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Effects of calcium on oxidation rates of apple mitochondria.

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EFFECTS OF CALCIUM ON OXIDATION RATES OF APPLE MITOCHONDRIA

A Thesis Presented

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ABSTRACT

The effects of Ca^{+2} on the oxidation rates of mitochondria extracted from the flesh of apples (Malus pumila Mill. cv Baldwin) were dependent upon the initial oxidation rates. If the oxidation rates were high, 10^{-4}M Ca^{+2} inhibited respiration. If they were low, 10^{-4} to 10^{-2}M Ca^{+2} stimulated respiration. It is proposed that stimulation was due to active Ca^{+2} accumulation by the mitochondria, and inhibition to curtailment of ATP turnover by ATPase; if this curtailment is great enough, it may mask the stimulatory effect of Ca^{+2} accumulation.

INTRODUCTION

In intact Baldwin apples an inverse relationship between Ca^{+2} content and fruit respiration has been shown (Bramlage et al., 1974, Faust and Klein, 1974, Faust and Shear, 1972). This relationship might express a direct effect of Ca^{+2} on the functioning of the mitochondria, site of the TCA cycle and terminal oxidation. Such is suggested by the following facts: (1) Ca^{+2} can stimulate "U-Factor" formation leading to uncoupling of oxidative phosphorylation and mitochondrial swelling (Wojtczak and Lehninger, 1961); (2) Ca^{+2} passively bound to membranes can alter membrane properties (Manery, 1966); (3) active accumulation of Ca^{+2} can lead to precipitation of inorganic phosphate (Pi), necessary for ATP formation (Elzam and Hodges, 1968). Therefore, studies were made of the effects of endogenous and exogenous Ca^{+2} on oxidation of mitochondria from apples the respiration rates of which were known to be influenced by Ca^{+2} content.

MATERIALS AND METHODS

Mature Baldwin apples were held at 0°C from October to June. Between January and June, mitochondria were isolated from the flesh of the fruits according to the procedure of Shirway and Bramlage, 1973. Mitochondrial reactions were carried out in a 2.3 ml glass chamber maintained at 25°C and fitted with a Clark oxygen electrode (Gilson Medical Electronic Co.). Polarizing voltage was maintained at 0.8 v, and oxygen uptake was recorded. All reaction media contained 0.25M sucrose, 5mM $MgCl_2$, 10mM TES (N-Tris (hydroxymethyl) methyl 2-amino ethane sulfonic acid) buffer, and 16mM succinate; in addition, 3 mg/ml bovine serum albumin and 2 mg/ml yeast extract were added immediately prior to assay. The reaction medium was maintained at pH 7.2.

Mitochondrial protein was determined by the method of Lowry et al. (1951), with bovine serum albumin standards. Calcium was determined by atomic absorption spectroscopy (Perkin-Elmer Model 214). Peel tissue was digested with nitric and perchloric acids. Mitochondrial preparations (0.5 ml) were digested in 30% H_2O_2 after addition of 1 drop of 5N H_2SO_4. The H_2O_2 was added in 2 ml aliquots until the sample no longer charred when evaporated to dryness. The digested sample was taken up in 10 ml of 1% $LaCl_3$. Phosphate content of mitochondrial preparations was determined by the procedure of Martin and Doty (1949).

RESULTS

Apples with relatively high peel Ca^{+2} levels yielded mitochondrial extracts which were significantly higher in Ca^{+2} than extracts from fruit with relatively low peel Ca^{+2} levels (Table 1). This relationship was found whether or not EDTA, a chelator of Ca^{+2}, was present in the extraction medium.

The mitochondrial extracts exhibited oxidation rates on succinate that were positively correlated with their Ca^{+2} levels (Figure 1). This correlation was significant when ELTA was present in the extraction medium ($r=0.93$; $df=4$); the correlation was high but not significant when EDTA was absent ($r=0.65$; $df=6$). Thus there appeared to be a relationship between endogenous Ca^{+2} levels of the mitochondrial extracts and their oxidation rates, but this relationship was opposite that between whole fruit respiration and endogenous Ca^{+2} levels, in which the relationship was significantly negative (Bramlage et al., 1974).

