The present results differ markedly from those reported in the literature[1,2]. This discrepancy is not easily explained. Unpublished evidence suggests that lactoyllactic acid and higher polymers in the preparation would escape intermediary oxidation but not recovery in urine by non-enzymatic method. The physiological significance of the metabolism of D-lactate may be largely based on the intestinal production of D,L-lactate by many kinds of bacteria[4,5]. It may also gain importance for the utilization of D-lactate by man and animals consuming fermented vegetable or milk diets.

*Zusammenfassung.* Mittels spezifischer Methoden wurde der Stoffwechsel von D-Lactat bei Ratten untersucht. Nach peroraler Gabe erschienen (entgegen den bisherigen Befunden) nur 1–2% im Harn. Nach i.p. Gabe wurde D-Lactat rasch oxydiert und binnen 6 h als $CO_2$ exspiriert (83.2%) sowie in Form von D-Lactat (3.8%) und Metaboliten (4.2%) im Harn ausgeschieden.

D. Giesecke and Angelika Fabritius

*Institut für Tierphysiologie der Universität, Veterinärstrasse 13, D–8 München 22 (Federal Republic of Germany), 28 March 1974.*

[4] R. S. Breed, E. G. D. Murray and N. R. Smith, *Bergey's Manual of Determinative Bacteriology* (Williams & Wilkens, Baltimore 1957).

[5] K. O. Stetter and O. Kandler, Arch. Mikrobiol. *94*, 221 (1973).

## Effect of Low Calcium Concentration on the Oxidation of NAD-Linked Substrates in Rat Liver and Tumor Mitochondria

The uptake of $Ca^{2+}$ and its effect on mitochondrial structures and functions has been extensively studied[1]. Recently Vinogradov et al.[2] have reported an inhibitory effect of high $Ca^{2+}$ concentrations on respiration of rat liver mitochondria in the presence of NAD-linked substrates.

In this paper we describe the effect of low $Ca^{2+}$ concentrations on rat liver and tumor mitochondria under different metabolic conditions. The results obtained indicate that $Ca^{2+}$ can play, in rat liver mitochondria, a significant role on the oxidation of NAD-linked substrates, depending on the energetic state of mitochondria upon addition of the cation. Moreover $Ca^{2+}$ has no effect on tumor mitochondria regardless of their energetic state.

*Materials and methods.* Rat liver mitochondria were prepared according to Chance and Hagihara[3]. Ehrlich ascites tumor cells mitochondria (Lettré-hyperdiploid strain) were isolated by the method of Kobayashi

[1] A. L. Lehninger, E. Carafoli and C. S. Rossi, in *Advances in Enzymology* (Ed. F. F. Nord; Interscience Publishers, New York 1967), vol. 29, p. 259.

[2] A. Vinogradov, A. Scarpa and B. Chance, Archs Biochem. Biophys. *152*, 646 (1972).

[3] B. Chance and B. Hagihara, in *Proc. 5th Int. Congr. Biochem., Moskow* (Ed. A. N. M. Sissakian; Pergamon Press, Oxford 1961), vol. 5, p. 3.

