Calcium Ion Cycling in Rat Liver Mitochondria

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1. Addition of *N*-ethylmaleimide to rat liver mitochondria respiring with succinate as substrate decreases both the initial rate of Ca^{2+} transport and the ability of mitochondria to retain Ca^{2+} . As a result, Ca^{2+} begins to leave the mitochondria soon after it has entered. Half-maximal effects occur at an *N*-ethylmaleimide concentration of about 100 nmol/mg of protein. 2. The efflux of Ca^{2+} induced by *N*-ethylmaleimide is not prevented by Mg^{2+} or by Ruthenium Red at concentrations known to prevent Ca^{2+} efflux when exogenous phosphate also is present. Swelling of mitochondria does not accompany *N*-ethylmaleimide-induced Ca^{2+} efflux. 3. Addition of Ca^{2+} to rat liver mitochondria in the presence of *N*-ethylmaleimide produces an immediate decrease in ΔE (membrane potential), which decreases further to only a slight extent over the next 8 min. Concomitant with this is an immediate increase and then levelling off of the $-59 \Delta \text{pH}$ (transmembrane pH gradient). 4. Preincubation of rat liver mitochondria with *p*-chloromercuribenzenesulphonate, which by contrast with *N*-ethylmaleimide is unable to penetrate the inner mitochondrial membrane, also prevents Ca^{2+} retention. The ΔE and $-59 \Delta \text{pH}$ respond to Ca^{2+} addition in a manner similar to that which occurs when *N*-ethylmaleimide is present. Subsequent addition of mercaptoethanol produces an immediate increase in both ΔE and $-59 \Delta \text{pH}$. At the same time Ca^{2+} is rapidly accumulated by the organelles. 5. The above data are interpreted as indicating that under the conditions of Ca^{2+} efflux seen here, the mitochondria retain their functional integrity. This contrasts with the uncoupling effect of Ca^{2+} seen in the presence of P_i , which generally leads to a loss of mitochondrial integrity. We suggest that a unique mechanism of Ca^{2+} cycling is able to take place when mitochondria have been treated with *N*-ethylmaleimide.

Several avenues of research now have provided considerable evidence that, apart from generating the bulk of the cell ATP, mitochondria in many tissues play an important biological role in controlling the concentration of intracellular Ca^{2+} (Bygrave, 1978). Recognition of this fact in turn has induced thought as to how mitochondrial Ca^{2+} transport itself might be regulated, especially since a number of metabolites and hormones are capable of influencing the transport system.

By analogy with the substrate-cycle concept discussed extensively by Newsholme & Crabtree (1976), we have begun to consider the control of Ca^{2+} fluxes across the inner membrane of mitochondria in terms of a Ca^{2+} -translocation cycle (Bygrave, 1978). Newsholme & Crabtree (1976) have suggested that the energy-requiring 'pump' and passive 'leak' components of ion-translocation systems in biomem-

branes may constitute a 'substrate' cycle, a view with which we are in full agreement. As pointed out by these authors, a particular attribute of substrate cycles is that they provide amplification and great sensitivity in metabolic regulation. In the present context also a great range of effectors of Ca^{2+} flux could be accommodated whether they be mass-action or allosteric in nature (Bygrave, 1978). The energy-requiring component of the mitochondrial Ca^{2+} -translocation cycle would be the Ruthenium Red-sensitive carrier and the operational equivalent of the 'leak' component would be the mechanism(s) involved in Ca^{2+} efflux from mitochondria. Whether the latter occur via a specific carrier, as suggested for example by Sordahl (1974), Cockrell (1976), Puskin *et al.* (1976) and Carafoli *et al.* (1977), or is explainable from considering only the known properties of the inward transporting system (Pozzan *et al.*, 1977), remains to be determined.

One ubiquitous metabolite especially influential on mitochondrial Ca^{2+} movements is P_i . The ability of the anion to increase both the initial rate and the

Abbreviations used: Δp , protonmotive force (proton electrochemical gradient); ΔE , membrane potential; $-z\Delta \text{pH}$, transmembrane pH gradient ($z = RT/nF \approx 59$ at 25°C).

extent of Ca^{2+} transport by mitochondria is well established (see Bygrave, 1977). This permeant anion can be transported into mitochondria on a *N*-ethylmaleimide-sensitive P_i/OH^- antiporter (Coty & Pedersen, 1974) or via a butylmalonate-sensitive $\text{P}_i/\text{dicarboxylate}$ antiporter (Johnson & Chappell, 1973). Whether the anion can be transported also on a La^{3+} -sensitive calcium/phosphate symporter (Moyle & Mitchell, 1977) is not yet established.

