

## CORRELATION BETWEEN GLUTAMATE AND $\text{Ca}^{2+}$ UPTAKE IN RAT LIVER MITOCHONDRIA

Roger DEBISE, Pierre GACHON\* and Roger DURAND<sup>†</sup>

Laboratoire de Biochimie, Université de Clermont-Ferrand II, BP 45, 63170 Aubière and \*Laboratoire de Pharmacologie Médicale, Formation de Recherche Associée à l'INSERM No. 10, UER de Médecine, 63001 Clermont-Ferrand-Cedex, France

Received 2 November 1977

### 1. Introduction

Rat liver mitochondria can accumulate large quantities of  $\text{Ca}^{2+}$  in the inner matrix compartment by an energy-linked process. It was demonstrated that this  $\text{Ca}^{2+}$  uptake is associated with a stoichiometric ejection of protons [1–3]. Anions such as phosphate, acetate and bicarbonate which permeate electro-neutrally can support and accompany this energy-linked  $\text{Ca}^{2+}$  transport in respiring rat liver mitochondria [4] and cause acidification of the matrix. In contrast, anions which permeate electrogenically without net transport of protons into matrix (nitrate, thiocyanate) do not accompany  $\text{Ca}^{2+}$  transport [4]. There is now a wide agreement that the driving force for  $\text{Ca}^{2+}$  uptake in the presence of permeant anions is the electrical component of the electrochemical proton gradient derived from respiration or ATP hydrolysis [5]. A controversy has been recently developed concerning the ratio  $\text{H}^+$  ejected/ $\text{Ca}^{2+}$  taken up, whether there are two (+) charges [6] or one (+) charge [7] when phosphate movements are inhibited.

We present here data showing that glutamate can accompany  $\text{Ca}^{2+}$  uptake in respiring rat liver mitochondria. Glutamate can enter rat liver mitochondria

by two transport systems: a glutamate/aspartate exchange [8–10] and a *N*-ethylmaleimide sensitive glutamate/ $\text{H}^+$  co-transport [11–15]. Our data are consistent with a co-transport glutamate/ $\text{Ca}^{2+}$  mediated by this last transport system.

### 2. Materials and methods

#### 2.1. Isolation of mitochondria

Rat liver mitochondria were isolated according to [16] in 0.25 M sucrose, 2 mM Tris-HCl, pH 7.4. Proteins were determined by the Biuret method [7].

#### 2.2. [ $^3\text{H}$ ] Glutamate incorporation

The technique used was derived from [12] and recently described in detail [18].

#### 2.3. $\text{Ca}^{2+}$ uptake

Atomic absorption determinations have been made with a Perkin Elmer spectrophotometer 420.

Changes in the extramitochondrial concentration of free  $\text{Ca}^{2+}$  were monitored by use of a  $\text{Ca}^{2+}$  selective electrode (Philips IS561) developed [19]. The electrode potentials were amplified by a Orion 801 A ionometer which drove a Sefram recorder.

Mitochondrial swelling, initiated by addition of  $100\text{ }\mu\text{M}$   $\text{Ca}^{2+}$ , were monitored at 546 nm in a cell photometer containing 1 ml 100 mM Tris-glutamate, pH 7.2.

**Abbreviations:**  $\text{P}_i$ , inorganic phosphate; Tris, Tris (Hydroxymethyl) aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Val, valinomycin; Rot, rotenone; Succ, succinate; Glu, glutamate; NEM, *N*-ethylmaleimide

<sup>†</sup> To whom reprint requests should be addressed

### 3. Results and discussion

#### 3.1. Stimulation of glutamate uptake by valinomycin and $\text{Ca}^{2+}$

Figure 1 shows that rat liver mitochondria, incubated in the presence of 5 mM potassium glutamate, absorb approx. 5–6 nmol glutamate/mg protein/min. This absorption is reduced 10–15% by rotenone [18] but is considerably increased by valinomycin which, in the presence of  $\text{K}^+$ , stimulated the transmembrane  $\Delta\text{pH}$  [20,21]. Addition of 50 natoms  $\text{Ca}^{2+}$ /mg protein

