Journal of Bioenergetics and Biomembranes, Vol. 22, No. 1, 1990

Regulation of Ca^{2+} Fluxes in Rat Liver Mitochondria by Ca^{2+}. Effects on Ca^{2+} Distribution

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Received July 10, 1989; revised August 9, 1989

Abstract

Rat liver mitochondria are able to temporarily lower the steady-state concentration of external Ca^{2+} after having accumulated a pulse of added Ca^{2+}. This has been attributed to inhibition of a putative $\Delta\psi$-modulated efflux pathway [Bernardi, P. (1984) *Biochim. Biophys. Acta* **766**, 277–282]. On the other hand, the rebounding could be due to stimulation of the uniporter by Ca^{2+} [Kröner, H. (1987) *Biol. Chem. Hoppe-Seyler* **369**, 149–155]. By measuring unidirectional Ca^{2+} fluxes, it was found that the uniporter was stimulated during the rebounding peak both under Bernardi's and Kröner's conditions, while no effects on the efflux could be demonstrated. The rate of unidirectional efflux of Ca^{2+} was not affected by inhibition of the uniporter. It appears likely that the rebounding is due to stimulation of the uniporter rather than inhibition of efflux.

Key Words: Calcium uniporter; calcium efflux; liver mitochondria; membrane potential; regulation of uniporter.

Introduction

Respiring rat liver mitochondria are able to lower the external Ca^{2+} concentration to around 1 μM (Saris and Åkerman, 1980). It is frequently observed that after uptake of an added aliquot of Ca^{2+}, the concentration is temporarily decreased even below this level. This phenomenon of rebounding is of interest since it indicates the presence of regulatory mechanisms in the mitochondrial transport of Ca^{2+}.

At the steady state the rates of influx and efflux of Ca^{2+} are, by definition, equal. A change in the steady-state level can be brought about either by

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a stimulation of influx or an inhibition of efflux (or a combination of these mechanisms). Bernardi (1984) interpreted the rebounding to an inhibition of efflux of Ca^{2+} by a $\Delta\psi$-modulated pathway[3] postulated on the basis of changes seen in the steady-state levels of Ca^{2+} under experimental conditions with slightly lowered $\Delta\psi$ (Bernardi and Azzone, 1982, 1983). In this particular case the lowering of $\Delta\psi$ would be brought about by influx of Ca^{2+} on the uniporter driven by the $\Delta\psi$, negative on the matrix side (Åkerman, 1978). The evidence in favor of this model was circumstantial and another mechanism involving stimulation of influx must also be considered, especially since there is ample evidence of the modulation of the uniporter activity; see reviews by Saris and Åkerman (1980) and Åkerman and Nicholls (1983). Thus, there is an inhibition by Mg^{2+}, making the Ca^{2+} uptake kinetics more sigmoidal (Åkerman 1977; Åkerman *et al.*, 1977; Bragadin *et al.*, 1979). Indeed, Bernardi (1984) found Mg^{2+} to inhibit the rebounding presumably by lowering the rate of Ca^{2+} influx sufficiently to allow the respiration to maintain the $\Delta\psi$. However, stimulation of uniporter activity by cations has also been reported. Stimulation by Ca^{2+} of Mn^{2+} transport by the uniporter in the presence of Mg^{2+} was observed early (Chance and Mela, 1966; Mela and Chance 1968; Vinogradov and Scarpa, 1973; Ernster *et al.*, 1978). These findings and the sigmoidicity were interpreted as indicating either two transport sites (Vinogradov and Scarpa, 1973) or the presence of a transport and a regulatory site (Ernster *et al.*, 1978). Mn^{2+} transport was stimulated also by spermine (Saris *et al.*, 1969).

