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Distribution of Phosphorus Compounds in Blowfly Thoracic Muscle

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Watanabe & Williams (1951) showed that the sarcosomes in the flight muscle of the blowfly *Phormia regina* are in many respects homologous with the intracellular mitochondria of vertebrate tissue, and therefore it seemed likely that the sarcosomes were the site of oxidative phosphorylation in insect muscle. This process has been demonstrated *in vitro* in sarcosomes from the blowfly *Calliphora erythrocephala* (Lewis & Slater, 1954) and the housefly *Musca domestica* (Sacktor, 1954). The phosphorus: oxygen (P:O) ratios, however, were lower than those which have been reported for rat-heart sarcosomes (Slater & Holton, 1954) and for mouse-liver mitochondria (Kielley & Kielley, 1951). The reason for this difference is not clear. Siekivitz & Potter (1955), working with rat-liver mitochondria, showed that although low P:O ratios were associated with the loss of nucleotides, mitochondria which gave a P:O ratio as high as 2.0 when oxidizing succinate contained only about 5% of the total adenine nucleotides in the liver

cells. Levenbook (1953), on the other hand, reported that 60% of the adenine nucleotides in the flight muscle of *P. regina* are retained by the isolated sarcosomes. In view of these findings we have attempted to identify the nucleotides and other phosphorus compounds in the thorax of *C. erythrocephala*, and have examined their distribution between the sarcosomes and the 'soluble' fraction from thoracic extracts. For this purpose we have used the 'labelled-pool' technique described by Winteringham, Bridges & Hellyer (1955), in which the phosphorus compounds are labelled *in vivo* with ^{32}P and subsequently isolated by paper chromatography.

MATERIALS AND METHODS

Adenosine triphosphate, adenosine diphosphate and muscle adenylic acid. These were purchased as the disodium salt, the sodium salt and the free acid respectively from Sigma Chemical Co., St Louis, Mo., U.S.A.

Fig. 1. Fractionation of a sarcosome suspension of blowfly thoraces. The extracts were stored at -15° immediately after preparation.

Diphosphopyridine nucleotide. This was purchased from Sigma Chemical Co. and L. Light and Co.

Glucose 6-phosphate and 3-phosphoglyceric acid. These were purchased as the barium salts from Sigma Chemical Co. Before use they were dissolved in 0.1N-HCl and the barium was removed by precipitation with an equivalent amount of sodium sulphate.

Sodium glycerophosphate. This was purchased from British Drug Houses Ltd.

Radioactive phosphorus. Carrier-free $H_3^{32}PO_4$ was purchased from The Radiochemical Centre, Amersham, Bucks.

Sarcosome-isolation medium. 0.318M-Sucrose; 0.009M-phosphate buffer (KH_2PO_4 - Na_2HPO_4 , pH 7.4); 0.01M-ethylenediaminetetra-acetate, pH 7.4.

Sarcosome-extraction media. (1) N-Formic acid in aq. 50% (v/v) ethanol. (2) Ethanol-ether (3:1, v/v).

Incorporation of [^{32}P]phosphate into the phosphorus compounds of the adult blowfly

Blowflies were reared at 23° and 80% relative humidity. Those used in the experiments were supplied with sucrose and water only. Flies were collected as required, immobilized by cooling, and placed in a 3 l. jar in which there were two small Petri dishes. One of these contained glucose and the other contained cotton wool saturated with 12 ml. of water containing 2 mc of [^{32}P]phosphate. Muslin was secured over the open end of the jar which was then kept at a constant temperature of 25° . The cotton wool was kept moist with water added from a pipette inserted through a small hole in the muslin. After a 48 hr. 'labelling' period the flies were transferred to another jar and

were supplied with glucose and water only, and kept at 25° for an 'equilibration' period of 48 hr. unless otherwise stated.

Isolation and extraction of sarcosomes

Forty flies were collected in a conical flask and immobilized by cooling. Any [^{32}P]phosphate adhering to the flies was removed by washing them first in 5% ethanol containing 1% of KH_2PO_4 , and then in aq. 1% KH_2PO_4 soln. They were then placed in an ice-cold dish lined with filter paper, which absorbed most of the moisture remaining on them. The heads and abdomina were removed, the thoraces collected in an ice-cold mortar and gently crushed in 3 ml. of isolation medium. The resulting brei was squeezed through six layers of muslin saturated with isolation medium, the filtrate being collected in a tube surrounded by crushed ice. A further 9 ml. of isolation medium was added to the residue, which was again squeezed and filtered. The combined filtrates (about 12 ml.) consisted of a suspension of sarcosomes and some broken myofibrils in a water-soluble thoracic extract, from which the sarcosomes were separated by differential centrifuging at 0° (see Fig. 1).

