STOICHIOMETRIC RELATIONSHIPS BETWEEN MITOCHONDRIAL ION ACCUMULATION AND OXIDATIVE PHOSPHORYLATION

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Earlier papers from this laboratory have described the massive accumulation of Ca^{++} and P_i by liver and kidney mitochondria, supported either by electron transport or ATP hydrolysis (Vasington and Murphy, 1961, 1962; Vasington, 1963; Lehninger, Rossi and Greenawalt, 1963). In these studies the ratio atoms Ca^{++} accumulated: atoms oxygen utilized (i.e. the Ca^{++}:O "accumulation ratio") was found to be of the same order of magnitude as the normal P:O ratio of oxidative phosphorylation, and may exceed it. These findings suggest that ion uptake and oxidative phosphorylation are related stoichiometrically.

This communication reports a detailed analysis of the stoichiometric relationships between the amounts of Ca^{++} and of P_i accumulated by respiring kidney and liver mitochondria, the amount of oxygen taken up in the presence of various respiratory substrates, and the number of phosphorylation sites in the respiratory chain.

In Table I are shown representative data obtained on the accumulation of Ca^{++} and P_i and the consumption of oxygen by rat kidney mitochondria oxidizing substrates characterized by different theoretical P:O ratios. Also shown are the actual ADP:O ratios for oxidation of these substrates, as determined in parallel tubes not containing Ca^{++} or ATP by the method

Table I

Molar relationship of Ca^{++} and P_i accumulation to phosphorylation sites

Reaction mixture contained 10mM Tris, pH 7.0, 80mM sodium chloride, 10mM magnesium chloride, 4mM sodium phosphate, pH 7.0, 3.33mM calcium chloride (labeled with Ca^{45}), 3mM sodium adenosine triphosphate, pH 7.0, 20mM sodium β-hydroxybutyrate, or 10mM sodium succinate, or 40mM sodium ascorbate and 0.3mM N,N,N',N'-tetramethyl-p-phenylenediamine as respiratory substrates, rat kidney mitochondria (4mg protein) in a total volume of 2 ml. Incubation was at 25 C. Ca^{++} and P_i uptake was measured as described earlier (Lehninger et al. 1963). Oxygen uptake was measured with the oxygen electrode. The ADP:O ratio was determined from the increment in uptake of oxygen given by addition of 0.12mM ADP to parallel test systems. Oligomycin was added at 1 μg per mg of mitochondrial protein. Uptake data in mμ atoms or mμ moles per min per mg protein.

Substrate and addition	Ca^{++} uptake	P_i uptake	O_2 uptake	Ca:O accumulation ratio	P_i:O accumulation ratio	ADP:O ratio
β-hydroxybutyrate	163	98	33	4.94	2.97	2.88
" + oligomycin	143	87	30	4.77	2.90	-
Succinate	292	208	109	2.70	1.91	1.91
" + oligomycin	261	201	96	2.73	2.09	-
Ascorbate	170	96	97	1.78	.99	.99
" + oligomycin	166	76	95	1.74	.80	-

of Chance and Williams (1956). The measurements were made in the initial stages of the accumulation reaction, before "saturation" of the mitochondria with Ca^{++} and P_i had been reached.

With D-β-hydroxybutyrate as substrate, the oxidation of which is normally accompanied by oxidative phosphorylation of ADP at a P:O ratio approaching 3.0, the measured Ca^{++}:O accumulation ratio was found to be 4.90, which is in substantial excess of the theoretical P:O ratio of 3.0 and of the measured ADP:O ratio of 2.88 estimated in a separate system containing no Ca^{++} or ATP. On the other hand, the P:O accumulation ratio (moles P_i accumulated per atom oxygen taken up) for β-hydroxybutyrate was 2.96, approximately equal to the measured as well as the theoretical P:O ratio. For succinate oxidation, which is characterized by a theoretical P:O ratio of 2.0, the measured Ca:O accumulation ratio was 3.30 and the P:O accumulation ratio 2.17; the measured ADP:O ratio was 2.09. For the oxidation of ascorbate in the presence of N,N,N',N',-tetramethyl-p-phenylenediamine as carrier, which is normally characterized by a P:O ratio of oxidative phosphorylation approaching 1.0 (Jacobs, 1960), the Ca^{++}:O and P:O accumulation ratios were 1.78 and 0.99 respectively, and the measured ADP:O ratio 0.95. Thus, in each case the P:O accumulation ratio approximates closely the measured ADP:O ratio of oxidative phosphorylation for the substrate used, as well as the theoretical P:O ratio. In each case also, the Ca^{++}:O accumulation ratio is substantially larger than the P:O ratio of oxidative phosphorylation; in a series of over 100 determinations, the Ca^{++}:O ratio in such experiments is about 1.67 times the P:O ratio of oxidative phosphorylation, a finding which confirms the observation of Vasington and Murphy (1962) that the Ca:O accumulation ratio in the presence of succinate may exceed the P:O ratio of oxidative phosphorylation.

