*Journal of Bioenergetics and Biomembranes, Vol. 36, No. 4, August 2004 (© 2004)*

# The Integration of Mitochondrial Calcium Transport and Storage

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*Received March 15, 2004; accepted May 7, 2004*

The extraordinary capacity of isolated mitochondria to accumulate $Ca^{2+}$ has been established for more than 40 years. The distinct kinetics of the independent uptake and efflux pathways accounts for the dual functionality of the transport process to either modulate matrix free $Ca^{2+}$ concentrations or to act as temporary stores of large amounts of $Ca^{2+}$ in the presence of phosphate. One puzzle has been the nature of the matrix calcium phosphate complex, since matrix free $Ca^{2+}$ seems to be buffered in the region of 1–5 $\mu$M in the presence of phosphate while millimolar $Ca^{2+}$ remains soluble in in vitro media. The key seems to be the elevated matrix pH and the third-power relationship of the $PO_4^{3-}$ concentration with pH. Taking this into account we may now finally have a model that explains the major features of physiological mitochondrial $Ca^{2+}$ transport.

**KEY WORDS:** Mitochondria; calcium; phosphate; membrane potential; tricalcium phosphate; pH; hydroxyapatite.

As so ably documented by the studies of Al Lehninger and his collaborators in the 1960s and 1970s, one of the most fascinating properties of isolated mitochondria is their seemingly enormous capacity (frequently in excess of 1 micromole/mg protein—corresponding to a total matrix concentration of 1 M) to accumulate and retain calcium. In their classic studies (reviewed in Lehninger, 1974; Lehninger *et al*., 1967; Rossi and Lehninger, 1964) some of the major features were established, including the role of phosphate as accompanying ion, effects on respiration, and the detection of associated proton movements, although this last was initially ascribed to the passive response to a primary $Ca^{2+}$ transport process rather than a manifestation of the chemiosmotic hypothesis. With the gradual realization that this latter provided a mechanism for the uptake of $Ca^{2+}$ via a uniport channel came a new problem, namely that estimates of the membrane potential $\Delta\psi$ in the region of 150 mV (Nicholls, 1974) and thus capable of maintaining a $10^5$ gradient of free $Ca^{2+}$ across the inner membrane led to the conclusion that a uniport would lead to an irreversible accumulation of the cation into the matrix. This paradox was resolved by the discovery of independent efflux pathways in heart and liver mitochondria (Crompton *et al*., 1978; Crompton and Heid, 1978) leading to the concept of a continuous cycling of $Ca^{2+}$ across the membrane utilizing the proton gradient either directly in liver mitochondria, via the $H^+/Ca^{2+}$ exchanger, or indirectly in the case of heart or brain mitochondria where a combination of $Na^+/Ca^{2+}$ and $H^+/Na^+$ exchangers were operative (Fig. 1).

This cycling of $Ca^{2+}$ led to a wealth of papers focussing on the ability of the pathways to transmit changes in cytoplasmic free $Ca^{2+}$, $[Ca^{2+}]_c$, into the matrix to control key metabolic enzymes including pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and NAD-linked isocitrate dehydrogenase (reviewed in Monje *et al*., 2001). However, it was still important to establish why mitochondria accumulated $Ca^{2+}$ rather than simply cycling it. Our own investigations began with the acquisition of a $Ca^{2+}$ selective macroelectrode and an investigation of the extent to which mitochondria could reduce the extramitochondrial free $Ca^{2+}$ concentration, $[Ca^{2+}]_e$ (Nicholls, 1978). Respiring liver mitochondria incubated in the presence of 30 nmol total $Ca^{2+}$/mg protein could lower $[Ca^{2+}]_e$ to about 0.8 $\mu$M while if $[Ca^{2+}]_e$ was lowered below this

