Phosphorylated Compounds in the Head and Thoracic Tissues of the Adult Housefly, *Musca domestica* L., during Flight, Rest, Anoxia and Starvation

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The labelled-pool technique (Winteringham, 1956) has been used for studying the effects of insecticides on the relative concentrations of the soluble phosphorus compounds of intact adult houseflies. To facilitate the interpretation of the data so obtained it was also necessary to study the effects of physical activity, starvation etc. alone. These effects are now reported and discussed in the light of current ideas about carbohydrate metabolism and its regulation in insects.

Insects derive energy mainly by the oxidation of endogenous carbohydrate and fat, in that order, but during sustained flight Diptera appear to utilize only carbohydrate (Chadwick, 1947; Clements, 1955; Krogh & Weis-Fogh, 1951; Kubista, 1958; Sacktor, 1955; Wigglesworth, 1949; Zebe, 1954).

Carbohydrate catabolism and its regulation in insect muscle have recently received particular attention (Chefurka, 1958*a*; Silva, Doyle & Wang, 1958). Of considerable interest have been the observations that insect flight muscle contains remarkably high and low concentrations respectively of α -glycerophosphate dehydrogenase and lactic acid dehydrogenase (Sacktor, 1955; Zebe & McShan, 1957). There was little accumulation of lactic acid under conditions of anaerobic glycolysis; instead there was an accumulation of α -glycerophosphate and possibly of pyruvate and acetate (Barron & Tahmisian, 1948; Chefurka, 1958*a*; Humphrey, 1949; Kubista, 1957, 1958; Winteringham, 1958). No less than 20% of the total acid-soluble phosphorus in the thoracic tissues (mainly flight muscle) of the resting adult housefly appears in the form of α -glycerophosphate (Winteringham, 1959). Kubista (1957) and Chefurka (1958*b*) suggested that α -glycerophosphate dehydrogenase replaced lactic acid dehydrogenase in the oxidation of reduced coenzyme I in glycolysing insect muscle. Bücher and his colleagues (Bücher & Klingenberg, 1958) and, later, Sacktor (1958) have proposed a central role for α -glycerophosphate in the control of carbohydrate metabolism in insect flight muscle.

MATERIALS AND METHODS

Diphosphopyridine nucleotide and crystalline α -glycerophosphate dehydrogenase (from muscle). These were pur-

chased from C. F. Boehringer und Söhne, Mannheim-Waldhof, Germany.

L- α -Glycerophosphate. The sodium salt was the kind gift of Dr R. W. Estabrook.

Insects and their treatment. Female adult houseflies (*Musca domestica* L.) not less than 3 days old at the time of extraction were used throughout. Unless stated otherwise in the tabulated results (below) the soluble phosphorus compounds were uniformly labelled with ^{32}P *in vivo*, as described earlier (Winteringham, Bridges & Hellyer, 1955), so that the specific radioactivity of the phosphorus of each compound was the same.

Extraction and paper-radiochromatographic analysis. After the soluble phosphorus pool had been labelled *in vivo*, groups of 10 adult female insects were normally immobilized by exposing them to cyclopropane for about 2 min., and were then plunged into liquid nitrogen to await dissection and extraction. The labelled compounds were extracted with ethanolic formic acid from the separated heads and thoraces (minus wing and leg appendages) with the slightly modified conditions already described (Winteringham, Hellyer & McKay, 1958). The extracted compounds were separated by chromatography and assayed radio-metrically (Winteringham *et al.* 1955, 1958). Any special treatment of the insects before extraction is explained in the tabulated results below.

Chemical identity of the labelled fractions. Cochromatographic tests with authentic triose phosphates which had not been available in the earlier work strongly suggested that the compound described in the earlier papers (Winteringham *et al.* 1955, 1958) as phosphoglycerate was in fact α -glycerophosphate (Winteringham, 1959). This was confirmed enzymically as follows.

The oxidation of α -glycerophosphate (α -GP) in the presence of diphosphopyridine nucleotide (DPN) with the formation of dihydroxyacetone phosphate (DAP) and reduced DPN (DPNH) is specifically catalysed by muscle α -glycerophosphate dehydrogenase according to the equation

with an equilibrium constant of 0.17×10^4 at pH 8.1. (McShan, 1949; Beisenherz, Bücher & Garbade, 1955). In the present work 0.1M-hydrazine sulphate as a trapping agent for DAP only changed the equilibrium constant to 2.4×10^2 at pH 8.1. However, in the presence of hydrazine and a large excess of DPN a trace of ^{32}P - α -GP would thus be largely converted into the acid-labile ^{32}P -DAP.

