Effect of Sodium and Potassium Ions on Mitochondrial Oxidative Phosphorylation. Studies with Arsenate*

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ABSTRACT: The effect of arsenate on the oxidative phosphorylation process has been studied in rat liver mitochondria incubated in media which contain ethylenediaminetetraacetate and either Na^+ or K^+. It was found that arsenate stimulated adenosine triphosphatase activity more effectively in mitochondria incubated in the sodium medium than in the potassium medium and that the activation of oxygen uptake by arsenate is less in mitochondria incubated with Na^+ than with K^+. The ^{32}P–adenosine triphosphate exchange reaction and its inhibition by arsenate are not affected differently by Na^+ or K^+. The rate of respiration of mitochondria incubated with K^+ and arsenate is increased by adenosine diphosphate and inhibited by inorganic phosphate, whereas the oxygen uptake of mitochondria incubated with Na^+ is not ostensibly affected by either adenosine diphosphate or inorganic phosphate. The respiration of mitochondria treated with arsenate and phosphate is stimulated by adenosine diphosphate and 2,4-dinitrophenol in the presence of K^+, but not Na^+. On the other hand, adenosine diphosphate inhibits the arsenate stimulated adenosinetriphosphatase activity in the sodium medium more effectively than in the potassium medium. These findings suggest that Na^+ and ethylenediaminetetraacetate induce in mitochondria an alteration which precludes the participation of P_i in mitochondrial oxidative phosphorylation.

Crane and Lipmann (1953) reported that arsenate uncoupled oxidative phosphorylation by a process that was gradual with time. Arsenate was also found to stimulate a latent ATPase activity in intact mitochondria and in submitochondrial particles (Azzone and Ernster, 1961; Wadkins, 1960), and inhibition of the ^{32}P–ATP exchange reaction by arsenate was also reported by Azzone and Ernster (1961). Estabrook (1961) found a stimulation of oxygen uptake by arsenate which could be increased by ADP. More recently, Ter Welle and Slater (1964) demonstrated, in agreement with Crane and Lipmann (1953) that the arsenate stimulated oxygen uptake could be inhibited by inorganic phosphate. Apparently, arsenate and phosphate compete for a site in the respiratory chain phosphorylation mechanism and according to the data of Ter Welle and Slater (1964), phosphate is the more successful competitor.

It has been reported that rat liver mitochondria present a loss of respiratory control and a low uptake of inorganic phosphate when the mitochondria are incubated in a medium which contains Na^+ and EDTA, on the other hand, when mitochondria are incubated with K^+ and EDTA, these functions are well preserved (Gómez-Puyou *et al.*, 1969a). In this work, arsenate was used as an agent to study the mechanism by which the substitution of K^+ by Na^+ produces the above-mentioned alterations.

The results indicate that an impairment of the entrance of inorganic phosphate into the oxidative phosphorylation sequence takes place when mitochondria are incubated with Na^+ and EDTA, but that the detrimental effect of Na^+ is localized to a step prior to the entrance of inorganic phosphate into the oxidative phosphorylation reaction. These findings would be in support of the early conclusions of Pressman and Lardy (1955) which indicated that potassium ions seem to be required for maximum phosphorylation rates by favoring the formation of a phosphorylated intermediate.

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Material and Methods

Rat liver mitochondria were isolated in 0.25 M sucrose and 1 mM EDTA at pH 7.3 according to the method of Schneider and Hogeboom (1950). Oxygen uptake was measured either polarographically (Yellow Springs Instrument Co.) or manometrically according to the conventional Warburg technique. Inorganic phosphate was determined in 6% trichloroacetic acid extracts according to the method of Martin and Doty as described by Lindberg and Ernster (1955).

The ^{32}P–ATP exchange reaction was studied in the following incubation mixture: 100 mM KCl or NaCl, 20 mM Tris-HCl (pH 7.3), 1.3 mM EDTA, 10 mM sucrose, 7.7 mM ATP, and 10 mM H_3PO_4 (adjusted to pH 7.3 with Tris) containing 10^5 cpm in a final volume of 1.0 ml for periods of time that varied between 5 and 10 min. The reaction was stopped with 6% trichloroacetic acid final concentration and the incorporation of ^{32}P into ATP was measured according to Plaut (1963).

