4. SKF 525 A inhibited the 'activation' of schradan by cockroach guts, as had previously been shown for rat-liver preparations.

5. With mice and cockroaches *in vivo*, SKF 525 A increased the concentration of paraoxon caused by the injection of parathion, and reduced the degradation of injected paraoxon.

6. It was concluded that the diverse effects of SKF 525 A on organophosphate toxicity in various species were due to the variations in the importance of inhibition of 'activating' as opposed to degrading enzymes.

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Mitochondria of the Ehrlich Ascites-Tumour Cell

2. DIPHOSPHOPYRIDINE NUCLEOTIDE- AND TRIPHOSPHOPYRIDINE NUCLEOTIDE-DEPENDENT OXIDATION OF ISOCITRATE*

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Studies of oxidative phosphorylation in Ehrlich ascites-cell mitochondria with α -oxoglutarate, L-glutamate, succinate and ascorbate as substrates were reported by Hawtreay & Silk (1960*a*). The present paper describes studies of isocitrate oxidation in the same tumour mitochondria and amplifies the findings of a preliminary report (Hawtreay & Silk, 1960*b*).

The mechanism of isocitrate oxidation has been investigated in considerable detail in normal-cell mitochondria, but little is known about the pathways which operate in malignant cells.

In rat-liver mitochondria and rat-heart sarcomeres the cofactor of isocitric dehydrogenase has been shown to be triphosphopyridine nucleotide (Kaplan, Swartz, Frech & Ciotti, 1956; Purvis, 1958*a, b*), although Ernster (1959) maintains that a diphosphopyridine nucleotide-dependent isocitric dehydrogenase is also present. Vignais, Vignais & Bartley (1957) have reported P:O ratios for diphosphopyridine nucleotide- and triphosphopyridine nucleotide-dependent isocitric dehydrogenases in the presence of intact rat-liver mitochondria, and both diphosphopyridine nucleotide- and triphosphopyridine nucleotide-dependent enzymes have been demonstrated in ox-adrenal mitochondria (Grant & Mongkolkul, 1958).

The cofactor requirement of isocitric dehydrogenase in tumour-cell mitochondria does not appear to have been correspondingly well investi-

* Part 1: Hawtreay & Silk (1960*a*).

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gated, although Reinafarje & Potter (1957) and Williams-Ashman (1953) have demonstrated a requirement for triphosphopyridine nucleotide in the Novikoff and Ehrlich tumours respectively.

Studies concerning pyridine nucleotide metabolism associated with *isocitrate* oxidation have indicated the presence of a transhydrogenase interconverting reduced triphosphopyridine nucleotide and diphosphopyridine nucleotide in normal-cell mitochondria (Kaplan *et al.* 1956; Purvis, 1958*a, b*), but this enzyme appears to be absent from Novikoff hepatoma-cell mitochondria (Reinafarje & Potter, 1957). Williams-Ashman (1953), on indirect evidence, using acetone extracts of whole tumour cells, concluded that the transhydrogenase enzyme is also absent from the Ehrlich ascites tumour.

The present investigation was undertaken to establish the pathway of *isocitrate* oxidation directly in Ehrlich ascites-cell mitochondria and to determine the cofactor requirement of *isocitric* dehydrogenase in these particulate preparations. Pyridine nucleotide metabolism and phosphorylations associated with *isocitrate* oxidation have also been investigated.

MATERIALS AND METHODS

Ehrlich ascites tumour. This tumour was kindly supplied by Dr K. Sugiura of the Sloan-Kettering Institute, New York, and has been maintained in Swiss albino mice which were used between the seventh and tenth day after inoculation.

Hexokinase. Crystalline material was supplied by General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. The enzyme was assayed by measurement of hexose disappearance according to Somogyi (1952), and by measurement of the disappearance of acid-labile phosphorus according to Crane & Sols (1955). Preparations had an activity of approx. 150 units/mg.

Alcohol dehydrogenase. This was supplied by General Biochemicals Inc. and was assayed according to the method of Racker (1955). Preparations had a specific activity of 1.1×10^5 units.

Cytochrome c. A 1% (w/v) solution in aq. 0.09% NaCl was supplied by General Biochemicals Inc., and contained 0.587 μ mole/ml. when assayed according to Umbreit, Burris & Stauffer (1957).

Adenosine 5'-phosphate and adenosine diphosphate. These were used as supplied by General Biochemicals Inc., and by the H.M. Chemical Co. of Los Angeles, Calif., U.S.A., respectively.

Amytal. Amytal (sodium salt of 5-isoamyl-5-ethylbarbituric acid) was supplied by Eli Lilly and Co., Indianapolis, Ind., U.S.A. Solutions were prepared in water.

Antimycin A. This was obtained from the Wisconsin Alumni Research Foundation, Madison, Wis., U.S.A. Solutions were prepared in aq. 50% ethanol and were used immediately.

Diphosphopyridine nucleotide. This was obtained from General Biochemicals Inc., and was assayed 85% pure by a modification of the method of Brodie (1955) with 10 mg.

of $\text{Na}_2\text{S}_2\text{O}_4$ per 1.0 ml. of 0.2M- Na_2HPO_4 as the reducing solution.

Triphosphopyridine nucleotide. This was obtained from General Biochemicals Inc., and was assayed as 70% pure according to the method of De Moss (1955).

D-Glucose 6-phosphate. This was supplied as the barium salt by General Biochemicals Inc., and converted into the sodium salt by treatment with Na_2SO_4 according to the method of De Moss (1955).

D-Glucose 6-phosphate dehydrogenase. The enzyme was used as supplied by General Biochemicals Inc.

isoCitrate. This was prepared from DL-*isocitric* acid lactone supplied by General Biochemicals Inc. The lactone (17.4 mg.) in 0.6 ml. of 0.5N-KOH was heated on a boiling-water bath for 10 min., cooled, neutralized to pH 7.4 with dil. HCl and adjusted to a final volume of 1.0 ml. with water. Solutions were stored at -30° and used within 7 days.