The isolated sarcosomes were homogenized at -15° in the ethanol-formic acid extraction medium, and the homogenate was centrifuged at 0° for 15 min. at 22 000 g. After a further extraction with ethanol-formic acid the residue was resuspended in ethanol-ether and allowed to stand for about 40 min. at 0° before being centrifuged. The details of the isolation and extraction procedures are given in Fig. 1. In one experiment a portion of extract A, and of extract C (Fig. 1), was treated with perchloric acid (final concn. 2%) and centrifuged at 0° for 10 min. at 22 000 g.

Paper-chromatographic techniques

Strips of Whatman no. 1 paper were soaked in 0.57N-HCl in 45% ethanol for 30 min. They were then soaked for a further 30 min. in aq. 5% NH_3 soln. and finally washed in six changes of glass-distilled water.

Portions of each extract (50 μl . or 100 μl .) were applied to the filter paper strip, and dried in a stream of cold air and chromatographed by the ascending technique in partially evacuated glass columns (Winteringham *et al.* 1955). The following solvent systems were used, and will be referred to in the text as solvents 1 to 9. Their composition is given in vol. %, unless otherwise stated. 1. 98% Formic acid-acetone-water (15:50:35). 2. Acetone-15% (w/v) trichloroacetic acid (65:35). 3. Aq. 2N- NH_3 soln.-methanol (30:70) (Burrows, Grylls & Harrison, 1952). 4. Methanol-98% formic acid-water (80:15:5). 5. Methanol-aq. NH_3 soln. (sp.gr. 0.88)-water (60:10:30) (Bandurski & Axelrod, 1951). 6. Phenol, water-saturated and containing 0.5% of 8-hydroxyquinoline (Hummel & Lindberg, 1949). 7. *iso*Propyl ether-90% formic acid (60:40) (Hanes & Isherwood, 1949). 8. 0.1M- KH_2PO_4 -Na $_2\text{HPO}_4$ (pH 6.8)-(NH_4) $_2\text{SO}_4$ -propan-1-ol (100 ml.:60 g.:2 ml.) (Bock, Ling, Morell & Lipton, 1956). 9. *n*-Butyric acid-water-NaOH (69 ml.:31 ml.:0.85 g.) (Wade & Morgan, 1955).

Chromatograms were developed at 5° for periods of 40-50 hr. in solvents 1, 2, 4 and 5, and at 25° for the same periods in solvents 3, 6, 7 and 8. In solvent 9 chromatograms were developed at 20° for 5 days, and were run on Whatman no. 3 paper which had been washed with *n*-formic acid and with water as described by Wade & Morgan (1954).

Location of known 'marker' compounds on chromatograms. One of the criteria used in establishing the identity of a ^{32}P -labelled compound was coincidence on the chromatogram with a known 'marker' compound, which was chromatographed together with the ^{32}P -labelled material. When nucleotides had been added as markers they were located by their absorption under ultraviolet light. Chromatograms which had been developed in solvent 6 were washed with xylol and light petroleum to remove phenol, which masks absorption by nucleotides (Hummel & Lindberg, 1949). Other added phosphorus compounds were located with a molybdate reagent as described by Burrows *et al.* (1952).

Radiometric scanning of paper chromatograms. The ^{32}P -labelled compounds were located on the chromatograms as described by Winteringham, Harrison & Bridges (1952). The chromatogram is passed automatically beneath a thin-end-window Geiger-Müller tube and the radioactivity recorded graphically. Each zone of radioactivity on the strip is represented by a peak on the graph, and the area enclosed by each peak is proportional to the amount of ^{32}P in the zone which it represents.

Ultraviolet-absorption spectra. Some fractions were extracted from several chromatograms with 2% perchloric acid and the ultraviolet absorption of the extract was measured against that of a similar extract from an equal number of blank chromatograms.

Estimation of total phosphorus in sarcosome suspensions

The total phosphorus content of the sarcosome suspensions from ' ^{32}P -labelled' flies could not be determined,

since these flies had been washed in a phosphate solution. An estimate of the phosphorus content of these suspensions was obtained by measuring the total phosphorus content of similar suspensions prepared from unwashed flies. A sample (0.1 ml.) of each suspension was heated together with 1 ml. of conc. HNO_3 for 30 min. and cooled. Concentrated H_2SO_4 (1 ml.) was added and the mixture heated to remove the HNO_3 . After dilution with about 20 ml. of water the sample was boiled to convert any pyrophosphate into orthophosphate. It was then cooled, and made up to 20 ml. with water and its phosphorus content determined according to Milton & Waters (1955). Parallel determinations were carried out on equal volumes of the sarcosome-isolation medium.