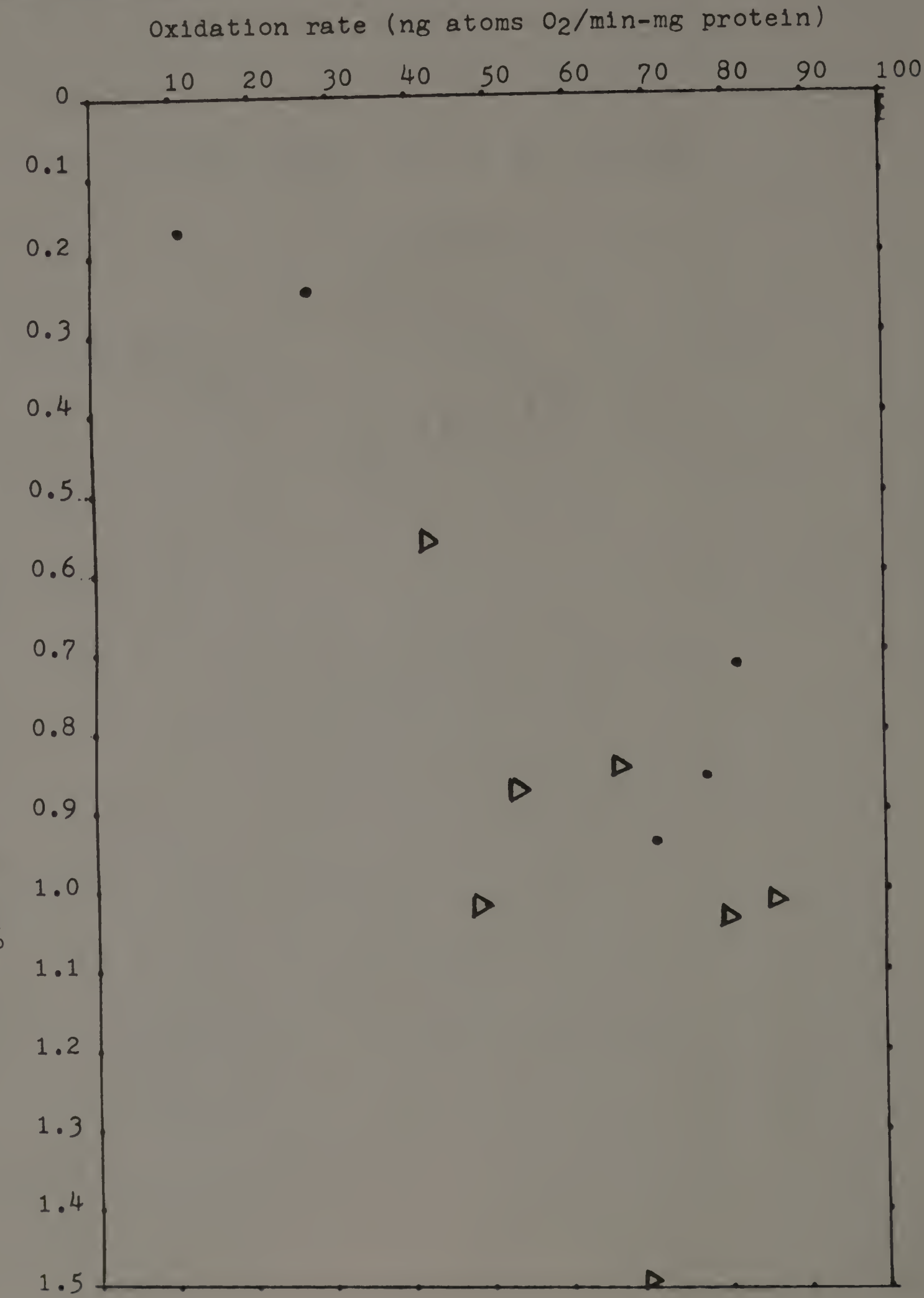
The effects of exogenous Ca^{+2} additions on the oxidative capacities of mitochondria were then examined. Only fruit with relatively high peel Ca^{+2} content were used as mitochondrial sources. When 10^{-4} M $CaCl_2$ was added to mitochondria extracted from apples stored for a relatively short time (3-4 months), succinate oxidation was reduced. In 6 experiments, this inhibition averaged 16%. However, by withholding sub-

Table 1. Calcium content of peel and of mitochondrial extracts from flesh of Baldwin apples. Mitochondria were extracted both with and without EDTA in the extraction medium.