Of more interest in this context is the stimulation of Ca^{2+} uptake by the uniporter at low steady state concentrations of Ca^{2+}. The set point of external Ca^{2+} was shifted to lower values by Mn^{2+} in the presence of Mg^{2+} and inorganic phosphate (Hughes and Exton, 1983; Allshire *et al.*, 1985, Allshire and Saris, 1986). Polyamines had the same effect (Nicchitta and Williamson, 1984; Allshire and Saris, 1986; Kröner, 1988). Of special significance is the stimulation of the uniporter by Ca^{2+} itself (Kröner 1986a,b, 1987). The stimulation persisted for minutes after Ca^{2+} had been removed and was observed in the presence of Mg^{2+} (Kröner, 1986, 1987). It is evident that such a hysteretic behavior could well account for a rebounding effect.

In this study we have measured unidirectional fluxes of Ca^{2+} in liver mitochondria both under the conditions of Bernardi (1984) and those of Kröner (1986a,b, 1987). We find that the rebounding is due to stimulation of Ca^{2+} influx on the uniporter.

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[3]Abbreviations: $\Delta\psi$, membrane potential; DMNTA, dimethylnitrilotriacetate; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; MOPS, 3-morpholinopropane sulfonic acid; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane.

Materials and Methods

Rat liver mitochondria were prepared by a conventional procedure as described by Allshire *et al.* (1985). Ca^{2+} uptake was measured as described by Kröner (1986a) using ^{45}Ca-labelled Ca^{2+} and DMNTA to buffer Ca^{2+} at 2 μM and Mg^{2+} when present at 1 mM. Ca^{2+} efflux was measured by using respiring mitochondria preloaded with ^{45}Ca and measuring the radioactivity in the mitochondria. For measurement of unidirectional fluxes, mitochondria were diluted tenfold with medium without disturbing the steady-state concentration of Ca^{2+} followed by filtration. The concentration of Ca^{2+} in the medium was measured with a Ca-sensitive electrode as described by Allshire *et al.* (1985). The amount of releasable Ca^{2+} was estimated by measuring the increase in Ca^{2+} in the medium following the addition of an uncoupling agent, FCCP. The quantity $\Delta\psi$ was measured with the aid of [^{14}C]$TMPP^+$ used at a concentration of 1.6 μM essentially as described by Bernardi and Azzone (1983) but for a shorter centrifugation step of 1 min. Protein was measured with a *biuret* procedure in the presence of cholate with bovine serum albumin as standard.

Radioactive substances were obtained from the Radiochemical Centre, Amersham, U.K. FCCP was a kind gift of Dr. P. G. Heutler. DMNTA was synthesized as described by Kröner (1986a). Ruthenium red was purified by recrystallisation as described by Fletcher *et al.* (1961).

Incubations were carried out at pH 7.0 and at 30°C in the Kröner medium (110 mM sucrose, 75 mM KCl, 5 mM acetate, 2 mM succinate, 5 μM rotenone, and $MgCl_2$ to give 1 mM in the presence of DMNTA and 10 mM TES buffer) or at room temperature (ca. 23°C) in the Bernardi medium (140 mM sucrose, 40 mM choline chloride, 10 mM Tris-MOPS, 1 mM P_i, 2 mM succinate, 5 μM rotenone, and 2 μg/ml oligomycin). For the measurement of unidirectional fluxes 10 mM DMNTA and 31 μM $CaCl_2$ were added to give 2 μM free Ca^{2+} in the presence of DMNTA.

Results

The conditions for obtaining a rebounding of external Ca^{2+} had to be found by varying the amount of Ca^{2+} added to the suspension of respiring mitochondria. Figure 1 shows that a rebounding was obtained both under the conditions of Kröner (1986a,b), Fig. 1A, and those of Bernardi (1984), Fig. 1B. Figure 1 also shows the general design of the experiment. After the establishment of a steady state in Ca^{2+} distribution, 30 μM Ca^{2+} was added. The mitochondria were able to quickly take up the added Ca^{2+} *plus* additional Ca^{2+} so that a higher *p*Ca was reported by the Ca-sensitive electrode.

