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DT DIAPHORASE

IV. COUPLING OF EXTRAMITOCHONDRIAL REDUCED PYRIDINE NUCLEOTIDE OXIDATION TO MITOCHONDRIAL RESPIRATORY CHAIN

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SUMMARY

Purified DT diaphorase from rat liver can support the aerobic oxidation of external DPNH and TPNH by isolated mitochondria when low concentrations of vitamin K_3 (2-methyl-1,4-naphthoquinone) are added. The oxidation is sensitive to dicoumarol which inhibits DT diaphorase, and to the respiratory-chain inhibitors, antimycin A and cyanide. The phosphate/oxygen ratio observed for the system is two. The reaction is specific with regard to the quinone; closely related naphthoquinones, such as 1,4-naphthoquinone, as well as various benzoquinones, are inactive. The possible significance of DT diaphorase in the regulation of the cellular levels of the extramitochondrial reduced pyridine nucleotides is discussed.

INTRODUCTION

DT diaphorase, a flavoenzyme catalyzing the oxidation of both DPNH and TPNH by artificial electron acceptors, in rat liver occurs in both mitochondria and microsomes, but the bulk of the enzyme is found in the soluble cytoplasm[1-3]. The relationship of the mitochondrial DT diaphorase to the terminal respiratory chain and to oxidative phosphorylation has been investigated in previous papers[4-7]. It was found[4-6] that this enzyme does not participate in respiration, and cannot mediate electron transfer between intramitochondrial DPNH or TPNH and the cytochrome system unless a catalytic amount of vitamin K_3 is added. Since DT diaphorase is strongly inhibited by dicoumarol[2,3], which uncouples oxidative phosphorylation[8], the question of a possible indirect function of the enzyme in the coupling of respiration to phosphorylation was also considered[1,3,4]. However, this possibility was eliminated on the evidence[7] that pigeon-liver mitochondria, which lack DT diaphorase, exhibit a fully efficient phosphorylation which is uncoupled by dicoumarol.

The studies reported in this paper represent an approach to the function of the DT diaphorase occurring in the extramitochondrial space of the liver cell. Since DT diaphorase occurs abundantly in the soluble cytoplasm[2,3] it might constitute a power-

Abbreviation: DCPIP, 2,6-dichlorophenolindophenol.

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ful means of oxidizing cytoplasmic reduced pyridine nucleotides with the possible function of mediating electron transfer between these coenzymes and the mitochondrial respiratory chain. This question has now been investigated by using purified DT diaphorase and isolated rat liver mitochondria as a system for oxidizing DPNH and TPNH. It was found that this system, when supplemented with a catalytic amount of vitamin K_3, catalyzes a rapid aerobic oxidation of both reduced pyridine nucleotides. The reaction, like the one previously observed with the intramitochondrial DT diaphorase[4,9], is specific for vitamin K_3, and is accompanied by a phosphorylation with a P/O ratio of 2. The possibility that this reaction pathway might constitute a control mechanism for the regulation of the levels of extramitochondrial reduced pyridine nucleotides is discussed. Part of this work has been presented in a preliminary form[9].

EXPERIMENTAL PROCEDURE

Materials

All materials used unless specified were commercial products. Vitamins K_1, K_2, and K_3 were obtained through the courtesy of F. Hoffman-La Roche, Inc., Basel. Coenzyme Q_0 was the kind gift of Professor D. E. GREEN, Madison, and coenzyme Q_{10} of Dr. J. LINKS, Amsterdam. The remainder of the quinones were kindly supplied by Dr. L. REIO, Stockholm. The alcohol dehydrogenase was a crystallized preparation in ammonium sulfate from Sigma Co. Purified glucose-6-phosphate dehydrogenase preparations from both Sigma Co. and Boehringer Co. were used without any observable difference in the reactions studied.

