Coupling of Oxidative Phosphorylation by Monovalent Cations*

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ABSTRACT: The effect of monovalent cations on the P:O ratios and respiratory rates of rat liver mitochondria with a K^+ content of 15 mM or less has been studied. The respiratory rate and the P:O ratios of these K^+-depleted mitochondria oxidizing NAD-dependent substrates diminish progressively as the concentration of sucrose in the incubation media is raised from 25 to 200 mM. The addition of the chloride salts of Li^+, K^+, Rb^+, Cs^+, and $(CH_3)_4N^+$ to K^+-depleted mitochondria incubated in 200 mM sucrose increases the P:O ratios; Li^+ is the most effective cation in the induction of oxidative phosphorylation, followed by K^+ and Rb^+. The effect of K^+ and Li^+ on oxidative phosphorylation is dependent on the time of incubation and on the concentration of added cations, and is associated with an increase in the intramitochondrial concentration of the cation. This is evidenced by the fact that maximal coupling of oxidative phosphorylation by Li^+ is attained when almost maximal swelling has taken place, and also by experiments which show that the favorable effect of K^+ on oxidative phosphorylation is enhanced by valinomycin. Simultaneously to the Li^+- and K^+- (plus valinomycin) induced increase in the P:O ratios an increase in the respiration rate is also observed. This latter effect is due at least in part, to a favorable effect of cations on the entrance of oxidizable substrates into the mitochondria, as demonstrated by experiments in which the level of NADH was measured. On the other hand, the mechanism by which cations increase the P:O ratio remains unknown.

For a number of years, the role of K^+ on oxidative phosphorylation has been a controversial subject. Although it is well established that K^+ facilitates the entrance of oxidizable substrates into mitochondria (Lynn and Brown, 1966; Harris *et al.*, 1967; Graven *et al.*, 1966; Kimmich and Rasmussen, 1967), its possible role on phosphorylation has not been completely elucidated, and the results from several independent groups of researchers are contradictory (Pressman and Lardy, 1952; Pressman and Lardy, 1955; Opit and Charnock, 1965; Blond and Whittam, 1965; Krall *et al.*, 1964; Smith and Beyer, 1967; Papa *et al.*, 1969). Nevertheless, some recent reports in K^+-depleted mitochondria strongly suggest that K^+ is directly involved in the reactions that lead to ATP formation during mitochondrial electron transport. In the latter studies, it has been found that the state 3:state 4 respiratory ratios are dependent on the intramitochondrial K^+ concentration. In this type of mitochondria, the addition of monovalent cations to the incubation mixture increases very significantly the state 3:state 4 respiratory ratios, largely due to an enhancement of the state 3 respiratory rate (Gómez-Puyou *et al.*, 1969a, 1970).

This beneficial effect of K^+ on the respiratory control of mitochondria has only been observed when NAD-dependent substrates are oxidized. With succinate as substrate, no clear cut effects of K^+ have been detected, suggesting that the action of K^+ is limited to site I of oxidative phosphorylation (Gómez-Puyou *et al.*, 1970). Further evidence in favor of this possibility has been obtained from studies made with submitochondrial particles. The respiratory rates of submitochondrial particles oxidizing NADH, but not succinate, are highly increased by including monovalent cations in the respiring media (Christiansen *et al.*, 1969; Pinto *et al.*, 1970). These latter findings, together with the observation that K^+ increases the state 3 respiratory rate in whole mitochondria, suggest that K^+ enhances the rate of synthesis of the energized state, or that K^+ may even be absolutely necessary for the formation of the energized state at site I of oxidative phosphorylation (Gómez-Puyou *et al.*, 1970; Pinto *et al.*, 1970).

This work presents evidence which indicates that monovalent cations increase both the rate of respiration and the P:O ratios of K^+-depleted mitochondria.

