REDUCTION OF ADDED DPN FROM THE CYTOCHROME C LEVEL IN SUBMITOCHONDRIAL PARTICLES

Hans Löw and Ivar Vallin

The Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden

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Reduction of pyridine nucleotides from the cytochrome c level with an external source of electrons has been described by Chance and Fugmann (1961) who used reduced cytochrome c in digitonin-treated pigeon heart mitochondria and by Packer and Denton (1962) who employed tetramethyl-p--phenylendiamine (TMPD) and ascorbate as the reductant in mammalian heart mitochondria. The energy supply for driving the reaction was, in the former case, exogenous ATP, and in the latter case, energy generated in the terminal energy conservation step. In both cases, reduction was limited to the enzyme level in that only the reduction of endogenous DPN was involved.

Penefsky (1962) has reported a system similar to that of Packer, in which the reduction of endogenous CoQ (ubiquinone) was studied.

In the present communication a system is described where externally added DPN is reduced by TMPD and ascorbate in submitochondrial particles from beef heart with added ATP as the source of energy.

Methods. Submitochondrial particles from beef heart were prepared as has been described by Linnane and Ziegler (1958) with the modifications introduced by Löw and Vallin (1962).

The formation of DPNH was followed by the change in optical density at 340 mu in a Beckman DK-2 spectrophotometer. The cuvette chamber was maintained at 30°C.

The incubation medium contained 50 mM TRIS-HCl, pH 8.0; 6 mM $MgCl_2$; 0.25 M sucrose; 1 mM KCN; 1 mM ATP; and 0.15 mg particle protein/ml. This

mixture was allowed to preincubate at 30°C until maximal reduction rate was achieved, usually between 6 - 12 minutes, whereupon the reaction was started by the addition of 0.3 mM TMPD, 5 mM ascorbate and 1.5 mM DPN in a final volume of 3.0 ml.

Only unfrozen particles have been used in this study, since freezing and subsequent thawing seemed to make the preparation considerably less stable.

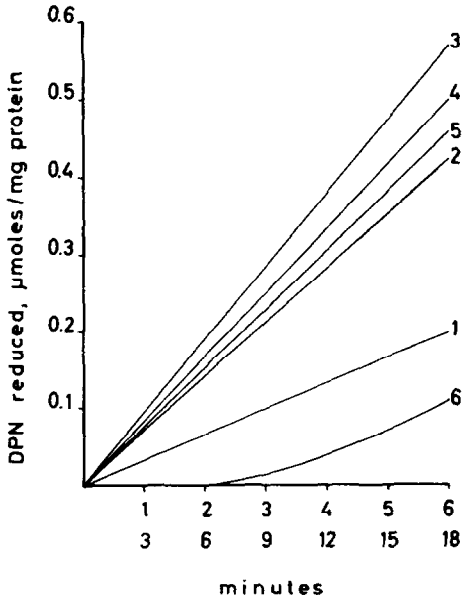

Figure 1. The effect of preincubation on the reduction of DPN from the cytochrome c level.
Conditions: as given in Methods. Incubation times: 1 = 6 minutes, 2 = 8 minutes, 3 = 9 minutes, 4 = 10 minutes and 5 = 12 minutes. 6 = no preincubation; curve 6 corresponds to the lower set of figures on the time axis.

Results. In fig. 1 the effect of preincubation on the rate of reduction of DPN is demonstrated. Maximal reduction rate was achieved after 9 minutes of preincubation. Extended treatment resulted in a decreased rate. With no preincubation, the reaction occasionally started after a time lag of several minutes, and the maximal rate attained was low. After preincubation, the reaction started without any lag and proceeded linearly for 5 to 10 minutes. The presence of the reduction system during preincubation

resulted in a decrease by 40 % of the rate which could be reached in the absence of the reduced dye. The maximal rate of reduction of DPN was 0.12 umoles/min/mg protein.

The reduction was completely dependent on the addition of Mg^{++}, the optimal concentration being 6 mM. Of the nucleotide triphosphates tested ATP alone could serve as the source of energy. The concentration of DPN giving the maximal rate of reduction was 1.5 mM. TPN could not be reduced.

Oligomycin inhibited the reduction; the concentration needed was the same whether the inhibitor was present during preincubation or not (Table I).

TABLE I
The effect of oligomycin and 2,4-dinitrophenol on the reduction of DPN from the cytochrome c level.

