A Quantitative Analysis of Super-Stoichiometric H\(^+\) Ejection and Ca\(^{2+}\) Uptake in Respiring Rat Liver Mitochondria*

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**SUMMARY**

Experiments described in this paper provide an explanation for the super-stoichiometry of respiration-dependent uptake of Ca\(^{2+}\) and ejection of H\(^+\) by mitochondria, i.e., Ca\(^{2+}\)/2e\(^-\) ratios greatly exceeding 2.0 per site and correspondingly increased H\(^+\)/2e\(^-\) ratios when the medium is devoid of phosphate. In rat liver mitochondria super-stoichiometry is evoked by Ca\(^{2+}\) concentrations in excess of 60 to 80 ng-ions per mg of protein, in an otherwise normal reaction medium of 120 mM KCl, pH 7.2. Super-stoichiometric H\(^+\) ejection and Ca\(^{2+}\) uptake is caused by a distinctly different process than the stimulation of oxygen consumption. Kinetic analysis shows that respiration-dependent Ca\(^{2+}\)-stimulated H\(^+\) ejection under conditions yielding super-stoichiometry is biphasic. It consists of (1) a large early burst of H\(^+\) release, exceeding 1400 ng-ions of H\(^+\) per mg per min, in which the H\(^+\)/2e\(^-\) ratio may be infinitely high; and (2) a slow phase stoichiometric with electron transport. Simultaneously, there is a large early burst of respiration-dependent Ca\(^{2+}\) binding, which may exceed 1000 ng-ions per mg per min, again with a very high Ca\(^{2+}\)/2e\(^-\) ratio, and a slow phase of Ca\(^{2+}\) uptake stoichiometric with respiration. Super-stoichiometric Ca\(^{2+}\)/2e\(^-\) and H\(^+\)/2e\(^-\) ratios are thus due to a rapid and large energy-dependent Ca\(^{2+}\) binding and H\(^+\) ejection superimposed on the slower, normal uptake of Ca\(^{2+}\) and ejection of H\(^+\) that is stoichiometric with electron transport. The extra Ca\(^{2+}\) uptake and H\(^+\) ejection in the early burst require a finite preceding period of State 4 electron transport, presumably for energization. Some 40 to 50 ng-ions of Ca\(^{2+}\) may be bound and up to 60 ng-ions of H\(^+\) may be ejected per mg of protein in the early burst under optimal conditions. Similar results are observed with Sr\(^{2+}\) uptake, but Mn\(^{2+}\) yields only the slow stoichiometric phase. Since the super-stoichiometry occurs in the absence of permeant anions and with mersalyl present to prevent cycling of mitochondrial phosphate, it is concluded that the fast extra Ca\(^{2+}\) binding and H\(^+\) ejection involve specific membrane-binding sites.

It is postulated that State 4 energization of the membrane causes changes in the affinity of these binding sites, leading to decreased affinity for H\(^+\) and increased affinity for Ca\(^{2+}\).

Considerable interest centers on the stoichiometry and mechanism of H\(^+\) ejection coupled to electron transport in mitochondria and in chloroplasts. Observations on mitochondria made with the oxidant pulse (1) method indicate that the number of H\(^+\) ions ejected per pair of electrons per energy-conserving site (hereafter designated the H\(^+\)/2e\(^-\) ratio) is 2.0. However, under various other conditions the H\(^+\)/2e\(^-\) ratio appears to exceed 2.0 (2-6). In particular, respiration-dependent Ca\(^{2+}\) accumulation by mitochondria has been widely observed to occur with a Ca\(^{2+}\)/2e\(^-\) ratio of 2.0 (7-10), equivalent to movement of 4 positive charges per pair of electrons per site; moreover, the H\(^+\)/2e\(^-\) ratio during Ca\(^{2+}\) uptake may approach 4 under some conditions (3, 4). The stoichiometry problem is further compounded by the unexplained "super-stoichiometry" of Ca\(^{2+}\) uptake and H\(^+\) ejection coupled to electron transport in rat liver mitochondria observed in the absence of phosphate, in which the Ca\(^{2+}\)/2e\(^-\) accumulation ratio may exceed two or three times the usual value of 2.0, with a proportional increase in the H\(^+\)/2e\(^-\) ejection ratio (11-16).

