Phosphorus Metabolism of the Housefly (*Musca domestica* L.) during Recovery from Anoxia

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Heslop, Price & Ray (1963) reported that adult houseflies were able to recover from long periods of anoxia. Even after 5 hr. in an atmosphere of nitrogen flies fully recovered and were able to fly. This suggested that the concentration of ATP had returned to normal in the thoracic tissues. Kubista (1961), on the other hand, found in cockroach-metathorax-muscle preparations that the concentration of ATP in preparations recovering from anoxia failed to reach that of the control.

The present investigations were concerned with the changes in phosphorus compounds in the thoracic tissues of adult houseflies during recovery from anoxia.

MATERIALS AND METHODS

Experimental animals. Adult houseflies, *Musca domestica*, were kept at 27° and fed on aq. 10% (w/v) glucose solution. Only males 3–4 days old were used in the experiments.

Treatment of flies. Flies were lightly anaesthetized with cyclopropane and separated into sexes on an ice-cold Petri dish. Males (20 or 40) were placed in cages made from wide-bore glass tubing with detachable gauze ends. Flies were allowed a period of at least 15 min. to recover from the anaesthetic and chill-coma. The control groups were then dropped into liquid nitrogen. All other groups were placed in a stream of oxygen-free nitrogen for 60 min. After anoxia groups of insects were either plunged at once into liquid nitrogen or first allowed to recover in fresh air before plunging into liquid nitrogen. When the recovery period was longer than 30 min. flies were transferred to larger cages; or, when the flies were left for 22 hr., they were transferred to 350 ml. glass jars and fed on aq. 10% (w/v) glucose. All the flies were killed by plunging them into liquid nitrogen.

Extraction of thoraces. The cages were opened under liquid nitrogen and the flies were divided into heads, thoraces and abdomina. The thoraces minus appendages were ground up in liquid nitrogen with 0.4N-perchloric acid in a pestle and mortar and the paste was transferred to a Potter-Elvehjem homogenizer at 0°. The pestle and mortar were 'rinsed' by grinding a further amount of 0.4N-perchloric acid in liquid nitrogen and the paste was transferred to the homogenizer. When 20 thoraces were extracted, 0.6 ml. of perchloric acid and a 'rinse' of 0.4 ml. were used; when 80 thoraces (two groups of 40) were extracted, 1 ml. of perchloric acid and a 'rinse' of 0.5 ml. were used. The thoraces were homogenized at 0° and centrifuged at 1500g for 10 min. at 0°. The supernatant was

decanted into chilled conical centrifuge tubes. The residue was extracted with 0.2N-perchloric acid (1 ml. for 20 thoraces and 2 ml. for 80 thoraces) and centrifuged as before. The supernatants were combined and neutralized with potassium hydroxide. The precipitated potassium perchlorate was removed by centrifuging at 1500g for 10 min. at 0°. The supernatant was made up to 10 ml.

Paper chromatography. One- and two-dimensional ascending chromatography was carried out with the following solvents: *A*, propan-2-ol-conc. aq. hydrochloric acid (sp.gr. 1.18)-water (85:22:18, by vol.); *B*, butan-1-ol saturated with water at 20°-conc. aq. ammonia (sp.gr. 0.88) (100:1, v/v) (Wyatt, 1955); *C*, acetone-formic acid-water (30:7:13, by vol.) (Burrows, Grylls & Harrison, 1952); *D*, isobutyric acid-aq. N-ammonia-0.1M-EDTA (125:75:2, by vol.) (Krebs & Wankmüller, 1953). Chromatograms were developed in solvents *A* and *B* for 18 hr. at 25°, in solvent *C* for 6 hr. at 5° and in solvent *D* for 48 hr. at 20°.

Chromatography of nucleotides and bases. Adenine and hypoxanthine were chromatographed two-dimensionally in solvent *A* and then in solvent *B* and located in ultraviolet light. A permanent record was obtained by making a contact print of the chromatogram under ultraviolet light on Ilford Document Paper and developing it in Copyphen.

The nucleotides were chromatographed two-dimensionally in solvents *C* and *B* in that order and also located in ultraviolet light. The separated nucleotides were finally eluted, re-concentrated on strips of Whatman no. 1 filter paper (Winteringham, 1953) and developed in solvent *D*.

