The Estimation of Adenosine Triphosphate and Related Compounds in Insect Tissue

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The report (Rockstein & Gutfreund, 1961) of some atypical ATP:ADP:AMP proportions in housefly thoracic tissues and the finding that there are wide discrepancies in the apparent ATP concentrations in insect tissues when these are determined in different ways (Heslop & Ray, 1961; J. P. Heslop, unpublished work) have prompted a re-examination of the techniques of freezing and extraction of insects and of the estimation of ATP in tissue extracts. The tissue chosen for this investigation was the housefly thorax but the findings are applicable equally to some, if not all, other insect tissues.

EXPERIMENTAL

Adult male houseflies, *Musca domestica* L., PIL strain, were fed on sugar and water only and were used 3–5 days after emergence. Nucleotides were purchased from Pabst Laboratories Inc., Milwaukee 5, Wis., U.S.A. Kits for 'ATP determination in blood' by an unpublished method of Bücher and Schuart, were purchased from Boehringer and Soehne, Mannheim, Germany. The kits contained phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase, D-3-phosphoglycerate (cyclohexylammonium salt) plus the necessary buffers and cofactors and were used according to the instructions supplied except that the volume of the reaction mixture was raised to 3 ml. without increasing the quantity of substrate. Curves of the decrease in extinction at 340 m μ with time as the reaction proceeded were plotted so that a correction could be made for 'drift'. Firefly tails were purchased from Schwartz BioResearch Inc., Mount Vernon, New York, U.S.A., and used for ATP determination as whole extracts in arsenate buffer according to the method of Strehler & Totter (1952). Orthophosphate and arginine phosphate were determined as by Heslop, Price & Ray (1963).

Treatment of tissue. (a) Standard procedure. Except where indicated flies were frozen in liquid nitrogen and divided into heads, thoraces and abdomina under liquid nitrogen. The thoraces (10 or 20), less legs and wings, were ground in a mortar with 0.5 ml. of 0.4N-perchloric acid and an excess

of liquid nitrogen and the resulting uniform paste was transferred to a chilled glass homogenizer standing in crushed ice. The mortar was scoured out with a second 0.5 ml. portion of perchloric acid and the resulting paste transferred to the homogenizer. The combined material was allowed to thaw in the ice bath and briefly homogenized. It was centrifuged at 2000g for 5 min. at 0° and the supernatant decanted into an ice-cold conical centrifuge tube. The residue was further extracted with two successive 1 ml. portions of 0.2N-perchloric acid by homogenization and centrifugation as described above and the combined supernatants were neutralized with potassium hydroxide. Time (30 min.) was allowed for the potassium chlorate to be precipitated, the suspension was centrifuged, and the supernatant made up to 5 ml. in a calibrated flask. The final extract contained 96–97% of the total acid-soluble phosphorus in the tissue before extraction.

(b) Variations on standard procedure. Houseflies (20) were cooled in ice and divided into heads, thoraces and abdomina. The thoraces were kept at 0° for 1 hr. and divided into two groups. Each group was then ground with 1 ml. of ice-cold 0.4N-perchloric acid in a previously chilled mortar and washed into a cold glass homogenizer with 0.2N-perchloric acid. The tissue was then extracted three times as described above. Other groups of flies were frozen in liquid nitrogen and transferred for about 2 min. to brine at –6°. From this they were placed in baths of melting ice containing various amounts of sodium chloride to give temperatures of –5° to –1° in 1° steps. After 1 hr. in one of the latter baths they were returned to liquid nitrogen to await dissection and extraction in the standard way. Control groups were kept in liquid nitrogen up to the time of extraction. The results of analysis of the thoraces of these flies are summarized in Table 1. They show that during a 1 hr. incubation period at temperatures below –3° the ATP and arginine phosphate in frozen housefly thoracic tissues were stable. When the incubation temperature was –2° or –1° there was extensive breakdown of these compounds in frozen tissues but there was little evidence of such breakdown in unfrozen thoraces

dissected from chilled flies even after 1 hr. at 0°. It is frequently necessary during experimental work to kill insects at a closely controlled time and freezing in liquid nitrogen achieves this object conveniently. The extraction procedure described above enables extracts with low and consistent orthophosphate content to be made from the frozen tissue.