Methods

Mitochondria. The technique for preparing mitochondria with a K^+ content of 15 mM or less has been described previously (Gómez-Puyou *et al.*, 1970); liver mitochondria prepared in 0.25 M sucrose and 1 mM EDTA are incubated in 10 mM glutamate, 100 mM NaCl, 20 mM Tris-HCl (pH 7.3), 10 mM H_3PO_4 (adjusted to pH 7.3 with Tris base), 1 mM EDTA, and 50 mM sucrose for 3 min at room temperature. The mixture is diluted with sucrose–EDTA and centrifuged. The mitochondrial pellet is washed twice with sucrose–EDTA and suspended in sucrose–EDTA for the following measurements.

Oxygen Uptake and P:O Ratios. The respiration of K^+-depleted mitochondria was measured with a Clark oxygen electrode at 25° in 3.0 ml of an incubation mixture whose composition is detailed under the individual experiments outlined in the Results section. For calculation of P:O ratios, an aliquot of the incubation media in which oxygen uptake was being measured was added immediately to a solution of trichloroacetic acid so as to give a 6% final concentration of the acid. Inorganic phosphate was determined in the trichloroacetic acid supernatant according to Sumner (1944). The error in the determination of inorganic phosphate was less than 3%.

Mitochondrial Swelling. The swelling of K^+-depleted mitochondria was studied in 3.0 ml of incubation media detailed in the individual experiments by following the decrease in the optical density at 520 nm with a DK 2 Beckman spectrophotometer; the reaction was started by adding mitochondria to the incubation mixture.

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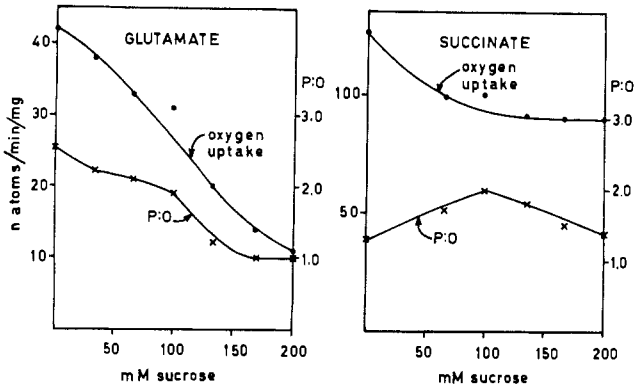

FIGURE 1: Oxygen uptake and P:O ratios of K^+-depleted mitochondria at various concentrations of sucrose. The incubating conditions were 4 mM Tris-HCl (pH 7.4), 5 mM glutamate or succinate, 2 mM H_3PO_4 (adjusted to pH 7.4 with Tris base), 1 mM EDTA, and the indicated concentrations of sucrose. The phosphorylation of ADP and the respiratory rates were calculated after the addition of 1.6 mM ADP which was added after 1-min incubation. When approximately 960 natoms of oxygen was consumed, an aliquot was taken for the assay of inorganic phosphate.

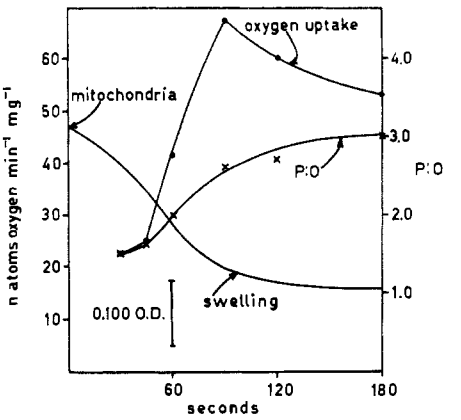

FIGURE 2: Effect of the time of preincubation of K^+-depleted mitochondria with LiCl on the respiratory rate and P:O ratios and mitochondrial swelling in LiCl. K^+-depleted mitochondria were incubated as in Table I with 20 mM LiCl; at the time indicated on the abscissa, 1.6 mM ADP was added; the respiratory rate and P:O ratio attained after the addition of ADP until only 10% of oxygen remained are plotted. The decrease in the optical density of K^+-depleted mitochondria was followed in a separate cuvet which contained an identical incubation mixture, in this case, no ADP was added. The amount of protein in the oxygen electrode experiments was 11.4 mg and in the swelling experiment 1.0 mg.

Reduction of NAD. The level of intramitochondrial NADH was measured according to the technique described by Bergmeyer (1965).

