Rebounds and Oscillations in Respiration-linked Movements of Ca\(^{++}\) and H\(^{+}\) in Rat Liver Mitochondria

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SUMMARY

Addition of Ca\(^{++}\) to rat liver mitochondria suspended in buffered 80 mM KCl or NaCl causes activation of respiration, uptake of Ca\(^{++}\), and ejection of H\(^{+}\), followed by return of the respiratory rate to a resting steady state in which there are no further net movements of Ca\(^{++}\) and H\(^{+}\). However, when the KCl concentration is only 20 mM, the Ca\(^{++}\)-induced respiratory jump is followed by a period in which the resting respiration remains constant at a low resting rate, but in which the inverse Ca\(^{++}\)-H\(^{+}\) exchange between mitochondria and medium undergoes oscillations. The oscillations are observed also after the accumulation of Sr\(^{++}\), but not after Mn\(^{++}\). They are maximal in media containing impermeant anions such as chloride, bromide, iodide, and thiocyanate. The oscillations are modified by buffer species and pH.

Oscillations in the Ca\(^{++}\)-H\(^{+}\) exchange do not occur in the presence of ATP, Mg\(^{++}\), succrose, or permeant anions such as phosphate or acetate. The oscillations appear to depend in part on reverse electron flow from succinate to nicotinamide adenine dinucleotide. Since the mitochondria appear to behave in a self-correcting manner, the oscillations in the inverse Ca\(^{++}\) and H\(^{+}\) movements are rationalized in terms of a feedback relationship between Ca\(^{++}\) influx and efflux.

When Ca\(^{++}\) (or Mn\(^{++}\) or Sr\(^{++}\)) is added to mitochondria respiring in State 4, particularly in the presence of phosphate, there is a large increase in respiratory rate, accumulation of Ca\(^{++}\), and ejection of H\(^{+}\) ions; these events are stoichiometrically related (cf. References 1 to 7). After such a respiratory "jump," the rate of oxygen uptake normally returns to the original resting level and remains constant in the ensuing steady state, in which there is no further net movement of the accumulated Ca\(^{++}\) or change of pH (8).

This paper describes a set of conditions in which the respiration-linked uptake of Ca\(^{++}\) and the reciprocal ejection of H\(^{+}\) exhibit very striking overshoot and rebound phenomena, often having the character of incipient, heavily damped oscillations. The rebound and return of the Ca\(^{++}\)-H\(^{+}\) exchange to a steady state were found to occur during the "resting" respiration following the Ca\(^{++}\)-induced respiratory jump. Such striking

 reversals of the energy-dependent Ca\(^{++}\)-H\(^{+}\) exchange occurring in the resting state, without change in the rate of respiration, appear to be caused by large fluctuations with time in the rate of Ca\(^{++}\) uptake, in the rate of Ca\(^{++}\) efflux, or in both (cf. References 9 and 10). Because these rebounds of the reciprocal Ca\(^{++}\)-H\(^{+}\) movements are apparently self-correcting, it is probable that Ca\(^{++}\) uptake and Ca\(^{++}\) efflux are linked in a feedback relationship.

Some of the findings described here have been presented in a preliminary communication (11).

EXPERIMENTAL PROCEDURE

Mitochondria were isolated from the livers of albino rats (Carworth Farms, Sprague-Dawley strain) by the 0.25 M sucrose procedure of Schneider (12); they were washed three times with cold sucrose. Oxygen uptake was measured polarographically with a Clark electrode (13) connected to a 10-mv recorder. The H\(^{+}\) movements were followed with a glass electrode linked to a Leeds and Northrop pH meter capable of scale expansion and a 5- to 12.5-mv Sargent model SR strip chart recorder. The scale was expanded so that a pH change of 0.1 yielded full scale deflection; the response of the electrode in each test medium was calibrated by additions of known amounts of HCl or NaOH. The rate of escape of KCl from the fiber junction of the salt bridge into the cuvette was determined by means of flame photometry. The maximum concentration of KCl attained in the medium by such leakage was 0.2 mM over the usual 3-min reaction period at 25°.