The sodium salts of ATP, ADP, and EDTA (Sigma Chemical Co.) adjusted to pH 7.3 with Tris base were used in all experiments. The amount of Na^+ added when these reagents were included in the incubation mixture does not affect the pattern of the results obtained. The arsenate used was H_3AsO_4 adjusted to pH 7.3 with Tris base.

Results

The Effect of Arsenate on the ATPase Activity and the ^{32}P–ATP Exchange Reaction of Mitochondria Incubated with Na^+ and K^+. The effect of different concentrations of arsenate on the ATPase activity of mitochondria incubated with Na^+ and K^+ as a function of time is shown in Figure 1. In agreement with Azzone and Ernster (1961), arsenate increased the

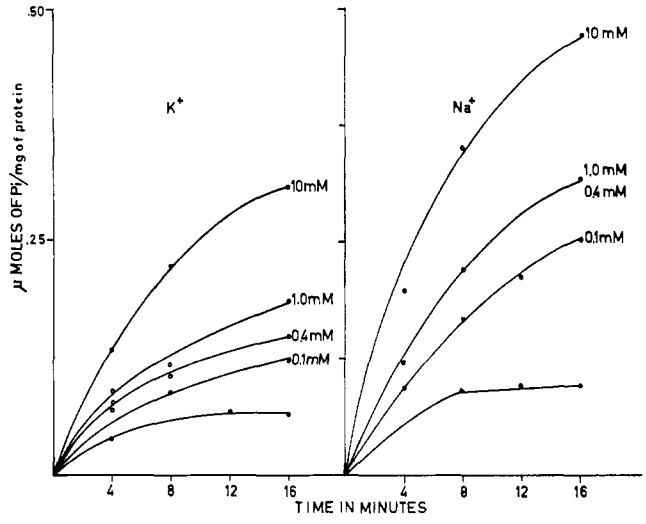

FIGURE 1: Effect of arsenate on the ATPase activity of mitochondria incubated with Na^+ and K^+. The incubation mixture contained 100 mM KCl or NaCl, 20 mM Tris-HCl (pH (7.3), 7.3 mM ATP, 80 mM sucrose, 0.3 mM EDTA, 40 mg of mitochondrial protein, and the indicated concentration of arsenate in a final volume of 7.5 ml. At the indicated time, aliquots were withdrawn and inorganic phosphate was determined. Incubation temperature, 25°. The same curve was obtained in the sodium medium with 0.4 mM arsenate and 1 mM arsenate.

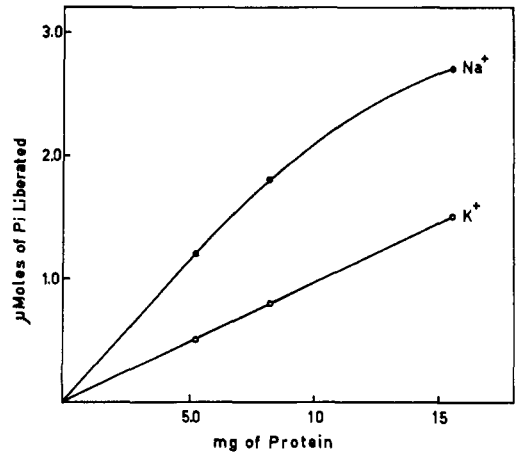

FIGURE 2: The arsenate-stimulated ATPase activity at various mitochondrial concentrations. The incubating conditions were: 100 mM KCl or NaCl, 20 mM Tris-HCl (pH 7.3), 1.3 mM EDTA, 80 mM sucrose, 7.3 mM ATP, 10 mM arsenate, and the indicated quantities of mitochondrial protein in a final volume of 1.5 ml. Incubation time, 10 min, temperature, 25°.

ATPase activity of mitochondria incubated with K^+, but a higher arsenate-stimulated ATPase activity was observed in the sodium medium. This different effect of Na^+ and K^+ on the arsenate-stimulated ATPase activity is independent of mitochondrial protein concentration (Figure 2) and is proportionally higher as the concentration of the respective salts is increased (Figure 3). The lack of linearity of the ATPase activity as a function of time is probably due to the inhibiting action of ADP, a phenomenon that will be described below.

