remove dissolved CO_2, there was an appreciable uptake of Ca^{2+} ions (curve *c*), and if the medium was briefly pre-gassed with CO_2/O_2 (1:19), most of the added Ca^{2+} ions were taken into the mitochondria (curve *d*). An addition of acetate (Ac) at 10 mM to curves (*a*)–(*c*) restored the Ca^{2+} uptake, and a brief (5 s) passage of pure CO_2 through the suspension induced a rapid uptake of the Ca^{2+} ions in curve *e*. The medium contained 120 mM-KCl, 10 mM-Tris/Hepes, pH 7.2, 3 mM-Tris/succinate, 1 μg of rotenone/ml and sufficient Arsenazo III to provide an A_{520} of 1.5. The protein concentration was 1.4 mg/ml for curves *a–d* and 1.0 mg/ml for curve *e*, which also had 0.16 mg of serum albumin/ml. The albumin was necessary to ensure complete uptake of the Ca^{2+} in this condition. [Parts of this Figure and of Fig. 2 have appeared elsewhere (Harris, 1978), and these are reproduced by courtesy of *Nature*.]

2 mol of acid/Ca^{2+} ion for uptakes exceeding about 50nmol/mg of protein. Considerable Ca^{2+} ion was initially taken up with little of the measured anion; Brand *et al.* (1976*a*) suggested that movement of adventitious phosphate and possibly the hydration of CO_2 were playing a part, because no inhibitors were used. By using heart mitochondria such experiments are simplified, because there is then no obvious contribution from CO_2 to Ca^{2+}-ion uptake. With an inhibitor of phosphate movement and with oligomycin present (which leads to both low internal ATP and inhibited adenosine triphosphatase activity) the generation of internal P_i from ATP is prevented; I show below that in these conditions even small Ca^{2+}-ion uptakes are accompanied by the stoicheiometric amounts of selected measured anions. By inducing HCl permeability with tributyltin acetate (Harris *et al.*, 1973; Skilleter, 1975), it is also possible to show 2 Cl^- ions accumulating per Ca^{2+} ion.

Such findings might be expected on grounds of chemical equivalence, since accumulation of a chemically appreciable amount of Ca^{2+} in an object as small, and with as limited a buffering power, as the mitochondrion could not be balanced by OH^- ion before alkaline lysis overtook the protein. In fact, very little Ca^{2+} ion is taken up in the absence of a permeant anion (Harris & Zaba, 1977), and a report showing appreciable uptakes (Bygrave *et al.*, 1977) could be explicable by CO_2 leading to $CaCO_3$ formation.

The Bromothymol Blue anion applied at low concentration is taken into mitochondria when Ca^{2+} ions are applied, provided that competing anions are virtually absent (Chance & Mela, 1966; Ghosh & Chance, 1970). Since the location of the Bromothymol Blue can be followed optically, it has been used to confirm the importance of endogenous phosphate and also to observe the behaviour of a model anion under conditions of exchange of internal K^+ ions for Ca^{2+} ions, which is an alternative mode of preserving electroneutrality.

Methods

The methods have all been described before. Preparation of rat heart mitochondria was carried out as in Harris & Zaba (1977), and that of rat liver mitochondria as in Harris *et al.* (1971); in each case the final wash was given in a solution without added chelating agent other than 0.05% serum albumin. The protein concentrations of the final suspensions were measured by a biuret reaction calibrated with serum albumin. The Ca^{2+}-ion uptake was monitored by observing the change in absorbance of Arsenazo III (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) between 685 and 675nm on an Aminco type D.W.2 dual-wavelength spectrophotometer as described by Harris (1977). Measurement of anion uptakes referred to in Fig. 5 was made on small samples separated in a bench centrifuge as used for the ATP measurements described by Harris (1977). ^{14}C-labelled material (The Radiochemical Centre, Amersham, Bucks., U.K.) was used for lactate, β-hydroxybutyrate and acetate measurements; ^{36}Cl was used for the Cl^--ion measurements. Bromothymol Blue absorbance changes were followed with the double-wavelength spectrophotometer by using the wavelength pair 685–618nm, but otherwise as described by Chance & Mela (1966).

