The Energy-linked Reaction of Calcium with Mitochondria*

BRITTON CHANCE

From the Johnson Research Foundation and the Departments of Biophysics and Physical Biochemistry, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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The reaction of divalent cations with mitochondria has been a subject of investigation from various standpoints for over 10 years (1). While a large number of contributions particularly from 1961 onward (see below) emphasized the accumulation of large amounts of calcium, this contribution and our previous work emphasize the reaction of low concentrations of divalent cations with phosphorylating mitochondria. These reactions lead to a short term activation of respiration that is followed by a restitution of respiratory control. Under these conditions, we found in 1955 the reaction of calcium to be similar to that with adenosine diphosphate (2) and unlike that with uncoupling agents (but cf. References 3–5).

Calcium activation of respiration in mitochondria was observed in 1953 by Siekevitz and Potter (3) and by Potter, Siekevitz, and Simonson (4), who reported approximately 3-fold stimulation of respiration on addition of 500 μM calcium to rat liver mitochondria. This observation was confirmed by Lindberg and Ernster (6). However, there was no indication that the respiratory activation was a reversible process, or that there was a calcium to oxygen stoichiometry. Also in 1953, Slater and Cleland (7) observed the tight binding of calcium to heart sarcosomes; the binding was so tight that the “isolated sarcosomes contained all the calcium in the heart muscle.” Our 1955 report (2) showed that the respiratory stimulation observed by Siekevitz and Potter (3) and by Potter, et al. (4) was not the uncoupling event pictured by Lehninger (5, 8) in which Ca++ by ADP plus Pi. Thus, the respiratory activation resembles the respiratory components that also closely resembled those caused by ADP plus Pi (15). Acetate now is found to replace phosphate (16, 17).

Changes of H+ concentration are caused by the phosphorylation of ADP and the dephosphorylation of ATP, and Swanson reported preliminary experiments on the phosphorylation reaction (21) with the glass electrode. We used the glass electrode and an indicator technique based upon bromthymol blue to follow the kinetics of uncoupling reactions in mitochondria (22). An increase of H+ concentration upon addition of manganese to mitochondria was observed by Bartley and Amoore (23). In the case of calcium, we noted the H+ increase to be very rapid and closely synchronized with the interval of stimulated respiration (22). Saris also recorded the H+ efflux by means of the glass electrode (18–20) and found Ca++:H+ to be about 1.2 in the presence of phosphate. H+ release was also determined by Brierley et al. (24, 25) and by Engström and DeLuca (26), who found the Ca++:H+ ratio to be 1.1.

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The uptake of Mn++ by mitochondria-rich tissues and by mitochondria themselves was shown isotopically with 55Mn++ by Maynard and Coutin (27). However, they were unable to demonstrate the uptake of Mn++ by mitochondria in vitro. They and Hindley and Fristeter (6) postulated a coenzyme function for Mn++. Chappell, Greville, and Cohn (12, 28) adopted the use of low cation concentrations (2) and found a similarity of Ca++- and Mn++-stimulated respiration in mitochondria. They measured Mn++ accumulation as well and found the Mn++:Pi stoichiometry to be 1.5 (12, 29) and, in addition, determined the Mn++:H+ value to be 1.1.

A number of studies carried out at high Ca++ concentrations have been carried out by Rossi, Lehninger, and Greenawalt (29, 30), who found an average value of 1.67 ± 0.3 for Ca++:Pi, and by Brerley et al. (24, 29), who found a value of 1.6. Other studies have been focused upon the mechanism by which the calcium might be accumulated through the formation of precipitates either as calcium phosphate (25) or as hydroxyapatite (29).

Studies of DeLuca and Engrström (31) and by Vasington and Murphy (32, 33) on calcium binding emphasized the maximal calcium uptake of rat kidney mitochondria. More recently Engrström and DeLuca report detailed studies at 0.3 mM Ca++ (26).

Control of calcium binding by the parathyroid hormone in rat liver mitochondria has been demonstrated (34). The site of Ca++ and Mn++ accumulation has been studied electron microscopically by Peachey (35), who finds the site of accumulation to be associated with the normal granules of the matrix space. He considers phospholipid as a binding site for low concentrations of Ca++ (cf. Reference 12) and suggests that binding to phosphate as calcium phosphate or hydroxyapatite (29) occurs when unusually large amounts of Ca++ are accumulated. Greenawalt, Rossi, and Lehninger (36) also find calcium in the matrix space and fail to find x-ray evidence for hydroxyapatite crystals (36).

Of considerable interest is the finding of DeLuca and Engrström (31) that the calcium accumulation by mitochondria is insensitive to the inhibitor oligomycin (37). Energy-linked reduction of DPN in mitochondria may also proceed in the absence of added phosphate and ATP (38) and in the presence of oligomycin (39, 40).

Thus, DPN reduction and divalent cation accumulation provide examples of energy-linked functions of mitochondria that are dependent not upon ATP itself, but instead upon nonphosphorylated intermediates produced by mitochondrial oxidations prior to the formation of ATP (11, 12, 20). Ca++ accumulation and DPN reduction can also be "driven" by added ATP (31) in a reaction that does not require added phosphate (29).

Interest in cation reactions with mitochondria is greatly enlarged by the finding of Sarie (20) that K+ is involved in the Ca++ reaction, and by the finding of Moore and Pressman (41) that valinomycin stimulates the uptake of K+ by rat liver mitochondria. Chappell and Crofts (42) suggest a similar effect of gramicidin. Christie et al. (43) have also demonstrated K+ uptake in mitochondria but do not add the antibiotics (see also Bartley and Davies (44)). Rasmussen, Fischer, and Arnaud find stimulation of K+ uptake is by parathyroid hormone (45). The emerging picture of a generalized function of mitochondria in ion accumulation invites a closer study of the role of the respiratory carriers in these reactions.

This paper presents details of our earlier report (2) with recent data on the stoichiometry of calcium and ADP, and provides, in addition, data on the rate of calcium uptake at various temperatures. The acid production associated with the calcium uptake (20, 22) has been measured with the sensitive glass electrode techniques (20, 46), and the reaction kinetics of oxygen utilization are also recorded. The affinity for calcium was also measured. The focus of this paper, however, is upon a comparison of the kinetics of these over-all reactions with the kinetics of the intermediates in electron transfer, cytochromes, flavoproteins, and pyridine nucleotides. Possible reaction mechanisms are discussed and a comparison is made of divalent cation uptake observed in vitro and the monovalent cation transport of various tissues (47).

**EXPERIMENTAL PROCEDURE**

*Preparations*—The rat liver and pigeon heart mitochondria were prepared by methods described previously (48, 49). Phosphate-free reaction media were employed: 20 mM Tris and 80 mM KCl for rat liver mitochondria; and 0.225 mM mannitol, 0.05 mM sucrose, and 0.01 Tris-chloride buffer, pH 7.4, for pigeon heart mitochondria.

Preparations of the sea gull mitochondria are described elsewhere (47) and the mitochondria were suspended in the mannitol-sucrose-Tris medium. If EDTA was employed in the preparation procedure, the last washing of the mitochondria was in 0.25 mM sucrose, especially when titrations with Ca++ were carried out.

**Optical Methods**—The use of the dual wave length spectrophotometer combined with a fluorometer has recently been described (49–51). Cytochrome b can be recorded in the region of the Soret band with little interference from α and ε (1 ε) for the State 3 to 4 to 3 transitions (9) recorded here. Also, the effect of the 430 μm flashes upon the fluorometer are avoided by the "time-sharing" principle described previously (49). In these experiments, we have recorded cytochrome b because it shows large changes in its steady state level upon addition of calcium as well as ADP (9). Light scattering changes are recorded by the "reference" beam of the dual wave length spectrophotometer, and suitable reference wave lengths for this purpose are 575 μm, as used in Fig. 11, or 540 μm.

The sensitivity of the fluorometer for DPNH was calibrated by absorbance measurements with the dual wave length spectrophotometer at 310 to 674 μm. The value of Δε340 – 374 = 4.7 mm−1 cm−1 was employed.

**Registration of Hydrogen Ion Changes**—A technique for continuous recording with both the dual wave length spectrophotometer and the glass electrode has been described on other occasions (46, 52). In recording the release of hydrogen ions by the mitochondria caused by calcium addition, the "suspension" error of the glass electrode discussed by Bartley and Davies (44) has been carefully considered and controlled by simultaneous kinetic studies of pH changes with ~10−3 μ bromthymol blue as an indicator. Such indicators are sensitive to "protein error" (53). These errors are investigated here and are found insignificant, probably because both the electrode and indicator methods are used as "null" devices, in which only small departures of pH from the initial value are recorded (~0.1 pH). The recordings are in terms of changes of hydrogen ion concentration rather than absolute concentration and are calibrated by the addition of known concentrations of hydrogen ions. The rapid response of the indicator technique, as rapid as mixing in the ouette, shows the response of the glass electrode technique to be
adequate for these experiments (54). The calibration and use of the pH technique with mitochondrial suspensions and with chromatophores as well is described elsewhere (20, 55, 56).

With bromothymol blue as an indicator, and based on measurements at a wave length of 620 mμ with respect to an appropriate reference wave length of 560 mμ, a typical value of ΔO.D.:ΔH+ is 0.052 mμ−1 cm−1 for a buffer concentration of 4 mμ Pι, and a protein concentration of 2 to 5 mμ per ml. This sensitivity is adequate for measuring pH changes due to the reaction of small concentrations (~100 mμ) of ADP and calcium with the mitochondria.

Measurements of Oxygen Utilization—Upon addition of low calcium concentrations (10 to 100 mμ) to relatively concentrated mitochondrial suspensions (2 mg of protein per ml), the interval of activated respiration may be as short as 3 seconds. Thus, accurate measurements of the respiratory rate in this interval require a response of the polarographic method in less than 1 second. This can be obtained with the vibrating platinum micro-electrode (57–59). Teflon- or polyethylene-covered electrodes may not be rapid enough for accurate measurement of the kinetics reported here. However, a single coat of collodion appears to be satisfactory from the standpoint of the response speed and gives a highly stable recording (50).

RESULTS

General Properties of Calcium-stimulated Respiration—Changes in respiratory activity and in the steady state oxidation-reduction levels of the respiratory carriers upon additions of low concentrations of calcium or ADP are indicated in Fig. 1A.3 In this case, rat liver mitochondria supplemented with succinate as substrate are employed. Cytochrome b oxidation is measured at 430 mμ with 410 mμ as the reference wave length; reduced pyridine nucleotide oxidation is measured from intensity of the fluorescence emission at 450 mμ in response to 366 mμ excitation. Time increases from left to right; the oxidation of cytochrome b and pyridine nucleotide components is recorded as an upward deflection, and oxygen concentration as measured polarographically by the vibrating platinum electrode, decreases as a downward deflection.

