IDENTIFICATION OF MAST CELLS IN THE SCANNING ELECTRON MICROSCOPE BY MEANS OF X-RAY SPECTROMETRY

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ABSTRACT

In mixed populations of rat peritoneal fluid cells, the mast cells can be differentiated from other cell types in the scanning electron microscope by virtue of the X-ray emission referable to their sulfur content. Both stationary probe and X-ray mapping are feasible; the amount of sulfur eliciting a signal is estimated to be about 8×10^{-18} g.

INTRODUCTION

Mast cells are characterized by numerous, large, metachromatic, cytoplasmic granules. The metachromasia is elicited by heparin, a highly sulfated mucopolysaccharide. Mast cells are ubiquitously distributed in the connective tissues of many animals, including man (9, 19). In various laboratory rodents, free mast cells are normally present in peritoneal and in pleural fluids, together with various other cellular types such as lymphocytes, macrophages, and eosinophils (16). In young animals, all these cells are approximately similar in size and shape, but the mast cells become progressively larger as the animal ages (17). Thus if older animals are selected, the larger mast cells can be tentatively identified in the scanning electron microscope merely on the basis of size. In this paper, it is shown that mast cells of the rat can also be identified in the scanning electron microscope by virtue of the X-ray fluorescence derived from their sulfur-rich granules.

Some of this material has appeared in abstract form (14).

MATERIALS AND METHODS

Peritoneal fluid was obtained from Sprague-Dawley male rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.; body weights about 225 g). For some preparations the fluid was air dried on a glass slide and the preparation was then coated with a thin film of carbon *in vacuo*. For other preparations, the cells were fixed in Karnovsky's mixed aldehyde formulation, post-fixed in osmium tetroxide, dehydrated in ethanol, air dried, and then coated with a thin film of gold-palladium *in vacuo*.