An important recent development in this area of study was the report of Harris & Zaba (1977) that mitochondria are unable to transport Ca^{2+} in the presence of *N*-ethylmaleimide and oligomycin (the latter being added to prevent the generation of internal P_i via adenosine triphosphatase). These data indicated that the Ca^{2+} transport occurring in the absence of added P_i was attributable to cycling of endogenous P_i via the above-mentioned P_i -transport systems in mitochondria and highlighted the critical role of P_i movement in mitochondrial Ca^{2+} fluxes.

We have confirmed the finding of Harris & Zaba (1977) that the initial rate of Ca^{2+} transport in liver and heart mitochondria is significantly decreased when cycling of P_i is prevented by *N*-ethylmaleimide (Bygrave *et al.*, 1977), but in further investigations we have observed that the prevention of P_i cycling induced a release of the accumulated Ca^{2+} , a finding that fully supports our earlier view (Reed & Bygrave, 1975) that the endogenous P_i decreases the rate of Ca^{2+} efflux from mitochondria.

In the present paper we describe some properties of Ca^{2+} cycling that exist under conditions where P_i cycling is restricted. In particular, evidence is presented that the mechanism of Ca^{2+} efflux seen in these conditions differs in significant respects from that which occurs in the presence of exogenous P_i .

Experimental

Isolation of mitochondria

The livers from rats (Wistar albino strain weighing approx. 200 g and killed by cervical dislocation) were rapidly removed and placed in ice-cold isolation medium containing 250 mM-sucrose, 5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and 0.5 mM-EGTA adjusted to pH 7.4 with KOH. The livers were minced with scissors and homogenized by two passes with a glass/Teflon tissue disintegrator (A. H. Thomas Co., Philadelphia, PA, U.S.A.; size C), which was motor-driven at 900 rev./min. The resulting suspension was made up to 80 ml with isolation medium and centrifuged at 900 g for 5 min in a Sorvall RC-2B refrigerated centrifuge fitted with a SS-34 rotor. The pellets were resuspended in the above medium and centrifuged at 800 g for 5 min. The combined supernatants were centrifuged at 4500 g for 5 min. The mitochondrial pellets were

washed twice with EGTA-free isolation medium by centrifuging at 4500 g for 5 min, and resuspended in this at a protein concentration of 70 mg/ml.

Mitochondrial protein concentration was assayed by the biuret method (Layne, 1957) after solubilization with deoxycholate (Jacobs *et al.*, 1956). Corrections were made for non-biuret colour and turbidity by subsequent cyanide treatment (Szarkowska & Klingenberg, 1963).

Protonmotive force

The protonmotive force (Δp) was determined by using the ion-distribution technique of Nicholls (1974). The incubation medium contained 150 mM-LiCl, 0.5 mM-KCl, 3 mM-Hepes (pH 7.4 with Tris), 5 mM-sodium succinate, 10 μM - $^{86}\text{RbCl}$ (0.12 $\mu\text{Ci/ml}$), 50 μM - ^{14}C methylamine (0.3 $\mu\text{Ci/ml}$), 50 μM -sodium ^{3}H acetate (1.2 $\mu\text{Ci/ml}$), 1 μM -rotenone and 0.5 μM -valinomycin. The scintillation fluid contained 6 g of butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole]/litre in toluene/2-methoxyethanol (3:2, v/v) containing 10% Biosolv BBS-3 (Beckman Instruments, Fullerton, CA, U.S.A.). Radioactivity was counted by using the three channels of the Packard Tri-Carb scintillation counter. Corrections were made for cross-over and background. The three discriminator control settings were such that cross-overs of 21 and 3.6% were allowed from the ^{32}P channel into the ^{14}C and ^3H channels respectively, and 26.8% was allowed from the ^{14}C channel into the ^3H channel. The components of Δp were calculated exactly as described by Nicholls (1974). A limiting matrix volume of 0.4 $\mu\text{l/mg}$ of mitochondrial protein was used for the calculations, as assumed by Nicholls (1974) and Mitchell & Moyle (1969).

