Mitochondria of the Ehrlich Ascites-Tumour Cell

ISOLATION AND STUDIES OF OXIDATIVE PHOSPHORYLATION

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Isolation of mitochondria from the Ehrlich ascites tumour is complicated by the fact that these cells have an extremely tough membrane which is not easily ruptured. The tumour cells can be disrupted by the application and sudden release of gas pressure (Nirenberg & Hogg, 1956), or by grinding in sucrose solution with all-glass or Teflon Potter homogenizers (Kun, Talalay & Williams-Ashman, 1951; Mutolo & Abrignani, 1958), or by vigorous shaking with quartz sand (Lindberg, Ljunggren, Ernster & Rēvész, 1953). These methods are, however, tedious and do not result in complete homogenization. Cytoplasmic material with included mitochondria remains attached to the nuclei in such sucrose homogenates, and hence a certain amount of the mitochondrial fraction is lost during initial centrifuging to remove the nuclei. Sonic oscillation has been applied to disrupt Ehrlich ascites cells (Davidson, Smellie, Keir & McArdle, 1958), but, since this technique may result in damage to the mitochondria, homogenization methods are to be preferred.

Unpublished work by E. Stotz and co-workers has shown that the membrane of ascites cells can be easily ruptured with a Dounce homogenizer (Dounce, Witter, Monty, Pate & Cottone, 1955) if these cells are previously swollen for 30–60 min. in diluted (1:3) Ringer-phosphate solution. The technique of lysing the cells into water has been applied by Littlefield & Keller (1957) to isolation of the microsome fraction from Ehrlich ascites-tumour cells.

In our experiments the cell membranes were pre-stressed in hypotonic solution and the cells subsequently disrupted into pure water by osmotic shock. Immediately after lysis, sufficient concentrated sucrose solution was added to the homogenate to give the usual 0.25 M-sucrose suspension, from which mitochondria were isolated in the customary manner. The method results in clean nuclei and mitochondria free of cytoplasmic material.

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Unlike liver mitochondria, those of the Ehrlich ascites cells do not appear to be damaged by contact over short periods with hypotonic solutions. With our method we have obtained preparations which show oxidative-phosphorylation activity with succinate, α -oxoglutarate and glutamate as substrates.

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 ninth and twelfth day after inoculation.

Hexokinase. Crystalline material was supplied by General Biochemicals Inc., 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 were found to have an activity of approximately 150 units/mg.

Cytochrome c. A 1% (w/v) solution in aq. 0.09% NaCl was supplied by General Biochemicals Inc., and found to contain 5.87×10^{-7} moles/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, California, respectively.

Adenosine triphosphate. This was used as the tetrasodium salt supplied by General Biochemicals Inc., and assayed 85% pure by the method of Crane & Sols (1955).

Diphosphopyridine nucleotide. This was obtained from General Biochemicals Inc., and was assayed 85% pure by a modification of the method of Brodie (1955) using 10 mg. of sodium dithionite/1.0 ml. of 0.2 M-disodium hydrogen phosphate as the reducing solution.

All other substrates and chemicals used were of analytical reagent grade.

Isolation of mitochondria. All operations were carried out at 0–4°. Mice were killed by cervical fracture and ascites fluid was withdrawn from the peritoneal cavity with a sterile syringe. The fluid was mixed immediately with sodium citrate solution [1 ml. of 3.8% (w/v) sodium citrate/10.0 ml.] and stored in ice. The cells were collected by centrifuging at 1800 g for 10 min. and washed once or twice by resuspending in iso-osmotic NaCl solution. They were then dispersed in hypo-osmotic ($\frac{1}{4}$ strength) Ringer-phosphate solution and allowed to stand 15–20 min. in the cold with occasional stirring. Phase-contrast microscopy at