Dioxan. The AnalaR grade of British Drug Houses Ltd. was purified by refluxing with excess of Na before distillation into dark-brown bottles for storage at -30° .

Ovalbumin. This was a twice recrystallized and freeze-dried sample obtained from L. Light and Co. Ltd., Colnbrook, Bucks, England.

Other chemicals. Nicotinamide, A.R. grade, was supplied by E. Merck, Germany. Ethanol, A.R. grade, and digitonin, A.R. grade, were supplied by British Drug Houses Ltd. Ordinary grades of 2,6-dichlorophenol-indophenol and 2,4-dinitrophenol were obtained from British Drug Houses Ltd. Chlorpromazine hydrochloride [2-chloro-10-(3-dimethylaminopropyl) phenothiazine hydrochloride] was supplied by May and Baker Ltd. All other chemicals were of analytical-reagent grade.

Isolation of Ehrlich ascites-cell mitochondria. These were isolated according to the procedure of Hawtre & Silk (1960*a*). The pH of the final suspension in 0.25M-sucrose was 6.9 as measured with the glass electrode.

Depletion of pyridine nucleotides. Mitochondria were depleted of their pyridine nucleotide content in one of two ways: (1) Incubation with sodium potassium phosphate buffer at 30° . To 1.6 ml. of mitochondrial suspension in 0.25M-sucrose was added 1.6 ml. of 0.25M-sucrose in 0.06M-sodium potassium phosphate buffer, pH 7.4. The mixture was gently homogenized with five passes of the tight plunger in a small Dounce homogenizer (supplied by Blassig Glass Specialties, Rochester, N.Y., U.S.A.). After homogenization, the mitochondrial suspension was incubated aerobically on a Warburg bath for 35–40 min. at 30° , and then placed immediately in ice. (2) Depletion by aging. The mitochondrial suspension in 0.25M-sucrose was aged for 5–6 hr. at 0° .

Preparation of rat-liver mitochondria depleted of pyridine nucleotides. Rat-liver mitochondria were isolated in 0.44M-sucrose according to the method of Dounce, Witter, Monty, Pate & Cottone (1955). The particles were twice washed in 0.25M-sucrose and were depleted of pyridine nucleotides by incubation in 0.05M-sucrose with 0.03M-phosphate buffer (pH 7.4) for 60 min. at 30° . A final suspension was prepared in 0.25M-sucrose and stored at 0° .

Mitochondrial-protein content. This was determined according to the biuret method of Cleland & Slater (1953) with an ovalbumin standard.

Digitonin treatment of mitochondria. Mitochondria were first depleted of pyridine nucleotides by incubation with

phosphate buffer. To 0.6 ml. of the final suspension in 0.25M-sucrose and 0.03M-phosphate buffer (pH 7.4) was added 0.6 ml. of a freshly prepared 1% digitonin solution. The preparation was thoroughly mixed and kept for 20 min. at 0° with occasional stirring. It was then assayed immediately. The mitochondria were apparently solubilized to some extent by digitonin treatment as evidenced by a clearing of the suspension.

Spectrophotometric assays with 2:6-dichlorophenol-indophenol and cytochrome c. Measurements were made in the 1 cm. cells of a Beckman Model DU spectrophotometer. The medium contained the following basic additions in a final volume of 3.0 ml.: 0.10M-sodium potassium phosphate buffer, pH 7.4; 3 mM-KCN; 3 mM-MgCl₂; 2.3 mM-DL-isocitrate (potassium salt; pH 7.4); 0.02M-nicotinamide; 36 μM-2:6-dichlorophenol-indophenol or 40 μM-cytochrome c; 1000–1500 μg. of diphosphopyridine nucleotide (DPN); 400–1000 μg. of triphosphopyridine nucleotide (TPN); Ehrlich ascites-cell mitochondria (0.20–0.31 mg. of protein) or rat-liver mitochondria (0.92 mg. of protein), both depleted of pyridine nucleotides. The following additions were made as required: reduced diphosphopyridine nucleotide (DPNH), generated from 1000 μg. of DPN by addition of 0.04 ml. of ethanol and 0.5 mg. of alcohol dehydrogenase to the medium; reduced triphosphopyridine nucleotide (TPNH), generated from 400–1000 μg. of TPN by addition of 1.7 mM-glucose 6-phosphate and 1.0 mg. of glucose 6-phosphate dehydrogenase to the medium; 0.19M-dioxan; 0.10M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)-acetate buffer (various pH values) in place of phosphate buffer; 2 mM-amytal; 0.2 mM-chlorpromazine; 0.2 mM-2:4-dinitrophenol; 5 μg. of antimycin A.

Cuvettes were examined visually after each reaction to determine whether agglutination or precipitation of the mitochondria had occurred. Reduction of 2:6-dichlorophenol-indophenol was followed at 600 mμ, and reduction of cytochrome c at 550 mμ.

Measurement of oxygen uptake and oxidative phosphorylation. The following medium in a volume of 2.36 ml. was placed in the main compartment of each Warburg flask (molarities refer to the final volume of 3.0 ml. after addition of the side-arm contents and mitochondrial suspension): 0.045M-sodium potassium phosphate buffer, pH 7.4; 34 mM-D-glucose; 15 mM-KF; 8 mM-MgCl₂; mM-adenosine 5'-phosphate (AMP); 2.5 mM-adenosine diphosphate (ADP); 2 mM-ethylenediaminetetra-acetic acid (EDTA); 0.01M-KCl; 62 mM-sucrose; 9.8 μM-cytochrome c; 3.3 mM-DL-isocitrate; 1.2 mM-DPN or TPN as required; 0.19M-dioxan as required. (All substrates and cofactors were neutralized to pH 7.4 with KOH and stored at –30° before use.) The side arms of each flask contained 70% of the total D-glucose, together with 1–3 mg. of crystalline hexokinase in a volume of 0.24 ml.