Methods

Rat-liver mitochondria were prepared by the procedure of SCHNEIDER AND HOGEBOOM[10] with minor modifications[11]. Purified DT diaphorase was prepared from the soluble fraction of rat-liver homogenate as described previously[3]. Oxygen consumption when measured manometrically was determined in small (5.0 ml volume) manometer vessels. The final volume was 1.0 ml. Polarographic measurements of the oxygen consumption were made with an open rotating cup and stationary platinium electrode according to the procedure of CHANCE AND WILLIAMS[12]. The volume was again 1.0 ml. Spectrophotometric determination of the oxidation of reduced pyridine nucleotide was performed with a recording Beckman DK-2 spectrophotometer using cuvettes of 1 cm light path as was described previously[3]. The reaction was followed at a wavelength of 340 mμ using an extinction coefficient (liter/mole/cm) of $6.22 \cdot 10^3$ (see ref. 13). Phosphate uptake was estimated according to the modified MARTIN AND DOTY procedure described by LINDBERG AND ERNSTER[14].

RESULTS

Stimulation of mitochondrial oxidation of extramitochondrial TPNH by vitamin K_3 and DT diaphorase

When TPNH, generated by the oxidation of glucose 6-phosphate with glucose-6-phosphate dehydrogenase, was incubated in the presence of fresh mitochondria, very little consumption of oxygen was observed as shown in Fig. 1. This finding is

in agreement with previous reports on the virtual inability of isolated mitochondria to oxidize catalytic concentrations of external TPNH[15-17]. The addition of vitamin K_3 to this system, however, produced a two- to threefold stimulation of the respiration. The respiration in the presence of vitamin K_3 was dicoumarol-sensitive and thus probably proceeds via the DT diaphorase of the mitochondria[4,5]. The addition of purified DT diaphorase to this system gave a further two- to threefold stimulation of the respiration. It may be pointed out that this rate of respiration was close

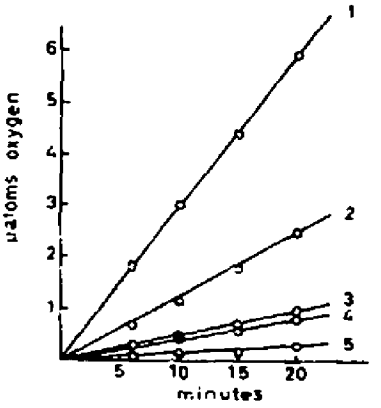

Fig. 1. Stimulation of the mitochondrial oxidation of extramitochondrial TPNH by vitamin K_3 and DT diaphorase. The complete system contained, per Warburg vessel, 20 µmoles glucose 6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 0.5 µmole TPN, 20 µmoles Tris buffer (pH 7.4), 12 µmoles orthophosphate (pH 7.4), 4 µmoles $MgCl_2$, 2 µmoles adenosine triphosphate, 24 µmoles glucose, an excess of yeast hexokinase, 50 µmoles sucrose, mitochondria from 200 mg rat liver, 0.01 µmole vitamin K_3, and an amount of 450-fold purified DT diaphorase (together with 1 mg serum albumin) capable of reducing 1 µmole DCPIP per minute. Final volume 1.0 ml. Temperature, 30°. Reading begun after 6 min thermoequilibration. 1, Complete system; 2, no DT diaphorase; 3, no DT diaphorase, no vitamin K_3; 4, no substrate; 5, no mitochondria.

to that obtained maximally with succinate or glutamate as substrate and may represent the limit of the cytochrome system to react with oxygen rather than that of TPNH to react with the cytochrome system. DT diaphorase in the absence of vitamin K_3 had no effect on the respiration.

The concentration of 10^{-5} M vitamin K_3 used in these experiments was not ascertained to be the minimal concentration required for the system; however, 10^{-6} M vitamin K_3 gave but very little stimulation of respiration either in the presence or absence of added DT diaphorase. Results from other experiments[4] have indicated that $5 \cdot 10^{-6}$ M is the minimal concentration required to support maximal respiration in a similar type of system.

It may be noted also that little or no oxygen consumption was observed in the absence of mitochondria, indicating that auto-oxidation of the vitamin K_3 was negligible under these conditions.

Effect of inhibitors on mitochondrial oxidation of extramitochondrial TPNH in the presence of vitamin K_3 and DT diaphorase

The role of the respiratory chain in the TPNH oxidation stimulated by vitamin

K_3 and DT diaphorase was determined with a number of inhibitors as shown in Table I. Amytal, which is assumed to act between DPNH and the cytochrome chain[18], showed no inhibition, and actually a slight and possibly significant stimulation of the respiration was observed. On the other hand, antimycin A, acting between cytochrome *b* and cytochrome *c*[19,20], and cyanide, inhibiting cytochrome oxidase, strongly inhibited this respiration. These results are in agreement with the report of COLPA-BOONSTRA AND SLATER[21] on the oxidation of reduced vitamin K_3 by mitochondria.

