The Behaviour of Pyridine Nucleotides of Mitochondria in a 'Saline Medium'

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The considerable volume of information obtained about the metabolism of the endogenous pyridine nucleotides of mitochondria has been derived from experiments in many different fields of research. Rarely have the investigations been concerned primarily with the reactions of the individual nucleotides themselves, though the work of Chance and his collaborators has thrown much light on the general pattern of behaviour of the mitochondrial coenzymes (for references to the need for investigations supplementary to this work see Chance & Baltscheffsky, 1958). Indeed, no systematic study of the reactions of the endogenous oxidized and reduced diphosphopyridine nucleotides and the oxidized and reduced triphosphopyridine nucleotides of mitochondria isolated by a standard procedure and subsequently incubated under a variety of experimental conditions has been reported. This paper describes an investigation of the reactivity of the pyridine nucleotides of mitochondria suspended in a 'saline medium' and the influence of the components of this medium on the behaviour of the coenzymes.

MATERIALS

5-Ethyl-5-isoamylbarbiturate (amytal). This was obtained from Eli Lilly and Co. Ltd., Basingstoke, and freshly prepared solutions of the sodium salt were used in each experiment.

2:4-Dinitrophenol. This was obtained from British Drug Houses Ltd., and stock solutions of this compound and of the metabolites used were prepared in 'saline medium' (see below) and stored at -15° before use.

Adenosine triphosphate. This was obtained as commercial products from L. Light and Co. Ltd., Colnbrook, Bucks, and Zellstoff-fabrik Waldhof, Pharmazeutische Abteilung, Wiesbaden, Germany.

All the other compounds used were commercial products. Reagents used in the analysis of pyridine nucleotides were as described by Bassham, Birt, Hems & Loening (1959).

METHODS

Preparation of media for the isolation and incubation of mitochondria. In experiments on the influence of nicotin-

amide on pyridine nucleotide metabolism, the basic medium was that of Chappell & Perry (1954), containing KCl (0.1M), 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer, pH 7.4 (0.05M), ethylenediaminetetraacetic acid (EDTA; 1 mM), MgSO_4 (5 mM) and adenosine triphosphate (ATP; 1 mM). The sucrose and 'saline' media were prepared as described by Birt & Bartley (1960*a*); the latter medium was that of Chappell & Perry (1954), to which nicotinamide (25 mM) had been added.

Preparation of mince. Rat-liver mince was prepared as described by Bassham *et al.* (1959) except that the final suspension was made in 'saline medium'.

Preparation of mitochondria. (a) Rat-liver mitochondria. These were prepared by the method described by Birt & Bartley (1960*a*), except that the removal of the 'fluffy layer' was less complete and there was more variation in the concentration of mitochondria in the final suspension.

(b) Rat-brain mitochondria. These were prepared by the method of Bellamy (1959) except that nicotinamide (25 mM) was added to the sucrose medium. The isolated particles were washed twice and finally suspended at 0° in the 'saline medium'.

(c) Fly-muscle mitochondria. These were prepared by the method of Sacktor (1953), except that nicotinamide (25 mM) was added to the isolation medium (sucrose, 0.25M, and bovine albumin, 1%). The isolated particles were washed twice and finally suspended at 0° in 'saline medium'.

Incubations. These were carried out in open conical flasks which were agitated gently during incubation at the appropriate temperature in a Dubnoff metabolic shaking incubator (manufactured by Precision Scientific Co., Chicago, Ill., U.S.A.).

Measurement of the distribution of the pyridine nucleotides between the mitochondria and the suspending medium. The mitochondria in a sample of the suspension were separated from the supernatant fluid by centrifuging at 0° for 5 min. at 26 000 g. Extractions of the whole suspension and the supernatant were made and the coenzyme content of both was determined. The intramitochondrial pyridine nucleotide was determined by difference.

Measurement of uptake of oxygen. Uptake of O_2 was measured in the Warburg apparatus. The values given in the tables are rates of O_2 consumption derived from the total uptake of O_2 at the times shown in the tables.

Measurements of pyridine nucleotides. Oxidized diphosphopyridine nucleotide (DPN^+), reduced diphosphopyridine nucleotide (DPNH), oxidized triphosphopyridine nucleotide (TPN^+) and reduced triphosphopyridine nucleotide (TPNH) and total levels of oxidized and reduced pyridine nucleotides were measured by the fluorimetric

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method of Bassham *et al.* (1959). All values for amounts of coenzyme are given to two significant figures or to the nearest 0.05 $\mu\text{m-mole/mg. dry wt.}$

Measurement of protein content of mitochondria. The dried mitochondrial pellets from which the dry matter of the mitochondrial suspensions was determined were wet-ashed and the N content of suitable dilutions measured by direct nesslerization. The mitochondrial protein was assumed to contain 16% of N.

RESULTS

Relative stability of the pyridine nucleotides of rat-liver mince and mitochondria. A portion of a rat-liver mince was suspended in 'saline medium' (1 g. fresh wt./10 ml. of suspension) and the suspension was sampled and kept at 0° while mitochondria were isolated from the rest of the preparation. Further samples of both mince and the isolated mitochondria (at 0°) were taken, the temperature of the two preparations was raised to 25° and incubation continued for 1 hr. before the final sampling. The estimations showed that, whereas there was no loss of pyridine nucleotide in the mince kept at 0°, there was a slight increase in the proportion of the total coenzymes in the

reduced form. During the incubation for 1 hr. at 25°, the rate of loss of the coenzymes was five times as great in the mince as in the mitochondrial suspension; the loss of reduced nucleotide was particularly great. Assuming that the mitochondria comprise 20% of the liver fresh weight and that the rate of loss of coenzymes was the same for the mitochondria in the two suspensions (both in the same medium), then the percentages of the total pyridine nucleotide present in the mitochondria at the beginning and end of the incubation were 34 and 89% respectively.

Influence of components of the isolation and incubation media on the pyridine nucleotides of mitochondria

Nicotinamide. Nicotinamide at 25 mM concentration in the sucrose medium used for isolation of mitochondria had little effect on the original levels of coenzymes in the isolated particles (Table 1). Though nothing is known about the extent to which the nicotinamide in the sucrose medium penetrates the mitochondria, it was found that the particles retained sufficient of it, even after the

Table 1. *Effect of nicotinamide on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$ Temperature of incubation, 25°; concn. of nicotinamide in both sucrose and Chappell-Perry media, 25 mM.

In this and subsequent tables, PN⁺ represents DPN⁺ + TPN⁺, PNH represents DPNH + TPNH and PN represents PN⁺ + PNH. For the meaning of the terms 'Absolute loss' (Abs. loss) and 'Relative loss' (Rel. loss) in this and subsequent tables, see text.

