PYRIDINE NUCLEOTIDES IN RAT-LIVER MITOCHONDRIA

I. THE DEMONSTRATION OF A NEW FORM OF DIPHOSPHOPYRIDINE NUCLEOTIDE AND OF TRIPHOSPHOPYRIDINE NUCLEOTIDE

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SUMMARY

1. In addition to DPN and DPNH, rat-liver mitochondria contain a diphosphopyridine nucleotide compound, which does not react with alcohol dehydrogenase.

2. DPN is liberated from this compound by incubation of the liver mitochondria with phosphate, ADP, or dinitrophenol, but not with ATP.

3. The compound was found together with DPNH in an alkali extract of fresh mitochondria. DPN was formed from both this compound and DPNH, when the extract was added to liver mitochondria, depleted of their pyridine nucleotides by incubation with phosphate in the absence of nicotinamide.

4. The new compound has the properties expected of DPN $\sim$ I, the postulated intermediate of oxidative phosphorylation.

5. A similar compound of triphosphopyridine nucleotide was also detected.

INTRODUCTION

In connexion with studies to determine whether mitochondrial isocitric dehydrogenase is a DPN- or a TPN-linked enzyme[1], it was necessary completely to deplete the mitochondria of their endogenous pyridine nucleotides. In the course of an investigation of the conversion of the reduced pyridine nucleotides to their oxidized form, which precedes the release of the oxidized form and its subsequent destruction, a new form of diphosphopyridine nucleotide was found. This was revealed by the increase in DPN reacting with alcohol dehydrogenase, over and above that accounted for by oxidation of DPNH to DPN, after incubation of mitochondria with phosphate, ADP or 2,4-dinitrophenol but not with ATP. A similar form of triphosphopyridine nucleotide was revealed by the increase in TPN reacting with TPN-specific isocitric dehydrogenase, over and above that accounted for by oxidation of TPNH to TPN. The new form of diphosphopyridine nucleotide has the properties expected of the compound DPN $\sim$ I

Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; ADP, ATP, adenosine di- and triphosphate; GSH, GSSG, glutathione and oxidized glutathione; Tris, tris(hydroxymethyl)aminomethane; fp, flavoprotein; P_i, inorganic phosphate.

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which has been postulated as an energy-rich intermediate of oxidative phosphorylation[2–4].

The interconversions of the various forms of diphosphopyridine nucleotide and of triphosphopyridine nucleotide including two transfer reactions between the new forms of TPN and DPN will be reported in a later paper. A preliminary report of some of this work has already appeared[5].

METHODS

Preparations and materials

Rat-liver mitochondria. Rat-liver mitochondria were prepared by the method of HOGEBOOM[6], following the procedure described by MYERS AND SLATER[7]. Depleted rat-liver mitochondria were prepared by incubation in 0.05 *M* sucrose, 40 m*M* phosphate for 45 min (*cf.* ref. [1]).

Enzyme preparations. Yeast alcohol dehydrogenase (analytical grade) was obtained from Boehringer. *TPN-specific isocitric dehydrogenase* was prepared from pig heart up to step 3 of the method of OCHOA[8]. *TPNH-specific GSSG reductase* was prepared from peas by the method of KAPLAN *et al*[9]. The purified enzyme used in this work did not oxidize DPNH.

Other preparations. The *ADP* used, obtained from Sigma Chemical Coy., contained 92 % (w/w) ADP (analysed enzymically[10]) and 2.2 moles inorg. P/100 moles ADP. *DPN* and *TPN* were obtained from Sigma Chemical Coy. and assayed for purity by yeast alcohol dehydrogenase and ethanol, and by TPN-specific isocitric dehydrogenase and isocitrate, respectively. Both were about 90–95 % pure. *DPNH* was prepared enzymically from DPN using yeast alcohol dehydrogenase and ethanol. *TPNH* was obtained from Sigma Chemical Coy., or prepared by reaction with stoichiometric amounts of TPN and D-isocitrate in the presence of TPN-specific isocitric dehydrogenase. It was assayed with GSSG and GSSG reductase. D-*Isocitric acid* was prepared from dimethyl-isocitric lactone. *GSSG* was prepared by oxidation of GSH (Boehringer, 93 % pure) by I_2.

Analytical methods

Inorganic phosphate was determined as described by MYERS AND SLATER[7]. *Protein* was determined by the biuret method as described by CLELAND AND SLATER[11].

Determination of pyridine nucleotides in rat-liver mitochondria

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 CIOTTI AND KAPLAN[12] and ASTRACHAN AND JACOBSON[13]. Fluorimetric determination of the oxidized DPN and TPN was carried out by a modification[12] of the methyl ethyl ketone method of CARPENTER AND KODICEK[14]. Fluorescence was measured on a COHEN[15] fluorimeter equipped with a nickel oxide glass filter in the incident beam, and a Euphor-glass filter between the solution and the photoelectric cell.

