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Super-stoichiometric Ratios between Ion Movements and Electron Transport in Rat Liver Mitochondria*

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SUMMARY

The number of Ca^{++} ions accumulated per pair of electrons passing through each energy-conserving site of the respiratory chain during Ca^{++}-induced respiratory jumps in isolated rat liver mitochondria (i.e. the Ca^{++}: $\sim$ ratio) is normally approximately 2.0 when the suspending medium contains 80 mM KCl or NaCl at pH 7.4, but no permeant anion. However, when the concentration of impermeant salts is raised to as high as 240 mM, or when the pH is raised to above 8.0, the Ca^{++} $\sim$ ratio increases to values exceeding 4.0 to 5.0, primarily because of a decrease in the amount of extra oxygen uptake evoked by addition of Ca^{++}. The ratio H^+ ejected: $\sim$ also increases in proportion to the Ca^{++}: $\sim$ ratio. Increase in sodium chloride concentration does not influence the adenosine diphosphate to oxygen ratio of oxidative phosphorylation. Such super-stoichiometric ratios of Ca^{++} uptake are almost completely dependent on electron transport, and occur in the presence of high concentration of *impermeant* anions such as chloride, bromide, iodide, and thiocyanate, but not when such salts are replaced with sucrose. In the presence of *permeant* anions, such as phosphate, the Ca^{++}: $\sim$ ratio remains constant at about 2.0. The possible causes of the apparently "flexible" stoichiometry of Ca^{++} uptake and H^+ ejection with electron transport in the absence of phosphate are discussed.

Several recent investigations have shown that approximately 2.0 Ca^{++}, Sr^{++}, or Mn^{++} ions may be accumulated by isolated mitochondria as a pair of electrons passes through each energy-conserving "site" of the respiratory chain (1–8). When inorganic phosphate is present in the medium it is accumulated with the divalent cation (2, 3, 6). It has also been found that for each Ca^{++} ion accumulated in the absence of phosphate, approximately 1 H^+ ion is ejected into the medium (8–12). These findings have led to the view that there is a fixed stoichiometry in the coupling between divalent cation accumulation, H^+ ejection, and electron transport, a stoichiometry which is apparently as exact as that of oxidative phosphorylation (1, 3, 7).

Recently we reported two sets of conditions under which the molar ratio of Ca^{++} accumulated to oxygen taken up may undergo significant variation, to values substantially higher than those previously observed, without significant change in the H^+:Ca^{++} ratio (13–15). Increasing the concentration of certain alkali metal salts, such as NaCl or KCl, or raising the pH of the medium from 7.2 to as high as 8.2, has been found to cause large increases in the Ca^{++}:O accumulation ratio when the medium does not contain inorganic phosphate. On the other hand, changes in salt concentration and pH were found not to influence the Ca^{++}:O accumulation ratio in the presence of inorganic phosphate (13). Changes in salt concentration and pH were also found to influence profoundly the rate of efflux of Ca^{++} from previously loaded mitochondria (14). C. Rossi and Azzone (16) have independently reported the occurrence of such super-stoichiometric ratios of Ca^{++} uptake in media of high salt concentrations or pH.

This communication reports critical details and additional experiments characterizing the phenomenon.

EXPERIMENTAL PROCEDURE

Rat liver mitochondria were isolated from livers of Sprague-Dawley albino rats (Carworth Farms) by the procedure of Schneider (17); they were washed twice with cold 0.25 M sucrose.

Oxygen consumption was measured with a Teflon-coated Clark oxygen electrode as described by Kielley and Bronk (18). Ca^{++} uptake was measured with the use of $^{45}Ca^{++}$ (25,000 cpm per μmole under thin window counting conditions). At the end of the respiratory jump, the mitochondria were quickly sedimented from the medium by means of a Beckman/Spinco Microfuge or filtered through a Millipore filter (AAW, 0.8 μ) fitted to a syringe with a Swinnex adapter (19). The $^{45}Ca^{++}$ remaining in the medium was then plated and counted. In nearly all the experiments the Ca^{++} was almost completely removed from the medium (more than 98.5%). In some experiments, as shown, removal of Ca^{++} was incomplete; in these cases counting was carried out to a 3% error. The amounts of $^{45}Ca^{++}$ accumulated in the experiments described were relatively large compared to the endogenous Ca^{++} present (approximately 10 nmoles per mg); furthermore, the endogenous Ca^{++} was found to have a very low efflux rate compared to the rate of accumulation of external Ca^{++}. Uptake

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of $^{45}Ca^{++}$ by the mitochondria therefore represented net uptake with only a negligible amount of exchange.