The uptake of \(^{44}\)Ca was measured after rapid filtration of aliquots of the mitochondrial suspension through Millipore filters (AAWP, pore size 0.8 μ), essentially as described by Rasmussen et al. (14). Aliquots (0.4 to 0.5 ml) of the suspension were withdrawn with a 5-ml Luer-Lok syringe, the needle was replaced with a Swinny adapter holding the Millipore filter, and the suspension was filtered by applying moderate pressure to the plunger of the syringe. The entire sampling operation was complete within 8 sec. Aliquots of the filtrates were plated and counted for \(^{44}\)Ca.

Protein concentration was measured by a biuret procedure. All the reagents used were analytical grade. Antimycin A and oligomycin were obtained from the Wisconsin Alumni Research Foundation. \(^{44}\)Ca (specific activity, 12.8 mC per mg) was purchased from the Nuclear Science and Engineering Company.
RESULTS

Rebound of Ca++-dependent H+ Ejection as Function of KCl Concentration—In the following description of the experimental findings, decreases in pH of the suspending medium following addition of divalent metal ions will be arbitrarily designated, for the sake of convenience and brevity, as indicating ejection of H+ from the mitochondria, and increases in pH as absorption of H+ from the medium by the mitochondria. A decrease in pH could equally well be due to absorption of OH− by the mitochondria and an increase in pH as ejection of OH−; in fact, movements of both H+ and OH− could be occurring simultaneously.

In Fig. 1 are shown two series of glass electrode traces of H+ movements after addition of Ca++ to rat liver mitochondria suspended in a simple medium of Tris-chloride, pH 7.65, 10 mM potassium or sodium succinate, and either KCl or NaCl; neither Mg++ nor inorganic phosphate was added to the system. The concentrations of KCl and NaCl were varied as shown. In the series of experiments in KCl media, it is seen that at the lowest KCl concentration (10 mM) addition of Ca++ to the suspension produced a very rapid initial ejection of H+, followed by a fast rebound corresponding to about 50% reabsorption of the ejected H+. The rebound was followed by a slower re-ejection of H+ which proceeded to a steady state in which the final pH was about equal to the pH attained in the first rapid ejection of H+. As KCl concentration was increased, the rebound decreased somewhat in magnitude. Above 80 mM KCl (not shown), there was essentially no rebound at all; after the first rapid phase of H+ ejection in such systems, the pH approached asymptotically a steady state in an approximately hyperbolic manner. At 10, 20, and 40 mM KCl, the molar ratio of H+ ejected (at the first peak) to Ca++ added (ΔH+/ΔCa++) was essentially constant at about 0.8. The rebound effect in such experiments is not caused by exhaustion of oxygen; the experiments were arranged so that less than 40% of the dissolved oxygen had been absorbed at the completion of a rebound cycle.

When KCl was replaced by equal concentrations of NaCl in the medium, the rebound effect was much smaller at 10 and 20 mM; at 40 mM NaCl no rebound was observed. This difference in response to KCl with respect to NaCl was consistently observed. Since addition of even small amounts of KCl to a system containing 10 mM NaCl was found to increase the magnitude of the rebound, it appears likely that the small rebounds seen in media containing 10 to 20 mM NaCl are due to the presence of some KCl in the medium, contributed in part by the efflux of intramitochondrial K+ that accompanies Ca++ accumulation (15) and in part to leakage of KCl from the salt bridge of the glass electrode system. The apparent dependence of the bounce effect on K+ of the medium is presumably related to the observation that the rate of efflux of accumulated Ca++ from mitochondria is much higher in a medium containing only K+ than in one containing only Na+ (16).

At NaCl or KCl concentrations above 80 mM, no trace of the bounce effect was observed under the conditions described.