The oxidized pyridine nucleotides were determined in the extracts of mitochondria obtained by adding 0.20 ml 40 % trichloroacetic acid to 1.25 ml of a suspension containing 0.25 ml of the mitochondrial preparation. After centrifugation, the supernatant was completely decanted and neutralized with 3.5 *N* NaOH, using

bromothymol blue as external indicator; 0.12 ml 3.5 *N* NaOH was usually required.

The reduced pyridine nucleotides were determined in alkali extracts of mitochondria. 1.4 ml 0.12 *M* Na_2CO_3* was added to a small homogenizing tube, corked, and placed in a boiling-water bath for 4 min. The mitochondrial aliquot (0.25 ml) was then quickly added to the homogenizing tube, the suspension homogenized with a Teflon plunger in the bath and left for 3–4 min. The sample was then completely transferred to a centrifuge tube, left at 0° for 10–20 min and centrifuged at low speed. The supernatant was completely decanted and then very carefully neutralized in the cold with 6 *N* HCl. Usually 2 drops were sufficient to bring the pH to 7.5–7.8. It is important that during the neutralization the pH does not go below 7.0 for even a very short period, since the reduced pyridine nucleotides are labile below this pH**.

To make the analyses specific for the oxidized and reduced forms of the pyridine nucleotide, specific enzymes were used. Two tubes were prepared for each determination; in one, trichloroacetic acid was added before the enzyme and in the other the enzymic reaction was completed before the addition of trichloroacetic acid.

(a) *DPN*. The reaction mixture contained 0.4 ml 0.25 *M* Tris, pH 10.1, 0.05 ml 95% ethanol, and various aliquots of a DPN solution or of the neutralized trichloroacetic acid extract of the mitochondria in a total vol. of 0.80 ml. An amount of alcohol dehydrogenase (0.05 ml of 20- to 40-fold diluted enzyme) sufficient completely to reduce DPN under these conditions, without reacting with TPN, was added and the reaction stopped with 0.15 ml 40% trichloroacetic acid after 15 min at room temperature (22°). In the control tube trichloroacetic acid was added at zero time.

(b) *TPN*. The reaction mixture contained 0.4 ml 0.25 *M* Tris, pH 7.2, 0.02 ml D-isocitric acid (3.0 μmoles), 0.01 ml 0.15 *M* Mg^{++}, and various aliquots of a TPN solution or of the neutralized trichloroacetic acid extract of mitochondria in a total volume of 0.84 ml. TPN-specific isocitric dehydrogenase (0.01 ml) was added to start the reaction. The reaction was stopped with 0.15 ml 40% trichloroacetic acid after 15 min at room temperature. In the control tube trichloroacetic acid was added at zero time.

(c) *DPNH*. The reaction mixture contained 0.25 ml 0.12 *M* Na_2CO_3-$NaHCO_3$, pH 10.2; 0.2 ml 1 *M* K phosphate, pH 7.5; 0.05 ml 0.5 *M* acetaldehyde, and various aliquots of a DPNH solution or of the neutralized alkali extract of mitochondria in a total volume of 0.84 ml. 0.008 ml of 40-fold or 80-fold diluted alcohol dehydrogenase*** was added and the reaction stopped with 0.15 ml 40% trichloroacetic acid after 3–5 min at room temperature. The amount of alcohol dehydrogenase used (40 units§)

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* In many of the experiments described in this paper, a Na_2CO_3 solution of pH 10.2 (presumably obtained by absorption of atmospheric CO_2) was used. With this solution, alkaline destruction of DPN and TPN is not complete. If freshly prepared Na_2CO_3 is used, the oxidized nucleotides are completely destroyed.

** Since this neutralization must be carried out very carefully, it is preferable to use one of the two following procedures, which have been found to give comparable results. (1) 1.5 ml of the supernatant is added to 0.9 ml 0.25 *M* Tris, pH 7.3 + 0.15 ml 1 *N* HCl, which is sufficient to bring the pH to 7.3 (checked with bromothymol blue). 0.7 ml of this solution can be used directly for the DPNH determination without adding phosphate or Na_2CO_3. (2) 0.3 ml of the unneutralized extract + 0.3 ml 1 *M* phosphate, pH 7.5, are used for the DPNH determination.

*** The alcohol dehydrogenase used in this study had been kept in the deep freeze for several months during which period it had been repeatedly thawed and refrozen. This treatment apparently resulted in the removal of a contaminating enzyme (see DISCUSSION).

