THE PYRIDINE NUCLEOTIDE CONTENT OF MITOCHONDRIA ISOLATED FROM EHRLICH ASCITES TUMOUR CELLS*

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SUMMARY

1. The addition of 1 m*M* DPN^+ had no effect on the rate of O_2 uptake with ascites-cell mitochondria oxidizing α-ketoglutarate or pyruvate + malate, and increased the rate of O_2 uptake with glutamate as substrate by only 17 %.

2. Direct analysis showed that the fresh mitochondria contain more DPN than rat-liver mitochondria and that 40 % of this is in the reduced form.

3. No TPN^+ and only small amounts of TPNH (0.5 μmole/g mitochondrial protein) were detected.

4. No evidence could be obtained that breakdown of mitochondrial DPN proceeds abnormally rapidly *in vitro*.

5. It is concluded that the reported DPN deficiency of tumour mitochondria as a class is due to the use of unsatisfactory isolation procedures.

INTRODUCTION

Investigations, inspired by WARBURG's concept (*cf.* ref. 2) of the impaired respiration in cancer cells, led around 1952 to the discovery that isolated tumour mitochondria require added DPN for the maximal oxidation of DPN-linked substrates under conditions in which liver mitochondria could do without[3,4]. On the basis of this "DPN effect" WENNER AND WEINHOUSE suggested that tumour mitochondria "do not bind DPN as strongly as (mitochondria of) certain normal tissues, and that this may result in a correspondingly higher level of this coenzyme in the soluble portion of the cell cytoplasm. It is further suggested that this phenomenon may account for the characteristically high glycolysis rates of intact tumour cells"[3].

Although the results on which the theory of WENNER AND WEINHOUSE is based have been confirmed by others[4–6], there are observations in the literature that are difficult to reconcile with it: With mitochondria from some tumours the "DPN effect" appears to be small[7] or even absent[8], while evidence has been presented that in mitochondria from other tumours the DPN requirement does not reflect a DPN deficiency of the mitochondria *in situ* but is caused by the rapid loss of DPN from the mitochondria *in vitro*[7–9]. Furthermore, CHANCE AND HESS[10] have recently demonstrated that the total pyridine nucleotide content of ascites-cell mitochondria,

* This work is part of the M.D. thesis of P. Borst, which was published in May, 1961 (see ref. 1).

determined with a semi-quantitative spectrophotometric method, is about half the value found for rat-liver mitochondria.

Although these observations argue against the general validity of the theory of WENNER AND WEINHOUSE, the experimental evidence is not conclusive and in recent reviews[11-13] of the field the matter has been left open. Moreover, the concept of WENNER AND WEINHOUSE has been incorporated in two recent theories[14,15] on the metabolic disturbance underlying malignant growth.

We have, therefore, reinvestigated this problem with mitochondria isolated from Ehrlich ascites-tumour cells, for which a marked DPN effect has recently been reported[6]. In this paper observations are presented on the effect of added DPN^+ on the rate of oxidation of DPN-linked substrates, on the DPN content of the mitochondrial preparation, and on the rate of loss of endogenous DPN by the mitochondria *in vitro*. The results demonstrate that our preparation of ascites-cell mitochondria is not deficient in DPN and that they do not lose their DPN abnormally rapidly *in vitro* even under adverse conditions. Since the TPN content of tumour mitochondria does not appear to have been studied previously, we have also determined TPN^+ and TPNH in our mitochondrial preparations. No TPN^+ and only small amounts of TPNH were found.

Some of these results have been briefly discussed elsewhere[16,17].

METHODS

Rat-liver mitochondria. Rat-liver mitochondria were prepared by the method of HOGEBOOM[18] following the procedure described by MYERS AND SLATER[19].

Washed ascites cells. The cells of an Ehrlich ascites tumour (kindly supplied by the late Prof. B. MENDEL) were harvested 6–9 days after inoculation of 0.2 ml of ascites fluid in Swiss mice. The ascites fluid of 6–10 mice was collected in approximately 50 ml ice-cold 0.9% NaCl + 1 m*M* EDTA (pH 7.0). The cells were filtered once through a loosely woven cheese-cloth to remove clots etc. and they were washed free from contaminating erythrocytes by repeated low-speed centrifugation in NaCl–EDTA. After the last wash the cells were taken up in 0.25 *M* sucrose (containing 1 m*M* EDTA, pH 7 in most experiments) and packed by centrifugation.

