a maximum rate of oxidation occurred decreased as the oxygen concentration was decreased.

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The Pyridine Nucleotide Metabolism of Mitochondria Incubated with and without Added Substrates and Metabolic Inhibitors

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The experiments described previously (Birt & Bartley, 1960*a*, *b*) have shown that the amounts and state of reduction of the endogenous pyridine nucleotides of rat-liver mitochondria are controlled by the components of the incubating medium. This paper is concerned with a general study of the effects of the temperature of incubation, the addition of various substrates, 2,4-dinitrophenol and amytal on the behaviour of the pyridine nucleotides in these particles; in particular, factors altering the dynamic steady-state levels of reduced nucleotide (by changing rates of oxidation or reduction of the coenzymes, or both) have been investigated.

MATERIALS AND METHODS

The chemicals used in the preparation of media for the isolation and incubation of rat-liver mitochondria and the details of the preparative procedure have been described previously (Birt & Bartley, 1960*a*). The mitochondria were isolated in a medium containing sucrose (0.25M) plus nicotinamide (25 mM) and incubated in a 'saline' medium containing KCl (0.1M), 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer (pH 7.4, 50 mM), MgSO₄ (5 mM), sodium ethylenediaminetetra-acetate (EDTA, 1 mM), adenosine triphosphate (ATP, 1 mM) and nicotinamide (25 mM).

The methods of incubation and estimation of O₂ uptake and pyridine nucleotide analysis were as described by Birt & Bartley (1960*b*) and Bassham, Birt, Hems & Loening (1959).

RESULTS

Changes in pyridine nucleotides during incubation without added substrates at different temperatures

A number of mitochondrial suspensions without added substrate were incubated at temperatures in the range 0–30° (Table 1). During 1 hr. at both 0° and 10°, there was no destruction of coenzyme and no decline in reduced pyridine nucleotide; indeed, at both temperatures there was a tendency for the amount of reduced diphosphopyridine nucleotide (DPNH) to increase slowly, though the level of reduced triphosphopyridine nucleotide (TPNH) did not change significantly. At and above 15°, however, the total coenzyme level declined by approximately 20% in 1 hr.; di- and tri-phosphopyridine nucleotides (DPN⁺ and TPN⁺) disappeared at similar rates. There was also a net loss of reduced coenzyme during 1 hr. The DPNH level usually fell, in 15–30 min., to a value below which little further decline occurred, but the oxidation of TPNH commonly continued throughout the hour of incubation, especially at 20° (similar data were obtained with mitochondria incubated at 25° without substrate). The last two columns of Table 1 indicate the relative changes occurring in the proportions of the di- and tri-phosphopyridine nucleotides present in the reduced form; values greater than 1.0 were the result of a decrease below the initial proportion of reduced coenzyme; values less than 1.0 of an increase over the initial proportion of reduced coenzyme. At 15° and above, the proportional change after incubation for 5 min. was always greater in the triphosphopyridine

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Table 1. *Effect of temperature on the oxidation of endogenous reduced nucleotides in different mitochondrial preparations*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$. Incubations were carried out in 'saline' medium, with gentle shaking. In this and subsequent tables DPN = sum of oxidized and reduced diphosphopyridine nucleotides ($\text{DPN}^+ + \text{DPNH}$); TPN = sum of oxidized and reduced triphosphopyridine nucleotides ($\text{TPN}^+ + \text{TPNH}$); $\text{PN}^+ = \text{DPN}^+ + \text{TPN}^+$; $\text{PNH} = \text{DPNH} + \text{TPNH}$; $\text{PN} = \text{PN}^+ + \text{PNH}$.

Temp.	Time (min.)	DPN	TPN	PN	DPNH DPN ⁺	TPNH TPN ⁺	PNH PN ⁺	at time 0 min.	
								$\frac{\text{DPNH}}{\text{DPN}^+}$	
								at time shown	at time shown
0°	0	1.90	0.86	2.76	0.49	7.5	1.1	1.0	1.0
	5	1.90	0.95	2.85	0.49	6.9	1.0	1.0	4.0
	15	2.20	0.91	3.11	0.59	10.4	1.1	0.83	2.7
	30	2.00	1.05	3.05	0.76	6.9	1.4	0.64	4.0
	60	2.10	0.76	2.86	0.70	3.8	1.0	0.70	7.2
10	0	1.55	1.05	2.60	0.64	4.0	1.3	1.0	1.0
	5	1.65	1.10	2.75	0.60	3.4	1.1	1.1	1.2
	15	1.90	0.98	2.88	0.99	3.3	1.4	0.65	1.2
	30	1.65	1.00	2.65	0.79	8.1	1.6	0.81	0.49
	60	1.85	0.88	2.73	1.03	3.4	1.5	0.62	1.2
15	0	2.15	2.00	4.15	0.97	6.6	2.1	1.0	1.0
	5	1.70	1.60	3.30	0.45	3.0	1.1	2.2	2.2
	15	1.60	1.56	3.15	0.21	0.6	0.37	4.6	11.0
	30	1.55	1.65	3.20	0.19	0.4	0.30	5.1	17.0
	60	1.50	1.50	3.00	0.24	0.5	0.35	4.0	13.0
20	0	2.00	1.95	3.95	0.71	6.4	1.8	1.0	1.0
	5	1.85	1.90	3.75	0.75	4.7	1.7	0.95	1.4
	15	1.65	1.50	3.15	0.27	1.4	0.65	2.6	4.6
	30	1.65	1.60	3.25	0.23	0.83	0.47	3.1	7.7
	60	1.70	1.75	3.45	0.25	0.50	0.37	2.8	13.0
30	0	2.45	2.30	4.75	1.10	4.1	1.9	1.0	1.0
	5	2.25	2.45	4.70	2.3	8.7	3.9	0.48	0.47
	15	2.35	2.10	4.45	0.54	1.2	0.8	2.0	3.4
	30	1.95	2.20	4.15	0.50	1.4	0.9	2.2	2.9
	60	1.75	1.35	3.10	0.04	0.12	0.1	28.0	34.0

Fig. 1. Effect of temperature on the time course of mitochondrial swelling. Samples of mitochondrial suspension (2 ml. in 0.25M-sucrose) were maintained for the times (Δ , 15 min.; $\bullet$, 30 min.; $\blacksquare$, 45 min.; $\bullet$, 60 min.) and temperatures indicated. The ratio of water to dry weight of the mitochondrial pellets obtained by high-speed centrifuging of the suspension was determined according to Werkheiser & Bartley (1957).

nucleotide, i.e. the ratio TPNH/TPN^+ declined more rapidly than the ratio DPNH/DPN^+ . Further, in all the experiments quoted, the amounts of

TPNH disappearing in each interval of time were greater than those of DPNH.