The melting and freezing points of many insect tissues from a variety of species have been elegantly measured by Salt (1956). They lie between -0.5° and -2.0° and frequently some parts of a single tissue thaw before others. The decomposition of labile phosphorus compounds in fly thoraces (Table 1) is therefore probably associated with thawing of the previously frozen tissue.

Estimation of phosphorus compounds of housefly thorax by the labelled-pool technique. Houseflies (18) were labelled by allowing them to feed for 24 hr. on glucose solution containing ^{32}P as orthophosphate (Winteringham, Bridges & Hellyer, 1955), anaesthetized with cyclopropane and transferred to wire-gauze cages. To remove external orthophosphate they were washed in repeated changes of water until no ^{32}P could be detected in the washings. They were then dried on filter paper and fed on 10 % glucose for a further 24 hr. Specific activities (Table 2) for orthophosphate, arginine phosphate, 7 min.-hydrolysable phosphate and stable acid-soluble phosphorus were determined on thoracic extracts from flies treated in a similar manner. They confirm the finding of Winteringham *et al.* (1955) that uniformity of labelling within the acid-soluble pool of phosphorus compounds is achieved by this method of labelling flies, and justify the calculation of metabolite concentrations in tissues from the percentage results for soluble ^{32}P and total acid-soluble phosphorus.

A perchloric acid extract of the thoraces was separated on paper chromatograms into four fractions (corresponding to fractions II, III, IV and V of Winteringham *et al.* 1955). Each fraction was recovered from the paper and rechromatographed by elution from Dowex 1 (formate) (as by Heslop & Ray, 1961). Certain compounds were tentatively identified by comparison of their chromatographic behaviour on paper and on Dowex 1 (formate) with that of known substances. Total acid-soluble phosphorus was separately determined and the concentrations of the tentatively identified metabolites (as $\mu\text{moles/g. wet wt. of thorax}$) were calculated (Table 3) on the assumption that thoraces without appendages made up 46.5 % of the whole fly weight. It is probable that all the peaks listed contain other compounds in addition to the one found, so the calculated tissue concentrations represent maximum values. The following metabolites have been determined on similar but unlabelled extracts by other methods: ortho-

phosphate, $5.6 \pm 0.77 \text{ s.d.}$ (nine batches of ten flies) $\mu\text{moles/g. of thoraces}$; arginine phosphate, 7.0 ± 1.23 (9) $\mu\text{moles/g.}$; glycerol 1-phosphate, 2.3 ± 0.48 (19) $\mu\text{moles/g.}$; ATP (Table 4). The identity of the chromatographic fraction that was taken to be ATP was further checked by comparing its ultraviolet-absorption spectrum with published data (Pabst circular OR 15), by checking the ratio of adenine (by extinction at 260 $m\mu$) to phosphorus (by chemical estimation after digestion with perchloric acid) in a portion of the fraction, and by showing that it reacted quantitatively with firefly-tail preparations and the system for phosphoglycerate-kinase assay.

Estimation of ATP in tissue extracts. It is clear from the results in Table 4 that there is a discrepancy between the values for ATP concentration in housefly thorax obtained with methods involving enzymes and those based on determina-

Table 1. *Orthophosphate, arginine phosphate and adenosine triphosphate in housefly thoraces variously treated before extraction*

Groups of ten flies were frozen in liquid nitrogen, incubated for 1 hr. at various temperatures, returned to liquid nitrogen and then extracted and analysed. ATP was estimated by the firefly-luciferin technique. Each figure is the mean of determinations on at least two groups of flies. For comparison two groups of flies were chilled in ice and cut into heads, thoraces and abdomina. The thoraces were left at 0° for 1 hr. and extracted in cold perchloric acid without freezing.

