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Flight-Muscle Mitochondria of *Lucilia cuprina* and *Musca domestica*

ESTIMATION OF THE PYRIDINE NUCLEOTIDE CONTENT AND OF THE RESPONSE OF RESPIRATION TO ADENOSINE DIPHOSPHATE

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The ability of many species of flying insects to increase their consumption of oxygen during flight and the factors controlling respiration during rest and activity have been the subject of much research (for a recent summary, see Sacktor, 1958; Bucher, Klingenberg & Zebe, 1958). The production of adenosine triphosphate by oxidative phosphorylation in the flight-muscle mitochondria is a most important aspect of energy transformation in such animals (Sacktor, 1954; Slater & Lewis, 1954), as in many other organisms. However, Sacktor and co-workers have described certain unusual

features of both the control of respiration with α -glycerophosphate, which is oxidized most readily by mitochondria from insect muscles (e.g. Chance & Sacktor, 1958), and in the composition of the electron-transport chain of particles isolated from thoraces of the fly *Musca domestica* L. With preparations from mature flies (3–7 days old) they found that the oxidation of α -glycerophosphate was independent of a supply of adenosine diphosphate (Sacktor, 1958; Sacktor & Cochran, 1958; Estabrook & Sacktor, 1958) and that pyridine nucleotide coenzymes were not detectable by the

spectrophotometric method used (Chance & Sacktor, 1958; see also Chance & Williams, 1955). Bucher *et al.* (1958) have queried the physiological significance of these findings; their preparations of mitochondria from the locust (*Locusta migratoria*) oxidized α -glycerophosphate more rapidly when adenosine diphosphate was present and contained a relatively high concentration of diphosphopyridine nucleotide (Klingenberg & Bucher, 1959), as did mitochondria isolated from the blowfly *Calliphora* (Price & Lewis, 1959).

This paper describes an investigation of both the concentration and reactivity of the pyridine nucleotides of flight-muscle mitochondria of *Musca* and the conditions in which the oxidation of α -glycerophosphate is controlled by adenosine diphosphate.

MATERIALS AND METHODS

Diphosphopyridine nucleotides, ADP, ATP (as sodium salts) and hexokinase (160 000 K.M. units/g.; Kunitz & McDonald, 1946) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other reagents used were of analytical grade.

Flies. Breeding of *Lucilia cuprina* Wied. was carried out by the method of L. Barton-Browne (unpublished work); adults were allowed to feed freely on sucrose and water and were kept at 25°. They were collected for preparation of mitochondria after inactivation at 5° for 5 min. Adults of *Musca domestica* L. were obtained from a single batch of pupae and treated in the same fashion.

Preparation of mitochondria. This was performed at 0–5°. Heads and abdomens were removed from the flies (mixed sexes) and thoraces were collected in a mortar containing an ice-cold suspension medium of 0.3 M-sucrose and 2 mM-sodium ethylenediaminetetra-acetate (EDTA). The tissue was crushed gently for about 2 min. with a pestle, the brei squeezed through four layers of muslin and the resulting suspension centrifuged for 3 min. at 150g. Mitochondria were sedimented from the supernatant fluid at 5000g for 10 min. Particles used in the experiments on respiratory control were washed twice, by resuspending in and centrifuging from the suspension medium, then made to the required volume with this medium; those required for pyridine nucleotide analyses were washed similarly in a saline medium (KCl, 0.173 M; EDTA, 2 mM). In one experiment, nicotinamide (50 mM) was added to this medium (see text). Microscopic examination of these preparations showed little contamination by either cytoplasmic debris or nuclei.

Measurement of respiration. The conventional Warburg technique was used with air as the gas phase. The final incubation medium (total volume, 2.5 ml., pH 7.2) contained 5 mM-MgCl₂, 5 mM-potassium phosphate buffer (pH 7.2), 1 mM-EDTA, 0.3 M-sucrose and the appropriate concentration of mitochondria; ADP (10 mM) or KCl (20 mM), together with the substrates (at pH 7.2), were added from the side arm after 10 min. preincubation at 25° unless otherwise noted in the Tables. In all the experiments the centre well contained 0.15 ml. of NaOH (2N) and filter paper.

q_{O_2} values are expressed as μ l. of O₂ consumed/mg. of protein/hr. The term 'respiratory control' has been used to imply that maximum rates of O₂ uptake with particular substrates require the presence of ADP. (For discussion of the use of this term, see Chance & Baltscheffsky, 1958.) The 'respiratory control ratio' is the quotient:

rate of O₂ uptake with ADP/rate of O₂ uptake without ADP.

