Stimulation of the respiration rate of rat liver mitochondria by sub-micromolar concentrations of extramitochondrial Ca^{2+}

John D. JOHNSTON and Martin D. BRAND

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

1. The respiration rate of rat liver mitochondria was stimulated by up to 70% when the extramitochondrial Ca^{2+} concentration was raised from 103 to 820 nM. This occurred when pyruvate, 2-oxoglutarate, or *threo*-(D_3)-isocitrate was employed as substrate, but not when succinate was used. 2. Ruthenium Red prevented the stimulation of mitochondrial respiration by extramitochondrial Ca^{2+} , showing that the effect required Ca^{2+} uptake into the mitochondrial matrix. 3. Starvation of rats for 48 h abolished the stimulation of mitochondrial respiration by extramitochondrial Ca^{2+} when pyruvate was used as substrate, but did not affect the stimulation of 2-oxoglutarate oxidation by extramitochondrial Ca^{2+} . 4. Our findings are in accord with proposals that oxidative metabolism in liver mitochondria may be stimulated by Ca^{2+} activation of intramitochondrial dehydrogenases.

INTRODUCTION

The action of glucagon, angiotensin II, vasopressin and α -adrenergic agonists on liver cells gives rise to an increased rate of respiration (reviewed by: Kraus-Friedmann, 1984; Denton & McCormack, 1985; Brand & Murphy, 1987). This increase is not a result of increased supply of ADP, since the ATP/ADP ratio of the mitochondrial matrix does not fall; rather it remains unchanged, or even increases (Siess *et al.*, 1977; Bryla *et al.*, 1977). These hormones also give rise to an increase in cytoplasmic Ca^{2+} concentration, from a basal value of 100 nM up to 600 nM (Murphy *et al.*, 1980; Charest *et al.*, 1983). Denton & McCormack (1985) and Hansford (1985) have proposed that an increase in cytoplasmic Ca^{2+} within this range results in an increase in mitochondrial-matrix Ca^{2+} concentration, which acts to stimulate respiration independently of the supply of ADP, thereby allowing the phosphorylation potential of the mitochondrial matrix to remain constant, or even to increase (see also Brand & Murphy, 1987).

The stimulation of mitochondrial respiration by Ca^{2+} is proposed to act via three matrix enzymes, i.e. 2-oxoglutarate dehydrogenase, NAD-linked isocitrate dehydrogenase and pyruvate dehydrogenase phosphate phosphatase (Hansford, 1985). Ca^{2+} decreases both the K_m value of NAD-linked isocitrate dehydrogenase for *threo*-(D_3)-isocitrate and the K_m value of 2-oxoglutarate dehydrogenase for 2-oxoglutarate (Denton & McCormack, 1980). Pyruvate dehydrogenase exists in both an active dephosphorylated form and an inactive phosphorylated form (Randle, 1986). Ca^{2+} stimulates pyruvate dehydrogenase phosphate phosphatase, which generates an increase in the active form of pyruvate dehydrogenase, and so increases the V_{max} of the complex (Hansford, 1985). The amount of active pyruvate dehydrogenase is decreased in starvation by the action of pyruvate dehydrogenase kinase, which itself is activated in starvation by pyruvate dehydrogenase kinase activator protein (Denyer *et al.*, 1986). Starvation also attenuates a rise in pyruvate dehydrogenase activity in

response to hormones such as glucagon and vasopressin (Randle, 1986).

Stimulation by extramitochondrial Ca^{2+} of the activity of these matrix enzymes and of the respiration rate has been observed in intact coupled mitochondria isolated from heart (Denton *et al.*, 1980), brown adipose tissue (McCormack & Denton, 1980) and white adipose tissue (Marshall *et al.*, 1984). The sensitivity to Ca^{2+} of the three matrix enzymes from liver is retained in mitochondrial extracts (McCormack, 1985b). Attempts to demonstrate that micromolar $[\text{Ca}^{2+}]_o$ can stimulate respiration of isolated liver mitochondria have, however, been unsuccessful (Hansford, 1985). McCormack (1985a,b) and Assimacopoulos-Jeannet *et al.* (1986) have used a Percoll-gradient procedure when isolating liver mitochondria to produce a preparation with low Ca^{2+} content, and demonstrated that the Ca^{2+} -sensitive intramitochondrial enzymes could be stimulated by an increase in $[\text{Ca}^{2+}]_o$, but experienced difficulty in demonstrating an increase in respiration.