To each Warburg vessel was added 0.4 ml. of the final suspension of mitochondria depleted of pyridine nucleotides, giving a protein concentration of between 0.60 and 0.88 mg./ml. after addition of the side-arm contents. The centre well of each flask contained 0.2 ml. of 40% (w/v) KOH for CO₂ absorption, and the gas phase was air. Uptake of O₂ was measured for 25–30 min. after equilibration for 9–12 min. at 30°. The reaction was stopped by addition of trichloroacetic acid and phosphate uptake was determined by the method of Fiske & Subbarow (1925) on cold trichloroacetic acid filtrates.

RESULTS

Cofactor requirement of isocitric dehydrogenase.

This was investigated with mitochondria depleted of their endogenous pyridine nucleotides by incubation in phosphate buffer. The effects of added DPN, TPN and a mixture of DPN + TPN on isocitrate oxidation were measured with 2:6-dichlorophenol-indophenol as electron acceptor (cf. Hawtrey & Silk, 1960b; Fig. 1). *iso*Citrate was not oxidized in the absence of added DPN or TPN, as expected, with mitochondria depleted of pyridine nucleotides, but oxidation occurred rapidly in the presence of DPN and at a slower rate in the presence of TPN. The initial rate of isocitrate oxidation with a mixture of DPN + TPN together was the same as that with DPN alone, but a slight decrease was observed after about 6 min.

DPNH produced *in situ* by the alcohol dehydrogenase reaction:

was very rapidly oxidized with 2:6-dichlorophenol-indophenol as electron acceptor.

TPNH, produced *in situ* by the glucose 6-phosphate dehydrogenase reaction:

was oxidized with 2:6-dichlorophenol-indophenol as acceptor, but at a much slower rate than DPNH.

Externally produced TPNH was oxidized at approximately twice the rate of TPNH generated from isocitrate oxidation in the presence of TPN.

An important finding with regard to the question of transhydrogenase activity in Ehrlich mitochondria was that added DPN did not increase the oxidation rate of externally generated TPNH.

Results obtained with cytochrome c as electron acceptor are shown in Fig. 1. The curves occupy the same relative positions as those obtained with 2:6-dichlorophenol-indophenol, except that *iso*-citrate was more rapidly oxidized in the presence of DPN + TPN than in the presence of DPN alone. Cytochrome c reduction was initially rapid with isocitrate in the presence of DPN but the rate decreased considerably after 2 min. This was consistently observed with different batches of mitochondria. In correspondence with the results with 2:6-dichlorophenol-indophenol as electron acceptor, externally generated TPNH was oxidized at a greater rate than the TPNH resulting from isocitrate oxidation in the presence of TPN.

The effect of cofactors on isocitrate oxidation was also examined with molecular oxygen as acceptor (cf. Hawtrey & Silk, 1960b; Fig. 2). Oxygen uptake was considerably greater with DPN

as cofactor than with TPN. In the presence of DPN+TPN, *isocitrate* oxidation occurred at a slower rate than with DPN alone. This finding parallels the result with 2:6-dichlorophenol-indophenol as electron acceptor.

In contrast with the results with 2:6-dichlorophenol-indophenol and cytochrome *c* as electron acceptors, it was observed that, with molecular oxygen as terminal acceptor, externally generated TPNH was oxidized at a much slower rate than the TPNH resulting from *isocitrate* oxidation in the presence of TPN. Mitochondria containing 1.91 mg. of protein absorbed only 0.51 μ g. atom of oxygen in the presence of TPNH externally generated from 1.2 mM-TPN, whereas 3.41 μ g. atoms of oxygen were absorbed in the presence of TPNH generated from *isocitrate* and an equivalent concentration of TPN.

Variation in diaphorase activity with coenzyme concentration. Mitochondria depleted of pyridine nucleotides by incubation in phosphate buffer were assayed for diaphorase activity by using various concentrations of DPN and TPN, with 2:6-di-

chlorophenol-indophenol as electron acceptor. Fig. 2 shows that up to a concentration of 0.67 mM-DPN the rate of increase in dye reduction was proportional to the amount of cofactor present. At higher concentrations of DPN a diminishing rate of increase was observed. Fig. 3 shows that with TPN as coenzyme the rate of dye reduction became maximal at a concentration of 0.3 mM-TPN.

The effect of TPN concentration on TPN-*isocitrate*-cytochrome *c* reductase activity is shown in Fig. 4. Maximum activity occurred at a concentration of 75 μ M-coenzyme, with inhibition of dye reduction above this molarity.

An increase in DPN concentration from 0.24 mM to 2.4 mM had no effect on the oxygen uptake of mitochondria which had been depleted of pyridine nucleotides by aging for 5 hr. in sucrose at 0° (Table 1).

Since albumins are reported to have the property of adsorbing inhibitors of respiration and oxidative phosphorylation (Wojtczak & Wojtczak, 1959), the oxygen uptake of pyridine nucleotide-depleted Ehrlich mitochondria was measured with 1.2 mM-DPN as cofactor in the presence of ovalbumin (10 mg./ml. of medium). A 13% decrease in DPN-linked activity was observed (Table 1).

Effect of pH on diaphorase activity. The effect of variation in pH on the DPN- and TPN-linked

Fig. 1. Reduction of cytochrome *c* by pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria in the presence of *isocitrate* and added cofactors. Cuvettes contained 0.26 mg. of mitochondrial protein. ○, *isoCitrate* + TPN; △, TPNH; ●, *isocitrate* + DPN; □, *isocitrate* + DPN + TPN.