Pre-treatment	Incubation temp.	Concn. ($\mu\text{moles/g.}$)		
		Ortho-phosphate	Arginine phosphate	ATP
Not frozen	0°	5.2	6.4	6.3
Frozen	-196	5.5	7.4	8.9
	-5	5.7	6.7	8.8
	-4	5.1	7.3	10.8
	-3	6.2	5.3	9.9
	-2	26.4	0.5	0.2
	-1	28.5	0.1	0.1

Table 2. *Relative specific activities of phosphorus compounds from housefly thoraces*

Conditions of ^{32}P -labelling are given in Table 3. Phosphorus was estimated by the method of Martin & Doty (1949) and ^{32}P was assayed on the blue solutions after these estimations. '7-min. P' refers to the orthophosphate released by 7 min. of hydrolysis in N-HCl at 100° after previous hydrolysis in 0.1 N-HCl at 100° for 1 min. The stable fraction was completely hydrolysed by boiling in conc. HClO_4 for 3 hr. before analysis.

Fraction	Sp. activity
Orthophosphate	1.00
Arginine phosphate	0.96
'7-min. P'	0.95
Stable, acid-soluble P	1.03

tion of adenine or of phosphorus after chromatographic separation. The value of 5 μ moles of ATP/g. of thorax based on adenine estimation is supported by data on the sum of acid-soluble adenine and hypoxanthine in the thoraces of 12 batches of anoxic flies. Ray & Heslop (1963) showed that during anoxia ATP is broken down to AMP plus IMP, but no further, and the ATP concentration calculated from these data (assuming ATP:ADP:AMP proportions 6.4:1.0:0) is 5.4 μ moles/g. of thorax.

In eight experiments where firefly-luciferin and phosphoglycerate-kinase estimations were carried

out on the same extracts, the firefly method gave results 8% higher than the kinase one and the difference was just significant by the *t* test ($P < 0.05$).

Five extracts were divided and one-half of each was hydrolysed for 1 min. in 0.1N-hydrochloric acid at 100° and then neutralized. The hydrolysed parts contained only 3% less 'ATP' than the untreated ones as determined by the firefly-luciferin and phosphoglycerate-kinase methods. It was concluded that arginine phosphate could not be responsible for the higher results obtained by using the methods involving enzymes.

Thorax extracts were prepared from normal flies and from flies which had been kept in oxygen-free nitrogen for 1 hr. Anoxia depletes the ATP, GTP and UTP in fly thorax and causes accumulation of nucleoside monophosphates (Heslop *et al.* 1963; Ray & Heslop, 1963). Amounts of ATP equivalent to 0.5 and 10 μ moles/g. were added to portions of both extracts and estimated by both enzymic methods. In all cases the added ATP was correctly estimated in the presence of the tissue extracts. It was concluded that there was no interfering substance in the extracts that could interfere with ATP estimations by these methods over the range of concentrations likely to be studied.

Mixtures of ATP, ADP, CTP, CDP, GTP, GDP, UTP, UDP, UDP-glucose and NAD in 0.1M-phosphate buffer were prepared to simulate housefly-thorax and cockroach-nerve extracts (based on the tissue concentrations in Table 3 and in Heslop & Ray, 1961, respectively). The 'ATP' in them was compared with that in buffered solutions of ATP at the same concentrations. The firefly-luciferin method gave results 10–15% high on 'artificial thorax extracts' and 22–30% high on 'artificial nerve extracts', with some evidence that the error might vary from one batch of firefly tails to another. The phosphoglycerate-kinase method gave results with

Table 3. *Phosphorus constituents of housefly thorax by the labelled-pool technique*

The acid-soluble phosphorus pool of 20 flies was uniformly labelled with 32 P and separated on paper chromatograms, followed by elution chromatography into 21 fractions of measurable size. Tissue concentrations of metabolites were calculated assuming that the total acid-soluble phosphorus in housefly thoraces is 45.4 μ g.atoms/g. of thorax. Owing to the probability that the chromatographic fractions include other substances than the one tentatively identified, the tissue concentrations quoted represent maximum values.