To maintain the hypothesis that the increase in the respiration rate of liver cells after hormone treatment is caused by raised cytoplasmic and matrix Ca^{2+} concentration leading to activation of the Ca^{2+} -sensitive enzymes, it is necessary to show stimulation of respiration of isolated liver mitochondria in response to appropriate $[\text{Ca}^{2+}]_o$. Using a simple technique which we have previously reported to produce a mitochondrial preparation with low Ca^{2+} content (Johnston & Brand, 1986), we ask the question: can an increase in $[\text{Ca}^{2+}]_o$ within the physiological range stimulate mitochondrial respiration?

EXPERIMENTAL

Female Wistar rats were fed on Diet 41B, obtained from Oxoid, Basingstoke, Hants., U.K. Animals were starved overnight (16 h) unless otherwise stated, and killed between 08:30 and 09:00 h. Liver mitochondria were prepared by the method of Chappell & Hansford

(1972) in 250 mM-sucrose/10 mM-Hepes/1 mM-EGTA, adjusted to pH 7.4 with KOH, at 4 °C. The following alterations were made to the protocol (Johnston & Brand, 1986): four centrifugations instead of three were performed at 12100 *g* for 10 min ($r_{\max}$ = 10.8 cm); the third and fourth centrifugations were carried out in 250 mM-sucrose/5 mM-Hepes/10 mM-NTA, adjusted to pH 7.25 with KOH, at 4 °C; between the third and fourth centrifugations mitochondria were incubated on ice in this medium at approx. 10 mg of protein/ml for 25 min. Mitochondrial preparations contained 1.26 ± 0.11 nmol of Ca^{2+} /mg of protein (fed animals), 1.41 ± 0.11 nmol of Ca^{2+} /mg (16 h-starved animals) and 1.31 ± 0.14 nmol of Ca^{2+} /mg (48 h-starved animals). Mitochondria prepared without the incubation procedure contained 3.75 ± 0.31 nmol of Ca^{2+} /mg of protein. Results are means $\pm$ S.E.M. for six preparations. Using a similar technique, Joseph *et al.* (1983) obtained similar results. Respiratory control ratios (State 3/State 4) of mitochondria were 6.47 ± 0.60 with 10 mM-glutamate/5 mM-malate, and 4.02 ± 0.19 with 10 mM-succinate in the presence of 3 μM -rotenone. Results are means $\pm$ S.E.M. for three preparations.

Mitochondrial protein was determined by a modified biuret method (Gornall *et al.*, 1949), with bovine serum albumin as standard.

Mitochondrial total Ca^{2+} content was measured with a Perkin-Elmer 380AA atomic-absorption spectrophotometer after mitochondrial Ca^{2+} had been extracted with 5% (w/v) trichloroacetic acid/1.5% (w/v) LaCl_3 (final concns.), and the denatured protein removed by centrifugation.

Mitochondrial respiration was measured with a Clark-type oxygen electrode. The basic incubation medium contained 70 mM-KCl, 80 mM-sucrose, 10 mM-NTA, 10 mM-Hepes, 5 mM- KH_2PO_4 , 5 mM- MgCl_2 ([free Mg^{2+}] = 0.34 mM), 5 mM- Na_2ADP , at 37 °C, adjusted to pH 7.25 with KOH. The basic incubation medium also contained 365 nmol of O/min as calibrated by the method of Robinson & Cooper (1970), and 6 μM total endogenous Ca^{2+} determined by atomic-absorption spectrophotometry. CaCl_2 was added from 10 mM stock solution to give the [free Ca^{2+}] required.