Fig. 2. Effect of added DPN on the rate of *isocitrate* oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria with 2:6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0.31 mg. of mitochondrial protein.

diaphorase activities associated with *isocitrate* oxidation was measured in tris-acetate buffers with 2,6-dichlorophenol-indophenol as electron acceptor. Mitochondria were depleted of pyridine nucleotides by incubation with phosphate buffer. Results in Fig. 5 show that the DPN-linked *isocitric* diaphorase has a pH optimum of 7.1 with a fall in activity on either side of this value. The TPN-linked *isocitric*-diaphorase system showed a pH optimum at 6.2 with an inflexion at pH 7.8. The activity at pH 6.2 was approximately two-thirds that of the DPN-linked diaphorase at

pH 7.1. Table 2 shows that in phosphate buffer at pH 7.4 both DPN- and TPN-dependent diaphorases were almost twice as active as in tris-acetate buffer at the same pH.

Effect of aging on diaphorase activity. Mitochondria which had been depleted of pyridine nucleotides by incubation with phosphate buffer at 30°, were aged over 84 hr. at 0° in 0.25M-sucrose plus 0.03M-phosphate buffer, pH 7.4. Samples of the mitochondrial suspension were removed, after intervals of 1, 24, 50, 72 and 84 hr., for assay of diaphorase activity with 2,6-dichlorophenol-indophenol as electron acceptor. The results in Table 3 show that DPN-dependent diaphorase activity

Fig. 3. Effect of added TPN on the rate of *isocitrate* oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria with 2,6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0.31 mg. of mitochondrial protein.

Fig. 4. Effect of added TPN on the rate of *isocitrate* oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria with cytochrome *c* as electron acceptor. Cuvettes contained 0.31 mg. of mitochondrial protein.

Table 1. *Effect of added diphosphopyridine nucleotide on isocitrate oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria*

Oxygen uptake was measured in Warburg manometers at 30°. Experimental conditions were as in the text. Ovalbumin, 10 mg./ml. of incubation medium.

System oxidized	Method of nucleotide depletion	Protein (mg./flask)	O ₂ uptake (μg.atoms)
DPN (2.4 mM) + <i>isocitrate</i>	Aging for 5 hr. in 0.25M-sucrose at 0°	2.63	7.92
DPN (0.24 mM) + <i>isocitrate</i>		2.63	8.10
DPN (1.2 mM) + <i>isocitrate</i>	Incubation with phosphate buffer at 30°	2.02	9.64
DPN (1.2 mM) + <i>isocitrate</i> + ovalbumin		2.02	8.44

Fig. 5. Effect of pH on (a) DPN-dependent and (b) TPN-dependent isocitric-dehydrogenase activity in Ehrlich ascites-cell mitochondria. The buffers were tris-acetate with 2:6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0.24 mg. of mitochondrial protein (depleted of pyridine nucleotides) and 1500 $\mu\text{g.}$ of DPN or 400 $\mu\text{g.}$ of TPN.

Table 2. *Diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent isocitric-diaphorase activities of pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria in phosphate and tris-acetate buffers*

Experimental conditions were as in the text. Cuvettes contained 0.18 mg. of mitochondrial protein and 1500 $\mu\text{g.}$ of DPN or 400 $\mu\text{g.}$ of TPN. The electron acceptor was 2:6-dichlorophenol-indophenol.

Buffer system	$\Delta E_{600 \text{ m}\mu}/10 \text{ min.}$	
	DPN + isocitrate	TPN + isocitrate
0.1 M- KH_2PO_4 - Na_2HPO_4 (pH 7.4)	0.478	0.172
0.1 M-Tris-acetate (pH 7.4)	0.283	0.100

dropped approximately 70% on storage for 84 hr. at 0° whereas that of the TPN-dependent enzyme system remained practically unchanged.

Phosphorylation associated with isocitrate oxidation. Results in Table 4 show that isocitrate oxidation is coupled to phosphorylation in the presence of both DPN and TPN as cofactor. In mitochondria completely depleted of pyridine nucleotides by incubation with phosphate buffer, P:O ratios associated with the DPN pathway lay between 1.11 and 1.31, whereas P:O ratios of 1.07 and 1.31 were measured with TPN as cofactor. P:O ratios

Table 3. *Effect of aging on the diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent isocitric-diaphorase activities of pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria*

Mitochondria were aged by storage in 0.25 M-sucrose at 0° for the times shown. Experimental conditions were as in the text. Cuvettes contained 0.26 mg. of mitochondrial protein and 1500 $\mu\text{g.}$ of DPN or 400 $\mu\text{g.}$ of TPN. The electron acceptor was 2:6-dichlorophenol-indophenol.

Time stored at 0° (hr.)	$\Delta E_{600 \text{ m}\mu}/12 \text{ min.}$	
	DPN + isocitrate	TPN + isocitrate
1	0.548	0.200
24	0.404	0.190
50	0.306	0.188
72	0.228	0.196
84	0.167	0.191

of 0.6–0.7 were measured for the DPN pathway in mitochondria depleted of pyridine nucleotides by aging for 5½ hr. in 0.25 M-sucrose at 0°. In no experiment was a P:O ratio greater than 1.31 observed for isocitrate oxidation.

Effect of respiratory-chain inhibitors and uncouplers on electron transport. The effects of amytal, chlorpromazine, 2:4-dinitrophenol and antimycin A on both DPN- and TPN-linked isocitrate oxidations were assayed with cytochrome c as electron

Table 4. *Phosphorylations associated with diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent isocitrate oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria*

Experimental conditions were as in the text. Results are shown for mitochondria depleted of endogenous pyridine nucleotides by two different methods. Temp., 30°.

