Reaction of Inorganic Phosphate with Mitochondrial Respiratory Chain*

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ABSTRACT: The response of respiratory chain components during transition of rat liver mitochondria to an inorganic phosphate depleted state and from this to an inorganic phosphate supplemented state has been studied. When electron flow is activated by addition of inorganic phosphate to inorganic phosphate depleted mitochondria (∼phosphate trap present) the responses of the respiratory carriers are the expression of two separate reactions: activation of oxidative phosphorylation by ∼phosphate formation and an oligomycin-insensitive reaction of inorganic phosphate with the respiratory chain. When reducing equivalents are fed to nicotinamide–adenine dinucleotide the latter reaction is characterized by flavoprotein oxidation and reduction of cytochrome *c*. With ascorbate (plus tetramethyl-*p*-phenylenediamine) as substrate inorganic phosphate causes oxidation of cytochrome *c*. The results suggest that the oligomycin-insensitive responses of respiratory carriers to inorganic phosphate are the expression of an energy load imposed by the uptake of inorganic phosphate by mitochondria on the oxidoreduction systems.

The response of the respiratory chain to the energy-linked reaction with ADP, ATP, Ca^{2+}, as well as other divalent cations, and to uncouplers of oxidative phosphorylation has been thoroughly investigated. Yet only fragmentary data are available on the interaction of P_i and the respiratory chain (Chance and Hagihara, 1961; Chance, 1965; Chance and Schöener, 1966a). P_i, besides its obvious role in oxidative phosphorylation, has marked effect on the energy-driven uptake of cations by mitochondria (Moore and Pressman, 1964; Chance, 1965; Rasmussen *et al.*, 1965; Chappell and Crofts, 1966; Azzi and Azzone, 1966). More recently the transport of anions in mitochondria has been on its own subject of study (Chappell and Haarhoff, 1966; Mitchell, 1966, 1968; Slater *et al.*, 1969). It has been proposed that in mitochondria the transport of anions is mediated by specific carriers *via* exchange diffusion with OH^- (Chappell and Crofts, 1966; Chappell and Haarhoff, 1967) or by symport with H^+ (Mitchell, 1968). Papa *et al.* (1969) have obtained evidence that the transport of P_i, but not that of a dicarboxylate anion, can be directly coupled to an OH^- counterflux (or H^+ symport). The problem of the energetics of anion uptake by mitochondria has also been raised (Chappell and Haarhoff, 1967; Mitchell, 1968; Slater, 1969; Van Dam and Kraaijenhof, 1969). This paper reports on the reaction of P_i with the respiratory chain of isolated rat liver mitochondria. Attention has been focused on particular changes of the redox state of flavoproteins and cytochrome *c* brought about by P_i supplementation to mitochondria under conditions of inhibition of oxidative phosphorylation. The results presented suggest that the uptake of P_i by mitochondria effects the energy pressure built up by the oxidoreductions of the respiratory chain. An account of part of this investigation has appeared (Quagliariello and Papa, 1966; Papa and Quagliariello, 1969).

Experimental Procedure

Preparations. Rat liver mitochondria were prepared by the method of Myers and Slater (1957) or by that of Chance and Hollunger (1961), pigeon heart mitochondria by that of Chance and Hagihara (1961). Mitochondria (3–6 mg of protein/ml) were incubated in a basic reaction medium containing 150 mM KCl, 20 mM Tris-HCl (pH 7.4), 25 mM sucrose (derived from the mitochondrial suspension), 1 mM $MgCl_2$, and 0.5 mM EGTA. The incubation was carried out at room temperature.

Analytical Methods. Protein was determined by the biuret method, P_i by the method of Lindberg and Ernster (1956). $NAD(P)^+$ and NAD(P)H were determined according to Klingenberg (1963).

Optical Methods. A dual-wavelength spectrophotometer was used to record the absorbance of nicotinamide nucleotides (340 *vs.* 374 mμ), flavoproteins (465 *vs.* 510 or 465 *vs.* 490 mμ), cytochrome *b* (562 *vs.* 575 mμ for the α band and 430 *vs.* 410 mμ for the Soret band), cytochrome *c* (550 *vs.* 540 mμ for the α band, or 418 *vs.* 405 mμ for the Soret band), and cytochrome *a* (605 *vs.* 630 mμ). Absorbancy of ubiquinone was recorded at 282 *vs.* 295 mμ. Changes in the fluorescence of nicotinamide nucleotides (excitation at 366 mμ, measurement at 450 mμ) and flavoproteins (excitation at 436 mμ, measurement at 570 mμ) were recorded simultaneously.

In some experiments the absorbancy of cytochrome *c* was recorded with a triple-beam apparatus with the measuring wavelength at 550 mμ and the two reference wavelengths at 530 and 570 mμ, respectively. The differential output (550 mμ *vs.* the average of the reference wavelengths) measured the absorbance of cytochrome *c* (D. Mayer and B. Chance, 1969, unpublished data).

Difference spectra of cytochromes were recorded at the

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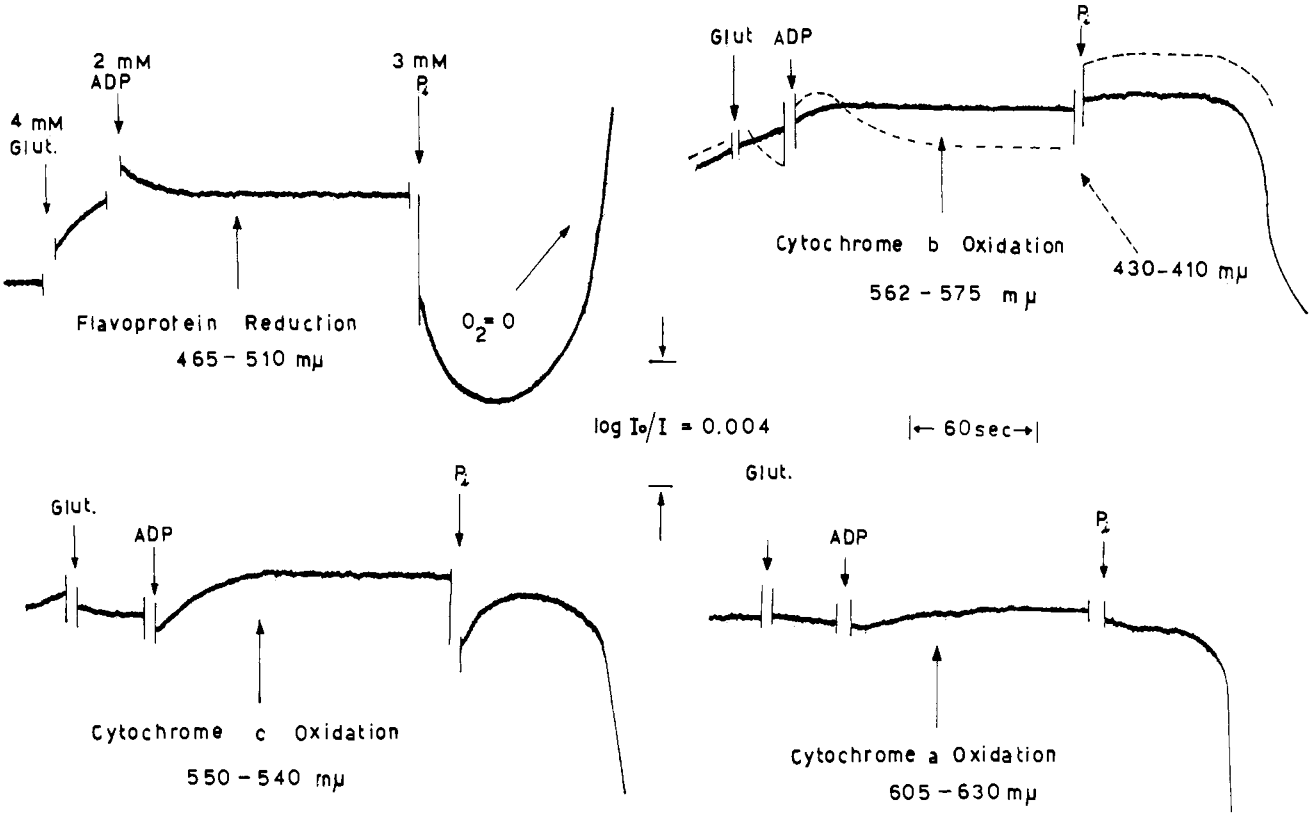

