COMBINED HISTOCHEMICAL AND X-RAY MICROANALYTICAL STUDIES ON THE COPPER-ACCUMULATING GRANULES IN THE MID-GUT OF LARVAL DROSOPHILA

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SUMMARY

The copper-containing granules in the mid-gut epithelium of larval Drosophila melanogaster were examined for acid phosphatase by combined histochemistry and energy-dispersive, X-ray microanalysis. After incubation, many of the granules were shown to contain simultaneously copper and sulphur (which are normal constituents), and lead and phosphorus (which are the detectable elements of the reaction product). Earlier work has been consolidated and extended and the evidence that the granules are formed as cytolysosomes is reviewed.

INTRODUCTION

It has been known for many years that various elements, including copper, accumulate in the mid-gut epithelium of some larval diptera (Poulson & Bowen, 1952; Poulson, Bowen, Hilde & Rubinson, 1952; Waterhouse, 1940, 1945; Waterhouse & Stay, 1955; Filshie, Poulson & Waterhouse, 1971). In a previous paper (Tapp, 1975) it was shown by X-ray microanalysis that the accumulated copper in larval Drosophila melanogaster is present in granules and is associated consistently with high concentrations of sulphur. Those observations confirmed directly and extended the earlier work of Poulson & Bowen (1952) and of Filshie et al. (1971) who had concluded from indirect evidence that the copper was concentrated into granules.

The nature of these granules was, however, uncertain. It was first thought that they might be mitochondria or parts of the Golgi apparatus (Poulson & Bowen, 1952). Later, ultrastructural studies (Filshie et al. 1971) showed that the cupophilic cells contained granules which appeared morphologically to be cytolysosomes, and that their number increased with increasing copper in the diet. It was granules of this type that were shown by X-ray microanalysis to contain copper (Tapp, 1975). Nevertheless, there was no histochemical evidence that the granules contained at least one acid hydrolase, which is indispensable if they are to be defined with certainty as lysosomes (Maggi, 1973).

The present investigation was designed to show histochemically the presence of an acid hydrolase in the granules and, by X-ray microanalysis, to demonstrate simultaneously the presence of copper and of the main elements of the histochemical reaction product in the same granule. Earlier observations (Tapp, 1975), which were
made almost entirely by wavelength-dispersive analysis, have been confirmed and extended in this study, which uses energy-dispersive analysis. Correlations between X-ray microanalysis and histochemistry have been attempted by many workers: the association of copper and a phosphatase in Wilson's disease was investigated by Goldfischer & Moskal (1966) and, more recently, Sohal, Peters & Hall (1976) have studied simultaneous analysis and histochemistry of mineralized concretions in the Malpighian tubules of the housefly.

MATERIALS AND METHODS

The methods used for culturing the larvae, for dissecting and detecting the copper-containing section of the mid-gut and the general preparation for electron microscopy have been described in detail previously (Tapp, 1975).

In this investigation the concentration of copper added to Alderson's medium (Alderson, 1957) was reduced slightly to 0.2 mg Cu per ml and was added as copper nitrate (77 mg Cu(NO₃)₂, 3H₂O per 100 ml medium) rather than as copper sulphate which was used in previous work. It should be emphasized that at this concentration the larvae show neither appreciable reduction in size nor increased mortality, compared with controls grown on a normal medium, whereas the very high concentrations of copper (10 mg Cu per ml medium) used by Filshie et al. (1971) in a different medium are toxic for Drosophila melanogaster when used in Alderson's medium.

Acid phosphatase was detected by a modification of Barka & Anderson's method (Barka & Anderson, 1962). The guts were dissected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (Millonig, 1961, 1962) and then transferred to 0.1 M Tris-maleate buffer (Pearse, 1968). The segments were then placed either in the incubation medium or the control medium (from which β-glycero-phosphate was omitted) and left at room temperature of 22 °C. Incubation times of 15, 30, 60 and 120 min were tried. After incubation the gut segments were washed with several changes of the same buffer at pH 5.1, dehydrated through graded ethanols and embedded.