In 10-min incubation and 100 mM K^+, the stimulation of ATPase activity by 10 mM arsenate varied between 0.19 and 0.1 μmole of inorganic phosphate per mg of mitochondrial protein. With Na^+, the activity was 50 or 100% higher in all experiments made. The favorable effect of Na^+, incomparison with K^+, on the arsenate-stimulated ATPase activity is observed to the same extent in mixtures which contained 80 and 140 mM sucrose. The arsenate-stimulated ATPase activity is slightly lower with 100 mM K^+ than in media which contained 20 mM Tris and this latter activity is similar to that obtained with 100 mM Tris. Between 0.3 and 1.3 mM EDTA, Na^+ also exerts a beneficial action on the arsenate-stimulated ATPase activity, lower concentrations of EDTA were not assayed since in the absence of EDTA a lack of reproducibility is observed in the results of experiments in which the different action of Na^+ and K^+ on oxidative phosphorylation is assayed (Gómez-Puyou *et al.*, 1969a). These latter experiments on the arsenate-stimulated ATPase activity are not shown.

The effect of arsenate on the ^{32}P–ATP exchange reaction of mitochondria (Boyer *et al.*, 1954; Boyer *et al.*, 1956) incubated with EDTA and Na^+ or K^+ was also studied. No significant or reproducible differences in the amount of phosphate incorporated into ATP in 5-min incubation (approximately 0.7 μmole/mg of protein) were observed between mitochondria incubated with Na^+ and K^+; the inhibiting effect of 8 mM arsenate and 0.8 mM arsenate, 50 and 25%, respectively, was not modified either by the two assayed cations. Na^+ and K^+ did not affect differently the exchange reaction of mitochondria incubated with antimycin A, with and without arsenate in the mixture.

The Effect of Arsenate on the Oxygen Uptake of Mitochondria Incubated with Na^+ and K^+. It has been reported that rat liver mitochondria incubated with sodium ions and EDTA have a high rate of respiration with NAD-dependent substrates, with succinate as substrate, the results reported were described as contradictory since no clear-cut difference was observed between mitochondria incubated with Na^+ and K^+ (Gómez-Puyou *et al.*, 1969a). The latter phenomenon has been

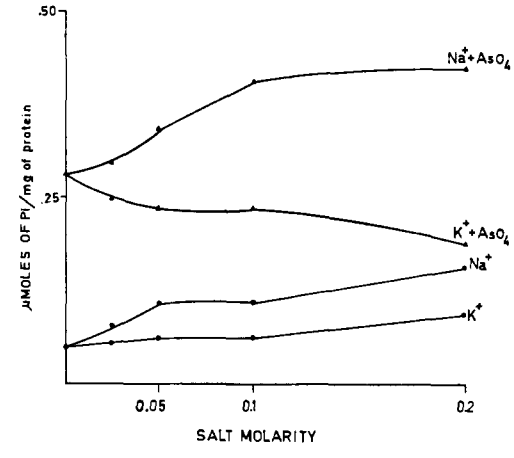

FIGURE 3: Effect of various concentrations of KCl and NaCl on the mitochondrial arsenate-stimulated ATPase activity. The incubation mixture contained KCl or NaCl at the indicated concentrations, 20 mM Tris-HCl (pH 7.3), 2 mM arsenate, 80 mM sucrose, 0.3 mM EDTA, 7.3 mM ATP, and 6.4 mg of mitochondrial protein in a final volume of 1.5 ml; incubation time, 15 min; temperature, 25°.