TABLE I

EFFECT OF SOME INHIBITORS ON THE MITOCHONDRIAL OXIDATION OF EXTRAMITOCHONDRIAL TPNH IN THE PRESENCE OF VITAMIN K_3 AND DT DIAPHORASE

Conditions were the same as in Fig. 1. The amounts of the additions were as follows: 1.0 μmole amytal, 1 μg antimycin A, 1.0 μmole KCN, and 10^{-3} μmole dicoumarol. Time measured, 20 min.

Additions (+) or omissions (−)		μatoms oxygen
Complete system		5.50
Complete system + amytal		6.59
Complete system + antimycin A		1.14
Complete system + cyanide		1.33
Complete system + dicoumarol		1.31
Complete system − DT diaphorase	vitamin K_3	1.12

Dicoumarol at a concentration of 10^{-6} M is a highly selective inhibitor for DT diaphorase[1,3,5] and gives no inhibition of normal mitochondrial respiration. The results in Table I show a nearly complete inhibition of the respiration stimulated by vitamin K_3 and DT diaphorase with this concentration of dicoumarol.

Comparison of mitochondrial oxidation of TPNH and DPNH in the presence of vitamin K_3 and DT diaphorase

Since DT diaphorase may react equally well with both DPNH and TPNH, a system for generating DPNH containing DPN, ethanol, and alcohol dehydrogenase was studied. As was reported elsewhere[3], the DT diaphorase has a low affinity for its substrates and dye reduction is slow when a generating system such as alcohol dehydrogenase and ethanol which is unfavorable to the production of reduced pyridine nucleotide is used. It was not surprising therefore that attempts to determine the rate of oxygen consumption supported by DT diaphorase in such a system gave low values compared with the glucose-6-phosphate dehydrogenase system.

In order to make a more satisfactory comparison, the oxidation of DPNH and TPNH was followed polarographically, using substrate amounts of the reduced pyridine nucleotides. Fig. 2 shows comparative traces of the oxygen consumption with TPNH and DPNH as substrates. It can be seen that under these conditions the two systems behaved identically in regards to stimulation of oxygen uptake by vitamin K_3 and DT diaphorase. The difference in the initial rate in the presence of reduced pyridine nucleotide alone was due to differences in the endogenous substrate present. The oxidation of DPNH showed no inhibition by amytal and the same