Protein estimation

The protein content of the washed sarcosomes was determined by the method of Gornall, Bardawill & David (1949).

RESULTS

Distribution of ^{32}P . Sarcosome suspensions were prepared from ^{32}P -labelled flies, with the sarcosome-isolation medium, and then fractionated as shown in Fig. 1. Equal volumes of each extract were pipetted on to filter-paper disks. These were placed, in turn, beneath a thin-end-window Geiger-Müller tube, and the radioactivity was measured over a 5 min. period. The results of two such experiments, in which the relative amount of ^{32}P in each extract was calculated, are shown in Table 1. It will be seen that more than 90% of the ^{32}P is in extract A (the 'soluble' fraction) while the combined sarcosome extracts (C, D and final residue) contain less than 5% of the total activity. It will also be seen that the distribution of ^{32}P between the extracts is similar in both the experiments.

Identification of ^{32}P -labelled compounds. The phosphorus compounds in extracts A, B, C and D were separated into a number of fractions by chromatographing 50 μl . portions of these extracts in solvent 1. The distribution of these fractions along the chromatogram is shown graphically in Fig. 2. Each fraction except A_1 and C_1 (see below) was eluted at 5° from the chromatogram on to another strip, as described by Winteringham *et al.* (1955), and chromatographed in a second solvent. By repetition of this procedure we were able to study the chromatographic behaviour of the fractions from extracts A, C and D in a series of solvent systems. The R_f values obtained, and the suggested composition of the fractions based on coincidence with 'marker' compounds, are shown in Fig. 3. Further evidence for the identity of the ^{32}P -labelled compounds in the extracts, together with some data on the distribution of ^{32}P among the compounds in each fraction, is given below.

Table 1. *Distribution of ³²P among extracts A, B, C, D and final residue, derived from a sarcosome suspension as shown in Fig. 1*

The first residue (see Fig. 1), which contained 1.2% of the ³²P in the sarcosome suspension, is not included in this table. The count was from 20 μ l. samples in Expt. 1, and from 10 μ l. samples in Expt. 2.

	Vol. of extract (ml.)	Count/min.	³² P in each extract (as % of ³² P in supernatant from sarcosome suspension)
Expt. 1. Extract A	11.6	11 009	92.03
Extract B	11.6	427	3.56
Extract C	2.1	1 261	1.90
Extract D	1.4	1 728	1.74
Final residue	4.2	255	0.77
Expt. 2. Extract A	8.5	4 715	92.44
Extract B	9.0	157	3.26
Extract C	1.8	464	1.92
Extract D	1.0	847	1.95
Final residue	1.6	123	0.43

Fig. 2. Distribution of ³²P-labelled material on chromatograms after chromatographing extracts A, B, C and D in solvent 1.

Extract A

Fraction A₁. This fraction remained at *R_f* 0 in solvent 1, and since it could not be eluted off the chromatogram further examination of its chromatographic properties in other solvents was not attempted. Even after the section of the chromatogram containing this fraction was heated in *N*-HCl at 100° for 7 min. no radioactivity could be detected in the hydrolysate. Furthermore the fraction was removed from the extract when this was deproteinized with perchloric acid.

We concluded from these results that fraction *A₁* contained either phosphoprotein or compounds, such as nucleic acids, tightly bound to protein. When Winteringham *et al.* (1955) chromatographed extracts of '³²P-labelled' housefly thoraces in solvent 1, they also observed a fraction remaining at *R_f* 0 which they attributed to phosphoprotein.

Fraction A₂. The adenosine triphosphate (ATP) fraction derived by chromatography of *A₂* in solvent 6 was heated together with ATP in *N*-HCl at 100° for 7 min. When the hydrolysate was chromatographed in solvent 1 it was found that the ³²P-labelled hydrolysis products coincided with those of ATP, and moreover that 66% of the ³²P coincided with orthophosphate. It was also found that the ultraviolet-absorption spectrum of *A₂* was that of an adenine nucleotide. The chromatographic evidence indicated that over 90% of the ³²P in *A₂* was accounted for by ATP, and about 6% by diphosphopyridine nucleotide (DPN).

