Mode of Insecticidal Action Studied with Labelled Systems

PHOSPHORYLATED COMPOUNDS IN THE MUSCLE OF THE ADULT HOUSEFLY, *MUSCA DOMESTICA*, L.

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Phosphorylated intermediates are involved in a ubiquitous and vital manner in nerve and muscle function. A disturbance in the normal distribution of these intermediates by an insecticide is likely, therefore, to be of significance in its mode of action. Disturbances in the distribution of phosphorylated intermediates in insect muscle have been studied by a combined radioactive tracer-paper chromatography technique. Insects were fed with radioactive phosphorus (^{32}P) so that the intermediates became labelled. The labelled compounds were extracted under conditions likely to preclude their decomposition and resolved by means of unidimensional paper chromatography. The paper chromatograms were then scanned radiometrically (Winteringham, Harrison & Bridges, 1952). In this way the ^{32}P -radioactivity (ordinate) was automatically plotted against distance along the paper chromatogram (abscissa) and the separated labelled fractions appeared as peaks of radioactivity. Provided the fractions so separated were isotopically labelled in a uniform manner the area enclosed by each peak of the radiochromatogram was proportional to the weight of labelled fraction present. By comparing radiochromatograms of normal and of poisoned tissues significant changes in the distribution of labelled metabolites can be determined. The absence of a change in distribution does not, of course, preclude any change in metabolic turnover rates. It seems likely, however, that any morbid changes in turnover rate would be reflected by a change in distribution. Some compounds, e.g. phosphoproteins, may not have become significantly labelled by the short-term feeding techniques used on account of relatively slow turnover rates. This paper is not concerned with these compounds. The techniques to be described are an attempt to study toxic action *in vivo* and to compare the results obtained with those which have been, or may be, predicted from experiments *in vitro*, e.g. with isolated enzyme systems. This paper describes the application of these techniques to the determination of the phosphorylated intermediates of the thoracic muscle of the adult housefly, *Musca domestica*, L. Later papers will describe the effects of insecticides

on the distributions found. For a preliminary communication concerning this work see Winteringham, Loveday & Hellyer (1953).

EXPERIMENTAL

^{32}P -labelled and unlabelled phosphorus compounds

Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and muscle adenylic acid (AMP). These were purchased as the sodium salt, the barium salt and as the free acid, respectively, from Light and Co.

Phosphoglyceric acid (PGA). This was purchased as the barium salt from Light and Co.

Ribose 5-phosphate (R 5-P) and glucose 6-phosphate (G 6-P). Samples of the barium salts supplied by the Schwarz Laboratories Inc., N.Y., were the gift of Mr C. J. Threlfall. Barium salts were converted into the sodium salts.

Glucose 1-phosphate (G 1-P). This was prepared as the potassium salt by Mr S. E. Lewis of this Laboratory, by the method of Sumner & Somers (1944).

^{32}P -labelled glucose 6-phosphate. Glucose was phosphorylated in the presence of inorganic phosphate and fresh baker's yeast (purchased locally from the Distillers Co. Ltd.) by the procedure of Neuberg & Lustig (1942), but the addition of fluoride was omitted and ^{32}P -labelled phosphate was substituted for ordinary phosphate. From the trichloroacetic acid (TCA) extract, free orthophosphate, PGA and hexose diphosphate (HDP) were separated as the magnesium and barium salts, respectively, by the method of Dubois & Potter (1943). The remaining solution which contained hexose monophosphates was freed of excess barium by sulphate precipitation and hydrolysed with N-HCl for 4 hr. at 100° to remove pyrophosphate and fructose 6-phosphate (F 6-P). Paper chromatographic analysis of the 'labelled' solution (see below) indicated the presence of two major fractions of R_f values respectively correct for G 6-P and PO_4^{3-} . The labelled G 6-P fraction was eluted from paper chromatograms and used as required.

^{32}P -labelled phosphoglyceric acid. Glucose was phosphorylated as before but fluoride was added to increase the yield of PGA. The labelled PGA was recovered as the barium salt (Dubois & Potter, 1943) and converted into the sodium salt.

^{32}P -labelled hexose diphosphate. This was isolated as the barium salt from the mixture used for preparing the PGA followed by conversion into the sodium salt. Paper chromatographic analysis indicated the presence of two compounds, the larger fraction behaving as HDP on the basis of

R_p value and rate of hydrolysis in $N-HCl$ at 100° . It was eluted from paper chromatograms as required.

^{32}P -labelled pyro- and meta-phosphates. These were prepared by heating ^{32}P -labelled Na_2HPO_4 and NaH_2PO_4 at 250° and at red heat (Partington, 1933).