Results

In the experiment of Figure 1, the P:O ratio and the respiratory rate of K^+-depleted mitochondria oxidizing glutamate and succinate were examined at increasing concentrations of sucrose in the incubation media. In the presence of glutamate, when the concentration of sucrose in the mixture was raised above 160 mM, the P:O ratios diminished to 1.0 or below (values as low as 0.6 have been detected). Simultaneously, the respiratory rate diminished to about 25 or 30% of the rate observed in the absence of sucrose. On the other hand, the respiratory rate and the P:O ratios of K^+-depleted mitochondria oxidizing succinate were affected very slightly by increasing concentrations of sucrose; the P:O ratio diminished to about 1.4 at 200 mM sucrose and the respiratory rate remained relatively unaffected (Figure 1). This experiment shows that sucrose, at relatively high tonicities, affects mainly site I of oxidative phosphorylation.

TABLE 1: Effect of Monovalent Cations on the Oxygen Uptake, P:O Ratios, and Swelling of K^+-Depleted Mitochondria.[a]

Salt Added	natoms of O Consumed min^{-1} mg^{-1}	P:O	$-\Delta OD$
	17	0.6	0
LiCl	83	3.0	0.240
KCl	21	2.0	0
RbCl	18	2.0	0
CsCl	19	1.7	0
TMACl	17	1.8	0

[a] The incubation mixture contained 200 mM sucrose, 4 mM Tris-HCl (pH 7.4), 5 mM glutamate, 2 mM H_3PO_4 (adjusted to pH 7.4 with Tris base), 1 mM EDTA, and 20 mM of the indicated salts. The phosphorylation of ADP and the respiratory rate were calculated after the addition of 1.6 mM ADP which was at 2-min incubation in the case of Li^+ and 5 min in the rest of the experiments. When approximately 960 natoms of oxygen was consumed, an aliquot was taken for the assay of inorganic phosphate. The measurement of the decrease in the optical density (OD) at 520 nm was made in the same incubation media, except that the amount of mitochondrial protein in the oxygen electrode vessel was 7.8 mg and in the swelling experiments 0.6 mg. The blank in the swelling experiments was an acid solution of methyl orange. TMACl = tetramethylammonium chloride.

As added monovalent cations significantly enhance the respiratory control of K^+-depleted mitochondria incubated in 50 mM sucrose or less and oxidizing NAD-dependent substrates (Gómez-Puyou *et al.*, 1969a, 1970), we tested the effect of the chloride salts of Li^+, K^+, Rb^+, Cs^+, and tetramethylammonium on the P:O ratios and respiratory rates of K^+-depleted mitochondria incubated with 200 mM sucrose and glutamate (Table I). All cations increased the P:O ratios of K^+-depleted mitochondria incubated at 200 mM sucrose; however, there were significant differences in the effectiveness of the various cations in increasing oxidative phosphorylation (Table I). The order of effectiveness was: $Li^+ > K^+ = Rb^+ > Cs^+$ = tetramethylammonium. The effect of Na^+ was not tested, since it has been found that Na^+ produces a partial uncoupling of oxidative phosphorylation (Gómez-Puyou *et al.*, 1969b; Sandoval *et al.*, 1970). The same salts had no effect on the P:O ratios and respiratory rates of K^+-depleted mitochondria oxidizing succinate at 200 mM sucrose (experiments not shown). The results of Table I also show that among the cations tested, Li^+ was the only one that induced significant mitochondrial swelling and high rates of respiration. These findings suggested that the uptake of cations was responsible for the induction of high P:O ratios and high rates of respiration.