this stage showed the ascites cells to be swollen but not lysed. Contaminating erythrocytes were completely lysed. The suspension was then centrifuged at 1800 *g* for 10 min. and the supernatant discarded. The cells (6–8 g. wet wt.) were immediately resuspended in ice-cold water (60 ml.) for exactly 3–0 min. by using a Dounce homogenizer with loose plunger (clearance approx. 0.001 in.) as supplied by Blaessig Glass Specialities, Rochester, N.Y., U.S.A. [To ensure complete lysis and removal of adhering cytoplasm from the nuclei, it is important to use 8–10 vol. of water/g. (wet wt.) of cells.] Concentrated sucrose solution was then quickly added to give the usual 0.25 *M*-sucrose suspension, which was further homogenized with 6–8 passes of the loose plunger, followed by 15 passes with the tight plunger (clearance approx. 0.0005 in.). Phase-contrast microscopy at this stage revealed a 90–95% homogenate in which the nuclei were free of cytoplasmic material. The pH of the homogenate measured at 25° with the glass electrode was 7.1.

Nuclei and cell debris were removed by centrifuging at 600–700 *g* for 20 min. The supernatant was carefully withdrawn and centrifuged 10 min. at 12 000–13 000 *g* in the high-speed head of the M.S.E. Major Refrigerated Centrifuge. The sediment from this centrifuging consisted of a firmly packed mitochondrial pellet with a so-called 'fluffy' layer loosely packed above it. The mitochondrial pellet was resuspended in 0.25 *M*-sucrose by using the homogenizer and again centrifuged for 10 min. at 12 000 *g*. Two such washings effectively removed the 'fluffy' layer and gave a uniform mitochondrial pellet which was finally resuspended in 0.25 *M*-sucrose [approx. 5–6 ml./g. (wet wt.)] equivalent to a concentration of 3–11 mg. of mitochondrial protein/ml. The pH of the final suspension measured with the glass electrode was 6.9. (In certain cases the mitochondrial suspension after the first washing was recentrifuged at 600–700 *g* for 10 min. to ensure complete removal of nuclei.)

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

Measurement of 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 con-

tents and mitochondrial suspension): 0.045 *M*-sodium potassium phosphate buffer, pH 7.4; 15 *mm*-KF; 8 *mm*-MgCl₂; 25 *mm*-adenosine diphosphate (ADP); *mm*-adenosine 5'-phosphate (AMP); 2 *mm*-ethylenediaminetetraacetic acid (EDTA); 34 *mm*-D-glucose; 0.01 *M*-KCl; 62 *mm*-sucrose; 0.24 *mm*-diphosphopyridine nucleotide (DPN); 7 *mm*-substrate; 9.8 *μm*-cytochrome *c*. (In measurement of α -oxoglutarate oxidation 0.02 *M*-malonate was added as an inhibitor. AMP, ADP and all substrates 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 a mitochondrial suspension in 0.25 *M* sucrose, giving a concentration of 0.4–1.5 mg. of protein/ml. of incubation medium 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. Oxygen uptake was measured for 25–30 min. after 13 min. equilibration at 30°. Phosphate uptake was determined by the method of Fiske & Subbarow (1925), on cold trichloroacetic acid filtrates. The oxygen uptake of the mitochondria (*q*_{O₂}) was expressed as μ l. of O₂/mg. of protein/hr. according to Slater & Holton (1954).

RESULTS

The results in Table 1 indicate that once-washed mitochondria have a low protein content and show low P:O ratios with succinate and α -oxoglutarate as substrates. The oxygen uptake per mg. of protein of once-washed mitochondria is, however, comparable with that of twice-washed mitochondria and is even higher when succinate is used as substrate. The yield of once-washed mitochondria corresponds to 6–8% of the wet weight of whole cells, while the yield of twice-washed material is equivalent to 4–6%. A second washing results in a uniform mitochondrial pellet free of 'fluffy' layer. Twice-washed mitochondria were thus used for the experiments recorded in Tables 2 and 3. A force of 12 000 *g* was found optimum for

Table 1. *Effect of washing on yield and oxidative phosphorylation of Ehrlich ascites-cell mitochondria*