Inhibitor	Concentration	% Inhibition
oligomycin	0.031 ug/mg protein	15
"	0.053 ug/mg protein	61
dinitrophenol [a]	$4 \cdot 10^{-6}$ M	47
" [a]	$6 \cdot 10^{-6}$ M	59
" [a]	$8 \cdot 10^{-6}$ M	72
dinitrophenol [b]	10^{-5} M	10
" [b]	$2 \cdot 10^{-5}$ M	43
" [b]	$3 \cdot 10^{-5}$ M	65

[a] DNP added to the preincubation mixture
[b] DNP added at start of the reaction
Conditions: as given in Methods.

2,4-Dinitrophenol caused an inhibition of the reaction. If present during preincubation, dinitrophenol was active in concentrations about 5 times lower than if added at the start of the reaction (Table I).

TABLE II
The effect of respiratory inhibitors on the reduction of DPN from the cytochrome c level.

Inhibitor	Concentration	% Inhibition
Amytal	0.12 mM	58
"	0.18 mM	74
"	0.24 mM	84
NQNO [a]	0.51 ug/mg protein	45
"	0.77 ug/mg protein	57
antimycin A	0.14 ug/mg protein	21
"	0.15 ug/mg protein	40
"	0.16 ug/mg protein	59
"	0.17 ug/mg protein	100
antimycin A [b]	0.17 ug/mg protein	0
" [b]	4.4 ug/mg protein	45

[a] 2-n-nonyl-4-hydroxyquinoline-N-oxide
[b] with 10 mM succinate as the reductant
Conditions: as given in Methods.

Data in Table II illustrate the effects of the respiratory inhibitors Amytal, NQNO and antimycin A. All three compounds were inhibitory, and the concentrations were similar to those needed for inhibition of the forward electron transport. It was immaterial whether or not these inhibitors were present during preincubation.

The mode of action of antimycin A in the reduction of DPN with succinate has been under debate (Chance and Hollunger, 1961; Hommes, 1962). A comparison was now made of the inhibition when ascorbate - TMPD and when succinate was used as the reductant. Relevant data are included in the latter part of Table II. As may be seen, concentrations of antimycin A that completely inhibited the reduction of DPN by TMPD -

ascorbate gave no inhibition of the DPN reduction by succinate. The amount of antimycin needed to give 50 % inhibition was about 30 times greater in the case of succinate than in the case of ascorbate and TMPD. The low concentration of antimycin A needed in the latter case corresponds well to that required for inhibition of the aerobic oxidation of succinate in heart particles (Löw and Vallin, 1962).

Discussion. The high antimycin sensitivity in the present system, where the cytochrome oxidase is terminally blocked with cyanide, indicates that the electrons enter the chain in the cytochrome c region. Thus the reduction, which is entirely dependent on the addition of ATP, seems to involve two of the three sites of phosphorylation, involved in the oxidation of DPNH.

The difference between the present system and the one where succinate serves as the source of electrons is clearest with respect to the antimycin A sensitivity. The great sensitivity of the ascorbate - TMPD system to antimycin A seems to justify our earlier viewpoint that when succinate is used as a reductant, the relatively weak antimycin A effect is most likely due to an unspecific effect (Löw, Krueger and Ziegler, 1961; Löw and Vallin, 1962), rather than to the specific electron transport inhibition (Chance and Hollunger, 1961; Hommes, 1962).

Hommes (1962) has demonstrated that when DPN is reduced by succinate, there is a lag phase which is abolished if the particles are preincubated with ATP prior to the addition of DPN. A similar though much longer lag phase was observed with the system described. Without preincubation, the reduction sometimes did not start at all, and even when it did the rate did not reach maximum. Preincubation with ATP might result in the building up of a highenergy intermediate, whose concentration is rate limiting for the reduction of DPN.

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REFERENCES

Chance, B. and Fugmann, U., Biochem. Biophys. Res. Comm., 4, 317 (1961)
Chance, B. and Hollunger, G., J. Biol. Chem., 236, 1562 (1961)
Hommes, F.A., Biochem. Biophys. Res. Comm., 8, 248 (1962)
Linnane, A.W. and Ziegler, D.M., Biochim. Biophys. Acta, 29, 630 (1958)
Löw, H., Krueger, H. and Ziegler, D.M., Biochem. Biophys. Res. Comm., 5, 231 (1961)
Löw, H. and Vallin, I., Biochim. Biophys. Acta (in press)
Packer, L. and Denton, M.D., Fed. Proc., 21, 52 (1962)
Penefsky, H.S., Biochim. Biophys. Acta, 58, 619 (1962)