The experiment of Figure 2 was designed to test whether cations outside the mitochondria were responsible for the higher P:O ratios and respiratory rates. Li^+ was chosen for

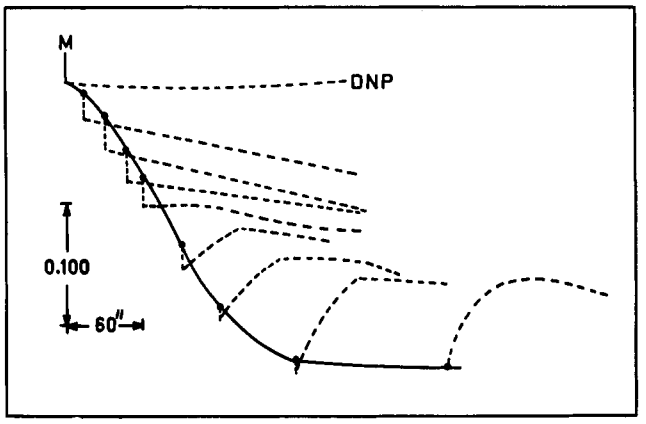

FIGURE 3: Effect of ADP on the Li^+-induced swelling of K^+-depleted mitochondria. The incubating conditions were as in Figure 2 for the swelling experiments; to separate but identical cuvets 0.8 mM ADP was added at the times indicated by the open circles and resulted in the traces shown by the dashed lines. The measurement of swelling was started by the addition of 0.9 mg of mitochondrial protein (M). In the trace marked DNP, 10^{-4} M 2,4-dinitrophenol was added at the beginning of the experiment. In all the swelling experiments including that with DNP the initial optical density attained after the addition of mitochondria was essentially identical.

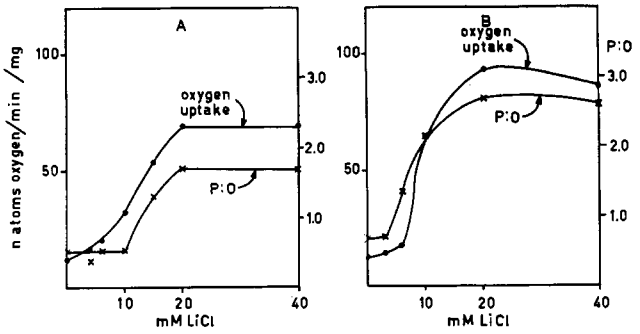

FIGURE 4: Effect of different concentrations of LiCl on the oxygen uptake and P:O ratios of K^+-depleted mitochondria. In part A, the incubating conditions were as in Table I, except that the indicated concentrations of LiCl were included. In part B, the experimental conditions were as in part A except that the tonicity of the media was maintained constant by varying the concentration of sucrose. In both experiments, ADP was added after 1-min of incubation.

these studies since it was the most effective cation in the induction of oxidative phosphorylation (Table I). If the presence of Li^+ outside the mitochondria were the factor responsible for the increase of P:O ratios and respiratory rates, it would have been expected that the same high P:O ratios would have been obtained regardless of the time of preincubation of mitochondria with the cation. However, it was found that the longer the time of preincubation of Li^+ with mitochondria, the higher the value of the P:O ratio until a maximal value was attained. This experiment suggests that external cations are not responsible for the induction of higher P:O ratios. Furthermore, the swelling trace of Figure 2, obtained with the same mitochondrial preparation and in conditions identical with those in which oxidative phosphorylation was measured, shows that maximal coupling was achieved when the mitochondria had undergone almost maximal swelling.

As the swelling of mitochondria may be an index of the uptake of cations into the mitochondria (Chappell and Crofts, 1966), the experiments described above suggest that the entrance of Li^+ into the mitochondria is responsible for the induction of high P:O ratios. However, the question arises as to why the P:O ratio is not increased by Li^+ when ADP is present in the incubation media. When ADP is added before 45-sec incubation of mitochondria with Li^+, a low P:O ratio is observed, even though the total time of incubation is close to 7 min. However, when ADP is added after 2-min incubation the P:O ratio rises almost to a maximum (Figure 2). These results suggest that, once the mitochondria are in the presence of ADP, Li^+ is not able to induce high P:O ratios. In the experiment of Figure 3 the swelling pattern of mitochondria incubated with Li^+ was followed and ADP was added at various intervals. At relatively short times of incubation, ADP inhibited completely the Li^+-induced mitochondrial swelling and at longer periods of incubation ADP induced a partial reversal of the Li^+-induced swelling. These results point to a competition between Li^+ uptake and oxidative phosphorylation, the latter being a more effective competitor, even in conditions of partial uncoupling. The inhibition of Li^+-induced swelling by 2,4-dinitrophenol (Figure 3) indicates that the uptake of Li^+ is energy dependent.

