Potassium and Proton Movements in Relation to the Energy-Linked Transport of Phosphate in Liver Mitochondria*

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ABSTRACT: The possible correlation that exists between phosphate transport, substrate oxidation, and the movements of K^+ and H^+ catalyzed by ionophorous antibiotics has been studied in mitochondria partially depleted of endogenous inorganic phosphate. Inorganic phosphate is required for valinomycin, nonactin, or monazomycin to reverse the efflux of K^+ and the light-scatter changes mediated by monensin A in mitochondria. The inhibition of glutamate + malate oxidation caused by monensin is overcome by valinomycin and low concentrations of inorganic phosphate. Alkali metal cation uptake induced by valinomycin-like antibiotics with monensin, is selectively supported by the oxidation of succinate, glutamate + malate, or β-hydroxybutyrate in the presence of phosphate. The net uptake of cation or water into mitochondria seems to be regulated by the content of intramitochondrial phosphate. An energy-linked uptake of inorganic phosphate supported by succinate, β-hydroxybutyrate, or glutamate + malate oxidation reverses the monensin-inhibited incorporation of $[^{32}P]P_i$ into adenosine triphosphate (ATP). The dinitrophenol-sensitive uptake of inorganic phosphate which occurs in presence of monensin is not accompanied by simultaneous K^+ uptake or H^+ efflux. An increase in the osmolarity of the medium above 340 mOsm also inhibits the aerobic translocation of phosphate and results in an inhibition of the incorporation of P_i into ATP. These inhibitory effects of hyperosmolarity, as well as those caused by monensin, are reversed by β-hydroxybutyrate, glutamate + malate, or succinate oxidation. It is concluded that the aerobic translocation of inorganic phosphate into mitochondria is not the consequence of primarily stimulated cation uptake or H^+ efflux. An explanation which attempts to rationalize the possible role of anion–anion-exchange processes in the regulation of phosphate transport, K^+ movements, and the incorporation of P_i into ATP is offered.

Various studies have been carried out on the possible mechanism by which oxidizable substrates, inorganic phosphate, and several other anions penetrate the mitochondrion. Harris *et al.* (1967a,b) advanced the hypothesis that the aerobic translocation of anions was dependent on the stimulation of cation influx and the subsequent charge neutralization due to secondary anion influx. Mitchell (1966, 1968) proposed that the uptake of an anionic substrate occurs through a proton–anion symport mechanism where protonation of the un-ionized acid could account for its translocation across the lipid membrane. In line with Mitchell's suggestion, Palmieri and Quagliariello (1969) observed that proton ejection from mitochondria was associated with stimulation of anion uptake and conversely, that the inhibition of anion uptake and efflux of endogenous anions apparently was the result of net proton uptake in respiratory-inhibited mitochondria. Data from Harris and Manger (1968a,b) appear to be consistent with the possibility that oxidizable substrates may be transported across mitochondrial membranes through an energy-dependent process which is not the reflection of energy-linked cation movements. Moreover, Hansford and Chappell (1968) have also described conditions where an energy-driven translocation of phosphate takes place in absence of net K^+ uptake.

Previous results obtained with neutral and monocarboxylic ionophorous antibiotics suggested that either cation uptake (Harris *et al.*, 1967a,b) or proton ejection (Palmieri and Quagliariello, 1969) could be primary phenomena subsequently followed by passive anion uptake. However, information described in the present work obtained from studies with the carboxylic antibiotic monensin A (Estrada-O *et al.*, 1967a–c) indicates that the translocation of saturating concentrations of some oxidizable substrates and inorganic phosphate is not a consequence of induced alkali metal cation uptake or proton efflux from mitochondria. Moreover it is shown, that under certain conditions, an energy-linked uptake of phosphate is necessary for the occurrence of alkali metal cation transport. Additional evidence in the present work suggests that the rate of incorporation of $[^{32}P]P_i$ into ATP in mitochondria depends not only on the transport of phosphate (Lardy *et al.*, 1967; Estrada-O *et al.*, 1967a) but also on the permeation or exchange of some oxidizable substrates through a mechanism not primarily associated with predicted movements of the mobile pool of alkali metal cations or protons.

Materials and Methods

Mitochondria were prepared from livers of male rats weighing 200 g as described by Johnson and Lardy (1967).

Preparation of Phosphate-Depleted Mitochondria. Mitochondria from 1 g of wet liver were incubated in 5 ml of a medium which contained: 2 mM NaADP, 2 mM β-hydroxybutyrate, 1 mM NaEDTA, 10 mM triethanolamine-Cl (pH 7.4), and 0.25 M sucrose at 30° for 10 min. The mitochondria

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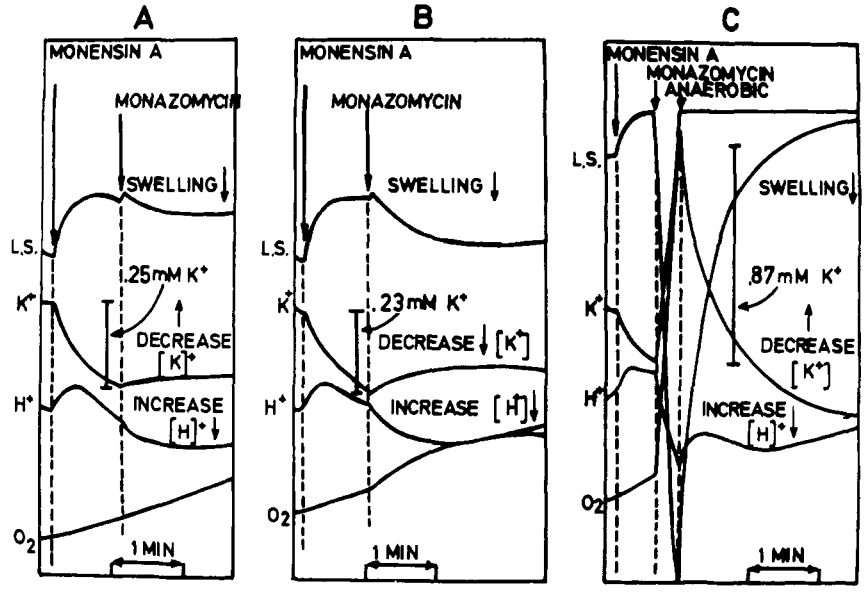