Even when there was a net oxidation of the reduced coenzyme after incubation for 1 hr. (i.e. at 15° and above), there was sometimes an initial transient increase in the amount of reduced nucleotides (Table 1, experiments at 20° and 30°), followed by their oxidation. This was most marked at 30°. The initial reduction of oxidized coenzymes in a mitochondrial suspension incubated without added substrate was enhanced greatly by increasing the concentration of mitochondria in the suspension. In all the experiments quoted in Table 1, the preparations contained 20–30 mg. of dry matter/ml.; the results obtained with a suspension having 45 mg. of dry matter/ml. showed that, on incubation, there was a large increase in the level of reduced coenzymes, and the restoration of the original ratio of reduced to oxidized nucleotide did not occur until after 30 min. The reduction of TPN^+ lagged behind that of DPN^+ but the ensuing oxidation of TPNH was more rapid than that of DPNH.

The series of incubations (Table 1) indicated that a rise from 0° to 10° did not affect the coenzymes

measurably, but that a rise of a further 5° produced all the changes characteristic of actively oxidizing mitochondria, though the final levels (after incubation for 60 min.) of reduced coenzymes were somewhat higher than at 20° and above. There were analogous temperature-dependent changes in the water content of rat-liver mitochondria suspended not in the 'saline' medium but in 0.25M-sucrose (Fig. 1). This is further evidence for a relationship between the maintenance of mitochondrial structure and the retention of reduced pyridine nucleotide (see Lehninger, Ray & Schneider, 1959).

All subsequent incubations were done at 25°, for although at this temperature the changes in oxidized and reduced coenzyme levels were sufficiently large to be measured accurately, the mitochondria did not undergo the almost complete loss of reduced nucleotides that occurred at 30°.

Oxygen uptake of mitochondria incubated in 'saline' medium without substrate

Table 2 presents the levels of oxidized and reduced coenzymes and the initial (calculated for the first 15 min.) and final (calculated for last 15 min.) rates of oxygen uptake. Expts. 1-4 show typical results obtained in the absence of substrate; the rate of oxygen uptake generally declined during the hour of incubation from an initial Q_{O_2} value of about 3. At the same time, the ratio of reduced to oxidized pyridine nucleotides diminished, but did not decrease below about 0.1. Exceptional experiments are Expts. 5-7, where there was an increase in the rate of oxygen consumption, and an almost complete disappearance of reduced coenzyme. In one experiment (Expt. 4) the rate of oxygen uptake declined although the final ratio of reduced to oxidized coenzyme was much less than 0.1. These data suggest that pyridine nucleotide-dependent respiration declines as the total amount of coenzyme and of endogenous substrate available to

reduce it decreases, and is later replaced by other types of oxidative processes.

In some experiments the initial rate of oxygen consumption was as high as 6-9 μ l./mg. dry wt./hr. There was no simple relationship between the initial rate of respiration and the ratio of reduced/oxidized coenzyme in the isolated mitochondria.

Effect of added substrates on the pyridine nucleotides and oxygen uptake of mitochondria

All the metabolites added (Table 3) increased the rate of oxygen consumption; considering only the stimulations produced by compounds dependent on DPN⁺ or TPN⁺ for their oxidation, the order of decreasing effectiveness was α -oxoglutarate, citrate, pyruvate plus malate, pyruvate, β -hydroxybutyrate and malate. The rates of oxygen consumption with the last-named four were constant during incubation for 1 hr.; that with citrate declined by 50%. α -Oxoglutarate, glutamate, pyruvate plus malate and β -hydroxybutyrate all increased the ratio of total reduced to total oxidized coenzyme over the initial value for isolated mitochondria before the first sample could be removed (at 'zero' time, usually 0.5-1.5 min. after addition of the substrate, at a temperature of about 5°), whereas citrate, malate and pyruvate did not. The greater the effect of the added substrate on the ratio of reduced to oxidized pyridine nucleotides, the greater was the effect on the rate of respiration in the first 15 min. Subsequently, all the substrates maintained the ratio for 1 hr. at higher values than that observed in the corresponding suspension without substrate; it was not possible to relate such decreases in the ratio to decreasing rates of respiration (see especially the experiments with citrate, malate and hydroxybutyrate, Table 3).

The effect of two substrates not dependent on pyridine nucleotides for their oxidation was also tested. One, choline, gave the maximum increase

Table 2. *Endogenous respiration and the ratios of reduced to oxidized mitochondrial nucleotides*

All values for pyridine nucleotide levels are given as μ m-moles/mg. dry wt.; for O_2 uptake, as μ l. of O_2 /mg. dry wt./hr. Temp. of incubation, 25°; all incubations were in 'saline' medium. See also protocol to Table 1.

Time (min.) ... Expt. no.	PN ⁺			PNH			PNH/PN ⁺			Uptake of O_2 at end of period	
	0	15	60	0	15	60	0	15	60	15	60
1	1.10	1.45	1.45	1.40	0.75	0.30	1.3	0.52	0.21	2.4	1.8
2	1.70	3.00	2.90	2.65	1.50	0.42	1.6	0.50	0.15	2.0	1.4
3	1.40	2.55	3.10	2.70	0.80	0.54	1.9	0.31	0.17	2.4	1.2
4	2.00	2.90	3.00	1.80	0.75	0.10	0.9	0.26	0.03	3.2	0.8
5*	2.00	3.10	3.20	1.70	0.30	0.16	0.9	0.10	0.05	0.1	3.0
6	1.35	2.45	2.40	1.85	0.74	0.14	1.4	0.30	0.06	3.0	6.0
7	1.85	3.15	3.30	2.60	0.76	0.10	1.4	0.24	0.03	2.4	2.8

* No ATP in 'saline' medium.

in respiration only after a lag phase; it had no effect on the ratio of reduced to oxidized coenzyme, which decreased as it did in the absence of added substrate. The second, succinate, produced a very pronounced increase in the rate of oxygen consumption during 15 min., though the increased level was not maintained throughout the incubation, and the decline in the respiration rate (68 % in 1 hr.) was greater than with any other substrate. This decline may have been due in part to the rapid removal of the substrate; if it is assumed that only succinate was oxidized, the data of Table 3, Expt. 3, show that about 53 % of the succinate was removed in 15 min. The production of even small amounts of oxaloacetate by the further metabolism of the fumarate produced may also have inhibited succinate oxidation. Succinate also produced the highest ratio of reduced to oxidized coenzyme observed in this series of experiments; the amount of reduced nucleotide had been greatly increased before the first sample could be taken. Higher levels of DPNH and TPNH were found

during incubation for 1 hr. with succinate than with any other substrate tested.