In some cases after chromatography in solvent *C* the nucleotides were eluted off the chromatogram in 0.1N-hydrochloric acid. The eluate was neutralized with 0.4N-potassium hydroxide and washed on to a Dowex 1 (formate form) column with water. The chromatogram was developed with formic acid-ammonium formate (Gilbert & Yemm, 1958).

Identification of bases and nucleotides. The bases and nucleotides were identified by their chromatographic behaviour and their ultraviolet-absorption spectra when measured against blanks prepared from paper treated in an identical manner. After chromatography in three solvents AMP and IMP were hydrolysed either in 72% (w/v) perchloric acid for 2 hr. at 100° or in 1N-hydrochloric acid for 10 min. at 100°. Orthophosphate was determined (Martin & Doty, 1949) in the perchloric acid hydrolysate, and the products of hydrolysis in hydrochloric acid were chromatographed in solvent *B*. The ribose 5'-phosphate was detected by a phosphate reagent (Hanes & Isherwood, 1949) and the base under ultraviolet light.

Determination of bases. After location on the chromatogram the zones were cut out and eluted overnight in 5 ml. of 0.1N-hydrochloric acid. The extinction of the eluate was

measured on a Unicam SP. 500 at 260 $m\mu$ against a blank prepared from paper treated in an identical manner.

Treatment of perchloric acid extracts for base ratio analysis. The neutralized extracts of 80 thoraces were evaporated to dryness in 30 ml. beakers in a vacuum desiccator over anhydrous calcium chloride and sulphuric acid. The residues were dissolved in 0.5 ml. of 0.1N-hydrochloric acid and decanted into hard-glass test tubes (1.2 cm. $\times$ 10 cm.). The beakers were washed out with a further 0.5 ml. of 0.1N-hydrochloric acid. The combined washings were evaporated to dryness and 50 μ l. of 72% (w/v) perchloric acid was added to each tube. The tubes were tightly stoppered with rubber bungs and heated on a boiling-water bath for 2 hr. (Marshak & Vogel, 1950). The digests were very charred and were neutralized with 0.4N-potassium hydroxide. The carbon and potassium perchlorate were sedimented by centrifuging at 1500g for 10 min. at 0°. The supernatants were decanted into beakers and evaporated to dryness. The residues obtained were dissolved in 0.1 ml. of 0.1N-hydrochloric acid, and 20 μ l. of each of these solutions was chromatographed two-dimensionally.

Determinations of adenosine triphosphate, arginine phosphate, glycerol 1-phosphate and orthophosphate. These were carried out as described by Heslop *et al.* (1963). The determinations were done on extracts of 20 thoraces.

RESULTS

Signs of recovery. Flies remained quite motionless during nitrogen anoxia. The first signs of recovery in fresh air were slight movements of the gut as seen through the abdominal surface after about 5 min. Leg movements occurred after 10 min. and the frequency of these movements increased until at 20 min. some of the flies were capable of walking. By 30 min. all the flies were on their feet and capable of flight. Flies subjected to 3 hr. of anoxia took longer to recovery but were all able to fly within 3½ hr.

Phosphorus compounds during recovery. After nitrogen anoxia groups were allowed to recover in fresh air for various times up to 19 hr. before being plunged into liquid nitrogen, extracted and analysed. The concentration of orthophosphate (Fig. 1), which had risen during the period of anoxia, fell dramatically during the first minute of recovery. It continued to fall and by 5 min. was significantly lower than the control (P less than 0.01 by the t test). By 20 min. the concentration had returned to normal.

During anoxia the ATP concentration fell to almost zero whereas the glycerol 1-phosphate concentration rose sharply (Fig. 2). Two stages may be recognized in the return of the concentrations of these compounds to their normal values on release from anoxia. The first stage was very rapid and occurred during the first minute of aerobic metabolism. In this period the glycerol 1-phosphate concentration fell from 18.5 to 10.5 μ moles/g. and the ATP concentration rose from 0.1 to 4.3 μ moles/g.

