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Stoichiometry of Respiratory Stimulation, Accumulation of Ca^{++} and Phosphate, and Oxidative Phosphorylation in Rat Liver Mitochondria*

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Isolated mitochondria of various tissues have been found to accumulate large amounts of Ca^{++} and phosphate from the medium in a process stoichiometrically dependent on high energy intermediates generated by the energy-conserving mechanisms of the respiratory chain (*cf.* References 1–18). In most of the cited investigations (*e.g.* References 2, 3, 5, 11, 13), the mitochondria were exposed to relatively high concentrations of Ca^{++} (2 to 4 mM), which are known to cause complete uncoupling of oxidative phosphorylation (*cf.* References 2, 19, 20), mitochondrial swelling (*cf.* Reference 21), and stimulation of the formation of endogenous, free fatty acids (22). Nevertheless, nearly exact stoichiometry of Ca^{++} uptake with electron transport could be demonstrated under these conditions (10, 11). Adenosine triphosphate is necessary in such experiments to support Ca^{++} uptake, for reasons which are not understood (1, 2, 5, 9–11, 23).

There is another set of observations in which much lower concentrations of Ca^{++} (*i.e.* 100 to 400 μM) have been employed. Siekevitz and Potter (19) and Potter, Siekevitz, and Simonson (20) showed that low concentrations of Ca^{++} greatly stimulated respiration of rat liver mitochondria. Chance showed that the addition of Ca^{++} to resting mitochondria in the absence of ADP produced a transient stimulation of respiration; the oxygen uptake returned to its original rate after a period of stimulation in a manner resembling the activation of respiration by ADP (23, 24). The addition of about 2.2 moles of Ca^{++} was required to stimulate the same amount of extra oxygen uptake as given by 1.0 mole of ADP. Chance (24) assumed that Ca^{++} was accumulated in the mitochondria stoichiometrically during the period of respiratory stimulation, but he reported no actual measurements of Ca^{++} uptake to support this important conclusion. A second finding made with low concentrations of Ca^{++} is that phosphate does not necessarily enter the mitochondria with Ca^{++} (4, 6, 7, 25); in contrast, experiments carried out with high concentrations of Ca^{++} showed that phosphate is required for Ca^{++} uptake and is accumulated with Ca^{++} (2, 5, 11). Apparently, ATP is not required for accumulation of low concentrations of Ca^{++} (4, 6, 7, 23–25), again in contrast with the experiments in which high concentrations of Ca^{++} were studied. It has been rather difficult to correlate this group of observations with those made on systems containing relatively high concentrations of Ca^{++}, since many of the experiments have been carried out under widely different conditions and are often briefly reported. Some of these points are crucial to an understanding of the mechanism of Ca^{++} and phosphate uptake and its relationship to oxidative phosphorylation.

In this paper a correlated study of all these aspects of Ca^{++} accumulation by rat liver mitochondria is presented, with special reference to the behavior of mitochondria in the presence of low concentrations of Ca^{++}. Quantitative measurements are reported of respiratory stimulation by Ca^{++} as determined by the oxygen electrode, accumulation of Ca^{++} and phosphate measured by isotopic methods, and the efficiency of oxidative phosphorylation either with the respiratory stimulation method or by enzymatic determination of the ATP formed. The specific roles of phosphate, ATP, and Mg^{++} were examined in detail, as were the action of oligomycin and atractyloside. From these correlated findings it is possible to reconcile some of the puzzling differences in behavior which apparently depend on Ca^{++} concentration. Furthermore, it is shown that both oxidative phosphorylation of ADP and oxidative accumulation of Ca^{++} may take place in the same population of tightly coupled mitochondria with nearly exact stoichiometry and without uncoupling or damage.

EXPERIMENTAL PROCEDURE

Mitochondria were isolated from livers of Wistar strain albino rats, essentially by the procedure of Schneider (26). They were washed twice with 0.25 M sucrose.

Oxygen consumption was measured with the Clark oxygen electrode, as described by Kielley and Bronk (27). Ca^{++} uptake was determined as described by Rossi and Lehninger (11). For measurement of small amounts of phosphate uptake, inorganic phosphate labeled with ^{32}P was added to the incubation medium. After incubation of the mitochondria, the system was quickly chilled to 0° and centrifuged for 4 minutes at 20,000 × *g*, the pellet was washed twice with cold 0.25 M sucrose and extracted with 5% perchloric acid, and the inorganic ^{32}P was determined after removal of ATP and ADP by adsorption on Norit. ATP was determined enzymatically by the hexokinase-glucose 6-phosphate dehydrogenase method of Steiner and Williams (28). Protein was determined by a biuret method (29).

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All reagents used were analytical grade. $^{45}CaCl_2$ and ^{32}P were purchased from Amersham Radiochemical Centre (England). Hexokinase (type IV) was obtained from Sigma Chemical Company, and glucose 6-phosphate dehydrogenase from Boehringer.

RESULTS

Stimulation of Respiration by Ca^{++}—The addition of low concentrations of Ca^{++} to rat liver mitochondria suspended in a medium containing only Tris buffer, pH 7.2, 10 mM $MgCl_2$, 80 mM NaCl, and β-hydroxybutyrate as substrate produces a sharp increase in the rate of oxygen uptake (Fig. 1). After a short period of nearly linear oxygen uptake, the rate abruptly returns to the initial rate. Under these conditions several successive additions of Ca^{++} may be made, each producing a transient stimulation of oxygen uptake, followed by an abrupt return to the initial rate. The mitochondria evidently remain undamaged and retain complete respiratory control. Such stimulation of the respiratory rate by Ca^{++} also takes place in the absence of added Mg^{++}, as shown in Fig. 1; furthermore, the respiratory rate returns again to the initial rate, after consumption of the same amount of extra oxygen. Respiratory stimulation by Ca^{++} in the concentrations used (150 μM) evidently does not require the addition of either Mg^{++} or phosphate to the medium. In contrast, both Mg^{++} and phosphate are required for so-called "massive" accumulation of Ca^{++} (2, 5, 11).

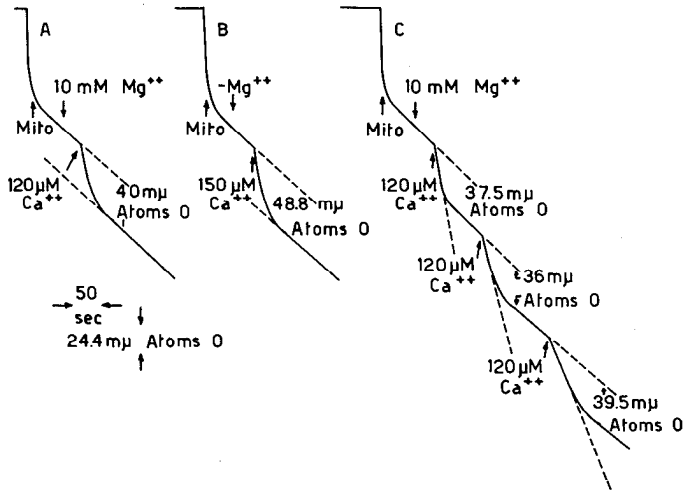

FIG. 1. The reaction system contained 20 mM Tris-HCl, pH 7.2, 80 mM NaCl, and 20 mM sodium DL-β-hydroxybutyrate as respiratory substrate. At the points indicated by the *arrows*, 7.4 mg of mitochondrial protein, 10 mM $MgCl_2$, and the CaCl were added successively. Total volume, 2.0 ml; temperature, 21°

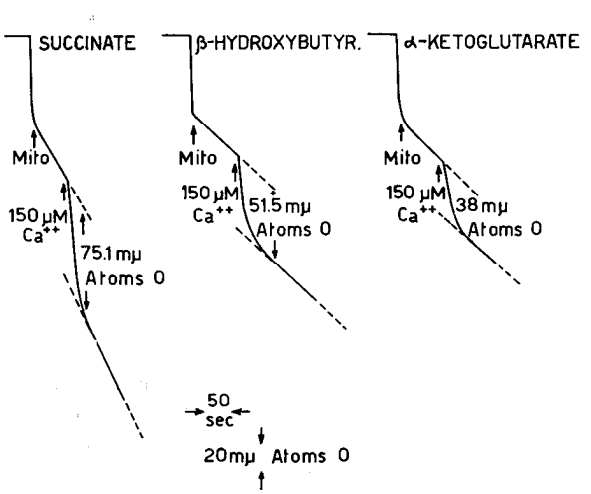

FIG. 2. The reaction system contained 20 mM Tris-HCl, pH 7.2, 80 mM NaCl, 10 mM $MgCl_2$, 10 mM sodium succinate, 20 mM sodium DL-β-hydroxybutyrate or 20 mM sodium α-ketoglutarate as respiratory substrates, and rat liver mitochondria (6 mg of protein) in 2 ml. Temperature, 21°. At the points indicated by the *arrows*, 150 μM $CaCl_2$ was added.

