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DT DIAPHORASE

III. SEPARATION OF MITOCHONDRIAL DT DIAPHORASE AND RESPIRATORY CHAIN

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SUMMARY

DT diaphorase, which was first purified from the soluble cytoplasmic fraction of rat liver, has been separated and purified from isolated mitochondria. The mitochondrial enzyme displays the same sensitivity to dicoumarol, reactivity with both DPNH and TPNH, and similar rates of reduction of various electron acceptors as the soluble-cytoplasmic enzyme. The residual mitochondrial fragments, free of DT diaphorase, exhibit a DPNH oxidase activity which resembles that of the respiratory chain of intact mitochondria in its sensitivity to amytal and antimycin A but which lacks phosphorylating capacity. Vitamin K_3 (2-methyl-1,4-naphthoquinone) when tested on the DT diaphorase-free submitochondrial particles induces no by-pass of the amytal-sensitive site of the DPNH oxidase, in contrast to the case with intact mitochondria. The addition of DT diaphorase to the particles is required for vitamin K_3 in order to accomplish such a by-pass. These and related findings are discussed in view of the various proposed functions of the different diaphorases in the pathways of mitochondrial oxidations.

INTRODUCTION

DT diaphorase, a flavo enzyme catalyzing the oxidation of both DPNH and TPNH by artificial electron acceptors, which was first detected in and isolated from the soluble fraction of rat-liver homogenates[1-3], is also present in mitochondria. This has raised the question of its relation to mitochondrial respiration and oxidative phosphorylation. As has been previously suggested[3,4] the similar properties of the vitamin K reductase reported by MÄRKI AND MARTIUS[5] indicate that the two enzymes are probably the same. MARTIUS *et al.*[5-8] have proposed that this enzyme functions in mitochondria as part of the phosphorylating respiratory chain between pyridine nucleotide and the cytochromes. The high dicoumarol sensitivity of the enzyme offered a basis for explaining the uncoupling action of dicoumarol on oxidative phosphorylation.

Abbreviation: DCPIP, 2,6-dichlorophenolindophenol.

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Studies reported in a preceding paper[9] have shown that a dicoumarol-sensitive enzyme can be demonstrated to function in respiration only provided that vitamin K_3 (2-methyl-1,4-naphthoquinone) is added to accept and mediate electrons to the respiratory chain and that amytal is present to block the normal respiration. The maximal P/O ratio for the dicoumarol-sensitive system was only two, in contrast to values approaching three, usually observed with the amytal sensitive oxidation of DPN-linked substrates. It has also been shown[10] that liver mitochondria from pigeon, an animal devoid of DT diaphorase, exhibit a normal extent of phosphorylation which is uncoupled by dicoumarol. These observations have led to the conclusion that DT diaphorase has no function in the phosphorylating respiratory pathway of mitochondria.

In this paper are reported the physical separation, recovery, and purification of the mitochondrial DT diaphorase from the amytal-sensitive DPNH oxidase system of the mitochondria, together with studies of the properties and interactions of the two enzymic entities.

Certain parts of this work have already been reported briefly[4,11].

EXPERIMENTAL PROCEDURE

Materials

Vitamins K_1, K_2, and K_3 were obtained through the courtesy of Hoffman and La Roche, Inc., Basel, Switzerland. Coenzyme Q_0 was the generous 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. All other reagents were commercial products.

Methods

Mitochondria were prepared from rat liver by a modification of the procedure of SCHNEIDER AND HOGEBOOM described previously[12]. Oxygen consumption when determined manometrically was measured in small (~5 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[13]. The volume was again 1.0 ml. Spectrophotometric determinations were carried out with a recording DK-2 Beckman Spectrophotometer using cuvettes of 1-cm light path as was described previously[3]. The reactions were followed by recording either the reduction of the acceptor at suitable wavelengths, such as with DCPIP, cytochrome *c*, and ferricyanide, or the oxidation of the reduced pyridine nucleotides at 340 mμ. The following extinction coefficients (liter/mole/cm) were used: $6.22 \cdot 10^3$ at 340 mμ for DPNH and TPNH (ref. 14), $21 \cdot 10^3$ at 600 mμ for DCPIP (ref. 15), $18.5 \cdot 10^3$ at 550 mμ for reduced minus oxidized cytochrome *c*[16]; and $1.00 \cdot 10^3$ at 418 mμ for ferricyanide[17]. Protein determinations were made with the biuret method. Phosphate was measured according to the modified MARTIN AND DOTY procedure described by LINDBERG AND ERNSTER[18].