Recently we reported (17) that mitochondria isolated from L1210 mouse leukemia cells show a particularly pronounced super-stoichiometry of H\(^+\) ejection during Ca\(^{2+}\) transport; the critical factor appeared to be the concentration of Ca\(^{2+}\) added to the medium. This observation led us to examine in more detail the effect of Ca\(^{2+}\) concentration and other factors on the super-stoichiometry and kinetics of H\(^+\) ejection and Ca\(^{2+}\) uptake in mitochondria from nonmalignant tissues. The experiments on rat liver mitochondria described in this paper, as well as others on L1210 tumor mitochondria (18), provide an explanation and basis for the super-stoichiometry effect.

It is shown here that respiration-dependent H\(^+\) ejection and Ca\(^{2+}\) uptake by rat liver mitochondria is a kinetically biphasic process under the conditions in which super-stoichiometry occurs. The excess H\(^+\) ejection and Ca\(^{2+}\) uptake in relation to electron transport are the result of a hitherto undescribed early, large burst of H\(^+\) ejection and Ca\(^{2+}\) uptake that is dependent on an energized state of the mitochondria, but that bears no simple stoichiometric relationship with electron transport. When this early burst of H\(^+\) ejection and Ca\(^{2+}\) uptake is superimposed on...
the normal ejection of H+ and uptake of Ca2+ that is stoichiometric with electron transport, it accounts for the super-stoichiometric H+/2e− and Ca2+/2e− ratios observed under certain conditions.

**EXPERIMENTAL PROCEDURE**

The oxygen concentration in the test system was sensed in most cases with a Clark-type electrode (Yellow Springs Instrument Co.); a vibrating platinum electrode was also used in some experiments. Changes in H+ concentration were followed with a Beckman combination glass electrode. Both oxygen and H+ concentration changes were measured simultaneously in the same closed vessel and the resulting responses recorded on strip charts with a Sargent DSRG recorder. The oxygen electrode traces were corrected for the lag in response to changes in oxygen concentration, following appropriate calibration with rapidly mixed oxygen-absorbing reagents. The magnitude of this correction was in any case not a serious consideration in interpretation of the kinetic differences between the rates of Ca2+-stimulated oxygen consumption and H+ ejection.

Net uptake of 46Ca2+ was measured by rapid manual sampling of the incubation mixture, filtration through double Millipore filters (0.45 μm), and scintillation counting of the 46Ca2+ remaining in the filtered medium. Measurements of Ca2+ uptake at 6-s intervals were possible.

For preparation of rat liver mitochondria, homogenates were prepared in 0.25 M sucrose and washed three times.

**RESULTS**

**Super-stoichiometry of H+ Ejection following a Ca2+ Pulse**—Fig. 1 shows a typical experiment on the time course of oxygen uptake and H+ ejection induced by a Ca2+ pulse in rat liver mitochondria respiring on succinate in the presence and absence of phosphate. The pH was 7.2 and the medium contained 120 mM KCl. The amount of Ca2+ added (160 ngs-ions per mg of protein) was relatively high, in the range found to cause super-stoichiometry in L1210 tumor mitochondria (17). In the presence of phosphate, the Ca2+/2e− ratio was 2.0 and the H+/2e− ratio was 1.95, normal values consistent with those observed in many laboratories (7–10). However, in the absence of phosphate, super-stoichiometric ratios were observed; the Ca2+/2e− ratio was 4.00 and the H+/2e− ratio was 6.00, over twice the values observed in the presence of phosphate. It is to be noted that the super-stoichiometry was observed in the presence of a nearly neutral medium (pH 7.2) and a normal salt concentration (120 mM KCl), in contrast to earlier experiments which showed that high medium pH and high salt concentrations were necessary to yield the super-stoichiometry effect in rat liver mitochondria (11–13).

The traces in Fig. 1 also show that no net H+ ejection occurred in the presence of the respiration-inhibitors antymycin A and rotenone or the proton-conducting uncoupling agent FCCP.† Thus, as shown before (11–15), the super-stoichiometry of H+ ejection is not simply the result of a nonspecific respiration-independent binding of Ca2+ (cf. Refs. 6 and 14), which in any case does not result in H+ ejection (19, 20).