Rates of O₂ uptake ($\mu\text{l. of O}_2\text{/mg. dry wt./hr.}$) at (i) end of 15 min., (ii) end of 60 min.:

	(a)	(i)	(ii)	(c)	(i)	(ii)
	(b)	6.4	3.7	(d)	4.4	0.9
		2.8	0.9		2.8	0.9

Mitochondria prepared in sucrose medium

Time (min.)	(a) Incubated in Chappell-Perry medium						(b) Incubated in Chappell-Perry medium plus nicotinamide ('saline medium')					
	PN ⁺	PNH	PN	PNH PN ⁺	Abs. loss (%)	Rel. loss (%)	PN ⁺	PNH	PN	PNH PN ⁺	Abs. loss (%)	Rel. loss (%)
0	0.70	1.70	2.40	2.4	27	—	1.75	1.55	3.30	0.90	0	—
15	0.75	1.25	2.00	1.7	39	17	2.40	0.85	3.25	0.35	2	2
60	0.40	0.75	1.15	1.9	65	52	2.10	0.30	2.40	0.14	27	27

Mitochondria prepared in sucrose-nicotinamide medium

Time (min.)	(c) Incubated in Chappell-Perry medium						(d) Incubated in Chappell-Perry medium plus nicotinamide ('saline medium')					
	PN ⁺	PNH	PN	PNH PN ⁺	Abs. loss (%)	Rel. loss (%)	PN ⁺	PNH	PN	PNH PN ⁺	Abs. loss (%)	Rel. loss (%)
0	0.60	1.70	2.30	2.8	30	—	1.40	1.75	3.15	1.3	4	—
15	0.73	1.30	2.03	1.8	39	12	2.10	0.75	2.85	0.36	14	10
60	0.52	1.05	1.57	2.0	52	32	2.10	0.17	2.27	0.08	31	28

washings and incubation in a medium containing no nicotinamide, for its characteristic effects to be seen [compare the stability of coenzymes and the respiration rates in (a) and (c), Table 1]. Accordingly, it was retained at the same concentration in the sucrose as in the ionic medium used for incubation.

The addition of nicotinamide (25 mM) to Chappell-Perry medium used for final suspension of the particles in these experiments increased the initial amounts of pyridine nucleotides found in the isolated mitochondria (Table 1), even though all the washings and resuspensions (time approx. 45 min.) in this medium before the first extractions of coenzymes were done at 0°. The values for 'absolute loss' in Table 1 (and subsequent tables) are based on the decrease below the highest initial total found in each experiment [e.g., in Table 1, 3.30 μ m-moles/mg. dry wt., in (b)]. The 'relative loss' (i.e. the percentage disappearance of the nucleotides in each individual mitochondrial suspension) was almost the same whenever the incubating medium contained nicotinamide, and only slightly greater when nicotinamide was present in the sucrose isolation medium alone (i.e. when it was absent from the incubation medium). When nicotinamide was omitted from both media, the loss during incubation was considerably greater.

Two other effects of nicotinamide were observed. First, it decreased the ability of mitochondria to maintain the nucleotides in the reduced state. This is shown in Table 1 [compare (a) and (b) and (c) and (d)]. Whereas the initial level of reduced coenzymes was similar in all four preparations, the final level in the presence of nicotinamide was relatively very small, so that the ratio of reduced coenzyme/oxidized coenzyme was greatly diminished. This diminution was enhanced by the protection of the oxidized coenzymes by the nicotinamide. Secondly, nicotinamide, whether present in the sucrose medium alone, in the 'saline medium' alone or in both, decreased the initial and final rates of endogenous respiration.

The influence of nicotinamide on mitochondria incubated with oxidizable substrate was tested in experiments with succinate and pyruvate (Tables 2 and 3).

Succinate. The stability of the coenzymes was affected in the same way by nicotinamide whether or not succinate was present, i.e. initial values were higher and the loss during incubation decreased. Values given in (b) and (d) of Table 2 illustrate the preferential protection of oxidized coenzymes. Succinate alone had little or no protective action; compare (a) and (b). At all times during incubation, the diminution produced by nicotinamide in

Table 2. *Effect of nicotinamide and succinate on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μ m-moles/mg. dry wt. Temperature of incubation, 25°; concn. of succinate, 10 mM; concn. of nicotinamide, 25 mM. See also protocol to Table 1.

Rates of O₂ uptake (μ l. of O₂/mg. dry wt./hr.) at (i) end of 15 min., (ii) end of 60 min.:

	(i)					(ii)						
	(a)	3.6		1.8		(c)	2.4		1.2			
	(b)	21.6		2.8		(d)	22.8		11.6			
Mitochondria prepared in sucrose-nicotinamide medium. Incubated in Chappell-Perry medium												
	(a) No substrate						(b) With succinate					
Time (min.)	PN+	PNH	PN	PNH PN+	Abs. loss (%)	Rel. loss (%)	PN+	PNH	PN	PNH PN+	Abs. loss (%)	Rel. loss (%)
0	0.4	2.9	3.30	7.3	19	—	0.29	2.8	3.09	9.7	25	—
15	0.85	2.1	2.95	2.5	25	11	0.18	3.0	3.18	17.0	22	0
60	0.60	1.4	2.00	2.3	51	39	0.31	1.8	2.11	5.8	49	31
Mitochondria prepared in sucrose-nicotinamide medium. Incubated in Chappell-Perry medium plus nicotinamide ('saline medium')												
	(c) No substrate						(d) With succinate					
Time (min.)	PN+	PNH	PN	PNH PN+	Abs. loss (%)	Rel. loss (%)	PN+	PNH	PN	PNH PN+	Abs. loss (%)	Rel. loss (%)
0	1.4	2.7	4.1	1.9	0	—	1.3	2.8	4.1	2.2	0	—
15	2.6	0.83	3.43	0.32	16	16	0.9	3.5	4.4	3.9	0	0
60	2.7	0.45	3.15	0.17	23	23	1.4	2.4	3.8	1.7	7	7

the ratios of reduced to oxidized coenzymes, demonstrated again in the absence of substrate, was found also with succinate; though with this substrate, the greater part of the effect was due to the preservation of the oxidized coenzymes by the nicotinamide, rather than to a lowering of the level of reduced coenzymes [compare 15 min. values in (b) and (d), Table 2]. The ratios produced by succinate during the incubation declined more rapidly in the absence of nicotinamide.

The effect of nicotinamide on the respiration with succinate was markedly different from that on endogenous respiration. The rate with the added substrate was virtually unaltered by nicotinamide at the beginning of incubation and was maintained at a higher level (fourfold) after 1 hr. (Table 2). In this experiment the differences in the final respira-

tion rates with succinate [(b) and (d)] were inversely proportional to the differences in the overall losses of coenzymes; the rate with nicotinamide was four times as great, and the loss of coenzymes four times less.

Pyruvate. Similar results were obtained with nicotinamide and pyruvate, which is much less effective than succinate in reducing pyridine nucleotides (Table 3). Nicotinamide stabilized the coenzymes and there was no decrease in the amounts of reduced nucleotide produced by the pyruvate. The preferential destruction of oxidized pyridine nucleotides in the absence of nicotinamide (compare 60 min. values, Table 3) caused the ratio of reduced coenzyme/oxidized coenzyme to increase throughout the hour of incubation.

Table 3. *Effect of nicotinamide and pyruvate on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$ and for O_2 uptake as $\mu\text{l. of O}_2/\text{mg. dry wt./hr.}$ Temperature of incubation, 25° ; concn. of nicotinamide 25 mM; concn. of pyruvate, 10 mM. See also protocol to Table 1.