Tentative identification	Percentage of acid-soluble 32 P	Concn. in tissue (μ moles/g.)
ATP	36.6	5.4
Orthophosphate	15.9	7.2
Arginine phosphate	12.1	5.5
Fraction IIIA	10.0	—
Glycerol 1-phosphate	7.1	3.2
ADP	3.8	0.9
Glucose 6-phosphate	2.4	1.1
Unidentified	2.2	—
GTP + UTP	1.8	0.3
CDP	1.2	0.3
UDP	0.6	0.1
UDP-glucose	0.5	0.1
GDP + ?	0.4	0.1
NAD	0.2	0.1
Unidentified fractions	each 1.0	—

Table 4. *Concentration of adenosine triphosphate in housefly thorax determined by different methods*

Some figures for cockroach nerve (Heslop & Ray, 1961) are included for purposes of comparison.

Method	Extraction solvent	ATP (μ moles/g. wet wt. of tissue) $\pm$ S.D. (no. of observations)	
		Fly thorax	Cockroach nerve
Labelled pool	50% Ethanol, N-formic acid	4.8 $\pm$ 0.8* (9)	1.5
	0.4N-HClO ₄	5.4	—
Adenine estimation	0.4N-HClO ₄	5.0 $\pm$ 1.2† (3)	—
Firefly luciferin	0.4N-HClO ₄	8.9 $\pm$ 1.1 (22)	2.7
Phosphoglycerate kinase	0.4N-HClO ₄	9.1 $\pm$ 0.7 (13)	—

* Calculated from data of Winteringham (1960), assuming that thoraces of female flies contain 45 μ g.atoms of P/g.

† Assuming that the ratio ATP:ADP is 6.4:1 and that other acid-soluble compounds of adenine are present in negligible amounts.

both extracts 2–8 % higher than on the corresponding ATP solutions.

The high results with the artificial nucleotide mixtures are due to the presence of nucleotide diphosphokinases and myokinase in the crude luciferin preparation (Balfour & Samson, 1959; J. P. Heslop, unpublished work). The same is probably true for the phosphoglycerate-kinase method as this preparation is not claimed to be absolutely specific for ATP.

An extract of housefly thoraces was made and found to contain 'ATP' equivalent to $9.1 \mu\text{moles/g.}$ of thorax by the kinase method ($9.7 \mu\text{moles/g.}$ by firefly luciferin). A portion of this extract containing $6 \mu\text{moles}$ of 'ATP' (kinase) was chromatographed by elution from Dowex 1 (formate) with the gradient previously employed. The adenine in the ATP peak was estimated by its ultraviolet absorption to total $3.4 \mu\text{moles}$. The amount of ATP in this peak was estimated by the phosphoglycerate-kinase method and found to be $3.6 \mu\text{moles}$. In previous experiments (Heslop & Ray, 1961) very high recoveries of nucleotides were consistently achieved from Dowex 1 columns. The difference between the ATP concentration found before and after chromatography is too large to be accounted for solely by interference from other nucleotides. It was therefore concluded that the ATP had been separated on the resin column from an interfering substance that was provisionally called 'compound X'.

Nature of compound X. The light-producing system in firefly lanterns is ATP-specific (McElroy & Green, 1956) and so is phosphoglycerate kinase. It would be expected then that compound X might donate one phosphate group to ADP and convert it into ATP, which would then react with the assay system. If compound X behaved in this way and contained only one phosphorus atom per molecule it would make up at least 8 % of the total acid-soluble phosphorus in housefly thorax and at least $5\frac{1}{2}$ % of the acid-soluble phosphorus in cockroach nerve. The only unidentified and untested substance that was present in sufficient quantity in either tissue to fulfil this function was the fraction IIIA (Table 3, and Heslop & Ray, 1961), which made up 10 % and 8.7 % of the acid-soluble phosphorus in the two tissues respectively. Fraction IIIA was prepared from fly thoraces by the paper-chromatographic procedure of Heslop & Ray (1961) and tested for its effect on both assay systems in the presence and absence of added ATP. In no case was there any sign that IIIA might behave as a phosphagen. In addition all the 'ATP' of the original housefly extract could be accounted for in the paper-chromatographic fraction II, by using the system for firefly-luciferin assay. It is suggested therefore that compound X does not contain phosphorus.