Ascites-cell mitochondria. Ehrlich ascites-cell mitochondria were obtained as described before[1,20]. In most experiments the washed cells were concentrated in two batches which were homogenized separately, and 0.25 *M* sucrose + 1 m*M* EDTA (pH 7) was used as the isolation medium instead of sucrose alone.

Enzyme preparations. Yeast alcohol dehydrogenase and liver glutamic dehydrogenase were obtained from Boehringer. TPN-specific isocitric dehydrogenase was prepared from pig heart up to step 3 of the method of OCHOA[21].

Special chemicals. All nucleotides were obtained from Sigma Chemical Co. DPNH was prepared enzymically from DPN^+, using yeast alcohol dehydrogenase and ethanol. DL-isocitric acid was prepared from dimethylisocitric lactone.

Analytical methods. O_2 uptake was measured at 25° with differential manometers as described previously[20]. Rates are expressed as Q_{O_2} (μl O_2/mg protein/h). Protein was determined by the biuret method in the modification of CLELAND AND SLATER[22] with crystalline egg albumin as standard.

DPNase activity. DPNase activity was measured as disappearance of DPN^+

at room temperature (20° ± 2) in the following medium: 0.05 *M* phosphate (pH 7.4); 0.025 *M* sucrose; 0.1 m*M* EDTA and $7.5 \cdot 10^{-4}$ *M* DPN^+. The reaction was stopped after 15 min by adding $HClO_4$ to a final concentration of 4%. The DPN^+ content of a suitable sample of the deproteinized supernatant, from which $HClO_4$ was removed as $KClO_4$ in the cold, was measured spectrophotometrically in a medium containing 0.3 *M* Tris–HCl buffer (pH 10.1), 3% ethanol and alcohol dehydrogenase. The reaction was started by adding the alcohol dehydrogenase and the increase in absorbancy at 340 mμ was read in a Zeiss spectrophotometer against a reference cuvette containing all components of the reaction medium except the sample. The reaction was mostly completed within 5 min. The DPNase activity was calculated from the difference in DPN^+ content of the reaction tube and a zero-time control.

Determination of pyridine nucleotides. The pyridine nucleotides, DPN^+, TPN^+, DPNH and TPNH, were determined by specific enzymic fluorimetric methods applied to acid and alkali extracts of mitochondria as described by JACOBSON AND ASTRACHAN[23] and CIOTTI AND KAPLAN[24]. Fluorescence was measured in a Photovolt Multiplier Fluorescence Meter Model 540 with filter B-Hg 1 as primary filter and filter B-470 as secondary filter. The detailed procedure followed in our experiments was that of PURVIS[25] with a few modifications introduced in our laboratory to improve the reliability[26]. The most important modifications were: Neutralization of the acid and alkali extracts was carried out in the presence of 0.1 *M* Tris buffer with a calculated amount of acid and alkali and great care was taken that the pH of the alkaline extract never went below 7 (*cf.* footnote on p. 437 of ref. 25). DPN^+ and DPNH were determined with alcohol dehydrogenase and TPN^+ with isocitric dehydrogenase. TPNH was determined by either of two procedures:

Procedure 1. DPNH + TPNH are determined in the neutralized alkaline extract with 5 m*M* NH_4Cl, 5 m*M* α-ketoglutarate, excess glutamic dehydrogenase and 0.1 *M* Tris-acetate buffer (pH 7.5). The value for TPNH is found as the difference of DPNH + TPNH and DPNH alone.

Procedure 2 (*cf.* ref. 26). DPNH + TPNH are converted into DPN^+ and TPN^+ as in procedure 1. After precipitating glutamic dehydrogenase with $HClO_4$ and removal of $HClO_4$ as $KClO_4$, TPN^+ is determined with isocitric dehydrogenase as described by PURVIS[25].

All manipulations with the alkali extract were done as rapidly as possible in the cold. Under these conditions no measurable loss of DPNH or TPNH by non-enzymic oxidation was encountered (*cf.* ref. 25). The development of the fluorescence was rigidly standardized and the alkaline incubation was carried out in the dark, which improved the reproducibility.

All values given in this paper were determined in duplicate or triplicate. Known amounts of enzymically active DPN^+, DPNH, TPN^+ and DPNH (used as standard for the DPNH + TPNH), taken in quadruplicate through the whole procedure, were used to calculate the amount of the corresponding pyridine nucleotide present in the extracts.