Upon addition of 72 mμ ADP to the succinate-glutamate-phosphate-supplemented mitochondria, respiration is activated to a rate of 4.2 mμ oxygen per second and proceeds until 26 mμ oxygen has been expended; the ADP:O2 value is 2.8, since succinate is the substrate. The rise of the trace, corresponding to the oxidation of cytochrome b and DPNH upon addition of ADP, is closely synchronized with the acceleration of respiration, and the fall of the traces is approximately half-maximal when the respiratory rate has subsided.

Upon addition of 102 mμ calcium, the respiratory rate is 5.5 mμ oxygen per second and respiration proceeds rapidly until 20 mμ oxygen is taken up. The Ca++:O2 value is 5.1. The Ca++:ADP value is 1.8. The response of the respiratory carriers to Ca++ is qualitatively indistinguishable from that due to ADP; slightly greater oxidation of cytochrome b and pyridine nucleotide occurs, as would be expected from the slightly greater respiratory activity elicited by calcium. The close relationship between the kinetics of DPNH and cytochrome b is characteristic of that between enzyme-substrate intermediates and the over-all reaction (60). But in mitochondria, a more complex system is involved in the case of cytochrome b and DPNH, the latter is clearly energy-dependent for its reduction (48), and the former is involved in the energy-linked pathway of mitochondria (61) and in the formation of a possible high energy intermediate (13, 62, 63). Thus, the rate at which these two components return to the State 4 level may be dependent upon the extent to which the concentrations of high energy compounds build up following the interval of activated respiration. This temporal discrepancy of cytochrome b and DPNH is somewhat greater with Ca++ than with ADP, presumably because Ca++ makes a greater energy drain on the mitochondria. This discrepancy becomes very large in the absence of added phosphate as discussed below.

Crossover Point for Calcium-activated Respiration—While the similarity of responses of cytochrome b and reduced pyridine nucleotide in Fig. 1A suggests a considerable similarity between the mechanism of respiratory activation by calcium and that elicited by addition of ADP plus phosphate, the "crossover point" (64, 65) for calcium addition would provide a more definitive indication that calcium activates electron transfer at the same energy conservation sites as do ADP plus phosphate (9). By repeating experiments similar to those of Fig. 1 at appropriate wave lengths for cytochromes a, b, c, flavoprotein, and reduced pyridine nucleotide, the steady state changes in response to calcium addition can be compared with those due to ADP. Table I gives the percentage changes of the oxidation-reduction state upon addition of either ADP or calcium to rat liver mitochondria supplemented with succinate at 26°C. These percentages represent the ratio of the optical density change caused by the addition of calcium or ADP to the total density change observed in the "completely oxidized" (State 2) to "completely reduced" (State 5) transition. In the case of the energy-linked pyridine nucleotide reduction, States 4 and 5 values are nearly identical (48).

With ADP as an activator of electron transport, cytochromes a and c are more reduced (+); cytochrome b, flavoprotein, and pyridine nucleotide are more oxidized (−). The same sequence is observed with calcium as activator, and only small quantitative differences are noted, i.e., a larger oxidation of reduced cytochrome b and reduced pyridine nucleotide is consistent with the greater rate of electron transfer obtained in the presence of calcium (cf. Fig. 1A). Thus, an identical crossover point between cytochromes b and c is obtained with calcium and with ADP, suggesting that these energy-conserving components react similarly with the two substances. We have observed that ubiquinone is 30% more oxidized in pigeon heart mitochondria in the presence of ADP and Pi (66), and Szarkowska and Klingenberg (67) have shown a similar oxidation with Ca++ addition in rat heart mitochondria. Although experiments with calcium have not been carried out in the presence of azide to identify the crossover point between cytochromes c and a (68) or under conditions that would reveal a crossover point between flavoprotein and DPNH (65), the similarity between the response of cytochrome c and flavoprotein to calcium and their response to ADP (Table I) suggests that these crossover points would also be operative with calcium as well. The data do not, however, rule out slightly different rates of calcium reaction with the different energy conservation sites; the data below appear to be consistent with kinetic differences between ADP and calcium.

Response to Calcium in Presence of Oligomycin—Figs. 1B and

1 This experiment was carried out in collaboration with Dr. B. Chappell in 1963.
Reaction of Calcium with Mitochondria

**Fig. 1.** A, effect of low concentrations of ADP or calcium upon respiratory activity (trace labeled \( \text{O}_2 \)) and the oxidation-reduction states of cytochrome \( b \) (trace labeled \( b' \)) and pyridine nucleotide (trace labeled PN) in a suspension of rat liver mitochondria. The upward deflections of the traces \( b \) and PN correspond to oxidation of reduced cytochrome \( b \) and reduced pyridine nucleotide. Time proceeds from left to right, and the concentration of the added agent is indicated in micromolar units. The increments of oxygen taken up in the activated state of respiration (State 3) is indicated as 28 and 20 \( \mu \text{M} \). The system contains 2 mg of protein per ml, 20 mM Tris-chloride, and 80 mM KCl in the reaction medium. The concentrations of phosphate and succinate are 5 mM. The volume of the reaction mixture is 3 ml, temperature 26\(^\circ\), pH 7.5 (Experiment 782A-3). B, the response of respiratory activity, cytochrome \( b \), and pyridine nucleotide in the presence of 11 \( \mu \text{g} \) per ml of oligomycin. Experimental conditions are similar to those of A except for increased sensitivity for cytochrome \( b \) and pyridine nucleotide. The oxygen traces are numbered with the amount of oxygen (micromoles per liter) used up upon each addition of calcium and with the initial rate of oxygen utilization (Experiments 784 to 785 III).

**Table I**

_Crossover point for Ca\(^{++}\)-activated electron transport in rat liver mitochondria_

The reaction mixture contained 0.25 mM sucrose, 0.01 mM phosphate, 0.01 mM succinate, 0.15 to 0.20 mM Ca\(^{++}\), and rat liver mitochondria (approximately 1 mg of protein per ml) (Experiments 474 and 476 II).

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<th>Change in steady state with</th>
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</tr>
<tr>
<td>Cytochrome ( a )</td>
<td>+7</td>
</tr>
<tr>
<td>Cytochrome ( c )</td>
<td>+9</td>
</tr>
<tr>
<td>Cytochrome ( b_0 )</td>
<td>-16</td>
</tr>
<tr>
<td>Cytochrome ( b_2 )</td>
<td>-39</td>
</tr>
<tr>
<td>Flavoprotein</td>
<td>-10</td>
</tr>
<tr>
<td>Pyridine nucleotide</td>
<td>-40</td>
</tr>
</tbody>
</table>

\( * \) Versene, 0.1 mM; Mg\(^{++}\), 0.5 mM.

2A illustrate responses to calcium in the presence of amounts of oligomycin sufficient to block the reaction with ADP and phosphate but not that with calcium and uncoupling agents (11, 31, 37, 46). No qualitative differences in the responses of cytochrome \( b \) and DPNH to calcium in the presence and absence of oligomycin are observed. Uncoupling agents would, of course, cause the same rapid oxidation of the respiratory carriers but the reaction would be irreversible. In fact, divalent cations are the only substances yet discovered that reversibly activate the oligomycin-blocked system. In Fig. 1B the amounts of the oxygen and the oxygen uptake rate are indicated by the numerals adjacent to the platinum electrode trace. The Ca\(^{++}\):\( \text{O}_2 \) values for the three additions are 5.6, 4.3, and 5.5 for succinate as a substrate, reasonably close to the values in the absence of oligomycin (Fig. 1A).

The charts show the high sensitivity of the optical method in detecting the response of the respiratory carriers to low concentrations of calcium (36 \( \mu \text{M} \) in Fig. 1B and 14 \( \mu \text{M} \) in Fig. 2A). Thus, it is now possible to titrate the mitochondria with calcium in a much lower concentration range than was previously possible. The response of cytochrome \( b \) in rat liver mitochondria is half-maximal at 50 \( \mu \text{M} \) (11). However, this procedure may actually over-estimate the half-maximal value, since the asymptote of the titration is difficult to reach because of the secondary uncoupling effects of large calcium concentrations.

In pigeon heart mitochondria (Fig. 2A), the high affinity for Ca\(^{++}\) is indicated by the response of cytochrome \( b \) to 14 \( \mu \text{M} \) Ca\(^{++}\); the half-maximal effect is observed with 40 to 45 \( \mu \text{M} \) Ca\(^{++}\) (11). The DPNH response can scarcely be observed at the lowest Ca\(^{++}\) concentration and the large increase of the DPNH response between 28 and 72 \( \mu \text{M} \) Ca\(^{++}\) relative to the cytochrome \( b \) response suggests a site selectivity for Ca\(^{++}\), favoring cytochrome \( b \). At 72 \( \mu \text{M} \) Ca\(^{++}\), the cyclic oxidation and reduction of cytochrome \( b \) and DPNH occur as in the case of rat liver mitochondria, and the oxygen trace shows a stimulation of respiration from 1.7 to 4.2 \( \mu \text{M} \) oxygen per second. The increment of oxygen uptake is 13 \( \mu \text{M} \), oxygen, a Ca\(^{++}\):

\( \text{O}_2 \) value of 5.5, similar to that for the rat liver mitochondria. These Ca\(^{++}\):

\( \text{O}_2 \) values are characteristic of succinate oxidation; glutamate is not rapidly oxidized in pigeon heart mitochondria.

**Response of Respiratory Carriers in Presence of Oligomycin and Amytal**—Fig. 2B shows responses to calcium in pigeon heart mitochondria in which both Amytal and oligomycin are present. In this case, the responses of cytochrome \( b \) are somewhat larger...
Fig. 2. Responses of reduced cytochrome b and pyridine nucleotide of pigeon heart mitochondria to additions of calcium in the presence of 11 μg per ml of oligomycin (A) and in the presence of 11 μg per ml of oligomycin and 1.4 mM Amytal (B). Pigeon heart mitochondria (1.6 mg per ml) was suspended in mannitol-sucrose-Tris medium and supplemented with 6.7 mM phosphate, 4 mM glutamate, and 4 mM succinate. The rates of oxygen utilization are indicated in micromoles per liter per second. Other conditions are as in Fig. 1 (Experiments 785 to 789 III).

It is of considerable theoretical interest to note in Fig. 2A where only oligomycin was present. This result is in accord with our previous work, which indicated that the response of cytochrome b to ADP is larger in the Amytal-inhibited system (61) in which DPN is no longer acting as an electron acceptor. At the lower concentrations of calcium, the response of DPNH is almost undetectable (69). Also, at each value of Ca++ concentration the response of cytochrome b is larger and of longer duration than the response observed in the absence of Amytal.

