Parathyroid Hormone and the Reactions of Mitochondria to Cations*

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ABSTRACT: The effect of parathyroid hormone upon the mitochondrial responses to magnesium and potassium addition have been studied and compared to those observed when calcium reacts with mitochondria. The hormone alters the responses of mitochondria to both Mg^{2+} and K^+. Either cation, in the presence of hormone, alters the mitochondrial responses to calcium. All three cations appear to be transported by the same mechanism. Hormone alters the permeability of the mitochondrial membrane to K^+ and Mg^{2+}. Most of the known effects of this hormone upon mitochondrial metabolism can be accounted for by this hypothesis. On the basis of this and other data a model is constructed to account for mitochondrial cation transport. Also, further verification of the thesis that the mitochondrial swelling associated with ion accumulation is an osmotic phenomenon has been obtained. The problems of the specificity of these hormonal effects and their physiological implications have been examined and discussed. Present evidence indicates that they are highly specific, and may be related to the physiologic effects of this hormone.

Considerable recent evidence indicates that the parathyroid hormone influences numerous parameters of mitochondrial metabolism (DeLuca *et al.*, 1962; Sallis *et al.*, 1963; Rasmussen *et al.*, 1964b; Fang and Rasmussen, 1964; Rasmussen *et al.*, 1964a; Aurbach *et al.*, 1964; Sallis and DeLuca, 1964, 1965; Rasmussen and DeLuca, 1963; Rasmussen, 1965). Most, but not all (Aurbach *et al.*, 1964), of these effects have been attributed to the effect of hormone upon ion exchanges. In fact, a major emphasis has been placed upon hormonally stimulated anion accumulation (Sallis *et al.*, 1963; Rasmussen *et al.*, 1964b), leading Sallis and DeLuca (1965) to propose a primary action of the hormone upon anion transport in spite of the fact that recent studies of the mitochondrial accumulation of calcium and manganese have emphasized the primacy of energy-linked cation transport with anion transport apparently a secondary and passive process (Chappell *et al.*, 1963, Rasmussen *et al.*, 1965; Chance, 1965). In view of these facts, and our previous observations concerning the effects of hormone upon magnesium and potassium transport (Sallis *et al.*, 1963; Rasmussen *et al.*, 1964a), a reconsideration of the effects of hormone upon cation transport has been undertaken. It is the purpose of this communication to describe the results of these studies. The results indicate that parathyroid hormone increases the permeability of the inner mitochondrial membrane to the cations, magnesium and potassium.

Methods

Rat liver mitochondria were prepared by a modification of the method of Schneider (1958). The rats were killed by decapitation. The livers were removed immediately, and placed in ice-cold 0.37 M sucrose–0.05 mM Tris–EDTA. Two-gram portions were added to 18 ml of 0.37 M sucrose–0.05 mM Tris–EDTA, and homogenized in a Potter–Elvehjem homogenizer. Nuclei were sedimented at 700*g* and mitochondria at 10,000*g*. The mitochondria were washed once in 0.37 M sucrose–0.05 mM EDTA and finally in 0.37 M sucrose and suspended in the latter solution. Pyridine nucleotide fluorescence was excited at 366 mμ and measured at 450 mμ (Chance, 1965; Chance and Hagihara, 1963). Oxygen consumption was measured with a vibrating platinum electrode (Chance and Hagihara, 1963). Light scattering was measured by recording absorbance at 540 mμ (Chance and Hagihara, 1963; Chance, 1965; Rasmussen *et al.*, 1965) and K^+ concentration with a Model 22 Radiometer pH meter, a suitable amplifier, and a Beckman cation specific electrode (No. 39137) and a standard reference electrode. The measurement of pyridine nucleotide fluorescence, light scattering, oxygen consumption, and in some cases K^+ concentration were carried out simultaneously[1] and recorded, after suitable amplification on a Type 5-124 recording oscillograph[2] or by employing the technique of Chance and Hagihara (1963). In the usual experiment, 0.5 ml of mitochondrial suspension, giving a final concentration

* From the Department of Biochemistry, University of Pennsylvania, Philadelphia, Pennsylvania. *Received August 20, 1965; revised November 22, 1965.* Supported by grants from the U. S. Public Health Services (AM-09650) and the U. S. Atomic Energy Commission (AT-30-1-3489).

[1] This instrument was constructed in the instrument and electronic shops of the Johnson Foundation. We are indebted to Dr. Britton Chance and Mr. Dieter Mayer for the design, construction, and operational readiness of this apparatus.

[2] Purchased from Consolidated Electrodynamics Corp., Pasadena, Calif.

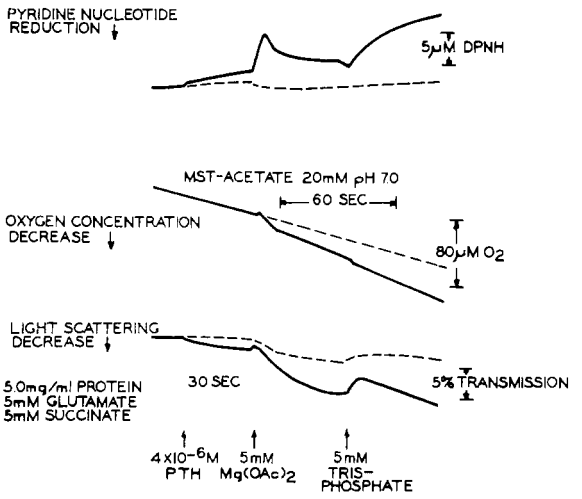

FIGURE 1: The response of mitochondria to the successive additions of $Mg(OAc)_2$ and Tris-phosphate in the absence (----) and presence (———) of 4×10^{-6} M parathyroid hormone in a mannitol-sucrose-Tris-acetate medium. Note that the addition of hormone (PTH) led to a slight decrease in light scattering and pyridine nucleotide fluorescence even before Mg^{2+} addition.

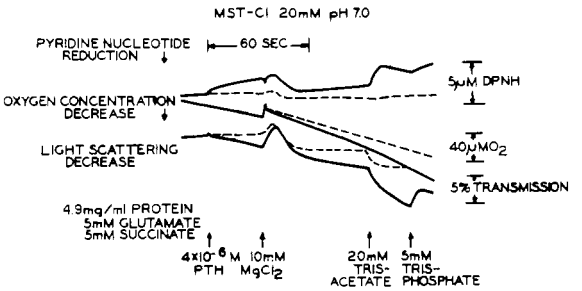

FIGURE 2: The response of mitochondria to the successive additions of $MgCl_2$, Tris-acetate, and Tris-phosphate in the absence (----) and presence (———) of parathyroid hormone in a mannitol-sucrose-Tris-chloride medium.

of 4–5 mg of mitochondrial protein per ml, was added to 5.0 ml of medium. Incubations were carried out at room temperature in mannitol-sucrose-Tris-acetate (MST-OAc)[3] or Tris-chloride (MST-Cl) media containing mannitol 225 mM, sucrose 75 mM, Tris glutamate 5 mM, Tris succinate 5 mM, and Tris-acetate or Tris-chloride, pH 7.0, 20 mM. Mitochondrial protein concentration was measured using the biuret method (Gornall *et al.*, 1949). Total DPNH (reduced pyridine

[3] Abbreviations employed are: MST, mannitol-sucrose-Tris; PTH, parathyroid hormone; DPNH, reduced diphosphopyridine nucleotide; OAc, acetate; BSA, bovine serum albumin; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