Incorporation of ^{32}P into the tissue phosphorus of adult houseflies in vivo

Housefly pupae were placed in breeding cages at 27° and provided with A.R. glucose and water *ad lib*. One- to five-day old adults which had been starved of food and drink for a few hr. were sexed while under mild cyclopropane anaesthesia and twenty-five females transferred to a 400 ml. Pyrex metabolism unit of the kind shown in Fig. 1. The B-50 cone (C) is closed at the top to form the floor of the unit. In the middle of this floor is a depression B or 'feeding-well' of about 1 ml. capacity. The well is charged with an absorbent wool plug moistened with 5% (w/v) A.R. glucose containing 1-1000 μC (according to the level of activity required) of carrier-free $^{32}PO_4^{3-}$ adjusted to pH 6 by the addition of $NaHCO_3$ to the sterilized solutions supplied by the Isotope Division of the Atomic Energy Research Establishment. Paper chromatographic analysis of the radioactive phosphate supplied showed that it consisted entirely of free orthophosphate. Occasionally a negligible trace of ^{32}P behaved as pyrophosphate. The metabolism unit was aerated (D and E) with dry CO_2 -free air at 3 ml./sec. The flies were allowed to feed, walk or fly for a 'labelling' period of 24 hr. The dry atmosphere encouraged copious drinking during this period. After 24 hr. the flies were anaesthetized by substituting cyclopropane for air at D for 2 min. The feeding-well was then replaced by one containing no radioactive phosphorus and the flies kept for a further 'metabolism' period of 24 hr. During 'labelling' and 'metabolism', which were conducted at 30° in a lighted cabinet, the wells were kept visibly moist by the addition of 5% (w/v) A.R. glucose from the reservoir A. Dead insects were removed at the end of the 'labelling' or 'metabolism' period. Usually all the insects survived and behaved normally.

Dispersion and extraction of tissue

The thorax of the housefly bears the legs and wings and is very rich in muscle. Extracts of whole thoraces representing muscle extracts were prepared as follows. At the end of the 'metabolism' period the flies were anaesthetized in cyclopropane for 2 min. and placed inside a small stainless-steel wire cage which was plunged into liquid nitrogen. The cage and contents were then rinsed by brief immersion in 50% aqueous ethanol containing 5% (v/v) orthophosphoric acid at -10° . In this way external contamination by carrier-free ^{32}P (as a result of walking over the feeding-well, etc.) was effectively removed. Paper chromatographic analysis (see below) showed that free orthophosphate only was present in this external rinse. The removal of external phosphorus was of importance. It was unlikely to have undergone isotopic dilution by tissue phosphates so that its presence in the extract would appear as an artificially high proportion of labelled PO_4^{3-} . The cage and contents were then successively rinsed in 50% aqueous ethanol alone and in acetone both at -10° . The washed and chilled flies were then transferred to isopentane at -70° to await dissection. Flies were transferred one at a time to a Petri dish chilled in

solid CO_2 . The heads, abdomens, and appendages were rapidly dissected off and the thoraces dropped into 1.8 ml. of N formic acid in 50% aqueous ethanol at -10° contained in a cooled all-glass 'homogenizer' of the type described by Potter (1949). Ten such thoraces were dispersed for 10 min. The dispersion was centrifuged at 5° and the supernatant stored at -15° . A further 1.8 ml. of ethanolic formic acid was added to the sediment and the dispersion repeated. This was repeated once more so that the pooled supernatants were almost exactly 5 ml. The total extract was concentrated to 1 ml. by subliming off the frozen solvent under high vacuum. The concentrate was finally centrifuged at room temperature and the slightly cloudy supernatant stored at -15° . The extracts contained acid-labile phosphorus compounds and for this reason the use of strong acids like TCA was avoided. The doubtless greater proportion of protein present as a result of this was without effect on the subsequent chromatographic separations. The presence of ethanol reduced the glycogen solubility and increased the solvent volatility for evaporation purposes.

Fig. 1

Fig. 2

Fig. 1. Metabolism chamber for feeding adult houseflies with ^{32}P -labelled phosphate solution. A, glucose reservoir; B, feeding-well depression in closed 'B-50' cone C; D, and E, inlet and outlet tubes for air circulation.

Fig. 2. Column for ascending paper chromatography under reduced pressure.

Finally formic acid was used in the principal solvent for chromatography so that its use for extraction avoided the presence of other acids which tend to interfere with the separations when present in the samples applied to paper chromatograms. Concentrates stored at -15° showed no changes in composition over several days. Assays of ^{32}P in the sediments (counted as suspensions) and in the extracts indicated that about 80% of the total tissue ^{32}P was recovered in the extracts. Most of this was recovered in the first 1.8 ml., which suggests that the residual ^{32}P was present as insoluble phosphoprotein, phospholipid, etc.

Paper-chromatographic techniques

Three of the solvents recommended by Burrows, Grylls & Harrison (1952) have been successfully adopted for separating the major phosphorylated intermediates of housefly muscle. The solvents used were made up by volume as follows: formic acid-acetone-water (14:60:26) (solvent no. 1); acetic acid-acetone-water (15:50:35) (solvent no. 2); 2N- NH_3 -methanol (30:70) (solvent no. 3).