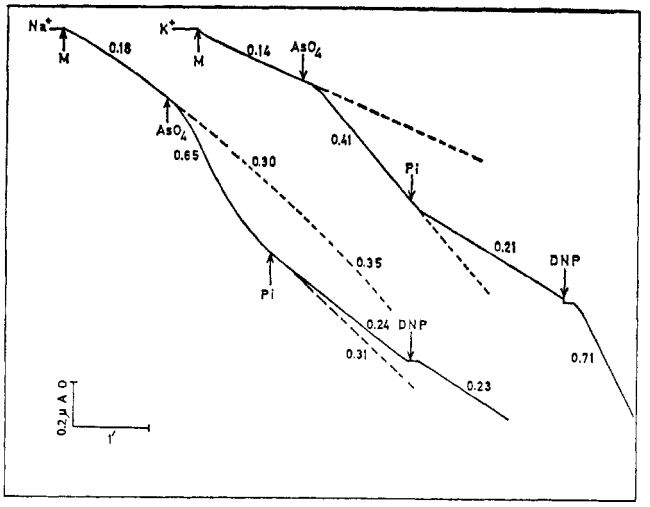

FIGURE 4: Effect of arsenate, inorganic phosphate, and 2,4-dinitrophenol on the oxygen uptake of mitochondria incubated with Na^+ and K^+. Respiration was measured polarographically in 5.0 ml of incubation mixture which contained 100 mM KCl or NaCl, 20 mM Tris-HCl (pH 7.3), 1 mM EDTA, 0.24 μM rotenone, 50 mM sucrose, 10 mM succinate, and 4.6 mg of mitochondrial protein. Where indicated mitochondria (M), 2 mM arsenate, 2 mM phosphate (P_i), and 10^{-4} M 2,4-dinitrophenol (DNP) were added. The broken lines represent the oxygen uptake without the addition of the indicated substance. The numbers on the side of the lines indicate the rate of respiration (μatoms/min); when the rate of respiration was not linear, the respiratory rates were calculated from the slope of the curve at the points where the numbers are shown; temperature, 25°.

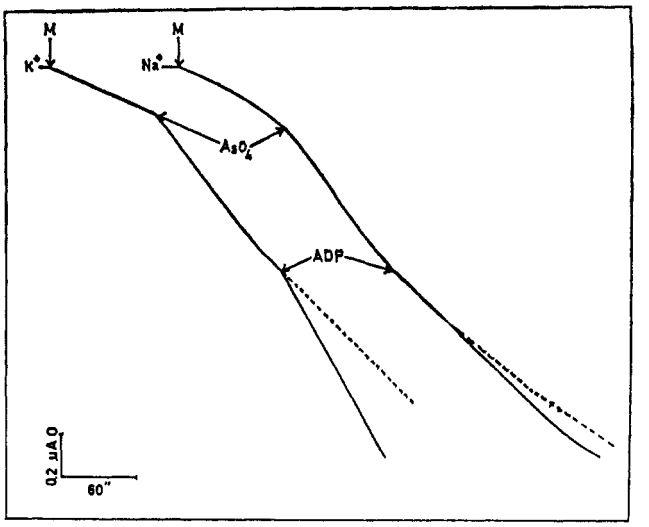

FIGURE 6: Effect of ADP on the arsenate-stimulated oxygen uptake of mitochondria incubated with Na^+ and K^+. The incubating conditions were as in Figure 4, except that the vessels contained 6.4 mg of mitochondrial protein; 0.6 mM arsenate and 0.54 mM ADP were added as indicated.

studied in more detail and it has been observed that during the first few minutes of the experiment, the rate of oxygen uptake is substantially higher in the sodium medium than in the potassium medium, but as the incubation time continues, a higher rate of oxygen uptake takes place in the mitochondria incubated with K^+ (Figures 4 and 5).

The effect of arsenate on the oxygen uptake of mitochondria has been extensively studied by several investigators (Crane and Lipmann, 1953; Estabrook, 1961; Ter Welle and Slater, 1964; Chappell and Crofts, 1965; Slater, 1966), and according to their data, arsenate in the absence of added inorganic phosphate and in the presence of K^+ induces a high rate of oxygen uptake. In mitochondria incubated with Na^+ and succinate, the addition of arsenate induces initially a rate of oxygen uptake which is significantly higher than that observed in mitochondria incubated with K^+ and arsenate (Figure 4). After this initial burst of respiration observed upon the addition of arsenate to mitochondria incubated with Na^+, the rate of respiration gradually diminishes and becomes significantly lower than that detected in mitochondria incubated with K^+ and arsenate (Figure 4). Indeed when the rate of oxygen uptake of mitochondria incubated in the same conditions was measured manometrically for longer periods of time, a higher rate of oxygen uptake was found in mitochondria incubated with K^+ and arsenate (Figure 5).

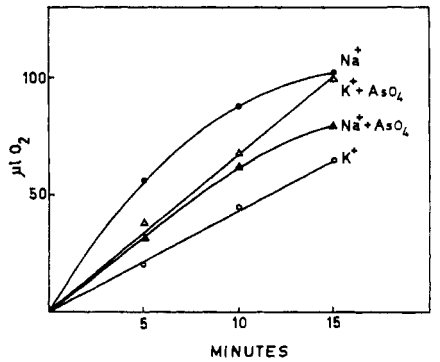

FIGURE 5: Effect of arsenate on the oxygen uptake of mitochondria incubated with Na^+ and K^+. Oxygen uptake was measured manometrically in the following incubation mixture: 100 mM KCl or NaCl, 20 mM Tris-HCl (pH 7.3), 1.2 mM EDTA, 80 mM sucrose, 10 mM succinate, and 15.8 mg of mitochondrialp rotein. After 7 min of thermoequilibrium, the first reading was made and 5 mM arsenate was added from the side arm. Final volume, 3.0 ml; temperature, 25°.

