STUDIES OF THE PHYSIOLOGY AND TOXICOLOGY OF BLOWFLIES

XIV. THE COMPOSITION, FORMATION, AND FATE OF THE GRANULES IN THE MALPIGHIAN TUBULES OF LUCILIA CUPRINA LARVAE

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Summary

Lucilia cuprina larvae possess two pairs of malpighian tubules. One pair, having a yellow appearance, is restricted to the posterior end of the body. The other pair commences at the posterior end of the body and runs forwards as far as the brain and then backwards on either side of the gut to enter it by a common duct. Whereas the ascending regions are greatly enlarged and have a white appearance caused by many small spherical granules in the lumen, the descending regions have the usual yellow appearance. The tubules are held in place at several points by fine strands of connective tissue.

A few small granules are present in the tubules of unhatched larvae. After hatching they increase rapidly in number and size and, when larvae have finished feeding, granules having a diameter up to about 16 μ are present. During pupal development the permeability of the cells of the granule-accumulating regions increases and granules are flushed down into the hindgut, where they contribute to the meconium to be excreted shortly after emergence of the adult. Although there still remains some differentiation of the tubules in the adult, granules do not accumulate unless a diet rich in calcium or magnesium salts is provided continuously.

Addition of calcium or magnesium to the larval diet leads to a great increase in granule accumulation and also to an increase in granule size (up to 20 μ). Addition of large amounts of phosphate depresses granule formation, presumably by binding calcium and magnesium in relatively insoluble form in the alimentary canal. Occasionally meat-fed larvae contain few or no granules.

The larval granules are soluble in dilute acids, and show birefringence, but no fluorescence. Analyses have shown that the granules contain large amounts of magnesium and calcium phosphates, some magnesium ammonium phosphate, large or small amounts of calcium carbonate, and a little magnesium carbonate. Both barium and strontium can be detected by a staining reaction and by spectrographic analysis in the granule-accumulating region. Magnesium and calcium are of about equal importance as granule constituents, although the composition of the granules is greatly influenced by the diet. On some diets, such as those extremely rich in calcium and relatively poor in phosphate, calcium carbonate became the preponderant constituent of the granules. Even under these circumstances the amount of respiratory carbon dioxide bound in granule form is less than 1 per cent. of that produced by the larvae.

Protein, sulphhydryl, chloride, sulphate, oxalate, lactate, potassium, and iron have not been detected in the granules and uric acid cannot be present in more than traces. On the other hand, urates are present in the yellow

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regions of the larval tubules and occur as small granules in the lumen throughout the tubules in the late pupa and adult. Riboflavin, copper, and sulphhydril are present in the tubule cells. There is no difference in the pH and Eh of the contents of the granule-accumulating and yellow regions.

Although several different types of larval granule may be distinguished visually, staining reactions suggest that each granule is normally a heterogeneous mixture of several salts, either laid down simultaneously, or in successive concentric layers.

Two enzymes associated with sites of calcification in vertebrates and in many invertebrates, namely carbonic anhydrase and alkaline phosphatase, appear to play no part in granule formation.

The conclusion is reached that the granules are merely waste products, formed as a result of the specialized nature of the cells in the accumulating region and stored there by virtue of the absence of peristalsis and of adequate flushing of water down the tubules.

I. INTRODUCTION

Accumulations of granules are characteristically present in one pair of malpighian tubules of many dipterous larvae, a feature which has been recorded frequently since the early observations by Dutrochet (1818) of milky vessels in larvae of the syrphid Eristalis tenax. The presence of granules, conferring a conspicuous white appearance upon the particular region of the tubules containing them, has led to much speculation, but to very little accurate information, upon their composition and function. Whereas some authors carried out no chemical tests whatever, a number (Batelli 1879; Krüger 1926; Müller 1924; Pantel 1898, 1914; Roubaud 1913; Vaney 1900) recorded solution of the granules in dilute acid, accompanied by evolution of gas, thereby demonstrating the presence of carbonate. Although it has always been assumed that calcium was combined with this carbonate to form the granules, the actual occurrence of calcium has only occasionally been demonstrated (Müller 1924; Pantel 1898; Roubaud 1913; Vaney 1900, 1902). Furthermore, while the occurrence of magnesium in the granules has been suggested on several occasions, there appears to be no recorded demonstration of its presence by chemical tests. The presence of calcium oxalate, phosphate, and urate in insect excreta has been recorded (Uvarov 1928; Wigglesworth 1939), but most reports are based on inadequate chemical evidence and there are no authentic records of the occurrence of these salts in the excreta of dipterous larvae. It is clear, therefore, that there is a need for accurate determinations of the identity and relative importance of the materials present in the granules. Furthermore, there is no information available on the factors influencing granule accumulation.

Three suggestions have been made in the literature regarding the function of these granules. They have been regarded as serving as a reserve for calcium (Vaney 1900, 1902), as having a respiratory function by virtue of the carbon dioxide they remove from the body (Keilin 1921), or as being merely an ordinary product of excretion (Pantel 1914). Certain specialized uses to which
accumulated granules are put are known in some dipterous families, such as the strengthening of the larval (Müller 1924) or puparial cuticle (Keilin 1921; Schutte 1921).

It will be shown below that, in L. cuprina, the granules represent merely a temporary storage of waste excretory products, amongst them respiratory carbonates, which are transferred from the tubules to the hindgut in the pupal stage to be excreted by the newly emerged adult.

II. METHODS

(a) Biological

L. cuprina larvae were bred under aseptic conditions at 30°C. on a medium consisting of 88 per cent. egg white, 11.7 per cent. dried baker's yeast, and 0.3 per cent. sodium chloride (Lennox 1939).

To obtain samples for chemical analysis, larvae were dissected under the least possible quantity of saline (Ephrussi and Beadle 1936) and the various tissues separated out on to small, previously weighed, pieces of coverslip. Identical paired samples of the granule-containing portions of the malpighian tubules were obtained by accumulating the granules from one of the two storage tubules of each larva on one coverslip and from the other on a second coverslip. Blood was collected in the manner already described (Waterhouse 1945b).

(b) Chemical

Inorganic calcium, magnesium, and phosphate* were extracted by treatment of the dried tissues with 20 per cent. trichloracetic acid for one hour at 37°C.

Phosphate was estimated colorimetrically by the method of Fiske and Subbarow (1925). Calcium was precipitated as oxalate after adjusting the pH to between 5 and 6 with ammonia, thereby reducing the danger of magnesium being occluded in the calcium precipitate. After centrifuging, the precipitate was washed with 2 per cent. ammonia in equal parts of ethyl alcohol, ether, and water (Wang 1935) and centrifuged again. The oxalate was then estimated by titration with permanganate. Magnesium was precipitated as the ammonium phosphate. This was washed in the same way as the calcium precipitate and its phosphate content estimated colorimetrically.

When information was required on total mineral content, the tissues were digested on a sand bath with concentrated sulphuric and nitric acids, or concentrated perchloric and nitric acids.

For the determination of carbonate, the volume of carbon dioxide liberated at 37°C. following treatment with 0.5N hydrochloric acid was measured manometrically. Ammonia was determined colorimetrically after the addition of Nessler's reagent to an acetic acid extract of the granule-accumulating regions.

* Although this is termed inorganic phosphate it includes esters (Umbreit, Burris, and Stauffer 1947).
III. The Larval Malpighian Tubules

In L. cuprina, as in other calliphorid larvae, there are two pairs of tubules, each pair opening into a short common duct leading into the alimentary canal (Fig. 1). One pair of tubules, that discharging into the left side of the alimentary canal, commences posteriorly and runs forward, one tubule on each side of the body, to about the level of the brain before turning sharply and running back down on either side of the gut to enter it at the end of the midgut. This turning point near the brain, which is designated as the “bend” region for convenience of later description, marks a rapid transition in the appearance of the tubules. The cells of the descending arms have the general appearance of a string of beads, the individual cells protruding into the body cavity. They are deep orange-yellow and contain many granular inclusions, but there is no solid material in the lumen. In sharp contrast, the cells of the ascending arms are far smoother in outline, they contain relatively few inclusions which are generally of a yellowish-brown colour and the lumen of the tubule is greatly enlarged, being stuffed with minute spherical granules (Plate 1, Figs. 1, 2, 5).

The second pair of tubules, discharging into the right side of the alimentary canal, are uniform throughout their entire length and closely resemble in appearance the descending arms of the pair just described. The two component tubules run backwards to the posterior end by a very winding course, their blind ends being joined by connective tissue to the respective blind ends of the anterior pair of tubules (Plate 1, Fig. 4). As a rule, the posterior pair of tubules contains no granules. The two short ducts by which the two pairs of tubules discharge into the gut are colourless and are formed of cells which do not protrude into the haemocoel. In larvae 2.5 days old, about 10 mm. in length, and of average weight 30 mg., each anterior tubule is about 20 mm. long and each posterior tubule about 16 mm.

The tubules are generally well tracheated, with the exception of the posterior two-thirds of the granule-accumulating regions which are comparatively poorly supplied. The tracheae are largely responsible for maintaining the tubules in position within the larva, although they are assisted in this by strands of connective tissue which are inserted in the body wall, the alary muscles or the midgut caeca. These strands of connective tissue (Fig. 1; Plate 1, Fig. 4) occur at the bend region, at the posterior end of the tubules, and at three intermediate positions (Waterhouse, unpublished data).

The histology of the tubules presents no unusual features. The yellow regions (Plate 1, Fig. 6) have an outer basement membrane, a rather uniform cytoplasm, and a distinct inner border, which is usually striated, but seldom appears free. Many of the granules which can be seen within the cells of living tubules are not visible in sections, having been dissolved by the fixatives. At times small vesicles of material can be seen protruding through the cell border, but these are probably fixation artefacts or degenerating cells (Wigglesworth 1931). The lumen of the tubules is usually bounded by two or three cells with rather indistinct boundaries.
The granule-accumulating region differs principally in the enormous distension of the lumen to hold granules. The cells are extremely flattened and thin, so that fine details are difficult to see. The narrow layer of cytoplasm is apparently uniform and basement membrane and inner cell border are very thin.