Calcium transport

Ca^{2+} transport was measured by the EGTA/Ruthenium Red quench technique (Reed & Bygrave, 1974). The reaction medium was held in a water-jacketed vessel with constant stirring and contained 150 mM-KCl, 3 mM-Hepes (pH 7.4), 5 mM-succinate and 1 μM -rotenone. The solution was left to equilibrate to a temperature of 15 or 25°C as indicated in Figure legends, after which time 1 mg of mitochondrial protein/ml was added. Then 25 μM - CaCl_2 (containing 0.5 μCi of $^{45}\text{Ca}^{2+}$) was added and the reaction terminated at the times indicated by transferring 100 μl into Eppendorf tubes containing ice-cold 150 mM-KCl, 100 μM -EGTA and 2 μM -Ruthenium Red. The quenched solutions were immediately centrifuged (Eppendorf Microfuge, 2 min at 12000 g), and samples of the supernatant transferred to vials containing 10 ml of scintillation fluid (as above) and counted for radioactivity to less than 1% error on a Packard Tri-Carb scintillation counter.

Swelling

Light-scattering by mitochondrial suspensions was measured by continuously recording the A_{520} with a Varian Techtron split-beam recording spectrophotometer.

Materials

All radiochemicals used were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals used were of analytical grade.

Results

Effect of N-ethylmaleimide on mitochondrial Ca²⁺ transport

Data in Fig. 1(a) show the influence of the permeant thiol-specific reagent *N*-ethylmaleimide (Gaudemer & Latruffe, 1975; LeQuoc *et al.*, 1976) on mitochondrial Ca²⁺ transport. The initial rate of transport was decreased by about 25% and the maximum uptake by about 35%. These results suggest that when cycling of P_i is restricted by *N*-ethylmaleimide, mitochondria have a limited capacity to accumulate Ca²⁺, a conclusion reached previously by Brand *et al.* (1976) and by Harris & Zaba (1977). Fig. 1(a) also shows that in addition to lowering the capacity of mitochondria for Ca²⁺, *N*-ethylmaleimide treatment prevented the organelle from retaining the ion; extrusion of Ca²⁺ sets in after about 2 min, suggesting an essential role of P_i translocation in the retention of accumulated Ca²⁺.

Data in Fig. 1(b) show the effect of various concentrations of *N*-ethylmaleimide on the initial rate of Ca²⁺ transport, on the extent of transport and on the amount of Ca²⁺ in the mitochondria 8 min after the addition of Ca²⁺. Half-maximal effect for all three events occurred at approx. 100 nmol of *N*-ethylmaleimide/mg of protein.

Effect of Mg²⁺ on the N-ethylmaleimide-induced efflux of Ca²⁺

Because the release of Ca²⁺ in the presence of exogenous P_i is invariably associated with the uncoupling of mitochondria, i.e. increased permeability of the inner membrane to H⁺ (Mitchell, 1966), we considered it important to examine the mechanism of Ca²⁺ efflux induced by *N*-ethylmaleimide, as seen in Fig. 1(a). The effects of Mg²⁺ were examined first, especially as the ion is known to inhibit the efflux of Ca²⁺ completely when added P_i is present (Haugaard *et al.*, 1969a; Drahota *et al.*, 1965). Data in Fig. 2(a) show that Mg²⁺ is unable to prevent efflux of Ca²⁺ in *N*-ethylmaleimide-treated rat liver mitochondria and as such provide the initial evidence that the efflux observed in the presence of P_i is different from that observed when P_i movements are inhibited. Data in Fig. 2(a) also confirm the finding of Åkerman *et al.* (1977) and of Hutson *et al.* (1976) that Mg²⁺ diminishes the initial rate of Ca²⁺ transport in rat liver mitochondria. Thus the inhibition by Mg²⁺ of the initial rate of Ca²⁺ transport occurs in both the absence and the presence of concomitant

Fig. 1. *Effect of N-ethylmaleimide on the transport of Ca²⁺ by rat liver mitochondria*

Mitochondria (1 mg/ml) were equilibrated aerobically at 15°C for 1 min, in 2.0 ml of medium containing 150 mM-KCl, 3 mM-Hepes (pH 7.4 with KOH), 5 mM-succinate and 1 μM-rotenone; 25 μM-CaCl₂ containing ⁴⁵CaCl₂ (0.5 μCi) was added and Ca²⁺ transport followed as described in the Experimental section. (a) Time course in the absence (○) or presence (●) of 200 μM-*N*-ethylmaleimide. (b) Influence of *N*-ethylmaleimide concentration on initial rate (i.e. Ca²⁺ in the mitochondria at 10 s) (●), maximum extent of transport (○) and amount of Ca²⁺ in the mitochondria after 8 min (▲).