The sections used for analysis were thinner than in previous work and usually showed a silver interference colour which represents a thickness of about 100 nm on the Peachy scale (Peachy, 1958). The sections were mounted on titanium grids and held by aluminium circlets in inserts of aluminium which had been machined specially into a standard, copper specimen rod. Analyses were performed in an EMMA-4 at ×16000 or ×25000, with a microprobe analysis area of less than 0.2 mm² in diameter on the surface of the section, with an accelerating voltage of 60 keV, and with a beam current of 60 nA. This beam current is only 15% of that used previously for wavelength-dispersive analyses with EMMA's spectrometers (Tapp, 1975). In this investigation a KEVEX energy-dispersive analyser was used. It was fitted with a 0.012-mm-thick window and had a detection area of 30 mm² and a resolution at 59 keV of better than 165 eV at 1 kHz. The signal was fed to a Link system 290 computer for processing and storage and spectrum plots were made on a Bryan X-Y recorder.

There are many problems in presenting the results of X-ray microanalysis and there is not yet a consensus of opinion about the best methods to apply. An approximate, quantitative method exists (Hall, 1971; Hall, Clarke Anderson & Appleton, 1973) and has been used on Drosophila gut (Tapp, 1975). It is, however, time-consuming to employ and has not yet been programmed for the Link computer. In this paper, the results are presented primarily as energy-dispersive spectra, which are histograms showing the number of X-ray quanta in each 20 eV band of a part of the spectrum. Such spectra are convenient for comparing the presence or absence of obvious element peaks between different areas of analysis. They can, however, be misleading when the relative sizes of a specific peak in 2 spectra are being compared, because the mass of the different analysis areas is unlikely to be the same. To help minimize this difficulty and to enable comparisons to be made in another way, the results have also been tabulated to show the number of X-ray quanta in each relevant, specific peak of the spectrum as a percentage of the 'white' or continuum radiation (which is proportional to mass) in the analysis area. This concept, and the way in which it is applied, is illustrated in Fig. 1.
Unless otherwise stated in the captions, all of the spectra shown in this paper have been collected over a live analysis time of 100 s and have not been smoothed. Severe peak overlap occurs between the sulphur K\textalpha peak and the lead M\textalpha peak. This means that sulphur levels cannot be compared between spectra if lead is present in one or both of them. The lead itself can, however, be compared by using the L\textalpha or L\textbeta peaks which occur in different parts of the spectrum. There is slight overlap between the following peaks: the K\textalpha peak of phosphorus and the K\textalpha peak of sulphur; the K\textalpha peak of silicon and the K\textalpha peak of phosphorus; the M\textalpha peak for lead and the K\textalpha peak for phosphorus. Peaks for titanium (K\textalpha and K\textbeta) and aluminium (K\textalpha) are always present in the spectra since these materials are used for the grid and its immediate surroundings. Their intensities vary both with the mass and composition of the analysis area and with its position relative to the titanium grid bars and the aluminium inserts. Variations in their sizes are not important in interpreting the results. Peaks on the spectra which are labelled simply by the element symbol are K\textalpha peaks.

Fig. 1. Diagrammatic representation of an idealized energy-dispersive spectrum showing 3 specific peaks. The range of the windows (shown by pairs of arrowheads below the abscissa) is set by the operator. The window for the continuum, which is proportional to mass in the analysis area, is set wide and at the right-hand end of the spectrum where there are unlikely to be specific main or subsidiary peaks for any elements present in the analysis areas. The total number of counts in the areas A (hatched), B (cross-hatched) and C (stippled) can be obtained from the computer. The 2 methods used in this paper to present the results are to give the actual energy spectra of the analysis areas and to give peak values representing A divided by C and multiplied by 100.

When two peaks overlap (e.g. 1 and 2), the setting of a window which will not include quanta from the adjacent peak is restricted and the precision of the analysis reduced. Although it is possible to set a very restricted window, as shown for peak 1, in practice some degree of overlap often occurs and must be borne in mind when comparing spectra.