The fraction behaving as ^{32}P - α -GP under the conditions of paper chromatography (Winteringham, 1959) equivalent to two thoraces was reconcentrated in a 1 mm. section of Whatman no. 1 paper (Winteringham, 1953) and dissolved in 0.5 ml. of water. On the basis of total soluble thoracic

phosphorus estimated by radioactivation (Winteringham *et al.* 1955), and the paper-radiochromatographic analysis of the particular extract used, 0.15 ml. of the above solution was equivalent to 0.013 μ mole of possible α -GP. Samples of 0.15 ml. were diluted to 3 ml. with water (2.85 ml.) which also contained hydrazine sulphate (250 μ moles), DPN (10 μ moles) and active or heat-inactivated α -glycerophosphate dehydrogenase (0.4 mg.) at pH 8.1 (0.05 M-2-amino-2-hydroxymethylpropane-1:3-diol buffer). The formation of DPNH was followed spectrophotometrically at 340 m μ , and in the presence of the active enzyme alone reached the equivalent of 0.013 μ mole of α -GP. This was in agreement with that estimated by paper-radiochromatographic and radioactivation analysis. The mixtures were finally acidified with conc. HCl and hydrolysed for 10 min. at 100°, and the hydrolysate was examined by paper-radiochromatography. Under these conditions any DAP formed enzymically would liberate its phosphorus as inorganic phosphate. The original 32 P behaved almost entirely as inorganic phosphate in the final radiochromatogram. These experiments thus confirmed that the fraction originally described as phosphoglycerate and representing some 20% of the total soluble thoracic phosphorus of the resting insect is L- α -glycerophosphate.

It is reiterated that in the tabulated results below the description of a 32 P-labelled fraction as a particular compound must not be taken to exclude the presence of other unidentified compounds in relatively small but possibly variable proportions. Fractions designated UC. 1, etc., are partly or completely resolved under the routine conditions of paper chromatography used but await identification. Recent work by the author's colleagues Mr R. G. Bridges and Mrs J. R. Beard has shown that the fast-running somewhat variable fraction earlier designated as UC. 4 (Winteringham *et al.* 1958) is almost certainly a soluble phospholipid fraction and is now so described in the tables. Up to 18% of the soluble thoracic 32 P has been extracted in this form from insects provided with dietary milk for 24–48 hr. preceding extraction.

The following abbreviations have been used in the tables and text: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), glucose 6-phosphate (G6-P), arginine phosphoric acid (Arg-P), inorganic phosphate (PO_4^{3-}), the phospholipid fraction recovered in ethanolic formic acid used for extraction (P lipid), unidentified phosphorus compounds (UC. 1, UC. 2 and UC. 3). Unresolved composite fractions have been indicated by brackets. Analyses of head and thoracic extracts from the same group of 10 insects is indicated by a common experiment number in the table.

RESULTS

Tabulation and significance of the data. The results are collected in Tables 1–6. The 32 P of each separated compound or fraction is expressed in units per cent of the total soluble 32 P recovered from the head (rich in nervous tissue, poor in muscle) or thorax (mainly flight muscle). Since the compounds are uniformly labelled, changes in the distributions of 32 P also represent changes in the relative tissue concentrations at the instant of chilling and extraction. As in the earlier work the

distribution of phosphorus found in a single experiment has been taken as the mean of the two paper-radiochromatographic analyses made. The significance of differences between the results of single experiments and the means of several experiments has been estimated by the *t* test and corresponding levels of probability (*P*) are indicated. In applying the *t* test it has been assumed that the variance in experiments with treated insects would be the same as that found in the corresponding groups of control experiments and indicated by the standard errors of the means in Tables 1 and 2. The use of the angular transformation for testing the significance of differences between mean values of less than 15% (see Bliss & Calhoun, 1954) led to increased values of *t*. Its use was therefore abandoned as a precautionary measure.