FIGURE 1: Effect of P_i on the redox state of flavoproteins and cytochromes in rat liver mitochondria. Mitochondria were incubated in the basic reaction medium. The additions are specified in the figure. Final volume, 3 ml. For other details, see under Experimental Procedure. The transient reduction of cytochrome *c* that is noted on adding 2 mM ADP is due to the P_i present in the commercial sample of ADP used. In fact there was a monophasic oxidation of cytochrome *c* when 0.2 mM ADP were added in the presence of glucose and hexokinase. ADP (2 mM) gave also an increase of the absorbance at 465 mμ, due to the slight absorption of ADP at this wavelength (compare with Figure 6).

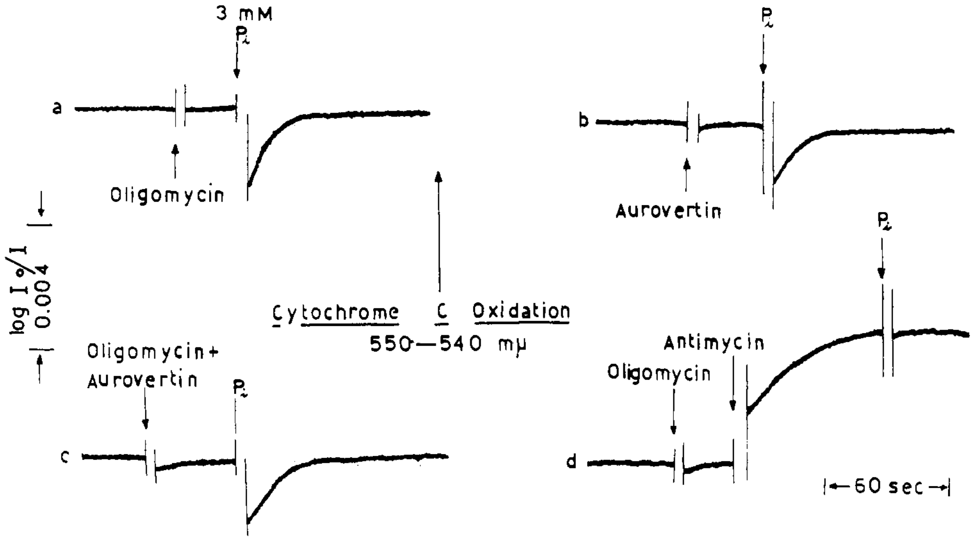

FIGURE 2: Effect of inhibitors on the response of cytochrome *c* absorbancy to P_i in rat liver mitochondria. Mitochondria were suspended in the basic reaction medium. After 2-min incubation with 5 mM glutamate and 2 mM ADP, the following inhibitors were added: (a) 30 μg of oligomycin, (b) 9 μg of aurovertin, (c) oligomycin and aurovertin, and (d) oligomycin and 1.5 μg of antimycin A. [P_i] was 3 mM. Final volume, 3 ml. For other details, see under Experimental Procedure.

