Anaerobic Metabolism in the Housefly, *Musca domestica* L.

By J. P. HESLOP, G. M. PRICE AND J. W. RAY

Biochemistry Department, Agricultural Research Council, Pest Infestation Laboratory, Slough, Bucks.

(Received 1 October 1962)

Adult and immature individuals of many insect species can survive anaerobic conditions for periods ranging from 4 hr. to 4 days, and even in a few cases for longer periods up to 56 days (see Brand, 1946, for numerous references). The present paper describes an investigation into some effects of anoxia on adult *Musca domestica*. A preliminary account of part of this work has been published (Heslop, Price & Ray, 1962).

MATERIALS AND METHODS

Experimental animals. Houseflies were reared at this Laboratory. Puparia were placed in cheese-cloth sleeves on wire frames for periods not exceeding 24 hr. and then removed. The adults that had emerged were fed on glucose and water only. Male flies were removed for experiment 3–6 days after emergence. Groups of 10 or 20 flies were subjected to chill-coma, placed in tared glass cages with wire-mesh ends and weighed. When flies were to be subjected to anoxia the cages were dropped into vessels previously flushed with oxygen-free nitrogen. The flow of nitrogen was maintained for at least 10 min. after introduction of the cage and then the vessel was re-sealed. Commercial oxygen-free nitrogen was further purified by bubbling it through either alkaline pyrogallol or a solution of 4.5 g. of sodium dithionite plus 0.5 g. of sodium anthraquinone- β -sulphonate in 25 ml. of *N*-potassium hydroxide. Flies were killed by plunging the cage into liquid nitrogen. After anoxia the transfer from anaerobic conditions to liquid nitrogen was made as quickly as possible to minimize the very rapid biochemical changes that take place when anoxic flies are returned to air (Ray & Heslop, 1963). The cages were opened under liquid nitrogen, and where appropriate the flies were divided into heads, thoraces and abdomina for separate analysis.

Preparation of extracts. A porcelain mortar was thoroughly chilled with liquid nitrogen and 0.5 ml. of 0.4*N*-perchloric acid was finely ground in it. More liquid nitrogen was added and the whole flies or parts thereof were ground to a uniform paste with the perchloric acid and transferred on a cold glass spatula to an homogenizer tube chilled in ice. The mortar was scoured out by grinding with a second 0.5 ml. of perchloric acid in the same way. The combined material was thawed and homogenized briefly at 0°. It was centrifuged at 2000*g* for 10 min. at 0° and the supernatant decanted into a centrifuge tube chilled in ice. The residue was re-extracted by homogenizing and centrifuging it with 1 ml. of 0.2*N*-perchloric acid at 0°. The combined supernatants were neutralized with potassium hydroxide solution at 0° and centrifuged as above. The final supernatant was diluted with water to 5 ml.

(10 flies) or 10 ml. (20 flies). The following determinations were then carried out.

Determination of adenosine triphosphate. This was carried out by the firefly-luciferin method of Strehler & Totter (1952), with firefly tails purchased from Schwartz Laboratories Inc.

Determination of orthophosphate. This was carried out as described by Martin & Doty (1949).

Determination of arginine phosphate. This was carried out by measurement of the orthophosphate released by 1 min. of hydrolysis at 100° in 0.1*N*-hydrochloric acid.

Determination of acid-soluble, phospholipid and residual phosphorus. After extraction of flies with perchloric acid at 0° the residue was further extracted three times at room temperature with chloroform-methanol (1:1, v/v) to remove phospholipids (Folch, Ascoli, Lees, Meath & LeBaron, 1951). The final insoluble residue and samples of both extracts were hydrolysed by boiling for 3 hr. with conc. perchloric acid. Orthophosphate determinations were performed on the hydrolysates.