Ejection and uptake of H^+ were followed with a glass electrode and Beckman Expandomatic pH meter equipped with a Sargent SR recorder. The absolute amounts of H^+ ion production or utilization were determined by calibration with internal standards of HCl or NaOH in each experiment, in the direction of the change measured.

RESULTS

Effect of Salt Concentration on Ca^{++}:~ Ratio—In a preceding communication (13) it was shown that addition of a standard amount of Ca^{++} to rat liver mitochondria suspended in a simple medium containing 10 mM succinate, 10 mM Tris-chloride (pH 7.4), and NaCl in concentrations increasing from 40 to 240 mM produced decreasing amounts of extra oxygen uptake, so that the accumulation ratio Ca^{++}:~ calculated from the extra oxygen uptake evoked increased from values of about 2.0 at 40 mM to 4.8 at 240 mM NaCl. The oxygen electrode traces showed that such variations in salt concentration produced little or no change in the rate of the resting respiration either before or after the Ca^{++} jump (13).

Data summarized in Table I give complete data on the amounts of Ca^{++} accumulated, the extra oxygen uptake evoked, and the total oxygen uptake in such experiments. Over 99% of the added Ca^{++} was accumulated during the respiratory jump, regardless of salt concentration. The extra oxygen uptake evoked by addition of Ca^{++} decreased with increasing NaCl concentration, as did the total oxygen uptake. The Ca^{++}:~ ratio, calculated on the basis of extra oxygen uptake, increased with salt concentration from a value of 1.6 at 40 mM NaCl to a value of 4.2 at 240 mM. When the Ca^{++}:~ ratio was calculated from the total oxygen uptake during the respiratory jump, the Ca^{++}:~ ratio rose from a low of 0.76 to a high of 1.90 at 240 mM NaCl.

It is probable that calculation of the Ca^{++}:~ ratio based on extra oxygen uptake more nearly represents the true stoichiometry between Ca^{++} uptake and electron transport. Although an earlier report from this laboratory has shown that resting respiration can support Ca^{++} uptake (20), the Ca^{++}:~ ratio of resting respiration is only about 0.12, or less than 10% of the efficiency observed in Ca^{++}-stimulated respiration. The resting or State 4 respiration thus may make only a very small contribution to the total Ca^{++} uptake. It is uncertain, however, whether that component of the total oxygen uptake during the jump which corresponds to the preceding resting rate has exactly the same coupling efficiency as the latter measured alone (20).

The decrease in extra oxygen uptake with increasing salt concentration is not an artifact caused by the effect of salt concentration on the solubility of oxygen in the medium or on the interaction of oxygen with the Teflon-coated cathode. Appropriate control experiments in which known amounts of sodium dithionite were added to absorb stoichiometric amounts of dissolved oxygen showed no significant differences in the electrode response as a function of salt concentration in the range examined. Furthermore, as will be seen below, the high salt concentration did not interfere with the observation of normal ADP:O ratios of oxidative phosphorylation, or of "normal" Ca^{++}:~ ratios of 2.0 when phosphate was present in the medium.

Although most of the Ca^{++}:~ ratios at 240 mM NaCl reported in this paper were in the range of 3.6 to 5.0 (on the basis of extra oxygen uptake), ratios even higher than this were observed when the salt concentration was increased still further. At 500 mM NaCl, values of Ca^{++}:~ as high as 8 to 10 were observed, although the ratios were difficult to determine accurately because of the very small extra oxygen uptake. The effects of very high salt concentrations and pH on Ca^{++} uptake and respiration are considered in more detail below.