The lumen of the common duct is bounded by several cells. Inner circular muscles and outer longitudinal muscles invest these cells. Their haemocoele boundary is richly supplied with tracheae and tracheoles which, however, do not become intracellular as they do in the adjoining midgut.

The granules of the accumulating region are transparent and colourless when viewed with transmitted light, but white by reflected light. They are generally spherical and, in larvae 2.5 days old on the control medium, range in diameter from 0.5 μ or less to about 10 μ, with the bulk of the materials in granules of 4 to 8 μ diameter. When mounted in balsam and examined under oil immersion a number of different types of granule can be clearly distinguished (Fig. 2; Plate 1, Figs. 1, 2, 5).
For instance, granules may have a uniform transparent interior; they may possess one or more centrally located dark granules, spherical or irregular in shape; they may possess one or more uniform or granular internal layers of different light-transmitting properties from the bulk of the granule; or they may be entirely composed of fine granular material. Occasionally irregular granules, which have been formed by the fusion of two or three smaller granules, may be seen. All of these types may have an indistinct or a distinct outer boundary, indicating different refractive indices of the surface layer.

Fig. 2.—Diagrammatic representation of some common types of granule.

IV. FORMATION AND FATE OF GRANULES IN THE TUBULES

(a) Fully Developed Eggs

Dissection of larvae removed from the egg membranes shortly before hatching demonstrated that granules were already present in small numbers in the lumen of the ascending arms of the anterior pair of malpighian tubules. The granules covered a range of sizes, the largest had a diameter a little under 2 μ, granules with a diameter of 1.8 μ were of frequent occurrence, and there were many with a diameter of 0.5 μ or less.

(b) Larvae

By the time larvae had fed on the artificial medium for a few hours, granules were clearly visible in large numbers in the ascending arms of the anterior pair of tubules. With increase in size and age, the granular deposits increased in quantity, and the size of the largest granules also increased rapidly.
When, after three or four days, larvae were ready to leave their food to pupate, spherical granules as large as 16 μ in diameter could be seen, with occasional irregular granules of somewhat greater width. Most, however, had a diameter of less than 10 μ. There was some variation from one individual to another in the amount accumulated and also in the appearance of the granular region. In some larvae the cell outline was irregular, with protruding cells alternating with constricted regions. Where the cells protruded, the lumen was enlarged and stuffed with granules, while the constricted regions coincided with a narrow lumen with less granular material. In other larvae the narrower regions were absent and the diameter of both tubule and lumen relatively uniform. This latter state occurred most frequently when the tubules were stuffed with granules.

A day or two after larvae ceased feeding, a pale green coloration of the contents of the granular regions of the tubules became apparent. This coloration increased in intensity up to about pupation and then remained unchanged. It was most intense in the posterior third or quarter of the granular region, becoming progressively weaker towards the anterior end. The material responsible is insoluble in alcohol (absolute or 70 per cent.), chloroform, ether, carbon tetrachloride, and acetone. The coloration was no more intense in larvae fed on blood or liver than on the artificial medium. As might be expected, therefore, tests for biliverdin and urobilin were negative. The material responsible for the coloration is apparently some by-product of metabolism produced in the prepupal stage shortly prior to pupation and may be related to the production of the phenols involved in the hardening of the puparium. Immersion of the intact tubule in distilled water caused initially a breakdown of the cells, accompanied by a gradual reduction in the intensity of the green coloration. The pigment, therefore, is somewhat soluble in water.

(c) Pupae

During the early stages of metamorphosis the general arrangement of the tubules is little altered, the granules remaining in the ascending arms of the anterior pair and retaining the greenish coloration acquired in the prepupal period. After the tubules have become visibly associated with the developing adult alimentary canal, discharge of the granules into the hindgut commences. This discharge begins shortly after a salmon pigmentation has appeared in the eyes and also after the dorsal thoracic bristles and those of the head have commenced to darken. It does, however, precede the darkening of the remaining bristles. The process of transference is a gradual one, being substantially complete by the time of emergence although, in the freshly emerged adult, there are sometimes small aggregates of granules which have not been discharged from the tubules. These may be present either in the former granule-accumulating regions, or on their way down the yellow portions of the tubules.

The factors initiating the transference of the granules are of some interest. The common ducts of each pair of tubules, which are well supplied with
muscles as in *Calliphora* larvae (Eastham 1925), undergo frequent peristaltic movements, which lead to the discharge of their contents into the hindgut. These movements influence directly only the granules which are now present in the adjoining portion of the tubules and could scarcely bring about their transfer from the granule-accumulating region. No peristalsis of the individual tubules occurs and their only movements appear to be bodily displacements due to peristalsis of the alimentary canal or to locomotion. An attractive hypothesis is that the fluid intake of the granule-accumulating region alters at the time of granule transfer, so that the granules are flushed down the tubules, a circulation of water being achieved by its reabsorption in the hindgut.

Evidence strongly supporting this view comes from two sources. Just at the stage at which granules have commenced to move towards the hindgut, the latter is often seen to contain a green fluid, but only very few granules. This would occur if the first flushings of the granule region dislodged a few granules, removing at the same time some of the green pigment. It would also supply a satisfactory explanation of the simultaneous appearance of the first few granules and of the pigment solution in the hindgut.

The second line of evidence comes from the injection of saline saturated with indigocarmine. No dye is taken up by the granule-accumulating regions, although the yellow regions rapidly absorb it and concentrate it in the lumen. In pupae, on the other hand, rapid dye uptake by the granule-accumulating region does occur. This is true even before the yellow regions have regained their function following reorganization. As soon as the yellow regions become functional again they too take up dye and both granules and dye are discharged into the hindgut. It is clear therefore that, in the early pupal period, the permeability to dyes of the cells of the granule-accumulating region has altered considerably, and one might well expect this to be associated with a greater capacity to take up fluid. Further proof of altered permeability comes from the observation that urates, which are absent from the larval accumulating region, but not from the remainder of the tubules, begin to appear in quantity in the accumulating regions at this stage in pupal development (see later).

One further observation may have some bearing on granule transfer. After larvae have been immersed for several hours in water, the accumulating regions commence to lose their characteristic white appearance and become more transparent. Addition of hypertonic saline to dissected larvae causes a return of the tubules to their normal appearance, indicating that the greater transparency was due to the accumulation of fluid in the lumen of the tubules. Presumably, the water-reabsorbing process has been interfered with by oxygen lack more than the water-secreting process. A parallel observation has been recorded by Gamo, Yamaguchi, and Nagai (1933), who found that the permeability of the midgut of the silkworm larva altered considerably if the larvae were subjected to oxygen lack. Distension with fluid would tend to produce discharge of the granules and, while this could not be observed in control larvae, a small proportion of the granules were discharged from the massive
deposits accumulated by larvae fed on calcium-enriched diets (see Section VI (a)). The poorer supply of oxygen during metamorphosis might favour the secretion of water into the accumulating region at a greater rate than it could be reabsorbed and this would result in discharge of the granules.

The amount of granular meconium present in the hindgut, even before more than a small fraction of the accumulated granules has been discharged, often exceeds the total originally present in the granule-accumulating regions. It is apparent, therefore, that the anterior pair of tubules has been discharging both newly formed and pre-existing granules. This is also suggested by the observation that, at times, portions of the yellow regions contain predominantly very small granules, but only very few larger ones, which would also be present if the granules came from the accumulating regions. Some time after discharge of granules has commenced from the anterior tubules, numerous small granules also appear in the posterior pair and are discharged into the hindgut. These granules (Plate 1, Fig. 3) are roughly spherical in outline, but are quite different from the regular spherical granules accumulated during larval life.

The increased fluid uptake by the tubules thus appears to serve two functions, that of discharging larval excretory products stored in the tubules and that of removing excretory products accumulated elsewhere in the body during the early stages of metamorphosis. It will be shown later (Section V (n)) that large quantities of urates are taken up by the tubules during this period, appearing first in the lumen in the form of small granules and subsequently contributing to the meconium.

(d) Adults

Shortly after emergence, most of the contents of the hindgut are expelled as meconium and the tubules now contain few granules. The larval granule-accumulating regions of the tubules can still be distinguished by their light pigmentation, smaller diameter, and smaller volume of the cells.

When adults are fed on a diet of sugar and water, scattered granules can often be seen in the blind ends of both pairs of tubules, as well as along their length. These granules occur more frequently and in greater numbers in the anterior pair of tubules than in the posterior pair and are small and not as uniformly spherical as the larval granules. There are good reasons for believing that, in the adult, granules are continually being formed and discharged. Thus, on normal diets, they do not increase in size or in number with increasing age, and it is possible to observe granules at any one time in all stages of discharge.

V. REACTIONS OF THE GRANULES

(a) Solubility

Granules from the tubules of fully grown larvae dissolved rapidly in concentrated mineral acids and slowly in dilute acids (e.g. N/15 hydrochloric acid). They also dissolved in 1 per cent. trichloracetic acid and in 1N acetic
acid. All types of granule appeared to dissolve at the same rate, so no separation of granules could be obtained by this means. Solution was accompanied by an evolution of gas, which was found by analysis to be carbon dioxide (see Section VIII). There is no doubt, therefore, that carbonates are present. Adult granules also dissolved in dilute acid with evolution of gas. Larval granules are not soluble in boiling water, alkalis, nor in any of the common organic solvents.

(b) Density

The density of the granules was determined by the float or sink method using tetrabromomethane (sp. gr. 2.95) diluted with carbon tetrachloride (sp. gr. 1.59). The values obtained lay between 2.3 and 2.8.

(c) Examination with Polarized Light

The granules are distinctly birefringent (Plate 1, Fig. 5) showing under crossed polars a black cross reminiscent of starch grains and of the calcospherites of the celery fly Acidia (Keilin 1921). This black cross remains stationary when the granules are rotated, indicating that the micelles are oriented normally to the periphery. The amount of light transmitted and the birefringence produced vary from granule to granule, indicating that the granules are not homogeneous.