Fraction A₃. After being chromatographed in solvent 3 this fraction was heated together with adenosine diphosphate (ADP) in *N*-HCl at 100° for 7 min. When the hydrolysate was chromatographed in solvent 1 it was found that the ³²P-labelled hydrolysis products coincided with those of ADP and that 50% of the ³²P was released as orthophosphate. These findings provide support for the chromatographic evidence which suggested that all the ³²P in *A₃* was accounted for by ADP.

Fraction A₄. When this fraction was chromatographed in solvent 3, it was resolved into three sub-fractions *A₄₁*, *A₄₂* and *A₄₃* (Fig. 3). *A₄₁* was again chromatographed in solvent 1, and on this occasion two portions (*R_f* 0.41 and 0.77) were obtained. The faster-running portion coincided with orthophosphate and accounted for 25% of the ³²P in *A₄*. The original chromatogram in solvent 1 showed that *A₄* did not contain any orthophosphate. Therefore the orthophosphate in *A₄₁* must have been produced by the breakdown of a labile phosphorus compound in *A₄*. It is possible that this labile compound is arginine phosphate (Baldwin & Needham, 1934), since its behaviour in solvents 1 and 3 is the same as that of a compound identified as arginine phosphate by Winteringham *et al.* (1955). The slower-running fraction derived from *A₄₁* has not

Fig. 3. Separation of ^{32}P -labelled compounds in extracts A, C and D by chromatography in a series of solvent systems. The solvent numbers and the R_F values of the labelled fractions are given, together with the marker compounds with which the labelled fractions coincided. The final identification is based on the chromatographic evidence shown in this figure and on the supplementary evidence given in the text. A_8 is composed of two subfractions which were never clearly separated in solvent 1. Ortho-P, orthophosphate.

been identified. It is not arginine phosphate since it was not hydrolysed by treatment with 0.01N-HCl at 37° for 4 hr. (Mende & Chambers, 1953).

The chromatographic behaviour of the unidentified portion of A_4 in solvent 1 (R_F 0.84) shows that it was not originally present in A_4 and therefore must have been produced by the breakdown of a labile compound in that fraction. We concluded that A_4 contains adenylic acid (AMP), glucose 6-phosphate (G6-P) and at least two labile compounds, one of which is possibly arginine phosphate.

Fraction A_5 . Orthophosphate accounted for 75% of the ^{32}P in this fraction, and the remainder was accounted for by 3-phosphoglyceric acid (PGA).

Extract C

Fraction C_1 . This fraction resembles fraction A_1 in that it remained at R_F 0 in solvent 1, could not be eluted off the chromatogram, was insoluble in N-HCl and was precipitated when the extract was treated with perchloric acid. Therefore we concluded that C_1 also contains either phosphoprotein or compounds tightly bound to protein.

Fraction C_2 . The 'ATP' fraction from solvent 6 (see Fig. 3) was heated together with ATP in N-HCl at 100° for 7 min. When the hydrolysate was chromatographed in solvent 1, the ^{32}P -labelled hydrolysis products coincided with those of ATP.

The 'DPN' fraction was heated together with DPN either in N-HCl at 100° for 7 min., or in N-NaOH at 25° for 18 hr. (Kaplan, Colowick & Barnes, 1951), and each hydrolysate was chromatographed in solvent 1. In both cases the DPN and the labelled fraction were completely hydrolysed, and the labelled hydrolysis products coincided with those of DPN.

The chromatographic evidence showed that 78% of the ^{32}P in C_2 is present in DPN and the remaining 22% in ATP.

Fraction C_3 . After being chromatographed in solvent 8 this fraction was heated together with ADP in N-HCl at 100° for 7 min. and the hydrolysate was chromatographed in solvent 1. Two labelled fractions containing equal amounts of ^{32}P were obtained, and these coincided with the hydrolysis products of ADP. We concluded from the chromatographic evidence that the major component of fraction C_3 is ADP.

Fraction C_4 . This fraction resembled A_4 in that it coincided with AMP and G6-P in solvent 1. In solvents 3 and 4, however, 85% of the ^{32}P coincided with AMP, but none with G6-P, which was separated from AMP in solvent 3. Therefore we concluded that the major component of fraction C_4 is AMP. The remaining 15% of this fraction has not been identified.

