ENDOGENOUS ADP OF MITOCHONDRIA, AN EARLY PHOSPHATE ACCEPTOR OF OXIDATIVE PHOSPHORYLATION AS DISCLOSED BY KINETIC STUDIES WITH C^{14} LABELLED ADP AND ATP AND WITH ATRACTYLOSIDE. [x)]

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Intact mitochondria have been shown to contain endogenous adenine nucleotides (Slater and Holton 1953, Siekevitz and Potter 1955, Pressman 1958, Klingenberg 1961, Heldt 1962). These seemed to be bound in a highly specific manner (Pfaff and Klingenberg 1964). There have been various studies on the function of the endogenous adenine nucleotides, however, no conclusive answer concerning their role in phosphate transfer was given.

Recently the role of endogenous guanine nucleotides in substrate-level phosphorylation was established using an ultramicro scale chromatography (Schnitger et al. 1959, Heldt 1963) and rate studies of P^{32} incorporation (Heldt et al. 1964). In the present communication it is attempted to differentiate between the phosphorylation rate of the endogenous and the exogenous adenine nucleotides. Thus the phosphorylation sequence which ultimately gives exogenous ATP, is determined. On this basis the point of action of atractyloside, an inhibitor of oxidative phosphorylation, (Vignais and Vignais 1962, Bruni et al. 1962) could be shown as interfering only with the phosphorylation of the exogenous adenine nucleotides. These results are inter-

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x) These data have been reported at the Sixth International Congress of Biochemistry, New York City 1964, in the Symposium on "Metabolism and its Control".

preted together with a specific translocation between the endogenous and exogenous adenine nucleotides as reported separately (Pfaff and Klingenberg 1964).

Results.

The incorporation of P^{32} into endogenous adenine nucleotides (curve I) is compared with the P^{32} incorporation into adenine nucleotides in the presence of added ADP (curve II). Controls by ion exchange chromatography established that the initial rise in curve I reflects a net formation of endogenous ATP. The following slow rise mainly reflects the incorporation of P^{32} into ADP and into the ß-position of ATP due to adenylate kinase activity. The initial rate of phosphorylation is not markedly increased in the presence of added ADP (curve II) and therefore can be attributed to endogenous ADP. The subsequent phosphorylation of the exogenous ADP proceeds after a short interval. This curve also reveals that the mitochondria were complete in respect to the amount of their bound adenine nucleotides because the amount of rapidly re - acting endogenous ADP is not increased by preincubation with added ADP.

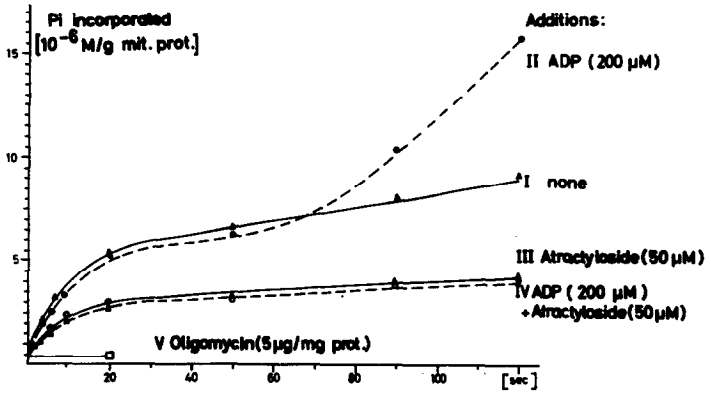

Fig. 1: Rat liver mitochondria (70 mg prot.) were kept anaerobic for 10 min. at 6° in a medium containing 0,3 M sucrose, 30 mM TRAP, pH 7,2, 2 mM EDTA, 8 mM succinate and additions as indicated. Total volume 10 ml. 1 min. after the addition of P^{32} orthophosphate (0,5 mM) phosphorylation was started by oxygen addition. Samples were taken, deproteinized by $HClO_4$ and analyzed for organic phosphate by the Nielsen-Lehninger method (1955).

Atractyloside has a small effect on the rate and extent of the phosphorylation of endogenous ADP (curve III), while it excludes the phosphorylation of exogenous ADP completely (curve IV). This marks the contrast to the action of oligomycin (curve V) which inhibits the oxidative phosphorylation of both the endogenous ADP (Slater et al. 1964, Heldt et al. 1964) and of the exogenous ADP (Lardy et al. 1958).