(d) Examination with Ultraviolet Light

When examined by ultraviolet light the granules showed no fluorescence; nor do undamaged tubules. Slight damage to the tubule cells, however, results in the diffusion into the surrounding fluid of a material having an intense yellow fluorescence. A chemical examination of tubules from liver-fed larvae has shown this material to be riboflavin. With approximately 1.5 \( \gamma \) per larva in the tubules, riboflavin appears to be present in a concentration greater even than that recorded by Metcalf (1943) for Periplaneta. Since riboflavin appears only in the immediate vicinity of damaged cells and since successive damage along the length of the tubules always produces fresh fluorescence, it is clear that the riboflavin is present within the cells and not in the lumen. Less fluorescent material is present in the cells of the granule-accumulating region than in the yellow regions and none is present in the common exit duct, except just at the junction of the two tubules. Since the intact tubules do not fluoresce, the riboflavin may be present in a reduced state. The pigment appears to be similar in many ways, although not in this respect, to the cytoplasmic form of riboflavin described in Periplaneta (Metcalf and Patton 1942; Metcalf 1943). Following the addition of large quantities of riboflavin to the diet (up to 60 \( \gamma \) per g.) the cells of the accumulating region sometimes show an orange fluorescence, whilst the remaining portions are unchanged. This may indicate that the oxidation-reduction potential is not as low in the cells of the accumulating region as elsewhere, although confirmation of this was not obtained in studies with redox dyes (see Section VIII(b)). In addition, the relatively poor tracheation of this region mentioned earlier (Section III) would not lead one to expect a higher Eh here than elsewhere.
(e) Phosphate

The cobalt sulphide visualization method for phosphate (Danielli 1946) was applied to sections of the accumulating regions and to smears of granules. An intense positive reaction was given by many of the granules (Plate 1, Fig. 1). Staining was generally uniform throughout the granule, but occasionally it was confined either to the central region or to the periphery. However, some granules of all types remained unstained so that, unless stained, phosphate-rich granules do not have a characteristic appearance. Sections of the yellow regions of the tubules did not stain for phosphate.

When granules from the calcium- and magnesium-enriched media shown in Table 7 were treated, only a small proportion of those from calcium-fed larvae and none of those from magnesium-fed larvae stained at all intensely. While it might be expected that the very large amounts of calcium carbonate present on the former diet would result either in many granules containing no phosphate, or granules in which any phosphate would be effectively masked, the absence of distinct staining of granules from magnesium-fed larvae where phosphate is the predominant anion in the granules cannot be explained.

(f) Sulphate

If sulphate is excreted by the granule-accumulating region the presence of calcium sulphate in the granules would be expected. Addition of barium chloride to a concentrated solution of the granules in dilute acetic acid produced no precipitate, indicating that sulphate was absent. Further, no reaction was given when the larval food contained large amounts of added sulphate. The granule-accumulating regions, therefore, are not involved in sulphate regulation.

(g) Chloride

The addition of silver nitrate to a concentrated solution of granules in dilute nitric acid did not produce a precipitate, indicating that chloride was absent.

(h) Calcium

Granules, smeared on a slide, were immersed for 10 minutes in a saturated aqueous solution of Gallamine blue which, according to Stock (1949) forms a specific purple lake with calcium. All, or almost all, of the granules became stained, the colour varying from very light to dark purple (Plate 1, Fig. 2). Sometimes inner inclusions or rings stained more deeply than outer layers of the granules and sometimes the reverse occurred. It appears that calcium is a component of all or almost all granules. Granules from larvae which had fed on media greatly enriched with magnesium and which contained far more magnesium than calcium (see Table 7) were found to stain very lightly or not at all. This provides good evidence that magnesium does not stain with Gallamine blue.

When dissected larvae were treated with the dye, the granule regions of the tubules stained purple, and the remainder of the body a non-specific blue.
(i) Magnesium

Titan yellow and Magneson II, dyes which produce characteristic absorption colours on magnesium hydroxide (B.D.H. 1939) were tested on dissected larvae made alkaline with dilute sodium hydroxide.

Titan yellow in aqueous solution produced the red coloration characteristic for magnesium in the yellow regions of the tubules even before addition of alkali, suggesting that the tubules have an alkaline reaction. After the addition of dilute alkali the granule region of the tubules and the cut edges of the cuticle also stained. The granules sometimes stained a light pink, sometimes remained colourless, suggesting either that there is little magnesium in the surface layers of the granules or that it is in some form which does not absorb the dye. Larvae fed on magnesium-enriched media gave similar reactions, except that the cells of the granule region of the tubules stained bright red and the granules pink. This staining was not confined to any particular type of granule. Solution of the granules in dilute acid, precipitation of magnesium as the hydroxide following addition of alkali, and addition of Titan yellow, produced a light pink precipitate from control larvae and a distinct red precipitate from magnesium-fed larvae.

Magneson II gave a rather more general coloration of the body than Titan yellow. The characteristic blue magnesium coloration was, however, most intense in the tubules and particularly in the granule-accumulating regions. Granules from control larvae stained a light blue, and those from larvae on magnesium-enriched media a darker blue. All granules stained similarly. Of the remaining portions of the body the hindgut stained more intensely than the midgut.

There is no doubt, therefore, that the malpighian tubules, and particularly the granule-accumulating regions, are richer in stainable magnesium than the other organs and tissues.

(j) Barium and Strontium

In third instar larvae fed on control medium, red staining after treatment with sodium rhodizonate (Waterhouse, unpublished data) was distinct in the granule-accumulating regions, and faint elsewhere in the tubules. Staining was most intense towards the posterior blind ends of the granule-accumulating regions and somewhat less intense near the bends. The intervening portion of the tubule, although stuffed with granules, either stained very lightly or not at all. Larvae fed on liver, brain, or muscle reacted similarly except for the occasional larva feeding on dehydrated mutton, which accumulated no granules (see Section VI(g)). These larvae did not stain at all.

Granules from a number of control larvae were mounted in sodium rhodizonate solution. A high proportion from some larvae were stained but, more frequently, only a very small proportion reacted. Sometimes no stained granules could be seen. All sizes and types of granules were stained at one time or another, although apparently identical granules from the same larvae remained unstained. The colour of the stained granules varied from pink to
dark red, being sometimes uniform throughout the granules, at others appearing only in the outer layers. There is strong evidence, therefore, that either barium or strontium, or both, are present at times in the granules. Staining occurred in a somewhat similar fashion in young second instar larvae, but the intensity was less.

The malpighian tubules of larvae fed on barium-enriched medium (0.5 ml. 1 per cent. barium chloride per 10 ml. medium) stained even more intensely than control larvae. All types of granules stained, but those which had an unhomogeneous interior composition reacted most frequently. Injection into the haemolymph of a dilute solution of barium chloride in saline, followed by rhodizonate treatment, resulted in fairly generalized light staining of the body with the granule region of the tubules almost always heavily stained and certain midgut cells sometimes distinctly stained. When barium is readily available, therefore, it is accumulated both in the granules and in the cells of the granular region in amounts greater than in control larvae.

The yellow portions of the malpighian tubules were seldom as intensely stained as the granule-containing regions. On several occasions barium-fed larvae had small deposits of granules in the lumen of the yellow tubules near their blind ends. These granules and the surrounding tubule cells stained more intensely with rhodizonate than the remainder of the yellow tubules. In pupae, as in larvae, staining occurred in the granule-accumulating portions of the tubules.

The malpighian tubules of larvae fed on strontium-enriched medium (0.5 ml. of 2.5 per cent. strontium chloride per 10 ml. medium) stained in a very similar fashion to those of larvae fed on barium-enriched medium, although the colour intensity was somewhat less, particularly in the yellow regions of the tubules. Nevertheless, it was more intense than encountered in control larvae. Examination of granules mounted in sodium rhodizonate solution showed that fewer were stained than with barium-fed larvae, but generally more than with controls. Most of the stained granules were medium-sized and there was often a good deal of intensely staining non-granular debris present. It appears, therefore, that strontium, too, can enter into the formation of the granules.

Definite evidence of the presence of both barium and strontium in control larvae was obtained by pretreatment with chromate (Waterhouse, unpublished data). Owing to the formation of unreactive barium chromate, this treatment prevented the characteristic intense coloration in tubules of larvae fed on barium-enriched medium. It did not interfere with tubule coloration of larvae on strontium-enriched medium, strontium chromate being capable of reacting with the rhodizonate solution. When this treatment was applied to control larvae or larvae fed on liver, some red coloration was usually observed in the granule-accumulating region, although this was not as intense as usual.

Finally, both barium and strontium were detected spectrographically in small amounts in the granules from control larvae, the former in a concentration between 0.005 and 0.001 per cent. dry weight, and the latter between 0.02 and 0.01 per cent. dry weight.
(k) Iron and Copper

The absence of stainable iron from the tubules, even when larvae have been fed on iron-enriched media, has already been described (Waterhouse 1940b). It is interesting to note, however, that, following the injection into the haemolymph of small amounts of saline saturated with ferric ammonium sulphate, the only portions of the body staining for iron by the Prussian blue reaction are the middle region of the midgut and the granule-accumulating portions of the tubules. In the latter, staining is generally most intense at the anterior and posterior ends and less intense towards the centre. Under these conditions stainable iron is not taken up by the yellow regions of the tubules.

Copper can generally be detected in the tubules of control larvae by the diethylidithiocarbamate reaction. It is present in the yellow portions, but also occurs at times in the granule-accumulating regions (Waterhouse 1945a). The ability of the cells of the granule region to take up copper from the blood was confirmed by injection into larvae of a dilute solution of copper sulphate in saline. The middle region of the midgut also stained more intensely than usual.

(l) Sodium and Potassium

Traces of sodium were detected in the granules by the zinc uranyl acetate test, but potassium was not detected either by sodium cobaltinitrite in acetic acid or by dipicrylamine (Feigl 1947).