Fraction C_5 . When this fraction was chromatographed in solvent 3, it was resolved into three fractions, each of which coincided with the KH_2PO_4 marker (Fig. 3). Three fractions were also obtained when ^{32}P -labelled orthophosphate was chromatographed together with KH_2PO_4 in this solvent. Curry (1953) has also observed the formation of several spots when orthophosphate is chromatographed in a neutral or an alkaline solvent, and has attributed this phenomenon to the separation of the three ionic forms. The fact that C_5 also gave rise to three fractions when chromatographed in solvent 3, and that each of these coincided with the phosphate marker, provides additional evidence that C_5 contains orthophosphate.

Fractions C_6 and D_6 . C_6 is the largest fraction in extract C. However, the fact that in solvent 1, an acid solvent, it ran further than orthophosphate suggested that it contained compounds not usually found in acid-soluble extracts from muscle. This suggestion was substantiated by the fact that an even larger amount of a similar fraction accounted for most of the ^{32}P -labelled material in extract D, the ethanol-ether extract (Fig. 2). The rate of hydrolysis of fraction C_6 in N-HCl at 100° was followed by taking 50 μl . samples of the hydrolysate at intervals up to 6 hr. and chromatographing these in solvent 1. Only one labelled product was found, and this did not coincide either with orthophosphate or with fraction C_6 . Furthermore, the amount of this product increased with time of hydrolysis (Fig. 4). Therefore it appeared that although C_6 was not removed from the chromatogram by boiling in N-HCl, it was slowly hydrolysed, with the production of a stable phosphorus compound which passed into solution. A similar compound was produced when fraction D_6 was hydrolysed in N-HCl. This finding, together with the behaviour of these fractions in solvent 1, suggested that their composition is similar and that they are

Fig. 4. Rate of liberation of ^{32}P -labelled hydrolysis product from fraction C_6 by N-HCl at 100°.

both derived from the same sarcosomal constituent, the greater part of which is extracted with the ethanol-ether medium. These observations suggested that fractions C_6 and D_6 contain phospholipids. This suggestion was supported by the fact that the ^{32}P -labelled hydrolysis product of both fractions coincided with glycerophosphate when chromatographed in solvents 1 and 3. It has been shown that the rate of hydrolysis of glycerophosphoric acid is markedly dependent on pH, and that it reaches a maximum at pH 4.0 (Bailly, 1942). The hydrolysates from both C_6 and D_6 were brought to pH 4.0, heated under 2 atm. pressure at 115° for 4 hr., and again chromatographed in solvents 1 and 3. After this treatment 74% of the labelled material coincided with orthophosphate in both solvents. This fact provided further evidence that the labelled hydrolysis product of both C_6 and D_6 is in fact glycerophosphoric acid. We concluded from the above evidence that both C_6 and D_6 contain phospholipids.

Extract B

This extract was obtained by washing the sarcosomes in the isolation medium. Although it has not been examined in detail, some indication of its composition was provided by chromatographing the extract in solvent 1 and comparing the radiochromatogram with those obtained from extracts A , C and D . The first fraction in extract B behaved like fractions A_1 and C_1 in that it remained at $R_f 0$. The second fraction coincided with ADP and the third with AMP, whereas in extracts A and C these compounds coincided with the third and fourth fractions respectively. This suggests that the second and third fractions in extract B correspond to fractions 3 and 4 respectively in extracts A and C , and that there is no fraction in extract B which corresponds to fraction 2, the fraction containing ATP and DPN in extracts A and C . The R_f value (0.65) of the last but one fraction in solvent 1 suggests that it corresponds to fraction 5 from extracts A and C , and therefore it may contain orthophosphate and PGA. The fact that the last fraction runs to the solvent front suggests that it corresponds to fraction 6 from extracts C and D .

Since extract B contains about 1.9% of the ^{32}P in the supernatant from the sarcosome suspensions, its ^{32}P could not all be accounted for by contamination with extract A , because 0.5 ml. of this extract would be required to account for that amount of ^{32}P , and the volume of the sarcosome pellet was not more than 0.1 ml. Furthermore, the presence in extract B of fraction 6, a fraction not present in extract A , but present in C and D , indicates that extract B contains ^{32}P -labelled material derived from the sarcosomes.

Uniformity of labelling

In preliminary experiments with blowflies the distribution of ^{32}P was examined in extract A and in the ethanol-formic acid extract of unwashed sarcosomes, prepared from three groups of flies after a 'labelling' period of 24 or 48 hr., and an 'equilibration' period of 48 or 120 hr. The results given in Table 2 show that in extract A , and in the sarcosome extract, the distribution of ^{32}P among the major fractions is similar in all three groups. Only in extract A from flies labelled for 24 hr. is there an indication that equilibrium has not been reached. In view of this finding the flies used in all other experiments were supplied with ^{32}P for 48 hr. and allowed to metabolize for 48 hr. before being extracted.