Results

The importance of CO_2 in determining the behaviour of liver mitochondria towards Ca^{2+} ions in the absence of phosphate movement is illustrated in Fig. 1. Mersalyl has been used to inhibit phosphate transfer (Tyler, 1970) and oligomycin to remove ATP; with either Diamox to inhibit carbonic anhydrase (as used by Elder & Lehninger, 1973) (curve *a*) or with medium pre-gassed with pure O_2 to remove CO_2 (curve *b*) there is little movement of added Ca^{2+} ions from the medium. With a medium equilibrated with the laboratory air there is a transfer of about 21.5nmol of Ca^{2+}/3.5mg of protein from medium to mitochondria (curve *c*). Prior gassing with CO_2/air (1:19) permitted removal of at least 35ng-ions of Ca^{2+} from the medium (curve *d*). Curve *e* shows the consequence of a brief exposure to a stream of 100% CO_2, which induces uptake of the Ca^{2+} ions from a previously inhibited suspension. The rate at which this uptake occurs compares with that obtained when phosphate is the added anion. It was necessary to supplement the suspension with albumin to obtain this result, because even a short exposure to Ca^{2+} ions under conditions of inhibited uptake otherwise prevents a sustained movement of Ca^{2+} ions into the particles. It seems probable that this effect is due to progressive damage caused by Ca^{2+}-ion-activated phospholipase activity. With heart mitochondria, no effect of CO_2 addition was found (results not shown).

The H^+ ions appearing in the medium when liver mitochondria took up Ca^{2+} ions in the presence of mersalyl and oligomycin from a medium pre-gassed with CO_2/O_2 (1:19) were close to $2H^+/Ca^{2+}$ (Fig. 2). In this condition there is, of course, a rapid drift of pH towards acidity because of hydration of the CO_2, but the immediate changes can be distinguished. Taken with Elder & Lehninger's (1973) conclusion that it is internal hydration of the CO_2 that takes place, the result implies a rapid counter-movement of protons against the Ca^{2+} ions.

Anion/Ca^{2+} ratios

To determine the stoicheiometry between the uptakes of Ca^{2+} ions and a given anion it is necessary