Mitochondria were incubated at 1 mg of mitochondrial protein/ml in a final volume of 3 ml. Endogenous rates of respiration ranged from 6 to 12 nmol of O/min per mg of protein. This varied from incubation to incubation, and was not noticeably affected by $[\text{Ca}^{2+}]_0$. Substrate was added after 5 min incubation. Rates of O_2 consumption were measured for 5 min and were linear during this time.

Apparent stability constants at pH 7.25 for Ca-NTA ($9.51 \times 10^3 \text{ M}^{-1}$), Ca-EGTA ($8.48 \times 10^6 \text{ M}^{-1}$), Mg-NTA ($1.69 \times 10^3 \text{ M}^{-1}$) and Mg-ADP ($7.93 \times 10^2 \text{ M}^{-1}$) were calculated from the absolute stability constants (Martell & Smith, 1977) as described by Fabiato & Fabiato (1979). Other complexes, e.g. Ca-ADP, did not contribute significantly to buffering.

Free Ca^{2+} and Mg^{2+} concentrations were calculated reiteratively by using the algorithm of Fabiato & Fabiato (1979) on a BBC microcomputer. Incubation media contained 6 μM -, 16 μM -, 26 μM -, 38 μM - and 51 μM - Ca^{2+} , giving calculated free Ca^{2+} concentrations of 103 nM, 252 nM, 410 nM, 594 nM and 820 nM respectively. To generate 0.75 nM free Ca^{2+} 1 mM-EGTA was added to the basic incubation medium. Endogenous Ca^{2+} was

always measured and taken into account when calculating free Ca^{2+} concentrations.

Standard CaCl_2 , pyruvic acid, Hepes, NTA and MgCl_2 were obtained from BDH, Dagenham, Essex, U.K. Na_2ADP was obtained from Boehringer Mannheim, Mannheim, West Germany. Succinic acid, sucrose, KCl, KOH and KH_2PO_4 were obtained from Fisons, Loughborough, Leics., U.K. EGTA, *threo*-(D₃)-isocitrate (potassium salt), rotenone and Ruthenium Red were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Ruthenium Red was purified by the method of Luft (1971). 2-Oxoglutaric acid was obtained from Koch-Light, Colnbrook, Bucks., U.K.

Statistical significance was assessed by two-tailed Student's *t* test on matched pairs.

RESULTS

Figs. 1(a), 2(a) and 3(a) demonstrate the dependence of respiration rate on the concentration of 2-oxoglutarate, pyruvate and succinate respectively at $[\text{Ca}^{2+}]_0$ ranging from 0.75 to 820 nM.

Fig. 1(a) shows that $[\text{Ca}^{2+}]_0$ has little effect on the mitochondrial respiration rate at 0.1 mM-2-oxoglutarate. At higher concentrations of 2-oxoglutarate, there is a distinct stimulation by $[\text{Ca}^{2+}]_0$, most pronounced between 0.7 mM- and 2.0 mM-2-oxoglutarate, indicating that the respiration rate is dependent not only on the concentration of 2-oxoglutarate, but also on $[\text{Ca}^{2+}]_0$. Above 2.0 mM-2-oxoglutarate there is a tendency for the lines to converge. The respiration rate reaches a maximum of 80 nmol of O/min per mg of protein.

Fig. 2(a) shows that at 0.1 mM-pyruvate $[\text{Ca}^{2+}]_0$ has little effect on pyruvate oxidation. At higher concentrations of pyruvate there is again a distinct stimulation by $[\text{Ca}^{2+}]_0$, which becomes more pronounced as pyruvate concentration increases, indicating that pyruvate oxidation depends not only on the concentration of pyruvate but also on $[\text{Ca}^{2+}]_0$. The respiration rate reaches a maximum of 37.5 nmol of O/min per mg of protein.

Fig. 3(a) shows that succinate oxidation gives the largest rate of respiration, 180 nmol of O/min per mg of protein above 1.5 mM-succinate. There is no discernible effect of varying the $[\text{Ca}^{2+}]_0$.

Data from Figs. 1(a), 2(a) and 3(a) are replotted in Figs. 1(b), 2(b) and 3(b) respectively to demonstrate the effects of $[\text{Ca}^{2+}]_0$ on the mitochondrial respiration rate.

