SUMMARY

1. Some conditions for the successful extraction of labile phosphorus compounds from frozen housefly tissue have been investigated.

2. Evidence has been presented for the existence in insect tissues of a non-phosphorylated substance which interfered with the estimation of ATP by two widely used assay systems.

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Glycerol 1-Phosphate Metabolism in the Housefly (*Musca domestica* L.) and the Effects of Poisons

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Recent papers have been concerned with the effects of anoxia and the insecticides DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] and dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4 α ,5,6,7,8,8 α -octahydro-exo-1,4-endo-5,8-dimethanonaphthalene) on the tissue concentrations of glycerol 1-phosphate and certain other phosphorylated metabolites in houseflies (Winteringham, 1960; Winteringham, Hellyer & McKay, 1960; Heslop, Price & Ray, 1963; Heslop & Ray, 1963; Ray & Heslop, 1963). During the first 20–30 min. of anoxia the concentration of glycerol 1-phosphate in the thoraces of houseflies increased sharply. The size of this increase was successively reduced as dieldrin poisoning was prolonged. At the same time after poisoning the steady-state concentration of glycerol 1-phosphate in the thoraces of treated, aerobic flies also declined. This paper describes results that provide an explanation for the observed effects of dieldrin and DDT poisoning on the accumulation of glycerol 1-phosphate during anoxia. The range of poisons studied has been extended to include 2,4-dinitrophenol, dinoseb (6-sec.-butyl-2,4-dinitrophenol), *n*-valone (2-*n*-valeryl-dane-1,3-dione), DFP (di-isopropyl phosphorofluoridate) and iodoacetate. The important observation of

Winteringham, Hellyer & McKay (1958) on the effect of injected iodoacetate in flies which implied (Winteringham, 1960) that there might be linkages between glycerol 1-phosphate dehydrogenase and glyceraldehyde phosphate dehydrogenase has been confirmed, and additional evidence for such linkage is presented.

MATERIALS AND METHODS

Experimental animals. Adult male houseflies, *Musca domestica* L., PIL strain, which had been fed on sugar and water for 3–5 days after emergence, were used. Cyclopropane anaesthesia was occasionally used as well as cold to immobilize the adult insects for handling. Insecticides were applied topically in acetone, and dinitrophenol and iodoacetate were injected in buffered aqueous solution from an Agla micrometer syringe with a 27 s.w.g. needle. The methods used for subjecting insects to anoxia and other handling techniques were described by Heslop *et al.* (1963).

Preparation and analysis of extracts. Orthophosphate, arginine phosphate, glycerol and glycerol 1-phosphate were determined as before (Heslop *et al.* 1963) save that three extractions of the ground and homogenized material were made instead of two, and that in the glycerol 1-phosphate estimations a graph of the change in extinction at 340 m μ against time was drawn for each estimation so that a cor-

reaction could be made for 'drift'. ATP was determined with phosphoglycerate kinase coupled to glyceraldehyde phosphate dehydrogenase, which were purchased in the form of kits 'for ATP determination in blood' (Boehringer und Soehne, Mannheim, Germany).

Respiration measurements. These were made by conventional manometric techniques or by means of an electrolytic respirometer (Winteringham, 1959).

All results are expressed as μ moles of metabolite/g. of appendage-free thorax $\pm$ s.d. The weight of the thoraces of male flies of this age and strain was found to be 46.5% of the weight of the whole insects.

RESULTS

In Table 1 are set out the results of glycerol 1-phosphate determinations on the thoraces from groups of houseflies taken from different generations. The concentration of thoracic glycerol 1-phosphate varies from one group of flies to another. For example, experiment D 19 differs significantly in this respect from D 30 ($P < 0.02$ by t test), D 32 ($P < 0.01$) and D 31 ($P < 0.001$). Although the thoracic glycerol 1-phosphate concentrations that

were found in D 31 were very much lower than those found in any other experiment with normal flies, the corresponding ATP and total acid-soluble phosphorus concentrations in the same extracts were indistinguishable from those in the thoraces of other flies with 'normal' glycerol 1-phosphate concentrations. It will be seen that the differences in thoracic glycerol 1-phosphate between generations are much larger than those between groups of flies taken from a single generation. The experiments reported below have been carried out each on a single generation of flies. Where an effect, for example, of a poison or of anoxia is reported, it has usually been confirmed with flies from at least one other generation.