Effect of Respiratory Inhibitors on Ca^{++}:~ Ratio—One interpretation that can be placed on the super-stoichiometric ratio of Ca^{++} uptake at high salt concentrations or pH is that the latter conditions may cause nonspecific adsorption of some of the added Ca^{++} by mitochondria, without coupled stoichiometric activation of electron transport. In fact, Rossi and Azzone (16) have suggested that this is the case. However, two sets of experiments in Table II indicate that very nearly all of the super-accumulation of Ca^{++} at 320 mM NaCl is prevented when respiratory inhibitors are present in the system. The data in Experiment 1, Table II, contrast the effects of antimycin A, rotenone, and 2,4-dinitrophenol on the amounts of Ca^{++} accumulated at 80 as opposed to 320 mM NaCl. Antimycin A at 0.2 μg per mg of mitochondrial protein, added to the system just before addition of Ca^{++}, inhibited about 85% of the Ca^{++} uptake at both 80 and 320 mM. Such additions of antimycin A did not give complete inhibition of respiration, as indicated by the oxygen electrode trace. More complete inhibition was given by the combination of antimycin A and rotenone, or by adding antimycin A to the suspending medium before the mitochondria, so that the latter were exposed to the inhibitor for at least 30 to 60 sec. There was an apparent tendency for Ca^{++} uptake to be slightly less sensitive to antimycin A and 2,4-dinitrophenol at 320 mM than at 80 mM NaCl in Experiment 1. The amount of inhibitor-insensitive Ca^{++} uptake at 320 mM evidently cannot begin to account for the super-accumulation of Ca^{++}. In other experiments under slightly different conditions (*e.g.* Experiment 2, Table II), and with a higher concentration of antimycin A, there was essentially complete inhibition of the super-accumulation of Ca^{++} at 320 mM NaCl. From these experiments it may be concluded that there is no significant amount of electron transport-independent binding of Ca^{++} under the conditions producing super-stoichiometry of Ca^{++} uptake.

Effect of NaCl on Oxidative Phosphorylation and Ca^{++}:ADP Ratio—In the paired experiments of Table III, the magnitude of

TABLE I

Effect of NaCl on oxygen uptake and Ca^{++} accumulation

The test system (1.9 ml) contained 10 mM sodium succinate, 10 mM Tris-chloride (pH 7.4), NaCl as shown, and rat liver mitochondria (5.0 mg of protein) at 24°. After 50 sec of incubation, 390 nmoles of $^{45}CaCl_2$ were added. Between 30 and 60 sec after return of the oxygen uptake to the resting level, the mitochondria were centrifuged and $^{45}Ca^{++}$ remaining in the supernatant medium was determined.

NaCl	Ca^{++} accumulated	Oxygen uptake		Ca^{++}:~	
		Extra	Total	Extra	Total
mM	*nmoles*	*natoms*			
40	385	122	356	1.60	0.76
80	387	93	195	2.10	1.0
160	386	63	138	3.1	1.40
240	385	47	104	4.2	1.90

TABLE II

Effect of inhibitors on Ca^{++} uptake

The system in Experiment 1 contained 5.0 mM sodium succinate, 5.0 mM Tris-chloride (pH 7.4), 80 or 320 mM NaCl as shown, and 5.0 mg of mitochondrial protein in a volume of 2.0 ml. Antimycin A was added at 0.2 μg per mg of protein; rotenone, at 1.0 μM; and 2,4-dinitrophenol, at 0.1 mM, 30 sec after the mitochondria, except where indicated. The respiratory jump was initiated by addition of 400 nmoles of $^{45}CaCl_2$. In Experiment 2 the system contained 10 mM sodium succinate, 10 mM Tris-chloride (pH 7.4), 80 or 320 mM NaCl, and 8.6 mg of mitochondrial protein. Antimycin A was added at 3.0 μg per mg of protein; rotenone, at 1.0 μM; and 2,4-dinitrophenol, at 0.1 mM, in all cases *before* the mitochondria. The amount of $CaCl_2$ added was 600 nmoles.

Inhibitor	NaCl	Ca^{++} uptake	Ca^{++}:~
	mM	*nmoles*	
Experiment 1			
None	80	400	2.12
Antimycin A	80	65	
Antimycin A (before)	80	8	
Antimycin A + rotenone	80	0	
2,4-Dinitrophenol	80	31	
None	320	392	5.32
Antimycin A	320	59	
Antimycin A (before)	320	68	
Antimycin A + rotenone	320	28	
2,4-Dinitrophenol	320	54	
Experiment 2			
None	80	593	2.06
Antimycin A	80	28	
2,4-Dinitrophenol	80	41	
None	320	595	3.95
Antimycin A	320	15	
2,4-Dinitrophenol	320	12	