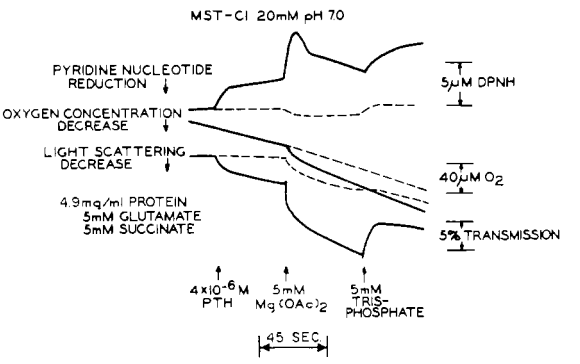

FIGURE 3: The response of mitochondria to the successive additions of $Mg(OAc)_2$ and Tris-phosphate in the absence (----) and presence (———) of parathyroid hormone in a mannitol-sucrose-Tris-chloride medium.

nucleotide) concentration of the mitochondrial suspensions was measured in an Aminco-Chance dual wavelength spectrophotometer (Chance and Williams, 1956). This instrument was also employed to measure changes in the oxidation-reduction states of the respiratory carriers using suitable wavelength pairs as described by Chance and Williams (1956). In these studies 0.3 ml of mitochondrial suspension was added to 2.7 ml of medium. The medium contained sucrose 320 mM, 2 mM $MgCl_2$, 9 mM Tris-glutamate, 10 mM Tris-chloride, pH 7.0, and 5×10^{-6} M parathyroid hormone. Magnesium was measured by atomic absorption spectrometry employing a Jarrell-Ash Model 82-360 spectrometer. Parathyroid hormone was prepared by the method of Hawker *et al.* (1966). It was dissolved in 0.001 M acetic acid immediately before use. In control experiments 0.001 M acetic acid was added in place of the hormone solution.

Results

In every instance the responses of mitochondria to the particular reagent were recorded in the absence as well as the presence of hormone. Typical results of magnesium addition in acetate and chloride media are shown in Figures 1 and 2, respectively. In the absence of hormone, magnesium acetate addition and the subsequent addition of phosphate had little effect upon oxygen consumption or pyridine nucleotide fluorescence but did lead to a decrease in light scattering. The usual effect was for magnesium to cause a slight reduction of pyridine nucleotide, and a small but significant decrease in light scattering (Figure 1). The addition of hormone brought about an immediate decrease in light scattering, a decrease in pyridine nucleotide fluorescence (pyridine nucleotide oxidation), and in some experiments a barely detectable increase in rate of O_2 consumption. The subsequent addition of magnesium acetate led to a burst of respiration followed by a sustained increase in rate of O_2 consumption, an immediate oxidation-reduction cycle of pyridine nucleotides, the reduction being only

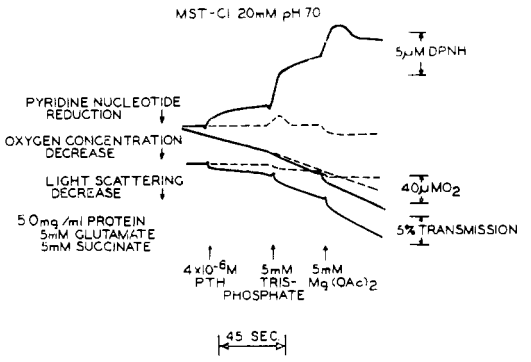

FIGURE 4: The response of mitochondria to the successive additions of Tris–phosphate and $Mg(OAc)_2$ in the absence (----) and presence (———) of parathyroid hormone in a mannitol–sucrose–Tris–chloride medium.

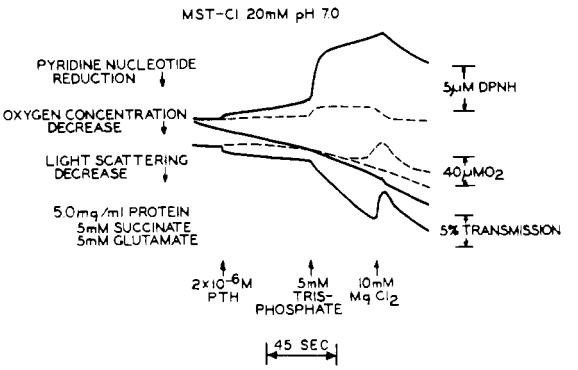

FIGURE 5: The response of mitochondria to the successive additions of Tris–phosphate and $MgCl_2$ in the absence (----) and presence (———) of parathyroid hormone in a mannitol–sucrose–Tris–chloride medium.

partial, followed by a more gradual reduction, and a significant decrease in light scattering. The subsequent addition of phosphate led to an oxidation of pyridine nucleotides, and an increase in light scattering without a marked change in rate of O_2 consumption. The extent of the decrease in light scattering was proportional to acetate concentration at any given concentration of magnesium, and was greater with higher magnesium concentrations.

The responses to magnesium were different in the chloride medium (Figure 2). In the absence of hormone, $MgCl_2$ produced a slight increase followed by a slight decrease in light scattering and a barely detectable oxidation–reduction of pyridine nucleotides. The addition of hormone led to a decrease in pyridine nucleotide fluorescence and a slight decrease in light scattering. The subsequent addition of $MgCl_2$ led to slight oxidation followed by significant reduction of the pyridine nucleotides, an initial increase in light scattering followed by a decrease, and in some instances a transitory increase in respiration. The subsequent addition of acetate led to a definite stimulation of respiration, a partial oxidation–reduction cycle of pyridine nucleotides, and a decrease in light scattering. Phosphate addition at this point produced mitochondrial contraction (increased light scattering) with a further increase in respiration.

The addition of magnesium acetate to mitochondria treated with hormone in a chloride medium led to changes (Figure 3) similar to those seen in Figure 1. However, under these conditions light scattering decreased less, the cycle of pyridine nucleotide oxidation–reduction was more complete, and the burst of respiration was followed by a return to a rate of respiration only slightly greater than control. Also, the subsequent addition of phosphate (Figure 3) led to a significant and sustained increase in light scattering. Over-all, the responses of the mitochondria to the sequential additions of magnesium acetate and phosphate under these conditions (Figure 3) were qualitatively similar to those observed after the addition of calcium acetate and phosphate to mitochondria in an acetate medium (see Figure 3, Rasmussen *et al.*, 1965).

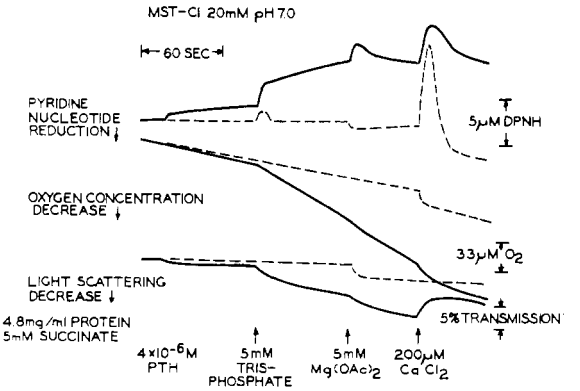

FIGURE 6: The response of mitochondria to the successive additions of Tris–phosphate, $Mg(OAc)_2$, and $CaCl_2$ in the absence (----) and presence (———) of parathyroid hormone in a mannitol–sucrose–Tris chloride medium in which succinate was the sole substrate.