Phosphorus compounds during rest. In Table 1 are the mean distributions of soluble phosphorus of the head tissues (11 groups of 10 flies of different generations) and of the thoracic tissues (49 groups of 10 flies representing 30 different generations). In all these experiments the insects were brought to a state of rest by the addition of cyclopropane to the air drawn through the metabolism chamber. An exposure of about 2 min. was usually adequate.

Phosphorus compounds during activity. In some experiments the insects were transferred to a small stainless-steel-gauze cage while under mild cyclopropane anaesthesia. They were then allowed to recover completely and were either standing or walking at the instant of plunging them into liquid nitrogen. The results are collected in Table 2. The only significant difference between the phosphorus distribution of the resting insect and that of the active insect was in the level of thoracic α -GP. The

Table 1. *Distribution of mean soluble phosphorus in the adult housefly at rest*

All insects were brought to rest by exposure to an air-cyclopropane mixture for about 2 min. before plunging them into liquid nitrogen and subjecting them to dissection, extraction, etc.

Fraction	32 P of fraction (% of total soluble 32 P recovered from head or thorax $\pm$ S.E.M.)	
	Head (11 expts.)	Thorax (49 expts.)
ATP + UC. 1	35.9 $\pm$ 2.1	34.7 $\pm$ 0.69
ADP	4.9 $\pm$ 0.7	3.7 $\pm$ 0.34
AMP + UC. 2	4.3 $\pm$ 0.4	3.6 $\pm$ 0.27
G 6-P	10.6 $\pm$ 0.9	9.1 $\pm$ 0.46
UC. 3	0.8	Trace
Arg-P	6.2 $\pm$ 0.5	10.5 $\pm$ 0.49
α -GP	13.0 $\pm$ 1.2	20.9 $\pm$ 0.66
PO_4^{3-}	20.9 $\pm$ 1.3	16.8 $\pm$ 0.67
P lipid	3.4	0.7
	100	100

mean level of 20.9% of the resting insect fell significantly to 12.7% on resumption of visible activity ($P < 0.01$). As in other experiments these results also show that a significant change in the distribution of thoracic phosphorus was not invariably followed by a corresponding change in the head tissues. This may be due to the predominance of muscle- and nervous-tissue sources of phosphorus respectively in these two regions.

Phosphorus compounds during flight. Four experiments were made in which groups of individual insects were freely suspended by fine wires tied round the constriction between thorax and abdomen or held by suction applied to the dorsal thorax through a 1 mm. capillary. Momentary contact of the legs sometimes stimulated these

insects into full captive flight. They were then immediately plunged into liquid nitrogen for extraction. The reluctance of some insects to attempt flight was apparently associated with their confinement to the relatively small glass metabolism chambers used or with the limited diet of aqueous glucose or both. In two experiments therefore the insects were transferred to a very large glass chamber and provided with milk, glucose and water *ad lib.* for several hours before the experiment. These insects had recovered their normal tendency to fly on stimulation. All the results are shown in Table 3. A consistent change which occurred on stimulating the non-anaesthetized insect into full flight was in the level of thoracic PO_4^{3-} , which rose significantly ($P < 0.01$) from a mean value of 20.4 (Table 2) to 28.0 (Table 3) during flight. Comparison of the data of Tables 2 and 3 suggest that the increase in PO_4^{3-} during flight was due to the breakdown of the composite fraction which contained Arg-P. No change in thoracic nucleotide levels or in any of the head compounds was indicated.

Phosphorus compounds during anoxia by drowning. Insects were transferred to stainless-steel-gauze cages while under cyclopropane anaesthesia and allowed to recover completely. They were then immersed in water containing a trace of Teepol wetting agent and were agitated to remove adhering air bubbles. In all cases struggles persisted for about 5 min. After different times the insects were quickly rinsed in acetone to remove superficial water and plunged into liquid nitrogen for extraction etc. The results are shown in Table 4.

The results suggest a rapid rise in the level of α -GP to a constant value of about 29% (mean level after 5 min. or more), which was significantly higher than that of the resting or active insect

Table 2. *Distribution of mean soluble phosphorus in the adult housefly during activity not involving flight*

No anaesthesia was used; all insects were standing, walking, etc., at the instant of death. In two experiments milk was added to the diet after labelling *in vivo*.