Method of nucleotide depletion	Cofactor		ΔO (μg .atoms)	ΔP (μ moles)	P:O
	DPN (m-moles)	TPN (m-moles)			
Incubated with phosphate buffer for 35–40 min. at 30°, then stored at 0°	—	1.2	3.00	3.22	1.07*
	—	1.4	2.46	3.22	1.31
	1.2	—	8.24	9.68	1.18*
	1.4	—	8.17	9.07	1.11
	2.4	—	5.28	6.93	1.31
Aged for 5½ hr. in 0.25M-sucrose at 0°	2.4	—	8.22	4.65	0.57
	2.8	—	8.55	5.60	0.66

* Similar results with three other mitochondrial preparations. Two further preparations showed satisfactory oxygen uptake but were devoid of phosphorylation activity.

Table 5. *Effect of respiratory chain inhibitors and uncouplers on electron transport associated with diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent isocitrate oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria*

Experimental conditions were as in the text. Cuvettes contained 0.35 mg. of mitochondrial protein and 1.0 mg. of DPN or TPN. Values of $\Delta E_{550 m\mu}$ are shown for the initial 2 min. Curves remained practically linear over 15 min. except with antimycin A. The electron acceptor was cytochrome c.

Inhibitor or uncoupler	Concn. (mM)	$\Delta E_{550 m\mu}$ over initial 2 min.	
		DPN + isocitrate	TPN + isocitrate
Control	—	0.239	0.030
Amytal	2.0	0.162	0.020
Chlorpromazine	0.2	0.237	0.057
2:4-Dinitrophenol	0.2	0.227	0.014
Antimycin A (5 μg .)	—	—	0.003
			(→ 0.030 after 15 min.)

acceptor. Mitochondria were completely depleted of pyridine nucleotides by incubation in phosphate buffer, but were not preincubated with the compounds under test. The reaction was assayed immediately after mixing with the inhibitor or uncoupler concerned. Curves were linear during the first 2 min. and remained practically so over 15 min. except in the case of antimycin A. Table 5 shows that 2 mM-amytal caused a 30 % decrease in both DPN- and TPN-dependent isocitrate oxidation. Chlorpromazine at a concentration of 0.2 mM had no effect on DPN-linked isocitrate oxidation, but caused an approximately twofold increase in TPN-dependent diaphorase activity. The uncoupling agent 2:4-dinitrophenol was also without effect on the DPN diaphorase, but decreased the activity of TPN-linked isocitrate oxidation by approximately 50 %. Antimycin A was tested only on the TPN-dependent system, which it failed to inhibit except during an initial period of 2 min. Thereafter cytochrome c reduction increased until the control value was reached after 15 min.

With 2:6-dichlorophenol-indophenol as electron acceptor, 2 mM-amytal had no effect on the rate of DPN-dependent isocitrate oxidation.

Effect of dioxan on electron transport. Oestrone and oestradiol have been shown to act as cofactors for the transhydrogenase enzymes in the high-speed supernatant fraction of rat-liver homogenates (Talalay & Hurlock, 1958). Solutions of these steroids in dioxan stimulated diaphorase activity associated with both DPN- and TPN-linked isocitrate oxidation in Ehrlich mitochondria, but to no greater extent than an equivalent concentration of pure dioxan. The effect of this solvent on isocitrate oxidation was accordingly examined in greater detail with mitochondria depleted of pyridine nucleotides by incubation in phosphate buffer.

Fig. 6 shows the stimulating effect of pure dioxan on both DPN- and TPN-diaphorase activities with 2:6-dichlorophenol-indophenol as electron acceptor. The TPN-dependent activity was enhanced to a greater extent than the DPN-dependent. Separate experiments showed that maximum stimulation of activity occurred at a concentration of 0.19M-dioxan.

Table 6 shows that, in contrast with the results with 2:6-dichlorophenol-indophenol as electron acceptor, dioxan caused a considerable lowering in

the activities of both DPN- and TPN-linked *isocitrate* oxidations when either cytochrome *c* or molecular oxygen was used as the terminal electron acceptor. This difference in effect is illustrated in Fig. 7 with reference to TPN-linked *isocitrate* oxidation. With 0.19M-dioxan, which affords maximum TPN-diaphorase activity when 2,6-dichlorophenol-indophenol is the electron acceptor, the activity with oxygen as acceptor was at its lowest level.

The effect of dioxan on *isocitrate* oxidation in normal rat-liver mitochondria was investigated for comparison with its effect on Ehrlich tumour mitochondria. The rat-liver mitochondria were depleted of pyridine nucleotides by incubation in phosphate buffer.

Fig. 6. Effect of 0.19M-dioxan on (a) TPN-dependent and (b) DPN-dependent *isocitrate* oxidation in Ehrlich ascites-cell mitochondria with 2,6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0.20 mg. of mitochondrial protein (depleted of pyridine nucleotides) and 1000 μ g. of DPN or 400 μ g. of TPN. $\circ$, Dioxan present; $\bullet$, dioxan absent.

Table 6: Effect of dioxan on diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent *isocitrate* oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria with cytochrome *c* and molecular oxygen as terminal electron acceptors

Experimental conditions were as in the text. Cuvettes contained: 40 μ M-cytochrome *c*; 0.20 mg. of mitochondrial protein; 1000 μ g. of DPN or TPN; and 0.19M-dioxan. Oxygen uptake was measured in Warburg manometers at 30°. Flasks contained: 1.90 mg. of mitochondrial protein; 1.2 mM-DPN or -TPN; and 0.19M-dioxan.