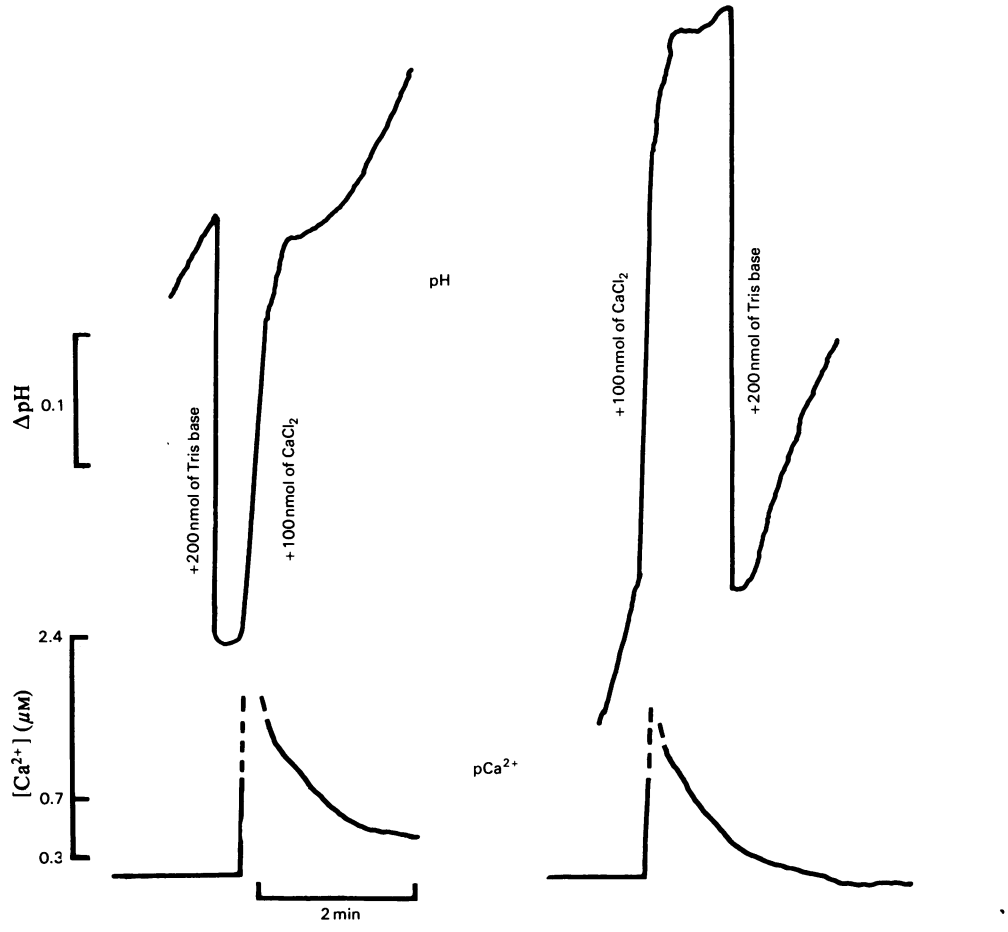

Fig. 2. *Production of acid when Ca^{2+} is taken up with CO_2*

The mitochondria were suspended in medium into which CO_2/O_2 (1:19) had been passed; the pH was adjusted with Tris base to 7.2 just before adding the mitochondria. Mersalyl was present at 20μM to inhibit phosphate movement and oligomycin at 2μg/ml was used to eliminate ATP as a source of phosphate. On addition of 100nmol of $CaCl_2$ with pH adjusted to 7.2 there is an immediate shift of pH towards acidity, and this was neutralized by adding 200nmol of Tris base, either after or before the $CaCl_2$. There is a continuous drift towards acidity because of hydration of the dissolved CO_2. A Ca^{2+}-sensitive electrode was used as a qualitative indicator to show that the Ca^{2+} had been taken up; the electrode is too sluggish to respond to the instantaneous Ca^{2+} concentration at the moment of addition. The medium was as in Fig. 1 with protein at 1.1mg/ml. The Ca^{2+}-electrode readings, which are on a log scale, were calibrated by using $CaCl_2$/nitrilotriacetic acid buffers (Reed & Bygrave, 1975).

to inhibit the movement of phosphate, whether present as such or potentially present as internal ATP, as well as to avoid participation of CO_2 when liver mitochondria are used. In the following experiments heart mitochondria were selected in order to avoid the latter interference. The movement of phosphate was inhibited with mersalyl, and ATP participation was inhibited with oligomycin. The heart mitochondria would still take up Ca^{2+} ions from media supplemented with lactate, acetate or β-hydroxybutyrate (Fig. 3). Additionally, and as reported by Stockdale *et al.* (1970) and Bygrave *et al.* (1977), the use of a tributyltin salt in a Cl^--ion-containing medium allows uptake of Ca^{2+} (Fig. 4). The numbers of equivalents of anions accumulated in the mitochondria were equal to the numbers of equivalents of Ca^{2+} ion added even for small additions (Fig. 5). Besides relating to heart and not to liver mitochondria, this result differs from Fig. 3 of Brand *et al.* (1976*a*) because the line passes through