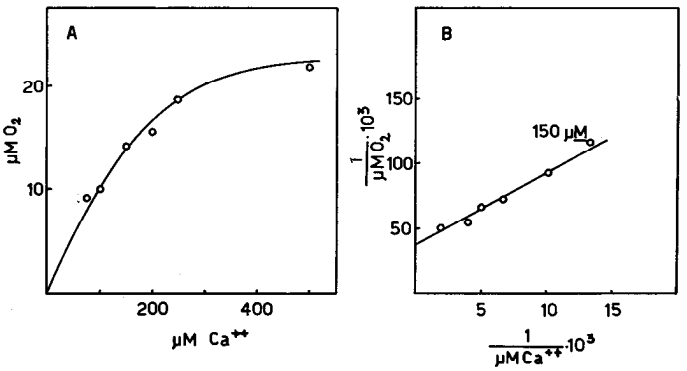

FIG. 3. *A*, experimental conditions as in Fig. 2. Sodium DL-β-hydroxybutyrate was the substrate. Mitochondrial protein, 6 mg; temperature, 21°. *B*, experimental conditions as in *A*.

Stoichiometry of Respiratory Stimulation by Ca^{++}—In Fig. 2 are shown oxygen electrode traces of the respiratory stimulation given by addition of Ca^{++} to rat liver mitochondria suspended in a medium containing respiratory substrates known to be oxidized with different P:O ratios: α-ketoglutarate, β-hydroxybutyrate, and succinate. Neither inorganic phosphate nor adenine nucleotide was added to the medium; however, Mg^{++} was present. Addition of 150 μM Ca^{++} in each case produced a prompt increase in the rate of oxygen uptake; after a period of nearly linear oxygen uptake, the rate abruptly returned to its original level. Extrapolation of the extra oxygen uptake evoked by Ca^{++} addition in the manner shown (*cf.* Chappell, Cohn, and Greville (7)) revealed that the ratio of millimicromoles of Ca^{++} added to millimicroatoms of extra oxygen uptake was 7.9 for α-ketoglutarate, 5.83 for β-hydroxybutyrate, and 4.0 for succinate. When these data were recalculated to allow for the difference in number of energy-conserving sites involved in the oxidation of each substrate, the ratio of molecules of Ca^{++} required per energy-conserving site activated was 1.98 for α-ketoglutarate, 1.94 for β-hydroxybutyrate, and 2.0 for succinate. Although Mg^{++} was added to the medium, its presence was unnecessary; it has no effect on the stoichiometry of respiratory release under these conditions (*cf.* Fig. 1).

It may be concluded that the magnitude of the extra oxygen uptake stimulated by Ca^{++} is stoichiometrically related to the number of energy-conserving sites traversed by pairs of electrons passing from substrate to oxygen. These data, obtained in experiments on simple media containing no phosphate, adenine nucleotides, or inhibitors, confirm the findings of Chance (24), who observed an average of about 2.2 molecules of Ca^{++} per site activated in test systems containing adenine nucleotides, phosphate, and oligomycin.

In Fig. 3 is shown a plot of the extra oxygen uptake evoked by Ca^{++} addition against the concentration of Ca^{++} added; the substrate was β-hydroxybutyrate. It is seen that the extra oxygen

uptake is approximately stoichiometric with Ca^{++} concentration up to about 150 μM Ca^{++}. It falls off somewhat at 250 μM Ca^{++}. Additions higher than 800 μM Ca^{++} no longer give increases in oxygen uptake, and the respiratory chain evidently is "saturated" with Ca^{++}. The slope of the line indicates that the ratio of millimicromoles of Ca^{++} added to millimicroatoms of extra oxygen uptake is between 5.76 and 6.95 and the ratio of Ca^{++} added per site activated is between 1.92 and 2.40, over the whole range of Ca^{++} concentration tested.

In Fig. 3*B* is shown a plot of the reciprocal of the extra oxygen uptake *versus* the reciprocal of Ca^{++} added. The linearity of the plot is suggestive of an enzyme-substrate relationship. The intercept in this experiment occurs at 154 μM Ca^{++}, at which one-half "saturation" of the energy-conserving sites is achieved. Similar experiments by Chance (24) yielded a value of about 45 μM, measured in a system containing phosphate and oligomycin. Although such measurements are a reflection of the high affinity of the energy-conserving sites for Ca^{++}, as Chance has pointed out (24), the values obtained depend not only on the concentration of Ca^{++} but also on the concentration of mitochondria. A concentration of 150 μM Ca^{++} may more than saturate the respiratory chains at a very small concentration of mitochondria, and yet give only half-saturation of a higher concentration. Thus, there is only a quasi-Michaelis-Menten relationship. However, the high affinity for Ca^{++} suggested by these experiments is confirmed below by comparison of respiratory activation by Ca^{++} and ADP.

Accumulation of Ca^{++} during Respiratory Stimulation—Table I gives representative data for three different respiratory substrates, showing that Ca^{++} is accumulated from the medium during the period of respiratory stimulation produced by Ca^{++}. The Ca^{++} accumulation was found to be stoichiometric with the respiratory stimulation; the average ratio of Ca^{++} added per site activated in these experiments was 2.01, and the ratio of Ca^{++} accumulated per site activated was 1.85. The accumulation of Ca^{++} does not require the presence of phosphate or adenine nucleotides, in contrast to the observations on test systems containing very high concentrations of Ca^{++} (*i.e.* 2 to 4 mM (2, 5, 11)).

Nonaccumulation of Phosphate during Ca^{++} Accumulation—In the experiments of Figs. 1, 2, and 3 and Table I, no phosphate was added to the medium, yet Ca^{++} was accumulated stoichiometrically. Furthermore, no significant net change of the small endogenous P_i content of the mitochondria accompanied Ca^{++} accumulation. In a typical experiment in which 300 mμmoles

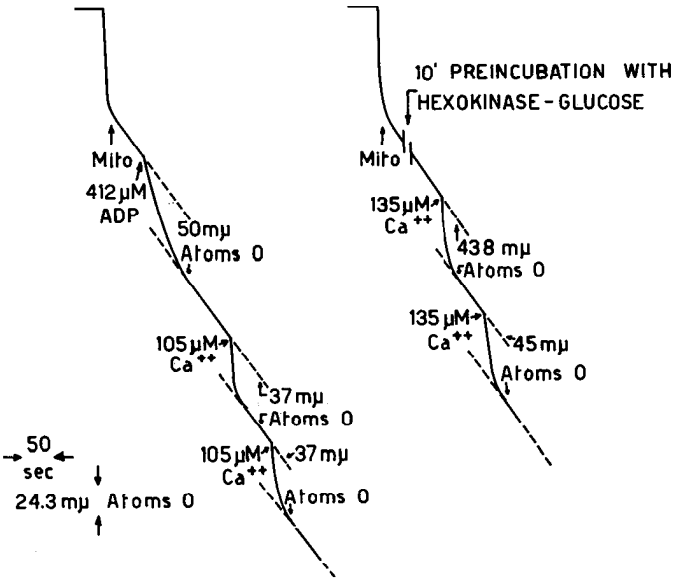

FIG. 4. Experimental conditions as in Fig. 2. Sodium DL-β-hydroxybutyrate was the substrate. Mitochondrial protein, 6 mg; temperature, 21°. The depletion by the hexokinase-glucose "trap" was carried out by pretreating the complete system with 100 μg of hexokinase and 10 mM glucose at 21° for 10 minutes.

of Ca^{++} were accumulated by an aliquot of mitochondria containing 6 mg of protein, the mitochondria contained 72 mμmoles of P_i before and 74 mμmoles of P_i after the accumulation, measured on perchloric acid extracts. A quantitative accumulation of P_i in the $Ca:P_i$ ratio of 1.67 (*cf.* Reference 11) would require the net uptake of 180 mμmoles of P_i during uptake of 300 mμmoles of Ca^{++}.