An attempt was made to follow the time course of the reduction of the coenzymes after the addition of succinate. However, even in 0.5 min. considerable reduction had occurred, and the ratio had risen to its maximum value after incubation for only 6 min. Thus the rapidity of the reaction prevents the accurate measurement of the increase in reduced coenzyme by the analytical method used (compare the results obtained with the recording spectrophotometer by Chance, 1954). When pyruvate was used as substrate (Table 8, column 11) the net reduction of the nucleotides was sufficiently slow to be followed.

Effect of substrate on the pyridine nucleotides on the 'fluffy layer'. The pyridine nucleotides of the 'fluffy layer' responded to added pyruvate and succinate in the same way as did the mitochondria, succinate being more effective in reducing the coenzymes than pyruvate; there was no decline in the ratio reduced/oxidized nucleo-

Table 3. *Pyridine nucleotides of mitochondria incubated with added substrates*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$; for O_2 uptake as $\mu\text{l. of O}_2/\text{mg. dry wt./hr.}$ Temp. of incubation, 25° ; concn. of all added substrates, 10 mM. The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

Expt. no.	Added substrate	Time (min.)	PN ⁺	PNH	PN	PNH/PN ⁺	Uptake of O_2 at end of time	Loss of nucleotides (%)
	Nil	0	1.40	2.15	3.55	1.55	—	—
		15	2.55	1.05	3.60	0.41	2.3	0
		60	2.60	0.37	2.96	0.14	1.5	17
1	Citrate	0	1.40	2.00	3.40	1.40	—	—
		15	0.72	2.80	3.52	3.90	16	0
		60	0.72	1.90	2.62	2.60	7.8	24
2	α -Oxoglutarate	0	1.00	2.80	3.80	2.80	—	—
		15	0.66	3.15	3.81	4.80	20	0
		60	1.25	1.25	2.50	1.00	—	34
2	Glutamate	0	1.20	2.65	3.85	2.20	—	—
		15	0.93	2.95	3.88	3.15	—	0
		60	1.45	1.10	2.55	0.76	—	33
3	Succinate	0	0.59	2.80	3.39	4.80	—	—
		15	0.44	2.65	3.09	6.00	14.2	9
		60	0.62	1.90	2.52	3.10	4.6	23
4	Malate	0	1.25	2.00	3.25	1.60	—	—
		15	1.20	2.15	3.35	1.80	6.6	0
		60	1.30	1.00	2.30	0.77	6.0	29
4	Pyruvate	0	1.30	1.90	3.20	1.50	—	—
		15	0.82	2.55	3.37	3.10	8.0	0
		60	0.75	2.10	2.85	2.80	7.8	12
5	Pyruvate plus malate	0	1.15	2.85	4.00	2.50	—	—
		15	0.81	2.80	3.61	3.50	12.5	10
		60	0.90	2.20	3.10	2.45	10.7	22
6	β -Hydroxybutyrate	0	1.50	3.25	4.75	2.15	—	—
		15	1.90	2.70	4.60	1.40	6.9	5
		60	2.85	1.05	3.90	0.37	4.8	19
6	Choline	0	1.90	2.55	4.45	1.35	—	—
		15	3.20	0.75	3.95	0.23	4.0	11
		60	2.65	0.42	3.07	0.16	6.9	31

tide during incubation for 15 min. without added substrate.

Reduction of di- and tri-phosphopyridine nucleotides by added substrates. Separate analyses of DPN⁺, DPNH, and TPN⁺ and TPNH during incubation with citrate, α -oxoglutarate, pyruvate and succinate showed that of the three substrates reacting directly with pyridine nucleotides only α -oxoglutarate had an immediate effect in reducing DPN⁺ and TPN⁺. The amount of TPNH formed was always small. Pyruvate and citrate had no immediate effect but reduced both DPN⁺ and TPN⁺ in 15 min. There was no clear evidence for a preferential reduction of TPN⁺ by citrate. The immediate reduction of the coenzymes after succinate addition involved both DPN⁺ and TPN⁺, as did the subsequent maintenance of a high ratio of reduced to oxidized coenzymes. With all four substrates, the amounts of TPN⁺ in the mitochondria after 15 min. were extremely small.

Reduction of pyridine nucleotides in mitochondria pre-incubated without substrate. When mitochondria were allowed to oxidize their endogenous coenzymes during pre-incubation for 30 min. at 25° (Table 4) before addition of β -hydroxybutyrate, succinate or pyruvate plus malate (10 mm), the levels of both DPNH and TPNH increased during a further 5 min. of incubation. With each substrate the greater increase was in DPNH. The ratios of reduced to oxidized coenzyme with succinate and pyruvate plus malate (4.3 and 3.8 respectively) were similar to those found after short incubation of freshly isolated mitochondria (see Table 3), but with β -hydroxybutyrate the ratio was lower (1.3 in this experiment compared with 2.15 in Table 3). Moreover, in an experiment in which pre-incubation

was continued for 40 min., the addition of 100 mm- β -hydroxybutyrate merely maintained, but did not increase, the levels of both DPNH and TPNH during a further 5 min. After 15 min. most of the coenzyme was oxidized. Thus it seems that whereas the enzymes responsible for dehydrogenating succinate and pyruvate plus malate retained their activity during pre-incubation, β -hydroxybutyric dehydrogenase was to some extent inactivated. The results of experiments on the reactivity of released nucleotides (Birt & Bartley, 1960a) and the rapid decline in the amount of reduced coenzyme described in Table 3 support this inference.

