salicylic acid 2·68; for the females the respective values were 1·81 and 4·79.

4. The relationship between glycogenolytic and metabolic-stimulant activity is discussed.

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The Distribution and Metabolism of Intra- and Extra-Mitochondrial Pyridine Nucleotides in Suspensions of Liver Mitochondria

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The work described in this paper was undertaken to study the penetration of pyridine nucleotides into mitochondria and the interconversion of one nucleotide into another in mitochondrial suspensions. The two methods required for this type of investigation have been developed in this laboratory. They consist of procedures for the rapid separation of mitochondria and medium (Werkheiser & Bartley, 1957) and the determination of the four forms of the pyridine nucleotides as described by Bassham, Birt, Hems & Loening (1959). With these techniques it has been possible to measure the extent of penetration of all four nucleotides into mitochondria at 0° and 25° and to obtain other information about the metabolism of pyridine nucleotides by mitochondria.

MATERIALS

Diphosphopyridine nucleotide (DPN^+) was obtained from Pabst Laboratories, Milwaukee, Wisconsin, U.S.A. It contained 91% of DPN^+ (by the alcohol-dehydrogenase method of Racker, 1950) and no triphosphopyridine nucleotide (TPN^+). Triphosphopyridine nucleotide was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. It contained 92% of TPN^+ when analysed by fluorimetry against a DPN^+ standard solution prepared from the DPN^+ described above. Reduced diphosphopyridine nucleotide (DPNH) was obtained from Sigma Chemical Co. and from C. F. Boehringer und Söhne, Mannheim, Germany. These products contained respectively 90% and 88% of DPNH, when analysed by fluorimetry. Reduced triphosphopyridine nucleotide (TPNH) was obtained from Sigma Chemical Co., and contained 92% of TPNH when analysed by fluorimetry. Adenosine triphosphate (ATP) was obtained as commercial preparations from L. Light and Co. Ltd, Colnbrook, Bucks and Zellstoff-fabrik Waldhof, Pharmazeutische Abteilung, Wiesbaden, Germany.

All reagents used in the analysis of pyridine nucleotides were as described previously (Bassham *et al.* 1959); all other reagents were obtained as commercial products.

Experimental animals. All the rats used were of the white Wistar strain.

METHODS

Preparation of media. For the fractionation of rat liver, a medium containing sucrose (0·25 M) and nicotinamide (25 mM) was prepared in glass-distilled water and was stored at 0–5° before use.

Sucrose media could not be used for the incubation of mitochondria with nucleotides because the hydrolysis products of sucrose formed during the acid extraction of the oxidized nucleotides interfered with the fluorimetric method of analysis. Instead, the medium of Chappell & Perry (1954) was freshly prepared from stock solutions of the components, stored at 0–5°; nicotinamide was added to give a final concentration of 25 mM. The phosphate content of the medium varied somewhat according to the preparation of ATP used. However, it never exceeded 0·7 mM. This concentration is only one-thirtieth of that used by Hunter, Malison, Bridgers, Schutz & Atchison (1959) to deplete mitochondria of their endogenous nucleotides. This medium will be referred to subsequently as the 'saline medium'.

Preparation of mitochondria. The entire tissue fractionation was carried out at 0–2° in an International refrigerated centrifuge, model PR 2. Rat-liver homogenates were prepared in the sucrose medium and the mitochondria isolated by the methods described by Werkheiser & Bartley (1957), with omission of the washing of the first residue. The mitochondrial pellet obtained after centrifuging for 20 min. at 2700 *g* was washed twice by successive resuspensions in 1 vol. of the ice-cold saline medium and centrifuging, first for 10 min. and finally for 5 min., at 8500 *g*. The copious fluffy layer was completely discarded at each decantation. The final mitochondrial residue was dispersed in the saline medium to give a suspension (hereafter 'stock suspension') containing 0·2–0·25 g. wet wt. of mitochondria/ml. The preparation was kept at 0° and the incubation begun as soon as possible.

Incubation. This was carried out in open conical flasks, which were agitated gently during the incubation, either packed in ice (0°) or immersed in water (25°) in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill., U.S.A.).

A sample (1·9 ml.) of the 'stock suspension' was pipetted into a conical flask immersed in ice and containing a measured volume (0·1 ml.) of saline medium. This suspension ('original mitochondrial suspension') was mixed thoroughly and two samples (0·5 ml.) were pipetted into chilled centrifuge tubes, one of which contained 0·05 ml. of [^{14}C]carboxypolyglucose (labelled polyglucose) (Werkheiser & Bartley, 1957). The mitochondria were packed in each tube by centrifuging at 26 000 *g* for 5 min., the supernatant fluid was decanted into chilled tubes and the pellets were blotted dry with filter paper. The pellet and supernatant fluid containing labelled polyglucose were used to measure the 'acid-insoluble dry matter' (*M*) of the mitochondria, and the extraparticulate fluid volume (*E*) of the pellet, as described by Werkheiser & Bartley (1957) and Amoore (1958). The second sample of supernatant fluid and second pellet were kept in a freezing bath (ethanol–CO_2) for subsequent measurement of the pyridine nucleotides; when the pellet was immersed in the bath, portions (0·2 ml.) of the 'original mitochondrial suspension' were pipetted into the acid and alkaline media for extraction of the nucleotides. Thus the 'original mitochondrial suspension' was allowed to stand at 0° for 8–9 min. before extraction.

As soon as the sampling of the 'original mitochondrial suspension' was complete, a suitable volume of the 'stock suspension' of mitochondria was pipetted into the incubation vessel, in which the appropriate amount of pyridine nucleotide had been dissolved in cold saline medium. The volume of medium was such that the dilution of the 'stock suspension' was the same as that in the 'original mitochondrial suspension'. The contents of the vessel were mixed rapidly and the timing of the incubation was commenced. The entire procedure described above for separation of mitochondrial pellet and supernatant fluid, and sampling of the suspension, was repeated at the appropriate times.

When the incubation was ended, the frozen mitochondrial pellets were resuspended in cold saline medium (2 ml.) and the pyridine nucleotides extracted from 0·5 ml. samples. The temperature of the suspension was kept at 0–2° before extraction. The pyridine nucleotide contents of the supernatant fluids were measured in 0·1 ml. samples.

The procedures described above permitted the measurement of the wet weight and dry weight (*M*) of the mitochondrial pellet, the extraparticulate (*E*) and intramitochondrial fluid volume (*I*) of the pellet, and the pyridine nucleotide content of the whole mitochondrial suspension, isolated mitochondrial pellet and supernatant fluid. The calculation of intramitochondrial nucleotide concentrations was based on the procedure of Werkheiser & Bartley (1957).