Relationship between metabolite concentration and physiological state. Houseflies (150) were subjected to three successive 1 hr. periods of anoxia, alternating with two 15 min. periods in air, and then left in air to recover. At 7 hr. 30 min. after release from anoxia 62% could fly, 7% could walk or make definite movements and 31% were still motionless. Batches of 7-20 flies were weighed, frozen and analysed for glycerol 1-phosphate and ATP (see Table 2). Some flies were flying spontaneously and were very active. These were captured without anaesthetic or cooling and comprised the first group. The remaining 'fliers' were individually observed to fly after stimulation by touch or nearby movement before capture. Out of 20 motionless flies kept for 76 hr. after release from anoxia two died and were rejected. The remainder looked normal, except that they did not move, and were found to use 1.24 ml. of oxygen/g./hr. immediately before freezing, compared with 2.86 ml./g./hr. for control flies actively moving about within the respirometer flask. No correlation was found between ATP concentration and ability to fly but there was less glycerol 1-phosphate in the thorax extracts made from motionless flies than in those which could fly.

Table 1. *Concentrations of glycerol 1-phosphate in the thoraces of normal healthy houseflies*

Groups of 7-20 active adult male flies were killed in liquid nitrogen. Glycerol 1-phosphate, expressed as μ moles/g. wet wt. of thorax $\pm$ s.d. (with the number of observations in parentheses), was determined on perchloric acid extracts. The experiments are in chronological order and in each experiment flies from a single generation were used.

Expt.	Glycerol 1-phosphate
D 19	2.6 ± 0.37 (4)
A 49	2.4 ± 0.15 (4)
D 28	3.4 ± 0.28 (3)
D 30	2.0 ± 0.19 (3)
D 31	0.7 ± 0.14 (3)
D 32	1.9 ± 0.09 (4)

Table 2. *Glycerol 1-phosphate and adenosine triphosphate in the thoraces of houseflies recovering from repeated anoxia*

Flies were subjected to three 1 hr. periods of anoxia alternating with two 15 min. periods in air and were then allowed to recover in air. Groups of 7-20 flies were selected according to symptoms at various times during the recovery period, killed in liquid nitrogen and the thoraces analysed.

Recovery time		Symptoms	ATP (μ moles/g.)	Glycerol 1-phosphate (μ moles/g.)
hr.	min.			
8	0	Very active; make frequent flights	4.9	3.3
8	0	Less active; all could fly	3.6	3.0
8	40	Could move; could not fly	4.4	4.6
8	40		4.9	3.7
8	40		5.8	2.7
8	40		4.1	1.6
76	0	Completely motionless	5.2	1.4
Control		Normal	9.0	2.4
(untreated)				

Effect of repeated anoxia. Flies from two successive generations were found to have identical thoracic glycerol 1-phosphate concentrations and could therefore be compared with one another. Groups (52) of ten flies were killed at various stages of a 5 hr. cycle of alternating 1 hr. periods of anoxia and recovery in air. At the end of each recovery period all of the flies were making movements and most of them could walk well and climb up vertical glass surfaces. Preliminary experiments showed that flies which can climb up glass after a period of anoxia were almost always capable of flight. It was found (Fig. 1) that the concentration of thoracic glycerol 1-phosphate rose during the first 20 min. of each period of anoxia but then declined throughout the rest of the 1 hr. period studied. Immediately the flies were returned to air the amount of thoracic glycerol 1-phosphate fell sharply. Other experiments on recovery from a single 30 min. period of anoxia have shown that the maximum rate of change in concentration of this glycerol 1-phosphate under these conditions is at least $1 \mu\text{mole/g.}$ of thorax/sec. Despite this extremely rapid change the concentration of glycerol 1-phosphate did not return to normal during any of the recovery periods studied. The resynthesis of ATP during recovery from anoxia ceases only 15 sec. after transfer of the insect from oxygen-free nitrogen to air. This corresponds to complete rephosphorylation of the AMP which accumulates in housefly thoraces during anoxia (Ray & Heslop, 1963). The relationship between the amount of ATP in the thoraces of the flies at the start of each anoxic period and the amount of glycerol 1-phosphate which accumulated during anoxia is discussed below.

Effect of iodoacetate. Six batches of ten flies were injected with $15 \mu\text{g.}$ of iodoacetic acid in sodium bicarbonate buffer, pH 7. Three batches were kept in air for 30 min., then in pure nitrogen for 30 min.

and then frozen in liquid nitrogen ('anoxic flies'). The others were frozen 1 hr. after injection ('aerobic flies'). The thoraces of the aerobic flies contained 0.5 ± 0.08 (3) μmole of glycerol 1-phosphate/g. and those of the anoxic flies 0.2 ± 0.2 (3) $\mu\text{mole/g.}$ The corresponding control value was $2.5 \mu\text{moles/g.}$ Although the thoracic ATP concentration in the aerobic flies was $0.1 \mu\text{mole/g.}$ there were 1.5 ± 0.52 (3) μmoles of ATP/g. in the thoraces of the anoxic flies, which indicates that a considerable amount of ATP must have been present in the aerobic flies at the start of anoxia and suggests that the failure of the anoxic flies to accumulate glycerol 1-phosphate was not due to inhibition of glycolysis by ATP-depletion.