Kinetic Relationships among Ca++ Uptake, H+ Ejection, and Respiratory Rate—In Fig. 2 is shown a set of curves comparing the time course of oxygen uptake, H+ ejection, and Ca++ uptake after the addition of Ca++ to mitochondria respiring in State 4 under conditions optimal for the bounce effect, i.e. 10 mM KCl with potassium succinate as substrate. After addition of Ca++ there was activation of respiration, ejection of H+, and uptake of Ca++.
Ca++; these were approximately synchronous, exactly as seen in normal, "nonbouncing" systems. As the respiration returned to the controlled or resting rate, ejection of H+ was essentially complete. Although the uptake of Ca++ appeared to be only about 75% complete at the point where respiration returned to the resting rate, the large error (~8 sec) in the sampling time may have caused failure to observe the point of maximum Ca++ uptake. After the respiratory jump was complete, the rate of respiration remained low and essentially constant. In this period both the rebound and the subsequent re-ejection of H+ took place, without detectable inflections in the resting rate of respiration. During the rebound of H+ ejection, there was loss of much of the newly accumulated Ca++ back to the medium. As re-ejection of H+ into the medium occurred, there was a second phase of Ca++ accumulation, both curves then proceeded toward a steady state. Thus the movements of H+ and Ca++ were reciprocal throughout all portions of the bounce phenomenon.

The large timing and sampling error involved in the Ca++ measurements, as well as the impossibility of obtaining more frequent samples, precludes more exact conclusions on the synchrony of the reciprocal H+ Ca++ movements. Although the peak in the Ca++ curve corresponding to release of accumulated Ca++ from the mitochondria appeared to precede the point of maximum H+ rebound, no conclusion can be drawn as to whether the depression in the Ca++ curve actually preceded, followed, or coincided with the H+ rebound, because of the sampling error. For the same reason the peak in the Ca++ curve corresponding to Ca++ ejection may not represent the point of maximum release of accumulated Ca++ back into the medium; the maximum Ca++ ejection may actually have been considerably larger. The measurements do suffice to demonstrate two important findings: (a) that the movements of H+ and Ca++ are reciprocal in direction during the rebound and re-ejection of H+ and (b) that the rebound and re-ejection of H+ occur during the resting state of respiration after the respiratory jump, without a significant change in the rate of the controlled respiration.

Finally, it may be mentioned that after such a bounce the mitochondria are still capable of responding to a second addition of Ca++ with an increase of respiratory rate, ejection of H+, and return to the resting rate of respiration. It is therefore clear that the bounce effect is not the result of some irreversible change but probably reflects a time lag in the normal chain of events in the coupling between electron transport, Ca++ uptake, and Ca++ efflux. Further evidence on this point is developed below.

Effect of Divalent Cations—On varying the amount of CaCl₂ added to a fixed concentration of mitochondria in a medium containing 10 mm KCl, it was found that fast H+ ejection, followed by a fast rebound, occurred at all concentrations of Ca++ added in the range from 12.5 to 150 mmoles of Ca++ per mg of protein; however, the character of the response varied with the amount of Ca++ added. Below 60 mmoles of Ca++ per mg of protein, there was rapid ejection of H+ followed by a fast rebound; the pH then remained at the level attained in the rebound. When the concentration of Ca++ was increased above 60 mmoles per mg of protein, there was an increasing tendency for re-ejection of H+ to occur after the rebound. At levels of 80 to 100 mmoles of Ca++ per mg of protein, after the rebound had taken place, the pH was completely re-ejected to the level of the original peak of H+ ejection, approximately as shown in Fig. 1. This amount of Ca++ corresponds approximately to the maximum capacity of rat liver mitochondria to take up Ca++ stoichiometrically with oxygen uptake in the absence of added phosphate (2, 4, 8).

When Sr++ (cf. References 6 and 17) and Mn++ were compared with Ca++, it was found that Sr++ gave more dramatic rebounds than Ca++ (Fig. 3). Addition of 80 mmoles of Sr++ per mg of mitochondrial protein produced an extremely rapid ejection of H+, yielding at the peak an H+:Sr++ ratio of about 0.80. The H+ ejection was followed by an almost equally rapid reabsorption of H+ by the mitochondria; at the end of this rebound, essentially all of the ejected H+ had been reabsorbed, the entire process of ejection and reabsorption being complete within less than 60 sec. Another cycle of H+ ejection and rebound often ensued in such experiments, but the second oscillations were heavily damped. On the other hand, it is highly significant that addition of Mn++ yielded no bounce effect (Fig. 3). Mn++ gives only a relatively low rate of activated respiration (6), and the curve in Fig. 3 shows only a low rate of H+ ejection, without a bounce effect. This finding strongly supports the view that the bounce effects seen with Ca++ or Sr++, which are very rapidly accumulated, are due to a time lag in the mechanisms by which ion uptake and efflux are held in adjustment. When the rate of divalent ion uptake is very slow, as in the case of Mn++ uptake, presumably influx and efflux mechanisms adjust themselves rapidly enough so that no bounce effect can be observed.