The windows set for collecting the X-ray quanta in the main, specific peaks of interest were P, K\textalpha (2020 eV), 1920–2120; S, K\textalpha (2320 eV), 2200–2440; Cu, K\textalpha (8060 eV), 7880–8240; Pb, L\textalpha (10560 eV), 10380–10740. The peak integrals obtained from the computer represent the sum of the counts in each 20 eV column of the histogram above a line drawn between the first and last window columns. If any columns within the window fall below the line, the differences between their heights and the window line are subtracted in summing the peak integral, but a peak integral which has a final negative value is recorded as zero. The computer also presents the total number of counts (peak plus background) in the entire histogram columns within the window. The continuum radiation was collected in a window set from 16000 to 19000 eV (Fig. 1).
RESULTS

Variations in methodology

The use of copper nitrate rather than copper sulphate as the copper additive in the diet produced no noticeable changes either in the accumulation of copper or in the ultrastructural appearance of the cells (Filshie et al. 1971; Tapp, 1975). The typical appearance of granules in a conventional thin, Araldite section is shown in Fig. 2. Fig. 4 shows energy-dispersive spectra from a larva fed copper nitrate, which confirm that the granules in the cuprophilic cells of the mid-gut contain large concentrations of copper and sulphur. It is clear, therefore, that the sulphur content of the granules does not originate from sulphate added to the diet.

The peaks for titanium and aluminium (the materials of the supporting grid and its surround) are always present in these spectra and are usually prominent. The aluminium peaks in the 2 energy-dispersive spectra shown in a previous paper (Tapp, 1975) were wrongly labelled as phosphorus – an element which is not present regularly in the granules.

Variability in the material

The general ultrastructure of typical, cuprophilic cells has been described and illustrated by Filshie et al. (1971) and Tapp (1975). They are bowl-shaped (although the section does not always pass through the depression of the bowl), have cytoplasm which is electron-dense in comparison to the surrounding, interstitial cells, and contain variable numbers of electron-dense granules. Although the number of granules increases when copper is added to the diet (Filshie et al. 1971), they are dispersed irregularly and it is not uncommon for a section to miss most or all of them. The presence of one, or a few granules in a fragment of cytoplasm is not by itself sufficient to identify the fragment as part of a cuprophilic cell since, when the diet is enriched with copper, some of the interstitial cells also contain granules which accumulate copper. Nevertheless, the granules in the interstitial cells are never as numerous nor as heavily laden with copper as those in adjacent cuprophilic cells.

The granules themselves vary in appearance when seen in conventional, thin, stained sections (Fig. 2). Although many are uniformly electron-dense, others contain inclusions and myelin forms; they sometimes give the impression of fusing together and it is possible to find appearances suggesting the formation of cytolyosomes. This

Fig. 2. Glutaraldehyde-, osmium tetroxide-fixed thin section stained with lead and uranium. Part of a cuprophilic cell showing typical electron-dense granules. The granules often occur in groups in the central part of the cell and frequently contain electron-lucent inclusions (arrows).

Fig. 3. Glutaraldehyde-fixed, unstained, thicker section which was used for analysis, after 1-h incubation for acid phosphatase. The micrograph shows a group of granules and smaller structures containing well localized acid-phosphatase precipitates. One granule (arrow) is acid-phosphatase negative. Groups of this type were used to produce the energy dispersive spectra shown in Fig. 7. It will be noticed that apart from the contrast given by the lead phosphate precipitate it is difficult to resolve many structures in the thicker, unstained sections used for analysis.
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detail is not visible in the thicker, unstained sections used for analysis and, under
analysis conditions, it is only possible to identify with certainty such prominent
structures as the nucleus, obvious granules, clear spaces in the cytoplasm and 'cyto-
plasm'.

Several hundred granules in the copper-accumulating region of the mid-gut have
now been analysed. Almost without exception the granules have contained large
amounts of both copper and sulphur, although the quantity of each which is present
and the proportion of one to the other vary from granule to granule. The 2 exceptions
encountered to date, although indistinguishable from typical copper-containing
granules in appearance, contained large concentrations of zinc (Fig. 5).

Small variations sometimes occur on the analysis traces from granules. Peaks for
silicon, phosphorus, chlorine, calcium and lead have been visible singly or in various
combinations in some spectra (Fig. 4). Although some of these peaks probably reflect
a genuine variation in composition, their significance is often uncertain and the values
obtained for the peak count expressed in terms of the background (Fig. 1 and Table 1)
are not only small but markedly variable with a one-step change (20 eV) in the
window setting. In other cases (silicon, chlorine) similar variations are occasionally
present in analysis spectra of the embedding medium and probably represent vari-
ations in the preparation and analysis techniques. Variations in sulphur content are
also found in analyses of the embedding medium and sometimes an increase in sulphur
occurs when sequential analyses are taken from the same spot. This variation is
probably the result of sulphur contamination during examination and analysis and
must introduce a small element of variability into the sulphur peaks obtained from
granules and the cytoplasm. Nevertheless, these variations, although of interest in
themselves, are small compared both with the copper and sulphur peaks of the main
granule constituents and with the lead and phosphorus peaks present after a successful
incubation for acid phosphatase. Their occurrence does not affect the main con-
clusions of this study.