It is improbable that the small amounts of endogenous P_i present in mitochondria are participating in the process of Ca^{++} accumulation. In the experiment shown in Fig. 4, 412 μM ADP was added to mitochondria supplemented with Mg^{++} in the absence of P_i. A brief respiratory response ensued, with a return of respiration to the initial rate. The extra oxygen uptake corresponded to an endogenous phosphate content of 13.7 mμmoles per mg of mitochondrial protein, assuming a theoretical P:O ratio of 3.0. This value is nearly exactly that measured chemically. It may be presumed that the mitochondria had been fully depleted of endogenous phosphate by the ADP addition; nevertheless, two subsequent additions of Ca^{++} elicited the usua

TABLE I

Stoichiometric relationships between activation of respiration and Ca^{++} accumulation

The test system contained 10 mM Tris-HCl (pH 7.2), 80 mM NaCl, 10 mM $MgCl_2$, 20 mM sodium DL-β-hydroxybutyrate, 10 mM sodium succinate, or 20 mM sodium α-ketoglutarate as respiratory substrates, and rat liver mitochondria (8 mg of protein) in 2 ml. The cyclic response of respiration induced by Ca^{++} was measured with the oxygen electrode. Temperature, 22°.

Substrate	Oxygen uptake	Ca^{++} added	Ca^{+} accumulated	Activation ratio[a]	Accumulation ratio[b]	Activation ratio (per site)[c]	Accumulation ratio (per site)[d]
	mμatoms	*mμmoles*					
β-Hydroxybutyrate	48.90	300	273	6.13	5.59	2.04	1.86
Succinate	74.00	300	275	4.05	3.72	2.02	1.86
α-Ketoglutarate	37.95	300	280	7.90	7.38	1.98	1.84

[a] Millimicromoles of Ca^{++} added per mμatom of extra oxygen uptake.
[b] Millimicromoles of Ca^{++} accumulated per mμatom of extra oxygen uptake.
[c] Molecules of Ca^{++} per site activated.
[d] Molecules of Ca^{++} accumulated per site.

stoichiometric respiratory stimulations, each with return to the initial rate.

In Fig. 4 is shown another experiment supporting this conclusion. Mitochondria were incubated for 10 minutes in the presence of respiratory substrate, Mg^{++}, and a hexokinase-glucose "trap" to deplete endogenous ATP. This procedure can also be expected to deplete endogenous inorganic phosphate, via phosphorylation of endogenous ADP. Following this incubation the addition of Ca^{++} produced the usual stoichiometric extra uptake of oxygen. These experiments, therefore, strongly indicate that endogenous phosphate plays no particular role in Ca^{++} accumulation.

These experiments fully support the general conclusion of Chappell *et al.* (6, 7) that divalent metal ions may be accumulated without simultaneous accumulation of phosphate. Such accumulation of Ca^{++} occurs in the absence of phosphate in the medium and when the Ca^{++} concentration is rather low, in contrast to earlier experiments on "massive" accumulation of Ca^{++}.

Effect of Inhibitors on Stimulation of Respiration by Ca^{++}—Data in Table II show that the stimulation of oxygen uptake and the stoichiometric accumulation of added Ca^{++}, *in the absence of inorganic phosphate*, are not affected by either oligomycin or atractyloside. Uptake of Ca^{++} from media containing high concentrations of Ca^{++} was earlier found to be insensitive to oligomycin (2, 3, 5, 11). Similarly, the presence of ADP itself does not alter the usual respiratory response to additions of Ca^{++}. It will be seen later, however, that *in the presence* of inorganic phosphate, oligomycin and atractyloside have rather different specific effects on Ca^{++} accumulation.

In Fig. 5*A* is shown the effect of arsenate on the respiratory stimulation given by Ca^{++}, in the absence of phosphate. Arsenate causes a decrease in the total extra oxygen uptake normally evoked by a given addition of Ca^{++} before respiration returns to the controlled state. For example, after addition of 150 μM Ca^{++}, the presence of 450 μM arsenate allowed the extra oxygen uptake of only 34 mμatoms of oxygen instead of the normally expected extra uptake of 53 mμatoms. The higher the arsenate concentration, the less extra oxygen is taken up. Such inhibition by arsenate is competitive with Ca^{++} added (Fig. 5, *B* and *C*); Fig. 5*C* shows a Lineweaver-Burk plot of data from a typical experiment demonstrating this point. The inhibition by arsenate is related to the specific and characteristic action of inorganic phosphate on Ca^{++} uptake and respiratory stimulation, to be described below. Our findings on the effect of arsenate do not appear to agree with those of Chappell *et al.* (7); their experiments are not reported in sufficient detail to permit further analysis.

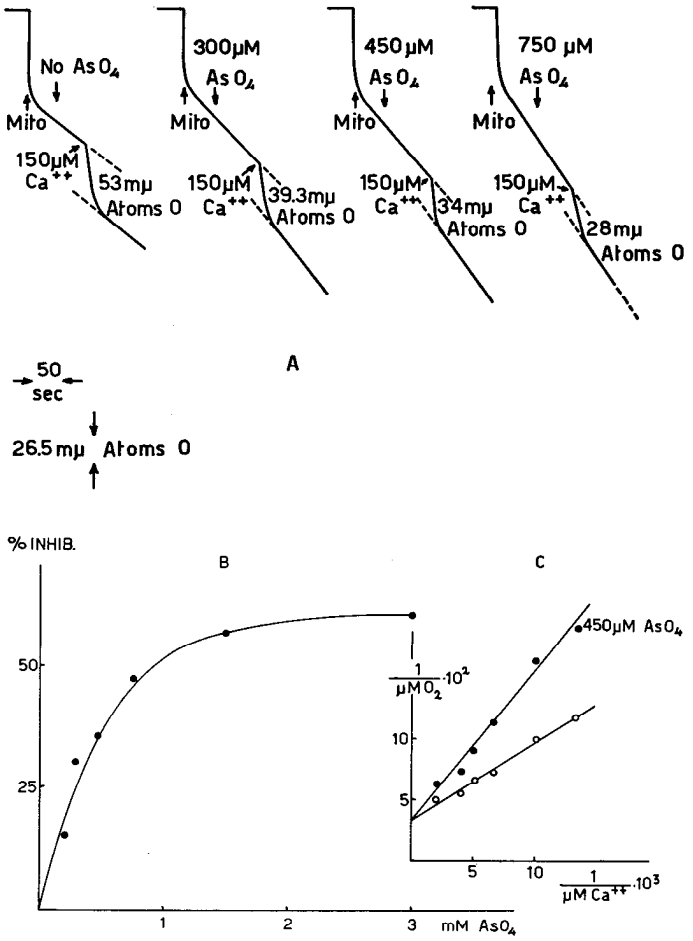

FIG. 5. *A*, the test system was that of Fig. 2, with sodium DL-β-hydroxybutyrate as substrate; 7 mg of mitochondrial protein were added. Sodium arsenate was added in the concentrations indicated. *B*, experimental conditions as in *A* of this figure; *C*, Lineweaver-Burk plot of typical experiments.