All of these observations are in keeping with the concept that cation transport is primary and active, whereas anion transport is secondary and passive. However, if the order of additions was reversed and phosphate added first (after hormone) a stimulation of respiration, a decrease in light scattering, and pyridine nucleotide oxidation occurred (Figures 4 and 5). The subsequent addition of magnesium acetate, Figure 4, or magnesium chloride, Figure 5, led to changes similar to those produced by these agents in the absence of phosphate (Figures 2 and 3). These mitochondrial responses to phosphate were particularly striking when succinate was the sole substrate (Figure 6). After phosphate addition (Figure 6), there was a considerable stimulation of respiration from 8.5 μM/g of protein per min to 17 μM/g of protein per min and decreased pyridine nucleotide fluorescence (oxidation) as well as a slight decrease

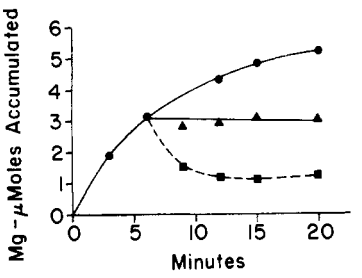

FIGURE 7: A plot of the mitochondrial accumulation of magnesium (micromole/milligram of protein) as a function of time. Mitochondria, equivalent to 4.6 mg/ml, were incubated in a medium containing 280 mM sucrose, 3 mM $Mg(OAc)_2$, 3 mM K_2HPO_4, 12 mM Na succinate, 15 mM imidazole buffer, pH 7.2, 0.5% bovine serum albumin, and 5×10^{-6} M parathyroid hormone. At 6 min, 10^{-3} M KCN (▲—▲) or 10^{-3} M KCN and 2×10^{-3} M ATP (■—■) were added to two separate vessels; a third (●—●) to which neither KCN or ATP were added served as control. Note that ATP addition led to prompt loss of mitochondrial magnesium. Bovine serum albumin was used to inhibit nonspecific mitochondrial swelling. Its presence was not necessary to demonstrate the present effects.

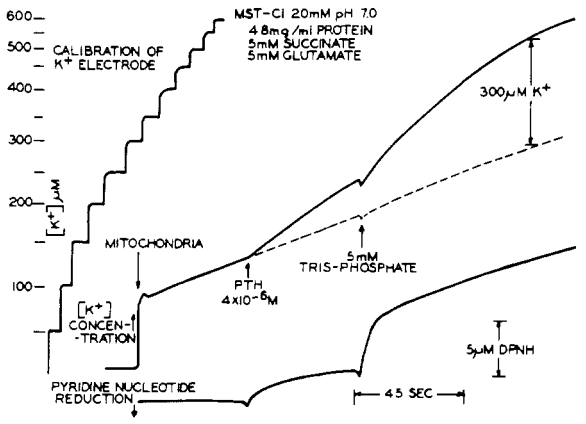

FIGURE 8: A plot of K^+ concentration and of pyridine nucleotide fluorescence as a function of time during the successive additions of parathyroid hormone, and Tris–phosphate to mitochondria in a mannitol–sucrose–Tris–chloride medium (———). The dashed line (------) indicates the K^+ concentration when 0.001 N acetic acid was added in place of hormone. Note that phosphate addition led to no increase in K^+ efflux in the control.

in light scattering. The subsequent addition of $Mg(OAc)_2$ led to a further slight increase in respiratory rate, a further decrease in light scattering, and a cycle of oxidation–reduction of the pyridine nucleotides. Addition of calcium chloride, at this point, produced a typical oxidation–reduction of pyridine nucleotides and a burst of respiration. However, when these responses to calcium were compared to those in the absence of hormone (dashed lines, Figure 6) it was quite clear that the rate of the calcium reaction was considerably slower in the presence of Mg^{2+} and hormone. Of particular interest was the fact that the addition of calcium in the presence of hormone, magnesium, and phosphate (Figure 6) led to a significant increase in light scattering, in spite of the fact that calcium usually leads to little change in light scattering in the presence of phosphate.

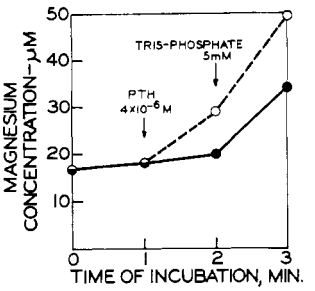

FIGURE 9: A plot of the magnesium concentration in the medium *vs.* time of incubation. Mitochondria (4.8 mg of protein/ml) were incubated in MST–Cl, pH 7.0 medium. Parathyroid hormone was added at 1 min to one set of mitochondria (○-----○) and Tris–phosphate to both control (●—●) and hormone-treated mitochondria (○-----○) after 2 min.

The above results suggest that either cation or anion can be actively accumulated. However, before reaching such a conclusion an alternative explanation was investigated. It was apparent from previous work that under many circumstances there is a bidirectional flux of magnesium and phosphate across the mitochondrial membrane and that under some conditions passive efflux was sufficient to prevent net accumulation of magnesium even though other evidence (increased rate of oxygen consumption) suggested that significant influx was taking place (Fang and Rasmussen, 1964). Furthermore, magnesium, accumulated by mitochondria in the presence of hormone, can be rapidly withdrawn from the mitochondria by the addition of ATP (Figure 7). Thus, it seemed possible that phosphate addition, particularly in the presence of hormone, might bring about magnesium and potassium efflux. This was found to be the case (Figures 8 and 9).

The addition of hormone to mitochondria in either a chloride (Figure 8) or acetate medium led to two immediate effects, potassium ejection and pyridine nucleotide oxidation. In fact, these responses were the most immediate observed after the addition of hormone. The subsequent addition of phosphate led to a further oxidation of pyridine nucleotide and a further ejection of K^+. The total increase in K^+ in the medium was 0.3 mM which was equivalent to a decrease of 10–15 mM within the mitochondria.

It was not possible to carry out similar experiments with magnesium because of the inability to monitor

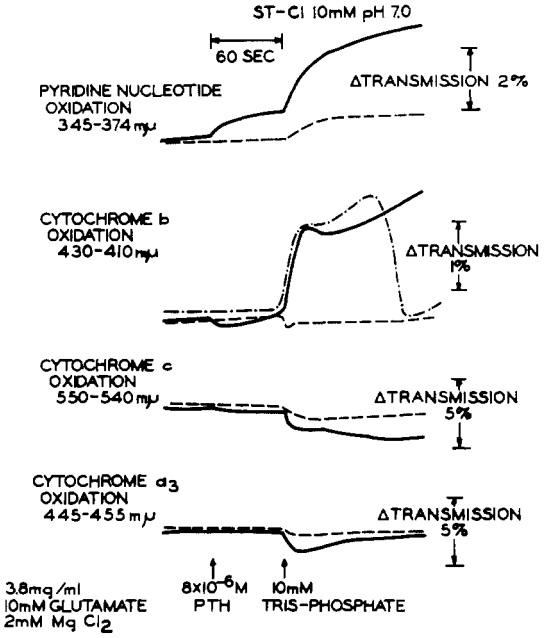

FIGURE 10: The changes of absorbance of pyridine nucleotide, cytochrome *b*, cytochrome *c*, and cytochrome a_3 to phosphate addition in the absence (-----) and presence (———) of parathyroid hormone, 5×10^{-6} M in a medium containing 320 mM sucrose, 2 mM $MgCl_2$, 9 mM Tris–glutamate, 10 mM Tris–chloride, pH 7.0. For comparison the change of absorbance of cytochrome *b* produced upon phosphate addition, in the absence of hormone but presence of 1 mM ADP, is plotted (—·—·—).

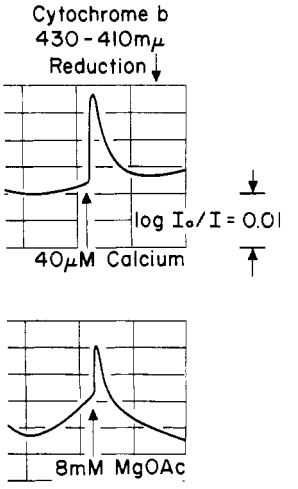

FIGURE 11: The response of cytochrome *b* to the addition of 40 μM Ca^{2+} (upper) and 8 mM magnesium acetate in the presence of 4×10^{-6} M parathyroid hormone (lower) in a medium containing 200 mM sucrose, 10 mM glutamate, 2 μg/ml of oliogmycin, 15 mM Tris–Cl, pH 7.0, 0.04 mM $MgCl_2$, 0.04 mM sodium phosphate, and mitochondria equivalent to 3 mg/ml of protein.