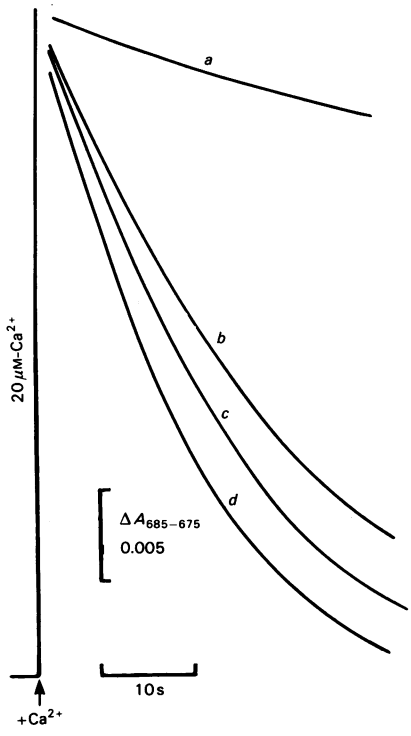

Fig. 3. *Uptakes of Ca^{2+} ions by heart mitochondria in the presence of salts of some organic acids*
The mitochondrial suspension was supplemented with mersalyl at 20μM and oligomycin at 2μg/ml, and the Ca^{2+}-ion uptake was measured after an addition of 20μM-$CaCl_2$. Without addition of the salt of an organic acid, some slow uptake (curve *a*) is probably due to endogenous fatty acids. Curves *b*, *c* and *d* show the uptakes that proceed in the presence of, respectively, lactate, β-hydroxybutyrate and acetate at 12mM as the Tris salts. The medium was as in Fig. 1 with protein at 1.8mg/ml.

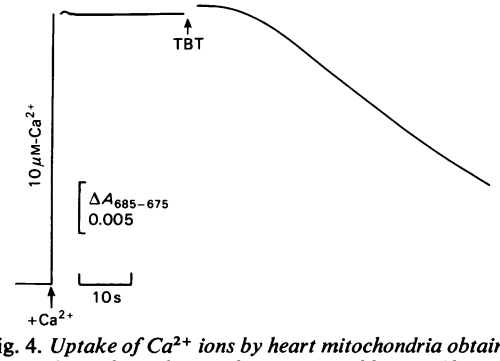

Fig. 4. *Uptake of Ca^{2+} ions by heart mitochondria obtained by making the membrane permeable to HCl*
With conditions the same as for the experiments of Fig. 3 there is little Ca^{2+}-ion uptake until the addition of tributyltin acetate (TBT) at 0.2μM. The protein was 0.7mg/ml.

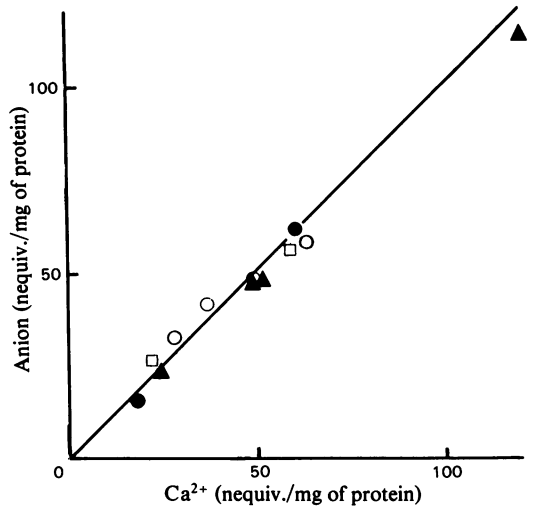

Fig. 5. *Stoicheiometry between Ca^{2+} uptakes and some anion uptakes in heart mitochondria*
Suspensions of heart mitochondria were supplemented with lactate (□), β-hydroxybutyrate (○) or acetate (●) at 12mM with 30–60nCi of ^{14}C-labelled compound/ml. Samples (0.5ml) were taken and centrifuged in small conical tubes in a Coleman Microfuge with a swing-out head. The supernatant was removed, and the pellet was washed with 120mM-KCl+10mM-Tris/Hepes and then extracted with 1.5M-$HClO_4$ to obtain a sample for measurement of the ^{14}C content of the pellet. A measured amount of $CaCl_2$ was then added to the suspension, and, after the Ca electrode showed that uptake of the Ca^{2+} was complete, a further similar sample was taken. The increment of anion was calculated from the additional ^{14}C found in the pellet; this was compared with the amount of Ca^{2+} added. For these three anions the medium was as in Fig. 1 with mersalyl (20μM) and oligomycin (2μg/ml). The Cl^- uptake (▲) was measured by using a medium containing 300mM-mannitol, 10mM-Tris/Hepes, 3mM-Tris/succinate and 20mM-KCl; the latter included a trace amount of $^{36}Cl^-$. The protein concentration was 1.8mg/ml.