Mitochondria prepared in sucrose-nicotinamide medium

Time (min.)	Incubated in Chappell-Perry medium plus pyruvate (10 mM)						Incubated in Chappell-Perry medium with nicotinamide ('saline medium') plus pyruvate (10 mM)					
	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O_2	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O_2
0	1.15	3.00	4.15	2.6	—	—	1.75	3.05	4.80	1.7	—	—
15	1.05	3.45	4.50	3.3	0	13.2	0.96	3.90	4.88	4.1	0	10.4
60	0.48	2.70	3.18	5.6	23	5.6	1.20	3.15	4.35	2.6	8	5.6

Table 4. *Effect of ethylenediaminetetra-acetic acid on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$, and for O_2 uptake as $\mu\text{l. of O}_2/\text{mg. dry wt./hr.}$ Temperature of incubation, 25° ; concn. of EDTA (sodium salt), 1 mM; concn. of pyruvate, 10 mM.

Incubation medium without EDTA: KCl, 0.1 M; tris-HCl buffer (pH 7.4) 0.05 M; MgSO_4 , 5 mM; ATP, 1 mM; nicotinamide 25 mM. See also protocol to Table 1.

Mitochondria prepared in sucrose-nicotinamide medium.
Incubated in medium without EDTA

Time (min.)	(a) No substrate						(b) With pyruvate					
	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O_2	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O_2
0	1.90	3.70	5.60	1.95	—	—	2.10	3.70	5.80	1.75	—	—
15	2.70	2.60	5.30	0.95	5	4.3	2.10	3.60	5.70	1.70	2	8.4
45	2.70	2.80	5.50	1.05	2	3.4	2.30	3.50	5.80	1.50	0	5.7

Mitochondria prepared in sucrose-nicotinamide medium.
Incubated in 'saline medium'

Time (min.)	(c) No substrate						(d) With pyruvate					
	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O_2	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O_2
0	2.10	3.50	5.60	1.65	—	—	2.30	3.40	5.70	1.50	—	—
15	2.80	2.10	4.90	0.75	13	3.9	2.70	2.40	5.10	0.90	14	5.4
45	2.80	2.10	4.90	0.75	13	3.4	2.20	2.70	4.90	1.25	17	5.3

Adenosine triphosphate. Without added substrate, the omission of ATP had little effect on either the oxidized or reduced coenzyme, the only apparent result being a slight oxidation of the reduced nucleotides. With succinate, no differences were detected when ATP was omitted. However, the initial respiration, both with and without succinate,

was increased by ATP. In the absence of ATP and without added substrate, the rate of oxygen uptake increased greatly during the incubation, and the level of reduced pyridine nucleotides fell to a very low value. Further reference to changes of this kind will be made later (see Birt & Bartley, 1960b).

Table 5. *Effect of magnesium on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$, and for O_2 uptake as $\mu\text{l. of O}_2/\text{mg. dry wt./hr.}$ Temperature of incubation, 25° ; concn. of magnesium, 5 mM; concn. of pyruvate, 10 mM.

Incubation medium without Mg^{2+} ions: KCl, 0.1 M; tris-HCl buffer (pH 7.4) 0.05 M; EDTA, 1 mM; ATP, 1 mM; nicotinamide, 25 mM. See also protocol to Table 1.

Mitochondria prepared in sucrose-nicotinamide medium.
Incubated in 'saline medium'

Time (min.)	(a) No substrate						(b) With pyruvate					
	PN ⁺	PNH	PN	PNH PN ⁺	Loss (%)	Uptake of O_2	PN ⁺	PNH	PN	PNH PN ⁺	Loss (%)	Uptake of O_2
0	1.40	2.60	4.00	1.9	—	—	1.35	2.80	4.15	2.1	—	—
15	1.90	1.80	3.70	0.9	8	3.0	0.55	3.45	4.00	6.3	4	9.0
60	3.15	0.30	3.45	0.1	14	1.1	1.15	2.60	3.75	2.3	10	7.0

Mitochondria prepared in sucrose-nicotinamide medium.
Incubated in medium without Mg^{2+} ions

Time (min.)	(c) No substrate						(d) With pyruvate					
	PN ⁺	PNH	PN	PNH PN ⁺	Loss (%)	Uptake of O_2	PN ⁺	PNH	PN	PNH PN ⁺	Loss (%)	Uptake of O_2
0	1.35	2.55	3.90	1.9	—	—	1.20	2.75	3.95	2.3	—	—
15	1.20	2.80	4.00	2.3	0	4.2	1.20	2.80	4.00	2.3	0	6.7
60	1.15	2.35	3.50	2.1	10	2.7	1.15	2.80	3.95	2.4	0	7.1

Table 6. *Effect of magnesium on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$, and for O_2 uptake as $\mu\text{l. of O}_2/\text{mg. dry wt./hr.}$ Temperature of incubation, 25° ; concn. of magnesium, 5 mM; concn. of succinate, 10 mM.

Incubation medium without Mg^{2+} ions: KCl, 0.1 M; tris-HCl buffer (pH 7.4) 0.05 M; EDTA, 1 mM; ATP, 1 mM; nicotinamide, 25 mM. See also protocol to Table 1.

Mitochondria prepared in sucrose-nicotinamide medium.
Incubated in 'saline medium'

Time (min.)	(a) No substrate						(b) With succinate					
	PN ⁺	PNH	PN	PNH PN ⁺	Loss (%)	Uptake of O_2	PN ⁺	PNH	PN	PNH PN ⁺	Loss (%)	Uptake of O_2
0	1.15	1.70	2.85	1.5	—	—	0.62	2.25	2.87	3.6	—	—
15	1.60	1.20	2.80	0.75	1	2.9	0.45	2.00	2.45	4.5	16	15.0
60	1.70	0.24	1.94	0.14	33	0.8	1.20	1.45	2.65	1.2	7	5.3

Mitochondria prepared in sucrose-nicotinamide medium.
Incubated in medium without Mg^{2+} ions

Time (min.)	(c) No substrate						(d) With succinate					
	PN ⁺	PNH	PN	PNH PN ⁺	Loss (%)	Uptake of O_2	PN ⁺	PNH	PN	PNH PN ⁺	Loss (%)	Uptake of O_2
0	1.05	1.70	2.75	1.6	—	—	0.74	2.00	2.74	2.7	—	—
15	1.05	1.40	2.45	1.3	11	4.3	0.65	1.80	2.45	2.8	12	14.8
60	1.15	1.20	2.35	1.0	14	3.3	1.20	1.40	2.60	1.2	6	5.5

Ethylenediaminetetra-acetic acid. The initial levels of total pyridine nucleotide in isolated mitochondria were unaffected by the addition of EDTA, but the proportion of reduced coenzyme was decreased (Table 4). This effect of EDTA in lowering the ratio of reduced coenzyme/oxidized coenzyme became more apparent after incubation for 15 min., especially in the presence of an added substrate (e.g. pyruvate). The stability of the nucleotides was somewhat greater in the absence of EDTA, perhaps because of the greater proportion of reduced coenzymes and the lower rate of leakage from the particles (see Table 11). The initial rates of oxygen uptake, both with and without added substrate, were decreased by EDTA, but the subsequent decline in the respiration rate was not as great as when EDTA was omitted.