Estimation of pyridine nucleotides. These were estimated by the fluorimetric method of Bassham, Birt, Hems & Loening (1959); with all preparations it was necessary to use the enzymic method for producing the 'blanks' (see Bassham *et al.* 1959). Pyridine nucleotide retention by the mitochondria in an incubated suspension was determined by the method of Birt & Bartley (1960a).

The following abbreviations have been used: DPN⁺, DPNH and DPN, oxidized, reduced and total diphosphopyridine nucleotides respectively; TPN⁺, TPNH and TPN, oxidized, reduced and total triphosphopyridine nucleotides respectively; PN⁺, PNH and PN, DPN⁺ plus TPN⁺, DPNH plus TPNH, DPN plus TPN respectively.

Estimation of protein. Protein was estimated by the method of Cleland & Slater (1953).

RESULTS

Pyridine nucleotide content of mitochondria isolated from the thoraces of Lucilia cuprina and Musca domestica. The amounts of the coenzymes found in isolated flight-muscle mitochondria are given in Table 1. The detection of TPN⁺ and TPNH was difficult, but as each experiment indicated the presence of at least one form of this nucleotide, the existence of very low concentrations of the co-enzyme in these mitochondria was established with reasonable certainty.

Nicotinamide increased the total recovery of the coenzymes slightly, by preserving DPN⁺ (Table 1). Because of the complications introduced by its addition in metabolic experiments (Birt & Bartley, 1960a), it was omitted from routine preparations.

Table 1. *Pyridine nucleotide content of fly-muscle mitochondria*

(a) Mitochondria from *Musca domestica*; values are the means of six estimations with different preparations. The range of values is given in parentheses. (b) Single estimation of *Musca* mitochondria prepared in a medium containing nicotinamide (50 mM). (c) Mitochondria from *Lucilia cuprina*; mean and range of three estimations. Flies used in all these experiments were 4–18 days old.

	Coenzyme content (μ m-moles/mg. of protein)		
	(a)	(b)	(c)
DPN ⁺	2.15 (1.7–2.4)	2.52	3.0 (1.5–3.8)
DPNH	0.20 (0.0–0.4)	0.15	0.05 (0.0–0.17)
DPN	2.35	2.67	3.05
TPN ⁺	0.03 (0.0–0.05)	0.01	0.11 (0.07–0.20)
TPNH	0.13 (0.0–0.20)	0.10	0.10 (0.0–0.20)
TPN	0.16	0.11	0.21

Table 2. *Effect of added substrates on the pyridine nucleotide content and respiration of Musca and Lucilia mitochondria*

Expts. 1-3 were with *Musca* mitochondria; Expts. 4 and 5 were with *Lucilia* mitochondria. Concn. of all substrates, except pyruvate (10 mM): 50 mM. Suspensions were incubated for 10 min. at 25° in the Warburg apparatus with the substrates shown. Flies used were 3-20 days old.

Expt. no.	Conditions of incubation	Coenzyme content (μ m-moles/mg. of protein)				q_{O_2} *
		DPN+ + TPN+	DPNH + TPNH	DPN + TPN	DPNH + TPNH DPN+ + TPN+	
1	No substrate; 0°; 0 min.	1.85	0	1.85	0	—
	Succinate	0.64	0.78	1.42	1.2	27
	α -Glycerophosphate	1.4	0	1.4	0	53
	Pyruvate plus malate	1.3	0.28	1.58	0.2	23
2	No substrate; 0°; 0 min.	1.40	0.04	1.44	0.0	—
	Succinate	0.90	0.34	1.24	0.37	73
	Succinate plus malate	0.95	0.20	1.15	0.21	7
	Glutamate plus malate	1.50	0	1.54	0	7
3	No substrate; 0°; 0 min.	2.40	0.7	3.17	0.3	—
	Succinate	1.38	0.8	2.18	0.6	34
	α -Glycerophosphate	0.90	0.97	1.87	1.1	50
4	No substrate; 0°; 0 min.	2.50	0.15	2.65	0.6	—
	Succinate	0.95	1.25	2.15	1.3	69
	α -Glycerophosphate	1.80	0.38	2.18	0.2	101
	Pyruvate plus malate	1.0	1.15	2.15	1.2	40
5	No substrate; 0°; 0 min.	2.85	0.20	3.05	0.07	—
	Succinate	1.90	1.10	3.0	0.6	30
	Succinate plus ATP	1.75	1.15	2.9	0.7	31
	α -Glycerophosphate	2.00	1.00	3.0	0.5	91
	α -Glycerophosphate plus ATP	1.85	1.05	2.9	0.6	93

* q_{O_2} values for oxidation of α -glycerophosphate are all low as the flasks contained 10-15 mg. of protein in a final volume of 2.5 ml.; the O_2 uptakes quoted for this series of experiments were measured with particles incubated in the saline medium (see text).