Fig. 1(b) demonstrates an increase in respiration rate when $[\text{Ca}^{2+}]_0$ is increased from 103 to 820 nM in the presence of 2-oxoglutarate. There is little or no change in the respiration rate when $[\text{Ca}^{2+}]_0$ is altered from 0.75 to 103 nM. There is a tendency for respiration to reach a plateau or even decrease when $[\text{Ca}^{2+}]_0$ is greater than 594 nM, but this does not always happen (see Fig. 5).

Fig. 2(b) demonstrates an increase in the rate of respiration in the presence of pyruvate when $[\text{Ca}^{2+}]_0$ is increased from 103 to 820 nM. As with oxidation of 2-oxoglutarate, there is little difference between respiration rates at 0.75 nM- and 103 nM- Ca^{2+} . The respiration rate tends to a plateau at $[\text{Ca}^{2+}]_0$ higher than 594 nM.

Fig. 3(b) demonstrates that increasing $[\text{Ca}^{2+}]_0$ from 0.75 to 820 nM has no effect on succinate oxidation. Succinate dehydrogenase is not known to be stimulated by Ca^{2+} (Hatefi, 1985), suggesting that the effects of $[\text{Ca}^{2+}]_0$ in Figs. 1 and 2 are not general, but are specific

Fig. 1. Effect of $[\text{Ca}^{2+}]_o$ on respiration rate of mitochondria at different concentrations of 2-oxoglutarate

Mitochondria were incubated as described in the Experimental section. Results are means $\pm$ S.E.M. [shown only in (b) for clarity] from three different preparations. In (a) respiration is plotted as a function of 2-oxoglutarate concentration, with the following $[\text{Ca}^{2+}]_o$ present: $\circ$, 0.75 nM; $\bullet$, 103 nM; $\square$, 252 nM; $\blacksquare$, 410 nM; ∇ , 594 nM; $\blacktriangledown$, 820 nM. Data from (a) are replotted in (b) to demonstrate respiration as a function of $[\text{Ca}^{2+}]_o$ at the following 2-oxoglutarate concentrations: $\circ$, 0.3 mM; $\bullet$, 0.5 mM; $\square$, 0.7 mM; $\blacksquare$, 1.0 mM; ∇ , 2.0 mM; $\blacktriangledown$, 3.5 mM. Statistical significance assessed against respiration rate at 0.75 nM $[\text{Ca}^{2+}]_o$ is shown only in (b): * $P < 0.05$, ** $P < 0.01$.

effects at the level of the dehydrogenases. We have also performed experiments with 0.1–5.0 mM-malate and 0.5–20 mM-D- β -hydroxybutyrate as substrate and found no stimulation of respiration up to 812 nM- Ca^{2+} (results not shown).

Fig. 4 shows respiration rate as a function of $[\text{Ca}^{2+}]_o$ with 0.5 mM-threo-D₃-isocitrate as substrate. Again, there

Fig. 2. Effect of $[\text{Ca}^{2+}]_o$ on respiration rate of mitochondria at different concentrations of pyruvate

Mitochondria were incubated as described in the Experimental section. Results are means $\pm$ S.E.M. [shown only in (b) for clarity] from three different preparations. In (a) respiration is plotted as a function of pyruvate concentration, with the following $[\text{Ca}^{2+}]_o$ present: $\circ$, 0.75 nM; $\bullet$, 103 nM; $\square$, 252 nM; $\blacksquare$, 410 nM; ∇ , 594 nM; $\blacktriangledown$, 820 nM. Data from (a) are replotted in (b) to demonstrate respiration as a function of $[\text{Ca}^{2+}]_o$ at the following pyruvate concentrations: $\circ$, 0.3 mM; $\bullet$, 0.5 mM; $\square$, 0.7 mM; $\blacksquare$, 1.0 mM; ∇ , 2.0 mM; $\blacktriangledown$, 5.0 mM. Statistical significance was assessed as in Fig. 1.