(m) Ammonia

Solutions used for the detection of ammonia were Nessler’s and Riegler’s reagents (Feigl 1937, 1947). Although Nessler’s reagent failed to produce any coloration of the tubules, Riegler’s reagent unmistakably indicated the presence of ammonia therein. Thus a distinct red coloration developed in the cells of the tubules with local variations here and there in the depth of color. Addition of saturated calcium hydroxide to render the solution slightly alkaline intensified the reaction and also prevented solution of the granules in the reagent. Many granules were stained light to dark yellowish-brown. This staining was not confined to any particular type of granule, but was more distinct in granules taken from stained tubules than in granules removed from the tubules and stained separately. It is clear, therefore, that ammonia either enters into the formation of the granules or, at least, is present in the fluid surrounding them and thus may be absorbed on to the granules during testing to produce staining. This agrees with the results of Lennox (1941), who showed quantitatively that more ammonia was present in the tubules than anywhere in the body except the hindgut. Unfortunately no distinction was drawn between the granule-accumulating portion and the remaining portion of the tubules. Furthermore, his analyses were based on aqueous extracts of organs, so that ammonia bound in the water-insoluble granules would not have been recorded.
Two principal tests were employed for the detection of uric acid. One was the intense blue colour produced by arsenophosphotungstic acid and sodium cyanide (Benedict 1922), the second employed the modified reagents of Newton (1937). Unfortunately neither of these methods is completely specific. Several other naturally occurring reducing agents produce a blue coloration which is, however, generally much less intense than that produced by uric acid. Thus Brown (1938a) found that cysteine and tyrosine gave faint colour reactions and Blauch and Koch (1937) added glutathione, ascorbic acid, cystine, glucose, and resorcinol to the list of reactive materials. Newton’s reagents do not react with phenols (e.g. tyrosine, resorcinol), but the remaining materials presumably still interfere if present in sufficiently high concentration in the tissues.

Since the uric acid reagents react with nickel-coated pins, fine glass rods were used for holding down the larvae. Several minutes were required for the colour to reach full intensity and it faded again after standing for some time. With Benedict’s reagents the blue coloration characteristic of uric acid appeared in the cut edges of the cuticle, in the skeletal muscles, and rather irregularly along the yellow portions of both anterior and posterior pairs of malpighian tubules. It occurred infrequently in the midgut, but more frequently in the hindgut, particularly just posterior to the entrance of the tubules. The hindgut contents were frequently stained a pale blue. A transient pale blue coloration was sometimes observed in the cells of granule-accumulating regions of the tubules, but more frequently no coloration occurred, even in larvae fed from hatching on media containing 5 per cent. uric acid. No coloration of the granules was ever observed. The cells of the granule-accumulating regions, in common with most other previously unstained tissues, did stain a light blue after injection of a saturated solution of uric acid in saline.

To determine whether the absence of staining was, perhaps, due to poor access of the reagents the granule-accumulating region was dissected out, dried, defatted with acetone, dried again, and the reagents added. The granules still failed to stain, although a similar treatment of previously unreactive fat body produced an intense blue coloration. When granule-containing portions of the tubules were treated with dilute hydrochloric acid and the reagents added, no more than a light blue colour ever developed, indicating that only slight traces of uric acid or urates could be present. This finding was confirmed with Newton’s reagents (Newton 1937) which produced a far more intense coloration of larvae than Benedict’s reagents, but no coloration of the granules or of a solution obtained by dissolving these in a sodium tungstate-sulphuric acid mixture.

Three confirmatory tests were performed. The reagent 2,6-dichloroquinone chloroimide (Fearon 1944) gave a golden-brown coloration in the same tissues which reacted above. The granules did not stain. Application of 0.25N silver nitrate produced blackening of cells and darkening of granules in the accumulating region, both changes being resistant to ammonia treatment;
on the other hand, the well-known Murexide test for uric acid gave a negative result. Both of these tests are regarded with suspicion by Lison (1936). Brown (1938a) found on analysis that, although uric acid was present in *L. sericata* larvae and that, although Benedict’s method indicated its presence in the excreta, uric acid was, in fact, not excreted as such, being first oxidized to allantoin. He suggested that an interfering substance in his analyses was probably tyrosine, produced from the casein of his larval medium. To sum up then, the absence of distinctive coloration from the accumulating region means that, if uric acid or urates are constituents of the granules, they must be present only in extremely small quantities.

In spite of the caution which must be observed in interpretation of results, some confidence was gained in the Benedict colour reaction by its application to pupae and to adults. Thus, in the late pupal stage, a very intense blue coloration was given by the fat body, the meconium, and both the yellow and the granule-accumulating regions of the malpighian tubules. Earlier, in studying events towards the end of pupal life, the appearance of small granules in both anterior and posterior pairs of tubules was described. The newly formed granules from the posterior pair were first tested for uric acid, being, to all appearances, identical with the newly formed granules of the anterior pair and uncontaminated, as are the latter, by larval granules. The newly formed granules differed markedly from the larval granules not only in appearance, but also by being sparingly soluble in 1N hydrochloric acid without evolution of gas, and by being soluble in 5 per cent. sodium cyanide. Furthermore, the cyanide solution of granules gave a very intense uric acid reaction, so that there is no doubt that they contain large amounts of uric acid or urates. Like the larval granules, however, they contain no protein (see (o)). Additional tests demonstrated that, late in pupal development, uric acid or urate granules are present in the granule-accumulating as well as in the yellow regions of the anterior pair of tubules. The larval granules, awaiting discharge from the granule-accumulating region, still do not react. Urate granules are also present in the adult, being predominantly in the yellow regions of the tubules and in the hindgut. Brown (1938a) has shown that there is a rapid rise in uric acid content during pupal development and a sharp fall on emergence, due to the excretion of uric acid in the meconium. Considerable quantities of uric acid are also produced and excreted during adult life. This coincides with the picture obtained histochemically.

(o) Protein

To determine whether protein entered into the formation of the granules as a nucleus for the deposition of salts, one large mass of granules was dissolved in 10 per cent. trichloracetic acid and another in 1N acetic acid. After allowing the slides to dry, ninhydrin in 50 per cent. glycerine was added and the slides heated. Although a faint blue coloration was seen at the edges of the dried deposit produced by acetic acid treatment, no staining of particles was observed. One must conclude, therefore, that protein is absent. In this
respect, the granules differ from the calcospHERites examined by Keilin (1921) in which an albuminoid stroma of calcoglobulin remained after acetic acid treatment. Small particles of organic material also remained after solution of the granules from the tubules of *Psychoda* larvae (Krüper 1930).

(p) Oxalate

In view of the many records of the presence of oxalic acid in excretory granules (Uvarov 1928; Wigglesworth 1939), mostly based upon the doubtful recognition of crystal structure, the sensitive aniline blue test (Feigl 1947) was performed. The tests were uniformly negative even when granules were examined which had been obtained from larvae growing on medium containing 0.5 per cent. oxalic acid.

(q) Lactate

Lactic acid could not be detected in the granules by the tests involving the production of characteristic colours with o- or p-hydroxydiphenyl and sulphuric acid.

(r) Sulphydryl

The recently described red stain (1 [4 chlormercuric phenyl azo] naphthol 2\(^{+}\) (Bennett 1948a, 1948b), which has a high specificity for sulphydryl groups) was used as a saturated solution in n-propyl alcohol. Most of the tissues and organs, including the yellow regions of the tubules, were heavily stained. Other tissues appeared only lightly stained, such as the tracheae and the cells of the granule-containing portion of the tubules, whilst the peritrophic membrane and cuticle (but not the underlying hypodermal cells) remained unstained. The granules did not stain. From these reactions it is clear that free sulphydryl groups do not occur in particularly high concentration in the granule-accumulating region of the tubules.

VI. **Effect on Granule Formation of Alterations to Diet**

(a) Calcium

The calcium content of the medium was increased by the addition of various soluble salts. A six-fold\(^{\dagger}\) addition of calcium caused an enlargement in diameter of the granule-accumulating region and its extension around the bend into the upper portion of the descending arms of the tubules. Deposits of granules increased further with increasing calcium concentration, adverse effects (growth retardation) first becoming noticeable with a 36-fold addition. These retarded larvae accumulated massive deposits of granules which occupied the upper half to two-thirds of the descending arms, in addition to the ascending arms of the tubules. A small accumulation of granules in the blind ends of the posterior pair of tubules was also a common feature. It was estimated that the amount of granular material which fully grown larvae, fed

\(^{\dagger}\) An x-fold addition means that x times as much of the element as was already present in the control medium was added.
on the 36-fold addition, had accumulated was about eight times that accumulated on control medium. It is a general feature that, as the calcium concentration in the medium increases, the ascending arms of the anterior tubules do not run straight back to the posterior end of the body as in control larvae, but are convoluted and this, with increased tubule diameter, provides the additional storage space. Granules having diameters up to 20 μ were not uncommon and there were many in the 10 to 15 μ range. The former are larger than are found in control larvae.

Adults fed a saturated solution of calcium gluconate accumulated many more granules of a larger average size than adults fed sugar solution. More granules were present in the blind ends of the anterior than of the posterior pair of tubules.

(b) Magnesium

A two-fold addition of magnesium as glycerophosphate resulted in a slight increase in the number of granules accumulated. Both a 12-fold addition of magnesium, which resulted in only very slight growth retardation, and a 20-fold addition, which caused severe retardation, produced large quantities of granules. A feature of similarity to larvae fed on calcium-enriched media was that the ascending arms of the tubules had several bends in them, thereby considerably increasing their length. However, on a magnesium-rich diet, not only was the region of the tubules near the blind end seldom enlarged in diameter, but there was a tendency for the distended portion of the descending arms to be filled with fluid and for relatively few granules to be present. The same situation was also commonly seen in the posterior third of the granule-accumulating region. This distension of the tubules with fluid appears to be characteristic of sublethal concentrations of magnesium in the food. As with calcium, there were small numbers of granules at times in the blind ends of the posterior pair of tubules.

Adults fed sugar solution containing magnesium glycerophosphate accumulated far more granules than those fed solution alone. These granules occurred in larger numbers in the anterior pair of tubules. Feeding either magnesium or calcium-rich foods, therefore, produces granule formation in adults, as in larvae, and the frequent absence of granules in adults is largely due to the low concentrations of these elements in their diet.

(c) Phosphate

The first distinct effect was with a three-fold addition of phosphate, when noticeably less granular material was formed. With an eight-fold addition, the overall picture was that of a considerable reduction in tubule diameter (granule-accumulating region only), while the deposits were markedly smaller than in control larvae. In particular, very few granules were present in the blind ends of the tubules. In some larvae, short regions of the tubules were relatively transparent, due to the presence in the lumen of fluid carrying few granules, a condition somewhat reminiscent of the far more extensive transparency associated with additions of magnesium. Larval growth was normal at this concentration of phosphate.
Adults fed sugar solution containing 2.5 per cent. potassium dihydrogen phosphate accumulated no granules.