Table 3. *Retention of pyridine nucleotides by mitochondria during incubation*

For experimental details see Materials and Methods section.

Nucleotide estimated in	Coenzyme content (μ m-mole/mg. of protein)								
	0°; 0 min.			No substrate; 25°; 15 min.			Succinate; 25°; 15 min.		
	PN+	PNH	PN	PN+	PNH	PN	PN+	PNH	PN
(a) Whole suspension	1.9	0.05	1.95	1.65	0	1.65	0.65	0.85	1.50
(b) Mitochondrial pellet	1.65	0	1.65	1.45	0	1.45	1.40	0	1.40
Percentage of total nucleotide in pellet	85			88			94		

Reactivity of the pyridine nucleotides of flight-muscle mitochondria. Brief incubation (10 min.) of *Musca* mitochondria with succinate or pyruvate plus malate always increased the ratio DPNH + TPNH/DPN+ + TPN+; glutamate alone, glutamate plus malate and malate plus succinate were not as effective. α -Glycerophosphate sometimes increased the ratio (two experiments in five) but only once was the reduction of the coenzymes greater than with succinate (Table 2).

The losses of pyridine nucleotide during the incubations were usually small (approx. 20%); the greatest loss was 44%, with α -glycerophosphate as substrate.

Similar results (Table 2) were obtained with mitochondria isolated from *Lucilia* thoraces; reduction of the coenzymes was achieved most readily with succinate, less completely with pyruvate plus malate or α -glycerophosphate.

By resedimenting the mitochondria after incubation for 15 min. either without substrate or with succinate and then determining the coenzyme content of the whole suspension and of the particles isolated from it, it was shown (Table 3) that over 85% of the pyridine nucleotides was retained by the mitochondria during this period of incubation in the saline medium, whether or not an oxidizable substrate was present (cf. Kaufman & Kaplan, 1960).

The rapid oxidation of the reduced nucleotides during centrifuging of the mitochondria (see Table 3) has been noted also with rat-liver mitochondria (Birt & Bartley, 1960b).

Effect of adenosine diphosphate on the oxidation of various substrates by flight-muscle mitochondria. The oxidation of glutamate and pyruvate plus malate was always increased by the addition of ADP, even when the preparations were made from flies up to 25 days old (Tables 4 and 10). The stimulation was not markedly affected by the concentration of mitochondria in the flask; thus in one experiment the respiratory-control ratios obtained initially with pyruvate plus malate and 1, 3 and 10 mg. of protein/flask were 1.8, 2.1 and 2.5 respectively; similar values were found with glutamate. However, the enhanced oxidation of pyruvate plus malate with ADP persisted for relatively longer periods of incubation at the lower concentrations of mitochondria (Table 4).

On the other hand, no stimulation by ADP of succinate oxidation occurred in any experiment;

frequently, there was an inhibition of O_2 uptake, sometimes as great as 34 % (Table 5).

The effect of ADP on the oxidation of α -glycerophosphate was more complex and depended on (a) the concentration of mitochondria and (b) the age of the flies.

(a) Four direct comparisons of the effects of ADP made with suspensions containing different amounts of the same mitochondrial preparation showed no clear evidence of respiratory control when the amount of protein/flask was between 2 and 5 mg.; in the corresponding systems with 0.2–1.6 mg. of protein/flask, ADP increased the O_2 consumption by at least 70 % (Table 6, Expts. 1–4; see also Tables 4 and 5). Respiratory control and the more pronounced requirement for ADP in relatively dilute suspensions were also demonstrated when an ADP-regenerating system was used (Table 7). The stimulation of O_2 uptake was greatest after about 15 min., presumably because hydrolysis of added ATP during the preincubation provided some ADP initially. Because of the

Table 4. *Effect of concentration of mitochondrial suspension on rates of oxygen uptake*

(a) No ADP; (b) ADP, 10 mM. Incubations were at 25° in the Warburg apparatus; all substrates (50 mM) and ADP were added from the side arm after 10 min.