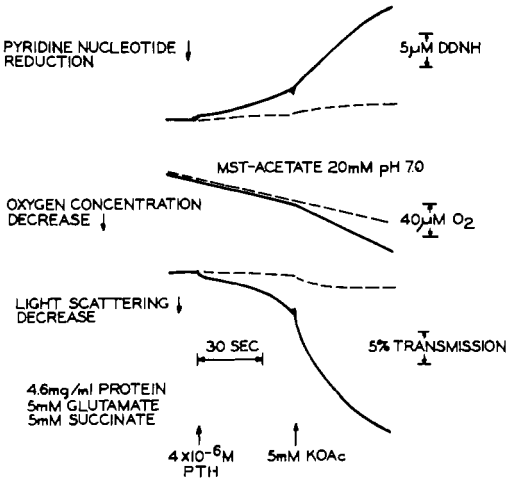

FIGURE 12: The response of mitochondria to the addition of 5 mM KOAc in the absence (- - - -) and presence (———) of 4×10^{-6} M parathyroid hormone in a mannitol–sucrose–Tris–acetate medium.

constantly the concentration of this cation. However, a different type of experiment was performed. Mitochondria were incubated in a mannitol–sucrose–chloride medium. At 1-min intervals aliquots were removed, chilled, centrifuged at 12,000*g* for 3 min, and the magnesium content of the supernatant measured. Hormone was added to one of two tubes after 1 min and phosphate added to both after another minute. The results are shown in Figure 9. It is apparent that hormone promoted Mg^{2+} release and this was augmented by phosphate addition. The magnesium concentration of the medium increased 0.03 mM in 3 min, equivalent to a decrease of 1–1.5 mM in the mitochondria.

The effect of parathyroid hormone upon the responses of the other respiratory carriers was also studied. The results are shown in Figure 10. The effect of phosphate addition, in the presence and absence of hormone, upon the absorbance of pyridine nucleotides, cytochrome *b*, cytochrome *c*, and cytochrome a_3 are recorded. Also shown is the response of cytochrome *b* to phosphate addition in the presence of ADP (without hormone). The initial cytochrome *b* response to phosphate addition in the presence of Mg^{2+} and hormone were quite similar to phosphate addition in the presence of ADP. Also the initial responses of the other respiratory carriers to phosphate addition, in the presence of Mg^{2+} and hormone, were those predicted from the original analysis of Chance and Williams (1956) concerning crossover points in oxidative phosphorylation.

These results imply that the coupling sites for Mg^{2+} transport are similar to those involved in oxidative phosphorylation. A similar conclusion has been reached by Chance (1965) concerning the coupling sites for calcium transport. In this latter regard, it is of interest that the response of cytochrome *b* to magnesium addition

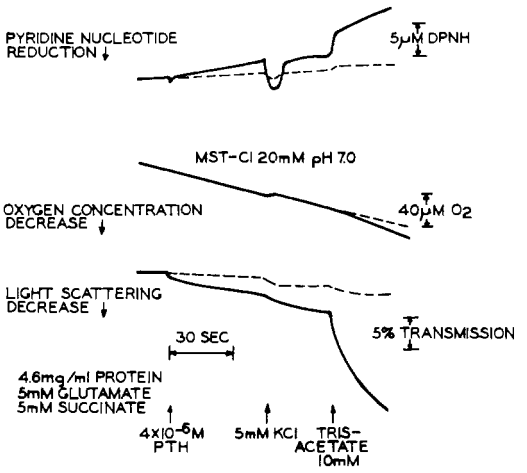

FIGURE 13: The response of mitochondria to the successive additions of 5 mM KCl and 10 mM Tris-acetate in the absence (- - - -) and presence (———) of 4×10^{-6} M parathyroid hormone in a mannitol-sucrose-Tris-chloride medium.

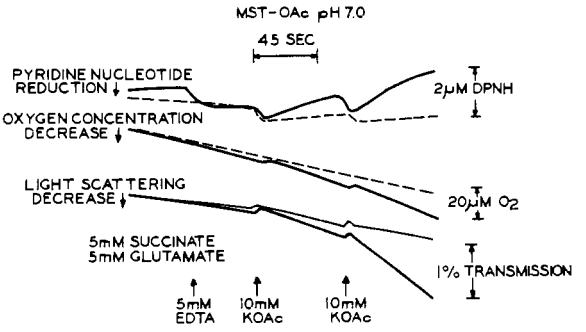

FIGURE 14: The response of mitochondria to the addition of potassium acetate in the absence (- - - -) and presence (———) of 5 mM EDTA in a mannitol-sucrose-Tris-acetate medium. Note that the scale used in this figure differs from that used in previous figures.

in the presence of hormone was similar to that observed upon calcium addition in the absence of hormone (Figure 11). The major difference in the pattern of the two responses was the continued reduction of cytochrome *b* after an initial oxidation in the hormone-magnesium system as compared to a cycle of oxidation-reduction upon calcium addition. Another important difference was that of concentration. The initial response to 8 mM $Mg(OAc)_2$ was similar to the response to 40μM $CaCl_2$.

The responses of mitochondria to potassium and hormone were also studied. Typical results are shown in Figures 12 and 13. In the presence of hormone, the addition of KOAc to mitochondria in an acetate medium led to a sustained increase in rate of oxygen consumption, a progressive increase in pyridine nucleotide fluorescence, and a marked decrease in light scatter-

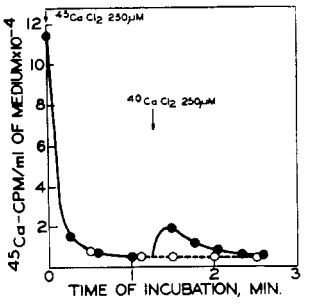

FIGURE 15: A plot of radiocalcium concentration in the medium as a function of time after the addition of 250 μM $CaCl_2$, labeled with 17.5 μc of ^{45}Ca, and then 1.25 min later a second addition of 250 μM unlabeled $CaCl_2$ (●—●) in a mannitol-sucrose-Tris-acetate medium. The dotted line (O- - - -O) represents the plot when the second addition was water alone.

ing (Figure 12). Under these circumstances, potassium was accumulated (Rasmussen *et al.*, 1964a). The addition of KCl in a chloride medium produced a small decrease in light scattering, a slight reduction-oxidation of pyridine nucleotides, and practically no change in respiration (Figure 13). The subsequent addition of acetate (Figure 13) or phosphate led to pyridine nucleotide oxidation, decrease in light scattering, and a stimulation of respiration.

Azzone and Azzi (1965) have noted that mitochondria treated with EDTA become more permeable to K^+. This observation plus the present ones concerning the hormonal effects upon the reactions of mitochondria to Mg^{2+} raised the possibility that the hormonal effect upon K^+ transport might be brought about by altering the Mg^{2+} binding properties of the membrane and thereby altering K^+ flux. No direct test of this hypothesis was possible. However, it was possible to test the effect of EDTA upon the reaction of mitochondria to K^+ under the present conditions. The results are shown in Figure 14. EDTA-treated mitochondria did respond to K^+ addition with a slight stimulation of respiration, of swelling, and of pyridine nucleotide oxidation. However, the effects were small (note that the scale on this figure differs from that on the preceeding ones).

Because of the many similarities between Mg^{2+} transport, in the presence of hormone, and of Ca^{2+} transport in its absence, a reinvestigation of hormone-stimulated ATP-supported uptake of magnesium was undertaken. It had been found previously that ATP did not support Mg^{2+} accumulation in the presence of hormone. However, a possible explanation of this lack of accumulation was apparent from studies such as that shown in Figure 7. It was possible that under the previous conditions, the bulk of the magnesium had been bound by the ATP and, therefore, not accumulated. By greatly increasing the magnesium concentration it was possible to demonstrate a hormonal stimulation of magnesium accumulation in the presence of Antimycin A and ATP (Table I). This result indicates a

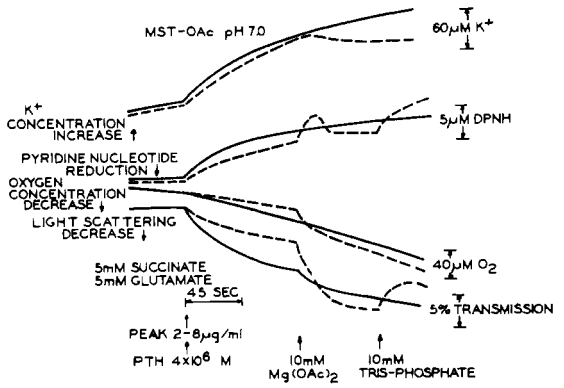

FIGURE 16: The response of mitochondria to the successive additions of 10 mM $Mg(OAc)_2$ and 10 mM Tris-phosphate in the presence of 8 μg/ml of peptide (peak 2) (————) compared to the responses seen in the presence of 4×10^{-6} M parathyroid hormone (– – – –) in a mannitol-sucrose-Tris-acetate medium.