Action of dinitrophenols and *n*-valone. After the injection of $1.8 \mu\text{g.}$ of 2,4-dinitrophenol in water the concentration of thoracic ATP was decreased to 3.6 ± 0.74 (3) $\mu\text{moles/g.}$ after 1 hr. and to 2.7 (2) $\mu\text{moles/g.}$ after $2\frac{1}{2}$ hr. The corresponding glycerol 1-phosphate concentrations were 1.8 ± 0.21 (3) and 1.5 (2) $\mu\text{moles/g.}$ respectively. The latter value was just significantly lower than the control ($P < 0.05$). Three groups of ten flies were injected with $1.8 \mu\text{g.}$ of dinitrophenol, kept in air for 30 min., placed in an atmosphere of nitrogen for a further 30 min., and then frozen. This lowered the thoracic ATP concentration to $0.1 \mu\text{mole/g.}$ and raised the concentration of glycerol 1-phosphate to 2.7 ± 0.45 (3) $\mu\text{moles/g.}$, which was significantly higher ($P < 0.05$) than the value obtained under aerobic conditions 1 hr. after the injection of dinitrophenol.

In view of the evidence discussed below that dinoseb and *n*-valone may have a similar mode of lethal action, the effect of these poisons on the concentrations of orthophosphate, arginine phosphate, ATP and glycerol 1-phosphate in housefly thoraces was measured 2 hr. or 5 hr. after the topical administration of different dosages (Tables 3 and 4).

Fig. 1. Effect of alternate exposure to anoxia in oxygen-free nitrogen and recovery in air on the concentrations of glycerol 1-phosphate (○) and ATP (●) in the thoracic tissues of adult male houseflies. Each point represents the mean of determinations on two to four groups of ten flies.

The data in Table 3 were obtained with flies from three different generations as indicated by the experiment designations. Those in Table 4 are all from one generation except for the control ATP (two generations), arginine phosphate (five generations) and orthophosphate (five generations). Preliminary experiments showed that the concentrations of these metabolites in fly thoraces do not vary widely from generation to generation as does the concentration of glycerol 1-phosphate and the data quoted may be compared with one another. The anoxic untreated control ATP, arginine phosphate and orthophosphate values from section (b) of Table 3 may be compared with the values in section (b) of Table 4. The concentration of thoracic glycerol 1-phosphate in untreated flies from four widely separated generations was found to be 18.4 ± 2.24 (10) μ moles/g. and may be used as control to the glycerol 1-phosphate figures in Table 4 (b). In preliminary toxicity trials 1 μ g. of dinoseb and 3 μ g. of *n*-valone both killed about 60 % of the houseflies used in 8 hr.

The actions of these two poisons differ. Although statistically significant breakdown of arginine

phosphate and accumulation of orthophosphate did occur in valone-poisoned flies, the effects were small compared with those of dinoseb. In addition, dinoseb brought about breakdown of ATP and altered the glycerol 1-phosphate concentrations. Two hours after treatment with 1 or 2 μ g. of dinoseb the concentration of glycerol 1-phosphate in thoraces of flies was significantly increased ($P < 0.001$ and < 0.01 respectively). Anoxia during the last 30 min. of the 2 hr. period of intoxication raised the glycerol 1-phosphate concentration in the groups dosed with 1 or 3 μ g. of dinoseb but had no such effect on the glycerol 1-phosphate of those treated with 2 μ g. When increases of glycerol 1-phosphate in dinoseb-treated flies were found as a result of anoxia they were much smaller than those found in untreated flies. Treatment with *n*-valone did not affect the thoracic glycerol 1-phosphate concentration of flies, nor did it modify the effect of anoxia on the concentrations of the metabolites studied.

The oxygen consumption of individual flies poisoned with dinoseb was measured in an electrolytic respirometer. The result of a typical experiment is shown in Fig. 7.

Table 3. *Effects of n-valone*

ATP, arginine phosphate, orthophosphate and glycerol 1-phosphate were determined on thoracic extracts from groups of ten male flies previously treated topically with *n*-valone in acetone. Results are expressed as μ moles/g wet wt. of thorax $\pm$ s.d. (with the numbers of observations in parentheses). Section (b) refers to flies that were made anoxic during the last 30 min. of the period of treatment.