TABLE II

Effect of oligomycin and atractyloside on respiration and Ca^{++} uptake

The test system was that shown in Table I. Sodium DL-β-hydroxybutyrate was the substrate. The additions were 2 mM sodium potassium phosphate buffer, pH 7.2, 150 mM $CaCl_2$, 150 mM ADP, 1 μg of oligomycin per mg of mitochondrial protein, 4 μg of atractyloside per mg of mitochondrial protein, and 6 mg of mitochondrial protein. Temperature, 21°.

System	Oxygen uptake	Ca^{++} uptake	Ca^{++} accumulated per mμatom of extra oxygen uptake	Ca^{++} accumulated per site activated
	mμatoms	*mμmoles*		*molecules*
Absence of inorganic phosphate				
Complete	51.2	278	5.43	1.81
+oligomycin	50.5	275	5.41	1.82
+atractyloside	51.0	277	5.43	1.81
+ADP	52.1	281	5.40	1.80
Presence of inorganic phosphate				
Complete	[a]	101		
+oligomycin	51.0	279	5.47	1.82
+atractyloside	[a]	97		
+ADP	51.4 [b]	280	5.45	1.82

[a] Extra oxygen uptake not measurable because the respiratory response induced by Ca^{++} continues indefinitely.

[b] Calculated by subtracting the extra oxygen uptake due to the oxidative phosphorylation of ADP.

Effect of Phosphate on Respiratory Stimulation by Ca^{++}—In the experiments reported up to this point, no phosphate was added to the medium. In the following experiments it is shown that phosphate has quite specific effects on respiratory stimulation and on Ca^{++} uptake. The experiments in Fig. 6 show the effect of added inorganic phosphate on respiratory stimulation by Ca^{++}. In the absence of added phosphate the usual stoichiometric response

of oxygen uptake to an addition of Ca^{++} occurs, followed by an abrupt return to the initial rate. In the presence of added phosphate, the respiratory stimulation by Ca^{++} also takes place, but the oxygen uptake fails to return to the initial resting state at the point expected from the stoichiometric relationship developed in the experiments in the absence of phosphate. It may be seen, however, that the rate of oxygen uptake does decline at the stoichiometric point but does not return completely to the initial rate. This point of inflection becomes less evident as phosphate concentration is increased. At concentrations of 2 mM and above, the stimulation of oxygen uptake by Ca^{++} continues indefinitely without any inflection at the point of stoichiometry. The presence of phosphate, therefore, abolishes the exact stoichiometry of stimulation of respiration by Ca^{++}; phosphate causes the respiration to remain in an activated state.

The data in Table II show that the stoichiometric accumulation of Ca^{++} by the mitochondria is also affected by the addition of phosphate to the medium. In the absence of added phosphate, Ca^{++} uptake is stoichiometric with the extra oxygen uptake, as was shown in Fig. 1 and Table I. However, on addition of 2.0 mM phosphate to the medium, not only is the oxygen uptake stimulated indefinitely but only a small fraction of the Ca^{++} added is taken up by the mitochondria.

These specific relationships between Ca^{++} and phosphate are not evident in the briefly reported studies of Chance (24) and Chappell *et al.* (4, 6, 7). Although Chance has reported stoichiometric stimulation of respiration by Ca^{++} in the presence of phosphate, his test system also contained adenine nucleotides or oligomycin (24); it will be seen below that these agents abolish the effect of phosphate described here. Although "massive" stoichiometric accumulation of Ca^{++} in the presence of added phosphate has been observed in several laboratories (2, 5, 11), such test systems also contained ATP, which was shown by Vasington and Murphy (2) to be required for uptake of Ca^{++} under these conditions. In the experiments reported here on the phosphate effect, no ATP was added to the medium.

Effect of Oligomycin and Atractyloside on Action of Phosphate—The experiments shown in Fig. 7 indicate that oligomycin abolishes both inhibitory effects of phosphate, namely, on Ca^{++} accumulation and on the return of Ca^{++}-stimulated respiration to the initial rate after a stoichiometric amount of extra oxygen has been taken up. In the presence of oligomycin the oxygen uptake stimulated by Ca^{++} in the presence of P_i returns abruptly and exactly to the resting rate after an amount of oxygen stoichiometric to the added Ca^{++} has been taken up. Since oligomycin has been shown to block the phosphorylation mechanism at the point of entry of inorganic phosphate (30, 31), it must be concluded that the uptake of phosphate into a covalent, energy-rich intermediate prevents accumulation of Ca^{++} in these amounts and thus allows respiration to be stimulated indefinitely by Ca^{++}. Oligomycin was earlier found not to inhibit respiration-supported "massive" uptake of Ca^{++} (2). The effect of phosphate on metal ion uptake will be considered further (see "Discussion").

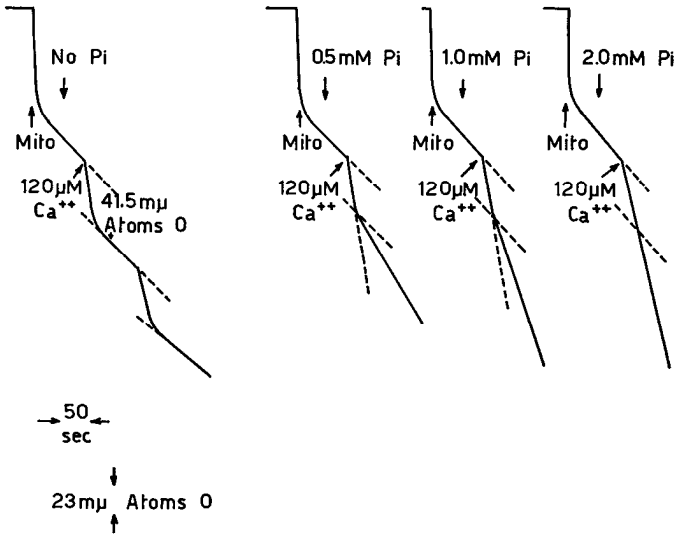

FIG. 6. Conditions as in Fig. 2. Sodium DL-β-hydroxybutyrate was the substrate; 6 mg of mitochondrial protein were added. Temperature, 22°.

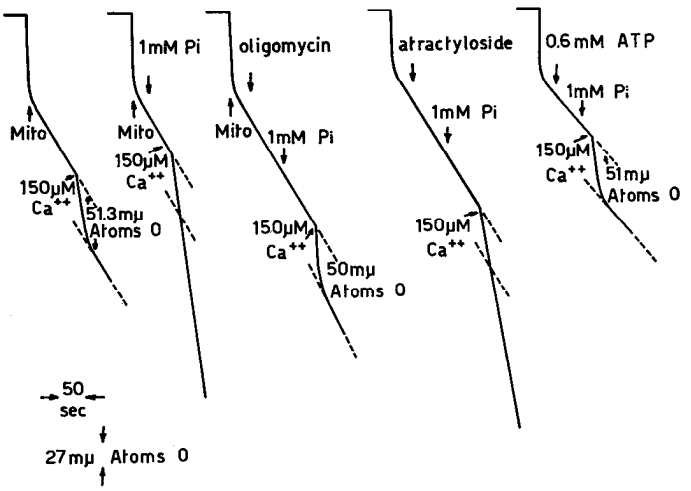

FIG. 7. Conditions as in Fig. 2; sodium DL-β-hydroxybutyrate was the substrate. When added, oligomycin was at a concentration of 1 μg per mg of mitochondrial protein, and atractyloside, at 4 μg per mg of mitochondrial protein. The system contained 9 mg of mitochondrial protein. Temperature, 21°.

The inhibitor atractyloside, on the other hand, does not abolish the characteristic effect of phosphate in prolonging Ca^{++} stimulation of respiration (Fig. 7, Table II), nor does it remove the inhibitory effect of phosphate on Ca^{++} accumulation. The action of atractyloside on the energy-coupling mechanism, therefore, is clearly different from that of oligomycin, in agreement with earlier indications reported by Bruni *et al.* (32) and by Vignais and Vignais (33), and with the recent demonstration by Bruni *et al.* (34) that atractyloside specifically inhibits Mg^{++}-dependent binding of adenine nucleotides to mitochondria, an action not given by oligomycin.