Magnesium. The initial amounts and proportions of the coenzymes in isolated mitochondria were unaffected by added magnesium (Tables 5 and 6). However, it promoted the oxidation of reduced pyridine nucleotide during incubation without added substrate, even though the rate of oxygen uptake, both initial and final, was higher in its absence. With pyruvate as substrate, both the initial stimulation of the uptake of oxygen and the reduction of the coenzymes were markedly decreased by omitting magnesium; with succinate, its omission produced only a slight decrease in the extent to which the coenzymes were reduced (compare amounts of reduced nucleotide present at 0 and 15 min. in Tables 5 and 6). Magnesium did not increase the stability of the coenzymes during incubation.

Amounts of pyridine nucleotides found in isolated mitochondria

After the investigations described above, all isolations of mitochondria were carried out in 0.25 M-sucrose plus 25 mM-nicotinamide, and all the suspensions were prepared in Chappell-Perry medium plus nicotinamide (25 mM), hereafter called 'saline medium', by the procedure described in the Methods section. The results of analyses of eight different mitochondrial suspensions prepared by this procedure are given in Table 7. The data refer to the original level of the coenzymes in mitochondria isolated and maintained at 0°, and are expressed on the basis of both dry weight and protein content. There was more diphosphopyridine nucleotide than triphosphopyridine nucleotide, and more variability in the amounts of the latter. The levels of DPNH and TPN⁺ were least variable. The values for the ratio DPNH/DPN⁺ ranged from 0.4 to 1.1 (mean 0.62) and for TPNH/TPN⁺ from 1.8 to 7.5 (mean 4.12). These values were all obtained from preparations used subsequently for the metabolic experiments described in

Table 7. *Amounts of pyridine nucleotides in isolated rat-liver mitochondria*

Values are given as (i) $\mu\text{m-moles/mg. dry wt.}$ and (ii) $\mu\text{m-moles/mg. of protein}$. The first series are the means of eight determinations $\pm$ S.E.M. The mitochondrial suspensions analysed contained 20–30 mg. of dry matter/ml.; the percentage of protein in the dry matter was 66%. In this and subsequent Tables, DPN represents DPN⁺ + DPNH and TPN represents TPN⁺ + TPNH. See also protocol to Table 1.

	(i)	(ii)
DPN ⁺	1.25 $\pm$ 0.03	1.9
DPNH	0.78 $\pm$ 0.01	1.2
DPN	2.03 $\pm$ 0.06	3.1
TPN ⁺	0.33 $\pm$ 0.01	0.5
TPNH	1.36 $\pm$ 0.09	2.1
TPN	1.69 $\pm$ 0.11	2.6
Total	3.72 $\pm$ 0.24	5.7
DPNH/DPN ⁺	0.62	—
TPNH/TPN ⁺	4.12	—
PNH/PN ⁺	1.35	—

this paper (contrast those used by Birt & Bartley, 1960a) and include particles derived from two fractions which appear in the 'mitochondrial' pellet after centrifuging in a sucrose-nicotinamide medium. The upper layer, lighter in colour and less firmly packed, resembled the 'fluffy layer' usually obtained in sucrose media but was more copious than in preparations made in the sucrose medium alone. In the routine isolation of mitochondria much, but not all, of the 'fluffy layer' was discarded. In one experiment a rigorous separation of the two fractions ('fluffy layer' and 'mitochondria') was carried out and the coenzyme levels were determined. The 'fluffy layer' contained less total pyridine nucleotide/mg. dry wt. but the proportions of the individual coenzymes were the same as in the mitochondrial fraction. The percentage protein of the two fractions was identical (66% of the dry matter).

Leakage of pyridine nucleotides from mitochondria suspended in 'saline medium'

The leakage of pyridine nucleotides from the mitochondria in a number of different incubated suspensions is shown in Table 8. In all but one experiment (Expt. 6) most of the coenzyme was retained by the mitochondria (or the particles of 'fluffy layer', Expt. 5) during periods of up to 1 hr. The released nucleotides were nearly all in the oxidized form, and were predominantly diphosphopyridine nucleotides. The exceptional experiment, an incubation for 1 hr. at 30° (Expt. 6), resulted in

Table 8. *Leakage of pyridine nucleotides from mitochondria in 'saline medium' without substrate*

For details of the measurement of the distribution of pyridine nucleotides between mitochondria and the suspending medium see text. Temperature of incubation in Expts. 1-5 was 25° and in Expt. 6 30°. See also protocol to Table 1.

Expt. no.	Time (min.)	PNH PN+ of whole suspension	PN (μ m-moles/ml. of suspension)						Percentage in supernatant fluid
			'In' mitochondria			In supernatant fluid			
			PN+	PNH	PN	PN+	PNH	PN	
1	15	1.7	32	60	92	3.2	0	3.2	3
	45	0.4	47	19	66	5.6	0.5	6.1	8
2	15	1.7	32	59	91	3.2	0	3.2	3
	45	0.4	47	19	66	5.6	0.4	6.0	8
3	50	0.46	28	21	49	4.7	0	4.7	9
4	45	0.46	35	29	64	9.9	0	9.9	13
5	15	—	—	—	—	4.6	0	4.6	—
5*	15	1.7	14	24	38	2.4	0	2.4	6
6	0	1.9	25	53	78	3	0	3	4
	60	0	2	0	2	47	0	47	96

* 'Fluffy layer.'

Table 9. *Leakage of pyridine nucleotides from mitochondria in 'saline medium' without substrate*

Temperature of incubation, 25°; for details of the measurement of the distribution of pyridine nucleotides between mitochondria and the suspending medium, see text. Uptake of O₂ was measured as μ l. of O₂/mg. dry wt./hr. See also protocol to Table 1.

		PN (μ m-moles/ml. of suspension)							
Time (min.)	PNH PN ⁺	'In' mitochondria			In supernatant fluid			Percentage in supernatant fluid	Uptake of O ₂
		PN ⁺	PNH	PN	PN ⁺	PNH	PN		
0	2.8	24	69	93	0.8	0	0.8	1	0
7	1.5	24	51	75	11	0	11	13	4.5
18	1.7	16	52	68	17	4	21	24	3.8
31	1.0	16	37	53	25	3	28	35	2.5
61	0.15	0.2	3	3.2	53	5	58	95	2.0

Table 10. *Influence of substrates and inhibitors on leakage of pyridine nucleotide from mitochondria*

Temperature of incubations, 25°; concn. of pyruvate and succinate, 10 mM; amytal, 1 mM; 2,4-dinitrophenol, 0.1 mM. The mitochondrial suspensions used in each experiment were derived from the same homogenate. See also protocol to Table 1.

Expt. no.	Substance added	PN in supernatant fluid after 1 hr. (μ m-moles/ml.)			Percentage of PN in supernatant fluid
		PN ⁺	PNH	PN	
1	Nil*	34	0	34	59
	Nil†	21	2	23	40
2	Nil	3.4	0.4	3.8	9
	Pyruvate	2.5	0.4	2.9	6
	Succinate	1.4	0.5	1.9	4
2‡	Nil	2.4	0	2.4	6
	Pyruvate	3.0	0	3.0	7
	Succinate	2.1	0	2.1	5
3	Nil	9.9	0	9.9	13
	Succinate	2.7	1.8	4.5	6
4	Nil	4.7	0	4.7	9
	Amytal	6.2	0	6.2	13
	2,4-Dinitrophenol	2.4	0	2.4	5

* Mitochondria prepared and incubated in 'saline medium'; final value for the ratio PNH/PN⁺ of whole suspension, 0.07.