Dose (μ g.)	Time (hr.)	Expt.	ATP	Arginine phosphate	Orthophosphate	Glycerol 1-phosphate
(a) Nil	—	D 28	8.8 ± 0.43 (3)	7.0 ± 0.53 (3)	5.2 ± 0.57 (3)	3.4 ± 0.28 (3)
3	2	D 28	9.2 ± 0.67 (3)	7.1 ± 1.31 (3)	5.4 ± 0.34 (3)	3.3 ± 0.19 (3)
3	5	D 27	7.0 ± 0.33 (3)	4.6 ± 0.51 (3)	6.3 ± 0.28 (3)	4.0 ± 0.24 (3)
6	2	D 28	8.2 (2)	5.9 ± 1.41 (3)	6.7 ± 1.76 (3)	3.4 ± 0.42 (3)
12	2	D 28	9.2 (2)	6.4 (2)	7.8 (2)	3.2 (2)
18	2	D 28	8.2 (2)	4.7 (2)	7.6 (2)	$*3.6 \pm 1.51$ (3)
(b) Nil	—	D 28	0.6 ± 0.57 (3)	0.4 ± 0.18 (3)	13.8 ± 0.66 (3)	17.6 ± 0.65 (3)
3	2	D 28	0.4 ± 0.06 (3)	0.4 ± 0.61 (3)	15.3 ± 1.82 (3)	18.8 ± 0.98 (3)
3	5	D 27	0.1 ± 0.06 (3)	0.2 ± 0.28 (3)	15.5 ± 1.79 (3)	15.7 ± 1.82 (3)
6	2	D 28	0.8 ± 0.19 (3)	1.0 ± 0.76 (3)	11.3 ± 1.10 (3)	18.5 ± 0.59 (3)
18	2	D 29	—	—	—	18.9

* Includes one figure from D 29.

Table 4. *Effect of dinoseb*

Thoracic concentrations of metabolites were measured on batches of ten flies 2 hr. after topical application of the insecticide in acetone and are expressed as μ moles/g. wet wt. of thorax $\pm$ s.d. (with the numbers of observations in parentheses). Section (b) refers to flies that were made anoxic during a 30 min. period immediately before they were killed in liquid nitrogen.

Dose (μ g.)	ATP	Arginine phosphate	Orthophosphate	Glycerol 1-phosphate
(a) Nil	9.1 ± 0.69 (13)	7.0 ± 1.23 (9)	5.6 ± 0.77 (9)	2.0 ± 0.19 (3)
1	6.0 ± 0.21 (3)	4.0 ± 0.33 (3)	13.3 ± 3.19 (3)	5.3 ± 0.75 (6)
2	4.3 ± 0.45 (3)	2.1 ± 0.50 (3)	19.2 ± 4.17 (3)	5.0 ± 1.75 (6)
3	2.4 ± 0.30 (3)	1.3 ± 0.96 (3)	26.1 ± 2.00 (3)	1.8 ± 0.37 (6)
(b) 1	0.7 ± 0.21 (3)	0	26.3 ± 0.55 (3)	9.7 ± 2.38 (3)
2	1.2 (2)	1.4 (2)	30.9 (2)	4.2 (2)
3	1.2 (2)	0.7 (2)	35.4 (2)	2.5 ± 0.42 (3)

Effects of DDT, di-isopropyl phosphorofluoridate and dieldrin. DDT ($1\mu\text{g.}$), DFP (0.5 or $4\mu\text{g.}$) or dieldrin ($0.2\mu\text{g.}$) was applied topically in acetone to flies that were then kept at $20-22^\circ$ for various periods and killed in liquid nitrogen. In some cases the flies were subjected to a period of anoxia immediately before they were killed. The time after treatment includes any period of anoxia. Figs. 2-4 show the thoracic glycerol 1-phosphate concentrations at different times after treatment with DDT, DFP or dieldrin. It is possible to obtain more homogeneous groups of flies with respect to symp-

toms of dieldrin poisoning by treating a large number of individuals and selecting groups of ten as they are 'knocked down' (i.e. just unable to stand). This has been done in the experiments reported and 'time after treatment' represents the sum of the average time ($2\frac{1}{4}$ hr.) between application of dieldrin and observed 'knock-down' and the controlled time between 'knock-down' and tissue freezing in liquid nitrogen.