the origin. In these conditions there appears to be little 'membrane binding' of the Ca^{2+} ions, though the cations and anions taken up may be associated with the internal protein.

These uptakes of Ca^{2+} ions along with weak acid anions were unaccompanied by appreciable or consistent acidification of the medium (Table 1). This is reasonable, since the acids used would be almost fully dissociated at the pH of the medium, so there would be little scope for proton production by further ionization in a somewhat more alkaline mitochondrial interior. Tests made with certain substrates including succinate, pyruvate and oxoglutarate, showed that little Ca^{2+} uptake was evoked

on making the addition to the metabolizing mitochondria. Glutamate and malate gave rise to appreciable removal of free Ca^{2+} ions by chelation, so it is not possible by this method to observe small movements of the Ca^{2+} into the mitochondria which might be produced on their addition.

Table 1. *Immediate changes of acidity of the medium in response to the addition of* 100*nmol of* $CaCl_2$ *(with pH adjusted to that of the medium) to an aerobic suspension of rat heart mitochondria in the presence of the Tris salts of some penetrant acids*

The medium contained 120mM-KCl, 20mM-Tris/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid], pH7.2, 3mM-Tris/succinate, 1μg of rotenone/ml and 2.2mg of mitochondrial protein/ml. The results of several independent experiments are tabulated. Mersalyl was present at 50μM and oligomycin at 2μg/mg of protein.

Penetrant acid as Tris salt	Concn. (mM)	Change of acidity in response to the $CaCl_2$ (ng-atoms of H^+)
β-Hydroxybutyrate	20	Nil, +15
Acetate	12.5	+17, −13, −5, −8
Propionate	7.5	Nil, nil, +10

Oxygen-pulse experiments

Brand *et al.* (1976*b*) have described a measurement of a H^+/O per site ratio depending on the transient acidification seen when a pulse of oxygenated medium is added to an anaerobic suspension supplemented with succinate and rotenone so that two phosphorylating sites are operative. They showed that phosphate had leaked from the mitochondria during the anaerobic phase, and accordingly tested the effect of *N*-ethylmaleimide as an inhibitor of phosphate movement on the ratio. It increased from 2 to 3 H^+ ions/O per site. The involvement of phosphate was further emphasized by showing that removal of phosphate by washing also raised the ratio to 3. It is suggested here from the result in Fig. 2 that the higher proton yield can have been due to a change from movement of P_i to movement of CO_2 with the Ca^{2+} ions.

It seemed of interest to aim in the other direction, that is, towards no H^+ production in response to oxygenation by using heart mitochondria in the presence of mersalyl and oligomycin. Adding pulses of oxygenated medium to these mitochondria did not alter the $[H^+]$ in the medium by more than 8nmol/ml in response to oxygen additions providing 82ng-atoms of O/ml. At the normal ratio of 2 Ca^{2+}/O (Chance, 1965) and with phosphate to yield 1 H^+