† Mitochondria prepared and incubated in medium without EDTA as in Table 4; final value for the ratio PNH/PN⁺ of whole suspension, 0.46.

‡ 'Fluffy layer'.

the almost complete release of the coenzymes from the mitochondria, and, at the same time, a very low final amount (below the limit of detection) of reduced nucleotide in the whole suspension. Infrequently, a similar leakage of pyridine nucleotides was obtained after incubation at 25°, and again the final ratio of reduced coenzyme to oxidized coenzyme in the whole suspension was very low. In the experiment of this kind described in Table 9, 35 % of the pyridine nucleotide was lost from the mitochondria in the first 30 min., while the ratio of reduced coenzyme to oxidized coenzyme in the whole suspension fell to one-third of its original value; in the last half-hour, a further 60 % of the coenzyme leaked out, and the ratio decreased to one-eighteenth of its original value. The respiration rate declined steadily during the entire incubation. It is noteworthy that small amounts of reduced pyridine nucleotide were detected in the incubating medium (compare Birt & Bartley, 1960*a*).

These experiments suggested a relationship between the maintenance of reduced nucleotide in the mitochondria and the rate of leakage. Further investigation (Table 10) showed that this relation was not a simple one, for the addition of a substrate which increased the amount of reduced coenzyme (e.g. pyruvate or succinate, Table 10) and of 2:4-dinitrophenol, which decreased the amount to the limits of detection, both apparently diminished the rate of leakage. EDTA, in Expt. 1 (Table 10) had decreased the ability of the mitochondria to maintain the level of reduced coenzymes throughout an incubation of 1 hr. without added substrate (compare Table 4), whereas amytal had at first increased then decreased this level; but both accelerated the release of coenzymes. Direct comparison of the leakage from mitochondria incubated alone and with 2:4-dinitrophenol or amytal showed that the increased rate of loss due to amytal occurred during the period of enhanced oxidation of the reduced nucleotide (the second 30 min. in Table 11); the slight decrease in the amount of coenzyme lost by leakage with 2:4-dinitrophenol also occurred in the second 30 min. of the incubation.

Reactivity of pyridine nucleotides
in the supernatant fluid

Nucleotides leaking from mitochondria into the supernatant fluid can act as hydrogen acceptors when substrate is added. In the experiment described in Table 12, two samples of the same mitochondrial suspension were shaken without substrate for 28 min., one at 0° and the other at 25°, and the distribution of the pyridine nucleotides between supernatant fluid and the mitochondria was determined for each sample. Pyruvate plus malate or β -hydroxybutyrate were added to

Table 11. Influence of 2:4-dinitrophenol and amytal on the leakage of pyridine nucleotides from mitochondria

All values for pyridine nucleotide levels are given as μ m-moles/ml. Temperature of incubation, 25°. See also protocol to Table 1.

Time (min.)		No addition						2:4-Dinitrophenol (0.1 mM)						Amytal (mM)					
		PN+	PNH	PN	PNH	PN+	PNH	PN	PNH	PN+	PNH	PN	PNH	PN+	PNH	PN	PNH	PN+	PNH
30	Whole suspension	43	18	61	0.42	48	0.5	48.5	0.01	34	28	62	0.82	29	28	5.2	0	5.2	0.97
	Supernatant fluid	3.8	0	3.8	—	4.0	0	4.0	—	5.2	0	5.2	—	15	0	8	—	—	—
	Mitochondria	39	18	57	0.46	44	0.5	44.5	0.01	29	28	57	0.97	29	28	5.2	0	5.2	0.97
	Percentage in supernatant fluid	9	0	6	—	8	0	8	—	49	0.5	49.5	0.01	49	0.5	11	0	11	0.01
60	Whole suspension	35	20	55	0.57	44	1	45	0.02	38	0	49	0.03	38	0.5	38.5	0.5	38.5	0.01
	Supernatant fluid	5.3	0	5.3	—	4.9	0	4.9	—	11	0	11	—	22	0	22	—	—	—
	Mitochondria	30	20	50	0.67	39	1	40	0.03	38	0.5	38.5	0.03	38	0.5	38.5	0.5	38.5	0.01
	Percentage in supernatant fluid	15	0	10	—	11	0	11	—	49	0.5	49.5	0.02	49	0.5	11	0	11	0.01

Table 12. Reactivity of pyridine nucleotides released from mitochondria

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/ml.}$ Concn. of β -hydroxybutyrate, pyruvate and malate, 10 mm. See also protocol to Table 1.

Addition	Temp. of pre-incubation or incubation (min.)	Whole suspension						Supernatant fluid						Mitochondria					
		PNH			Loss (%)			PNH			PNH			PNH			PNH		
		PNH	PN	PNH	PNH	PNH	PNH	PNH	PNH	PNH	PNH	PNH	PNH	PNH	PNH	PNH	PNH	PNH	PNH
Nil	0	20.5	48	68.5	2.3	—	—	—	—	—	20.5	48	68.5	20.5	48	68.5	20.5	48	68.5
Nil	0	24	46	70	1.9	0	10.4	0	10.4	0	13.6	46	59.6	13.6	46	59.6	13.6	46	59.6
Nil	25	30	26.5	56.5	0.9	18	10.1	0.9	11.0	0.09	19.9	25.6	45.5	19.9	25.6	45.5	19.9	25.6	45.5
β -Hydroxybutyrate	25 (from 0°)	24	40.5	64.5	1.7	6	9.4	0.9	10.3	0.10	14.6	39.6	54.2	14.6	39.6	54.2	14.6	39.6	54.2
Pyruvate plus malate	25 (from 0°)	17	45	62	2.7	9	3.7	6.9	10.6	1.9	13.3	38.1	51.4	13.3	38.1	51.4	13.3	38.1	51.4
β -Hydroxybutyrate	25 (from 25°)	22	25	47	1.1	31	9.5	1.3	10.8	0.14	12.5	23.7	36.2	12.5	23.7	36.2	12.5	23.7	36.2
Pyruvate plus malate	25 (from 25°)	18	42	60	2.3	12	6.5	4.3	10.8	0.66	11.5	37.7	49.2	11.5	37.7	49.2	11.5	37.7	49.2

portions of each of the pre-incubated suspensions, the mixtures were incubated for 15 min. at 25° and the distribution of the nucleotides between supernatant fluid and mitochondria was again determined. The addition of pyruvate plus malate resulted in a hydrogenation of the coenzymes in the supernatant fluid, more especially when the pre-incubation had been at 0°. The amounts of pyridine nucleotide released into the supernatant fluid during pre-incubation for 28 min. without substrate were the same at both 0° and 25°; there were no great changes in these amounts during the 15 min. of incubation with added substrate, though the levels of total nucleotide in the whole suspensions declined by up to 13%.

It is apparent that ratios of reduced to oxidized mitochondrial nucleotide assessed by analysis of whole suspensions of mitochondria will be in error because of leakage from the particles during incubation. Usually, the ratios measured in this way will be lower than the true values, because the leakage of oxidized coenzyme exceeds that of reduced coenzyme. This can be seen by comparing ratios of reduced to oxidized nucleotide for 'Whole suspension' and 'Mitochondria' in Tables 11 and 12.