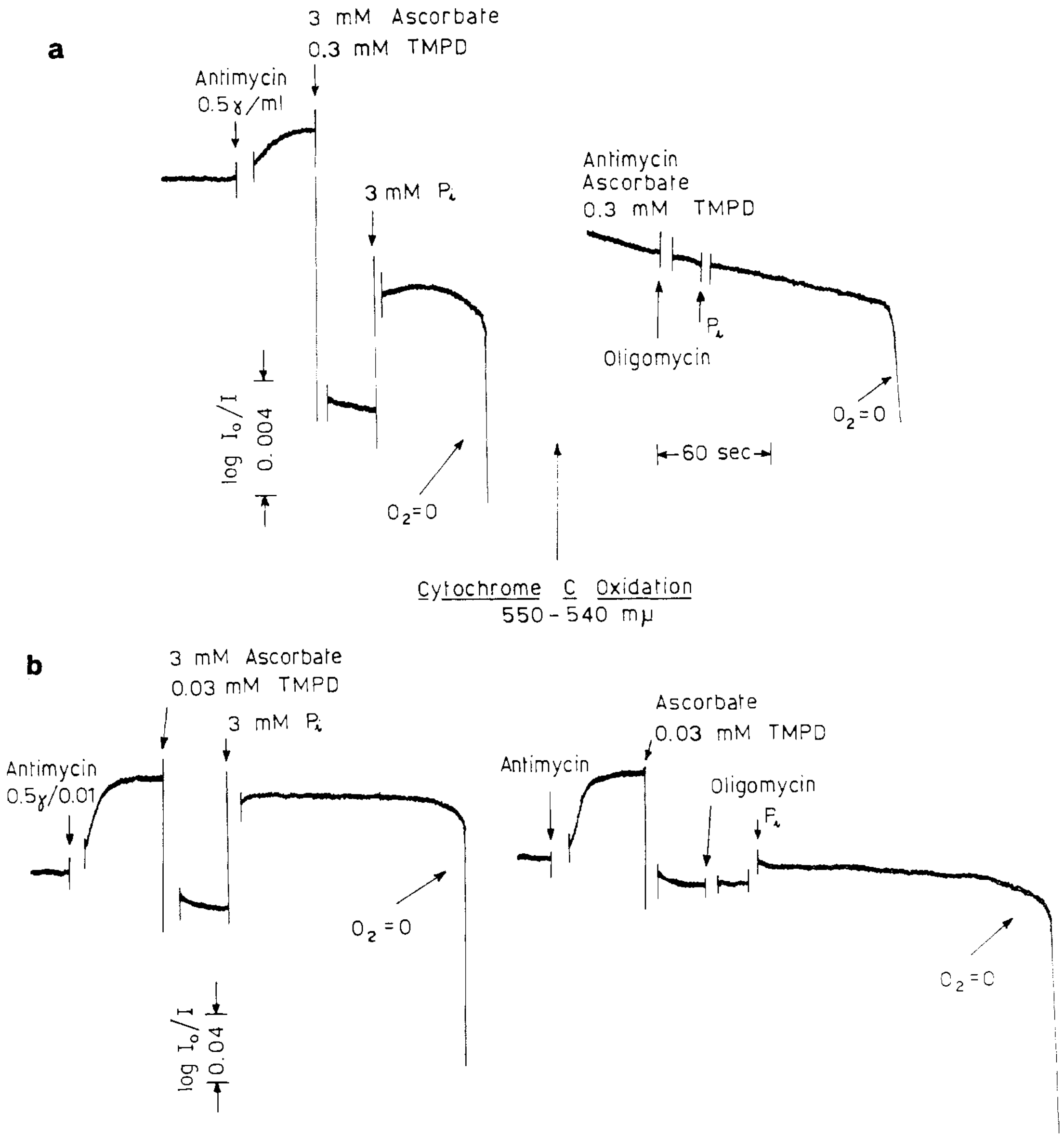

FIGURE 3: Effect of P_i on cytochrome *c* absorbancy in rat liver mitochondria with TMPD (plus ascorbate) as respiratory substrate. Mitochondria were incubated in the basic reaction medium. After 2-min preincubation with 5 mM glutamate and 2 mM ADP, 1.5 μg of antimycin was added followed by TMPD (plus ascorbate). Final volume, 3 ml. The same results presented in this figure were observed when cytochrome *c* oxidoreduction was monitored in the Soret region at the wavelength pair 418 *vs.* 405.

temperature of liquid nitrogen (Estabrook, 1956; Chance and Schöener, 1966b). Since the repeatibility of a cytochrome peak with the instrument used is within ±10 Å, this value gives the tolerance of the cytochrome peaks.

Uptake of P_i by Mitochondria. $[^{32}P]P_i$ was added, in the presence of an excess of oligomycin, to mitochondria depleted of endogenous P_i by preincubation with β-hydroxybutyrate and a ~P trap. Three minutes after the addition of $[^{32}P]P_i$, mitochondria were separated from the suspending medium and centrifuged into $HClO_4$ as described by Pfaff (1965). The amount of $[^{32}P]P_i$ in the matrix space was calculated from the radioactivity in the $HClO_4$ extract and corrected for the $[^{32}P]P_i$ radioactivity of sucrose-permeable space and adherent medium. These were determined with $[^{14}C]$sucrose.

Potentiometric Recording in the Presence of TMPD. A platinum electrode has been employed (Caswell and Pressman, 1968) to record changes in the redox state of TMPD[1] in response to its interaction with the respiratory chain.

Measurement of Oxygen Uptake. Respiratory activity was measured polarographically with a vibrating platinum electrode.

Results

Effect of P_i on Electron Transport. Freshly isolated rat liver mitochondria contain 20–40 nmoles of P_i/mg of protein.

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[1] Abbreviation used is: TMPD, tetramethyl-*p*-phenylenediamine.

