INHIBITION OF SUCCINATE OXIDATION BY DPN AND DPN-ANALOGUES IN SUB-MITOCHONDRIAL SYSTEMS.*)

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While studying the effects of DPN-analogues on mitochondrial respiration and oxidative phosphorylation, we found a marked reduction of oxygen uptake coupled to the oxidation of succinate besides effects on DPN-dependent oxido-reduction reactions. Similar results describing an effect of DPN on succinate oxidation have been obtained previously by Griffiths (1962). These observations prompted a study of the effect of a variety of DPN-analogues on the succin-oxidase of different mitochondrial systems. This communication describes the inhibition of succinate oxidation in preparations of mitochondrial fragments.

The results so far obtained indicate that the extent of the inhibition depends critically on the state of the mitochondrial system and is most pronounced in an " uncoupled " preparation.

Preparation and Methods

The majority of the experiments has been performed with mitochondrial membrane preparations in which the phosphorylative capacity had been depressed by ultrasonic treatment. Sonic disruption of mitochondria was achieved at $0^{\circ}C$ with a M.S.E. sonic oscillator at 20 Kc and 50 w. Heart mitochondria were sonicated for 35 sec, liver mitochondria for 2 min. The liver particles resemble the fragments described by Wise and Lehninger (1962). Phosphorylating sub-mitochondrial particles from heart were prepared by the modified method of Linnane and Ziegler (1958). Manometric assays were carried out in standard Warburg flasks at 37° for heart and 25° for liver systems (Neubert and Hoffmeister, 1960). The composition of the reaction mixture is specified in the figure legends. ATP formation was estimated using the "hexokinase trap" as phosphate disappearence. Phosphate was determined by the method of Fiske and Subbarow.

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The 6-aminonicotinamide-and 4-acetylpyridine-analogues were synthesized as described earlier (Brunnemann,Coper and Herken,1962) using a brain microsomal system. The other pyridine nucleotides were obtained from Pabst Laboratories.

Results and Discussion

Earlier work has shown that succinate can play a part in the reduction of DPN. This effect may be achieved by means of the energy-linked reversal of oxidative phosphorylation (Chance and Hollunger,1960 ; Klingenberg and Slenczka,1959) or by the reduction of acetoacetate under conditions where the phosphorylating processes are completely uncoupled (Krebs and Eggleston,1962).

We have undertaken to investigate the possibility whether DPN has any effect on the succinate-linked part of the electron transport chain.

The representative data (Table 1) show that 3-acetylpyridine-adenine-dinucleotide (3-APAD),3-pyridinealdehyde-adenine-dinucleotide (3-PAAD) and 3-acetylpyridine-deaminoadenine-dinucleotide (3-APXD) as well as DPN itself strongly decrease the succinoxidase activity of the sub-mitochondrial heart and liver systems. Thionicotinamide-adenine-dinucleotide (TNAD) and nicotinamide-deaminoadenine-dinucleotide (NXD) have so far been found only effective in liver systems. In some preparations 6-aminonicotinamide-adenine-dinucleotide (6-ANAD) and nicotinamide-mononucleotide (NMN) showed an inhibition of varying intensity, being ineffective in others,while 4-acetylpyridine-adenine-dinucleotide (4-APAD), 3-pyridinealdehyde-deaminoadenine-dinucleotide (3-PAXD) and TPN were comparatively ineffective in all experiments.

The fact of an increased inhibition with time, after a short lag phase (Figure 1) suggested the accumulation of an inhibitor. In non-phosphorylating particles the possibility has been excluded that DPN and its analogues are causing the inhibition by making possible the accumulation of oxalacetate (Neubert, 1963b).

Analysis of the incubation mixture showed that under certain conditions marked absorption changes occured in the 290-300 mμ region,when DPN,3-APAD, 3-PAAD, 3-APXD or 6-ANAD were incubated together with the succinate.

On the other hand, no characteristic changes in the absorption spectra could be detected in the presence of 3-PAXD and 4-APAD, compounds which failed to inhibit the oxygen uptake coupled with succinate oxidation.

Since the absorption spectrum of the unidentified DPN-compound exhibited similar characteristics as described by Chaykin,Meinhart and Krebs(1956) for the " hydrated " form of $DPNH_2$, " DPNH-X " was prepared by the method of Pfleiderer and Stock (1962) and added directly to the succinate-oxidizing system. Under these conditions the inhibition als occured but without any lag phase.