Effect of 2:4-dinitrophenol on the pyridine nucleotides of mitochondria

It has been suggested that the mechanism by which phosphorylation exerts a control on the respiration of mitochondria involves reactions of the pyridine nucleotides (see Chance & Williams, 1956). Preliminary experiments showed that in the presence of 2:4-dinitrophenol, pyruvate was less effective in maintaining the level of reduced pyridine nucleotides than was succinate. This was further tested with three different concentrations of dinitrophenol in the presence of succinate or pyruvate. At all concentrations dinitrophenol diminished the formation of reduced pyridine nucleotide on addition of substrate, but this effect was much greater with pyruvate than with succinate, especially at 10 μ M-dinitrophenol. With this concentration (Table 5) when succinate was present the pyridine nucleotides were 95 % reduced after incubation for 15 min. and 72 % after 45 min. With pyruvate the comparable figures were 88 and 7.5 % respectively. Even with

Table 4. *Reduction by β -hydroxybutyrate, succinate and pyruvate plus malate of the pyridine nucleotides of mitochondria pre-incubated without added substrate*

All values for pyridine nucleotide levels are given as μ m-moles/mg. dry wt. Temp. of incubation, 25°; concn. of β -hydroxybutyrate, succinate, pyruvate and malate each 10 mm. The mitochondria were pre-incubated in 'saline' medium alone for 30 min. at 25°, and the suspension was sampled before the addition of the substrates. Final samples for analysis were taken after a further incubation for 5 min. at 25°. See also protocol to Table 1.

	No substrate (30 min.)	β -Hydroxy- butyrate (35 min.)	Succinate (35 min.)	Pyruvate plus malate (35 min.)
DPN ⁺	1.52	1.28	0.48	0.70
DPNH	0.76	1.04	1.76	1.56
DPN	2.28	2.32	2.24	2.26
$\frac{\text{DPNH}}{\text{DPN}^+}$	0.50	0.81	3.6	2.2
TPN ⁺	0.53	0.42	0.26	0.10
TPNH	0.97	1.23	1.44	1.48
TPN	1.50	1.65	1.70	1.58
$\frac{\text{TPNH}}{\text{TPN}^+}$	1.8	2.9	5.5	14.8

mm-dinitrophenol the pyridine nucleotides remained about 30 % reduced throughout the incubation in the presence of succinate, whereas with pyruvate only 11.5 % of the nucleotides was in the reduced form after 15 min. and 5 % after 45 min. The increases in the respiration rates after 15 min. expressed as a percentage increase over the rate for mitochondria incubated without substrate (Table 5) produced by 1 mm-, 0.1 mm- and 10 μ M-dinitrophenol were, for pyruvate, 2, 70 and 125 % respectively; for succinate, the corresponding values were 180, 260 and 250 %. All these rates decreased after 45 min. Throughout the incubation the maximum stimulation of respiration with

pyruvate was produced by 10 μ M-dinitrophenol, and with succinate by 0.1 mm-dinitrophenol.

In this experiment with succinate there was a 38-fold difference in the ratios of reduced to oxidized pyridine nucleotides but only a 25 % difference in the respiration rate (compare values with succinate + mm- and 10 μ M-dinitrophenol); a sevenfold difference in this ratio occurred without difference in the respiration rate (compare values with 0.1 mm- and 10 μ M-dinitrophenol).

Further experiments with both substrates and different concentrations of dinitrophenol confirmed the general observations made above though the magnitude of some of the effects differed (Tables 6

Table 5. *Effect of different concentrations of 2:4-dinitrophenol and succinate or pyruvate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μ m-moles/mg. dry wt.; for O₂ uptake as μ l. of O₂/mg. dry wt./hr. Temp. of incubation, 25°; concn. of pyruvate and succinate, 10 mm. The initial mitochondrial suspension (0°, 0 min.) contained PN⁺, 1.67; PNH, 3.20; PN, 4.87; PNH/PN⁺, 1.9. 'Percentage loss' is the percentage decrease on the total amount of coenzyme below the initial total: 4.87 μ m-moles/mg. dry wt. Without added substrate, the rates of O₂ consumption at 15 min. and 45 min. were 9.8 μ l./mg. dry wt./hr. and 4.8 μ l./mg. dry wt./hr. respectively. The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

Substrate	Concn. of dinitrophenol (mm)	15 min.						45 min.					
		PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O ₂	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O ₂
Pyruvate	1	3.70	0.49	4.19	0.13	14	10.0	3.80	0.20	4.00	0.05	18	5.1
	0.1	2.80	1.25	4.05	0.45	17	16.4	3.20	0.20	3.40	0.06	29	5.4
	0.01	0.90	3.50	4.40	3.9	10	22.0	3.75	0.30	4.05	0.08	17	9.9
Succinate	1	3.05	1.45	4.50	0.48	8	27.6	3.20	1.20	4.40	0.38	9	18.4
	0.1	1.25	3.15	4.40	2.5	10	35.2	1.65	3.00	4.65	1.8	5	26.2
	0.01	0.25	4.55	4.80	18.0	0	34.5	1.30	3.30	4.60	2.5	6	18.5

Table 6. *Effect of 2:4-dinitrophenol and succinate or pyruvate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μ m-moles/mg. dry wt.; for O₂ uptake, as μ l. of O₂/mg. dry wt./hr. Temp. of incubation, 25°; concn. of succinate and pyruvate, 10 mm. 'Percentage loss' is the percentage decrease in the total amount of coenzyme below the highest initial total: 4.90 μ m-moles/mg. dry wt. The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

Time (min.)	Succinate										
	No addition						With dinitrophenol (0.1 mm)				
	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O ₂	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)
0	1.25	3.60	4.85	2.9	—	—	2.35	2.10	4.45	0.90	—
15	0.63	3.90	4.53	6.2	6	14.6	2.90	1.90	4.80	0.66	0
60	1.50	2.50	4.00	1.7	17	9.6	2.85	1.00	3.85	0.35	14
Time (min.)	Pyruvate										
	No addition						With dinitrophenol (0.1 mm)				
	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)	Uptake of O ₂	PN ⁺	PNH	PN	PNH/PN ⁺	Loss (%)
0	1.70	2.95	4.65	1.7	—	—	2.35	2.55	4.90	1.1	—
15	1.25	3.55	4.80	2.8	0	8.0	3.50	0.52	4.02	0.15	18
60	1.40	2.55	3.95	1.8	14	9.0	2.90	0.23	3.13	0.08	36