The observations on oligomycin thus explain why Chance (24) was able to observe cyclic stimulation of oxygen uptake by Ca^{++} in the presence of phosphate; the very large amounts of oligomycin added (11 μg per ml) abolished the deleterious effect of phosphate.

Effect of ATP on Respiratory Stimulation by Ca^{++} in Presence of Added Phosphate—In Fig. 7 is shown a typical experiment which shows that ATP, like oligomycin, also is able to abolish the deleterious action of phosphate on the stoichiometry of respiratory stimulation by Ca^{++}. Other experiments to be described below show that the presence of ATP also allows stoichi-

ometric accumulation of Ca^{++}. Mg^{++} is required for the full effect of ATP in reversing the action of phosphate.

The action of ATP described here, which must also account for some of Chance's findings (*cf.* Fig. 1 in Reference 24), clarifies what has appeared to be a significant difference between Ca^{++} uptake from media of low and high Ca^{++} concentrations. In brief, it is now clear that ATP is required to obtain stoichiometric uptake of Ca^{++}, whatever its concentration in the medium, as long as the medium also contains phosphate.

Sequential Accumulation of Ca^{++} and Oxidative Phosphorylation —In Fig. 8 it is seen that Ca^{++} stimulates respiration stoichiometrically in the presence of phosphate following a cycle of respiratory stimulation and oxidative phosphorylation after an addition of ADP. Presumably, the ATP formed in the first cycle protects the succeeding respiratory stimulation by Ca^{++} from the deleterious effect of P_i. The reverse sequence of events can also be observed. After mitochondria have accumulated Ca^{++} following a typical respiratory stimulation in the absence of phosphate, they are still capable of yielding normal respiratory stimulation on addition of ADP + P_i. The ADP:O ratio of 2.82 observed in this experiment (β-hydroxybutyrate was the substrate) clearly shows that the accumulation of small amounts of Ca^{++} by mitochondria in the absence of phosphate does not cause uncoupling of oxidative phosphorylation nor does it impair respiratory control by ADP, in contrast to the effects of much higher concentrations of Ca^{++}.

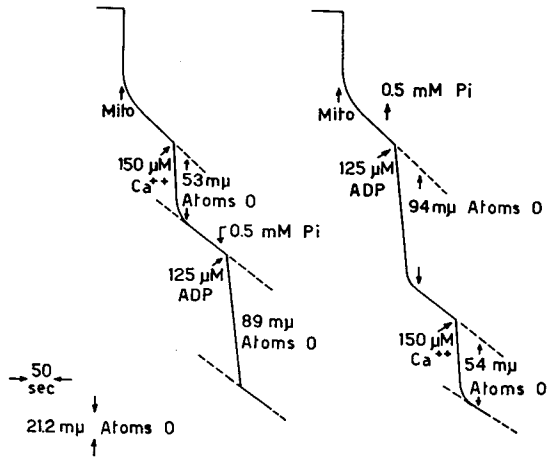

FIG. 8. The conditions were as in Fig. 2. Sodium DL-β-hydroxybutyrate was the substrate; 7.4 mg of mitochondrial protein added. Incubated at 21°.

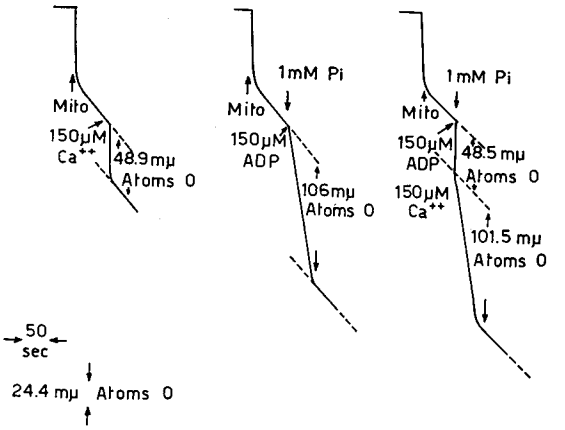

FIG. 9. Conditions as in Fig. 2; sodium DL-β-hydroxybutyrate was the substrate, and 6 mg of mitochondrial protein were added. Temperature, 21°.

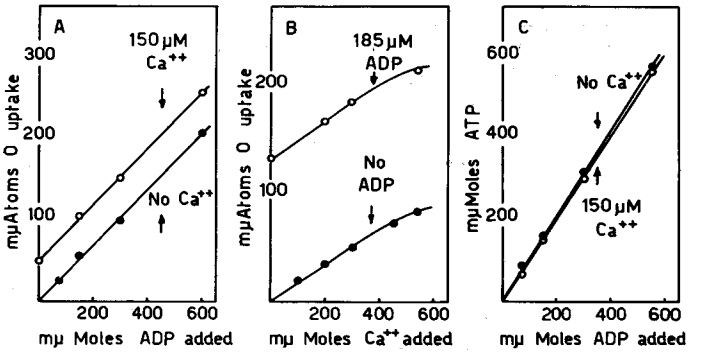

FIG. 10. *A*, conditions as in Fig. 2, with sodium DL-β-hydroxybutyrate as respiratory substrate. Mitochondrial protein, 6.5 mg; temperature, 21°. The reaction mixture was supplemented with 0.5 mM sodium potassium phosphate buffer, pH 7.2, in experiments carried out in the presence of ADP. The oxygen uptake is plotted as a function of ADP concentration. ●——●, without $CaCl_2$; ○——○, with 150 mM $CaCl_2$. *B*, the conditions were as in *A*, with varying concentrations of $CaCl_2$. The oxygen uptake is plotted as a function of $CaCl_2$ concentration. ●——●, without ADP; ○——○, with 185 mM ADP. *C*, the conditions were as in *A*. The ATP synthesis is plotted as a function of ADP concentration. ●——●, without $CaCl_2$; ○——○, with 150 mM $CaCl_2$.

Stoichiometric Accumulation of Ca^{++} and Oxidative Phosphorylation of ADP Following Simultaneous Addition of Ca^{++} and ADP —In Fig. 9 are shown the respiratory responses given in replicate experiments by Ca^{++} alone (in the absence of P_i), by the combination ADP + P_i, and, finally, by the simultaneous addition of *both* Ca^{++} and the combination ADP + P_i. The addition of 300 mµmoles of Ca^{++} alone gives an extra oxygen uptake of 48.9 mµatoms. The addition of 300 mµmoles of ADP (in the presence of P_i) produced an extra oxygen uptake of 106 mµatoms. The combined addition of 300 mµmoles of Ca^{++} and 300 mµmoles of ADP + P_i yielded an extra oxygen uptake of 150.0 mµatoms of oxygen uptake, which may be compared with the sum 48.9 + 106 = 154.9 mµatoms of oxygen uptake produced by separate additions of Ca^{++} and ADP. The respiratory stimulation produced by Ca^{++} and ADP + P_i added together is, therefore, completely additive with respect to total extra oxygen uptake. Furthermore, the respiration returned precisely to the original rate.

The concentration of P_i added in such experiments was kept relatively low in order to avoid any deleterious effect of P_i on the stoichiometry between stimulation of respiration and accumulation of Ca^{++}. Under these conditions, as is shown below, both oxidative phosphorylation and Ca^{++} uptake proceeded with nearly exact stoichiometry.

The data collected in Fig. 10*A* indicate that the respiratory stimulation of 50.0 mµatoms of oxygen given by 300 mµmoles of Ca^{++} remains the same within experimental error when ADP additions varying from 75 to 600 mµmoles are made simultaneously. A similar experiment carried out with ADP held constant at 370 mµmoles and the Ca^{++} addition varied from 75 to 600 mµmoles also showed (Fig. 10*B*) the completely additive stoichiometry of the respiratory responses given by Ca^{++} and ADP. Finally, in Fig. 10*C* is also shown an experiment illustrating the additional point that the presence of 150 µM Ca^{++} does not in-

hibit the measured accumulation of ATP from ADP added over the range 75 to 600 mμmoles.