The cytoplasm also shows variations in composition from area to area, with phos-
phorus, sulphur and copper being the elements most frequently encountered (Figs.
4, 5). The copper content varies from a very low level, which can be explained as
copper background, to as much as 50% of the copper present in some adjacent
granules. Such high copper levels are, however, rare and it is possible that they are
found in analysis areas which contain a fragment of a granule, either mature or in
process of formation. The most common situation is for a small and variable, but
significant, copper peak to be present (Tapp, 1975). Cytoplasmic phosphorus varies
mainly with the presence of mitochondria in the analysis area.

Fig. 4. Three spectra from a copper-containing granule, adjacent cytoplasm and near-
by supporting medium (A, B, C, respectively), from a larva fed on a medium enriched
with copper nitrate. The granule differs significantly in composition from both the
cytoplasm and the Araldite in containing high concentrations of sulphur and copper.
The analysis spectrum of the granule is interesting in showing small peaks for silicon
and phosphorus - elements which are not regularly present (compare the control
spectrum of Fig. 6).
Histochemistry

An incubation period of 60 min appeared to give optimal results (Fig. 3); a visibly detectable precipitate was well localized to some granules and to smaller structures which, in the cuprophilic cells, were often associated with clusters of granules or scattered sparsely in the cytoplasm, particularly the apical cytoplasm. These acid-phosphatase-positive, smaller structures were far more numerous in the interstitial cells than in the cuprophilic cells themselves and tended to occur as vesicles or irregular, small spaces in the apical half of the cells. A shorter incubation period still
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gave a detectable precipitate in some of the smaller positive structures (particularly in the interstitial cells) but many of the granules failed to give an obvious positive response. After longer periods of incubation a precipitate frequently appeared on the nuclear membrane, on the basal invaginations of the cells, and irregularly within the cytoplasm. Control material, which had been left in the incubation medium minus β-glycerophosphate, showed no visible precipitate in any part of the cell.

X-ray microanalysis showed that granules containing a precipitate were copper-containing granules which also displayed specific peaks for lead and phosphorus—the two detectable elements in the lead phosphate reaction product of the acid phosphatase technique. Copper-containing granules from the controls did not show these prominent lead and phosphorus peaks, although sometimes there was a small lead peak which suggested that some non-specific lead-staining had occurred (Fig. 6).

The amount of precipitate present on copper-containing granules was very variable and, although the majority of them contained some precipitate, it was not uncommon to find a granule with a high copper content and no visible precipitate (Fig. 3) even after 2 h of incubation. The smaller structures in the cytoplasm which contained a precipitate gave X-ray spectra with prominent specific peaks for lead and phosphorus. They usually showed a small copper peak, always much smaller than the copper peak of the most prominent copper-containing granules but varying in magnitude from vesicle to vesicle. Analysis spectra for a copper-containing granule with a visible precipitate, for one without a visible precipitate, and for a vesicle with a precipitate are shown in Fig. 7.

Data from the spectra presented in Figs. 2–5 are shown and compared in Table 1.

**DISCUSSION**

The presence of acid phosphatase in most of the copper-containing granules strengthens the case that they are cytolysosomes. To summarize the evidence: granules in the cuprophilic cells sometimes contain myelin figures and fragments of other organelles (Filshie et al. 1971, and Fig. 2); the granules contain concentrations of copper and sulphur (Tapp, 1975, and Figs. 4, 6, 7); a majority of the copper-containing granules give a positive acid-phosphatase reaction (Figs. 3, 6, 7). The detection of copper concentrations in some cytoplasmic areas, the increased number of granules with increased copper in the diet (Filshie et al. 1971), and the small and irregular variations in granule composition (particularly of phosphorus, sulphur, chloride and calcium, which are normal constituents of other cell organelles and cytoplasm), are all consistent with the cytolysosome hypothesis.