Table 7. *Effect of 2:4-dinitrophenol and succinate or pyruvate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$; for O_2 uptake as $\mu\text{l. of O}_2/\text{mg. dry wt./hr.}$ Temp. of incubation, 25° ; concn. of succinate and pyruvate, 10 mM. 'Percentage loss' is the percentage decrease in the total amount of coenzyme below the highest initial total: $4.15 \mu\text{m-moles/mg. dry wt.}$ The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

Succinate												
Time (min.)	No addition						With dinitrophenol (10 μM)					
	PN+	PNH	PN	PNH PN+	Loss (%)	Uptake of O ₂	PN+	PNH	PN	PNH PN+	Loss (%)	Uptake of O ₂
0	0.81	3.30	4.11	4.1	—	—	1.05	3.10	4.15	3.0	—	—
15	0.78	3.05	3.53	3.9	7	15.6	1.10	2.90	4.00	2.6	3	21.2
60	0.96	2.00	2.96	2.1	28	5.5	1.10	1.85	2.95	1.7	28	13.0
Pyruvate												
Time (min.)	No addition						With dinitrophenol (10 μM)					
	PN+	PNH	PN	PNH PN+	Loss (%)	Uptake of O ₂	PN+	PNH	PN	PNH PN+	Loss (%)	Uptake of O ₂
0	1.45	2.35	3.80	1.6	—	—	1.65	2.10	3.75	1.3	—	—
15	0.89	2.80	3.69	3.2	2	9.6	0.69	2.90	3.60	4.2	4	14.4
60	1.05	2.15	3.20	2.1	19	5.1	1.25	1.95	3.20	1.5	15	12.0

Table 8. *Effect of 2:4-dinitrophenol, pyruvate, malate and pyruvate plus malate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$; for O_2 uptake as $\mu\text{l. of O}_2/\text{mg. dry wt./hr.}$ Temp. of incubation, 25° . The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

Substrate added	Concn. of dinitrophenol (mm)	15 min.					45 min.				
		PN ⁺	PNH	PN	PNH PN ⁺	Uptake of O ₂	PN ⁺	PNH	PN	PNH PN ⁺	Uptake of O ₂
Nil	Nil	—	—	—	—	6.0	2.2	2.1	4.3	1.0	2.8
Pyruvate (10 mm)	Nil	2.1	1.9	4.0	0.9	10.2	1.6	2.6	4.2	1.6	12.0
Pyruvate (10 mm)	0.1	3.7	0.15	3.85	0.04	19.3	3.7	0.18	3.88	0.05	9.0
Malate (10 mm)	Nil	1.2	3.5	4.7	2.9	6.6	1.3	3.1	4.4	2.4	2.4
Malate (10 mm)	0.1	1.2	3.6	4.8	3.0	22.6	1.7	2.3	4.0	1.4	22.2
Pyruvate (10 mm) plus malate (1 mm)	0.1	1.8	2.9	4.7	1.6	13.8	3.2	0.25	3.45	0.08	9.6
Pyruvate (10 mm) plus malate (10 mm)	0.1	0.6	4.2	4.8	7.0	22.6	1.6	2.4	4.0	1.5	20.4

and 7). Dinitrophenol (10 μM) decreased slightly the proportion of reduced coenzyme produced by succinate and pyruvate. With dinitrophenol (0.1 mM) succinate was less effective in reducing the pyridine nucleotides than in the experiment already described (Table 5), though it produced a greater amount of reduced coenzyme than did pyruvate under the same conditions (Tables 6 and 7). Both concentrations of dinitrophenol increased the respiration rate with succinate; but only the lower concentration had this effect with pyruvate, dinitrophenol (0.1 mM) actually decreasing the initial rate.

In summary, although the response of pyridine nucleotides to different concentrations of dinitrophenol and succinate or pyruvate is somewhat variable, it was apparent that:

(i) The reduction of the coenzymes was more

readily inhibited by dinitrophenol (1 mM–10 μM) in the presence of pyruvate than in the presence of succinate. Respiration with succinate was always stimulated by both 0.1 mM- and 10 μM -dinitrophenol, but with pyruvate, concentrations of 0.1 mM and greater may depress respiration.

(ii) It was possible to obtain high values (2.5–18) for the ratio reduced/oxidized coenzyme with succinate plus 0.1 mM- and 10 μM -dinitrophenol.

Effect of dinitrophenol with pyruvate plus malate. As pyruvate plus malate produce high ratios of reduced to oxidized pyridine nucleotides (see Table 3), the effect of dinitrophenol in combination with these two substrates was tested. Table 8 shows that:

(i) Dinitrophenol (0.1 mM) had a relatively small effect on the reduction of pyridine nucleotides by malate after either 15 or 45 min. (compare Table 6).

(ii) When malate (1 mM) was added with pyruvate (10 mM) and dinitrophenol, the level of reduced nucleotide was maintained only during 15 min. of incubation; whereas with 10 mM-malate and -pyruvate the amount of reduced nucleotide was almost identical, even after 45 min., with that produced by pyruvate without dinitrophenol. These results suggested that a decreased availability of C₄ acids was responsible, at least in part, for the effect of dinitrophenol on the reduction of the coenzymes by added pyruvate (see Discussion).

(iii) The rate of respiration with malate was less than with pyruvate (see also Table 3) but the stimulation of this rate by dinitrophenol (0.1 mM) was greater, especially after longer times (45 min.) of incubation. The stimulated rate was not increased when pyruvate was present also.

(iv) Thus in the absence of dinitrophenol, malate was much less effective than malate plus pyruvate in stimulating respiration and reducing pyridine nucleotides, but there was a close similarity, both in respiration rate and changes in pyridine nucleotides, between the behaviour of mitochondria incubated in malate with DNP and malate plus pyruvate with DNP.