RESULTS

Extraction of DT diaphorase from isolated mitochondria

After unsuccessful attempts to use various common methods of chemical and physical disruption of mitochondria, a virtually complete extraction and recovery of the mitochondrial DT diaphorase was obtained with an adaption of the procedure used by KIELLEY AND KIELLEY[18] for the preparation of mitochondrial adenosine triphosphatase. Freshly prepared rat liver mitochondria, washed free of cytoplasmic contamination by repeated washing with 0.25 M sucrose, were suspended in cold 0.003 M phosphate buffer (pH 7.5) and the suspension was homogenized in the cold with a Super-Thurrax blendor for 2 min. The treated suspension was centrifuged at 20 000 × *g* for 10 min. The supernatant was saved and the sediment was resuspended in 0.003 M phosphate buffer and homogenized once again with the blendor.

TABLE I

DIAPHORASE ACTIVITIES OF MITOCHONDRIAL SUBFRACTIONS

Expt.	Fraction	Substrate	Diaphorase activity (μmoles DCPIP reduced/min/g liver)			
			No addition	8 mg Tween-20	10^{-6} M dicoumarol	8 mg Tween-20 + 10^{-6} M dicoumarol
1	Mitochondria	DPNH	3.93	3.93	3.57	2.69
		TPNH	0.71	1.61	0.36	0.07
	Final supernatant	DPNH	0.57	1.18	0.20	0.21
		TPNH	0.70	1.25	0.11	0.11
	Light pellet	DPNH	0.86	0.73	0.86	0.73
		TPNH	0.03			
	Heavy pellet	DPNH	1.34	1.01	1.34	1.01
		TPNH	0.04			
	Recovery (final supernatant + light and heavy particles)	DPNH	2.77			
		TPNH	0.77			
2	Final supernatant	DPNH	0.30	0.54		0
		TPNH	0.34	0.64		0
	Light pellet	DPNH	0.43	0.43		0.43
		TPNH	0	0		0
3	Final supernatant	DPNH	0.28	0.70		0.05
		TPNH	0.30	0.71		0.04
	Light pellet	DPNH	0.50	0.50		0.46
		TPNH	0.01	0.04		0.01
4	Final supernatant	DPNH	0.27	0.64		
		TPNH	0.28	0.66		
	Light pellet	DPNH	0.45	0.50		
		TPNH	0.03	0.06		
5*	Final supernatant I	DPNH	0.33			
		TPNH	0.27			
	Light pellet I	DPNH	0.39			
		TPNH	0.01			
	Final supernatant II	DPNH	0.15			
		TPNH	0.11			
	Light pellet II	DPNH	0.13			
		TPNH	0			

* In this experiment, the two low-speed supernatants, obtained after the two consecutive Thurrax-treatments, were not pooled but centrifuged separately.

The centrifugation at 20 000 × *g* was repeated and the sediment now obtained was designated the "heavy pellet". The two supernatant fractions were combined and centrifuged at 105 000 × *g* for 30 min to give a final supernatant fraction and a "light pellet". For subsequent studies the two sedimented pellets were resuspended in 0.003 M phosphate buffer.

In Table I is shown the diaphorase activity with DPNH and TPNH in the various fractions from several experiments. It can be seen that only the final supernatant fraction contained appreciable TPNH diaphorase activity and this was accompanied by a nearly equal DPNH diaphorase activity. Both activities were stimulated

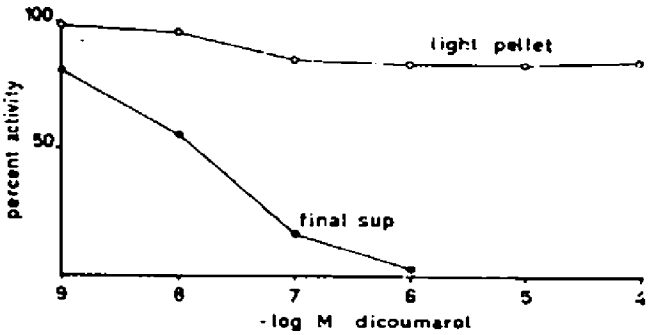

Fig. 1. Comparison of the sensitivity to dicoumarol of the final supernatant fraction and light pellet fraction from mitochondria. Preparation of the submitochondrial fractions was as described in text. Assay conditions were the same as in Table I.