It has already been shown that the ^{32}P -labelled ATP and ADP fractions from extract A are uniformly labelled, since on hydrolysis for 7 min. in N-HCl 66 and 50% respectively of the total ^{32}P of these nucleotides was liberated as ^{32}P orthophosphate. Further evidence that equilibrium had been reached was obtained as follows. The total phosphorus content of the whole-muscle suspension prepared from 39 ' ^{32}P -labelled' flies was estimated to be 1.500 mg., which is the mean of four determinations (1.622, 1.295, 1.521, 1.565 mg.) made on sarcosome suspensions prepared from similar groups of 'unlabelled' flies (see Methods). With this value the amount of phosphorus in extract A was calculated on the basis of ^{32}P distribution. Further, by measurement of the areas enclosed by the peaks on the graphs we were able to calculate the percentage of phosphorus accounted for by the ATP

Table 2. Effect of time on the distribution of ^{32}P in extract A and in an extract of unwashed sarcosomes

Extract A and the unwashed sarcosomes were obtained as shown in Fig. 1. The fractions 1-5 (extract A) and 1-6 (ethanol-formic acid sarcosome extract) were obtained when these extracts were chromatographed in solvent 1 (Fig. 2). The ^{32}P in each fraction is expressed as a percentage of the ^{32}P in the extract.

'Labelling' period (hr.)	'Equilibration' period (hr.)	Extract A				Sarcosome extract				
		1	2+3	4	5	1	2+3	4	5	6
24	48	—	45.6	10.1	44.3	7.0	21.2	7.1	30.9	33.8
48	48	4.6	38.4	14.0	43.0	4.9	23.9	6.5	33.7	31.0
48	120	5.0	39.8	17.2	38.0	8.6	21.3	7.0	30.8	32.3

fraction in extract *A*, and from these data a value of 0.36 mM was obtained for the concentration of ATP in this extract. A second value for the concentration of ATP in extract *A* was obtained by a more conventional method. Preliminary experiments showed that by the procedure used to obtain the absorption spectra, 80 % of the ATP applied to a chromatogram could be recovered in the perchloric acid extract. After allowing for the 20 % loss during chromatographic and elution procedures, and by the use of a value of 14.7×10^3 for the extinction coefficient (Pabst Laboratories, 1956), we obtained a value of 0.31 mM for the concentration of ATP in extract *A*. This value agrees closely with that calculated on the basis of ^{32}P distribution. Agreement between these two values could be obtained only if isotopic equilibrium had been reached and if the phosphorus compounds in the sarcosome suspension are uniformly labelled. Further evidence that this is so is the close agreement between the value of 4.8 $\mu\text{moles/g.}$ of sarcosomal protein for the DPN content of the sarcosomes, calculated on a ^{32}P -distribution basis, and the value of 4.7 $\mu\text{moles/g.}$ of protein which we obtained by the enzymic method of Racker (1950) as modified by Holton (1955).

The weights of the phosphorus compounds in the 'soluble' fraction (extract *A*) and in the washed sarcosomes (extracts *C + D*), calculated on the basis of ^{32}P distribution, are shown in Table 3.

DISCUSSION

It was shown by Winteringham *et al.* (1955) that the acid-soluble compounds in the thoracic muscle of the housefly, *Musca domestica*, can be uniformly labelled with ^{32}P when the insects are supplied with water containing carrier-free $^{32}\text{PO}_4^{3-}$. With blow-

flies we have found that the phosphorus compounds become uniformly labelled and that isotopic equilibrium is reached when the flies have been supplied with carrier-free $^{32}\text{PO}_4^{3-}$ for 48 hr. and kept for a further 48 hr. on a phosphate-free diet. Therefore we were able to use the distribution of ^{32}P as a basis for calculating the amounts of the major phosphorus compounds which have been identified in the muscle extracts.

Sarcosome suspensions were prepared from 'labelled' flies and the sarcosomes were isolated by differential centrifuging under conditions which would be expected to retain their ability to carry out oxidative phosphorylation (Lewis & Slater, 1954). The supernatant (extract *A*) represents the 'soluble' fraction and contains over 90 % of the phosphorus in the sarcosome suspension. Chromatographic analysis showed that the major phosphorus compounds in this extract were the same as those found by Winteringham *et al.* (1955) in housefly thoraces which had been extracted at -15° . Although arginine phosphate was not identified in our extracts, there was present in extract *A* a labile compound whose chromatographic behaviour resembled that of arginine phosphate. Compounds which have been identified in extract *A* include ATP, ADP, AMP, DPN, G6-P, PGA and orthophosphate. There are several other labelled compounds in this extract which were detectable on the chromatograms, but these have not been identified.