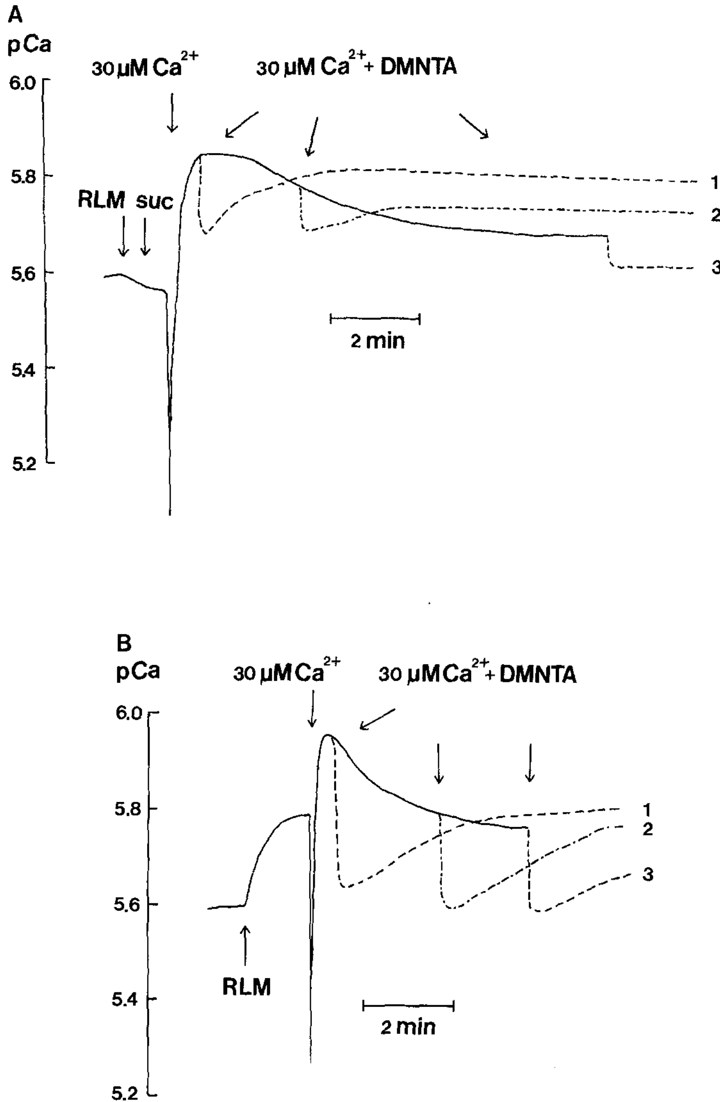

Fig. 1. The rebounding of Ca^{2+}. Rat liver mitochondria (RLM), 1 mg protein/ml, and 30 μM Ca^{2+} were added as indicated. After the addition of 2 mM succinate (suc) and 30 μM Ca^{2+} as indicated, 5 mM DMNTA and 30 μM Ca^{2+} were added at arrows at the peak of rebounding (1), during the return to the steady-state level before the first addition of Ca^{2+} (2), and when the steady-state level was almost reached (3). The traces after the addition are indicated by broken lines. (A) Kröner's medium (1987); (B) Bernardi's medium (1984). For details, see Materials and Methods.