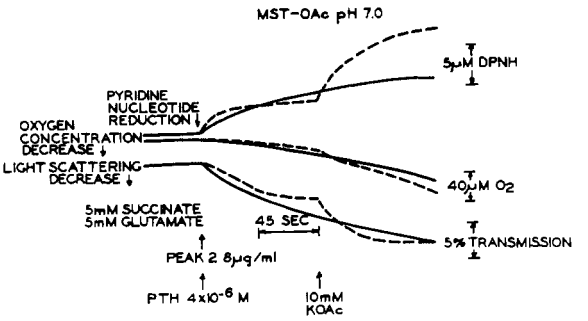

FIGURE 17: The response of mitochondria to the addition of 10 mM KOAc in the present of 8 μg/ml of peak 2 (————) compared to the response observed in the presence of 4×10^{-6} M parathyroid hormone (– – –).

TABLE I: Magnesium Content of Control and Parathyroid Hormone Treated Mitochondria.[a]

Time of Incubation (min)	μg/mg Mitochondrial Protein	
	Control	Hormone Treated
5	2.2	3.3
20	2.7	3.7

[a] Mitochondria, 3 mg/ml, were incubated in sucrose 150 mM, Tris-ATP 5 mM, $MgCl_2$ 30 mM, Tris-phosphate 10 mM, pH 7.0, Antimycin A 4 μg/ml, and parathyroid hormone 4×10^{-6} M, at 32°.

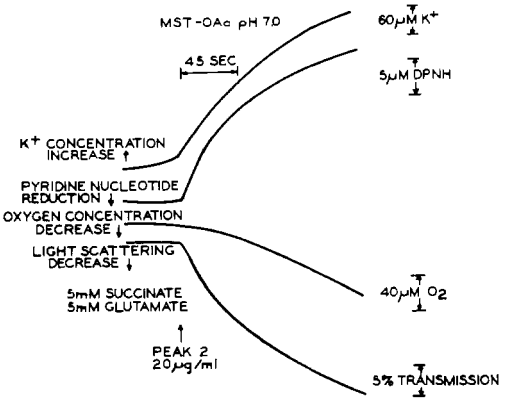

FIGURE 18: The response of mitochondria to 20 μg/ml of peak 2 in a mannitol-sucrose-Tris-acetate medium.

further similarity between the calcium transport system and the present one.

Another point of difference was the apparent lack of Ca^{2+} efflux after accumulation as compared to a significant Mg^{2+} efflux (Fang and Rasmussen, 1964). A reinvestigation of the calcium transport system demonstrated that calcium efflux does occur. It was possible to demonstrate this efflux by first adding labeled calcium to mitochondria, allowing it to accumulate, and then making a second addition of unlabeled calcium. This second addition of calcium led to a transitory release of labeled calcium (Figure 15).

In earlier publications (DeLuca *et al.* 1962; Fang *et al.* 1963; Sallis *et al.* 1963), it was reported that none of the other common peptide hormones produce effects similar to parathyroid hormone upon isolated mitochondria. The macrocyclic antibiotics, Valinomycin (Moore and Pressman, 1964; Pressman, 1965; Ogata and Rasmussen, 1966), and Gramicidin (Chappell and Crofts, 1965) have effects which, on cursory examination, are similar to those produced by hormone (Rasmussen, 1964a). However, their effects are confined to alterations in mitochondrial responses to monovalent cations. Neither antibiotic influences the mitochondrial responses to Mg^{2+}. Another substance of particular interest is a polypeptide, peak 2, isolated from bovine parathyroid glands (Rasmussen *et al.*, 1964c; Hawker *et al.*, 1966). This highly basic peptide was found to mimic some of the effects of parathyroid hormone upon isolated mitochondria *in vitro* but none of its effects *in vivo* (Rasmussen *et al.*, 1964c). Therefore, its effect upon various mitochondrial responses was examined further. Some similarities but several important differences were noted between these responses and those observed in the presence of parathyroid hormone. As shown in Figure 16, the addition of 8 μg/ml of peak 2 to mitochondria in a MST-OAc medium led to immediate swelling, pyridine nucleotide oxidation, K^+ ejection, and a slight increase in respiration. These responses were qualitatively similar to those produced by hormone, and a concentration of peak 2 was selected so that a similar degree of pyridine nucleotide oxidation was obtained as that produced by 4×10^{-6} M parathyroid hormone. In the presence of this concentration

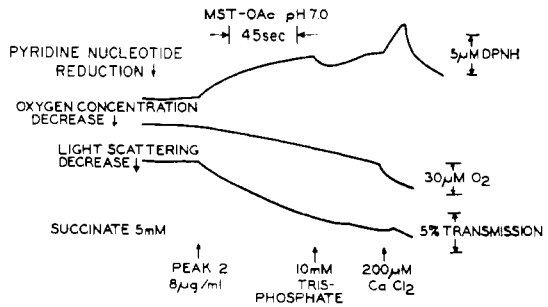

FIGURE 19: The response of mitochondria to the successive addition of 10 mM Tris–phosphate and 200 μM $CaCl_2$ in the presence of 8 μg/ml of peak 2. Compare with Figure 6.

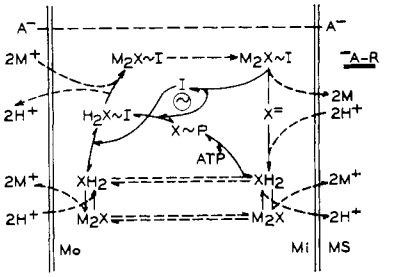

FIGURE 20: A model of the cation pump of the inner mitochondrial membrane where $M_2X\sim I$ or $H_2X\sim I$ are the energized forms of the carrier and XH_2 and M_2X the nonenergized form. Mo is the outer surface of the inner membrane; Mi, the inner surface; MS, the matrix space; A^-, the permeable anion; $^-A–R$, the fixed polyanion in the matrix space; and $X\sim P$ is the phosphorylated high energy intermediate of oxidative phosphorylation. Solid lines (———) represent reactions between components, and dashed (– – – –) transport steps. Note particularly that the carrier in the nonenergized form can move in either direction across the membrane but that the energized carrier can only move from outside to inside.

of peak 2 the subsequent addition of magnesium and then of phosphate to mitochondria had no effects upon pyridine nucleotide fluorescence, respiratory rate, or rate of swelling (Figure 16), in contrast to the striking effects of these ions in the presence of hormone (Figure 1). Similarly, potassium addition produced no changes in swelling, pyridine nucleotide fluorescence, or respiratory rate (Figure 17), as it did in the presence of parathyroid hormone (Figure 12). Furthermore, the addition of a higher concentration of peak 2 in the absence of added cation led to progressive and uncontrolled K^+ ejection, mitochondrial swelling, and pyridine nucleotide oxidation (Figure 18). Similar changes were not seen with comparable increases in parathyroid hormone concentration. An additional difference was that phosphate addition in the presence of hormone led to significant responses (Figure 6) but had little effect in the presence of peak 2 (Figure 19). Of particular interest is the fact that the peak 2 treated mitochondria are responsive to Ca^{2+} (Figure 19) even though not responsive to K^+ or Mg^{2+}. Similar but less extensive studies were carried out with polylysine. Mitochondrial responses to Mg^{2+}, K^+, and phosphate were similar to those noted with peak 2 and clearly different from those noted with parathyroid hormone.