The effect of different concentrations of Li^+ on the P:O ratios and oxygen uptake was assayed (Figure 4A); 20 mM LiCl induced maximal rates of respiration and maximal P:O ratios. Similar results were obtained when these experiments were repeated at constant tonicities by varying the concentration of sucrose (Figure 4B).

The results up to this point suggest that the entrance of Li^+ into the mitochondria promotes higher respiratory rates and P:O ratios. However, the results of Table I show that K^+, Rb^+, Cs^+, and tetramethylammonium increase the P:O ratio without inducing appreciable mitochondrial swelling. Thus it was considered possible that the latter cations did not induce high P:O ratios because of a limited permeability of the mitochondria. This possibility was tested with the aid of valinomycin. Valinomycin has been reported to increase the uptake of K^+ into the mitochondria (Moore and Pressman, 1964; Pressman, 1965; Chappell and Crofts, 1966), thus the entrance of K^+ into the mitochondria was promoted by the addition of different concentrations of the cationophile valinomycin. The P:O ratios and respiratory rates of K^+-depleted mitochondria incubated with K^+ and different concentrations of valinomycin are shown in Table II. Both parameters are increased in the presence of valinomycin. The effect of valinomycin also depends on the concentration of added K^+ as shown in Table III.

The valinomycin-mediated K^+ uptake was tested by measuring mitochondrial swelling under conditions identical to those in which oxidative phosphorylation was assayed. Valinomycin + K^+ in these conditions induced swelling much more rapidly than Li^+ (compare Figure 5 with the swelling trace of Figure 2). The greater rate of swelling with K^+ + valinomycin correlates with a more rapid induction of oxidative phosphorphorylation. The P:O ratios obtained with valinomycin + K^+ are around 1.7 if ADP is added at 45-sec incubation, when swelling has reached almost its maximal extent.

There is another difference between the swelling processes which are induced by Li^+ and by K^+ + valinomycin. Unlike the Li^+-induced swelling, the valinomycin + K^+-induced swelling is not inhibited by ADP; nevertheless, it is blocked by 2,4-dinitrophenol (Figure 5). Apparently, in the presence of valinomycin + K^+, the energy-dependent K^+ movements

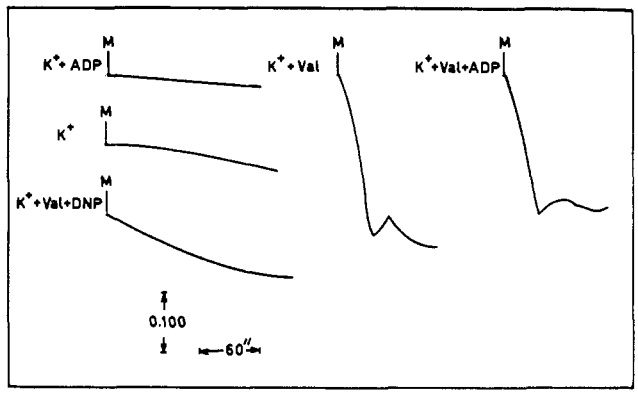

FIGURE 5: Effect of valinomycin and ADP on the swelling of K^+-depleted mitochondria. The experimental conditions were as in Table III except that the mixture contained 20 mM KCl and where indicated 12 ngrams of valinomycin/mg of protein, 10^{-4} M 2,4-dinitrophenol (DNP), and 1.6 mM ADP. In all cases, the reaction was started by the addition of 1.0 mg of mitochondrial protein (M) to the incubation media and in all cases the initial optical density was essentially the same.