Addition of 5.0 mm MgCl₂ damped or completely prevented

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**Fig. 3. Effect of Sr++, Ca++, Mn++.** The test system contained 10 mm potassium succinate; 10 mm KCl; 10 mm Tris-chloride, pH 7.63; and rat liver mitochondria (5.0 mg of protein) in a volume of 1.9 ml. Divalent ions were added as the chlorides at 80 mmoles per mg of protein. The temperature was 25°C.
the bounce normally given by Ca++ in experiments such as those described in Fig. 3.

*Effect of Inorganic Phosphate and Other Anions*—Fig. 4 shows the effect of the addition of inorganic phosphate on the H+ bounce effect in a medium containing 10 mM KCl, after addition of Ca++. The medium also contained oligomycin to eliminate swelling of mitochondria and discharge of Ca++ caused by phosphate; under these conditions phosphate is accumulated with Ca++ (2). It is seen that the presence of inorganic phosphate abolished the rebound of H+ ejection. Control experiments with oligomycin in the absence of phosphate showed that oligomycin alone does not modify the H+ rebound (see below). Similar inhibition of the bounce was given by arsenate. The prevention of the rebound by phosphate may be related to the fact that phosphate is accumulated with Ca++, a process which ultimately leads to deposition of an amorphous form of calcium phosphate having the composition of hydroxyapatite (cf. Greenawalt, Rossi, and Lehninger (18)). Since Ca++ is more stably retained in mitochondria in the presence of phosphate than in its absence (2, 8), the inhibitory effect of phosphate on the rebound in H+ ejection may be caused by the tendency of the deposited calcium phosphate to remain in the mitochondria in an insoluble form. From this consideration it appeared possible that the rebound phenomenon is related, in part at least, to the nature of the anions that are present in the medium and that may be accumulated with Ca++.

In Fig. 5 are shown experiments with some other anions, all tested in the presence of oligomycin. The addition of iodide, bromide, formate, and thiocyanate as potassium salts to the 10 mM KCl medium produced no significant change in the bounce of H+ ejection following addition of Ca++; these anions, like chloride, are presumably impermeable (cf. 9, 19, 20). Addition of acetate, however, completely abolished the rebound and thus acted like phosphate. Acetate has been reported to be accumulated with Ca++, and its accumulation leads to mitochondrial swelling (20). These experiments thus support the view that the bounce effect depends in part on the nature of the anions in the medium and their movements between the mitochondria and the medium.

ATP at 1 to 5 mM damped the bounce, with or without added Mg++. The addition of ATP and Mg++ to mitochondria accumulating Ca++, particularly when phosphate is present, is known to cause retention of Ca++ by the mitochondria in a particularly stable form having a low efflux rate (2, 8, 10).

*Effect of Inhibitors of Respiration and Phosphorylation*—The effects of cyanide, antimycin A, rotenone, and Amytal on H+ movements after Ca++ addition were examined in a system containing 10 mM KCl, with succinate as substrate (Fig. 6). The top tracing shows the typical bounce in H+ ejection after addition of Ca++. The second and third tracings show that cyanide and antimycin A abolished completely the ejection of H+, demonstrating that at least the first phase of H+ ejection is completely dependent on electron transport from succinate to oxygen. Such experiments, however, do not prove that the second H+ ejection occurring after the H+ rebound is also respiration-linked. It is possible that only the first ejection is respiration-linked; the second phase of H+ ejection could be essentially a passive movement of H+ consequent on some other process. This point was examined by adding antimycin A, not at the beginning of the experiment, but at the peak of the rebound of H+ ejection (Fig. 6). The tracing shows that the second ejection of H+ was also blocked by antimycin A and is therefore respiration-linked.