One of the most interesting parameters of mitochondrial metabolism which should be investigated under the present conditions are alterations in hydrogen ion concentration. An attempt was made to accomplish this in the present investigation. However, it was not possible because of technical difficulties. The buffering capacities of the added solutions were considerable and their pH altered upon dilution. Hence, it was impossible not to observe a pH shift of a magnitude sufficient to obscure the changes which might be expected to accompany ion transfer. Another approach to this problem will be necessary.

Discussion

Three important conclusions can be drawn from the present and previous results: (1) the transport of magnesium across the inner mitochondrial membrane in the presence of parathyroid hormone has many similarities to calcium transport in its absence; (2) in mitochondria, cation transport appears to be active and primary, anion transport secondary and passive; and (3) a major effect of the parathyroid hormone is to increase the permeability of the mitochondrial membrane to magnesium and potassium, thereby lowering the apparent K_m for the energy-linked influx of these ions across this membrane.

A comparison of previous results (Rasmussen *et al.*, 1965; Chance, 1965) with those recorded in Figures 1, 2, 3, and 11 indicates the striking similarity between the responses of mitochondria to calcium and to magnesium in the presence of hormone. For example, the results shown in Figure 3 of the present work and Figure 3 of the previous (Rasmussen *et al.*, 1965) are qualitatively similar. There are, however, some differences. The addition of calcium in an acetate medium led to a burst of respiration followed by a return to the control rate. It was accompanied by a complete oxidation–reduction cycle of the pyridine nucleotides. Magnesium acetate addition was followed by a burst of respiration and incomplete oxidation–reduction cycle. There was a second component to the response to magnesium acetate which was missing from the calcium responses. This was a sustained increase in the rate of O_2 consumption and a sustained increase in pyridine nucleotide oxidation. However, this difference is probably a reflection of the difference of the solubilities of the salts these two cations form within the mitochondria, the difference in their rates of efflux from the mitochondria, and the difference in their affinities for the mitochondrial cation carrier. The continued respira-

tory stimulation in the case of magnesium is probably a consequence of the continual efflux and reaccumulation of significant quantities of magnesium from the mitochondria (Fang and Rasmussen, 1964), in contrast to the small rate of calcium efflux (Figure 15). A second difference was the response to the subsequent addition of phosphate. In both cases phosphate addition led to mitochondrial contraction without a stimulation of respiration. However, contraction was not as marked with magnesium (Figure 3) as with calcium (Rasmussen *et al.*, 1965), and the pyridine nucleotides became more oxidized after phosphate addition in the present system but more reduced in the calcium system.

The results recorded in Figures 10 and 11 and Table I substantiate the similarities between Mg^{2+} and Ca^{2+} transport because both activate the same coupling sites as does ADP, and both are supported by energy derived either from ATP hydrolysis (Table I) or substrate oxidation. However, there is one point of difference which has not been resolved. The ratio of Ca^{2+} accumulated/oxygen consumed (Ca^{2+}/O) is in the range of 6.0 for calcium (Chance, 1963, 1965); whereas, even when efflux is accounted for, the highest ratios of Mg^{2+} influx/O are 3.0 (Fang and Rasmussen, 1964). This may mean either that the method used to estimate efflux is invalid and underestimates this parameter, and thereby Mg^{2+} influx, that both entry and exit of magnesium are energy-linked, or that the affinity of the cation carrier for magnesium is so low that only 1 mole of magnesium is bound per mole of carrier in contrast to 2 of calcium. Also, there appears to be some difference in anion specificity for the binding of these two cations (Rasmussen, 1965). This observation plus the fact that it had not been possible to demonstrate ATP-supported Mg^{2+} accumulation in the presence of parathyroid hormone had led to the postulate that Mg^{2+} and Ca^{2+} were transported by different carriers which compete for the same source of energy (Rasmussen, 1965). The obvious effect which Mg^{2+} has upon the reaction of mitochondria with calcium in the presence of hormone (Figure 6) is compatible with either view. However, the present evidence has resolved most of the major differences and indicates a striking similarity between the reaction of mitochondria to calcium, and to magnesium in the presence of parathyroid hormone. These similarities make our previous arguments less compelling, particularly when it is realized that any change in the membrane, such as that produced by parathyroid hormone, can possibly alter the rates of anion as well as cation penetration.

In view of the present results, it is difficult to accept the conclusion of Sallis and DeLuca (1965) that hormone has a direct effect upon anion transport. Their results have been confirmed in the present study (Figure 6) but their conclusion seems unwarranted in view of the results shown in Figures 8 and 9. The most plausible explanation of these findings is that after hormone addition but before phosphate addition some cation has leaked out of the mitochondria. Phosphate addition leads to a further cation, magnesium and potassium, efflux which is sufficient, in the presence of hormone, to bring about an increased rate of cation influx with a consequent stimulation of respiration. However, it is well to point out that the present results do not completely exclude the possibility that hormone alters phosphate permeability. It is clear from the present work and the previous work with calcium (Rasmussen *et al.*, 1965) that the permeability of the membrane to particular anions can greatly modify the response of the mitochondria to cation, *i.e.*, modify the activity of the cation pump. For example, an agent which increased the Cl^- permeability of the mitochondrial membrane would alter the response of the mitochondria to calcium in a chloride medium. It was possible to test this possibility by studying the effect of hormone upon the response of mitochondria to sulfate. When calcium accumulates in an acetate medium, mitochondrial swelling occurs (Rasmussen *et al.*, 1965). The subsequent addition of phosphate leads to contraction with exchange of phosphate for intramitochondrial acetate. Sulfate will not bring about a similar effect. It was reasoned that if hormone altered anion permeability and allowed sulfate to enter the mitochondria, then hormone addition should allow sulfate to replace phosphate in causing acetate ejection and mitochondrial contraction in the calcium system. Hormone did not alter the response to sulfate. Hence, the only available evidence indicates that hormone does not specifically alter the permeability of the membrane to anion.

It is noteworthy that the effect of phosphate, in the presence of hormone, was greatest when succinate was the sole substrate. This is in keeping with our previous observations concerning the greater effects of hormone upon ion accumulation in the presence of succinate (Fang and Rasmussen, 1964). It would seem to imply a unique role of DPNH, the DPN^+ (diphosphopyridine nucleotide) coupling site, or substrate phosphorylation in maintaining a higher degree of respiratory control and membrane integrity.

Our previous (Rasmussen *et al.*, 1964a) and present observations (Figure 12 and 13) indicate that parathyroid hormone influences the permeability of the mitochondrial membrane to potassium and that there is competition between K^+ and Mg^{2+} transport. The evidence is insufficient to determine whether K^+ and Mg^{2+} are transported by the same carrier. Considerably more experimentation is necessary to decide this question, but it is clear that the responses to K^+ in the presence of hormone could be accounted for if this were the case, particularly when considered in the light of recent studies with the antibiotic Valinomycin and its effects upon K^+ and Ca^{2+} transport (Ogata and Rasmussen, 1966). On the other hand, the possibility exists that a primary hormonal effect upon Mg^{2+} binding could be responsible for the altered K^+ permeability (Azzone and Azzi, 1965; and Figure 14). This could explain why ATP-supported K^+ uptake is readily demonstrable in the presence of Valinomycin (Pressman, 1965; Ogata and Rasmussen, 1966), but extremely difficult to demonstrate in the presence of parathyroid hormone (Rasmussen *et al.*, 1964a) particularly if one considers the evidence of Lee and Ernster (1965) that low

concentrations of magnesium are important in the terminal phosphorylating steps. It is of considerable interest that Mg^{2+} addition to hormone-treated mitochondria leads to a cessation of hormone-induced K^+ efflux (Figure 16).