Each flask contained the following in a total volume of 3.0 ml.: mitochondrial suspension in 0.25 *M*-sucrose, 0.4 ml.; sodium potassium phosphate buffer (pH 7.4), 45 *mm*; D-glucose, 34 *mm*; KF, 15 *mm*; MgCl₂, 8 *mm*; ADP, 2.5 *mm*; AMP, *mm*; EDTA (pH 7.4), 2 *mm*; KCl, 0.01 *M*; sucrose, 67 *mm* (with succinate as substrate), or 62 *mm* (with α -oxoglutarate as substrate); succinate, 0.01 *M*, or α -oxoglutarate, 6.6 *mm*; cytochrome *c*, 9.8 *μm*; 1–2 mg. of crystalline hexokinase. (In experiments with α -oxoglutarate as substrate, malonate (0.02 *M*), DPN (2.4 *mm*) and nicotinamide (0.02 *M*) were added in addition.) Temp. 30°. Equilibration time 13 min. Manometric flasks were maintained at 0° before each experiment.

No. of washings	Mitochondria		Succinate		α -Oxoglutarate	
	Yield [% (wet wt.) of whole cells]	Protein content [mg./g. (wet wt.)]				
			P:O	<i>q</i> _{O₂}	P:O	<i>q</i> _{O₂}
1	6–8	14.6	0.26	146.0	0.82	46.8
2	4–6	26.0	1.44 ± 0.20*	105.9 ± 22.2*	2.38†	53.8†

* Average of thirteen different mitochondrial preparations.

† Average of two different mitochondrial preparations.

obtaining a firmly packed mitochondrial pellet with high protein content. The final mitochondrial suspension in 0.25M-sucrose was apparently homogeneous when examined under the phase-contrast microscope.

Mitochondrial preparations showed q_{O_2} values in the range 77–149 with succinate as substrate (Table 2). A final concentration of 0.24 mM-DPN was present in each flask, although this is probably not required for the oxidation of succinate. The P:O ratios with succinate as substrate were measured for 15 of the mitochondrial preparations by using an active hexokinase and were found to lie within the range 1.01–1.82. Six of the preparations showed P:O ratios in the range 1.54–1.82.

With ascorbate, mitochondria showed q_{O_2} values in the range 54–66, but it proved impossible to measure P:O ratios with this substrate. Inorganic phosphate was liberated, instead of esterified, possibly owing to the operation of the myokinase reaction combined with a considerable degree of uncoupling at the cytochrome *c* level of oxidative phosphorylation. Similar results were obtained with or without the addition of 0.24 mM-DPN. In each experiment with ascorbate, a control determination was carried out on the same batch of mitochondria with succinate as substrate. This was not only to ensure the functional integrity of the mitochondrial preparation, but to provide additional information regarding the phosphorylation site(s) which appear to be uncoupled in tumour-cell mitochondria.

In experiments with α -oxoglutarate as substrate (Table 3), mitochondria showed q_{O_2} values of approximately 30 in media unfortified with DPN. Addition of 0.24 mM-DPN had little effect on the q_{O_2} , but 2.4 mM-DPN (with 0.02M-nicotinamide) raised the q_{O_2} by approximately 100%, with a concomitant increase in P:O ratio. It is noteworthy that 0.02M-nicotinamide alone raised the P:O ratio, but was apparently without effect on the q_{O_2} in any experiment.

A similar DPN effect was observed with L-glutamate as substrate (Table 3). Addition of 0.24 mM-DPN to the medium raised the q_{O_2} by 115%, while a 400% increase was observed on addition of 1.2 mM-DPN with 0.02M-nicotinamide. The P:O ratio was also increased by addition of 1.2 mM-DPN with 0.02M-nicotinamide, but the enhancement was less than was observed on similar fortification of the medium with α -oxoglutarate as substrate.

Fig. 1 shows the change in oxygen uptake per mg. of protein of various mitochondrial preparations with four different substrates over experimental periods of 30–35 min. Curves are included to show the effect of DPN and nicotinamide additions in the oxidation of α -oxoglutarate and L-glutamate.