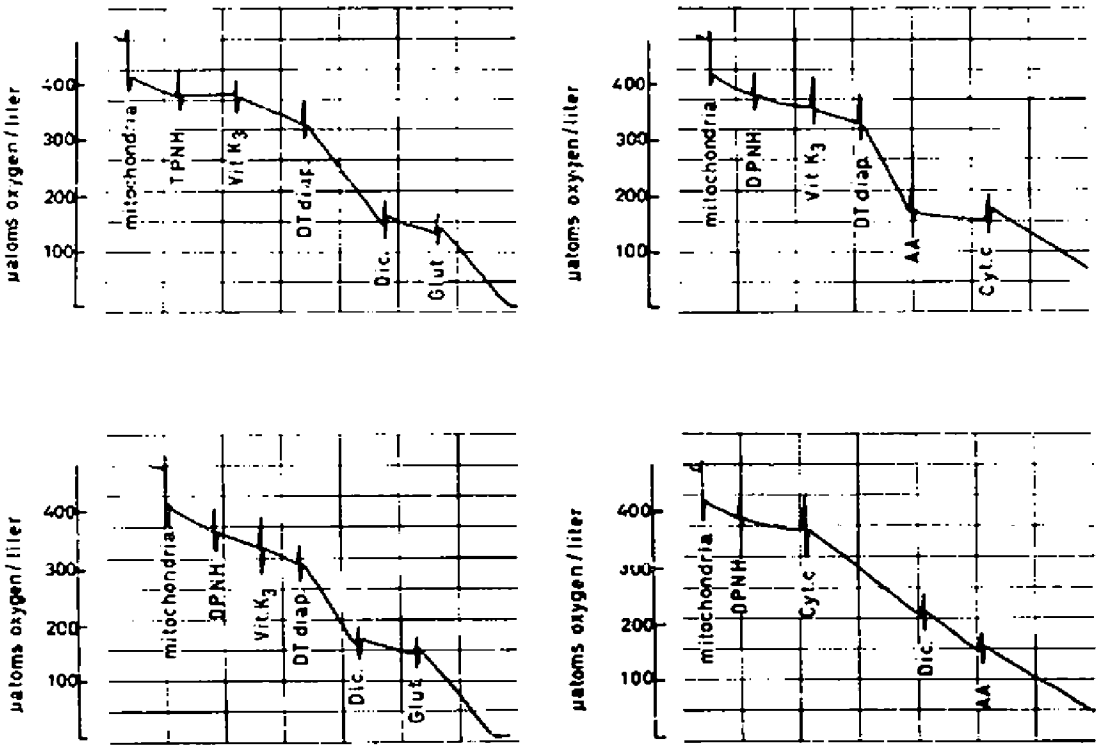

Fig. 2. Comparison of the mitochondrial oxidation of extramitochondrial TPNH and DPNH mediated by vitamin K_3 and by cytochrome *c*. The medium contained 20 μmoles Tris buffer (pH 7.4), 20 μmoles orthophosphate (pH 7.4), 4 μmoles $MgCl_2$, 6 μmoles glucose, 1 μmole ATP, excess yeast hexokinase, 100 μmoles KCl, 50 μmoles sucrose, and 1 mg serum albumin in 1.0 ml. Mitochondria from 200 mg rat liver were used. The amounts of the additions were as follows: 1.0 μmole TPNH or DPNH, $5 \cdot 10^{-3}$ μmole vitamin K_3, purified DT diaphorase capable of reducing 1 μmole DCPIP per minute, $5 \cdot 10^{-3}$ μmole dicoumarol, 1 μg antimycin A, 10 μmoles glutamate, and $6 \cdot 10^{-2}$ μmole cytochrome *c*. Final volume, 1.2 ml.

sensitivity to dicoumarol and antimycin A as did the TPNH system, both in manometric and polarographic experiments. This clearly distinguished this pathway from the DPNH oxidation in the presence of cytochrome *c* reported by LEHNINGER[22] which shows no sensitivity to dicoumarol or antimycin A. No appreciable oxidation could be observed with TPNH in the presence of cytochrome *c*.

Sensitivity of the vitamin K_3-mediated oxidation of TPNH by mitochondria in the presence of DT diaphorase to antimycin A

In Fig. 3 is shown the titration of mitochondrial respiration by antimycin A with glutamate as substrate and with the oxidation of glucose 6-phosphate mediated by vitamin K_3 and DT diaphorase. Although the latter system showed slightly less sensitivity than the normal oxidation of glutamate, both systems were inhibited maximally by the same level of antimycin A, supporting the view that the same

site of inhibition is involved in both systems. It must therefore be presumed that the electrons from the TPNH enter the respiratory chain at or before the normal site of antimycin A inhibition.

Reaction of DT diaphorase and vitamin K_3 with a succinic oxidase preparation

A further attempt to elucidate the site and nature of the interaction of vitamin K_3 and DT diaphorase with the respiratory chain was made by studying the reaction

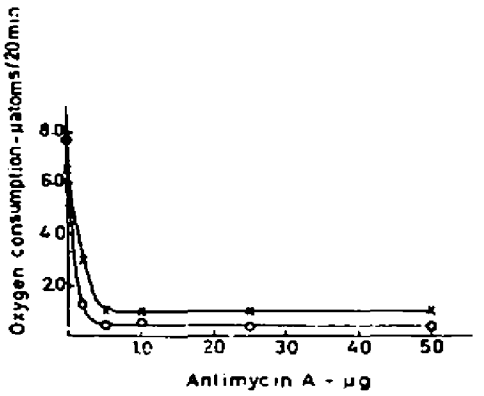

Fig. 3. Effect of antimycin A on the mitochondrial oxidation of glutamate and oxidation of extramitochondrial TPNH mediated by vitamin K_3 and DT diaphorase. The complete system contained, per Warburg vessel, 10 µmoles glutamate or 10 µmoles glucose 6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 0.5 µmole TPN, 20 µmoles Tris buffer (pH 7.4), 20 µmoles orthophosphate (pH 7.4), 4 µmoles $MgCl_2$, 2 µmoles ATP, 24 µmoles glucose, excess yeast hexokinase, 100 µmoles KCl, 50 µmoles sucrose, $5 \cdot 10^{-3}$ µmole vitamin K_3, purified DT diaphorase (together with 1 mg serum albumin) capable of reducing 1 µmole DCPIP per minute and mitochondria from 200 mg rat liver. Final volume, 1.0 ml. Temperature, 30°. Readings began after 6 min thermoequilibration. ○—○, glutamate; ×—×, glucose 6-phosphate + glucose-6-phosphate dehydrogenase + TPN.