RESULTS

DPN effect

Experiments on the maximal effect of added DPN^+ on the rate of O_2 uptake of ascites-cell mitochondria oxidizing DPN-linked substrates are summarized in Table I.

The Q_{O_2} values are in the same range as those obtained for rat-liver mitochondria and only with glutamate as substrate was a small DPN effect (17%) regularly observed, which was only 4% when initial rates were compared. The reason for the anomalous results with glutamate is not clear*, and has not been further investigated in view of the experiments described below.

TABLE I

THE EFFECT OF DPN^+ ADDITION ON THE RATE OF O_2 UPTAKE BY ASCITES-CELL MITOCHONDRIA OXIDIZING DPN-LINKED SUBSTRATES

The O_2 uptake in parallel flasks, with and without DPN^+, was determined as described previously[20]. In most experiments the medium for measuring P/O ratio's (reaction time mostly 30 min) was used but in two experiments conditions for measuring ADP or P_i-respiratory control were used[20]. The values represent the data obtained with 10 different mitochondrial preparations, 2 of which were prepared in sucrose.

Substrate	*No. of expts.*	*Added cytochrome c (μM)*	*Mean maximal Q_{O_2} without added DPN+*	*Maximal stimulation by 1 mM DPN+ (%)*
0.02 *M* α-ketoglutarate	1	0	78.5	+ 1
0.02 *M* α-ketoglutarate + 0.02 *M* malonate	2	0	59.3	— 6
0.02 *M* L-glutamate	3	40	81.0	+ 17
0.02 *M* L-glutamate	1	0	64.5	+ 18
0.02 *M* pyruvate + 2 m*M* L-malate	3	40	86.6	+ 2

Determination of DPN in fresh ascites-cell mitochondria

The results of analyses of the DPN^+ and DPNH contents of 7 preparations of ascites-cell mitochondria are given in Table II. For comparison similar data are given for rat-liver mitochondria analysed by the same procedure in the same period of time (Spring, 1960). The maximal Q_{O_2} values obtained with DPN-linked substrates are similar for the two types of mitochondria. It is clear from Table II that ascites-cell mitochondria contain even more DPN on a protein basis than rat-liver mitochondria. This difference becomes even more marked when one experiment with the tumour mitochondria—which gave a clearly anomalous result—is omitted. The mean value for total DPN then becomes 5.9 with a range of 5.1–6.6 μmoles/g protein. There was no significant difference between the DPN content of mitochondria isolated in sucrose, sucrose–EDTA or sucrose–nicotinamide. The latter observation shows that the DPNase present in the mitochondrial preparation is unable to break down the endogenous mitochondrial DPN during the isolation procedure.

The high percentage reduction of DPN in the fresh ascites-cell mitochondria indicates that the oxidation of endogenous substrate is still under ADP respiratory

* We have found that under the conditions of our experiments the pathway of glutamate oxidation is similar to that observed in rat-heart sarcosomes, rat-liver mitochondria and pigeon-breast-muscle sarcosomes, namely a quantitative conversion to aspartate by means of a transamination to oxaloacetate followed by that part of the Krebs-cycle lying between α-ketoglutarate and oxaloacetate[27,28,1]. It is therefore difficult to understand why a small DPN effect was repeatedly observed with glutamate as substrate but not in one experiment with α-ketoglutarate without malonate. More experiments are required to decide whether the small difference found is significant and to determine its cause.

TABLE II

THE DPN CONTENT OF FRESH ASCITES-CELL AND RAT-LIVER MITOCHONDRIA

The ascites-cell mitochondria were isolated either in 0.25 *M* sucrose or in 0.25 *M* sucrose + 1 m*M* EDTA (pH 7) or in 0.25 *M* sucrose + 0.02 *M* nicotinamide (pH 7) as indicated in the table. The values are expressed as means with range in brackets.

