Regulation of Oxidative Phosphorylation in Mitochondria by External Free Ca\textsuperscript{2+} Concentrations*

Rafael Moreno-Sánchez‡
From the Departamento de Bioenergética, Centro de Investigaciones en Fisiología Celular, UNAM. Apartado Postal 70-600, 04510 México, D.F.

The rate of oxidative phosphorylation was studied in rat liver mitochondria incubated with free Ca\textsuperscript{2+} concentrations that range from 10^{-8} to 5 \times 10^{-6} M. The highest rate was observed between 0.5-1.0 \mu M Ca\textsuperscript{2+}. ATP synthesis was measured by polarographic and spectrophotometric techniques and by uptake of radiolabeled inorganic phosphate. The concentration of Ca\textsuperscript{2+} at which maximal rates of ATP synthesis take place is modified by Mg\textsuperscript{2+} and phosphate. The dependence of oxidative phosphorylation on Ca\textsuperscript{2+} was observed with α-ketoglutarate, glutamate + malate, and succinate, but not with β-hydroxybutyrate. At 10^{-9} M Ca\textsuperscript{2+} there is a continuous exit of endogenous Ca\textsuperscript{2+}, while with 10^{-6} M Ca\textsuperscript{2+}, intramitochondrial Ca\textsuperscript{2+} levels remained constant throughout time. Apparently the control of the level of internal Ca\textsuperscript{2+} by external Ca\textsuperscript{2+} modulates the rate of oxidative phosphorylation. Uncoupler-stimulated respiration also depends on Ca\textsuperscript{2+} concentration, even though at 10^{-9} to 10^{-6} M Ca\textsuperscript{2+} the rate of oxidative phosphorylation is lower than the rate of uncoupled respiration. The contribution of the ADP/ATP carrier and the ATP synthase to the kinetic regulation of ATP synthesis at 10^{-9} and 10^{-6} M Ca\textsuperscript{2+} was evaluated by titrations with carboxyatractyloside and oligomycin, respectively. The contribution of the carrier and the synthase to the regulation of the final rate of ATP synthesis was different at the two concentrations of Ca\textsuperscript{2+}; therefore, the concentration of extramitochondrial Ca\textsuperscript{2+} influences the overall kinetics of oxidative phosphorylation.

Oxidative phosphorylation and Ca\textsuperscript{2+} uptake in mitochondria are two processes that depend on energy of electrochemical H\textsuperscript{+} gradients. In competition studies (1, 2) it has been shown that mitochondria preferentially use the energy of H\textsuperscript{+} gradient for Ca\textsuperscript{2+} uptake rather than for the phosphorylation of ADP. On the other hand, it has been reported that oxidative phosphorylation is inhibited in mitochondria that have a high content of endogenous Ca\textsuperscript{2+}, i.e. smooth muscle (3), heart (4, 5), uterus (6), tumor (7-9), and brain (10), which suggests that the level of matrix Ca\textsuperscript{2+} may affect directly the activity of intramitochondrial enzymes involved in ATP synthesis. The exact mechanism of the inhibition by internal Ca\textsuperscript{2+} is not well known, but in liver and uterus mitochondria it has been observed that Ca\textsuperscript{2+} affects the functioning of the ADP/ATP carrier (6, 11, 12). More recently it was found that internal Ca\textsuperscript{2+} induces a unidirectional efflux of adenine nucleotides (13) which apparently diminishes the activity of the translocator, thus inducing low rates of oxidative phosphorylation in Ca\textsuperscript{2+}-loaded mitochondria (13). It has also been suggested that Ca\textsuperscript{2+} prevents the release of inhibiting action of the ATPase inhibitor protein from mitochondrial ATPase (14, 15).

Interestingly, Robertson et al. (16) reported that in heart mitochondria external Ca\textsuperscript{2+} in the range of 10^{-10} to 10^{-6} M induces a dual effect on oxidative phosphorylation, either an activation of approximately 25% (between 0.1-1.0 \mu M) and a strong inhibiting effect with >1.0 \mu M regardless of the presence of Mg\textsuperscript{2+}. In this work the effect of external Ca\textsuperscript{2+} concentrations lower than 1.0 \mu M on oxidative phosphorylation was studied. The results showed that by varying external Ca\textsuperscript{2+} concentrations, it is possible to observe different rates of steady-state ATP synthesis. At these different rates, the contribution of the adenine nucleotide translocase and the ATP synthase to the control of rate of oxidative phosphorylation (17, 18) was evaluated. It was found that the level of external Ca\textsuperscript{2+} induces important modifications of the overall kinetics of oxidative phosphorylation.

MATERIALS AND METHODS
Preparation of Mitochondria—Female Wistar rats weighing 180-200 g and fasted for 38-44 h were killed by decapitation. The liver was extracted and washed twice and homogenized with 250 mM sucrose, 5 mM HEPES, 0.5 mM EGTA (SHE), pH 7.0, in the cold. The homogenate was centrifuged to 7000 \times g for 10 min. The mitochondrial pellet was washed with SHE, resuspended, and incubated with 0.5% bovine serum albumin for 5 min in ice with occasional stirring; subsequently the mixture was diluted 20 times and centrifuged to 7000 \times g for 10 min. The sediment was washed and resuspended in SHE to a concentration of 50-80 mg/ml.