Fig. 2. Effect of Mg^{2+} and Ruthenium Red on the efflux of Ca^{2+} induced by *N*-ethylmaleimide

The reaction media were as described in Fig. 1: (a) 200 μM -*N*-ethylmaleimide in the absence (○) or presence (●) of 2 mM- $MgCl_2$; (b) 200 μM -*N*-ethylmaleimide present (○), and Ruthenium Red (200 pmol/mg of protein) was added at the point indicated with the arrow (●).

P_i movement across the inner mitochondrial membrane.

Data in Fig. 2(b) provide the second piece of evidence that Ca^{2+} efflux occurring in the absence of P_i cycling is different from that occurring in its presence. Ruthenium Red, which is reported to prevent Ca^{2+} efflux from mitochondria when P_i is present (Carafoli *et al.*, 1977; Luthra & Olson, 1977), had no effect on that occurring when *N*-ethylmaleimide is first preincubated with the mitochondria.

Changes in the protonmotive force in the presence of *N*-ethylmaleimide and Ca^{2+}

Fig. 3(a) shows how the addition of Ca^{2+} to mitochondria incubated with succinate alters the electrical and chemical components of the proton-

Fig. 3. Effect of *N*-ethylmaleimide and Ca^{2+} on the protonmotive force

Mitochondria (1 mg/ml) were equilibrated aerobically at 25°C for 2 min in 4.0 ml of medium containing 150 mM-LiCl, 0.5 mM-Hepes, 5 mM-succinate, 10 μM -⁸⁶RbCl, 50 μM -[¹⁴C]methylamine, 50 μM -sodium [³H]-acetate, 1 μM -rotenone and 0.5 μM -valinomycin. At zero time 25 μM - $CaCl_2$ was added and at appropriate intervals samples were filtered. The filter papers were dissolved in the scintillation fluid and the protonmotive force (a) was calculated as described in the Experimental section. (b) Ca^{2+} transport was followed under identical conditions, but in the absence of radiochemicals, by using ⁴⁵Ca (see the Experimental section), except that KCl in the quench mixture was replaced by LiCl. (a) —, Control; ----, +200 μM -*N*-ethylmaleimide; ▲, protonmotive force; ○, membrane potential; ●, -59 ΔpH. (b) ○, Control; ●, +200 μM -*N*-ethylmaleimide.

Fig. 4. Effect of *p*-chloromercuribenzenesulphonate on the protonmotive force

Incubation conditions were identical with those described in Fig. 3. (a) —, Control; ---, 100 μM-*p*-chloromercuribenzenesulphonate; ▲, protonmotive force; ○, membrane potential; ●, -59 ΔpH. (b) ---, +100 μM-*p*-chloromercuribenzenesulphonate; ···, after the addition (arrowed) of 1 mM-mercapto-

ethanol; ▲, protonmotive force; ○, membrane potential; ●, -59 ΔpH. (c) ○, Control; ●, +100 μM-*p*-chloromercuribenzenesulphonate; ▲, after adding 1 mM-mercaptoethanol at arrow.

After a 120 s preincubation with *N*-ethylmaleimide, the Δ*E* and -59 ΔpH were about 10 and 5 mV lower respectively than the control values. Addition of Ca²⁺ at zero time produced a rapid decrease in Δ*E*, the change being about twice that of the control. The Δ*E* then remained at the new value without showing any sign of returning to the original value. The -59 ΔpH also increased on addition of Ca²⁺ and the increase was approximately twice that of the control and then remained constant for the duration of the experiment. The Δ*p* remained at much the same value for the duration of the experiment. These findings suggest that the inner mitochondrial membrane has not been damaged by *N*-ethylmaleimide treatment and therefore that the inability of mitochondria to retain Ca²⁺ is not associated with a loss in mitochondrial integrity.

Fig. 3(b) shows that under the conditions of the above experiment, Ca²⁺ movements were qualitatively similar to those shown in Fig. 1(a).