§ One unit is the amount of enzyme in a volume of 3.0 ml required to decrease the absorbancy by 0.01/min at pH 7.5, in the presence of excess DPNH and acetaldehyde.

was sufficient completely to oxidize the DPNH with negligible oxidation of TPNH. In the control tube trichloroacetic acid was added at zero time.

(*d*) *TPNH*. The reaction mixture contained 0.25 ml 0.25 *M* Tris, pH 7.4; 0.05 ml (5 μmoles) GSSG, pH 7.4, and various aliquots of a TPNH solution or of the neutralized alkali extracts of mitochondria in a total volume of 0.75 ml. 0.1 ml GSSG reductase* was added to start the reaction and the reaction stopped with 0.15 ml 40% trichloroacetic acid after 15 min at room temperature. In the control tube trichloroacetic acid was added at zero time.

After the completion of the various enzyme reactions, the fluorescence was developed by adding 0.2 ml methyl ethyl ketone followed by 0.6 ml 3.5 *N* NaOH. After 5 min at room temperature, 6 ml 0.4 *N* HCl was added and the tubes left to stand for a further 5 min. After checking that the pH was below 3, the tubes were placed in a boiling-water bath for 5 min, cooled and read in matched fluorimetric tubes. Known amounts of enzymically active DPN taken through the whole procedure were used to prepare a standard curve relating fluorescence intensity to amount of DPN. This curve was linear up to about 0.01 μmole DPN.

In the DPN and TPN determinations, the decrease in fluorescence brought by the enzymic reaction is equal to the amount of oxidized pyridine nucleotide present. (The reduced form is completely destroyed by treatment with trichloroacetic acid or with boiling HCl in the procedure used to develop the fluorescence.) Usually, but not always, the total fluorescence (corrected for the fluorescence of the reagents) obtained with the trichloroacetic acid extract of the mitochondria without reaction with enzymes was equivalent to the (DPN + TPN) content, as calculated from the separate analyses.

In the DPNH and TPNH determinations, the increase in fluorescence brought about by the enzymic reaction is equal to the amount of reduced pyridine nucleotide present.

The results of some tests of the reliability of these analytical methods are shown in Table I. None of the procedures were disturbed by the presence of the other three

TABLE I

TEST OF SPECIFICITY AND RECOVERIES OF THE VARIOUS PYRIDINE NUCLEOTIDES IN THE ANALYTICAL PROCEDURES

Known amounts of pyridine nucleotides were treated according to the various analytical procedures, either alone or added to freshly prepared liver mitochondria.

Pyridine nucleotide added	*Recovery (%) in analytical procedure for*			
	DPN	*DPNH*	*TPN*	*TPNH*
DPN – alone	103	0	0	0
– to mitochondria	97	—	—	—
DPNH – alone	0	99	0	0
– to mitochondria	—	98	—	—
TPN – alone	0	0	92	0
TPNH – alone	0	0	0	93
– to mitochondria	—	—	—	104

* Some preparations of GSSG reductase contain DPNH-oxidizing enzymes. The preparation used in these experiments did not oxidize DPNH in the presence of the alkali extract.

substances measured (care must be taken to choose an amount of alcohol dehydrogenase which completely oxidizes DPNH, without appreciably affecting TPNH. HOLZER[17] has shown that commercial preparations of yeast alcohol dehydrogenase contain as an impurity a TPNH-reacting alcohol dehydrogenase). The recoveries were quantitative within the limits of accuracy of the method. The reliability of the method is illustrated in Fig. 1.

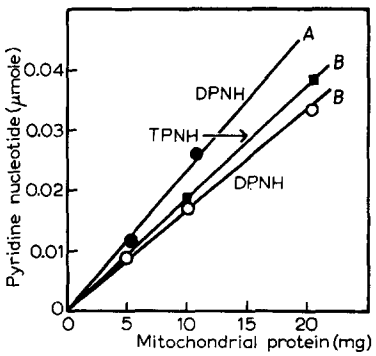

Fig. 1. Amounts of reduced pyridine nucleotides found in the alkali extract from different amounts of mitochondria. A and B represent different mitochondrial suspensions.