Measurement of pyridine nucleotides. DPN^+, DPNH, TPN^+ and TPNH were measured by the fluorimetric method described by Bassham *et al.* (1959). Dilutions of the extracts, when necessary, were made with 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 8·2.

Measurement of protein content of mitochondria. The acid-insoluble dry matter of the mitochondrial pellets was wet-ashed and the N content of a suitable dilution measured by direct nesslerization. The mitochondrial protein was assumed to contain 16% of N.

Measurement of chloride. Chloride was measured by the method of Sanderson (1952) as modified by Amoore & Bartley (1958).

RESULTS

Endogenous pyridine nucleotides in mitochondria. The calculation of the values for the endogenous pyridine nucleotide content of mitochondria (Table 1) have been obtained by analysis of resuspended mitochondrial pellets and are based on the 'acid-insoluble dry matter' of the mitochondria. About 66% of this dry matter is protein, so that values/mg. of protein will be 1½ times as great as those quoted, i.e. total amount of diphosphopyridine nucleotide, 3·8 μmoles/mg. of protein, of

Table 1. *Endogenous pyridine nucleotide content of mitochondria*

Values are means from seventeen different mitochondrial preparations ± S.E.M.

Nucleotide	Amount (μm-moles/mg. dry wt.)
DPN^+	2·02 ± 0·06
DPNH	0·44 ± 0·04
DPN^+ + DPNH	2·46 ± 0·07
TPN^+	1·26 ± 0·10
TPNH	1·08 ± 0·09
TPN^+ + TPNH	2·34 ± 0·12

Table 2. *Oxidation of endogenous pyridine nucleotides by mitochondrial suspensions*

Concentrations listed under the heading Direct were obtained by analysis of the resuspended mitochondrial pellet and those listed under Difference by analysis of the original mitochondrial suspension and of the suspending fluid after sedimentation of the mitochondria. All manipulations were at 0°. For details, see text.

Expt. no.	Concn. of nucleotide in intramitochondrial water (μm-moles/ml.)									
	DPN+		DPNH		TPN+		TPNH		Total	
	Direct	Difference	Direct	Difference	Direct	Difference	Direct	Difference	Direct	Difference
1	1060	840	190	110	850	490	420	530	2520	1970
2	930	510	130	790	670	230	460	920	2190	2440
3	590	340	170	560	430	90	390	780	1560	1770
4	740	750	130	430	490	150	330	950	1690	2280

Table 3. *Changes in the internal water content of mitochondria in the presence of added coenzymes at* 0°

I/M, Internal water (ml.) of the mitochondria/dry wt. (g.) of mitochondria. Original, *I/M* calculated before addition of the nucleotide; (*a*) *I/M* calculated immediately after addition; (*b*) *I/M* calculated 7 or 30 min. after addition. Chloride space, chloride concn. in mitochondrial water/chloride concn. of supernatant fluid expressed as a percentage.

Nucleotide added	Concn. (mM)	*I/M*			Chloride space		
		Original	(*a*)	(*b*)	Original	(*a*)	(*b*)
DPN+	0·9	2·5	2·5	2·6	92	89	108
	1·6	2·7	2·7	2·8	79	83	83
	7·8	2·4	2·4	2·3	60	65	99
	14·1	2·2	2·2	—	84	108	—
DPNH	0·74	3·1	2·5	2·5	101	86	76
	0·8	2·1	2·3	2·0	77	79	64
	1·85	2·2	2·3	2·2	84	79	82
	6·7	2·3	2·3	2·3	76	74	79
	6·7	2·4	1·7	—	70	44	—
	9·2	2·7	2·0	—	84	80	—
TPN+	0·57	2·4	2·1	2·1	—	—	—
	1·55	3·2	3·1	2·8	100	96	84
	3·8	2·4	2·2	2·3	57	73	73
	11·2	2·2	2·3	—	84	110	—
TPNH	0·85	2·6	2·6	2·5	61	67	66
	1·09	2·5	2·4	2·2	88	82	80
	2·0	2·2	2·4	1·9	100	96	82
	7·15	2·2	1·9	1·8	85	—	75
	8·0	2·4	2·2	—	70	76	—
	12·8	2·7	2·2	—	84	75	—

Table 4. *Changes in the internal water content of mitochondria in the presence of added coenzymes at* 25°

Original, *I/M* calculated for mitochondria before addition of nucleotide, and at 0°; figures in parentheses refer to *I/M* values of these mitochondria after 30 min. incubation at 0°. (*a*) *I/M* immediately after nucleotide addition at 25°; (*b*) *I/M* measured after incubation for 7 min. or 30 min. (*). Chloride space is defined in Table 3.

Nucleotide added	Concn. (mM)	*I/M*			Chloride space		
		Original	(*a*)	(*b*)	Original	(*a*)	(*b*)
DPN+	0·9	2·5 (2·6)	—	2·5*	92 (89)	—	108*
	7·8	2·7	2·8	2·6	82	75	75
	10·3	3·0	3·0	2·8	88	94	87
TPN+	1·6	3·2 (2·8)	—	2·8*	100 (96)	—	88*
	7·7	2·7	2·9	2·8	82	84	78
	10·4	3·0	2·9	3·0	88	96	108

triphosphopyridine nucleotide, 3·6 μmoles/mg. of protein. The mean value for the ratio internal water content of the mitochondria to dry weight (*I/M*) is about 2·4, hence the original concentration of both di- and tri-phosphopyridine nucleotides in the mitochondrial water is about mM, assuming that all of the nucleotide is in solution. Although the amounts of di- and tri-phosphopyridine nucleotide are approximately equal the variability in the latter is much greater, and more of it is recovered in the reduced form. However, no precise information about the ratios of oxidized and

Table 5. *Total losses of pyridine nucleotides in mitochondrial suspensions*

Values given for percentage concn. are (*a*) for suspensions incubated for periods of either 15 or 30 min. at 0° and (*b*) for suspensions incubated for a further 30 min. at 25°. DPN, Values for DPN+ plus DPNH; TPN, values for TPN+ plus TPNH.