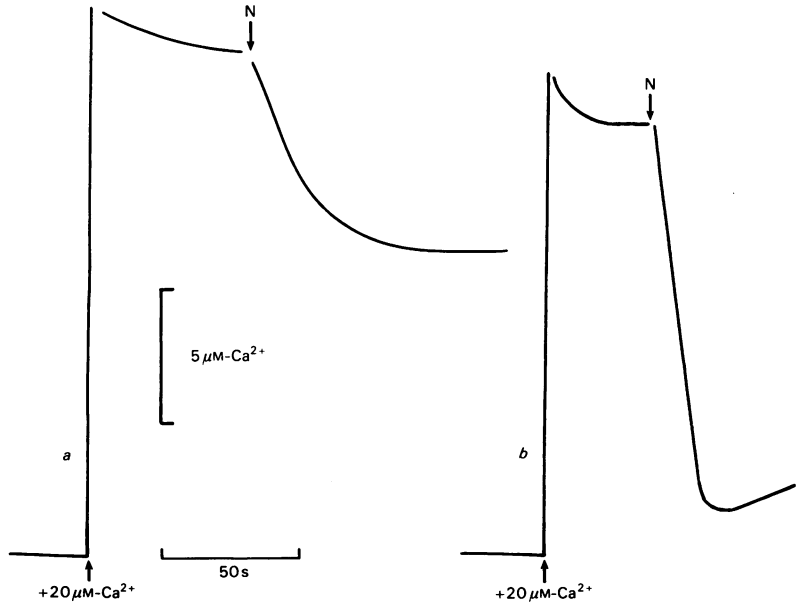

Fig. 6 *Uptakes of* Ca_2^+ *mediated with nigericin*

Suspensions of (*a*) heart and (*b*) liver mitochondria supplemented with mersalyl (40μM) and oligomycin (2μg/ml) did not take up appreciable amounts of Ca^{2+} until an addition of nigericin at N (to 25ng/ml) was made. The media were as for Fig. 1, pre-gassed with O_2, with the protein at concentrations of (*a*) 0.8 and (*b*) 1.3 mg/ml. Ca^{2+} uptake was determined as described in Fig. 1.

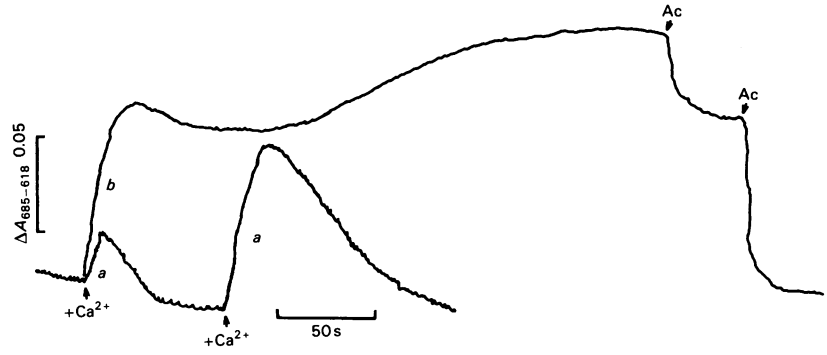

Fig. 7. *Effect of mersalyl on the uptake of Bromothymol Blue in response to Ca^{2+}*
The differential absorbance of a suspension of liver mitochondria was measured between 685 and 618 nm in the presence of Bromothymol Blue added to 4 μM. Trace *a*, obtained without mersalyl, shows a transient rise due to Bromothymol Blue uptake when a first addition of Ca^{2+} (to 40 μM) is made and a larger but still transient rise in response to a second addition. Trace *b*, obtained with mersalyl present at 20 μM, shows a larger and prolonged uptake of the dye in response to the Ca^{2+} addition, which in this experiment is eventually discharged by adding acetate (Ac) first to 1.25 and then to 6.25 mM. The medium was as described in Fig. 1, but contained Bromothymol Blue instead of Arsenazo III.