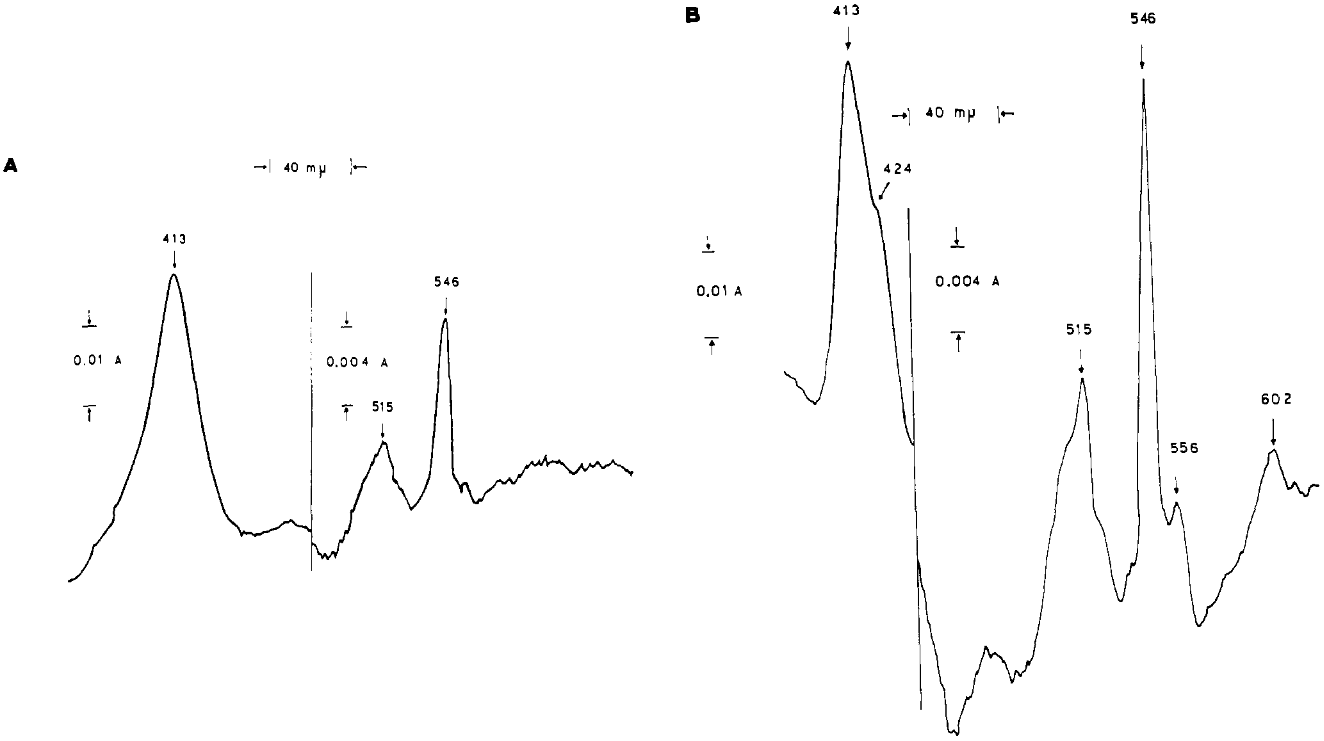

FIGURE 4: Spectral studies. (A) Spectrum representing the difference between P_i-depleted and P_i-supplemented rat liver mitochondria with glutamate as respiratory substrate. Mitochondria were incubated in the basic reaction medium. After 2-min preincubation with 5 mM glutamate, 20 mM glucose, 0.3 mM ADP, and 5 units of hexokinase, 10 μg of oligomycin was added. Final volume, 1 ml. One minute after oligomycin addition, 0.4 ml of the mitochondrial suspension was brought into the references cell and rapidly frozen. The remainder was supplemented with 5 mM P_i, brought into the measuring cell, and frozen within a few seconds. Optical path length, 2 mm. For other details, see Experimental Procedure. (B) Spectrum representing the difference between P_i-depleted and P_i-depleted, succinate-supplemented rat liver mitochondria. Mitochondria were incubated in the basic reaction medium. After 2-min preincubation with 20 mM glucose 0.3 mM ADP and 5 units of hexokinase, 0.4 ml of the mitochondrial suspension was brought into the reference cell and rapidly frozen, the remainder was supplemented with 5 mM succinate and frozen 30 sec thereafter. Optical path length, 2 mm.

This was removed by a 2–3-min incubation with a respiratory substrate and a ~P trap (ADP, AMP, or ADP–glucose–hexokinase; see also Borst and Slater, 1961). The depletion of P_i caused inhibition of respiration and reduction of nicotinamide nucleotides. The subsequent addition of P_i gave an abrupt activation of respiration and a change of the steady state of NAD in the direction of oxidation. Phosphate (250 μM) gave half-maximal activation of respiration (Chance and Hagihara, 1961). Figure 1 shows the behavior of other components of the respiratory chain. Depletion of P_i caused oxidation of flavoproteins and cytochrome *c*. The changes in the absorbancy at the wavelength pair 562 *vs.* 575 mμ indicated a small oxidation of cytochrome *b*. In the Soret region an initial oxidation followed by reduction (similar to that was described for nicotinamide nucleotides) was apparent. Cytochrome *a* showed an oxidation which ensued after a small reduction. After 2 min the steady-state reduction levels were nicotinamide nucleotides, 80% (NAD, 45%; NADP, 95%); flavoprotein, 18%; cytochrome *b*, 14%; cytochrome *c*, 7%, and cytochrome *a*, 0%.

The addition of P_i to depleted mitochondria caused a change of the steady state of flavoproteins and cytochrome *b* in the direction of oxidation and a small reduction of cytochrome *a*. These responses are analogous to those of the state 4–state 3 transition (Chance and Williams, 1956). The absorbancy change at 550 *vs.* 540 mμ revealed, on the other hand, an anomalous response of cytochrome *c*. P_i caused an abrupt increase of absorbancy at 550 mμ *vs.* that at 540 mμ. This change, which indicates cytochrome *c* reduction, went to completion in the mixing time and was followed by a slower change in the opposite direction. The change in the steady state was in the direction of reduction. The response of cytochrome *c* described contrasted with that observed in state 4–state 3 transition. In the latter case ADP caused monophasic oxidation of cytochrome *c* (see Chance and Williams, 1956). The initial response at 465 *vs.* 510 mμ was equally rapid as that at 550 *vs.* 540 mμ. Since cytochromes *b* and *a* show no significant fast response and since the absorbancy changes at 465 and 550 mμ are equally large, it seems reasonable to identify the fast reaction with the flavoprotein–cytochrome *c* couple acting through the antimycin A site and cytochrome c_1.

Effect of Inhibitors on the Response of Cytochrome c to P_i. Figure 2 shows that the P_i-induced transient reduction of cytochrome *c* was insensitive to oligomycin (trace a) and aurovertin (trace b) as well as to the two antibiotics added together (trace c). The antibiotics practically abolished activation of respiration by P_i. The response of cytochrome *c* was abolished by antimycin (trace d). Triple-beam recordings (see Experimental Procedure) showed that the addition of 30 μM P_i to P_i-depleted, glutamate-supplemented mitochondria, gave, in the presence of oligomycin, a marked transient

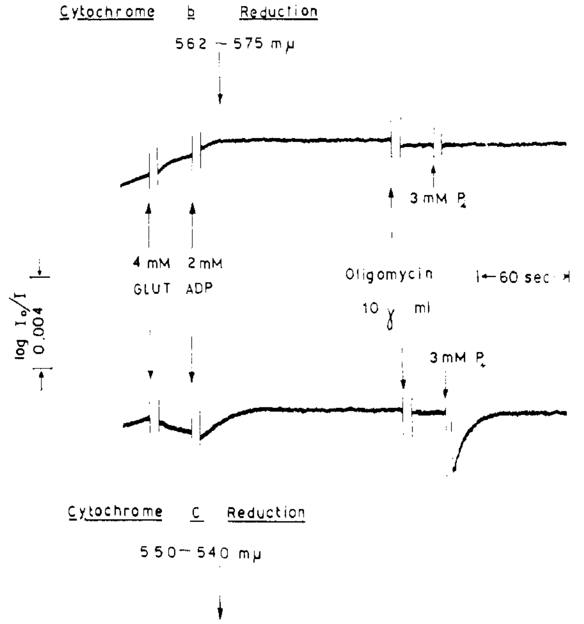

FIGURE 5: Effect of P_i on cytochromes *c* and *b* absorbancy in rat liver mitochondria supplemented with glutamate as respiratory substrate. Mitochondria were incubated in the basic reaction medium. The additions are specified in the figure. Final volume, 3 ml.

increase of cytochrome *c* absorbancy. A titration showed that 30–50 μM P_i gave half-maximal effect on cytochrome *c*. When ascorbate plus TMPD (in the presence of antimycin) was used as respiratory substrate the activation of electron flow by P_i caused an immediate oxidation of cytochrome *c*. No oxidation of cytochrome *c* was observed in the presence of oligomycin with 300 μM TMPD (Figure 3a). However when the availability of electrons to cytochrome *c* was limited by lowering the TMPD concentration (30 μM), the P_i-induced oxidation of cytochrome *c* resulted to be partly insensitive to oligomycin (Figure 3b).

