EVIDENCE FOR THE PARTICIPATION OF ENDOGENOUS GUANOSINE TRIPHOSPHATE IN SUBSTRATE LEVEL PHOSPHATE TRANSFER IN INTACT MITOCHONDRIA.

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Guanosine and inosine diphosphate (GDP and IDP) have been shown to be possible phosphate acceptors in the succinic thiokinase reaction in mammalian kidney and heart, (Mazumder, Sanadi and Rodwell 1960):

Succinyl CoA + XDP → Succinat + XTP + CoA (1)

X = inosine or guanosine

The nucleotide specifity of reaction (1) has been studied only in isolated enzyme preparations. The difference of K_m between guanine and inosine nucleotides was shown to be small. Thus the question as to which nucleotide system,(the inosine or the guanine system) is the physiological participant in the phosphate transfer reactions (1) remained open.

In order to answer this question, the role of guanine and inosine nucleotides in intact liver mitochondria was studied by following the incorporation of P^{32} into phosphate compounds of mitochondria. The basis of the experiments was the ultramicro scale ion exchange chromatography for separation of phosphate compounds (Schnitger et al. 1959, Heldt, 1963). This was combined with simultaneous chart recording of ultraviolet absorption, analysis of total phosphorous content and radioactivity. Exact data on radioactivity were obtained by digital recording. The P^{32} labelled compounds were identified by comparing the chromatographic behaviour of the collected fractions with added components on thin layer radio electrophoresis and

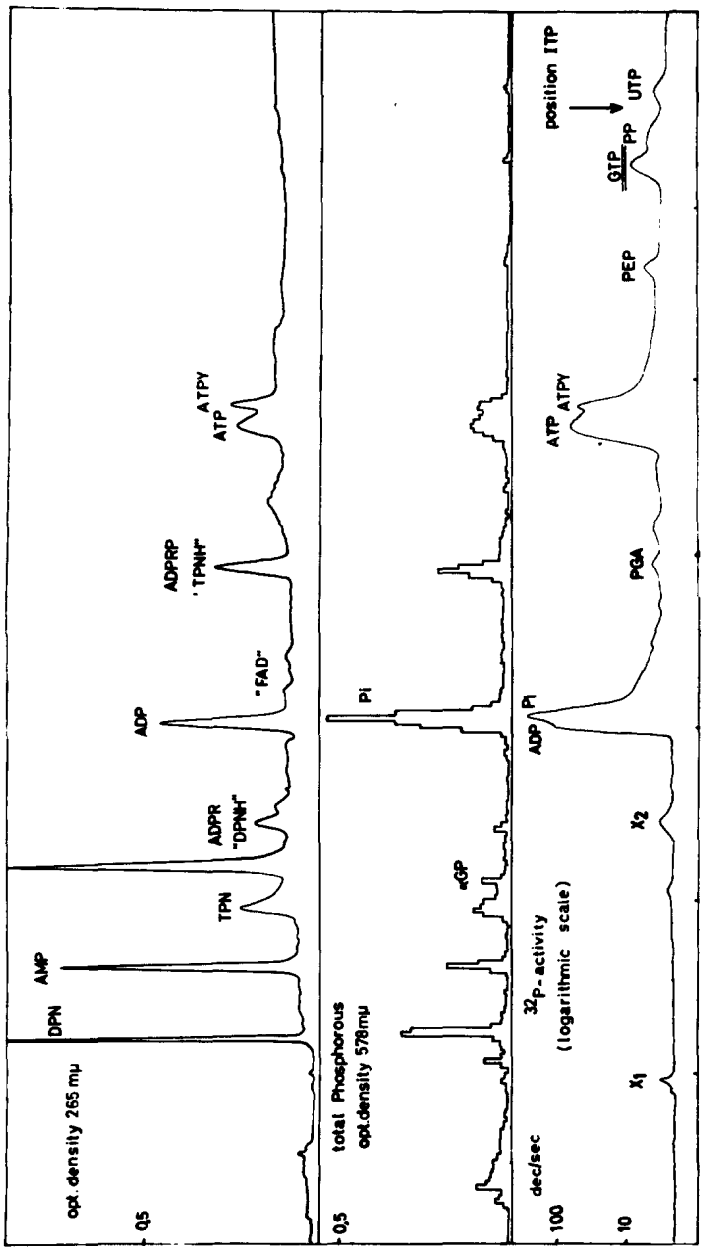
thin layer radio chromatography in various systems (for details see Heldt, Jacobs and Klingenberg 1964).

Results.

1. Substrate level phosphorylation was studied by aerobic incubation of mitochondria with ketoglutarate and P^{32}-orthophosphate. Dinitrophenol (DNP) and oligomycin were added to eliminate respiratory chain phosphorylation. Fig. 1 shows the ion exchange chromatogram of the acid soluble extract. There is P^{32} incorporation into ADP, ATP, inorganic pyrophosphate (P-P), GTP, uridine triphosphate (UTP), phosphoenolpyruvate (PEP), phosphoglyceric acid (PGA), and the unidentified compounds X_1 and X_2. ATPY represents a different form of ATP, most probably having been formed artificially during chromatography. No radioactive peak is found at the position were ITP would appear in our chromatographic system. The absence of ITP in liver mitochondria was confirmed in a number of similar experiments under various conditions of incubation.