Determination of L-glycerol 1-phosphate. This was carried out by a modification of the method of Bublitz & Kennedy (1954). The reaction mixture contained: EDTA (66 mM), hydrazine (0.5 M), NAD (1.5 mM) pH 9.4, crystalline glycerol 1-phosphate dehydrogenase (20 μ g./ml.) plus extract equivalent to about one-third of a fly/ml. The increase in absorption at 340 m μ was measured after addition of the enzyme and incubation for 1 hr. at 37°. Both the NAD and the enzyme were purchased from Boehringer und Soehne, Mannheim, Germany.

Determination of glycerol. This was carried out as described by Lambert & Neish (1950).

Determination of adenine and hypoxanthine. Adenine and hypoxanthine were isolated by paper chromatography from hydrolysed perchloric acid extracts as described by Ray & Heslop (1963) and determined by their ultraviolet absorption.

RESULTS

When exposed to oxygen-free nitrogen houseflies undergo a short period of hyperactivity and then start to falter and will fall from glass surfaces within 30–45 sec. After 45–90 sec. all collapse and remain motionless. The symptoms of flies on recovery from anoxia are described by Ray & Heslop (1963) and demonstrate that the effects of anoxia described below are not lethal.

The effect of anoxia on the concentrations of orthophosphate, ATP and arginine phosphate is shown in Fig. 1. The flies were weighed before the start of anoxia and the results are expressed as μ moles/g. based on that weight. The concentrations

of ATP and arginine phosphate fell rapidly during anoxia and reached very low values within 30 min. The concentration of orthophosphate continued to rise even after the substantially complete breakdown of ATP and arginine phosphate. At 120 min. it was 13.0 ± 0.3 (three experiments), which was significantly higher than that at 60 min. of anoxia, when it was 12.0 ± 0.6 (six experiments) $\mu\text{moles/g.}$ (P less than 0.01).

The concentrations of glycerol and of glycerol 1-phosphate in whole flies after different periods of

anoxia are shown in Fig. 2. There was a rapid rise in the glycerol 1-phosphate concentration during the first 20–25 min. of anoxia but no further rise after that time. There was no evidence that the abrupt cessation of glycerol 1-phosphate accumulation at 25 min. of anoxia was due to further metabolism to glycerol, although the possibility that there may be some production of glycerol from glycerol 1-phosphate in thoraces during the first 25 min. of anoxia is indicated by the results in Table 1.

Table 1 shows the effect of anoxia on orthophosphate, glycerol 1-phosphate, acid-soluble phosphorus and glycerol concentrations in heads, thoraces and abdomina. The average weight of each fly in a sample of 50 taken from the culture used for this experiment was 14.24 mg., of which heads

Fig. 1. Effect of anoxia on ATP (○), arginine phosphate (△) and orthophosphate (●) in whole houseflies. Each orthophosphate point represents one determination of a batch of 10 flies. One arginine phosphate determination was done with each orthophosphate determination but only means are shown for the sake of clarity. Each ATP point represents the mean of at least two determinations on two to four extracts of batches of 10 flies. Experimental details are given in the text.

Fig. 2. Effect of anoxia on glycerol (○) and glycerol 1-phosphate (●) in whole houseflies. Each point represents one determination on a batch of 10 flies. Experimental details are given in the text.

Table 1. *Effect of anoxia on the concentrations of orthophosphate, glycerol 1-phosphate, glycerol and acid-soluble phosphorus in heads, thoraces and abdomina of houseflies*

Each value represents one determination on a batch of 10 thoraces. The amounts given are those found in 1 g. wet wt. of tissue. Experimental details are given in the text.