Low-Temperature Spectra. The spectrum characteristics of the anomalous response of cytochrome *c* and the possible response of other cytochromes to P_i (+oligomycin) were examined by low-temperature spectra. The spectrum of Figure 4a represents the difference between P_i-depleted and P_i-supplemented mitochondria. The spectrum clearly shows the appearance of the absorption bands of cytochrome *c* having peaks at 546, 515, and 413 mμ. No other absorption band could be detected. The specificity of the cytochrome *c* response immediately raises the question of whether its absorbancy change does really represent reduction of the cytochrome. In fact it could also be possible that the increase in cytochrome *c* absorbancy is due to some conformational change of the hemoprotein (Chance, 1967). An answer to this was sought by comparing the absorption spectrum of cytochrome *c*, as it appears upon P_i treatment, with that obtained with other reactants. The low-temperature spectrum of Figure 4b represents the difference between P_i-depleted and P_i-depleted succinate-supplemented mitochondria. The addition of succinate caused the appearance of the absorption bands of cytochrome *c* with peaks at 546, 515, and 413 mμ. These peaks have the same position as those obtained with P_i. Succinate caused also reduction of cytochromes *b* and *a*.

Reaction of P_i with Other Respiratory Carriers. The double-beam traces of Figure 5 show that the transient reduction of cytochrome *c*, caused by P_i (+oligomycin), was not accompanied by any detectable change in the redox state of cytochrome *b*. The same is apparent from the difference spectrum of Figure 4. The effect of P_i on the redox state of ubiquinone was also examined. This was done by monitoring with a dual-wavelength spectrophotometer the absorbance changes at 282 *vs.* 295 mμ. Under the condition used no significant effect of P_i (added in the presence of oligomycin to P_i-depleted rat liver mitochondria) on the redox of ubiquinone could be detected.

As noted above the kinetic correlation between the initial response at 465 *vs.* 510 and that at 550 *vs.* 540 mμ (see Figure 1) suggests that flavoproteins may be the reductant of cytochrome *c* in the P_i-induced transition. In the experiment of Figure 6 the redox state of flavoproteins and NAD was monitored fluorometrically. Mitochondria were incubated in the standard reaction mixture with glucose and hexokinase. After reduction of NAD and flavoproteins by α-oxoglutarate, arsenite was added to inhibit the reduction of lipoamide dehydrogenase flavoprotein by this substrate. The oxidation of α-oxoglutarate is in fact promoted by P_i in an oligomycin-insensitive fashion (Tager *et al.*, 1969). It can be seen that arsenite caused marked oxidation of both NAD and flavoproteins. β-Hydroxybutyrate which feeds reducing equivalents directly to NAD, reduced NAD, and flavoproteins. ADP clearly gave oxidation of flavoproteins and NAD. The addition of P_i, in the presence of oligomycin, caused flavoprotein oxidation. This was accompanied by a smaller oxidation of NADH. The addition of tetrachlorotrifluoromethylbenzimidazole (Jones and Watson, 1965) which releases the inhibition by oligomycin, caused a large oxidation of both NADH and flavoproteins.

Potentiometric Recording of the Effect of P_i. In the experiment of Figure 7 a platinum electrode potentiometric measurement of TMPD-supplemented mitochondria was recorded simultaneously with the absorbancy of cytochrome *c*. Caswell and Pressman (1968) have shown that TMPD, added in catalytic concentrations to a mitochondrial suspension gives rapid response at a platinum electrode and equilibrates rapidly with a respiratory chain component. These authors were able to correlate the potential of TMPD with the response of cytochrome *c*. Due to interference of TMPD at the α band of cytochrome *c*, the absorbancy of the latter was followed in the Soret region (420 *vs.* 400 mμ). Glutamate gave an increase of absorbancy at 420 *vs.* 400 mμ and a decrease of the potential of TMPD. ADP caused a significant deflection of the two traces, both in the opposite direction. Oligomycin was then added, followed 1 min later by P_i. This caused an abrupt increase of absorbancy followed by a slower decrease. The absorbancy changes induced by P_i are specifically due to cytochrome *c* (see the difference spectrum of Figure 4). These were accompanied by a decrease of the TMPD potential followed by a slower secondary increase. P_i caused an increase of absorbancy of cytochrome *c* and a decrease of TMPD potential also when other NAD-linked respiratory substrates (β-hydroxybutyrate or α-oxoglutarate) or succinate (plus glutamate) were used. However with succinate the response to P_i was smaller. Also when the KCl medium was replaced by a sucrose–Tris medium P_i gave a similar reduction of cytochrome *c* and decrease of

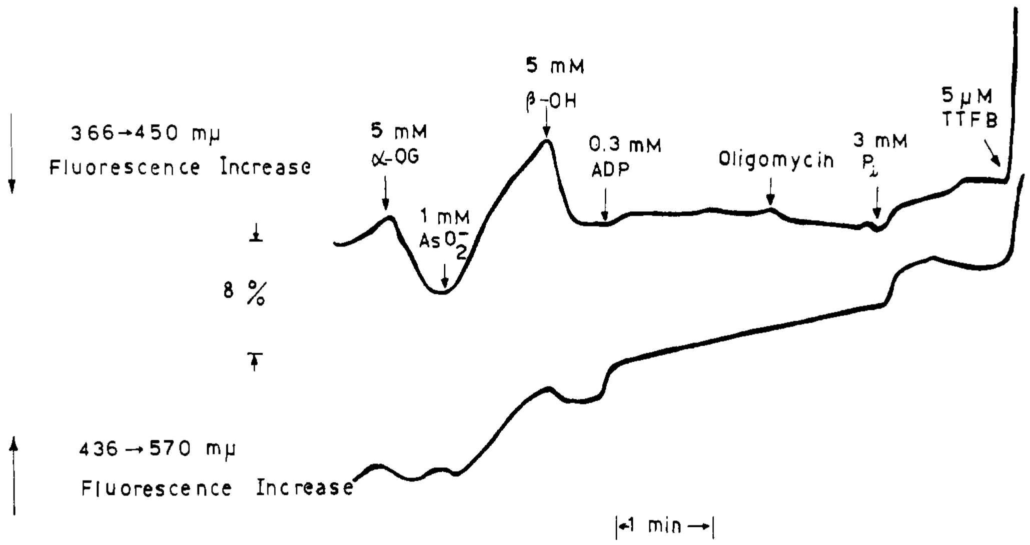

FIGURE 6: Changes of fluorescence of flavoproteins (excitation at 436 mμ, measurement at 570 mμ) and nicotinamide nucleotides (excitation at 336 mμ, measurement at 540 mμ) upon depletion and subsequent addition of P_i in rat liver mitochondria. Mitochondria were incubated in the basic reaction mixture containing, in addition, 20 mM glucose and 15 units of hexokinase. The other additions are specified in the figure. Oligomycin, 10 μg; final volume, 1 ml. For other details, see Experimental Procedure.