The effects of rotenone and Amytal, which block electron transport between NAD and flavoprotein, were also tested with succinate as substrate. It was found that these inhibitors did not block respiration-dependent H+ ejection on addition of Ca++, as was expected, since they do not block oxidation of succinate. However, both inhibitors had the effect of damping the bounce, often almost completely, in concentrations that are known to inhibit electron transport. These findings suggest that the bounce effect in H+ ejection is in part due to some reversal of electron transport from succinate to NAD after the initial ejection of H+; such reverse electron flow is known to be blocked by rotenone and Amytal (21, 22).

Oligomycin did not influence the bounce effect at all (Fig. 6). This finding is in agreement with earlier observations that oligomycin does not interfere with the energy-linked uptake of Ca++ (2, 23, 24).

*Effect of Respiratory Substrate*—In Fig. 7 is shown a comparison of β-hydroxybutyrate and succinate as respiratory substrates in supporting H+ ejection on addition of Ca++. The experiment was carried out in a medium of 10 mM KCl and 10 mM Tris chloride, pH 7.6; 78 mMoles of CaCl2 were added per mg of mitochondrial protein. It is seen that succinate supported the usual

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**Fig. 4.** Effect of inorganic phosphate. The basic system was that described in Fig. 3. Inorganic phosphate was added at 0.6 mM and oligomycin at 0.2 μg per mg of protein. The divalent cation was Ca++. The temperature was 25°.

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bounce in H⁺ ejection, followed by re-ejection of H⁺. On the other hand, when β-hydroxybutyrate was the substrate, only the rapid H⁺ ejection and H⁺ re-absorption phases took place; re-ejection of H⁺ was never found to occur with this substrate. This finding confirms the suggestion made above that the rebound and re-ejection of H⁺ may be associated in some manner with the known capacity of succinate to bring about energy-linked reduction of mitochondrial NAD. Variation in the concentration of potassium succinate from 0.5 to 10 mM produced no change in the kinetics or amplitude of the Ca²⁺-H⁺ exchange.

Effect of Sucrose—The addition of sucrose to the standard medium of 10 mM KCl, 10 mM potassium succinate, and 10 mM Tris-chloride, pH 7.63, had the effect of damping and preventing the H⁺ rebound. The damping tendency of sucrose was evident at 25 mM; at sucrose concentrations of 100 mM and above, no rebound behavior was ever observed. It is clear from this finding why the rebound and re-ejection of H⁺ may not have been observed in similar experiments reported from other laboratories, since other investigators have reported using reaction systems containing from 100 to 250 mM sucrose or mannitol (cf. References 4, 20, 25, and 26). Striking inhibitory effects of sucrose on respiration have been reported by Johnson and Lardy (27) and on partial reactions of oxidative phosphorylation and mitochondrial contraction by Lehninger (28).

Effect of Temperature—The bounce effect in H⁺ ejection is very sensitive to temperature. Most of the observations reported above were made at 25°. However, the bounce was highly damped or did not occur at all at temperatures in the range between 4° and 15°. At these temperatures the usual ejection of H⁺ after the addition of Ca²⁺ was observed. However, there was little or no tendency for the H⁺ to be reabsorbed at these lower temperatures. This observation also is consistent with the view that the bounce effect is due to a time lag in the feedback adjustment of Ca²⁺ influx and efflux rates. It is also consistent with our earlier finding that Ca²⁺ efflux has a relatively high temperature coefficient (8) and is thus not a purely passive diffusion-controlled process.

Effect of pH—The bounce effect is highly dependent on the pH of the medium (Fig. 8). The bounce (i.e. rebound followed by re-ejection of H⁺) was most striking and complete at pH 7.4. At pH 7.0 very little rebound occurred. When the pH was raised to 8.0, there was an extremely rapid rebound of H⁺, with reabsorption of at least 90% of the ejected H⁺; however, only a very slow re-ejection of H⁺ took place. At pH 8.5 there was a
sharp spike of $H^+$ ejection and reabsorption; at the end of the rebound significantly more $H^+$ had been absorbed than was originally ejected. However, the pH remained at this level in a steady state with no tendency to re-ejection of $H^+$. These striking effects of pH on the bounce effect are probably related to the characteristic effects of pH on the accumulation (9) and efflux (10) of $Ca^{++}$. It may also be noted that the ratio $\Delta H^+: \Delta Ca^{++}$ at the peak decreased with increasing pH.