The preparations were then examined first by secondary electron detection mode in order to select areas containing mast cells which were identified by virtue of their size and granular component. These same areas were then further examined in one of two ways for X-ray analysis, namely: (a) By using the beam as a stationary probe ($\times$ 400 magnification, 15 kV, and 10^{-10} A; beam diameter at the object about 30 nm) in a JEOL SEM U3 coupled to an Edax solid state analyzer (Edax International Inc., Prairie view, Il.). The emitted X rays were collected for 30 s. For mast cells, the beam was positioned squarely over the peripheral cytoplasm and so as to avoid the centrally located nucleus. Nuclei were not recognizable in nonmast cells. As a control, the probe was placed on a cell- and debris-free area of the glass slide, about 50-100 μm away from any cell; or (b) By using the beam as a scanning probe in a Kent Cambridge Stereoscan, model S4-10 (Kent Cambridge Scientific, Inc., Morton Grove, Ill.), equipped with a lanthanum hexaboride gun and coupled to a virtually identical Edax
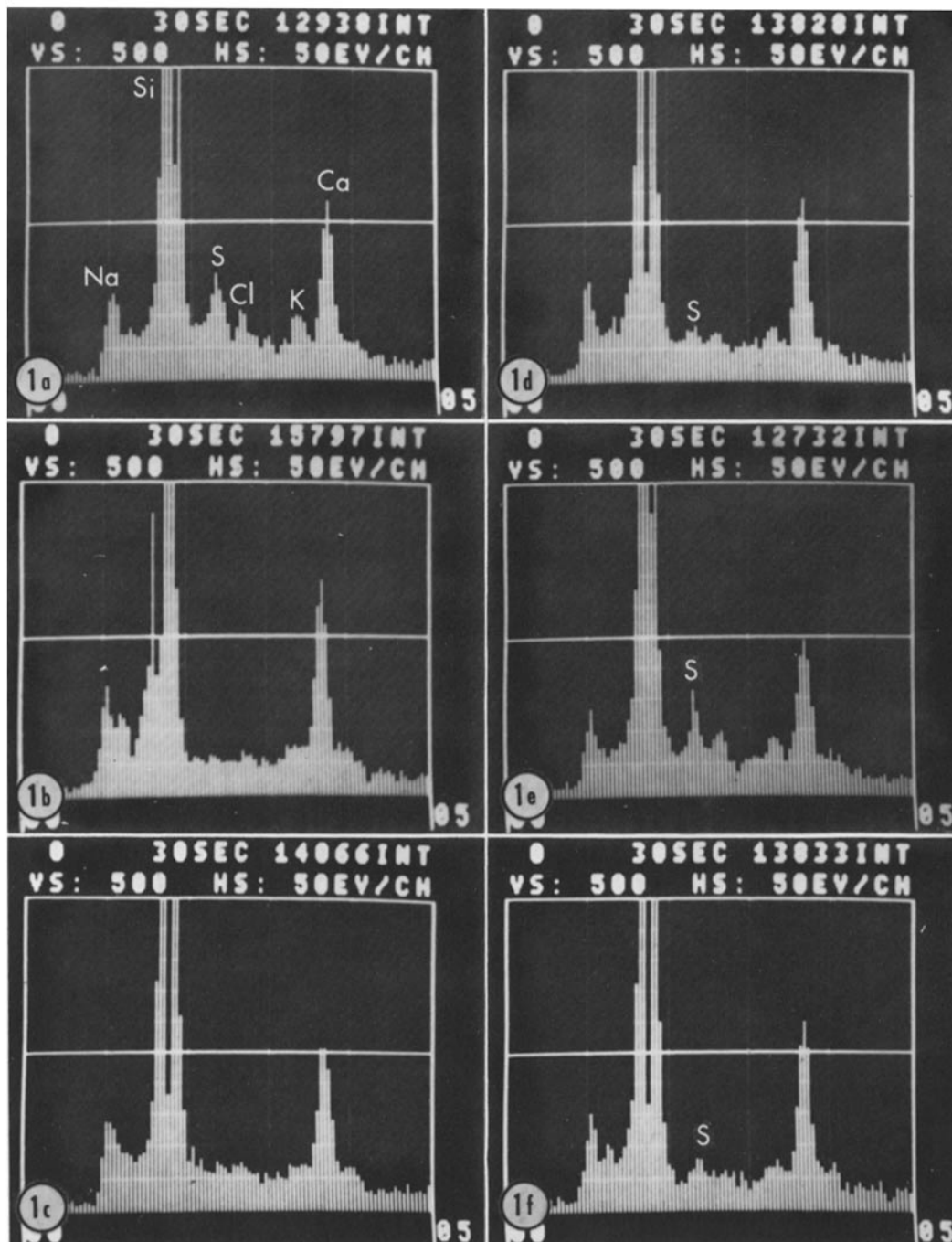
FIGURE 1   X-ray spectra of rat peritoneal fluid cells (air-dried, unfixed preparation) obtained with a stationary probe (JEOL U3 SEM. × 500. Tilt angle = 0°). (a) and (e), Mast cells; (c), (d), and (f), nonmast cells; (b), area of glass slide free of cells or debris. The most prominent peaks in all spectra are those for Na Kα, Si Kα, and Ca Kα; their presence in (b) verifies that they are elicited by the glass slide support. Peaks for K Kα, and Cl Kα + Kβ are vaguely suggested in some of the small (nonmast) cells. A sulfur Kα peak is evident in some and suggested in other cellular spectra (a, d, e, f), but is conspicuously absent from the glass control spectrum (b). Among the cellular spectra, that S Kα peak is far more prominent for the mast cells (a, e), than for nonmast elements (d, f).

solid state analyzer (resolution 180 eV; 10-mm² detector with a 1.5-inch collimator). The beam was scanned four times over the same field at 100 s per frame, and the X rays emitted at 3,200 ± 100 eV were used to construct a sulfur distribution map for that field. The cathode ray tube image was recorded on PN 55 polaroid film (Polaroid Corp., Cambridge, Mass.).

RESULTS

Unfixed cells collapsed severely onto the slide. By secondary electron detection, the smaller cells could not be identified by type even though they are known to include macrophages, lymphocytes, eosinophilic granulocytes, and small mast cells. Large mast cells were often recognizable by virtue of their cytoplasmic granules which could be discerned by greatly increasing the tilt angle. A depression was often present in the central area where, as is known from light microscopy, the nucleus is normally located. Other large cells, some perhaps mast cells also, could not be identified with confidence. Fixed cells, on the other hand, were not collapsed. The preparation method used did not preserve the surface projections of mast cells that are so characteristic of transmission micrographs. On the other hand, the collapse of the plasmalemmal folds probably helped to bring the underlying granules into evidence. The various fixed cells tended to cluster and overlap each other. The cell size range was narrower than for unfixed preparations, and mast cells were recognizable as much by virtue of their granules as by size.