Whatman no. 1 paper, purchased in rolls of strip 1.125 in. wide, was used throughout. The paper was washed by soaking it overnight in 0.1N-HCl followed by successive rinsing in dilute ammonia and glass-distilled water. Washed 160 cm. long strips were folded double and pairs of samples (10–50 $\mu\text{l.}$) of the concentrate were applied at starting lines 7 cm. above the fold. The free ends of the paper were pinned together and a small glass weight was placed in the fold of the strip which was then hung in an all-glass column of the type shown in Fig. 2. Each column contained 100 ml. of the required solvent and the strip was immersed so that the starting lines were about 5 cm. above the solvent. The column was then exhausted at a water pump to ensure immediate saturation of the column with the solvent vapour. Ascending chromatography proceeded for 48 hr. at 5° for solvent 1 and at 25° for solvents 2 and 3. The solvent usually ascended 50 cm. or so above the starting lines. When strips were removed from the columns they were placed in a desiccator, dried *in vacuo* over soda-lime and charcoal, and stored at -15° until required for radiometric scanning (see below) or for chemical tests. After having located an incompletely resolved fraction by radiometric scanning, the paper containing it was cut out and attached to a new strip of paper. The fraction was then re-concentrated into a narrow band on the new strip by a simple elution technique (Winteringham, 1953). Except for the slowest running fractions complete re-concentration was achieved in 24 hr. at 5° in a mild draught. In the present work the eluting solvent consisted by volume of 0.1% redistilled formic acid in 50% aqueous acetone. The re-concentrated fraction was resolved in a second solvent by the procedures described.

The behaviour of a particular fraction under the conditions of acid hydrolysis was studied by suspending the paper section containing the fraction (marked off lightly in pencil during radiometric scanning) in acid at the required temperature. At intervals suitable samples were chromatographed in the usual way. In interpreting the subsequent radiochromatograms, account was taken of the acidity of the sample used. The presence of a strong acid invariably caused a fraction to run with a high R_f value.

Location of known compounds added to paper chromatograms. Unlabelled nucleotides were located and determined by neutron activation (see below) or by their inhibition of

the fluorescence in ultraviolet light at 2540Å (Holiday & Johnson, 1949). Labelled compounds were located and determined by the radiometric scanning techniques described below. Unlabelled compounds were detected chemically on paper by the acetone-dip technique of Burrows *et al.* (1952), using a modification of the molybdate reagent of Hanes & Isherwood (1949).

Radiometric scanning of paper chromatograms. The ^{32}P -labelled fractions resolved by paper chromatography were located and assayed quantitatively by an automatic scanning technique which has been described in detail elsewhere (Winteringham *et al.* 1952). In the present work the fractions separated were isotopically labelled completely and apparently uniformly (see below) so that the relative areas enclosed by the peaks of the plotted radiochromatograms (measured by planimeter) represented the relative proportions in which the fractions were present.

The Geiger-Müller tubes used possessed 1 in. end-windows (2 mg./cm.²). Tests with known amounts of ^{32}P -labelled PO_4^{3-} run on Whatman no. 1 paper indicated that about 20% of the ^{32}P -disintegrations were recorded. Geometrical factors were the principal cause of inefficiency, window absorption and self-absorption by the paper being negligible for ^{32}P .

Neutron activation of paper chromatograms. Neutron activation was employed (i) for estimating the relative $^{31}\text{P}/^{32}\text{P}$ ratios of the labelled fractions, (ii) for the detection of unlabelled phosphorus compounds, and (iii) for the estimation of the total phosphorus content of the extracts. Paper chromatograms of labelled or unlabelled extracts were irradiated in the Harwell pile (BEPO) at a neutron flux of 10^{11} neutrons/cm.²/sec. for 120 hr. Great care was taken not to contaminate the strips (e.g. by fingering) before irradiation. In the present work the ^{32}P concentration varied from 10^{-2} to $10\mu\text{C}/\text{thorax}$ (mean wt. of thorax was 7 mg.). This corresponded to 3.4×10^{-8} to $3.4 \times 10^{-6}\mu\text{g.}$ of isotopic $^{32}\text{P}/\text{thorax}$. The total weight of natural ^{31}P (natural isotopic abundance is 100%) was estimated by the activation of unlabelled extracts to be of the order of $10\mu\text{g.}/\text{thorax}$. The labelling process therefore results in a negligible increase in tissue phosphorus. In practice 'carrier-free' ^{32}P may have undergone isotope dilution by a factor of 300 by phosphorus present in the irradiated target, reagents employed for extraction etc. (For this information the authors are indebted to Mr Brian Payne of the Isotope Division, Harwell.) The increase in tissue phosphorus would still be less than 0.1%. It follows that the ratio of the net increase in ^{32}P activity on activation to the original ^{32}P activity due to labelling is a measure of the $^{31}\text{P}/^{32}\text{P}$ ratio in the separated fraction. It was calculated that $1\mu\text{g.}$ of ^{31}P would give rise to about 6000 β -particles/sec. [by the $^{31}\text{P}(n, \gamma)^{32}\text{P}$ -reaction] and that this could be assayed to within a few parts per cent by scanning a week or so after removal from the pile. This was confirmed experimentally by the irradiation of known weights of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ run on paper chromatograms.

The irradiation of insect extracts resolved on paper chromatograms gave rise to radioactive isotopes other than ^{32}P . ^{24}Na and ^{42}K were identified by their energetic β - and γ -emission and by their short half-lives. It was interesting to note that these metals ran quite separately from the PO_4^{3-} as free cations in acid solvents when applied as the salts. Emitters of soft β -rays which had relatively long half-lives were also produced and probably consisted largely of ^{35}S arising by the $^{35}\text{Cl}(n, p)^{35}\text{S}$ -reaction with chlorides doubtless

present in the extracts. Irradiated chromatograms were scanned 1 week after removing them from the pile by which time ^{24}Na and ^{42}K had decayed to insignificance. By scanning through a 28 mg./cm.² aluminium screen the soft β -particles were effectively screened off. The remaining activity was due entirely to ^{32}P on the basis of half-life determinations over a period of 5 weeks.