RESULTS

Effect of incubation of mitochondrial suspensions on concentrations of various forms of pyridine nucleotides

Figs. 2–4 describe experiments in which the mitochondria were incubated for various times at 30° with 40 m*M* phosphate (pH 7.3) in 0.05 *M* sucrose (Figs. 2A, 3 and 4) or with 1.9 m*M* ADP, 2 m*M* phosphate, 33 m*M* nicotinamide in 0.25 *M* sucrose (Fig. 2B). Fig. 2A shows that the "depletion" of diphosphopyridine nucleotide

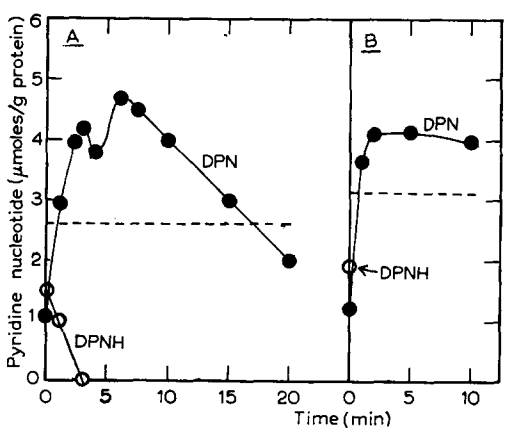

Fig. 2. Formation of DPN and disappearance of DPNH by incubation of mitochondria with phosphate or ADP. The mitochondrial suspension was gently shaken in air at 30°. A. Mitochondrial protein, 2.4 mg/ml; sucrose, 0.05 *M*; phosphate, pH 7.2, 40 m*M*. B. Mitochondrial protein, 5.6 mg/ml; sucrose, 0.25 *M*; ADP, pH 7.2, 1.9 m*M*; phosphate, pH 7.2, 2 m*M*; nicotinamide, 33 m*M*. The horizontal dotted lines show the (DPN + DPNH) at zero time.

from the mitochondria consists of at least two phases: first, the conversion of DPNH to its oxidized form, and secondly, destruction of the DPN.

The oxidation of TPNH occurred somewhat more slowly but in a similar manner (Figs. 3 and 4). The initial rates of DPN and TPN appearance shown in Fig. 4 are 1.8 and 0.8 μmoles/g protein/min, respectively. The relative rates are in good agreement with previous work which showed that the pyridine nucleotide transhydrogenase activity of depleted heart and liver mitochondria is slightly less than one-half the activity of the DPNH oxidase system[1].

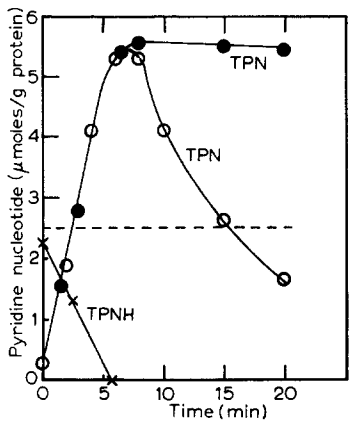

Fig. 3. Formation of TPN and disappearance of TPNH by incubation of mitochondria with phosphate. The mitochondrial suspension was gently shaken in air at 30°. O—O, ×—×, mitochondrial protein, 6.4 mg/ml; sucrose, 0.05 *M*; phosphate, pH 7.2, 40 m*M*. ●—●, same with nicotinamide, 33 m*M*. The horizontal dotted line shows the (TPN + TPNH) at zero time.

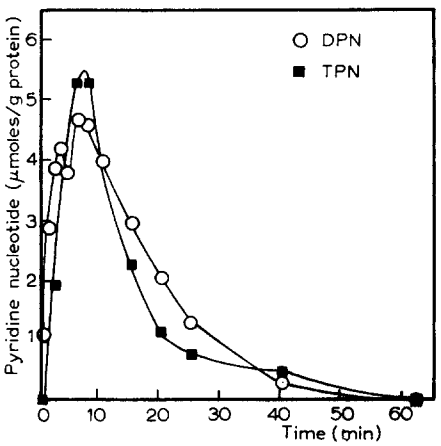

Fig. 4. Destruction of DPN and TPN by incubation of rat-liver mitochondria (6.1 mg protein/ml) in 0.05 *M* sucrose, 40 m*M* phosphate.

KAUFMAN AND KAPLAN[18] have shown that release of the oxidized pyridine nucleotide from the mitochondria into the supernatant occurs before destruction by the DPNase. Nicotinamide inhibits the destruction of the oxidized nucleotides (Figs. 2B and 3). It is believed by JACOBSON AND KAPLAN[19] that the DPNase which is responsible for the destruction of the oxidized pyridine nucleotides is of microsomal origin and is due to microsomal contamination of the mitochondrial preparation. This may possibly explain the fact that the time necessary for complete destruction of the pyridine nucleotides shown in Fig. 4 (about 60 min at 30°) is much greater than the 15 min reported by HUNTER AND FORD[20] and KAPLAN *et al.*[21].