Terminal electron acceptor...	Cytochrome <i>c</i>	Oxygen
System oxidized	$\Delta E_{550m\mu}/$ 2 min.	(μ g.atoms/mg. of protein/hr.)
DPN + <i>isocitrate</i>	0.165	10.13
DPN + <i>isocitrate</i> + dioxan	0.052	4.13
TPN + <i>isocitrate</i>	0.024	3.21
TPN + <i>isocitrate</i> + dioxan	0.010	1.70

Table 7 shows that with 2,6-dichlorophenol-indophenol as electron acceptor, 0.19M-dioxan caused a depression of 40 % in diaphorase activity associated with TPN-dependent *isocitrate* oxidation. The rate of dye-reduction in the presence of DPN was the same as the control value for *isocitrate* without cofactors, and was unaffected by

Table 7. Effect of dioxan on *isocitrate* oxidation in pyridine nucleotide-depleted rat-liver mitochondria with 2,6-dichlorophenol-indophenol as electron acceptor

Preparation of mitochondria and experimental conditions were as in the text. Cuvettes contained: 0.92 mg. of mitochondrial protein; 400 μ g. of DPN or TPN; and 0.19M-dioxan. TPNH was externally generated from 400 μ g. of TPN by the glucose 6-phosphate-dehydrogenase reaction.

System oxidized	$\Delta E_{600 m\mu}/8$ min.
Control. <i>isocitrate</i> (no cofactor)	0.010
DPN + <i>isocitrate</i>	0.016
DPN + <i>isocitrate</i> + dioxan	0.018
TPN + <i>isocitrate</i>	0.380
TPN + <i>isocitrate</i> + dioxan	0.223
DPN + TPN + <i>isocitrate</i>	0.464
DPN + TPN + <i>isocitrate</i> + dioxan	0.514
TPNH	0.386
TPNH + dioxan	0.483

Fig. 7. Effect of dioxan concentration on TPN-dependent *isocitrate* oxidation in Ehrlich ascites-cell mitochondria with 2,6-dichlorophenol-indophenol ($\circ$) and molecular oxygen ($\bullet$) as electron acceptors. Cuvettes contained 0.20 mg. of mitochondrial protein (depleted of pyridine nucleotides) and 400 μ g. of TPN. Oxygen uptake was measured in Warburg manometers containing 1.2 mM-TPN.

addition of dioxan. Addition of DPN to the TPN-dependent isocitrate-oxidase system caused a 21 % increase in diaphorase activity, which was enhanced a further 14 % by addition of 0.19M-dioxan. Externally added TPNH was oxidized at the same rate as TPNH generated from isocitrate oxidation in the presence of TPN, but dioxan, in contrast with its depressant effect on TPN-dependent isocitrate oxidation, caused a stimulation of 25 % in the oxidation rate of externally added TPNH.

Effect of digitonin on electron transport. Both DPN- and TPN-linked isocitrate-diaphorase activities were assayed in digitonin-treated mitochondria with 2:6-dichlorophenol-indophenol as electron acceptor. The results are shown in Fig. 8, together with control curves obtained with the same batch of mitochondria which had been depleted of pyridine nucleotides but which had not been treated with digitonin.

Digitonin treatment stimulated DPN-dependent isocitrate-diaphorase activity, but was without effect on the TPN-linked system.

Fig. 8. Effect of digitonin on DPN-dependent and TPN-dependent isocitrate oxidation in Ehrlich ascites-cell mitochondria with 2:6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0.29 mg. of mitochondrial protein (depleted of pyridine nucleotide content and pre-treated with digitonin) and 1500 μ g. of DPN or 400 μ g. of TPN. ●, DPN and digitonin; ○, DPN; Δ, TPN with and without digitonin.

DISCUSSION

It may be concluded from this study that mitochondria of the Ehrlich ascites cell possess both DPN- and TPN-dependent isocitric-dehydrogenase enzyme systems. This is in contrast with rat-liver mitochondria, which have been shown to contain only a TPN-specific isocitric dehydrogenase (Table 7) in agreement with Kaplan *et al.* (1956) and with Purvis (1958*a, b*). Of the two isocitrate oxidation pathways in Ehrlich mitochondria, the DPN-dependent system is some three to four times as active as its TPN counterpart. This may possibly be connected with the observation that TPN concentrations are very low in tumour cells [Wenner & Weinhouse (1953) and Glock & McLean (1957)].

The observation that isocitrate oxidation with all electron acceptors is more rapid in the presence of DPN + TPN than in the presence of TPN alone, probably represents an additive effect since separate DPN- and TPN-linked pathways exist in Ehrlich mitochondria. If separate pathways did not exist, then it would be necessary to postulate the operation of a transhydrogenase enzyme transferring hydrogen from TPNH to DPN, yielding DPNH, which is very rapidly oxidized (cf. Hawtrey & Silk, 1960*b*; Fig. 1). Since addition of DPN did not stimulate the oxidation rate of externally added TPNH (Hawtrey & Silk, 1960*b*), it may be concluded that the transhydrogenase pathway is either absent or of little importance in Ehrlich mitochondria. The necessity for a pyridine nucleotide transhydrogenase is apparently obviated by the existence of a direct pathway to oxygen for reoxidation of TPNH generated from TPN during isocitrate oxidation. On different experimental grounds Williams-Ashman (1953) also concluded that transhydrogenase activity is absent in Ehrlich cells, whereas Reinafarje & Potter (1957) failed to detect the enzyme in Novikoff hepatoma cells. Findings regarding transhydrogenase activity in a variety of cell types have been summarized by Kit & Griffin (1958).

Since external TPNH is more rapidly oxidized with 2:6-dichlorophenol-indophenol and cytochrome *c* as electron acceptors than TPNH generated from TPN during isocitrate oxidation, it would seem that the dehydrogenase reaction is rate-limiting in TPN-dependent oxidation. Since the oxidation rates of external and isocitrate-generated TPNH are both lower with cytochrome *c* as acceptor than with 2:6-dichlorophenol-indophenol, it would seem that a further rate-limiting step may occur between cytochrome *c* and the flavin at which 2:6-dichlorophenol-indophenol accepts electrons. However, since with molecular oxygen as acceptor, external TPNH is much more slowly oxidized than isocitrate-generated TPNH, it

may well be that separate oxidation pathways exist for externally and internally generated TPNH.