Histological distribution of tissue ^{32}P

The autoradiographic technique described by Winteringham, Harrison & Hammond (1950) was used to study the histological distribution of ^{32}P in the fly thorax before extraction. In this way the danger of leaching by the autoradiographic technique itself was avoided.

Thoraces prepared for extraction were intercepted at the isopentane stage and placed upon a degassed paraffin wax (m.p. 58°) platform contained in a short-path freeze-sublimation unit under high vacuum. The unit was submerged in a bath of ethyl oxalate slush at -40° to -50° (Mendelow & Hamilton, 1950). When dehydration was complete the tissue was embedded by melting the wax platform, blocked out, and sectioned at 10 μ . Consecutive sections were stained (Heidenhain's haematoxylin and eosin) and autoradiographed (Kodak fine grain stripping emulsion), respectively. A constant difficulty was that of cutting undamaged sections. The tissue was not fixed chemically and almost invariably the relatively brittle cuticle broke away. This has happened in the typical section shown in Fig. 4.

RESULTS

Qualitative and quantitative composition of the extract

The most useful initial chromatographic fractionation was obtained in solvent 1. A typical radiochromatogram obtained with this solvent is shown in Fig. 3. Five major fractions, I_1 , II_1 , III_1 , IV_1 and V_1 (right subscript indicates the solvent number) were resolved. Fraction III_1 was sometimes partly resolved into two fractions but rarely sufficiently well for quantitative analysis (e.g. see Fig. 3). At least three distinct compounds were present in this fraction in any case. The major fractions ran with mean R_f values of 0, 0.15, 0.37, 0.57 and 0.70, respectively. Fraction II_1 could be resolved in solvent 2 into two fractions, I_2 and II_2 (the latter usually present in small proportion or absent) of mean R_f values 0.14 and 0.34, respectively. Fraction III_1 was further resolved by solvent 3 into three fractions, I_3 , II_3 and III_3 of mean R_f values 0, 0.31 and 0.46, respectively.

These fractions have been tentatively identified by comparing their R_f values with those published for known compounds (Burrows *et al.* 1952), by co-chromatography with authentic compounds, and by their behaviour on acid hydrolysis. There is evidence that compounds other than those identified were present in small proportions but their identification was not considered practicable or necessary at the present stage of the work. When a fraction is

described therefore as a particular compound it must be understood that this does not exclude the possible presence of other compounds in relatively small amounts.

Fraction I_1 . This largely disappeared from extracts which were centrifuged at 6000 g for 60 min. before chromatography. It was insoluble in N-HCl at 100° when the paper containing the fraction was suspended in the acid for 7 min. It was concluded that I_1 was probably phosphoprotein in suspension and has not therefore been included in the total acid-soluble ^{32}P . Added ^{32}P -labelled metaphosphate remained at R_f 0 in solvent 1 but clearly was absent from fraction I_1 because of the relative ease with which metaphosphate undergoes hydrolysis.

Fraction II_1 . Added ATP or ADP ran in this fraction, the latter being the faster when they were added to the sample prior to chromatography in solvent 1. Hydrolysis of fraction I_2 (prepared by resolving II_1 in solvent 2) for 10 min. in N-HCl at 100° liberated two-thirds of the ^{32}P which ran as PO_4^{3-} in solvent 1. Added ATP co-chromatographed exactly with I_2 . Hydrolysis of II_2 for 10 min. in N-HCl at 100° liberated one-half of the ^{32}P as PO_4^{3-} . Added ADP ran with II_2 . The hydrolysis of the combined fraction II_1 ($\text{I}_2 + \text{II}_2$) for 10 min. in N-HCl at 100° gave rise to PO_4^{3-} and a major derivative which co-chromatographed exactly with added R5-P in solvent 1, the combined fraction running with an R_f value of 0.50. A minor derivative co-chromatographed exactly with AMP under the same conditions. II_1 was selectively hydrolysed in the total extract in the presence of a suitably buffered potato apyrase prepared according to Lee & Eiler (1951). It was concluded that I_2 and II_2 were respectively ATP and ADP and that the α -, β -, and γ -P atoms were uniformly labelled. On a routine basis II_1 was used as an estimate of the total ATP + ADP. Resolution of II_1 in solvent 2 was used for estimating the ATP/ADP ratio (usually very high). A check on this ratio and on the uniformity of labelling was made by the hydrolysis of II_1 (after reconcentrating) for 10 min. at 100° in N-HCl followed by a determination of the ratio

$$\frac{{}^{32}\text{P as PO}_4^{3-}}{{}^{32}\text{P as AMP + R 5-P}}$$

Fraction III_1 . Labelled or unlabelled G 6-P co-chromatographed exactly with the faster running peak of III_1 (see Fig. 3). The added G 6-P ran exactly with III_3 (prepared by resolving III_1 in solvent 3). Labelled HDP and G 1-P ran either just too fast or too slowly in solvent 1 to suggest their normal presence in III_1 . Labelled pyrophosphate ran with III_1 and remained at R_f 0 with I_3 in solvent 3. However, the rate of hydrolysis of none

of the separated components of III_1 corresponded to that of pyrophosphate, and its presence in III_1 in significant amount was ruled out.