The most interesting feature of Fig. 2 is that during the incubation with 40 m*M* phosphate in hypotonic medium or with 1.9 m*M* ADP, 2 m*M* phosphate in isotonic medium, the content of DPN reacting with yeast alcohol dehydrogenase increased to a value above the (DPN + DPNH) found at zero time. The extra DPN must be derived from some precursor.

The extra DPN was also formed by incubation with 2,4-dinitrophenol. Table II compares the formation of "extra" DPN, by incubation in isotonic medium with ADP and phosphate or with dinitrophenol, with that formed with 40 m*M* phosphate in hypotonic medium. Fig. 5 summarizes the result of an experiment in which the

TABLE II

FORMATION OF "EXTRA" DPN BY INCUBATION OF LIVER MITOCHONDRIA UNDER DIFFERENT CONDITIONS

The mitochondria were incubated for 10 min at room temperature with shaking in 0.05 *M* or 0.25 *M* sucrose containing 0.03 *M* nicotinamide and the additions shown. The pH was 7.4 in each case.

Addition	*Final sucrose (M)*	*Incubation time (min)*	*Diphosphopyridine nucleotide (μmoles/g protein)*			
			DPN	*DPNH*	*DPN + DPNH*	*"extra" DPN**
None	0.25	0	0.66	1.95	2.61	1.52
None	0.25	10	1.21	1.50	2.71	1.44
Phosphate (1.6 m*M*)	0.25	10	2.38	0.86	3.24	0.89
ADP (1.6 m*M*) + phosphate (1.6 m*M*)	0.25	10	3.99	0.00	3.99	0.14
Dinitrophenol (8 μ*M*)	0.25	10	3.84	0.00	3.84	0.29
Phosphate (40 m*M*)	0.05	10	4.13	—	—	—

* "Extra" DPN = total DPN as determined by incubation with 40 m*M* phosphate (4.13) — (DPN + DPNH).

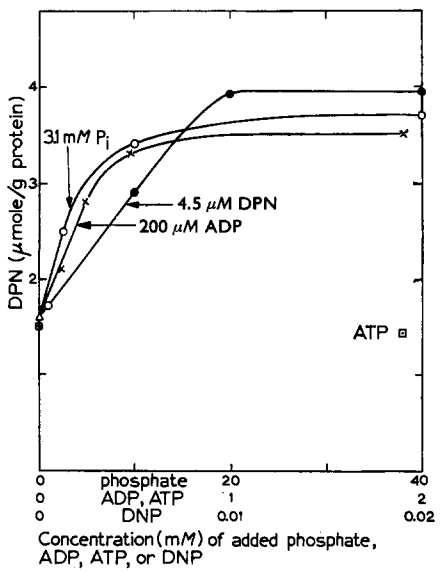

Fig. 5. Effect of concentration of phosphate, ADP and dinitrophenol on the formation of DPN from DPNH and "extra" DPN. Mitochondrial suspension (5.2 mg/ml) incubated for 5 min at 30° in the presence of 0.23 *M* sucrose, 10 m*M* nicotinamide and the indicated concentrations of phosphate, ADP, ATP or dinitrophenol (DNP) at pH 7.2. The arrows show the concentrations required for half-maximal appearance of DPN. ⊞, not-incubated mitochondria; △, mitochondria incubated with no addition.

amount of DPN formed in 5 min at 30° was studied as a function of concentration of phosphate, ADP, and 2,4-dinitrophenol. The concentrations giving half-maximal appearance of DPN in this time were 3.1 m*M* phosphate, 200 μ*M* ADP and 4.5 μ*M* dinitrophenol. In other experiments, carried out in the presence of 2 m*M* phosphate, half-maximal appearance of DPN was obtained with 70 μ*M* ADP. No DPN was formed by incubation with 1.9 m*M* ATP.

Concentrations of different forms of pyridine nucleotides in freshly prepared liver mitochondria

The concentrations of the six forms of the pyridine nucleotides in freshly prepared rat-liver mitochondria are given in Table III. Since significant differences in the DPNH and TPNH concentrations were found in mitochondria prepared in Amsterdam and Brandeis, both sets of values are given. These differences will be discussed in a following paper. The difference between the oxidized pyridine nucleotide after incubation, and the sum of oxidized and reduced nucleotide before incubation, was in every case highly significant ($P < 0.001$).

TABLE III

AVERAGE CONCENTRATION OF VARIOUS FORMS OF PYRIDINE NUCLEOTIDE IN RAT-LIVER MITOCHONDRIA

The values given (μmoles/g protein) are the means $\pm$ standard error of mean.