Also shown in Table 1 are the effects of oligomycin on the Ca:O and P:O accumulation ratios. Vasington and Murphy (1962), Lehninger et al (1963), and Brierley et al (1963) have shown that oligomycin does not

significantly inhibit ion uptake when it is supported by respiration; on the other hand oligomycin does inhibit ion uptake when it is supported by ATP hydrolysis. Data in Table 1 show that oligomycin inhibits oxygen and ion uptake only very slightly and does not significantly alter the P:O and Ca:O accumulation ratios. The failure of oligomycin to inhibit ion uptake significantly demonstrates that the ion accumulation under the conditions in Table 1 is supported entirely by electron transport, with negligible contribution from ATP present in the medium. In many similar experiments, rat liver and rat kidney mitochondria have been found to give essentially identical results on the stoichiometry of Ca^{++} and P_i accumulation and of oxidative phosphorylation.

The data presented here thus show that accumulation of approximately 1.67 atoms of Ca^{++} and 1.0 molecules of P_i is driven by transfer of a pair of electrons through each phosphorylation site of the respiratory chain. It appears most probable that the non-integral value 1.67 for the Ca^{++}:2e accumulation ratio per phosphorylation site is directly related to the chemical composition of the calcium phosphate salt which is deposited in the mitochondria. Over 100 measurements on the molar Ca:P ratio of the accumulation have yielded an average value of 1.67 ± 0.08, which is essentially identical with the Ca:P ratio of 1.67 for calcium hydroxyapatite: $\underline{[Ca_3(PO_4)_2]}_3 \cdot Ca(OH)_2$ (Lehninger *et al* (1963)).

There are at least two possible general mechanisms for accumulation of calcium phosphate linked to the phosphorylation sites of the respiratory chain: (a) Ca^{++} is the actively transported ion, and P_i follows "passively" and (b) P_i is the actively transported ion, and Ca^{++} follows "passively". It is possible also that <u>both</u> Ca^{++} and P_i are actively translocated. The data reported here cannot conclusively prove which mechanism describes the process. However the nearly exact molar equality of the P:O <u>accumulation</u> ratios and the measured or theoretical P:O ratio of oxidative phosphorylation is consistent with the view that it is the phosphate anion which is actively transported by action of the

phosphorylation sites in the respiratory chain. The non-integral value of 1.67 for the Ca^{++}:2e accumulation ratio does not necessarily exclude a mechanism of active translocation of Ca^{++}, but is more consistent with the view that Ca^{++} follows "passively" in such amounts as to form a stable salt of calcium phosphate insoluble at the pH of the intramitochondrial milieu. Isolated rat liver, kidney, or heart mitochondria are now known to accumulate K^{+}, Ca^{++}, Mg^{++}, Mn^{++}, Sr^{++}, and Ba^{++}, (cf. Werkheiser and Bartley (1957); Brierley et al (1962); Chappell et al (1962); Chappell and Greville (1963)) as well as alkylguanidines (Pressman, 1962), in respiration-dependent processes. Despite the diversity of cations accumulated by mitochondria, phosphate is accumulated as the major counter-anion in each case. Translocation or uptake of phosphate therefore appears to be a common denominator in cation uptake as well as in oxidative phosphorylation.

Certain other observations must be explained before it may be concluded that phosphate rather than the cation is the active translocated ion. Oligomycin, which is known to block incorporation of P_i into a chemical linkage in normal oxidative phosphorylation, does not inhibit respiration-dependent accumulation of phosphate. However it is possible that the accumulation of phosphate does not involve actual formation of a covalent phosphate derivative by an oligomycin-sensitive reaction, but rather some energy-linked physical or chemical change in structure or properties which normally precedes the oligomycin-sensitive step. Although Chappell and Greville (1963) have found that $Mn^{++}$and Ca^{++} may be accumulated without phosphate under certain conditions, we have observed that in the very early stages of Ca^{++} uptake supported by succinate oxidation, the Ca^{++}:P ratio is usually lower than 1.67 and then approaches but does not exceed this value as more Ca^{++} and P_i are accumulated.

Full details of experiments on the stoichiometry of Ca^{++}, P_i, H^{+}, OH^{-} and oxygen utilization as well as ATP hydrolysis in intact mitochondria from different tissues and in digitonin fragments of rat liver

mitochondria (cf. Vasington, 1963) will be presented for publication elsewhere.

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