Nucleotide added	Initial measured concn. of nucleotides in whole suspension (mM)		Concn. of nucleotides in whole suspension (percentage of initial value)			
			(*a*)		(*b*)	
	DPN	TPN	DPN	TPN	DPN	TPN
DPN+	0·92	0·036	104	92	61	6
	1·38	0·14	115	107	—	—
	6·80	0·56	111	102	—	—
TPN+	0·24	0·59	96	105	112	100
	0·52	1·60	98	94	138	75
	1·20	2·70	92	96	—	—
DPNH	0·74	0·20	113	100	—	—
	2·40	0·37	82	130	81	43
	7·80	0·50	91	175	—	—
TPNH	0·32	0·77	122	102	—	—
	0·66	2·0	86	96	119	75
	0·85	6·90	110	107	—	—

Table 6. *Total losses of pyridine nucleotides in supernatant during incubation with mitochondrial suspensions*

DPN, Values for DPN+ + DPNH; TPN, values for TPN+ + TPNH.

Nucleotide added	Initial measured concn. of nucleotides in supernatant fluids (mM)		Concn. of nucleotides in supernatant fluids after 15 min. at 25°; (percentage of initial value)	
	DPN	TPN	DPN	TPN
DPN+	10·3	0	90	0
	7·8	0·11	87	118
TPN+	4·3	10·4	82	102
	3·0	7·7	50	95

reduced coenzymes in the mitochondria as originally isolated can be obtained from these figures, because the preparation of the pellet for analysis (centrifuging, resuspension for sampling) causes considerable alteration in these ratios. Table 2 presents values obtained at comparable times for DPN+, DPNH, TPN+ and TPNH in the mitochondria by analysis of the resuspended pellet and of the original suspension of mitochondria. Generally (e.g. Expts. 2 and 3) there was more oxidized and less reduced coenzyme in the pellet; infrequently (e.g. Expts. 1 and 4) this relationship did not hold. In consequence, the ratios of reduced to oxidized coenzyme (see Table 1) are lower in the mitochondrial pellet than in the original suspension.

Effect of incubation of mitochondria with added nucleotides

Effect on water content of mitochondria. DPN+ and TPN+ at the concentrations used produced virtually no change in the proportion of water to dry matter during incubation at either 0° or 25°. DPNH at both higher and lower concentrations sometimes caused a pronounced shrinkage (for example, in one of the quoted experiments at 6·7 mM, I/M decreased by 29% in 15 min.; at 0·74 mM, by 19% in 30 min.). TPNH also caused shrinkage, but the greatest reduction in I/M (18·5% at 12·8 mM) was less than that found with DPNH. It should be noted that in all experiments with TPNH the supernatant fluid contains some DPNH also (e.g. with TPNH 12·8 mM, concn. of DPNH was 0·3 mM).

Effect on the 'chloride space' of mitochondria. At 0°, in the presence of both DPN+ and TPN+, the proportion of mitochondrial water penetrated by chloride ('chloride space') usually did not change or increased (Tables 3 and 4); this was true at 25° also, except in one experiment with DPN+ (7·8 mM) when there was a slight decrease. On the other hand, during the shrinkage produced by DPNH, the 'chloride space' diminished, indicating a selective decrease in the intramitochondrial volume available to chloride. Slight decrease in the 'chloride space' occurred during incubation with TPNH.

Losses of pyridine nucleotides during incubation. The rate of loss of endogenous nucleotides is low (approximately 20% destroyed on incubation for 1 hr. at 25°; Birt, unpublished work), and Table 5 shows that the total coenzyme of mitochondrial suspensions to which either di- or tri-phosphopyridine nucleotides have been added has a similar stability. The greatest loss, in 30 min. at 0°, was 18% of the diphosphopyridine nucleotides when the original concentration of DPNH in the supernatant fluid was 2·4 mM. The rate of destruction was somewhat greater at 25° but analysis of the supernatant fluid of suspensions to which DPN+ or

Table 7. *Dephosphorylation of triphosphopyridine nucleotide and reduced triphosphopyridine nucleotide by mitochondrial suspensions*

All experiments commenced at 0°. Original percentage composition is that of TPN^+ or TPNH; (*a*) is that immediately (0–2 min.) after addition of nucleotide; (*b*) is that 15–30 min. after addition, still at 0°; (*c*) is that after incubation at 25° from 30 to 60 min. The calculated initial concentration is obtained from the fluorimetric analysis of the solid nucleotides, and the total initial value of added nucleotide, estimated fluorimetrically. Note that this table gives no estimate of the amounts of coenzymes destroyed in the supernatant fluid. Supernatant fluid in experiments with TPN^+ contains only TPN^+ and DPN^+.

		Percentage composition of supernatant fluid							
		TPN				DPN			
Nucleotide added	Calc. initial concn. (mM)	Original	(*a*)	(*b*)	(*c*)	Original	(*a*)	(*b*)	(*c*)
TPN^+	14·8	91	71	—	—	9	29	—	—
	5·0	91	69	69	—	9	31	31	—
	0·68	91	76	76	73	9	24	24	32
TPNH	12·4	92+3*	95+2	—	—	4+1*	2+1	—	—
	7·9	92+3	94+4	—	—	4+1	1+1	—	—
	7·3	92+3	91+2	90+3	—	4+1	6+1	6+1	—
	2·5	92+3	80+0	64+17	8+57	4+1	20+1	12+7	6+29
	1·3	92+3	78+7	59+19	—	4+1	11+4	12+10	—

* Values joined by + are for the reduced and oxidized forms respectively of the di- and tri-phosphopyridine nucleotides.

Table 8. *Dephosphorylation of triphosphopyridine nucleotides by mitochondrial suspensions*

Experiments were done at 25°. Original percentage composition, as described in Table 7; (*a*) as described in Table 7, at 25°; (*b*) 15 min. after addition of TPN^+, at 25°. TPN, Values for $TPN^+ + TPNH$; DPN, values for $DPN^+ + DPNH$.

		Percentage composition of supernatant fluid					
		TPN			DPN		
Nucleotide added	Calc. initial concn. (mM)	Original	(*a*)	(*b*)	Original	(*a*)	(*b*)
TPN^+	13·4	91	71	72	9	29	24
	9·7	91	72	68	9	28	14

TPN^+ were added in concentration above 3 mM showed recoveries greater than 50% after 15 min. (Table 6).

Dephosphorylation of TPN^+ and TPNH in the supernatant fluid. At 0° there was an initial rapid dephosphorylation of added TPN^+ (Table 7), the proportion dephosphorylated being approximately constant (roughly 30%) and independent of the original concentration. No further dephosphorylation was observed, except when the original concentration of TPN^+ was low (0·68 mM) and the temperature was raised to 25° for 30 min. When the entire incubation was at 25° and the concentration of TPN^+ high, the same proportion of TPN^+ disappeared in the first few minutes and there was no subsequent dephosphorylation (Table 8).