FIGURE 1: The effect of monensin, monazomycin, and phosphate or acetate on the rate of glutamate + malate oxidation, light-scattering changes, and the transport of K^+ and H^+ in liver mitochondria. A downward deflection of the light-scattering trace (L. S.) is associated with swelling of the mitochondria. An upward deflection in the K^+ or H^+ tracing represents a decrease in the concentration of these ions in the medium or an uptake of K^+ or H^+ by the mitochondria. The medium contained: 10 mM KCl, 1 mM $MgCl_2$, 10 mM triethanolamine–HCl (pH 7.4), 180 mM sucrose, and mitochondria equivalent to 1.4 mg of N in 5-ml volume at 28°. Monazomycin and monensin were added at a concentration of 1.3×10^{-7} and 2×10^{-7} M, respectively. Other additions were as follows. Panel A: 10 mM acetate–triethanolamine and 10 mM glutamate. Identical tracings were observed when acetate was replaced by 10 mM phosphate or when glutamate was replaced by equal amounts of L-malate, pyruvate, citrate, or ketoglutarate; panel B: 5 mM glutamate + 5 mM L-malate and 10 mM of either acetate, formate, or butyrate–triethanolamine; panel C: 5 mM glutamate + 5 mM malate and 10 mM phosphate–triethanolamine. The mitochondria were partially depleted of P_i as described in Methods.

were sedimented at 19,000*g* for 10 min in 0.08 M sucrose–0.25 M mannitol. They lost approximately 30% of its original content of P_i by this treatment (from approximately 14.5 to 10 μmoles of P_i per g of protein).

Measurement of K^+ and H^+ Movements, Oxygen Consumption, and Light-Scattering Changes in Mitochondria. A continuous recording of oxygen consumption, light-scattering changes, and variations in the extramitochondrial concentration of K^+ and H^+ was carried out using an apparatus designed, developed, and constructed by Chance, Mayer, and Pressman (Pressman, 1965, 1967; Graven *et al.*, 1966). Independent measurements of light-scattering changes were performed in some experiments with a Beckman DK-2 recording spectrophotometer.

Measurement of the Incorporation of $[^{32}P]P_i$ into ATP. $[^{32}P]P_i$ orthophosphate was separated from other phosphate-containing compounds by the method of Martin and Doty (1947) as modified by Falcone and Witonsky (1964). In the experiments in which the exchange of $[^{32}P]P_i$ between P_i and ATP was measured in absence of substrates, the exchange rates were corrected for recycling (Boyer *et al.*, 1956) on the assumption that only the terminal phosphate moiety of ATP freely exchanged with orthophosphate.

Measurement of the Total Accumulation of P_i into Mitochondria. Rat liver mitochondria were incubated in 2.0 ml of the reaction mixture described in Figure 6 and Table I. After 5-min incubation at 30°, 1 ml of the reaction mixture was rapidly cooled and gently layered over 6.0 ml of 0.88 M sucrose and the mitochondria were isolated essentially as described by Pressman (1958) and Brierley *et al.* (1963). After addition of 5% trichloroacetic acid, P_i was extracted by the procedure of Falcone and Witonsky (1964) and measured in the organic solvent phase by determination of the radioactivity when $[^{32}P]P_i$ was used or chemically by the method of Martin and Doty (1947) as modified by Lindberg and Ernster (1965).

Other Analytical Procedures. Radioactivity measurements were made with a Nuclear-Chicago gas-flow low-background counter equipped with a micromil window. $[^{32}P]P_i$ was obtained from Volk Radiochemical Co. (Burbank, Calif.) or from Tracerlab (Waltham, Mass.). The antibiotics monazomycin, monensin A, nigericin, and nonactin used in these experiments were a kind gift from Dr. Henry A. Lardy. Valinomycin was obtained from Calbiochem.

Results

Mitochondria partially depleted of endogenous P_i by ADP were used in all experiments. The stimulation of ion movements catalyzed by valinomycin-like antibiotics in presence of monensin was found to be absolutely dependent on added phosphate. As shown in Figure 1, monazomycin overcomes the monensin-induced K^+ efflux, light-scatter changes, and inhibition of oxygen uptake. This effect is dependent on the oxidizable substrate present and the anionic composition of the mixture. Panel A of Figure 1 shows the effects of 2×10^{-7} M monensin and 1.3×10^{-7} M monazomycin in medium which contains K^+, acetate, and glutamate. Addition of monensin, prior to monazomycin, causes K^+ efflux, proton uptake, and mitochondrial contraction; the