by Tween and inhibited by 10^{-6} M dicoumarol in a manner typical of DT diaphorase[2,3]. In contrast, the DPNH diaphorase activities of both the "heavy" and "light" particulate fractions were unaffected by these agents. The sharp difference in dicoumarol sensitivity between the DPNH diaphorase activity of the supernatant fraction and the light pellet is shown in Fig. 1 where it may be seen that the activity of the pellet was not inhibited by even 10^{-4} M dicoumarol.

On the basis of specificity towards the pyridine nucleotides it would appear that the recovery of the DT diaphorase in the final supernatant fraction as measured by the activity with TPNH was 100% and that of the residual DPNH-specific diaphorase in the particulate fractions about 70%.

This extraction procedure appears to be more selective than the sonication procedure used by STEIN AND KAPLAN[20] which solubilized both diaphorases, according to the chromatographic patterns obtained by these authors. The various other methods of extraction which were tried in this laboratory, such as acetone treatment, freezing and thawing, hypotonic extraction, and treatment with detergents, gave either similarly non-selective extraction of both diaphorases or incomplete extraction of the DT diaphorase*.

* After precipitation of mitochondria with acetone, and subsequent extraction of the precipitate with water, about 50% of the DT diaphorase, but no DPNH diaphorase, came into solution. After freezing and thawing and subsequent extraction, 70–75% of the DT diaphorase but no DPNH diaphorase, came into solution. The DT diaphorase thus obtained, however, was not stimulated to the usual extent upon addition of Tween. After treatment with water for 2 h at 30°, about 50% of the DT diaphorase was extracted from the mitochondria together with about 40% of the DPNH diaphorase.

Purification and properties of mitochondrial DT diaphorase

DT diaphorase was purified from the final supernatant fraction of the homogenized rat-liver mitochondria by the method described for the purification of the enzyme from the soluble cytoplasm of rat liver[3]. When the mitochondrial supernatant fraction was subjected to chromatography on a DEAE-cellulose column, a single peak of diaphorase activity was obtained which contained both TPNH and DPNH diaphorase activity, as is shown in Fig. 2. This purified enzyme exhibited the same properties as the soluble-cytoplasmic enzyme, that is, inhibition by dicoumarol, activation by albumin and Tween, and the same reactivity with various electron

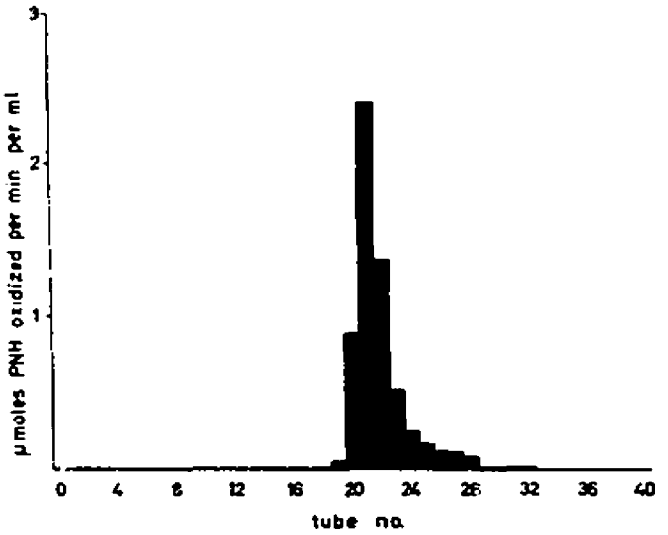

Fig. 2. Separation of the mitochondrial DT diaphorase on DEAE-cellulose column. Assay conditions were the same as in Table 1. Each fraction was tested with both DPNH and TPNH and the activities were equal within the range of 5%.

acceptors. With regard to all the properties tested the two enzymes appear to be identical. However, from a recent report of MÄRKI AND MARTIUS[21] it appears that the two enzymes may differ in certain physico-chemical properties.