TPNH was dephosphorylated at a slower rate than TPN^+ (Table 7). The percentage disappearance varied inversely with the original concentration and increased with time and was much faster at 25°. The ultimate products of the reaction were either DPNH or, especially at 25°, DPN^+, but the activity of enzymes destroying these two nucleotides was such that the amount of diphosphopyridine nucleotide appearing was not equal to the amount of triphosphopyridine nucleotide disappearing.

Oxidation of reduced pyridine nucleotides in the supernatant. At 0°, and at all concentrations used, DPNH was oxidized during incubation. The total amount oxidized at 0° was approximately the same, 0·016 μmole/mg. dry wt. in 30 min., independent of the original concentration, and the rate, measured as the percentage oxidized between times *b* and *c* (30 min. at 0°) and *c* and *d* (30 min. at 25°), was increased roughly fivefold by raising the temperature to 25° (Table 9). Oxidation of TPNH (products either TPN^+ or DPN^+) followed the same pattern, but the rates at both 0° and 25° were only one-half to two-thirds of the corresponding values for DPNH.

The oxidation of nucleotides in the supernatant fluid held finally in the mitochondrial pellet was greatly accelerated during the process of centrifuging and resuspension for sampling. The extent of this oxidation can be seen by comparing the amounts of reduced nucleotides in the supernatant fluid in the pellet [calculated from the concentration

of reduced nucleotide in the bulk of the supernatant fluid and the measured volume (E) of extraparticulate fluid] with the total amounts of nucleotide found by direct analysis of the pellet (Table 10). In all experiments with DPNH, the extent of oxidation was so great that the total DPNH of the pellet was much less than that anticipated in the supernatant fluid of the pellet alone, even though all manipulations of the mitochondria before extractions were carried out at 0°. Oxidation could not be prevented, though it was decreased, by heating the pellet at 100° for 2 min. before resuspension.

The rapid oxidation of DPNH sometimes appeared to induce an oxidation of TPNH, so that although the total amount of TPN^+ in the mitochondria was unchanged, a similar discrepancy occurred between TPNH expected and TPNH found in the pellet. Analyses of samples after incubation with TPNH sometimes, though not always, showed the same effect of oxidation in the pellet, though the disagreement between TPNH expected and TPNH found was never so great as in the corresponding experiments with DPNH.

Dephosphorylation of intramitochondrial TPN^+ and TPNH. The extent of this dephosphorylation

Table 9. *Oxidation of added pyridine nucleotides by mitochondrial suspensions in the supernatant fluid*

Initial concentration was calculated as before. (*a*) Percentage oxidized in solid nucleotide added; (*b*) and (*c*), percentage oxidized at 0° after 0–2 min. and after 15 or 30 min. (*) of incubation respectively; (*d*) percentage oxidized after 30–60 min. at 25°. DPN^+ alone appears after addition of DPNH; the values joined by + in the Table for TPNH additions refer to $TPN^+ + DPN^+$.

Added nucleotide	Calc. initial concn. (mM)	Percentage of added nucleotide oxidized in supernatant fluid (*a*)	(*b*)	(*c*)	(*d*)
DPNH	9·0	4	8	—	—
	7·2	4	9	13	—
	2·1	4	13	26*	97
	0·9	4	24	49	—
	0·9	4	18	45	—
TPNH	8·0	3+1	3+2	—	—
	7·2	3+1	2+1	3+1	—
	2·5	3+1	4+2	16+7*	61+3
	1·3	3+1	8+4	28+11	—

Table 10. *Oxidation of added pyridine nucleotides by mitochondrial suspensions in the pellet*

Mitochondrial suspension at the temperature shown was sampled at various times; analytical figures given are for the whole pellet and figures (E) are calculated for the measured volume of supernatant fluid entrapped in the pellet.

Added nucleotide	Calc. initial concn. (mM)	Temp. of suspension	Time of incubation (min.)	Source of pyridine nucleotide	Amount of coenzyme (μm-moles) DPN^+	DPNH	TPN^+	TPNH
DPNH	7·3	0°	0	Pellet	640	65	92	37
				E	38	540	1	53
	7·3	0	15	Pellet	640	79	73	76
				E	59	550	2	57
	8·0	0	0	Pellet	410	104	68	60
				E	42	410	1	92
	2·1	0	0	Pellet	150	28	45	28
				E	12	56	0	8
	2·1	25	60	Pellet	160	21	55	24
				E	62	5	1	6
TPNH	7·9	0	0	Pellet	88	170	96	370
				E	6	7	15	410
	7·2	0	15	Pellet	68	73	105	370
				E	4	18	85	70
	2·4	0	0	Pellet	120	71	140	170
				E	4	33	6	120
		25	60	Pellet	130	24	180	20
				E	29	6	64	8

Table 11. *Changes in intramitochondrial pyridine nucleotides on addition of di- or tri-phosphopyridine nucleotide*

Expts. 5 and 6 were done with one preparation of mitochondria, incubated with the nucleotides at 25°. All other experiments were at 0°. (*a*) Before addition of nucleotide; (*b*) immediately after addition; (*c*) 15 or 30 min. after addition.

Expt. no.	Nucleotide added	Nucleotide measured	Concn. in supernatant fluid (mM)			Amount in mitochondria (μm-moles/mg. dry wt.)		
			(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
1	DPN+	DPN+	0	1·6	1·5	1·70	1·60	2·40
		DPNH	0	0	0	0·56	0·68	0·47
		TPN+	0	0·090	0·10	0·87	0·88	1·20
		TPNH	0	0	0	1·58	1·30	1·10
2	DPN+	DPN+	2	7·8	7·9	2·50	5·20	6·70
		DPNH	0	0·008	0·004	0·34	0·48	0·34
		TPN+	1	0	0	1·30	1·90	1·90
		TPNH	0	0·016	0·008	1·20	0·96	0·23
3	TPN+	DPN+	3	0·350	0·380	1·60	2·00	2·50
		DPNH	0	0	0	0·40	1·40	2·30
		TPN+	0	1·6	1·600	1·20	0·73	0·38
		TPNH	0	0	0	0·64	1·40	1·40
4	TPN+	DPN+	8	1·7	1·7	2·30	2·70	2·70
		DPNH	0	0	0	0·15	0·27	0·25
		TPN+	2	3·8	3·8	0·75	5·20	4·90
		TPNH	0	0	0	0·86	0·74	1·00
5	DPN+	DPN+	0	7·8	6·8	2·0	5·70	8·8
		DPNH	0	0	0	0·48	0·17	0
		TPN+	0	0·11	0·13	1·20	1·80	2·90
		TPNH	0	0	0	1·70	0·50	0·00
6	TPN+	DPN+	0	3·0	1·5	2·0	0·00	2·60
		DPNH	0	0	0	0·48	0·90	0·87
		TPN+	0	7·7	7·3	1·20	11·7	16·3
		TPNH	0	0	0	1·70	1·20	1·30

Table 12. *Changes in intramitochondrial nucleotides on addition of reduced di-or tri-phosphopyridine nucleotide*

All experiments were at 0°. (*a*) Before addition of nucleotide; (*b*) immediately after addition; (*c*) 15 or 30 min. after addition. Where values for DPN+ + DPNH and TPN+ + TPNH are bracketed, oxidation in the pellet prevented separate estimation of the oxidized and reduced forms.