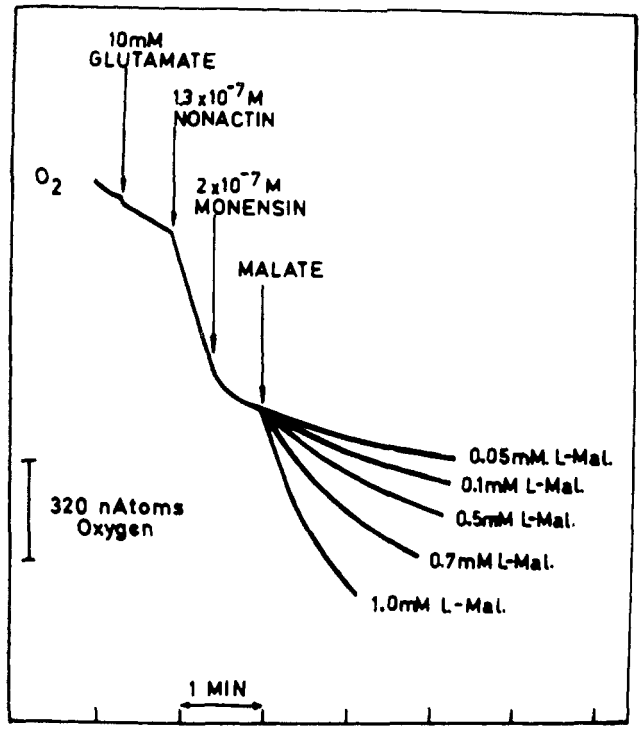

FIGURE 2: The quantitative requirements of L-malate to reverse the monensin-inhibited glutamate oxidation previously stimulated by nonactin in mitochondria. The reaction mixture contained: 15 mM KCl, 10 mM glutamate–triethanolamine, 1 mM $MgCl_2$, 10 mM triethanolamine–HCl (pH 7.4), 5 mM phosphate–triethanolamine, 180 mM sucrose, 1.3×10^{-7} M nonactin, 2×10^{-7} M monensin, and 2 mg of mitochondrial N in 5-ml volume at 28°. L-Malate was added at the indicated concentrations. The mitochondria were partially depleted of endogenous P_i as described in methods.

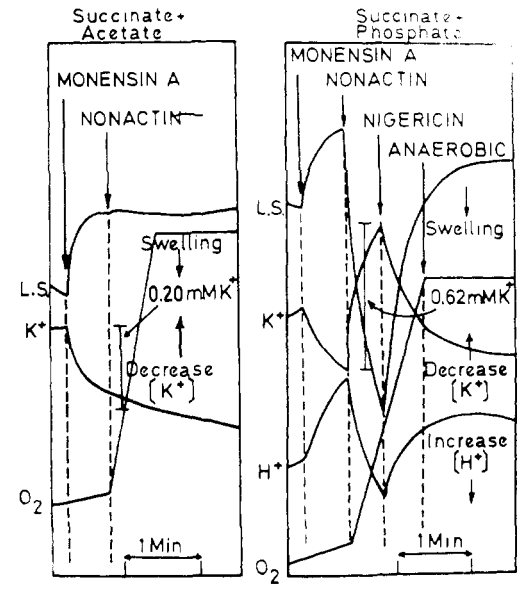

FIGURE 3: The effect of acetate or phosphate on the rate of succinate oxidation, light-scattering changes, and K^+ and H^+ movements effected under the influence of monensin, nonactin, and nigericin in mitochondria. The reaction mixture contained: 15 mM KCl, 1 mM $MgCl_2$, 10 mM triethanolamine–HCl, 10 mM succinate–triethanolamine, 10 mM of either acetate (left panel), or phosphate–triethanolamine (right panel), 180 mM sucrose, and 1.8 mg of mitochondrial N in 5-ml volume at 28°. Monensin, nonactin, and nigericin were added at the concentration of 2×10^{-7}, 1.3×10^{-7}, and 1×10^{-7} M, respectively. The mitochondria were partially depleted of P_i as described in Methods.

further addition of monazomycin does not affect these tracings nor does it stimulate the oxidation of glutamate. Almost identical results are found when glutamate is replaced by 10 mM L-malate, pyruvate, citrate, or α-ketoglutarate or else when acetate is substituted by 10 mM phosphate, arsenate, formate, or butyrate. Monazomycin is also unable to reverse cation efflux and inhibition of substrate oxidation mediated by monensin (panel B of Figure 1) when the pair 5 mM glutamate + 5 mM L-malate is used as oxidizable substrate with acetate, formate, or butyrate as accompanying anions. On the other hand, monazomycin fully overcomes the ion movements and light-scatter changes induced by monensin when acetate, formate, or butyrate are replaced by phosphate in the presence of glutamate + malate. Monazomycin simultaneously stimulates oxygen uptake, H^+ efflux, and K^+ influx. The subsequent anaerobiosis causes rapid loss of the accumulated cation. The nonactin homologs as well as valinomycin also reverse the ion movements caused by monensin under the conditions displayed in panel C of Figure 1. After the efflux of cations caused by monensin, glutamate and L-malate have to be present simultaneously with phosphate in order to be oxidized. The quantitative requirements of L-malate needed to reverse the inhibition of glutamate oxidation caused by monensin in the presence of phosphate and nonactin are shown in Figure 2. Half-maximal stimulation of oxygen uptake is obtained with 0.5 mM L-malate; 0.3 mM of added phosphate causes half-maximal stimulation of oxygen uptake in similar experiments carried out with excess of glutamate and L-malate (10 mM each). D-Malate does not replace L-malate in these conditions.