of the vitamin K_3–DT diaphorase system with the respiratory chain of a succinic oxidase preparation. The succinic oxidase was prepared from rat-liver mitochondria with 0.3% deoxycholate by the method of SIEKEVITZ AND WATSON[23]. It was found necessary to give further washing with the deoxycholate than was described in order to reduce the DPNH oxidase activity. An experiment is shown in Table II where the DPNH oxidase activity was 35% of the succinic oxidase activity. This respiration was sensitive to both amytal and antimycin A. In the presence of a TPNH-generating system the preparation with DT diaphorase and vitamin K_3 gave a respiration of 70% of the succinate respiration. This respiration had lost amytal sensitivity, but remains at least 60% sensitive to antimycin A. This system showed all the characteristics of the intact mitochondrial system and suggests that the electrons from vitamin K_3 may enter the respiratory chain at a site common to both the succinic oxidase and DPNH oxidase chains. A probable site would be cytochrome *b* which has been implicated also in the vitamin K_3-mediated bypass of the amytal inhibition in mitochondrial respiration[4].

TABLE II

OXIDATION OF TPNH BY A SUCCINIC OXIDASE PREPARATION IN THE PRESENCE OF DT DIAPHORASE AND VITAMIN K_3

Conditions were the same as in Fig. 3. The amounts of the substrates were 10 μmoles succinate, 10 μmoles glutamate, 100 μmoles ethanol, 10 μmoles glucose 6-phosphate, 1 μmole DPN, 1 μmole TPN, 1000 units alcohol dehydrogenase, and 0.6 unit glucose-6-phosphate dehydrogenase. The amounts of the additions were 1 μg antimycin A, 2 μmoles amytal, excess DT diaphorase with 1 mg serum albumin, and $5 \cdot 10^{-3}$ μmole vitamin K_3. Time measured, 20 min.

Substrate	Additions	μatoms oxygen
Succinate		3.84
Succinate	Antimycin A	0.26
Glutamate		0.13
Glutamate + DPN		0.65
Glutamate + DPN	Antimycin A	0.41
Ethanol + DPN + alcohol dehydrogenase		1.32
Ethanol + DPN + alcohol dehydrogenase	Amytal	0.48
Ethanol + DPN + alcohol dehydrogenase	Antimycin A	0.64
Glucose 6-phosphate + TPN + glucose-6-phosphate dehydrogenase		0.26
Glucose 6-phosphate + TPN + glucose-6-phosphate dehydrogenase	DT diaphorase	0.58
Glucose 6-phosphate + TPN + glucose-6-phosphate dehydrogenase	Vitamin K_3	0.87
Glucose 6-phosphate + TPN + glucose-6-phosphate dehydrogenase	DT diaphorase + vitamin K_3	2.73
Glucose 6-phosphate + TPN + glucose-6-phosphate dehydrogenase	DT diaphorase + vitamin K_3 + amytal	2.36
Glucose 6-phosphate + TPN + glucose-6-phosphate dehydrogenase	DT diaphorase + vitamin K_3 + antimycin A	1.04

Specificity for vitamin K_3 in the stimulation of mitochondrial oxidation of TPNH in the presence of DT diaphorase

A large number of related naphthoquinones and benzoquinones were tested in order to determine whether they might replace vitamin K_3 in mediating the oxidation of extramitochondrial TPNH through the DT diaphorase to the respiratory chain. Although most of these quinones have been observed to accept electrons from DT diaphorase in the presence of reduced pyridine nucleotide, it was found that only vitamin K_3 was appreciably active in the DT diaphorase-catalyzed bypass of the amytal inhibition of DPN-linked oxidation in mitochondria[4]. As can be observed in Table III, the same surprising specificity was found in the DT diaphorase-catalyzed oxidation of extramitochondrial TPNH by mitochondria. Other than vitamin K_3, the only quinone to give an appreciable stimulation of TPNH oxidation in the presence of DT diaphorase was 2-hydroxy-3-methyl-1,4-naphthoquinone and this was markedly less than the oxidation in the presence of vitamin K_3. The benzoquinones, including coenzyme Q_0, were again completely inactive in this system as tested under these conditions.