The thicker, unstained sections used for analysis lack contrast and it is not possible to distinguish areas of possible cytolysosome formation. Nevertheless, when thinner, stained sections are examined by conventional electron microscopy, not only do many granules contain inclusions, but it is possible to find areas in which small granules and vesicles are apparently fusing and where the morphology does suggest cytolysosome formation. It is possible that the cytoplasmic areas which show a high copper content on analysis contain such structures which go unrecognized in the sections used.
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The smaller vesicles and clear spaces which show a strong, positive acid-phosphatase reaction but which usually contain little or no copper are probably lysosomes at an early stage in their cellular evolution. The observation that they are far more numerous in the interstitial cells (which contain few copper granules) than in the cuprophilic cells (which contain many) is consistent with the possibility that young lysosomes in cuprophilic cells are constantly being used in the formation of cytolysosomes. The copper-containing granules which do not show an obvious, positive acid-phosphatase reaction can also be explained in terms of lysosome evolution if assumed to be residual bodies, derived from cytolysosomes, in which enzyme activity has declined. In support of this view, they contain very high concentrations of copper, usually higher than adjacent granules showing a positive reaction, and it is known that similar residual bodies in other tissues show little or no enzyme activity (Maggi, 1973). An alternative interpretation, however, is that the mass fraction of the copper in the reactive granules has been lowered by the local addition of the mass of the histochemical reaction product—a possibility which is discussed later.

Most of the observations can be interpreted, therefore, in terms of the formation and evolution of cytolysosomes in the cuprophilic cells. Nevertheless, the direct evidence relates only to some, mature granules and it remains possible that at least some of the others may have a different origin. It is not known how the copper enters the cells nor what foci are involved in the early stages of concentration. Neither is it known why copper should accumulate so heavily in the cuprophilic cells of the mid-gut nor whether this accumulation serves any physiological function.

The use of X-ray microanalysis combined with a suitable histochemical technique has 2 significant advantages: the composition of the section where a reaction product accumulates can be determined, and the composition of the product can be analysed. In the first case, granules which show an acid-phosphatase reaction can be shown

Fig. 6. Three spectra showing 2 typical results from 1-h incubated (A) and 1-h control (c) granules in the histochemical experiments, compared with a lead phosphate standard (n). During the recording of the lead phosphate spectrum, the beam current was reduced until the counting rate (650 Hz) was comparable with the range of counting rates (400–900 Hz) frequently encountered in analysing copper-containing granules.

The incubated granule shows prominent peaks for both lead and phosphorus, which are absent from the control. Sometimes, a small Pb Lα peak is found in the controls which may indicate a degree of non-specific lead staining. A small copper peak is present even in the copper-free, lead standard. This is a background effect caused by X-rays and electrons emitted from the lead striking copper components in and around the specimen chamber. Its height increases with the beam current (and the counting rate) but is never more than a fraction (about one tenth) of the Pb Lα peak.

It should be noticed that although the height of the copper peak in the incubated granule is greater than that in the control, it is the control granule which contains more copper in terms of the mass of the analysis area (Table 1). This difference in mass is reflected in the spectra by the different heights of the white or continuum radiation. Furthermore, the height of the copper peak in the incubated granule will have been increased by the fluorescent effect of lead X-rays.
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Table 1. The percentage ratio of specific peak counts to total counts in a selected range of the continuum for the spectra shown in Figs. 4-7

<table>
<thead>
<tr>
<th>Specimen</th>
<th>P</th>
<th>S</th>
<th>Cu</th>
<th>Zn</th>
<th>Pb</th>
</tr>
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<tbody>
<tr>
<td>Fig. 4</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Granule</td>
<td>2</td>
<td>66</td>
<td>64</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>5</td>
<td>29</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Araldite</td>
<td>0</td>
<td>14</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Fig. 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granule</td>
<td>20</td>
<td>153</td>
<td>15</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>13</td>
<td>66</td>
<td>6</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Granule (incubated)</td>
<td>18</td>
<td>—</td>
<td>55</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>Granule (control)</td>
<td>20</td>
<td>—</td>
<td>5</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Fig. 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granule</td>
<td>0</td>
<td>89</td>
<td>83</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Granule (A.P. positive)</td>
<td>18</td>
<td>—</td>
<td>33</td>
<td>2</td>
<td>54</td>
</tr>
<tr>
<td>Granule (A.P. negative)</td>
<td>4</td>
<td>170</td>
<td>156</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>'Lysosome'</td>
<td>28</td>
<td>—</td>
<td>4</td>
<td>0</td>
<td>77</td>
</tr>
</tbody>
</table>