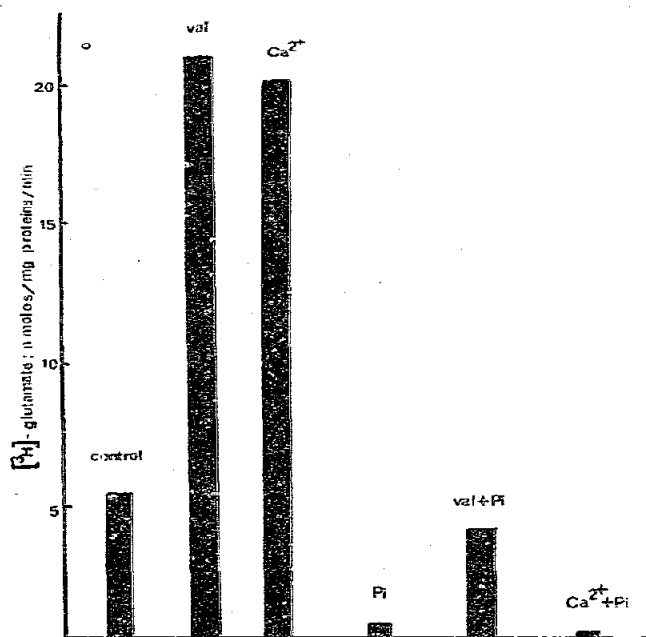


Fig. 1. Stimulation of glutamate accumulation by valinomycin and  $\text{Ca}^{2+}$ . Mitochondria, 2 mg, were preincubated in Eppendorf cups in 1 ml medium (200 mM sucrose, 5 mM  $\text{MgCl}_2$ , 10 mM Hepes-KOH buffer, pH 7.4 and [<sup>14</sup>C]sucrose). Glutamate transport was initiated by adding [<sup>3</sup>H]glutamate ( $\text{K}^+$  salt), 5 mM final concentration, and stopped by rapid centrifugation with an Eppendorf 3200 microcentrifuge. The supernatant was discarded and the pellet dissolved in 0.2 ml formic acid. The radioactivity (<sup>3</sup>H and <sup>14</sup>C) of the pellet was measured with an Intertechnique SL 40 scintillation counter in the POPOP/PP0 system. The [<sup>14</sup>C]sucrose permeable space was found to range from 1.5  $\mu\text{l}$  to 2  $\mu\text{l}$ /mg proteins. For each experiment, [<sup>3</sup>H]glutamate in the matrix space (sucrose impermeable) was calculated by subtracting [<sup>3</sup>H]-glutamate present in the sucrose space. Valinomycin 20 ng/mg protein,  $\text{Ca}^{2+}$  50 natoms/mg proteins, Pi 5 mM.

increases glutamate uptake to the same extent as the valinomycin- $\text{K}^+$  system.

In contrast, addition of 5 mM  $\text{P}_i$  stops glutamate uptake whether  $\text{Ca}^{2+}$  is present or not. When valinomycin is also present,  $\text{P}_i$  considerably reduces glutamate uptake (4 nmols [<sup>3</sup>H]Glu/mg proteins, instead of 22). It has been clearly established that phosphate uptake by mitochondria is controlled by the transmembrane  $\Delta\text{pH}$  [22,23]. In the presence of  $\text{Ca}^{2+}$  or of valinomycin there appears to be competition between the phosphate/ $\text{H}^+$  and the glutamate/ $\text{H}^+$  co-transporters in relation to the transmembrane  $\Delta\text{pH}$ . The presence of  $\text{Ca}^{2+}$  favours the phosphate/ $\text{Ca}^{2+}$  co-transport.

A small part of the reduction in radioactivity measured in the presence of  $\text{P}_i$  may be due to a  $\text{P}_i$ / [<sup>3</sup>H]dicarboxylate exchange resulting from [<sup>3</sup>H]-glutamate metabolism.

#### 3.2. The effect of glutamate on $\text{Ca}^{2+}$ uptake

##### 3.2.1. Mitochondrial $\text{Ca}^{2+}$ determined by atomic absorption

Figure 2 shows that, in the absence of any added substrate, the mitochondria contain about 5 natoms