In the sarcosomes about 13 % of the phosphorus is accounted for by the nucleotides ADP, AMP and DPN. Much of the remaining phosphorus is accounted for by phospholipids, which are probably constituents of the sarcosome membrane, as has been suggested for rat-liver mitochondria by Siekivitz & Watson (1956). The composition of the sarcosome residue has not been examined, but it seems probable that it would contain nucleic acids, since only small amounts of these compounds would have been extracted with our media.

Levenbook (1953) has reported values, calculated on a dry-weight basis, for the adenine nucleotide content of the thoracic muscle of the blowfly *Phormia regina*. When these were recalculated on a wet-weight basis, a value of 72 % for the water content of the muscle being used (Levenbook & Williams, 1956), the following values were obtained (mg./g. of wet muscle): ATP, 4.55; ADP, 0.87; AMP, 1.13. We obtained comparable values (ATP, 4.1; ADP, 2.05; AMP, 0.51) by combining the amounts of adenine nucleotides in the soluble fraction and in the sarcosome extracts (Table 3). After measuring the absorption at 260 $\text{m}\mu$ of extracts of isolated sarcosomes, Levenbook (1953) concluded that 60–70 % of the adenine nucleotides in the muscle were present in the sarcosomes.

Table 3. *Distribution of phosphorus compounds in blowfly thoracic muscle*

The 'soluble' fraction (extract *A*) and the sarcosome extracts (*C + D*) were obtained as shown in Fig. 1. The phospholipid content of the sarcosomes was calculated by multiplying the value for 'phospholipid phosphorus' by 25.

Compound	Content (mg./g. of wet muscle)	
	'Soluble' fraction (extract <i>A</i>)	Sarcosome extracts (<i>C + D</i>)
ATP	4.09	0.01
ADP	2.01	0.04
AMP	0.45	0.06
DPN	0.50	0.06
G6-P	1.55	—
PGA	1.70	—
Phospholipid	—	1.91
PO_4^{3-}	3.10	0.02

This result is in contrast with our findings, which suggest that less than 3% of the adenine nucleotides are present in the sarcosomes, even if the nucleotides in extract *B* were derived solely from the sarcosomes.

Insect sarcosomes are believed to be homologous with vertebrate mitochondria. In order therefore to compare our values for the phosphorus compounds in blowfly sarcosomes with those of other workers for these compounds in vertebrate sarcosomes and mitochondria, we have recalculated our values (mg./g. of muscle in Table 3) in terms of μ moles/g. of sarcosomal protein. Our value for the DPN content (4.8μ moles) is similar to that obtained by Holton (1955) for rat-heart sarcosomes (6.0μ moles), and also to the values of Jacobson & Kaplan (1957) for rat-liver mitochondria and rat-kidney mitochondria (2.0 and 6.4μ moles respectively). Our value of 4.2μ moles for the ADP content is about one-fifth of that obtained by Slater & Holton (1953) for pig-heart sarcosomes (20.0μ moles), but it should be noted that in the vertebrate preparation ADP was not estimated directly. In blowfly sarcosomes ATP and ADP together account for 6.1μ moles of '7 min.-hydrolysable phosphorus'. This is about one-third of the amount (17.2μ moles) found in mouse-liver mitochondria by Kielley & Kielley (1951). The value we obtained for inorganic phosphate (10.0μ moles) is about one-sixth of that obtained by Cleland (1957) for rat-heart sarcosomes (64.0μ moles). For AMP we obtained a value of 8.8μ moles. The percentages of the total phosphorus of blowfly sarcosomes accounted for by acid-soluble phosphorus (24%) and by lipid phosphorus (60%) are similar to those accounted for (21 and 56% respectively) by these constituents in rat-liver mitochondria (Kennedy & Lehninger, 1949).

We have concluded that the phosphorus compounds in blowfly sarcosomes are similar to those found by other workers in vertebrate sarcosomes and mitochondria. However, the concentrations of the energy-rich compounds ADP and ATP in relation to protein are lower in the insect sarcosomes than in the vertebrate preparations.

SUMMARY

1. When blowflies (*Calliphora erythrocephala*) were supplied with $^{32}\text{P}\text{O}_4^{3-}$ for 48 hr. and left for a further 48 hr. on a phosphate-free diet, the phosphorus compounds in the fly became uniformly labelled and isotopic equilibrium was attained.