Fraction	³² P of fraction (% of total soluble ³² P recovered from head or thorax $\pm$ S.E.M.)	
	Head (7 expts.)	Thorax (9 expts.)
ATP + UC. 1	32.0 $\pm$ 2.6	32.2 $\pm$ 1.9
ADP	5.0 $\pm$ 0.7	4.7 $\pm$ 0.8
AMP + UC. 2	4.0 $\pm$ 0.9	2.5 $\pm$ 0.6
G 6-P	8.7 $\pm$ 1.0	8.3 $\pm$ 1.2
UC. 3	2.0	0.7
Arg-P	9.3 $\pm$ 2.5	13.4 $\pm$ 1.7
α -GP	13.0 $\pm$ 1.4	12.7 $\pm$ 1.3
PO_4^{3-}	21.7 $\pm$ 1.6	20.4 $\pm$ 1.7
P lipid	4.3	5.1
	100	100

Table 3. *Distribution of soluble phosphorus in the adult housefly during flight*

Insects were plunged into liquid nitrogen during captive flight. In Expts. 3 and 4 the insects were first conditioned by supplementary dietary milk and by the provision of adequate flying space.

Expt. no.	...	³² P of fraction (% of total soluble ³² P recovered from head or thorax)					Mean values (thorax only)		
		Head (4)	Thorax						
			(1)	(2)	(3)	(4)			
Fraction									
ATP + UC. 1		38.7	51.6	28.9	37.2	34.8	41.0		
ADP		4.2		5.5	0	6.1			
AMP + UC. 2	}	7.9	8.6	2.1	16.8	5.8	16.2		
G 6-P		Trace		8.4		0			
UC. 3				0					
Arg-P				6.2		16.7			
α-GP		13.7	10.2	15.6	9.4	7.9	10.8		
PO ₄ ³⁻		21.6	29.6	27.2	26.5	28.7	28.0		
P lipid		Trace	0	6.1	10.1	Trace	4.0		
							100		

($P < 0.01$). There was a delayed but obvious breakdown of the ATP+ADP fraction with the formation of PO_4^{3-} . Although barely significantly different from that of the resting insect ($0.1 > P > 0.05$) the high AMP value after 300 min. suggests that AMP is another product of nucleotide breakdown. The level of Arg-P after 300 min. was significantly lower than that of the resting insect ($P < 0.05$). Thus extended anoxia caused a drastic breakdown of high-energy-phosphorus compounds but this breakdown might have been a post-mortem change.

Phosphorus compounds during starvation. A common effect of insecticides in adult houseflies is

Table 4. *Effects of drowning on the distribution of thoracic soluble phosphorus in the adult housefly*

Non-anaesthetized insects were submerged in air-free water containing a wetting agent, for different times, before extraction.

Expt. no. ...	³² P of fraction (% of total soluble ³² P recovered from the thorax)		
	(1)	(2)	(3)
Duration of anoxia (min.)	5	120	300
Fraction			
ATP + UC. 1	30.7	3.9	5.9
ADP			
AMP + UC. 2	13.3	16.6	7.0
G 6-P			5.0
UC. 3			0
Arg-P			2.8
α-GP			27.7
PO_4^{3-}	29.1	42.1	49.2
P lipid	0	5.2	2.4

loss of locomotive ability and co-ordination so that the insect is unable to feed or drink, exhausts its endogenous food and becomes desiccated. Experiments were therefore made to determine the effects of starvation alone. The results are summarized in Table 5.

It will be seen that, provided that the insects were behaving normally up to the time of extraction, starvation and the contingent desiccation had no effect. The distributions of head and thoracic phosphorus of Expt. 1 were not significantly different from those of the normal resting insect (Table 1). At the stage of prostration (Expt. 2), however, there was a significant fall ($P < 0.01$) in the head ATP, the phosphorus appearing as PO_4^{3-} . That this was not some degenerative change associated with the onset of death was shown by its complete reversal by glucose injection and recovery of the insect (Expt. 3). Expt. 2 also demonstrated that changes in the head and thoracic tissues may occur independently (see above) because there was no significant change in thoracic phosphorus at prostration. Expts. 2 and 3 may be compared with those of Table 2 because the insects were not immobilized with cyclopropane.

