

## The Incorporation of $^{32}\text{P}$ into Mitochondrial Thiamine Diphosphate\* in the Presence of Different Substrates and Inhibitors

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The incorporation of radioactive phosphate into intramitochondrial TDP has been studied in a series of investigations<sup>1-4</sup>. The incorporation was found to depend on the simultaneous metabolism of pyruvate or succinate, whereas  $\alpha$ -ketoglutarate was less effective<sup>4</sup>. These results are partly in agreement with those of Bartley<sup>5</sup>, who, when incubating a kidney cyclophorase enzyme complex with synthetic TDP and  $^{32}\text{P}_i$  found a somewhat higher incorporation in the presence of pyruvate and succinate than when using  $\alpha$ -ketoglutarate. However, in Bartley's experiments the incorporation was slow compared with that observed by me, since added thiamine phosphates do not take part in mitochondrial phosphate metabolism to the same degree<sup>3</sup> as intramitochondrial TDP. That the incorporation of  $^{32}\text{P}$  into TDP was higher with succinate than with  $\alpha$ -ketoglutarate was surprising, for since the investigations of Auhagen<sup>6</sup> and Lohmann and Schuster<sup>7</sup>, TDP has been known especially to function as a coenzyme in the decarboxylation of  $\alpha$ -ketoacids. However the incorporation with succinate as a substrate might depend on succinate being transformed into pyruvate in the mitochondria and, when the pyruvate thus formed was oxidatively decarboxylated, an exchange of the phosphate in TDP might take place. If this were the case fumarate, which is primarily formed when succinate gives rise to pyruvate, should have the same effect as succinate on the incorporation into TDP. Results from an experiment aiming to answer this question are seen in Table 1. The experimental condi-

tions were the same as in the previous paper<sup>3</sup> except that the concentration of phosphate buffer present in the mitochondrial suspension was twice that of the earlier amount (3 ml 0.5 M, pH 7.4, instead of 1.5 ml) and pyruvate and  $\alpha$ -ketoglutarate were replaced by succinate or fumarate. From the table it is seen that when fumarate is metabolized the incorporation of  $^{32}\text{P}$  into intramitochondrial TDP is slower than when succinate is present, the incorporation being of the same extent as that during incubation without added substrate. Thus the incorporation into TDP with succinate as a substrate does not depend on succinate being transformed into pyruvate.

Amytal (5-ethyl-5-isoamylbarbiturate), in a concentration of 1.8 mM has been shown by Ernster *et al.*<sup>8</sup> to inhibit completely hydrogen transfer from mitochondrial dehydrogenases to oxygen in DPN-linked oxidation systems, while at the same time having no effect on the aerobic oxidation of succinate, or on the phosphorylations coupled to it. This is explained as depending on the action of amytal on the electron transport pathway between FAD and Slater's factor at a site not present in the succinate oxidation pathway.

Incubating mitochondria in the same way as in the experiments described in Table 1 with succinate and amytal gave the same or only slightly lower incorporation of  $^{32}\text{P}$  into TDP than when amytal was omitted (Table 2). The oxidation of fumarate was completely inhibited with the same concentration of amytal. This provides further evidence that the incorporation into TDP is not caused only by oxidative decarboxylation of the pyruvate eventually formed from succinate.

Succinic dehydrogenase has been shown not to contain TDP. Up to now the only known reaction including succinate which requires the presence of TDP is the decarboxylation of succinate to propionate found by Whitely<sup>9</sup> to occur in certain bacteria, and the carboxylation of propionate recently shown by Lardy and Adler<sup>10</sup> to occur in liver mitochondria. However, the reverse reaction of the propionate carboxylation they found to proceed slowly, at least in extracts of mitochondria, making this reaction less probable as a possible explanation of the results above.

Sodium fluoride (final concentration 0.02 M), when used in my experiments, inhibited the incorporation into TDP completely and reduced the oxygen consumption to

\* Abbreviations: TDP, thiamine diphosphate; ADP, adenosine diphosphate; DPN, diphosphopyridine nucleotide; DNP, 2,4-dinitrophenol; FAD, flavin adenine dinucleotide;  $^{32}\text{P}_i$ , radioactive inorganic phosphate; P/O = the ratio of  $\mu$ moles esterified phosphate to  $\mu$ atoms consumed oxygen.

*Table 1.* Incorporation of radioactive phosphate into intramitochondrial TDP in the presence of succinate or fumarate. Experimental conditions see text. Volume of sample 14.22 ml. Two ml of this volume were transferred to Warburg vessels for determination of the  $O_2$ -consumption. Incubation temp. 30°C. Incubation time 25 min. No synthetic TDP was present during incubation. In order to facilitate the isolation of the intramitochondrial TDP three mg were, however, added after the reaction had been stopped with TCA. The radioactivity of intramitochondrial TDP was estimated according to values given in an earlier paper<sup>3</sup>.

Substrate	TDP counts/ $\mu$ g hydrolyzable P	Oxygen consumption $mm^3/25$ min/ $2$ ml sample	P/O
Succinate	$1.45 \times 10^4$	151	2.45
Fumarate	$0.66 \times 10^4$	145	2.28
None	$0.72 \times 10^4$	40	1.86

*Table 2.* The effect of amytal on the incorporation of radioactive phosphate into intramitochondrial TDP with succinate as a substrate. Experimental conditions see Table 1. Incubation time 20 min. When amytal is present its final concentration is 1.8 mM.

Substrate	Inhibitor	TDP counts/ $\mu$ g hydrolyzable P	Oxygen consumption $mm^3/20$ min/ $2$ ml sample	P/O
Succinate	None	$4.6 \times 10^4$	133	2.3
Succinate	Amytal	$4.4 \times 10^4$	135	2.1
None	None	$2.1 \times 10^4$	48	1.9

20 %. DNP (final concentration  $10^{-4}$  M) had no effect on the incorporation. The substrate was in both cases pyruvate.

Ochoa<sup>11</sup> when investigating the enzymic synthesis of TDP in animal tissues found that NaF in a concentration of 0.04 M only slightly affected the synthesis. This may be taken as additional evidence, aside from that given earlier<sup>2</sup>, that the incorporation of  $^{32}P$  into TDP, reported above, is not only the result of a mere synthesis of TDP from thiamine present in the mitochondria.

The results with DNP are in agreement with those of Ogata *et al.*<sup>12</sup> who found no inhibition with DNP of the transfer of  $^{32}P$  from labelled TDP to ADP in a liver cyclophorase system.

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