Peel Ca^{+2} ppm	EDTA in extraction medium	Mitochondrial Ca^{+2} ug/mg protein[a]
1057	Yes	0.82 ± 0.06
463	Yes	0.35 ± 0.15
1057	No	1.07 ± 0.07
463	No	0.75 ± 0.10

[a] Each value is the mean of 3 replicates, with standard errors indicated.

Figure 1. Relationship between mitochondrial endogenous Ca^{+2} and the oxidation rate. Circles, extract contained EDTA; triangles, extract devoid of EDTA. Reaction medium as in Table 2.



strate, i.e. adding the succinate 4 minutes after the addition of mitochondria rather than prior to the mitochondria, the Ca^{+2} effect was reversed. Now concentrations as high as 10^{-3}M $CaCl_2$ stimulated oxidation. In 6 experiments, stimulation from 10^{-3}M $CaCl_2$ averaged 32%.

In later experiments, mitochondria were extracted from apples stored for a relatively long time (7-8 months). Mitochondria from these fruit were not inhibited by Ca^{+2}, but rather, were stimulated by 10^{-2} to 10^{-4}M $CaCl_2$ (Table 2). When ATP (0.1 mole) was added to the reaction medium, the stimulation from 10^{-3} and 10^{-2}M Ca^{+2} was reduced. If substrate was withheld from these mitochondria extracted from the older fruit, their initial oxidation rates declined, and as was expected, Ca^{+2} was a more effective stimulant (Table 3).

Mitochondria stimulated by Ca^{+2}, both those from which substrate had been withheld and those extracted from apples stored for 7-8 months, had a common factor, namely, initially low oxidation rates (Table 4). On the other hand, mitochondria inhibited by Ca^{+2} (those from short term apples) had much higher initial oxidation rates. Thus, whether Ca^{+2} stimulated or inhibited seemed to depend on the initial oxidation rate of the mitochondria. Statistics confirmed this; there was a highly significant negative correlation ($r=-0.72$; $df=14$) between initial oxidation rate and the Ca^{+2} effect on oxidation.

Table 2. Effects of 3 Ca^{+2} concentrations and ATP (0.1 mole) on mitochondrial oxidation. The reaction medium contained 0.25M sucrose, 5mM $MgCl_2$, 10mM TES buffer, 16mM succinate, 3mg/ml bovine serum albumin and 2mg/ml yeast extract. In experiments with ATP, the ATP was added before the mitochondria. Ca^{+2} was added approximately 4 minutes after addition of the mitochondria (1-2mg of mitochondrial protein).

Ca^{+2} concentration	ATP	% Stimulation [a]
10^{-2}M	No	55
10^{-3}M	No	45
10^{-4}M	No	17
10^{-2}M	Yes	21
10^{-3}M	Yes	16
10^{-4}M	Yes	14

[a] Each value is the mean of 6 experiments.

Table 3. Effect of substrate withholding on the oxidation rate and the Ca^{+2} effect of mitochondria extracted from apples of long storage. Reaction medium as in Table 2.

Treatment	Initial oxidation rate ng atoms O_2/ min-mg protein	Ca^{+2} concentration	% Stimulation [a]
Substrate added	44	10^{-2}M	33
before mitochondria	49	10^{-3}M	11
	50	10^{-4}M	12
Substrate added	32	10^{-2}M	48
4 minutes after	31	10^{-3}M	50
mitochondria	33	10^{-4}M	57

[a] Each value is the mean of 4 experiments.

Table 4. Effect of storage length and substrate withholding on mitochondrial oxidation rates and Ca^{+2} effect. Reaction medium as in Table 2.

Treatment	Initial oxidation rate ng atoms O_2/ min-mg protein	Ca^{+2} concentration	% Stimulation [a]
Short storage [b]	77	10^{-4}M	-16%
Short storage + substrate withheld	30	10^{-3}M	32%
Long storage	37	10^{-4}M	17%
Long storage	31	10^{-3}M	44%

[a] Each value is the mean of 6 experiments.
[b] Short storage equals 3-4 months; long storage equals 7-8 months.