Expt. 4 shows that when the condition of the prostrated insects had deteriorated to the point where glucose therapy was ineffective (1 hr. or more after becoming prostrate) there was an obvious breakdown in ATP and accumulation of PO_4^{3-} in both head and thoracic tissues.

Phosphorus compounds under various conditions. It was possible that the difference in thoracic α-GP levels in the so-called resting insect (Table 1) and in

Table 5. *Effects of starvation on the distribution of soluble phosphorus of head and thorax in the adult housefly*

Expt. 1. Insects were deprived of food and water for 5–10 hr. and finally were anaesthetized with cyclopropane, plunged into liquid nitrogen etc. All insects behaved normally up to the time of extraction. *Expt. 2.* Insects were starved to the point of complete prostration (10–12 hr.: on backs, no wing or leg movements). Individual insects were transferred to liquid nitrogen without anaesthesia immediately after becoming prostrate. *Expt. 3.* Exactly as in (2) but prostrate insects were intrathoracically injected with aqueous glucose (200 μg.). They were transferred to liquid nitrogen 1 hr. after injection of glucose, when all the insects had recovered and were behaving normally. *Expt. 4.* Insects were starved to death (20–24 hr.; no recovery on glucose injection).

Expt. no. ...	³² P of fraction (% of total soluble ³² P recovered from the head or thorax)							
	Head				Thorax			
	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Fraction								
ATP + UC. 1	35.9	14.8	28.4	9.4	29.9	27.8	28.1	11.9
ADP	± 4.7	± 5.8	± 8.6	± 2.6	± 6.8	± 4.6	± 6.1	± 3.5
AMP + UC. 2	22.2	3.8	7.4	3.9	2.4	30.4	27.0	4.9
G 6-P		14.8	11.0	7.0	8.8			7.5
UC. 3		Trace	Trace	4.1	0.7			0.6
Arg-P		3.7	5.1	3.7	12.4			3.6
α-GP		8.2	12.4	8.5	21.8			5.9
PO_4^{3-}	21.1	42.4	19.1	49.1	14.8	21.5	17.1	53.9
P lipid	5.1	6.5	8.0	11.7	2.4	4.6	10.0	8.2

the active insect (Table 2) was not due to a fall in α -GP during activity but due to an accumulation as a result of some pharmacological or toxic effect (e.g. anoxia) of the anaesthetic itself. This seemed unlikely, however, because if the insect were deeply anaesthetized for more than 3 hr. by exposure to air-cyclopropane mixture there was no further change in head or thoracic phosphorus distribution beyond that which occurred on cessation of activity after 2 min. (cf. Expt. 1 of Table 6 with the results of Table 1). Similarly, deep anaesthesia with 1:2-dichloroethane vapour had no significant effect on the α -GP level (Expt. 2). It has already been shown that under conditions of anoxia there is a marked further rise in thoracic α -GP (Table 4).

In studying the effects of insecticides these were invariably applied topically in acetone solution to the dorsal surface of the thorax or were injected intrathoracically in water or acetone while the insect was under mild cyclopropane anaesthesia. For this purpose an Agla micrometer syringe (Burroughs Wellcome and Co., London) fitted with a 27 s.w.g. needle was always used. Numerous experiments have shown that provided that the insect was not injured (as shown by its rapid recovery of normal activity) there was no significant effect on phosphorus distribution. The results of Expts. 3 and 4 of Table 6 are typical. Insects which showed any sign of injury as a result of treatment or handling were never used in the preparation of extracts. Other experiments (Winteringham *et al.* 1955) have shown that extension of the metabolism time from 24 to 120 hr. had no effect on thoracic phosphorus distribution. Higher but variable proportions of ^{32}P were sometimes

found in the P-lipid fractions of the thoraces of insects supplied with dietary milk (mean value 8.9 %), but there were no effects on the distribution of ^{32}P between the remaining fractions.