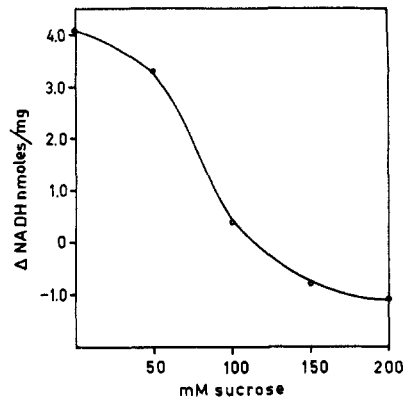

FIGURE 6: Effect of sucrose on the NADH levels of K^+-depleted mitochondria. K^+-Depleted mitochondria (17.6 mg of protein) were incubated in 2 mM H_3PO_4 (adjusted to pH 7.4 with Tris base), 5 mM glutamate, 1 mM EDTA, 4 mM Tris-HCl (pH 7.4), and the indicated concentrations of sucrose in a final volume of 1.0 ml. The reaction was stopped with alcoholic KOH after 3-min incubation. The values expressed in the graph are the difference between the NADH level detected in the absence of glutamate and those encountered in its presence. In the absence of added glutamate 2.0 nmoles of NADH/mg of protein was detected.

are more effective competitors than the phosphorylation of ADP, while the reverse situation seems to prevail in the case of Li^+ uptake (Figure 3).

The results at this stage of the investigation indicated that high respiratory rates and P:O ratios are attained when a cation is accumulated. However, Rb^+, Cs^+, tetramethylammonium, and K^+, in the absence of valinomycin, increase the P:O ratios without significantly affecting the respiratory rate (Table I). Therefore, it is possible that the low rates of respiration observed with K^+, Rb^+, Cs^+, and tetramethylammonium could be due to a limited uptake of substrates. This possibility was tested by measuring the reduction of intramitochondrial NAD by added glutamate. According to Chappell and Robinson (1968), the reduction of NAD by added substrates is a measure of substrate availability to the respiratory chain.

Figure 6 shows that an increase in the concentration of sucrose diminishes the reduction of NAD by glutamate. Further, the addition of Li^+, K^+, Rb^+, Cs^+, and tetramethylammonium increases the level of NADH in mitochondria incubated in 200 mM sucrose (Table IV). The latter results are in agreement with previous reports that show that monovalent cations facilitate the uptake of substrates into the mitochondria (Lynn and Brown, 1966; Harris *et al.*, 1967; Graven *et al.*, 1966; Kimmich and Rasmussen, 1967). However, the results of Figure 2, and Table II and Figure 5 show that when the cation is accumulated high P:O ratios and respiratory rates are attained. These findings indicate that cations have a dual role in K^+-depleted mitochondria.

TABLE II: Effect of Valinomycin on the P:O Ratios and Respiratory Rates of K^+-Depleted Mitochondria.[a]

ngrams of Valinomycin mg^{-1}	P:O	natoms of O Consumed mg^{-1} min^{-1}
	0.6	19.4
0.66	1.3	21.3
1.3	1.6	31.5
2.6	1.6	49.0
3.2	1.7	50.3
6.4	1.8	59.9
12.8	1.8	111.1
48.0	0.7	105.0

[a] The incubating conditions were 20 mM KCl, 2 mM H_3PO_4 (adjusted to pH 7.4 with Tris base), 1 mM EDTA, 4 mM Tris-HCl (pH 7.4), 5 mM glutamate, 200 mM sucrose, and the indicated concentrations of valinomycin. The final volume was 3.0 ml. The respiratory rates and P:O ratios are those obtained after the addition of 1.6 mM ADP (after 45-sec incubation) until 90% of oxygen had been consumed.

TABLE III: Effect of KCl and Valinomycin on the P:O Ratios and Respiration of K^+-Depleted Mitochondria.[a]

	−Valinomycin		+Valinomycin	
KCl (mM)	P:O	natoms of O Consumed min^{-1} mg^{-1}	P:O	natoms of O Consumed min^{-1} mg^{-1}
	0.7	12	0.7	12
4	0.9	16	1.4	35
10	1.2	16	1.6	35
20	1.3	20	1.7	47
40	1.0	22	1.7	41

[a] The incubating conditions were 2 mM H_3PO_4 (adjusted to pH 7.3 with Tris base), 1 mM EDTA, 4 mM Tris-HCl, 5 mM glutamate, 200 mM sucrose, 2.6 ngrams of valinomycin/mg of mitochondrial protein, and the indicated concentrations of KCl. The P:O ratios and respiratory rates expressed are those attained after the addition of 1.6 mM ADP (45-sec incubation) until about 10% of oxygen remained.