Wenner and associates have shown that intact Ehrlich ascites cells do not oxidize externally generated TPNH with molecular oxygen as terminal electron acceptor (Wenner, Hackney & Herbert, 1957; Wenner, Hackney & Moliterno, 1958; Wenner & Hackney, 1959; Wenner, 1959). From studies of the hexose monophosphate shunt pathway these authors concluded that pyruvate is the natural electron acceptor for TPNH oxidation in the tumour cell. The existence of a TPNH-cytochrome *c* reductase in the Novikoff hepatoma has been demonstrated by Birns, Essner & Novikoff (1959).

The rate of *isocitrate* oxidation with DPN as cofactor remained linear over 10 min. with 2:6-dichlorophenol-indophenol as electron acceptor, and over 22 min. with molecular oxygen as acceptor (Hawtreys & Silk, 1960*b*). However, with cytochrome *c* as acceptor, DPN-linked oxidation was strongly inhibited after an initial period of 2 min. (Fig. 1). This may be due to inhibition of further cytochrome *c* reduction by an accumulation of reduced cytochrome *c*. Alternatively, it may be due to reoxidation of reduced cytochrome *c* despite cyanide blocking of cytochrome oxidase. This could establish a steady level, in which reduction is balanced by oxidation.

The amount of coenzyme required to saturate both the DPN- and TPN-dependent *isocitric* dehydrogenases of Ehrlich mitochondria varied according to the electron acceptor used. Thus with 2:6-dichlorophenol-indophenol as acceptor, 1.35 mM-DPN afforded maximum enzyme activity (Fig. 2), but with molecular oxygen as acceptor only 0.24 mM-DPN was required to attain maximal respiration (Table 1). Similarly with TPN, a concentration of 0.3 mM was adequate to saturate the system with 2:6-dichlorophenol-indophenol as electron acceptor (Fig. 3), whereas only 75 μ M-TPN was required to afford maximum activity with cytochrome *c* as terminal acceptor (Fig. 4).

The existence of separate DPN- and TPN-dependent *isocitric* dehydrogenases in Ehrlich mitochondria was corroborated by determination of pH-activity curves for the two enzymes in tris-acetate buffers with 2:6-dichlorophenol-indophenol as electron acceptor. The DPN-dependent enzyme exhibited a pH optimum of 7.1 with a fall in activity on either side of this value, whereas the TPN-dependent system showed a pH optimum at 6.2 with an inflexion at pH 7.8 (Fig. 5). Rat-liver lactic dehydrogenase exhibits similar properties (Navazio, Ernster & Ernster, 1957) in that the enzyme is both DPN- and TPN-dependent, with pH optima of 7.6 and 6.0 for the two cofactors respectively. The difference between the diaphorase

activities of Ehrlich mitochondria in phosphate and tris-acetate buffers is not readily explicable, but is not without parallel (Silk, Hawtreys & Macintosh, 1958).

A further distinction between the DPN- and TPN-linked *isocitric*-dehydrogenase systems in Ehrlich mitochondria was established by aging experiments. Two-thirds of the DPN-diaphorase activity was lost on 84 hr. storage at 0°, whereas the TPN-linked system was little affected under the same conditions (Table 3). Plaut & Sung (1954) have reported that the DPN-dependent *isocitric* dehydrogenase of animal tissues is much more labile than the corresponding TPN-dependent enzyme. However, Williams-Ashman (1953) has observed a decrease in TPN-linked *isocitric* dehydrogenase activity on storage of a bicarbonate extract prepared from acetone-dried powders of whole Ehrlich ascites cells.

Phosphorylation is apparently coupled to electron transport during both DPN- and TPN-dependent *isocitrate* oxidation, but very low P:O ratios were observed for this process. The highest measured was 1.31 (Table 4). In rat-liver mitochondria and heart sarcosomes, phosphorylation occurs only after transfer of electrons from TPNH to DPN via a transhydrogenase (Kaplan *et al.* 1956), but in Ehrlich mitochondria, which do not contain a transhydrogenase, phosphorylation is associated with the direct TPNH to oxygen pathway (Table 4).

The similar inhibitory action of amytal on both DPN- and TPN-cytochrome *c*-reductase activities in Ehrlich mitochondria might indicate an effect on a common flavin (Table 5). The failure of amytal to affect DPN-linked diaphorase activity with 2:6-dichlorophenol-indophenol as electron acceptor is contrary to its effect on intact rat-liver mitochondria, where, at the same concentration, it is reported to block DPN-linked oxidations completely through an action on the electron-transfer chain between DPN and flavoprotein (Purvis, 1958*b*; Ernster, Jalling, Löw & Lindberg, 1955; Chance, 1956).

Chlorpromazine exerts a stimulating effect on TPN-dependent *isocitrate* oxidation in Ehrlich mitochondria, but has no effect on DPN-linked oxidation (Table 5). In rat-liver mitochondria, chlorpromazine inhibits DPN-linked oxidations by 50% when cytochrome *c* is used as electron acceptor (Dawkins, Judah & Rees, 1959).

The effect of 2:4-dinitrophenol on TPN-cytochrome *c*-reductase activity is in keeping with its action on this system isolated from bovine heart muscle (Lang & Nason, 1959), but its lack of effect on the DPN-dependent system is unexpected (Table 5).