Table 2. *Oxidation and associated phosphorylations with succinate and ascorbate respectively as substrates*

Each flask contained the following in a total volume of 3.0 ml.: mitochondrial suspension in 0.25 M-sucrose, 0.4 ml.; sodium potassium phosphate buffer (pH 7.4), 0.45 mm; D-glucose, 34 mm; KF, 15 mm; $MgCl_2$, 8 mm; ADP, 2.5 mm; AMP, mm; EDTA (pH 7.4), 2 mm; KCl, 0.01 M; sucrose, 67 mm (in experiments without DPN), or 62 mm (in experiments with DPN); succinate, 0.01 M, or ascorbate, 6.6 mm; cytochrome c, 9.8 μ M; 1-2 mg. of crystalline hexokinase. Temp. 30°. Equilibration time 12-15 min. Manometric flasks were maintained at 0° before each experiment.								
Expt.	DPN additions (m-moles)	Incubation time (min.)	Wt. of protein/flask (mg.)	ΔO (μ g. atom)	ΔP_i (μ mole)	P:O	q_{O_2}	Remarks
Succinate	0.24	32	2.17	-7.36	-4.95	0.67	78.8	P:O ratios low owing to use of hexokinase with low activity
	0.24	32	2.17	-7.16	-4.78	0.67	76.9	
	0.24	32	2.17	-9.26	-7.73	0.84	99.6	
	(No KF added)	33	2.29	-6.64	-6.71	1.01	65.6	—
2	None	32 $\pm$ 4	2.64 $\pm$ 0.78	-(10.97 $\pm$ 1.63)	-(15.82 $\pm$ 3.71)	1.44 $\pm$ 0.21	105.9 $\pm$ 22.2*	Average of 13 different mitochondrial preparations
3	0.24	35	2.83	-11.15	-13.03	1.17	83.9	—
Ascorbate	None	33	2.29	-6.70	+0.93	—	66.2	Same mitochondrial preparation as used for Expt. 2 with succinate as substrate
	None	33	2.29	-6.58	+4.66	—	65.0	
	0.24	35	2.83	-7.14	+2.81	—	53.8	
								Same mitochondrial preparation as used for Expt. 4 with succinate as substrate
* Average deviation from mean.								

* Average deviation from mean.

Each flask contained the following in a total volume of 3.0 ml.: mitochondrial suspension in 0.25M-sucrose, 0.4 ml.; sodium potassium phosphate buffer (pH 7.4), 45 mM; D-glucose, 34 mM; KF, 15 mM; $MgCl_2$, 8 mM; ADP, 2.5 mM; AMP, mM; EDTA (pH 7.4), 2 mM; KCl, 0.01M; sucrose, 67 mM (in experiments without DPN), or 62 mM (in experiments with DPN); succinate, 0.01M, or ascorbate, 6.6 mM; cytochrome *c*, 9.8 μ M; 1–2 mg. of crystalline hexokinase. Temp. 30°. Equilibration time 12–15 min. Manometric flasks were maintained at 0° before each experiment.

Table 3. *Oxidation and associated phosphorylations with α -oxoglutarate and L-glutamate respectively as substrates*

Each flask contained the following in a total volume of 3.0 ml.: mitochondrial suspension in 0.25M-sucrose, 0.4 ml.; sodium potassium phosphate buffer (pH 7.4), 45 mM; D-glucose, 34 mM; KF, 15 mM; $MgCl_2$, 8 mM; ADP, 2.5 mM; AMP, mM; EDTA (pH 7.4), 2 mM; KCl, 0.01 M; sucrose, 62 mM; α -oxoglutarate, 6.6 mM, or L-glutamate, 6.6 mM; malonate, 0.02 M; cytochrome c, 9.8 μ M; 1–2 mg. of crystalline hexokinase. (In experiments with L-glutamate as substrate, malonate was omitted from the incubation medium.) Temp. 30°. Equilibration time 13 min. Manometric flasks were maintained at 0° before each experiment.