For example, the duration of the "cycle" (P_una - F_una ; see Equation 1 below) is doubled. Some response of DPNH is observed at 72 μM Ca++ in spite of 1.4 mM Amytal. This result is in accord with our general view that Amytal is an inhibitor of the reaction of ADP and phosphate with the high energy mitochondrial intermediates, but it is not a complete inhibitor of the reaction with Ca++ which presumably acts at a point closer to the respiratory chain than the site of Amytal inhibition (69). Thus, Ca++ "breaks through" the Amytal block as in the case of our previous studies with uncoupling agents (69).

It is of considerable theoretical interest to note in Fig. 2B that cycles of the respiratory carriers are observed in the absence of measurable changes of respiratory rates (70). First, succinate oxidation is partly blocked by Amytal (69), and acceleration of respiration upon addition of Ca++ is not possible. Second, the Ca++ addition acts on the oxidation-reduction state of the carriers largely by altering the level of the high energy intermediates instead of by changing the flux through the electron transport system. This result emphasizes the general idea that the oxidation-reduction states of the carriers may respond either to changes of flux or to changes in the level of high energy intermediates of the chain, as in the case of cytochrome b and DPNH.

These two records illustrate the reactions of calcium with intermediates closer to the respiratory chain than those involved in the phosphorylation of ADP. The specific responses of the respiratory carriers to the addition of Ca++ under these conditions verify that energy loads are imposed upon mitochondrial high energy intermediates.

Speed of State 4 to 3 Transition—Since calcium activates respiration to a higher degree than do ADP and phosphate, it is of interest to determine whether or not the State 4 to 3 transition is more rapid in the presence of calcium than in the presence of ADP and P1. Klingenberg (71) finds at 18° that the State 4 to 3 transition for DPNH has a half-time of 0.6 second with Ca++ and 1.7 seconds with ADP. In our experiments at 23°, addition of calcium or ADP in a cuvette gives insufficiently rapid mixing. We have, therefore, employed the regenerative flow apparatus for this measurement (72). Experimental results are shown in Fig. 3A (ADP addition) and Fig. 3B (calcium addition). In this apparatus the mitochondria are diluted, supplemented with succinate and phosphate, and placed in the main syringe. ADP is added by means of the auxiliary syringe of 0.0125 the volume of the main syringe. The initial concentration of ADP is 8 mM; the final concentration in the observation tube is 100 μM. The syringes are driven pneumatically (73). The moment of starting the flow is indicated by the downward deflection of the bottom trace of Fig. 3, and the sharp break to the horizontal portion of the trace indicates when the flow stops. The measured time during the flow in this apparatus corresponds to approximately 2 milliseconds after mixing, and the stopping time is about 10 milliseconds. The upward deflection of both traces when the flow stops indicates the progress of the reaction in which cytochrome b becomes more oxidized following the addition of ADP (100 μM) or calcium (100 μM). From the time markers on the top trace (small marks, 0.1 second) it is apparent that the steady state change is half complete in about 0.07 second for both ADP and Ca++ addition at 26°. In short, we find that both ADP...
and Ca++ cause very rapid changes of the steady state level of cytochrome b. The initial slopes of the reactions with ADP and Ca++ differ slightly, the rate being about 30% more rapid with Ca++ than with ADP. The reaction rates for cytochrome b are much faster than those for DPNH reported by Klingenberg (71), and the DPNH kinetics reported by him should be re-examined with the rapid flow apparatus. Preliminary experiments with pigeon heart mitochondria also show a rapid response of cytochrome b, and it is probable that rat heart mitochondria would respond similarly in view of the similarity of steady state responses to Ca++ of rat and pigeon heart mitochondria.

Stoichiometric Aspects—The activation of respiration by ADP or by Ca++ provides a method for determining the relative stoichiometry of Ca++ and ADP. The effects of calcium and ADP were compared on the basis of the amount of oxygen taken up according to the oxygen electrode during the sequential additions of ADP and calcium. Values of the Ca++:ADP of 2.2 were obtained previously (2, 11).

This comparison is made more accurately in terms of the area under the curve of the “cycle” of oxidation and reduction of the respiratory carrier. The validation for employing the area under the oxidation-reduction curve of an enzyme-substrate intermediate in the calculation of the amount of substrate expended was provided in 1943 (60) when the simple formula for computing the area under the curve for the enzyme-substrate intermediate was derived (k2 is the first order constant for the velocity of breakdown of the intermediate; Pmax is its maximal concentration; x0 is the concentration of the substrate added; and t1/2 is the time from 1/2 rise to 1/2 fall in the concentration of the intermediate).

\[ k_2 = x_0 / P_{max} t_{1/2} \]

The equation was tested in that case by mechanical differential analyzer solutions for the enzyme-substrate kinetics and subsequently, in greater detail, by mathematical analysis (14). This method, applied to our first traces of 1955,9 gives a Ca++:ADP value of 1.7, which is consistent with subsequent studies presented here or obtained by chemical methods (29, 30).

Measurements of Oxygen Utilization—By measurement of the oxygen utilized on calcium addition in the preceding figures, we have observed Ca++:O2 values of 4.3 to 5.6 with an average value of 5.2 for succinate oxidation. Fig. 4 indicates a series of experiments over a wide range of Ca++ concentration. Increasing amounts of calcium are added to rat liver mitochondria and the fast oxygen uptake is measured by a platinum electrode, the rat liver mitochondria being supplemented by succinate and glutamate. The data are recorded in the presence and absence of added phosphate. The initial slope of the trace closely corresponds to Ca++:O2 = 6 as compared with the value of 5.2 above. The larger value is due to the glutamate present. It is apparent that the Ca++:O2 value will reflect the phosphorylative activities of the mitochondria and hence will be a variable quantity, whereas the Ca++:ADP value should be a true stoichiometric factor and is, therefore, to be preferred in calculation of the stoichiometry.

The maximal amount of oxygen that is expended during the interval of calcium-stimulated respiration is indicated to be 80 μM in the presence of phosphate and 20 μM in the absence of phosphate in Fig. 4. Converting these values to a protein basis, we obtain 60 and 15 μmoles of oxygen per mg of protein. At 5 calcium atoms per oxygen atom these values convert, respectively, to 300 and 75 mmoles of Ca++ per mg of protein. These values correspond to a smaller uptake of cation than is obtained in the presence of ATP where about 3 μmoles per mg of protein are accumulated (32).

Effect of Temperature—The effect of temperature upon the efficiency of the calcium reaction has been investigated between 26° and 44° (Table II). In this table ADP:O2, Ca++:O2, and Ca++:ADP values are included. The Ca++:O2 values increase

8 The substrate present in the mitochondrial preparation employed in the 1955 (2) experiment was not mentioned in the further discussion of this experiment in 1963 (11); 10 mM glutamate was present. The respiratory control of the guinea pig liver preparation has been noted elsewhere (10).
only slightly from 26–38° but decrease to less than half at 44°. The Ca++:ADP values are approximately constant at 26° and 38° but drop at 44°. This may well indicate a change in the calcium stoichiometry. However, kinetic factors also are involved; for example, at 44° the respiratory activity with calcium falls relative to that with ADP.

Kinetics of Hydrogen Ion Production, Oxygen Utilization, and Reduced Pyridine Nucleotide—It was observed some time ago (20, 22) that a rapid hydrogen ion release accompanies the activation of respiration by calcium; an experiment illustrating this point is indicated in Fig. 5. This record illustrates the use of the glass electrode in recording hydrogen ion changes due to ADP and calcium and shows the relationship of these effects to oxygen utilization and to the responses of the respiratory carriers. Guinea pig kidney mitochondria are treated with 4 mM succinate (arrow), and energy-linked DPN reduction occurs, giving an increase of absorption at 340 relative to 374 µ. Upon addition of ADP, the DPNH is rapidly oxidized (State 3) and returns to the initial highly reduced state (State 4) as the ADP is exhausted.

The glass electrode trace, after a brief deflection due to an inequality of the pH of the ADP solution and the reaction medium, runs parallel to the oxygen trace and indicates an uptake of hydrogen ions due to the phosphorylation of ADP. The oxygen utilization is 54 µ and hydrogen ion uptake is 160 µ corresponding to ADP:O₂ of 3.7 and ADP:H⁺ of 1.3 (compared with 1.1, pH 7.4, 26° (26)) for guinea pig kidney mitochondria with succinate or a substrate. Upon addition of 133 µ calcium, DPNH is oxidized and respiration is accelerated. Now there is a release of hydrogen ions which occurs almost as rapidly as hydrogen ions were taken up during phosphorylation of ADP. The increment of oxygen is 186 µ and of H⁺ concentration change is 120 µ. This change in the kinetics of hydrogen ion concentration occurs in spite of the similarities in the response of respiration and of respiratory carriers, and underlines the basic difference between the phosphorylation of ADP and the accumulation of cations. The Ca++:O₂ value is 8.3; the Ca++:H⁺ value is 1.1, and the Ca++:ADP value is 2.2. The value of Ca++:H⁺ approximates that obtained by Saris (20), by Brierley, Murer, and Backmann (75), and by Engstrom and DeLuca (26).

The second addition of calcium fails to stimulate the respiration to the extent of the first addition but does give the same Ca++:O₂ and Ca++:H⁺ values. Again the responses of the respiratory carriers clearly indicate the energy load imposed by the calcium reaction, although respiration is only slightly accelerated.

Measurements of Acid Production with Indicator Method—In our study of the glass electrode method it was considered essential to employ an independent method to ensure that “suspension error” (44) would not have a significant effect. The colorimetric method for mitochondrial suspensions has been employed. In

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**TABLE II**

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<th>Temperature</th>
<th>ADP+O₂</th>
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<td>µmole/mg protein/min</td>
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Fig. 5. The uptake and release of hydrogen ions in oxidative phosphorylation and calcium accumulation. Beginning at the left-hand portion of the chart, and reading from top to bottom, traces are oxygen utilization, reduced pyridine nucleotide (PN), and glass electrode recordings. As indicated in the right hand margin of the chart, a decrease of pH and oxidation of reduced pyridine nucleotide are indicated as an upward deflection and a decrease of oxygen concentration is indicated as a downward deflection. The optical path length is 1 cm and the change of DPNH concentration is computed from the increment of extinction coefficient 4.7 nm⁻¹ cm⁻¹. The rate of oxygen utilization and of hydrogen ion release or accumulation is computed from independent calibrations with HCl and expressed by the numbers adjacent to the traces in terms of micromoles per liter per second. Guinea pig kidney mitochondria: reaction medium contains 0.25 M sucrose, 4 mM phosphate, and 5 mM magnesium, pH 7.4; 4 mM succinate was added as indicated in the figure. The experiment was carried out in collaboration with Dr. Helen Conrad Davies and is recorded with the same equipment as for Fig. 1 of Reference 46 (Experiment 756-1 II).