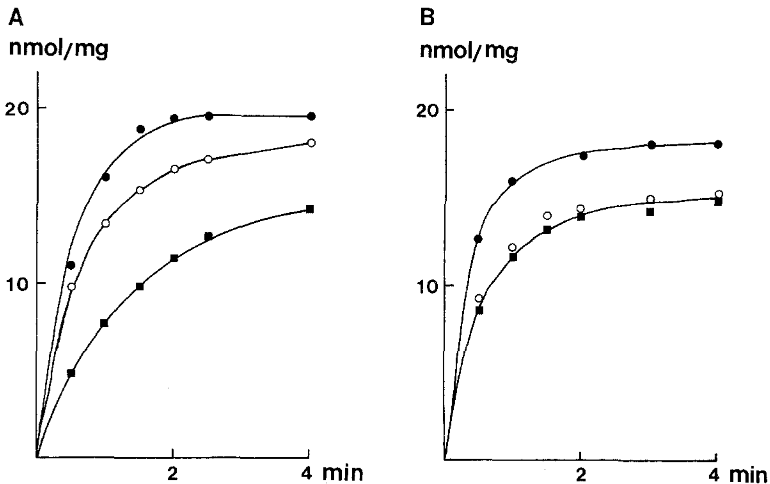

Fig. 2. Ca^{2+} influx during Ca^{2+} rebounding. Conditions were as in Fig. 1. Unidirectional influx was studied by using ^{45}Ca-labelled Ca^{2+} with the addition of 5 mM DMNTA and 30 μM Ca^{2+} to buffer Ca^{2+} at 2 μM. The radioactivity of mitochondria separated by ultrafiltration through 0.45 μm filters was measured. Filled circles, DMNTA and Ca^{2+} added at "1" in Fig. 1; open circles, DMNTA and Ca^{2+} added at "2" in Fig. 1; squares, DMNTA and Ca^{2+} added at "3" in Fig. 1. (A) Kröner's medium; (B) Bernardi's medium.

In the Kröner medium, A, pCa levels slowly returned toward the original steady-state level, while in Bernardi's medium, B, changes were faster. Addition of DMNTA plus ^{45}Ca and 30 μM Ca^{2+} to bring the free Ca^{2+} concentration to 2 μM caused a shift to lower pCa values. These additions were made at various points of the rebounding and allowed an estimation of initial rates of Ca^{2+} influx. It is seen that when the additions were made at the peak of rebounding, mitochondria were still able to change the Ca^{2+} concentration toward lower values despite the presence of the calcium buffer while the changes were smaller or absent when the additions were made when the original steady state was being approached. Additions were made at the peak of the rebounding, 1; when the peak was decaying, 2; and when the steady-state was being approached, 3.

In Fig. 2 the mitochondrial Ca^{2+} contents are shown for the experiments of Fig. 1. The rising curves show the increase in ^{45}Ca-labelled Ca^{2+} in the mitochondria and are thus a measure of the influx rates. The rates at the rebounding peak (filled circles) are clearly the highest, followed by these at the decay of the peak (open circles) and at the end of the decay (squares), both under Kröner's conditions, A, and under those of Bernardi, B. Figure 3 shows the behavior of ^{45}Ca-preloaded mitochondria in parallel experiments. There was no inhibition of efflux during the rebounding peak. Ruthenium red or Ca^{2+}-chelating agents could not be used since they might influence efflux

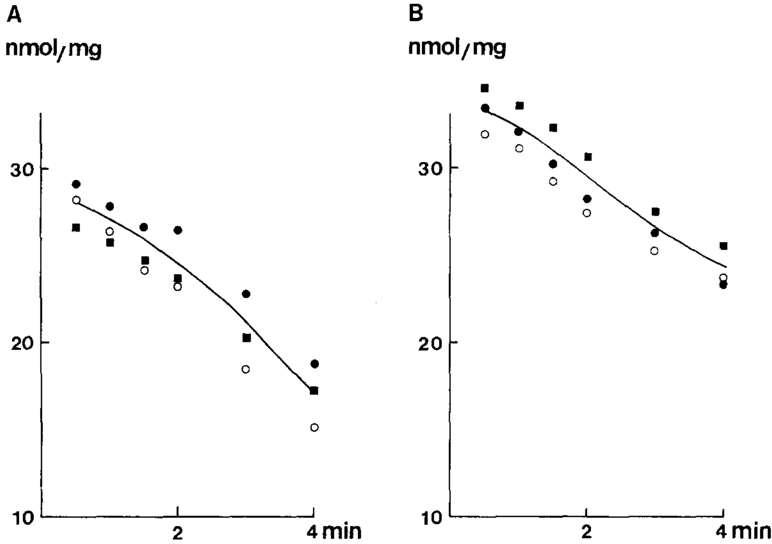

Fig. 3. Unidirectional Ca^{2+} efflux during Ca^{2+} rebounding. Conditions and symbols are as in Fig. 2 but for the addition of ^{45}Ca with the first addition of Ca^{2+}. At the points corresponding to "1"–"3" in Fig. 1, the mitochondrial suspension was diluted tenfold with the medium containing 5 mM DMNTA *plus* enough Ca^{2+} to keep the *p*Ca level unchanged during the dilution; thereafter, samples were filtered and the radioactivity of the mitochondria measured. (A) Kröner's medium; (B) Bernardi's medium.