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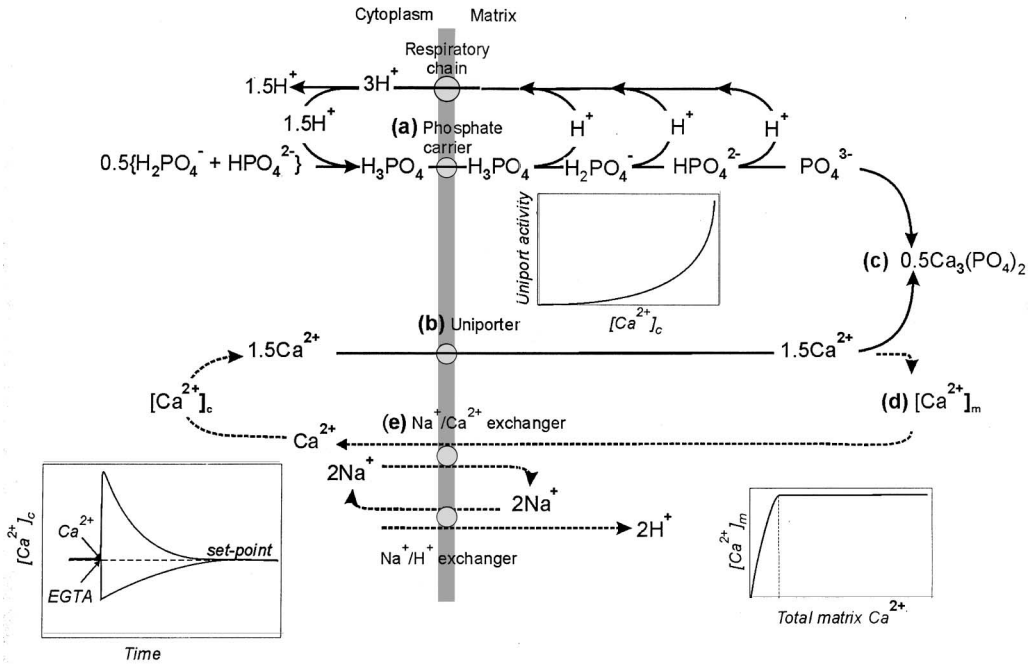


**Fig. 1.** Schematic of the ion movements involved in the net accumulation (solid arrows) and steady-state cycling (dashed arrows) of $Ca^{2+}$. (a) The phosphate carrier transports $H_2PO_4^-$ in exchange for $OH^-$ but this is formally equivalent to the electroneutral transport of $H_3PO_4$. Because three proton dissociations are required to from $PO_4^{3-}$ the concentration of this species is inversely proportional to the cube of the proton concentration in the matrix (Chalmers and Nicholls, 2003). (b) The uniport activity increases as the 2.5 power of cytoplasmic free $Ca^{2+}$ concentration $[Ca^{2+}]_c$ (Zoccarato and Nicholls, 1982), see insert. (c) The tricalcium phosphate complex forms when its ion activity product is exceeded. Because the concentration of $PO_4^{3-}$ increases with pH, the solubility of $Ca^{2+}$ decreases and is about 2 $\mu$M when matrix pH is about 7.7 and external total phosphate is about 5 mM (Chalmers and Nicholls, 2003). (d) The matrix free $Ca^{2+}$ concentration, $[Ca^{2+}]_m$, varies with total matrix $Ca^{2+}$ until about 10 nmol/mg is accumulated and the tricalcium phosphate complex start to form. In this initial region matrix $Ca^{2+}$ can regulate tricarboxylic acid enzymes. Once the complex forms, $[Ca^{2+}]_m$ is invariant with matrix $Ca^{2+}$ load and the cytoplasmic $Ca^{2+}$ buffering mode is seen (see insert). (e) The $Na^+/Ca^{2+}$ exchanger is controlled by $[Ca^{2+}]_m$; when the matrix is in cytoplasmic buffering mode (>10 nmol $Ca^{2+}$/mg accumulated) mitochondria seek to accumulate (or release matrix $Ca^{2+}$ to restore a set-point at which the kinetics of uptake via the uniporter exactly balance efflux via the $Na^+/Ca^{2+}$ exchanger (Nicholls, 1978).

value by the addition of a chelator there was a slow release of matrix $Ca^{2+}$ until this same value was attained (Nicholls, 1978). It thus seemed that this represented a value at which a dynamic steady-state equilibrium cycling was achieved.