	No. of expts.	*μmoles/g protein*		
		DPN+	*DPNH*	*DPN+ + DPNH*
Ascites-cell mitochondria				
Sucrose	2	4.1 (3.8–4.4)	1.8 (1.5–2.1)	5.9 (5.8–5.9)
Sucrose–EDTA	4	3.0 (1.5–4.1)	2.3 (1.8–2.7)	5.3 (3.3–6.6)
Sucrose–nicotinamide	1	3.6	1.8	5.4
Total	7	3.4 (1.5–4.4)	2.1 (1.8–2.7)	5.5 (3.3–6.6)
Rat-liver mitochondria	10	1.4 (0.7–2.2)	2.3 (1.0–4.3)	3.7 (2.1–5.0)

control (*cf.* refs. 29 and 30). This suggests that the mitochondrial preparation is of good quality, in agreement with other results obtained with these mitochondria[1,17,20].

Determination of TPN in fresh ascites-cell mitochondria

In contrast to the high total DPN content, the TPN content of fresh ascites-cell mitochondria is low—only one tenth of that of rat-liver mitochondria (Table III). TPN^+ could not be demonstrated at all. Small amounts of TPNH were demonstrated by two slightly different procedures. In the first TPNH was determined as the difference of TPNH + DPNH and DPNH alone. Since only small amounts of TPNH are present, this difference method is inaccurate. In the second procedure the alkaline extract was neutralized, TPNH was converted into TPN^+ with glutamic dehydrogenase and TPN^+ was determined directly. This is the more accurate method.

TABLE III

THE TPN CONTENT OF FRESH ASCITES-CELL AND RAT-LIVER MITOCHONDRIA

The values for rat-liver mitochondria have been taken from Table I of ref. 26; they were obtained with procedure 1 by Mr. B. WINTER (TPN^+, 4 expts. and TPNH, 13 expts.). Of the 5 preparations of ascites-cell mitochondria analysed by procedure 1, two were isolated in sucrose, and one in 0.25 *M* sucrose + 0.02 *M* nicotinamide (pH 7.0) sucrose–EDTA. There was no significant difference between results obtained with the three different isolation media. The two preparations analysed by procedure 2 were isolated in sucrose–EDTA. All values are expressed as means with range in brackets.

	No. of expts.	*μmoles/g protein*		
		TPN+	*TPNH*	*TPN+ + TPNH*
Ascites-cell mitochondria				
Procedure 1	5	0.0 (—0.1–0.3)	0.4 (—0.3–1.0)	0.5 (—0.4–1.3)
Procedure 2	2	0.0 (0.0–0.0)	0.4 (0.2–0.5)	0.4 (0.2–0.5)
Total	7	0.0 (—0.1–0.3)	0.4 (—0.3–1.0)	0.5 (—0.4–1.3)
Rat-liver mitochondria		0.3	4.9	5.2

The value of 0.4 μmole found for TPNH in the absence of any detectable TPN^+ is not unreasonable in view of the high degree of reduction of the DPN. It has been shown[29,30] that in tightly-coupled mitochondrial preparations the percentage reduction of TPN is always higher than that of DPN (*cf.* values for fresh rat-liver mitochondria of Tables II and III). Using the data of KLINGENBERG AND SLENCZKA[29] for the relation between percentage reduction of DPN and TPN, it can be calculated that with 40% reduction of DPN (Table II) at least 85% of TPN will be in the reduced form. The concentration of TPN^+ corresponding to 0.4 μmole TPNH would not be detectable by the procedure used.

To exclude the possibility that the TPN content was underestimated by the conversion of TPN^+ to DPN^+ during the preparation of the mitochondria, the fate of TPN^+ added to the mitochondria was studied. As Table IV shows there was no measurable breakdown in the presence of nicotinamide.

Recently PURVIS[25] has reported the presence in rat-liver mitochondria of hidden or "extra" forms of DPN and TPN which cannot be determined with the usual methods. These "extra" forms of the pyridine nucleotides are converted into the oxidized forms when the mitochondria are incubated at room temperature in hypotonic phosphate buffer containing nicotinamide. Appreciable amounts of "extra" DPN or TPN were not detected in our ascites-cell mitochondria by PURVIS's[25] procedure.

Rate of breakdown of intramitochondrial DPN

Table IV shows that an appreciable amount of DPN^+ was left in the mitochondria after a 30-min hypotonic incubation. This was rather surprising in view of the alleged labile character of tumour mitochondria in general and the indication from the literature that tumour mitochondria tend to lose their DPN rapidly *in vitro*. Further experiments were therefore carried out. The results shown in Tables V and VI demonstrate that the slow breakdown of mitochondrial DPN cannot be explained by lack of DPNase activity but must be due to the fact that ascites-cell mitochondria release their DPN only slowly into the medium even when shaken in hypotonic phosphate buffer at room temperature (*cf.* ref. 31).