In Table III is shown a complete tabulation of data on four separate experiments, in which the respiratory stimulation produced by Ca^{++}, by ADP, and by the two combined is given, as well as actual measurements of Ca^{++} uptake by the isotopic method and enzymatic measurements of the ATP accumulated, determined by the hexokinase-glucose 6-phosphate dehydrogenase assay. The substrate was β-hydroxybutyrate in each case. The data show not only that the respiratory stimulation given by Ca^{++} and by ADP is completely additive, but also that the amount of Ca^{++} taken up by the mitochondria agrees closely with the amount calculated from the respiratory stimulation, and that the amounts of ATP actually formed agree closely with the amounts calculated from the ADP:O ratios. When both Ca^{++} and ADP are added to mitochondria it is clear that both oxidative phosphorylation of ADP and Ca^{++} accumulation may take place additively, each with the exact stoichiometry shown when tested separately. The ATP:O and Ca:O ratios are nearly maximal and agree with those shown in Figs. 1 to 5 and Table I. Finally, it may be seen that the amount of Ca^{++} accumulated is an average of 1.85 molecules per site activated, with the range 1.77 to 1.94; the ATP formed per site is in the range of 0.90 to 0.94.

Other data already summarized in Table I indicate that during α-ketoglutarate and succinate oxidation similar relationships hold; thus, all three energy-conserving sites of the respiratory chain show identical properties. It is significant that the dinitrophenol-insensitive, substrate-linked phosphorylation step associated with oxidation of α-ketoglutarate to succinate also appears to be capable of supporting accumulation of Ca^{++} ions.

TABLE III

Stoichiometry between Ca^{++} accumulated, ATP formed, and extra oxygen uptake

The conditions were those of Table I. Sodium DL-β-hydroxybutyrate was the substrate; 1 mM sodium potassium phosphate buffer, pH 7.2, was added only when ADP was added. Protein, 6 mg; temperature, 21°.

Ca^{++} added	Ca^{++} accumulated	Oxidative phosphorylation: ADP added	Oxidative phosphorylation: ATP formed	Extra oxygen uptake	ATP formed per mμatom of extra oxygen uptake[a]	Ca^{++} accumulated per mμatom of extra oxygen uptake[b]	Ca^{++} accumulated per site activated[b]
mμmoles		*mμmoles*		*mμatoms*	*mμmoles*		*molecules*
		75	73	26	2.75		
300	280			52		5.34	1.78
300	283	75	74	71	2.80	5.40	1.77
		150	152	54	2.81		
300	290			50		5.80	1.93
300	288	150	149	106	2.80	5.76	1.91
		300	291	106	2.75		
300	273			49		5.69	1.89
300	280	300	295	149	2.78	5.84	1.94
		600	581	213	2.71		
300	291			51		5.68	1.89
300	289	600	587	260	2.76	5.76	1.92

[a] Calculated by subtracting the oxygen uptake due to the Ca^{++}-activated respiration.

[b] Calculated by subtracting the oxygen uptake due to the oxidative phosphorylation of ADP.

TABLE IV

Stoichiometry of phosphate and Ca^{++} accumulation

The experimental conditions were arranged as in Table I. Sodium DL-β-hydroxybutyrate was the substrate. The additions were 2 mM sodium potassium phosphate buffer, pH 7.2, 175 μM $CaCl_2$, 400 μM ADP, and 1 μg of oligomycin per mg of mitochondrial protein. Mitochondrial protein, 6 mg; temperature, 22°.

System	Ca^{++} added	Oxygen uptake	P_i accumulated	P_i accumulated per mμatom of extra oxygen uptake[a]	P_i accumulated per site activated[a]
	mμmoles	*mμatoms*	*mμmoles*		*molecules*
Complete	350	60			
$+P_i$	350	[b]			
$+P_i$ + ADP	350	68	215	3.16	1.05
$+P_i$ + ADP + oligomycin	350	65	205	3.15	1.05

[a] Corrected for zero time.

[b] Not measurable because the respiratory response induced by Ca^{++} continues indefinitely.

More detailed experiments on this specific point will be presented for publication elsewhere.

Accumulation of P_i with Ca^{++} and Its Dependence on ATP—Accumulation of P_i accompanies accumulation of Ca^{++} in experiments in which both Ca^{++} and ADP + P_i are added simultaneously, as is seen in Table IV. In the absence of added ADP, Ca^{++} stimulates respiration indefinitely and only small amounts of P_i are accumulated. When ADP is phosphorylated to ATP, the latter blocks the usual action of phosphate and supports uptake of both Ca^{++} and P_i (Table IV). Oligomycin does not interfere with either Ca^{++} (2) or P_i (11) accumulation during "massive" accumulation of Ca^{++}.

It may be concluded that Ca^{++} accumulation is not accompanied by accumulation of P_i in the absence of ATP; in the presence of ATP, phosphate is accumulated in an amount stoichiometric with both Ca^{++} and the number of energy-conserving sites in the respiratory chain. These findings thus reconcile the properties of the Ca^{++} accumulation process as studied in systems containing low as opposed to high Ca^{++} concentrations.

Sequence of Respiratory Activation and Affinity of Chain for Ca^{++} and ADP—That Ca^{++} has a higher affinity for the respiratory chain than ADP (*cf.* References 7, 24) is evident in experiments of Fig. 9, in which simultaneous additions of Ca^{++} and ADP + P_i were made to activate respiration. The shape of the oxygen uptake curve supports the conclusion that when a mixture of Ca^{++} and ADP is added, respiration is stimulated first and exclusively by the Ca^{++} until essentially all the Ca^{++} is accumulated; then the respiration is stimulated by ADP with formation of ATP. The oxygen uptake curve after simultaneous addition of both Ca^{++} and ADP usually shows a point of inflection after the uptake of an amount of oxygen which is stoichiometric with the Ca^{++} added, but not stoichiometric with the ADP added. After the inflection, an additional amount of oxygen is taken up, at a slower rate, which is stoichiometrically related with the ADP added. This effect has been seen repeatedly under the conditions described.

DISCUSSION

The observations presented in this paper permit some clarification and reconciliation of a number of observations made in the

different approaches to the study of Ca^{++} accumulation by mitochondria which have been employed in different laboratories. Some of these points are crucial to an understanding of the mechanism and stoichiometry of the process.

The type of experiments described here, in which rather low concentrations (*i.e.* 100 to 500 μM) of Ca^{++} were added to the suspending medium, permits observation of a greater number of important relationships between ion transport, electron transport, and oxidative phosphorylation than is possible in the so-called "massive" accumulation experiments (*cf.* References 1, 2, 5, 10), in which mitochondria were suspended in media containing rather high concentrations of Ca^{++} (*i.e.* 2 to 4 mM). Thus, rather precise stoichiometric relationships between the stimulation of oxygen uptake by Ca^{++} and by ADP, the accumulation of Ca^{++}, the accumulation of P_i, and the formation of ATP have been observed under conditions of low Ca^{++} concentration. Furthermore, the mitochondria are evidently undamaged by low concentrations of Ca^{++}, retain normal respiratory control, and exhibit maximum P:O ratios of oxidative phosphorylation even after two or more respiratory stimulations by Ca^{++} or by ADP.

There are a number of points of similarity in experiments carried out with high as opposed to low concentrations of Ca^{++}. Most important is the fact that the stoichiometry between Ca^{++} and phosphate accumulation and electron transport is nearly exactly the same in both types of system (*i.e.* about 1.67 to 1.90 moles of Ca^{++} accumulated, and about 1.0 mole of P_i accumulated as each pair of electron equivalents traverses each of the energy-conserving sites). Thus, the experiments reported here completely confirm our early studies (10, 11). In addition, Ca^{++} accumulation supported by electron transport in the presence of either high or low concentrations of Ca^{++} is insensitive to oligomycin. Finally, in both systems, Ca^{++} fails to be accumulated stoichiometrically in the presence of phosphate unless ATP is present.