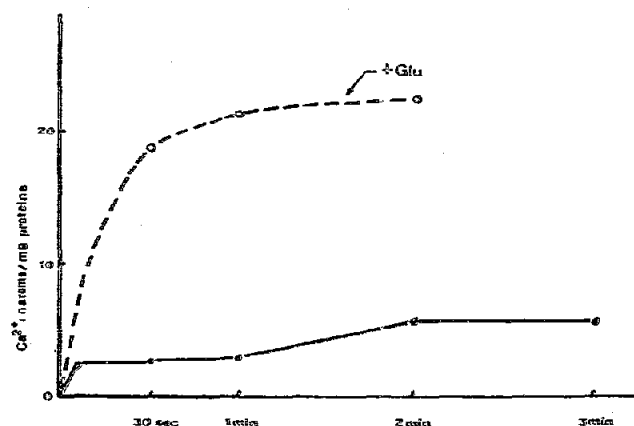


Fig. 2. Effect of glutamate on  $\text{Ca}^{2+}$  fixation. Mitochondria, 2 mg, were incubated in the same conditions as for glutamate uptake assays (fig. 1).  $\text{Ca}^{2+}$ , 50 natoms/mg protein, was initially present in the medium and the reaction was initiated by adding mitochondria and stopped by centrifugation at various times. In each case the pellet was dissolved in 0.2 ml formic acid for  $\text{Ca}^{2+}$  determination by atomic absorption. (—●—●—)  $\text{Ca}^{2+}$  incorporation. (---○---○---)  $\text{Ca}^{2+}$  incorporation with 5 mM Glu.

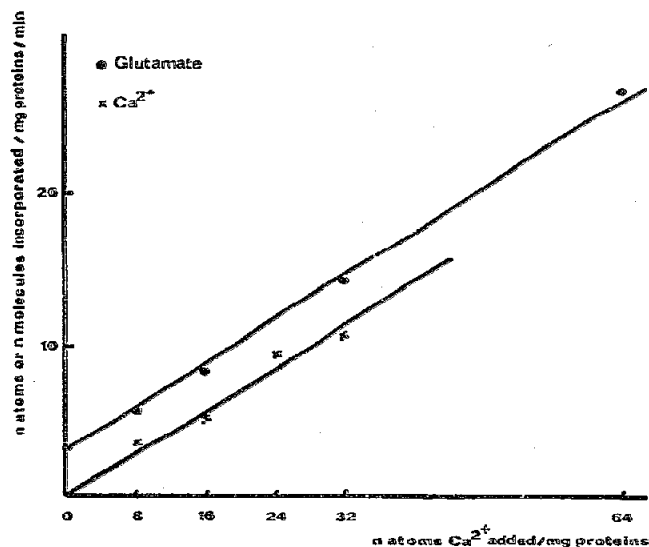


Fig.3. Correlation between glutamate and  $\text{Ca}^{2+}$  incorporation.  $[\text{}^3\text{H}]\text{Glutamate}$  and  $\text{Ca}^{2+}$  incorporation were determined in conditions described in fig.1 ( $[\text{}^3\text{H}]\text{Glu.}$ ) and fig.2 ( $\text{Ca}^{2+}$ ). (—●—●—) Glutamate accumulation. (—×—×—)  $\text{Ca}^{2+}$  accumulation.

$\text{Ca}^{2+}$ /mg protein. In the presence of 5 mM glutamate they accumulate up to 22 natoms  $\text{Ca}^{2+}$ /mg protein.

Figure 3 shows that there is a linear regression of the quantities of  $\text{Ca}^{2+}$  and  $[\text{}^3\text{H}]\text{glutamate}$  absorbed and the concentration of  $\text{Ca}^{2+}$  in the incubation medium.

### 3.2.2. Change in extramitochondrial free $\text{Ca}^{2+}$ concentration determined by a specific $\text{Ca}^{2+}$ electrode

In the absence of any added substrate mitochondria fix relatively little  $\text{Ca}^{2+}$  (fig.4A). The addition of 5 mM glutamate induces active penetration of mitochondria by  $\text{Ca}^{2+}$  and after a few minutes all the available  $\text{Ca}^{2+}$  is fixed. This raises the question of whether, in such an experiment, glutamate is involved only as a substrate generating the energy for  $\text{Ca}^{2+}$  transport or as an anion, indispensable to  $\text{Ca}^{2+}$  transport, before it is oxidised.

In fig.4B it can be seen that the addition of succinate in the presence of rotenone (protonmotive force generator) induces a slight  $\text{Ca}^{2+}$  penetration. In contrast, in the same conditions, glutamate addition gives rise to complete  $\text{Ca}^{2+}$  fixation. Figure 4C shows