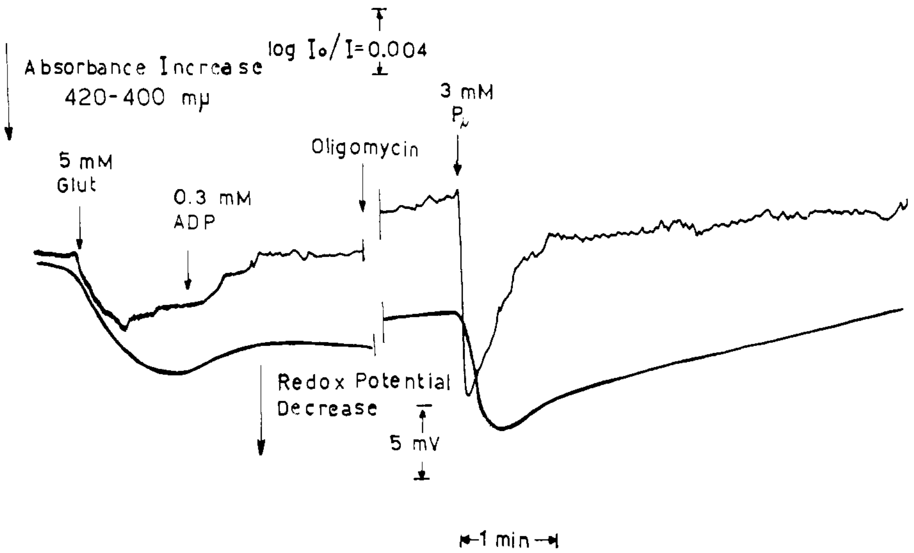

FIGURE 7: The effect of P_i on the absorbancy of cytochrome *c* and the platinum electrode potential in TMPD-supplemented rat liver mitochondria. Respiratory substrate: glutamate. Mitochondria were incubated in the basic reaction medium with the following additional components: 13 μM TMPD, 20 mM glucose, and 15 units of hexokinase. The other additions are specified in the figure. Oligomycin, 30 μg; final volume, 2.5 ml. For other details, see Experimental Procedure. Note that the changes of the redox potential were slower than that of the absorbancy change. The delay is, at least partly due to the slow equilibration of TMPD at the electrode. In fact it was considerably decreased by using an electrode with a larger surface.

TMPD potential; however, in this case, the secondary reoxidation was much smaller.

It was shown, in Figure 2, that when an NAD-linked substrate was used, antimycin A completely abolished the P_i-induced reduction of cytochrome *c*. Figure 8 shows that, with glutamate as substrate, antimycin, which caused oxidation of cytochrome *c* and a large increase of the redox potential of TMPD, did not prevent the P_i-induced decrease of TMPD potential, nor did it prevent, in the presence of TMPD, the P_i-induced reduction of cytochrome *c*. It has been shown (Lee *et al.*, 1965; Packer and Mustafa, 1966) that TMPD can establish a by-pass over the antimycin A sensitive site of the respiratory chain. Thus, in the presence of antimycin, TMPD accepts reducing equivalents from a component in the respiratory chain located before the antimycin-sensitive site and donates them to cytochrome *c*. The changes in the TMPD potential and cytochrome *c* absorbance shown in Figure 8 indicate that P_i activates the flow of reducing equivalents along the TMPD shunt. Further support to this is given below. The recording of flavoprotein absorbance of

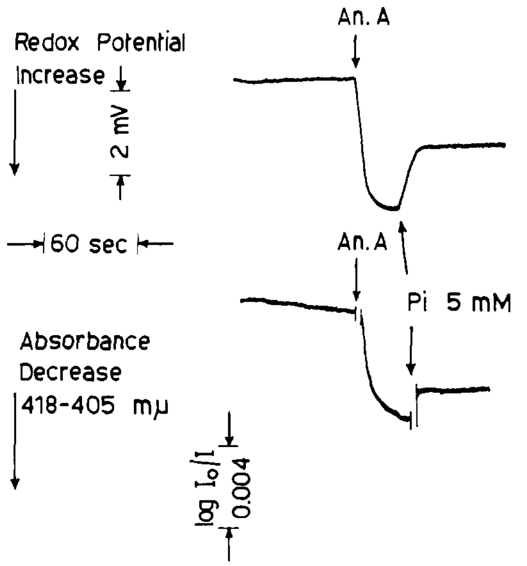

FIGURE 8: The effect of P_i in the presence of antimycin A, on the absorbance of cytochrome *c* and the platinum electrode potential in TMPD-supplemented rat liver mitochondria. Respiratory substrate 5 mM glutamate. Mitochondria were incubated in the basic reaction medium with the following additional components, 10 μM TMPD, 20 mM glucose, 15 units of hexokinase, and 0.2 mM ADP. After 3-min preincubation 30 μg of oligomycin (not shown) and 3 μg of antimycin A were added. Final volume, 3 ml. For other details, see Experimental Procedure.

Figure 9 shows that the addition of P_i to P_i-depleted mitochondria, caused in the presence of TMPD and oligomycin an oxidation of flavoprotein which was not abolished by antimycin A. It appears, therefore, that, at least in the presence of antimycin, TMPD can equilibrate not only with cytochrome *c* but also with component(s) before the antimycin-sensitive site. It is also evident from these experiments that P_i can promote electron flow from flavoprotein not only to cytochrome *c* but also to an artificial acceptor.

Specificity of the P_i Effect. Figure 10 shows that arsenate was able to replace P_i in promoting the transient reduction of cytochrome *c* when added in the presence or absence of oligomycin to P_i-depleted mitochondria. Acetate, on the other hand, was unable to do so.

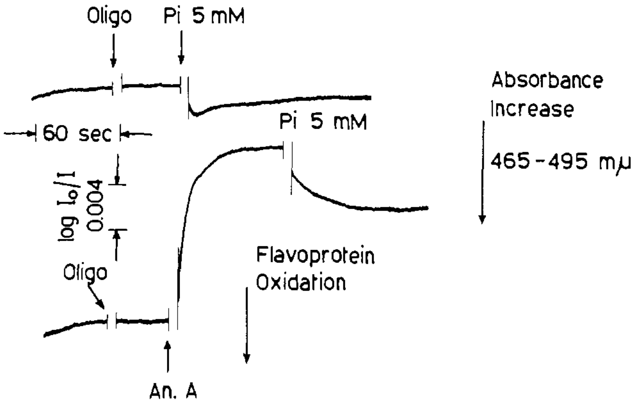

FIGURE 9: The effect of P_i on the absorbance of flavoprotein in TMPD-supplemented rat liver mitochondria. The experimental conditions were those described in the legend to Figure 8.