The equilibrium $[Ca^{2+}]_e$, which we termed the "set-point" was invariant when the total $Ca^{2+}$ load was varied from 10 to 50 nmol/mg protein (Nicholls, 1978). However above this value the capacity of the mitochondria to accumulate $Ca^{2+}$ was severely curtailed and the set-point rose. Net accumulation of $Ca^{2+}$ would be predicted to lower $\Delta\psi$ and raise $\Delta$pH as net proton extrusion occurs in compensation for the uptake of $Ca^{2+}$. The only reason why 50 nmol/mg protein of $Ca^{2+}$ could be accumulated without affecting the set-point was because of the presence of endogenous phosphate in the preparation. When this was depleted by preincubating mitochondria with glucose and hexokinase or by inhibiting the phosphate transporter with *N*-ethylmaleimide much less $Ca^{2+}$ could be taken up before the set-point was raised (Nicholls, 1978; Zoccarato and Nicholls, 1982). In theory, both acetate and phosphate could serve as compensatory permeant anions to prevent the build-up of $\Delta$pH and drop in $\Delta\psi$; however their effects were distinct. With acetate the set-point rose slowly with $Ca^{2+}$ load (Nicholls, 1978; Zoccarato and Nicholls, 1982) whereas with additional phosphate as permeant anion the set-point remained remarkably constant as total $Ca^{2+}$ was varied from 10 to several hundred nmol/mg (Nicholls and Scott, 1980). As investigated by Rossi and Lehninger (1964) matrix $Ca^{2+}$ in the presence of phosphate appears to form some type of osmotically inactive complex, whereas calcium acetate is soluble. This raised the possibility that the remarkable invariance of the set-point over the range from 10 to 500 nmol/mg $Ca^{2+}$ (Chalmers and

Nicholls, 2003) was due to the kinetic balance between the activity of the uniporter at a given $[Ca^{2+}]_e$ and an activity of the efflux pathway that was essentially independent of total matrix $Ca^{2+}$ load over this range because the free matrix $Ca^{2+}$, $[Ca^{2+}]_m$, was buffered at a constant value by the supposed $Ca^{2+}$-phosphate complex.

This hypothesis was tested in a subsequent study (Zoccarato and Nicholls, 1982). Without making any assumptions about the chemical nature of the $Ca^{2+}$-phosphate complex, a constant ion activity ("solubility") product should mean that $[Ca^{2+}]_m$ would decrease when the matrix free phosphate concentration increased. This latter is surprisingly easy to control. Because the dominant pathway for Pi transport across the inner membrane is the electroneutral phosphate transporter (Palmieri *et al*., 1996), then if $\Delta$pH does not change the concentrations of the different ionized forms of Pi free in the matrix will be proportional to the external Pi concentration. This was tested directly by monitoring the net rate of $Ca^{2+}$ efflux from liver mitochondria following addition of ruthenium rd to inhibit the uniporter. $Ca^{2+}$ efflux rates decreased from 7 nmol/min/mg protein from mitochondria phosphate depleted by glucose/hexokinase to 0.5 nmol/min/mg protein in the presence of 3 mM external Pi (Zoccarato and Nicholls, 1982). Mitochondrial membrane potential did not change when Pi was increased, but the set-point decreased from 0.78 $\mu$M to 0.55 $\mu$M.