Expt.	Special additions	Incubation time (min.)	Wt. of protein/flask (mg.)	ΔO (μ g. atom)	ΔP_i (μ mole)	P:O	q_{O_2}
α -Oxoglutarate							
1	—	35	1.98	-3.17	-5.87	1.85	34.1
2	0.24 mM-DPN	32	4.35	-5.80	-11.28	1.95	31.1
3	2.4 mM-DPN + 0.02 M-nicotinamide	35	1.98	-5.41	-12.97	2.40	58.3
4	0.02 M-Nicotinamide	35	2.12	-2.61	-6.43	2.47	26.2
	2.4 mM-DPN + 0.02 M-nicotinamide	35	2.12	-4.92	-11.59	2.36	49.4
L-Glutamate							
1	1.2 mM-DPN + 0.02 M-nicotinamide	30	2.46	-6.03	-10.97	1.83	60.9
	—	30	2.46	-1.48	-1.48	1.00	15.0
2	0.24 mM-DPN	30	1.56	-2.36	—	—	37.6

Fig. 1. Oxygen uptake of mitochondria in the presence of various substrates and cofactors. Incubation medium as in Tables 1–3 with 0.01M-succinate (○), 6.6 mM-L-glutamate + 1.2 mM-DPN + 0.02 M-nicotinamide (▽), 6.6 mM- α -oxoglutarate + 2.4 mM-DPN + 0.02 M-nicotinamide (□), 6.6 mM-ascorbate (●), 6.6 mM- α -oxoglutarate (△), 6.6 mM-L-glutamate (▲). Temp. 30°.

DISCUSSION

If carefully followed, the method of isolation of ascites-tumour mitochondria described offers

several advantages over previous techniques (Nirenberg & Hogg, 1956; Kun *et al.* 1951; Mutolo & Abrignani, 1958, and Lindberg *et al.* 1953). The use of Dounce homogenizers, which need not be removed from the ice bath during the isolation procedure, is convenient for proper control of temperature. The homogenization of pre-stressed cells is relatively simple in comparison with the prolonged grinding of unswollen cells required in previous isolation techniques.

The mitochondria obtained showed no tendency to agglutinate in water or 0.25M-sucrose and appeared to be functionally intact, as evidenced by the oxidative phosphorylation activity found with a variety of substrates. Unlike those of liver, the particulates showed no endogenous oxygen uptake. A yield of 4–6% of the original wet weight of cells as twice-washed mitochondria is considered satisfactory on account of the low mitochondrial content of tumour cells (Kit & Griffin, 1958).

In view of the finding by Fonnesu & Davies (1956) that AMP prevents swelling of liver mitochondria, certain experiments were carried out in which the Ehrlich cells were lysed into water containing mM-AMP. Mitochondria obtained by this modification did not, however, show any difference in oxidative phosphorylation capacity from others isolated without addition of AMP.

The fact that once-washed mitochondria showed low P:O ratios with succinate and α -oxoglutarate (Table 1), combined with the observation that twice-washed mitochondria showed a lower q_{O_2} with succinate than once-washed mitochondria (Table 1), would indicate the presence of either

contaminating ATP-ases or uncoupling agents in once-washed mitochondrial preparations. Twice-washed mitochondria were accordingly used for all subsequent experiments in Tables 2 and 3.

In a medium based on that of other workers, the oxidation of succinate by Ehrlich-tumour mitochondria was accompanied by a marked esterification of inorganic phosphate (cf. Kun *et al.* 1951; Lindberg *et al.* 1953). P:O ratios determined with succinate lay within the range 1.01–1.82. The variation may be due to slight differences in age or the amount of contaminating erythrocytes, leucocytes, etc., in each batch of ascites fluid used. Minor variations of experimental technique during the isolation procedure may also account for the range of results. The P:O ratios compare favourably with the values of 1.4, obtained by Lindberg *et al.* (1953), and 1.2, obtained by Kun *et al.* (1951), for Ehrlich-ascites mitochondria.

The theoretical P:O ratio of 2.0 for succinate was never obtained, even in the highly fortified medium of Table 2. The presence of fluoride decreased the q_{O_2} of one batch of mitochondria from 99 to 78 in accord with the hypothesis of Slater (1954) that formation of a phosphate-fluoride complex inhibits succinic dehydrogenase in mitochondria. Addition of 0.24 mM-DPN to the phosphorylating medium probably served to maintain structural integrity of the mitochondria but, as might be expected, it was without effect on the q_{O_2} and P:O ratios with succinate as substrate. Ehrlich ascites-cell mitochondria showed q_{O_2} values with succinate which, per mg. of protein, were comparable with those of kidney, but which were in general above those of other normal cells, e.g. liver and brain (Wenner & Weinhouse, 1953).