the extra oxygen uptake elicited by addition of Ca^{++} and by ADP were compared at each concentration of NaCl in the medium. The data show that, as salt concentration was increased from 40 to 320 mM NaCl, the usual increase in the Ca^{++}:O accumulation ratio occurred; the ratio rose from 5.0 to 11.7, based on extra oxygen uptake. On the other hand, the extra oxygen uptake elicited by addition of fixed amounts of ADP remained essentially constant. At 40 mM NaCl the ADP:O ratio was 2.0, and at 320 mM NaCl it was 2.1. Varying the NaCl concentration in this range under these conditions therefore did not influence the stoichiometry of oxidative phosphorylation, but did cause large variations in the quantitative relationship between Ca^{++} accumulation and electron transport.

It has been proposed that the molar ratio of Ca^{++} accumulated to ADP phosphorylated, extrapolated from such paired experiments, is the most valid way of correcting ion accumulation measurements for energy "leakage" (7). The data in Table III show that the Ca^{++}:ADP ratio increased from 2.5 to 5.6 when salt concentration was increased from 40 to 320 mM. A pair of electrons moving from succinate to oxygen can cause phosphorylation of only 2 molecules of ADP to ATP, regardless of salt concentration, but may cause net accumulation of from 4 to 12 or more Ca^{++} ions as NaCl concentration is increased.

Effect of Sucrose on Ca^{++}:~ Ratio—When the NaCl of the medium was replaced with sucrose, and the sucrose concentration was varied over the range from 160 to 480 mM (Fig. 1), there was no significant effect on the Ca^{++}:~ accumulation ratio. The effect of NaCl on the Ca^{++}:O ratio therefore cannot be attributed simply to changes in the tonicity of the medium. At 480 mM sucrose some inhibition of both resting and activated respiration occurred, but there was no significant effect on the Ca^{++}:~ ratio.

Effect of Different Salts on Ca^{++}:~ Ratio—Data in Fig. 1 show that replacement of NaCl with increasing concentrations of LiCl or KCl also yielded increases in the Ca^{++}:~ ratio. Data collected in Fig. 2 show the effect of replacing chloride by other anions. KCl, KBr, and KCNS gave similar increases in the

TABLE III

Effect of NaCl concentration on Ca^{++}:O, ADP:O, and Ca^{++}:ADP ratios

The test system for measurement of the Ca^{++}:O ratio was exactly as described in Fig. 1; 400 nmoles of $^{45}CaCl_2$ were added to initiate the jump. The test system for measurement of the ADP:O ratio contained 10 mM sodium succinate, 10 mM Tris-chloride (pH 7.4), NaCl in the concentrations shown, 2.0 mM $MgCl_2$, and 2.0 mM phosphate; to start the jump, 200 nmoles of ADP were added. The temperature was 24°. All data were calculated on the basis of extra oxygen uptake. A single preparation of mitochondria was used; at each level of NaCl the ADP jump was carried out on a separate aliquot of mitochondria.

NaCl	Ca^{++} accumulated	Ca^{++}:O	ADP:O	Ca^{++}:ADP	Ca^{++}:~
mM	*nmoles*				
40	393	5.0	2.0	2.5	1.3
80	392	6.7	1.9	3.5	1.8
160	393	7.3	1.9	3.9	2.0
240	356	7.9	2.0	4.0	2.0
320	374	11.7	2.1	5.6	2.8

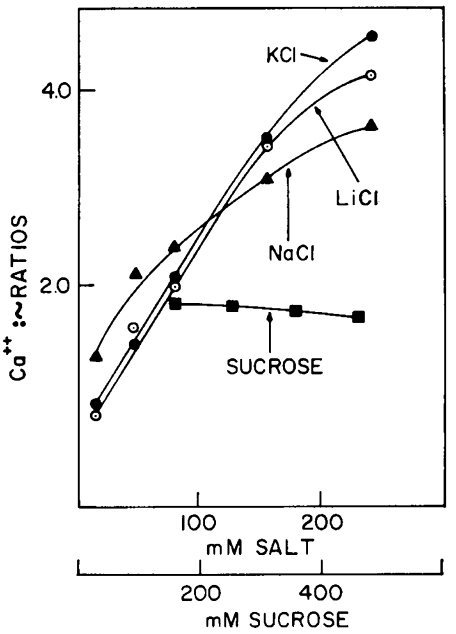

FIG. 1. Effect of sucrose, K^+, and Li^+ on the Ca^{++}:~ ratio. The test system (1.9 ml) contained 5.0 mg of mitochondrial protein, 10 mM sodium succinate, 10 mM Tris-chloride (pH 7.4), and salts or sucrose as shown. The temperature was 24–25°. To start the jump, 390 mmoles of $^{45}CaCl_2$ were added. Data were calculated on the basis of extra oxygen uptake.