In the flies not subjected to anoxia dieldrin poisoning brought about a significant reduction in thoracic glycerol 1-phosphate concentration, which confirmed the previous finding of Heslop & Ray (1963). Intoxication with DDT for 4 hr. brought about a barely significant ($P < 0.05$) reduction of thoracic glycerol 1-phosphate concentration in one experiment out of two. A similar reduction 4 hr. after treatment with $0.5\mu\text{g.}$ of DFP was found in one experiment out of three. The dosage of DFP was increased to $4\mu\text{g.}$ in an attempt to resolve this point but no significant effect on glycerol 1-phosphate could be found. It was concluded that although there may be effects of DDT and DFP on the concentration of this metabolite in housefly thoraces they are too small to be measured with the technique used.

Less glycerol 1-phosphate accumulated during 30 min. of anoxia in the flies poisoned with dieldrin DDT and DFP than in normal ones. This effect became more marked as the time of poisoning was increased and it could have been due merely to slowing of glycolytic processes as the symptoms of poisoning advanced. To test this point, groups of

Fig. 2. Glycerol 1-phosphate in the thoracic tissues of houseflies treated with $0.2\mu\text{g.}$ of dieldrin by topical application in $1\mu\text{l.}$ of acetone. $\circ$, Flies left in air throughout; $\bullet$, flies kept under anoxic conditions during the last 30 min. of the period of treatment. Each point is the mean of determinations on two groups of ten flies taken from a single generation.

Fig. 3. Concentrations of glycerol 1-phosphate in the thoracic tissues of houseflies at different times after treatment with $1\mu\text{g.}$ of DDT applied in acetone to the dorsal thorax. $\circ$, Flies left in air throughout; $\bullet$, flies made anoxic for the 30 min. period immediately before killing. Each point is the mean of determinations on three or six batches of ten flies taken from a single generation.

Fig. 4. Glycerol 1-phosphate in the thoracic tissues of houseflies treated topically with $0.5\mu\text{g.}$ ($\circ$, $\bullet$) or $4\mu\text{g.}$ (Δ , $\blacktriangle$) of di-isopropyl phosphorofluoridate in acetone. Open symbols refer to aerobic flies and filled-in ones to flies that were made anoxic during the 30 min. period immediately before they were killed. Each point is the mean of determinations on three to six groups of ten flies.

Fig. 5. Glycerol 1-phosphate concentrations in the thoracic tissues of flies poisoned for 4 hr. with 1 μ g. of DDT (■), 0.5 μ g. of di-isopropyl phosphorofluoridate (●), or 0.2 μ g. of dieldrin (○). The zero anoxia points of the DDT and DFP curves coincided. Various periods of anoxia were included in the 4 hr. period of treatment and the flies (three to six groups of ten flies for each point) were killed in liquid nitrogen while they were still anoxic.

poisoned flies were killed after various periods of anoxia and the glycerol 1-phosphate was determined to give curves (Fig. 5) analogous to the first period of anoxia in Fig. 1. The initial rate of rise of glycerol 1-phosphate concentration after the onset of anoxia was not slower in the poisoned than in the untreated flies. In the flies treated with DDT and dieldrin the failure of glycerol 1-phosphate to continue accumulating to its normal anoxic maximum after 10 min. suggest a corresponding failure of glycolysis.

DISCUSSION

Concentrations of metabolites in tissues. Ray & Heslop (1963) showed that the abnormally low concentration of ATP in the thoraces of flies that had recovered from a period of anoxia was due to a reduction of total acid-soluble adenine and not to abnormal ATP:ADP:AMP proportions. It is possible that the thoracic ATP is divided into compartments, as for example the 'storage' and 'turn-over' ATP postulated for rat abdominal wall muscle by Hohurst, Reim & Bartels (1962). If this is so, it follows from the results in Table 2 that enough ATP to allow normal function of the flight muscle is present in each compartment after the sequence of alternate periods of anoxia and recovery in air which has been used in these experiments. It is suggested therefore that the decreased ATP concentrations in the thoraces of 'recovered' flies after anoxia represent normal ATP distribution with alteration of the amount of ATP only.