No. of preparations	*Prepared in*	*Diphosphopyridine nucleotide*				
		Not incubated			*Incubated**	*"extra" DPN***
		DPN	*DPNH*	*DPN + DPNH*	*DPN*	
49	Amsterdam	1.37 $\pm$ 0.064	1.69 $\pm$ 0.07	3.06 $\pm$ 0.08	4.16 $\pm$ 0.06	1.10
14	Brandeis	1.51 $\pm$ 0.066	1.26 $\pm$ 0.12	2.77 $\pm$ 0.15	4.14 $\pm$ 0.11	1.37
Total 63		1.41 $\pm$ 0.042	1.60 $\pm$ 0.06	3.01 $\pm$ 0.07	4.15 $\pm$ 0.05	1.14

No. of preparations	*Prepared in*	*Triphosphopyridine nucleotide*				
		Not incubated			*Incubated**	*"extra" TPN***
		TPN	*TPNH*	*TPN + TPNH*	*TPN*	
18	Amsterdam	0.25 $\pm$ 0.12	2.25 $\pm$ 0.15	2.50 $\pm$ 0.15	5.00 $\pm$ 0.17	2.50
13	Brandeis	0.25 $\pm$ 0.15	3.34 $\pm$ 0.30	3.59 $\pm$ 0.32	5.01 $\pm$ 0.08	1.42
Total 31		0.25 $\pm$ 0.09	2.71 $\pm$ 0.18	2.96 $\pm$ 0.18	5.00 $\pm$ 0.12	2.04

* For 7–15 min at 30° in the presence of 33 m*M* nicotinamide and (i) 40 m*M* phosphate (pH 7.2) in 0.05 *M* sucrose, (ii) 1.9 m*M* ADP (pH 7.2) in 0.05 *M* sucrose, (iii) 1.9 m*M* ADP (pH 7.2), 2 m*M* phosphate (pH 7.2) in 0.05 *M* or 0.25 *M* sucrose, or (iv) 10 μ*M* 2,4-dinitrophenol in 0.05 *M* or 0.25 *M* sucrose.

** "Extra" DPN = (DPN after incubation with phosphate, ADP or dinitrophenol) — ((DPN + DPNH) before incubation). Similarly "Extra" TPN = (TPN after incubation with phosphate, ADP or dinitrophenol) — ((TPN + TPNH) before incubation).

The frequency distribution of the amount of "extra DPN" in the 63 preparations is given in Fig. 6. Only two preparations did not show any "extra" DPN.

The presence of "extra" DPN in the alkali extract

The difference in (DPN + DPNH) found at zero time and the DPN found after incubation with phosphate, ADP or dinitrophenol could be due to either (a), the "extra" DPN is stable to treatment with trichloroacetic acid and/or Na_2CO_3, but does not react with alcohol dehydrogenase, or (b), it is destroyed during the preparation of both the acid and alkali extracts.

The possible presence of the "extra" DPN in the acid and alkali extracts of mitochondria was tested by adding the neutralized extracts together with ADP and phosphate to mitochondria, previously incubated with phosphate in the absence of nicotinamide in order to deplete them of pyridine nucleotides. Using mitochondria,

the amount of "extra" DPN in which had been increased by incubation with α-ketoglutarate[5], about 90% of the "extra" DPN was recovered in the alkali extract (Table IV). None was recovered in the acid extract.

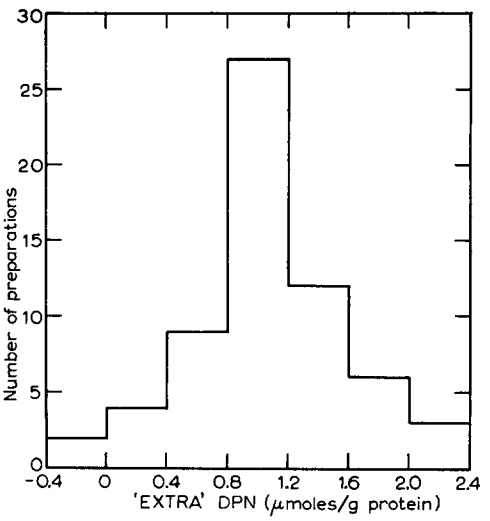

Fig. 6. Frequency distribution of the amount of "extra" DPN found in all preparations of rat-liver mitochondria analysed.

TABLE IV

Different aliquots of a mitochondrial suspension were incubated for (a) 1.5 min at 0° with no addition; (b) 1.5 min at 0° with 10 m*M* α-ketoglutarate; (c) 7 min at 30° with 1.9 m*M* ADP, 15 m*M* phosphate, 33 m*M* nicotinamide, pH 7.2. After these incubations, acid and alkali extracts were made as described in the METHODS. After measuring the DPN and DPNH contents of the neutralized extracts, aliquots of extracts (a) and (b) were added together with ADP (1.9 m*M* final concentration), phosphate, pH 7.2 (15 m*M*), and nicotinamide (33 m*M*) to mitochondria, previously almost depleted of their pyridine nucleotide, by incubation in 0.05 *M* sucrose, 40 m*M* phosphate, pH 7.2, for 45 min at 30°. After incubation of this mixture at 35° for 11 min, trichloroacetic acid was added and the DPN content determined.