(d) Mixtures of Calcium, Magnesium, and Phosphate

Larvae grown on media containing two-fold additions of both calcium and phosphate were indistinguishable from control larvae in amount of granular deposit. Since a two-fold addition of calcium alone produced an increase in granular material it appears that the increased phosphate partially inhibited granule formation. Similarly, although a two-fold addition of calcium and magnesium produced a slightly greater accumulation of granules than a two-fold addition of either calcium or magnesium alone, a two-fold addition of calcium, magnesium, and phosphate produced about the same amount of granular material as with calcium alone.

On the other hand, when a three-fold addition of calcium and magnesium and a 1.5-fold addition of phosphate was made, the tubules were far better filled than those of control larvae. It is clear that there is a rather complicated interrelation between the identity and both relative and absolute concentrations of various substances in the diet in their effect on granule accumulation. This is clarified in the discussion.

(e) Barium or Strontium

The addition of barium or strontium to the medium in concentration sufficient to produce growth retardation appeared to increase slightly the amount of granules accumulated. Both metals are toxic in concentrations (about 0.002 per cent. and 0.003 per cent. dry weight respectively) very much lower than the concentrations of calcium (0.04 per cent.) and magnesium (0.12 per cent.) normally present in the control medium. The amounts of barium and strontium available to the larvae would not, therefore, be expected to produce greatly increased numbers of granules unless these metals were accumulated preferentially.

(f) Carbonate or Bicarbonate

The addition of sodium carbonate or bicarbonate to the medium had no effect on granule accumulation. The carbonate apparently did not prevent any appreciable amounts of the calcium and magnesium of the medium being absorbed from the alimentary canal. However, with carbon dioxide already available in the larval tissues in excess over needs for granule formation (see Section IX), additional carbonate or bicarbonate would scarcely be expected to produce any effect.

(g) Meat-fed Larvae

Larvae fed on sheep’s liver, brain, or muscle accumulate relatively small quantities of granules. Occasional larvae fed on reconstituted dehydrated mutton accumulated none at all. Poor accumulation of granules is no doubt due to the lower concentrations of calcium and magnesium in these diets.
(h) Citrate

Citrate has frequently been used as a decalcifying agent. However, concentrations of citric acid and sodium citrate which caused some growth retardation of larvae depressed granule formation only slightly, if at all.

(i) Oxalate

Oxalic acid, incorporated in the medium in a concentration sufficient to produce slight growth retardation, had no apparent effect upon granule accumulation. It was thought that the formation of relatively insoluble calcium oxalate in the food might depress granule formation by lowering the amount of calcium available.

(j) Phloridzin

Phloridzin had no effect upon granule formation. Gutman, Warwick, and Gutman (1941) reported that phloridzin prevented calcification of cartilage by inhibiting a phosphorylative glycogenolytic enzyme system, but this system is evidently unimportant in granule formation.

(k) Sulphanilamide

Sulphanilamide, in concentrations sufficient to cause some growth retardation, had no effect on granule formation. The granules formed dissolved in dilute acid with the evolution of carbon dioxide in the same way as control granules. The effect of feeding sulphanilamide was investigated because this material has been shown to inhibit carbonic anhydrase, which catalyses the production of carbonate at sites of calcification in vertebrates (Mann and Keilin 1940). Since sulphanilamide had no effect on granule formation, either carbonic anhydrase is not important in this process or the sulphanilamide did not reach the granule-accumulating regions. The former is supported by the failure to detect carbonic anhydrase by the method of Philpot and Philpot (1936) either in ground-up larvae or in extracts of the malpighian tubules.

VII. The pH and EH of the Tubules

(a) pH Indicators

Saturated solutions of indicators were injected into larvae or added to dissections (Table 1).

The contents of the yellow regions of the tubules have a pH of 7.4 to 7.8, the pH often varying slightly, but irregularly, along the length of the tubules. The cells have a similar or slightly lower pH, as can sometimes be seen after prolonged irrigation with indicator solutions. Indicators are taken up by the granule-accumulating region only after lengthy contact and then principally in the portion immediately adjoining a yellow region. It is possible that much of the indicator has diffused in from these yellow regions. In all cases the colour of the indicator was the same in the accumulating regions as in the yellow regions or slightly more alkaline.
A feature of these tests was the rapid uptake of indicator by the midgut, the various regions of which exhibited pH values already noted (Waterhouse 1940a). The midgut caeca, which did not stain when indicator media were fed, stained following injection and had the same pH as the anterior midgut. Indicators were also absorbed by the isolated hindgut, being visible in the lumen and in some, but not all, of the hindgut cells.

The adult tubules have a slightly lower pH than those of larvae, namely 7.2 to 7.6. Occasionally some regions, which varied from adult to adult, but which generally occurred towards the outlet into the gut, had a pH lower than the bulk of the tubules. The reason for these apparently more acid regions was not determined, but they may have been due to the presence of urates. This is suggested by the acid pH of the meconium (6.2 to 6.5), urates (presumably acid) having been added to the slightly alkaline larval granules to form the bulk of the solid material in the meconium. The portions of the adult tubules corresponding to the larval granule-accumulating regions did not take up indicators nearly as readily as the remainder of the tubules but, when indicator was visible, values similar to the remainder of the tubules were obtained.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Larvae 2.5 Days Old</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Malpighian Tubules</td>
<td>Blood</td>
</tr>
<tr>
<td></td>
<td>Yellow Region</td>
<td>Granule Region</td>
</tr>
<tr>
<td>Brom-cresol purple</td>
<td>&gt; 6.4</td>
<td>&gt; 6.4</td>
</tr>
<tr>
<td>Phenol red</td>
<td>7.4-7.8</td>
<td>7.4-7.8</td>
</tr>
<tr>
<td>Meta-cresol purple</td>
<td>About 7.8</td>
<td>About 7.8</td>
</tr>
<tr>
<td>Cresol red</td>
<td>&lt; 7.8</td>
<td>About 7.8</td>
</tr>
<tr>
<td>Range</td>
<td>7.4-7.8</td>
<td>7.4-7.8</td>
</tr>
</tbody>
</table>

(b) *Eh* Indicators

One per cent. solutions in saline (or saturated solutions if the solubility was lower) of the indicators shown in Table 2 were injected into larvae. These were generally taken up rapidly by the tubules. After large doses, indicators were also often taken up by the gut cells and fat body. The addition of hydro-sulphite or peroxide was sometimes useful in deciding whether the indicator was in the oxidized or reduced state.

Taking pH 7.6 as the mean value for the larval tubules, an Eh in the range 0.13 volts to 0.16 volts is indicated. The granule-containing regions of the tubules were never as intensely coloured as the yellow regions owing, presumably, to a slower dye uptake, but no difference in the character of the coloration could be observed. In spite of the rather poorer supply of tracheae to the granule-accumulating regions, oxidizing conditions are apparently similar to those in the yellow regions. No confirmation was therefore obtained.
of the indication from riboflavin uptake that the Eh was higher in the granule region than elsewhere in the tubules. The potential of the blood is 0.14 to 0.17 volts. This value is close to that of 0.12 volts determined with indicators for Sarcophaga larva by Dennell (1947).

VIII. CHEMICAL ANALYSES

Analyses were carried out on the granule-containing portion of the mal-pigian tubules (referred to as “M.T.—granules” in the tables), as well as on various other larval tissues for comparison. The yellow portions of both pairs of tubules were pooled for analysis. Table 3 gives individual analyses for calcium, magnesium, and phosphorus for larvae from six different cultures. Cultures 1 to 4 were dissected when larvae were 2.5 days old, culture 5 at 3.5 days, and culture 6 at 4.5 days. As there was no apparent difference between these age groups the results were averaged.

### Table 2

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Eh of Tubules (pH 7.6)</th>
<th>Eh of Blood (pH 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thionine</td>
<td>&gt; 0.043</td>
<td>&gt; 0.050</td>
</tr>
<tr>
<td>1-Naphthol 2-sodium sulphonate indo</td>
<td>&gt; 0.073</td>
<td>&gt; 0.088</td>
</tr>
<tr>
<td>2,6-dichlorphenol</td>
<td>&gt; 0.087</td>
<td>&gt; 0.099</td>
</tr>
<tr>
<td>1-Naphthol 2-sulphonate indophenol</td>
<td>&gt; 0.094</td>
<td>&gt; 0.101</td>
</tr>
<tr>
<td>Toluylene blue</td>
<td>(&gt; &gt; 0.130*)</td>
<td>(&gt; &gt; 0.140*)</td>
</tr>
<tr>
<td>Sodium 2,6-dibromobenzenone</td>
<td>(&lt; 0.150)</td>
<td>(&lt; 0.168)</td>
</tr>
<tr>
<td>3-methoxyindophenol</td>
<td>(&lt; 0.173)</td>
<td>(&lt; 0.200)</td>
</tr>
<tr>
<td>o-Cresol indophenol</td>
<td>&lt; 0.186</td>
<td>&lt; 0.185</td>
</tr>
<tr>
<td>m-Cresol indophenol</td>
<td>&lt; 0.184</td>
<td>&lt; 0.200</td>
</tr>
<tr>
<td>Phenol indophenol</td>
<td>&lt; 0.184</td>
<td>&lt; 0.200</td>
</tr>
<tr>
<td>o-Brom-phenol indophenol</td>
<td>&lt; 0.184</td>
<td>&lt; 0.200</td>
</tr>
<tr>
<td>Range</td>
<td>0.13 to 0.16</td>
<td>0.14 to 0.17</td>
</tr>
</tbody>
</table>

* All values except these taken from Hewitt (1936); these approximate values were arrived at by analogy with pH curves for the other indicators.

An important factor, leading to variation in analyses of granules, is their contamination by constituents from the encircling cells. These form only a small proportion of the total weight when the tubules are well filled with granules, but a far higher proportion when the tubules are less well filled. It follows that granules of identical composition will give different percentage values depending on the ratio of weight of granules to weight of cell constituents. This factor, however, does not account for the entire variation found.