Pyridine nucleotides of mitochondria isolated from rat brain

The applicability of the methods for extraction and estimation of pyridine nucleotides, and the amounts and susceptibility to experimental control of the oxidized and reduced coenzymes of the mitochondria of rat-brain tissue, have been examined briefly. The total amount of pyridine nucleotide found in isolated brain mitochondria was considerably lower than that found in liver mitochondria (compare Tables 7 and 13): there was approximately one-third of the amount of diphosphopyridine nucleotide and one-fiftieth of the amount of triphosphopyridine nucleotide. The initial proportion of reduced coenzymes was extremely low, both DPNH and TPNH occurring at the limit of detection in a suspension containing 20 mg. dry wt. of mitochondria/ml.

During the incubation of brain mitochondria in 'saline medium' alone there was little change in the proportion of reduced coenzyme, but considerable loss (30%) of the total pyridine nucleotide occurred. The incubation of brain mitochondria for 15 min. with succinate, glutamate, pyruvate or α -oxoglutarate demonstrated marked differences in the ability of these compounds to affect the ratio of reduced to oxidized coenzymes (Table 14). The level of reduced coenzymes was greatly increased by succinate and glutamate, but was not altered by either pyruvate or α -oxoglutarate.

Table 13. *Levels of endogenous pyridine nucleotides in isolated brain mitochondria*

All values for pyridine nucleotide levels are the means of the analyses of three preparations of mitochondria; they are given as $\mu\text{m-moles/mg. dry wt.}$; the suspensions of mitochondria contained 20–30 mg. of dry matter/ml. See also protocol to Table 1.

DPN ⁺	DPNH	DPN	TPN ⁺	TPNH	TPN	$\frac{\text{PNH}}{\text{PN}^+}$
0.62	0.05	0.67	0.01	0.02	0.03	0.11

Table 14. *Effect of added substrates on the pyridine nucleotides of brain mitochondria*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$ Temperature of incubation, 25°; concn. of pyruvate, glutamate, α -oxoglutarate and succinate, 10 mM. See also protocol to Table 1.

Substrate added	Time (min.)	PN ⁺	PNH	PN	$\frac{\text{PNH}}{\text{PN}^+}$
Nil	0	0.65	0.11	0.76	0.17
Nil	15	0.44	0.09	0.53	0.20
Pyruvate	15	0.45	0.08	0.53	0.18
Glutamate	15	0.42	0.22	0.64	0.52
α -Oxoglutarate	15	0.54	0.10	0.64	0.19
Succinate	15	0.29	0.27	0.56	0.93

Table 15. *Comparison of the pyridine nucleotide content of mitochondria prepared for sampling by two different methods*

Values under (i) are taken from Birt & Bartley (1960*a*) and those under (ii) are from this paper (Table 7). Values for DPN (DPN⁺ + DPNH) and TPN (TPN⁺ + TPNH) are given as $\mu\text{m-moles/mg. of protein.}$

	(i)	(ii)
DPN	3.7	3.1
TPN	3.6	2.6
$\frac{\text{DPNH}}{\text{DPN}^+}$	0.22	0.62
$\frac{\text{TPNH}}{\text{TPN}^+}$	0.86	4.12

DISCUSSION

Two series of values obtained in investigations described in this and a preceding paper (Birt & Bartley, 1960*a*) for the amounts of pyridine nucleotides in isolated mitochondria are given in Table 15. All the suspensions used contained nicotinamide, which decreases the losses of coenzyme occurring during the isolation even at 0°. For the first series, mitochondrial preparations containing relatively large amounts of 'fluffy layer' were analysed; the sampling of these suspensions involved a short time (2 min.) at a temperature of about 10° (when the substrate was added and mixed) and was therefore somewhat slower than in the experiments from which the second series was derived. Hence the lower total amount of coenzyme/mg. dry wt. in the former series. In both series the variability in the level of triphosphopyridine nucleotide was greater than that of the diphosphopyridine nucleotide perhaps because of the greater permeability of the

mitochondrial membrane to the former (Birt & Bartley, 1960*a*).

The two series also showed markedly different proportions of oxidized and reduced coenzyme; the mean values of the ratios DPNH/DPN⁺ and TPNH/TPN⁺ were 0.62 and 4.12 (series 1) and 0.22 and 0.86 (series 2) respectively. The values shown in Table 15 were obtained (i) by analyses of resuspended mitochondrial pellets and (ii) by analyses of the original mitochondrial suspensions. The ratio TPNH/TPN⁺ was much greater than the ratio DPNH/DPN⁺ in both these analyses, but the constituents of the media used for isolation and suspension may influence the relative proportions of oxidized and reduced coenzymes greatly. For example, both nicotinamide and EDTA (in the sucrose or 'saline' media) decreased the ratio of reduced to oxidized pyridine nucleotide. It has been shown previously that this ratio can be altered also by varying the method of preparation of the particles (see Birt & Bartley, 1960*a*). Thus the precise significance of the proportions of reduced coenzyme in freshly prepared mitochondria cannot be established without consideration of the preparative procedure used.

Stability of pyridine nucleotides during incubation

Although the pyridine nucleotides of mitochondria are more stable than those of whole-tissue preparations (compare Bassham *et al.* 1959) in the great majority of experiments there was some loss of coenzyme during both the isolation and incubation for 1 hr. at 25°. In the early stages of the isolation, before the washing of the particles was completed, the rate of loss was rapid (compare values for 'absolute loss', Table 1) and it is likely that much of the destruction was due to the

extremely active nucleotide-splitting enzymes of contaminants of the mitochondrial fraction, especially the microsomes; nicotinamide, which inhibits DPN⁺ nucleosidase, an enzyme of relatively low activity in the mitochondria themselves (Jacobson & Kaplan, 1957a), was most effective at this time. Mitochondria can lose pyridine nucleotides to the suspending medium, even at 0° (see Table 12), and a vigorous destruction of the coenzymes in the supernatant fluid may result in continuing leakage and therefore in an appreciably lower total level of coenzyme in the original whole suspension. It is possible that during the subsequent manipulations the mitochondrial nucleotide-splitting enzymes are themselves responsible for much of the loss that occurs, because (i) the rate of destruction at 25° was relatively low, and was less readily influenced by nicotinamide, (ii) in the washed suspensions in 'saline' little of the pyridine nucleotide was initially extramitochondrial, though there was always some leakage, and (iii) even when there was appreciable destruction in the whole suspension, the level in the supernatant fluid remained approximately constant (see Tables 11 and 12). The supernatant fluid may, however, retain some capacity for destroying coenzymes. Added pyridine nucleotides disappear at a low rate (Birt & Bartley, 1960a) and when the leakage of coenzymes from the mitochondria was very pronounced the rate of destruction was sometimes increased (Table 9, compare the first 31 and the last 30 min.).

