THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 243, No. 7, Issue of April 10, pp. 1514-1522, 1968
Printed in U.S.A.

Ion Transport in Liver Mitochondria

IV. THE RELATIONSHIP BETWEEN ION TRANSLOCATION AND ELECTRON TRANSPORT*

(Received for publication, July 7, 1967)

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SUMMARY

Addition of small amounts of Ca^{++} to liver mitochondria incubated in the absence of phosphate or acetate results in an inhibition of respiration. The inhibition of respiration is reversed by increasing the concentration of substrate anions in the medium, and is enhanced by increasing the ionic strength or the pH of the medium. The H^+:O and Ca^{++}:O ratios are increased severalfold when measured under conditions of inhibited respiration. Furthermore, mitochondria which have entered a state of inhibited respiration continue to take up Ca^{++} and to release H^+. It is concluded that the above stoichiometries in the absence of phosphate or acetate are determined by both uninhibited and inhibited respirations. The higher the concentration of Ca^{++}, the larger is the contribution of the inhibited respiration. In the presence of acetate, the Ca^{++} to total oxygen ratio is in the region of 2.0 to 2.5 with succinate as substrate.

The inhibition of respiration is related to the increase of the intramitochondrial pH as followed kinetically with the bromthymol blue technique. The bromthymol blue response is dependent on the rate of influx of anions. Increasing the ionic strength and pH enhances the bromthymol blue response, presumably by restraining the influx of anions.

The mechanism by which the inhibition of respiration causes the superstoichiometric ratios is discussed.

In earlier studies on the accumulation of divalent cations by rat liver mitochondria, it was reported that about 2.0 Ca^{++} or Mn^{++} ions may be taken up per pair of electrons passing through each energy-conserving site of the electron transport chain (1–6). This stoichiometry was unchanged whether or not permeant anions such as phosphate were present in the medium. Concomitant with the uptake of Ca^{++}, H^+ was ejected. The H^+:Ca^{++} ratio was about 0.7 in the presence, and about 1.0 in the absence, of phosphate (6–9). In the presence of acetate it decreased to 0.2 (10). More recently, however, it was reported independently from two laboratories that the Ca^{++}:O and the H^+:O ratios could undergo a considerable increase (11–14). Among the conditions giving rise to superstoichiometric Ca^{++}:O ratios was the increase of the pH of the medium and of the ionic strength. The increase of the Ca^{++}:O ratio was abolished by the addition of permeant anions (12, 14). These findings have been taken as evidence to support the conclusion that a fixed relationship between electron transport and ion translocation does not exist (13, 15).

In the present report we will show that the observation of a constant stoichiometry of 2 $Ca^{++}/\sim$ in the absence of phosphate has been accidental, presumably because of a large uptake of the anions added as substrates or released from the mitochondria. In fact, if Ca^{++} is taken up in the absence of anions, a state of inhibited mitochondrial respiration is induced, and high H^+:O and Ca^{++}:O ratios are observed.

In view of the present results we conclude that when the stoichiometries of Ca^{++} translocation are measured in the absence of permeant anions, part of the H^+ release and of the Ca^{++} uptake occurs under conditions of inhibited respiration, and this results in an overestimation of the Ca^{++}:O and H^+:O ratios. The inhibition of electron transfer during Ca^{++} uptake has been found to be related to an increased absorbance of the pH indicator, bromthymol blue.

EXPERIMENTAL PROCEDURE

Materials and Methods—Rat liver mitochondria, prepared in 0.25 M sucrose-0.5 mM EGTA[1] and resuspended in EGTA-free 0.25 M sucrose, were used throughout. The experimental details have been described in previous papers (16, 17). H^+ release was measured with a Beckman glass electrode and a Beckman pH meter. Oxygen uptake was measured polarographically with a Clark electrode. H^+ release and oxygen consumption were recorded simultaneously. Ca^{++} uptake was measured by using $^{45}CaCl_2$. The supernatants were counted after rapid centrifugation with the Beckman/Spinco microfuge. Unless otherwise stated, separation of the mitochondria from the medium, for the measurement of Ca^{++} uptake, was initiated im-

* This investigation was aided by grants from the National Research Council and from NATO (No. 273).

[1] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

mediately after stimulation of respiration had ended. All experiments were carried out at 20°.

The intramitochondrial pH was followed by the technique proposed by Chance and Mela (18, 19). Bromthymol blue was used at a concentration of 5 μM, about 1 μmole per g of protein. A dual wave length spectrophotometer built in the workshop of the Medical School, University of Bristol, was used. The spectrophotometer was equipped with interference filters, at 623 and 700 mμ A Kodak wratten filter type 33 was put between the cuvette and the photomultiplier to reduce interference by cytochrome *a*. In other experiments the increase of titratable alkalinity of the mitochondria after Ca^{++} uptake was measured according to the technique described by Rossi, Bielawski, and Lehninger (20).

RESULTS

As reported in a previous communication (11), the amount of oxygen taken up, following the addition of a given amount of Ca^{++}, by liver mitochondria incubated aerobically in an isoosmotic sucrose or NaCl medium, decreased concomitantly with the increase of pH of the incubation medium. During the course of the investigation it was noted, however, that the extent of inhibition of the oxygen uptake, due to the increased alkalinity, was not constant. The effect of the increase of pH was greater at 500 than at 250 μM Ca^{++} and disappeared completely at low Ca^{++} concentrations. Furthermore, the Ca^{++} concentration at which the effect of pH on respiration was observed increased as the amount of mitochondrial protein in the medium was raised.

Effect of Ca^{++} Concentration on Inhibition of Respiration—A titration of the amount of oxygen taken up *versus* Ca^{++} added has been reported by Rossi and Lehninger (5). A constant Ca^{++}:O ratio was observed between 16 and 70 μg of Ca^{++} ions per g of protein. Amounts of Ca^{++} higher than 80 μg of Ca^{++} ion per g of protein gave only a slight stimulation of the respiration, and it was suggested that the respiratory chain was "saturated." Ca^{++} uptake was not measured in that experiment. In the following it will be assumed that the "saturation" phenomenon corresponds to the state of inhibited respiration denoted by Chance (21) and Chance and Schoener (22) as "state 6." The question will, however, be discussed later more in detail.