Oxygen Consumption—Oxygen uptake of mitochondria incubated in 3 ml of incubation basis mixture that contained 130 mM KCl, 20 mM HEPES, 2 mM MgCl\textsubscript{2}, 2 mM K phosphate, 5 mM succinate, and Ca\textsuperscript{2+}-EGTA buffer, pH 7.20, was recorded by means of an oxygen electrode (Yellow Springs Instrument Co.)

Ca\textsuperscript{2+}-EGTA Buffers—To calculate the free Ca\textsuperscript{2+} concentration, the stability constants and the program described by Fabiato and Fabiato (19) were used. This program considers the contribution of all the ligands and metals ions that were included in the experiments. The final concentration of EGTA was 2.0 mM, and the pH of the fresh medium was carefully adjusted to 7.20.

Ca\textsuperscript{2+} Transport—For assays of Ca\textsuperscript{2+} fluxes the procedure in Ref. 13 was utilized. Briefly, mitochondria were incubated in the conditions described under "Results" with 4 Ca\textsuperscript{2+} (2-4 \times 10^5 pmol/mg). At the desired times aliquots were withdrawn and filtered through

* This study was supported in part by a grant from CONACyT, México and the Organization of American States to Dr. A. Gómez-Puyou. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Fellow of Universidad Nacional Autónoma de México.
Sartorius filters of a pore diameter of 0.22 μm. The filter was washed with 1 ml of cold 130 mM KCl, 5 mM Tris-HCl, pH 7.4, and counted for radioactivity after solubilization with 5 ml of the scintillation liquid Tritosol (20).

Intramitochondrial Ca²⁺ Content—This measurement was made by atomic absorption spectrophotometry, using a Perkin-Elmer (model 560) spectrophotometer. Mitochondria were separated from the medium by rapid centrifugation in a Beckman microfuge B. The pellet was extracted for Ca²⁺ determination with 1.5% (v/v) HCl, 1% (v/v) LaCl₃, 25 mM KCl, and denatured protein eliminated by centrifugation. The supernatant was used for the assays.

Spectrophotometric ATP Determination—This was carried out according to Lamschweizer and Trausnicht (21). Mitochondria were incubated under the conditions described under “Results.” At fixed times aliquots of 2 ml were transferred to cuvettes that contained the necessary compounds for the spectrophotometric determination of ATP (21).

Uptake of ³²P into ATP—Mitochondria were incubated under the indicated conditions with ³²P, (approximately 0.5 μCi/μmol). At predetermined times the reaction was stopped with 6% trichloroacetic acid (final concentration). After centrifugation of denatured protein an aliquot of the supernatant was withdrawn and 32Pi extracted as described originally by Lindberg and Ernster (22) with n-butyl acetate. The aqueous phase was used for assay of ³²P, incorporated into ATP by assay of Cerenkov radiation.

RESULTS

Ca²⁺ Uptake at Various External Free Ca²⁺ Concentrations—The experiments detailed in Table I show that the intramitochondrial level of Ca²⁺ of respiring mitochondria is importantly affected by the extramitochondrial Ca²⁺ concentration. At 10⁻⁹ M external Ca²⁺, the intramitochondrial level of Ca²⁺ diminishes progressively by approximately 70% of that of the starting preparation. In mitochondria incubated with 10⁻⁶ M Ca²⁺, the level does not change significantly, while at an external Ca²⁺ concentration of 5 × 10⁻⁶ M the intramitochondrial level increases. The addition of ADP which induces a state 4 to state 3 transition results in a further increase in Ca²⁺ uptake in mitochondria incubated with 5 × 10⁻⁶ M Ca²⁺ but does not modify appreciably the level of internal Ca²⁺ when mitochondria are incubated with lower external Ca²⁺ concentrations. In agreement with Nichols (23), it would appear, that at 10⁻⁴ M external Ca²⁺, the rate of Ca²⁺ influx would equal that of Ca²⁺ efflux, while at concentrations below or above 10⁻⁶ M external Ca²⁺ either Ca²⁺ efflux or influx would proceed until a different steady state is reached. As the concentration of internal Ca²⁺ may influence ATP synthesis (3-16) oxidative phosphorylation by mitochondria incubated in the experimental conditions of Table I was studied.

| Time | 10⁻⁴ M | 10⁻³ M | 5 × 10⁻⁶ M |
|------|--------|--------|------------|
| 1 min| 20.4 ± 1.8 (4) | 19.4 ± 1.5 (2) | 27.2 ± 7.3 (3) |
| 3 min| 11.7 |
| 5 min| 10.8 ± 1.1 (4) | 22.2 ± 3.3 (4) | 31.1 ± 8 (2) |
| 7 min| 6.0 |
| 10 min| 5.9 ± 2.4 (4) | 29.2 ± 2 (3) | 85.9 ± 8.3 (2) |

Synthesis of ATP at Various Ca²⁺ Concentrations—Mitochondria incubated in state 4 conditions for 5 min with different oxidizable substrates and then given ADP show a distinct rate of state 3 respiration that depends on the concentration of Ca²⁺ in the medium (Table II). With succinate, glutamate + malate, and α-ketoglutarate as oxidizable substrates, higher rates of oxygen uptake are observed with 10⁻⁶ M than at 10⁻⁹ M Ca²⁺. With all these substrates, 5 × 10⁻⁶ M Ca²⁺ induces a strong diminution of the state 3 respiration rate; with β-hydroxybutyrate the diminution starts to be observed at 10⁻⁸ M Ca²⁺.