Concn. of mitochondrial protein (mg./flask)	Age of flies (days)	Time of incubation (min.)	Uptake of O_2 (μ l./mg. of protein/hr.)							
			Pyruvate plus malate		Glutamate		Succinate		α -Glycerophosphate	
			(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
10	4	0–5	35	73	2.6	7.2	67	70	—*	—
		15–20	15	8	4.0	9.4	75	67	0	0
3	6	0–5	22	56	1.2	6.8	84	81	120	125
		15–20	18	34	2.2	7.4	72	62	79	78
1.1	4	0–5	—	—	—	—	30	30	90	312
		10–20	25	45	1	5	35	33	215	428
0.6	4	0–5	} Not estimated {				52	50	210	435
		50–60					45	45	200	135

* Uptake of O_2 not measurable with this concentration of protein/flask; see text.

Table 5. *Effect of concentration of mitochondrial suspension in demonstration of respiratory control*

(a) and (c) No ADP; (b) and (d) ADP, 10 mM. Both substrates were initially 50 mM and, with the ADP, were added to the appropriate vessels at the beginning of the experiment without preincubation.

In Expts. A and C, after 25 min., ADP (10 mM) was added to (a), α -glycerophosphate (finally 25 mM) was added to (b). In Expt. B substrates and ADP were added after 25 min. Flies were 2 days old.

Time (min.)	Uptake of O ₂ (μl./mg. of protein/hr.)											
	A				B				C			
	3.9 mg. of protein/flask				3.9 mg. of protein/flask				0.39 mg. of protein/flask			
	α-Glycero- phosphate		Succinate		α-Glycero- phosphate		Succinate		α-Glycero- phosphate		Succinate	
	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
0-5	170	150	79	52	0	0	0	0	40	550	0	0
15-20	15	29	79	55	0	0	0	0	40	420	30	30
30-35	7	170	93	73	200	230	57	62	220	380	30	30
40-45	0	94	87	73	36	26	70	80	300	380	30	30

Table 6. *Comparison of respiratory-control ratios obtained with different concentrations of the same mitochondrial suspensions*

Respiratory-control ratio			
$= \frac{\text{O}_2 \text{ uptake with } \alpha\text{-glycerophosphate plus ADP}}{\text{O}_2 \text{ uptake with } \alpha\text{-glycerophosphate alone}}$			
during first 5 min. of incubation.			
Expt. no.	Age of flies (days)	Concn. of protein (mg./flask)	Respiratory-control ratio
1	8	4.8	0.91
		1.6	2.3
2	2	3.9	0.95
		0.4	13.7
3	5	2.3	1.3
		0.7	6.0
4	—	1.1	3.1
		0.6	4.0
		0.2	∞
5	14	4.3	0.83
		1.4	0.74
6	22	1.1	1.1
		0.4	1.3

high activity of the α -glycerophosphate oxidase of these mitochondria valid estimations of respiratory rates could not be made when the concentration of protein exceeded about 5 mg./flask, as the rate of O_2 uptake approached the limit imposed by the equipment used; in none of the experiments described above was the maximum respiratory rate greater than 75 % of the experimentally determined capacity of the apparatus.

The sequence in which the particles were exposed to ADP and α -glycerophosphate also influenced the initial q_{O_2} . The O_2 uptake of mitochondria preincubated in α -glycerophosphate rose quickly (in less than 5 min.) to the maximum rate after the addition of ADP; addition of α -glycerophosphate to mitochondria preincubated in ADP produced a slower rise, reaching a maximum in 10–15 min. (Table 8). Also the respiratory rate with α -glycerophosphate alone or with ADP was higher after preincubation for 25 min. at 25° without substrate (Table 5).

Respiratory control was also demonstrated with α -glycerophosphate and suspensions of *Musca* mitochondria containing about 1 mg. of protein/flask. The initial respiration was stimulated approximately twofold during incubation in both the sucrose and (less consistently) the saline media.

(b) Even with an appropriate concentration of mitochondria, pronounced stimulations of respiration did not always follow the addition of ADP. As the age of flies increased it became more difficult to demonstrate respiratory control (Tables 6, 8 and 9). The loss of this requirement for ADP

was not prevented by preparing and incubating the particles in a medium containing serum albumin (1 %), which binds endogenous uncoupling agents (Hulsmann, Elliott & Slater, 1960), nor by incubation in a hypo-osmotic medium (0.12M with respect to sucrose).

