3. The data obtained have been compared with the results previously reported from the application of the same fractionation technique to guinea-pig-liver microsome material labelled *in vivo*. The pattern of incorporation into the subfractions found in the cell-free system is quite different from that found in the intact liver cell: in particular, the behaviour of the proteins associated with RNA is markedly different.

4. Changes which occur in the microsome material as a consequence of incubation have been investigated: an appreciable loss of RNA was found to occur. The significance of the incorporation of [¹⁴C]amino acids into microsome protein in this cell-free system has been discussed.

5. A brief study was also made of the changes in the specific activity of both the soluble protein and of the trichloroacetic acid-soluble material of the cell-sap fraction after incubation of this cell-free system with [¹⁴C]amino acids.

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REFERENCES

- Allfrey, V. G., Daly, M. M. & Mirsky, A. E. (1953). *J. gen. Physiol.* **37**, 157.
- Askonas, B. A., Simkin, J. L. & Work, T. S. (1957). *Symp. biochem. Soc.* no. 14, p. 32.
- Bhargava, P. M., Simkin, J. L. & Work, T. S. (1958). *Biochem. J.* (in the Press).
- Canellakis, E. S. (1957). *Biochim. biophys. Acta*, **23**, 217.
- Frame, E. G., Russell, J. A. & Wilhelmi, A. E. (1943). *J. biol. Chem.* **149**, 255.
- Gale, E. F. & Folkes, J. P. (1955). *Biochem. J.* **59**, 675.
- Hultin, T. (1950). *Exp. Cell Res.* **1**, 376.
- Humphrey, J. H. & Sulitzeanu, D. (1958). *Biochem. J.* (in the Press).
- Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.
- Mütting, D. & Wortmann, V. (1954). *Biochem. Z.* **325**, 448.
- Russell, J. A. (1944). *J. biol. Chem.* **156**, 467.
- Sachs, H. & Waelsch, H. (1956). *Biochim. biophys. Acta*, **21**, 188.
- Siekevitz, P. (1952). *J. biol. Chem.* **195**, 549.
- Simkin, J. L. (1955). *Commun. 3rd Int. Congr. Biochem., Brussels*, p. 74.
- Simkin, J. L. (1957). *Biochem. J.* **66**, 6p.
- Simkin, J. L. & Work, T. S. (1957). *Biochem. J.* **65**, 307.
- Ullmann, A. & Straub, F. B. (1955). *Acta physiol. hung.* **8**, 279.
- Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.
- Winnick, T., Friedberg, F. & Greenberg, D. M. (1948). *J. biol. Chem.* **175**, 117.
- Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.
- Zamecnik, P. C., Keller, E. B., Littlefield, J. W., Hoagland, M. B. & Loftfield, R. B. (1956). *J. cell. comp. Physiol.* **47**, suppl. 1, 81.

Polyphosphates Excreted by Wax-Moth Larvae (*Galleria mellonella* L. and *Achroia grisella* Fabr.)

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During their studies on the biochemistry of *Galleria mellonella*, the greater wax-moth, Niemierko (1950) and Niemierko & Niemierko (1950*a, b*) observed that these organisms excreted large amounts of an acid-labile phosphate which was not present in their food. They identified this material as inorganic polyphosphate, and showed that 85 % of the total orthophosphate consumed was excreted in this form. Polyphosphates are also formed by *Achroia grisella*, the lesser wax-moth, but not by a number of other insects, suggesting that the formation of these compounds is connected with the ability to digest and assimilate wax.

Although the polyphosphates are widespread in bacteria, moulds and algae (Schmidt, 1951), Niemierko's work is, so far, the only convincing identification of these compounds in material

derived from higher animals. Niemierko & Niemierko (1950*a, b*) present some evidence that they were not produced by intestinal bacteria. It has been claimed that small amounts of polyphosphates occur in the fat bodies of other insects (Heller, Karpiak & Zubikowa, 1950), but details of the identification were not given. As large amounts of the excreta of both wax-moths were available, it seemed worth while to confirm the results of Niemierko & Niemierko and to attempt to identify the polyphosphates present by ion-exchange chromatography.