Fig. 1 shows a curve of Ca²⁺ concentration versus rate of respiration with succinate as substrate. Maximal stimulation of state 3 respiration was observed with 10⁻⁶ M Ca²⁺ (Fig. I A). From preparation to preparation, the maximum was found to vary between 0.5-1.0 μM Ca²⁺, but in all cases, higher Ca²⁺ concentrations the rate of state 3 respiration falls sharply. Even though state 4 respiratory rates increase progressively from 24 natsoms O mg⁻¹ min⁻¹ at 10⁻⁹ to 34 at 10⁻⁶ M Ca²⁺, the large variations in state 3 respiration observed between these concentrations of Ca²⁺ are most likely due to changes in the rate of oxidative phosphorylation. Indeed the ADP/O ratios are not modified by these concentrations of Ca²⁺, and the respiratory control values at 10⁻⁹ and 10⁻⁶ M Ca²⁺ are 3.6 and 4.6, respectively (see data of Fig. 1 A). Moreover, the direct assay of ATP formed in the various incubation media showed that ATP formation parallels the rate of oxygen consumption (Fig. 1 B). For example, with the polarographic method it was observed that state 3 respiration is approximately 33% higher at 10⁻⁹ M than at 10⁻⁷ M Ca²⁺, while the spectrophotometric assay of ATP, in the presence of 10 mM AMP, indicated that ATP synthesis was 25% higher at 5 × 10⁻⁷ M than at 10⁻⁷ M Ca²⁺.

The effect of different concentrations of Ca²⁺ on ATP synthesis was also measured by assays of the phosphorylation of ADP by inorganic phosphate [³²P]. Mitochondria preincubated for 5 min with 5 × 10⁻⁶ M Ca²⁺ show a higher synthesis of ATP, than when preincubated with 10⁻⁹ M Ca²⁺. At 5 × 10⁻⁶ M Ca²⁺ synthesis of ATP is depressed (Table III). Thus the results of Fig. 1 and Tables I to III indicate that there is a critical concentration of Ca²⁺ at which oxidative phosphorylation takes place at maximum rates. The strong inhibition of oxidative phosphorylation by high Ca²⁺ concentrations (5 × 10⁻⁶ M) observed in Fig 1 and Tables II and III is probably due to a massive accumulation of Ca²⁺, a phenomenon which has been extensively documented (Refs. 3-16 and see also Table I).

Characteristics of the Effect of External Ca²⁺ on Oxidative Phosphorylation—The rate of oxidative phosphorylation de-

| Substrate | Rate of state 3 respiration |
|-----------|-----------------------------|
| Succinate | 10⁻⁷ M Ca²⁺ | 10⁻⁹ M Ca²⁺ | 5 × 10⁻⁶ M Ca²⁺ |
| Glutamate + malate | 84 | 124.8 | 16.3 |
| α-Ketoglutarate | 38.4 | 86.4 | 19.7 |
| Succinate + rotenone (3 μM) | 95.2 | 156.1 |
| β-Hydroxybutyrate | 45.6 | 30.2 | 12 |
**Ca²⁺ and Oxidative Phosphorylation**

**Fig. 1.** Stimulation by external free Ca²⁺ of state 3 respiration. A, mitochondria (3 mg) were added to 3 ml of basic medium of incubation (see "Materials and Methods") with 5 mM succinate as substrate and the concentrations of Ca²⁺ shown. After 5 min, 250 µM ADP was added and the rate of respiration recorded. The values reported are the mean ± S.D. of 4 observations. B, the rate of respiration was measured as indicated in A. Spectrophotometric ATP determination was made according to Ref. 21. Mitochondria (1 mg/ml) were incubated for 5 min, and aliquots were transferred to cuvettes with the necessary compounds plus 0.5 mM ADP for ATP determination (21). Respiration was carried out at 30 °C and ATP determination at 23 °C. The unit of x axis of the inset is 1 M Ca²⁺; ATP synthesis is nmol min⁻¹ mg⁻¹.

**TABLE III**

| Time (min) | 10⁻⁸ M | 5 x 10⁻⁷ M | 5 x 10⁻⁸ M |
|------------|--------|------------|------------|
| Ca²⁺       | Ca²⁺   | Ca²⁺       | Ca²⁺       |
| 1          | 301.3  | 280.6      | 74.3       |
| 5          | 118.7  | 202.6      | 43.9       |
| 10         | 48     | 148.6      | 43.9       |

**Fig. 2.** Effect of the time of preincubation with different Ca²⁺ concentrations on rate of respiration. Experimental conditions were as indicated in Fig. 1A, except that at the times shown, 250 µM ADP was added. The numbers on the traces indicate ATP synthesis (nmol mg⁻¹ min⁻¹) determined spectrophotometrically as described in Fig. 1B.