Reconstitution of the vitamin K_3-mediated pathway of electron transport in submitochondrial particles

When the submitochondrial particles (the light pellet fraction) were incubated aerobically with DPNH as substrate a respiration was obtained which was sensitive to amytal (and also to antimycin A). Added vitamin K_3, as shown in the oxygen electrode record in Fig. 3, could not overcome the amytal inhibition, in contrast to the case previously described with isolated intact mitochondria[9]. However, further addition of purified DT diaphorase fully restored the respiration, and the oxygen uptake was now completely sensitive to dicoumarol.

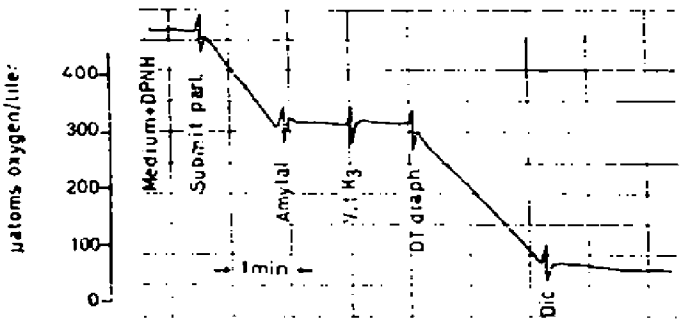

Fig. 3. Restoration of DPNH oxidation by submitochondrial particles in the presence of amytal by vitamin K_3 and DT diaphorase. The medium contained 1.0 mM DPNH, 0.07% serum albumin, and 0.05 M orthophosphate (pH 7.5). Submitochondrial particles (light pellet) from 200 mg liver were used. The amounts of the additions were as follows: 2.0 µmoles amytal, $5 \cdot 10^{-3}$ µmoles vitamin K_3, DT diaphorase capable of reducing 1 µmoles DCPIP/min, and 10^{-3} µmole dicoumarol. Final volume, 1.2 ml. Oxygen consumption was followed polarographically. Temperature, 25°.

Specific requirement for vitamin K_3 in the reactions of DT diaphorase with the respiratory chain of submitochondrial particles

The submitochondrial particles offered an opportunity to test the specificity for the quinone required in coupling the DT diaphorase to the respiratory chain free from the considerations of membrane permeability[9,22]. In Table II is shown an experiment using TPNH as substrate, added DT diaphorase, and various quinones to mediate electron transport to the light pellet fraction from liver mitochondria. It may be seen that the same specificity towards vitamin K_3 is shown in this system as was observed in the intact mitochondria[9].

Reactivity and amytal sensitivity of the respiratory chain diaphorase with various electron acceptors

Now that it seemed to be established that reduced vitamin K_3 can readily react with the submitochondrial particles the question arose why vitamin K_3 alone, without further addition of purified DT diaphorase, did not overcome the amytal inhibition in the experiment shown in Fig. 3. Does not the DPNH-specific diaphorase component of the particles react with vitamin K_3, or, if it does, is this reaction sensitive to amytal? The two considerations are relevant in view of recent reports by RINGLER *et al.*[23] about the relative unability of purified DPNH dehydrogenase from beef-heart mitochondria to react with vitamin K_3, and by SLATER *et al.*[21] who have shown that the DPNH–vitamin K_3 reductase of KEILIN AND HARTREE heart-muscle preparations is partially amytal sensitive.

In Table III is shown the ability of a variety of quinones and other electron acceptors which have been used in the study of the DT diaphorase[3] to accept electrons from the diaphorase of the residual respiratory chain. As was seen previously (Table I) DCPIP reacted well with both diaphorases studied; however, ferricyanide, which reacted only slowly with DT diaphorase, gave very high reaction rates with

TABLE II

THE EFFECT OF VARIOUS QUINONES ON THE OXIDATION OF TPNH BY SUBMITOCHONDRIAL PARTICLES IN THE PRESENCE OF DT DIAPHORASE

The basic system contained 150 μmoles orthophosphate (pH 7.5), 0.3 μmole TPNH, 2 mg serum albumin, and submitochondrial particles from 100 mg rat liver. The amounts of the additions were 0.6 mg cytochrome *c*, excess purified DT diaphorase, and $2.5 \cdot 10^{-3}$ μmoles of quinone added in 25 μl ethanol. Total volume was 3.0 ml. Temperature, 20°. Reaction followed at 340 mμ