TABLE IV

THE BREAKDOWN OF DPN^+ AND TPN^+ BY ASCITES-CELL MITOCHONDRIA IN THE PRESENCE OF NICOTINAMIDE

Fresh ascites-cell mitochondria, prepared in sucrose–EDTA, were shaken at room temperature for 30 min in a medium containing 0.05 *M* phosphate buffer (pH 7.4), 0.05 *M* sucrose and 0.2 m*M* EDTA. The following additions were then made: nicotinamide to a final concentration of 0.03 *M*, approx. 10 μmoles of TPN^+/g protein and enough phosphate buffer to keep the phosphate concentration 0.05 *M*. Immediately after these additions samples were taken (sample 1) and analysed for DPNH + TPNH, DPN^+ and TPN^+. After a further 10-min shaking at room temperature sample 2 was taken. Since only small samples were used for the analyses of reduced pyridine nucleotides, the two values given are not significantly different from 0.

Sample	μmoles/g protein		
	DPNH + TPNH	DPN^+	TPN^+
1	−0.1	2.5	12.6
2	+0.3	2.4	12.8

TABLE V

COMPARISON OF THE RATE OF BREAKDOWN OF ENDOGENOUS AND EXOGENOUS DPN^+ BY ASCITES-CELL MITOCHONDRIA

Fresh ascites-cell mitochondria, prepared in sucrose–EDTA, were shaken at room temperature (20° ± 2) in a medium containing 0.05 *M* phosphate buffer (pH 7.4), 0.05 *M* sucrose and 0.2 m*M* EDTA. After 10 min, sample 1 was analysed for DPN^+ and DPNH, and 40.9 μmoles DPN^+/g protein were added. After shaking for another 10 min sample 2 was taken.

Sample	*μmoles/g protein*	
	DPN^+	*DPNH*
1	3.6	1.3
2	3.0	

TABLE VI

THE RATE OF BREAKDOWN OF ENDOGENOUS AND EXOGENOUS DPN^+ BY ASCITES-CELL MITOCHONDRIA

Fresh ascites-cell mitochondria, prepared in sucrose–EDTA, were shaken at room temperature (20° ± 2) in a medium containing 0.05 *M* phosphate buffer (pH 7.4), 0.05 *M* sucrose and 0.2 m*M* EDTA. At the times indicated samples of this mixture were analysed for DPN^+ and DPNH. DPNase activity was determined on the sample taken after 15-min incubation.

Time (min)	*μmoles/g protein*			*DPNase activity (μmoles/g protein 15 min)*
	DPN^+	*DPNH*	*DPN^+ + DPNH*	
0	4.1	2.2	6.3	
15	2.1	0.0	2.1	34
30	1.2	0.0	1.2	
45	0.4	0.0	0.4	

Determination of TPNH in washed ascites cells

GLOCK AND MCLEAN[32] have shown that tumours as a class contain little TPN and in some of them, including the Krebs ascites-cell tumour, they could not demonstrate the presence of any form of TPN at all (total TPN content < 3 μg/g tissue). Employing a fluorimetric method similar to that used in the present paper IBSEN *et al.*[33] found low but significant amounts of TPN^+ in washed Ehrlich ascites cells. The presence of TPNH was not studied. However, when 2-deoxy-D-glucose was added, the TPN^+ content of the cells rose sharply to a value more than half the DPN^+ content. This indicates that large amounts of TPNH were present in the washed cells. Since this raised the remote possibility that part of this TPNH, originally present in the mitochondria, was converted to DPNH during the isolation of the mitochondria we have determined the TPNH content of the washed ascites cells used in our work. A direct analysis of TPNH in the alkaline extract of the cells proved impossible with the methods available. The main difficulty is that with low amounts of protein the determination is not sensitive enough, while a more concentrated alkaline extract (> 3 mg protein) becomes very gelatinous when heated. Concentration of a dilute extract is not feasible owing to the non-enzymic oxidation of reduced pyridine nucleotides during this procedure. Therefore, procedure 2 (see METHODS) was used. A dilute alkaline extract was neutralized, the TPNH converted to TPN^+ with glutamic dehydrogenase, and the dilute extract was then concentrated by freeze drying and TPN^+ determined as usual.