In Fig. 1 it is seen that the concentration of Ca^{++} at which inhibition of respiration appeared was dependent on the concentration of succinate in the medium. Four additions of 24 μg of Ca^{++} ions per g of protein to mitochondria incubated with 0.4 mM succinate gave a respiratory stimulation of 4.9, 1.8, 1.4, and 0.7 μg atoms of oxygen per g of protein. The total oxygen uptake was 8.8 μg atoms per g of protein. On the other hand, with 20 mM succinate, subsequent additions of the same amount of Ca^{++} gave a respiratory stimulation of 6.3, 4.6, 2.1, and 1.8 μg atoms of oxygen per g of protein, with a sum of 14.8 μg atoms per g of protein. The lesser respiratory stimulation at 0.4 mM succinate was not due to lack of substrate, since the respiration was stimulated by the addition of acetate.

A decrease of respiratory stimulation by successive additions of Ca^{++} was also observed upon increasing the NaCl concentration from 0.1 to 0.3 M. Fig. 2 shows that at 0.1 M NaCl, three additions of 24 μg of Ca^{++} ion per g of protein gave respiratory bursts of 5.1, 3.2, and 2.5 μg atoms of oxygen per g of protein, for a total of 10.8 μg atoms per g of protein. On the other hand, at 0.3 M NaCl, three Ca^{++} additions gave a respiratory stimulation of 3.2, 0.6, and 0.6 μg atoms of oxygen per g of protein, for a total of 4.4 μg atoms per g of protein. It is to be noted that the difference in respiratory stimulation between 0.1 and 0.3 M NaCl was about 40% at the first addition and 80% at the second and third additions. At lower Ca^{++} concentrations, the increase of the ionic strength did not inhibit respiration. Results similar to those reported in Fig. 2 were obtained when the pH of the medium, instead of the ionic strength, was increased. Inhibition of respiration appeared at lower Ca^{++} concentrations at pH 8 that at pH 7. However, in the case of increase of pH as with an increase of ionic strength, no inhibition of respiration was observed at low Ca^{++} concentrations.

The amount of oxygen consumed by the mitochondria before the rising of the respiratory inhibition set in was greater if Ca^{++} were added in a divided dose instead of a single pulse. Fig. 3 shows that addition of 12.7 μg of Ca^{++} ion per g of protein caused a stimulation of oxygen uptake of about 1.8 μg atoms of oxygen per g of protein and a H^+ release of 10 μg of H^+ ion per g of protein, with a H^+:O ratio of 5.6. Subsequent additions of the same amount of Ca^{++} gave about the same respiratory stimulation, 2.1 and 2.0 μg atoms of oxygen per g of protein, and a

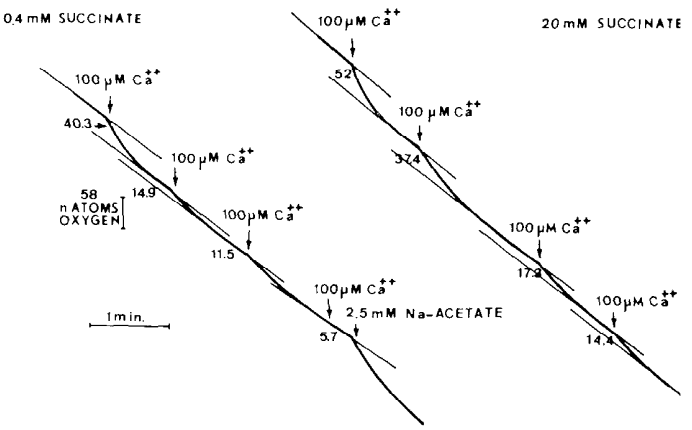

FIG. 1. Stimulation of respiration by Ca^{++} at various succinate concentrations. Experimental conditions were as follows: 0.25 M sucrose; 5 mM Tris-HCl, pH 7.3; 5 μM rotenone; 8.2 mg of mitochondrial protein; final volume, 2 ml. Concentrations of succinate were as indicated in the figure.

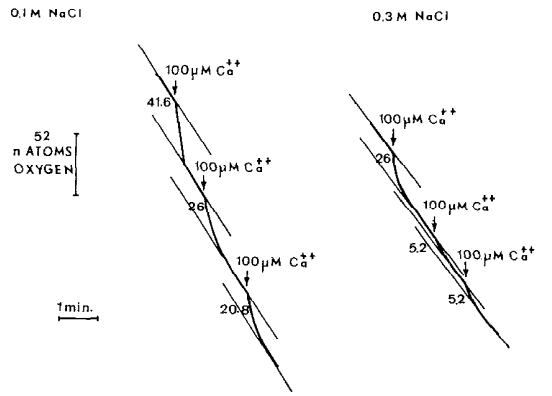

FIG. 2. Stimulation of respiration by Ca^{++} at various NaCl concentrations. Experimental conditions were as follows: 5 mM Tris-HCl, pH 7.4; 4 mM sodium succinate; 5 μM rotenone; 8.2 mg of mitochondrial protein. Concentrations of NaCl were as indicated in the figure; final volume, 2 ml.