Additions	*μmoles TPNH oxidized/min $\times 10^3$*
None	6
Cytochrome *c*	13
DT diaphorase	6
Vitamin K_3	11
DT diaphorase + vitamin K_3	178
DT diaphorase + 1,2-naphthoquinone	34
DT diaphorase + 1,4-naphthoquinone	26
DT diaphorase + 2-hydroxy-1,4-naphthoquinone	8
DT diaphorase + 2-methyl-3-hydroxy-1,4-naphthoquinone	3
DT diaphorase + *p*-benzoquinone	5
DT diaphorase + 2-methylbenzoquinone	5
DT diaphorase + 2,6-dimethylbenzoquinone	3
DT diaphorase + coenzyme Q_0	5
DT diaphorase + vitamin K_1	3
DT diaphorase + vitamin K_2	3
DT diaphorase + coenzyme Q_{10}	3

TABLE III

CAPACITY OF VARIOUS QUINONES AND OTHER COMPOUNDS TO ACT AS TERMINAL ELECTRON ACCEPTORS IN SUBMITOCHONDRIAL DPNH OXIDASE, AND THE AMYTAL SENSITIVITY OF THESE REACTIONS

The test system contained submitochondrial preparations ("light pellet") from 200 mg liver, 0.1 mM DPNH, 0.02 M phosphate buffer (pH 7.5). The acceptors were added in final concentrations of 0.04 mM, except ferricyanide which was added in final concentration of 1 mM. Oxygen uptake was blocked with 0.3 mM cyanide. Final volume 3 ml. The oxidation of DPNH was followed at 340 mμ in a recording Beckman DK-2 spectrophotometer.

Electron acceptor	*μmoles DPNH oxidized/min/g liver*		
	Without amytal	*With 2 mM amytal*	*Per cent inhibition*
Oxygen	0.13	0.03	81
Vitamin K_3	0.16	0.08	52
2-Methyl-benzoquinone	0.35	0.12	64
Coenzyme Q_0	0.36	0.22	40
1,4-Naphthoquinone	0.40	0.36	12
p-Benzoquinone	1.22	1.16	5
1,2-Naphthoquinone	1.28	1.06	18
DCPIP	0.45	0.37	18
Ferricyanide	1.10	0.90	18

the DPNH diaphorase of the respiratory chain. This is in agreement with the observations of SINGER *et al.* [23,25] on their purified DPNH dehydrogenase.

The reactivity of the various quinones was quite variable. Vitamin K_3 showed little reactivity with the respiratory chain as compared with either DCPIP or 1,4-naphthoquinone. It is of interest to observe, in fact, that the 2-methyl-substituted quinones tested had markedly lower rates of reaction than the analogous unsubstituted compounds. A distinction of the 2-methyl-substituted quinones can also be seen from the sensitivity of the reaction to amytal. The reduction of 2-methyl-substituted quinones appears to be more sensitive to amytal in terms of per cent inhibition than that of the unsubstituted quinones. This is more clearly shown in Fig. 4 with vitamin K_3 and 1,4-naphthoquinone where the inhibition of vitamin K_3

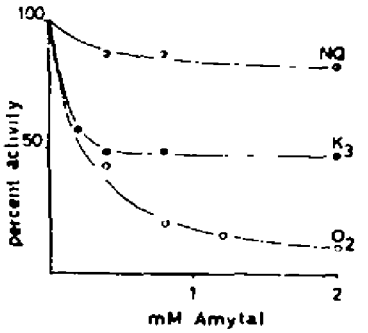

Fig. 4. Comparison of the amytal sensitivity of vitamin K_3 and 1,4-naphthoquinone as terminal electron acceptors for the submitochondrial DPNH oxidase. Conditions were the same as in Table III. In the reaction with the quinones 0.04 mM vitamin K_3 or 1,4-naphthoquinone in the presence of 0.33 mM KCN was added.

reduction reached a level of 55% as compared with only 20% with the 1,4-naphthoquinone. The respiratory chain itself was inhibited 90% as measured with oxygen under these conditions.

It thus appears that the lack of effect of vitamin K_3 alone in overcoming the amytal inhibition of the DPNH oxidase of the submitochondrial particles may be the resultant of a relatively poor reactivity of the DPNH specific diaphorase with vitamin K_3 and a partial sensitivity of this reaction towards amytal. In addition, the structural state of the preparation proved to be of importance for both its ability to react with vitamin K_3 as terminal electron acceptor and its sensitivity to amytal. In the experiment shown in Table IV, for example, the oxidation rate with vitamin K_3 was only 25% of that with oxygen; however, the former could be increased roughly to the level of the latter by freezing and thawing. The same treatment also largely abolished the sensitivity of the DPNH oxidase to amytal.