Ca²⁺ transport (24, 25) by diminishing carrier affinity, and Robertson et al. (16) observed that Mg²⁺ shifted the inhibiting effect of Ca²⁺ on state 3 respiration to higher concentration.
**Table IV**

*Respiration stimulated by consecutive additions of ADP at different Ca\textsuperscript{2+} concentrations*

Mitochondria were incubated in conditions described in Table I. At the times shown, consecutive additions of 240 nmol of ADP were made.

| Time | Succinate | Glutamate + malate |
|------|-----------|--------------------|
|      | Rate of respiration | Rate of respiration |
|      | 10\textsuperscript{-9} M | 10\textsuperscript{-9} M | 10\textsuperscript{-9} M | 10\textsuperscript{-9} M |
| State 3 | State 4 | State 3 | State 4 | State 3 | State 4 |
| min | | | | | | | |
| 1 | 175.2 | 44 | 159.8 | 44 | 104.1 | 30 | 110.4 | 25 |
| 3 | 132 | 38.4 | 144 | 42.2 | 105.6 | 28.8 | 105.6 | 23 |
| 5 | 72 | 24 | 132 | 39.8 | 87.8 | 27.8 | 103.2 | 21.6 |
| 7 | 44.6 | 21.6 | | 39.8 | 59 | 25.9 | 93.6 | 24 |

**Table V**

*Effect of Ca\textsuperscript{2+} on uncoupler-stimulated respiration*

Mitochondria were incubated at the times and Ca\textsuperscript{2+} concentrations shown. At this time, 0.33 mM carbonyl cyanide p-trifluoromethoxyphenylhydrazone was added and the rate of respiration recorded. The numbers in parenthesis indicate the rate of state 3 respiration obtained in the same conditions with 390 μM ADP.

| Time | Rate of uncoupled respiration |
|------|-------------------------------|
|      | 10\textsuperscript{-9} M | 10\textsuperscript{-9} M |
| min | Ca\textsuperscript{2+} | Ca\textsuperscript{2+} |
| 1.5 | 272 (152) | 244 (156) |
| 5.0 | 157 (84) | 212 (144) |

---

in porcine heart mitochondria. The concentration of Ca\textsuperscript{2+} required to induce maximal stimulation of state 3 respiration in the experimental conditions described in this work is also affected by Mg\textsuperscript{2+}. In the absence of Mg\textsuperscript{2+} the same pattern of results shown in Fig. 2 was obtained, except that maximal rates of state 3 respiration were obtained with 0.3 μM Ca\textsuperscript{2+} (data not shown). The shift in Ca\textsuperscript{2+} concentration required to induce maximal rates of oxidative phosphorylation could be related to an effect of Mg\textsuperscript{2+} on the null point between influx and efflux of Ca\textsuperscript{2+} as described by various authors (Refs. 23, 26, and 27; for a review see Ref. 28).

It was also observed that the effect of Ca\textsuperscript{2+} on oxidative phosphorylation is modified by the concentration of inorganic phosphate. With the optimal Ca\textsuperscript{2+} concentration (10\textsuperscript{-4} M), the rate of state 3 respiration was further increased when inorganic phosphate was varied from 0.5 to 10 mM. The rate of resting respiration decreased approximately 30% in the same range of inorganic phosphate (data not shown).

Thus from the experiments described, it appears that there is a level of endogenous Ca\textsuperscript{2+} which would be essential for maximal rates of oxidative phosphorylation.

**Uncoupled Respiration of Mitochondria Incubated with 10\textsuperscript{-9} and 10\textsuperscript{-4} M Ca\textsuperscript{2+}**—In an attempt to understand why the rate of oxidative phosphorylation is higher at 10\textsuperscript{-9} than 10\textsuperscript{-4} M external Ca\textsuperscript{2+}, the rate of uncoupler-stimulated respiration was studied (Table V). It was observed that in mitochondria exposed to 10\textsuperscript{-9} M Ca\textsuperscript{2+} the rate of respiration decreased as the time of preincubation was increased. In mitochondria incubated with 10\textsuperscript{-4} M Ca\textsuperscript{2+}, the rate of respiration remained more or less constant regardless of the length of the preincubation. These observations indicate that external Ca\textsuperscript{2+} has an effect on the rate of electron flow and/or the transport of oxidizable substrates into the mitochondria. However, this does not seem to account completely for the different rates of ATP synthesis observed at the two Ca\textsuperscript{2+} concentrations, since the rate of uncoupler-stimulated respiration is higher than that of state 3 (Table VI), notwithstanding the concentration of Ca\textsuperscript{2+}. This suggests that the presently described effect of different Ca\textsuperscript{2+} concentrations on the rate of ATP synthesis is most likely due to an action on the phosphorylation of ADP or on the adenine nucleotide translocase.