Detailed analysis of pyridine nucleotides in the

presence of dinitrophenol. The decrease in the observed levels of reduced pyridine nucleotides in the presence of dinitrophenol was due to a diminution in the amounts of both DPNH and TPNH. After incubation for 15 min. without added substrate, the ratio of reduced to oxidized nucleotide had declined from 0.9 to 0.04; TPNH disappeared more rapidly than DPNH. With pyruvate and dinitrophenol there was an increase after incubation for 15 min. in the amount of DPNH only; succinate increased both that of DPNH and TPNH slightly.

Effect of 5-ethyl-5-isocamylbarbiturate (amytal) on the pyridine nucleotides of mitochondria

The respiratory inhibitor amytal (Ernster, Jalling, Löw & Lindberg, 1955) enhanced the reduction of pyridine nucleotide occurring after incubation for 15 min. without substrate or with pyruvate, but not that with succinate (Table 9). Longer incubations showed that without added substrate the effect of amytal was to maintain the concentration of reduced pyridine nucleotides during the early part of an incubation, but to accelerate their oxidation later; sometimes the maintenance occurred only after an initial lag.

Table 9. *Effect of amytal without added substrate, and with pyruvate or succinate, on the mitochondrial pyridine nucleotides*

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

	No substrate			Pyruvate		Succinate	
	Zero time (no amytal)	15 min. (no amytal)	15 min. (amytal)	15 min. (no amytal)	15 min. (amytal)	15 min. (no amytal)	15 min. (amytal)
PN+	1.40	2.50	1.30	2.70	0.75	0.30	0.46
PNH	3.25	1.50	2.90	1.65	3.40	3.85	3.95
PN	4.65	4.00	4.20	4.35	4.15	4.15	4.41
PNH							
PN+	2.3	0.6	2.2	0.6	4.5	12.8	8.6
Loss (%)	—	14	9	6	11	11	5

Table 10. *Effect of amytal without added substrate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as $\mu\text{m-moles/mg. dry wt.}$; temp. of incubation, 25°; concn. of amytal, 1 mM. See also protocol to Table 1.

	Zero time				15 min.				30 min.			
	PN+	PNH	PN	PNH PN+	PN+	PNH	PN	PNH PN+	PN+	PNH	PN	PNH PN+
Without amytal	2.20	2.40	4.60	1.1	1.55	2.60	4.15	1.7	2.30	1.20	3.50	0.52
With amytal	—	—	—	—	1.50	2.40	3.90	1.6	1.60	2.40	4.00	1.50
	45 min.				60 min.							
	PN+	PNH	PN	PNH PN+	PN+	PNH	PN	PNH PN+				
Without amytal	2.30	0.83	3.13	0.36	2.35	0.90	3.25	0.38				
With amytal	2.00	1.60	3.60	0.80	3.05	0.41	3.46	0.13				

Thus in the experiment described in Table 10, amytal was without effect after 15 min. (compare Table 9), it doubled the proportion of reduced coenzyme (relative to the control suspension at comparable times) after both 30 and 45 min. and halved it after 60 min. Pre-incubation with amytal for 15 min. at 0° did not influence the response to the inhibitor during subsequent incubation at 25°. During the pre-incubation there was no loss of pyridine nucleotide but the proportion of the total coenzyme reduced increased from 63 to 71 % in the presence of the amytal.

With added pyruvate also, the maintenance of the level of reduced pyridine nucleotide by amytal was transient. The enhanced accumulation of reduced coenzyme was most pronounced after about 5 min. of incubation. Thereafter the difference in the pyridine nucleotides of the suspensions with and without amytal diminished, until after 1 hr. the levels of reduced nucleotide were the same in both.

DISCUSSION

The experiments described illustrate how the amounts of the four pyridine nucleotides within the mitochondria vary with the experimental conditions. The proportions of the pyridine nucleotides can be affected by changes in the structure of the mitochondria or by competition between substrates for the terminal pathway of oxidation. Thus the abrupt increase in the rate of oxidation of the pyridine nucleotides with increase of temperature is probably due to a structural change since the rate of swelling also shows this abrupt change.

The effect of substrate competition is illustrated by the experiments with added succinate. It is likely that reducing equivalents donated by succinate and by DPNH will compete for common rate-limiting components of the electron-transport chain (compare Wu & Tsou, 1955); judged by the rate of oxygen uptake succinate is an extremely effective hydrogen donor and will therefore compete successfully with other substrates. High levels of reduced coenzyme will therefore be rapidly established and maintained. The effectiveness of succinate as an inhibitor of DPNH oxidation is illustrated by the rapid increase in the amounts of reduced pyridine nucleotides (usually maximal by the time of the first analysis) which occurred when this substrate was added. Chance & Hollunger (1957) explain similar observations as the reversal of oxidative phosphorylation resulting from increases in the amounts of high-energy phosphates formed during the oxidation of succinate. They report that uncoupling agents prevent this reduction, whereas in some of the experiments described in this paper (e.g. Tables 5 and 6), even after 15 min. in 0.1 mM-dinitrophenol, a concen-

tration which not only prevents the synthesis of ATP during succinate oxidation by mitochondria suspended in 'saline' medium but also decreases the pre-existing level (Klemperer, 1957), succinate can maintain a high level of reduced pyridine nucleotide. It seems unlikely that, under these conditions of incubation, the reduction of coenzyme results from a hydrogen transfer from succinate mediated by a high level of ATP. A decrease in the rate of oxidation of reduced pyridine nucleotides by respiratory-chain competition is a more likely cause of the observed increase in the proportion of reduced coenzyme. The relatively small total amount of reducing equivalents required to produce the extra reduced coenzyme formed was probably derived from a hydrogen donor with an oxidation-reduction potential favouring reduction of pyridine nucleotide (e.g. endogenous substrate or malate produced from succinate), rather than from succinate itself.

Effect of dinitrophenol and amytal on the pyridine nucleotides of mitochondria

During the course of incubation for 1 hr., dinitrophenol always shifted the dynamic steady state of the pyridine nucleotides towards oxidation. The results in Tables 5-8 revealed a distinct difference between the ability of pyruvate on the one hand and succinate or malate on the other to maintain reduced pyridine nucleotides in the presence of dinitrophenol. It is possible that the effects of dinitrophenol on pyruvate oxidation were not due solely to the uncoupling of respiration and phosphorylation, but also to a decrease in the rate at which pyruvate itself was oxidized. In the experiments where dinitrophenol (at concentrations of 1 and 0.1 mM) had the greatest influence on the reduced coenzymes, the rate of oxygen consumption with pyruvate was also decreased by the uncoupling agent (Table 6). These effects could be reversed by the addition of malate, either at 10 mM or, for a short time only, at 1 mM concentration, because the availability of oxaloacetate limited pyruvate oxidation and therefore coenzyme reduction. The inhibition of respiration which sometimes followed the addition of dinitrophenol (0.1 mM or greater) may have been due to an impaired ability of the mitochondria to synthesize C₄ intermediates from pyruvate (Utter & Kurahashi, 1954; Bartley & Avi-Dor, 1955).