In order to eliminate the possibility that this specificity was due to a selective permeability of the mitochondrial membrane, the experiments were repeated with

TABLE III

THE EFFECT OF VARIOUS QUINONES ON THE MITOCHONDRIAL OXIDATION OF EXTRAMITOCHONDRIAL TPNH IN THE PRESENCE OF DT DIAPHORASE

Conditions were the same as in Fig. 3. Each Warburg vessel contained 20 μmoles glucose 6-phosphate, 0.5 unit glucose-6-phosphate dehydrogenase (Sigma), 0.5 μmole TPN, 20 μmoles Tris buffer (pH 7.4), 20 μmoles orthophosphate (pH 7.4), 4 μmoles $MgCl_2$, 2 μmoles adenosine triphosphate, 24 μmoles glucose, an excess of yeast hexokinase, 100 μmoles KCl, 50 μmoles sucrose, an amount of DT diaphorase (with 1 mg serum albumin) capable of reducing 1 μmole vitamin K_3 per minute and mitochondria from 200 mg rat liver. The amount of quinone was 0.01 μmole added in 10 μl ethanol. Time measured, 20 min. Final concentration of cytochrome *c*, $6 \cdot 10^{-5}$ M.

Quinone	μatoms oxygen	
	Expt. 1	*Expt. 2*
None	0.77	0.57
Vitamin K_3	0.15	1.26
1,2-Naphthoquinone	1.03	
1,4-Naphthoquinone	0.87	
2-Hydroxy-1,4-naphthoquinone	0.85	
2-Methyl-3-hydroxy-1,4-naphthoquinone	1.45	
p-Benzoquinone	0.98	0.57
2-Methylbenzoquinone		0.44
2,6-Dimethylbenzoquinone		0.38
Coenzyme Q_0		0.60
Vitamin K_1		0.55
Vitamin K_2		0.67
Cytochrome *c*	1.08	

TABLE IV

ESTERIFICATION OF PHOSPHATE ACCOMPANYING THE MITOCHONDRIAL OXIDATION OF EXTRAMITOCHONDRIAL TPNH MEDIATED BY DT DIAPHORASE AND VITAMIN K_3

Conditions were the same as in Fig. 3. The amounts of the additions were 2 μmoles amytal and 0.1 μmole 2, 4-dinitrophenol. Phosphate uptake was estimated according to the modified MARTIN AND DOTY procedure[11]

	Additions (+) or omissions (−)	μatoms oxygen	μmoles phosphate	P/O
1. Complete system		3.40	3.30	0.95
	+ 2,4-Dinitrophenol	3.28	0.30	0.09
	− DT diaphorase, vitamin K_3	0.19	0.44	
2. Complete system		5.08	2.59	0.51
	+ Amytal	5.57	1.29	0.23
	+ 2,4-Dinitrophenol	4.90	0.03	0.01
	− DT diaphorase, − vitamin K_3	0.40	0.44	—
	DT diaphorase, vitamin K_3, + amytal	0.50	0.10	—
3. Complete system		6.58	3.28	0.50
	+ Amytal	6.17	0.99	0.16
	+ 2,4-Dinitrophenol	6.23	0.12	0.02
	− DT diaphorase, − vitamin K_3	1.56	0.93	—
	− DT diaphorase, − vitamin K_3, + amytal	1.04	0.04	—

the submitochondrial DPNH oxidase preparation previously described[5] using added TPNH and purified DT diaphorase. As was reported in the preceding paper[5], a similar picture of specificity was obtained.

Esterification of phosphate in the vitamin K_3-mediated oxidation of TPNH by mitochondria in the presence of DT diaphorase

The determination of P/O ratios for the oxidation of TPNH in the DT diaphorase–vitamin K_3 supplemented system by the usual manometric measurement of oxygen and direct determination of phosphate was very erratic. Several experiments are shown in Table IV. The ratios varied from 0.45 to almost 1.00. This phosphorylation was surprisingly sensitive to amytal and highly sensitive to dinitrophenol. These low values observed for this system are similar to the values observed by COLPA-BOONSTRA AND SLATER[21] for the oxidation of reduced vitamin K_3 by sarcosomes, and by JACOBS *et al.*[24,25] for the oxidation of extramitochondrial DPNH in the presence of various electron mediators.