rates on the putative $\Delta\psi$-gated pathway by raising $\Delta\psi$ (Åkerman, 1978). The $\Delta\psi$ was estimated under Kröner's conditions at points corresponding to the sampling at the rebounding peak (condition 1 in Fig. 1) and when the steady-state was approached again (condition 3 in Fig. 1); see Table I. Changes were slight at most, without statistically significant differences, while Bernardi (1984) reported that $\Delta\psi$ was clearly lowered during the rebounding.

Table I. $\Delta\psi$ during Ca^{2+} Rebounding[a]

Conditions	$\Delta\psi$ (mV)	Standard error
During rebounding peak	202.6	3.1
+ DMNTA + Ca^{2+}	194.6	3.6
After return to steady state	212.3	3.2
+ DMNTA + Ca^{2+}	193.5	3.1

[a]Experimental conditions were as in Fig. 1A but in the presence of 1.6 μM [^{14}C]-$TMPP^+$. Mitochondria were removed at the rebounding peak, immediately after the addition "1" in Fig. 1, just before and just after addition "3" in Fig. 1. The $\Delta\psi$ was calculated by the Nernst equation from the distribution of $TMPP^+$ as described in Materials and Methods. The data represent the data from three experiments using different preparations.

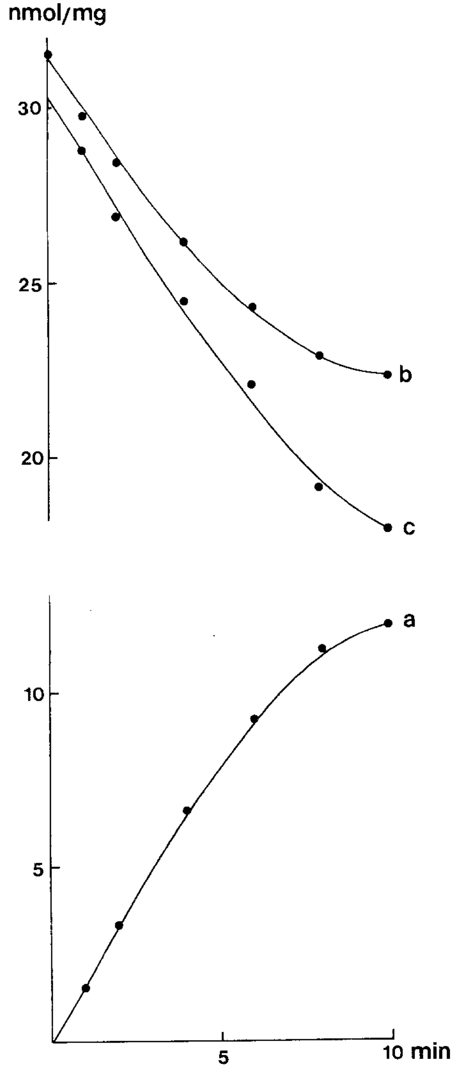

Fig. 4. Ca^{2+} efflux in the presence and absence of influx. Mitochondria, 1 mg protein/ml, were incubated in Kröner's medium. After 1 min 31 μM $CaCl_2$ was added in the experiments "b" and "c." After 2 min, 2.5 mM DMNTA was added. After a further 1 min the experiment was started by the following additions: in "a," 31 μM labelled with ^{45}Ca and DMNTA to a final concentration of 5 mM; in "b," the same as in "a," but without ^{45}Ca; in "c," 1 μM ruthenium red added before the second addition of Ca^{2+} or the second addition omitted.