Expt.		*1*			*2*		
Incubation		*(a)*	*(b)*	*(c)*	*(a)*	*(b)*	*(c)*
Acid extract							
DPN (μmoles/g protein)							
– before incubation with	(i)	2.89	1.62	3.90	1.76	0.97	4.23
– after depleted mitochondria	(ii)	2.83*	—	—	—	0.93*	—
Alkali extract							
DPNH (μmoles/g protein)	(iii)	0.59	0.16	—	1.69	1.46	—
DPN (μmoles/g protein)							
– before incubation with	(iv)	0.64	0.42	—	—	0.16	—
– after depleted mitochondria	(v)	1.38	2.52	—	—	3.18	—
– formed by incubation with depleted mitochondria	(vi)	0.74	2.10	—	—	3.02	—
– accounted for by DPNH	(vii)	0.59	0.16	—	—	1.46	—
– derived from precursor	(viii)	0.15	1.94	—	—	1.56	—
"Extra" DPN (μmoles/g protein)							
– calculated**	(ix)	0.42	2.12	—	0.76	1.80	—
(viii)/(ix) × 100		36	91	—	—	87	—

* Corrected for DPN in depleted mitochondria.

** (i) in incubation (c) *minus* [(i) + (iii)] in incubations (a) or (b), respectively.

DISCUSSION

The fact that the total DPN found after incubating the mitochondria exceeded the (DPN + DPNH) before incubation could be explained in two ways: (a) the analytical

method underestimates the DPNH content of fresh mitochondria; (b) DPN is liberated from some precursor during the incubation. Before discussing the latter possibility it is advisable to list the evidence that the analytical procedure was sound.

(1) DPNH and TPNH added to liver mitochondria were recovered quantitatively (Table I).

(2) The amounts of DPNH and TPNH found in the alkali extract were proportional to the amount of mitochondria used (Fig. 1).

(3) To investigate the possibility that DPNH was oxidized by the DPNH oxidase system in the mitochondria before this enzyme system was inactivated in the Na_2CO_3 at 100°, experiments were carried out in which 1.8 m*M* Amytal or 2 m*M* KCN were added to the mitochondria prior to preparing the extracts. These inhibitors of the DPNH oxidase system did not bring about any increase in the DPNH content.

(4) Experiments which will be described in a following paper showed that when mitochondria were incubated with α-ketoglutarate, succinate or glutamate, the DPN content decreased with either no change or a slight decrease in the DPNH content. There appears no reason to expect that addition of these substrates would cause a further under-estimation of the DPNH content. The addition of β-hydroxybutyrate caused an increase in both the DPN and DPNH contents so that (DPN + DPNH) became almost equal to the DPN liberated by incubation with phosphate and ADP. There is no reason to expect that addition of β-hydroxybutyrate could improve the estimation of the DPNH.

(5) It will also be shown in a following paper that incubation of liver mitochondria with 0.8 m*M* Amytal and ADP causes the disappearance of DPNH with little change in DPN. Again, there is no reason to expect that this concentration of Amytal would cause a further under-estimation of the DPNH content. As already mentioned, a higher concentration of Amytal (1.8 m*M*) had no effect on the DPNH content.

(6) The alkali extract of mitochondria was found to contain a substance, not DPNH, which yielded DPN on incubation with mitochondria previously depleted of their own pyridine nucleotide (Table IV).

It is concluded, therefore, that the extra DPN formed by incubation of mitochondria with phosphate, ADP or dinitrophenol is due to liberation of DPN from some precursor. Although the nature or the function of this precursor is not yet established, the type of substance which causes the liberation of DPN suggests that it is connected with oxidative phosphorylation. In fact, it has the properties expected of a diphosphopyridine nucleotide compound DPN ~ I which has been postulated as an energy-rich intermediate of oxidative phosphorylation[3,4,22], undergoing the following reactions:

$$\mathrm{DPN}\sim\mathrm{I} + \mathrm{X} \rightleftharpoons \mathrm{X}\sim\mathrm{I} + \mathrm{DPN} \quad (1)$$

$$\mathrm{X}\sim\mathrm{I} + \mathrm{P_i} \rightleftharpoons \mathrm{X}\sim\mathrm{P} + \mathrm{I} \quad (2)$$

$$\mathrm{X}\sim\mathrm{P} + \mathrm{ADP} \rightleftharpoons \mathrm{X} + \mathrm{ATP} \quad (3)$$

$$\mathrm{X}\sim\mathrm{I} + \mathrm{H_2O} \xrightarrow{[\text{(dinitrophenol)}]} \mathrm{X} + \mathrm{I} \quad (4)$$