The phosphorus, calcium, and magnesium contents of the granular region of the tubules is, on the average, 20 to 30 times higher than that of other
portions of the larva. Indeed, although comprising only 0.9 per cent. of the dry weight of the larva, the granule region contains 28 per cent. of the inorganic phosphorus, 45 per cent. of the calcium, and 36 per cent. of the magnesium. There is, therefore, no room for doubt that the granules are rich in these elements. It should also be noted that, on the average, they contain slightly more magnesium than calcium. The relatively high content of the yellow region of the tubules and the invariably higher content of the hindgut than the midgut for these three elements is readily explained by the assumption that these elements are continually being absorbed from the haemolymph by the malpighian tubules and that, while portion is retained in granular form, the remainder is excreted. Selective removal of other constituents from the food in the midgut might account for higher concentrations of these materials in the hindgut, but not in the yellow regions of the tubules.

The accumulation of phosphate in the granules was further demonstrated by feeding larvae on medium containing radio-active P\textsuperscript{32}. The granule-accumulating region of the tubules gave a count which was at least 15 times that of the yellow region and which also indicated a far higher phosphorus concentration than in the remaining tissues. A comparison was made (Table 4) of the total and inorganic phosphorus contents of the various tissues.

While it is clear that the granular region of the tubules contains some organically bound phosphorus the amount present is far less than the amount of inorganic phosphorus, and probably comes from the cells surrounding the granules. On the other hand, in the remaining organs and tissues, organically bound phosphorus is present in far greater amount than inorganic phosphorus.

**Table 4**

TOTAL AND INORGANIC PHOSPHORUS CONTENTS OF TISSUES OF *L. CUPRINA* LARVAE CULTURES 7 TO 10

<table>
<thead>
<tr>
<th></th>
<th>7</th>
<th></th>
<th>8</th>
<th></th>
<th>9</th>
<th></th>
<th>10</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M.T.—granules</td>
<td>13.4</td>
<td>12.6</td>
<td>17.0</td>
<td>11.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.T.—yellow regions</td>
<td>1.45</td>
<td>0.61</td>
<td>1.48</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hindgut</td>
<td>1.62</td>
<td>0.54</td>
<td>1.56</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midgut</td>
<td>1.08</td>
<td>0.20</td>
<td>1.69</td>
<td>0.37</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cuticle + muscles</td>
<td>1.06</td>
<td>0.29</td>
<td>1.14*</td>
<td>0.26*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat body</td>
<td>0.99</td>
<td>0.13</td>
<td>1.11</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>0.99</td>
<td>0.35</td>
<td>1.11</td>
<td>0.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole larva</td>
<td>1.22*</td>
<td>0.48*</td>
<td>1.10†</td>
<td>0.44†</td>
<td>1.41**</td>
<td>1.29‡</td>
<td>0.33§</td>
<td></td>
</tr>
<tr>
<td>Av. larval wt. (mg.)</td>
<td>28.6</td>
<td>33.4</td>
<td>31.5</td>
<td>14.2</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Moisture (%)</td>
<td>79.5</td>
<td>76.1</td>
<td>77.5</td>
<td>79.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Average of 2 analyses. † Average of 6 analyses.
** Average of 3 analyses. ‡ Average of 16 analyses.
§ Average of 4 analyses.
By contrast, figures for total calcium and magnesium were no higher than those for inorganic calcium and magnesium, indicating that only very small amounts of these elements can be present in organic combination.

Table 5 gives results of analyses of the granule-containing portion of the malpighian tubules for phosphorus, calcium, magnesium, and carbonate. On the average, 3 per cent. of the dry weight of the tubules was due to carbonate. In the course of these experiments it was found that all of the gas evolved was absorbed by potassium hydroxide and hence was carbon dioxide.

Table 5
ANALYSIS OF THE GRANULE-ACCUMULATING REGION OF LARVAL MALPIGHIAN TUBULES CULTURES 4 TO 6 AND 11

<table>
<thead>
<tr>
<th></th>
<th>11</th>
<th>4</th>
<th>6</th>
<th>5</th>
<th>Av.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>14.7</td>
<td>13.6</td>
<td>12.3</td>
<td>11.1</td>
<td>12.9</td>
</tr>
<tr>
<td>Ca</td>
<td>7.1*</td>
<td>6.2</td>
<td>7.2</td>
<td>7.4*</td>
<td>7.0</td>
</tr>
<tr>
<td>Mg</td>
<td>8.6*</td>
<td>11.3</td>
<td>9.2</td>
<td>10.0*</td>
<td>9.8</td>
</tr>
<tr>
<td>CO₃</td>
<td>1.6*</td>
<td>5.1</td>
<td>3.3</td>
<td>2.4</td>
<td>3.1</td>
</tr>
<tr>
<td>Residue from P analysis</td>
<td>12</td>
<td>19</td>
<td>22</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Residue from Ca and Mg analyses</td>
<td>7</td>
<td>15</td>
<td>25</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Av. larval wt. (mg.)</td>
<td>28.6</td>
<td>21.1</td>
<td>27.8</td>
<td>36.1</td>
<td></td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>83.1</td>
<td>80.1</td>
<td>79.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (days)</td>
<td>2.5</td>
<td>2.5</td>
<td>3.5</td>
<td>4.5</td>
<td></td>
</tr>
</tbody>
</table>

* Average of 2 analyses.

To obtain some information on the amount of insoluble residue, which has been referred to earlier as a source of variation when calculating percentages for the granules, the coverslips carrying the samples were dried and reweighed after treatment with trichloracetic acid, giving figures which varied from 7 to 25 per cent. The vigorous evolution of carbon dioxide following acid treatment could be seen at times to result in fragments of tubule being dislodged from the coverslip. More reliance should, therefore, be placed on the higher than on the lower values.

Table 6
ANALYSES OF THE ARTIFICIAL MEDIUM

<table>
<thead>
<tr>
<th></th>
<th>% Dry Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>Total Inorganic Ca Inorganic Mg Inorganic</td>
</tr>
<tr>
<td></td>
<td>0.68* 0.40† 0.04† 0.12†</td>
</tr>
<tr>
<td>Moisture content</td>
<td>77%</td>
</tr>
</tbody>
</table>

* Average of 3 analyses. † Average of 4 analyses. ‡ Average of 8 analyses.

Analyses of the medium (Table 6) showed that it was about as rich in inorganic phosphorus as whole larvae, but contained less organic phosphorus, calcium, and magnesium.
In Table 7, typical analyses are shown of the granule-containing portion of the tubules from larvae grown on media to which various salts have been added. It should be noted that the figures for content per larva are not exactly proportional to those for per cent. dry weight. This is due to the variation from larva to larva in the weight of granules accumulated and the fact that tubules from different larvae were used for the various analyses.

**Table 7**

**The Effect on the Composition of the Granules of Enriching the Medium with Salts**

<table>
<thead>
<tr>
<th>% Dry wt.</th>
<th>Ca 24x</th>
<th>Ca 21x, P 1.5x</th>
<th>Mg 12x, P 4.5x</th>
<th>P 3x</th>
<th>P 6x</th>
<th>Av. for Control Larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>7.1</td>
<td>6.9</td>
<td>13.7</td>
<td>12.2</td>
<td>12.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Ca</td>
<td>29.2*</td>
<td>20.0</td>
<td>1.7</td>
<td>11.0</td>
<td>6.3*</td>
<td>7.5</td>
</tr>
<tr>
<td>Mg</td>
<td>1.7</td>
<td>0.7</td>
<td>14.6</td>
<td>10.8</td>
<td>7.4*</td>
<td>9.6</td>
</tr>
<tr>
<td>CO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>21.8</td>
<td>18.1</td>
<td>4.3</td>
<td>4.7</td>
<td>8.9</td>
<td>3.1</td>
</tr>
<tr>
<td>γ per Larva</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>19.6</td>
<td>24.8</td>
<td>22.4</td>
<td>3.4</td>
<td>3.6</td>
<td>6.1</td>
</tr>
<tr>
<td>Ca</td>
<td>106</td>
<td>67</td>
<td>4.6</td>
<td>3.4</td>
<td>2.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Mg</td>
<td>6.4</td>
<td>2.4</td>
<td>21.3</td>
<td>3.4</td>
<td>2.4</td>
<td>5.4</td>
</tr>
<tr>
<td>CO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>85*</td>
<td>75.0</td>
<td>5.0</td>
<td>1.7</td>
<td>2.8</td>
<td>2.1</td>
</tr>
<tr>
<td>% Residue from</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>–</td>
<td>7.4</td>
<td>4.4</td>
<td>15</td>
<td>23.9</td>
<td></td>
</tr>
<tr>
<td>Ca and Mg</td>
<td>–</td>
<td>–</td>
<td>7.7</td>
<td>–</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>Av. larval wt. (mg.)</td>
<td>29.1</td>
<td>28.5</td>
<td>25.4</td>
<td>26.3</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>–</td>
<td>–</td>
<td>82.7</td>
<td>81.0</td>
<td>83.0</td>
<td></td>
</tr>
<tr>
<td>Salt used</td>
<td>Calcium gluconate</td>
<td>Calcium glycerophosphate</td>
<td>Magnesium glycerophosphate</td>
<td>KH₂PO₄</td>
<td>KH₂PO₄</td>
<td>Av. for Control Larvae</td>
</tr>
<tr>
<td>Age (days)</td>
<td>3.5</td>
<td>5.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

* Average of 2 analyses.

Addition of calcium to the diet clearly increased very greatly the amount of both calcium and carbonate in the tubules and this is almost certainly due to an increased amount of calcium carbonate. Although the percentage of magnesium present is far less than on control media, the actual amount present per larva falls within the range observed for larvae on control media (see Table 3), so that accumulation of magnesium has apparently not been interfered with. Whereas the percentage of phosphorus present is somewhat less than for control larvae, the actual amount per larva is considerably increased and one can therefore conclude that calcium phosphate is a constituent of the granules. Since no phosphate was added in the enrichment of medium with calcium gluconate it is clear that, in larvae on control media, phosphate is available in excess over requirements for granule formation, other factors (of which the concentration of calcium is one) limiting the amount of phosphate deposited.