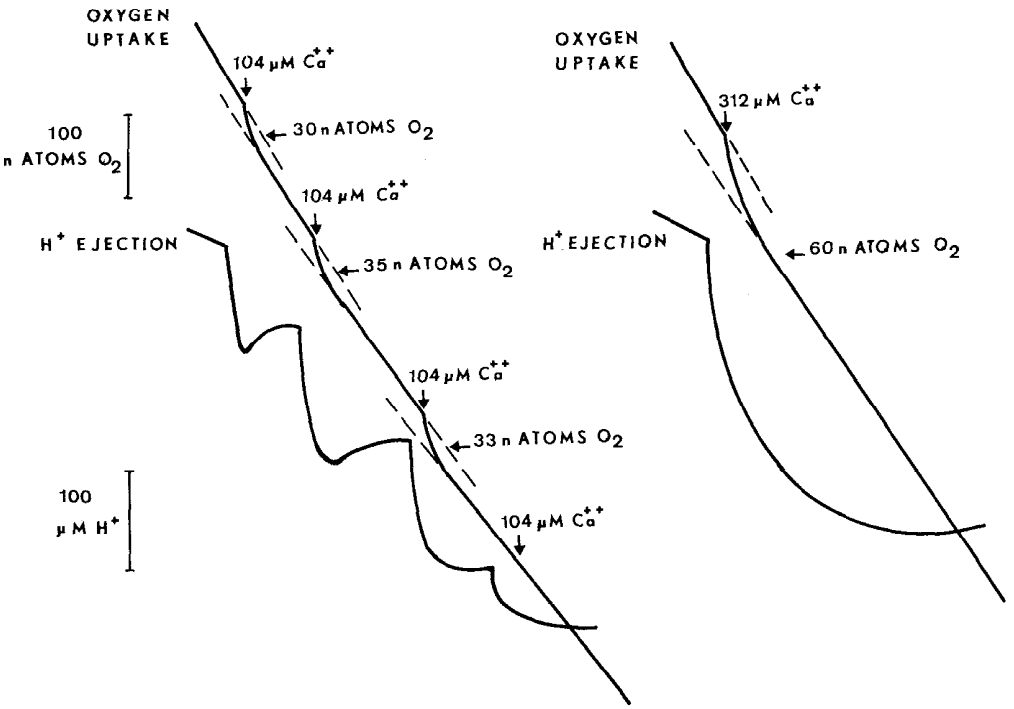

FIG. 3. Amount of oxygen uptake according to whether single or separate Ca^{++} additions are made. Experimental conditions were as follows: 0.1 M NaCl; 5 mM Tris-HCl, pH 8.0; 2 mM sodium succinate; 5 μM rotenone; 16.4 mg of mitochondrial protein; final volume, 2 ml.

higher H^+ release, 14 and 15.5 μg of H^+ ion per g of protein with H^+:O ratios of 6.7 and 7.7, respectively. At the last addition of Ca^{++} respiratory stimulation was almost absent, whereas a considerable stimulation of H^+ release was still observed. The total oxygen burst during the first three additions of Ca^{++} was 5.9 μg atoms of oxygen per g of protein, and the sum of the H^+ was 39 μg of H^+ ion per g of protein. In the parallel experiment of Fig. 3, the same amount of Ca^{++}, 38 μg ions per g of protein, was added in a single pulse. The oxygen taken up was 3.7 μg atoms of oxygen per g of protein. It is seen in this experiment that the release of protons continued after the mitochondria had entered a state of inhibited respiration. The total H^+ released was 36 μg of ion per g of protein. The experiment of Fig. 3 thus shows that, although the total amount of H^+ released was about the same, and all the Ca^{++} was presumably taken up, the amount of oxygen consumed was 50% higher when three, instead of one, additions of Ca^{++} were made.

Carafoli, Rossi, and Lehninger (23) have shown that Ca^{++} can be taken up by the mitochondria under a state of resting respiration. Fig. 4 shows that a large proton release and Ca^{++} uptake is observed in mitochondria which have entered a state of inhibited respiration. In agreement with the experiment of Fig. 3, the H^+ release did not level off concomitantly with the inhibition of the respiration but continued for a considerable length of time. Ca^{++} was also taken up parallel to the release of protons, as indicated by the data for Ca^{++} uptake reported in Fig. 4*A*. In order to observe the phenomenon described in Figs. 3 and 4*A* it is necessary to add an amount of Ca^{++} to the mitochondria, such that an excess of Ca^{++} remains in the medium after the mitochondria have entered the state of inhibited respiration (Fig. 4*B*). In the case of the experiment of Fig. 4*B*, this amount was above 40 μg of Ca^{++} ion per g of protein.

H^+:O and Ca^{++}:O Ratios—In the following the oxygen uptake will be considered equivalent to ~ production, according to the stoichiometry of 2~/O with succinate as substrate. In Fig. 5 are reported H^+ release, Ca^{++} uptake, and total and Δ oxygen consumption after addition of various Ca^{++} concentrations to mitochondria incubated at pH 7 and 8.2. It is seen that at pH 7 Ca^{++} uptake was proportional to the amount of Ca^{++} added in the range between 14 and 140 μg of Ca^{++} ion per g of protein. H^+ was released in a constant proportion to the uptake of Ca^{++}, except at the lower Ca^{++} concentrations where the appearance of a "bounce" in the H^+ electrode trace (see Fig. 3 and Reference 17) resulted in a decrease of the H^+:Ca^{++} ratio. On the other hand, the uptake of oxygen was related to the uptake of Ca^{++} and the release of H^+ in a very special fashion. In fact, at 14 μg of Ca^{++} ion per g of protein, the amount of oxygen uptake was 7.3 μg atoms (total) and 4.3 μg atoms (Δ) per g of protein. In the range between 14 and 140 μg of Ca^{++} ion per g of protein, the consumption of oxygen increased slightly in relation to the amount of Ca^{++} added and thus also to the Ca^{++} taken up and the H^+ released. The H^+:O and Ca^{++}:O ratios calculated on the slope of the oxygen values, total or Δ, in the range between 14 and 140 μg of Ca^{++} ion per g of protein were about 3 Ca^{++}/~ and about 4.5 H^+/~ (Fig. 6). In other words, the H^+:O and Ca^{++}:O ratios observed at 14 μg of Ca^{++} ion per g of protein were much lower (about one third) than the ratios observed in the range 14 to 140 μg of Ca^{++} ion per g of protein. Since, as will be shown below, the higher Ca^{++}:O and H^+:O ratios are abolished by permeant anions, which also remove inhibition of respiration, we shall assume that the two different stoichiometric ratios reflect an uptake of Ca^{++} and a release of H^+ in a state either of inhibited or of uninhibited respiration. In Fig. 6 are also reported the H^+:O and Ca^{++}:O ratios calculated on the basis of the total or Δ oxygen uptake measured at each Ca^{++} concentration. The H^+:O and Ca^{++}:O ratios increased with the increase of the