TABLE VII

THE DPNH AND TPNH CONTENT OF WASHED EHRLICH ASCITES CELLS

Washed ascites cells were taken up in water and rapidly added to a boiling solution of Na_2CO_3 (final concn. 0.106 *M*) and heated for 3 min in a boiling-water bath. The final protein concentration in the extract was approx. 3 mg/ml. The viscous solution was cooled in ice, and Tris–HCl (pH 7.4) to a final concentration of 0.08 *M* was added. Under rapid stirring with a magnetic stirrer 1 *N* HCl was slowly added until the pH was between 8.0 and 8.5. α-Ketoglutarate (final concn. 5 m*M*), NH_4Cl (10 m*M*) and excess glutamic dehydrogenase were then added and the solution was stirred for 10 min at room temperature. $HClO_4$ was then added to a final concn. of about 4 % and the protein was removed by centrifugation. The deproteinized supernatant was neutralized with 5 *N* KOH and the $KClO_4$ was centrifuged off in the cold. The supernatant (about 100 ml) was freeze-dried, taken up in a small volume of water, and DPN^+ and TPN^+ were determined as described under METHODS.

Expt.	*μmoles/g protein*	
	DPNH	*TPNH*
4.8.60	0.12	0.10
11.8.60*	0.53	0.03

* 10 m*M* glucose present during washing of the cells.

The results of two experiments with this procedure are given in Table VII. For comparison and as a check on the method, DPNH was also determined. Although the results obtained should be interpreted with some reservation, they support the conclusion of GLOCK AND MCLEAN[32] that only small amounts of TPNH are present in ascites cells.

It should be noted that the method used here to estimate small amounts of TPNH in large amounts of material may be more useful when used in conjunction with sensitive spectrophotometry (*cf.* ref. 29). When fluorimetry is used the increase in sensitivity obtained by concentrating the dilute extract is partly offset by the increase in the blank fluorescence.

DISCUSSION

The experiments described in this paper demonstrate that freshly isolated ascites-cell mitochondria are not deficient in DPN. In addition evidence is presented that these mitochondria are not "leaky" and that they do not lose their DPN exceptionally rapidly *in vitro*. This evidence can be summarized as follows:

(a) The high DPN content of the fresh mitochondria makes it unlikely that DPN is lost during the isolation procedure. This means that at 0° the mitochondria retain their DPN for at least 1.5 h, even though subjected to mechanical and osmotic stress.

(b) With most of the substrates tested DPN^+ had no influence on the rate of oxidation during the whole 30-min reaction period. This means that under these conditions sufficient DPN is retained by the mitochondria for an optimal functioning of the Krebs cycle.

(c) When the mitochondria are shaken at room temperature in hypotonic phosphate buffer, more than 30 min is required to deplete them of endogenous DPN. Since DPNase activity is high, it must be assumed that even under adverse conditions the mitochondria lose their DPN only slowly.

There is good indirect evidence that the results obtained with isolated ascites-

cell mitochondria are representative for the mitochondria in the intact cell. In the first place, an artefactual increase in DPN content during the isolation procedure is highly unlikely. Conversion of TPN into DPN could never be quantitatively important because ascites cells contain very little TPN. Moreover, the isolated mitochondria do not convert added TPN^+ into DPN^+ at a measurable rate. Adsorption of cytoplasmic DPN^+ to the mitochondria would probably not lead to an increase in the DPN content found because the DPNase would break it down during the isolation procedure, and incorporation of cytoplasmic DPN^+ into mitochondria only occurs with damaged mitochondria at high DPN^+ concentrations[34,35]. Secondly, results obtained with intact ascites cells have revealed no deficiency in DPN-linked mitochondrial oxidations. Thirdly, recent experiments on the effect of H_2O_2 and carcinostatics on the metabolism of ascites cells provide suggestive evidence that ascites-cell mitochondria do not easily lose their DPN to the cytoplasm *in vivo* (*cf.* discussion in ref. 17).

Our results concerning the presence of TPN in ascites-cell mitochondria are less clear-cut than those for DPN The amounts of TPNH found are small and rather variable. It would therefore be desirable to confirm our results with a double-beam spectrophotometer.