Effect of Buffers—Since experiments above show that the bounce effect is influenced by both cations (Na$^+$ and K$^+$) and anions (phosphate and acetate) in the medium, it appeared likely that it would also be influenced by specific buffer cations or anions, or possibly the corresponding free acid or base species. The results of a series of experiments comparing the bounce effect at pH 7.5 in media buffered with different conjugate acid-base pairs at 10 mM are shown in Fig. 9. It is seen that Tris ($pK' = 8.3$ at 20°C) and glycylglycine ($pK' = 8.4$) buffers yielded bounces that were indistinguishable kinetically; in both cases the bounce was followed by re-ejection of $H^+$ to the original level attained in the first ejection. However, the kinetic data of the bounces observed in media containing two new buffers described by Good et al. (29), namely N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (Buffer H) and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Buffer T) are quite different.

The curves in Fig. 9 show that Buffer II ($pK' - 7.55$) also supported a bounce effect, but it was a slower and more striking effect. Reabsorption of newly ejected $H^+$ was slower but much more complete than in the case of the Tris buffer; re-ejection of $H^+$ also proceeded more slowly, but it ultimately reached a level equal to that attained at the first ejection of $H^+$. Buffer T ($pK' = 7.50$) also yielded a rebound, somewhat slower, but more complete than that supported by the Tris buffer; however, after the rebound essentially no re-ejection of $H^+$ took place.

These results indicate that different buffer cations and anions (or the free base or acid species) may greatly modify the ion movements between mitochondria and medium during or after respiratory jumps induced by addition of $Ca^{++}$. At least two mechanisms appear open for such effects: either the buffer cation or anion may influence the efflux rate of $Ca^{++}$, as appears to be the case for K$^+$, or these ions (or their free acid or base species) may also move across the mitochondrial membrane, actively or passively, in synchrony with or after the movement of $Ca^{++}$.

It has recently been found (29) that phosphorylating oxidation of succinate by bean mitochondria is greatly influenced by the buffer system employed; the universally used Tris buffer system was found to be quite inhibitory compared to Buffers H and T, which yielded maximum rates of oxidation.

**Fig. 6.** Effect of respiratory inhibitors. The medium contained 10 mM potassium succinate; 10 mM Tris chloride, pH 7.4; 10 mM KCl; and rat liver mitochondria (5.0 mg) in 1.9 ml. The inhibitors added were 1.0 mM KCN, 1 mM rotenone, 1.0 mM Amytal, 2 $\mu$g of antimycin A per mg of protein, and 0.2 $\mu$g of oligomycin per mg of protein. CaCl$_2$ (390 mmoles) was added at points indicated by arrows. The temperature was 25°C.

**Fig. 7.** Effect of respiratory substrate. The reaction vessel contained, in a final volume of 1.9 ml, 10 mM Tris-Cl, pH 7.4; 20 mM KCl; 10 mM potassium succinate or pL-3-hydroxybutyrate ($\beta$-OH); and 5.0 mg of mitochondrial protein. At points indicated by arrows, 390 mmoles of CaCl$_2$ were added. The temperature was 25°C.
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FIG. 8. Effect of pH. The reaction medium contained 10 mM Tris-chloride at pH values shown, 10 mM KCl, 10 mM potassium succinate, and 5.0 mg of mitochondrial protein in a final volume of 1.9 ml. At the points indicated by the arrows, 450 mmoles of CaCl₂ were added. The temperature was 25°.

FIG. 9. Effect of different buffers. At points shown by the arrows, 400 mmoles of CaCl₂ were added to the system containing 10 mM KCl; 5.0 mM potassium succinate; the buffers shown (see the text), at a concentration of 10 mM, all adjusted to pH 7.5; and 5.0 mg of mitochondrial protein in a total volume of 2.0 ml. The temperature was 25°. HEPES, Buffer H; TES, Buffer T.