Fraction I_3 (separated from III_1 in solvent 3) was slowly hydrolysed in 0.03N-HCl at 28° but not in the presence of 0.30% ammonium molybdate. This suggested the presence of the invertebrate phosphagen, arginine phosphoric acid (Arg-P), in I_3 (and therefore in III_1) and the absence of creatine phosphoric acid (Meyerhof, 1928). I_3 was completely hydrolysed to PO_4^{3-} in 0.01N-HCl at 37° in 4 hr. This also suggested that I_3 was Arg-P and not G 1-P (Meyerhof & Lohmann, 1928; Mende & Chambers, 1953). Free arginine was apparently present in the extract but ran faster than III_1 under the same conditions. Hydrolysis of III_1 invariably gave rise to a positive ninhydrin-reacting substance which, under the paper chromatographic conditions for separating arginine from the majority of amino acids (aqueous acetone solvent of Bentley & Whitehead, 1950) behaved as arginine. The formation of free arginine on hydrolysing III_1 was confirmed by Weber's (1930) modification of the Sakaguchi reaction for guanidine derivatives. It was concluded that I_3 was Arg-P. Mild hydrolysis of III_1 indicated that Arg-P ran with the slower peak of III_1 .

AMP ran with the slowest-running part of III_1 in solvent 1. It appeared to co-chromatograph exactly with II_3 when the combined fraction ($\text{III}_1 + \text{AMP}$) was run in solvent 3. When this combined fraction was re-run in solvent 1 it was clear however that part of II_3 was not AMP. This was apparently due to the presence of an unidentified compound in II_3 (provisionally designated U.C.1). In some experiments in which the ATP was apparently decomposed *in vivo* (e.g. as a result of methyl bromide poisoning), the slower running part of III_1 and II_3 (separated from III_1) was largely increased at the expense of II_1 . II_3 then co-chromatographed exactly with AMP in solvent 3 and on re-running the combined fraction in solvent 1. U.C.1 was now apparently completely masked by the AMP which clearly constituted most of II_3 in these circumstances. Hydrolysis of II_3 for 3 hr. in N-HCl at 100° liberated 30–60% of the ^{32}P -activity as PO_4^{3-} . The presence of two unidentified derivatives (R 5-P?) was indicated. Similar patterns of hydrolysis products were obtained whether II_3 was separated from normal extracts or from extracts in which the ATP had largely decomposed *in vivo* as above. This suggested that U.C.1 might be related to AMP. Hydrolysis of III_3 for 3 hr. in N-HCl at 100° liberated 4–16% of the ^{32}P as PO_4^{3-} . This was consistent with the co-chromatographic experiments which strongly indicated that III_3 and G 6-P were identical.

It was concluded that III_1 consisted of Arg-P, AMP, G 6-P and an unidentified compound U.C.1.

On a routine basis III_1 has been used for estimating the combined fraction and resolution in solvent 3 for estimating the separated fractions Arg-P (I_3), AMP + U.C.1 (II_3) and G 6-P (III_3).

Fraction IV_1 . This fraction co-chromatographed exactly with unlabelled or ^{32}P -labelled PGA in solvent 1 and the combined fraction was not resolved on re-running in solvent 3. The chromatographic tests in solvent 3 were not entirely satisfactory. The added 'PGA', though unresolved in solvent 1 and co-chromatographing exactly with IV_1 , gave rise to two or more fractions (located chemically) when re-run in solvent 3. However, one of these (R_f 0.62) co-chromatographed exactly with the eluted IV_1 . Fraction IV_1 was completely stable to 3 hr. hydrolysis in N-HCl at 100°. Bandurski & Axelrod (1951), using an acid solvent similar to solvent 1, found that PGA ran 0.79 times as fast as PO_4^{3-} , which compares well with the value 0.81 found for fraction IV_1 in the present work. It was concluded that fraction IV_1 was PGA.

Fraction V_1 . This fraction co-chromatographed exactly with ^{32}P -labelled orthophosphate or inactive phosphate. On re-running in solvent 3, fraction V_1 remained at R_f 0, but Burrows *et al.* (1952) reported an R_f value of 0.31 for sodium orthophosphate. This anomaly was apparently due to the fact that in the present work the phosphate

Fig. 3. Tracing of an original radiochromatogram illustrating the primary paper-chromatographic fractionation in formic acid-acetone solvent (solvent 1) of ^{32}P -labelled compounds extracted from housefly thorax. Peaks of radioactivity have been inked in for clarity. Fraction II_1 may be resolved in acetic acid-acetone solvent (solvent 2). Fraction III_1 may be resolved in ammonia-methanol solvent (solvent 3). The tentative identification of the fractions is indicated in the figure.

Table 1. *Distribution of ^{32}P in ethanolic-formic acid extracts of thoracic tissues of Musca domestica adults*

Fraction	³² P of fraction as % of the total soluble ³² P	Range	Remarks
ATP	37.2	31.7-46.8	ADP; 0-10%. By solvent-2 analysis
ADP	1.2		
Arg-P	10.9	18.6-30.8	Arg-P by solvent-3 analysis (13.4% by hydrolysis)
AMP + U.C. 1	6.9		
G 6-P	7.8		
PGA	20.8	18.5-24.0	—
PO ₄ ³⁻	15.2	10.6-21.1	—
	100.0		

was applied by elution of the free PO_4^{3-} ion separated first in an acid solvent, the eluted PO_4^{3-} then being held back in solvent 3 presumably by trace metals of the paper or extract (magnesium, calcium, etc.). Fraction V_1 was selectively precipitated from the extract by magnesium acetate at pH 8.2 although variable proportions of the ATP fraction were co-precipitated (cf. Lee & Eiler, 1953). Fraction V_1 was unchanged by hydrolysis and was concluded to be free PO_4^{3-} .