It is possible that an open ring form of DPN and its analogues may be responsible for the observed inhibitory effect. Following the procedure devised

Table 1

Inhibitory effect of pyridine nucleotides on succinate oxidation

Assay conditions : 30 µmoles K-phosphate-buffer, pH 7,4 ; 16,5 µmoles $MgCl_2$; 60 µmoles KCl ; 26 µmoles Na-succinate ; 10 µmoles ADP ; 100 µmoles glucose ; 20 µmoles Tris-HCl, pH 7,4 ; 1 mg Hexokinase (Pabst Laboratories) and 1-10 mg submitochondrial protein were added to each manometer flask.
The pyridine nucleotides were present in the incubation mixture in concentrations between 0,3 - 0,5 mM. Final volume 2,5 ml. Incubation time 10-15 min.
The oxidation rate is expressed in µatoms / 15 min.

Assay system	Sonic fragments *							Lyophilized mitochondria **	
	Rat liver		Rat heart			Sheep heart		Rat liver	Rat heart
	Expt.No		Expt.No			Expt.No			
	I	II	I	II	III	I	II		
Control	4,2	1,8	5,8	3,8	4,9	5,4	8,3	5,8	7,6
+ DPN	2,2	0,8	2,3	1,0	2,9		3,0	2,0	5,1
+ 3-APAD	1,3			0,9	2,8	2,7			2,4
+ 3-PAAD	2,2		2,5		3,5			2,1	3,0
+ NXD	1,8				4,7				
+ 3-APXD		0,5	1,7						
+ 3-PAXD		1,5	5,3						
+ TNAD	2,3				4,6				
+ 6-ANAD	2,7	1,7	5,2				4,8	5,8	
+ 4-APAD			5,7	3,7					
+ NMN		1,5	4,6		4,8			5,5	
+ TPN		1,8		3,4					

* P/O 0,1-0,5 ** P/O 0,0

by Pfleiderer and Stock (1962) with 3-glyceraldehydephosphate-dehydrogenase the sub-mitochondrial fragments were analyzed on a charcoal column for any bound DPN-compounds (Chaplain,1963).When succinate and DPN had been present in the reaction mixture, the formation of a DPN-derivative related to the open ring form of DPN reported by Burton(1960) was noted. Using the spectrophotometric constants for the primary acid product of $DPNH_2$ quoted by Chaykin et al. (1956) a concentration of 0,2 mµmole/mg protein was calculated. The formation of this DPN compound absorbing in the 290 mµ region was observed concomitantly with the progressive inhibition on succinate-oxidation, even under conditions where no accumulation of the " DPNH-X " type compound in the reaction medium was detectable. When 3-APAD or 3-PAAD had been present initially together with succinate under the described incubation conditions enzyme-bound derivatives of these analogues absorbing in the 300 mµ region were detected which may also represent open ring forms.