Typical X-ray emission spectra obtained by the stationary probe approach are shown in Fig. 1. Most prominent in all spectra are peaks for sodium Kα and Kβ (unresolved; average 1,739 eV), and calcium Kα (3,690 eV). All cells (Fig. 1 a, c–f), but not the cell-free control area (Fig. 1 b), display definite peaks for potassium Kα (3,312 eV), chlorine Kα and Kβ (unresolved: 2,621 and 2,815 eV, respectively), and sulfur Kα (2,307 eV). The sulfur peak is substantially more prominent for mast cells (Fig. 1 a and e) than for nonmast elements (Fig. 1 c, d, and f). Comparable results were obtained with the stationary probe whether the cells were fixed and uncollapsed, or unfixed and collapsed.

Sulfur distribution maps also proved feasible. Fig. 2 shows a field at × 100 magnification imaged by secondary electron mode (unfixed preparation). Numerous cells are present, including two large mast cells and an almost as large cell that looks much like nearby nonmast elements. That same

field is shown in Fig. 3, but imaged by sulfur mapping.[1] Only the two cells confidently thought to be mast cells are revealed beyond question. Nonmast elements are ill defined by sulfur mapping: a slight increase of signal over background seems to outline their distribution in the field of view, in agreement with the stationary probe data which also appeared to detect some sulfur in these cells, but resolution is almost nil because of the high background and consequent low signal-to-noise ratio. One of the mast cells is shown at × 5,000 direct magnification in Fig. 4. The central area, exhibiting lower signal density, represents the nucleus and probably the Golgi area. There may even be a suggestion of granule-like concentrations of signal points within the cytoplasm, but it is unlikely that these represent individual granules. The nuclear area, when viewed through a mask so as to facilitate visual discrimination, definitely presents a greater signal density than does the bremsstrahlung background outside the cell boundaries; this is probably due to cytoplasmic granules' overlapping the nucleus or being trapped below it, rather than to nucleoplasm composition.

DISCUSSION

In stained smears or sections examined by light microscopy, mast cells are readily identified by virtue of the metachromatic tinctorial properties of their granules. As a result, they can easily be located when present as a minor component of mixed populations even if undistinguished by size or shape. One of the problems inherent in scanning electron microscopy is to identify cells within the scanning electron microscope so that, in mixed populations, the cells of interest may be selected for special study, and particular surface features then related to specific cell types. This presents a definite problem with mast cells because these can at times display extensive hyaloplasmic veils that would obscure underlying cytoplasmic granules (10 12, 15), or because the telltale granules may collapse and become barely recognizable when unfixed cells are air dried (13). Yet collapsed, unfixed cells may be particularly suitable for some