On the other hand, experiments with high Ca^{++} concentrations in the medium do not permit evaluation of the stoichiometry of respiratory stimulation and control, nor do they allow observation of simultaneous or sequential oxidative phosphorylation of ADP. High concentrations of Ca^{++} completely uncouple formation of ATP, even in the presence of an extremely active hexokinase-glucose "trap" (13); in addition, high Ca^{++} concentrations cause mitochondrial swelling (21) and the stimulation of the formation of free fatty acids (22), which are themselves potent uncoupling agents. These damaging effects of high levels of Ca^{++} thus obscure some of the significant relationships between ion transport and electron transport.

The experiments with low concentrations of Ca^{++} reported here reveal two other important points. The first is the fact that Ca^{++} may be accumulated stoichiometrically with electron transport without the simultaneous accumulation of phosphate. In contrast, phosphate accumulation always occurs with Ca^{++} accumulation when the Ca^{++} concentration is high (10, 11). Usually in media containing high concentrations of Ca^{++}, the reactions are allowed to go so far that the capacity of the normal binding sites is greatly exceeded and the bound Ca^{++} is evidently "trapped" as insoluble, amorphous deposits of calcium phosphate, in the presence of ATP and Mg^{++} in the medium. Because of the large extent and rapidity of massive Ca^{++} accumulation, the fact that Ca^{++} may be actively accumulated without phosphate has been missed in the experiments with high Ca^{++} in the medium.

Our findings, therefore, fully confirm the general conclusions of Chappell *et al.* (7) that the divalent cations may be accumulated in an active process prior to entry of phosphate. They make untenable the hypothesis that phosphate accumulation is necessary for accumulation of Ca^{++} and that the active process may actually be phosphate translocation rather than cation translocation, which was a permissible interpretation of the experiments carried out with high concentrations of Ca^{++} (*cf.* Reference 11).

The second important point established by the experiments with low concentrations of Ca^{++} is the fact that phosphate accumulation occurs with Ca^{++} accumulation only when the medium also contains ATP. The experiments also suggest that Mg^{++} is required primarily not for Ca^{++} uptake and release of respiration, but rather for the accumulation of P_i and support of this process by ATP. If ATP is not present in the medium, the presence of phosphate disturbs the Ca^{++} uptake or retention mechanism, so that respiration is stimulated indefinitely and Ca^{++} is no longer accumulated quantitatively. The accumulation of Ca^{++} thus occurs by some interaction of Ca^{++} with a high energy intermediate generated by electron transport at some stage before phosphate enters into covalent linkage, since oligomycin prevents the discharging action of phosphate on Ca^{++} uptake.

When phosphate is present in the medium (in the absence of ATP or oligomycin) it either prevents the binding of Ca^{++} or causes the discharge of bound Ca^{++}, so that a large part of the added Ca^{++} remains free in the medium, and thus continues to activate the respiratory chain. It is the continuous escape or discharge of free Ca^{++} caused by the presence of phosphate that causes the indefinite, noncyclic stimulation of respiration by Ca^{++}, without return to the resting rate. This effect of P_i in the absence of ATP is not evident in the experiments reported by Chance (24), for example, because of the fact that his system usually contained oligomycin or ATP, which prevent this action of phosphate.

It is now possible to specify more closely the basis of the curious requirement of ATP noted in the earlier experiments on massive accumulation of Ca^{++} (2, 5, 9, 11). It is clear that ATP is not required for Ca^{++} uptake *per se*, but rather for the prevention of the opposing or deleterious action of inorganic phosphate. The prevention of the phosphate effect by ATP also results in stoichiometric accumulation of the phosphate together with Ca^{++}. The requirement of ATP has been suggested earlier to be on the structure of the mitochondria (5, 8); however, oligomycin inhibits mitochondrial contraction by ATP (35) whereas the specific effect of ATP in supporting phosphate uptake, and with it, Ca^{++} uptake, is not affected by oligomycin. In fact, oligomycin can *replace* ATP. Recently it has been found that, during Ca^{++} accumulation by mitochondria, ATP also is bound, in the ratio of about 1 mole per 6 to 10 moles of Ca^{++} (36); the uptake of adenine nucleotides has cofactor requirements similar to those of Ca^{++} uptake. This finding, as well as that of Bruni *et al.* (34), namely, that binding of ATP to mitochondria may be inhibited by atractyloside, may be related to the specific role of ATP in supporting Ca^{++} uptake dependent on electron transport.

Finally, the data reported here indicate that the sequence of steps in active Ca^{++} uptake postulated by Chance (24) and also by Chappell (7) may have to be modified. They have suggested that in the first step Ca^{++} enters mitochondria rapidly and "passively," a reasonable postulation on which our data shed no particular light. In the second phase, Chance postulated that Ca^{++}

activated respiration, without being accumulated, in a sustained manner by attacking a high energy intermediate; this stage is presumed to occur without interaction with phosphate. In the third stage, phosphate is believed to enter the scheme and it is viewed as combining with Ca^{++} to form insoluble deposits of calcium phosphate and thus removing Ca^{++} from further activation of respiration, causing a cessation of respiration.

Our findings are not compatible with this scheme, since they show that Ca^{++} alone in the absence of phosphate may activate respiration and be accumulated stoichiometrically; endogenous P_i was shown to play no detectable role in this stage of Ca^{++} uptake. We therefore propose that in the second stage Ca^{++} activates respiration and accumulates quantitatively, perhaps with discharge of H^+ ions, without intervention of phosphate. When phosphate is present it can be accumulated with the Ca^{++} only if ATP is present, but phosphate accumulation is not necessary for the Ca^{++} uptake; this is suggested to be a third step in the process. A fourth step can be recognized. Mitochondria have only limited capacity to bind Ca^{++} actively in the absence of phosphate (about 100 to 150 mμmoles per mg of protein), perhaps because of the limited number of specific, respiration-activated binding sites. Massive amounts of Ca^{++} can be accumulated far beyond the capacity of these binding sites, but only if phosphate is available and only if ATP is also present. The Ca^{++} is then sequestered as insoluble, amorphous, electron-dense deposits of calcium phosphate (12, 37, 38).

Isolated mitochondria can actively accumulate a number of divalent metal ions in addition to Ca^{++}, namely, Mg^{++} (39, 40), Mn^{++} (4, 6, 7), Sr^{++} (4, 6, 7, 41, 42), and Ba^{++} (4, 6, 7). Although there are a number of common properties and features in the uptake of these ions, there are also some differences which may prove to be highly significant in further analysis of the mechanism of mitochondrial ion transport. In particular, the effect of ATP in supporting accumulation of the metal ion and of phosphate appears to differ strikingly. ATP is not required for phosphate accumulation during massive accumulation of Mg^{++} by beef heart mitochondria (39, 40), or during the smaller scale accumulation of Mn^{++} and phosphate by rat liver mitochondria (4, 6, 7). In the case of Sr^{++} accumulation, phosphate and ATP have different effects in the early and later stages of this process; nevertheless, they are essentially similar to those involved in Ca^{++} accumulation (41, 42). The effects of ATP and phosphate described in this paper are probably not general features in the mechanism of uptake of all divalent ions. It is also relevant that these ions differ among themselves in another respect; for example, Mg^{++} and Mn^{++} have long been known to stabilize mitochondrial structure against swelling (*cf.* review (21)), an effect also given by Sr^{++} (41, 42). On the other hand, high concentrations of Ca^{++} can cause profound swelling and release of free acids (21, 22).

Further analysis of the mechanism of accumulation of divalent ions by mitochondria should include consideration of the results of recent investigations. Among these are the observation that Ca^{++} may be accumulated by mitochondrial "ghosts" incapable of oxidative phosphorylation of ADP (14), the fact that ATP, ADP, or both are also bound by mitochondria during Ca^{++} accumulation (36), and the finding that early stages in the uptake of Mn^{++} are accompanied by significant changes in the proton relaxation rate of the hydrated Mn^{++} ion (7).

SUMMARY

1. Addition of low concentrations of Ca^{++} (150 μM) to rat liver mitochondria supplemented with a respiratory substrate elicits an increase in the rate of oxygen uptake followed by a return to the resting rate; about 2.0 molecules of Ca^{++} give the same respiratory stimulation as 1.0 molecule of adenosine diphosphate. This cyclic stimulation by Ca^{++} does not require the presence of Mg^{++}, phosphate, or adenosine triphosphate; it is not affected by oligomycin.