Changes in the protonmotive force in the presence of *p*-chloromercuribenzenesulphonate and Ca²⁺

Because the mechanism of action of *N*-ethylmaleimide on phosphate transport involves irreversible formation of a covalent bond, it was thought that further details about the involvement of P_i movements in the present work might be gained from using the non-penetrating thiol-group inhibitor *p*-chloromercuribenzenesulphonate, whose inhibitory effects also are known to be readily reversed by mercaptoethanol. Preliminary experiments indicated that maximal effects of the inhibitor on inducing Ca²⁺ efflux were obtained at a concentration of approximately 100 nmol/mg of protein (data not shown).

As shown in Fig. 4(a), addition of 100 μM-*p*-chloromercuribenzenesulphonate lowered the Δ*E* by about 65 mV. Addition of Ca²⁺ produced a rapid decrease in Δ*E* the change in which was about twice that of the control. The Δ*E* progressively decreased also, by contrast with the control. The -59 ΔpH increased only slightly after addition of Ca²⁺ when *p*-chloromercuribenzenesulphonate also was present; this progressively diminished with time. The proton-

motive force also decreased steadily with time, so that after 8 min its magnitude was only about 115 mV.

Fig. 4(c) shows that Ca^{2+} transport as measured under conditions similar to those described for Fig. 4(a) was only slight in the presence of *p*-chloromercuribenzenesulphonate; any Ca^{2+} accumulated was soon released into the medium.

Addition of mercaptoethanol to the *p*-chloromercuribenzenesulphonate-treated mitochondria (Fig. 4b) induced a rapid increase in both components of the protonmotive force. The increase in ΔE was more rapid and by 1 min had gone from 75 to about 120 mV, whereas that of the $-59 \Delta \text{pH}$ rose quickly from 65 to 85 mV and then gradually increased to about 120 mV after 5 min.

At the same time Ca^{2+} was rapidly accumulated by the mitochondria (Fig. 4c).

Two features of the experiment are particularly noteworthy. The first is that because mercaptoethanol was able to reverse the inhibitory effect of *p*-chloromercuribenzenesulphonate on Ca^{2+} transport and the establishment of the protonmotive force, it is likely that the *p*-chloromercuribenzenesulphonate treatment did not greatly influence the functional integrity of the inner mitochondrial membrane. The second point is that even though the ΔE attained a value of only about 120 mV after the addition of mercaptoethanol (i.e. some 30 mV less than that of the control), the amount of Ca^{2+} accu-

mulated (Fig. 4c) was about the same as that of the control and was retained by the mitochondria. This raises the possibility that the ΔE is not the rate-limiting factor in Ca^{2+} accumulation and retention by the mitochondria, at least under the conditions used. The marked increase in Δp is also noteworthy.

Changes in mitochondrial volume in the presence of N-ethylmaleimide

The efflux of Ca^{2+} seen in the presence of added P_i is associated with a large amplitude swelling of mitochondria (Haugaard *et al.*, 1969a; Chappell & Crofts, 1965).

Fig. 5 shows that addition of Ca^{2+} to mitochondria incubated with *N*-ethylmaleimide produced a slight contraction as seen in the control; see also Chappell & Crofts (1965). However, after such contraction there was no further absorbance change. The data show too that addition of Ca^{2+} to mitochondria treated with *p*-chloromercuribenzenesulphonate resulted in slight swelling which, although it increases gradually with time, was arrested immediately after the addition of mercaptoethanol.

These findings thus provide a third piece of evidence that Ca^{2+} efflux occurring in the absence of P_i movements is different from that occurring in the presence of exogenous P_i .

Discussion

Data in this and in a previous paper (Bygrave *et al.*, 1977) confirm the findings of Brand *et al.* (1976), Moyle & Mitchell (1977) and Harris & Zaba (1977) that the presence of *N*-ethylmaleimide in the reaction medium restricts Ca^{2+} transport into mitochondria isolated from rat liver. A second major consequence of *N*-ethylmaleimide treatment of mitochondria revealed in this and the earlier work (Bygrave *et al.*, 1977) is that the organelle no longer is able to retain accumulated Ca^{2+} . Thus *N*-ethylmaleimide treatment has a dual effect on Ca^{2+} -cycling in rat liver mitochondria; it prevents Ca^{2+} influx and promotes Ca^{2+} efflux.