**Effect of Ca2+ Concentration on Amounts of H+ Ejected and Oxygen Consumed**—Since earlier experiments on L1210 tumor mitochondria (17) indicated that Ca2+ concentration is critical in the super-stoichiometry of H+ ejection, the effect of the initial Ca2+ concentration on the amounts of H+ ejected, Ca2+ accumulated, and oxygen consumed in respiratory jumps induced in rat liver mitochondria by Ca2+ was examined under conditions otherwise identical with those in Fig. 1. Succinate was the substrate; phosphate was not added. The measurements were taken after the rate of oxygen consumption had returned to the pre-Ca2+ State 4 rate, as indicated in the traces in Fig. 1. The amount of Ca2+ added was varied from 20 to 1000 ngs-ions of Ca2+ per mg of protein. The collected results are plotted in Fig. 2. It is seen that the maximum amount of extra oxygen consumption induced by pulses of Ca2+ is about 11 ngs-atoms per mg of mitochondrial protein; this maximum was attained by addition of about 40 ngs-ions of Ca2+ per mg of protein. However, this amount of Ca2+ is insufficient to yield maximum ejection of H+ and maximum Ca2+ binding, which required substantially higher initial concentrations of Ca2+. Stimulation of oxygen consumption and ejection of H+ therefore appear to be the result of two different Ca2+-dependent processes differing in their affinity for Ca2+, as in the case of L1210 tumor mitochondria (17); such an effect is also evident in the report of Rossi and Azzone (4).

To show the extent of the deviation of Ca2+-induced H+ ejection from strict stoichiometry with oxygen uptake, Fig. 2 also includes a dashed line showing the expected amount of H+ ejected, assuming that the H+/2e− ratio is 2.0. When the Ca2+ concentration is higher than about 40 to 60 ngs-ions per mg of protein the H+/2e− ratios become significantly greater than 2.0. The maximum H+/2e− ratio observed was about 4.0 in this set of experiments and the maximum amount of Ca2+ bound, regardless of the Ca2+ concentration added, was about 80 ngs-ions per mg of protein (7). The ratio of ngs-ions of H+ ejected to ngs-ions of Ca2+ bound varied from about 1.1 to about 1.7 in such experiments, depending on the Ca2+ concentration. Again, the H+ ejection and Ca2+ binding observed at the higher concentrations of Ca2+ in such experiments were completely dependent on energy-coupled electron transport; antymycin A + rotenone or FCCP yielded complete inhibition of all H+ ejection and all Ca2+ binding.

† The abbreviations used are: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.
H+/O ratio of 4.0 with succinate, assuming 2H+ are ejected per site.

Time Course of H+ Ejection and Oxygen Consumption Stimulated by Ca2+.—Since Ca2+-induced H+ ejection and Ca2+-induced respiratory stimulation show different dependence on Ca2+ concentration, it appeared possible that these two processes might also differ with respect to their course with time. Precise comparisons were therefore made of the time course of H+ ejection and oxygen consumption following pulsed additions of Ca2+. The oxygen and H+ traces in Fig. 3 (left) show that there is a significant lag period (not instrumental) before the rate of oxygen consumption increases toward the maximum Ca2+-stimulated rate. However, there is no lag period in the Ca2+-induced H+ ejection, which is very rapid; half or more of the total H+ ejection occurred before any significant increase in the rate of oxygen consumption took place. The fast early phase of H+ ejection was followed by a slower phase which was proportional to the rate of oxygen consumption. These kinetic relationships are more clearly demonstrated by the loess plot in Fig. 3 (left), in which the ratio of ng-ions of H+ ejected to ng-atoms of oxygen consumed for successive 6-s intervals (the ΔH+/ΔO ratio) is plotted against time. This ratio is very high in the first 2 to 3 s, in excess of 50, but levels off after 6 s to a value of about 4.0 to 4.5 throughout the subsequent 6-s intervals. This experiment shows that the early burst of Ca2+-induced H+ ejection is very large and bears no simple stoichiometric relationship to the amount of oxygen consumed; indeed, in several such experiments the ΔH+/ΔO ratio in the first seconds was found to approach infinity. After the early burst the subsequent ejection of H+ is apparently stoichiometric with electron transport, with a H+/2e- ratio of approximately 2. The super-stoichiometric value of the H+/2e- ratio for the entire jump period is caused by the large early burst of H+ ejection, superimposed on the slower, normal H+ ejection associated with electron transport induced by Ca2+, since the cumulative H+/O ratio for successive 6-s intervals during the complete jump, as well as a plot of the cumulative H+/O ratio (dashed curve), Right, time course of H+ absorption and extra oxygen consumption during an ADP-induced respiratory jump. The test system (2.0 ml) consisted of 120 mM KCl, 3.0 mM Hepes buffer, pH 7.2, 2.0 mM succinate, 0.5 mM phosphate, and mitochondria (5 mg of protein). ADP (100 moles) was added at the point shown. Shown below the O2 and H+ traces is a plot of the ratio of ng-ions of H+ absorbed to ng-atoms of oxygen consumed (ΔH+/ΔO) for successive 6-s intervals during the complete jump. The ΔH+/ΔO plots are on the same time scale as the oxygen and H+ traces. The scales for the traces are in ng-atoms of oxygen or H+. 