**Control of the Rate of ATP Synthesis by the Adenine Nucleotide Translocator and by the ATP Synthase**—It has been previously proposed that the rate of oxidative phosphorylation is limited by the activity of the adenine nucleotide translocator (29-32). However, this postulation has not been entirely accepted (Refs. 33, and 34; for a review see Ref. 35). Since different steady-state rates of ATP synthesis can be induced by varying external Ca\textsuperscript{2+} concentrations, it was possible to explore the contribution of the adenine nucleotide translocator to the process, in an attempt to define the role of Ca\textsuperscript{2+} in the control of the rate of ATP synthesis. To this purpose a titration of the rate of state 3 respiration with a specific irreversible inhibitor of the translocator (36) was carried out. Fig. 3 shows the effect of different amounts of carboxyatractyloside on state 3 respiration of mitochondria incubated with 10\textsuperscript{-9} and 10\textsuperscript{-4} M external Ca\textsuperscript{2+} with succinate and glutamate + malate as substrates (Fig. 3, A and B, respectively). A marked difference of the effect of carboxyatractyloside on the rate of state 3 respiration is observed at a concentration of carboxyatractyloside below 70 pmol/mg, respiration at 10\textsuperscript{-9} M Ca\textsuperscript{2+} being more sensitive to carboxyatractyloside than at 10\textsuperscript{-9} M Ca\textsuperscript{2+}.

According to Tager and co-workers (17, 18), the degree of

---

**Figure 3. Inhibition of the state 3 respiration by carboxyatractyloside at two Ca\textsuperscript{2+} concentrations.** Mitochondria (5 mg) were incubated in the conditions described in Fig. 1A with the amounts of carboxyatractyloside (CAT) indicated. After 5 min of preincubation with succinate plus rotenone (A) or glutamate plus malate (B), 240 μM ADP was added and the rate of respiration recorded. O, 10\textsuperscript{-9} M Ca\textsuperscript{2+}; C, 10\textsuperscript{-4} M Ca\textsuperscript{2+}.

**Table VI**

*Control strength of the adenine nucleotide translocator and ATP synthase on oxidative phosphorylation at 10\textsuperscript{-9} and 10\textsuperscript{-4} M external Ca\textsuperscript{2+}*

The control strength (Ci) was calculated according to Ref. 17 using the data of Figs. 3 and 4. Uninhibited oxidative phosphorylation (Fo) was calculated from the rate of state 3 respiration and its respective ADP/O ratio.

| Substrate | External Ca\textsuperscript{2+} |
|-----------|-------------------------------|
|           | Translocase | ATP synthase |
|           | 10\textsuperscript{-9} M | 10\textsuperscript{-4} M | 10\textsuperscript{-9} M | 10\textsuperscript{-4} M |
| Succinate + rotenone | Fo Ci Fo Ci | 124 0.10 163 0.46 | 138 0.20 214 0.05 |
| Glutamate + malate  | Fo Ci Fo Ci | 119 0.27 197 0.66 |
control of a step in a metabolic pathway may be estimated by measuring the initial slope of the inhibition curve and relating it to the uninhibited and fully inhibited activity. The results of the calculations are shown in Table VI. Clearly there is significant difference in the control of the rate of oxidative phosphorylation by the translocator at the two Ca²⁺ concentrations studied, independently of the substrate used. Its contribution to the overall control of the rate of oxidative phosphorylation is about 4.6 and 2.4 times higher with succinate than with glutamate-malate, respectively, at 10⁻⁷ M than at 10⁻⁸ M Ca²⁺.

The contribution of the ATP synthase to the control of oxidative phosphorylation was judged by assaying the sensitivity of state 3 respiration to oligomycin of mitochondria incubated with 10⁻⁷ and 10⁻⁸ M Ca²⁺. The results of Fig. 4 show that regardless of the rate of state 3 respiration, the inhibition curve by increasing concentrations of oligomycin is highly sigmoidal; nevertheless, it is of interest to point out that 10⁻⁷ M Ca²⁺, the degree of control of oxidative phosphorylation exerted by the ATP synthase is higher than at 10⁻⁸ M Ca²⁺. Moreover, according to the criterion employed to estimate the control exerted by the ATP synthase and the translocase, it would appear that with 10⁻⁸ M external Ca²⁺, the two enzymes possess a similar quantitative effect on the regulation of the rate of ATP synthesis.

**DISCUSSION**

The effect of Ca²⁺ on oxidative phosphorylation has been extensively studied, and there is general agreement that at relatively high amounts of Ca²⁺ accumulated, oxidative phosphorylation is inhibited (3–16). In this work these results have been confirmed, but in addition it was found that there is a concentration of Ca²⁺ at which maximal rates of ATP synthesis (as determined by the polarographic method or by direct assay) take place. In our standard experimental conditions this is around 10⁻⁸ M Ca²⁺, but it may vary with the presence or absence of Mg²⁺ and with the concentration of phosphate in the medium.

It may be considered that the lower rates of ATP synthesis observed at 10⁻⁸ M Ca²⁺ in comparison to those observed with 10⁻⁶ M Ca²⁺ are due to damage of mitochondria. In this respect, it is to be noted that between 10⁻⁶ and 10⁻⁴ M external Ca²⁺ concentration, similar ADP/O ratios are attained, which indicates that the permeability properties of the mitochondria are not affected.