The tentative identification of the principal fractions is indicated in Fig. 3.

Mean composition of muscle extract. On the basis of this tentative identification the mean composition of several extracts made over a period of 3 months from groups of adult flies of different generations together with the ranges of variation found are summarized in Table 1.

Replicate analyses made on the same extract usually agreed to within 2 % (total ^{32}P) of the mean value for each major fraction separated in solvent 1 provided the fractions were reasonably well resolved.

Uniformity of labelling ; total acid-soluble phosphorus

It was possible that the phosphorylated intermediates extracted were not uniformly labelled isotopically. For example, Flock & Bollman (1944) found a much more rapid incorporation of ^{32}P into the terminal phosphorus atom (P') of ATP than into the P^{α} and P^{β} atoms. In the present work the nucleotide was apparently uniformly labelled. An estimate of the relative $^{31}\text{P}/^{32}\text{P}$ ratios of the separated fractions was made by neutron activation of paper chromatograms of labelled extracts. This ratio for the PO_4^{3-} fraction could not be used for

comparison because the unavoidable contamination of the labelled extract by inactive phosphate during the external rinse of the flies in dilute phosphoric acid gave an artificially high $^{31}\text{P}/^{32}\text{P}$ ratio in this fraction. The average results of two experiments in which labelled strips were allowed to decay and then activated are summarized below.

The results indicate the presence of a higher proportion of ^{31}P in the original nucleotide fraction than in the others. It is significant, however, that in the ADP and other fractions the $^{31}\text{P}/^{32}\text{P}$ ratios are in reasonable agreement. If in fact the high $^{31}\text{P}/^{32}\text{P}$ ratio of II_1 were due to incomplete labelling of the nucleotide fraction, the highest ratio would have been expected in the ADP fraction, because of its higher P^α content. It was concluded that while there was no evidence of incomplete labelling of the nucleotide in these experiments there was evidence for the presence of an unlabelled phosphorus compound in the nucleotide fraction behaving as ATP in solvents 1 and 2.

Limited acid hydrolysis of the resolved ATP and ADP fractions, respectively, liberated two-thirds and one-half the total ^{32}P -activity as free PO_4^{3-} . This would only be expected for uniformly labelled nucleotides. There were no differences in the proportions of the total ^{32}P -activity running as ATP in groups of flies which had been allowed to metabolize 24, 48, 72 and 120 hr., respectively. This also suggests that isotopic equilibrium among the labelled intermediates was achieved 24 hr. after labelling.

As a precautionary measure the nucleotide fractions have been hydrolysed on a routine basis and the fraction of total ^{32}P -activity liberated as PO_4^{3-} used as a check on the uniformity of labelling. Evidence of incomplete labelling would be taken into account in the final analysis and reported.

Fraction	...	...	...	...	...	II ₁	I ₂	II ₂	III ₁ (Arg-P + AMP + G 6-P + U.C. 1)	IV ₁
¹ P/ ³² P ratio: (that of 'PGA' taken as unity)						(ATP + ADP)	(ATP)	(ADP)		(PGA)
						1.2	1.3	0.9	0.9	1.0

Histological distribution of ^{32}P

Since an extract of whole thorax has been used as an indicator of the distribution of phosphorylated intermediates of muscle it was of interest to determine the histological ^{32}P -distribution in transverse sections of the thorax. A typical stained transverse section and the corresponding autoradiograph made from the neighbouring section are shown in Figs. 4 and 5. ^{32}P -Concentrations appear as dark zones in the autoradiograph. A fairly uniform distribution of ^{32}P can be seen in the indirect flight muscle blocks in the upper half of the autoradiograph (*A*) while a relatively higher but localized concentration occurs in the walls of the stomach and proventriculus seen as elliptically shaped organs in the lower half of the autoradiograph (*B*). The higher concentration in the gut was confirmed by dissecting out gut and muscle separately from a labelled insect under cooled light petroleum. The tissue was dried and weighed on tared cover slips and the ^{32}P -activity assayed by direct counting under a thin end-window Geiger-Müller tube. The ^{32}P -activities of gut and muscle tissue gave respectively 75 and 30 net counts/min./ μg . dry tissue. A pair of extracts was made of a horizontally bisected thorax so that one extract was of dorsal tissue rich in muscle and the other of ventral tissue containing the gut, ventral nerve ganglia, etc. Chromatographic analyses showed that these extracts were of very similar composition with some evidence of a higher

ATP content of the gut-containing tissue. It was concluded that the use of extracts of entire thorax fairly represented the distribution of phosphorylated intermediates in the thoracic muscle.