Nonactin and similar ionophorous antibiotics are also able to overcome ion movements mediated by monensin in a medium which contains succinate and phosphate. Nonactin stimulates succinate oxidation after monensin, without reversing cation efflux, proton uptake, and light-scatter changes mediated by the latter compound in a mixture which contains 10 mM acetate, formate, propionate, or butyrate (left panel of Figure 3). The uncoupling of succinate oxidation caused by nonactin in the presence of monensin (left panel of Figure 3) has similar mechanistic implications to the uncoupling caused by valinomycin and nigericin in medium free of added P_i (Pressman *et al.*, 1967). On the other hand, when "penetrant" anions such as acetate or formate (Chappell and Haarhoff, 1967) are substituted by inorganic phosphate (right panel of Figure 3), nonactin not only stimulates succinate oxidation but completely reverses the cation outflow and the proton uptake which had been previously induced by monensin. Almost identical phosphate-dependent effects are observed when 10 mM β-hydroxybutyrate is used instead of succinate.

At concentrations of extramitochondrial K^+ of less than 45 mM, ion fluxes caused by both monensin A or dianemycin are reversed by valinomycin-like antibiotics. This process is dependent on the oxidizable substrate added and on P_i (Figures 1, 3, and 4). On the other hand, ion movements induced by nigericin or compound HLR-206 (Lardy

et al., 1967; Pressman, 1968; Henderson *et al.*, 1969; Estrada-O and Dorschner, 1968) are not counter acted by valinomycin-like antibiotics under similar experimental conditions. Data to be published elsewhere (C. Gómez-Lojero and S. Estrada-O, in preparation) indicates that differences in the association constants for the various antibiotic–cation complexes (Pressman, 1968) could explain differences in their effects upon mitochondria.

The regulation by P_i of mitochondrial volume changes linked with K^+ movements in mitochondria partially depleted of endogenous P_i is shown in Figure 4. Trace A of Figure 4 indicates that monensin added after valinomycin completely reverses light-scatter changes in a medium containing K^+, acetate, or formate and either glutamate, pyruvate, L-malate, citrate, or α-ketoglutarate. The reversal of the light-scatter trace by monensin is unaffected by two subsequent additions of 5 mM P_i. When glutamate is substituted by succinate, β-hydroxybutyrate, or glutamate + malate in medium which contains acetate or formate, monensin not only overcomes to the basal value of the light-scatter trace mediated by valinomycin but induces an increase in the light scatter of a magnitude which is equivalent to the net swelling caused by valinomycin (trace B of Figure 5). In mitochondria which had not been depleted of phosphate the monensin does not cause this large increase in light scatter. Thus, it appears that the endogenous phosphate level is of importance in the regulation of the net movements of cation or water into mitochondria. Different to the results displayed in trace A of Figure 5, the addition of 5 mM P_i on top of the contraction caused by monensin (trace B of Figure 5) induces a large decrease of the light-scatter trace. In medium which already contains succinate and phosphate (trace C of Figure 5), monensin causes a rapid reversal of the light-scatter decrease mediated by valinomycin which is immediately followed by a large swelling cycle.

In an attempt to explain the above-described data, it was considered that the uptake of P_i but not that of the other "penetrant" anions such as acetate, formate, propionate, or butyrate (Chappell and Haarhoff, 1967) was obligatorily coupled to the oxidation of succinate, β-hydroxybutyrate, or glutamate + malate in presence of monensin. Therefore, it was thought that the inhibition of phosphate uptake into the mitochondria by carboxylic antibiotics (Estrada-O *et al.*, 1967a; Lardy *et al.*, 1967) could be reversed by the oxidation of the above substrates. We have also suggested that monocarboxylic antibiotics inhibit the $[^{32}P]P_i$–ATP-exchange reaction as a consequence of their ability to prevent the accumulation of phosphate into mitochondria (Lardy *et al.*, 1958, 1967; Estrada-O *et al.*, 1967a). Accordingly, the possible effect of various oxidizable substrates to overcome the inhibition by carboxylic antibiotics of this exchange reaction was studied. This reaction was considered as a monitor of the ability of oxidizable substrates to facilitate phosphate entry during antibiotic-induced efflux of K^+. These results are displayed in Figure 5. In these experiments the incorporation of $[^{32}P]P_i$ into ATP was measured under conditions that permitted observations of the sum of effects of the penetration of $[^{32}P]P_i$ and of ATP into mitochondria and the exchange reaction proper. Panel A of Figure 5 shows the temporal sequence of the incorporation of $[^{32}P]P_i$ into ATP in absence of carboxylic antibiotics or added oxidizable substrates. The addition of monensin, approx-