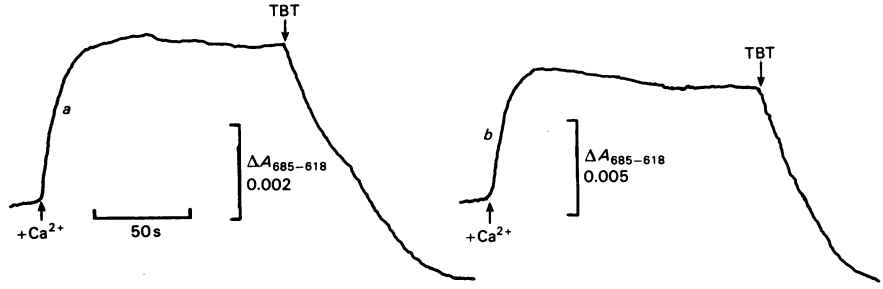

Fig. 8. *Discharge of Bromothymol Blue on induction of μCl permeability*
The uptake of Bromothymol Blue was induced by adding Ca^{2+} ions (40 μM) to suspensions of (*a*) heart and (*b*) liver mitochondria in the presence of mersalyl (20 μM) and oligomycin (2 μg/ml). On addition of tributyltin acetate to 0.8 μM (at TBT), the dye is discharged. Taken with the results of Figs. 4 and 5 showing that the tributyltin induces an uptake of Ca^{2+} accompanied with Cl^-, it appears that the competing Cl^-, present at much higher concentration, expels the internal dye anion. The medium was as in Fig. 1 with Bromothymol Blue at 4 μM instead of Arsenazo III. The protein concentrations were (*a*) 0.8 mg/ml and (*b*) 1.1 mg/ml. The absorbance difference was measured between 685 and 618 nm.

ion per Ca^{2+} ion, the 82 ng-atoms of O would have yielded 164 nmol of H^+ ions.

Cation exchange as a mechanism for preserving electroneutrality during Ca^{2+} uptake

Although the main purpose of this paper is to emphasize that an electroneutral uptake of Ca^{2+} with its equivalent of anions normally occurs, it seemed important for completeness to mention an alternative mechanism. Scarpa & Azzone (1970) showed that Ca^{2+} uptake could be induced by valinomycin at the expense of running the internal K^+ out down a gradient. Alternatively uptake of Ca^{2+} ions by respiring mitochondria when limited by anion supply can be restarted with nigericin (see Fig. 4 of Rottenberg & Scarpa, 1974). Estrada-O *et al.* (1972) have illustrated the simultaneous addition of Ca^{2+} and nigericin, which caused an efflux of K^+ with uptake of Ca^{2+} and little change of external pH. The effects of nigericin on anion-limited Ca^{2+} movements into heart and liver mitochondria suggest that the cation exchanges are limited to a movement of 20–25 nequiv./mg of protein (Fig. 6). This mode of uptake will be shown in the next section to be accompanied by a discharge of weak acid, that is by a presumed acidification in contrast with the alkalinization supposed to accompany uptake with anions (Addanki *et al.*, 1968).