DISCUSSION

When houseflies were frozen in liquid nitrogen and then allowed to warm above -3° , decomposition of ATP and arginine phosphate occurred. Such decomposition could be avoided by ensuring that intimate contact between the tissue and the denaturing extractant was achieved before the temperature was permitted to rise. This phenomenon has been recognized for some time [e.g. by Fowler & Lewis (1958), who considered the extraction of acetylcholine from frozen insect tissue, and by Lin, Cohen & Cohen (1958), who studied the extraction of labile phosphates from frozen mammalian brain], but unfortunately the necessary precaution to ensure that the substances extracted are those that were present in the frozen tissue before extraction is still frequently neglected in recent work. An example from insect biochemistry is the work of Rockstein & Gutfreund (1961). They found ATP:ADP:AMP proportions in the thoraces of 3–4-day-old male houseflies of 0.6:0.4:4.9. In the present study proportions 6.4:1.0:0 were found. It is highly significant that Rockstein & Gutfreund found a marked decline in the activity of magnesium-activated adenosine triphosphatase in mitochondria prepared from the thoraces of aging flies, especially after the ninth to tenth days, and that in 15–16-day-old flies they found ATP:ADP:AMP proportions 6.2:1.3:1.1. The extraction procedure after freezing of the flies that these authors used differed significantly from that reported above in one respect only. The frozen flies were placed in a Teflon-glass homogenizer at 0° and then homogenized in cold 0.6N-perchloric acid.

The concentration of orthophosphate is easy to determine in aqueous extracts and is the most sensitive indicator of breakdown within the acid-soluble phosphorus pool.

Evidence has been presented for the existence in insect tissue of a substance provisionally named 'compound X', which behaves like ATP towards the assay systems based on phosphoglycerate kinase coupled with glyceraldehyde phosphate dehydrogenase and the whole firefly-tail extracts in arsenate buffer. Compound X did not contain phosphorus and was stable to 1 min. of hydrolysis in 0.1N-hydrochloric acid at 100° . On these grounds none of the known 'high-energy' compounds can be suggested as a possible identity for compound X. Speculation about the role of this compound in insect tissue would be unfruitful at this stage but it must be emphasized that there is a strong possibility that it will interfere with analysis whenever the ATP concentration of insect material is studied by the systems for firefly-luciferin or phosphoglycerate-kinase assay.

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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REFERENCES

- Balfour, W. M. & Samson, F. E. (1959). *Arch. Biochem. Biophys.* **84**, 140.
- Fowler, K. S. & Lewis, S. E. (1958). *J. Physiol.* **142**, 165.
- Heslop, J. P., Price, G. M. & Ray, J. W. (1963). *Biochem. J.* **87**, 35.
- Heslop, J. P. & Ray, J. W. (1961). *J. Insect Physiol.* **7**, 127.
- Lin, S., Cohen, H. P. & Cohen, M. M. (1958). *Neurology*, **8**, suppl. 1, 72.
- McElroy, W. D. & Green, A. A. (1956). *Arch. Biochem. Biophys.* **64**, 257.
- Martin, J. B. & Doty, D. M. (1949). *Analyt. Chem.* **21**, 965.
- Ray, J. W. & Heslop, J. P. (1963). *Biochem. J.* **87**, 39.
- Rockstein, M. & Gutfreund, D. E. (1961). *Science*, **133**, 1476.
- Salt, R. W. (1956). *Canad. J. Zool.* **34**, 1.
- 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.

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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-