Under those conditions, where no respiratory control was observed (i.e. with concentrated mitochondrial suspensions or with mitochondria from older flies), addition of ADP often caused an inhibition of O_2 uptake with α -glycerophosphate (see Tables 5 and 9; compare values for succinate oxidation, Table 5). In some of these experiments, ADP decreased the initial O_2 uptake by about 30 %, but maintained respiration at a greater rate after 30–40 min.

Comparison of maximum rates of oxygen uptake with different substrates. As ADP is required for maximum rates of oxidation of all the substrates tested, except succinate, the comparisons of relative oxidase activities have been made between systems containing ADP.

1. There was no detectable endogenous respiration with or without ADP (Table 5).

2. Despite the impossibility of using a wide range of concentrations of mitochondria in suspensions oxidizing α -glycerophosphate, higher q_{O_2} values were obtained with this substrate and more dilute suspensions of the particles (Tables 6 and 7). The maximum rate recorded was 750 $\mu\text{l.}/\text{mg.}$ of protein/hr. (0.19 mg. of protein/flask); rates of 200–300 $\mu\text{l.}/\text{mg.}$ of protein/hr. were obtained with 1.0–1.5 mg. of protein/flask. Mitochondria from older flies usually oxidized α -glycerophosphate more slowly (compare Tables 8 and 10). The initial rates of O_2 uptake were the same with 100, 50 and 25 mM- α -glycerophosphate but were lower by about 80 % with 5 mM; i.e. under the conditions used the oxidase system was saturated between 5 and 25 mM. Neither a decrease in the tonicity of the incubation medium nor addition of serum albumin (1 %) influenced the rates of respiration greatly.

3. The rates of oxygen uptake with all the other substrates tested were lower than with α -glycerophosphate (Table 4). The maximum q_{O_2} with pyruvate plus malate was 73; with succinate, the maximum was 93 (mean value in seven experiments, 63). Very low q_{O_2} values were obtained with malate or glutamate (about 3 and 10 respectively). Combination of malate with succinate (without ADP) depressed the O_2 uptake to about one-quarter of that with succinate alone (Table 2, Expt. 2). Dilution of the mitochondria did not markedly influence the rates with any of these substrates, though there was some decrease in O_2 uptake as the flies aged (Table 10).

Thus the observed respiratory activities of the *Lucilia* mitochondria were similar to those ob-

Table 7. *Comparison of the effect of adenosine diphosphate and of an adenosine diphosphate-regenerating system on the oxidation of α -glycerophosphate*

Concn. of α -glycerophosphate, 50 mM; concn. of ADP, 10 mM. The ADP-generating system comprised, in the main compartment of the flasks, ATP (2 mM), hexokinase (0.1 mg.) and, in the side arm, glucose (final concn. 70 mM). In the control, hexokinase was replaced with serum albumin. In the experiments with the ADP-regenerating system, glucose and α -glycerophosphate were tipped in after 10 min. preincubation; in the other experiments, ADP and α -glycerophosphate were added together after 10 min. Readings were commenced at the time of tipping. Flies were 2 days old.

Time (min.)	Uptake of O ₂ (μ l./mg. of protein/hr.)									
	Concn. of protein (mg./flask)									
	1.1		0.55		0.19		1.1		0.19	
	-	+ADP	-	+ADP	-	+ADP	-	+Hexo- kinase	-	+Hexo- kinase
0-5	140	440	125	505	0	740	430	530	360	570
15-20	185	390	185	640	84	750	190	420	270	680
45-55	220	300	140	320	42	415	250	230	135	595

Table 8. *Effect of preincubation with adenosine diphosphate or α -glycerophosphate on respiratory rate*

(a) and (b): Preincubated without either ADP or α -glycerophosphate, then α -glycerophosphate was added to (a) and α -glycerophosphate plus ADP to (b). (c): Preincubated with ADP, α -glycerophosphate added. (d) and (e): Preincubated with α -glycerophosphate, then ADP was added to (d); nothing was added to (e). Concn. of α -glycerophosphate, 50 mM; concn. of ADP, 10 mM.

Expt. no.	Age of flies (days)	Time of incubation (min.)	Uptake of O ₂ (μ l./mg. of protein/hr.)				
			(a) Nothing	(b) Nothing	(c) ADP	(d) α -Glycero- phosphate	(e) α -Glycero- phosphate
1	4	(Preincubation)*	0	0	0	125	125
		5	20	110	160	295	210
		10	120	250	260	295	145
		15	160	250	260	250	165
		20	200	295	320	295	210
2	22	(Preincubation)*	0	—	0	60	—
		5	96	—	48	280	—
		10	145	—	180	240	—
		15	195	—	280	280	—
		20	240	—	280	280	—

* Values given are for the 5 min. period preceding the addition of the reactants.