The experiment shown in fig. 2 is designed to decide whether endogenous ADP is phosphorylated prior to, simultaneously with, or after exogenous ADP. For this purpose C^{14} labelled ADP was added exactly at the same time as the phosphorylation was started with oxygen. If endogenous ADP, which is unlabelled, is phosphorylated prior to the added labelled ADP, the specific activity of the ATP formed should be

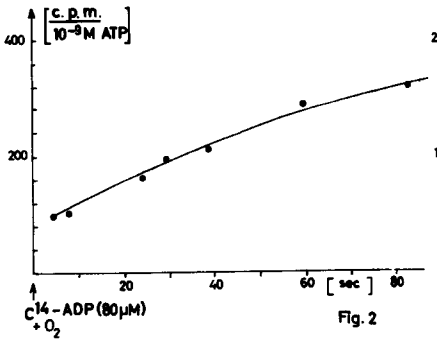

Fig. 2

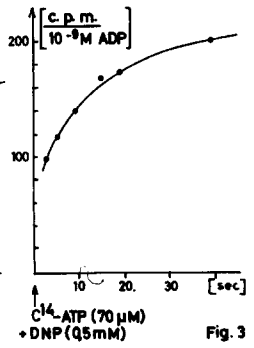

Fig. 3

Fig. 2: Rat liver mitochondria (108 mg prot.) in a final volume of 20 ml were incubated according to fig. 1. 1,5 uC C^{14}-ADP (final concentration 80 µM) was added together with oxygen. The adenine nucleotides in the resulting extracts were separated and quantitatively measured by ion exchange chromatography. The C^{14} activity of the separated fractions was measured by a liquid scintillation counter. The specific C^{14} activity was referred to the amount of ATP formed, i.e. ATP measured at the time, minus ATP measured before the start with oxygen.

Fig. 3: Rat liver mitochondria (140 mg prot.) in a final volume of 10 ml were incubated aerobically at 18°. At the time indicated 0,5 mM dinitrophenol and 0,25 µC C^{14} ATP (final concentration 70 µM) were added. For methods cf. legend of fig. 2.

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low at the beginning and rise until it reaches equilibrium and vice versa. If the exchange between exo-

genous and endogenous ADP is much faster than phosphorylation, the specific activity of the ATP formed should be constant from the beginning in this case the experiment would not yield any decision.

The result of our experiment clearly shows a rise of the specific activity of ATP which means that endogenous ADP is phosphorylated prior to added ADP.

The same sequence can be shown for a reverse action of the ADP-kinase, the dinitrophenol stimulated hydrolysis of added ATP (fig. 3). The specific C^{14} activity of the ADP formed rises after the addition of a mixture of dinitrophenol and C^{14} ATP. This demonstrates that endogenous ADP is split prior to exogenous ATP.

Discussion.

The demonstration that the endogenous ADP is phosphorylated prior to the exogenous ADP amplifies the question as to the mechanism of how the ultimate formation of exogenous ATP is achieved. There may be, for example, an often suggested transphosphorylation step or an exchange of the total molecules. An exchange between endogenous and exogenous adenine nucleotides of mitochondria - apart from any phosphorylation reaction - could be demonstrated recently (Pfaff and Klingenberg 1964). Furthermore, this exchange was inhibited by atractyloside.

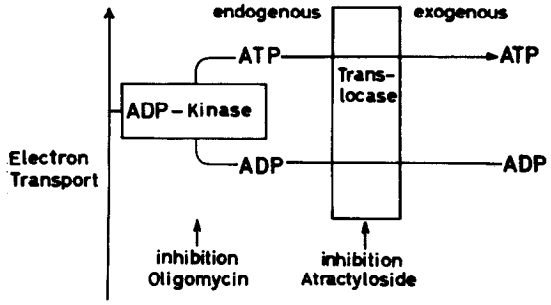

Fig 4

The combined results lead to a reaction sequence for the overall phosphorylation of exogenous adenine nucleotides as shown in figure 4. The phosphorylation of ADP takes place in an inner compartment of the mitochondria, which probably is the "matrix" space. The endogenous adenine nucleotides are bound there, possibly by the structural protein (Hultin and Richard-

son 1964). In the first reaction bound ADP becomes phosphorylated by the ADP-kinase of the respiratory chain. In the second reaction, the binding place of the formed ATP is taken by exogenous ADP. This exchange may be due to a specific protein, which may tentatively be called a "translocase".

Bruni et al. (1964a, 1964b) have shown that atractyloside inhibits the "binding" of ADP in mitochondria and suggested, that it acts at the site of the phosphorylation of ADP. Recently, however, atractyloside was found to inhibit the specific translocating exchange between the exogenous and endogenous adenine nucleotides (Klingenberg and Pfaff 1964). The present findings that atractyloside only inhibits phosphorylation of the exogenous ADP are consistent with its inhibiting action on the exchange of the adenine nucleotides. This is in contrast to the assumption that atractyloside acts on the ADP-phosphorylating enzymes. It has been shown independently by Kemp and Slater (pers. comm.) and Chappel and Crofts (pers. comm.) that atractyloside does not inhibit P^{32} incorporation into endogenous ATP.

Thus atractyloside is a valuable tool in studying the aspects of compartmentation in the phosphate transfer. Evidence for the exchange reaction and phosphate transfer sequence is obtained by kinetic analysis alone. However, the finding of an inhibitor greatly substantiates these conclusions. It supports the hypothesis that the exchange step is catalyzed by a specific "translocase".

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