Discussion

The main difficulty in investigating the role of K^+ in oxidative phosphorylation has been the impossibility of achieving a clear separation of the action of K^+ on substrate permeability from its possible action on the oxidative phosphorylation reactions. Thus, many of the results previously reported on the role of K^+ on oxidative phosphorylation may be explained by evoking an action of cations on substrate permeability, (Pressman and Lardy, 1952, 1955; Gómez-Puyou *et al.*, 1969a). However, two short communications on studies of submitochondrial particles show that monovalent cations increase the rate of respiration of submitochondrial particles oxidizing either β-hydroxybutyrate or NADH, but not succinate (Christiansen *et al.*, 1969; Pinto *et al.*, 1970). As no clear-cut permeability barrier to β-hydroxybutyrate and NADH have been detected in submitochondrial particles, these data suggested that monovalent cations were involved more directly in energy transfer at site I (Pinto *et al.*, 1970). Furthermore, some recent studies on K^+-depleted mitochondria also suggested that monovalent cations could be absolutely necessary for oxidative phosphorylation to take place at site I (Gómez-Puyou *et al.*, 1970). In this work, it has been shown that monovalent cations may induce high rates of respiration and maximal P:O ratios in K^+-depleted mitochondria oxidizing a NAD-dependent substrate, but not succinate.

It is most probable that the cation-induced increase in the respiratory rate is due, at least in part, to an increase in the entrance of oxidizable substrates into the mitochondria. The evidence for this assumption is that the inhibition of the glutamate-dependent reduction of NAD induced by increasing sucrose concentrations is reversed by monovalent cations. Li^+ is the most effective of all the cations tested in overcoming this inhibition. The above findings would be in agreement with reports in other laboratories in which cations have been shown to exert a beneficial action on substrate transport (Lynn and Brown, 1966; Harris *et al.*, 1967; Graven *et al.*, 1966; Kimmich and Rasmussen, 1967).

In K^+-depleted mitochondria oxidizing glutamate, the P:O ratios that approach 3.0 at 50 mM sucrose progressively diminish to 1.0 or below when the concentration of sucrose is gradually raised to 200 mM. The explanation for this phenomenon may be a matter of different properties of the membrane at high and low tonicities. For instance, at 50 mM sucrose, added K^+ increases state 3 : state 4 ratios (Gómez-Puyou *et al.*, 1969a, 1970), while at 200 mM sucrose the beneficial effects of K^+ on oxidative phosphorylation have to be mediated by valinomycin. Furthermore, the valinomycin-induced K^+ uptake which is energy independent at low sucrose concentrations (Gómez-Puyou *et al.*, 1970), becomes energy dependent at high sucrose concentrations.

The results of this work show that internal cations are responsible for the induction of the high P:O ratios and that the external cations apparently do not contribute a great deal to this induction. The evidence for the above conclusion is twofold. First, the P:O ratios at different concentrations of added K^+ are lower than those attained in the presence of the same concentrations of K^+ and a suboptimal concentration of valinomycin. Second, maximal P:O ratios are achieved in the presence of Li^+ only when the mitochondria have undergone maximal swelling of Li^+ uptake.

Unfortunately, it has not been possible to determine the concentration of internal cation required for maximal P:O ratios. The reason for this is that the relatively high concentrations of Li^+ (20 mM) that are needed to induce P:O ratios that approach 3.0 preclude the assay of the amount of Li^+ that has gone into the mitochondria. With low concentrations of added K^+ (2 mM) and valinomycin, the uptake of about 300 nmoles of K^+/mg of protein have been detected. However, it is conceivable that only a small proportion of the cation that has gone into the mitochondria would become membrane bound. The larger part would become part of the osmotically active space as revealed by the large swelling detected with valinomycin + K^+. It is possible that only the membrane-bound cations would be functionally active in oxidative phosphorylation. In this respect, it has been reported that Li^+ is a surface binding agent (Massari and Azzone, 1970).