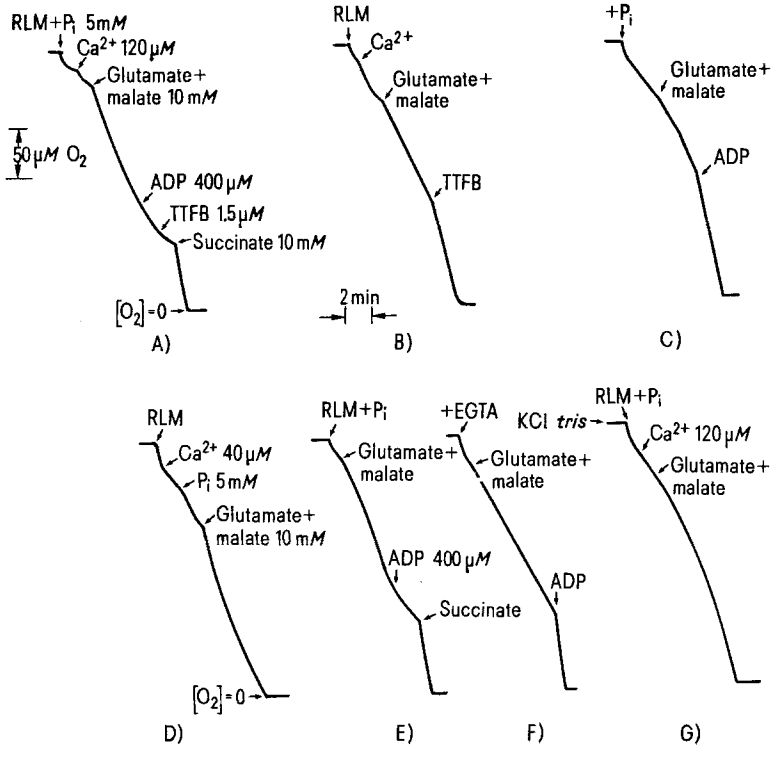


Fig. 1. Polarographic recordings of $O_2$ uptake in rat liver mitochondria showing the effect of $Ca^{2+}$, in the presence of $P_i$, on NAD-linked substrates respiration. 0.1 ml of mitochondrial suspension, containing 5–6 mg protein (A, B, C, D and G) or 2.5–3 mg protein (E and F), were added to 2.4 ml of an isotonic medium composed by: 0.225 *M* mannitol, 0.075 *M* sucrose and 0.01 *M* MOPS (morpholino propane sulfonic acid) pH 7.4, except in G, where the medium used was 0.08 *M* KCl and 0.02 *M Tris* pH 7.4. All the additions are reported on the figure. EGTA concentration: 1.0 m*M*.

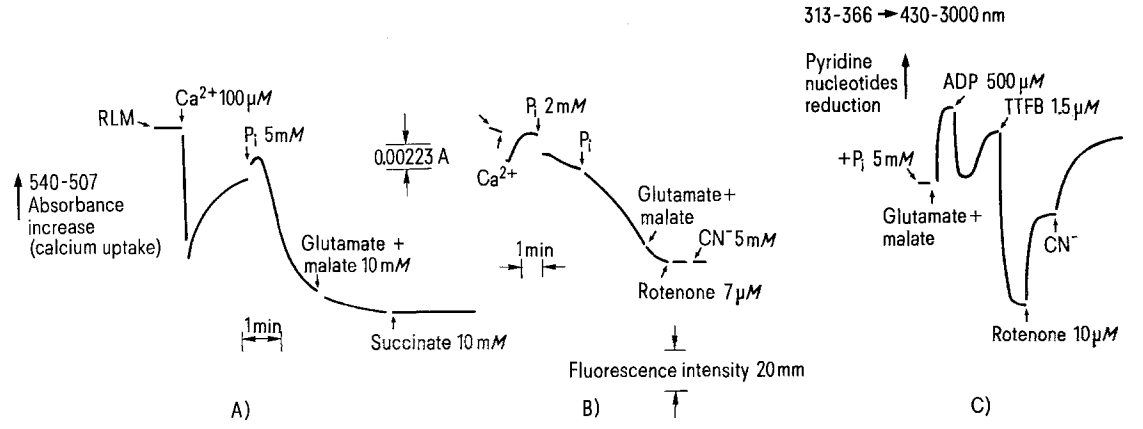


Fig. 2. Calcium uptake kinetic (A) and pyridine-nucleotides redox changes (B and C) in rat liver mitochondria. In A the wavelength pair 540–507 nm was used. The downward deflection, after $Ca^{2+}$ addition, indicates murexide-$Ca^{2+}$ complex formation, whereas the upward deflection means cation dissapearance from the medium. Murexide conc. 50 $\mu M$. Protein conc. 2.0 mg/ml. Final volume 2.5 ml. Medium composition see Figure 1. B) and C) fluorimetric measurements of steady state changes of pyridine-nucleotides. The excitation was at 313–366 nm (primary filter) and the emitted fluorescence wasmeasured, after passing through another filter (secondary filter), above 430 nm. Suspending medium, protein conc. and final volume as in A.