All indications are that the swelling observed in the present instance had an osmotic basis similar to that proposed previously (Rasmussen *et al.*, 1964a, 1965) and to that observed by others (Dilley, 1964; Chappell and Crofts, 1965; Tedeschi and Hegarty, 1965). Of particular note was the calcium-induced contraction observed (Figure 6) after Ca^{2+} was added to mitochondria pretreated with hormone, phosphate, and magnesium. The most likely explanation of this change is that magnesium and phosphate were accumulated and existed, in part, in an osmotically active form within the mitochondria. When calcium was added it was accumulated with a resultant complexing of phosphate and mitochondrial contraction.

The two proposals that (1) parathyroid hormone alters the permeability of the membrane to magnesium and (2) magnesium and calcium are transported by a similar mechanism lead to the necessary consequence that parathyroid hormone should decrease the rate and extent of calcium transport in the presence of magnesium. Preliminary results (Figure 6) indicate that this is the case. This provides a rational basis for the effect of hormone upon calcium release, but not for a specific effect of vitamin D upon hormonally dependent calcium release (DeLuca *et al.*, 1962). This particular aspect of parathyroid action is being reinvestigated in the light of these more recent findings.

Most of the known facts concerning ion transport in mitochondria (Chappell *et al.*, 1963; Chance, 1963; Chance and Yoshioka, 1965; Chance, 1965; Judah *et al.*, 1965; Rossi and Lehninger, 1964; Chappell and Crofts, 1965; Lehninger, 1962; Rasmussen *et al.*, 1965; Rasmussen and DeLuca, 1963; Saris, 1963) and the effects of parathyroid hormone (DeLuca *et al.*, 1962; Sallis *et al.*, 1963; Rasmussen *et al.*, 1964b; Fang and Rasmussen, 1964; Rasmussen *et al.*, 1964a; Aurbach *et al.*, 1964; Sallis and DeLuca, 1964, 1965), Valinomycin (Moore and Pressman, 1964; Pressman, 1965; Ogata and Rasmussen, 1966), and Gramicidin (Chappell and Crofts, 1965) can be accounted for by assuming that all cations are transported by a common carrier located in the inner mitochondrial membrane (Tedeschi, 1959; Parsons, 1965; Klingenberg, 1963) which can exist either in an energized ($H_2X\sim I$) or nonenergized form (H_2X) (Figure 20). When in the energized form, the carrier is capable of transporting cations into the matrix space but not in the opposite direction. When in the nonenergized state the carrier can shuttle ions in either direction. The second component of the system is the location of fixed anionic sites within the matrix space. In the presence of an impermeant anion, *e.g.*, chloride, cation uptake is limited by the concentrations of fixed intramitochondrial anionic sites and of energized carrier in the membrane. H^+ evolution in a chloride medium is a consequence of the combination of cation with carrier; in a phosphate medium it is due to the intramitochondrial reaction

$$3Ca^{2+} + 2HPO_2^{2-} \rightleftharpoons Ca_3(PO_4)_2 + 2H^+$$

In this view, the mitochondrial membrane is relatively permeable to H^+, and the distribution of protons depends upon the distribution of electrical and chemical gradients across the membrane. Another point of view has been expressed (Mitchell, 1961; Chappell and Crofts, 1965). These authors propose that the movement of protons is the primary energy-linked reaction, and that cation movements are secondary to the electrochemical gradient produced by the separation of H^+ and OH^- across the membrane.

There are difficulties with either model. The most important one for the present model is the hypothetical nature of $X\sim I$. The present model depends heavily upon the chemical reality of this substance. On the other hand, the hydrogen pump model does not account for several important experimental facts. In an acetate medium, the addition of calcium leads to accumulation of acetate, little H^+ evolution, a burst of respiration, and mitochondrial swelling (Rasmussen *et al.*, 1965). The subsequent addition of phosphate leads to an exchange of acetate for phosphate, and H^+ evolution due presumably to the formation of $Ca_3(PO_4)_2$. This H^+ is ejected rapidly from the mitochondria without evidence of energy utilization. Second, in order to explain the effects of anions it is necessary to postulate an OH^-–anion exchange. However, the H^+ pump model requires that OH^- within the mitochondria is greater than outside. Hence, the addition of A^-, in large concentration outside with a nonpermeant cation, should drive the exchange because of the large concentration gradients across the membrane. This has not been demonstrated. The further prediction can be made that the presence of permeant anions should decrease the rate of ATP synthesis. Another point which is not fully accounted for by the H^+ pump model is the fact when mitochondria are treated with Valinomycin (Ogata and Rasmussen, 1966) and then both K^+ and Ca^{2+} are added, no K^+ accumulation takes place until nearly all the Ca^{2+} is accumulated.

One of the characteristics of the present proposal is a two-way flux of cations. This is certainly true for Mg^{2+} and K^+, but did not appear to hold for Ca^{2+}. However, by appropriate experimental design it was possible to demonstrate Ca^{2+}-induced Ca^{2+} efflux under conditions of membrane integrity (Figure 15). This result coupled with our previous observation (Ogata and Rasmussen, submitted for publication, 1965) of Ca^{2+}-induced K^+ efflux, in the presence of Valinomycin, indicates that there is no obligatory H^+ ejection when cation is accumulated and constitutes important further evidence against the proposal that the primary basis of cation accumulation is an outwardly directed H^+ pump. Further support for the present model comes from our previous evidence showing that K^+ and Ca^{2+} compete for passive efflux as well as active influx when Valinomycin is present (Ogata and Rasmussen, 1966).

This model represents a slight modification of that proposed previously for calcium transport (Rasmussen *et al.*, 1965). The difference is that in the present model the inhibited state (state 6, Chance, 1965), observed when calcium reacts with mitochondria in a chloride medium, is reached when all of X is in the form of $M_2X\sim I$ rather than, as proposed previously, when all of X is in the form of M_2X. The main difference is that the reaction of calcium with mitochondria in a chloride medium is described by the equation

$$6Ca^{2+} + 3H_2X\sim I \rightleftharpoons 6H^+ + 3Ca_2(X\sim I)^{2+} \quad (1)$$

rather than as previously proposed (Rasmussen *et al.*, 1965)

$$6Ca^{2+} + H_2X\sim I \rightleftharpoons 6H^+ + 3I + 3(Ca_2X)^{2+} \quad (2)$$

and the addition of acetate leads to the further reactions

$$3Ca_2(X\sim I)^{2+} + 6A^- \rightleftharpoons 6(CaA)^+ + 3X^{2-} + 3I \quad (3)$$

$$3X^{2-} + 6H^+ \rightleftharpoons 3H_2X \quad (4)$$

rather than

$$3(Ca_2X)^{2+} + 6A^- \rightleftharpoons 6(CaA)^+ - 3X^{2-} \quad (5)$$

$$3X^{2-} + 6H^+ \rightleftharpoons 3H_2X \quad (6)$$

The over-all reaction in an acetate medium would be identical with that previously described (Rasmussen *et al.*, 1965). This present formulation accounts for the fact that when the inhibited state is reached calcium does not leak out of the mitochondria. If the inhibited state obtained when all of X was in the form Ca_2X, then calcium would leak out of the mitochondria and exchange for H^+ at the outer membrane. Hence, the present model better explains the available facts. It also accounts for the responses of the respiratory carriers in state 6. For example, at the cytochrome *b* coupling site when all of X and I are in the form of $M_2X\sim I$ cytochrome *b* would be in a more highly oxidized state as is observed in state 6 (Chance, 1965; Rasmussen *et al.*, 1965).

A simple explanation of the changes in pyridine nucleotide fluorescence observed under the various conditions employed in the present study is not possible, although most of the changes can be accounted for on the simple proposal that the degree of pyridine nucleotide oxidation is proportional to the concentration of $M_2X\sim I$.