The failure of antimycin A to inhibit TPN-dependent activity, except during an initial period of 2 min., is in contrast with its effect on most

normal-cell mitochondria, in which it acts as a potent blocker of electron transport (Chance, 1956). The unusual effect in Ehrlich mitochondria may be due to inactivation of the antimycin A. The TPN-cytochrome *c* reductase isolated from bovine heart muscle by Lang & Nason (1959) is, however, insensitive to antimycin A. The effects of dioxan on electron transport in Ehrlich mitochondria are the opposite of its effects on normal rat-liver mitochondria. Thus with 2:6-dichlorophenol-indophenol as electron acceptor, dioxan enhanced both DPN- and TPN-dependent *isocitric*-dehydrogenase activities in the tumour particulates (Fig 6), but depressed TPN-dependent diaphorase activity in rat-liver mitochondria, which do not contain a DPN-dependent enzyme (Table 7). The effect of dioxan apparently depends upon the electron acceptor used, since it depressed both the DPN- and TPN-linked *isocitric*-dehydrogenase activities of Ehrlich mitochondria, with either cytochrome *c* or molecular oxygen as terminal acceptor (Table 6, Fig. 7). In normal rat-liver mitochondria, dioxan caused a stimulation of *isocitric*-dehydrogenase activity in the presence of DPN + TPN, and also an increase in the oxidation rate of externally generated TPNH, although it depressed the oxidation rate of TPNH generated from *isocitrate* oxidation in the presence of TPN. The action of dioxan may be similar to that of acetone treatment, which Ernster (1958) has found to enhance the TPN-diaphorase activity of rat-liver mitochondria.

Digitonin has been used by Kaplan, Colowick & Neufeld (1953) to extract transhydrogenase activity in a non-sedimentable form from bovine heart mitochondria, whereas Stein, Kaplan & Ciotti (1959) have shown that digitonin causes an increase in the transhydrogenase activity of pigeon-liver mitochondria. Vignais & Vignais (1957) have reported similar effects of digitonin on reaction rates in mitochondria. The DPN-dependent *isocitric*-dehydrogenase activity of Ehrlich mitochondria was enhanced by digitonin treatment, which was without effect on the TPN-dependent diaphorase (Fig. 8).

The effects of digitonin, dioxan, amytal, chlorpromazine, 2:4-dinitrophenol and antimycin A on electron transport in Ehrlich mitochondria require further study with a variety of electron acceptors before the mechanism of *isocitrate* oxidation in these tumour particulates will be fully understood.

SUMMARY

1. Ehrlich ascites-cell mitochondria possess both diphosphopyridine nucleotide- and triphosphopyridine nucleotide-dependent *isocitric* dehydrogenases, in contrast with rat-liver mito-

chondria, which contain only the triphosphopyridine nucleotide-specific enzyme. Both dehydrogenase systems in Ehrlich mitochondria are linked to oxygen and the diphosphopyridine nucleotide-dependent enzyme is some three to four times as active as its triphosphopyridine nucleotide-specific counterpart.

2. Pyridine nucleotide transhydrogenase is completely absent from Ehrlich ascites-cell mitochondria.

3. With 2:6-dichlorophenol-indophenol and cytochrome *c* as electron acceptors, externally added reduced triphosphopyridine nucleotide is more rapidly oxidized than reduced triphosphopyridine nucleotide generated from *isocitrate* oxidation in the presence of triphosphopyridine nucleotide. With molecular oxygen as acceptor, externally added reduced triphosphopyridine nucleotide is oxidized more slowly than *isocitrate*-generated reduced triphosphopyridine nucleotide. Thus the dehydrogenase reaction appears to be rate-limiting in triphosphopyridine nucleotide-dependent *isocitrate* oxidation, whereas a further rate-limiting step may occur between cytochrome *c* and the flavin at which 2:6-dichlorophenol-indophenol accepts electrons.

4. The dependence of *isocitric*-dehydrogenase activity on coenzyme concentration has been determined with 2:6-dichlorophenol-indophenol, cytochrome *c* and molecular oxygen as electron acceptors.

5. Determination of pH-activity curves in tris buffers showed that diphosphopyridine nucleotide-dependent *isocitric* dehydrogenase has a pH optimum of 7.1 with a fall in activity on either side of this value, whereas the triphosphopyridine nucleotide-dependent system has a pH optimum of 6.2 with an inflexion at pH 7.8.

6. Mitochondria stored for 84 hr. at 0° lose two-thirds of their diphosphopyridine nucleotide-diaphorase activity, whereas triphosphopyridine nucleotide-diaphorase activity is unaffected.

7. Electron transfer resulting from both diphosphopyridine nucleotide and triphosphopyridine nucleotide-dependent *isocitrate* oxidation is coupled to phosphorylation, but it was not possible to obtain P:O ratios higher than 1.31.

8. The effects of digitonin, dioxan, amytal, chlorpromazine, 2:4-dinitrophenol and antimycin A on electron transport in Ehrlich ascites-cell mitochondria have been determined with 2:6-dichlorophenol-indophenol, cytochrome *c* or molecular oxygen as terminal acceptors. The effects have been contrasted with those on normal rat-liver mitochondria.

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A Determination of K_s , the Substrate Constant, for Serum Cholinesterase

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K_s , the substrate constant (Dixon & Webb, 1958), has not previously been determined for esterases. Slater (1955) has suggested that if the constant k_3 , governing the rate of breakdown of the enzyme-substrate complex, can be varied independently of K_s , then the plot of K_m against V , the maximum reaction velocity, will give K_s on extrapolation to the K_m axis. In this paper the alcohol-

activation and reversible inhibition of the hydrolysis of certain nitrophenyl carboxylic esters by human serum cholinesterase have been employed to evaluate K_s by the use of the suggestions, but not the procedure, of Slater (1955).

Knowledge of K_s is particularly important when evaluating the substrate-specificity pattern of esterases since it is K_s , and not K_m , that is a