Our figures on the total pyridine nucleotide content of isolated ascites-cell mitochondria are essentially in agreement with those briefly reported by CHANCE AND HESS[10]. Employing a semi-quantitative spectrophotometric method these authors have found that the concentration of pyridine nucleotide relative to cytochrome *a* in rat-liver mitochondria was twice that in ascites-cell mitochondria. Since the cytochrome *a* content on a protein basis was equal for the two types of mitochondria, they have considered the possibility that their ascites-cell mitochondria had been damaged during the isolation procedure. However, more than half of the pyridine nucleotide of rat-liver mitochondria consists of TPN (refs. 29, 35; see Table I in ref. 26), while ascites-cell mitochondria contain little TPN (Table II). Our pyridine nucleotide value for ascites-cell mitochondria relative to rat-liver mitochondria is therefore only slightly higher than that of CHANCE AND HESS.

The reason why other workers have consistently obtained mitochondria deficient in DPN is not known. WEINHOUSE[11] has suggested several possibilities, all based on the idea that there is a fundamental difference in DPN metabolism between tumours and normal tissues. In view of the results presented in this paper we prefer the hypothesis that the difference found is accidental and due to the fact that most tumours are poor starting material for the isolation of good mitochondria. Factors probably involved—some of which have been discussed in recent papers of EMMELOT and coworkers[36,37]—are:

(a) Toughness of the cells. Unsatisfactory results have also been obtained with normal tough tissue like muscle.

(b) Necrotic areas containing damaged mitochondria.

(c) Low yield of the isolation procedure due to toughness of the tissue and the low mitochondrial count of tumours (reviewed in ref. 12). This may adversely affect the quality of the mitochondrial preparation since mitochondria tend to age rapidly in diluted suspensions. The reasons for this are not yet understood, but the loss of stabilizing factors on dilution[38,39] and the rapid oxidation of endogenous substrates in dilute suspensions are probably contributing factors.

(d) The release of uncoupling long-chain fatty acids during the isolation procedure[37,38,40–43]. Although this release requires aging with rat-liver mitochondria, free fatty acids are already present in the fresh mitochondrial preparation prepared from other tissues, such as some preparations of insect sarcosomes[44,45]. Evidence that this may also be the case for some preparations of tumour mitochondria has been obtained by DEVLIN AND PRUSS[46], by EMMELOT and coworkers[37,47] and by one of us[1]. The fatty acids may be derived from damaged mitochondria (*cf.* (b)), from lipid material adsorbed onto the mitochondria during homogenization (*cf.* refs. 37, 47), or from contaminating microsomes (*cf.* data for rat-liver microsomes in refs. 38 and 40)*.

(e) The use of unsatisfactory isolation media. EMMELOT[8] has shown that, with most tumours studied, sucrose–EDTA gave better results than sucrose alone, which has been used by most other workers.

(f) The use of rat-liver mitochondria as the standard of reference for "normality". Since liver does not have the disadvantages mentioned, comparison of liver and tumour mitochondria will always reveal "abnormalities". However, judged by these criteria most preparations of mitochondria isolated from rat heart, skeletal muscle, insect flight-muscle and brain and even some types of liver mitochondria (*cf.* ref. 36) will probably also turn out to be "abnormal", which indicates that the criteria employed are unsatisfactory.

In summary, we believe that the DPN effect found by others is not due to a biochemical abnormality of the tumours studied but reflects the technical difficulties encountered in the isolation of tumour mitochondria. This conclusion is in line with the important work of WEINHOUSE and coworkers, CHANCE AND HESS and others (*cf.* discussion in ref. 1) on intact tumour cells which has revealed no consistent abnormality in the oxidative metabolism of tumours.

Even if this conclusion is not accepted it is clear from the results reported in this paper that theories based on the idea that tumour mitochondria are deficient in DPN or tend to lose their DPN abnormally rapidly *in vitro* do not hold for our strain of ascites-cell tumour. Since a general theory which has no general validity is untenable we propose that the concept of the DPN deficiency of tumour mitochondria be abandoned.

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* Experiments of EMMELOT[8,48] with a series of tumours have demonstrated that mitochondrial preparations with a high ATPase activity and DPN requirement invariably showed high DPNase activity, while low DPNase activity was associated with a partly latent ATPase and a low DPN requirement. If the assumption is made that in tumours the DPNase is localized in the microsomes as it is in the normal tissues studied[49,50], EMMELOT's results would provide strong evidence that microsomal contamination is responsible for the instability of some preparations of tumour mitochondria.

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