The second phase was much slower and at the end of 2 hr. the glycerol 1-phosphate concentration was still twice that found in the normal control and the ATP concentration had remained unchanged during this time. At 19 hr. the concentrations of both substances had returned to the values found in the normal fly. The concentration of arginine phosphate, which fell during anoxia, rose less rapidly than the ATP concentration during recovery. The rate of increase was maintained and by 15–20 min. the concentration had reached that found in the normal control.

Base ratios during recovery. After extraction and hydrolysis of extracts of 80 thoraces and chromatography as described in the Materials and Methods section the control group gave one main ultra-violet-absorbing zone which was identified as

Fig. 1. Changes in the concentration of orthophosphate occurring in the housefly thorax during recovery from 1 hr. of anoxia. Each point represents a determination of a sample from an extract of 20 thoraces. Experimental details are given in the text.

Fig. 2. Changes in the concentrations of glycerol 1-phosphate (Δ), arginine phosphate ($\square$) and adenosine triphosphate ($\circ$) occurring in the housefly thorax during recovery from 1 hr. of anoxia. Each point represents a determination made on a sample from an extract of 20 thoraces. Experimental details are given in the text.

adenine, and flies subjected to anoxia gave two main ultraviolet-absorbing zones identified as adenine and hypoxanthine. The adenine after 1 hr. of anoxia represented 62 % of the bases recovered after perchloric acid hydrolysis. The bulk of the remainder was hypoxanthine with traces of other bases too small to determine. During the first hour of recovery this picture remained unchanged, adenine representing 65 and 62 % of the bases at 30 and 60 min. in air respectively. At 19 hr. the hypoxanthine had almost disappeared.

Identification of adenosine monophosphate and inosine monophosphate as breakdown products of adenosine triphosphate. The purine bases obtained after 1 hr. of anoxia and during the first hours of recovery are adenine and hypoxanthine. Their parent compounds before hydrolysis could be base, nucleoside or nucleotide. Extracts were chromatographed on two-dimensional sheets in solvent *C*. The control extract gave one main zone of absorption at R_F 0.06, corresponding to nucleotide triphosphates, and a faint spot at R_F 0.38. The extract from flies subjected to 1 hr. of anoxia gave a faintly absorbing zone at R_F 0.06 and the main zone at R_F 0.38, but in the extract from flies that had been allowed a 2 hr. recovery period after 1 hr. of anoxia the absorption was divided more evenly between the zone at R_F 0.06 and 0.38, the former being the larger. Hypoxanthine and adenine have R_F values of 0.63 and 0.53 respectively in solvent *C*, and therefore were not present in the extract. When developed in solvent *B* all the absorbing zones failed to move. This behaviour excluded nucleosides, as they have R_F values greater than 0 in solvent *B* (Wyatt, 1955). From these observations it was concluded that the absorbing substances could only be nucleotides. The zones with R_F 0.38 were chromatographed in solvent *D*. The extract from flies exposed to 1 hr. of anoxia now gave two absorbing zones, at R_F 0.31 and 0.54, and the extract from flies that had been allowed a 1 hr. recovery period gave one zone at R_F 0.29. The faster-running zone behaved like AMP under identical conditions and the slower one like IMP. On elution the spots gave ultraviolet-absorption spectra corresponding to AMP and IMP.

IMP and AMP obtained from an extract from flies exposed to 1 hr. of anoxia after chromatography in three solvents yielded 4.7 and 3.9 μg . of phosphorus as compared with 5.4 and 4.6 μg . of phosphorus calculated from the absorption spectra. The IMP and AMP obtained in solvent *D* from an extract from flies subjected to 1 hr. of anoxia and then allowed a 2 hr. recovery period were also hydrolysed in 1*N*-hydrochloric acid for 10 min. at 100° and chromatographed in solvent *B*. The absorbing zones obtained after hydrolysis gave a chromatographic pattern similar to that of the

free base, leaving a phosphate-positive area at the point of application as would be expected if it were ribose 5'-phosphate.