Expt. no.	Nucleotide added	Nucleotide measured	Concn. in supernatant fluid (mM)			Amount in mitochondria (μm-moles/mg. dry wt.)		
			(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
1	DPNH	DPN+	0·007	0·270	0·540	1·90	4·2	5·7
		DPNH	0	1·85	1·20	0·55		
		TPN+	0·003	0·005	0·12	1·50	3·2	3·0
		TPNH	0	0·180	0·29	1·40		
2	DPNH	DPN+	0·005	0·58	0·94	2·40	5·8	6·5
		DPNH	0	6·70	6·40	0·44		
		TPN+	0·005	0·06	0·06	1·90	2·5	3·1
		TPNH	0	0·66	0·72	0·97		
3	TPNH	DPN+	0·005	0·05	0·10	2·00	2·4	2·8
		DPNH	0	0·09	0·14	0·67	2·0	1·20
		TPN+	0·003	0·13	0·33	0·85	1·8	1·8
		TPNH	0	0·85	0·53	1·30	0·67	1·4
4	TPNH	DPN+	0·006	—	0·09	1·80	—	2·4
		DPNH	0	—	0·44	0·46	—	2·1
		TPN+	0·006	—	0·20	1·30	—	3·6
		TPNH	0	—	7·10	1·40	—	2·6
5	TPNH	DPN+	0·01	—	0·13	2·10	—	9·3
		DPNH	0	—	0·14	0·87	—	
		TPN+	0	—	0·29	1·30	—	1·8
		TPNH	0	—	8·0	0·98	—	

varied, but in every experiment with TPN^+ and TPNH there was an increase in the total amount of diphosphopyridine nucleotide in the mitochondria (Table 11, Expts. 3, 4, 6; Table 12, Expts. 3, 4, 5). Sometimes there was also an increase in the TPN^+ (Table 11, Expts. 3, 6; Table 12, Expt. 4). The temperature of incubation did not have a marked influence on the rate of dephosphorylation (compare Table 11, Expt. 6; Table 12, Expt. 5).

Oxidation of intramitochondrial nucleotides. The occurrence of a rapid oxidation in the packed mitochondrial pellet of endogenous (Table 2) and added (Table 10) reduced nucleotides has already been discussed. Similarly, DPNH and TPNH entering the mitochondria were readily oxidized (Table 12). This oxidation always prevented the measurement of the levels of individual intramitochondrial coenzymes (DPN^+ and DPNH, and sometimes TPN^+ and TPNH) after incubation with DPNH, and occasionally after incubation with TPNH. The rapid oxidation of TPNH in one experiment (Table 12, Expt. 5) was accompanied by considerable dephosphorylation. However, it is always possible to calculate the increases in total di- and tri-phosphopyridine nucleotides of the mitochondria.

Changes in amounts of intramitochondrial nucleotides. During incubation with DPN^+, at both higher and lower concentrations, there was an increase in the amount of DPN^+ in the mitochondria, no change in TPN^+ and a decrease in both DPNH and TPNH. These changes were found at 0° and at 25°, when the loss of reduced pyridine nucleotide was accelerated.

Incubation with TPN^+ always increased the amount of DPN^+ in the mitochondria, and sometimes that of the TPN^+. At 0°, DPNH and TPNH were either maintained or increased during incubation; at 25°, the decline in the reduced nucleotides was less rapid than with added DPN^+.

The amount of DPN^+ always increased during incubation with either DPNH or TPNH; DPNH did not affect the level of TPN^+, whereas TPNH in some experiments increased it.

Permeability to added pyridine nucleotides

Table 13 presents values for the permeability of different mitochondrial preparations to DPN^+, DPNH, TPN^+ and TPNH, each at three different concentrations. Permeability has been calculated as the ratio of the total increase in the concentration of intramitochondrial pyridine nucleotides to the total increase in the concentration of extramitochondrial nucleotides, expressed as a percentage. The choice of total values as giving the most useful measure of permeability was determined by the transformations which both extra- and intramitochondrial coenzymes undergo during incubation. All the values for increases in concentration have been corrected for changes in the water content/unit dry weight of the mitochondria, so that swelling and shrinking, which were usually slight, do not influence the results obtained.

Comparison of the results obtained with DPN^+ and TPN^+ shows that at each concentration, except the lowest, the permeability to TPN^+ was greater; in the exceptional experiment, the external concentration of TPN^+ was lower than the initial internal concentration. If the permeabilities are calculated from the external concentration of the particular nucleotide being investigated (i.e. either DPN^+ or TPN^+) rather than the total external concentration, the relative permeability

Table 13. *Permeability of mitochondria to nucleotides*

Permeability, total increase in concentration of intramitochondrial pyridine nucleotides/total increase in concentration of extramitochondrial nucleotides, expressed as percentage. All values have been corrected for swelling or shrinking of the mitochondria during incubation. (*a*) Permeability immediately after addition of nucleotide at 0°; (*b*) permeability 15 or 30 min. after addition of nucleotide at 0°; (*c*) permeability after incubation for 30–60 min. at 25°.

Nucleotide added	Expt. no.	Concn. of added nucleotide (total) (mM)			Permeability (%)		
		(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
DPN^+	1	0·90	0·91	0·60	22	12	16
	2	1·7	1·6	—	0	6	—
	3	7·8	7·9	—	17	23	—
TPN^+	4	0·75	0·75	0·80	0	102	6
	5	1·9	2·0	2·00	28	53	37
	6	5·5	5·5	—	40	38	—
DPNH	7	1·1	1·0	—	61	65	—
	8	2·3	2·2	2·4	37	71	100
	9	8·0	8·1	—	13	21	—
TPNH	10	1·1	1·1	—	74	86	—
	11	2·7	2·5	2·6	91	110	77
	12	—	7·8	—	—	44	—

to TPN is even greater. For example, permeabilities calculated in this way immediately after the addition of the nucleotides are (*a*) for DPN^+ unchanged (Table 13, Expts. 2 and 3) and (*b*) for TPN^+ increased from 28 to 34% (Table 13, Expt. 5) and from 40 to 58% (Table 13, Expt. 6). Thus the correct figures for permeability to TPN^+ will always be somewhat higher than those shown in Table 13, whereas the correct values for DPN^+ will be almost identical with those of the table.