DISCUSSION

Metabolic regulation during flight. There is evidence that in Diptera the blood trehalose provides an immediate carbohydrate reserve for the flying insect, that it is broken down as far as pyruvate through the classical Embden-Meyerhof pathway, and oxidized by means of the Krebs cycle (Chefurka, 1958*a*; Evans & Dethier, 1957; Winteringham, 1957, 1958). The assumption of full flight leads to a dramatic increase in respiration (Sacktor, 1958) and this in turn suggests critical changes at certain rate-limiting steps of oxidative metabolism. A much simplified but likely scheme of trehalose catabolism and associated ATP synthesis is illustrated in Fig. 1. A remarkably high rate of α -GP oxidation through a structurally bound DPN-independent oxidase (Fig. 1, reaction 5) from locust-wing muscle led Bücher and his colleagues (Bücher & Klingenberg, 1958) to postulate an α -GP cycle (see Fig. 1). In the conventional mechanism of respiratory control (Lardy, 1955), ADP and PO_4^{3-} arising from an energy-dissipating process such as the rapid contractions of flight muscle provide the necessary substrates for oxidative phosphorylation and the contingent flow of electrons through the respiratory chain. In the postulated α -GP cycle this would permit the rapid oxidation of α -GP by the structurally-bound DPN-independent oxidase shown as reaction 5 (Fig. 1).

Table 6. *Effects of extended anaesthesia, acetone and water injections on distribution of soluble phosphorus in the adult housefly*

Expt. 1. Insects were deeply anaesthetized by continuous exposure to an air-cyclopropane mixture for 3.5 hr. and finally were removed to fresh air for about 30 min. until there were slight movements, to ensure that dead insects were not included. *Expt. 2.* Deep anaesthesia was induced by exposure to 1:2-dichloroethane vapour-air mixture for 6 min. *Expt. 3.* Flies were anaesthetized and extracted 2 hr. after injection of 1 $\mu\text{l.}$ of acetone. *Expt. 4.* Flies were anaesthetized and extracted 2 hr. after injection of 2 $\mu\text{l.}$ of water. Insects were allowed 48 hr. metabolism after labelling *in vivo*.

		^{32}P of fraction (% of total soluble ^{32}P recovered from the head or thorax)					
		Head		Thorax			
Expt. no.	...	(1)	(4)	(1)	(2)	(3)	(4)
Fraction							
ATP + UC. 1		37.3	31.9	30.6	35.1	36.7	30.5
ADP		± 2.2		± 2.0	0	0	± 3.7
AMP + UC. 2	}	27.0	22.9	33.0	4.0	0	1.8
G 6-P					9.0	11.6	6.5
UC. 3					0	0	0
Arg-P					5.6	13.5	19.0
α -GP		14.4	17.6	17.0	15.9	15.1	15.1
PO_4^{3-}		19.1	19.4	17.4	30.4	23.1	23.4
P lipid		Trace	8.2	Trace	0	Trace	0

mechanism of control of α -GP-oxidase activity by an endogenous chelating agent in the flight muscle: 'During rest, glycerol-3-P oxidase is in an inhibited state... in flight, the inhibition is reversed and this brings about the high respiratory rate characteristic of the glycerol-3-P cycle.' Whether the α -GP cycle is under respiratory control as suggested by Bücher or controlled by the action of an endogenous chelating agent as suggested by Sacktor it will be seen that in either event α -GP might be expected to accumulate during rest and to become depleted during flight. The present work has shown that the level of α -GP fell significantly in the transition from cyclopropane-induced rest to the activity associated with walking or standing, but there was no further significant fall on the assumption of full flight. There was, however, a significant rise in the level of free PO_4^{3-} and this would be consistent with respiratory control in the intact flying insect. The methods used were probably insufficiently sensitive to detect small increases in amounts of phosphate acceptors such as ADP, although the fall in the fraction containing arginine phosphoric acid suggests that free arginine may also have increased during flight.

Effects of anoxia. If, in insect flight muscle, the DPN-dependent α -GP dehydrogenase (Fig. 1, reaction 8) replaces lactic acid dehydrogenase (reaction 7) in the reoxidation of DPNH during anaerobic glycolysis, an accumulation of α -GP would be expected under the same conditions because reaction 5 would be inoperative and reaction 7 absent. Kubista (1957) has demonstrated such an accumulation in the isolated thoracic muscles of the cockroach *Periplaneta americana*. A similar accumulation has now been found in the muscle of the intact housefly but, unlike the results of Kubista, there was also an increase in inorganic phosphate as a result of the breakdown of high-energy phosphates.