The rather modest change in set-point accompanying a 16-fold decrease in efflux rate indicates a very steep dependency of the uniporter on $[Ca^{2+}]_e$. This was investigated directly by an experiment in which $Ca^{2+}$ was infused into mitochondrial incubations at varying rates and $[Ca^{2+}]_e$ was monitored until it achieved a constant value, implying that the net rate of $Ca^{2+}$ accumulation by the mitochondria equalled the rate of the infusion and hence that the activity of the uniporter was equal to the infusion rate plus the activity of the efflux pathway. By varying the infusion rate the activity of the uniporter was determined to vary as the 2.5th power of $[Ca^{2+}]_e$. The contrast between this high dependency for uptake and the virtual independency of the efflux pathway on matrix $Ca^{2+}$ load (due to the buffering of $[Ca^{2+}]_m$) is sufficient to account for the remarkable constancy of the set-point over this 50-fold range of total matrix $Ca^{2+}$ from 10 to 500 nmol/mg.

It should be emphasized in these studies that care was taken to reproduce the physiological conditions existing in the cytoplasm as accurately as possible, and in particular including adenine nucleotides in the incubation media. As evidenced from the literally thousands of publications on the permeability transition (PT), it is all too easy to incubate mitochondria in sucrose under nonphysiological conditions, omit the natural protective action of exogenous adenine nucleotides, subject the unfortunate organelles to a massive bolus of $Ca^{2+}$ and watch them swell. This is not to denigrate carefully controlled experiments designed to reproduce oxidative stress in, for example cardiac reperfusion models (Crompton, 1999), but rather to emphasize the importance of realizing that mitochondria operate in a controlled environment.

We have recently revisited the question of the nature of the matrix stored $Ca^{2+}$ (Chalmers and Nicholls, 2003) in order to answer a number of outstanding questions. In particular we were interested in confirming that the $Ca^{2+}$-phosphate complex in the matrix was able to maintain $[Ca^{2+}]_m$ at a constant value independent of total $Ca^{2+}$ load and attempting to determine the factors that control the maximal $Ca^{2+}$ loading capacity of the matrix. We were concerned to eliminate the bioenergetic loads associated with conventional bolus additions of the cation which result in a sudden demand on the proton gradient, transient depolarization, increased respiration, and changes in $\Delta$pH. Because each of these parameters changes rapidly during a bolus addition it is difficult to distinguish the precise factors that are for example responsible for defining the maximal capacity of the matrix to retain $Ca^{2+}$. Instead, we adapted the infusion technique discussed above in order to slowly load the matrix with $Ca^{2+}$ with negligible consequences for the mitochondrial bioenergetics.

Liver mitochondria in the presence of ADP and oligmycin were able to accumulate 800 nmol $Ca^{2+}$/mg over a period of 10 min before $[Ca^{2+}]_e$ rose precipitously indicating the onset of a PT. No change in mitochondrial membrane potential occurred during the infusion until the onset of the transition. Some increase in NAD(P)H fluorescence was noted, together with an increase in light-scattering. It should be emphasized in this context that what is usually referred to as "swelling" is actually measured as a decrease in light scattering caused by a decrease in the difference in refractive index between the matrix and the medium as the latter enters and dilutes the former. The increased light scattering during matrix $Ca^{2+}$ loading is most likely due to the light-scattering properties or increased matrix refractive index due to the formation of the matrix $Ca^{2+}$-phosphate complex. Importantly, during the $Ca^{2+}$ loading no increase in the level of reactive oxygen species could be detected, if anything $H_2O_2$ production decreased during $Ca^{2+}$ loading (Chalmers and Nicholls, 2003).

The PT inhibitor cyclosporin A enhances the $Ca^{2+}$ loading capacity of liver mitochondria in the presence of ADP by 2.5-fold, while oxidation of endogenous NADH by the addition of acetoacetate decreases the loading capacity by a similar extent (Chalmers and Nicholls, 2003). Interestingly acetoacetate is still effective in lowering

loading capacity in the presence of cyclosporin A and ADP.