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3 Experiments were done in collaboration with Dr. Helen Conrad Davies in 1957.

4 In our preliminary experiments in 1955 (2) we noted that the responses of DPNH and of respiratory activity to Ca++ were equal or greater than those to ADP with guinea pig liver mitochondria (10). This is not the case with the guinea pig kidney mitochondria preparation used in this series of experiments. The calcium-stimulated rate was less than the ADP-stimulated rate, probably because the mitochondria were more sensitive to the inhibition phenomena of Reference 2 than the liver preparations. Nevertheless, the preparation is suitable for a demonstration of the responses of hydrogen ion release to Ca++ or ADP.

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**Fig. 6** Bromothymol blue is employed to indicate changes of hydrogen ion concentration in response to the addition of ADP and calcium. Addition of 200 µM ADP and its phosphorylation causes a hydrogen ion decrease of two large scale divisions. Upon addition of 166 µM calcium, the trace deflects abruptly upward (two large scale divisions); the hydrogen ion release is identical with the hydrogen ion uptake caused by 200 µM ADP. The calibration shows the effect of 90 µM phosphoric acid; 65 µM HCl gave the same deflection corresponding to an ADP:H⁺ value of 1.2 and a Ca++:H⁺ value of 1.0.

A second addition of 166 µM calcium gives an increment of approximately 16 scale divisions, but the ATPase has been activated so that a continuous production of acid occurs. Thus, the third and fourth additions of calcium scarcely accelerate the slow production of hydrogen ions, and there appears to be a limit to
In this case, cytochrome b and DPNH decline only slowly from oxidations. Independent measurements of the hydrogen ion release (76). There is probably no qualitative differences in the response of mitochondria to Ca++ and Sr++. (12). This experiment indicates the need for substrate for the proton release from the mitochondria, whether or not phosphate is present.

Substrate can be an important factor in the amount of calcium uptake by mitochondria, as illustrated by Fig. 7B (see also Reference 20). In State 4 (succinate-glutamate) the two curves essentially duplicate Fig. 7A. In State 1, the slope is approximately the same but only 100 μM is taken up when a plateau is reached. This experiment indicates the need for substrate for the proton release from the mitochondria, whether or not phosphate is present.

**Reactions with Calcium in Absence of Added Phosphate**—In Fig. 8, rat liver mitochondria, supplemented with succinate but free of added phosphate, respond rapidly to the addition of 188 μM calcium which gives increased oxidation of reduced pyridine nucleotide and of cytochrome b. The small rapid burst of respiration reaches a velocity of 6.1 μM O₂ per second but at the end of 5 seconds has subsided to the State 4 rate. It is important to note that ADP causes a scarcely measurable effect under these conditions. Independent measurements of the hydrogen ion release show it to be closely synchronized with the respiratory activity. In this case, cytochrome b and DPNH decline only slowly from their oxidized states and the reduced state for cytochrome b is reached only after approximately 25 seconds. Reduced pyridine nucleotide proceeds to a more highly fluorescent state which may be due to either increased light scattering or tighter binding of the reduced pyridine nucleotide (see below). With the second addition of 158 μM calcium, an even shorter burst of respiration is observed at a rate of 5.1 μM O₂ per second. In this case, the carriers, cytochrome b and reduced pyridine nucleotide, fail to return to their initial reduced states (cytochrome b declining only slightly and DPNH becoming more oxidized). Here we have a distinctive discrepancy between the oxidation state and electron flow rate; the energy state of the mitochondria here appears to be a dominant factor in the control of the oxidation-reduction level of pyridine nucleotide and cytochrome b.

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**Fig. 6.** Illustrating the use of the indicator method for the measurement of the kinetics of hydrogen ion accumulation in oxidative phosphorylation on addition of ADP and hydrogen ion release from the mitochondrial suspension on addition of calcium. Guinea pig kidney mitochondria are suspended in the same medium as in Fig. 5 and supplemented with 4 mM glutamate. Approximately 10 μM bromthymol blue, initial pH approximately 7.2. Reference and measuring wave lengths as well as absorbance calibration are indicated in the diagram (Experiment 760-2,3 II).

**Fig. 7. A,** measurement of the extent of hydrogen ion release in rat liver mitochondria by the glass electrode technique (see Fig. 5) in the presence and absence of phosphate (2 mM). The reaction medium is 20 mM Tris-HCl, pH 7.4, and 80 mM KCl; substrate, 2 mM succinate-2 mM glutamate (Experiment RO-429,410 III). B, the effect of metabolic state and the presence and absence of phosphate upon the hydrogen ion release of rat liver mitochondria, as measured by the glass electrode. Mitochondria in State 1 (endogenous substrate only) with or without 2 mM phosphate as indicated in the diagram. State 4 mitochondria supplemented with 2 mM succinate-2 mM glutamate with or without 2 mM phosphate. The sensitivity of the medium to changes of hydrogen ion concentration were calibrated individually in the four studies. Other conditions as in Figs. 5 and 7A (Experiment RO-410 III).
At this point the addition of 1.8 mm phosphate causes an abrupt jump of cytochrome b and DPNH to a highly oxidized state, together with a burst of respiration at 5.0 μM O₂ per second. As the rapid respiration rate subsides, the reduction of cytochrome b proceeds rapidly to the initial reduced state and is closely followed by the reduction of pyridine nucleotide. It should be noted that while cytochrome b returns nearly to the initial reduced state, pyridine nucleotide returns to a somewhat less fluorescent state than after the first addition of calcium. The third addition of calcium (103 μM) elicits respiration at 5.6 μM O₂ per second and a cycle of cytochrome b oxidation-reduction of cytochrome b and DPNH.

These data suggest that oxidation-reduction states of the respiratory carriers unrelated to the electron transfer activity can be obtained in the phosphate-depleted cytosol. The small burst of respiration observed on the addition of calcium in the absence of added phosphate represents the utilization of an endogenous material. The fact that a second, later addition of Ca²⁺ causes a second burst of respiration suggests that not all the material is depleted in the first addition of calcium.

While the initial addition of calcium to the mitochondria results in a state in which pyridine nucleotide is more fluorescent and cytochrome b is highly reduced, a different state is achieved after the second addition of calcium. Presumably, these two different states are representative of the two different effects of calcium, one involving its uptake by the mitochondria, and the other involving its uncoupling of the mitochondria. The highly fluorescent state after a first addition of calcium is identified with the uptake of Ca²⁺, the depletion of an endogenous substance, and the binding of Ca²⁺ to the available sites. The lower fluorescent state following the second addition of Ca²⁺ indicates that the amount of calcium added has exceeded the calcium-binding capacities of the mitochondria, the free calcium acting as an uncoupling agent. This view is strengthened by the effect of phosphate addition; both cytochrome b and pyridine nucleotide return to their initial reduced states. Also, addition of ethylene glycol tetraacetate to the uncoupled state causes a rapid return of the carriers to their levels after the first addition of Ca²⁺.

In pigeon heart mitochondria, supplemented with succinate and glutamate, the addition of 158 μM calcium in the absence of added phosphate causes a small amount of rapid respiration (Fig. 9A); only 5 μM O₂ (compared with over 10 μM in Fig. 8) is expended since the pigeon heart mitochondria are deficient in phosphate relative to the rat liver mitochondria. Cytochrome b and DPNH jump to steady states which are stable in this case and show very little tendency to return to their initial reduced states. Addition of 3.6 mm phosphate causes both carriers to become oxidized. Simultaneously, the respiration rate increases to 3.3 μM O₂ per second. Following the slowing of respiratory activity, both carriers return to a less reduced state. A second addition of 150 μM calcium gives a respiratory activation of 3.5 μM O₂ per second and corresponding cycles in cytochrome b and DPNH.

The right-hand portion of Fig. 9 illustrates a response of the Amytal-blocked system to calcium in the absence of added phosphate. The initial addition of 158 μM calcium causes a small oxygen utilization and a very large and rapid oxidation of cytochrome b, the characteristic response of cytochrome b in the Amytal-blocked system (61). There is no measurable oxidation of DPNH, and there is no indication of return of cytochrome b or DPNH trace to the reduced state. Upon addition of 3.6 mm phosphate, cytochrome b is rapidly oxidized. Also, a small DPNH oxidation occurs. With the slowdown of respiration, there is a reduction of cytochrome b and DPNH. A second addition of 158 μM calcium causes a large cycle of cytochrome b and a small cycle of DPNH together with stimulated respiration. Thus, pigeon heart mitochondria show an even greater discrepancy between the oxidation-reduction state and the respiratory activity in the phosphate-depleted mitochondria.

**Response of Herring Gull Mitochondria to Calcium**—It is of considerable interest to compare the response to calcium of liver, kidney, and heart mitochondria with the response of mitochondria from the organ which has the highest sodium transport activity yet recorded (47). We recorded in Fig. 10A the response of the respiratory carriers of a suspension of mitochondria isolated from the salt gland of the herring gull to ADP and calcium. A response of cytochrome b and DPNH to 140 μM ADP is a typical oxidation-reduction cycle, and the rate of ADP phosphorylation calculated from Equation 1 corresponds to 6.0 μM per second. A second addition of 72 μM Ca²⁺ gives a shorter cycle of oxidation, and the rate of the calcium uptake, calculated as before, is

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**Fig. 8.** Responses of respiratory carriers and respiratory activity to additions of calcium in the absence of added phosphate. Aerobic rat liver mitochondria, 3 mg of protein per ml in 80 mm KCl, 20 mm triethanolamine-HCl, pH 7.4, 25°. Additions of reagents are indicated in the diagram as are the sensitivity calibrations. The initial rates of oxygen utilization are indicated in units of micromoles per liter per second (Experiment 782A-7 111).
**Reaction of Calcium with Mitochondria**

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**Fig. 9.** Responses of respiratory carriers and respiration rate of pigeon heart mitochondria to calcium in the absence of added phosphate. Pigeon heart mitochondria (1.5 mg of protein per ml) suspended in mannitol-sucrose-Tris reaction medium. Glutamate (4 mm) and succinate (4 mm) are present. The increments of oxygen expended in the rapid phase of respiration are indicated in units of micromoles per liter. The rates of oxygen utilization are indicated in units of micromoles per liter of oxygen per second in all cases except for the first addition of calcium in A and B where the rates cannot be accurately measured. Experiment carried out in collaboration with Dr. Brian Chappell (Experiment 77-3, 5 III).

**Fig. 10.** Responses to ADP and calcium of the respiratory carriers of mitochondria prepared from the salt gland of herring gull mitochondria in the presence (left) and absence (right) of phosphate. Mitochondria are suspended in mannitol-Tris-sucrose medium pH 7.4, 33°. In A, protein concentration is 0.90 mg per ml. In B, protein concentration is 0.93 mg per ml. Other conditions similar to those of Figs. 8 and 9 (Experiment 782-21, 22 III).