The present model assumes that $X\sim I$, the energized form of the cation carrier, is identical with $X\sim I$, the high energy intermediate of oxidative phosphorylation. However, as noted previously (Rasmussen *et al.*, 1965) an alternate explanation is that they are distinct species related only in so far as $X\sim I$ of oxidative phosphorylation is the source of energy for the cation carrier. An estimate of the concentration of this cation carrier, $X\sim I$, was made in previous work (Rasmussen *et al.*, 1965) based upon the response of mitochondria to calcium in the presence of Oligomycin, and either Antimycin or KCN. This estimate was 4–16 mμmoles/mg of protein and is similar to the estimate of the concentration of the high energy intermediate of oxidative phosphorylation obtained by ATP "jump" (Schachinger *et al.*, 1960). An alternate means of calculating the concentration of energized carrier was possible on the basis of a previous observation (Figure 4 in Rasmussen *et al.*, 1965). The first addition of calcium to mitochondria in a chloride medium led to $Ca^{2+}/O = 6.6$, whereas the addition of acetate after the inhibited state was reached led to a $Ca^{2+}/O = 5.6$. The over-all value is 6.1. This could mean that some $X\sim I$ existed when calcium was added. Hence, upon calcium addition, O_2 consumption was only necessary for the formation of new $X\sim I$ accounting for the high value of 6.6. Acetate addition led to removal of all calcium from $X\sim I$ and the hydrolysis of $X\sim I$. The low value of 5.6 is due to the extra O_2 needed to reestablish the initial concentration of $X\sim I$. Calculated on this basis, $X\sim I$ was 12 mμmoles/mg of protein. From these results it was possible to conclude that a similar pool of high energy intermediates are available for either cation transport or oxidative phosphorylation, but none of the presently available data can determine whether the cation carrier and nonphosphorylated intermediate are identical.

The effect of parathyroid hormone can be explained by assuming one of two actions. The hormone either alters the permeability of the membrane to magnesium and potassium, thereby altering the rate at which these ions penetrate some barrier and interact with the carrier, or the hormone alters the K^+ and Mg^{2+} binding properties of the carrier, *i.e.*, it lowers the apparent K_m for Mg^{2+} and K^+ binding to carrier (Rasmussen, 1964).

The data indicate that if the hormone does interact directly with the carrier to alter its ion binding properties it does so in a very specific manner which does not lead to a significant change in its, the carrier's, ability to bind calcium. Operationally these two possible modes of action would be identical in their effects. However, the subtle difference is that in the first one there is no direct interaction between hormone and carrier, whereas in the second a direct interaction is postulated. For the present both will be included under the same term and the effect of hormone will be described as one of altering the permeability of the mitochondrial membrane to K^+ and Mg^{2+}.

The responses of the mitochondria to the addition of a particular cation will be controlled by a number of complexly interrelated factors: (1) the rate of penetration of the cation through the membrane to the carrier; (2) the affinity ($1/K_m$) of cation for carrier; (3) the state and activity of the electron transport and oxidative-phosphorylation systems; (4) the predominant substrate being metabolized; (5) the concentration of cations on both sides of the membrane; (6) their rates of membrane penetration and their carrier affinities; (7)

the concentrations and membrane permeabilities of the anions present; and (8) the types of possible chemical reactions between cations and anions on each side of the membrane. Also, as pointed out elsewhere (Rasmussen *et al.*, 1964a), the expression of an inherent transport property is determined by the ionic environment in which the membrane is placed. This proposition has received dramatic affirmation by the recent studies of Kipnis (1965) concerning the influence of changes of the ionic environment upon the transport properties of the cell membrane. Thus, it becomes of considerable importance to distinguish between the functional capabilities which are inherent in the particular membrane structure and the functional properties of the membrane which are the restricted set of capabilities expressed in a particular environment. When viewed in this light it is possible to explain the fact that the reactions of mitochondria with calcium *in vitro* appear to be considerably more rapid than their reactions *in vivo* (Chance, 1965). This in turn points up the very real difficulty in relating mitochondrial activity *in vitro* to their activity *in vivo*. It is worth reemphasizing (Rasmussen *et al.*, 1964a) that practically none of the studies on isolated mitochondria have been carried out in media which, in composition, approach the intracellular milieu in which they normally function. Yet in the light of this most recent evidence it becomes increasingly important to do so if an understanding of the physiological functions of the various mitochondrial transport systems are to be achieved.

Practically all the known effects of parathyroid hormone upon mitochondria, including the data of Aurbach *et al.* (1964) and that of Sallis and DeLuca (1964, 1965), can be accounted for in terms of its effects upon cation transport, the associated movements of anions, the associated uncoupling of electron transport from oxidative phosphorylation, and the accompanying mitochondrial swelling. The observations of Aurbach *et al.* (1964) concerning an effect of hormone upon $^{14}CO_2$ production from succinate 1,4-^{14}C can be explained by the uncoupling effect of the hormonally stimulated ion transport. A careful study of this system in this laboratory has shown a striking correspondence between its properties and those of the ion transport systems altered by hormone.

It is not yet possible to define the physiological significance of these hormonal effects upon ion fluxes across a subcellular membrane, but one striking fact is their specificity. Aurbach (1965) has suggested that these effects are similar to those produced by a number of basic polypeptides including a basic peptide, peak 2, isolated from bovine parathyroid glands (Rasmussen *et al.*, 1964c; Hawker *et al.*, (1966). Although this substance does promote phosphate accumulation (Rasmussen *et al.*, 1964c), the present results (Figures 15–19) indicate that the basis for its action is considerably different from that of parathyroid hormone. Similar studies have been performed with polylysine with qualitatively similar results. The important characteristics of the mitochondrial responses to these basic substances are an immediate increase in mitochondrial swelling, respiration, K^+ ejection, and pyridine nucleotide oxidation regardless of the anionic composition of the medium, and a lack of significant response to either Mg^{2+} or K^+ addition in terms of pyridine nucleotide fluorescence, respiratory activation, or rate of swelling. Particularly noteworthy is the complete lack of respiratory activation by Mg^{2+} or K^+ addition in spite of the fact that Ca^{2+} addition will lead to activation.

It has not been possible to determine the precise mode of action of these basic polypeptides but it is of interest that the mitochondria often agglutinate after their addition, a phenomenon not normally observed with parathyroid hormone. It could be argued that the most immediate effect of hormone upon light scattering, K^+ ejection, and pyridine nucleotide oxidation (Figures 3, 8) are relatively nonspecific because they are similar to those seen upon the addition of peak 2 (Figures 16–19) or polylysine. Even if this were the case, it is clear that the subsequent responses are quite different. However, as noted above, a small increase in the concentration of peak 2 or polylysine leads to progressive and marked changes in the mitochondria (Figure 13), whereas a comparable increase in hormone concentration does not produce similar exaggerated changes. Thus, it appears unlikely that even the immediate effects of the hormone are similar to those of the basic polypeptides. This is substantiated by the fact that $Mg(OAc)_2$ addition did not alter the rate of K^+ efflux from mitochondria treated with peak 2 but led to a prompt cessation of K^+ efflux from hormone-treated mitochondria (Figure 16).

The present results leave no doubt that the effects of parathyroid hormone upon isolated mitochondria are highly specific and not solely related to the basic character of this hormonal polypeptide. They constitute further important evidence in support of the thesis (Rasmussen and DeLuca 1963; Rasmussen, 1965) that this hormone interacts directly with membranes and that these interactions account for some, at least, of its physiological effects.

Acknowledgments

We are deeply indebted to Dr. Britton Chance for his assistance in the design, construction, and operational readiness of some of the apparatus used in these studies as well as for his critical appraisal of the results. We wish to thank Miss Anita Köllner for expert technical assistance, and Mr. Charles Hawker for the preparation of the parathyroid hormone used in this work.

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