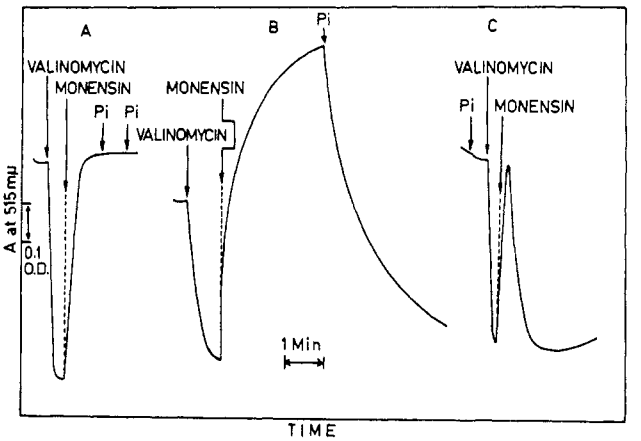

FIGURE 4: The effect of monensin and phosphate or acetate on the light-scattering changes mediated by valinomycin in presence of glutamate or β-hydroxybutyrate. The basic reaction mixture contained 15 mM KCl, 1 mM $MgCl_2$, 10 mM triethanolamine–HCl (pH 7.4), 180 mM sucrose, and 0.5 mg of mitochondrial N in 3-ml volume at 25°. Prior to the addition of the antibiotics, the vessels also contained 10 mM glutamate plus 10 mM acetate–triethanolamine in experiment from tracing A; 10 mM β-hydroxybutyrate plus 10 mM acetate–triethanolamine in that from tracing B and 10 mM β-hydroxybutyrate plus 5 mM phosphate–triethanolamine in tracing C. Where indicated P_i was added at a final concentration of 5 mM. Valinomycin and monensin were added at a concentration of 1.3×10^{-7} and 1×10^{-7} M, respectively.

imately 1.5 min after the initiation of the exchange reaction (panel B of Figure 5), causes a complete and instantaneous inhibition of the incorporation of $[^{32}P]P_i$ into ATP. Further addition of β-hydroxybutyrate 4 min after monensin, rapidly overcomes the inhibitory effect observed and returns the exchange reaction to the control values. The independent addition of glutamate or L-malate does not alter the block caused by monensin. Nevertheless, when L-malate is added after glutamate (panel C of Figure 5) or *vice versa*, an immediate reversal of the $[^{32}P]P_i$–ATP incorporation block takes place. As seen in panel D of Figure 5, neither α-ketoglutarate nor pyruvate are able to overcome the inhibitory effect of monensin on the measured parameter; however, it is apparent that succinate restores the exchange reaction to the control values observed in absence of monensin or substrate. Results identical with those obtained with monensin A in these experiments are also found with nigericin or dianemycin. This is entirely compatible not only with the fact that the oxidative phosphorylation of succinate, β-hydroxybutyrate, or glutamate + malate is unaffected by carboxylic antibiotics (Lardy *et al.*, 1958; Henderson and Chappell, 1967; Pressman *et al.*, 1967) but also with the possibility that the oxidation of the above substrates could stimulate an energy-linked uptake of phosphate into mitochondria in presence of monensin. The direct demonstration of this latter proposal is shown in Figure 6. In these experiments, the effect of different oxidizable substrates was tested on the net inhibition of phosphate accumulation produced by monensin in mitochondria. A continuous measurement of K^+ and H^+ movements was also carried out in separate vessels which were subjected to the same experimental conditions and where samples were taken after 5 min to measure the net accumulation of phosphate into mitochondria. It is observed that

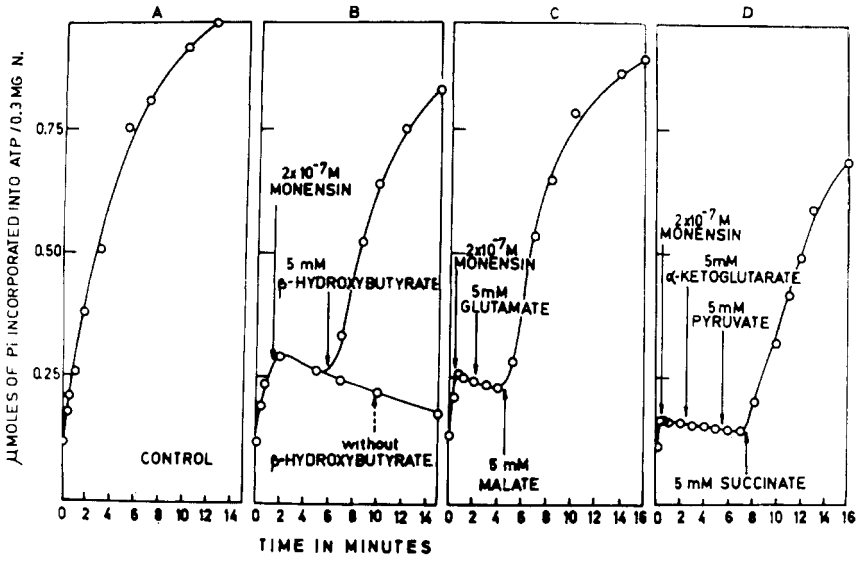

FIGURE 5: The effect of monensin and oxidizable substrates on the rates of incorporation of [^{32}P]P_i into ATP in liver mitochondria. The reaction mixture contained: 6 mM Tris–ATP pH 7.4 (triethanolamine), 5 mM inorganic phosphate–triethanolamine containing 15,000 cpm of [^{32}P]P_i, 15 mM KCl, 180 mM sucrose, 10 mM triethanolamine–HCl, and mitochondria equivalent to 0.3 mg of mitochondrial N in 1.0-ml final volume at 30°. All substrates were neutralized with triethanolamine and added at a concentration of 5 mM; monensin was added at 2×10^{-7} M. The reaction was initiated by the addition of [^{32}P]P_i-labeled orthophosphate; it was stopped at the indicated times by addition of 6% trichloroacetic acid. The tracing shown in panel A represents the rate of incorporation of [^{32}P]P_i into ATP in absence of oxidizable substrates or monensin. The mitochondria were partially depleted of endogenous P_i as described.