Some further properties of the submitochondrial DPNH oxidase

The light pellet from the mitochondrial fractionation resuspended in 0.003 M phosphate buffer, exhibits, as has already been mentioned, a DPNH oxidase activity.

TABLE IV

EFFECT OF FREEZING AND THAWING ON THE ACTIVITY OF THE SUBMITOCHONDRIAL DPNH OXIDASE

Conditions were the same as in Table III. The amounts of the additions were as follows: 1.0 mM amytal, 0.33 mM KCN and 0.04 mM vitamin K_3.

Additions	μmoles DPNH oxidized/min/g liver	
	Before freezing	After freezing
None	0.143	0.113
Amytal	0.033	0.090
KCN	0.033	0.040
Vitamin K_3 + KCN	0.047	0.090

Table V shows the DPNH oxidase and succinic oxidase activities of this fraction, and, in addition, the DPNH–cytochrome *c* reductase and succinic–cytochrome *c* reductase activities. The succinic oxidase activity was low, only about 30% of the DPNH oxidase activity. The succinic–cytochrome *c* reductase activity was even weaker when compared to the very high DPNH–cytochrome *c* reductase activity.

The DPNH oxidase activity was sensitive, besides amytal, also to antimycin A

TABLE V

COMPARISON OF DPNH AND SUCCINATE AS SUBSTRATES FOR THE LIVER SUBMITOCHONDRIAL FRAGMENTS PREPARED ACCORDING TO KIELLEY AND KIELLEY WITH OXYGEN AND WITH CYTOCHROME *c* AS ELECTRON ACCEPTORS

The medium for oxygen measurement contained 1.0 mM DPNH or 10.0 mM succinate, 0.03 M orthophosphate (pH 7.5), and submitochondrial preparation (light pellet) from 200 mg liver. Final volume, 1.0 ml. Oxygen consumption was followed polarographically. The medium for cytochrome *c* reductase measurement contained 0.1 mM DPNH or 10.0 mM succinate, 0.05 mM cytochrome *c*, 0.05 M orthophosphate (pH 7.5), and submitochondrial preparation (light pellet) from 200 mg liver. Final volume, 3.0 ml. Cytochrome *c* reduction was followed spectrophotometrically at 550 mμ in the presence of cyanide. Temperature, 25°.

Substrate	Activity, μ c/min/g liver	
	Oxygen	Cytochrome *c*
DPNH	0.161	0.300
Succinate	0.054	0.016

(Table VI). The addition of cytochrome *c* to this system stimulated the respiration three or four fold, and the stimulation of the respiration was largely unaffected by amytal and antimycin A. This suggests the presence of an "external" type of DPNH–cytochrome *c* reductase, earlier described in mitochondria[26–31].

The preparation exhibited no phosphorylation under any of the above conditions, addition of DT diaphorase, with or without vitamin K_3, did not alter this state of affairs. It did exhibit a high adenosine triphosphatase activity[19] (see Table VII).

TABLE VI

PROPERTIES OF DPNH OXIDASE ACTIVITY OF LIVER-MITOCHONDRIAL FRAGMENTS PREPARED ACCORDING TO KIELLEY AND KIELLEY (1953)

Conditions were the same as in Table III. The amounts of the additions were as follows: 1 mM amytal, 0.8 μg/ml antimycin A, 0.33 mM KCN, 0.01 mM cytochrome *c*

Addition	μmoles DPNH oxidized/min/g liver	
	Without cytochrome *c*	With cytochrome *c*
None	0.100	0.460
Amytal	0.033	0.233
Antimycin A	0.033	0.223
KCN	0.010	0.020

Effect of desaminothyroxine

Desaminothyroxine, which is an inhibitor of the DT diaphorase[3], was also found to strongly inhibit the submitochondrial DPNH oxidase. As is shown in Fig. 5, this inhibitor, in contrast to amytal, inhibited the diaphorase activity of the particles as measured with DCPIP as acceptor to the same extent as the oxidase activity. Interestingly, however, the DPNH cytochrome *c* reductase activity was only slightly inhibited by desaminothyroxine in these concentrations. Furthermore, when measured