When the question was reinvestigated by means of an oxygen electrode, it was

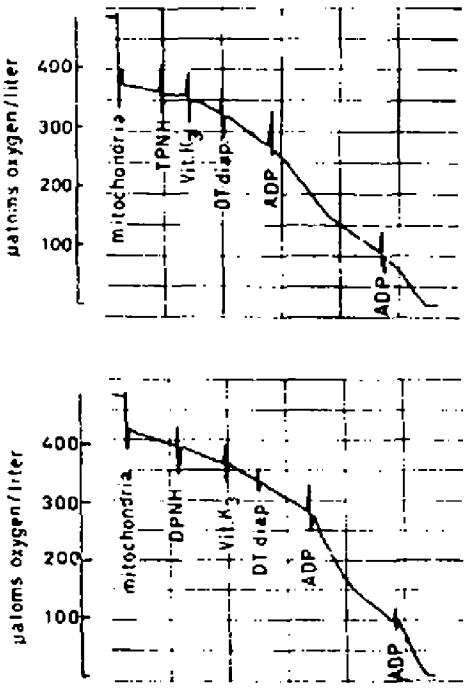

Fig. 4. Esterification of phosphate and respiratory control during the mitochondrial oxidation of TPNH and DPNH mediated by vitamin K_3 and DT diaphorase as measured by the polarographic method of CHANCE AND WILLIAMS[30]. The medium contained 20 μmoles Tris buffer (pH 7.4), 20 μmoles orthophosphate (pH 7.4), 4 μmoles $MgCl_2$, 1 μmole ATP, 100 μmoles KCl, 50 μmoles sucrose, and 1 mg serum albumin in 1.0 ml. Mitochondria from 200 mg rat liver were used. The amounts of the additions were as follows: 1.0 μmole DPNH, $5 \cdot 10^{-3}$ μmole vitamin K_3, purified DT diaphorase capable of reducing 1 μmole DCPIP per minute, and 0.30 μmole ADP. Final volume, 1.2 ml. Temperature, 25°.

observed that the system exhibited a respiratory control in which there was a three- to fourfold stimulation of respiration by the addition of phosphate acceptor. This respiratory control, significantly, is similar to that observed with succinate as substrate (see however ref. 26). Utilizing this respiratory control, it was possible to determine the P/O ratio for this system by the method of CHANCE AND WILLIAMS[12]. The trace of such an experiment is shown in Fig. 4. The P/O ratios obtained in this manner were 2.0 with both TPNH and DPNH as substrate, in agreement with the results obtained for the vitamin K_3-mediated mitochondrial oxidation of glutamate in the presence of amytal[4].

DISCUSSION

The properties determined for the oxidation of extramitochondrial reduced pyridine nucleotides in the system studied in this paper appear to be closely similar to those observed for the vitamin K_3-mediated bypass of the amytal-sensitive site of the mitochondrial respiratory chain which has also been shown to involve DT diaphorase[4,5]. An important difference is that whereas in the case of intramitochondrial pyridine nucleotides the mitochondrial DT diaphorase serves as a link to vitamin K_3, in the present system external DT diaphorase is needed. In both cases, there is a specific requirement for vitamin K_3, indicating that the sites of entrance of the electrons into the respiratory chain are identical for the two systems, located probably at the level of cytochrome *b*. These properties are also in agreement for the most part with those reported for the oxidation of reduced vitamin K_3 by COLPA-BOONSTRA AND SLATER[21]. JACOBS *et al.*[24,25] reported that coenzyme Q_9 added to mitochondrial suspensions stimulated an antimycin A-sensitive oxidation of DPNH, while vitamin K_3 stimulated an oxidation of DPNH which was insensitive to antimycin A. However, these authors found it necessary to use concentrations of the quinones which were 50-fold higher than those used in the experiments reported here, and therefore a comparison of their results with ours is not readily feasible.