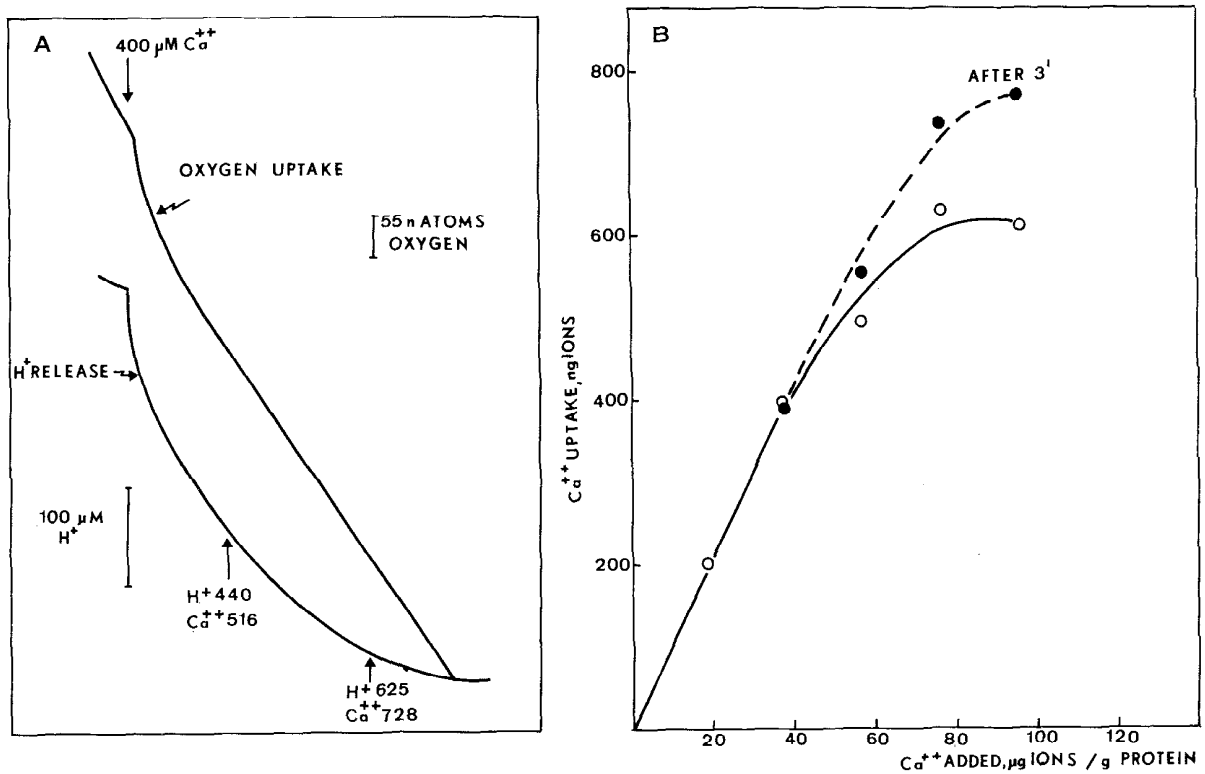

FIG. 4. *A* and *B*, Ca^{++} uptake and proton release in an inhibited state of respiration. Experimental conditions were: *A*, 0.1 M NaCl; 5 mM Tris-HCl, pH 8.4; 5 μM rotenone; 10 mM sodium succinate; and 10 mg of protein; *B*, 50 mM NaCl; 125 mM sucrose; 2.5 mM sodium succinate; 5 mM Tris-HCl, pH 8.0; 5 μM rotenone; and 10.4 mg of mitochondrial protein; final volume, 2.0 ml.

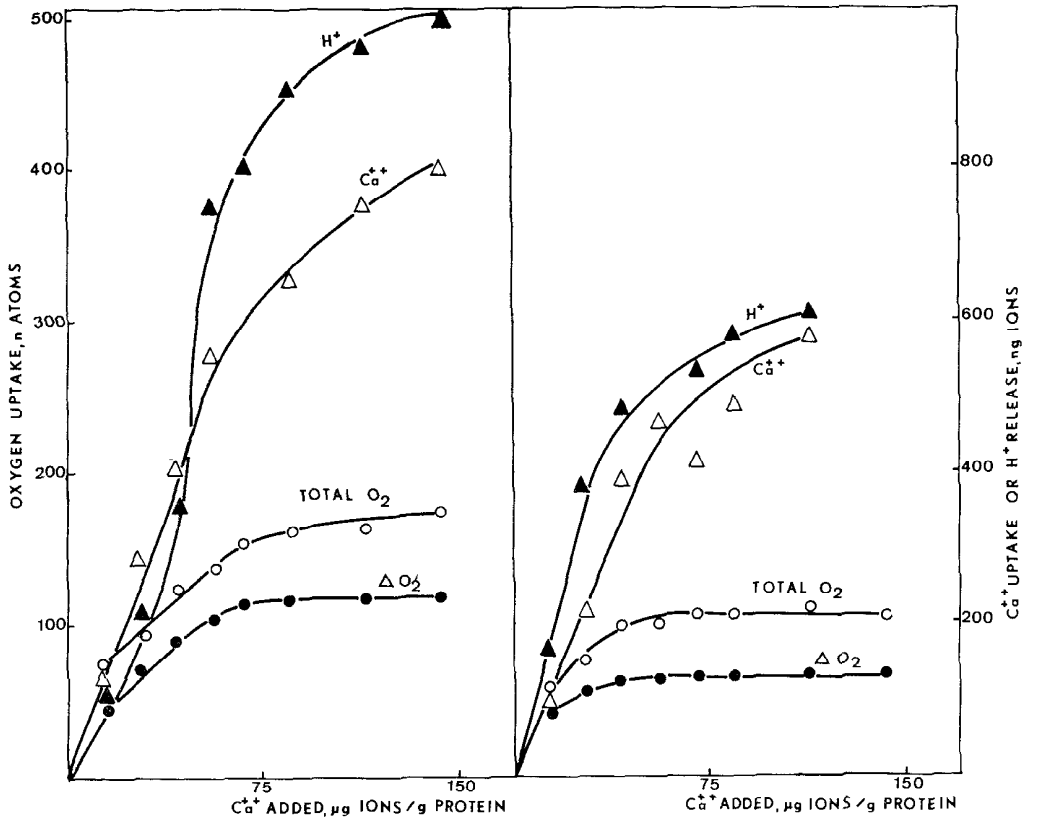

FIG. 5. Ca^{++} and oxygen uptake and H^+ release at various Ca^{++} concentrations. Experimental conditions were as follows: 0.25 M sucrose; 3 mM Tris-HCl, pH 7.0 (*left*) or 8.3 (*right*); 13 mM succinate; 3 μM rotenone; and 10.5 mg of mitochondrial protein; final volume, 3 ml.