No evidence was obtained for the consistent release of substantial amounts of pyridine nucleotide from an 'inhibited' form during incubation with dinitrophenol (contrast Purvis, 1958; compare Baessler & Pressman, 1959).

The results obtained with amytal confirm the observations made by Ernster *et al.* (1955) and Chance & Hollunger (1957). However, it is

apparent that the time taken for amytal to exert its maximum effect in enhancing the reduction of pyridine nucleotides is variable, and that the effect itself is transient, being followed by changes in the mitochondrial behaviour considered as 'degenerative'.

Respiration and the pyridine nucleotide metabolism of mitochondria incubated without substrate

The rate of endogenous respiration of the mitochondria in the medium is relatively low, possibly due in part to the presence of EDTA and nicotinamide (compare Ziegler & Linnane, 1958). In four experiments in which the values for DPNH (as $\mu\text{m-moles/mg. dry wt.}$) were 0.86, 0.72, 0.48 and 0.44 with total reduced pyridine nucleotides 2.8, 2.4, 1.6 and 1.9 the initial respiratory rates ($\mu\text{l. of oxygen/mg. dry wt./hr.}$) were 4.3, 3.6, 1.3 and 1.5 respectively. Thus the amounts of both DPNH and total reduced pyridine nucleotide show a parallelism with the initial rates of oxygen uptake.

There was usually sufficient endogenous substrate to maintain or increase the level of reduced pyridine nucleotides for a limited time (up to 1 hr. at 15–25°). When there was an increase in respiration on prolonged incubation (compare Expts. 4 and 5, 6 and 7, Table 2; Expt. 6, Table 3) the pyridine nucleotides were almost completely oxidized. This relative independence of the amounts of reduced coenzyme and respiration rate suggests that the substrates being oxidized are not capable of reducing the pyridine nucleotides. It is possible that these changes are reflexions of mitochondrial 'degeneration' (Birt & Bartley, 1960a; Sacktor, 1954; compare also the increased ease of penetration of similar mitochondria by DPNH, Birt & Bartley, 1960b). The 'degenerative' changes were prevented by substrate and accelerated by amytal.

Effect of added substrate on the rate of respiration and reduction of pyridine nucleotides

The stimulation of the initial oxygen uptake that occurred on addition of substrate was directly related to the total measured level of reduced pyridine nucleotide (Table 3), and the relative magnitudes of the increases in the respiration rate with different substrates (with the exception of β -hydroxybutyrate) were the same as those reported by Ziegler & Linnane (1958) and D. Bellamy (unpublished work) for mitochondria in sucrose. The differences in the rates of net reduction of the coenzymes by different substrates could be due to an interaction of the pyridine nucleotides and enzymes released into the supernatant fluid, differences in the permeability of the mitochondria to different substrates, or differences in the activity of various dehydrogenases. Although certain enzymes are readily liberated from mitochondria in

a form in which they can react with coenzymes and added substrates (Birt & Bartley, 1960a), the rapid and considerable reduction of pyridine nucleotide with succinate and β -hydroxybutyrate (both dehydrogenated by enzymes that are firmly bound to the mitochondria) and the slow reduction by malate (whose dehydrogenase can be made soluble) make it unlikely that much of this initial reduction occurs in the supernatant fluid. Ziegler & Linnane (1958), using the oxygen uptake of the mitochondria as a criterion for the ingress of a substrate, claim that undamaged particles in 0.5M-sucrose are impermeable to citrate and malate, but permeable to succinate, α -oxoglutarate, β -hydroxybutyrate, glutamate and pyruvate plus malate. The data of Table 3 can be reconciled with these observations, and it is possible that if the differences in the permeability of the mitochondria are indeed responsible for the observed differences in oxygen uptake the differences in the speed with which the coenzymes respond to the added substrate may also be attributed to this cause. However, Amoore (1958) has shown that mitochondria in 0.25M-sucrose at 0° have equal permeability to pyruvate, succinate and citrate. It would seem that a direct comparison of the penetration of different substrates into mitochondria at 25° is necessary for the resolution of this point. The slow reduction by pyruvate is greatly accelerated by the addition of malate, which is itself slowly dehydrogenated and is presumably without effect on the permeability to pyruvate. It is possible therefore that at least some of the variation in the rates of reduction is due to the different activities of the individual dehydrogenases under the conditions of incubation.

The oxidation of reduced coenzyme is unaffected by choline, which is oxidized by a flavoprotein (Rendina & Singer, 1958). Phosphorylation may occur during this oxidation (Rothschild, Cori & Barron, 1954), which therefore appears to be linked in some way to the electron-transport chain, though not in the same way as is succinate.

It has been shown that when there was a net reduction of pyridine nucleotides, the level of DPNH increased more rapidly than that of TPNH (see Table 1). In these experiments it was difficult to observe well-defined increases in the amount of TPNH, because very little of the triphosphopyridine nucleotide was present initially as TPN^+ . However, experiments in which the level of TPNH was decreased by pre-incubation at 25° before the addition of substrate showed that succinate and pyruvate plus malate rapidly reduced both DPN and TPN; β -hydroxybutyrate was much less efficient in reducing the coenzymes, but it did increase the level of DPNH and, very slightly, that of TPNH. In all of these experiments the amount of DPNH produced was greater than that of TPNH.