Given ascorbate as substrate, Ehrlich ascites-cell mitochondria showed q_{O_2} values of 54–66 in phosphorylating media, with or without addition of DPN. In one case oxygen uptake was equivalent to that of the succinate control which was run on the same batch of mitochondria. In no instance could a P:O ratio be determined with ascorbate as substrate. Instead of phosphate esterification a net increase of inorganic phosphate was consistently observed. Even the high concentration of fluoride present in the medium was apparently without effect in preventing adenosine triphosphatase activity at the phosphorylation level concerned in ascorbate oxidation (cf. Emmelot & Bos, 1957).

Failure to achieve the theoretical P:O ratio of 2.0 during succinate oxidation would indicate either that the two phosphorylating sites between succinic dehydrogenase and cytochrome a_3 (oxidase) are both partially uncoupled, or that one of the sites is uncoupled to a considerable extent. The experiments with ascorbate in which no phosphate

esterification could be found would suggest that it is the cytochrome c phosphorylation site that is very largely uncoupled in Ehrlich ascites-cell mitochondria (cf. Copenhaver & Lardy, 1952; Slater, 1955; Hunter, 1951; Nielsen & Lehninger, 1954 and Chance & Williams, 1956). This may, of course, be true only for mitochondria prepared by the method described in this paper. Uncoupling of the cytochrome c phosphorylation site has not been observed in normal cell mitochondria such as those of liver (cf. Nielsen & Lehninger, 1954).

Studies with substrates that require DPN for their oxidation revealed interesting data on the functioning of Ehrlich ascites-cell mitochondria. Thus, in the absence of added DPN or the diphosphopyridine nucleotidase inhibitor nicotinamide, mitochondria oxidized α -oxoglutarate with low q_{O_2} values and P:O ratios which were little affected by addition of 0.24 mM-DPN to the medium. However, in experiments where the concentration of DPN was raised to 2.4 mM and nicotinamide was also added, a marked stimulation in both q_{O_2} and P:O ratio was observed. The highest P:O ratio for α -oxoglutarate oxidation (P:O 2.7) was obtained in the presence of nicotinamide alone without DPN addition.

Low oxygen uptakes and P:O ratios were obtained with L-glutamate as substrate. Addition of 0.24 mM-DPN to the medium caused a marked increase in q_{O_2} , but an even more striking increase in both q_{O_2} and P:O ratio was observed on addition of 1.2 mM-DPN together with nicotinamide.

These results clearly confirm the DPN requirement of tumour mitochondria discovered by Wenner & Weinhouse (1953), i.e. the Wenner-Weinhouse effect. These workers found that oxidation of pyruvate and citric acid-cycle metabolites by tumour mitochondria was greatly enhanced by the addition of DPN. The effect was, moreover, specific for DPN and could not be simulated by adding any other known cofactor. Wenner & Weinhouse suggested that tumour mitochondria may be less efficient in binding DPN than are their counterparts in non-neoplastic tissues, and that this may result in a correspondingly high level of DPN in the cytoplasm of the tumour cell. The finding of Branster & Morton (1956), that the rate of DPN synthesis in isolated tumour nuclei was approximately one-fifth of that in corresponding normal cell nuclei, would suggest that tumour mitochondria are unable to obtain an adequate supply of the coenzyme for proper biological functioning in the living cell, although the lower nuclear DPN synthesis may even be adequate for the limited number of mitochondria in tumour cells. The possibility also exists that DPN transport between the nucleus and mitochondria may be impaired in tumour cells.