Super-stoichiometry of H+ ejection by rat liver mitochondria was also observed with three-site substrates such as glutamate or pyruvate + malate and with the one-site system ascorbate + tetramethylephenediamine. Moreover, super-stoichiometry was also observed in the absence of added substrate, when respiration was occurring at the expense of endogenous mitochondrial substrates (see below).

Time Course of El+ Ejection and Oxygen Consumption Stimulated by Ca2+—Since Ca2+-induced H+ ejection and Ca2+-induced respiratory stimulation show different dependence on Ca2+ concentration, it appeared possible that these two processes might also differ with respect to their course with time. Precise comparisons were therefore made of the time course of H+ ejection and oxygen consumption following pulsed additions of Ca2+. The oxygen and H+ traces in Fig. 3 (left) show that there is a significant lag period (not instrumental) before the rate of oxygen consumption increases toward the maximum Ca2+-stimulated rate. However, there is no lag period in the Ca2+-induced H+ ejection, which is very rapid; half or more of the total H+ ejection occurred before any significant increase in the rate of oxygen consumption took place. The fast early phase of H+ ejection was followed by a slower phase which was proportional to the rate of oxygen consumption. These kinetic relationships are more clearly demonstrated by the loess plot in Fig. 3 (left), in which the ratio of ng-ions of H+ ejected to ng-atoms of oxygen consumed for successive 6-s intervals (the ΔH+/ΔO ratio) is plotted against time. This ratio is very high in the first 2 to 3 s, in excess of 50, but levels off after 6 s to a value of about 4.0 to 4.5 throughout the subsequent 6-s intervals. This experiment shows that the early burst of Ca2+-induced H+ ejection is very large and bears no simple stoichiometric relationship to the amount of oxygen consumed; indeed, in several such experiments the ΔH+/ΔO ratio in the first seconds was found to approach infinity. After the early burst the subsequent ejection of H+ is apparently stoichiometric with electron transport, with a H+/2e- ratio of approximately 2. The super-stoichiometric value of the H+/2e- ratio for the entire jump period is caused by the large early burst of H+ ejection, superimposed on the slower, normal H+ ejection associated with electron transport induced by Ca2+, since the cumulative H+/O ratio (Fig. 3, left) is very high at the beginning of the jump and progressively falls to an overall or time-integrated value for the entire jump of about 9.6 or 4.8 H+ per site. As a control experiment, to show that instrument response was not a controlling factor in the early burst of Ca2+-induced H+ ejection, the time course of oxygen consumption and H+ absorption during respiratory jumps induced by ADP was followed under closely similar conditions (Fig. 3, right). It is seen that the ADP-stimulated increase in oxygen consumption begins simultaneously with H+ absorption and the two processes remain in proportion throughout the jump. The ratio of ng-ions of H+ absorbed to ng-atoms of oxygen consumed (ΔH+/ΔO) for successive 6-s intervals was constant at about 1.58, or 0.79 per site throughout the entire jump, including the first 6-s interval, in close agreement with the expected H+/ADP stoichiometry of oxidative phosphorylation at this pH (21).  

Biphasic Nature of Ca2+-induced H+ Ejection—The time course of H+ ejection induced by Ca2+ could be resolved into two kinetically distinct phases. Use of a relatively low, suboptimal concentration (0.2 mM) of the respiratory substrate succinate, to slow down the rate of electron transport and thus the rate of the second phase of H+ ejection, i.e., that portion stoichiometric with electron transport, yielded distinctly biphasic H+ ejection traces following addition of Ca2+ (Fig. 4). There was an early rapid burst of H+ ejection following the addition of Ca2+, which ended...
in a distinct inflection at which the rate of H+ ejection decreased sharply and became approximately proportional to the rate of oxygen consumption. Biphasic H+ ejection was also observed when no substrate was added, electron flow then arising from endogenous substrates. The point of inflection in H+ ejection was particularly evident when the Ca*+ concentration was relatively high (Fig. 4).

