# RESPIRATION-DEPENDENT ACCUMULATION OF INORGANIC PHOSPHATE AND $Ca^{++}$ BY RAT LIVER MITOCHONDRIA

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Earlier work in this department has shown that rat kidney mitochondria (Vasington and Murphy, 1961, 1962) as well as digitonin fragments of rat liver mitochondria (Vasington, 1963) actively accumulate very large amounts of $Ca^{++}$ from the suspending medium during respiration. $Ca^{++}$ accumulation is inhibited by dinitrophenol and other uncoupling agents, as well as by cyanide and antimycin A, showing its dependence on energy-coupling mechanisms and on respiration. About 3.0-4.0 μatoms of $Ca^{++}$ are accumulated by intact kidney mitochondria per μatom of oxygen taken up. Uptake of $Ca^{++}$ by isolated kidney mitochondria has also been observed by DeLuca and Engstrom (1961).

This communication reports that inorganic phosphate of the medium is the specific and major anion accompanying the active uptake of $Ca^{++}$ by rat liver mitochondria, that $Ca^{++}$ and $P_i$ enter in a definite molar ratio, and that $P_i$ does not accumulate significantly in liver mitochondria when $Ca^{++}$ is replaced in the medium by other cations. Data in Table I (Experiment 1) show the rapid net accumulation of both $Ca^{++}$ and $P_i$ by rat liver mitochondria incubated with succinate, $P_i$, ATP, $MgCl_2$, NaCl, and tris buffer pH 7.0; the accumulation was measured in extracts of the mitochondrial pellet sedimented from the incubation medium. The

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Table I

Accumulation of $P_i$ accompanying uptake of $Ca^{++}$ in rat liver mitochondria

The system for Exp. 1 contained 10 mM succinate, 10 mM $MgCl_2$, 10 mM tris HCl (pH 7.4), 80 mM NaCl, 4 mM $CaCl_2$ labeled with $Ca^{45}$, 3.0 mM ATP, 4.0 mM $P_i$ (pH 7.4), and rat liver mitochondria (3.0 mg protein) in 3.0 ml. In Exp. 2 succinate was omitted and ATP was 15 mM. Incubated at 30° for times shown. Tubes were chilled, centrifuged at 13,000 x g for 4 minutes, and mitochondria washed once with cold 0.25 M sucrose. $Ca^{++}$ uptake was measured according to Vasington and Murphy (1962) and $P_i$ by the Fiske-Subbarow method in trichloroacetic acid extracts of the washed mitochondria.

| | $O_2$ uptake μatoms | Net $Ca^{++}$ accumulation μmoles | Net $P_i$ accumulation μmoles |
|---|---|---|---|
| 1. Complete - 5 min. | | 2.6 | 1.4 |
| " -10 min. | | 3.2 | 2.1 |
| " -15 min. | | 3.8 | 2.3 |
| Complete (20 min.) | 2.1 | 5.8 | 2.7 |
| Mitochondria omitted | | 0.00 | 0.00 |
| $Ca^{++}$ omitted | | (0.0) | 0.1 |
| $Ca^{++}$ + ATP omitted | | (0.0) | 0.1 |
| $Mg^{++}$ omitted | | 0.0 | 0.1 |
| ATP omitted | | 0.3 | 0.2 |
| Succinate omitted | | 1.1 | 0.6 |
| Complete + 0.1 mM DNP | | 0.1 | 0.1 |
| " + 1 mM cyanide | | 0.1 | 0.1 |
| " + oligomycin (1.5 r/ml) | | 5.4 | 2.9 |
| 2. Complete (15 mM ATP) | | 7.92 | 4.51 |
| " + oligomycin | | 0.54 | 0.39 |

amounts of $Ca^{++}$ and $P_i$ accumulated in the complete system by rat liver mitochondria (up to 2.2 μmoles $Ca^{++}$ and 1.6 μmoles $P_i$ per mg protein at saturation) are many-fold greater than the normal content of freshly isolated mitochondria. The data show that the accumulation of $P_i$ and $Ca^{++}$ by mitochondria requires the presence of ATP, $Mg^{++}$ and succinate. In the absence of $Ca^{++}$ (or $Ca^{++}$ + ATP) no significant $P_i$ uptake occurred. Dinitrophenol and cyanide blocked uptake of both $Ca^{++}$ and $P_i$. No experimental conditions were found which supported large $P_i$ uptake without $Ca^{++}$ uptake, or $Ca^{++}$ uptake without $P_i$ uptake. Net oxidative phosphoryla-

tion of ADP does not occur in the complete test system when it is supplemented with yeast hexokinase and glucose, as was shown earlier for kidney mitochondria (Vasington and Murphy, 1962); the added $Ca^{++}$ uncouples phosphorylation of ADP completely.

Under the conditions shown in Exp 1, oligomycin did not block uptake of either $P_i$ or $Ca^{++}$ at a concentration known to inhibit oxidative phosphorylation. On the other hand, when the ATP concentration was raised to 15 mM or higher (Exp. 2), the requirement for respiratory substrate was no longer evident and $Ca^{++}$ and $P_i$ accumulation were completely blocked by oligomycin, indicating that the accumulation mechanism may be driven by ATP alone under favorable circumstances.