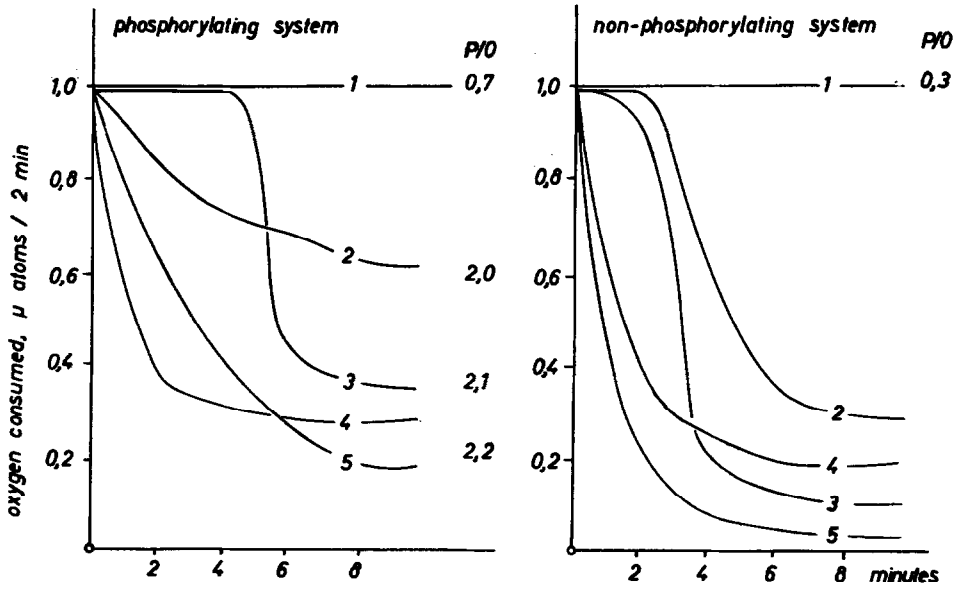

Fig.1. Inhibition of succinoxidase by pyridine nucleotides

Beef heart sonic fragments. Experimental conditions as in Table 1. All pyridine nucleotides were added in a final concentration of 0,5 mM. 1) Control, 2) 3-PAAD, 3) DPN, 4) " DPNH-X", 5) 3-APAD.

The formation of a pyridone form may be a third possibility for an inhibitor. Such a pyridone form of DPN might be the intermediate next in line to the identified 290 mμ compound. Kaplan (1951) has postulated the following mechanism: $DPNH_2$ + DPN-pyridone ⟶ 2 DPN. Preliminary experiments have shown that $DPNH_2$, although itself not affecting the oxidation of succinate(Neubert,1963b), was able to bring about a reversal of the effect caused by the different DPN-compounds (Neubert,1963b; Chaplain,1963). The removal of the pyridones would therefore steadily decrease the inhibitor concentration as reflected by a restoration of the rate of oxygen uptake with succinate as substrate. In the suggested reaction sequence $DPNH_2$-linked substrates would control the rate of succinate-oxidation in intact mitochondrial systems.

The experiments with non-phosphorylating systems (Tables 1 + 2, Fig.1) argue against the possibility of a link between the succinate oxidase part of the electron transport chain and DPN-dehydrogenase in some kind of energy-linked reversal of oxidative phosphorylation (Löw,Krueger and Ziegler,1961). However, since the incubation mixture and the submitochondrial particles have been found to contain reduced forms of DPN, succinate oxidation must provide the reducing equivalents for DPN by some means. In such a reaction mechanism succinate dehydrogenase would produce its own inhibitor in the presence of DPN

Table 2

Effect of pyridine nucleotides on succinate-coupled oxidative phosphorylation in submitochondrial systems.

The incubation conditions were the same as described in Table 1. 2,4 DNP, 0,1 mM, was added if indicated, Incubation time 10-15 min.

Assay system	Oxygen uptake (in μmoles)	P/O
Rat liver Control	3,9	0,2
+ 3-APAD	1,3	1,0
Rat heart Control	6,0	0,4
+ DPN	2,0	1,0
+ 2,4-DNP	6,0	
+ DPN + 2,4-DNP	1,6	
Control	4,0	0,3
+ DPN	1,2	1,0
+ 3-APAD	1,1	1,1

The degree of inhibition was markedly higher in experiments carried out with uncoupled mitochondria and sub-mitochondrial systems, while phosphorylating preparations in general showed a smaller inhibitory effect by the DPN-derivatives (Fig.1). Under experimental conditions in which any phosphorylative capacity had been left in the preparations the disappearence of inorganic phosphate from the incubation medium remained constant although succinate-oxidation was suppressed by DPN or 3-APAD, giving rise to an increase in P/O ratio (Table 2, Fig. 1). This confirms observations by Griffiths (1962) performed with DPN.

In several experiments where the initial phosphate uptake coupled to succinate oxidation was low, an increase of net phosphorylation was observed.

These findings suggest that only part of the succinate oxidation which is not coupled to phosphorylation is sensitive to the DPN compounds. This view of selective inhibition of non-phosphorylating electron transport from the oxidation of succinate may be supported by the observation that certain cortical steroids inhibit the oxidation of succinate by thyroxine-treated liver mitochondria without any change in net phosphorylation where suitable conditions increase the P/O ratios to normal values (Neubert, 1963).

We interpret our experimental findings as providing suggestive evidence for the existence of a site in the succinic dehydrogenase which binds DPN or one of its derivatives. Under these conditions the accumulation of an DPN- intermediate may provide a control mechanism making succinate oxidation more efficient in the absence of DPN reducing substrates.

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