There remains the problem of why the entrance of monovalent cations into the mitochondria induces high P:O ratios. Two possible explanations would be: the uptake of cations could induce a pH gradient through a H^+ cation antiport which would be favorable for oxidative phosphorylation (Mitchell, 1968) or cations could be necessary for a change in some structure involved in oxidative phosphorylation. Although this problem cannot be clarified with our data, the following points should be taken into consideration. Although the natural intramitochondrial cation is K^+, the results of this paper and a preceeding one with K^+-depleted mitochondria (Gómez-Puyou *et al.*, 1970), as well as the studies with submitochondrial particles (Christiansen *et al.*, 1969; Pinto *et al.*, 1970), indicate that almost any monovalent cation may replace K^+. This would seem to cast some doubt on the possibility that the cation binds to a specific locus, rather, it would seem that the internal cations exert a more general action, perhaps the establishment of a pH gradient. However, in whole mitochondria, it has been reported that Na^+ produces loss of respiratory control and partial uncoupling of oxidative phosphorylation, this action of Na^+ is apparently competitive with K^+ (Gómez-Puyou *et al.*, 1969b; Sandoval *et al.*, 1970) which would seem to indicate that there is a certain specificity on the site of action of cations. The latter observation would argue in favor of the possibility that cations act on a specific locus.

TABLE IV: Effect of Cations on the Reduction of NAD in K^+-Depleted Mitochondria.[a]

Salt Added	nmoles of NADH/mg of Protein
	3.3
LiCl	6.0
KCl	6.4
RbCl	6.0
CsCl	5.4
TMACl	6.4

[a] The experimental conditions were as in Figure 6, at 200 mM sucrose; the incubating mixture contained in addition 20 mM of the indicated salts; (TMACl = tetramethylammonium chloride).

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Inhibition and Kinetic Mechanism of Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase*

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ABSTRACT: The patterns of product inhibition by NADH, dead-end inhibition by nitrate, α-glycerophosphate, and 3-phosphoglycerate, and substrate inhibition by 3-hydroxypropionaldehyde-3-P permit deduction of the kinetic mechanism of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase as ordered, with NAD, aldehyde, and phosphate or arsenate adding in that order, and acid release preceeding NADH release. The rate-limiting step with glyceraldehyde-3-P as substrate is NADH release. When 3-hydroxypropionaldehyde-3-P is used as substrate, with arsenate some previous step is equally rate limiting, while with phosphate a previous step is much slower, and the steady-state level of E·NADH is reduced sixfold from that present with arsenate. The kinetic constants with phosphate or arsenate are reported for eight aldehydes which act as substrates.

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD oxidoreductase phosphorylating, EC 1.2.1.12) is capable of catalyzing a number of different reactions; however, the most important one from the physiological point of view is the oxidative phosphorylation of D-glyceraldehyde 3-phosphate (GAP).[1] The enzyme is not specific for GAP, but can catalyze the oxidative phosphorylation of other aldehydes such as D-glyceraldehyde, propionaldehyde, and acetaldehyde. While the enzyme is specific for the hydrogen acceptor, NAD, it is possible to replace the inorganic orthophosphate by arsenate and so convert the phosphorolysis to an irreversible arsenolysis. It has also been established that oxidation precedes phosphorolysis and that under certain conditions the two steps can be separated from one another (Velick, 1954; Boyer and Segal, 1954; Krimsky and Racker, 1963). For example, in the absence of phosphate the oxidation of GAP leads to an acyl-enzyme in which a 3-phosphoglyceryl thiol ester is found (Velick and Hayes, 1953; Segal and Boyer, 1953; Velick and Furfine, 1963).

The kinetic mechanism of this enzyme is of particular interest because (a) it has been investigated by three different groups of workers who came to different and apparently con-

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[1] Abbreviations used are: GAP, D-glyceraldehyde-3-phosphate; HPAP, 3-hydroxypropionaldehyde-3-phosphate; PGA, 3-phosphoglyceric acid.