TABLE I: P_i Uptake by Rat Liver Mitochondria and Reduction of Cytochrome *c*.[a]

Additions	$[^{32}P]P_i$ in the Matrix (nmoles)	P_i-Induced Cytochrome *c* Reduction[b]
None	255	18×10^{-3}
DNP, 100 μM	108	0
Mersalyl, 170 μM	16	0
Mersalyl plus cysteine, 1 mM	257	18×10^{-3}

[a] Mitochondria (6.8 mg/ml) were preincubated 3 min in the standard reaction mixture containing in addition: 5 mM β-hydroxybutyrate, 0.2 mM ADP, 20 mM glucose, and 15 units of hexokinase. Oligomycin (10 μg) was then added, followed 60 sec later by 3 mM P_i. The other additions were made 30 sec before that of P_i. The uptake of $[^{32}P]P_i$ and the reduction of cytochrome *c* were followed in separate samples. The final volume for the uptake determination was 1 ml, that for the absorbance recordings, 3 ml. [b] Absorbance increase at 550 *vs.* 540 mμ log I_0/I.

Correlation of the Reaction of P_i with the Respiratory Chain and Its Accumulation by Mitochondria. The experiment of Table I shows that P_i, added in the presence of oligomycin, to P_i-depleted mitochondria, was actively accumulated by mitochondria. The uptake of P_i was severely inhibited by dinitrophenol. Mersalyl blocked almost completely the penetration of P_i, into the mitochondria (see Tyler, 1969; Meijer and Tager, 1969). Both dinitrophenol and mersalyl suppressed the P_i-induced reduction of cytochrome *c*. Cysteine prevented completely the inhibitory effect of mersalyl on both P_i penetration (see also Tyler, 1969, and Fonyo and Bessmann, 1968) and cytochrome *c* reduction. DNP and mersalyl also abolished the P_i-induced decrease of TMPD potential. It should be noted that mersalyl at the concentrations used (20–25 nmoles/mg of protein) abolishes completely the activation of respiration brought about by P_i addition in the presence of ADP and absence of oligomycin, but has only a small effect on the uncoupler-stimulated respiration. This indicates that the effect of mersalyl is, at these concentrations, largely confined to the phosphate penetration (Tyler, 1969). Accumulation of P_i by oligomycin-treated mitochondria occurs also when ascorbate (plus TMPD and antimycin) is used as respiratory substrate (Papa *et al.*, 1969).

Discussion

Depletion of endogenous P_i by incubation with respiratory substrates and a ~P trap, brings the respiratory chain of mitochondria into an inhibited state that is characterized by defined steady-state redox levels of the respiratory carriers. This state differs from state 4 (the state which is induced by depleting ADP (Chance and Williams, 1956), as well as from

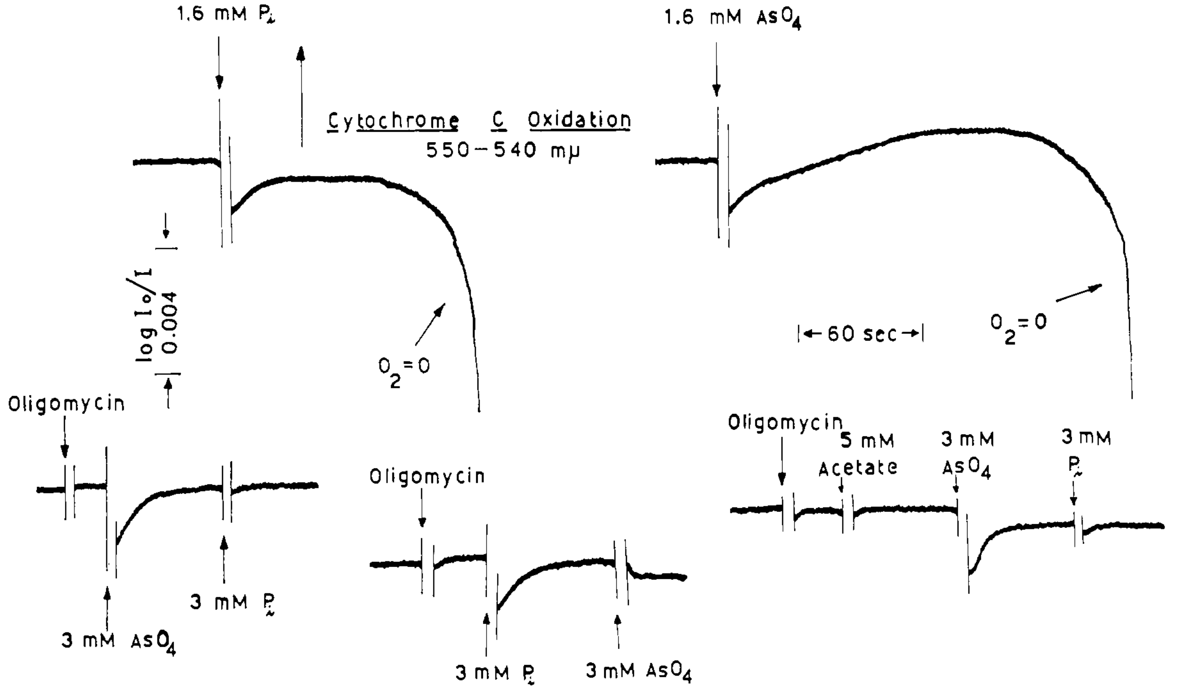

FIGURE 10: Effect of anions on the absorbancy of cytochrome *c* in rat liver mitochondria. Mitochondria were incubated in the basic reaction medium. After 2-min preincubation with 5 mM glutamate and 2 mM ADP the additions specified in the Figure were made. Oligomycin, 30 μg; final volume, 3 ml.