The present studies show that near-maximal effects of *N*-ethylmaleimide on Ca^{2+} -cycling were obtained at approx. 150 nmol/mg of protein. This value may be compared with that of approx. 90 nmol/mg of protein obtained by Hutson (1977), who examined the effect of *N*-ethylmaleimide on the steady-state kinetics of energy-dependent Ca^{2+} transport in rat liver mitochondria. Haugaard *et al.* (1969b) studied the effect of 5,5'-dithiobis-(2-nitrobenzoic acid) on Ca^{2+} transport by rat liver mitochondria and observed that near-maximal inhibition of influx occurred at a concentration of approx. 50 nmol/mg of protein. No effect of the thiol-group inhibitor was seen on Ca^{2+} efflux, presumably because of the inclusion in the reaction medium of ATP, which

Fig. 5. Volume changes of mitochondria during Ca^{2+} transport in the absence and presence of *N*-ethylmaleimide or *p*-chloromercuribenzenesulphonate

Conditions were as in Fig. 3, except that the reaction was started with $0.5 \mu\text{M}$ -valinomycin. After 2 min incubation, $25 \mu\text{M}$ - CaCl_2 was added. $\circ$, Control; $\bullet$, $+200 \mu\text{M}$ -*N*-ethylmaleimide; $\blacktriangle$, $+100 \mu\text{M}$ -*p*-chloromercuribenzenesulphonate; at the point indicated by the arrow, 1 mM-mercaptoethanol was also added ($\triangle$).

itself would induce Ca²⁺ retention by mitochondria. According to these workers, *N*-ethylmaleimide produced effects similar to those of 5,5'-dithiobis-(2-nitrobenzoic acid).

As pointed out in the introduction, anion-transport systems whose activities influence Ca²⁺ retention by mitochondria include the P_i- and adenine nucleotide-translocation systems (Bygrave, 1978); each is sensitive to thiol-group inhibitors. In the present experiments the activities of each should have been minimal, since the concentrations of *N*-ethylmaleimide required to inhibit P_i transport via the P_i/OH⁻ exchange is in the range 30 nmol/mg of protein (Coty & Pedersen, 1974) to approx. 90 nmol/mg of protein (Klingenberg *et al.*, 1974). Approximately 60 nmol/mg of protein is required to inhibit the P_i/P_i exchange when butylmalonate also is present to inhibit the P_i/dicarboxylate exchange (Meijer *et al.*, 1970) and near-maximal inhibition of adenine nucleotide translocation occurs at *N*-ethylmaleimide concentrations of 30 nmol/mg of protein (Leblanc & Clauser, 1972; Vignais & Vignais, 1972). The *N*-ethylmaleimide-induced efflux of Ca²⁺ cannot be attributed on the basis of the present experiments solely to an action of the inhibitor on P_i and adenine nucleotide movements, but some assessment of the likelihood can be made.

Studies by Sabadie-Pialoux & Gautheron (1971) indicate that the number of free thiol groups in rat liver mitochondria approach 90 nmol/mg of protein, but this number can vary according to the experimental conditions used. Addition of ADP plus P_i to well-coupled mitochondria, for instance, leads to an increase by some 30% of free thiol groups (Sabadie-Pialoux & Gautheron, 1971). This could reflect a conformational change in the (inner) mitochondrial membrane that is *N*-ethylmaleimide-sensitive (Leblanc & Clauser, 1972; Vignais & Vignais, 1972). Other conditions under which the reactivity of thiol groups in mitochondria is altered include the anionic composition of the reaction medium, the pH, and the metabolic status of the mitochondria (see Scott *et al.*, 1970; Klingenberg *et al.*, 1974). Moreover, evidence exists that in addition to reacting with thiol groups, *N*-ethylmaleimide reacts with side-chain amino groups that contain active hydrogens (Brewer & Riehm, 1967).

Thus although in the first instance it would seem unlikely that inhibition of P_i transport alone contributes to the induced Ca²⁺ efflux, the possibility may not be so unrealistic when consideration to the above is given. It is worth recalling also that the interaction of Ca²⁺ with rat liver mitochondria produces changes in the microscopic appearance of the organelle (Greenawalt *et al.*, 1964), which also might lead to unmasking of thiol groups.