The extent to which added substrates protected the coenzymes was variable. In nine out of 15 experiments there was a decrease in the rate of destruction after the addition of substrates with DPN-dependent dehydrogenases, though a similar effect was seen in only three out of eight experiments with succinate, and not at all in the one experiment with choline. The protective action may be due to (i) decreased leakage of coenzymes, (ii) an increase in the level of reduced coenzymes, which are relatively more stable (the explanation given for a similar effect of substrate in minces and homogenates), or (iii) a more rapid turnover of the coenzymes during the accelerated operation of the electron-transport chain itself. Although the first factor may be of some importance it does not appear to be a sufficient explanation, because amytal can increase the leakage without increasing the destruction of coenzymes, and succinate consistently decreased the leakage but not the destruction. Moreover 2,4-dinitrophenol, apparently without effect on leakage, increased the rate of destruction, though whether the loss occurred before or after the release of coenzyme from the mitochondria was not determined. The second and third factors, however, both appeared to influence

the stability of the coenzymes. Succinate, which was capable of reducing a greater proportion of the pyridine nucleotides than any other substrate tested, was less effective in inhibiting the destruction of the coenzymes than was pyruvate (the protective action was shown in six out of 12 and 10 out of 12 experiments respectively). On the other hand, losses produced by 2,4-dinitrophenol (see Birt & Bartley, 1960b) were seen more commonly with added pyruvate as substrate (in 10 out of 12 experiments) than with succinate (in 0 out of 12 experiments); 2,4-dinitrophenol stimulated the respiration with pyruvate less than with succinate, but lowered the level of reduced pyridine nucleotide more rapidly with the former. Thus it seems that the rapid passage of electrons along the complete respiratory chain from DPNH to oxygen together with a maintained high level of reduced pyridine nucleotide is necessary for the maximum preservation of the coenzymes. These conditions were fulfilled when there was an excess of substrate oxidized by pyridine nucleotide-dependent dehydrogenases. The coenzymes of the mitochondria would then be fully utilized in binding the substrates and accepting hydrogen, and especially in the presence of nicotinamide, the ability of the splitting enzymes to compete successfully for the nucleotides would be greatly impaired.

Effect of constituents of the isolation and incubation media on pyridine nucleotides

Nicotinamide was always added to the media used for isolation and incubation. The following effects on the mitochondria were observed: (i) The sedimentation characteristics of the particles were altered, and the proportion of 'fluffy layer' increased; when the mitochondria were exposed to nicotinamide in the sucrose only, they acquired and retained sufficient of it to influence their subsequent reactions, even when the 'saline medium' had no nicotinamide. (ii) The destruction of the coenzymes, particularly the oxidized forms, was decreased (compare Bassham *et al.* 1959). (iii) The reduction of pyridine nucleotides by endogenous but not added substrates (at 10 mM concentration) was prevented. (iv) The rate of endogenous respiration was lowered by 30–50% but that with added substrate was decreased very little (compare Bassham *et al.* 1959).

The mechanism of the action of nicotinamide (and other compounds) as inhibitors of nucleosidases has been studied by Kaplan *et al.* (Zatman, Kaplan & Colowick, 1953; Zatman, Kaplan, Colowick & Ciotti, 1954), who have shown that it replaces the OH⁻ ion of water as an acceptor for the adenine-ribose-phosphate-phosphate-ribose group, i.e. it can be bound on an enzyme surface at a site normally occupied by the nicotinamide

residue of the oxidized coenzyme. It seems likely that all the observations listed above can be explained by assuming that nicotinamide is bound in this way at the reactive sites of other enzymes. Thus it may be retained by washed mitochondria, attached to and influencing the action of the enzymes concerned with the oxidation and reduction of pyridine nucleotides. Normally, with endogenous substrate, the dehydrogenase systems are not saturated, for both the level of reduced coenzyme and the oxygen consumption increase greatly when substrates are added. The coenzymes, as their turnover in the cyclic oxidation-reduction processes is not proceeding at its maximum rate, can be replaced at the binding sites by nicotinamide, so that the proportion of reduced coenzymes and the endogenous respiration are both diminished. When substrate is in excess, the opportunities for nicotinamide to replace the bound coenzyme will be greatly decreased, and the inhibitor has no significant influence on either the reduced coenzyme or the respiratory rate. Jacobson & Kaplan (1957*a*) have shown that nicotinamide depresses the rate of oxidation of extramitochondrial DPNH, and this, too, is probably due to the binding of the inhibitor at sites normally reacting with pyridine nucleotide.

Adenosine triphosphate was almost without effect on the pyridine nucleotides, but apparently preserved the mitochondria from changes regarded as degenerative (see Birt & Bartley, 1960*b*).

Magnesium added to mitochondrial suspensions facilitates the coupling of oxidation and phosphorylation (Chance & Williams, 1956) and its omission may result in a rapid increase in the rate of oxygen uptake after incubation for a few minutes (Baltscheffsky, 1957). Two separate effects of magnesium on mitochondria in the 'saline medium' were observed. One, evident when no substrate was added, was to promote the oxidation of the reduced pyridine nucleotides; in its absence, the increased oxygen consumption (cf. Baltscheffsky, 1957) did not result in the usual progressive oxidation of the coenzymes (Tables 5 and 6). The second effect of magnesium was to facilitate the reduction of pyridine nucleotides and to permit the stimulation of oxygen uptake by added pyruvate (Table 5), probably because pyruvic decarboxylase requires magnesium for its maximum activity (Green, 1952); on the other hand, magnesium had little effect on the behaviour of mitochondria incubated with succinate, though neither the increase nor the decline in the ratio of reduced to oxidized pyridine nucleotide was as pronounced when it was omitted (Table 6). From all these data, it appears that magnesium is necessary for the oxidation of DPNH by the respiratory chain, perhaps being required for the binding of some mitochondrial component

essential for oxidative phosphorylation (see Kielley & Bronk, 1957; Linnane & Ziegler, 1958). The most striking feature of the experiments without added magnesium was the length of time during which the reduced coenzymes were retained. This effect was not observed in any other experiments at 25° and suggests that the linkage between DPNH (and therefore TPNH; see Birt & Bartley, 1960*b*) and all the oxidative pathways has been broken; Green (1959) has reported that magnesium is required for the binding of DPN⁺ by mitochondria, and the EDTA of the suspending medium may remove sufficient of the endogenous magnesium to destroy the binding sites involved. There are a number of conflicting reports about the influence of added EDTA on suspensions of mitochondria (for summary, see Schneider, 1959). There was no evidence for a degeneration of the particles incubated in the media without EDTA. Its addition will lower slightly the concentration of free magnesium in the 'saline medium'; hence, as pyruvate oxidation requires magnesium, the ability of pyruvate to reduce the coenzymes may be decreased and the oxygen uptake with this substrate diminished. However, as it did not prevent the oxidation of reduced nucleotides when no substrate was added (though it retarded it appreciably) there was still sufficient free magnesium in the medium for the operation of the electron-transport chain, which also requires magnesium. Thus the concentration of magnesium needed to activate this chain may be lower than that required for maximum activity of pyruvic decarboxylase.

The results obtained in this investigation of the influence of various constituents of the suspending medium emphasize the dependence of the proportions of oxidized and reduced coenzyme in isolated mitochondria on the method of preparation. Thus two of the components of the 'saline medium', nicotinamide and EDTA, lowered the original level of reduced coenzymes and decreased the extent to which reduction occurred during incubation. The addition of nicotinamide, especially, may account for much of the discrepancy between the ratios of reduced to oxidized coenzyme measured by Chance & Williams (1955) and those reported here. Differences in the time elapsing before measurements are made and in the manner of incubating the mitochondria may also be important in this regard.