Under anaerobic conditions ATP is converted into AMP and IMP, and glycerol 1-phosphate

Fig. 6. The thoracic ATP of houseflies was depleted (Fig. 1) by repeated anoxia and the ATP concentration before anoxia compared with the increase in glycerol 1-phosphate 20 min. (curve A) and 30 min. (curve B) after the start of a period of anoxia. Other groups of flies were poisoned and in some cases subjected to 30 min. of anoxia immediately before they were killed. In these flies the thoracic ATP in treated aerobic flies is compared with the increase in thoracic glycerol 1-phosphate in comparable groups of 30 min.-anoxic flies treated for the same length of time. Untreated controls were similarly compared. It is important to consider the results in relation to the appropriate control (see text). ▲, Flies 2 hr. after topical treatment with 1, 2 or 3 μ g. of 6-sec.-butyl-2,4-dinitrophenol in acetone or the appropriate control; ▼, 2 hr. after injection of 1.8 μ g. of dinitrophenol; □, 4 hr. after 0.2 μ g. of dieldrin was applied topically in acetone or acetone alone; ■, 4 hr. after 1 μ g. of DDT (the appropriate control point corresponded with that of the dieldrin experiment and is not shown); △, 4 hr. after 4 μ g. of di-isopropyl phosphorofluoridate in acetone, and ▽, its appropriate control. Each point represents the mean of determinations on thoraces from two to six batches of eight to ten flies.

accumulates. The accumulation of glycerol 1-phosphate stops at about the same time as the ATP concentration is decreased to zero. At this time there is a substantial accumulation of orthophosphate in the thoraces (Heslop *et al.* 1963). It is suggested that glycolysis ceases after 20–25 min. of anoxia because no more ATP is available for phosphorylation of hexose or hexose phosphate. The amount of glycerol 1-phosphate accumulating

during 20 min. (curve A) and 30 min. (curve B) of anoxia has been compared with the ATP concentration at the start of anoxia (Fig. 6). Curve A has been extrapolated to zero ATP concentration. This extrapolation indicates that in the unpoisoned fly some of the ATP produced by anaerobic glycolysis is used to satisfy the demands of the Embden-Meyerhof pathway for ATP.

Accumulation of glycerol 1-phosphate in poisoned flies during anoxia. Dinoseb, dieldrin, DDT and, to a lesser extent, DFP poisoning reduce the accumulation of thoracic glycerol 1-phosphate which anoxia causes in houseflies. J. P. Heslop (unpublished work) has been unable to find any accumulation of glycerol in DDT-poisoned flies as a result of anoxia. It is suggested that the results in Fig. 6 indicate that under anaerobic conditions these poisons interfere with the phosphorylation steps of glycolysis probably by causing increased breakdown of ATP.

Hewlett (1963) has studied the toxicities of mixtures of dinoseb and *n*-valone to adults of *Alphitobius laevigatus*, the lesser mealworm beetle, and showed that the mortalities were consistent with simple similar joint action as defined by Hewlett & Plackett (1959). This implies that the two insecticides exert similar effects at a common site of action. Gostick (1961), working on the same species, showed that dinoseb, DDT, dieldrin and an organophosphorus insecticide, malathion, all stimulated oxygen uptake by intact adult insects but that *n*-valone did not. There is almost no similarity between the effects of dinoseb and *n*-valone on the concentrations of orthophosphate, arginine phosphate, ATP or glycerol 1-phosphate in the thoraces of intact houseflies under any of the conditions studied. If Hewlett's conclusion is correct, both the stimulation of oxygen consumption of aerobic flies and the inhibition of glycerol 1-phosphate accumulation in anoxic flies after dinoseb poisoning are secondary effects of the poison.

Effect of iodoacetate. Under aerobic conditions, poisoning with a large dose of iodoacetate has been shown to reduce the thoracic glycerol 1-phosphate concentration of houseflies (Winteringham *et al.* 1958; Winteringham, 1960). Baranowsky (1949) has shown that rabbit-muscle glycerol 1-phosphate dehydrogenase is not directly inhibited *in vitro* by iodoacetate. The most probable explanation for the reduction of thoracic glycerol 1-phosphate after iodoacetate poisoning is that glycerol 1-phosphate dehydrogenase is indirectly inhibited by the reduced availability of NADH₂ resulting from the direct inhibition of glyceraldehyde phosphate dehydrogenase (Winteringham, 1960). Alternatively it was possible that the very active glycerol 1-phosphate-oxidase system in dipteran flight muscle might be able to oxidize all the glycerol

1-phosphate formed under conditions of inhibited glycolysis, but in this case anoxia should bring about a rise in glycerol 1-phosphate concentration in iodoacetate-poisoned flies. This did not occur. Evidence is presented above that there was some ATP in the thoraces of the poisoned flies at the start of the period of anoxia to support glycolysis. It was concluded that the failure of iodoacetate-poisoned flies to accumulate glycerol 1-phosphate during anoxia was due to inhibition of glycerol 1-phosphate dehydrogenase as a result of tight linkage with glyceraldehyde phosphate dehydrogenase through the coenzyme. Other evidence supports this conclusion. Mr R. G. Bridges (personal communication) injected large quantities of [¹⁴C]-glycerol into houseflies and found that radioactivity was rapidly incorporated into glycerol 1-phosphate, trehalose and glycogen. However, when massive injections of [1-¹⁴C]acetate were made into houseflies (Mr G. M. Price, personal communication) no labelling of hexoses could be detected, indicating that glycolysis cannot be reversed by large injections of acetate. If there were tight linkage between triose phosphate and glycerol 1-phosphate dehydrogenases, reversal of glycolysis would imply reversal of the whole of the respiratory chain of the glycerol 1-phosphate-oxidase system. This is most unlikely and suggests that glycolysis in housefly flight muscle is functionally irreversible at the triose phosphate-dehydrogenase step.