Since the passage of electrons in TPN-linked systems proceeds through a transhydrogenation reaction between TPNH and DPN to form TPN and DPNH, followed by the DPNH oxidase reaction[21,1], a similar TPN precursor was not expected. However, Fig. 3 shows the presence in liver mitochondria of a compound which

liberates TPN during incubation. The significance of this compound is discussed in a following paper.

Reactions (1)–(4) are consistent with the breakdown of DPN ~ I brought about by incubation with inorganic phosphate, ADP or dinitrophenol, and with the fact that ATP does not cause this breakdown. The concentrations of phosphate, ADP and dinitrophenol found to give half the maximum liberation of DPN from DPNH and "extra" DPN are comparable with those found by CHANCE AND CONNELLY[23] and CHANCE AND HOLLUNGER[24] to be necessary for half-maximum oxidation of intramitochondrial DPNH, measured spectrophotometrically (1 mM phosphate[23]; 56 μM ADP, in the presence of 10 mM phosphate[23]; 3.0 μM dinitrophenol[24]).

The finding that ADP itself is able to bring about the decomposition of "extra" DPN without the addition of P_i can be explained on the basis of reactions (1)–(4) by the fact that the mitochondrial preparations contain sufficient inorganic phosphate for the reaction. Our preparations contain about 5 μmoles P_i/g protein, more than the total of "extra" DPN + "extra" TPN.

The fact that relatively high concentrations of inorganic phosphate in the absence of ADP cause the appearance of DPN is probably not due to the presence of sufficient X in the mitochondria, but is rather due to an "uncoupling" action of phosphate, *i.e.* incubation with phosphate causes the hydrolysis of X ~ P or X ~ I.

The ratios of the total triphosphopyridine nucleotide to diphosphopyridine nucleotide and of TPNH to TPN given in Table III agree closely with the values reported by GLOCK AND MCLEAN[25]. The total DPN content agrees with the values reported by HOLTON *et al.*[26] after incubation with ADP. Although most other workers (*e.g. cf.* refs. [25,27]) have reported DPNH to DPN ratios of 0.5, the higher ratio in Table III agrees with the conclusions of CHANCE AND WILLIAMS[4] that tightly coupled mitochondria have high DPNH/DPN ratios.

The only complete study of the DPN, DPNH, TPN and TPNH content of liver mitochondria (expressed as μmoles/g protein) reported in the literature is that of JACOBSON AND KAPLAN[27] who found DPN, 1.22; DPNH, 0.54; TPN, 0.4; TPNH, 3.92. The ratio (TPN + TPNH)/(DPN + DPNH) differs quite markedly from the values given in this paper and from those of GLOCK AND MCLEAN[25]. The TPNH content is higher and the DPNH value lower than our values. These differences may possibly be due to there being more "extra" DPN and less "extra" TPN in the mitochondrial preparations used by JACOBSON AND KAPLAN. A significant difference in the concentrations of the reduced pyridine nucleotides between mitochondria prepared in different laboratories is shown in Table III. These differences are reflected in the amounts of "extra" pyridine nucleotides found.

In its stability to alkali and instability to acid, "extra" DPN resembles DPNH. However, unlike the latter it does not yield DPN on reaction with acetaldehyde* and

* Recently[16] it has been found that commercial preparations of alcohol dehydrogenase (both Boehringer and Worthington) contain an enzyme which catalyses the liberation of DPN from "extra" DPN in the presence of acetaldehyde. This enzyme, which is possibly an aldehyde dehydrogenase, disappeared on storage (during which the enzyme preparation was repeatedly thawed and refrozen). A small amount (30–40 units) of the fresh preparation is sufficient to oxidize all the DPNH in 1 min, with negligible effect on the "extra" DPN. (This was ascertained by comparison with measurements made with malic dehydrogenase + oxaloacetate, which reacts only with DPNH). A larger amount (150–200 units) reacts with nearly all the "extra" DPN in 5 min. Extracts of *Clostridium kluyverii* catalyse the liberation of both DPN from "extra" DPN, and of TPN from "extra" TPN, in the presence of acetaldehyde.

alcohol dehydrogenase. The properties of this "extra" DPN thus bear some resemblance to those of addition compounds of DPN which are substituted on the 4-carbon atom[28-31]. The possible nature of DPN ~ I will be further discussed in a following paper.

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