9.5 µm per second. The ratio of these two quantities, Ca++:ADP, is 1.6, a value in accord with those previously obtained for the other types of mitochondria. In other words, these mitochondria isolated from the highly active sodium transport system are no more or less efficient than those obtained from other tissues.

The effect of phosphate is illustrated in Fig. 10B, and it is seen in the absence of added phosphate that calcium causes oxidation of the two carriers as in the case of the heart mitochondria. Addition of 3.6 mM phosphate causes further oxidation of the two carriers and a return of cytochrome b almost to the initial level. Pyridine nucleotide, however, remains in a highly oxidized state, probably because of an uncoupling effect of some calcium remaining in the reaction medium.

**Relationship to Light Scattering Changes**—A better understanding of the phenomena illustrated by Fig. 8 is afforded by measurement of the light scattering changes in the mitochondria. As has recently been pointed out (1, 77, 78), two types of swelling may occur in mitochondria: the large amplitude studies, which have been described by Tapley (79), or the low amplitude swelling and contraction effects (77, 78). It is of interest that the energy-linked calcium-binding phenomenon is closely associated with the low amplitude changes and has little to do with the larger slow changes studied by other workers (1, 79). An experiment on this point is indicated in Fig. 11, in which pigeon heart mitochondria are used because heart sarcosomes are generally more appropriate for studying light scattering changes (77). The
of this component. There follow two additions of 103 trace, which is seen to lie at a higher level after the cyclic response

enough to cause a deflection of the base-line for the cytochrome b

servations. The light scattering changes, "low amplitude swell-

nucleotide and cytochrome b, in accordance with the previous ob-

duction of calcium an abrupt oxidation of DPNH and reduced cyto-

addition of 1.3 mM phosphate. The cycle of oxidation and re-

cumulative respiration by ADP as well and show increased light scattering in the active phase of respiration (State 3). This is a further similarity between the mitochondrial response to ADP and low concentrations of calcium. Shortly after the second addition of 158 μM calcium, the capacity of the mitochondria to accumulate calcium is exceeded; after an initial increase, the light scattering abruptly decreases and the trace proceeds off scale because of large amplitude calcium-induced swelling (1, 79). The pyridine nucleotide fluorescence diminishes to a level even lower than that observed in State 3. After a partial "cycle," the cytochrome response proceeds off scale because of the effect of the large light scattering change. In the presence of phosphate, the low amplitude swelling and contraction effects are closely related to the primary reaction of calcium with the mitochondria. The large amplitude changes appear to be related to secondary effects of Ca++, such as uncoupling of oxidative phosphorylation and damage to the mitochondrial membranes.

DISCUSSION

This paper emphasizes the reversible nature of calcium-stimu-
lated respiration and identifies it with the energy load of ion ac-

mulation imposed upon energy conservation intermediates of

the mitochondria. Since the reaction of calcium requires the most rapid functional depletion of high energy intermediates in

the mitochondria, its study reveals characteristics not only of ion accumulation processes of unusual interest, but of the kinetic re-

ponses of the electron transport and oxidative phosphorylation system to sudden energy demands. Both the experimental evi-

dence on the physical and chemical nature of the reaction and the role of the mitochondrial structure in the reaction are discussed.

Lastly, an attempt is made to evaluate whether the reaction is of

physiological significance.

General Characteristics of Reaction of Calcium with Mitochon-

dria—Under the experimental conditions employed in this study, four distinctive phenomena occur simultaneously upon addition of low concentrations of calcium or ADP and P1 to mitochondrial suspensions Coincident with the addition of either of these substances (in phosphate-supplemented mitochondria): (a) the

pigeon heart mitochondria are supplemented with succinate,
glutamate, and ATP to produce a highly reduced state (State 4).

We have observed differences in the response of mitochondria
to calcium addition in the absence of added phosphate depending upon two factors, the amount of an endogenous substance and
the energy state of the mitochondria (the latter is dependent to a
certain extent upon the endogenous substrates in the mitochon-
drial preparation). In Fig. 9, it is observed that on the first addition of calcium an abrupt oxidation of DPNH and reduced cyto-

chrome b occurs, and in Fig. 11 it is observed that a reduction of
cytochrome b and pyridine nucleotide occurs due to the added

ATP. It should be noted, however, that there is a very brief
"cycle" of oxidation of both these components which precedes their reduction (Fig. 11). Separate experiments similar to those of Fig. 9A showed that the state immediately obtained in Fig.

11A upon addition of calcium was obtained in roughly 1 minute under the conditions of Fig. 9A. In short, the difference between
the two experiments is attributed to the added ATP which per-

mits the completion of the Ca++ reaction in the absence of added phosphate (Steps 4 and 5 of Fig. 13). The light scattering in-

creases, as indicated by the downward deflection of the 575 μM trace. A second addition of 103 μM calcium still causes no appreciable oxidative response of the carriers but does cause small increases of fluorescence and light scattering. Activation of respiration and oxidation of the carriers is caused by the addition of 1.3 mM phosphate. The cycle of oxidation and re-
duction of pyridine nucleotide occurs although the State 4 value corresponds to less fluorescence, as was observed in the case of

Fig. 9. The light scattering change is definitely delayed with re-

spect to the carrier response (77), and in the course of ½ minute the light scattering considerably decreases. This change is large
eough to cause a deflection of the base-line for the cytochrome b trace, which is seen to lie at a higher level after the cyclic response of this component. There follow two additions of 103 μM calcium which cause cyclic oxidation and reduction of pyridine nucleotide and cytochrome b, in accordance with the previous ob-
servations. The light scattering changes, "low amplitude swell-
ing and contraction effects," are typical for cyclic activation of

Light scattering is measured in terms of absorbance changes at

575 μM (middle trace). Cytochrome b is measured at its α band

(562 to 575 μM), and pyridine nucleotide is measured fluoromet-

rically, 3 mg of protein per ml, pH 7.4, 23° (Experiment 782B-24

III).
The rate, which has been measured by rapidly mixing these substances with State 4 mitochondria, shows half-times of 70 milliseconds at 33°C. These short activation times indicate that any intervening processes between the arrival of calcium at the outer membrane of the mitochondria and its subsequent reaction with respiratory enzymes of the crista are either nonexistent or non-rate-limiting. The high speed of this reaction also indicates capabilities of the mitochondria for rapid participation in physiological functions, not only in the restoration of ATP levels but also in ion uptake processes in rapidly responding systems, as in contractile tissues.

Even at the time of our preliminary report in 1955, differences in the reaction of calcium and ADP with mitochondria were apparent. For example, the respiratory activity was approximately 50% greater in the presence of calcium provided the calcium concentrations were low enough not to cause respiratory inhibition (2, 11). Even from these data, it was apparent that calcium is more competent kinetically than ADP and probably reacts closer to the energy-conserving sites. While calcium has been classed as an uncoupling agent ever since 1949 (5), it was not apparent until our 1955 experiments that a basic difference existed. While both reactions impose a load upon the energy-conservation sites in mitochondria and cause a State 4 to 3 transition accompanied by an activation of respiration, low concentrations of calcium are expended in ion accumulation while the uncoupling agent, insofar as can be determined, is not. Also, in the reaction with the uncoupling agent, energy is dissipated, while in the reaction with calcium, energy is conserved in terms of ion accumulation (81).

The most striking difference between the calcium and ADP reactions is their phosphate requirements. The respiratory stimulation obtained in Fig. 8 on calcium addition in the absence of phosphate is nearly identical with that after addition of 1.8 mM phosphate; no comparable ADP response can be obtained in these mitochondria without added phosphate. Apparently the endogenous phosphate (~0.1 μmole per mg of protein) is not available, and a reactant other than phosphate is responsible for the primary reaction of Fig. 8. Recent data suggest that acetate and a number of anions can substitute for phosphate (16, 17).

Another striking dissimilarity between the effects of calcium and of uncoupling agents is their titration characteristics in altering the steady states of the respiratory carriers and the respiratory control. One may titrate the oxidation-reduction states and the respiratory rate with uncoupling agents (14) in a continuous fashion obtaining rectangular hyperbolas for the control characteristics (82). With uncoupling agents the respiration rate is constant unless oxalacetate accumulation occurs. With calcium, the respiration rate and steady states of the carriers can be titrated only in the transient phase, before calcium is expended. Thereafter, the steady states of the carriers and respiratory rate may be even slower prior to the addition of calcium. In short, there is no evidence whatsoever for a partial uncoupling by low concentrations of calcium. At higher concentrations of calcium the uncoupling event is observed. When the initial concentration of calcium exceeds a certain critical level and the respiratory rate, instead of accelerating further, somewhat slows down, reduced pyridine nucleotide is further oxidized and the respiration continues well beyond the point at which one would have expected all the calcium to have been accumulated. It is this event that has many points of similarity with that already described for uncoupling agents, dibromophenol in particular (46). It is probable under these circumstances that the mitochondria have become "leaky" and that calcium is being lost by mitochondria as rapidly as it is being accumulated.

Under appropriate experimental conditions, additions of either calcium or ADP can cause respiratory inhibition. In the ADP-inhibited state (83) a high degree of DPNH oxidation leads to an inhibition of electron transport. In the analogous situation observed on the addition of Ca++ or Mn++ to mitochondria buffered with Tris-chloride in the absence of phosphate, there is an oxidation of DPNH and respiratory inhibition. In this state an accumulation of a "high energy" form of cytochrome b is observed, a condition termed State 6 (13). It is apparent that the reaction mechanisms for ADP and calcium differ under these conditions since ADP-inhibited respiration is reactivated by the addition of glutamate, and this is not the case with Ca++ and Mn++, where phosphate addition reactivates respiration.

Another difference in the reaction of calcium and ADP discussed in detail below is the difference in the stoichiometric ratios to the high energy intermediate; 2 calcium atoms are required to...
cause a respiratory stimulation equivalent to that caused by 1 ADP molecule. The simplest explanation is that there is a common intermediate that has two binding sites for calcium and one site for ADP and phosphate; the energy requirement for the phosphorylation of ADP is double that for the accumulation of calcium.

Light scattering changes accompany the uptake of calcium by mitochondria and are similar to those observed upon addition of ADP (77, 78). In Tris-chloride medium and in the presence of phosphate, these changes are categorized as small amplitude shrinking and swelling phenomena in contrast with the large amplitude phenomena observed when the calcium accumulation exceeds the capabilities of the mitochondria producing very large amplitude light scattering decreases (1).

A difference in the light scattering properties has been revealed in recent experiments in acetate medium where calcium accumulation, instead of causing shrinkage, causes very rapid swelling (16, 17). Here the difference is attributed to the fact that an anion to which the mitochondria are permeable accompanies calcium accumulation, increases the amount of osmotically active material in the mitochondria, and causes the entry of water. This phenomenon has not yet been observed in ADP phosphorylation.