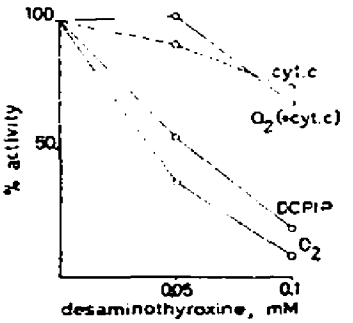

Fig. 5. Effect of desaminothyroxine on the oxidation of DPNH by submitochondrial fragments with oxygen, cytochrome *c*, and DCPIP as electron acceptors. Conditions were the same as in Table III. In the case of oxygen as electron acceptor, either no further additions were made (line marked O_2) or 0.005 mM cytochrome *c* was added (line marked O_2 (+ cyt. *c*)).

in the presence of catalytic amounts of purified cytochrome *c*, the DPNH oxidase activity of the particles was likewise only slightly inhibited. That this effect of cytochrome *c* was not due to a binding or inactivation of the inhibitor is shown in Table VII where the effect of desaminothyroxine on the adenosine triphosphatase activity of the particles (which has been shown to be sensitive to desaminothyroxine[32]) was measured in both the presence and absence of DPNH and cytochrome *c*. It may be seen that the presence of DPNH and cytochrome *c* in the medium did not alter the sensitivity of the adenosine triphosphatase activity to desaminothyroxine (nor did

TABLE VII

COMPARISON OF EFFECTS OF DESAMINOTHYROXINE ON DPNH DIAPHORASE, DPNH–CYTOCHROME *c* REDUCTASE AND ATPASE ACTIVITIES OF MITOCHONDRIAL FRAGMENTS PREPARED ACCORDING TO KIELLEY AND KIELLEY[19]

ATPase activity was determined as described in ref. 33. Other experimental conditions as in Fig. 5

Reaction	Per cent inhibition by 10^{-4} M desaminothyroxine
DPNH diaphorase (in presence of ATP and Mg^{2+})	81
DPNH–cytochrome *c* reductase (in absence of ATP and Mg^{2+})	18
DPNH–cytochrome *c* reductase (in presence of ATP and Mg^{2+})	74
ATPase (in absence of DPNH and cytochrome *c*)	84
ATPase (in presence of DPNH and cytochrome *c*)	76

the presence of ATP and Mg^{2+} alter the insensitivity of the DPNH–cytochrome *c* reductase activity). It may be added that all the quinones mentioned in Table III, and also ferricyanide, oxidized DPNH with this preparation in desaminothyroxine-sensitive way, similar to DCPIP. These findings indicate that cytochrome *c* on one hand, and the quinones, ferricyanide and DCPIP on the other, react with two separate enzymes in the preparation.

DISCUSSION

In the course of investigating the properties and function of DT diaphorase, or vitamin K reductase with which it appears to be identical[3,4], the possibility suggested by MARTIUS *et al.*[5-8] of a function for this enzyme in the normal phosphorylating respiratory chain of mitochondria was considered. Experiments reported in the preceding paper[9] on the sensitivity of DPN-linked mitochondrial respiration to dicoumarol in the presence and absence of vitamin K_3 offered strong evidence against the possibility of such a function of this enzyme. As was reported in the present paper, it has now been possible to separate the respiratory chain as a DPNH oxidase from the soluble DT diaphorase. The properties of amytal and antimycin A sensitivity, usually associated with the main phosphorylating chain of mitochondria[26,27,30,34,35], remained with the DPNH oxidase. Furthermore, with the DPNH oxidase in the absence of soluble DT diaphorase it was no longer possible to overcome the amytal inhibition of DPNH oxidation by the addition of vitamin K_3.

Study of the two diaphorases involved in these pathways suggested that while the mitochondrial DT diaphorase had properties which were identical with the cytoplasmic enzyme[2,3], that is, no specificity towards pyridine nucleotides, high sensitivity to dicoumarol, etc., the diaphorase of the respiratory chain showed properties which were in agreement with those observed by SINGER *et al.*[23,25] for the purified DPNH dehydrogenase, namely, specificity for DPNH, insensitivity towards dicoumarol, high reactivity with ferricyanide, and low reactivity with vitamin K_3. This is completely consistent with the function of the DPNH dehydrogenase, rather than the DT diaphorase, as the diaphorase of the primary respiratory chain of mitochondria.