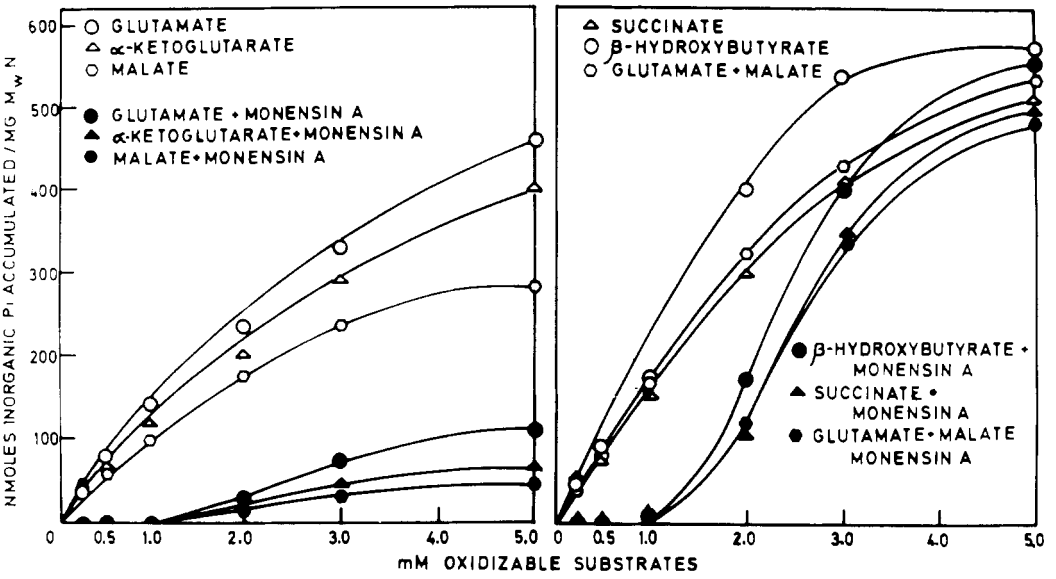

FIGURE 6: The effect of monensin and increasing concentrations of oxidizable substrates on the net accumulation of P_i by liver mitochondria. The reaction mixture contained: 15 mM KCl, 5 mM P_i–triethanolamine containing 10,000 cpm of [^{32}P]P_i, 7.5 mM $MgCl_2$, 10 mM triethanolamine–HCl, the indicated oxidizable substrates at a concentration of 5 mM 180 mM sucrose, 2×10^{-7} M monensin, and mitochondria equivalent to 2.0 mg of N in 2-ml volume. After 10-min incubation at 30°, the mitochondria were isolated and P_i was separated and determined as described. The zero time substracted from the experimental vessels contained 5×10^{-5} M 2,4-dinitrophenol and 2 μg/ml of antimycin.

monensin almost completely inhibits the accumulation of phosphate in mitochondria respiring with endogenous substrates or with increasing concentrations of glutamate, pyruvate, α-ketoglutarate, or L-malate (left panel of Figure 6). On the other hand, when these substrates are replaced by succinate, β-hydroxybutyrate, or glutamate + malate (right panel of Figure 6) the accumulation of phosphate is restored as a function of substrate concentration to the values observed in absence of monensin. It was also observed that the K^+ efflux and the H^+ uptake mediated by monensin remain unaffected along the experiments where an important accumulation of inorganic phosphate occurred. Other experiments have indicated that the ability of succinate to stimulate the accumulation of phosphate in presence of monensin is

TABLE I: Effect of Increasing Concentrations of KCl on the Substrate-Dependent Uptake of P_i by Liver Mitochondria.[a]

Oxidizable Substrate Added	Additive KCl (mM)				
	0	25	50	100	150
	nmoles of P_i accumulated/mg of mitochondrial N[b]				
Glutamate	395	425	280	150	72
L-Malate	320	360	190	95	43
α-Ketoglutarate	370	390	240	102	48
Succinate	380	412	380	383	378
β-Hydroxybutyrate	425	480	420	405	400

[a] The mitochondria used in these experiments were partially depleted of endogenous inorganic phosphate by ADP as described in Methods. Mitochondria equivalent to 2.3 mg of N were incubated for 10 min at 30° in 2.0 ml of media containing: 5 mM phosphate–triethanolamine (pH 7.4) containing 15,000 cpm of [^{32}P]P_i, 7.5 mM $MgCl_2$, 10 mM triethanolamine–HCl (pH 7.4), 160 mM sucrose, 10 mM oxidizable substrates, and KCl at the indicated concentrations. The mitochondria were isolated and inorganic phosphate was separated from other phosphate-containing compounds and determined as described in Methods. [b] The average values from four individual experiments which agreed closely are presented.

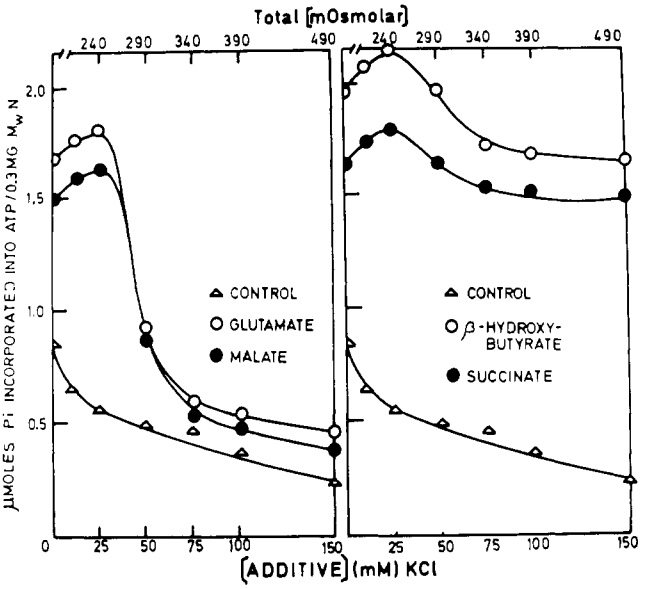

FIGURE 7: The effect of oxidizable substrates and increasing concentrations of KCl on the incorporation of [^{32}P]P_i into ATP in liver mitochondria. Experimental conditions were essentially the same as described in Figure 5, except for the omission of carboxylic antibiotics and for the evaluation of the indicated concentrations of KCl on the measured reaction. The milliosmolarity values shown in the upper portion of each panel represent the total osmolarity of the media where the concentration of alkali metal cation was added to that of the different components of the reaction mixture.