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[1] Although detector resolution was nominally 180 eV, the S Kα and S Kβ peaks were probably not resolved for a window setting of 2,300 ± 100 eV. Nominal detector resolution is in reference to iron Kα, i.e., 6,398 eV; it probably improves to about 140 eV for energies centered around the sulfur peak, and thus the window is more accurately 2,300 ± 170 eV, or wide enough to span the 158 eV separating the Kα and Kβ peaks of sulfur.
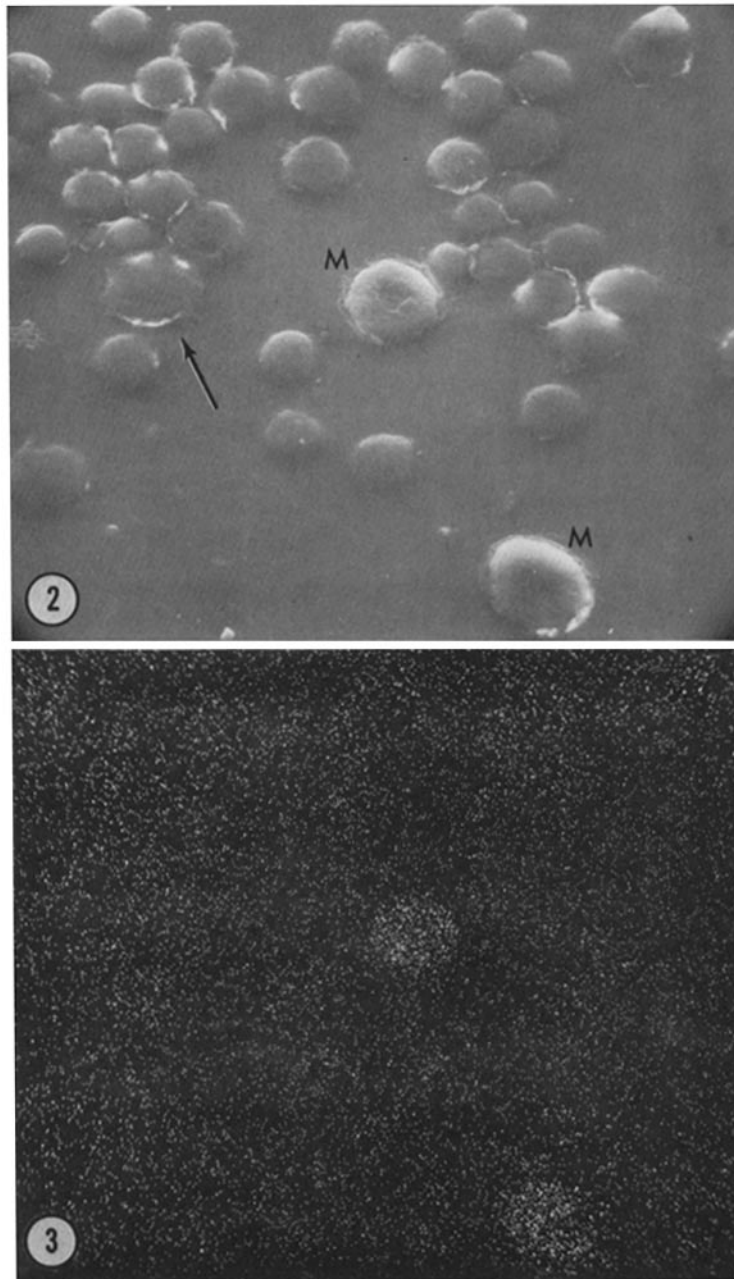
FIGURE 2 Rat peritoneal fluid cells imaged by scanning electron microscopy in secondary electron mode. Of the 43 cells displayed, two (M) stand out by virtue of their large size and faint granularity and, by analogy to preparations viewed by light microscopy, must be identified as mast cells. One other large cell (arrow) lacks granularity and is unidentified as to type. The slightly oval shape of the cells, all foreshortened in the same direction, results from tilting of the preparation required to enhance detector efficiency and to improve the apparent relief of these extremely collapsed cells (air-dried, unfixed preparation). Note slight central depression exhibited by the mast cells. Note also that the glass areas between cells are almost free of cell debris or other extraneous matter. (Stereoscan. × 1,000. Tilt angle = 33°.)
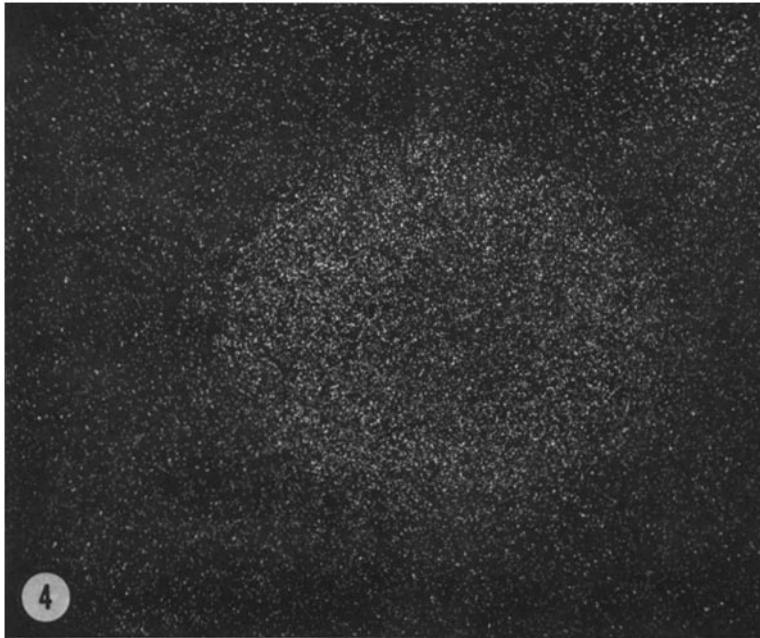
FIGURE 3   X-ray distribution map for sulfur over same area shown in Fig. 2. The two mast cells stand out with particular clarity. The unidentified large cell does not. (Stereoscan. × 1,000. Tilt angle = 33°.)