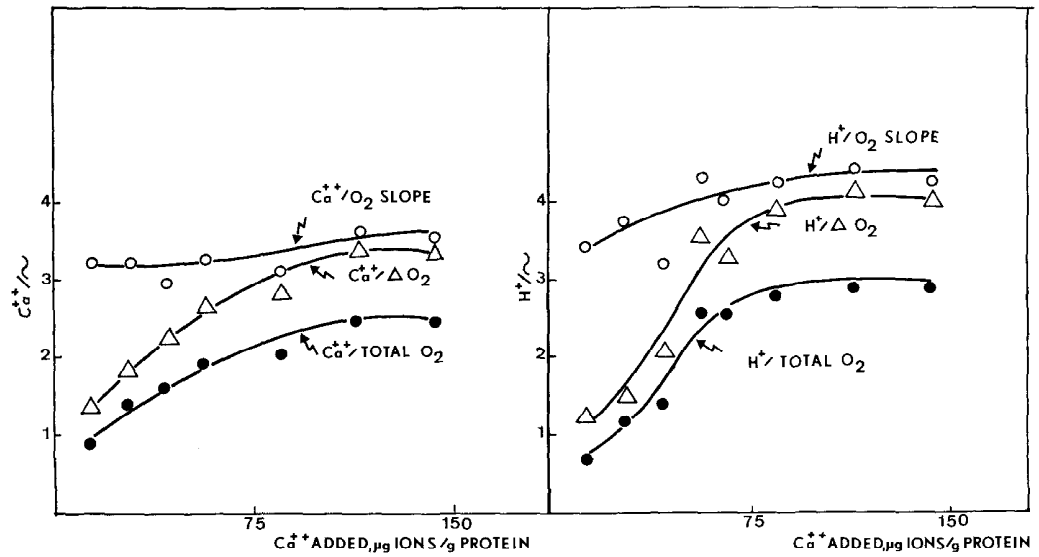

FIG. 6. Ca^{++}:O and H^+:O ratios. The ratios are calculated on the basis of the data of Fig. 5 (pH 7.0)

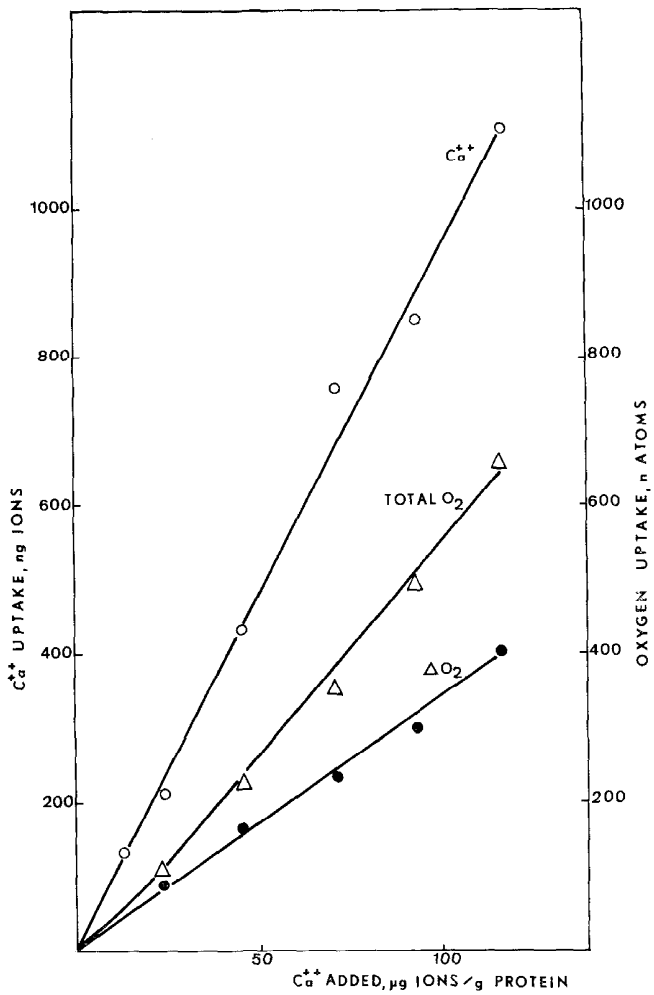

FIG. 7. Ca^{++} and oxygen uptake in the presence of acetate at various Ca^{++} concentrations. Experimental conditions were as follows: 0.25 M sucrose; 3.3 mM Tris-HCl, pH 8.0; 13 mM succinate; 16 mM acetate; 3 μM rotenone; and 10 mg of mitochondrial protein.

amount of Ca^{++} added, this being due to the fact that, at the higher Ca^{++} concentrations, a larger part of the Ca^{++} uptake and H^+ release occurs in a state of inhibited respiration. Since the choice of Δ or total oxygen uptake is open to uncertainty (*cf.* "Discussion"), both values are reported with the assumption that the H^+:O or Ca^{++}:O ratio lies within this range. In Fig. 5 (*right*) are also reported H^+ release, Ca^{++} uptake, and oxygen uptake at pH 8.3. At pH 8.3 relatively low H^+:O and Ca^{++}:O ratios were also observed at 14 μg of Ca^{++} ion per g of protein and higher ratios above this Ca^{++} concentration. The H^+:O and Ca^{++}:O ratios calculated on the basis of the slope of the oxygen values were about 7 $H^+/\sim$ and 5 $Ca^{++}/\sim$, respectively. Thus, from the results presented in Figs. 5 and 6, it would seem that high stoichiometric H^+:O and Ca^{++}:O ratios are observed at pH 7.0 as well as at 8.2. The fact that superstoichiometric ratios were observed first at the higher pH or ionic strength is only due to the circumstance that these conditions facilitate the occurrence of inhibition of respiration. However, even at pH 7.0, the uptake of Ca^{++} can be considered to occur under conditions of uninhibited respiration only in a very limited range of Ca^{++} concentrations.