EXPERIMENTAL

The excreta of both moths were provided by Dr L. Bailey. The moths were cultured at either 35° (*G. mellonella*) or room temperature (*A. grisella*) in large glass jars containing

Table 1. *Barium precipitation of acid-labile phosphates*

To 2 ml. samples containing 60–200 $\mu\text{g.}$ of P was added 3N-acetate buffer (2 ml.) and saturated $\text{Ba}(\text{NO}_3)_2$ (1 ml.). The mixture stood on ice overnight and was then centrifuged at 2000 g for 15 min. The precipitate was dissolved in N-HCl and the acid-labile phosphate estimated after the barium had been removed as sulphate. The pH of the supernatant solution was measured by means of a glass electrode.

pH	Per cent precipitated			
	Acid-labile phosphate in extract of excreta of <i>G. mellonella</i>	Tripolyphosphate	Trimetaphosphate	Tetrametaphosphate
3.5	0	0	0	0
3.8	14	—	—	—
4.0	—	45	0	0
4.1	30	—	—	—
4.5	—	70	0	0
4.7	50	77	1	0
5.1	74	90	2	0
5.5	—	—	4	0
5.9	92	—	—	—

pieces of brood-comb. After about 2 months, when the brood-comb had almost disappeared, the moths and larvae were removed. The detritus left in the bottles was a dry mixture of insect excreta, unconsumed brood-comb and cocoons. The latter two materials could be distinguished by eye and were removed as far as possible, leaving a black granular material, the bulk of which was excreta.

Orthophosphate and total phosphate were estimated by the modification of the method of Kuttner & Lichtenstein described by Holden & Pirie (1955). Acid-labile phosphate was estimated as orthophosphate after being heated in N-HCl for 10 min. at 100°.

Chromatography of polyphosphates was carried out on Dowex 1 (100–200 mesh, 8% cross-linkage) in the manner described by Peters & Rieman (1956). The eluents used in this method were: (1) 0.25M-KCl, (2) 0.40M-KCl, (3) 0.50M-KCl, all buffered at pH 5.0 with 0.005M-acetate buffer; and (4) 3.0N-HCl, which was allowed to stand overnight in contact with the resin in order to hydrolyse any tenaciously absorbed polyphosphates.

RESULTS

Phosphorus accounts for 0.6–0.7% of the dry weight of the excreta of *G. mellonella*. Repeated extractions with water or 5% trichloroacetic acid solution removed only about 68% of this total, and the residue could not be dissolved even after heating for 15 min. on a boiling-water bath with either N-sodium hydroxide or N-hydrochloric acid. About 10% of the soluble fraction was orthophosphate and 73% was hydrolysed to this form after boiling for 10 min. with N-hydrochloric acid. The excreta of *A. grisella* contained about 1.3 times as much extractable phosphate as that of *G. mellonella*, but a larger proportion of it (30–40%) was orthophosphate.

The acid-labile phosphate in the extracts could be precipitated by barium salts (Table 1) under conditions similar to those described by Wiame (1949). Although these results varied a little from

Fig. 1. Chromatography of extracts of the excreta of *G. mellonella*. An aqueous extract (5 ml.; 900 $\mu\text{g.}$ of total phosphorus) was adjusted to pH 5.0 and chromatographed on a Dowex 1 column. Fractions of the effluent (10 ml.) were collected, and samples (3 ml.) taken for total phosphate (●) and orthophosphate (○) estimations.

experiment to experiment, probably according to the concentration of the precipitable phosphate, there was never any precipitation below pH 3.5, and it neared completion only at about pH 5.5. This suggests the behaviour of low- rather than high-molecular-weight polyphosphates (Krishnan & Damle, 1956). Tripolyphosphate behaved in a similar manner (Table 1), whereas the cyclic tri- and tetra-metaphosphates were not precipitated at all under these conditions.

Lead is a more effective precipitant than barium, and nearly all the acid-labile phosphate in the extracts was precipitated at pH 3 by lead acetate.

Aqueous extracts of the excreta could be chromatographed successfully on Dowex 1 columns, without any preliminary purification. The capacity of the resin for the phosphate in this solution was, however, small, and less than 1 mg. of total phosphate could be applied. Fig. 1 shows the result of a typical experiment on the material from *G. mellonella*. The phosphate has been resolved into

four components: one is orthophosphate (*B*); two are acid-labile phosphates (*C* and *D*), whose positions suggest that they are pyrophosphate and tripolyphosphate respectively; and one (*A*) is an unidentified acid-stable phosphate. In addition to these, a small amount of phosphate was tenaciously absorbed by the resin and was eluted only on treating the column with 3*N*-hydrochloric acid as described above. This material may consist of polyphosphates containing more than five phosphorus groups (Peters & Rieman, 1956).