DISCUSSION

The findings reported in this paper are best rationalized and discussed in terms of recent observations on the dynamics of Ca++ accumulation and retention during and after Ca++-induced respiratory jumps in the absence of phosphate (8, 11, 30). In such experiments, nearly all the added Ca++ is rapidly accumulated during the period of stimulated respiration; the respiratory jump is then followed by a resting phase in which the rate of oxygen uptake is low and constant and in which the accumulated Ca++ remains in the mitochondria (8). The accumulated Ca++, however, is not irreversibly sequestered in the mitochondria but exists in a dynamic steady state in which continuous and presumably passive efflux of Ca++ is counterbalanced by active Ca++ uptake coupled to the resting respiration (8). That so called resting respiration is capable of supporting Ca++ accumulation has been directly demonstrated by Carafoli, Rossi, and Lehninger (30). The efflux rate of Ca++ from previously loaded mitochondria, measured under conditions in which it is unopposed by active Ca++ uptake, may vary widely depending on conditions; it is decreased by increasing concentrations of NaCl and by increasing pH, and is increased by K+ and by the presence of phosphate (10). On the other hand, there is evidence that the rate or efficiency of active Ca++ uptake may also undergo variation depending on NaCl concentration, pH, and the presence of phosphate (9). The steady state level of Ca++ existing in the mitochondria at the end of the respiratory jump thus depends on the uptake and efflux rates, which in turn depend on the concentration of certain anions and on the pH, among other factors. Under normal or nonbouncing conditions such steady states remain constant with time, presumably because both the Ca++ influx and efflux rates remain constant with time.

In this paper a set of conditions has been defined in which the Ca++ influx or efflux rate in the resting respiratory state following a Ca++-induced jump evidently does not remain constant over...
accumulated actively high rate of Ca++ efflux may more than equal the rate of respiratory jump is over, however, and the Ca++ uptake rate greater than during the following resting state. When the normal rate would scarcely be evident, since the activated state in a "normal" or nonbouncing system. During the respiratory jump itself, an increase in Ca++ efflux rate to 2 to 3 times the normal rate would scarcely be evident, since the activated state of respiration is from 4 to 6 times as fast as the resting respiration and it is perhaps 8 fold more efficiently coupled to Ca++ uptake; the Ca++ uptake rate during the jump is thus some 40 to 60 times greater than during the following resting state. When the respiratory jump is over, however, and the Ca++ uptake rate abruptly decreases to as little as \( \frac{1}{2} \) its former rate, then a relatively high rate of Ca++ efflux may more than equal the rate of Ca++ uptake in the early part of the resting period. Loss of accumulated Ca++ to the medium and reabsorption of H+ would then be expected to take place and could cause the rebound in the H+ ejection curve. If the efflux rate now gradually decreases, finally stabilizing at the normal rate, the system would be expected to return from the peak of the H+ bounce back to a condition where Ca++ efflux and influx rates come into balance, at that level of Ca++ uptake and H+ ejection that existed at the end of the period of rapid H+ ejection. Thus the efflux effects described in this paper could be accounted for as the resultant of a constant stoichiometry between Ca++ uptake and electron transport, combined with a decreasing rate of efflux of the Ca++ with time. Since it has been shown that efflux rates can in fact vary with salt concentration and pH (9, 10), this explanation appears to be the simplest that can be offered for such a bounce effect. However, not all the observations reported are in accord with this one-variable hypothesis. Although the bounce effect is dependent on some variables known to influence efflux rate, such as salt concentration and pH (9, 10), it is also dependent on some other variables that influence primarily the energy-linked Ca++ accumulation. For example, the full bounce effect occurs only when succinate is the substrate, but not with \( \beta \)-hydroxybutyrate; in the presence of the latter there is a rebound, but there is no return to the state attained just after the jump. Furthermore, the damping action of rotenone and Amytal on the bounce in succinate-supported systems also indicates that a respiration-linked event, presumably the Ca++ accumulation rate or efficiency, is a major determinant of the bounce effect. The damping effect of succrose, which is known to inhibit some aspect of respiratory energy coupling (27, 28), also suggests that the rate of respiration-dependent uptake of Ca++ may undergo changes with time, particularly since succrose has been found to have no significant effect on the rate of efflux of Ca++. These observations thus support the view that both the rate of Ca++ uptake and the rate of Ca++ efflux may undergo changes with time and could thus operate together to yield the bounce effects described.