Effect of Anions—Carafoli *et al.* (14) have reported that superstoichiometric H^+:O and Ca^{++}:O ratios are not observed in the presence of permeant anions. Fig. 7 shows a comparison of the Ca^{++} and oxygen uptakes with mitochondria incubated at pH 8.0, as measured in the presence and absence of acetate. It appears that in the presence of acetate an almost constant proportion between Ca^{++} and oxygen uptake was obtained in the range between 0 and 100 μg of Ca^{++} ion per g of protein. The $Ca^{++}/\sim$ ratio was about 1 to 1.5; that is, it was close to the value observed, in the experiments of Figs. 5 and 6, at the lowest Ca^{++} concentrations. Thus, the effect of permeant anions was that of extending the range of constant proportion between Ca^{++} and oxygen uptake.

When succinate was omitted from the incubation medium and the uptake of Ca^{++} occurred at the expense of the oxidation of endogenous substrates, high Ca^{++}:O ratios began to appear at much lower Ca^{++} concentrations, *e.g.* at 5 μg of Ca^{++} ion per g of protein. On the other hand, when mitochondria were stored for some hours, a larger amount of Ca^{++} was taken up without

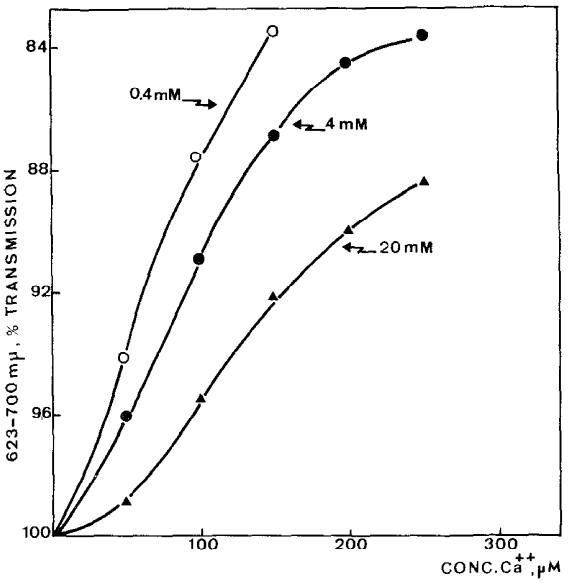

FIG. 8. Effect of the succinate concentration on the bromthymol blue response. Experimental conditions were as follows: 0.25 M sucrose; 12.5 mM Tris-HCl, pH 7.5; 5 μM bromthymol blue; and 8.2 mg of mitochondrial protein. Concentration of succinate as indicated in the figure.

superstoichiometric Ca^{++}:O or H^+:O ratios. These observations indicate that when anions such as acetate or phosphate are not added to the medium, the concentration Ca^{++} at which inhibition of respiration appears, and thus also the superstoichiometric ratios, is dependent on the concentration of the substrate anions added or of the endogenous anions released from the mitochondria during storage.

Intramitochondrial pH during Cation Uptake—Since the pH of the external aqueous phase drops during cation uptake, it might be expected that the pH of the inner mitochondrial phase rises. In fact, an increase of pH of the dissolved mitochondrial pellet was observed by Brierley *et al.* (24). The increase of intramitochondrial pH has been followed kinetically by Chance and Mela, by adding bromthymol blue as intramitochondrial pH indicator (18, 19); by Rossi *et al.* (20) and Gear *et al.* (25), by titrating the increase of intramitochondrial alkalinity; and later by Addanki, Cahill, and Sotos (26), by using the weak acid, 5,5-dimethyl-2,4-oxazolidinedione. Although the results are in apparent qualitative agreement, questions have been raised concerning the use of bromthymol blue in this fashion (25). In our laboratory we have obtained evidence that supplying to, or removing energy from, mitochondria treated with bromthymol blue causes opposite effects on the absorbance changes depending on whether mitochondria are incubated in the presence or absence of permeant cations. These results could be interpreted either to indicate that the change of the intramitochondrial pH, as a function of the energy supply, is dependent on the presence of permeant cations (assuming that bromthymol blue does measure the intramitochondrial pH), or that the responses of this indicator are an expression of the degree of binding of the indicator. When our work was already completed, it came to our knowledge that Mitchell and Moyle[2] have attempted to localize the indicator in the mitochondria. According to Mitchell and Moyle, bromthymol blue moves from the inner to the outer mitochondrial phase during changes of the functional state of the mitochondria.

[2] Personal communication.

In the experiments reported below, bromthymol blue has been used to follow the kinetics of the intramitochondrial pH changes in relation to the respiratory activity. In control experiments, carried out in the absence of this indicator, it was ascertained that, under the conditions of the experiments of Figs. 8 through 10, no light-scattering change interfered with the BTB responses.

In agreement with the findings of Chance and Mela (18), the bromthymol blue response was abolished when a permeant anion such as acetate was present in the medium. The effect of acetate was dependent on three factors: the concentration of acetate, the pH, and the ionic strength of the medium. The effect of acetate decreased when its concentration was diminished from 10 to 2 mM. Furthermore, the effect of acetate was diminished when the pH of the medium was increased from 7 to 8 or when the ionic strength of the medium was increased. When permeant anions, such as acetate or phosphate, were absent, the bromthymol blue response was correlated with the concentration of succinate, the pH, and the ionic strength of the medium.