et al.[4]. Morris hepatoma 3924A mitochondria were prepared following PEDERSEN et al.[5]. Oxygen consumption was measured polarographically with a Clark oxygen electrode. $Ca^{2+}$ uptake kinetics were followed using murexide[6] (ammonium purpurate) in a dual wavelength/Split-beam Aminco Chance spectrophotometer. Redox changes of pyridine nucleotides were recorded fluorimetrically in an Eppendorf filter fluorometer. All the experiments were performed at room temperature (about 22 °C). The reagents used were of analytical grade.

*Results and discussion.* Figure 1 shows the effect that low $Ca^{2+}$ concentrations, in the presence of $P_i$ (inorganic phosphate), exert on the oxidation of NAD-linked substrates in rat liver mitochondria. When glutamate plus malate (10 m$M$) or other NAD-linked substrates (e.g. pyruvate and $\beta$-hydroxybutyrate) are added to mitochondrial suspension pretreated with $P_i$ (5 m$M$) and 120 or 40 $\mu M$ $Ca^{2+}$ (traces A and D respectively) the rate of oxygen consumption increases. However, in a short lapse of time, the respiration becomes progressively inhibited (same traces) and cannot be further stimulated by either ADP or the uncoupler TTFB (tetrachloro-2-trifluoromethylbenzimidazole), whereas the addition of succinate restores $O_2$ consumption (trace A). Indeed, control experiments have been performed using succinate alone as substrate: in this case there is no detectable inhibition of respiration by $Ca^{2+}$ (not shown). Moreover, under particular experimental conditions (mitochondrial prot. conc. half that normally used), we have found that also the endogenous $Ca^{2+}$ can cause the inhibition (trace E), which is in fact removed by EGTA (ethylene glycol di(aminoethyl)-tetra-acetic acid), a specific $Ca^{2+}$ chelating agent (trace F). $K^+$, which slows down $Ca^{2+}$ entry into mitochondria[6–8] is also able to prevent the inhibition (trace G). The results so far described clearly demonstrate that $Ca^{2+}$ at low concentrations, is able to depress mitochondrial oxidation of NAD-linked substrates, whereas it does not affect succinate utilization. Traces B and C represent control experiments where $Ca^{2+}$ and $P_i$ have been added separately.

We have also studied mitochondrial $Ca^{2+}$ uptake and PN redox changes under the conditions described above. The results are reported in Figure 2. The addition of $P_i$ to $Ca^{2+}$ treated mitochondria provokes a complete

[4] S. KOBAYASHI, B. HAGIHARA, M. MASUZUMI and K. OKUNUKI Biochim. biophys. Acta, *113*, 421 (1966).
[5] P. L. PEDERSEN, J. W. GREENEWALT, T. L. CHAN and H. P. MORRIS, Cancer Res., *30*, 2620 (1970).
[6] L. MELA and B. CHANCE, Biochemistry, *7*, 4059 (1968).
[7] E. J. HARRIS, J. D. JUDAH and K. AHMED, in *Current Topics in Bioenergetics* (Ed. D. R. SANADI; Academic Press, New York 1966), vol. 1, p. 255.
[8] C. ROSSI, A. AZZI and C. F. AZZONE, J. biol. Chem., *242*, 951 (1967).