inhibited by antimycin or by 2,4-dinitrophenol, whereas that of β-hydroxybutyrate or glutamate + malate is prevented by antimycin, rotenone, or 2,4-dinitrophenol. Oligomycin does not inhibit the substrate effects.

These results not only substantiate our previous suggestion that the carboxylic antibiotics inhibit the [^{32}P]P_i–ATP exchange reaction (Lardy *et al.*, 1958) by preventing the uptake of phosphate into mitochondria (Lardy *et al.*, 1967; Estrada-O *et al.*, 1967a) but they also indicate that the phosphate uptake which occurs in the presence of carboxylic antibiotics and either succinate, glutamate + malate, or β-hydroxybutyrate is not obligatorily coupled to simultaneous alkali metal cation uptake. Moreover, 3 mM of both EDTA or EGTA[1] do not inhibit the incorporation of [^{32}P]P_i into ATP which is associated to the K^+ efflux induced by carboxylic antibiotics in the presence of succinate. These findings suggest that the translocation of phosphate which takes place in the above experimental conditions is not primarily dependent on the uptake of divalent cations into mitochondria. Moreover, it seems that an energy-linked uptake of phosphate may be able to drive the accumulation of alkali metal cations in a system which contains neutral and carboxylic antibiotics as well as an oxidizable substrate such as β-hydroxybutyrate (Figures 1, 3, and 4).

Since Chappell *et al.* (1967, 1968) and Mitchell and Moyle (1969) have observed an osmotic translocation of phosphate in respiratory-inhibited mitochondria, it is of interest to describe the effect of osmolarity on the aerobic uptake of phosphate into ATP and into mitochondria. These results also have a bearing on the reported inhibition of the incorporation of [^{32}P]P_i into ATP caused by hyperosmolarity (Lehninger *et al.*, 1957). Figure 7 indicates that the magnitude of the incorporation of [^{32}P]P_i into ATP in the mitochondria depends on the osmolarity change of the medium and on the presence of certain oxidizable substrates. In the first place, it is apparent that the incorporation of [^{32}P]P_i into ATP which occurs in a media which contain β-hydroxybutyrate is higher than the value found in the presence of other oxidizable substrates. This observation parallels the fact that β-hydroxybutyrate is the substrate which promotes the highest accumulation of P_i into the mitochondria as compared with other substrates (right panel of Figure 6). The increase in the concentration of sucrose or the chloride salts of K^+, Na^+, or Cs^+ up to 240 mOsm does not have significant effect on the incorporation of [^{32}P]P_i into ATP that takes place in the presence of glutamate, L-malate, β-hydroxybutyrate, or succinate. On the other hand, the omission of oxidizable substrates from media of increasing osmolarity results in a progressive decrease of the exchange reaction, as originally described by Lehninger *et al.* (1957). The increase in salt or sucrose concentration from 240 to 340 mOsm causes an almost complete block of the measured reaction in a media which contains glutamate or L-malate. However, in agreement with the data obtained on the effect of monensin on the uptake of phosphate into mitochondria (Figure 6), the incorporation of [^{32}P]P_i into ATP which occurs in presence of succinate or β-hydroxybutyrate is largely insensitive to the inhibitory effect of high concentrations of K^+, Na^+, or Cs^+. As shown in Table I, it is also apparent that hyper-

[1] Abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ester)-*N*,*N*′-tetraacetic acid.

osmolarity decreases the net accumulation of inorganic phosphate into mitochondria in the presence of glutamate, malate, or α-ketoglutarate while it does not inhibit this phenomenon in that of succinate or β-hydroxybutyrate. Therefore, it appears that cation efflux caused by carboxylic antibiotics and the hyperosmolarity of the medium, share the ability to inhibit the incorporation of [^{32}P]P_i into ATP by preventing the transport or the exchange of phosphate by the mitochondria. In both cases this inhibition is prevented by the stimulation of phosphate uptake coupled to the oxidation of succinate, β-hydroxybutyrate, or glutamate + malate.