The lowest oxidation rates observed in these experiments were those for which data are presented in Table 2. It is noteworthy that all 3 levels of Ca^{+2} used (10^{-4}, 10^{-3}, 10^{-2}M) were equally effective in stimulating oxidation, and that the addition of ATP significantly reduced the Ca^{+2} stimulation (Table 2).

To determine the specificity of the Ca^{+2} effect on oxidation, 10^{-3}M $CaCl_2$, KCl, $MgCl_2$, $MnCl_2$, and $SrCl_2$ were added to mitochondria extracted from apples stored 8 months. Oxidation was not stimulated by $MgCl_2$, $MnCl_2$, or KCl, but was stimulated by both $CaCl_2$ and $SrCl_2$. Thus, the response was not a general salt effect.

Although mitochondria from fruit stored 7-8 months exhibited lower oxidation rates than those from fruit stored 3-4 months, there was no effect of aging on the Respiratory Control Ratios of the mitochondria (Table 5). However, Pi level within the extracts was distinctly different; Pi was significantly lower in extracts from the older fruit.

Table 5. Phosphate levels and respiratory control ratios of mitochondria extracted from apples stored for short and long terms.

Length of storage months	Phosphate level[a] (umoles Pi/mg protein)	RCR[b] State 3 respiration / State 4 respiration
3-4	0.064 ± 0.006	1.5
7-8	0.041 ± 0.005	1.6

[a] Each value is the mean of 3 replicates, with standard errors indicated.
[b] Each value is the mean of 6 experiments.

DISCUSSION

Intact Baldwin apples deficient in Ca^{+2} exhibit accelerated rates of respiration (Bramlage et al., 1974), but we have found (Table 1) that the mitochondria from these fruits show the reverse relationship: lessened oxidation rates with lower endogenous Ca^{+2}. Thus, it appears that the accelerated respiration rates of Ca^{+2}-deficient apples are due to extramitochondrial influence. Perhaps this influence involves membrane permeability. Membranes are negatively charged at physiological pH (Manery, 1966). Calcium, by binding with these sites, changes the electrical properties by decreasing the net charge (Manery, 1966). This binding would also dehydrate the membrane by causing loss of water of hydration (Manery, 1966). Both of these changes could markedly alter membrane configuration and hence permeability properties. Faust and Klein (1974) showed that incubation of disks of apple tissue in $CaCl_2$ solutions subsequently reduced the uptake of uracil and valine by the disks.

The addition of exogenous Ca^{+2} to apple mitochondria produced conflicting results. Under some conditions, oxidation was stimulated, while under other conditions it was inhibited. The key to understanding these data seems to be connected with the initial oxidation rates of the mitochondria (Figure 2).

The presence of a respiratory stimulant other than Ca^{+2}, possibly an ATPase, could account for the differing Ca^{+2} ef-