Addition of magnesium increased considerably the amount accumulated per larva of magnesium and phosphate and, to a lesser extent, the amount of
carbonate. The amount of calcium accumulated was unchanged. Magnesium phosphate and magnesium carbonate are evidently constituents of these granules, which were also found to contain about 0.5 per cent. ammonia. If all of this ammonia is present, as might be expected, as the insoluble magnesium ammonium phosphate, this would bind only 5 per cent. of the magnesium present. These larvae each contained about 0.48 \( \gamma \) ammonia in granule form, which is considerably more than control or liver-fed larvae (about 0.068 \( \gamma \)) or calcium-fed larvae (about 0.088 \( \gamma \)). These latter figures correspond to an ammonia content in the granules falling within the range 0.1 to 0.3 per cent. It is clear, therefore, that only a small amount of the magnesium is present as magnesium ammonium phosphate.

Addition of phosphate had little effect upon the percentage composition of the granule region. This may be taken as confirmatory evidence that phosphate is available in excess in the control medium. The amounts accumulated per larva in the granule region of these larvae were equivalent to, or lower than, those at the lower ends of the ranges indicated in Table 3 for control larvae, general confirmation of the earlier observation that the addition of phosphate tends to inhibit granule formation.

Several analyses have been carried out on granules from larvae fed on sheep's liver (Table 8). Cultures A and B gave percentage values reminiscent of those on media rich in calcium (Table 7), in that both calcium and carbonate

<table>
<thead>
<tr>
<th>Table 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANALYSIS OF GRANULE-ACCUMULATING REGION OF LARVAE FED SHEEP'S LIVER AT 27°C.</strong></td>
</tr>
<tr>
<td>Cultures</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>% Dry wt.</td>
</tr>
<tr>
<td>P</td>
</tr>
<tr>
<td>Ca</td>
</tr>
<tr>
<td>Mg</td>
</tr>
<tr>
<td>CO(_3)</td>
</tr>
<tr>
<td>γ per Larva</td>
</tr>
<tr>
<td>P</td>
</tr>
<tr>
<td>Ca</td>
</tr>
<tr>
<td>Mg</td>
</tr>
<tr>
<td>CO(_3)</td>
</tr>
<tr>
<td>% Residue from</td>
</tr>
<tr>
<td>P</td>
</tr>
<tr>
<td>Ca and Mg</td>
</tr>
<tr>
<td>Av. larval wt. (mg.)</td>
</tr>
<tr>
<td>Moisture (%)</td>
</tr>
<tr>
<td>Age (days)</td>
</tr>
</tbody>
</table>

* Average of 2 analyses.

were abundant, while phosphorus and magnesium were present in relatively small amounts. On the other hand, culture C gave values not unlike those on the artificial medium, and cultures D and E indicated that, at times, more magnesium is present than calcium.
In all cases the weight of granules present per larva was considerably smaller than in larvae fed on the artificial medium. The extremely large variation in the relative amounts of material present is almost certainly due to the rather variable composition of liver (average for several analyses shown in Table 11). Further analyses of larvae fed on animal tissues were not performed since the main factors influencing the composition of the larval granules had been determined using the more uniform artificial medium. In addition, when only small quantities of granules are present, the mineral content of the cells of the tubules becomes an important interfering factor in the analyses.

IX. Discussion

It has been established that the granules accumulated in the tubules of *L. cuprina* larvae contain calcium and magnesium phosphates and carbonates. It is highly probable also that part of the magnesium present occurs as magnesium ammonium phosphate. However, quite apart from the low values for ammonia found by analysis it is clear that most of the magnesium cannot be bound in this fashion because, if all of the magnesium were so bound, the granule region of control larvae, with an average of 9.4 per cent. magnesium, would contain about 95 per cent. magnesium ammonium phosphate, which would leave no place for the calcium salts. The relative amounts of calcium and magnesium in the granules varies with their relative concentrations in the food. When fed the standard artificial medium, which contains three times as much magnesium as calcium (Table 6), slightly more magnesium than calcium is present in the granules. A food containing equal weights of calcium and magnesium would therefore be expected to result in more calcium than magnesium being accumulated in the granules. However, since many animal tissues contain more magnesium than calcium (Table 11; Takamatsu 1936) calcium and magnesium are probably of about equal importance as normal granule constituents. Certainly the proportion of magnesium present is greater than has been imagined by earlier workers and contrasts strikingly with figures for bone in which 40 to 50 times more calcium is present than magnesium (Duckworth, Godden, and Warnock 1940). The contents of phosphorus, calcium, and magnesium in whole larvae and in larval blood recorded in the present analyses are of the same order of magnitude as those already recorded for other insects, including several Diptera (Table 9), so that there is nothing peculiar about *L. cuprina* larvae in this respect.

Specific staining reactions have failed to restrict any particular salt to any of the various granule types and it must therefore be concluded that all salts may occur in all types of granule. The appearance of certain granules suggests that they are built up of concentric layers of differing composition, although each layer may be either a homogeneous or heterogeneous mixture of salts. On certain highly enriched diets, however, it is possible that single-salt granules occur.
The presence of barium and strontium is perhaps surprising at first sight, particularly because of the toxicity of the former, although in fact it might quite reasonably have been expected. Any mechanism for the uptake of calcium and magnesium from the haemolymph would be expected to operate also for barium and strontium. Nevertheless, similar accumulations of barium and strontium do not appear to have been recorded in other animals.

It is worth considering whether measurements of the respiratory quotient of granule-accumulating dipterous larvae will give values affected by the calcium and magnesium contents of their food. According to Hitchcock and Haub (1941) 7.7 litres of carbon dioxide are produced per 100 g. Phormia larvae from the time they leave their food until adults emerge. At least as much must have been produced during larval development. Further, from the figures of Cousin (1932), one can calculate that large, feeding larvae of L. sericata produce an average of 440 ml. carbon dioxide per 100 g. larvae per hour, while small larvae respire at the rate of 660 ml. carbon dioxide per 100 g. per hour. In the present experiments, larvae fed on a diet enriched 24 times with calcium, which accumulated more carbonate-containing granules than on any other diet examined (see Table 10), bound in 3.5 days only a little over 100 ml. of carbon dioxide per hour.

### Table 9

**PHOSPHORUS, CALCIUM, AND MAGNESIUM CONTENTS OF DIPTERA**

<table>
<thead>
<tr>
<th>Species</th>
<th>g. per 100 g. Dry Tissue</th>
<th>P</th>
<th>Total</th>
<th>Inorganic</th>
<th>Ca</th>
<th>Mg</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcophaga carnaria larvae on meat</td>
<td>0.87</td>
<td>Tomita and Kumon* 1936</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calliphora erythrocephala larvae on meat</td>
<td>0.76</td>
<td>Khouvine and Gregoire* 1940</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lucilia sericata larvae—</td>
<td>0.70-0.76</td>
<td>Hobson 1935</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lucilia sericata larvae—</td>
<td>0.77</td>
<td>Baldwin and Needham* 1933</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drosophila adults from larvae fed—</td>
<td></td>
<td>Rubinstein, Lwowa, &amp; Burlakowa 1935</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal diet</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>deficient diet</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chironomus tentans larvae</td>
<td>1.18</td>
<td>Birge and Juday* 1922</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lucilia cuprina larvae artificial medium</td>
<td>1.2</td>
<td>Present analyses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These figures were calculated from data provided by the respective authors.
dioxide in granule form per 100 g. larvae, which would certainly introduce much less than a 1 per cent. error in respiratory quotient determinations. On most diets, including animal tissues, the error involved would be still less than on this calcium-rich diet.

TABLE 10

<table>
<thead>
<tr>
<th>Medium</th>
<th>Age of Larvae (days)</th>
<th>Av. CO₂/100 g. Larvae Wet Weight (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>3.5</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>4.5</td>
<td>3</td>
</tr>
<tr>
<td>Enriched P 3x</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>Enriched P 6x</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>Enriched {Mg 12x, P 4.5x}</td>
<td>2.5</td>
<td>8</td>
</tr>
<tr>
<td>Enriched {Ca 21x, P 3x}</td>
<td>5.5</td>
<td>111</td>
</tr>
<tr>
<td>Enriched Ca 24x</td>
<td>3.5</td>
<td>123</td>
</tr>
</tbody>
</table>

On the other hand, calliphorid larvae excrete large amounts of ammonia in close proximity to the most important respiratory openings, the posterior spiracles, which may seriously disturb a measurement of the respiratory quotient (Brown 1938b) by the formation of ammonium carbonate. It is possible, therefore, that even more carbon dioxide was produced than was recorded by Cousin (1932) and by Hitchcock and Haub (1941). Certainly, immersion of larvae in acids results in bubbles of carbon dioxide being produced from the anus and from the surface of the cuticle surrounding it. This is not due to the solution of granules, which are absent from these situations.

It is clear both from feeding experiments and from analyses that the quantity of granules accumulated and their composition depend upon the composition of the diet. On many of the diets more phosphate is present and, on all of the diets, more carbon dioxide is available within the larva than can be found in granule form; under these conditions the amounts of calcium and magnesium present limit granule formation. The smaller accumulations of granules in larvae fed on meat compared with those on the artificial medium would appear to reflect the somewhat lower concentration of calcium and magnesium and the equal or higher concentration of phosphorus in meat. Indeed, it appears probable that the low concentration in the larval food and the consequent small number of granules formed must have been responsible for the omission of Lowne (1890) and Pérez (1910) to mention the presence of granules in their extensive works on Calliphora erythrocephala larvae. Pérez did observe
the accumulation of granules in the pupal period, but these clearly correspond to the urate-rich granules formed in *L. cuprina* pupae.