Finally, the reactivities of calcium and ADP with mitochondria at low temperature differs dramatically; the ADP reaction is almost completely inhibited at 7°C while that with calcium proceeds (32, 33). These three methods make possible the determination of four stoichiometric coefficients: Ca++:O, Ca++:phosphate, Ca++:H+, and Ca++:ADP. Although the last quantity does not represent the ratio of two substances which react with one another in calcium accumulation, it does represent the ratio of stoichiometric coefficients of calcium and ADP in their stimulation of oxygen utilization or in causing oxidation-reduction cycles of the respiratory carriers. The value of Ca++:ADP is easy to determine with precision since the end point of the pH change; however, it is customary to determine the Ca++:H+ value from the ratio of the initial amount of calcium added and the amount of NaOH needed to return the pH initially to the value prior to the addition of Ca++. One advantage of the Ca++:H+ value is that it is not a function of the phosphorylation efficiency, and values of approximately 1 obtained here are in general agreement with the value of Saris (1.2) (19), Engström and DeLucia (1.1) (26), and Brierley et al. (0.9) (75). The possibility that the production of H+ is not a constant parameter of the reaction of calcium with the mitochondria has been suggested in the work of Saris who finds that Ca++:H+ is lower (0.9) at high K+ concentrations and is 2.5 in the absence of potassium. This change is in agreement with our results in the absence of phosphate where the Ca++:H+ value is 1.7 (Fig. 7A). In acetate medium Ca++:H+ values of 2 to 3 can be obtained (16, 17). These results suggest that the Ca++:H+ value depends largely on the nature and function of the anion present. The fact that hydrogen ions are produced in condition, and determinations can be made with a double beam spectrophotometer at calcium concentrations in the region of 10^-6 to 10^-4 M.

The Ca++:ADP value is perhaps the most constant parameter of the four since it is independent of phosphorylation efficiency and has, as a matter of fact, been used to determine the efficiency of calcium uptake in mitochondria of little known phosphorylation properties (47). This method may also be independent of the relative ratio of the calcium and ADP reactions over even larger ranges than those investigated here. Values determined from the quotient of Ca++:O and ADP:O gave in 1955 a value for Ca++:ADP of 2.2 (2) and in 1959 a value of 2.1. The average of the determinations here is 1.9. If Equation 1 is used, the average of all values is 1.7 and the average value for the two methods is 1.8. The Ca++:ADP values are converted to a Ca++:O2 value of 6.5 by an ADP:O2 value of 3.6 for succinate or to a Ca++:O value of 11 by an ADP:O value of 6.2 for glutamate as a substitute for rat liver mitochondria (57).

The Ca++:O value is used by a number of workers because of the ease with which it is determined from the addition of a known amount of calcium and the measurement of oxygen uptake in the interval of stimulated respiration (57). It suffers, however, from a disadvantage compared with the Ca++:ADP determination; namely, the determination becomes inaccurate with small concentrations of calcium because of the shortness of the interval of stimulated respiration and the inaccuracies in determining the changes of small concentrations of oxygen. In addition, the Ca++:O value depends upon the phosphorylation efficiency of the mitochondria. A typical Ca++:O value is 5.8 (15); a Mn++:O value is 5.4 (12). In this work we have used succinate as the principal electron donor and find lower Ca++:O values than those who used DPN-linked substrates. For example, in Fig. 1, the Ca++:O value is 2.6 (Ca++:ADP = 1.8) for rat liver mitochondria oxidizing succinate, and in Fig. 5, the Ca++:O value is 3.8 for guinea pig kidney mitochondria oxidizing glutamate. It is obvious that the Ca++:O value is a highly sensitive indicator of mitochondrial efficiency, and it is recommended that it be used for this purpose, and not for the determination of the efficiency of Ca++:ADP

The H+ ejection appears to be closely synchronized with the interval of stimulated respiration (20) in reaction media such as that used here where chloride is the anion. The end point for the reaction of calcium or ADP may be determined from the end point of the pH change; however, it is customary to determine the Ca++:H+ value from the ratio of the initial amount of calcium added and the amount of NaOH needed to return the pH initially to the value prior to the addition of Ca++. One advantage of the Ca++:H+ value is that it is not a function of the phosphorylation efficiency, and values of approximately 1 obtained here are in general agreement with the value of Saris (1.2) (19), Engström and DeLucia (1.1) (26), and Brierley et al. (0.9) (75). The possibility that the production of H+ is not a constant parameter of the reaction of calcium with the mitochondria has been suggested in the work of Saris who finds that Ca++:H+ is lower (0.9) at high K+ concentrations and is 2.5 in the absence of potassium. This change is in agreement with our results in the absence of phosphate where the Ca++:H+ value is 1.7 (Fig. 7A). In acetate medium Ca++:H+ values of 2 to 3 can be obtained (16, 17). These results suggest that the Ca++:H+ value depends largely on the nature and function of the anion present. The fact that hydrogen ions are produced in

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6 A further study of Sr++ accumulation has recently appeared (70).
the presence or absence of phosphate suggests that hydrogen ion formation in the action of accumulated calcium with the phosphate is of secondary consequence to the primary release of hydrogen ions in accumulation of calcium. For this reason the hydrogen ion ejection seems to be less desirable than the Ca\(^{2+}\):ADP value.

The Ca\(^{2+}\):phosphate value has been determined most recently by Rossi and Lehninger (15) to be 1.6, and Mn\(^{2+}\):phosphate ratio has been determined by Chappell et al. (12) to be 1.8. A large number of determinations of the Ca\(^{2+}\):phosphate value have been carried out with extremely high calcium concentrations (29), and surprisingly enough the ratios \((1.67 \pm 0.03)\) are in the range obtained with the low calcium concentrations. As in the case of the Ca\(^{2+}\):O value, the Ca\(^{2+}\):phosphate value is dependent upon the phosphorylation efficiency of the mitochondria. In addition, the Ca\(^{2+}\):phosphate suffers from the drawback of the Ca\(^{2+}\):H\(^+\) value; the phosphate reaction can no longer be considered to be a primary step of calcium accumulation but a secondary reaction of accumulated calcium; calcium and phosphate need not be partners in stoichiometric reactions under all conditions.

In summary, we prefer the Ca\(^{2+}\):ADP value, since it relates the relative efficiencies with which the mitochondria react with calcium and ADP, and is a value which should be constant for a wide variety of preparations over wide ranges of conditions. Furthermore, it is probably the physiologically important value which determines the efficiency with which mitochondria participate in reactions with ADP and calcium in vivo. Finally, it can be determined with highest precision and over the widest range of conditions. It is therefore recommended that this value be used in describing the efficiency of the calcium reaction with mitochondria.

**Site of Action of Calcium**—The site of action of calcium can be considered from the biochemical and from the structural viewpoint. From the biochemical, it is of importance to define accurately the site in the sequence of energy-conservation reactions where calcium acts. From the standpoint of biological structure, it is important to know the place in the mitochondria where calcium is accumulated.

**Biochemical Aspects**—The most general result of our studies on the site of action of calcium is afforded by the crossover data in the State 4 to 3 transition observed on the addition of low concentrations of calcium. The fact that the crossover point observed for calcium addition is the same as that observed for ADP addition (64, 65) indicates that calcium interacts with the same energy conservation sites as does ADP, the only difference being a quantitative one; namely, it interacts more rapidly. Thus there is no "special calcium site" which is specifically involved in ion transport and not involved in oxidative phosphorylation. This is an important observation, not only for the mechanism of calcium action, but also for validation of comparison of the Ca\(^{2+}\):O\(_2\) value with the ADP:O\(_2\) value and for a calculation of the calcium transported "per site" (15). The possibility that the DPNH-flavin site provides a more effective intermediate for calcium accumulation than the other sites is suggested by the Amytal inhibition of the rapid accumulation of small concentrations of calcium (11). It is not clear that this represents a real difference in Ca\(^{2+}\) and ADP site specificity; the kinetic difference might well be observed with ADP if it stimulated electron transport to the extent that is observed in the presence of calcium.

The evidence presented here that calcium can react rapidly with the carrier intermediate in the absence of added phosphate suggests that the site of calcium action is at the level of the non-phosphorylated intermediate \((X \sim I)\), and this is the hypothesis that we prefer. However, it is important to point out that a calcium reaction at the \(X \sim P\) level must release the phosphate,

\[
Ca^{2+} \rightarrow X \rightarrow P \iff Ca^{2+} \sim X + P
\]

provided there is an acceptor for \(Ca^{2+}\); \(P\) is then recycled to form \(X\) or \(P\) again. This mechanism is, however, distinct from those in which calcium phosphate precipitation is postulated (33). Here calcium binding to the mitochondrial components is postulated, and their concentration limits the amount of calcium taken up in the absence of added phosphate (12).

The question does not seem further to be resolved by our early observations (2) that calcium stimulates respiration to a greater extent than does ADP or by the observation of DeLuca and Engström (31) that calcium accumulation is insensitive to oligomycin. The mechanism above would be operative in spite of inhibition of ATPase and exchange reactions.

In summary, calcium reacts with all three energy conservation sites of mitochondria, and at a point in the energy transfer pathway which is at the level of either the nonphosphorylated or phosphorylated intermediate. In the formulation presented below, the calcium reaction with the \(X \sim I\) intermediate will be indicated, particularly to emphasize that reaction with phosphate does not appear to be an important part of the calcium reaction, but not to eliminate the possibility of an \(X \sim P\) reaction in which the phosphate is recycled.\(^{16}\)

**Structural Aspects**—The role of mitochondrial structure in the function of calcium accumulation in mitochondria is identified in two ways. First, by a delineation of the integrity of mitochondrial structure necessary for calcium accumulation, and second, by electron micrographic evidence of the site of calcium accumulation.

Treatment of mitochondria with ultrasound yields submitochondrial fragments which so far have not yet been shown to carry out a calcium accumulation or to show calcium stimulation of respiration (75, 85).\(^{11,12}\) Such submitochondrial fragments may contain intact inner membrane subunits (86); pretreatment with 1 mM phosphate is required to remove the projecting units of rat liver mitochondria by sonic disruption (87). Nevertheless, no calcium accumulation or calcium stimulation of respirations is observed with such particles.

The recent observation (88) that digitonin particles accumulate calcium, although in reduced amounts compared with intact mitochondria, is difficult to interpret, since most authors did not report stimulation of respiration by calcium in the digitonin particles, nor have we found calcium stimulation of respiration of similar preparations which show respiratory control with ADP and \(P_I\) (16).\(^{6}\) The report is of further interest, since these digitonin particles are stated by Lehninger et al. (88) to be insensitive to uncoupling by excessive amounts of calcium. It is

\(^{9}\) Obviously, a \(Ca^{2+}\):P\(_i\) could be determined as is \(Ca^{2+}\):ADP, except the low affinity of mitochondria for \(P_i\) makes this impractical.