Phosphate recoveries from the column were quantitative (90–100 %). The approximate amounts of the various phosphates present were: component *A*, 3 %; component *B*, 14 %; component *C*, 23 %; component *D*, 44 %; fraction retained by the resin, 16 %. Extracts of *A. grisella* contained the same phosphates in similar amounts, but with somewhat more orthophosphate and less of the material retained on the resin. The proportions were: *A*, 2 %; *B*, 29 %; *C*, 23 %; *D*, 40 %; residue, 6 %. The bulk of the water-soluble phosphates present in both cases (60–70 %) were therefore small-chain linear polyphosphates. No cyclic metaphosphates were detected.

The identification of components *C* and *D* as pyrophosphate and tripolyphosphate respectively is based mainly on their behaviour on the chromatogram, and their quantitative conversion into orthophosphate on boiling with *N*-hydrochloric acid for 10 min. They were both also quantitatively converted into orthophosphate by a purified preparation of an unspecific pea-phosphatase (Pierpoint, 1957). Although adenosine diphosphate and adenosine triphosphate behave in a similar manner to *C* and *D* during the chromatography they can clearly be distinguished by their absorption at 260 $m\mu$. If all the absorption at this wavelength associated with *C* and *D* were due to the presence of adenosine derivatives, it could not account for more than a few per cent of the total phosphate present in these components.

Measurement of the absorption at 260 $m\mu$ of the fractions of eluate revealed the presence of two peaks of absorbing materials. The first was in fractions 4–7, and corresponded to material that had been washed straight through the column. These tubes contained the bulk of the brown coloration that was present in the initial extracts, and their ultraviolet-absorption spectrum did not have any distinct maxima. The second peak was in fractions 23–29. Its ultraviolet-absorption spectrum had maxima at 235 and 290 $m\mu$ and closely resembled that recorded for uric acid (Johnson, 1951). The ratio of absorption at these two wavelengths was 0.87, while that for a commercial sample of uric acid dissolved in eluent 1 was 0.90.

DISCUSSION

The present work confirms the excretion of polyphosphates by the wax-moth larvae, and suggests that the bulk of them are small straight-chain compounds. It throws no light on the possible role of the intestinal bacteria in the formation and degradation of these compounds. It is possible that the particular phosphates observed were the result of a bacterial breakdown of high-molecular-weight polyphosphates which took place in the moths' intestines. It is less likely that they were the result of such a breakdown taking place after the excreta were voided, because of the extreme dryness of the excreta. They contain only 1 or 2 % of water and thus make an unfavourable medium for enzyme action or bacterial growth. The similarity in phosphate composition of the excreta of the two insects also argues against this latter breakdown; moreover, phosphatase activity could not be detected in aqueous extracts of the excreta.

Recently Wojtczak (1956) has analysed the phosphates excreted by starving larvae of *G. mellonella*, with results somewhat different from those described here. About half the acid-labile phosphate present was pyrophosphate, and the rest consisted mainly of a more highly polymerized compound which was hydrolysed to trimetaphosphate during paper chromatography in acid solvents. This polyphosphate gave a metachromatic effect with toluidine blue. No such metachromatic effect could be observed with the smaller amounts of highly polymerized polyphosphates detected in the present work, but this may have been due to masking by the brood-comb pigments present in the excreta of non-starving wax-moths.

There have been reports that the cells of the ventriculus of the honeybee contain a large number of phosphate-rich granules (Bailey, 1955; Day, 1949). An attempt was therefore made to detect polyphosphates in trichloroacetic extracts prepared from the mid-gut of 243 honeybees. Large amounts of orthophosphate and an acid-stable phosphate that emerged from the column before the orthophosphate were observed, but no polyphosphates. If they were present at all they must have accounted for less than 2 % of the total phosphate.

SUMMARY

1. The excreta of wax-moth larvae (*Galleria mellonella* L. and *Achroia grisella* Fabr.) feeding on brood-comb contains approximately 0.5–1.0 % of its dry weight as phosphorus. About half of this is present as water-soluble acid-labile phosphates, and about a third is not extracted with water, boiling acid or boiling alkali.