The rate of unidirectional efflux of Ca^{2+} was independent of the activity of the uniporter. In the experiments shown in Fig. 4 Ca^{2+} was added in two consecutive aliquots. Line *a* represents an experiment in which the second addition of Ca^{2+} was labeled with ^{45}Ca. It shows unidirectional Ca^{2+} uptake. Line *b* shows the corresponding experiments in which mitochondrial Ca^{2+} was labeled by including ^{45}Ca in the first addition of Ca^{2+}. It shows the unidirectional efflux during the uptake of the second aliquot of Ca^{2+}. Line *c* shows the efflux of Ca^{2+} when the uniporter was inhibited by ruthenium red or the influx of Ca^{2+} prevented by chelation to DMNTA. The initial rates of efflux were the same in the experiments depicted by lines *b* and *c*, while the divergence with time was due to the dilution of mitochondrial label due to the influx of Ca^{2+}; see line *a*.

The $\Delta\psi$ was measured by the $TMPP^+$ technique under Kröner's conditions (Table I). The $\Delta\psi$ was high throughout the experiment—ca. 200 mV with no statistically significant changes during the experiments.

Discussion

The data presented in Fig. 1 show that a rebounding of Ca^{2+} can be obtained under a variety of conditions. Bernardi and Azzone (1983) and Bernardi (1984) reported that changes in the membrane potential by various means influenced the distribution of Ca^{2+} across the inner membrane in liver mitochondria. A change in the distribution could be due to changes in the rates of influx or/and efflux of Ca^{2+}. The authors preferred to interpret their data as indicating a stimulation of the efflux rate above a $\Delta\psi$ of ca. 130 mV. Below this $\Delta\psi$ there is an ohmic relationship between the rate of influx and the $\Delta\psi$ (Nicholls, 1978). Evidence in support of this interpretation is coherent but circumstantial: the similarities in dependence of the Na^+-independent Ca^{2+} efflux rate and the rebounding upon temperature and pH. The inhibition of rebounding by Mg^{2+} was explained as being due to inhibition of the rate of Ca^{2+} influx and thereby on the lowering of the $\Delta\psi$ (Bernardi, 1984). The time-related changes in Ca^{2+} distribution could be explained by the slow recovery of $\Delta\psi$ after a pulse of Ca^{2+} (Åkerman, 1978).

On the other hand, Kröner (1986a,b, 1987) has shown that the uniporter activity is stimulated by small amounts of Ca^{2+} and that this effect exhibits hysteresis, i.e., the effect persists for several minutes after the concentration of Ca^{2+} has been reduced by uptake into the mitochondria. Such a behavior could well account for the rebounding phenomenon. Ca^{2+} changes the kinetics of the uniporter from the pronounced sigmoidicity seen in the presence of Mg^{2+} to a more hyperbolic dependence of rate upon concentration of Ca^{2+} (Kröner 1987). This indeed could be expected on the basis of the

mutual stimulation of Ca^{2+} and Mn^{2+}—a Ca^{2+} analogue—transport by the uniporter under appropriate conditions (Hughes and Exton, 1983; Allshire *et al.*, 1985; Allshire and Saris, 1986).

The question then arises, whether the rebounding under Bernardi's (1984) conditions also could be due to stimulation of influx rather than inhibition of efflux. The main difference between the conditions of Bernardi (1984) and those of Kröner (1986,a,b, 1987) relate to the presence of 1 mM Mg^{2+} in the medium employed by the latter and its absence in the medium used by Bernardi (1984). The mechanisms under these different conditions might be different, with an effect upon efflux occurring under Bernardi's conditions (1984) and an effect on the influx under Kröner's (1986,a,b, 1987). This question can be resolved by measuring unidirectional fluxes of Ca^{2+}. Indeed, the technique of Kröner (1986,a,b, 1987) to use ^{45}Ca and a Ca^{2+} buffer added at specified times and measuring initial rates is equivalent to measuring the unidirectional influx rate. Using this approach, we show (Fig. 2) that there was a clear stimulation of influx on the uniporter also under Bernardi's conditions. The stimulation of the uniporter by the Ca^{2+} analogue Mn^{2+} appeared to be by relieving the inhibitory action of Mg^{2+} (Hughes and Exton, 1983; Allshire *et al.*, 1985). However, Kröner (1986a,b) found a stimulation of the uniporter by Ca^{2+} even without added Mg^{2+}.