When the ultraviolet-absorbing fractions separated on paper in solvent *C* were chromatographed on Dowex 1 (formate form) the results supported the conclusions arrived at above. The control gave a main peak of absorption corresponding to ATP and only traces of material in the AMP and IMP positions. The extract from flies subjected to 1 hr. of anoxia gave two main peaks, one corresponding to AMP and the other to IMP. When these fractions were evaporated to dryness, digested for 2 hr. at 100° in perchloric acid, neutralized and chromatographed for bases (see the Materials and Methods section), the absorbing zones after elution in 0.1*N*-hydrochloric acid gave ultraviolet-absorption spectra corresponding to adenine and hypoxanthine respectively. In the extract from flies allowed 1 hr. recovery period after 1 hr. of anoxia the AMP peak had disappeared, and the ATP reappeared leaving the IMP unchanged.

DISCUSSION

The rapid depletion of glycerol 1-phosphate on returning the flies to aerobic conditions demonstrates the rapidity of penetration of oxygen and the efficiency of the glycerol 1-phosphate-oxidase system. The rapid initial fall in glycerol 1-phosphate concentration gives way to a very much slower rate of disappearance of the substrate after 1 min. in air. The return of physical activity of the animal and the return to the normal physiological concentration of arginine phosphate occur together. During anoxia ATP is broken down and AMP and IMP accumulate.

In the anoxic housefly IMP accumulates only during the first 30 min. of anoxia, and thereafter remains at a constant concentration for up to 3 hr. The ATP concentration at the end of the first 30 min. of anoxia has fallen to a very low value (see Heslop *et al.* 1963; Fig. 2). This strongly suggests that the adenylate deaminase responsible for the breakdown of AMP to IMP in the housefly requires the presence of ATP to activate it. Adenylate deaminases of this type have been described by Lyubimova & Matlina (1954) associated with myosin and by Weil-Malherbe & Green (1955) in the microsomal fraction of ox brain. They both found that ATP in its normal physiological concentration activated the enzyme but that in its absence the enzyme was virtually inactive. This would perhaps explain the failure of Gilmour & Calaby (1953) to find any adenylate deaminase associated with locust myosin.

Kubista (1961) found that IMP was the main breakdown product of ATP in his anoxic cockroach

preparations. The concentration of ATP fell only to 50 % of the normal (Kubista, 1959). This would be sufficient to activate the adenylate deaminase and would account for his finding that only a trace of AMP was present, if a type of enzyme is present in the cockroach similar to that suggested for the housefly.

During recovery the AMP present in the fly is rapidly rephosphorylated to give ATP. Sacktor & Cockran (1957) have shown *in vitro* that housefly flight-muscle sarcosomes are capable of dephosphorylating IDP, so the reverse reaction should perhaps be possible. IMP is neither rephosphorylated nor reaminated during the first 2 hr. of recovery. Parnas (1928) describes the reamination of IMP in muscle at the expense of nitrogen contained in the amino acid fraction. Subsequently it has been shown that IMP is the normal precursor of AMP in nucleotide synthesis in a wide range of organisms, e.g. in yeast (Carter & Cohen, 1956), in *Escherichia coli* (Lieberman, 1956) and in rabbit bone-marrow extracts (Abrams & Bentley, 1955). The requirements for this reaction are GTP and L-aspartic acid. The concentration of L-aspartic acid falls during anoxia (G. M. Price, personal communication) and remains at a very low value during the first hour of recovery (G. M. Price & J. W. Ray, unpublished work). At 2 hr. the concentration has returned to normal, but at this time the IMP concentration remains unchanged and the ATP concentration has failed to rise any further. There still remains the possibility that the other cofactor GTP is depleted during anoxia and not resynthesized very rapidly. Evidence obtained in a single Dowex 1 (formate form) chromatogram for an extract of flies that had been allowed to recover for 1 hr. after anoxia for 1 hr. failed to show the joint GTP and UTP peak whereas it could be readily observed in the control extracts.

SUMMARY

1. When whole flies are subjected to anoxia ATP is broken down in the thorax and AMP and IMP accumulate.

2. AMP is rapidly phosphorylated during recovery from anoxia but IMP remains unchanged for at least 2 hr.

3. The return of normal motor activity in the fly parallels the resynthesis of arginine phosphate.

4. The concentration of glycerol 1-phosphate returns to normal in two stages, the first being very rapid and the second slow.

5. The orthophosphate concentration falls to below normal during recovery for 5 min. but by 20 min. is about normal.

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