2. The composition of the water-soluble phosphates excreted by *G. mellonella* was found by

anion-exchange chromatography to be orthophosphate (14%), acid-stable phosphate (3%), pyrophosphate (23%), tripolyphosphate (44%), and a fraction retained by the resin (16%). This last fraction probably consists of more highly condensed polyphosphates.

3. The composition of the water-soluble phosphates excreted by *A. grisella* was orthophosphate (29%), pyrophosphate (23%), tripolyphosphate (40%), acid-stable phosphate (2%), and the fraction retained by the resin (6%).

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REFERENCES

- Bailey, L. (1955). *Parasitology*, **45**, 86.
 Day, M. F. (1949). *Aust. J. sci. Res. B*, **2**, 31.
 Heller, J., Karpiak, St. & Zubikowa, I. (1950). *Nature, Lond.*, **166**, 187.
 Holden, M. & Pirie, N. W. (1955). *Biochem. J.* **60**, 53.
 Johnson, A. E. (1951). *Biochem. J.* **51**, 133.
 Krishnan, P. S. & Damle, S. P. (1956). *Appl. Microbiol.* **4**, 179.
 Niemierko, S. (1950). *Acta Biol. exp., Varsovie*, **15**, 91.
 Niemierko, S. & Niemierko, W. (1950a). *Acta Biol. exp., Varsovie*, **15**, 111.
 Niemierko, S. & Niemierko, W. (1950b). *Nature, Lond.*, **166**, 288.
 Peters, T. V. & Rieman, W. (1956). *Analyt. chim. acta*, **14**, 131.
 Pierpoint, W. S. (1957). *Biochem. J.* **65**, 67.
 Schmidt, G. (1951). In *Phosphorus Metabolism*, vol. 1, p. 443. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
 Wiame, J. M. (1949). *J. biol. Chem.* **178**, 919.
 Wojtezak, A. B. (1956). *Acta Biol. exp., Varsovie*, **17**, 235.

A Method for the Determination of Non-Haem Iron in Bone Marrow

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There are many reports in the literature (Rath & Finch, 1948; Masshoff & Gruner, 1951; Hutchison, 1953) of investigations into the iron content of bone marrow from human subjects by various histological techniques. Most of the work has been done with smear preparations or sections of fixed tissue from material obtained at biopsy and stained with acidified potassium ferrocyanide. A deposit of ferri-ferrocyanide (Prussian blue) indicates the presence of iron-containing material. It is well recognized that only haemosiderin will be detected in this way, the Prussian blue being formed on the surface of the haemosiderin particles.

Granick (1954) originally postulated that iron was utilized in the synthesis of haemoglobin only from ferritin and that haemosiderin represented deposits of iron in excess of metabolic requirements. In these circumstances the presence of haemosiderin would imply the presence of adequate amounts of iron and the Prussian blue test could be used as a rough estimate of the body stores of iron. Shoden, Gabrio & Finch (1953), however, have found a simultaneous labelling of both ferritin and haemosiderin after administration of radioactive iron. Similarly, during haemopoiesis they found that iron from both ferritin and haemosiderin was utilized. These findings would suggest the necessity

of estimating the total quantity of iron in the bone marrow rather than that fraction present as haemosiderin.

Cavayé Hazen (1949) and others have reported the results of the estimation of iron in bone marrow obtained at necropsy in a variety of diseases. Relatively large amounts of tissue were required for the analysis which was performed on a wet ash of the material.

As it was desirable to use biopsy material it was decided to develop a method requiring only a small quantity of marrow tissue. It has been shown by Krause (1943) and by McCoy & Schultze (1944) that the lipid content of marrow tissue is variable. Dietz (1949) has studied the relationship between nitrogen, lipid and water content in bone marrow from several species. From these results it would appear that any analysis based on tissue weight, either wet or dry, would be unreliable. Davidson, Leslie & White (1947) have stated that with moderate quantities of marrow tissue (30–60 mg.) the values for deoxyribonucleic acid are the least satisfactory of their results, the amount of phosphorus being too small for accurate analysis. It was therefore decided to try to relate the iron content of the marrow to the protein content and this has proved satisfactory. The method for the