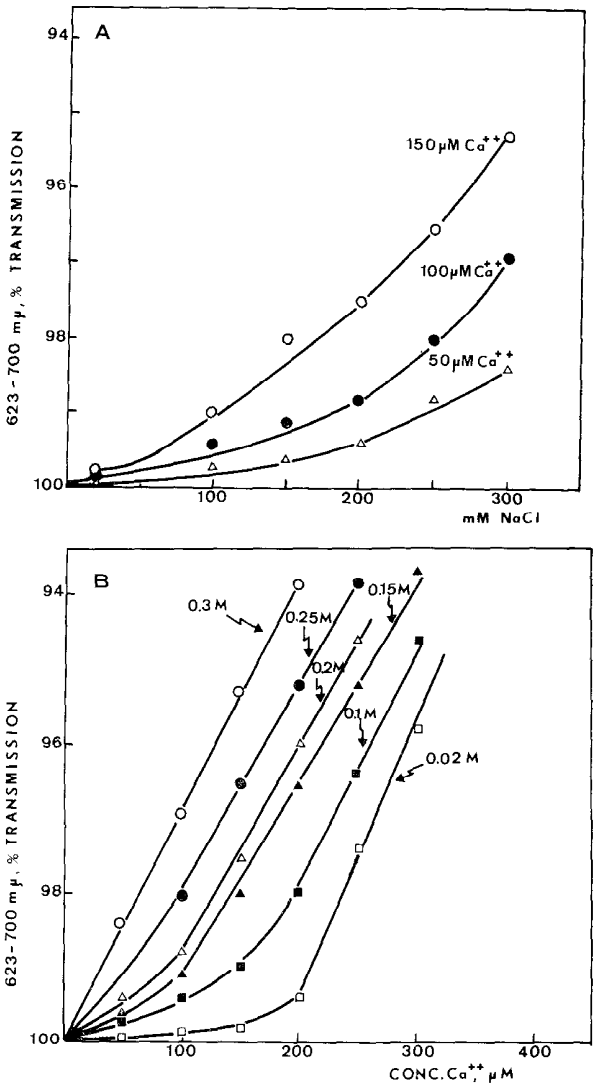

FIG. 9. *A* and *B*, The bromthymol blue response at various NaCl concentrations. Experimental conditions were as follows: 20 mM sodium succinate; 12.5 mM Tris-HCl, pH 7.4; 5 μM bromthymol blue; and 8.5 mg of mitochondrial protein. Concentration of NaCl as indicated in the figure.

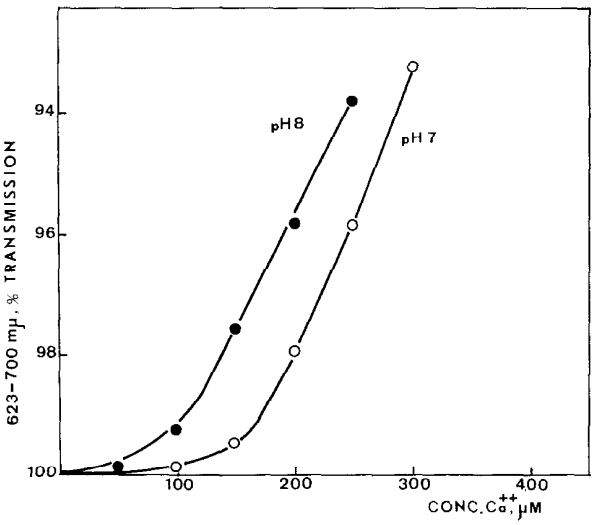

FIG. 10. The bromthymol blue response at various pH values of the incubation medium. Experimental conditions were as follows: 20 mM sodium succinate, 12.5 mM Tris-HCl at the pH indicated, 0.25 M sucrose, 5 μM bromthymol blue, and 8.5 mg of mitochondrial protein.

Fig. 8 shows that addition of 12 μg of Ca^{++} ion per g of protein to mitochondria incubated in 0.25 M sucrose, in the presence of 20 mM succinate, resulted only in a slight bromthymol blue response. Indeed, after an initial increase, the absorbance decreased again almost to the initial value (compare with the oxygen trace of Fig. 1). Subsequent additions of 12 μg of Ca^{++} ion per g of protein resulted in a larger increase of absorbance which, however, also tended to decline. The values reported in the figure are those measured 60 sec after the addition of Ca^{++}. At the lower succinate concentrations, the extent of bromthymol blue response per each addition of 12 μg of Ca^{++} ion per g of protein was much more pronounced. Thus, high succinate concentrations appear to abolish the response of this indicator in a manner similar to that observed with acetate.

Fig. 9*A* shows the effect of NaCl concentration on the bromthymol blue response in the presence of 20 mM succinate. A slight response, after addition of 12 μg of Ca^{++} ion per g of protein, was observed at 0.1 M NaCl, and a more marked one at 0.3 M NaCl (compare with the oxygen trace of Fig. 2). After addition of 36 μg of Ca^{++} ion per g of protein the bromthymol blue response increased with the increase of the NaCl concentration in the medium. As seen in Fig. 9*B*, the effect of increasing the NaCl concentration was mainly that of lowering the concentration of Ca^{++} at which the bromthymol blue response appeared and, thus, inversely to reduce the level below which Ca^{++} uptake occurred with little or no increase of absorbance. Indeed, at 0.3 M NaCl the absorbance increased at 12 μg of Ca^{++} ion per g of protein. At the lower NaCl concentrations a progressively increasing lag occurred in the response of bromthymol blue as the Ca^{++} concentrations were increased. However, after the intial lag the extent of increase of absorbance per amount of Ca^{++} added was roughly comparable at the various NaCl concentrations.

Increasing the pH of the medium from 7 to 8 resulted also in a lowering of the concentration of Ca^{++} at which the bromthymol blue response was observed. However, the extent of increase of absorbance per unit of Ca^{++} added was constant at high and low pH values (Fig. 10).

DISCUSSION

Calculation of Oxygen Uptake—The calculations of the H^+:O and Ca^{++}:O ratios are open to uncertainty because of the interpretation of the role of the resting respiration during the phase of activated respiration. Chappell, Cohn, and Greville (2) suggested plotting the oxygen values *versus* the cation concentrations and then calculating the cation to oxygen ratio on the basis of the slope. This procedure is equivalent to taking the Δ oxygen uptake and has been mostly used by subsequent authors. Rossi and Azzone (11) suggested taking the total oxygen uptake by showing that a significant amount of Ca^{++} could enter the mitochondria on account of the resting respiration. Carafoli *et al.* (14), on the other hand, on the basis of experiments in which Ca^{++} is taken up without activation of the respiration, consider that the efficiency of Ca^{++} uptake is much lower (about 10%) in state 4 than in state 3 respiration. Therefore they maintain that the choice of the ΔO_2 does not cause a significant error in the calculation of the stoichiometric ratios. The reasoning of Carafoli *et al.* is difficult to accept because the efficiency of Ca^{++} translocation is compared at two different Ca^{++} concentrations. We think that the extent of utilization of the state 4 respiration is dependent on the relative K_m and rate constants of the reaction for cation penetration and of those causing the energy leaks. Ca^{++} is a very efficient competitor in utilizing the state 4 respiration, provided its concentration is sufficiently high. The present argument is similar to that used to explain the normal P:O ratios in mitochondria with low respiratory control.