FIGURE 4   Higher magnification X-ray mapping of the mast cell at the center of Fig. 2. The central nuclear area can be discerned. × 5,000.

studies because this procedure precludes dissolution of cellular components into fixatives, wash fluids, or other reagents, and because a collapsed cell causes less scattering of beam electrons, thus improving resolution of X-ray mapping. Cell size is a possible criterion for identification purposes (17) but is not applicable to all mast cells: those of young animals are no larger than the nonmast cells in the heterogeneous cell populations of peritoneal or pleural fluids; small mast cells are regularly present among the majority of large ones found in older animals; and the relative size relationships can be obscured when the macrophages become hypertrophied, "activated," or "angry" (reference 8 and accompanying discussion). Last, mast cells can be stained differentially as a function of granule maturation by a procedure involving alcian blue and safranin (20), presumably as a function of progressive sulfation of granular polysaccharide components (2). Heparin is an acid mucopolysaccharide, the polysaccharide fragment consisting of sulfated glucosamine and uronic acids (a mixture of glucuric and L-iduronic acids) subunits. When fully sulfated, the polymer contains about 2.5 sulfates per repeating disaccharide

subunit in the form of both sulfate esters (O-sulfate) and sulfamate (N-sulfate) groups (18). Carboxyl groups are also present in the uronic acids of the heparin and in the protein with which the heparin is complexed (1, 7, 21). Thus staining properties probably derive from the numbers and the binding strength of these various negatively charged groups with the positively charged chromophore ions of the dyes. But some of these sites may also be unavailable through binding to protein components of the granule, or by virtue of steric hindrance referable to conformation of the proteinpolysaccharide molecule that may itself vary with granule maturation. These considerations have yet to be explored. X-ray analysis would reflect absolute sulfur content irrespective of molecular conformation, complexing, or steric considerations, and may therefore contribute to a better understanding of the tinctorial changes involved in mast cell maturation, whatever this entails or signifies.

X-ray analysis of single cells is clearly informative. Of the several elements revealed by the stationary probe approach, three are equally prominent in control and in cellular spectra alike,

namely sodium, silicon, and calcium. For this reason, these can be ascribed to the microscope slide support rather than to the cells, oxides of these elements being major constituents of glass.[2] This indicates that the probe penetrated the entire cell as well as some of the underlying substrate. The presence of peaks for potassium, sulfur, and chlorine in conjunction with the various cell types lends confidence to the data while attesting to the sensitivity of the method. That the peaks for potassium and chlorine seem more prominent for the mast cells may not be significant, because they rise to less than twice background, a rule of thumb criterion to distinguish signal from noise in X-ray spectra. A more accurate interpretation of the data requires computer-assisted technology that was not available. Most of the cytoplasmic areas of mast cells should represent granular substance, however, and it is not clear why chlorine (if that peak is real) might be associated with it. It may be artifactitious, perhaps a result of extracellular fluid's being drawn to the cell during the drying process. Theoretically, mast cells, being larger, may collapse into a thicker heap than nonmast elements, and thus might present more bulk per unit of beam area. If this is so, all the peaks should be equally emphasized, but this is not the case and the extreme flattening of mast cells seen in the secondary electron image also suggests this is unlikely to account for the difference in peak heights for mast cells as compared to nonmast cells. Nevertheless, this conclusion remains subject to confirmation by further experiments.