	Time of anoxia (min.)	Orthophosphate (μmoles/g.)	Glycerol 1-phosphate (μmoles/g.)	Glycerol (μmoles/g.)	Acid-soluble phosphorus (μg.atoms of P/g.)
Heads	0	3.2	2.3	0.8	16.8
	25	6.7	4.0	0.8	16.8
	60	6.5	2.9	0.7	24.0
	120	7.5	3.9	0.8	16.8
	180	7.2	3.1	0.9	16.8
Thoraces	0	4.7	1.7	0.6	40.2
	25	10.5	14.1	1.3	37.2
	60	7.7	13.9	1.3	38.8
	120	13.2	13.0	1.4	38.4
	180	13.5	12.9	1.6	38.4
Abdomina	0	14.2	0.8	5.8	26.5
	25	17.0	2.9	7.5	25.8
	60	18.8	3.9	6.0	29.2
	120	19.0	3.0	5.7	25.8
	180	17.9	2.6	6.6	24.3

Table 2. *Effects of anoxia on the acid-soluble, phospholipid and residual phosphorus pools of whole male 5-day-old houseflies*

The flies were kept after pupation on a glucose and water diet. The results are expressed as $\mu\text{g. atoms of P/g. wet wt.}$ before anoxia. Each value represents one determination on a batch of ten thoraces. Experimental details are given in the text.

Time of anoxia (min.)	Acid-soluble phosphorus ($\mu\text{g. atoms of P/g.}$)	Phospholipid phosphorus ($\mu\text{g. atoms of P/g.}$)	Residual phosphorus ($\mu\text{g. atoms of P/g.}$)
0	19.0	23.3	7.1
2	21.8	25.3	7.4
4	19.6	24.8	7.9
8	22.1	25.8	8.1
12	22.1	24.6	5.4
16	21.5	25.6	7.7
20	21.8	26.8	5.1
24	19.9	26.3	8.8
28	19.3	27.8	5.9
32	18.6	29.2	5.7
36	21.1	30.0	11.1
40	20.3	28.9	8.6

Table 3. *Adenine and hypoxanthine in hydrolysed perchloric acid extracts of housefly thoraces after anoxia*

The amounts of hypoxanthine and adenine are those found in 1 g. wet wt. of thorax. Each value represents one determination on a batch of ten thoraces. Experimental details are given in the text.

Time of anoxia (min.)	Hypoxanthine ($\mu\text{moles/g.}$)	Adenine ($\mu\text{moles/g.}$)
0	Trace	6.6
30	1.7	3.9
60	2.4	4.1
90	2.9	3.7
120	2.4	3.4
180	2.0	3.5

accounted for 14 %, thoraces for 55 % and abdomina for 31 %. Hence 88 % of the accumulation of glycerol 1-phosphate and 71 % of the increase in orthophosphate observed after 25 min. of anoxia were due to changes occurring in the thoraces. Other experiments have shown that over 75 % of the ATP and arginine phosphate of the whole fly is located in the thorax. Quantitatively, therefore, the most important changes in these phosphorus compounds during anoxia occur in the thorax.

The total phosphorus in the acid-soluble, phospholipid and residual phosphorus pools was determined after different periods of anoxia (Table 2). No evidence was found for any net transfer of phosphorus from one pool to another during anoxia.

The breakdown of ATP was studied by measuring the amount of adenine and hypoxanthine in hydrolysed perchloric acid extracts of thoraces of normal and anoxic flies (Table 3). The concentration of hypoxanthine rose and that of adenine fell during the early stages of anoxia, but the concentration of acid-soluble adenine did not fall further as anoxia was prolonged.

DISCUSSION

During the first 30 min. of anoxia there is a decrease in the concentration of ATP in whole *M. domestica*. In the thorax (where most of this insect's ATP is located) the concentration of acid-soluble compounds of adenine falls and that of acid-soluble compounds of hypoxanthine rises. Ray & Heslop (1963) have shown that the breakdown products of ATP in housefly thorax during anoxia are AMP and IMP, and they offer a tentative explanation for this observation.