The modulation of the rates of oxidative phosphorylation by concentrations of external Ca²⁺ below 1.0 μM is of interest, since this is apparently the range in which Ca²⁺ concentration varies within the cell under physiological conditions (26). In order to determine the rate of ATP synthesis, the results of this work also show that upon exposure of mitochondria to concentrations of external Ca²⁺ of 10⁻³ M (a concentration at which oxidative phosphorylation is about 30% lower than at 10⁻⁶ M Ca²⁺), the amount of internal Ca²⁺ falls to a level of about 10 nmol/mg of protein (after 5 min of incubation). While during incubation with 10⁻⁶ M external Ca²⁺, the amount of intramitochondrial Ca²⁺ is poised at a level of approximately 20 nmol mg⁻¹, a concentration approximately equal to that of the starting preparation. Following Nicholls (23), at concentrations of 10⁻⁹ M external Ca²⁺, a membrane potential of ~192 mV would be required to retain internal Ca²⁺ (as derived from the Nernst equation assuming an activity coefficient of internal Ca²⁺ of 0.1). Since in conditions similar to those of the present work, the mitochondrial membrane potential is of the order of 120–170 mV negative inside (37, 38), Ca²⁺ release would be a thermodynamically favored process. Using the same considerations, in mitochondria incubated with 10⁻⁶ M external Ca²⁺ and in which maximal rates of ATP synthesis were detected, the inward and the outward movements of Ca²⁺ would be in near equilibrium.

The present data show that the rate of ATP synthesis in mitochondria decreases in a time-dependent process when incubated at 10⁻⁸ M Ca²⁺ in comparison to that attained at 10⁻⁶ M Ca²⁺. Apparently as Ca²⁺ leaks out of the mitochondria, the rate of oxidative phosphorylation gradually decreases. Moreover, in conditions in which internal Ca²⁺ is maintained at a constant level of approximately 20 nmol/mg of protein, the rate of respiration is not ostensibly affected. Thus it would appear that the level of internal Ca²⁺ as modulated by the extramitochondrial Ca²⁺ concentrations influences the rate of oxidative phosphorylation.

An attempt has been made to explore how the internal concentration of Ca²⁺ affects the overall kinetics of ATP synthesis. It has been observed that, similar to the state 3 respiratory rates, electron transport of uncoupled mitochondria is faster at 10⁻⁸ than at 10⁻⁹ M Ca²⁺. This Ca²⁺-dependent process can be related to the higher rate of ATP synthesis observed with 10⁻⁶ M Ca²⁺, but it is noteworthy that the rate of state 3 respiration is lower than uncoupled respiration, regardless of the Ca²⁺ concentration in the medium. This indicates a priori that state 3 respiration is more importantly limited by either the phosphorylation of ADP and/or by the activity of the adenine nucleotide translocator.

An analysis of the inhibition curve of state 3 respiration as induced by oligomycin revealed that the control exerted by the ATP synthase was higher at 10⁻⁸ than at 10⁻⁶ M Ca²⁺. However, it was also observed that to attain a steep change in the slope of the inhibition curve more oligomycin was required at 10⁻⁹ M Ca²⁺. The latter would suggest that a different number of active enzymes would be operating at the two concentrations of Ca²⁺ studied; indeed it has been previ-
ously reported that Ca\textsuperscript{2+} levels may affect the interaction of the inhibitor protein with the ATP synthase (14, 15), thus affecting the number of functional enzymes. Therefore, the overall data obtained with oligomycin suggest that the analysis of the control excreted by an enzyme on a metabolic pathway as described by Kacser and Burns (39, 40) and by Heinrich and Rapoport (41, 42) would seem to require an experimental evaluation of the extent to which an enzyme that undergoes reversible transitions affects its degree of control on the pathway. The ATP synthase would seem to be a good system to test the reported theoretical considerations.

The titrations of state 3 respiration with carboxyatractyloside showed that the degree of control exerted by the translocase is significantly different in the presence of 10\textsuperscript{-6} than with 10\textsuperscript{-5} M Ca\textsuperscript{2+}, being several times higher at 10\textsuperscript{-6} than at 10\textsuperscript{-5} M Ca\textsuperscript{2+}. Thus, the modification of the rate of oxidative phosphorylation parallels a change of the kinetic control exerted by the translocase, which suggests that other kinetic transitions are occurring in other steps of coupled ATP synthesis when the amount of mitochondrial Ca\textsuperscript{2+} is varied. In other words, if the degree of control exerted by the ADP/ATP carrier is different at the two levels of Ca\textsuperscript{2+} studied, it follows that the quantitative contribution of the other steps of oxidative phosphorylation would also necessarily undergo modification. In fact, it is relevant that the Ca\textsuperscript{2+}-dependent variations in the control, as evaluated from the initial slope of inhibition curves (17, 18, 39–42), exerted by the ATP synthase and the translocase occur in opposite directions, i.e., when the control exerted by the ATP synthase is higher (10\textsuperscript{-5} M external Ca\textsuperscript{2+}), the control by the translocator is relatively low. The kind of kinetic relation where a change in the degree to which one enzyme is rate limiting results in an opposite directed change in the degree to which the other enzyme becomes limiting has been studied by Stoner and Sirak (43). They concluded that such a sequential coupling relation is established between ADP transport and the phosphorylation reaction during oxidative phosphorylation.