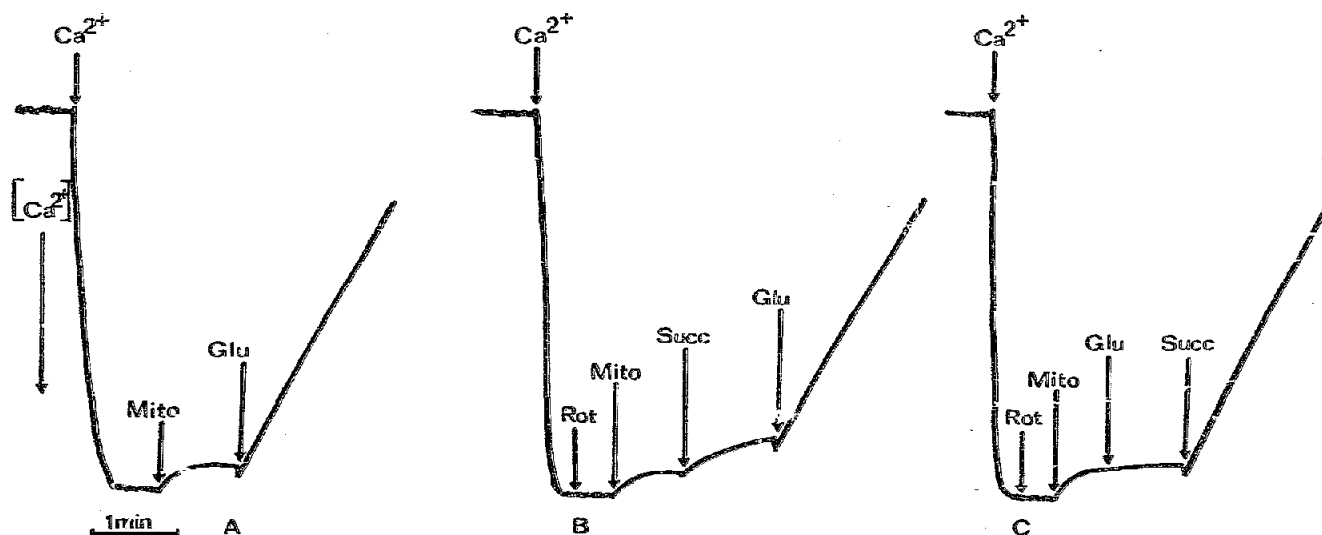


Fig.4. Effect of glutamate on extramitochondrial  $\text{Ca}^{2+}$  concentration measured with a  $\text{Ca}^{2+}$  specific electrode. Mitochondria, 1 mg, were incubated in 5 ml medium (20 mM sucrose, 20 mM KCl, 10 mM Hepes-KOH, pH 7.2).  $\text{Ca}^{2+}$  (100 natoms) was added before mitochondria. Changes in  $\text{Ca}^{2+}$  concentration were monitored with a  $\text{Ca}^{2+}$ -specific electrode Philips connected to a Orion 801 A ionometer with a Sefram recorder. Glutamate 5 mM, succinate 5 mM, rotenone 1  $\mu\text{g}$ .

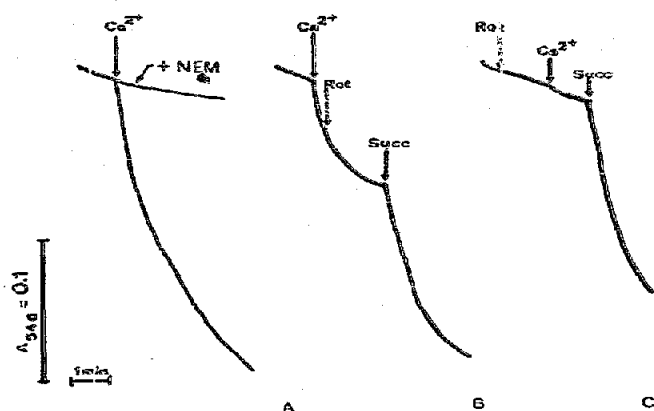


Fig.5. Energy-linked swelling induced by  $\text{Ca}^{2+}$  of rat liver mitochondria in Tris glutamate. Mitochondria, 1 mg, were added to 1 ml 0.1  $\mu\text{M}$  Tris-glutamate, pH 7.2.  $\text{Ca}^{2+}$  100 nM, NEM 100 nmol, succinate 5 mM, rotenone 1  $\mu\text{g}$ .

that glutamate added in the presence of rotenone, which inhibits its oxidation, does not result in  $\text{Ca}^{2+}$  fixation. Following succinate addition results immediately in calcium glutamate uptake.

### 3.2.3. Mitochondria swelling induced by calcium glutamate

When the mitochondria are incubated in the presence of 100 mM Tris-glutamate, no swelling is observed. The addition of 100  $\mu\text{M}$   $\text{Ca}^{2+}$  induces swelling which can be inhibited by *N*-ethylmaleimide (fig.5A).