Table 9. *Effect of age of flies on respiratory-control ratios with α -glycerophosphate*

Respiratory-control ratio

$$= \frac{\text{O}_2 \text{ uptake with } \alpha\text{-glycerophosphate plus ADP}}{\text{O}_2 \text{ uptake with } \alpha\text{-glycerophosphate alone}}$$

for 5 min. period preceding the times shown.

Age of flies (days)	Concn. of protein (mg./flask)	Respiratory-control ratios		
		5 min.	15 min.	30 min.
2	1.1	3.1	2.8	2.3
	0.6	6.5	7.5	2.4
4	1.1	3.5	2.1	0.9
10	1.4	1.9	1.1	1.1
20	1.8	0.9	0.8	0.9
	0.6	1.2	—	1.5
22	1.1	0.9	1.1	0.9
	0.4	2.0	1.1	1.1

tained with preparations from the housefly (Sacktor, 1958) and the locust (Bucher & Klingenberg, 1958), in that α -glycerophosphate was oxidized more readily than the other substrates tested. The particles were unable to oxidize pyruvate at the relatively high rates reported by Gregg, Heisler & Remmert (1959) and ATP did not affect the O₂ uptake with succinate (compare Azzone, Ernster & Klingenberg, 1960).

Stability of the respiratory rate. Whereas the rate of oxidation of α -glycerophosphate was much greater initially than that of any other substrate tested, the difference diminished during incubation since the O₂ uptake with α -glycerophosphate often declined and ceased. This occurred most readily when the ratio of the concentration of α -glycerophosphate to that of the mitochondria was relatively low (e.g. Table 5). A similar change did not

Table 10. *Effect of age of flies on respiratory-control ratios with various substrates*

Concn. of all substrates, 50 mM; conc. of ADP, 10 mM. R.C. ratio, respiratory-control ratio (see Table 9); q_{O_2} is the maximum rate of O_2 uptake observed, expressed as $\mu\text{l./mg. of protein/hr.}$

Age of flies (days)	Time of incubation (min.)	Pyruvate plus malate		Glutamate		α -Glycerophosphate	
		R.C. ratio	q_{O_2}	R.C. ratio	q_{O_2}	R.C. ratio	q_{O_2}
5	0-5	1.3	35	1.4	10	3.5	360
	15-30	2.0	—	3.0	—	1.6	—
	30-60	1.5	—	3.5	—	0.7	—
25	0-5	—	10.8	—	3.2	—	180
	15-30	1.6	—	3.0	—	1.2	—
	30-60	1.8	—	3.1	—	0.8	—

occur with any other substrate. When mitochondria were incubated with α -glycerophosphate (25 mM) and ADP until respiration ceased, addition of more substrate, but not of ADP, restored the original O_2 uptake (Table 5).

These results suggest that, in the manometric experiments, the cessation of respiration was due to the formation of such an amount of dihydroxyacetone phosphate that the ratio 'concentration of α -glycerophosphate/concentration of dihydroxyacetone phosphate' (calculated from the O_2 uptake) reached a value of about 3; in seven experiments, with initial concentrations of the substrate from 25 to 100 mM, O_2 uptake stopped when the value of this ratio was between 2.7 and 3.4. Estabrook & Sacktor (1958) have reported that dihydroxyacetone phosphate did not limit the oxidation of α -glycerophosphate (measured polarographically), even when the ratio was as low as 0.25. In view of the other differences in the respiratory behaviour of comparable preparations estimated by different techniques, this discrepancy is perhaps also due to differing experimental conditions.

When incubation was continued until respiration ceased, the total O_2 uptake by suspensions containing the same amounts of α -glycerophosphate and mitochondria was the same, despite differences in rates during the incubation. Thus in some experiments, the 'stabilizing' of the respiratory activity by the addition of ADP was probably due to an inhibition of O_2 uptake (see above) and the consequent delay in increasing the concentration of dihydroxyacetone phosphate to the value at which respiration stops.