is little difference in respiration rates at 0.75 nM- Ca^{2+} and 103 nM- Ca^{2+} , respiration increases over the range 103–594 nM- Ca^{2+} , and reaches a plateau at higher $[\text{Ca}^{2+}]_o$. Fig. 4 also demonstrates that the increase in respiration associated with increasing $[\text{Ca}^{2+}]_o$ can be prevented by including Ruthenium Red, a potent inhibitor of mitochondrial Ca^{2+} uptake (Nicholls & Akerman, 1982), in the incubation medium. This shows that the stimulation of respiration depends on entry of Ca^{2+} into the mitochondrial matrix. Similar sensitivity to

Fig. 3. Effect of $[Ca^{2+}]_o$ on respiration rate of mitochondria at different concentrations of succinate

Mitochondria were incubated as described in the Experimental section with $3 \mu M$ -rotenone. Results are means $\pm$ S.E.M. [shown only in (b) for clarity] from three different preparations. In (a) respiration is plotted as a function of succinate concentration, with the following $[Ca^{2+}]_o$ present: $\circ$, 0.75 nM; $\bullet$, 103 nM; $\square$, 252 nM; $\blacksquare$, 410 nM; ∇ , 594 nM; $\blacktriangledown$, 820 nM. Data from (a) are replotted in (b) to demonstrate respiration as a function of $[Ca^{2+}]_o$ at the following succinate concentrations: $\circ$, 0.1 mM; $\bullet$, 0.3 mM; $\square$, 0.5 mM; $\blacksquare$, 1.5 mM; ∇ , 3.0 mM. Statistical significance was assessed as in Fig. 1.

Ruthenium Red is shown with 2-oxoglutarate (Fig. 5) and pyruvate (Fig. 6).

Fig. 5 demonstrates that 48 h starvation of animals before killing them has no effect on mitochondrial respiration rate with 1 mM-2-oxoglutarate as substrate. The activity of 2-oxoglutarate dehydrogenase in heart is not known to be affected by starvation (McCormack *et al.*, 1982).

The rate of respiration with pyruvate, on the other hand, depends on the extent of starvation of the animal

Fig. 4. Effect of $[Ca^{2+}]_o$ on respiration rate of mitochondria respiring on 0.5 mM-threo-(D₃)-isocitrate

Mitochondria were incubated as described in the Experimental section in the absence ($\circ$) or the presence ($\bullet$) of $1 \mu M$ -Ruthenium Red. Results are means $\pm$ S.E.M. for three different preparations. Statistical significance was assessed as in Fig. 1.

Fig. 5. Effect of dietary status of rats on the respiration rate of mitochondria respiring on 1 mM-2-oxoglutarate

Mitochondria obtained from fed ($\circ$), overnight-starved ($\bullet$) or 48 h-starved ($\square$) rats were incubated as described in the Experimental section. Mitochondria obtained from overnight-starved rats were also incubated as described in the Experimental section in the presence of $1 \mu M$ -Ruthenium Red ($\blacksquare$). Results are means $\pm$ S.E.M. for three different preparations. Statistical significance was assessed as in Fig. 1.

(Fig. 6). Increasing $[Ca^{2+}]_o$ from 103 to 820 nM increased the respiration rate of mitochondria from both fed animals and animals starved overnight for 16 h. Although there is no difference in the rates of respiration

Fig. 6. Effect of dietary status of rats on the respiration rate of mitochondria respiring on 1 mM-pyruvate

Mitochondria obtained from fed (○), overnight-starved (●) or 48 h-starved (□) rats were incubated as described in the Experimental section. Mitochondria obtained from overnight-starved rats were also incubated as described in the Experimental section in the presence of 1 μM -Ruthenium Red (■). Results are means $\pm$ S.E.M. for three different preparations. Statistical significance was assessed as in Fig. 1.

between fed and starved animals at 0.75 nM-[Ca²⁺]_o, mitochondria obtained from animals starved for 48 h failed to show Ca²⁺-sensitivity of pyruvate oxidation when [Ca²⁺]_o was raised from 103 to 820 nM. These results are consistent with a relative increase in the activity of pyruvate dehydrogenase kinase compared with pyruvate dehydrogenase phosphate phosphatase in starvation, masking any stimulation by [Ca²⁺]_o (Randle, 1986).