Rotenone addition also causes inhibition whereas succinate re-establishes swelling (fig.5B). Calcium glutamate penetration is thus an energy-requiring process. When glutamate oxidation is inhibited by rotenone, succinate addition after  $\text{Ca}^{2+}$  results in glutamate- $\text{Ca}^{2+}$  transport (fig.5C).

## 4. Conclusions

Our results suggest that there is cotransport of glutamate and  $\text{Ca}^{2+}$  in rat liver mitochondria and that the process requires energy. This hypothesis is supported by the following facts:

1. Phosphate, whose active transport is linked to the

transmembrane  $\Delta\text{pH}$ , is a powerful inhibitor of glutamate translocation. In our experimental conditions, phosphate utilizes the transmembrane  $\Delta\text{pH}$  to its own advantage.

2. The glutamate/ $\text{Ca}^{2+}$  co-transport is inhibited if glutamate oxidation is blocked by rotenone. This inhibition is removed if succinate is added to the incubation medium.
3. The inhibition of glutamate/ $\text{Ca}^{2+}$  co-transport by NEM suggests that the glutamate/ $\text{H}^+$  transport system is involved.

## Acknowledgements

Thanks are due to DGRST (convention 76.7.1171). We are grateful to Professor Duchene-Marullaz for the use of the Perkin Elmer atomic absorption spectrometer.

## References

- [1] Chappel, J. B., Cohn, M. and Greville, G. D. (1963) in: Energy Linked Functions of Mitochondria (Chance, B. ed) pp. 219-231, Academic Press.
- [2] Chance, B. (1965) *J. Biol. Chem.* 240, 2729-2748.
- [3] Rossi, C. S., Bielawski, J. and Lehninger, A. L. (1966) *J. Biol. Chem.* 241, 1919-1921.
- [4] Lehninger, A. L. (1974) *Proc. Natl. Acad. Sci. USA* 71, 1520-1524.
- [5] Rottenberg, H. and Scarpa, A. (1974) *Biochemistry* 13, 4811-4817.
- [6] Reynafarje, B. and Lehninger, A. L. (1977) *Biochem. Biophys. Res. Commun.* 77, 1273-1279.
- [7] Moyle, J. and Mitchell, P. (1977) *FEBS Lett.* 73, 131-136.
- [8] Azzi, A., Chappel, J. B. and Robinson, B. H. (1967) *Biochem. Biophys. Res. Commun.* 29, 148-152.
- [9] La Noue, K. F. and Fischler, M. E. (1974) *J. Biol. Chem.* 249, 7522-7528.
- [10] La Noue, K. F., Meijer, A. J. and Brouwer, A. (1974) *Arch. Biochem. Biophys.* 161, 544-550.
- [11] Meijer, A. J., Brouwer, A., Reijngoud, D. J., Hoek, J. B. and Tager, J. M. (1972) *Biochim. Biophys. Acta* 283, 421-429.
- [12] Meyer, J. and Vignais, P. M. (1973) *Biochim. Biophys. Acta* 325, 375-394.
- [13] Bradford, N. M. and McGivan, J. D. (1973) *Biochem. J.* 134, 1023-1029.

- [14] Debise, R. and Durand, R. (1974) *Biochimie* 56, 161-170.
- [15] Hoek, J. B. and Njogu, R. M. (1976) *FEBS Lett.* 71, 341-346.
- [16] Johnson, D. and Lardy, H. A. (1967) in: *Methods in enzymology*, Vol. X, pp. 94-96, Academic Press.
- [17] Gornall, A. G. and Bardawill, L. H. and David, M. M. (1949) *J. Biol. Chem.* 177, 751-766.
- [18] Debise, R., Briand, Y., Durand, R., Gachon, P. and Jeminet, G. (1977) *Biochimie* 59, 497-508.
- [19] Ammann, D., Giuggi, M., Pretsch, E. and Simon, W. (1975) *Anal. Lett.* 8, 705-720.
- [20] Mitchell, P. and Moyle, J. (1969) *Eur. J. Biochem.* 7, 471-484.
- [21] Padan, E. and Rottenberg, H. (1973) *Eur. J. Biochem.* 40, 431-437.
- [22] Mitchell, P. and Moyle, J. (1969) *Eur. J. Biochem.* 9, 149-155.
- [23] Palmieri, F., Quagliariello, E. and Klingenberg, M. (1970) *Eur. J. Biochem.* 17, 230-238.