Inhibition by *N*-ethylmaleimide of succinate oxidation, and therefore the driving force for Ca²⁺

transport, seems unlikely on two grounds; first, the Δp is only slightly decreased in the presence of the compound (Fig. 3), and, second, addition of tributyltin to *N*-ethylmaleimide-treated mitochondria, induces an immediate influx of Ca²⁺ into mitochondria (Bygrave *et al.*, 1977). It has been suggested that specific *N*-ethylmaleimide-sensitive groups exist close to the Ca²⁺ carrier (Bygrave *et al.*, 1977; Hutson, 1977); our data do not rule out the possibility that the thiol-group inhibitor interacts directly with these.

Previous studies in many laboratories have shown that efflux of Ca²⁺ from mitochondria occurs as a result of 'damage' to the mitochondrial inner membrane. Such efflux is associated, for example, with swelling of mitochondria, an increase in adenosine triphosphatase activity, and concomitant loss of ability of the mitochondria to carry out oxidative phosphorylation (see Saris, 1963; Bygrave & Reed, 1970). These events, which are considerably enhanced by the presence of P_i, are attributable to the uncoupling effect of Ca²⁺ on mitochondrial energy-linked functions originally observed by Lehninger (1949).

For this reason it was important to assess the 'integrity' of the mitochondria after Ca²⁺ efflux under the present experimental conditions. The experiments revealed (Figs. 3–5) that swelling of the organelle does not accompany Ca²⁺ release, nor does the protonmotive force change to an extent that reflects a complete loss of impermeability to H⁺, a finding that would provide evidence of membrane 'damage'. Indeed, the almost complete reversibility of the system in terms of swelling, Ca²⁺ transport and the protonmotive force were clearly demonstrable with the appropriate additions of *p*-chloromercuribenzenesulphonate and mercaptoethanol.

Further evidence that the mechanism of Ca²⁺ efflux seen in the present report differs from that occurring in the presence of P_i was the finding that Mg²⁺ and Ruthenium Red, each of which inhibits Ca²⁺ efflux in the presence of P_i (Ernster, 1956; Haugaard *et al.*, 1969a; Sordahl, 1974; Luthra & Olson, 1977; Carafoli *et al.*, 1977), were ineffective in preventing Ca²⁺ efflux under the present incubation conditions. Moreover, other experiments in this laboratory (V. Prpic, T. L. Spencer & F. L. Bygrave, unpublished work) have shown that glucagon administration to rats *in vivo*, which offsets the uncoupling action of Ca²⁺ on isolated mitochondria described above, is without effect on the Ca²⁺ efflux seen here (see also Dorman *et al.*, 1975).

The assemblage of these data thus shows that the mechanism of efflux of Ca²⁺ from *N*-ethylmaleimide-treated mitochondria is distinctly different from that seen in the presence of P_i cycling. The present report certainly emphasizes the important and possibly vital physiological role played by thiol groups in the movement of Ca²⁺ across the inner mitochondrial

membrane (Lehninger, 1974; Reed & Bygrave, 1975; Bygrave, 1977; Smith & Bygrave, 1978), especially in regard to their interaction with the Ca^{2+} -translocation cycle located in that membrane (Bygrave, 1978).