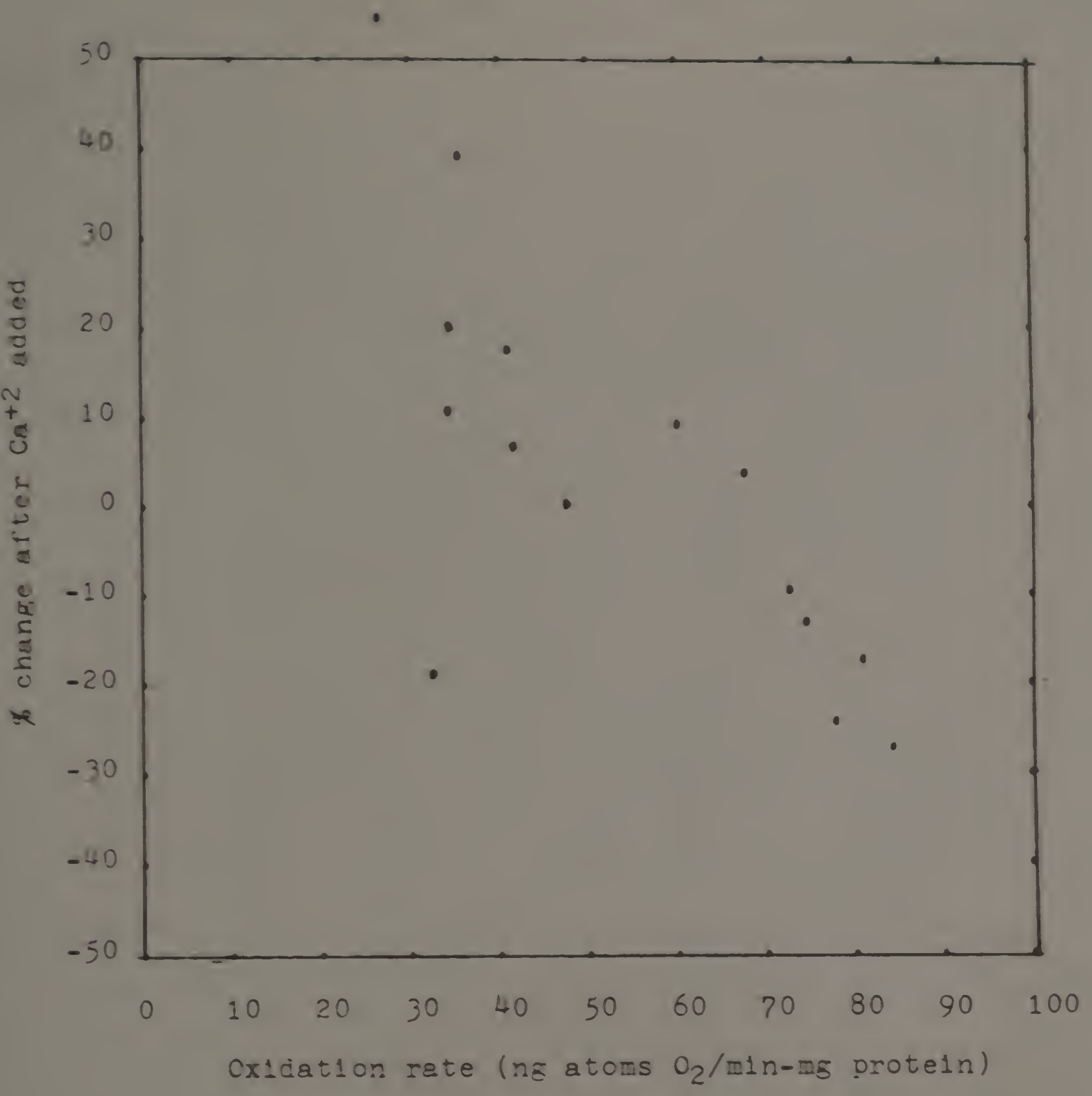


Figure 2. Effects of 10^{-4}M $CaCl_2$ addition on mitochondrial oxidation rates. Mitochondrial extracts were from both short and long term apples of relatively high endogenous Ca^{+2} levels. Reaction medium as in Table 2.

fect. Mitochondria with high oxidation rates would reflect rapid turnover of ATP; those with low rates would reflect slow turnover of ATP. Turnover rate by ATPase would depend on the adenylate and Pi supply within the mitochondria. The higher the internal adenylate and Pi supply, the more ATP that can be formed, and the more ATP formed, the more that can be degraded by the ATPase. The data support this hypothesis; mitochondria with high oxidation rates had significantly more Pi than those with low rates (Table 5).

Gamble and Hess (1966) showed that respiration is necessary to maintain the intramitochondrial Pi supply. Thus, when substrate is withheld, Pi may passively diffuse out of the mitochondria, making them similar to mitochondria of initially low Pi content. Both types of mitochondria have low initial oxidation rates and both types respond positively to Ca^{+2}. This stimulation is probably due to Ca^{+2} accumulation. Other researchers (Rasmussen et al., 1965, Hodges and Hanson, 1965) have shown that mitochondria can accumulate Ca^{+2} by means of an energy-requiring process that drives electron transport.