The presence of a third pathway of electron transport from DPNH was also observed, that of the DPNH–cytochrome *c* reductase first observed by LEHNINGER[26]. This pathway was characterized by insensitivity to the respiratory inhibitors, amytal and antimycin A[26,30]. The enzymes involved in this pathway could now be further differentiated by unreactivity with quinones and by relative insensitivity towards dicoumarol and desaminothyroxine. Although the DPNH–cytochrome *c* reductase activity was found in the pellet containing the amytal and antimycin A-sensitive DPNH oxidase activity, this does not rule out the possibility of contamination with the microsomal enzyme[26] which might follow the fractionation of the pellet. Attempts to remove and recover the DPNH–cytochrome *c* reductase activity from both mitochondria and the DPNH oxidase particle by the method of JACOBS AND SANADI[31] were unsuccessful.

It may also be worthwhile to note that the entire TPNH diaphorase activity of the mitochondria can be attributed to the soluble DT diaphorase. In the residual light pellet fraction very little activity with TPNH could be determined using DCPIP, cytochrome *c*, or oxygen as the final electron acceptor. This suggests that the previously reported mitochondrial TPNH oxidase[37–41] and TPNH–cytochrome *c* reductase[42–44] activities may result from the combined interaction of transhydrogenase and the DPNH diaphorase of the respiratory chain[38,45], or from fortuitous reaction of the DT diaphorase with the respiratory chain[46], possibly through the means of a naturally occurring quinone.

Another aspect of the work reported here raises some question as to the nature of the inhibition of the respiratory chain by amytal. Spectrophotometric studies on intact mitochondria have suggested that amytal acts upon the respiratory chain between DPNH and flavoprotein[47,48]. On the other hand, however, studies on submitochondrial systems[49] have indicated that, in this case at least, amytal did not block the reduction of flavin. Furthermore, amytal inhibits the mitochondrial oxidation of choline[27], a system which does not involve DPN[50]. Recently SINGER *et al.*[23,25] have reported that the purified DPNH dehydrogenase was completely insensitive to amytal.

The work reported here confirms this latter observation on the DPNH diaphorase of an intact amytal-sensitive DPNH oxidase using DCPIP, ferricyanide, and various quinones as electron acceptors. When, however, quinones substituted with a methyl group in the 2-position of the ring were used as electron acceptor, inhibition by amytal was observed, to the extent, at least, of 40–60%, but in addition the maximal rate of oxidation was low with these quinones. These data, therefore, become open to two interpretations. The 2-methyl-substituted quinones may share the property of reacting with the flavoprotein in a manner which is sensitive to inhibition by amytal. Alternatively, it is possible that the 2-methyl-substituted quinones lack the ability to react with the flavoprotein in an amytal-insensitive manner; that is to say, these quinones, in contrast to the unsubstituted quinones, can react only via an amytal-sensitive pathway rather than directly with the flavin. The latter possibility is supported by the very low reaction rates of vitamin K_3 observed with the purified DPNH dehydrogenase[23,25]. It is impossible, however, to clearly distinguish between the two alternatives at this time. Nevertheless, in either case there may be inferred a possible significance to the 2-methyl-substituted quinones in the normal function of the DPNH diaphorase.

It must be concluded that while no evidence has been observed for the function of the vitamin K reductase of MARTIUS *et al.*[5-8] in the phosphorylating electron transport chain, the possible function of vitamin K itself has not been eliminated. A role of vitamin K in electron transport-coupled phosphorylation has been suggested not only by MARTIUS *et al.*[7,8,51], but also by work with irradiation of both bacterial systems[52,53] and liver mitochondria[54,55]. This work suggests a possible site of vitamin K function between the respiratory-chain diaphorase and cytochrome *b*. Although vitamin K has been reported absent from heart sarcosomes[56], it has been reported present in various mitochondrial preparations[57,58]. The work reported here, while open possibly to other interpretations as well, is not in disagreement with these concepts. Both the specificity in the oxidation of the reduced quinone and the apparent requirement of 2-methyl-substitution in the quinonoid ring for amytal sensitivity in the reduction of the quinone by the diaphorase of the respiratory chain would be in agreement with the location of such a quinone at this site. The question, however, must obviously remain open to further consideration.

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