The transfer of hydrogen from extramitochondrial NADH₂ to intramitochondrial oxidase systems can be mediated by 'shuttles' based on diffusible substances, which can be reduced outside and oxidized within the mitochondria (Boxer & Devlin, 1961). In flight muscle of the blowfly (*Phormia regina*) Sacktor & Dick (1962) point out that malate and glycerol 1-phosphate dehydrogenases are present in the cytoplasm, but they were unable to achieve more than a slight temporary stimulation of the oxidation of NADH₂ by teased flight-muscle preparation when oxaloacetate was added. In contrast, addition of dihydroxyacetone phosphate in catalytic quantities greatly stimulated oxidation of NADH₂ by such preparations. This suggests that it is unnecessary to propose any structural basis for a linkage between triose phosphate and glycerol 1-phosphate dehydrogenases. If there were only one active shuttle system for hydrogen transfer from NADH₂ into housefly flight-muscle mitochondria a tight functional linkage between the enzymes would necessarily follow.

Steady-state concentration of glycerol 1-phosphate. In the thoraces of normal houseflies the concentration of glycerol 1-phosphate can vary widely (Table 1). The differences between one generation and another are very much larger than those between groups of flies taken from a single genera-

tion and show no relationship to flight ability. Abnormally high thoracic glycerol 1-phosphate concentrations are found in flies that have recovered from anoxia and appear to be quite normal (Ray & Heslop, 1963). The finding by Heslop & Ray (1963) that thoracic glycerol 1-phosphate can vary between different strains of houseflies may be merely an example of this sort of variability. It need not indicate a real difference between the strains.

Insecticidal poisoning can affect the glycerol 1-phosphate concentration in the thoraces of aerobic flies. Dieldrin and larger doses of dinitrophenols cause it to fall and small doses of dinitrophenols bring about a rise (Fig. 2 and Table 4). Novotny, Kubista & Foustka (1963) have found increases in glycerol 1-phosphate in preparations of cockroach metathorax 5 min. after perfusion with dinitrophenol and have shown that they were not due to partial anaerobiosis. No increase of glycerol 1-phosphate was found 40 min. after perfusion with dinitrophenol. Novotny *et al.* (1963) treated their preparations with dinitrophenol and in some cases simultaneously placed them in oxygen-free nitrogen for 10 min. Their results and those of Novotny (1960) demonstrated an increased rate of both aerobic and anaerobic glycolysis after poisoning with dinitrophenol. The surge of respiration in dinitrophenol poisoning (and presumably the uncoupling action) occurs very soon after treatment (Novotny *et al.* 1963; Fig. 7), and any reversal of the uncoupling effect at the high dosage studied is improbable, but both increases and decreases of thoracic glycerol 1-phosphate can be found in treated flies 2 hr. after treatment with different doses. This indicates that the cause of the dinitrophenol effects on the concentration of glycerol 1-phosphate in the thoraces of aerobic flies must lie

outside the respiratory chain and this is supported by the fact that Novotny (1960) found similar effects of dinitrophenol on the rates of both aerobic and anaerobic glycolysis.

Other pathways of glycerol 1-phosphate metabolism. If flies were subjected to anoxia the concentration of glycerol 1-phosphate in the thorax increased during the first 20–30 min. If, however, the period of anoxia were prolonged for a further 30–40 min. then the concentration of glycerol 1-phosphate in the thorax started to fall (Fig. 1). A similar disappearance occurs in whole flies (Heslop *et al.* 1963), indicating that it is not due to transport of glycerol 1-phosphate to other parts of the insect. Since (a) there is no evidence that glycerol 1-phosphate is converted into glycerol after 25 min. of anoxia (Heslop *et al.* 1963), (b) there is no significant incorporation of labelled glycerol or glycerol 1-phosphate into fats (R. G. Bridges, personal communication) and (c) it is not likely that glycolysis could be reversed when the concentration of ATP is very low and the glycerol 1-phosphate-oxidase system is completely inhibited, it is suggested that an additional pathway for glycerol 1-phosphate catabolism must exist.