2. Sarcosome suspensions were prepared from the thoraces, and fractionated by differential centrifuging into a 'soluble' and a sarcosome fraction.

3. The sarcosomes were extracted with ethanol-formic acid and then with ethanol-ether.

4. The ^{32}P -labelled compounds in the 'soluble' fraction and in the sarcosome extracts were examined by paper-chromatographic techniques.

5. In the 'soluble' fraction, which contained over 90% of the phosphorus in the whole-muscle suspension, the following compounds were identified: adenosine tri- and di-phosphate, adenylic acid, diphosphopyridine nucleotide, glucose 6-phosphate, 3-phosphoglyceric acid and orthophosphate.

6. The sarcosomes contained adenosine tri- and di-phosphate, adenylic acid, diphosphopyridine nucleotide, orthophosphate and phospholipid.

7. A comparison is made of the amounts of phosphorus compounds in blowfly sarcosomes with those reported for vertebrate sarcosomes and mitochondria.

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Observations on the Molecular Weight and Chemical Composition of Nisin A

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Nisin is an antibiotic normally made up of a mixture of peptides containing the following amino acids: alanine, glycine, serine, aspartic acid, valine, methionine, leucine, isoleucine, histidine, lysine, proline, lanthionine and β -methyl-lanthionine.

Some of the single peptides received a preliminary investigation by Berridge, Newton & Abraham (1952), who worked with commercial material prepared at great cost for therapeutic experiments. Cheeseman & Berridge (1957) described a more economical method of preparation of material which was intended for structural investigations. This report describes the separation of the major peptide by countercurrent distribution, a determination of its molecular weight and the derivation of some possible amino acid sequences.

The determination of amino acids by elution from paper chromatograms was used to derive a minimum molecular weight. This was in good agreement with the maximum molecular weight calculated from diffusion coefficients. Thus a reasonable estimate for actual molecular weight could be made.

The molecule as a whole proved too complex for a total determination of the amino acid sequence at this stage, but the structures of several small portions of the polypeptide chain isolated by partial hydrolysis in acid were determined, end-group analysis being used.

EXPERIMENTAL

Solvents for countercurrent distribution. Unless otherwise stated, all solvents employed were of A.R. grade (British Drug Houses Ltd.). The butan-1-ol was tested before use for the presence of peroxides (Newton & Abraham, 1950), and was considered free of peroxides if no red colour developed when the alcohol was added to a freshly prepared solution of pure KSCN and $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$.

Any positive samples found were redistilled over zinc powder before use.

Berridge *et al.* (1952) had used a solvent system consisting of methanol, butan-1-ol and acetate buffer at pH 3.0, in which nisin had an overall partition coefficient close to unity. This system had two advantages for its use. First, the partition coefficient at the beginning was close to unity; and other things being equal the maximum resolving power is obtained from a limited number of transfers when the geometric mean of the partition coefficients of the components of a mixture approaches unity (Craig & Craig, 1956). Secondly, at the pH used, some of the groups in the material appeared to be partly ionized and differences in solubility due to differences in the dissociation constants of the ionizable groups may occur under such conditions. The system had, however, a serious disadvantage from the preparative aspect which was that the nisin was not very soluble. It was therefore necessary to produce a new solvent which did not have this disadvantage, but retained the two advantages mentioned above. A mixture consisting of butan-1-ol, water, acetic acid and aq. NH_3 soln., with the pH of the aqueous phase at 2.8 to 3.0, was found to give satisfactory results. It was made by mixing 5 l. of water, 3.75 l. of butan-1-ol and 1.24 l. of acetic acid. The pH of the aqueous phase was adjusted by addition of conc. aq. NH_3 soln. to the mixture, and the solvent was kept overnight to reach equilibrium.

Apparatus. This consisted of approximately 100 units of normal design (cf. Craig, Hausmann, Ahrens & Harfenist, 1951), except that the second or decantation chamber (B) was similar to the first (A), having the same volume and having a decantation tube also at the same height (Fig. 1). Thus it was also possible to have bottom phase in chamber B. A complete cycle of operations therefore involved the addition of top phase to A, equilibration, separation, decantation of top phase into B, equilibration, separation and decantation. For one-half of the equilibrations the bottom phase in B was alone. This system had the advantage that for a given capital outlay and space practically double the usual number of units could be obtained, but there was the disadvantage that more transfers were required to effect a given separation than would be