On the other hand, mitochondria with high oxidation rates were inhibited by Ca^{+2}. These high rates could be possibly due to a high rate of ATP turnover. It is proposed that Ca^{+2} accumulation may still be stimulating oxidation, but that this stimulation may be masked by the inhibition due to Ca^{+2} curtailment of ATP turnover. This could occur

since the mitochondrial Ca^{+2} carrier is probably one of the high energy precursors of ATP, either I~X (Rasmussen et al., 1965) or X~P (Hodges and Hanson, 1965). By binding with the ATP precursor, Ca^{+2} would reduce ATP synthesis and hence its turnover by ATPase. If the breakdown of Ca:I~X or Ca:X~P is slower than the formation plus degradation of ATP, then oxidation will be inhibited.

With the lowest oxidation rates observed in these experiments, all 3 levels of Ca^{+2} used (10^{-4}, 10^{-3}, 10^{-2}M) were equally effective in stimulating oxidation. This indicates that 10^{-4}M Ca^{+2} was a saturating amount. In mitochondria with low oxidation rates, the Ca^{+2} stimulation was significantly reduced by the presence of exogenous ATP which will increase ADP availability through ATP turnover.

If mitochondria have rapid ATP turnover, then their respiratory control should be low. And it was; the highest respiratory control ratio observed was 1.8. It is not that these mitochondria are uncoupled; they do synthesize ATP. Rather, they are loosely coupled (Racker, 1970); i.e., they produce ATP but they degrade it as soon as it is formed. Perhaps, in some cases, loosely coupled mitochondria are the in vivo condition. Thermogenic brown fat mitochondria from newborn and cold-stressed guinea pigs possess (Christiansen, 1970) all criteria of loose coupling as described by Ernster and Luft (1963).

The divalent cations, Ca^{+2} and Sr^{+2}, were equally effec-

tive in stimulating oxidation. Mg^{+2} and Mn^{+2} were not, and neither was K^{+}. Similar results were obtained with animal mitochondria (Lenninger, 1970). Animal mitochondria can actively accumulate Ca^{+2},Sr^{+2} and Mn^{+2} but not Mg^{+2} which can not penetrate the mitochondrial membrane. K^{+} can only be accumulated in the presence of ionophorous antibiotics, such as valinomycin and gramicidin.

Since all of these cations were added as chloride salts and some of them resulted in no change, it appears that what we regard as a "Ca^{+2} effect" was not actually a "chloride effect".

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APPENDIX

Calcium can affect plant mitochondria in a number of ways. In the absence of bovine serum albumin, Ca^{+2} can stimulate "U-Factor" formation leading to uncoupling of oxidative phosphorylation and swelling of the mitochondria (Wojtczak and Lehninger, 1961). Calcium also can promote extramitochondrial NADH oxidation, probably by releasing some rate-limiting step (Miller et al., 1970). Calcium passively bound to membranes can alter their properties (Manery, 1966), and active accumulation of Ca^{+2} within mitochondria can stimulate respiration. The latter process in corn mitochondria requires inorganic phosphate (Hanson et al., 1965).

During swelling of rat liver mitochondria induced by Ca^{+2}, direct analytical measurements indicated a parallel intramitochondrial formation of U-Factor, a heat stable, isooctane-soluble uncoupling and swelling agent of fatty acid nature (Wojtczak and Lehninger, 1961). If BSA were present, the swelling due to Ca^{+2} was prevented, due to BSA binding of fatty acids.

It is well known that Ca^{+2} stimulates enzymic hydrolysis of neutral fats and phospholipids. In some cases, this stimulation is due to removal of products, i.e. fatty acids, which form insoluble Ca^{+2} salts, rather than a true activation of enzymic hydrolysis. With U-Factor formation, however, Ca^{+2} seems to be a true activator, since BSA, which is also a good acceptor for fatty acids, did not stimulate U-

Factor formation (Wojtczak and Lehninger, 1961).

Plant mitochondria readily oxidize exogenous NADH. This oxidation is stimulated by salts in general and by divalent cations in particular. Hackett (1961) found that the greater divalent cation stimulation can not be explained in terms of greater ionic strength alone. He suggested that the stimulation was due to either an increased NADH permeability or the release of some limiting step in the respiratory chain.

Miller et al. (1970) rejected the first suggestion. They reasoned that if divalent cations alter membrane permeability, this should affect the oxidation of all substrates and not just NADH. However, their experimentation showed this not to be true. Nevertheless, there remained the possibility that divalent cations were affecting a specific NADH transport system.