**Rapid Ca*+ Uptake during Early Phase of H+ Ejection**—Since the conditions that yield super-stoichiometry of H+ ejection also lead to super-stoichiometric Ca*+/2e- ratios (11-13), it appeared possible that the early rapid burst of H+ on adding Ca*+ to respiring rat liver mitochondria is accompanied by an equivalent burst of Ca*+ binding by the mitochondria. Using a rapid sampling method it was possible to measure the uptake of Ca*+ from the medium at short intervals during the Ca*+-induced respiratory jump. Data of a typical experiment are shown in Fig. 5. It is seen that Ca*+ binding, like H+ ejection, is also biphasic. By the time of the first measurement (6 s after Ca*+ addition) over half of the total Ca*+ uptake had already occurred; the rate of the Ca*+ uptake after 6 s was clearly much lower and proportional to oxygen uptake, as is shown by the plot of ΔCa*+/ΔO for successive 6-s intervals. In the first 6-s interval the ΔCa*+/ΔO ratio was about 54, equivalent to a Ca*+/2e- ratio of 27. The integrated or cumulative Ca*+/O ratio was about 8.8 for the entire jump, equivalent to an over-all Ca*+/2e- ratio of 4.2. Although the manual sampling method did not allow more measurements of Ca*+ concentration in the early phase, it is clear from a number of such experiments that the initial fast burst of Ca*+ uptake can account for the super-stoichiometric ratios of Ca*+ uptake.

The ratio of ng-ions of H+ ejected to ng-ions of Ca*+ bound in the early phase was found to approach 1.7 to 1.8. It therefore appears likely that the added Ca*+ is causing ejection of close to an electrically equivalent number of H+ ions from protonated sites in the mitochondria in the early burst.

**Requirement of Preceding Period of Electron Transport for Fast Burst of H+ Ejection**—When the sequence of addition of Ca*+ and respiratory substrate was varied (Fig. 6), it was found that the fast burst of H+ ejection evoked by addition of Ca*+ occurs only after a preceding period of State 4 electron transport.
When rat liver mitochondria were incubated in the absence of succinate and with rotenone to inhibit endogenous respiration, no H⁺ ejection occurred on addition of Ca²⁺, as expected from the experiments in Fig. 1. If succinate was added first, followed by 60 s later by Ca²⁺, then the usual fast burst of H⁺ ejection occurred, followed by a slower phase of H⁺ ejection proportional to oxygen consumption (Fig. 6). However, when Ca²⁺ was added first to the rotenone-inhibited mitochondria, followed by succinate 60 s later, there was no fast burst of H⁺ ejection on addition of the succinate, only the slow stoichiometric phase. Similarly, when Ca²⁺ and succinate were added simultaneously, only the slow phase of H⁺ ejection ensued. From these experiments it is concluded that the mitochondrial reservoir of H⁺ that yields the rapid, nonstoichiometric ejection of H⁺ on addition of Ca²⁺ must first be filled or energized by a preceding period of State 4 electron transport.

Experiments were then carried out to determine the time required to fill this reservoir. Additions of Ca²⁺ were made to rotenone-treated mitochondria at various times after addition of a low concentration (0.2 mM) of succinate; the H⁺ ejection traces were recorded at high chart speeds (5 to 10 inches per min) and carefully analyzed for the initial rate of H⁺ ejection. In the lower part of Fig. 6 is a normalized composite of the H⁺ traces summarizing the results. It is seen that the amount and rate of H⁺ ejection in the initial rapid burst increased with an increase in the time elapsing between addition of succinate and subsequent addition of Ca²⁺. They became maximal when this interval was about 36 s at 25°. When the succinate concentration was raised to 2.0 mM in such experiments, the time required for maximal early H⁺ ejection was reduced to ~12 s.