A tentative outline scheme of the reactions of glycerol 1-phosphate is given in Scheme 1. Some

Scheme 1. Outline of the known reactions of glycerol 1-phosphate *in vivo*. Reactions ultimately dependent on ATP are indicated by *.

of these reactions (into phospholipids and neutral fats) have not yet been confirmed in houseflies. Under conditions of anoxia, when reaction (a) is blocked, the pathway (b) leading to glycerol 1-phosphate (Kubista, 1957, 1959; Bücher & Klingenberg, 1958) must cease to operate after 20–25 min. of anoxia unless the glycerol 1-phosphate subsequently produced is further metabolized (see Fig. 2). The most probable further metabolite is glycerol, but, although there was some evidence (Table 1) that glycerol was formed in the thorax during the first 25 min. of anoxia, further metabolism by this route did not occur after this time. It is not inconceivable that glycerol 1-phosphate might be converted into phospholipid or into triglycerides (for references see Kennedy, 1957). However, the failure to find any movement of phosphorus between the acid-soluble and phospholipid pools (Table 2) and the severe depletion of 'high-energy' phosphates under anoxic conditions make it very unlikely that incorporation into lipids could account for the pattern of glycerol 1-phosphate accumulation observed. It is suggested that in anoxia glycerol 1-phosphate is an end product of glycolysis, that it is not derived from or converted into lipid, and that the pathway leading to its production ceases to operate after 25 min. of anoxia.

In anoxic houseflies orthophosphate accumulates, as has been reported by Winteringham (1960). During the first 30 min. of anoxia the accumulation of orthophosphate is associated with the breakdown of ATP and arginine phosphate. Further increases in the orthophosphate concentration do occur, however, during the second hour of anoxia, and may continue even later. The results of Winteringham (1960) suggest that glucose 6-phosphate may be one of the substances catabolized to release orthophosphate. The accumulation of orthophosphate in anoxic flies is in marked contrast with the findings of Kubista (1959) in cockroach-metathorax preparations. Kubista found decreases in the concentrations of 1 min.-hydrolysable phosphate and 3 hr.-hydrolysable phos-

phate of the order of only 50 % during 40 min. of anoxia, but that the concentration of orthophosphate did not change. All the phosphorus that could have been released by breakdown of 'high-energy' compounds was accounted for as glycerol 1-phosphate.

Quantitatively the most important changes induced by anoxia in the concentrations of the compounds studied occur in the thorax. A transverse section through this body region (Winteringham, Bridges & Hellyer, 1955) shows that the bulk of the thorax consists of indirect flight muscle. It is therefore suggested that biochemical changes in this tissue account for the effects of anoxia reported above.

SUMMARY

1. The effects of various periods of anoxia on the tissue concentrations of glycerol 1-phosphate, orthophosphate, adenosine triphosphate, arginine phosphate, acid-soluble phosphorus, phospholipid phosphorus, residual phosphorus and glycerol have been studied in adult male *Musca domestica*.

REFERENCES

- Brand, T. von (1946). *Anaerobiosis in Invertebrates*. Normandy, Mo., U.S.A.: Pubbl. Biodynamica.
- Bublitz, C. & Kennedy, P. (1954). *J. biol. Chem.* **211**, 951.
- Bücher, T. & Klingenberg, M. (1958). *Angew. Chem.* **70**, 552.
- Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951). *J. biol. Chem.* **191**, 833.
- Heslop, J. P., Price, G. M. & Ray, J. W. (1962). *Biochem. J.* **82**, 24p.
- Kennedy, E. P. (1957). *Annu. Rev. Biochem.* **26**, 119.
- Kubista, V. (1957). *Nature, Lond.*, **180**, 549.
- Kubista, V. (1959). *Marburger Sitzungsberichte*, **81**, 17.
- Lambert, M. & Neish, A. C. (1950). *Canad. J. Res. B*, **28**, 83.
- Martin, J. B. & Doty, D. M. (1949). *Analyt. Chem.* **21**, 965.
- Ray, J. W. & Heslop, J. P. (1963). *Biochem. J.* **87**, 39.
- Strehler, B. L. & Totter, J. R. (1952). *Arch. Biochem. Biophys.* **40**, 28.
- Winteringham, F. P. W. (1960). *Biochem. J.* **75**, 38.
- Winteringham, F. P. W., Bridges, P. M. & Hellyer, G. C. (1955). *Biochem. J.* **59**, 13.