Both COLPA-BOONSTRA AND SLATER[21], for the oxidation of reduced vitamin K_3, and JACOBS *et al.*[24,25], for the oxidation of DPNH through various added electron mediators, have reported esterification of phosphate accompanying respiration; however, in all cases, the P/O ratio was lower than 1 even though the reactions were sensitive to antimycin A. This is in agreement with the P/O ratios observed manometrically in this work. Even when corrections were applied for the uncoupling effects of vitamin K_3 and the antimycin A-insensitive respiration, the values obtained approach a P/O ratio of only 1. However, when the ratio was determined over a short time interval by oxygen electrode measurement, a value of 2 was indicated. This is more in accordance with the value anticipated for a respiration which exhibits a sensitivity to antimycin A. The disagreement of this value with the ratios obtained manometrically may be due to the longer period of measurement required with the latter method, allowing either an uncoupling of phosphorylation by vitamin K_3 or a decreased sensitivity to antimycin A and greater reactivity with lower members of the respiratory chain.

As has been pointed out by numerous authors[27-30], the levels of oxidized and reduced pyridine nucleotide may be of great importance in the regulation of meta-

bolic processes in the cell. It was observed by GLOCK AND MCLEAN[31], as well as by various others[32,33], that in most tissues the levels of DPNH are rather low compared to DPN, while in the case of the triphosphopyridine nucleotides the reverse is true. This is related perhaps to the teleological generalization that diphosphopyridine nucleotide is functionally associated with oxidative-catabolic reactions and triphosphopyridine nucleotide with reductive synthesis. A most important consideration in this connection concerns the mechanisms involved in the oxidation of extramitochondrial reduced pyridine nucleotides by the mitochondrial respiratory chain[20,34-37]. These mechanisms are to date not fully understood.

Reduced pyridine nucleotides added externally to mitochondria are not oxidized appreciably[15,22,38], a phenomenon usually attributed to an "impermeability" of the mitochondrial membrane to these compounds. Added cytochrome *c* may, at least with mitochondria from certain tissues, induce a high rate of oxidation of external DPNH[39]. This pathway differs from that involved in the oxidation of intramitochondrial DPNH in that it is largely insensitive to amytal and antimycin A and is capable only of a limited extent of phosphorylation[18,22]. Since there is so far no evidence for the occurrence of extramitochondrial cytochrome *c*[39,40], the physiological significance of this pathway remains unclear.

More recently it has been suggested that substrates such as α-glycerol phosphate[41-43], or malate[37,44], which have dehydrogenases located both in the soluble cytoplasm and in the mitochondria, may act as carriers of the DPNH hydrogen across the mitochondrial membrane. Also it has been observed that β-hydroxybutyrate may stimulate the oxidation of DPNH by mitochondria through a mechanism which is not yet understood[45]. All these pathways are specific for DPN, and no mechanism for the oxidation of extramitochondrial TPNH has yet been suggested except through transhydrogenation to DPNH[46-49].

The presence of a diaphorase oxidizing both reduced pyridine nucleotides in the soluble cytoplasm of the cell, together with the observation that this enzyme in the presence of certain quinones can couple the oxidation of the nucleotides to the mitochondrial respiratory chain, offers another alternative pathway of extramitochondrial reduced pyridine nucleotide oxidation which does not involve a requirement for cytochrome *c* and which would allow for the oxidation of TPNH as well. Furthermore, since the level of this enzyme is high in most tissues, the rate of reduced pyridine nucleotide oxidation would be dependent upon the levels of the quinone available in the cytoplasm, thus offering a sensitive control mechanism for the oxidation of these nucleotides. WENNER[50] has demonstrated such an influence of vitamin K on the regulation of the glycolytic shunt in ascites tumor cells. Moreover, it has recently been shown in this laboratory[51] that addition of vitamin K_3 to ascites tumor cells in the presence of glucose prevents the inhibition of respiration by amytal and abolishes the Crabtree effect. In both WENNER's and our system the vitamin K effect was sensitive to dicoumarol, indicating the involvement of DT diaphorase. A report by JOSHI *et al.*[52] on the mitochondrial oxidation of TPNH in the presence of soluble cytoplasm may also involve a similar system. However, in order to assess the possible role of DT diaphorase in regulating cellular pyridine nucleotide levels, and in general to understand the physiological function of this enzyme, it will be necessary to first find its natural electron acceptor. Efforts to this end are now in progress[53].

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