Moreover, the observed difference between the rates of uncoupled electron transport and state 4 respiration at 10\textsuperscript{-6} and 10\textsuperscript{-5} M external Ca\textsuperscript{2+} suggests the existence of a Ca\textsuperscript{2+}-induced modification of substrate transport and/or electron transfer. Therefore, it is suggested that variations in the extramitochondrial Ca\textsuperscript{2+} concentration, within the limits that exist in living cells (26), modify quantitatively the contribution of the multiple control points (17, 18) that are involved in the overall kinetics of ATP synthesis positing the system at various rates of oxidative phosphorylation.

Along this line, measurements of the activity of several intramitochondrial dehydrogenases (44–46) and electron probe analysis of heart and other muscle cells (47) indicate that the possible physiological range of intramitochondrial Ca\textsuperscript{2+} is of the order of 1–5 nmol/mg of mitochondrial protein. In our experimental conditions, the Ca\textsuperscript{2+} levels are near the aforementioned values which suggests that Ca\textsuperscript{2+} exerts a physiological role on the regulation of oxidative phosphorylation. Moreover, a dependence of the activity of carbamoyl phosphate synthase and pyruvate carboxylase on internal Ca\textsuperscript{2+} levels in a range similar to that attained here has been reported (11, 48).

At the moment it is not possible to ascertain the mechanism through which the internal concentrations of Ca\textsuperscript{2+} induce the aforementioned changes of the kinetics of oxidative phosphorylation, but it is interesting that there are reports that indicate that the amount of intramitochondrial adenine nucleotides is affected by the internal concentration of Ca\textsuperscript{2+} (13, 49, 50), and recently it was reported that prolonged incubation of mitochondria increase the control strength exerted by the adenine nucleotide translocator (51) which apparently is associated with a diminution in the intramitochondrial pool of adenine nucleotides. However, it is necessary to consider that variations in the level of other endogenous components may account for the presently described observations. With respect to Mg\textsuperscript{2+}, it is important to point out that in liver mitochondria with a content of approximately 30 nmol of Mg\textsuperscript{2+}/mg of protein incubated for 5 min in mixtures similar to these employed here, Masini et al. (52) observed a loss of approximately 3 nmol of Mg\textsuperscript{2+}/mg. Thus variations in Mg\textsuperscript{2+} levels would not seem to be an important factor in the presently described effects of Ca\textsuperscript{2+} on oxidative phosphorylation.

In relation to the contribution of the ATP synthase to the overall process of ATP synthesis, it must be recalled that the action of the natural ATPase inhibitor protein on the ATP synthase appears to be modulated by the concentrations of Ca\textsuperscript{2+} in the mitochondria (14, 15). Therefore, it would seem that Ca\textsuperscript{2+} concentration in the exterior and/or interior of the mitochondria induces important modifications of the kinetic characteristics of many of the steps involved in the total process of ATP synthesis.

Acknowledgments—I would like to gratefully acknowledge the helpful discussions of Dr. A. Gómez-Puyou. The collaboration of Guadalupe Ramirez in typing the manuscript is acknowledged.