Magnitude of H⁺ Reservoir—The maximum amount of H⁺ that can be ejected in the first early burst from rat liver mitochondria by Ca²⁺ was determined by extrapolation from the H⁺ ejection traces, under various conditions. Maximum early H⁺ ejection, in excess of 50 ng-ions of H⁺ per mg of protein, required a period of 30 to 60 s of aerobic preincubation with succinate, relatively high substrate concentration (2.0 mM succinate), and addition of at least 60 ng-ions of Ca²⁺ per mg of protein. However, extrapolation from the H⁺ electrode traces was not completely satisfactory because of some merging of the fast and slow phases, particularly at high concentrations of Ca²⁺.

The most satisfactory determinations of the magnitude of the early H⁺ burst were made in the presence of mersalyl, an inhibitor of the phosphate-hydroxide antiporter of the inner membrane (22), to suppress the slow stoichiometric phase of H⁺ ejection and Ca²⁺ uptake, which is in part a reflection of the cycling of small amounts of phosphate arising from the endogenous pool in the mitochondrial matrix, driven out of the matrix by exchange with the succinate used as external substrate, via the dicarboxylate-phosphate exchange (23). The results are shown in Fig. 7. It is seen that the rapid early burst of H⁺ ejection still takes place in the presence of mersalyl without significant inhibition, whereas the subsequent slow phase of H⁺ ejection is greatly diminished in both rate and total extent. The early burst of H⁺ ejection and Ca²⁺ uptake thus is not due to rapid entry of endogenous phosphate into the matrix as counterion for the Ca²⁺; rather it proceeds independently of the mersalyl-sensitive phosphate carrier. Moreover, this type of experiment allowed a more accurate determination of the amount of H⁺ ejected from the mitochondria in the early burst, as a function of Ca²⁺ concentration. Fig. 7 shows that the magnitude of the burst increases with Ca²⁺ concentration until a maximum ejection of about 45 ng-ions of H⁺ per mg of protein is reached, which is evoked by Ca²⁺ added at 60 ng-ions per mg of protein.

Rate of Early Burst of H⁺ Ejection—From experiments in which Ca²⁺ concentration and substrate concentration were varied and fast chart speeds were employed to improve the accuracy of extrapolation, it was possible to approximate the maximum rate of H⁺ ejection in the early burst. Data for succinate and pyruvate + malate as substrate are given in Table I, which also shows the apparent Kₐ(Ca²⁺) for the early H⁺ ejection, uncorrected for binding of Ca²⁺ by components of the reaction medium. The maximum observed rate of H⁺ ejection in the early burst exceeded 1400 ng-ions of H⁺ per mg of protein per min, with either succinate or pyruvate + malate, or at least 6 times the rate of the slow stoichiometric phase of H⁺ ejection in the presence of succinate, a ‘fast’ substrate, and about 15 times the rate of the slow stoichiometric phase of H⁺ ejection coupled to oxidation of pyruvate + malate, which is representative of the over-all rate of the tricarboxylate cycle oxidations. Presumably the rates of the fast and slow phases of Ca²⁺ binding were in approximate proportion to the rates of H⁺ ejection. Assuming a H⁺/Ca²⁺ ratio of 1.4, the rate of the early Ca²⁺ binding is calculated to be about 1000 ng-ions per mg of protein per min.

Effect of Sr²⁺ and Mn²⁺—Sr²⁺ and Mn²⁺ are accumulated by respiring mitochondria under conditions supporting Ca²⁺ transport (review, see Ref. 9). Addition of Sr²⁺ to rat liver mitochondria gave biphasic H⁺ ejection curves very similar to those given by Ca²⁺ pulses. However, the addition of Mn²⁺ does not yield an early burst of H⁺ ejection and thus does not show superstoichiometry under these conditions (Fig. 8). The H⁺/2e⁻ ratio (succinate) was found to be about 2.0 to 2.2 following Mn²⁺ addition in the absence of phosphate. Earlier work has shown

![Fig. 7. Effect of Ca²⁺ concentration on the amount of H⁺ ejected in the early phase. The test system was as shown in Fig. 5, with the addition of mersalyl (50 μM).](https://www.jbc.org/)

### Table I

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that Mn$^{2+}$ is taken up more slowly than Ca$^{2+}$ by respiring mitochondria (9, 24) and that Mn$^{2+}$ uptake is promoted by simultaneous addition of Ca$^{2+}$ (24).