The arsenate-stimulated respiration is inhibited by inorganic phosphate (Crane and Lipmann, 1953; Ter Welle and Slater, 1964; Slater, 1966), presumably through a competition between phosphate and arsenate in which the former is a more favorable competitor (Ter Welle and Slater, 1964). This effect of phosphate on the arsenate-stimulated oxygen uptake was studied in mitochondria incubated with Na^+ and K^+ (Figure 4). In the potassium medium, phosphate inhibited the mitochondrial respiration stimulated by arsenate much more effectively than in the sodium medium. However, it should be taken into account that due to the low stimulatory action of arsenate in the sodium medium, the inhibiting effect of phosphate may be difficult to evaluate.

Estabrook (1961) reported that ADP enhanced the arsenate-stimulated oxygen uptake of mitochondria incubated with K^+, probably by removing endogenous phosphate and thereby eliminating a competition reaction with arsenate. This effect of ADP was confirmed in mitochondria incubated with K^+ and arsenate (Figure 6); however, ADP failed to increase sig-

TABLE I: Phosphorylation of ADP in Mitochondria Incubated with Na^+ and K^+.[a]

	P_i Uptake (μmoles)	
	K^+	Na^+
Glutamate	11.4	7.1
Glutamate + arsenate	2.0	0.5
Succinate	7.5	0.2
Succinate + arsenate	4.2	0

[a] The incubation mixture contained 0.1 M KCl or NaCl, 20 mM Tris-HCl (pH 7.3), 1.2 mM EDTA, 80 mM sucrose, 10 mM H_3PO_4 adjusted to pH 7.3 with Tris, 10 mM substrate, 7 mM ADP, and 13.3 mg of mitochondrial protein in a final volume of 3.0 ml. The mixture contained 6 mM arsenate where indicated. The reaction was started by the addition of ADP. Incubation time with glutamate 18 min, with succinate 13 min; temperature, 25°.

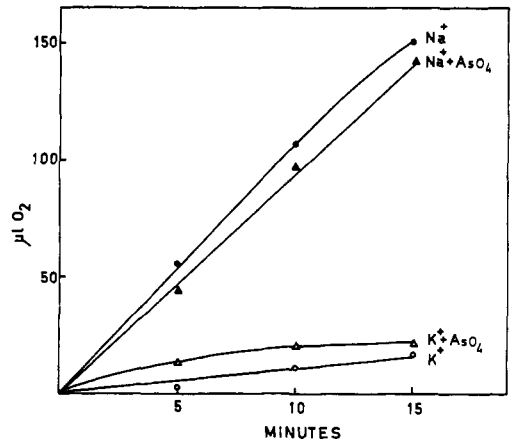

FIGURE 7: Effect of arsenate on the oxygen uptake of mitochondria incubated with Na^+ and K^+. The incubating conditions were as in Figure 5 except that 10 mM glutamate was used as substrate and the preparation contained 17.3 mg of mitochondrial protein.

nificantly the rate of respiration of mitochondria incubated with Na^+ and arsenate (Figure 6).

Relation between the ATPase Activity and the Respiration of Mitochondria Incubated with Arsenate and Na^+ or K^+. The results obtained indicate that significant differences on the effect of arsenate in mitochondria are encountered when the particles are incubated with Na^+ or K^+. In an attempt to investigate the mechanism involved in these findings, the following experiments were carried out.