REFERENCES
1. Rossi, C. S., and Lehninger, A. L. (1964) J. Biol. Chem. 239, 3971–3980
2. Vercesi, A., Reynafarje, B., and Lehninger, A. L. (1978) J. Biol. Chem. 253, 6379–6385
3. Vallières, J., Scarpas, A., and Sonnlo, A. P. (1975) Arch. Biochem. Biophys. 170, 669–669
4. Jacobus, W. E., Tiozzo, R., Lugli, G., Lehninger, A. L., and Carafoli, E. (1979) J. Biol. Chem. 250, 7863–7870
5. Sordahl, L. A. (1975) Arch. Biochem. Biophys. 167, 104–115
6. Malmström, K., and Carafoli, E. (1977) Arch. Biochem. Biophys. 652, 677–666
7. Thorne, R. F. W., and Bygrave, F. L. (1974) Biochem. J. 144, 575–588
8. Villalobo, A., and Lehninger, A. L. (1980) J. Biol. Chem. 255, 2457–2464
9. Abou-Khalil, S., Abou-Khalil, W. H., and Younis, A. A. (1981) Arch. Biochem. Biophys. 209, 460–464
10. Roman, I., Clark, A., and Swanson, P. D. (1981) Membr. Biochem. 10, 1–9
11. Foldes, M., and Barritt, G. J. (1977) J. Biol. Chem. 252, 5372–5380
12. Gómez-Puyou, A., Tuena de Gómez-Puyou, M., Riapp, M., and Carafoli, E. (1979) Arch. Biochem. Biophys. 194, 399–404
13. Moreno-Sánchez, R. (1983) Biochim. Biophys. Acta 724, 278–285
14. Tuena de Gómez-Puyou, M., Gavilanes, M., Gómez-Puyou, A., and Ernster, L. (1980) Biochim. Biophys. Acta 592, 396–405
15. Hilleler, L., Muchiri, P. M., Nordenbrand, K., and Ernster, L. (1983) FEBS Lett. 154, 247–250
16. Robertson, S. P., Potter, J. D., and Rouslin, W. (1982) J. Biol. Chem. 257, 1743–1748
17. Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R., and Tager, J. M. (1982) J. Biol. Chem. 257, 2764–2767
18. Tager, J. M., Wanders, R. J. A., Groen, A. K., Kunz, W., Bohnensack, R., Kuster, U., Letko, G., Bohme, G., Duszyński, J., and Woitzek, L. (1983) FEBS Lett. 151, 1–9
19. Fabiato, A., and Fabiato, F. (1979) J. Physiol. (Paris) 75, 463–505
20. Fricker, U. (1975) Anal. Biochem. 63, 555–559
21. Lamprecht, W., and Trautschold, J. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) Verlag Chemie, Weinheim/Bergstr, FRG
22. Lindberg, O., and Ernster, L. (1956) Methods Biochem. Anal. 3, 1–22
23. Nicholls, D. G. (1978) *Biochem. J.* **176**, 463–474
24. Hutson, S. M., Pfeiffer, D. R., and Lardy, H. A. (1976) *J. Biol. Chem.* **251**, 5251–5258
25. Hutson, S. M. (1977) *J. Biol. Chem.* **252**, 4539–4545
26. Murphy, E., Coll, K., Rich, T. L., and Williamson, J. R. (1980) *J. Biol. Chem.* **255**, 6601–6608
27. Becker, G. L. (1980) *Biochim. Biophys. Acta* **591**, 234–239
28. Nicholls, D., and Akerman, K. (1982) *Biochim. Biophys. Acta* **683**, 57–88
29. Lemasters, J. J., and Sowers, A. E. (1979) *J. Biol. Chem.* **254**, 1248–1251
30. Kunz, W., Bohnensack, R., Böhme, G., Küster, U., Letko, G., and Schönfeld, P. (1981) *Arch. Biochem. Biophys.* **209**, 219–229
31. Jacobs, W. E., Moreadith, R. W., and Vandegaer, K. M. (1982) *J. Biol. Chem.* **257**, 2397–2402
32. Wanders, R. J. A., Groen, A. K., Meijer, A. J., and Tager, J. M. (1981) *FEBS Lett.* **132**, 201–206
33. Forman, N. G., and Wilson, D. F. (1982) *J. Biol. Chem.* **257**, 12908–12915
34. Forman, N. G., and Wilson, D. F. (1983) *J. Biol. Chem.* **258**, 8649–8655
35. Erecinska, M., and Wilson, D. F. (1982) *J. Membr. Biol.* **70**, 1–14
36. Vignais, P. V. (1976) *Biochim. Biophys. Acta* **456**, 1–38
37. Wilson, D. F., and Forman, N. G. (1982) *Biochemistry* **21**, 1438–1444
38. Zoratti, M., Pietrobon, D., and Aozzone, G. F. (1983) *Biochim. Biophys. Acta* **723**, 59–70
39. Kacser, H., and Burns, J. A. (1973) *Symp. Soc. Exp. Biol.* **27**, 65–104
40. Kacser, H., and Burns, J. A. (1979) *Biochem. Soc. Trans.* **7**, 1149–1160
41. Heinrich, R., and Rapoport, T. A. (1974) *Eur. J. Biochem.* **42**, 89–95
42. Heinrich, R., and Rapoport, T. A. (1974) *Eur. J. Biochem.* **42**, 97–105
43. Stoner, C. D., and Sirak, H. D. (1979) *J. Bioenerg. Biomembr.* **11**, 113–146
44. Hansford, R. G., and Castro, F. (1981) *Biochem. J.* **198**, 525–533
45. Coll, K., Joseph, S. K., Corkey, B. E., and Williamson, J. R. (1982) *J. Biol. Chem.* **257**, 8696–8704
46. Hansford, R. G., and Castro, F. (1982) *J. Bioenerg. Biomembr.* **14**, 361–376
47. Somlyo, A. P., Somlyo, A. V., Shuman, H., Scarpa, A., Endo, M., and Inesi, G. (1981) in *Calcium Phosphate Transport Across Biomembranes* (Bronner, F., and Peterlik, M., eds) pp. 87–93, Academic Press, New York
48. Meijer, A. J., van Woerkom, G. M., Steinman, R., and Williamson, J. R. (1981) *J. Biol. Chem.* **256**, 3443–3446
49. Zoccarato, F., Rugolo, M., Siliprandi, D., and Siliprandi, N. (1981) *Eur. J. Biochem.* **114**, 195–199
50. Harris, E. J., and Chen, M. S. (1982) *Biochem. Biophys. Res. Commun.* **104**, 1264–1270
51. Grunwald, R., and Lemasters, J. J. (1984) *Fed. Proc.* **43**, 1877
52. Masini, A., Cecarelli-Stanza, D., and Muscatello, U. (1983) *J. Bioenerg. Biomembr.* **15**, 217–234