Suitable figures for comparison with the artificial medium for the mineral content of sheep, cattle, and most other animal tissues and organs do not appear to be available, but what information can be calculated from the literature suggests that they are of the same general order of magnitude as the figures

<table>
<thead>
<tr>
<th>Table 11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COMPARISON OF THE MINERAL CONTENTS OF MAMMALIAN TISSUES AND OF THE ARTIFICIAL MEDIUM</strong></td>
</tr>
<tr>
<td>g./100 g. Dry Wt.</td>
</tr>
<tr>
<td>Ca</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Human muscle*</td>
</tr>
<tr>
<td>Human blood*</td>
</tr>
<tr>
<td>Human brain*</td>
</tr>
<tr>
<td>Human liver*</td>
</tr>
<tr>
<td>Sheep blood†</td>
</tr>
<tr>
<td>Sheep blood‡</td>
</tr>
<tr>
<td>Sheep liver</td>
</tr>
<tr>
<td>Artificial medium</td>
</tr>
</tbody>
</table>

* Calculated from Shohl 1939.
† Calculated from Hobson 1935.
‡ Calculated from Ray 1939.

for the human body (Table 11). Human muscle, for instance, contains about half as much calcium and magnesium as the medium. Since addition of calcium or magnesium salts to sheep or beef muscle considerably increases the amount of granular deposit, it can only be in having too low a concentration of these minerals that meat is unsuitable for granule accumulation. The different amounts of both calcium and magnesium available in the food for the two groups of species may possibly explain the accumulation of calcospherites in the fat body of leaf-mining Agromyzidae (Henneeguy 1897; Keilin 1921), but not in predatory species (Thorpe 1930), and this may also hold for the reverse situation existing in the Limnobiinae (Tipulidae) where larvae of some predaceous species contain granules in their malpighian tubules, while plant-eating species do not (Könemann 1924).

Nor is it particularly surprising, as has been mentioned in the literature, that the larvae of *Auchmeromyia* (Calliphoridae), which feed only on the blood of mammals, should accumulate granules, since calcium is present in blood in higher concentration on a dry weight basis than in the artificial medium. Admittedly, magnesium is present in about a quarter the concentration and phosphorus in less than half the concentration present in the artificial medium. However, even if phosphorus is not present in excess of metabolic requirements, and Hobson (1935) states that growth of *L. sericata* larvae on blood is improved by the addition of phosphate, there must be all the carbon dioxide required to fix any calcium available for granule formation. Furthermore, it has been found
that, in *L. cuprina* larvae, granule formation is partly inhibited by high concentrations of phosphate in the diet, although the larvae are otherwise normal. This effect is probably due to the retention of much of the calcium and magnesium in the alimentary canal as insoluble phosphates, and is overcome by enriching the medium with calcium or magnesium.

In addition to an adequate supply of the materials needed for granule formation, there must also be a specialized region of the malpighian tubules where granules can form and from which they are not flushed continuously into the hindgut. There are, for example, many blood-suckingian insects which do not accumulate granules. In *L. cuprina* and in other calliphorid larvae the accumulating regions can easily be distinguished in general appearance from the remainder of the tubules, even when portions within the region contain no granules. The epithelium in the granule region of the larval tubules appears to be specially adapted to secrete into the lumen a number of materials including magnesium, calcium, and other alkaline earth metals as well as phosphates and bicarbonates or carbonates. Urates and several dyes, which are principally taken up by the yellow regions of the tubules, are not absorbed. While aqueous solutions are probably necessary for the transportation of the materials into the lumen, water must be rapidly reabsorbed or the granules would tend to be flushed out.

An analogous secretion of certain dyestuffs into the lumen of the tubules, followed by reabsorption by the cells which secreted them, has been noted in *Forficula, Periplaneta*, and *Dermestes* by Lison (1942). Certainly in *L. cuprina* a marked difference exists in the rate at which water is discharged into the lumen of the two regions. Thus, when tubules are placed in hypotonic saline, water uptake forces the granules further into the blind ends of the tubules and not in the direction of discharge. From this it might be thought that the granule regions are merely blind ends of the tubules into which the yellow regions direct excretory products for storage. This is not so since, under normal conditions, no packing of the granules into the blind ends can be observed. In addition, granules already in the blind ends of the tubules increase in size with increasing age of the larva, so that additional layers must be added *in situ* in spite of an intervening length of tubule packed with granules. Furthermore, following injection, iron is taken up by the granule-accumulating regions and not by the yellow regions.

In larvae fed on diets enriched with suitable calcium and magnesium salts a functional modification of the tubules, dependent upon the composition of the food, appears to have taken place. Granules are accumulated in the descending arms of the anterior tubules, a location in which they are not found in meat-fed larvae. These granules are not excreted as soon as they are formed as would be expected if the cells of this region retained their usual capacity to discharge materials rapidly into the lumen for transport to the hindgut. Instead, they are retained and increase in size as in the true accumulating regions. Experiments with dyes indicate that the permeability of
the granule-containing "yellow" regions has diminished to that of the true granule-accumulating regions. Furthermore, following injection, iron is now taken up by these cells which hitherto failed to absorb it.

Within the single pair of tubules involved in granule accumulation a different location of granules has been noted in different groups of dipterous larvae; for example, the entire tubule in Stratiomyia (Vaney 1900), the ascending tube in Lucilia, the distal part only of the ascending tube in Thrixion (Pantel 1898), and in the dilated middle region in Ptychoptera (Pantel 1914). This varied distribution is probably closely associated with the occurrence of a specialized tubule epithelium, but is also dependent upon the composition of the food, since it has been shown in Lucilia that granules may either be almost completely absent (on some meat diets), or present in the anterior half of the descending tubule as well as in the ascending arms after feeding on calcium- and magnesium-rich diets.

In adults there is less differentiation of the tubules although, in L. cuprina, the region corresponding to the larval granule-accumulating portion can still be distinguished from the rest by the smaller amount of yellow pigment present. Accumulations in adult flies have been recorded only in Drosophila (Eastham 1925), which were feeding on the same food as the larvae. Certainly, granules were absent in wild Drosophila adults caught in Canberra. In adult L. cuprina, granules are normally absent, but may be present if the diet contains quantities of calcium and magnesium salts. When present, they are more numerous in the relatively unpigmented region of the tubules than elsewhere, indicating that these regions are still specially adapted for granule formation. The discharge of granules from adult tubules after transfer from a mineral-rich to a mineral-poor diet indicates that two factors are responsible for the normal absence of accumulations in the adult. The first is the inadequate amount of calcium and magnesium salts available from the diet and the second is that, as granules are formed, they are continually being discharged from the tubules.

There is no support for the statement by Stewart (1934) that L. sericata larvae exude calcium carbonate through their body wall and, indeed, Stewart himself gives no adequate evidence that this may occur. Roubaud (1913) states that granules are excreted continuously by Auchmeromyia larvae and this may be true. In L. cuprina on diets which produce very large accumulations, causing invasion of granules into the yellow regions of the tubules, some are excreted but, if this does occur in larvae fed meat or the artificial medium, it is certainly unusual. There is no reason to doubt, however, that some calcium is normally excreted, although not in granule form. Neither the larval cuticle nor the empty pupal cases effervesce when immersed in acid as they would if calcium carbonate were continuously exuded through the cuticle. Furthermore, passage of calcium carbonate in this manner would not be expected on the basis of the structure of the cuticle. There is no indication of any solution of granules on moulting (Müller 1924) or on metamorphosis,
as Keilin (1921) described for the calcospherites in the tubules of Acidia, and none are used to impregnate the puparium.

In vertebrates, and in many invertebrates, carbonic anhydrase commonly occurs at sites of calcification and has been found to catalyse the production of carbonate ions, whereby carbon dioxide is made freely available for combination as calcium carbonate. Inhibition of this enzyme by oral administration of a number of materials, including sulphanilamide, has been shown to inhibit egg shell formation in the domestic fowl (Benesch, Barron, and Mawson 1944; Gutawska and Pozzani 1945; Mann and Keilin 1940). Addition of sulphanilamide to the food had no apparent effect on granule formation or on the deposition of carbonates in L. cuprina larvae and carbonic anhydrase could not be detected in the tubules or in the larval tissues. This is in agreement with several workers (Florkin 1935; Kreps and Chenykaeva 1942; Sobotka and Kann 1941), who have recorded that carbonic anhydrase is either absent from insects or present in extremely small amounts. If carbonic anhydrase were to occur in insects at all, it might well be expected in the granule-accumulating region of blowfly larvae, but even here it was found to be absent. Its absence favours the elimination of carbon dioxide without hydration and may be responsible for the fact that so little is bound in the granules. Absence of carbonic anhydrase, at least from many insects, renders somewhat less probable the scheme suggested by Wigglesworth (1931) for the formation of uric acid crystals in the malpighian tubules. In this scheme, carbonate is required to displace urate from its cation, resulting in free uric acid.

Alkaline phosphatase is invariably present in quantity at calcification sites in vertebrates (Moog 1946), but does not appear to be present in the cells of the granule region of the tubules, although it occurs abundantly in several other parts of the body (Day 1949).

It might appear then that granule formation occurs in L. cuprina as a deposition of salts from solutions concentrated within the lumen of specialized regions of the tubules, without the intervention of at least two of the enzymes which are important in the calcification of the exo- and endoskeletal structures in other animals.

No evidence has been obtained in the present experiments that the granules of L. cuprina have any other function than that of storage excretion. While it is true that some respiratory carbon dioxide is bound in the granules, this mode of elimination of carbon dioxide cannot be important to the larva as was suggested by Keilin (1921), since less than 1 per cent. of the amount produced is bound in granule form even under favourable conditions. Also, normal larvae can be obtained which contain few granules, or none, and hence little or no bound carbon dioxide. The fact that normal larvae lacking granules can produce normal adults is good reason to believe that granules are not indispensable to larval physiology. The further fact that any granules which may have accumulated are completely eliminated by the newly emerged adult leads one to the conclusion that granules are merely waste products.
stored (by virtue of the absence of peristalsis and of adequate water circulation down the tubules) during larval and pupal life in amount and composition depending upon the nature of the food.

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PHYSIOLOGY AND TOXICOLOGY OF BLOWFLIES. XIV 111


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EXPLANATION OF PLATE 1

Fig. 1.—Granules, from liver-fed larva, stained for phosphate. Concentric rings within granules in Figures 1 and 2 are mainly due to differences in properties of the various layers and not to purely optical effects.

Fig. 2.—Granules from larva fed on calcium-enriched artificial medium (24 x Ca as gluconate). Stained with Gallamine blue.

Fig. 3.—Pupal granules from posterior pair of malpighian tubules.

Fig. 4.—Posterior blind end of granule-accumulating region showing the terminal filament and two strands of connective tissue attached at one end to tubule and at the other to a branch of an alary muscle.

Fig. 5.—Granules from larva fed on the artificial medium, photographed under crossed polars.

Fig. 6.—Transverse section of yellow region of malpighian tubule.
WATERHOUSE.—STUDIES OF THE PHYSIOLOGY AND TOXICCLOGY OF BLOWFLIES. XIV