2. Rat liver mitochondria were incubated for 10 sec. Table 1 shows the amount of Pi uptake (measured as Pi^{32}-incorporation) of ATP and GTP under the influence of various inhibitors. Without any uncouplers or inhibitors being added, nearly all bound P^{32} is found as endogenous ATP^{32}, and only a minor fraction as endogenous GTP^{32}. This reflects the small guanine nucleotide content of mitochondria, which amounts to about 3% of the adenine nucleotide content. Further only about 20% of the total guanine nucleotides are rapidly phosphorylated in connection with substrate level phosphate transfer. (Heldt, unpubl.). Thus only this portion is labelled during the short time of incubation employed in the experiments described here. The addition of DNP resulted in 99% inhibition of ATP labelling, but inhibited P^{32}-incorporation into GTP only by one third. If DNP was added together with oligomycin, GTP-labelling was even higher than in the control experiment, while ATP labelling showed 95% inhibition. It has been shown by Chappel

Fig. 1:
Ion exchange chromatogram from rat liver mitochondria



Rat liver mitochondria (7,0mg prot.) were incubated aerobically 4 min. at 18° in the presence of DNP(0,05mM),oligomycin(50μg),ketoglutarate(7,5mM),P^{32}phosphate (0,5mM),sucrose(0,25M),EDTA(1mM),triethanolamine-HCl pH 7,2(30mM)in a total volume of 2 ml.The mitochondria were separated from the suspension medium by silicon layer filtering centrifugation and deproteinized by $HClO_4$(Pfaff,unpubl.).The acid soluble extract was analysed on Dowex 1x8(1mm x 200cm).A continuous linear gradient was achieved by a special pumping device(Schnitger,unpubl.),the mixing vessel contained 22ml H_2O and the reservoir 9M HCOOH + 0,9M $HCOONH_4$.Flow rate: 0,55ml/h.

Table I

Effect of DNP, oligomycin, arsenite and arsenate on phosphorylation of mitochondrial GTP.

Rat liver mitochondria were incubated in a medium according to the first experiment, but omitting DNP and oligomycin. The anaerobic mitochondria were added to the medium which had been made oxigen free by washing it with nitrogen. After 20 sec., phosphorylation was begun by bubbling oxigen through the mixture, after another 10 sec. perchloric acid was added. Additions were made as indicated, in the case of oligomycin the mitochondria were preincubated with it for 4 min. before addition to the medium.

Expt No	Additions	Pi-uptake (10^{-9} Mol/g prot.)	
		ATP	GTP
I	control	1910	15
	DNP (0,1 mM)	26	10
	DNP (0,1 mM) + oligomycin (7μg/mg prot.)	96	19
	DNP (0,1 mM)+oligomycin(7μg/mg prot.)+arsenite (1 mM)	42	5
	oligomycin (7μg/mg prot.)	85	20
II	control	3100	59
	arsenate (5 mM)	650	2

and Greville (1961) that substrate level phosphorylation can be maintained to some extent in the presence of oligomycin + DNP. Our results agree with these findings. Oligomycin alone had a similar effect;again activity of P^{32}-GTP was higher than in the control experiment and P^{32}-incorporation into ATP was very low. It may be also noted, that this experiment shows the very strong inhibition of respiratory chain linked phosphorylation of endogenous adenine nucleotides by oligomycin. Arsenite, which inhibits ketoglutarate oxidation, lowered GTP labelling considerably. Arsenate was found to be a very potent inhibitor of GTP-formation, however it did not lower ATP-formation to the same extent. This finding suggests that arsenate has a stronger effect on substrate level phosphate transfer than on oxidative phosphorylation.

Discussion

Substrate level phosphate transfer in mitochondria has been studied so far only on the basis of the secondary ATP formation. As shown in this paper, it is possible to study this phosphorylation on the primary step by following the prior phosphorylated product GTP. By the use of P^{32}-labelling it was possible to detect in mitochondria traces of endogenous GTP which otherwise would have been too small for direct measurement. Applying this sensitive method of detection, a participation of ITP in the metabolism of rat liver mitochondria was not observed. This result contradicts an earlier report by Siekevitz a. Potter (1955) about rat liver mitochondria showing P^{32}-incorporation into endogenous ITP. GTP labelling on the other hand showed all the characteristics (noninhibition by oligomycin and DNP and inhibition by arsenite and arsenate) one would expect from an intermediate of substrate level phosphate transfer. The results described in this paper allow the conclusion that GTP alone is the product of the succinic thiokinase reaction in rat liver mitochondria.

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