Permeability to TPNH is greater than that to DPNH at comparable concentrations. Application of corrections for dephosphorylation increased the value for permeability to TPNH slightly, but not as greatly as for TPN^+, because of the lower rate of dephosphorylation of TPNH. Similarly, the calculated value of the permeability to DPNH will be increased by using the external concentration of DPNH alone, but the increase is not sufficient to abolish the difference between the permeabilities to the two nucleotides. For example, the corrected permeability to DPNH (see Table 13, Expt. 9) is 26% instead of 21% after 15 min. at 0°; to TPNH (Table 13, Expt. 12) it is 48% instead of 44%.

The permeability to all four nucleotides generally increased on incubation at 0°, and did not change markedly when the temperature was raised to 25°.

There was no evidence for the adsorption of DPN^+, TPN^+ or TPNH, but the permeability (as previously defined) to DPNH increased as the external concentration decreased, suggesting considerable adsorption of this coenzyme. Therefore direct comparisons between the permeabilities of di- and tri-phosphopyridine nucleotides were made by incubating different samples of the same mitochondrial suspension with high concentrations of DPN^+ and TPN^+, and DPNH and TPNH. These experiments (Table 14) confirmed the results already described (Table 13) at 0°; further, the same marked difference was demonstrated at 25°, at which temperature the mitochondria are capable of metabolic activities.

Effect of change in mitochondria on permeability to nucleotides. The demonstration of the difference in permeability to di- and tri-phosphopyridine nucleotides was dependent on the condition of the mitochondria in the original suspension. Thus increase in the original value of I/M, decrease in the initial proportion of reduced nucleotide (especially DPNH) and loss of ability to maintain reduced nucleotides during incubation, all of which can be considered as degenerative changes, were observed to accompany a considerable increase in the permeability to DPN^+ and DPNH, whereas that to TPN^+ and TPNH did not change (Table 15). This alteration in relative permeability was found only when all of

Table 14. *Permeability of mitochondria to pyridine nucleotides: direct comparisons*

Permeability is defined in Table 13. All values are corrected for swelling or shrinking of the mitochondria during incubation. (*a*) Values immediately after addition of nucleotide; (*b*) values after 15 min.

Expt. no.	Nucleotide added	Temp. of incubation	Concn. of added nucleotide (total) (mM)		Permeability (%)	
			(*a*)	(*b*)	(*a*)	(*b*)
1	DPN^+	0°	14	—	25	—
	TPN^+		16	—	41	—
2	DPNH	0	8·8	—	0	—
	TPNH		8·5	—	37	—
3	DPN^+	25	7·9	6·9	12	28
	TPN^+		11	8·8	30	60

Table 15. *Comparison of permeability of different preparations of mitochondria*

Mitochondria in Expts. 2 and 4 had undergone the degenerative changes described in the text. Expts. 1 and 2, incubation at 0°; Expts. 3 and 4, incubation at 25°. Permeability was measured immediately after addition of the nucleotides. DPN and TPN refer to total di- and tri-phosphopyridine nucleotides respectively.

	Mitochondria before addition of nucleotide			Percentage of original total reduced nucleotide retained on incubation with				Permeability (%)	
		Percentage of nucleotide reduced		DPN		TPN			
Expt. no.	I/M	DPN	TPN	2 min.	15 min.	2 min.	15 min.	DPN^+(H)	TPN^+(H)
1 (DPNH, TPNH)	2·4	42	42	—	—	—	—	0	37
2 (DPNH, TPNH)	2·7	8	69	—	—	—	—	47	40
3 (DPN^+, TPN^+)	2·7	25	59	30	0	62	64	12	28
4 (DPN^+, TPN^+)	2·95	13	44	0	0	88	0	39	27

the three variations in the original mitochondrial suspension had occurred and never when only one was detected.

DISCUSSION

Description of some properties of mitochondria prepared in saline medium. The initial water content (I/M mean 2·4 approximately) of mitochondria in the saline medium was higher than those of mitochondria suspended in sucrose (e.g. 1·6–1·9, Werkheiser & Bartley, 1957), and the proportion of dry matter in the mitochondria is therefore somewhat lower (approximately 30%).

The 'chloride space' (range 63–100%) of the original mitochondrial suspension in saline medium was usually larger after comparable times of incubation than that of mitochondria in sucrose plus chloride (34–74%; Amoore & Bartley, 1958), indicating that there had been an increase in the permeability to chloride after transfer from sucrose.

Endogenous pyridine nucleotides in mitochondria. Published values for the levels of the coenzymes of rat-liver mitochondria show considerable variation. Table 16 summarizes recent work.

With the mitochondria used in this investigation (66% protein) the total level of pyridine nucleotide/mg. of protein was 7·3 μmoles (Table 16), a value close to those of Jacobson & Kaplan (1957*a*) and Chance & Williams (1955). The figures given by Glock & McLean (1956) and Maley & Lardy (1955) are much lower, as are the initial levels quoted by Purvis (1958). The values in parentheses in Table 16 are for the levels of di- and tri-phosphopyridine nucleotide after release of the 'inhibited forms' of these coenzymes (Purvis, 1958). If the distinction drawn by Purvis between 'inhibited' and free forms of DPN and TPN is applicable to the nucleotides of the mitochondria in the saline medium, it would seem that the extraction procedure used removes both forms of the coenzymes. Thus the total amount of pyridine nucleotide present is measured, irrespective of its intramitochondrial location [compare the evidence presented by Lehninger, Wadkins, Cooper, Devlin & Gamble (1958) and Chance & Baltscheffsky (1958) for the binding of coenzymes to particular dehydrogenase systems].

Glock & McLean (1956) and Purvis (1958) find approximately equal amounts of di- and tri-phosphopyridine nucleotides in liver mitochondria, as described also in this paper; whereas Jacobson & Kaplan (1957*a*) report a considerable excess of TPN^+.