Since oscillatory or "hunting" phenomena are characteristic of feedback control systems in which there is some hysteresis or lag, the findings described here suggest the occurrence of a feedback relationship between the rate of passive efflux and the rate of active, energy-linked uptake of Ca++ in the mitochondria that enables the system to act in a self-correcting manner, which it evidently does in bouncing systems that return to normal steady states. In nonbouncing conditions (i.e., \( \geq 80 \) mm NaCl or KCl) there appears to be no detectable lag in the adjustment of efflux or influx rates. However, under bounce conditions, the system adjusts itself relatively slowly in seeking the final steady state. Since the oscillation is a heavily damped one, the feedback relationship is fairly closely coupled. However, in other experiments to be reported elsewhere, in which ATP hydrolysis rather than respiration was the driving force for Ca++ accumulation, the feedback relationship was found to have sufficient hysteresis to allow a series of oscillations in Ca++ uptake to occur.¹

The observations described in this paper extend the growing number of cases described in which enzyme systems exhibit oscillatory behavior (for examples, see References 31 through 35). In the case described here, the rebounds and oscillations involve an enzyme system operating in conjunction with a membrane system, in which enzyme-activated movements of ions take place across the mitochondrial membrane. It appears possible that the effects described here are related to the oscillatory ATPase activity and K+ movements in valinomycin-treated mitochondria that have been briefly reported by Lardy and Craven (36) and Pressman (37), respectively.

The observations reported here also may provide further insight into the origin and direction of movements of H+ ions generated during electron transport in mitochondria, particularly when respiration is activated by addition of divalent metal ions such as Ca++, Sr++, and Mn++, or by monovalent ions such as K+ and Na+ in the presence of antibiotics such as valinomycin and gramicidin (25, 26). Electron transport in the forward direction, i.e., from substrate to oxygen, is now known to result in H+ ejection (cf. References 25 and 38). The experiments described here show that the rebound of H+ ejection (actually an uptake of H+) occurring when succinate is substrate is largely blocked by Amytal or rotenone, suggesting that reverse electron transfer from succinate to NAD may cause uptake of H+ from the medium. This finding would be expected if the H+ ejection during forward electron transport is caused by asymmetric location of the electron carrier enzyme molecules in the mitochondrial membranes in such a way that H+ generation occurs on the outer side of the membrane only. However, it appears unlikely that the rebound of H+ ejection observed in our experiments can be wholly accounted for by passage of electrons from succinate to endogenous intramitochondrial NAD, since rat liver mitochondria contain only about 7 mmoles of NAD per mg of protein, whereas the usual rebound in H+ ejection observed in the course of these experiments was in the neighborhood of about 30 mmoles of H+ absorbed per mg of protein. Nevertheless, these considerations warrant further investigation of the changes in the

¹ J. Bielawski, F. Werblin, J. Peterson, and A. L. Lehninger, unpublished data.
oxidation-reduction state of the mitochondrial pyridine nucleotides during such bounces, as well as of the H+ movements during reverse electron transport.

The finding that the permeant anions phosphate and acetate (9, 19, 20) eliminate the bounce, which is most conspicuous in a medium containing the impermeant Cl− anion (or the similar Br−, I−, or CNS− anions), indicates that there may be a transient deficit of anions within the mitochondrion after Ca++ uptake occurs, a stress that could be relieved by the rapid efflux of Ca++ and uptake of H+ that occurs during the rebound.

Finally, the observations reported on the effect of different buffers on the bounce effect, taken together with the demonstration by Good et al. (29) that the buffer species may profoundly influence mitochondrial respiration and phosphorylation, suggest that considerable care must be employed in choice of buffers in examination of systems in which ion transport activities are taking place. It appears possible that at least part of the effect of certain buffers in such systems may be caused by active or passive movements of buffer cations or anions (or their free acids or bases) into mitochondria, experiments on such effects are under way.

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Rebounds and Oscillations in Respiration-linked Movements of Ca\(^{++}\) and H\(^{+}\)
in Rat Liver Mitochondria
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