Inhibition of Respiration and Bromthymol Blue Response—Inhibition of electron transfer after addition of cations was first reported by Chance (21). Chance and Schoener (22) attributed the inhibited state of electron transfer both to the reaction of the cytochromes with the cations and to the alkalinization of the cristal membrane as measured with bromthymol blue. Carafoli *et al.* (14) observed that high concentrations of impermeant anions and high pH often resulted in an inhibited state of respiration and therefore suggested that the inhibition of respiration be related to the superstoichiometric ratios. Mitchell (28) has suggested that the inhibition of respiration denoted as state 6 is due to alkalinization of the mitochondrial interior. According to Chance and Mela (19), up to 40 μmoles of Ca^{++} per g of protein may be accumulated by the mitochondria without intramitochondrial pH changes.

Analysis of the factors which affect the bromthymol blue response was within the scope of the present work. Our experiments show that this response is related to an inhibition of respiration; both are enhanced when the concentration of succinate is decreased, and when the pH of the incubation medium or the NaCl concentration is increased. The effects of the increase of pH and ionic strength could be due to an increased extraction of protons from the mitochondria before addition of Ca^{++}. Protons however should be originated by the inner and not by the outer mitochondrial space, in order that the extraction of protons, occurring during "equilibration," interfere with the subsequent aerobic proton release and Ca^{++} translocation. Thus, this explanation requires that the mitochondrial membrane be permeable to protons. Evidence for an impermeability of the membrane to protons has been produced by Mitchell and Moyle (27). The alternative explanation, that the inhibition of respiration is dependent on the rate of anion influx, is supported by two observations. First, there is a correlation between the inhibition

of respiration and the concentration of succinate in the medium. Succinate has been shown to be taken up together with calcium or K^+ (17, 29–31). Second, it has been shown that the accumulation of succinate into the mitochondria is inhibited at alkaline pH (17, 31). The inhibition of electron transfer after addition of Ca^{++} at high NaCl concentrations is suggested to be caused by inhibition of the penetration of succinate. Thus, when other permeant anions, such as phosphate or acetate, are absent, the rate of respiration is dependent on the entrance of the endogenous released anions and of succinate. All agents or conditions which restrain the entrance of succinate also lower the concentration of Ca^{++} at which the inhibition of respiration appears.

Stoichiometry of Ion Translocation—Carafoli *et al.* (14) emphasized that the common denominator in bringing about the superstoichiometric behavior was the presence of high concentrations of impermeant anions, which would be OH^- in the case of the pH change. According to Carafoli *et al.* (14), increased concentrations of external impermeant anions alter the potential of the mitochondrial membrane; thus more favorable conditions for Ca^{++} uptake are created. The prevention of the superstoichiometry by permeant anions was attributed to a collapsing effect on the abnormally high potential of the membrane. The present data indicate that the common denominator of the conditions which result in the superstoichiometric Ca^{++}:O and H^+:O ratios is the inhibition of respiration. This is, in turn, dependent on the interplay of two factors: the amount of Ca^{++} and of permeant anions taken up.

In a previous paper (16) we have denoted Ca^{++} as a permeant cation, which enters the inner mitochondrial space in the absence of metabolism, although its distribution at the two sides of the membrane is profoundly affected by the metabolic state. The force driving the accumulation of permeant cations may be an electrical potential, as proposed by Mitchell (28). However, the generation of the membrane potential is accompanied, according to the chemiosmotic hypothesis, by a pH differential, the latter being neutralized when coupled translocations of anions take place. It can be calculated from the internal mitochondrial buffering power (32) that the uptake of 13 μg of Ca^{++} ion per g of protein concomitantly with the release of 13 μg of H^+ ion should be followed by an increase of the intramitochondrial pH of 1 unit. From the data in Figs. 5 and 6 it would seem that an increase of 1 pH unit is already capable of causing an inhibition of the respiration. In fact, the H^+:O and Ca^{++}:O ratios, obtained by an addition of Ca^{++} above 14 μg of Ca^{++} ion per g of protein, were high, about 3 Ca^{++} and 4.5 $H^+/\sim$.

The role of anions under these conditions becomes critical. In fact, with permeant anions large amounts of Ca^{++} can be accumulated without superstoichiometries. On the other hand, superstoichiometries are observed when the rate of anion influx does not keep pace with the rate of cation influx. High pH or ionic strength, by restraining the influx of anions, facilitate the superstoichiometric ratios. However, superstoichiometries occur not only at high pH and ionic strength. We suggest that when amounts of Ca^{++} higher than 15 μg of ion per g of protein are added, in the absence of phosphate and acetate, to mitochondria incubated at pH near 7, the stoichiometries are presumably obtained under condition of partial inhibition of respiration and are therefore overestimated. Respiration, although inhibited during the preceding Ca^{++} uptake, may again be stimulated by a subsequent Ca^{++} addition, since the intramitochondrial pH declines in the time interval between the Ca^{++} additions because of the anion diffusion.

Cockrell, Harris, and Pressman (33) have reported high stoichiometric ratios during K^+ uptake supported by ATP hydrolysis. Studies are in progress in our laboratory to determine whether the same factors, shown above to cause high Ca^{++}:O ratios, may also affect the stoichiometry of K^+ uptake.

The mechanism by which inhibition of respiration is able to cause the high stoichiometric ratios is not yet understood. It may be imagined that the low solubility of the Ca^{++} salts at high pH values plays a significant role in causing the high stoichiometric ratios. A larger contribution of metabolism-independent Ca^{++} binding may also be expected under conditions in which respiration is inhibited and the intramitochondrial concentration of Ca^{++} exceeds 10 mM. Further investigation is in progress to evaluate the role of these factors.

Acknowledgments—The authors wish to thank Dr. Peter Mitchell for discussing in detail the present work and for providing useful information about the bromthymol blue technique. The authors also wish to thank Mr. Luciano Pregnolato for expert technical assistance and Dr. Cristiano Rossi for collaboration in an earlier phase of the investigation.

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