SUMMARY

1. The concentration of glycerol 1-phosphate in normal houseflies was found to vary widely from one generation to another.

2. Some insecticides have been shown to affect the thoracic glycerol 1-phosphate and ATP concentrations in aerobic flies.

3. The amount of glycerol 1-phosphate that accumulates in the thoraces of unpoisoned flies during anoxia has been shown to be related to the ATP in the thoraces at the start of anoxia. This relationship was upset by poisoning with dinitrophenol, 6-*sec.*-butyl-2,4-dinitrophenol, DDT, dieldrin and di-isopropyl phosphorofluoridate, but not with 2-*n*-valerylindione-1,3-indane.

4. The effect of injected iodoacetate on glycerol 1-phosphate and ATP in housefly thoraces under aerobic conditions and after anoxia has been studied.

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Fig. 7. Oxygen consumption of individual flies after topical treatment with 1 μ l. of acetone (—) or 1 μ g. of dinoseb in 1 μ l. of acetone (---).

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The Effect of Ultrasonic Vibrations on the Protein-Synthesizing Activity of Microsome Preparations from Rat Liver

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One approach for the preparation of cell fragments with biological activity is the application of ultrasonic vibrations. In studies on protein synthesis this method has been used on mouse-ascites-tumour cells (Martin, Malec, Coote & Work, 1961) and on calf-heart mitochondria by Kalf & Simpson (1959) and Kroon (1963). In previous work on the synthesis of serum albumin by liver microsomes it was noticed that a marked clearing of a suspension of liver microsomes could be achieved by the application of ultrasonic vibrations. From this work it seemed possible that such treatment of liver microsomes might provide a particulate fraction, active in protein synthesis but possessing a simpler morphological structure. It was also possible that an active soluble preparation might be produced.

The present paper shows that treatment of liver microsomes by ultrasonic vibrations has an inhibitory effect on their ability to incorporate ^{14}C -labelled amino acids into protein. Similar treatment of ribonucleoprotein particles has a less marked effect and seems to be mainly associated with the release of a ribonucleic acid fraction important for protein synthesis.

A preliminary report of some aspects of this work has been given by Decken & Campbell (1961). Ogata, Watanabe, Morita & Sugano (1962) have confirmed these findings and have produced evidence for the metabolic specificity of ribonucleic acid released from liver ribonucleoprotein particles by ultrasonic treatment (Sugano, Ogata, Mirota, Tominaga & Watanabe, 1962).

Some aspects of the present work have been reported in a preliminary form (Campbell & Cooper, 1963).

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MATERIALS AND METHODS

Chemicals. The dipotassium salt of ATP, the sodium salt of GTP, and NADH and NADP⁺ were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Pyruvate kinase, the silver barium salt of PEP,† the barium salt of glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Solutions of the free acid of PEP were prepared before use and adjusted to pH 7.4 (glass electrode) with N-KOH. Solutions of the free acid of glucose 6-phosphate were prepared and adjusted to pH 6.5 (glass electrode) with N-KOH. Tris and GSH were obtained from the British Drug Houses Ltd., Poole, Dorset. The sodium salt of deoxycholic acid was obtained from Merck A.-G., Darmstadt, Germany, the non-ionic detergent Lubrol W from Imperial Chemical Industries Ltd., Manchester, and polyU from Miles Laboratories, Stoke Poges, Slough, Bucks.

Radioactive amino acids, uniformly labelled L-[^{14}C]-leucine (45.6 mc/m-mole) and L-[^{14}C]-phenylalanine (61.9 mc/m-mole), were obtained from The Radiochemical Centre, Amersham, Bucks., and [^3H]polyU was kindly given by Dr T. Hultin.

Animals. Rats (150–200 g. body wt.) were Wistar albino strain highly inbred at this Institute. Regenerating liver was obtained from rats 2 days after partial hepatectomy by the method of Higgins & Anderson (1931). The rats were kept without food for approx. 18 hr. before being killed by a blow on the head followed by decapitation.

Tissue preparations. Rat liver was homogenized in medium A containing MgCl_2 (10 mM), tris buffer (35 mM) (pH 7.8 at 25°), KCl (25 mM) and sucrose (0.15 M). Microsomes, RNP particles, cell sap and pH 5 fraction were prepared as described by Decken & Campbell (1962). When necessary, 'washed' microsomes were prepared by resuspending the pellet from 4 g. of liver in medium A (11 ml.)

† The abbreviations used are: PEP, phosphoenolpyruvic acid; polyU, polyuridylic acid; RNP, ribonucleoprotein.