Ca^{++}:~ ratio (succinate) as their concentration in the medium was increased. However, acetate had a rather different effect. High concentrations of acetate yielded considerable swelling and loss of respiratory control, in agreement with observations of Rasmussen, Chance, and Ogata (21) and Chance (7), who found

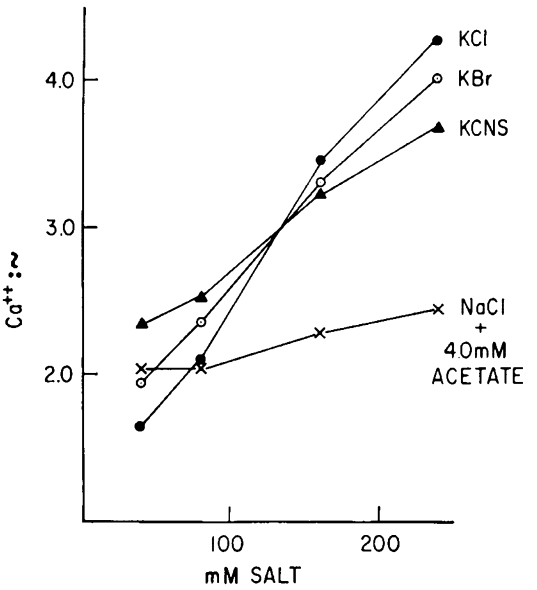

FIG. 2. Effect of anions on the Ca^{++}:~ ratio. Details are exactly the same as in Fig. 1. The data on acetate were obtained from systems in which NaCl was varied, with acetate held constant at 4.0 mM.

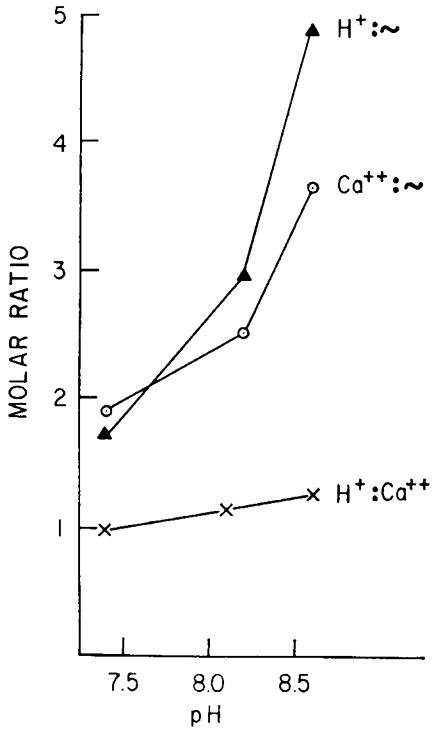

FIG. 3. Effect of pH on the uptake of Ca^{++} and ejection of H^+. The system (2.0 ml) contained mitochondria (5.0 mg of protein), 10 mM sodium succinate, 10 mM Tris-chloride at the initial pH given, and 80 mM NaCl. At zero time, 400 nmoles of $^{45}CaCl_2$ were added. The temperature was 25°.

that acetate is permeant through the mitochondrial membrane, whereas chloride is not. It was therefore not possible to measure the effect of replacing NaCl with equivalent amounts of sodium acetate. However, when a low concentration of sodium acetate (4.0 mM) was added to systems in which NaCl concentration was varied, the acetate had the effect of preventing most of the usual increase in the Ca^{++}:~ ratio caused by increasing NaCl concentration (Fig. 2). The effect of the permeant anion acetate is thus very similar to the effect of phosphate, in the presence of which the Ca^{++}:~ ratio was found to remain fixed at 2.0, regardless of NaCl concentration (13).

These findings indicate that when impermeant anions such as Cl^-, Br^-, and CNS^- are present in the medium, increasing their concentrations increases the magnitude of the Ca^{++}:~ ratio if there are no permeant anions in the medium. On the other hand, the Ca^{++}:~ ratio tends to remain nearly constant, regardless of the concentration of impermeant anions, when permeant anions such as phosphate or acetate are present in the medium.