The nature of the $Ca^{2+}$-phosphate complex and its ability to buffer $[Ca^{2+}]_m$ that was predicted by the earlier studies on the set-point were investigated by loading rat brain mitochondria with the low affinity fura2-FF. As the total matrix load was varied either by the addition of $Ca^{2+}$ or EGTA it became apparent that there was a discontinuity at about 10 nmol total $Ca^{2+}$/mg protein. Above this value $[Ca^{2+}]_m$ was virtually invariant with total load, whereas below 10 nmol/mg $[Ca^{2+}]_m$ varied as a linear function of total $Ca^{2+}$. This resolves a longstanding debate between advocates of matrix $Ca^{2+}$ as a means of transmitting hormonal and metabolic changes in cytoplasmic $Ca^{2+}$ into the matrix (Hansford, 1994; McCormack and Denton, 1990) and those including ourselves who had emphasized the $Ca^{2+}$ buffering function of the mitochondrion (Nicholls and Åkerman, 1982). It is apparent that the mitochondrion is beautifully adapted to either role. When $[Ca^{2+}]_c$ is maintained below the set-point there is too little matrix $Ca^{2+}$ to form the $Ca^{2+}$-phosphate complex and $[Ca^{2+}]_m$ varies with $[Ca^{2+}]_c$, allowing for a messenger role for the cation and the control of the citric acid cycle. When however $[Ca^{2+}]_c$ rises, even briefly, above the set-point then sufficient matrix loading occurs for the formation of the $Ca^{2+}$-phosphate complex.

Studies with fluorescent $Ca^{2+}$ indicators using either isolated mitochondria (Al Nassar and Crompton, 1986; Davis *et al*., 1987; Lukacs and Kapus, 1987; McCormack *et al*., 1989; Moreno and Hansford, 1988) or intact cells (numerous studies employing rhod-2 and related indicators) have reported surprisingly low values for $[Ca^{2+}]_m$- in the range 1–5 $\mu$M under a variety of conditions. Indeed in the regulatory range (0–10 nmol total $Ca^{2+}$ /mg) the effects on citric acid cycle enzymes are consistent with $[Ca^{2+}]_m$ values of 0.5–2 $\mu$M (Hansford and Castro, 1982). In our study $[Ca^{2+}]_m$ for brain mitochondria remained in the range 2–3 $\mu$M when total $Ca^{2+}$ was increased from 10 to 500 nmol/mg (Chalmers and Nicholls, 2003). Thus the earlier prediction that $[Ca^{2+}]_m$ should be essentially independent of total matrix $Ca^{2+}$ (Zoccarato and Nicholls, 1982) was confirmed. Furthermore, the expected inverse relationship between external Pi concentration and $[Ca^{2+}]_m$ was also found. Interestingly cyclosporin A, although it more than doubled the maximal loading capacity of the matrix did not affect the stability of the $Ca^{2+}$-phosphate complex.

A major puzzle has been how to reconcile the apparent properties of the matrix $Ca^{2+}$-phosphate complex with that of known complexes in solution. Physiological cell incubation media contain *millimolar* $Ca^{2+}$ in solution in the presence of millimolar Pi, and yet in the matrix some form of osmotically inactive complex forms when $[Ca^{2+}]_m$ rises above 1–5 $\mu$M. Furthermore, in vitro calcium phosphate complexes once formed are notoriously difficult to redissolve, whereas the addition of a protonophore to $Ca^{2+}$-loaded mitochondria leads to an extremely rapid efflux of $Ca^{2+}$ via reversal of the uniporter and phosphate separately via the phosphate transporter (Zoccarato and Nicholls, 1982). Finally, if the gradient of free $Ca^{2+}$ across the inner membrane is really so low (say 0.5 $\mu$M outside and 2 $\mu$M inside), as is the gradient of free Pi (defined by the low $\Delta$pH) why is the addition of protonophore so effective in triggering massive and rapid $Ca^{2+}$ release?