DISCUSSION

The flight-muscle mitochondria of the two flies *Lucilia* and *Musca* contain similar total amounts of pyridine nucleotide. The coenzymes were almost completely oxidized in the freshly isolated particles and about 95% of the total was DPN^+ . Slightly higher amounts of pyridine nucleotide have been found in the mitochondria of the locust (Klingen-

berg & Bucher, 1959) and the blowfly (Price & Lewis, 1959) and of some vertebrate muscles (e.g. for heart sarcosomes, see Holton, 1955). The high concentration of DPN relative to TPN in such particles (and in the entire thoraces of the flies; unpublished data) contrasts with the values obtained with mitochondria and homogenates of tissues such as liver and mammary glands of higher animals (e.g. see Birt & Bartley, 1960b; McLean, 1958), and is further support for the suggestion that the main function of DPN is in ATP production, whereas TPNH is used preferentially for reductive syntheses. As there is apparently some DPN-nucleosidase activity in the tissues (Table 1), the greater amount of coenzyme in the *Lucilia* preparations may reflect the shorter time required to prepare sufficient thoraces for the isolation of mitochondria for the experiments. The greater speed of preparation was also decisive in the choice of *Lucilia* for experiments on respiratory control.

Reduction of pyridine nucleotides by added substrate. All the substrates tested were capable of increasing the amount of reduced coenzyme in the mitochondria though only succinate was always effective. Even with this substrate the ratio $\text{DPNH} + \text{TPNH}/\text{DPN}^+ + \text{TPN}^+$ varied greatly in different experiments (compare Expt. nos. 2 and 4, Table 2). α -Glycerophosphate reduced the pyridine nucleotides less consistently (Table 2; cf. Bucher & Klingenberg, 1958). Two factors may account for this difference. First, it has been assumed that the reduction of DPN by succinate and α -glycerophosphate is due to a reversal of oxidative phosphorylation (Chance & Hollunger, 1957; Bucher & Klingenberg, 1958); alternatively, it has been proposed that an increased net reduction of the coenzymes by the malate produced from succinate is caused by an inhibition of DPNH oxidation during the simultaneous oxidation of the succinate (Birt & Bartley, 1960c). With α -glycerophosphate, only the endogenous substrate of the mitochondria would be available for the reduction and, as Klingenberg & Bucher (1959) have shown that the concentration of this material may decline rapidly in isolated

mitochondria, it is possible that the relatively greater variability in the extent of reduction with α -glycerophosphate is due to differences in the amount of DPN-reducing substances retained in different mitochondrial preparations.

Secondly, Klingenberg & Bucher (1959) have suggested that saline media are not well adapted to preserving mitochondrial structure and that reduction of pyridine nucleotides by α -glycerophosphate is a relatively labile function of the particles. Thus, whereas on this criterion the *Musca* mitochondria may be considered to be 'damaged', having lost their ability to reverse oxidative phosphorylation with α -glycerophosphate as substrate, on other criteria proposed by these authors (respiratory control with α -glycerophosphate, retention of coenzymes) they are 'undamaged'. Moreover, they have retained the ability to reduce pyridine nucleotides during the oxidation of succinate. While it is not possible with the data of this paper to decide which explanation of the results is correct, all the observations reported are compatible with the suggestion that the increase concentration of reduced coenzyme is a consequence of diminished opportunity for coenzyme oxidation.

The respiration of *Musca* mitochondria suspended in saline was not invariably increased by ADP. The attempt to increase the rate of this reduction by adding malate with succinate was vitiated by the pronounced inhibition of electron transport resulting, possibly by the rapid formation of oxaloacetate (Tyler, 1954).

Respiratory control by adenosine diphosphate. The results reported in this paper emphasize that with mitochondrial preparations the detection of respiratory control by ADP can depend completely on the conditions used in the experiment. An investigation of factors influencing the response of various mitochondrial oxidases to ADP has recently been published by Bellamy & Bartley (1960), who also concluded that different methods of estimating respiratory control can give widely differing results. The finding that, with mitochondria prepared from relatively young flies, the demonstration of a control of α -glycerophosphate oxidation by ADP was dependent on suitable dilution of the mitochondrial suspension, is in accord with their suggestion that the stimulation of uptake of oxygen by this phosphate acceptor may be facilitated by the equilibration of intramitochondrial solutes during shaking with the incubation medium. It is known that the mitochondria of another fly (*Calliphora*) contain ADP and ATP, at least some of which can be released into a buffered suspension medium (Price & Lewis, 1959). In the least concentrated suspensions of particles used in this work, the loss of ADP during equilibration in the incubation medium may be sufficient to lower the

intramitochondrial concentration below that required for maximum oxidation of α -glycerophosphate. In more concentrated suspensions, where this would not occur, it is possible that the ADP added may limit the entry (or release) of substrates (Packer & Golder, 1960), and therefore depress the uptake of oxygen.