**Effect of La$^{3+}$ and Ruthenium Red—La$^{3+}$ (25, 26) and ruthenium red (27), which block respiration-dependent Ca$^{2+}$ binding and transport by rat liver mitochondria, also prevent the respiration-dependent super-stoichiometric early burst of H$^+$ ejection that follows a pulse of Ca$^{2+}$ (experiments not shown). In the case of La$^{3+}$ this result was predictable from an earlier investigation (25).

**Discussion**

The experiments described in this paper demonstrate that the excess ejection of H$^+$ and uptake of Ca$^{2+}$ occurring in the super-stoichiometry phenomenon in the absence of added phosphate is largely if not entirely caused by a hitherto undescribed early burst of H$^+$ ejection and Ca$^{2+}$ binding, preceding the normal rate of translocation of these ions that is stoichiometric with oxygen consumption. The early burst appears to consist of a very rapid uptake of Ca$^{2+}$ from the medium and an ejection of H$^+$ that requires energization by electron transport but that is not stoichiometric with it.

The concentration of Ca$^{2+}$ in the suspending medium is an important determinant of the magnitude of the super-stoichiometric early burst of H$^+$ ejection and Ca$^{2+}$ uptake. Ca$^{2+}$ pulses up to about 40 ng-ions per mg of mitochondrial protein, which produce maximum amounts of extra oxygen consumption, yield normal stoichiometry between H$^+$ ejection, Ca$^{2+}$ binding, and oxygen consumption, i.e. the H$^+/2e^-$ and the Ca$^{2+}/2e^-$ ratios are about 2.0 when determined at the end of the jump in oxygen consumption, in accordance with many earlier observations (7–9). At concentrations of Ca$^{2+}$ above 40 ng-ions per mg of protein, the over-all H$^+/2e^-$ and Ca$^{2+}/2e^-$ ratios begin to increase to values of 4 to 5 or more, since the super-stoichiometric early burst requires a higher concentration of Ca$^{2+}$ than the stimulation of oxygen uptake, as reported earlier for L1210 tumor mitochondria (17). This observation suggests that the Ca$^{2+}$ binding sites responsible for the super-stoichiometric exchange of Ca$^{2+}$ for bound H$^+$ are not identical with those stimulating oxygen consumption. The concentration of Ca$^{2+}$ in the medium thus must be added to the other factors known to induce super-stoichiometry. However, earlier observations indicate that high pH and salt concentration may exert their effects through a very different mechanism than Ca$^{2+}$, namely, by inhibiting the efflux of Ca$^{2+}$ from previously loaded mitochondria (12).

The data reported here also demonstrate that maximum super-stoichiometry is observed early in the period of Ca$^{2+}$-induced stimulation of oxygen consumption, particularly before the slow super-stoichiometric phase of H$^+$ ejection and Ca$^{2+}$ binding has begun. At this early point the H$^+/2e^-$ ratio may exceed 50 and approach infinity, with comparably high Ca$^{2+}/2e^-$ ratios. Thus, the H$^+/2e^-$ and Ca$^{2+}/2e^-$ ratios (when phosphate is absent) may vary over a wide range, from the normal values of about 2.0 to infinitely high values, depending on (a) the concentration of Ca$^{2+}$ and (b) the time span over which the H$^+$ ejection and Ca$^{2+}$ are measured.

The super-stoichiometric ion exchanges are not the result of respiration-independent binding of Ca$^{2+}$, which has been established to proceed without release of H$^+$ from mitochondria (19, 20). Since Ca$^{2+}$ binding and H$^+$ release occur together in the super-stoichiometric phase, it appears possible that in respiring mitochondria the binding of a large amount of Ca$^{2+}$ causes the H$^+$ binding sites in the membrane to undergo a large decrease in affinity for H$^+$, so that H$^+$ is released to the medium. Such a change in affinity for H$^+$ coupled with binding of Ca$^{2+}$ may be the reflection, as postulated elsewhere (28), of a membrane Bohr effect, analogous to the Bohr effect of hemoglobin, in which binding of oxygen causes a decrease in the affinity for H$^+$.