In all the analyses quoted the ratio $TPNH/TPN^+$ is much greater than the ratio $DPNH/DPN^+$. However, these ratios are of doubtful significance, as they may reflect only the conditions in which the mitochondria were isolated (see Dickens, Glock & McLean, 1959). It would appear that the oxidation of endogenous nucleotides occurring when the mitochondria are sedimented during the isolation procedure (as in the experiments described in Table 2) will result in decrease in the ratio of reduced to oxidized coenzymes, if no reversal of the process occurs during resuspension.

Changes in mitochondrial properties after addition of pyridine nucleotides. The shrinkage of mitochondria with DPNH and TPNH is probably due to a complex series of reactions, especially in view of the recent work of Lehninger, Ray & Schneider (1959), which ascribes a particular role to DPNH in the maintenance of the structure of the mitochondrial membrane. It is possible that the shrinkage is mediated by a transhydrogenation to the endogenous nucleotides in the presence of added DPNH and TPNH; however, as the hydrogen

Table 16. *Concentrations of endogenous pyridine nucleotides in mitochondria*

Authors	Concn. (μm-moles/mg. of protein) DPN	TPN	Total pyridine nucleotide	Remarks
(1) Maley & Lardy (1955)	—	—	Approx. 2·9	Calculated from value for total pyridine nucleotide/mg. of mitochondrial N
(2) Chance & Williams (1955)	—	—	Approx. 7·1	Calculated from difference spectrum for anaerobic mitochondria and protein content of mitochondrial suspension
(3) Glock & McLean (1956)	1·9	1·8	3·7	Calculated from pyridine nucleotide and N values given for unwashed mitochondria
(4) Jacobson & Kaplan (1957*a*)	2·0	4·3	6·3	Calculated from values given as g./mg. of protein
(5) Purvis (1958)	2·7 (4·3)	2·4 (4·9)	5·1 (9·2)	Quoted directly
(6) This paper (1960)	3·7	3·6	7·3	—

transfer to the endogenous nucleotides is more marked with TPNH than DPNH, whereas the shrinkage is less so, it seems more likely that the fall in values of I/M is due specifically to the addition of DPNH itself. The relatively smaller shrinkage found with TPNH may be due to its partial transformation into DPNH. The decrease in 'chloride space' produced by DPNH (and TPNH) is further evidence for a profound change in the structural organization of the mitochondria exposed to these two nucleotides.

Metabolism of pyridine nucleotides added to mitochondrial suspensions. Jacobson & Kaplan (1957*b*) found that rat-liver mitochondria contain DPN^+ and DPNH pyrophosphatases of low activities (approximately one-tenth of that of the microsomes) but were unable to detect DPN-nucleosidase activity. Sung & Williams (1952) report slight DPN-nucleosidase activity. Our results are consistent with these findings. Added DPN^+, DPNH and TPNH disappeared slowly during incubation, at rates comparable with those for the loss of endogenous coenzymes, but TPN^+ was rapidly dephosphorylated in the early stages of the incubations (compare the dephosphorylation of added ATP by rat-liver mitochondria at 0° described by Amoore, 1958). This may be due to the action of the non-specific phosphatases of either the mitochondria (e.g. alkaline phosphatase; Allard, de Lamirande, Faria & Cantero, 1954) or contaminants derived from the soluble part of the cell. The latter seems likely to account for at least some of the dephosphorylation, as the endogenous TPN^+ is much more stable. The rate of dephosphorylation was far greater initially but the small losses occurring during incubation with low concentrations of TPN^+ at 25° suggest that the process does continue at a much reduced rate.

The more rapid oxidation in the supernatant fluid of added DPNH, compared with that of added TPNH, agrees with the findings of Joshi, Newburgh & Cheldelin (1957) and Baessler & Pressman (1959). The absolute rate of oxidation was low: in the first 30 min. of incubation at 0° with 2·1 mM-DPNH the rate of consumption of oxygen required to account for the measured disappearance of DPNH was 0·18 μl./mg. dry wt./hr., rising to 1·0 μl./mg. dry wt./hr. when the temperature was raised to 25°. (These values may be compared with the rate of oxygen consumption required to account for the loss of endogenous reduced nucleotides when mitochondria were incubated at 25° without substrate: approximately 0·13 μl./mg. dry wt./hr.) Oxidation (assessed by the loss of DPNH balanced by a gain in DPN^+) occurred even when the permeability of the mitochondria was extremely low, perhaps by a process akin to the non-phosphorylating pathway of Lehninger (1955–56). He has reported that mitochondria in sucrose will oxidize DPNH readily only when they have been pretreated in such a way as to increase their permeability; but Maley (1957), who incubated mitochondria with DPNH (and ethanol) in the medium, found a much more vigorous oxidation without pretreatment. This worker notes, however, that the presence of the ethanol may alter the permeability to nucleotides. The great increase in the rate of oxidation of reduced nucleotides which occurs when the mitochondria are packed during centrifuging was surprising, as it was thought that the relatively anaerobic conditions would promote further reduction. It is known, however, that both DPNH and TPNH are oxidized non-enzymically in solutions of free flavins (Singer & Kearney, 1950; Bassham *et al.* 1959), and the oxidation in the pellets is at least partly non-enzymic, as it cannot be prevented by heating. During the packing of the pellet, the concentrations of compounds (including flavins) released from damaged particles will increase greatly, and the rate of oxidation is accelerated correspondingly. This cannot, however, be a valid explanation for the greater rate of oxidation of the endogenous nucleotides, which are retained in the mitochondria during centrifuging.

It has been shown that a number of transformations of added pyridine nucleotides proceed in the supernatant fluid of the incubated mitochondrial suspension. These must result from the release of catalytic compounds (e.g. flavins or enzymes) from the particulate material, or occur at the surface of the mitochondria.

Permeability of mitochondria to nucleotides. Because of the complexity of the transformation which the nucleotides undergo during incubation with mitochondria it is convenient to summarize the information obtained by a diagram (Fig. 1). This illustrates all the reactions which have been observed during incubations with all four coenzymes; in any particular experiments with a given nucleotide, at least some, but usually not all, of the possible transformations of the nucleotide were seen. Mitochondria have a low permeability to both DPN^+ and DPNH. DPNH is strongly adsorbed and is oxidized in the mitochondria. DPN^+ that enters the mitochondria does not appear to influence the behaviour of the endogenous reduced nucleotides, and is never itself reduced. Thus it apparently does not enter into the 'pool' of functional endogenous coenzymes. It is not possible to draw any analogous conclusions about the behaviour of DPNH entering the mitochondria, but the permeability to this nucleotide was usually lower than to DPN^+.