Although Na^+ and K^+ do not affect differently the ^{32}P–ATP exchange reaction, the results of Table I show that the phosphorylating capacity of the mitochondria is different in the sodium medium than in the potassium medium in the presence of EDTA and in the absence of added Mg^{2+}. With Na^+, a lower capacity to phosphorylate ADP is observed both with glutamate and succinate as substrates; in these conditions, arsenate completely abolishes the extremely low succinate-supported phosphorylation and produces extremely low levels of phosphorylation in the case of glutamate where appreciable phosphorylating capacity is detected in the controls. Apparently, although adequate respiratory activity remains (Figures 5 and 7), the phosphorylating reactions of the mitochondria are more sensitive to arsenate in the presence of sodium ions. Indeed, mitochondria, in the presence of arsenate, Na^+, and glutamate, have a rate of oxygen uptake which exceeds by far the oxygen uptake of mitochondria incubated in the same conditions with K^+ (Figure 7). This high rate of respiration with arsenate, Na^+, and glutamate is due mainly to the high oxygen uptake observed in the absence of arsenate (Gómez-Puyou *et al.*, 1969a) and not to a stimulatory action of arsenate, rather, arsenate produces a small depression of respiration in mitochondria incubated with Na^+ (Figure 7).

These experiments indicated that the lower levels of phosphorylation in mitochondria incubated with Na^+ and arsenate were probably responsible for the higher arsenate-stimulated ATPase activity in mitochondria. This assumption was substantiated by the experiments of Figure 4, the addition of 2,4-dinitrophenol or ADP induces a high rate of respiration when mitochondria are incubated with K^+ in the presence of arsenate and inorganic phosphate (Slater, 1966); however, 2,4-dinitrophenol (or ADP) fails to release the respiration of mitochondria incubated with Na^+ in the same conditions (Figure 4).

The low rate of phosphorylation in mitochondria incubated with Na^+ could be due to a defect in the entrance into the oxidative phosphorylation sequence of inorganic phosphate, ADP, or both. It is probable that the entrance of ADP is unimpaired since ADP inhibits strongly the arsenate-stimulated ATPase activity of mitochondria incubated in the sodium medium (Table II). On the other hand, inorganic phosphate fails to inhibit the small stimulation of respiration induced by arsenate in mitochondria incubated with Na^+ (Figure 4) and 2,4-dinitrophenol which acts on a nonphosphorylated intermediate (Lehninger *et al.*, 1959) fails to release the respiration of mitochondria incubated in the sodium medium with arsenate and phosphate (Figure 4). Thus, it is probable that a step prior to the entrance of phosphate into the oxidative phosphorylation sequence is the one which will be affected by exposing mitochondria to Na^+ and EDTA.

TABLE II: Effect of ADP on the Arsenate-Stimulated ATPase Activity of Mitochondria Incubated with Na^+ and K^+.[a]

	P_i Liberated (μmoles)	
	K^+	Na^+
	0.3	0.9
Arsenate	1.6	3.5
Arsenate + ADP	1.2	2.0
ADP	0.2	0.3

[a] The incubating conditions were: 100 mM KCl or NaCl, 20 mM Tris-HCl (pH 7.3), 7.3 mM ATP, 1.3 mM EDTA, 80 mM sucrose, 10 mM arsenate, and 6.6 mM ADP where indicated; the preparation contained 10 mg of mitochondrial protein; final volume, 1.5 ml; incubation, 10 min; temperature 25°.