The binding of 40 to 50 ng-ions of Ca$^{2+}$ in the super-stoichiometric phase, which is about equal to the amount of Ca$^{2+}$ bound by respiring rat liver mitochondria in the absence of permeant anions, as indicated by Chance and Yoshioka (29) and Gear et al. (30), may be related to our earlier finding (20) that respiration-inhibited rat liver mitochondria show two classes of binding sites for Ca$^{2+}$, high affinity sites ($K_m = 0.025$ μM) that are small in number ($n = 1$ to 2 ng ions of Ca$^{2+}$/per ng of protein) and low affinity sites ($K_m = 100$ μM) that are much more numerous ($n = 40$ ng ions of Ca$^{2+}$/per ng of protein). The agreement between the number of low affinity Ca$^{2+}$ sites and the number of Ca$^{2+}$ ions bound during super-stoichiometry suggests that these low affinity sites are involved in the super-stoichiometric binding of Ca$^{2+}$ in respiring mitochondria. If this is the case, the linkage between the Ca$^{2+}$-binding sites and the H$^+$-binding sites is such that in the absence of electron transport H$^+$ is not released when Ca$^{2+}$ is bound whereas in the respiring, energized state, H$^+$ release does take place as Ca$^{2+}$ is bound.

Of particular interest is the observation that a finite, relatively long period of preceding State 4 respiration is required for maximum super-stoichiometric ejection of H$^+$ (and presumably binding of Ca$^{2+}$). There are at least two possible explanations for this unusual time dependence. The first is that the specific mitochondrial sites from which H$^+$ ions are released on addition of Ca$^{2+}$ may be unprotonated when respiration is inhibited and gain protons only from the H$^+$ that is translocated from the matrix stoichiometrically with electron transport, whatever the mechanism of the latter process. If this is the case, the number of protons released by the Ca$^{2+}$ pulse might be expected to correspond with the number of protons translocated across the membrane during the minimum period of state 4 energization, assuming a H$^+/2e^-$ ratio of 2.0. In the experiment of Fig. 6, 36 s of state 4 respiration, equivalent to about 7 mg-atoms of oxygen consumed and 28 ng-ions of H$^+$ transported per mg of protein, were required to yield a maximal burst of about 40 ng-ions of H$^+$ per mg of mitochondrial protein. These figures are not in exact agreement but they suggest that such an explanation should not be dismissed at present.

Another possible explanation is that a finite period of State 4 respiration is required for the mitochondria to undergo some ultrastructural change into a conformation in which all the protonated sites are energized and competent to release H$^+$ to the medium when Ca$^{2+}$ is added. Hackenbrock's studies (31) of
orthodox - condensed ultrastructural transitions of mouse liver mitochondria indicate that a rather considerable period of State 4 respiration is required for essentially all the mitochondria to undergo the condensed → orthodox transition. The binding sites for H⁺ and Ca²⁺ in the orthodox conformation characteristic of State 4 may well differ in affinities from those in the condensed structural state of mitochondria. Further evidence supporting this type of explanation is the finding of Hackenbrock and Caplan (32) that the membrane conformation when Ca²⁺ is bound by mitochondria, K₉(Ca²⁺) for stimulation of oxygen consumption is about 8 µM, whereas in rat liver mitochondria under identical conditions it is at least 50 µM Ca²⁺; both figures are uncorrected for content.

Finally, we may consider the differences between the Ca²⁺-binding properties of L1210 ascites tumor mitochondria (17, 18) and the rat liver mitochondria studied here. The L1210 mitochondria show a significantly greater rate of respiration-dependent stoichiometric Ca²⁺ transport than rat liver mitochondria, owing to their higher rate of succinate oxidation. They are also some 6-fold more sensitive to stimulation of respiration by Ca²⁺ than rat liver mitochondria. In the tumor mitochondria, Kₑ(Ca²⁺) for stimulation of oxygen consumption is about 8 µM, whereas in rat liver mitochondria under identical conditions it is at least 50 µM Ca²⁺; both figures are uncorrected for compartmentalization of Ca²⁺ by other components of the medium. The tumor mitochondria also show a more pronounced degree of super-stoichiometric Ca²⁺ transport than the condensed state. A survey of the occurrence of super-stoichiometry in mitochondria from various normal and malignant tissues is under way.

To be described elsewhere are studies showing that the early burst of H⁺ ejection and Ca²⁺ binding may occur under intracellular conditions, i.e., when both phosphate and Mg²⁺ are present.

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A Quantitative Analysis of Super-Stoichiometric H\(^+\) Ejection and Ca\(^{2+}\) Uptake in Respiring Rat Liver Mitochondria

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