The values were calculated according to the method illustrated in Fig. 1 and described under 'Methods'. Because of the very marked overlap between the peaks for S Kα and Pb Mα no figures for sulphur are given in incubated, positive material. Figures for lead are calculated from the Pb Lα peak.

simultaneously to be copper-containing granules. In the second case, the precipitate can be shown to increase both the lead and the phosphorus composition of the granules and to be therefore, at least in part, a genuine reaction product. This combination of techniques would also allow a genuine lead phosphate precipitate to be distinguished in a granular, electron-dense structure and allows a check on the possible occurrence of non-specific lead staining. X-ray microanalysis has also been used to detect histochemical reaction products which are not electron-dense (Ryder & Bowen, 1974).

Energy-dispersive, rather than wavelength-dispersive analysis, has the great advantage of showing at a glance the composition of the analysis area. It has revealed that small variations in composition occur even in the embedding medium. It is possible that some of these variations arise from contamination (silicon from the knife edge or as an irregular, self-detection phenomenon in the silicon detector; sulphur from the microscope column) or from a failure to mix homogeneously the constituents of the Araldite (chloride). Such small variations do not affect our own conclusions but in problems where they might interfere it would be necessary to

Fig. 7. A comparison of an acid-phosphatase positive, copper-containing granule (A), an acid-phosphatase negative, copper-containing granule (B) and an acid-phosphatase positive vacuole (C) ('lysosome') from closely adjacent regions of the same cell. A positive reaction is seen visually as a precipitate (see Fig. 3) and as prominent P and Pb Lα peaks on the spectra. The difference in mass between the analysis areas containing lead phosphate and the one which does not is reflected in the height of the continuum. It can be seen that the positive granule and the 'lysosome' give prominent Pb Lα and P Kα peaks which are not obtained from the negative granule.
reduce or exclude them by using a diamond knife, sulphur-free oils and greases in the column, and a chloride-free embedding medium (Van Steveninck, Van Steveninck, Hall & Peters, 1974; Spurr has now produced a chloride-free version of his original low-viscosity embedding medium; Spurr, 1969). It is also possible to reduce sulphur contamination significantly by using an efficient cold-finger (Jessen, Peters & Hall, 1974).

In conclusion, it is worth drawing attention to some of the general considerations which affect electron-microprobe analysis. Composite peaks are often encountered on energy-dispersive spectra and, although it is possible to separate and distinguish the overlapping curves of several elements (Birks, 1971), it is often easier and better to separate them by using wavelength-dispersive analysis simultaneously. Used in this way the 2 methods of analysis are complementary and the ability to use them both simultaneously is a particular advantage offered by EMMA-4.

The introduction of a heavy element, such as lead, during the acid-phosphatase reaction, alters the analysis spectrum in several ways. Not only do the specific peaks for lead appear but, because of X-rays and electrons secondarily emitted from the lead, both the continuum radiation and the specific peaks of elements lighter than lead are enhanced by a fluorescent effect (Goodhew, 1976). These effects can be considerable and pose particular difficulties for quantitative work.

In EMMA-4 the electron beam is not completely collimated and coherent and, even when the probe is focused at the centre of an empty square of a titanium grid, characteristic peaks for titanium and for the grid surround (aluminium) are obtained. When a specimen is inserted, these peaks increase in intensity and, in addition, it is usually possible to detect a small ‘background’ peak for copper which is used for many components in and around the specimen chamber. The aluminium from which the inserts and circlips were machined was, in fact, an aluminium alloy containing traces of copper and is likely to have been the main source of this ‘copper background’. If the specimen contains a heavy element, such as lead, the background effects will be increased by a fluorescent effect and, for this reason, it is difficult to detect confidently with the specimen holder used small quantities of copper in structures which contain a positive lead phosphate precipitate for acid phosphatase. However, the mature, copper-containing granules studied in this paper contain so much copper that no ambiguity exists.

REFERENCES

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