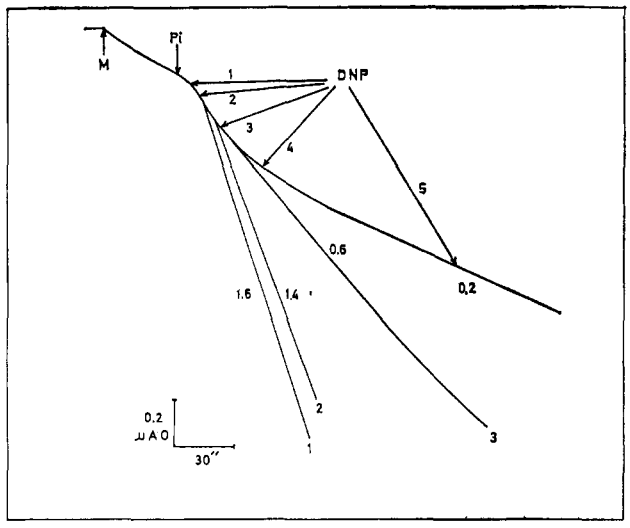

FIGURE 8: Effect of inorganic phosphate and 2,4-dinitrophenol on the oxygen uptake of mitochondria incubated with Na^+. Oxygen uptake was measured polarographically in the following incubation mixture: 100 mM NaCl, 20 mM Tris-HCl (pH 7.3), 1 mM EDTA, 50 mM sucrose, 10 mM succinate, 0.24 μM rotenone, and 4.8 mg of mitochondrial protein (M) in a final volume of 5.0 ml. Where indicated, mitochondria and 2 mM H_3PO_4 (adjusted to pH 7.3 with Tris) were added. 2,4-Dinitrophenol (DNP) (10^{-4} M) was added to different vessels treated with mitochondria and phosphate at the time indicated by the arrows numbered 1 to 5; this addition resulted in different respiratory rates which correspond to the respiration traces indicated by their respective numbers. The fourth and fifth addition of dinitrophenol did not alter the oxygen uptake trace. The numbers indicate the actual rate of oxygen uptake.

In view of this conclusion, the short but significant increment in the rate of respiration observed upon the addition of arsenate to mitochondria incubated with Na^+ became of particular interest (Figure 4). It was found that the addition of inorganic phosphate to mitochondria incubated in the same conditions produced a similar increase in the oxygen uptake which was followed by a rate slightly higher than the initial state 4 rate. Dinitrophenol added at various times after the addition of phosphate induced rates of respiration which diminished as the time of contact of mitochondria with phosphate increased (Figure 8).

Discussion

It has been reported that rat liver mitochondria incubated in the absence of added Mg^{2+} in a sodium medium supplemented with EDTA present a lack of respiratory control and a low capacity to phosphorylate ADP. In these conditions, the rate of the aerobic oxidation of NAD-dependent substrates was found to be increased to an extent which varied between 70 and 100% of the rate induced by 2,4-dinitrophenol in mitochondria incubated with K^+ (Gómez-Puyou *et al.*, 1969a). These findings suggested that Na^+ and EDTA produce an alteration of a mitochondrial structure which is required for appropriate respiratory control. Since the effect of arsenate on mitochondria has been extensively studied (Crane and Lipmann, 1953; Chan *et al.*, 1960; Wadkins, 1960; Azzone and Ernster, 1961; Estabrook, 1961; Ter Welle and Slater, 1964) and since arsenate has the further advantage of its resemblance to inorganic phosphate, but with a lower stability of arsenylated compounds, it has been used in the present studies in order to gain some insight into the nature of the mechanism involved in the loss of the phosphorylating ability of mitochondria incubated with Na^+ and EDTA.

It has been found that arsenate produces different quantitative effects on mitochondria which depend on the cationic composition of the medium. The higher arsenate-stimulated ATPase activity that is observed in mitochondria incubated with Na^+ in comparison with the activity observed in mitochondria incubated with K^+ and arsenate, according to the reasoning of Ter Welle and Slater (1964), could be ascribed to a lower phosphorylation of ADP, which in turn could be due to a lower oxidation of endogenous substrates or to a lower efficiency of the phosphorylation reactions. With succinate as substrate, it is true that in experiments that last for the time of incubation used in the assay of the ATPase activity, mitochondria, in the presence of Na^+ and arsenate, consume less oxygen than mitochondria incubated with K^+ and arsenate. However, with glutamate as substrate, the oxygen uptake of mitochondria incubated with Na^+ and arsenate is considerably higher than in the presence of K^+ and arsenate. Thus, it is possible to conclude from these experiments that the rate of oxidation of endogenous substrates is not the only factor that is responsible for the higher arsenate-stimulated ATPase activity in mitochondria incubated with Na^+. Rather, it is possible that Na^+ and K^+ affect differently one or several of the steps involved in the formation of ATP during electron transport. The high rate of respiration detected with glutamate and arsenate in the sodium medium indicates that the availability of oxidizable substrates is not limiting.

Except for an initial and unspecific burst of respiration which occurs upon the addition of arsenate or phosphate to mitochondria, the aerobic oxidation of succinate is not ostensibly increased by arsenate in mitochondria incubated with Na^+. In agreement with Crane and Lipmann (1953) and Ter Welle and Slater (1964), it was found that with K^+ in the mixture, inorganic phosphate inhibited the arsenate-stimulated oxygen uptake probably by competing with arsenate. On the other hand, the small stimulation of oxygen uptake produced by arsenate in mitochondria incubated in the sodium medium was hardly affected by added phosphate. Although the low inhibiting action of phosphate on the oxygen uptake of mitochondria incubated with Na^+ and arsenate may be difficult to evaluate due to the low stimulatory action of arsenate, the latter results may be interpreted assuming that the small effect of the two compounds is due to the fact that in mitochondria incubated with Na^+ and EDTA, the entrance of arsenate and phosphate into the appropriate step of the phosphorylation reaction is either absent or highly diminished.