Leakage of pyridine nucleotides from mitochondria during incubation

The leakage of pyridine nucleotides from mitochondria incubated under various conditions has been described by several workers and investigated by direct measurement of the coenzymes by Kaufman & Kaplan (1959). They report an almost

complete loss of nucleotide during the incubation of mitochondria at 30° in 0.25 M-sucrose and 0.02 M-phosphate. Nicotinamide did not affect the leakage, but substrates, especially succinate, decreased it. The leakage of coenzymes from mitochondria incubated in the 'saline medium' at 25° without added substrate was much less complete. This was probably due to several factors, namely, the difference in temperature (in the one experiment at 30° in 'saline medium' leakage was extremely rapid, though increasing the temperature from 0° to 25° had little effect on the loss), the low concentration of inorganic phosphate and the presence of ATP and magnesium. EDTA, which assists in the retention of coenzymes by mitochondria incubated at 30° in 0.25 M-sucrose, even after almost complete oxidation of the nucleotides (Lester & Hatefi, 1958), had the reverse effect as a component of the 'saline medium'. It is possible that EDTA, by efficiently binding calcium, which is present in isolated mitochondria at the same concentration as magnesium (Bartley & Amoore, 1958), alters the mitochondrial structure sufficiently to permit a redistribution of the pyridine nucleotides between the mitochondria and the suspending medium. Occasionally, substantial leakage occurred in the 'saline medium', but only in combination with other changes interpreted as indicating the breakdown of the mitochondria. The ability of added substrates to diminish the leakage was also observed in this medium, but an apparently similar effect of 2:4-dinitrophenol may be due to an accelerated destruction of the coenzymes in the supernatant fluid, especially during the early stages of the incubation (see Table 11). In this experiment (Table 11), if the difference between the amounts of total pyridine nucleotide in the whole suspensions with and without 2:4-dinitrophenol was due to destruction of released coenzymes, the leakage in 2:4-dinitrophenol during 30 min. was about 28% of the total rather than 8%. Amytal always increased the leakage after 1 hr.

These results are in agreement with the conclusions of Lester & Hatefi (1958) and Corwin & Lipsett (1959), that the oxidation of the pyridine nucleotides is not the sole factor controlling leakage, though the nearer to completion this oxidation becomes (i.e. when the ratio of reduced to oxidized coenzyme falls below 0.1) the more rapid is the loss. The degenerative changes occurring in mitochondria with low levels of reduced nucleotide (see Birt & Bartley, 1960b) may include the oxidation of some mitochondrial component necessary for the binding of the coenzyme, as supposed by Lester & Hatefi (1958); also the breakdown of mitochondrial 'compartmentation' may increase the accessibility of the coenzyme binding-sites to

known competitors for attachment, e.g., ATP, calcium and phosphate.

The difference in the ability of β -hydroxybutyrate and pyruvate plus malate to reduce the pyridine nucleotides liberated into the suspending medium may be due to the difference in the firmness of the binding of the dehydrogenases to the mitochondrial structure. Unlike β -hydroxybutyric dehydrogenase, the enzymes responsible for dehydrogenating pyruvate plus malate were less active if the pre-incubation was at 25° rather than at 0°. It is likely that the reduction of the coenzymes in the supernatant fluid was due mainly to liberated malic dehydrogenase, which may undergo partial denaturation during the shaking for 28 min. at 25° without substrate.

Pyridine nucleotides of mitochondria of other tissues

A short study of the coenzymes in the mitochondria of other tissues gave an indication of the general applicability of the method of analysis to the study of mitochondrial nucleotides. No difficulties were found with the two preparations analysed. Mitochondria from the thoracic muscle of the fly *Phormia terraenovae* contained no detectable pyridine nucleotide [for other estimates of the coenzyme content of insect-muscle mitochondria see Chance & Sacktor (1958); Price & Lewis (1959)]. Rat-brain mitochondria, however, contained DPN⁺ and small amounts of DPNH, TPN⁺ and TPNH. The values obtained were in fair agreement with those of Jacobson & Kaplan (1957b) and showed that the ratio of reduced to oxidized coenzymes in these mitochondria is extremely low, though it could be increased by incubation with a number of substrates, especially succinate. In view of the proposed role for triphosphopyridine nucleotide in rat-liver mitochondria (Birt & Bartley, 1960b) it is of interest that the brain particles, which have a low transhydrogenase activity (Vignais & Vignais, 1957; Humphrey, 1957), have a very low concentration of triphosphopyridine nucleotide also.

SUMMARY

1. The amounts and behaviour of the pyridine nucleotides of rat-liver mitochondria are shown to depend on the method of preparation and the constituents of the suspending medium. In the present work, the tissue was fractionated in a sucrose-nicotinamide medium and the mitochondria were incubated in a 'saline medium'.

2. The rate of loss of the mitochondrial pyridine nucleotide was about one-tenth of that of the coenzymes of the mince from which the particles were isolated.

3. Nicotinamide increased the initial amounts and stabilized the pyridine nucleotides of the

isolated mitochondria during incubation at 25°. The endogenous respiration and the capacity of the particles to maintain reduced coenzyme were both decreased. With nicotinamide the proportions of reduced coenzyme produced by succinate and pyruvate were lowered, but the respiration rates were unaltered.

4. Ethylenediaminetetra-acetic acid decreased by about 7% the initial proportions of reduced coenzyme and diminished the reduction of the nucleotides by both endogenous substrate and added pyruvate. The rate of destruction was increased about fourfold by ethylenediaminetetra-acetate, and the respiration rate, though initially lower, was also more stable.

5. Magnesium was required for the oxidation of reduced pyridine nucleotide during incubation without added substrate and for the reduction of the coenzymes by added pyruvate. The initial rate of respiration without added substrate and with pyruvate were lower by about 25% when magnesium was present.

6. Analyses of freshly isolated mitochondria at 0° showed that the ratio of reduced diphosphopyridine nucleotide/oxidized diphosphopyridine nucleotide ranged from 0.4 to 1.1 (mean 0.62) and that of reduced triphosphopyridine nucleotide/oxidized triphosphopyridine nucleotide ranged from 1.8 to 7.5 (mean 4.12). The levels of reduced diphosphopyridine nucleotide and oxidized triphosphopyridine nucleotide were least variable. The 'fluffy layer' of these preparations contained less total pyridine nucleotide/mg. dry wt., but the same proportion of each individual nucleotide as the rest of the particles.

7. The rate of leakage of coenzymes from the mitochondria at 25° was usually low, about 10% in 1 hr.; it was increased (up to ninefold) when there was considerable oxidation of nucleotides. Added pyruvate and succinate decreased the leakage occurring in 1 hr. but amyntal and ethylenediaminetetra-acetic acid increased it. The released pyridine nucleotide was reduced by pyruvate plus malate and β -hydroxybutyrate.

8. Rat-brain mitochondria had lower levels of all the coenzymes, particularly of the triphosphopyridine nucleotides. The initial ratio of reduced coenzyme to oxidized coenzyme was relatively low (0.11–0.17) and was increased by incubation with

succinate and glutamate (up to sixfold), but not with pyruvate or α -oxoglutarate.

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