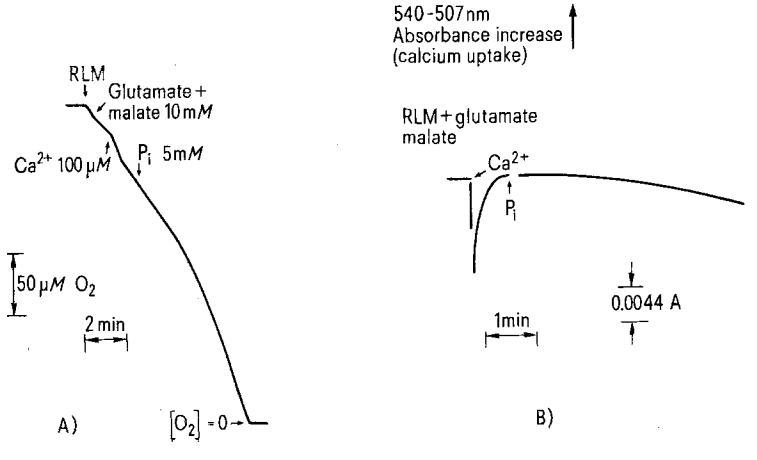


Fig. 3. Polarographic recordings of oxygen consumption (A) and calcium uptake kinetic (B) in rat liver mitochondria pretreated with NAD-linked substrates before $Ca^{2+}$ and $P_i$ addition. Protein conc. 2.0 mg/ml. Final volume 2.5 ml. Medium composition as in Fig. 1. For further $Ca^{2+}$ uptake kinetic explanations see Figure 2. Murexide (B) 50 $\mu M$.

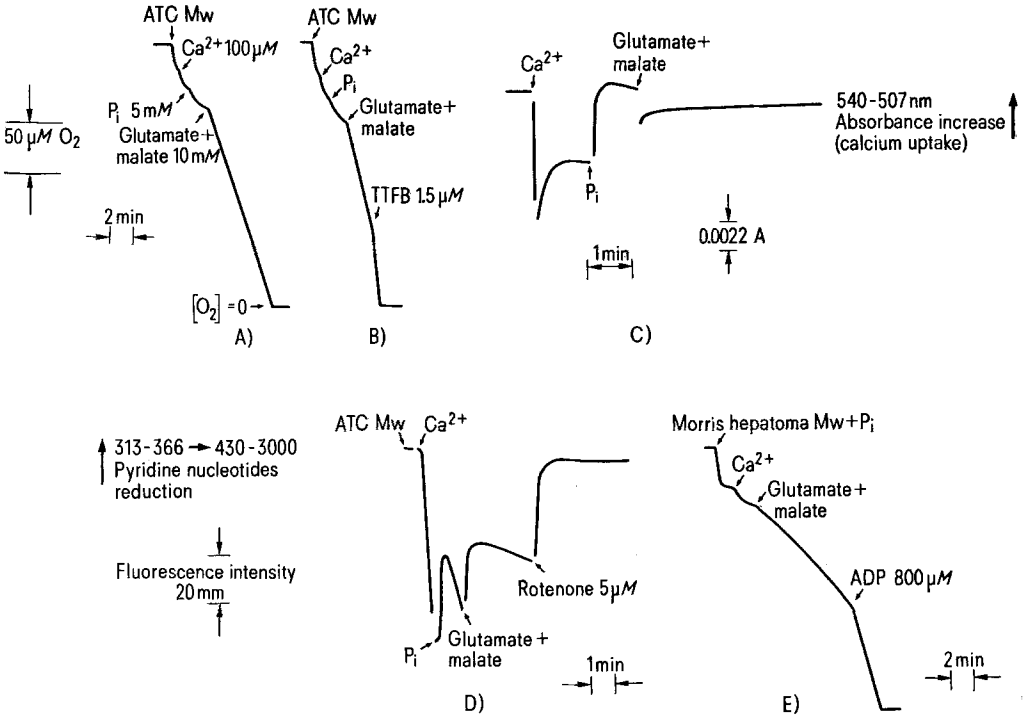


Fig. 4. A, B, C and D; respiration, $Ca^{2+}$ uptake and PN kinetics of isolated Ehrlich ascites tumor cells (ATC) mitochondria. E) respiration of Morris hepatoma 3924 A mitochondria. Tumor mitochondria were suspended in the same medium described in Figure 1. The final volume was 2.5 ml in all cases. Protein concentration: 2.0 mg protein/ml (A, B and E); 1.0 mg protein/ml (C); 2.2 mg protein/ml (D).