Miller et al. (1970) proposed that divalent cations were probably stimulating a flavoprotein which directly reduced exogenous NADH. That there might be such a flavoprotein was suggested by the fact that divalent cations did not affect the reduction of endogenous NADH (produced by malate-pyruvate oxidation). That this stimulation occurred early in the respiratory chain was indicated by the fact that divalent cations did not affect succinate oxidation and therefore were probably affecting the respiratory chain before cytochrome b.

There are two types of passive calcium binding: (1) low

affinity binding which is half-saturated at 100 uM Ca^{+2}, and (2) high affinity binding which is half-saturated at 0.025 uM Ca^{+2} (Van Dam and Meyer, 1971). The low affinity binding seems to depend on phospholipids (Scarpa and Azzi, 1968) and there is evidence suggesting that it is involved in the process of Ca^{+2} translocation (Scarpa and Azzone, 1968).

High affinity binding is uncoupler sensitive and this fact led Reynafaye and Lehninger (1969) to conclude that high affinity binding sites may represent specific membrane carriers for Ca^{+2} translocation. This idea has been disputed by Mela and Chance (1969); for Ca^{+2} translocation is lanthamide sensitive and the number of lanthamide sensitive sites is one order of magnitude lower than the uncoupler sensitive sites.

At physiological pH, the mitochondria are negatively charged and each of the negative sites is surrounded by water of hydration (Manery, 1966). When Ca^{+2} binds to these sites, it causes a change in electrical properties by decreasing the net charge. This binding also dehydrates the membrane by causing loss of water of hydration. Both of these changes could markedly alter membrane configuration and hence permeability properties.

In human red blood cells and brain slices, Ca^{+2} is needed to maintain membrane permeability. Once the normal permeability of red blood cells to electrolytes is lost by lactose treatments, only Ca^{+2} can restore it (Bolingbroke

and Maizels, 1959). Furthermore, K^+-depleted brain slices will not reaccumulate K^+ if Ca^{+2} is absent (Gardos, 1961). This is so even in the presence of the transport-supporting metabolites glucose and glutamate.

Calcium accumulation within mitochondria is an active process driven by energy derived from either coupled respiration or ATP hydrolysis (Van Dam and Meyer, 1971). Hodges and Hanson (1965) showed that corn mitochondria can accumulate Ca^{+2} in the presence of phosphate. The kinetics of these systems were determined by Elzam and Hodges (1968). The optimum pH was about 7.5 and 8.0 for the ATP- and substrate-driven systems, respectively. Both systems also show similar temperature optima of 30° to 35°. The energies of activation, determined on the basis of Arrhenius plots, were 14.6 kcal/mole for the substrate-driven system and 10.97 kcal/mole for the ATP-driven system. On the basis of linear Lineweaver-Burk plots the Km's for Ca^{+2} accumulation for the substrate- and ATP-driven systems were 0.37mM and 1.0mM, respectively, and the Vmax's were 4.15 umoles/mg N/min and 0.50 umoles/mg N/min, respectively. On a molar basis the relative effectiveness of 3 inhibitors on substrate-driven transport is DNP$>$oligomycin$>$azide. For the ATP-driven system the relative effectiveness of the inhibitors is oligomycin$>$azide$>$DNP.

The Ca^{+2}:Pi ratio for both systems approximated 1.6:1, which was similar to that found for animal mitochondria and

suggests the deposition of hydroxyapatite inside the mitochondria (Elzam and Hodges, 1968). Magnesium, manganese and barium ions acted as strong competitors of Ca^{+2} transport.

As far back as 1949, Ca^{+2} was classified as an uncoupler of oxidative phosphorylation. In these early experiments, Ca^{+2} was added in massive amounts which led to sustained respiratory stimulation, similar to that given by DNP (Siekevitz and Potter, 1953). In later experiments, Ca^{+2} was added in lesser concentrations. This led to a temporary burst of respiration, followed by a return to State 4 respiration, the "resting state" (Chance, 1963). In these temporary bursts of respiration Ca^{+2} is still in a sense an "uncoupler", for when respiratory energy is driving Ca^{+2} accumulation, it is unavailable for ATP synthesis (Hanson, 1972). However, upon completion of Ca^{+2} accumulation, ATP can once more be formed. ATP synthesis and Ca^{+2} accumulation are, therefore, alternative processes; they do not occur at the same time but rather sequentially.