Radiation and age effects

No attempt has been made to calculate the radiation dose in röntgen units received by the insects because of the difficulty of defining the geometrical conditions. A fly was not only exposed to radiation by self-absorption of the beta particles of its own tissue but to radiation from neighbouring flies, from the feeding-wells, and to that back-scattered by the walls of the metabolism unit. Tissue concentrations of ^{32}P were calculated, however, from the results of calibrating the scanning unit with known amounts of ^{32}P separated on paper chromatograms. These concentrations were compared with the distribution of labelled intermediates determined in seventeen different groups of ten flies in which the calculated ^{32}P concentrations varied from 0.002 μC to 1.01 $\mu\text{C}/\text{mg}$. wet tissue at the time of 'labelling'. There was apparently no relation between the small variations in distribution found and the radiation levels as indicated by the concentration of ^{32}P in the tissue. The ages of the same fly groups was either 3 days or 6-7 days at the time of extraction. The mean (ATP + ADP)-value (% total ^{32}P) was higher (42.2 %) for the older flies than for the 3-day flies (36.9 %) but the difference was not significant ($P > 0.05$). It was concluded that any radiation or

Fig. 4

Fig. 4. Photomicrograph of transverse section through thorax of adult housefly stained in Heidenhain's haematoxylin. *A*, indirect flight muscle; *B*, gut. ($\times 25$).

Fig. 5

Fig. 5. Autoradiograph showing histological distribution of ^{32}P in transverse section through thorax of adult housefly (cf. Fig. 4). *A* corresponds to indirect flight muscle; *B* corresponds to gut. Darker zones indicate higher ^{32}P concentrations. ($\times 25$).

Table 2. *Distribution of labelled intermediates in thoracic muscle of flies at different levels of motor activity and cyclopropane anaesthesia*

State of test flies at instant of death	All figures are % of total soluble ^{32}P							
	Full flight		Crawling only		1 hr. continuous anaesthesia		5 hr. continuous anaesthesia	
	Control	Test	Control	Test	Control	Test	Control	Test
Fraction								
ATP + ADP	38.0	51.6	46.4	45.5	29.2	27.0	36.0	29.4
Arg-P + G 6-P + AMP + U.C. 1	19.4	8.6	20.4	15.6	27.4	30.6	18.0	30.7
PGA	31.4	10.2	18.0	11.4	32.7	22.7	34.2	14.7
PO_4^{3-}	11.2	29.6	15.2	27.5	10.7	19.7	11.8	25.2
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

age effects were small compared to the natural variation in phosphorus distribution from one fly group to another.

Effects of cyclopropane anaesthesia

To bring all the insects used in these studies to a common state of rest they were anaesthetized in cyclopropane for 2 min. immediately before chilling, extracting, etc. Possible effects of cyclopropane were investigated by determining the distribution of labelled intermediates of 'test' groups of flies which were at different levels of motor activity or anaesthesia at the instant of death (plunging into liquid nitrogen). The results are summarized in Table 2. 'Controls' refer to groups of flies from the same generation, 'labelled' and 'metabolized' simultaneously with the test flies and subjected to the standardized 2 min. anaesthesia.

It will be seen that motor activity is associated with a reduction in the Arg-P fraction and increase in PO_4^{3-} . The fraction (ATP + ADP) during full flight was the highest ever recorded and therefore significant. Other effects believed to be significant are the increase in PO_4^{3-} and depletion in PGA during activity or extended anaesthesia. The evident accumulation of and depletion of Arg-P with relatively constant ATP during rest and activity respectively are consistent with current views of muscle biochemistry (Mommaerts, 1951; Greenberg, 1952). Finally the results suggest that the brief cyclopropane anaesthesia normally used causes no abnormal distribution of labelled intermediates.

DISCUSSION

Meyerhof & Lohmann (1928), Meyerhof (1928), and Needham, Needham, Baldwin & Yudkin (1932) have shown that the phosphagen of many invertebrate species is arginine phosphoric acid (Arg-P). Baldwin & Needham (1934) found Arg-P in the thoracic tissue of *Calliphora* and *Lucilia* species of blowfly. The presence of Arg-P as well as free arginine in thoracic tissue extracts of the adult housefly *Musca domestica* has been demonstrated in the present work. It is therefore surprising that Pratt (1950) was unable to detect free arginine in

Table 3. *Comparison of phosphorylated intermediates found in the resting muscle of the adult housefly (present work) and in the rat (LePage, 1948)*

Compound	Phosphorus of compound expressed as % of total acid-soluble phosphorus	
	Housefly	Rat
ATP	37.2	33.6
ADP	1.2	2.4
Phosphagen	10.9	33.7
AMP	<6.9	3.2
G 6-P	7.8	5.2
PGA	20.8	2.9
PO_4^{3-}	15.2	15.3
Other compounds	>0.0	3.7
	100.0	100.0

the haemolymph of *Musca domestica* adults. Insect and mammalian ATP appear to be identical (Albaum & Kletzkin, 1948; Calaby, 1951). The other phosphorylated intermediates of housefly muscle suggested by the present work are well-known extractives of mammalian muscle, and it is interesting to compare the overall distribution found with that reported by LePage (1948) for resting rat muscle. This has been done in Table 3. The original figures of LePage, which were in μmoles of intermediates per 100 g. of wet tissue, have been converted into g. of equivalent phosphorus per 100 g. of total acid-soluble phosphorus accounted for.

Notable are the relatively low phosphagen and high PGA of the insect. Meyerhof (1928) found as much as 75 % of the total acid-soluble phosphorus present as Arg-P in resting Crustacean muscle.