release of the cation, not reversed by the addition of both glutamate plus malate and succinate (trace A), together with a strong oxidation of PN, not modified even by rotenone and cyanide (trace B). Trace C shows the redox limits of the system, obtained by the uncoupler (TTFB) and cyanide, to prove that changes in light scattering do not simulate the effect observed.

The irreversible PN oxidation and the previously described inhibition of respiration indicate that low $Ca^{2+}$ concentrations, in the presence of $P_i$, produce a damaging effect on rat liver mitochondrial respiratory chain, namely at NADH-dehydrogenases level. In addition, the fact that succinate, though stimulating respiration (Figure 1: traces A and E), fails to induce $Ca^{2+}$ uptake (Figure 2: trace A) indicates that in these conditions mitochondria become uncoupled.

Figure 3 shows the results of experiments in which $Ca^{2+}$ and $P_i$ have been added to mitochondria pretreated with NAD-linked substrates. Indeed, in these conditions, different phenomena can be observed: there is no inhibition of respiration (trace A) and $Ca^{2+}$ taken up is retained within mitochondria throughout the whole observation time.

At the moment we have no complete explanation for the complex effects that low $Ca^{2+}$ concentrations exert on rat liver mitochondria. Following VINOGRADOV et al. [2], who reached almost similar results using very high $Ca^{2+}$ concentrations (500 $\mu M$), it could be said that the increased mitochondrial membrane permeability to PN, caused by $Ca^{2+}$, is responsible for the entire phenomenon. Indeed, in our case, such a conclusion seems to be rather incomplete because of the different effect $Ca^{2+}$ elicits in mitochondria oxidizing endogenous or exogenous substrates. On the basis of these observations, we can conclude that $Ca^{2+}$, in the presence of $P_i$, displays its damaging effect only when it comes in contact with mitochondrial membranes in a low energy state (absence of added substrates).

Figure 4 summarizes all the results obtained from experiments carried out on Ehrlich ascites tumor cells (ATC) and Morris hepatoma mitochondria. Tumor mitochondria pretreated with $Ca^{2+}$ 100 $\mu M$ and $P_i$ 5 m$M$ show, after addition of NAD-linked substrates, a linear activation of respiration (trace A, B and E); in the same conditions as rat liver mitochondria, they retain all $Ca^{2+}$ accumulated (trace C) and display a regular PN reactivity (trace D).

To conclude, low $Ca^{2+}$ concentrations, in the presence of $P_i$, inhibit rat liver mitochondria respiration, whereas they are not active on mitochondria of Ehrlich ascites tumor cells and Morris hepatoma. The mechanism of such an inhibition has not been clarified. The differences found in the behaviour of tumor cells mitochondria with respect to those of rat liver are considerable; at the present, however, it is difficult to say which role changed reactivity of the tumor cells mitochondria plays in the whole cell metabolic features [9].

*Riassunto.* È stato osservato che basse concentrazioni di $Ca^{2+}$ possono esercitare un ruolo importante sull'ossidazione dei substrati NAD-dipendenti in mitocondri di fegato di ratto in rapporto allo stato energetico di questi, mentre non mostrano alcun effetto su mitocondri di tumore nelle stesse condizioni sperimentali.

M. L. EBOLI, A. CITTADINI and T. TERRANOVA

*Institute of General Pathology, Catholic University, via Pineta Sacchetti 644, I-00168 Roma (Italy),*
*28 January 1974.*

[9] Acknowledgments. This work has been partly supported by a grant from Consiglio Nazionale delle Ricerche and Ministero della Pubblica Istruzione, Italy. We thank Mr. CARLO MASSACCI for his help in the performance of this work.