2. Concurrently with stimulation of respiration, the Ca^{++} added is accumulated by the mitochondria; about 1.9 molecules of Ca^{++} are accumulated per energy-conserving site activated. No phosphate accumulation accompanies Ca^{++} accumulation under these circumstances.

3. The presence of inorganic phosphate permits respiratory stimulation by Ca^{++}, but the oxygen uptake then continues indefinitely at the stimulated rate, without return to the original rate, and very little Ca^{++} is accumulated. This action of inorganic phosphate is prevented by ATP or by oligomycin; in the presence of the latter agents, the usual stoichiometric relationship between oxygen uptake and Ca^{++} uptake occurs. Atractyloside does not reverse the effect of phosphate.

4. Oxidative phosphorylation of ADP and oxidative accumulation of Ca^{++}, each occurring with stoichiometric respiratory stimulation, can take place sequentially in a single aliquot of mitochondria in either sequence of addition of Ca^{++} and ADP + P_i, indicating that accumulation of Ca^{++} does not damage respiratory control or the phosphorylation mechanisms.

5. When Ca^{++} and ADP + P_i are added simultaneously to mitochondria in the presence of respiratory substrate and Mg^{++}, both accumulation of Ca^{++} and phosphate and phosphorylation of ADP take place stoichiometrically with the electron transport. The extra oxygen uptakes elicited by Ca^{++} and by ADP + P_i are completely additive. About 1.9 moles of Ca^{++} and about 1.0 mole of phosphate were accumulated per pair of electron equivalents activating each of the energy-conserving sites of the respiratory chain.

6. The affinity of Ca^{++} for the energy-conserving sites of the respiratory chain is higher than that of ADP. When both are added simultaneously, Ca^{++} stimulates respiration first and exclusively. After all the Ca^{++} added has been accumulated, ADP activates respiration and is phosphorylated.

7. From these observations it appears that activation of the energy-conserving site by Ca^{++}, accompanied by accumulation of Ca^{++}, is the first stage in ion accumulation. This does not require phosphate. When phosphate is present in the medium, it may also be accumulated with the Ca^{++}, but only if ATP + Mg^{++} are present in the medium. The findings thus reconcile observations made in systems having high and low concentrations of Ca^{++}. Ca^{++} in excess in the medium does not disturb the stoichiometric relationship between accumulation of Ca^{++} and phosphate with electron transport, but it does obscure the stoichiometry with respiratory control and with the sequence and relationship of the separate steps, and it uncouples phosphorylation.

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REFERENCES

1. Vasington, F. D., and Murphy, J. V., *Federation Proc.*, **20**, 146 (1961).

2. Vasington, F. D., and Murphy, J. V., *J. Biol. Chem.*, **237**, 2670 (1962).
3. DeLuca, H. F., and Engstrom, G. W., *Proc. Natl. Acad. Sci. U. S.*, **47**, 1744 (1961).
4. Chappell, J. B., Greville, G. D., and Bicknell, K. E., *Biochem. J.*, **84**, 61P (1962).
5. Brierley, G. P., Murer, E., and Green, D. E., *Science*, **140**, 60 (1963).
6. Chappell, J. B., and Greville, G. D., *Federation Proc.*, **22**, 526 (1963).
7. Chappell, J. B., Cohn, M., and Greville, G. D., in B. Chance (Editor), *Energy-linked functions of mitochondria*, Academic Press, Inc., New York, 1963, p. 219.
8. Vasington, F. D., *J. Biol. Chem.*, **238**, 1841 (1963).
9. Lehninger, A. L., Rossi, C. S., and Greenawalt, J. W., *Biochem. and Biophys. Research Communs.*, **10**, 444 (1963).
10. Rossi, C. S., and Lehninger, A. L., *Biochem. and Biophys. Research Communs.*, **11**, 441 (1963).
11. Rossi, C. S., and Lehninger, A. L., *Biochem. Z.*, **338**, 698 (1963).
12. Greenawalt, J. W., Rossi, C. S., and Lehninger, A. L., *J. Cell Biol.*, in press.
13. Carafoli, E., Rossi, C. S., and Lehninger, A. L., *J. Biol. Chem.*, **239**, 3055 (1964).
14. Vasington, F. D., and Greenawalt, J. W., *Biochem. and Biophys. Research Communs.*, **15**, 133 (1964).
15. Brierley, G. P., in B. Chance (Editor), *Energy-linked functions of mitochondria*, Academic Press, Inc., New York, 1963, p. 237.
16. Saris, N. E., *Acta Chem. Scand.*, **17**, 882 (1963).
17. Reynolds, E. S., Thiers, R. E., and Vallee, B. L., *J. Biol. Chem.*, **237**, 3546 (1962).
18. Sallis, J. D., DeLuca, H. F., and Rasmussen, H., *J. Biol. Chem.*, **238**, 4098 (1963).
19. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **201**, 1 (1953).
20. Potter, V. R., Siekevitz, P., and Simonson, H. C., *J. Biol. Chem.*, **205**, 893 (1953).
21. Lehninger, A. L., *Physiol. Revs.*, **42**, 467 (1962).
22. Wojtczak, L., and Lehninger, A. L., *Biochim. et Biophys. Acta*, **51**, 442 (1961).
23. Chance, B., in C. Liebecq (Editor), *Proceedings of the third International Congress of Biochemistry, Brussels, 1955*, Academic Press, Inc., New York, 1956, p. 300.
24. Chance, B., in B. Chance (Editor), *Energy-linked functions of mitochondria*, Academic Press, Inc., New York, 1963, p. 253.
25. Slater, E. C., and Cleland, K. W., *Biochem. J.*, **55**, 566 (1953).
26. Schneider, W. C., in W. W. Umbreit, R. Burris, and J. E. Stauffer (Editors), *Manometric techniques*, Burgess Publishing Company, Minneapolis, 1957, p. 188.
27. Kielley, W. W., and Bronk, J. R., *J. Biol. Chem.*, **230**, 521 (1958).
28. Steiner, D. F., and Williams, R. H., *J. Biol. Chem.*, **234**, 1342 (1959).
29. Layne, E., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. III*, Academic Press, Inc., New York, 1957, p. 450.
30. Lardy, H. A., Johnson, D., and McMurray, W. C., *Arch. Biochem. Biophys.*, **78**, 587 (1958).
31. Huijing, F., and Slater, E. C., *J. Biochem. (Tokyo)*, **49**, 493 (1961).
32. Bruni, A., Contessa, A. R., and Luciani, S., *Biochim. et Biophys. Acta*, **60**, 301 (1962).
33. Vignais, P. V., and Vignais, P. M., *Biochim. et Biophys. Acta*, **60**, 284 (1962).
34. Bruni, A., Luciani, S., and Contessa, A. R., *Nature*, **201**, 1219 (1964).
35. Neubert, D., and Lehninger, A. L., *Biochim. et Biophys. Acta*, **62**, 556 (1962).
36. Carafoli, E., and Lehninger, A. L., *Biochem. and Biophys. Research Communs.*, **16**, 66 (1964).
37. Brierley, G. P., and Slautterback, D. B., *Biochim. et Biophys. Acta*, **82**, 183 (1964).
38. Peachey, L. D., in S. S. Breese (Editor), *Fifth International Congress for Electron Microscopy*, Academic Press, Inc., New York, 1962, p. 00-3-.
39. Brierley, G. P., Bachmann, E., and Green, D. E., *Proc. Natl. Acad. Sci. U. S.*, **48**, 1928 (1962).
40. Brierley, G. P., Murer, E., Bachmann, E., and Green, D. E., *J. Biol. Chem.*, **238**, 3482 (1963).
41. Carafoli, E., Weiland, S., and Lehninger, A. L., *Biochim. et Biophys. Acta*, in press.
42. Carafoli, E., *Biochim. et Biophys. Acta*, in press.