A study of the time course of coenzyme reduction showed that when there was a net reduction of pyridine nucleotides the level of DPNH increased more rapidly than TPNH. Conversely, when the level of reduced coenzyme decreased, TPNH was oxidized faster than DPNH. As the four forms of the pyridine nucleotides are in a state of dynamic equilibrium involving reversible transhydrogenation (Stein, Kaplan & Ciotti, 1959), the following reactions may occur:

Our experiments indicate that reactions (1) and (2) proceed when dehydrogenation is rapid relative to the rate of electron transport from DPNH to oxygen; reaction (3) occurs when dehydrogenation is relatively slow. Thus variations in the level of DPNH are minimized (see Table 1, and Birt & Bartley, 1960*a*), whether there is an excess or deficiency of substrate and TPNH acts as a reserve of reducing power, which is supplemented when the oxidation of substrates exceeds the capacity of the electron-transport chain and is depleted when there is little substrate. Since TPNH and TPN^+ readily pass through the mitochondrial membrane the function of the TPN^+ -linked isocitric dehydrogenase may be to maintain TPNH despite a constant leakage; conversely TPNH made extramitochondrially may under some circumstances supplement the mitochondrial reserve.

Despite this interchange of the four forms of the pyridine nucleotides it seems that they do not have free access to the transhydrogenase within the particles since the quotient $[(\text{TPNH} \times \text{DPN}^+)/(\text{TPN}^+ \times \text{DPNH})]$ is much greater than 1 (e.g. 6.7 from the results in Table 1; compare 0.7 in the presence of transhydrogenase isolated from ox heart by Kaplan, Colowick & Neufeld, 1953). The binding of different 'pools' of coenzyme by the dehydrogenases and the respiratory chain, and a pool of 'free' nucleotide (possibly containing more of the triphosphopyridine nucleotide) would be sufficient to account for this compartmentation.

As the activity of the transhydrogenase of rat-liver mitochondria is sufficient to allow rapid and reversible transfers of hydrogen between DPNH and TPNH (Stein *et al.* 1959), it is apparent that no conclusions about the oxidation and reduction of particular intramitochondrial coenzymes can be drawn without assays for all four pyridine nucleotides.

SUMMARY

1. A study has been made of the effect of temperature, added substrate and metabolic inhibitors on the amounts of oxidized and reduced di- and tri-phosphopyridine nucleotides in rat-liver mitochondria suspended in a 'saline' medium. The particles of the 'fluffy layer' behaved essentially in the same way as the mitochondria.

2. At and below 10°, the levels of reduced pyridine nucleotides increased slowly; reduced diphosphopyridine nucleotide accumulated more rapidly than reduced triphosphopyridine nucleotide during incubation with added substrate. Net oxidation (sometimes after an initial reduction) of coenzyme was detected only when the temperature was 15° and above, the rate of disappearance of reduced triphosphopyridine nucleotide being greater than that of reduced diphosphopyridine nucleotide. In more concentrated (twofold) suspensions of mitochondria the initial reduction was more pronounced.

3. Without substrate, the Q_{O_2} of the preparations (measured after 15 min. at 25°) was usually about 3 and the respiration rate declined during further incubation for 1 hr.

4. Added α -oxoglutarate, citrate, pyruvate plus malate, pyruvate, β -hydroxybutyrate and malate increased both the levels of reduced diphosphopyridine nucleotide and reduced triphosphopyridine nucleotide (maximum increase in ratio of reduced/oxidized coenzymes from 1.55 to 4.80, with added α -oxoglutarate; minimum increase from 1.55 to 1.80 with added malate) and the rates of oxygen uptake (maximum increase 8.5-fold, with α -oxoglutarate; minimum increase threefold, with malate), above the values found with freshly isolated mitochondria. All the substrates except citrate, pyruvate and malate reduced the nucleotides appreciably in less than 2 min.

5. Succinate produced the highest amounts of reduced coenzyme observed in suspensions of mitochondria, altering the levels in less than 30 sec. The increase in the ratio of reduced/oxidized coenzyme was about fourfold.

6. If the reduced nucleotides of isolated mitochondria were partially oxidized by pre-incubation for about 30 min. at 25° without added substrate, re-reduction of diphosphopyridine nucleotide (and, more slowly, triphosphopyridine nucleotide) could be demonstrated after the addition of pyruvate plus malate, β -hydroxybutyrate and succinate.

7. The reduction of mitochondrial pyridine nucleotides in the presence of pyruvate or without added substrate was less when dinitrophenol was added at concentrations of 10 μM or greater. Dinitrophenol (0.1 mM) was less effective in blocking reduction if malate was added with the pyruvate.

8. The reduction of coenzymes by malate and succinate was much less sensitive to dinitrophenol than that by pyruvate; relatively high ratios (2.5–18) of reduced to oxidized coenzyme could be obtained with concentrations of dinitrophenol of 10 μ M and 0.1 mM.

9. The maintenance of reduced coenzyme by endogenous substrate and pyruvate was enhanced for a short time by amytal, the effect usually being maximal after about 30 min. of incubation. Subsequently there was a rapid decline in the amount of reduced coenzyme.

10. On the basis of the results obtained, it is proposed that the function of mitochondrial reduced triphosphopyridine nucleotide is that of a reserve of reducing power, which is supplemented when the oxidation of substrate exceeds the capacity of the electron-transport chain and which is utilized in the preservation of the level of reduced diphosphopyridine nucleotide when there is little oxidizable substrate. The significance of the data obtained with dinitrophenol for the formulation of the mechanism of the reduction of mitochondrial coenzymes by succinate and the evidence obtained for 'compartmentation' of these coenzymes is also discussed.

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Exchange of Potassium Ions across a Concentration Difference by Isolated Rat-Liver Mitochondria

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Several experiments have been described which suggest that isolated mitochondria are capable of active transport (Spector, 1953; Macfarlane & Spencer, 1953; Stanbury & Mudge, 1953; Bartley & Davies, 1954; Mudge, 1955; Price, Fonnesu & Davies, 1956; Gamble, 1957; Bartley & Amoore, 1958). Nevertheless, it is difficult to prove that a given tissue can perform active transport. Rosenberg (1954) emphasized the necessity of showing that transport of solute from lower to higher electrochemical potential had occurred. The

electrochemical potential should include terms representing both the chemical-concentration difference, and the electrical-potential difference, existing across the membrane (Ussing, 1949). Nothing seems to be known about the potential across the mitochondrial membrane, so the present argument is limited to consideration of the chemical-concentration differences. Even within these restricted terms of reference, however, the above-mentioned experiments are not entirely convincing, because of the following considerations. Spector (1953), Stanbury & Mudge (1953), Mudge (1955) and Gamble (1957) measured move-

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