DISCUSSION

The aim of this work was to investigate the effect of an increase in [Ca²⁺]_o within the physiological range on the respiration of coupled liver mitochondria. All experiments were carried out in the presence of physiological extramitochondrial concentrations of Mg²⁺ and Na⁺, which inhibit Ca²⁺ uptake by and promote Ca²⁺ egress from mitochondria respectively (Nicholls & Akerman, 1982).

When [Ca²⁺]_o was increased from 103 to 820 nM, the rate of respiration of mitochondria isolated from animals starved overnight was stimulated by up to 30% with pyruvate, 60% with 2-oxoglutarate, and 70% with *threo*-(D₃)-isocitrate, but there was no stimulation of the respiratory rate when succinate was the substrate. These results are consistent with an increase in [Ca²⁺]_o causing a rise in intramitochondrial [Ca²⁺], which in turn activates 2-oxoglutarate dehydrogenase, NAD-linked isocitrate dehydrogenase and pyruvate dehydrogenase phosphate phosphatase.

An increase in [Ca²⁺]_o from 0.75 to 103 nM has no

effect on the rate of respiration when 2-oxoglutarate, pyruvate, *threo*-(D₃)-isocitrate or succinate is employed as substrate. There is therefore a threshold above which [Ca²⁺]_o must first rise before stimulation of the Ca²⁺-sensitive intramitochondrial enzymes (and of the respiratory rate) occurs.

When [Ca²⁺]_o was increased above 594 nM, mitochondrial respiration rate tended to a plateau. This presumably represents an optimum value of [Ca²⁺]_o above which an increase fails to stimulate the respiratory rate, rather than Ca²⁺-induced damage to the mitochondria, since succinate oxidation rates remained constant.

Starvation of rats for 48 h before death resulted in the loss of Ca²⁺-sensitivity of pyruvate oxidation, yet the sensitivity of 2-oxoglutarate oxidation was maintained, implying that the distribution of Ca²⁺ across the mitochondrial inner membrane was unaffected by starvation. These results are consistent with an increase in the activity of pyruvate dehydrogenase kinase relative to pyruvate dehydrogenase phosphate phosphatase in starvation, thereby preventing stimulation of pyruvate dehydrogenase by increasing [Ca²⁺]_o (Randle, 1986).

Hansford (1985) reported that the respiration rate of isolated liver mitochondria was unaffected by [Ca²⁺]_o. There may be several factors contributing to the difficulties experienced by others in demonstrating an increase in respiration rate in response to [Ca²⁺]_o. We have used mitochondria prepared in such a way that their endogenous Ca²⁺ content is approx. 1 nmol/mg of protein, similar to that found *in vivo* (Somlyo *et al.*, 1985). Higher Ca²⁺ contents may mask the effect, as suggested by Hansford (1985). We have worked at 37 °C instead of 25 °C or 30 °C, and indeed have been unable to show any effect of sub-micromolar Ca²⁺ concentrations at lower temperatures (J. D. Johnston & M. D. Brand, unpublished work). We have reported respiration rates between 0.1 mM and 5.0 mM substrate. Had we taken the ratio of respiration rates at 0.125 mM and 5.0 mM substrate as reported by Hansford (1985) and Marshall *et al.* (1984), stimulation of respiration by Ca²⁺ would not have been so apparent, e.g. Fig. 1(a). We have also relied on endogenous malate instead of added malate. Each of these factors may be important. Our results differ from those of Moreno-Sanchez (1985), who found that oxidation of glutamate/malate, 2-oxoglutarate and succinate and ATP synthesis were sensitive to [Ca²⁺]_o. His results, however, were obtained with a mitochondrial preparation with a high endogenous Ca²⁺ content (> 20 nmol/mg of protein), making interpretation difficult.