Our observation that Ehrlich ascites-cell mitochondria show a high P:O ratio for α -oxoglutarate oxidation without added DPN, but in the presence of the nicotinamide alone, would suggest that these tumour mitochondria may indeed have sufficient bound DPN for α -oxoglutarate oxidation, but will function satisfactorily only if the bound DPN is not broken down by diphosphopyridine nucleotidase action. A high diphosphopyridine nucleotidase activity in tumour-cell mitochondria has been observed by many workers (Morton, 1958; Kun *et al.* 1951; Weinhouse, 1955).

SUMMARY

1. An improved method is described for the isolation of Ehrlich-tumour mitochondria. The ascites cells are pre-stressed by swelling in hypotonic solution and are then disrupted into pure water by osmotic shock. Addition of concentrated sucrose solution to the homogenate immediately after lysis gives a 0.25 M-sucrose suspension, from which mitochondria can be isolated by differential centrifuging in the customary manner.

2. The method affords clean mitochondria which are free of adhering cytoplasmic material and are functionally intact, as evidenced by their oxidative phosphorylation activity shown with succinate, α -oxoglutarate and L-glutamate as substrates.

3. Studies with succinate and ascorbate as substrates have provided evidence which suggests that the cytochrome *c* phosphorylation site is uncoupled to a considerable extent in Ehrlich ascites-cell mitochondria.

4. Ehrlich ascites-cell mitochondria exhibit a pronounced diphosphopyridine nucleotide requirement for maximal oxidative phosphorylation activity with α -oxoglutarate and L-glutamate as substrates (Wenner-Weinhouse effect). Evidence suggests that the diphosphopyridine nucleotidase inhibitor, nicotinamide, may possibly be more effective than diphosphopyridine nucleotide itself in raising the P:O ratio.

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REFERENCES

- Branster, M. V. & Morton, R. K. (1956). *Biochem. J.* **63**, 640.
- Brodie, A. F. (1955). *Methods in Enzymology*, vol. 2, p. 693. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.
- Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
- Copenhaver, J. H. & Lardy, H. A. (1952). *J. biol. Chem.* **195**, 225.
- Crane, R. K. & Sols, A. (1955). *Methods in Enzymology*, vol. 1, p. 277. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Davidson, J. N., Smellie, R. M. S., Keir, H. M. & McArdle, H. (1958). *Nature, Lond.*, **182**, 589.
- Dounce, A. L., Witter, R. F., Monty, K. J., Pate, S. & Cottone, M. A. (1955). *J. biophys. biochem. Cytol.* **1**, 139.
- Emmelot, P. & Bos, C. J. (1957). *Brit. J. Cancer*, **11**, 148.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Fonnesu, A. & Davies, R. E. (1956). *Biochem. J.* **64**, 769.
- Hunter, F. E. (1951). *Phosphorus Metabolism*, vol. 1, p. 297. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
- Kit, S. & Griffin, A. Clark (1958). *Cancer Res.* **18**, 630.
- Kun, E., Talalay, P. & Williams-Ashman, H. G. (1951). *Cancer Res.* **11**, 855.
- Lindberg, O., Ljunggren, M., Ernster, L. & Rēvész, L. (1953). *Exp. Cell Res.* **4**, 243.
- Littlefield, J. W. & Keller, E. B. (1957). *J. biol. Chem.* **224**, 13.
- Morton, R. K. (1958). *Nature, Lond.*, **181**, 540.
- Mutolo, V. & Abrignani, F. (1958). *Brit. J. Cancer*, **12**, 285.
- Nielsen, S. O. & Lehninger, A. L. (1954). *J. Amer. chem. Soc.* **76**, 3860.
- Nirenberg, M. W. & Hogg, J. F. (1956). *J. Amer. chem. Soc.* **78**, 6210.
- Slater, E. C. (1954). *Biochem. J.* **58**, i.
- Slater, E. C. (1955). *Biochem. J.* **59**, 392.
- Slater, E. C. & Holton, F. A. (1954). *Biochem. J.* **56**, 28.
- Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques and Tissue Metabolism*, p. 301. Minneapolis: Burgess Publishing Co.
- Wenner, C. E. & Weinhouse, S. (1953). *Cancer Res.* **13**, 21.
- Weinhouse, S. (1955). *Advanc. Cancer Res.* **3**, 269.