\(^{11}\) B. Chance, unpublished data.

\(^{16}\) E. Racker, personal communication.
obviously of great interest to determine whether calcium accumulation by rapid reaction with high energy intermediates of the energy transfer system occurs in the digitonin particles or whether the effect is similar to that observed by Slater and Cleland (7) in their studies of heart sarcosomes.

One important difference in the structure of the disrupted particles and intact mitochondria is the loss of material from the matrix space and of continuity of the inner membrane. This result in itself calls special attention to the inner membrane of mitochondria as the structural element barrier across which the calcium is accumulated.

Recent electron micrographic studies (35, 36) identify sites of accumulation of high concentrations of calcium within the mitochondria. There is general agreement that calcium accumulation occurs in the matrix space (36), and Peachey identifies the site with the “normal granules” of the matrix space (35).

The electron micrographs of Fig. 12 (courtesy of D. F. Parsons) illustrate possible sites for calcium localization in the matrix of intercristal space (ICS) of flight muscle mitochondrion (upper figure). In addition, the accumulation may well be in the base membrane structure (M). In the lower figure rat liver mitochondria are supplemented with 1 mM Ca++. The membrane structure now appears highly distorted and enlarged, as compared with intact membranes. We interpret this result to indicate that calcium has interacted with the base membrane itself. This type of distortion is also observed without added phosphate and at calcium concentrations less than those which cause light scattering decreases in the mitochondria. A possible interpretation of these data is that there are primary and secondary steps in calcium accumulation, that observed at low concentration being associated with the base to which the inner membrane subunits are attached and at higher concentrations with large areas similar to those described by Peachey (35) and Chance and Estabrook (88). Greenawalt and Vasington also suggest that specific sites on the cristae and the inner membrane are involved (90).

**Reaction Mechanisms for Calcium Accumulation**—Since kinetics, stoichiometry, chemical specificity, and intramitochondrial localization of the calcium reaction have been considered separately, it is now appropriate to outline a hypothesis which is consistent with these diverse data. Such a hypothesis cannot be considered to be a definitive reaction mechanism, but at least it provides a convenient framework for compiling the existing experimental data and for designing further experiments. This hypothesis is based upon the observation of other workers, particularly Saris (20) and Chappell et al. (12), and represents an evolved form of our earlier scheme (11). The first step in calcium accumulation is the passive diffusion of cations through the outer membrane of the mitochondria to appropriate primary binding sites of the inner membrane. This initial step would correspond to the calcium binding observed by Slater and Cleland (7) or the calcium retained by mitochondrial membranes observed by Saris (20) or the primary decrease of proton relaxation time of manganese accumulation measured by the spin-echo method (12). The reaction does not cause a measurable spectroscopic change. This initial binding site is also consistent with Klingenberg’s calculations (71) that indicate a binding site for calcium of an area larger than that corresponding to the outer membrane. Lastly, preliminary evidence that calcium can be accumulated by mitochondria in which the outer membrane is no longer intact (90) further supports this viewpoint. This reaction is indicated as Step 1 of Fig. 13 where 4 calcium ions are indicated to have become attached to the surface of the inner membrane or to the extensions of the inner membrane which form the cristae. This site for the initial binding of calcium is postulated to be the site of the crista which contains the projecting subunits. These inner membrane subunits are free of cytochrome (87, 91) but probably contain the ATPase “coupling factor” of Racker (92, 93). While this ATPase is oligomycin-insensitive itself, in the presence of a suitable factor, it becomes oligomycin-sensitive.

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13 B. Chance and D. Parsons, unpublished observations.
Therefore, we do not visualize the calcium reaction occurring at the level of the ATPase itself, but farther along the energy transfer chain at a point designated by \(X \sim I\), or \(X \sim P\), as discussed above.

In order to be consistent with the stoichiometry that \(X \sim I\) reacts in a 1:1 fashion with ADP and phosphate (64), but reacts in a 2:1 stoichiometry of calcium (2), we indicate two binding sites on \(X \sim I\) by the formulation \(Ca_2(X-I)\). Protons may not be directly involved, but they are so indicated here to be consistent with proton release on binding of calcium.

The second step then proceeds with the formation of an intermediate of the type \(Ca_2(X-I)\). We have indicated 2 protons to be released here, in accordance with a \(Ca^{++}:H^+\) ratio of 1. This value is observed with low concentrations of calcium in the absence of phosphate and in the presence of chloride.

The question of whether or not protein phosphate is formed in the reaction of calcium with \(X \sim P\) or \(X \sim I\) might be inferred from changes in phosphohistidine concentration on reaction of calcium with mitochondria (94). It is a matter of interest that X and I might be inferred or X and I might be recycled to function in "vectorial" transport of the carrier intermediate across the wall of the cristae.

Transport of the carrier intermediate across the wall of the cristae is indicated in Step 3. Any mechanism for "mobile carrier function" proposed in connection with other transport mechanisms is applicable here. We have already considered rotation and translation of these cytochromes to function in "vectorial" electron transport (64), and here we may add to this idea the possibility that the axis for electron and ion transport may be orthogonal. Thus, electron transport is in the plane of the membrane (either parallel or perpendicular to the long axis of the cristae) and cation transport is perpendicular to it. The large absorbance change observed in cytochrome b upon addition of cations to mitochondria (13) mentioned in the paragraph below can as well be attributed to cytochrome function in ion transport.

Rotation or translation of reduced cytochrome b may serve to bring the cation from one side of the cristae to the other.

For the sake of clarity we have indicated separately the transport and energy changes in Steps 3 and 4, but it is probable that they occur simultaneously. Step 4 indicates that the calcium is bound to phospholipid (12, 35) or other components of the cristae. At the same time the low energy components of the \(X \sim I\) compound are released and respiration is stimulated. Step 4 is identified by a large increase of proton relaxation time (12) and with a large absorbance increase of DPNH in cytochrome b under conditions of Fig. 11. It should be noted that the cytochrome changes are probably more accurate indicators of Step 4 than is the spin-echo method (12); the latter averages the effects of the four possible species of manganese: free \(Mn^{++}\) outside the mitochondria (\(e_0 = 1\)), membrane-bound \(Mn^{++}\) (\(e_0 = 5\) to 6), phospholipid-bound \(Mn^{++}\) of unknown \(e_0\), and phosphate-bound \(Mn^{++}\) (\(e_0 = 0.1\)).

Step 4 corresponds to two states of the respiratory carriers depending upon whether \(X\) and \(I\) accumulate or are recycled to form \(X \sim I\). In the first case, Step 4 is observable spectroscopically when the binding sites of Step 4 are occupied, when phosphate or acetate is absent, and when Steps 5 and 6 are not possible. Step 4 would correspond to a highly oxidized state of the respiratory carriers, particularly DPNH, as illustrated by Figs. 8, 9, and 10. In the second case, \(X \sim I\) accumulates and cations saturate Steps 2 and 3. Then Step 4 is identified spectroscopically by an increased fluorescence of reduced pyridine nucleotide (clearly shown in Fig. 8), which distinguishes Step 4 and Step 5, in which the fluorescence level is less (see Fig. 8).

It is possible that the proton release of Step 2 contributes to the oxidized state of the respiratory carriers by decreasing the intramitochondrial pH at the site of cation accumulation. Steps 2 and 3 are not spectroscopically identifiable in the presence of anion to which the mitochondria are permeable such as phosphate or acetate (17).

The relationship of Step 4 to State 6, observed especially clearly with manganese addition in the absence of phosphate...
and leading to DPNH oxidation and to the formation of a highly absorbing species of cytochrome b (13) is clarified by the considerations above where \( X \) and \( I \) can be recycled until Steps 2 and 3 are saturated with the cation and all the intermediates are in the \( X \sim I \) form. This is supported by the data of Fig. 11 which show a very brief cytochrome b oxidation on adding Ca++ in the presence of ATP and in the absence of added phosphate, followed by an increased reduction of cytochrome b.

At this point two reactions may ensue: first in Step 5, the reaction with phosphate causing a release of calcium from the mitochondrial membranes and the precipitation of calcium phosphate or hydroxyapatite (36) indicated here for 3 of the 4 Ca++ atoms. The inner membranes are freed of a calcium deposit by this reaction, and the larger calcium accumulation observed in the presence of phosphate is possible (32, 33). As the reaction is here formulated, additional hydrogen ions are released. The over-all \( \text{Ca}^{++} \cdot \text{H}^{+} \) value at this point of 0.6 is significantly lower than the value of 1 observed experimentally. A more complete equation is now available that gives \( \text{Ca}^{++} : \text{H}^{+} = 1 \) (17).

It should be noted that the steps listed as "4" here and as "2B" in our earlier publication (11) are identical and indicate calcium transport without the need for the expenditure of phosphate in the \( \text{Ca}^{++} \) accumulation. This mechanism appears adequately to explain the similar experimental results of Rosci and Lehniger notwithstanding their comments to the contrary (15) which are apparently due to a misunderstanding of our mechanism (11).

An alternative reaction mechanism (Step 6) is provided by more recent experiments which suggest that carboxylic acids such as acetate may enter the mitochondria with the calcium and neutralize the hydrogen ions released in Step 2 or 3 (16, 17). Whether the acetate reacts as well with the bound calcium cannot be determined at present, although preliminary estimates of the stoichiometry indicate 1 calcium atom per acetate atom (17).

The possibility that this over-all mechanism can function for monovalent cations was suggested some time ago by Saris' work (20) and has been stimulated by the work of Moore and Pressman (41), Rasmussen (45), and Christie et al. (43). Lastly, the chemical and structural relationships of the three sites of energy conservation and of cation transport are closely related to the biological potential divider proposed by R. E. Davies (95).

**Possibilities for Physiological Function**—Three aspects of the calcium reaction may be briefly considered in relation to the possibility of its functional activity in vivo. The first is the structural problem; namely, can the ionic gradients accumulated in mitochondria be communicated with suitable sources and sinks for ion accumulation? Second is the problem of thermodynamic requirements. For example, is a calcium pump efficient enough to accumulate a high ratio of \( \text{Ca}^{++} \) inside to the value outside? Third, there are the kinetic problems. Does the calcium reaction operate sufficiently rapidly to participate significantly in ionic redistributions to meet the needs of physiological function? These are the problems which have already been considered (albeit piecemeal) in connection with mitochondrial function in energy metabolism of the cell. They are, however, competing reactions that require a quantitative rather than a qualitative analysis. For example in the case of energy metabolism, it is necessary to determine what portion of the ADP phosphorylation is due to mitochondrial and glycolytic activity. Although structural factors have not yet been of great importance, certainly stoichiometric and kinetic factors have been of significance in determining whether ADP is phosphorylated by glycolytic or mitochondrial activities (in competition with glycolytic activity, with other phosphorylative activities, or with energy stores in the cell). Ultimately, such problems are resolved by direct determinations in vivo, as for example, afforded by mitochondrial response to single contractions in intact muscle (96-99).