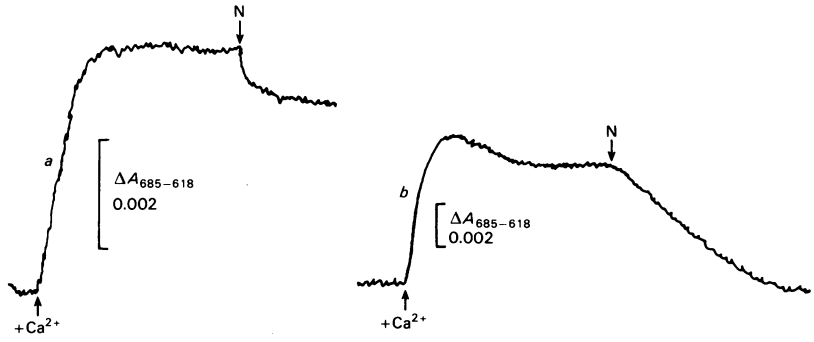

Fig. 9. *Uptake and discharge of Bromothymol Blue on addition respectively of Ca^{2+} ions and of nigericin*
The uptake of Bromothymol Blue was induced as in Figs. 7 and 8 by adding Ca^{2+} ions (40μM) to suspensions of (*a*) heart and (*b*) liver mitochondria in media supplemented with mersalyl at 20μM and oligomycin at 2μg/ml. On addition of nigericin (at N, to 40ng/ml) there is a partial or complete discharge of the dye. Taken with the record of Ca^{2+} movement induced by nigericin in Fig. 6 it appears that, although the ionophore permits Ca^{2+} uptake to proceed in quantity, there is also a shift towards acidity in the interior so that the dye anion is protonated and so no longer retained. The medium was as in Fig. 1, but containing Bromothymol Blue at 4μM instead of Arsenazo III; protein concentrations and wavelength settings were as in Fig. 8.

Response of Bromothymol Blue accumulation to agents affecting Ca^{2+} uptake

The 'binding' or uptake of a number of anionic dyes has been shown to increase when Ca^{2+} is taken by the mitochondria (Colonna *et al.*, 1972). As noted by Chance & Mela (1966), the increased absorbance associated with dye uptake is small and transient in response to a first limited Ca^{2+} addition, whereas a second addition provides a larger and longer-lasting response (Fig. 7, curve *a*). Since it is proposed that endogenous phosphate plays an important role in accompanying Ca^{2+} and such entry of competing anion would displace the Bromothymol Blue from the mitochondria, it is interesting that mersalyl both increases and prolongs the uptake of the dye (Fig. 7, curve *b*). Addition of a permeant anion (acetate) leads to discharge of the dye.

In the presence of mersalyl the retention of the dye by the mitochondria provides a sensitive indicator for either entering competing anions or of conversion of the dye anion into the protonated acid, which is not accumulated. Fig. 8 illustrates the discharge of the dye by Cl^- after tributyltin acetate has been used to mediate Cl^- permeation. Induction of K^+ permeability with nigericin also leads to discharge of some of the dye anion (Fig. 9). Since nigericin is known to induce internal acidification (Pressman *et al.*, 1967), it is reasonable to ascribe this to protonation of the dye anion.

Discussion

The principal aim of these experiments has been to show that there is a simple explanation for the frequent appearance of protons in variable yield per Ca^{2+} ion taken up by mitochondria. It is that phosphate and CO_2 are commonly present; in liver mitochondria both can give rise to acidity when they accumulate respectively as $Ca_3(PO_4)_2$ and $CaCO_3$ with yields of $1H^+$ ion and $2H^+$ ions per Ca^{2+} ion. The slight excess of the Ca^{2+} ion/P_i ratio above the theoretical value of 1.5, noted by Rossi & Lehninger (1963), may have occurred because of some $CaCO_3$ formation. The limited quantity of internal phosphate generated by Ca^{2+}-ion-stimulated hydrolysis of internal ATP (Harris & Berent, 1969) is also a source of H^+ ions, but this process can be inhibited with oligomycin (Brand & Lehninger, 1975).

When phosphate movement and generation are prevented, and CO_2 hydration is absent as a source of carbonate, as is the case with heart mitochondria, then Ca^{2+} ions can be taken up with their equivalent of anions of weak organic acids without shift of pH (Table 1). This Ca^{2+} ion-dependent anion uptake is likely to be of metabolic importance, because more energy is obtainable by oxidizing the anion than is needed to accumulate the Ca^{2+} ion.

The results obtained with Bromothymol Blue as model anion show that conditions of apparent internal alkalinization (anion uptake) or of apparent acidification (anion discharge) can both be associated with uptakes of Ca^{2+} ions.

Thanks are due to the British Heart Foundation for continuing support and to Dr. M. C. W. Evans of the Department of Botany, University College London, for the use of the spectrophotometer. Tributyltin acetate was a gift from Dr. J. Cremer of the M. R. C., Carshalton. I am grateful for advice on presentation to Professor H.

Baum and to Dr. B. Banks. Professor A. L. Lehninger, in correspondence, commended to me the importance of measuring the anion/Ca^{2+}-ion ratios in heart mitochondria.

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