SUMMARY

1. The adult housefly, *Musca domestica* L., has been fed with carrier-free $^{32}\text{PO}_4$. The radioisotope was incorporated into the acid-soluble phosphorylated compounds of thoracic tissue. These compounds were extracted and separated on paper chromatograms and assayed by an automatic radiometric scanning technique.

2. The major fractions separated have been tentatively identified as ATP, ADP, AMP, glucose

6-phosphate, arginine phosphoric acid, phosphoglycerate and free orthophosphate.

3. Evidence was obtained that all the P atoms of the ATP and ADP fractions were equally labelled.

4. The histological distribution of the ^{32}P was studied by an autoradiographic technique. A relatively high concentration of ^{32}P in the gut of the thorax was found but appeared to be of the same chemical composition as that of the muscle.

5. The effects of motor activity and cyclopropane anaesthesia upon the distribution of labelled intermediates appeared to be consistent with current views in muscle biochemistry.

6. The phosphorylated compounds of resting housefly and mammalian muscle have been compared. Relatively low phosphagen and high phosphoglycerate levels in the insect were noted.

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REFERENCES

- Albaum, H. G. & Kletzkyn, M. (1948). *Arch. Biochem.* **16**, 333.
- Baldwin, E. & Needham, D. M. (1934). *J. Physiol.* **80**, 221.
- Bandurski, R. L. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.
- Bentley, H. R. & Whitehead, J. K. (1950). *Biochem. J.* **46**, 341.
- Burrows, S., Grylls, F. S. M. & Harrison, J. S. (1952). *Nature, Lond.*, **170**, 800.
- Calaby, J. H. (1951). *Arch. Biochem. Biophys.* **31**, 294.
- Dubois, K. P. & Potter, V. R. (1943). *J. biol. Chem.* **147**, 41.
- Flock, E. V. & Bollman, J. L. (1944). *J. biol. Chem.* **152**, 371.
- Greenberg, D. M. (1952). *Phosphorus Metabolism*, vol. II, p. 14. Baltimore: Johns Hopkins Press.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Holiday, E. R. & Johnson, E. A. (1949). *Nature, Lond.*, **163**, 216.
- Lee, K.-H. & Eiler, J. J. (1951). *Science*, **114**, 393.
- Lee, K.-H. & Eiler, J. J. (1953). *J. biol. Chem.* **203**, 705.
- LePage, G. A. (1948). *Cancer Res.* **8**, 193.
- Mende, T. J. & Chambers, E. L. (1953). *Arch. Biochem. Biophys.* **45**, 105.
- Mendelow, H. & Hamilton, J. B. (1950). *Anat. Rec.* **107**, 443.
- Meyerhof, O. (1928). *Arch. Sci. biol., Napoli*, **12**, 536.
- Meyerhof, O. & Lohmann, K. (1928). *Biochem. Z.* **196**, 49.
- Mommaerts, W. F. H. M. (1951). *Phosphorus Metabolism*, vol. I, p. 551. Baltimore: Johns Hopkins Press.
- Needham, D. M., Needham, J., Baldwin, E. & Yudkin, J. (1932). *Proc. Roy. Soc. B*, **110**, 260.
- Neuberg, C. & Lustig, H. (1942). *J. Amer. chem. Soc.* **64**, 2722.
- Partington, J. R. (1933). *A Text-Book of Inorganic Chemistry*, p. 624. London: Macmillan and Co.
- Potter, V. R. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. p. 136. Minneapolis: Burgess Publishing Co.
- Pratt, J. J. (1950). *Ann. ent. Soc. Amer.* **43**, 573.
- Sumner, J. B. & Somers, G. F. (1944). *Arch. Biochem.* **4**, 11.
- Weber, C. J. (1930). *J. biol. Chem.* **86**, 217.
- Winteringham, F. P. W. (1953). *Nature, Lond.*, **172**, 727.
- Winteringham, F. P. W., Harrison, A. & Hammond, J. H. (1950). *Nature, Lond.*, **165**, 149.
- Winteringham, F. P. W., Harrison, A. & Bridges, R. G. (1952). *Nucleonics*, **10**, 52.
- Winteringham, F. P. W., Loveday, P. M. & Hellyer, G. C. (1953). *Biochem. J.* **55**, xxxiii.

The Biological Action of Substances Related to Thyroxine

10. THE DE-IODINATION OF THYROXINE IN THYROIDECTOMIZED RATS*

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In previous communications (Wilkinson & MacLagan, 1953, 1954; Wilkinson, Sprott, Bowden & MacLagan, 1954) it has been shown that, in intact rats, the output of urinary iodide after a single injection of ^{131}I -labelled thyroxine or triiodothyronine is considerably reduced by the simultaneous administration of butyl 4-hydroxy-3:5-diiodobenzoate (BHDB). In contrast with these

results, Roche, Deltour & Michel (1953) reported that, in thyroidectomized rats, BHDB brought about an increase in the urinary iodide excretion after a single dose of radioactive thyroxine. Our work has therefore been extended to thyroidectomized animals in an endeavour to explain these conflicting results.

A brief report of some of the results has been published elsewhere (Wilkinson & Feetham, 1954).

* Part 9, Wilkinson & MacLagan (1954).