**Structural Aspects**—While calcium accumulation may function effectively for the intracellular accumulation of monovalent or divalent cations released or required for intracellular function as, for example, in relaxation activity of heart by diffusion from source to sink and return within the cell, the possibilities for extracellular function appear to be limited by the lack of adequate information on the continuity of the plasma membrane and the space bounded by the inner membrane of the mitochondria in which it is believed that ions are accumulated. Evidence is now accumulating for the existence of cytoplasmic microtubules in mammalian cells (100). These tubules are approximately 100 A in diameter and are located not only generally through the cytoplasm but are seen in close proximity to the outer membrane of mitochondria (101). The possibility that these tubules connect to the inner membrane of the mitochondria and serve the function of communicating extracellular concentrations of ions directly to the mitochondrial membrane is one that may have deep implications with regard to the functional role of mitochondria in vivo.

In such communications between loci for intramitochondrial ion accumulation and the extracellular space, the arrangement of mitochondria adjacent to the deep invaginations in the excretory duct of tissues specialized for ion transporting such as the sea gull salt gland (47, 102) allows ionic concentrations from the plasma to enter the cell membrane and to be concentrated in the interior of the mitochondria and excreted in the lumen of the duct of the salt gland. These speculations are intended in no way to ignore the function of the sodium-activated ATPase of the endoplasmic reticulum which has been found in relatively high concentration in the sea gull salt gland, but it is more to reemphasize that any cation pump of the mitochondria requires an appropriate structural organization for it to be effective.

While the function of mitochondria in intracellular ion accumulation does not require complex structures described above, there are certain kinetics and stoichiometric aspects of calcium accumulation that must be considered, for example, in order that calcium might function as a relaxation mechanism in heart.

**Thermodynamic Aspects**—The question of the thermodynamic potential achieved in ion accumulation by mitochondria will ultimately be resolved by measurements of electrode potentials in various states of mitochondria. In the absence of such information, tentative estimates may be obtained from the ratio of calcium concentration accumulated in the mitochondria to that remaining outside. Obviously, enormous concentrations of calcium can be precipitated inside mitochondria as calcium phosphate (several micromoles per mg of protein (33)), but this is unlikely to be of physiological significance. Saris makes a detailed calculation and obtains 30 mM as the intramitochondrial calcium concentration for 97 \( \mu \text{M} \) added Ca++ (20). A simple calculation from Fig. 8 indicates that 158 \( \mu \text{M} \) Ca++ is accumulated by 3 mg of protein per ml in the absence of phosphate and in the presence of chloride as the anion. With the approximate significance

\[ \text{F. Jóbsis, personal communication.} \]
The ability to respond rapidly to calcium does not ensure that calcium will be expended in the interval, for example, between contractions of the heart. One may compute the maximum rate of calcium utilization for concentrations of calcium which saturate the respiratory activity by referring to Table II which gives the turnover number of cytochrome c in calcium accumulation to be 25 per second at 38°. For rat heart mitochondria, the value is approximately double this at 38.9°. If we multiply the number of calcium atoms per cytochrome c taken up by the cytochrome c content of tissue, we can calculate an intracellular mitochondrial calcium concentration. For rat heart, a concentration of 50 μM cytochrome c per kg, wet weight, is appropriate and the calcium uptake rate is 50 × 50 μM calcium per second, or 2.5 mmol calcium per second (see Reference 97, 98, 104, 105). Calcium release for rat heart may be calculated on the assumption that 1 mole of calcium is released per mole of ATP broken down, corresponding approximately to 300 μmoles of calcium per kg per twitch. At a steady heart rate of 200 beats per minute, we obtain 1 mM calcium per kg, wet weight, per second. Thus the calcium capability for saturating values of calcium is 2.5 times that required by 200 beats per minute. In addition to the calcium load, the mitochondria would be required to carry an ADP load of approximately equal magnitude. Ca++ and ADP would compete for respiratory activity, Ca++ reacting preferentially because of the greater respiratory stimulation and its reaction with a site closer to the chain than ADP. The preferential reaction is not a matter of affinity as suggested by Rossi and Lehninger (15). The affinity for ADP is greater than for Ca++ (20 compared with 45 μM for pigeon heart mitochondria (49)).

The third question is whether the calcium concentration will be reduced to an adequately low level to permit relaxation between contractions. One type of calculation is based upon the values of t₁ off computed from Equation 1. The calcium concentration at t₁ off is approximately the Kₐ value. From the heart cytochrome c turnover number of 50 per second at 38° and for x₀ = 300 μM calcium and Pₘₐₓ = 50 μM cytochrome c, t₁ off is about 0.1 second, corresponding to a contraction rate of 600 per minute. While this value is also high compared to usual rates for rat heart, calcium concentration corresponding to the Kₐ value (45 μM for pigeon heart mitochondria) is still very high compared to the calcium concentration required for relaxation of muscle fibers (about 10⁻⁷ M (103)).

Obviously, the calcium accumulation activity of the mitochondria would be supplemented by that of the endoplasmic reticulum, and at the present time it is as difficult to indicate the proportions of the total activity that each of the individual activities might share, a situation analogous to the sharing of ADP phosphorylation by mitochondrial and glycolytic activities. It is apparent, however, from the work of the late José Ramirez (97) that heart strips from the toad show cytochrome c responses to individual contractions which are fused one with the other at frequencies of 2 per second. In preliminary experiments in rapid recording of fluorescence changes in the perfused heart the responses of DPNH fluorescence to individual contractions can be observed at frequencies of approximately 1 per second. These observations appear at first sight to fail to confirm the calculations above and suggest that the kinetic responses of components of the respiratory chain in vivo may be slower than those computed from data obtained in vitro.

Lastly, one would expect to find in a beating heart that employs mitochondrial calcium accumulation partial inhibition of mitochondrial activity by oligomycin. Preliminary observations on perfused heart indicate incomplete reduction of DPN on adding 2 μg per ml of oligomycin to the perfusate. A reduction of 85% is obtained with oligomycin and a further 20 per cent reduction is obtained with Amytal.

In summary, the discrepancy between the calculated cycling of cytochromes based upon measurements in vitro and those actually observed in vivo show a significant difference; the time to complete the uptake of ADP and calcium in vivo appears longer than would be expected on the basis of data obtained in vitro. In the ADP reaction, the creatine kinase "buffers" the ADP concentration so that maximal activity of the mitochondria is not elicited in a single twitch (106). In calcium accumulation, similar factors may be operative but their identities are unknown. The problem of calcium release from the binding sites, either in the endoplasmic reticulum or in the mitochondria themselves, remains unsolved.

**SUMMARY**

1. The addition of low concentrations of calcium to suspensions of mitochondria gives an interval of stimulated respiration that is proportional to the amount of calcium added, a response qualitatively resembling that to added adenosine diphosphate and phosphate. The response of guinea pig liver mitochondria (2) has been confirmed and extended to mitochondria prepared from rat liver, guinea pig kidney, pigeon heart, and sea gull salt gland.

2. Respiratory rates during calcium-activated respiration in rat liver and pigeon heart mitochondria may exceed those obtained in the presence of ADP and phosphate by over 50%.

3. The efficiency of calcium utilization by mitochondria is evaluated by four criteria; Ca++:ADP is most useful and can be determined from Ca++:O₆ divided by ADP:O₆ or from a simple equation for enzyme-substrate intermediates (Equation 1).

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13 J. R. Williamson and D. Jamieson, unpublished observations.
14 J. R. Williamson, personal communication.
The two methods give 1.9 and 1.7, respectively, for a variety of conditions and preparations at 25°; the average Ca++:ADP is 1.8. This value is independent of the mitochondrial efficiency, while the Ca++:O2 values will vary with the efficiency. The Ca++:H+ value is measured by the glass electrode and by an indicator method and closely approximates 1.0 with chloride as an anion, but increases to 1.7 in the absence of phosphate, and thus is not constant.

4. The response of the respiratory carriers to Ca++ is unaffected by concentrations of oligomycin which prevent their response to ADP plus phosphate. This response identifies the energy donor in the calcium reaction as a precursor of ATP. The response of DPNH to calcium in Amytal- and oligomycin-treated mitochondria is largely but not completely eliminated, indicating some breakthrough of calcium at the Amytal-blocked point (see also Reference 50). The calcium reaction is appropriately identified as an energy-linked function of mitochondria involving their internal high energy intermediates.

5. The hydrogen ion release accompanying the reaction of calcium with the mitochondria has been found to be closely synchronized with the interval of stimulated respiratory activity and with the oxidation-reduction cycles of the respiratory carriers. Hydrogen ion release comes early in the calcium reaction with mitochondria.

6. A crossover point between cytochrome b and c is observed with calcium as with ADP, and the steady state changes of the respiratory carriers upon the addition of calcium are similar to those caused by ADP, taking into account the greater activation of respiration by calcium. It is concluded that calcium affects all the sites of oxidative phosphorylation affected by ADP and phosphate.

7. The speed of response of cytochrome b in the State 4 to 3 transition caused by calcium is very rapid; the half-time is 70 milliseconds at 23°.

8. The response of oxygen utilization and of the respiratory carriers in the absence of added phosphate shows two states. At low calcium concentrations, a reduced state of cytochrome b and a highly fluorescent state of DPNH is obtained. At higher calcium concentrations, a partial uncoupling of the mitochondria with consequent high oxidation states in the carriers is seen. Addition of phosphate rapidly restores the carriers to their usual reduced state, following the completion of calcium uptake.

9. Light scattering changes accompany calcium accumulation and are similar to those that accompany the phosphorylation of ADP. The increased light scattering caused by the Ca++ reaction is reversible and is of low amplitude.

10. The role of cytochrome b and DPNH in the cation accumulation is considered in relation to mitochondrial structure and special roles for high energy intermediates of both these carriers in cation transport normal to the plane of electron transport is suggested.

11. An over-all mechanism for Ca++ accumulation in the mitochondrial crista is presented, and the possibility that the primary events do not require phosphate is considered.

12. The role of mitochondrial structure in Ca++ accumulation is considered, and this need distinguishes Ca++ accumulation from oxidative phosphorylation of ADP. The location of calcium accumulation is considered in terms of the current views on the localization of the cytochrome chain in mitochondrial membranes.

13. The possibilities for the functioning of calcium accumulation as a relaxing factor in heart tissue have been considered, and the kinetic, thermodynamic, and stoichiometric factors are consistent with the possibility of such a function. The biological structure required for intra- and extracellular ion accumulation is defined.

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