state 6 (that induced by depleting P$_i$ with low concentration of divalent cations (Chance and Schöener, 1966a)). The transition to state 6 shows oxidation of NADH and a considerable reduction of cytochrome *b* as revealed by a large increase of absorbancy at 562 mμ. The depletion of P$_i$ with a $\sim$P trap is accompanied by reduction of NAD and does not show any significant reduction of cytochrome *b* at 562 mμ. The reactivation of electron flow by addition of P$_i$ to mitochondria, depleted with the $\sim$P trap and glutamate, causes responses of NADH, flavoproteins, cytochrome *b*, and cytochrome *a* (Figure 1) which are qualitatively analogous to those of state 4–state 3 transition (Chance and Williams, 1956). On the other hand, cytochrome *c* shows an anomalous response characterized by an abrupt increase of absorbancy followed by a slower decrease. This response of cytochrome *c* is antimycin sensitive. On the contrary it is insensitive to high concentrations of oligomycin and of aurovertin as well as to the combined addition of the two antibiotics. At the concentrations used here these antibiotics inhibit completely the [^{32}P]P$_i$–ATP exchange and the exchange of ^{18}O between P$_i$ and H$_2$O (Lardy *et al.* 1964). Furthermore the inhibitory effects of the two antibiotics on the exchange reactions are additive (Lardy *et al.*, 1964). The apparent K_m for P$_i$ in the oligomycin insensitive reduction of cytochrome *c* is about 30 μM. This is one order of magnitude smaller that the K_m for the activation of respiration by P$_i$ (250 μM). It appears therefore that when electron flow through the respiratory chain is activated by addition of P$_i$ to P$_i$-depleted mitochondria ($\sim$P trap present) the responses of the respiratory carriers are the expression of two separate but superimposed reactions: (i) activation of oxidative phosphorylation by $\sim$P formation, a reaction, which is inhibited by oligomycin (Ernster and Lee, 1964; Ernster *et al.*, 1967; Ter Welle and Slater, 1967); (ii) an oligomycin-insensitive reaction of P$_i$ with the respiratory chain, revealed by the anomalous response of cytochrome *c*. Low-temperature difference spectra showed that the absorption spectrum of cytochrome *c*, that appears on addition of P$_i$ (+oligomycin) to P$_i$-depleted mitochondria, is identical with that appearing upon reduction of cytochrome *c* by succinate. Thus it seems unlikely that P$_i$ is a ligand of cytochrome *c* heme. In the presence of oligomycin cytochrome *b* does not respond to the addition of P$_i$ (Figures 4a and 5). Since flavoprotein does not show the sharpening and enhancement phenomena of the cytochromes, it does not show in the low-temperature spectrum as a trough at 460 mμ. It is noteworthy however that cytochrome *c*$_1$ does not show clearly in this spectrum at 552–553 mμ and further investigation on this point are required. The fast kinetics of the absorbancy changes caused by P$_i$ addition at 465 *vs.* 510 mμ (flavoprotein oxidation) may be associated with the initial absorbancy change at 550 *vs.* 540 mμ (cytochrome *c* reduction) (Figure 1). Also in the presence of oligomycin P$_i$ causes oxidation of flavoproteins. This suggests a close association between the fast oxidation of flavoprotein and the fast reduction of cytochrome *c*. That P$_i$ activates electron flow in the flavoprotein cytochrome *c* region of the respiratory chain is also evident from the potentiometric recording of TMPD-supplemented mitochondria. These recordings show that the oxidation of flavoprotein and the reduction of cytochrome *c* induced by P$_i$, were accompanied by a decrease of the TMPD potential (TMPD reduction). The fact that also in the presence of antimycin P$_i$ activated electron flow from flavoprotein to TMPD and cytochrome *c* (Figures 8 and 9) indicates that P$_i$ acts primarily by promoting oxidation of flavoproteins. When ascorbate (plus TMPD) was used as respiratory substrate P_i caused cytochrome *c* oxidation.

There appear to be two extremely rapid reactions of the respiratory chain components with anions and cations; the reaction described here, where phosphate causes oxidation of flavoproteins and reduction of cytochrome *c*, and that described elsewhere (Chance, 1965; Chance and Schöener, 1966a) in which calcium caused an abrupt oxidation of cytochrome *b*, and a concomitant reduction of cytochrome *c*. The reaction of P_i was in this investigation routinely studied in the presence of EGTA and $MgCl_2$. It was also insensitive to an excess of EGTA added alone. This seems to rule out the possibility that the effects of P_i are due to endogenous Ca^{2+}. Also the failure of a response of cytochrome *b* in the phosphate reaction does not allow a unified explanation for this and the Ca^{2+} reaction.

From the energetic point of view the data presented show that the addition of P_i to P_i-depleted mitochondria, under conditions of inhibition of oxidative phosphorylation effects the energy pressure established by the oxidoreductions of the components of the respiratory chain (see also Hansford and Chappell, 1968). With NAD-linked substrates and succinate, and under the prevailing conditions, this effect of P_i manifests itself at the level of the second coupling region of the respiratory chain. The effect of P_i is more pronounced with NAD-linked substrates rather than with succinate. It is possible that this is due to the different redox pressure of the respiratory carriers established by NAD-linked substrates and succinate. With ascorbate (plus TMPD and antimycin) as respiratory substrate, the addition of P_i induced oxidation of cytochrome *c*. This indicates an energy load imposed by P_i on the third coupling site in the cytochrome *c* oxygen span of the respiratory chain. At high TMPD concentrations (300 μM) no oxidation of cytochrome *c* upon P_i addition was observed when oxidative phosphorylation was blocked by oligomycin. However when the availability of electrons to cytochrome *c* was made limiting by lowering the TMPD concentration an oligomycin-insensitive P_i-induced oxidation of cytochrome *c* could be observed. Thus, although the oxidation of cytochrome *c* induced by P_i, in the presence of ascorbate (plus TMPD), appears to be preeminently due to ATP synthesis, it is also evident that P_i can effect also the third coupling site of the chain by an oligomycin-insensitive reaction.

The data of Table I show a correlation between the P_i-induced reduction of cytochrome *c* and the accumulation of this anion by mitochondria. As a possible mechanism for the oligomycin-insensitive reaction of P_i with the respiratory chain it is proposed that the redox transitions of the respiratory chain are the consequence of the transport of P_i from the extra to the intramitochondrial space. As reported in the introduction Papa *et al.* (1969) have produced evidence that the translocation of P_i across the mitochondrial membrane can be coupled to an OH^- counterflux (or H^+ symport). It is known that in intact mitochondrial electron transfer along the respiratory chain can be accompanied by proton ejection in the surrounding medium. Various mechanisms have been proposed to explain this proton translocation (Mitchell, 1966; Chappell and Crofts, 1966; Chance and Mela, 1966a; Van Dam and Kraaijenhoff, 1969; Williams, 1970). The translocation of P_i across the mitochondrial membrane and the flow of electrons along the respiratory chain could be coupled through flux of protons in the mitochondrial membrane. Through this coupling the uptake of P_i by mitochondria could impose an energy load on the oxidoreductions of the respiratory chain. Extensive accumulation of P_i would require a concomitant uptake of cations. In the absence of the latter, P_i induces only a transient energy load on the redox pressure of the respiratory chain components without any significant effect on the overall electron flow.

Evidence has been reported that the addition of P_i, arsenate, or acetate to P_i-depleted mitochondria causes mitochondrial acidification (Chance and Mela, 1966b,c; Papa *et al.*, 1969). P_i and arsenate but not acetate, even at a concentration of 10 mM, induce cytochrome *c* reduction. This indicates that the effect of P_i on electron flow cannot be simply explained by mitochondrial acidification. According to Chappell and Haarhoff (1967) a specific carrier mediates the translocation of P_i and arsenate across the mitochondrial membrane, but not that of acetate. Evidently electron flow does respond to ion flux only when this is mediated by specific systems.

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