Further evidence which substantiates this possibility was obtained when the effect of ADP was assayed on the arsenate-stimulated ATPase activity and on the arsenate-stimulated oxygen uptake. ADP, in the presence of K^+, as reported by Estabrook (1961), increases the arsenate-stimulated oxygen uptake, probably by removing inorganic phosphate and thereby eliminating a competition reaction with arsenate. In the sodium medium, ADP apparently enters quite adequately the phosphorylation reaction since it inhibits the arsenate-stimulated ATPase activity of mitochondria incubated with Na^+ and yet, in mitochondria incubated with Na^+ and arsenate, ADP failed to produce an important activation of res-

piration. This lack of effect of ADP on the arsenate-stimulated oxygen uptake would suggest that ADP does not stimulate respiration because of its inability to remove phosphate. According to the theories of oxidative phosphorylation, phosphate, before reacting with ADP, must be elevated to a high-energy state. ADP would not be able to remove inorganic phosphate because in mitochondria incubated with Na^+ and EDTA, this step would be impaired. Thus, the failure of ADP to increase the arsenate-stimulated oxygen uptake would be indirect evidence of a diminished or nonexistence of the phosphorylated intermediate in mitochondria incubated with Na^+.

In this respect, it should be mentioned that the ^{32}P–ATP exchange reaction nor the inhibiting action of arsenate was affected differently by Na^+ or K^+. Within the context of the chemical theory (Slater, 1953; Lehninger *et al.*, 1959) or the chemiosmotic hypothesis (Mitchell, 1966), the ^{32}P–ATP exchange reaction comprises the final steps of the oxidative phosphorylation sequence, presumably, Na^+ and K^+ would not affect differently these steps. A conclusion which is substantiated by the observed inhibiting effect of ADP on the arsenate-stimulated ATPase activity of mitochondria incubated with Na^+.

Perhaps the best evidence for the postulation that arsenate and phosphate do not enter the oxidative phosphorylation reaction in mitochondria incubated with Na^+ is provided by the finding that phosphate produces the same effect as arsenate on mitochondrial respiration (Figure 8). Obviously, arsenate does not increase respiration by forming an unstable arsenylated compound, but by a process which is currently under study and probably due to ion movements in the mitochondria. The relevant point here is that these results indicate that the entrance of arsenate or phosphate into the phosphorylation sequence is highly diminished in mitochondria incubated with Na^+. Furthermore, the same experiment shows that the sensitivity to 2,4-dinitrophenol diminishes as the time of exposure of mitochondria to phosphate increases; it is during this interval that an alteration of oxidative phosphorylation is taking place. The locus of which can be localized to a site between electron transport and the point of action of dinitrophenol.

The nature of this alteration cannot be ascertained at the present time within the postulations of the chemiosmotic hypothesis or the chemical theory. However, it is probable that the detrimental action of Na^+ and EDTA on oxidative phosphorylation is due at least in part to its effect on the potassium content of the mitochondria and on a possible substitution of K^+ by Na^+ on a reaction which would respond differently to Na^+ and K^+; evidence which shows that Na^+ and K^+ affect differently state 4 respiratory activity has been presented before (Gómez-Puyou *et al.*, 1969b). Mustafa *et al.* (1966) found that EDTA increased the permeability of the mitochondria to Na^+ and K^+ and Settlemire *et al.* (1968) reported that the addition of EDTA to mitochondria incubated with Na^+ produces the extrusion of K^+ from the mitochondria. It has also been shown that mitochondria partially depleted of K^+ have low respiratory responses to ADP and dinitrophenol which may be significantly increased by added K^+ (Gómez-Puyou *et al.*, 1969b). Although the favorable action of K^+ on the entrance of oxidizable substrates into the mitochondria is well recognized (Graven *et al.*, 1966; Lynn and Brown, 1966; Harris *et al.*, 1967), the data presently reported would provide further evidence to the hypothesis that K^+ is required for the proper functioning of a reaction that takes place between the electron transfer step and the site of action of 2,4-dinitrophenol.

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