We conclude that the respiration rate of isolated liver mitochondria in the presence of pyruvate, 2-oxoglutarate or *threo*-(D₃)-isocitrate may be stimulated by [Ca²⁺]_o in the physiological range. This is consistent with the proposal that hormonal stimulation of O₂ consumption in liver is caused by elevated cytoplasmic and matrix [Ca²⁺], leading to stimulation of 2-oxoglutarate dehydrogenase, NAD-linked isocitrate dehydrogenase and pyruvate dehydrogenase (Denton & McCormack, 1985).

J.D.J. is indebted to Foulkes Foundation Fellowship, London, Wolfson College, Cambridge, and EAJ, Dunfermline, for financial assistance. We thank Mr. Duane Lakin-Thomas, Mr. Mark Leach, Miss Gina Allgood and Mrs. Mary George for technical assistance.

REFERENCES

- Assimakopoulos-Jeannet, F., McCormack, J. G. & Jeanrenaud, B. (1986) *J. Biol. Chem.* **261**, 8799–8804
- Brand, M. D. & Murphy, M. P. (1987) *Biol. Rev. Cambridge Philos. Soc.*, in the press
- Bryla, J., Harris, E. J. & Plumb, J. A. (1977) *FEBS Lett.* **80**, 443–448
- Chappell, J. B. & Hansford, R. G. (1972) in *Subcellular Components: Preparation and Fractionation* (Birnie, G. D., ed.), 2nd edn., pp. 79–81, Butterworths, London
- Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 8769–8773
- Denton, R. M. & McCormack, J. G. (1980) *FEBS Lett.* **119**, 1–8
- Denton, R. M. & McCormack, J. G. (1985) *Am. J. Physiol.* **249**, E543–E554
- Denton, R. M., McCormack, J. G. & Edgell, N. J. (1980) *Biochem. J.* **190**, 107–117
- Denyer, G. S., Kerbey, A. L. & Randle, P. J. (1986) *Biochem. J.* **239**, 347–354
- Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- Gornall, A. G., Bardawill, C. J. & Davis, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Hansford, R. G. (1985) *Rev. Physiol. Biochem. Pharmacol.* **102**, 1–72
- Hatefi, Y. (1985) *Annu. Rev. Biochem.* **54**, 1015–1070
- Johnston, J. D. & Brand, M. D. (1986) *Biochem. Soc. Trans.* **14**, 1182
- Joseph, S. K., Coll, K. E., Cooper, R. H., Marks, J. S. & Williamson, J. R. (1983) *J. Biol. Chem.* **258**, 731–741
- Kraus-Friedmann, N. (1984) *Physiol. Rev.* **64**, 170–253
- Luft, J. H. (1971) *Anat. Rec.* **171**, 347–368
- Marshall, S. E., McCormack, J. G. & Denton, R. M. (1984) *Biochem. J.* **218**, 249–260
- Martell, A. E. & Smith, R. M. (1977) *Critical Stability Constants*, vol. 1, Plenum Press, New York
- McCormack, J. G. (1985a) *FEBS Lett.* **180**, 259–264
- McCormack, J. G. (1985b) *Biochem. J.* **231**, 581–595
- McCormack, J. G. & Denton, R. M. (1980) *Biochem. J.* **190**, 95–105
- McCormack, J. G., Edgell, N. J. & Denton, R. M. (1982) *Biochem. J.* **202**, 419–427
- Moreno-Sanchez, R. (1985) *J. Biol. Chem.* **260**, 12554–12560
- Murphy, E., Coll, K. E., Rich, T. L. & Williamson, J. R. (1980) *J. Biol. Chem.* **255**, 6600–6608
- Nicholls, D. G. & Akerman, K. (1982) *Biochim. Biophys. Acta* **683**, 57–88
- Randle, P. J. (1986) *Biochem. Soc. Trans.* **14**, 799–806
- Robinson, J. & Cooper, J. M. (1970) *Anal. Biochem.* **33**, 390–399
- Siess, E. A., Brocks, D. G., Lattke, H. K. & Wieland, O. H. (1977) *Biochem. J.* **166**, 225–235
- Somlyo, A. P., Bond, M. & Somlyo, A. V. (1985) *Nature (London)* **314**, 622–625

Received 15 December 1986/3 March 1987; accepted 20 March 1987