Effect of pH on Ca^{++}:~, H^+:Ca^{++}, and H^+:~ Ratios—Super-stoichiometric ratios between Ca^{++} uptake and electron transport are also observed when the pH of the suspending medium is increased from the usual 7.4 to 8.2, with salt concentration held constant at 80 mM (13). Data collected in Fig. 3 show that both the Ca^{++}:~ and the H^+:~ ratios doubled when the pH was increased from 7.4 to 8.6, whereas the H^+:Ca^{++} ratio rose by only about 25%. The Ca^{++}:~ values were calculated on the basis of extra oxygen uptake; H^+:Ca^{++} ratios were extrapolated from glass electrode traces. The Ca^{++} accumulation data were obtained 60 sec after completion of the respiratory jump. As in the case of the experiments with increasing NaCl concentration, the effect of increasing the pH of the medium was to cause a decrease in the extra oxygen uptake evoked by Ca^{++} addition. It was reported earlier (13) that in the presence of phosphate the Ca^{++}:~ ratio remains constant at all pH values in this range.

Completeness of Accumulation of Added Ca^{++}—Under certain circumstances favoring super-stoichiometric behavior, the added Ca^{++} was found to be incompletely accumulated. When β-hydroxybutyrate was the substrate, Ca^{++} added at the level of 80 nmoles per mg of mitochondrial protein was virtually completely accumulated at NaCl concentrations up to 320 mM, or at pH values up to 8.0. When succinate was the substrate, Ca^{++} accumulation was often significantly less than complete at the highest salt concentrations, or at the highest pH values (pH 8.5). When both NaCl concentration and the pH of the medium were elevated, incomplete accumulation of added Ca^{++} occurred with either substrate. Under these conditions the Ca^{++}:O respiratory activation ratio (*i.e.* the ratio of Ca^{++} added to extra oxygen uptake evoked) may be substantially higher than the true Ca^{++}:O accumulation ratio. Similarly, the H^+:Ca^{++} ratio may vary considerably depending on whether it is calculated from the amount of Ca^{++} accumulated or added.

Inhibited State of Respiration Induced by High Salt Concentration and pH—When a Ca^{++}-induced respiratory jump took place in media in which both pH and salt concentration are elevated to levels at which each alone would cause super-stoichiometric behavior, an inhibited state of respiration often was found to ensue. Typical experiments in Fig. 4 show that, in a medium of pH 8.0 containing 240 mM NaCl, addition of Ca^{++} produced the usual acceleration of oxygen uptake. However, instead of returning to approximately the original resting respiration in the usual way (13), the rate of oxygen uptake "overshot" and became

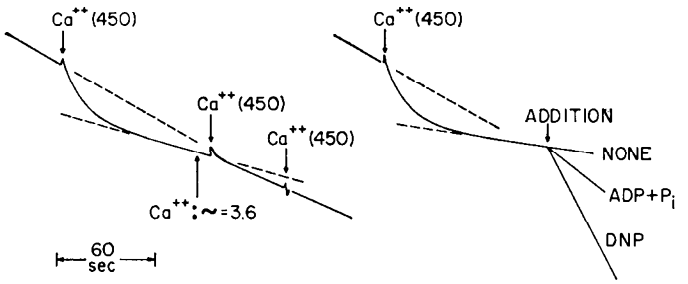

FIG. 4. Induction of an inhibited state of respiration. The test system contained in 2.0 ml of 240 mM NaCl, 10 mM sodium succinate, 10 mM Tris-chloride (pH 8.0), and rat liver mitochondria (5.0 mg of protein). To start the jump, 450 nmoles of $CaCl_2$ were added. Further additions of 450 nmoles of $CaCl_2$ were made in the experiment on the *left*. In the experiment recorded on the *right*, 250 nmoles of ADP + 1.0 mM phosphate or 50 nmoles of 2,4-dinitrophenol (*DNP*) were added.

significantly less than the initial State 4 rate; in some experiments it virtually ceased. A second and third addition of Ca^{++} gave little or no stimulation of respiration, whereas in normal circumstances (3) repeated additions of Ca^{++} would lead to a succession of respiratory jumps with nearly exact stoichiometry. That the mitochondria in such an inhibited state are not irreversibly damaged is also shown in Fig. 4. Addition of either dinitrophenol or ADP plus phosphate to mitochondria in such an inhibited state promptly released the inhibition.