Discussion

The problem of determining which transport process, anion or cation, is primarily energy linked has been the subject of conflicting interpretations in the literature. Some reports indicate that carboxylic acid substrates or P_i penetrate into the mitochondrion driven by an energy-dependent cation influx (Harris *et al.*, 1967a,b), by the ejection of protons (Mitchell, 1968; Palmieri and Quagliariello, 1969), and by a mechanism not primarily related to energy-linked cation movements (Harris and Manger, 1968a–c). The information presented in this paper has shown that when the mitochondrial accumulation of alkali metal cations is inhibited by monensin A, an energy-linked uptake of P_i supported by the penetration and the further oxidation of some substrates, allows valinomycin, nonactin, or monazomycin to stimulate the uptake of K^+ into mitochondria. Moreover, it is also apparent that the accumulation of phosphate into the mitochondria not necessarily has to be driven by energy-linked movements of the mobile pool of cations or protons. The continuous monitoring of K^+ and H^+ movements carried out along the experiments described in Figure 6 has shown that the accumulation of phosphate occurring in the presence of monensin and succinate, β-hydroxybutyrate, or glutamate + malate is effected simultaneously with the efflux of K^+ and the uptake of H^+ induced by the carboxylic antibiotic. Moreover, recent data from our laboratory (Estrada-O and Calderón, 1970) have shown that monensin or nigericin catalyze a nonenergy-dependent Ca^{2+}–K^+ exchange across mitochondrial membranes. Addition of phosphate and substrates such as glutamate, pyruvate, or L-malate does not result in the net accumulation of Ca^{2+} or phosphate in the presence of the carboxylic ionophore. However, when the above substrates are replaced by β-hydroxybutyrate, succinate, or glutamate + malate in the presence of phosphate and monensin or nigericin, a significant accumulation of Ca^{2+} and phosphate associated to the efflux of K^+ from mitochondria results. These latter findings suggest that an energy-dependent uptake of phosphate facilitates the accumulation of Ca^{2+} into mitochondria. Similar conclusions are derived from the data described in Figures 1, 3, and 4 from this paper, which indicate that an energy-linked uptake of phosphate facilitates the accumulation of K^+ into these subcellular particles. Thus, it is apparent that conditions exist where cation translocation is secondary to energy-linked P_i transport. This proposal differs from the previous suggestions by Harris *et al.* (1967a,b) which predict that the uptake of P_i is the consequence of energy-linked cation uptake into mitochondria. Also, different to Palmieri and Quagliariello (1969) who found an apparent correlation between H^+ influx and inhibition of inorganic phosphate uptake in respiratory-inhibited mitochondria, the present work indicates that phosphate uptake may take place simultaneous to the influx of H^+ in respiring mitochondria. It is likely that under certain circumstances, a primarily induced energy-dependent uptake of phosphate in exchange for an internal substrate anion (Chappell and Haarhoff, 1967) such as L-malate (Papa *et al.*, 1969; de Jong *et al.*, 1969) could facilitate the translocation of alkali metal cations in exchange for protons across mitochondrial membranes. This suggestion does not contradict the proposal by Mitchell (1968) that proons generated in the respiratory chain during substrate oxidation, could be used in a symport type of mechanism for the protonation and subsequent translocation of P_i into mitochondria.

Data showing that the oxidation of relatively high concentrations (10 mM) of succinate or β-hydroxybutyrate occurs independently of the addition of phosphate and of the movements of K^+ or H^+ (Figure 3) apparently places the uptake of saturating concentrations of these anions in the category of the phosphate transport mechanism suggested in this paper. On the other hand, it is clear that the oxidation of high concentrations of glutamate, malate, pyruvate, citrate, α-ketoglutarate, or the glutamate + malate pair (Figures 1 and 2) requires not only K^+ or H^+ movements (Graven *et al.*, 1966) but also of the presence of inorganic phosphate. The explanation for this phosphate requirement may lie in the fact that the oxidation of glutamate which takes place *via* the oxidation of α-ketoglutarate (Azzone and Ernster, 1961) as well as the entry of α-ketoglutarate and malate into mitochondria are dependent on the supply of phosphate (Chappell *et al.*, 1967). Therefore, it is apparent that our data support the concept suggested by Henderson *et al.* (1969) indicating that loss of internal phosphate caused by carboxylic antibiotics (Estrada-O *et al.*, 1967a; Lardy *et al.*, 1967) or uncouplers plus ammonium ion (Henderson *et al.*, 1969) could be responsible for the substrate specific inhibition mediated by these compounds (Lardy *et al.*, 1958; Graven *et al.*, 1966). Additional support for this proposal are the data which indicate that relatively high concentrations of P_i completely overcomes the inhibition of glutamate oxidation caused by carboxylic antibiotics in mitochondria (Estrada-O *et al.*, 1967b).

The results presented in Figures 6–7 and Table I show that succinate, β-hydroxybutyrate, or glutamate + malate reverse the inhibition of the mitochondrial accumulation of phosphate as well as the inhibition of the incorporation of [^{32}P]P_i into ATP mediated by monensin or by hyperosmolarity. It is likely that in conditions of hyperosmolarity, paralleling the conditions where K^+ efflux is caused by monensin, the above substrates but not α-ketoglutarate or glutamate, penetrate into the mitochondrion supporting an energy-linked uptake of inorganic phosphate accompanied by the ejection of an internal anion which restores the inhibited incorporation of [^{32}P]P_i into ATP. This suggestion is substantiated by the fact that hyperosmolarity blocks the oxidation of glutamate, pyruvate, α-ketoglutarate, or L-malate but not that of succinate or β-hydroxybutyrate (Johnson and Lardy, 1958; Astmon and Davies, 1967).

Lehninger *et al.* (1957) explained the inhibition of the [^{32}P]P_i–ATP exchange caused by hyperosmolarity by postu-

lating the interaction of high concentrations of K^+ or sucrose with an enzyme component involved with the energy-conserving mechanism of mitochondria. The results discussed in the present work indicate that such inhibition is caused by preventing the access of phosphate into mitochondria. This conclusion, as well as data obtained with monensin, supports the view that rates of incorporation of $[^{32}P]P_i$ into ATP are not only a function of the translocation of P_i (Lardy *et al.*, 1967; Estrada-O *et al.*, 1967a) but also of the permeation or exchange of some oxidizable substrates carried out through a mechanism not primarily dependent on previously postulated movements of the mobile pool of alkali metal cations or protons.

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