The fact that ATP hydrolysis also supports Ca^{+2} accumulation suggests that an intermediate of oxidative phosphorylation is responsible for Ca^{+2} transport (Rasmussen et al., 1965, Hodges and Hanson, 1965). Hodges and Hanson (1965) proposed that this intermediate was X P, since in corn mitochondria Ca^{+2} accumulation did not occur unless phosphate was present. Rasmussen et al. (1965), working

with rat liver mitochondria, found that phosphate was not essential for Ca^{+2} accumulation. They proposed that it is I~X, the non-phosphorylated intermediate, that causes Ca^{+2} transport.

Both of these theories are based on the Chemical Theory of oxidative phosphorylation. This theory was first proposed in principle by Slater (1953) and elaborated in detail by Lehninger (1953-1954) and Chance and Williams (1956). The hypothesis states that passage of a pair of electrons from each of 3 specific carriers in the respiratory chain to the next is coupled to the formation of an energy-rich bond, presumably an an anhydride linkage, between one of the two electron carriers and a third entity, an unidentified "coupling factor". This high-energy intermediate, directly or indirectly, can react with phosphate to form a phosphorylated high-energy intermediate which can donate its phosphate to ADP (Lehninger et al., 1967). This is illustrated in the following reactions, where C represents carrier, I represents inhibitor, and X represents unknown.

$$C\sim I \;\&\; X \rightleftharpoons C \;\&\; I\sim X$$

$$I\sim X \;\&\; Pi \rightleftharpoons X\sim P \;\&\; I$$

$$X\sim P \;\&\; ADP \rightleftharpoons X \;\&\; ATP$$

It was noted that the accumulation of Ca^{+2} led to the ejection of hydrogen ions (Saris, 1963). The appearance of the hydrogen ions can be explained by the modified Chemical Theory as follows. The high-energy intermediate is an acid

anhydride (Hanson *et al.*, 1972). The water that is released upon its formation is separated into ions. The hydrogen ions are ejected to the outside of the mitochondria - the hydroxyl ions to the inside.

The Chemiosmotic Theory is a second theory of how respiration is coupled to oxidative phosphorylation; its chief proponent is the British biochemist Mitchell. According to this theory, the energy derived from respiration is conserved in a separation of charges, hydrogen ions on the outside of the mitochondria, hydroxyl ions on the inside (Mitchell, 1966). In the Chemical Theory, there was likewise a separation of charges. The Chemiosmotic Theory differs from the Chemical Theory in that there is no formation of a chemical high-energy intermediate; thus, the hydrogen and hydroxyl ions do not come from the formation of an acid anhydride.

The Chemiosmotic Theory has further been described in a review article by Lehninger *et al.*(1967) who states: "There are several steps in the electron transport chain which involve either uptake or formation of protons. Mitchell has proposed that the enzymes catalyzing these steps are arranged in the plane of a hydrogen ion impermeable membrane in a folded manner, geometrically speaking, so as to form three 'loops'. The active sites of the enzymes carrying out the three H^+-yielding reactions are proposed to be oriented in the plane of the membrane so as to eject protons to the outside, and the active sites of the enzymes catalyzing the

three H^+-absorbing reaction steps are oriented so as to extract protons from the intramitochondrial compartment. The three oxidation-reduction loops are thus functionally equivalent to the three energy-conserving sites in the traditional coupling hypothesis; in effect each loop is an energy-conserving 'site'."

According to the Chemiosmotic Theory, Ca^{+2} accumulation is an accidental feature of mitochondrial activity. Calcium freely diffuses into the mitochondria without the intervention of a carrier, since it is being exchanged for the ejected hydrogen. The accumulated Ca^{+2} would, however, collapse the transmembrane potential which in turn would stimulate respiration (Lehninger et al., 1967).

It has been shown that K^+ effluxing from mitochondria, moving down a concentration gradient of at least 200, resulted in ATP synthesis (Massari and Azzone, 1970). It is probable that a Ca^{+2} efflux can have the same result. Thus, Ca^{+2} accumulation probably is an energy storage phenomemon.

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