Permeability to the diphosphopyridine nucleotides may be increased by altering the properties of the mitochondria used (see also Lehninger, 1951),

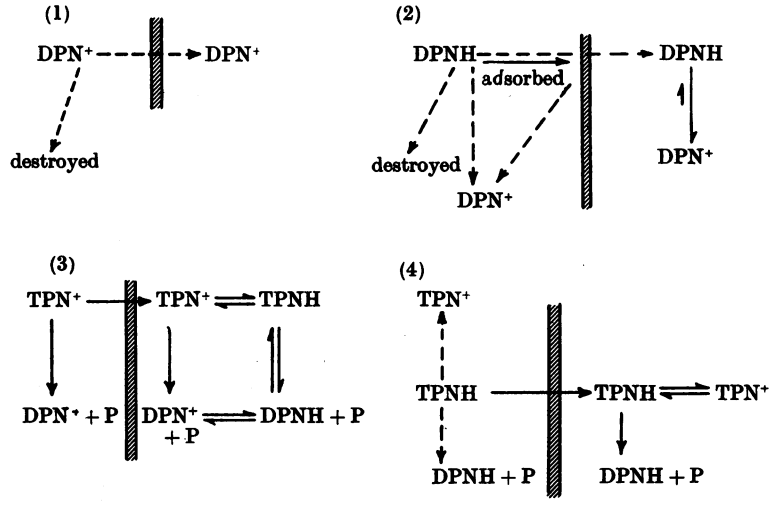

Fig. 1. Transformations of pyridine nucleotides incubated with mitochondria. Broken lines indicate slow reactions, continuous lines rapid reactions. The hatched area represents the mitochondrial membrane, the areas to the left and right of it the extra- and intra-mitochondrial environments respectively. Reactions which have been described for a particular nucleotide in (1) are not repeated in (2), (3) or (4), in which the nucleotide appears. P indicates the liberation of orthophosphate.

and was more than doubled in preparations with higher I/M, lower original level of reduced pyridine nucleotide and decreased ability to maintain reduced nucleotides. These changes are consistent with swelling and disruption of the mitochondrial structure, processes which, when induced by pretreatment with phosphate, produce mitochondrial suspensions which have lost most of their endogenous nucleotides but have the ability to incorporate large quantities of DPN^+ (Hunter *et al.* 1959). The final concentration of DPN in these mitochondria may be much greater than the original concentration, and at least some of the reincorporated nucleotide is bound in such a way as to function in oxidative phosphorylation. In our experiments, the degree of disorganization of the mitochondria and the loss of endogenous nucleotides during preparation (estimated by comparing the initial amounts of the coenzymes with the values of Table 1), though much less, were sufficient to permit a relatively great uptake of DPN^+ and DPNH.

Both TPN^+ and TPNH enter the mitochondria readily. TPN^+ increases or maintains the level of reduced nucleotides (TPNH and DPNH) at both 0° and 25°, and so appears to have access to sites involved in intramitochondrial oxidation and reduction. However, as the rate of dephosphorylation of TPN^+ entering the mitochondria was much higher than that of the endogenous TPN^+, it is also apparent that the intramitochondrial location of much of the TPN^+ is distinct from that of the endogenous TPN^+. It is not possible to make similar observations about TPNH.

The experiments described in this paper give direct experimental support to the notion that isolated undamaged mitochondria can exclude added DPN^+ and DPNH from participation in intramitochondrial processes of oxidation and reduction. Some binding of the nucleotide and alteration of mitochondrial structure may occur with DPNH. TPN^+ and TPNH, on the other hand, can enter the mitochondria readily and participate in oxidations and reductions. Thus it seems that there is a possibility of the passage of hydrogen, available either for energy production via transhydrogenation in the mitochondria or for reductive syntheses, across the mitochondrial membrane.

SUMMARY

1. The distribution of added and endogenous pyridine nucleotides between rat-liver mitochondria and the medium in which they were suspended has been studied. The distribution of the added coenzymes between the suspending fluid and the mitochondria has been interpreted in terms of the permeability of the mitochondria to the pyridine nucleotides.

2. The amounts of endogenous nucleotides (as μmoles/mg. dry wt.) were: DPN^+ 2·02; DPNH 0·44; TPN^+ 1·26; TPNH 1·08. Of the dry matter of the mitochondria, 66% was protein. Considerable oxidation of the reduced nucleotides occurred when the particles were sedimented.

3. Addition of DPNH and TPNH sometimes produced a shrinkage of the mitochondria, the

maximum decrease in the proportion of water to dry matter in the particles being 29% in 15 min. with DPNH (6·7 mM) and 18·5% with TPNH (12·8 mM). During shrinkage, there was a selective decrease in the 'chloride space' of the mitochondria.

4. Loss of added nucleotides was always slight at 0°, the greatest amounting to 18% disappearance of DPNH (originally 2·4 mM) in 30 min. The maximum rate of loss was approximately trebled when the temperature was raised to 25°.

5. TPN^+ and TPNH were dephosphorylated, the former more rapidly; the products appeared in both the mitochondria and supernatant fluid.

6. DPNH was oxidized slowly (approximately 0·032 μmole/mg. dry wt./hr.) in the suspension, but much more rapidly when the mitochondria were sedimented. The rate of oxidation of TPNH under similar conditions was approximately two-thirds of the corresponding values for DPNH.

7. After addition of DPN^+, DPNH, TPN^+ and TPNH at concentrations greater than 5 mM to different suspensions, the mitochondria contained DPN^+ and DPNH at concentrations 0–25% and TPN^+ and TPNH at concentrations 37–41% of that in the supernatant fluid. This difference in 'permeability' was demonstrated over a wide range (2–15 mM) of concentrations and at both 0° and 25°. Only DPNH was adsorbed by the mitochondria.

8. TPN^+ and TPNH permeating the mitochondria enter the 'pool' of functional nucleotides.

9. Degenerative changes in the original mitochondrial preparations (swelling, low level of endogenous reduced nucleotides and inability to maintain reduced nucleotides) resulted in a greatly increased 'permeability' to the diphosphopyridine nucleotides.

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Lipid Synthesis by Human Leucocytes *in vitro*

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Synthesis of lipid by human and rabbit erythrocytes *in vitro* has been reported by several investigators (Altman, Watman & Salomon, 1951; Altman 1953; James, Lovelock & Webb, 1957; Marks, Johnson, Hirschberg & Banks, 1958; Rowe, 1959; Turner, 1958). In all these studies blood, sometimes with the 'buffy coat' removed after centrifuging, has been incubated with [^{14}C]acetate. After incubation the cells, plasma or whole blood have been extracted for lipids and this lipid extract

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