The inhibited state of respiration described here evidently is related to the conditions that produce super-stoichiometric ratios of Ca^{++} uptake to electron transport, *i.e.* high concentration of an impermeant anion and a high pH. The inhibited state resembles in some respects that described by Chance (22), *i.e.* "State 6," which is also released by addition of dinitrophenol or ADP. However, the conditions under which State 6 was observed (22) are rather different from those described here.

DISCUSSION

The data reported in this paper, as well as those reported earlier (13, 14), indicate that the molar relationship between the amount of Ca^{++} accumulated and the extra or total oxygen taken up during a Ca^{++}-induced respiratory jump is not a fixed stoichiometric entity under all conditions. The $Ca^{++}:\sim$ ratio may vary considerably, from values of less than 2.0 to values exceeding 5.0 or more, when no phosphate is present in the suspending medium. Such increases in the $Ca^{++}:\sim$ ratio are caused by increasing the pH of the medium or increasing the concentrations of salts having impermeant anions such as chloride, bromide, or iodide. On the other hand, when inorganic phosphate is present in the medium, then the $Ca^{++}:\sim$ accumulation ratio remains nearly constant at about 2.0, regardless of variations of pH or salt concentration, within limits (13, 14). The constancy of the $Ca^{++}:\sim$ ratio in the presence of phosphate in the experiments reported here, as well as in earlier studies (1–5), reaffirms earlier conclusions that mitochondrial uptake of Ca^{++} in the presence of phosphate proceeds in strict stoichiometry with oxygen uptake, and that this stoichiometry is as characteristic as is the ADP:O ratio of oxidative phosphorylation (1–3, 7). It is particularly noteworthy that constant stoichiometry of Ca^{++} uptake with electron transport was observed in massive loading experiments in the presence of phosphate (1), in which the mitochondria retained no respiratory control and only the *total* oxygen uptake, measured manometrically, was used in the calculations. Such massive loading experiments are not complicated by the problem of deciding the proper manner of calculating the $Ca^{++}:\sim$ ratio from respiratory jump experiments, *i.e.* whether on the basis of extra or total oxygen uptake.

Rossi and Azzone (16) have independently observed that high concentrations of NaCl and high pH in the medium lead to super-stoichiometric ratios between Ca^{++} and oxygen uptake. The only discrepancy is their conclusion that the H^+:O ratio does not change when salt concentration or pH is increased, whereas our findings show that the H^+:O ratios increased in proportion to the increase in the Ca^{++}:O accumulation ratio. This discrepancy may be more apparent than real, since the experimental conditions were different. For example, rotenone was present in their system. Furthermore, it is not clear whether Ca^{++}:O activation or accumulation ratios were measured. As data in Table IV show, these ratios are not necessarily identical. Other experimental factors may also be critical in comparison of the data, such as the ratio of Ca^{++} to mitochondrial protein and the time of sampling in measurements of Ca^{++} uptake.

Quantitative interpretation of the flexible relationship between Ca^{++} uptake and electron transport is rendered rather difficult because of the complex dynamics of Ca^{++}-stimulated respiratory jumps. Although 99% or more of the added Ca^{++} may be accumulated in such a jump, in the ensuing steady state of resting respiration there is continuous efflux of Ca^{++} to the medium opposed by continuous energy-dependent uptake (12). It is the dynamic balance between the influx and efflux rates of Ca^{++} in this resting steady state that is a major determinant of the fraction of added Ca^{++} that is retained (20). The observed or *net* $Ca^{++}:\sim$ ratio, such as was measured in this investigation, may be the resultant of an *intrinsic* $Ca^{++}:\sim$ stoichiometry, counterbalanced by an efflux process. Another complication is that the observed $Ca^{++}:\sim$ ratio is greatly different during the jump and during the ensuing steady state. During State 4 or resting respiration, the observed $Ca^{++}:\sim$ ratio is normally only 0.2 to 0.3, compared with the value of about 2.0 observed during the jump (20). Moreover, it has been found that the amount of Ca^{++} retained during the resting respiration after a jump may undergo large rebounds or oscillations under certain conditions, without change in the rate of respiration (23, 24). Variability

TABLE IV

Effect of pH and NaCl concentration on completeness of uptake of Ca^{++}

The system contained 10 mM DL-β-hydroxybutyrate, 10 mM Tris-chloride at the pH shown, NaCl at the concentrations shown, and 5.0 mg of mitochondrial protein. The total volume was 1.9 ml. To initiate the jump, 435 nmoles of $CaCl_2$ were added. The Ca^{++} uptake was measured 60 sec following completion of the jump.