It is interesting that the effect of external Ca^{2+} on the uniporter is discernible long after the Ca^{2+} concentration in the medium has been lowered by uptake. How this is brought about is not clear at present. A conformational change in the uniporter, induced by Ca^{2+}, might exhibit a high degree of stability, possibly by trapping the modulating Ca^{2+} ion. Another possibility is that the effect on the uniporter is mediated by phospholipids whose state is changed by binding of Ca^{2+}.

Acknowledgments

This study was supported by a grant from the Finnish Society of Sciences and Letters. The skillful technical assistance of Mrs Kaija Niva is gratefully acknowledged.

References

Åkerman, K. E. O. (1977). *J. Bioenerg. Biomembr.* **9**, 65–72.
Åkerman, K. E. O. (1978). *Biochim. Biophys. Acta* **502**, 359–366.
Åkerman, K. E. O., and Nicholls, D. G. (1983). *Rev. Physiol. Biochem. Pharmacol.* **95**, 149–201.
Åkerman, K. E. O., Wikström, M. K. F., and Saris, N.-E. L. (1977). *Biochim. Biophys. Acta* **464**, 287–294.

Allshire, A., and Saris, N.-E. L. (1986). In *Manganese in Metabolism and Enzyme Function* (V. L. Schramm and F. C. Wedler, eds.), Academic Press, pp. 51–63.
Allshire, A., Bernardi, P., and Saris, N.-E. L. (1985). *Biochim. Biophys. Acta* **807**, 202–209.
Bernardi, P. (1984). *Biochim. Biophys. Acta* **766**, 277–282.
Bernardi, P., and Azzone, G. F. (1982). *FEBS Lett.* **139**, 13–16.
Bernardi, P., and Azzone, G. P. (1983). *Eur. J. Biochem.* **134**, 377–383.
Bragadin, M., Pozzan, T. and Azzone, G. F. (1979). *Biochemistry* **18**, 5972–5978.
Chance, B., and Mela, L. (1966). *Biochemistry* **5**, 3220–3223.
Ernster, L., Nakazawa, T. and Nordenbrand, K. (1978). In *The Proton and Calcium Pumps* (G. F. Azzone *et al.*, eds.), Elsevier/North Holland, Amsterdam, pp. 163–176.
Fletcher, J. M., Greenfield, B. F., Hardy, C. J., Scargill, D., and Woodhead, J. C. (1961). *J. Chem. Soc.*, 2000–2006.
Hughes, B. P., and Exton, J. H. (1983). *Biochem. J.* **212**, 773–782.
Kröner, H. (1986a). *Biol. Chem. Hoppe-Seyler* **367**, 483–493.
Kröner, H. (1986b). *Arch. Biochem. Biophys.* **251**, 525–535.
Kröner, H. (1987). *Biol. Chem. Hoppe-Seyler* **369**, 149–155.
Kröner, H. (1988). *Arch. Biochem. Biophys.* **267**, 205–210.
Mela, L., and Chance, B. (1968). *Biochemistry* **7**, 4059–4063.
Nicchitta, C. V., and Williamson, J. R. (1984). *J. Biol. Chem.* **259**, 12978–12963.
Nicholls, D. G. (1978). *Biochem. J.* **176**, 463–474.
Saris, N.-E., and Åkerman, K. E. O. (1980). *Curr. Top. Bioenerg.* **10**, 103–179.
Saris, N.-E. L., Wikström, M. F., and Seppälä, A. J. (1969). In *Mitochondria, Structure and Function* (L. Ernster and Z. Drahota, eds.), Academic Press, London and New York, pp. 363–368.
Vinogradov, A., and Scarpa, A. (1973). *J. Biol. Chem.* **248**, 5527–5531.