It is not possible to investigate the nature of the matrix $Ca^{2+}$-phosphate complex directly, due to its instant dissociation when the mitochondria are disrupted and to the ability of $Ca^{2+}$-phosphate complexes to change during fixation or drying for physicochemical analysis. Thus hydroxyapatite can be detected in fixed and desiccated samples, but it is generally accepted that this is an artifact. In an artificial cytoplasm in the presence of ATP, amorphous $Ca_3(PO_4)_2$ is initially formed when millimolar $Ca^{2+}$ is titrated in, particularly at alkaline pH (Wuthier *et al*., 1985).

Some limits on the stoichiometry of the matrix complex can be reached by accurate determination of the ratio of $Ca^{2+}$ accumulated to net protons extruded during the uptake. These classic studies by Lehninger and colleagues produced values close to 1.0 $H^+/Ca^{2+}$ (Lehniger *et al*., 1967). While it was not initially realized that this was a "chemiosotic" proton extruded by the respiratory chain the analysis remains valid. This ratio is consistent with the formation of $Ca_3(PO_4)_2$ (Fig. 1) whereas hydroxyapatite with a formula $Ca_5(PO_4)_3OH$ would give a ratio of 1.1. While these ratios are perhaps too close to allow unambiguous discrimination, other forms such as $CaHPO_4$ (ratio 0.5) and $Ca(H_2PO_4)_2$ (ratio-1) can be eliminated.

The clue both to the low $Ca^{2+}$ solubility in the matrix and the rapid efflux initiated by protonophore appears to lie in the pH gradient across the inner membrane. The highly active phosphate carrier equilibrates the transported species $H_2PO_4^-$ with $OH^-$ and thus accumulates the anion as a function of the $\Delta$pH. Two further dissociations of $H_2PO_4^-$ to $HPO_4^{2-}$ and $PO_4^{3-}$ are required before $Ca_3(PO_4)_2$ is formed and both of these dissociations are dependent on the matrix pH, with the final result that the concentration of the $PO_4^{3-}$ species is dependent on the third power of the $\Delta$pH at constant external phosphate.

The solubility of a salt is governed by the ion activity product (solubility product). Values reported in the literature for the calcium phosphate complexes are somewhat variable, but values of $3 \times 10^{-30}$ for amorphous $Ca_3(PO_4)_2$ and $1 \times 10^{-59}$ for hydroxyapatite are representative. From

the pKs for the dissociation of the phosphate anionic forms it can be calculated that the transmembrane gradient of the $PO_4^{3-}$ anion varies as the third power of the pH gradient, so that an increase in matrix pH from 7 to 8 would increase the matrix $PO_4^{3-}$ concentration by 1000-fold. Since the ion activity product is a constant, this means that taking an example of mitochondria incubated in the presence of 5 mM total external Pi the saturating $Ca^{2+}$ concentration in equilibrium with tricalcium phosphate in the matrix would decrease from about 100 $\mu$M at pH 7 to 1 $\mu$M at pH 8, while the corresponding saturating $Ca^{2+}$ concentrations in equilibrium with hydroxyapatite would be about 15-fold lower (Chalmers and Nicholls, 2003).

The best fit with the experimental data for $[Ca^{2+}]_m$ would be consistent with tricalcium phosphate in the matrix of respiring mitochondria at a pH of about 7.7, when saturating $[Ca^{2+}]_m$ would be calculated to be in the region of 2 $\mu$M. Thus, the low values reported for $[Ca^{2+}]_m$ are entirely consistent with the physical chemistry of calcium phosphate complexes at alkaline pH. This also provides an explanation for the rapid efflux of $Ca^{2+}$ and Pi on their respective carriers when protonophores are added, since this is associated with a dramatic matrix acidification. This would decrease the concentration of $PO_4^{3-}$, destabilizing the complex, and increasing $[Ca^{2+}]_m$ to about 100 $\mu$M, the high $Ca^{2+}$ gradient thus driving the rapid efflux of the cation.