Visual inspection of the spectra in Fig. 1 suggests that sulfur, chlorine, and potassium peaks are approximately similar for various nonmast cells; however, they fail to satisfy the criterion that, in the absence of computer processing, a signal requires a peak height of about twice background for positive identification. On the other hand, this criterion is met by the sulfur peak of mast cells, and is approached for the chlorine and potassium peaks of these same cells. The latter (K) appears to have the same height for all the cells sampled. Thus by visual inspection the S/Cl and S/K ratios appear greater for mast cells than for nonmast

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[2] Composition data, obtained from industry sources for the specific microscope slides used, are as follows: Al_2O_3, 1.5%; CaO, 7%; MgO, 4%; SiO_2, 72%; Na_2O, 15%; B, Fe, and K, traces.

cells. These various statements must be taken as very tentative, however. The spectra of Fig. 1 were obtained from unfixed, collapsed cells. The thickness of these collapsed cells is more closely related to cellular diameters than to cell volume and, because the electron probe sampled a very small area of the collapsed cell, the data are referable more to the probe-excited volume (including the glass slide below) than to cellular mass. It might be tempting to attach significance to absolute peak heights and to the ratio of one peak to another. This would be unwarranted, however, because quantification of electron probe X-ray data is a very complex matter (3). Among other factors contributing to uncertainty in quantification are the following: (a) Electrons lose energy more rapidly in a low atomic number (z) matrix since light elements are more rapidly ionized. This would produce a lower intensity for the same concentration of an element in a light matrix than in a heavier one; the high content of sulfur ($z = 16$) and zinc ($z = 30$) in mast cells as compared to protoplasm in general (hydrogen, $z = 1$; carbon, $z = 6$; nitrogen, $z = 7$; oxygen, $z = 8$) suggests their "matrix" may differ from that of the other cell types in peritoneal fluid. (b) X rays are absorbed in the specimen matrix en route to the X-ray detector and different elements have different mass absorption coefficients, each of which varies with the energy of the X-ray. (c) The radiation from one element is capable of exciting atoms of another element, and thus can give rise to secondary fluorescence which will increase the intensity measured from that element. And this does not take account of bremsstrahlung effects which are not constant across the energy spectrum. Complex mathematical computations and approximations are necessary for even a semiquantitative approach to spectral data. The required computer instrumentation was not available for the present study.

That mast cells can indeed be singled out by X-ray analysis is clearly established in this study, although it remains to be shown that all of them can be so demonstrated equally under various situations. The amount of sulfur actually detected was remarkably small. The heparin content of rat mast cells has been reported as $39 \pm 8 \ \mu g / 10^6$ cells (4). As a first approximation the selected mast cells collapsed onto the slide covered a circular area of about 20 μm in diameter. Bovine heparin

consists of about 10% sulfur (4, 6), and rat heparin is apparently of similar composition (5, 18).[3] The electron beam used for the stationary probe approach was about 30 nm in diameter. On the basis of these data it can be calculated that the amount of sulfur "sampled" by the beam cross section was about 8×10^{-18} g. Yet it should be pointed out that neither the stationary probe nor the scanning mode approach was pushed to its limit of detectability. For the stationary probe approach only 30 s of collection time was used and no attempt was made to isolate the signal from background noise by computer technology. For the scanning probe approach, a range of concentrations was detected, the area of the nucleus (from which granules are excluded) being distinctly set off not only from the rest of the cytoplasm but also somewhat from the bremsstrahlung "noise" elicited from the glass slide support. And that bremsstrahlung background noise could presumably be reduced substantially by judicious selection of a more suitable support.

Both the instrumentation and its use in biology are relatively new. This study points to the potential that their approach may offer. It suggests that much could be done if full advantage were taken of available technology, especially if preparative methods were so designed as to optimize instrumental capability. There is a tendency among biologists to downgrade the scanning electron microscope, perhaps because it does not afford the resolution of the transmission electron microscope. It is hoped that this communication will help in redressing this misguided concept by emphasizing how these two instruments rather complement each other, and that the scanning electron microscope and associated technologies have much to offer indeed. It is probable that cell types other than mast cells may similarly be distinguished by virtue of their rich endowment of a distinctive element or, conversely, that quantitative elemental analysis on single cells may prove feasible and may aid in the interpretation of cytochemical or tinctorial properties of mast cells or other cell types.

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[3] The data quoted were determined experimentally. Theoretically, the sulfur content of purified heparin calculated for 2.5 SO_4 groups per heparin disaccharide is 12.9%. Because a portion of the heparin in vivo is likely to be less than fully sulfated, it seems more appropriate and conservative to calculate detection sensitivity on the basis of actual analyses. Theoretical values would be 1.29 times better, i.e., about 5.7×10^{-18} g. With the JEOL instrument, used for stationary probe measurements on which these calculations are based, the beam struck the object normal to the support plane (tilting angle = 0° for X-ray analysis) and therefore no angular correction is needed.

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