The molar ratio of $Ca^{++}$ and $P_i$ accumulated in the mitochondrial pellet was found to be =1.8. The $Ca^{++}$:$P_i$ ratio of $Ca_3(PO_4)_2$ is 1.5, of $CaHPO_4$ is 1.0, of $Ca(H_2PO_4)_2$ is 0.5 and of hydroxyapatite is 1.67. The amount of $Ca^{++}$ and $P_i$ accumulated, when calculated on the basis of molar concentration in the intramitochondrial water, may be as high as 0.5 M and far exceeds the solubility product of hydroxyapatite. Most of the accumulated $P_i$ measured by the Fiske-Subbarow method is true inorganic phosphate; however the identity of a minor esterified form of P is under investigation.

$P_i$ and $Ca^{++}$ uptake was not supported when ATP was replaced by UTP, GTP, ITP, or AMP, nor by EDTA, spermine, or pyrophosphate. $Ca^{++}$ could not be replaced in supporting $P_i$ uptake by spermine, $NAD^+$, cytochrome c, $K^+$, $Na^+$, and $NH_4^+$. Tests carried out under the exact conditions used by Brierley, Bachmann, and Green (1962) to demonstrate accumulation of $Mg^{++}$ and $P_i$ by beef heart mitochondria showed that $Mg^{++}$ was less than 20% as active as $Ca^{++}$ in supporting $P_i$ uptake in rat liver mitochondria.

Isotopic experiments showed that at high $P_i$ concentrations (12-20 mM), the $P_i$ of the medium was the precursor of well over 90% of the accumulated $P_i$. Some $P^{32}$ from terminal-labeled $ATP^{32}$ also entered the mitochondria without passing through the inorganic phosphate pool, but its amount

was independent of the amount of $P_i$ entering. No other anions, including $Cl^-$, $SO_4^=$, $NO_3^-$, glycerophosphate, and pyrophosphate etc., were found to replace or compete with phosphate in entering liver mitochondria with $Ca^{++}$.

Electron micrographs of thin sections of pellets of washed $Ca^{++}$- and $P_i$-loaded mitochondria fixed with osmium tetroxide showed them to consist of two distinct types of about equal proportions (cf. Amoore and Bartley,(1958)). One type appeared to be somewhat swollen but otherwise normal. However the other type was strikingly different. Although the surrounding membranes and some cristae were visible, the shape of these mitochondria was distorted and irregular. A common alteration was the apparent contraction of the matrix and the sometimes very wide separation of inner and outer surrounding membranes. The most singular finding in the latter mitochondria was the presence of numerous electron-opaque masses often having diameters as large as 500-1000 A, which were largely located near the periphery of the matrix. It is possible that these masses are greatly enlarged forms of the often-observed "dense granules" of mitochondria, which Peachey (1962) has suggested may bind divalent cations such as $Sr^{++}$ and $Ba^{++}$. Mitochondria incubated in test systems from which ATP or $Ca^{++}$ had been omitted were essentially normal and showed no dense granules.

The $Ca^{++}$ and $P_i$ accumulation mechanism of rat liver and rat kidney mitochondria is clearly different from the ATP-dependent $Ca^{++}$ uptake by skeletal muscle microsomes (Hasselbach and Makinose, 1961; Ebashi and Lipmann, 1962; Molnar and Lorand, 1962), which does not require respiratory substrates. Rat liver microsomes show no $Ca^{++}$ or $P_i$ uptake when tested under the experimental conditions optimal for uptake of these ions by liver mitochondria. As pointed out above, the $Ca^{++}$ and $P_i$ accumulation system of rat liver and kidney mitochondria also appears to be different from the system causing uptake of $Mg^{++}$ and $P_i$ in beef heart mitochondria, which Brierley et al. (1962)

have indicated to be inhibited by $Ca^{++}$. They have found that the presence of an active phosphate acceptor system prevents accumulation of $Mg^{++}$ and $P_i$; on the other hand $P_i$ and $Ca^{++}$ uptake in rat liver mitochondria requires ATP or ADP. The $Ca^{++}$:O ratio in liver mitochondria is about 3.0, whereas the $Mg^{++}$:O ratio in heart mitochondria is about 0.75. Our findings on uptake of $P_i$ and $Ca^{++}$ do not appear to be explained by the type of mechanism proposed by Brierley et al., i.e. that the phosphate taken up in the presence of $Mg^{++}$ by respiring beef heart mitochondria has two alternative fates: either it is transferred to ADP to form ATP (i.e. oxidative phosphorylation) or it promotes translocation and binding of $Mg^{++}$, in such a way that ion transport and ATP formation are inversely related. The relationships between $P_i$ uptake by mitochondria and the uptake of $Ca^{++}$, $Mg^{++}$, $Mn^{++}$ (Chappell et al (1962)), $K^+$ (Gamble (1957) and other cations are under further investigation.

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