The $Ca^{2+}$-transport properties of isolated mitochondria are sufficient to account for the major features observed for in situ mitochondrial $Ca^{2+}$ transport. In particular mitochondria only appear to sequester significant amounts of $Ca^{2+}$ when $[Ca^{2+}]_m$ rises above 0.5 $\mu$M, corresponding rather nicely with the set-point observed for isolated mitochondria (Werth and Thayer, 1994). Isolated mitochondria release $Ca^{2+}$ when the external $Ca^{2+}$ concentration falls below the set-point (Nicholls, 1978) and this behavior can be observed in cultured neurons recovering after a transient cytoplasmic $Ca^{2+}$ load, where the recovery to basal $[Ca^{2+}]_c$ is delayed by a shoulder consistent with an unloading of the temporarily accumulated cation to the cytoplasm (Werth and Thayer, 1994).

In conclusion, the mitochondrial $Ca^{2+}$ transport and sequestration properties are perfectly adapted to accumulate, store, and release large amounts of the cation. The pioneering work of Al Lehninger and his colleagues laid the essential groundwork from which these studies have evolved.

## ACKNOWLEDGMENT

This research was supported in part by a grant (NS41908) from the National Institute for Neurological Disorder and Stroke.

## REFERENCES

Al-Nassar, I., and Crompton, M. (1986). *Biochem. J.* **239**, 31–40.
Chalmers, S., and Nicholls, D. G. (2003). *J. Biol. Chem.* **279**, 19062–19070.
Crompton, M. (1999). *Biochem. J.* **341**, 233–249.
Crompton, M., and Heid, I. (1978). *Eur. J. Biochem.* **91**, 599–608.
Crompton, M., Moser, R., Ludi, H., and Carafoli, E. (1978). *Eur. J. Biochem.* **82**, 25–31.
Davis, M. H., Altschuld, R. A., Jung, D. W., and Brierley, G. P. (1987). *Biochem. Biophys. Res. Commun.* **149**, 40–45.
Hansford, R. G. (1994). *J. Bioenerg. Biomembr.* **26**, 495–508.
Hansford, R. G., and Castro, F. (1982). *J. Bioenerg. Biomembr.* **14**, 361–376.
Lehninger, A. L. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1520–1524.
Lehninger, A. L., Carafoli, E., and Rossi, C. S. (1967). *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 259–320.
Lukacs, G. L., and Kapus, A. (1987). *Biochem. J.* **248**, 609–613.
McCormack, J. G., Browne, H. M., and Dawes, N. J. (1989). *Biochim. Biophys. Acta* **973**, 420–427.
McCormack, J. G., and Denton, R. M. (1990). *Biochim. Biophys. Acta* **1018**, 287–291.
Monje, M. L., Phillips, R., and Sapolsky, R. (2001). *Brain Res.* **911**, 37–42.
Moreno, S., and Hansford, R. G. (1988). *Biochem. J.* **256**, 403–412.
Nicholls, D. G. (1974). *Eur. J. Biochem.* **50**, 305–315.
Nicholls, D. G. (1978). *Biochem. J.* **176**, 463–474.
Nicholls, D. G., and Åkerman, K. E. O. (1982). *Biochim. Biophys. Acta* **683**, 57–88.
Nicholls, D. G., and Scott, I. D. (1980). *Biochem. J.* **186**, 833–839.
Palmieri, F., Bisaccia, F., Capobianco, L., Dolce, V., Fiermonte, G., lacobazzi, V., Indiveri, C., and Palmieri, L. (1996). *Biochim. Biophys. Acta* **1275**, 127–132.
Rossi, C. S., and Lehninger, A. L. (1964). *J. Biol. Chem.* **239**, 3971–3980.
Werth, J. L., and Thayer, S. A. (1994). *J. Neurosci.* **14**, 346–356.
Wuthier, R. E., Rice, G. S., Wallace, J. E., Weaver, R. L., LeGeros, R. Z., and Eanes, E. D. (1985). *Calcif. Tissue Int.* **37**, 401–410.
Zoccarato, F., and Nicholls, D. G. (1982). *Eur. J. Biochem.* **127**, 333–338.