These two different effects of ADP may explain the stimulation or inhibition of uptake of oxygen observed under different conditions of incubation with ADP. Moreover, it is apparent that mitochondria preincubated in ADP or exposed simultaneously to ADP and α -glycerophosphate attain the maximum rate of oxygen uptake more slowly than those preincubated in α -glycerophosphate alone. No evidence was obtained for inhibition by ADP of the oxidation of any other substrate except succinate, and with this compound no stimulation was ever detected. Thus a change in the total extramitochondrial concentration of ADP may alter the structure and therefore the permeability of mitochondria in such a way as to produce a relatively selective exclusion of particular substrates, especially those which are themselves phosphorylated.

Slater & Lewis (1954) have shown that 2:4-dinitrophenol increased the respiration of *Calliphora* mitochondria oxidizing α -oxoglutarate only if the insects were relatively young, and Sacktor & Cochran (1958) reported that mature flies (3–7 days) do not exhibit respiratory control with α -glycerophosphate. With the experimental procedures described in this paper, respiratory control was always detectable with all substrates tested (except succinate) and flies up to 10 days old, and, in the initial stages of incubation, often with flies up to 1 month old. In general, however, the influence of ADP was less apparent with the older flies, both the dependence on ADP and the rates of oxidation of particular substrates declining. With pyruvate plus malate and glutamate, respiratory control was not lost in the mitochondria of older flies. The reason for the changes on aging is not known, but a decline in the rate of oxidation of certain compounds by liver mitochondria isolated from aging rats has been attributed to an increasing sensitivity of the particles to 'damage', resulting in inactivation of particular oxidases (Weinbach, 1959).

Bucher & Klingenberg (1958) have described a decrease in respiratory control during the aging of isolated locust mitochondria oxidizing α -glycerophosphate, but a similar change was not found during the relatively brief preincubation without substrate described in Table 2; indeed, in this experiment there was evidence for a slight increase in respiratory control after the preincubation (Table 5; cf. Bellamy & Bartley, 1960).

The results presented in this paper provide clear evidence that, given suitable experimental conditions and methods of estimation, the oxidation of α -glycerophosphate (and other substrates) by *Lucilia* mitochondria is controlled in part by the availability of ADP; and that pyridine nucleotides occur in the respiratory chain of two species of fly. They also underline some of the difficulties encountered in interpreting information obtained with isolated mitochondrial suspensions and the dependence of the observed behaviour of such systems on the conditions of preparation and incubation. In particular, it would seem to be premature to conclude that the control of the rate of respiration in the flight-muscle mitochondria of these insects is of a type radically different from that proposed for mitochondria from other organisms.

SUMMARY

1. The amounts and reactivity of the pyridine nucleotides of the flight-muscle mitochondria of the flies *Musca* and *Lucilia* have been estimated by a fluorimetric method and some factors influencing the rates of oxidation of various substrates have been investigated.

2. Particles freshly isolated from both flies contain about 2.5–3.0 μ m-moles of total coenzymes/mg. of protein, almost all in the oxidized form. About 95 % of the total is oxidized diphosphopyridine nucleotide.

3. Incubation with succinate, pyruvate plus malate and (less consistently) α -glycerophosphate increased the amount of reduced coenzyme. The maximum reduction observed was of 58 % of the total, with succinate.

4. Not more than 15 % of the pyridine nucleotides were lost from the particles in 15 min. incubation with or without substrate.

5. Adenosine diphosphate increased the respiration of dilute suspensions (about 0.4 mg. of protein/ml.) of mitochondria prepared from flies less than 2 weeks old and oxidizing tricarboxylic acid-cycle intermediates or α -glycerophosphate. Stimulations greater than tenfold were obtained.

6. With more concentrated suspensions (1.2–2.0 mg. of protein/ml.) or older flies, oxidation of α -glycerophosphate was no longer increased by adenosine diphosphate and was sometimes decreased.

7. q_{O_2} values (with adenosine diphosphate) were much higher for α -glycerophosphate (200–750 μ l. of oxygen/mg. of protein/hr.) than for succinate

(about 65), pyruvate plus malate (70), malate (3) or glutamate (10).

8. The results emphasize that the respiratory behaviour of mitochondrial preparations is influenced greatly by the techniques used for incubation and estimation.

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