Effects of SH reagents. In a previous paper (Winteringham *et al.* 1958) it was reported that iodoacetate or excessive doses of methyl bromide vapour caused a fall in the phosphoglycerate level of adult houseflies. This effect should now be described as a fall in α -GP, which, however, would still be consistent with the inhibition of triose phosphate dehydrogenase *in vivo*. Inhibition of the SH-dependent triose phosphate dehydrogenase (Fig. 1, reaction 6) would reduce the supply of DPNH for reaction 8. Unless reaction 5 were equally sensitive to the SH reagent a depletion of α -GP would then occur.

SUMMARY

1. The presence of α -glycerophosphate representing some 20% of the total soluble phosphorus

of the thoracic tissues of the resting intact adult housefly has been confirmed enzymically.

2. The transition from cyclopropane-induced rest to normal activity was associated with a fall in thoracic α -glycerophosphate. A further fall on the assumption of full flight was not apparent but the level of inorganic phosphate rose significantly.

3. Anoxia due to drowning caused an accumulation of thoracic α -glycerophosphate and a slower breakdown of adenosine triphosphate with the formation of inorganic phosphate and possibly adenosine monophosphate.

4. Starvation to the point of prostration caused a fall in head adenosine triphosphate which could be reversed by injection of aqueous glucose.

5. Extended cyclopropane anaesthesia, injected water or acetone were apparently without effect on phosphorus distribution.

6. Changes in head and thoracic phosphorus compounds could occur independently in the same insect.

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A Hydrolytic Procedure for the Identification and Estimation of Individual Phospholipids in Biological Samples

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Procedures which have been developed for the assay of individual phospholipids within a mixed-lipid sample isolated from tissues, usually depend upon a total hydrolysis of the phospholipids followed by separate determinations of the hydrolysis products, e.g. choline, phosphorus, inositol, serine, ethanolamine and sphingosine. Not only does this require the setting up of a large number of individual time-consuming assays, but the method suffers from the disadvantage that it fails to distinguish between the plasmalogens and the usual di-acylated phospholipids, e.g. between choline plasmalogen and lecithin.

The present method is based on one developed some years ago for the examination of phospholipid fractions by paper chromatography after mild alkaline hydrolysis (Dawson, 1954*a*). Such a hydrolysis results in the removal of the fatty acids from the alkali-labile phospholipids, leaving recognizable water-soluble fragments (mainly the corresponding esters of glycerophosphoric acid, e.g. glycerylphosphorylcholine) which can be subsequently separated and estimated by paper chromatography. This procedure has been simplified so that quantitative recoveries of the deacylated phospholipid fragments can be achieved, and extended so that the alkali-stable phospholipids (defined in this paper as the phosphorus remaining soluble in lipid solvent after alkaline hydrolysis) can also be examined. Thus the alkali-stable phospholipids are subjected to mild acid hydrolysis when the deacylated plasmalogens break down,

giving a long-chain aldehyde and their corresponding water-soluble phosphodiester. Finally the residual phospholipids are hydrolysed in methanolic hydrochloric acid, when sphingomyelin gives phosphorylcholine and sphingosylphosphorylcholine (Rennkamp, 1949; Dawson, 1958*a*).

The method requires the use of only a single assay procedure (for phosphorus) and it is possible to resolve and assay complex lipid samples within 2 days.

EXPERIMENTAL

Preparation of lipid samples

The procedure of Folch, Lees & Sloane-Stanley (1957) was mainly followed. The tissue samples were homogenized in 24 vol. of CHCl_3 -methanol (2:1, v/v) in a high-speed blender. After standing for 30 min. with stirring, the suspension was centrifuged and filtered. The filtrate was shaken for 2-3 min. with 0.2 vol. of 0.9% NaCl soln. After centrifuging the emulsion, or leaving it overnight at 0°, the upper aqueous layer containing any water-soluble phosphorus impurities was removed and discarded. The lower layer was taken to dryness *in vacuo*, dissolved in an equal volume of CHCl_3 , analysed for total phosphorus and stored at -20° until required.

As the subsequent alkaline hydrolysis of the phospholipids was performed with a limited amount of alkali, the method was not directly applicable to lipid extracts containing large amounts of neutral fat and low concentrations of phospholipid (< 40%, w/w), e.g. extracts of adipose tissue or fatty livers. With such extracts it is first necessary to separate the phospholipids with a simple silicic acid column or by other means (Borgström, 1952; Barron & Hanahan, 1958).