References

- Åkerman, K. E. O., Wikström, M. K. F. & Saris, N.-E. (1977) *Biochim. Biophys. Acta* **464**, 287–294
- Brand, M. D., Chen, C. H. & Lehninger, A. L. (1976) *J. Biol. Chem.* **251**, 968–974
- Brewer, C. F. & Riehm, J. P. (1967) *Anal. Biochem.* **18**, 248–255
- Bygrave, F. L. (1977) *Curr. Top. Bioenerg.* **6**, 259–318
- Bygrave, F. L. (1978) *Biol. Rev. Cambridge Philos. Soc.* **53**, 43–79
- Bygrave, F. L. & Reed, K. C. (1970) *FEBS Lett.* **7**, 339–342
- Bygrave, F. L., Ramachandran, C. & Smith, R. L. (1977) *FEBS Lett.* **83**, 155–158
- Carafoli, E., Crompton, M. & Malmstrom, K. (1977) *Eur. Symp. Hormones and Cell Regulations 1st* (Dumont, J. & Nunez, J., eds.), pp. 157–166, North-Holland Publishing Co., Amsterdam
- Chappell, J. B. & Crofts, A. R. (1965) *Biochem. J.* **95**, 378–386
- Cockrell, R. S. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1759
- Coty, W. A. & Pedersen, P. L. (1974) *J. Biol. Chem.* **249**, 2593–2598
- Dorman, D. M., Barritt, G. J. & Bygrave, F. L. (1975) *Biochem. J.* **150**, 389–395
- Drahota, Z., Carafoli, E., Rossi, C. S., Gamble, R. L. & Lehninger, A. L. (1965) *J. Biol. Chem.* **240**, 2712–2720
- Ernster, L. (1956) *Exp. Cell Res.* **10**, 704–720
- Gaudemer, Y. & Latruffe, N. (1975) *FEBS Lett.* **54**, 30–34
- Greenawalt, J. W., Rossi, C. S. & Lehninger, A. L. (1964) *J. Cell Biol.* **23**, 21–38
- Harris, E. J. & Zaba, B. (1977) *FEBS Lett.* **79**, 284–290
- Haugaard, N., Haugaard, E. S. & Lee, N. H. (1969a) *Proc. K. Ned. Akad. Wet. Ser. C* **72**, 1–15
- Haugaard, N., Lee, N. H., Kostrzewa, R., Horn, R. S. & Haugaard, E. S. (1969b) *Biochim. Biophys. Acta* **172**, 198–204
- Hutson, S. M. (1977) *J. Biol. Chem.* **252**, 4539–4545
- Hutson, S. M., Pfeiffer, D. R. & Lardy, H. A. (1977) *J. Biol. Chem.* **251**, 5251–5258
- Jacobs, E. E., Jacobs, M., Sanadi, D. R. & Bradley, L. B. (1956) *J. Biol. Chem.* **223**, 147–156
- Johnson, R. N. & Chappell, J. B. (1973) *Biochem. J.* **134**, 769–774
- Klingenberg, M., Durand, R. & Guerin, B. (1974) *Eur. J. Biochem.* **42**, 135–150
- Layne, E. (1957) *Methods Enzymol.* **3**, 447–454
- Leblanc, P. & Clauser, H. (1972) *FEBS Lett.* **23**, 107–110
- Lehninger, A. L. (1949) *J. Biol. Chem.* **178**, 625–644
- Lehninger, A. L. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1520–1524
- LeQuoc, D., LeQuoc, K. & Gaudemer, Y. (1976) *Biochem. Biophys. Res. Commun.* **68**, 106–113
- Luthra, R. & Olson, M. S. (1977) *FEBS Lett.* **81**, 142–146
- Meijer, A. J., Groot, G. S. P. & Tager, J. M. (1970) *FEBS Lett.* **8**, 41–44
- Mitchell, P. (1966) *Biol. Rev. Cambridge Philos. Soc.* **41**, 445–502
- Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **7**, 471–484
- Moyle, J. & Mitchell, J. (1977) *FEBS Lett.* **73**, 131–136
- Newsholme, E. A. & Crabtree, B. (1976) *Biochem. Soc. Symp.* **41**, 61–109
- Nicholls, D. G. (1974) *Eur. J. Biochem.* **50**, 305–315
- Pozzan, T., Bragadin, M. & Azzone, G. F. (1977) *Biochemistry* **16**, 5618–5625
- Puskin, J. S., Gunter, T. E., Gunter, K. K. & Russell, P. R. (1976) *Biochemistry* **15**, 3834–3842
- Reed, K. C. & Bygrave, F. L. (1974) *Biochem. J.* **140**, 143–155
- Reed, K. C. & Bygrave, F. L. (1975) *Eur. J. Biochem.* **55**, 497–504
- Sabadie-Pialoux, N. & Gautheron, D. (1971) *Biochim. Biophys. Acta* **234**, 9–15
- Saris, N. E. L. (1963) *Soc. Sci. Fenn.* **28**, 1–59
- Scott, K. M., Knight, V. A., Settlemire, C. T. & Brierley, G. P. (1970) *Biochemistry* **9**, 714–723
- Smith, R. L. & Bygrave, F. L. (1978) *Biochem. J.* **170**, 81–85
- Sordahl, L. A. (1974) *Arch. Biochem. Biophys.* **167**, 104–115
- Szarkowska, L. & Klingenberg, M. (1963) *Biochem. Z.* **338**, 674–697
- Vignais, P. V. & Vignais, P. M. (1972) *FEBS Lett.* **26**, 27–31