NaCl	pH	Ca^{++} uptake	Removal of added Ca^{++}	Extra O_2 uptake	$Ca^{++}:\sim$ accumulation ratio	$Ca^{++}:\sim$ activation ratio
mM		*nmoles*	%	*natoms*		
80	7.0	434	99.7	57	2.5	2.5
80	7.4	434	99.7	70	2.1	2.1
80	8.0	421	96.8	40	3.5	3.6
240	7.4	434	99.7	40	3.6	9.7
240	8.0	338	77.6	15	7.5	18.2
160	8.5	185	42.5	8	4.6	
240	8.5	200	46.0	0		

in the *observed* Ca^{++}: $\sim$ ratio could therefore be caused either by changes in the *intrinsic* Ca^{++}: $\sim$ stoichiometry or by changes in the Ca^{++} efflux rate; possibly both could vary. We have reported that changes in pH and salt concentration may in fact cause large changes in the rate of efflux of previously accumulated Ca^{++} (14). Increasing the concentration of NaCl from 80 mM to 240 mM caused over 50% inhibition of the Ca^{++} efflux rate, and increasing the pH from 7.2 to 8.0 caused a 60% inhibition of the Ca^{++} efflux (14). These decreases in Ca^{++} efflux rates caused by increased NaCl concentration or pH might account for at least some of the increase in the over-all observed Ca^{++}: $\sim$ ratios, but it is difficult to evaluate the magnitude of this contribution because of the complex dynamics of the over-all process.

There is a possible common denominator in the two variables, namely pH and NaCl concentration, that bring about super-stoichiometric behavior. Both variables involve changes in the concentration of an impermeant anion, which would be OH^- in the case of pH changes. The mitochondrial membrane has been shown to be impermeable to H^+ and OH^- ions (25, 26, 27), as well as to chloride, bromide, and iodide (21). It is less likely that the cationic components of the medium are involved in producing super-stoichiometric ratios, since experiments not shown here indicate that NH_4Cl is about as active as NaCl; NH_4^+ does penetrate the mitochondrial membrane, according to Chappell and Crofts (28). The association of the phenomenon of super-stoichiometric behavior with the concentration of impermeant anions raises the possibility that the effect reflects the balance of electrical charges across the mitochondrial membrane, and thus the membrane potential. Actually, it appears possible that there is a change in membrane potential following a respiratory jump induced by Ca^{++} in the absence of permeant anions, since for each Ca^{++} entering only 1 H^+ ion is ejected (8–12), and no other ion movements appear to occur in large enough amounts to compensate (29–32). Increase in the concentration of external impermeant anions can place increased electrical stress on the membrane and thus alter its potential (*cf.* Reference 33). The entry of Ca^{++} in abnormally high amounts may be the result of the tendency of the membrane system to adjust to or compensate for the electrical stress on the membrane. Whatever the cause of the super-stoichiometric effect, it requires occurrence of coupled respiration. The prevention of the super-stoichiometric effect by permeant anions may be the result of the collapse of an abnormally high membrane potential induced by the high external concentration of impermeant anion. Large Donnan effects influencing the equilibrium of permeant cations across the membrane can, of course, be produced by variations in the concentration of external impermeant anions.

More recent experiments (*cf.* Reference 34), to be described in detail in following communications, indicate that addition of freshly prepared mitochondria to media containing impermeant salts such as NaCl causes pronounced ejection of H^+ ions and other acid-base changes which are a reflection of the salt concentration and the pH of the medium. Such endogenous acid-base changes, which precede addition of Ca^{++} in the experiments on super-stoichiometric behavior, appear to condition the subsequent response of the mitochondria to the Ca^{++} (34). The important effect of the order of addition of Ca^{++} and mitochondria in such experiments is also implicit in the recent communication of Rossi, Azzi, and Azzone (35).

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