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# The Interior of an "Unaltered" Intracellular Structure–the Zymogen Granule–Viewed with High Resolution X-Ray Microscopy

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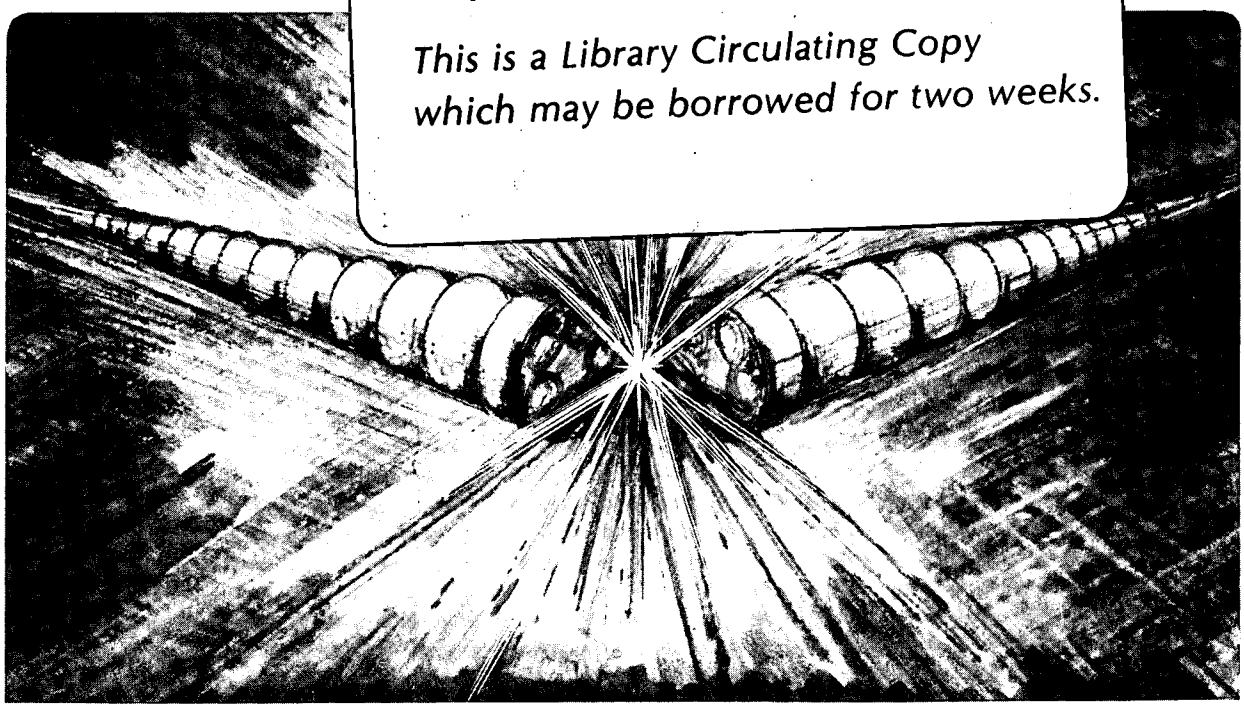

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# THE INTERIOR OF AN "UNALTERED" INTRACELLULAR STRUCTURE

# -THE ZYMOGEN GRANULE-

# VIEWED WITH HIGH RESOLUTION X-RAY MICROSCOPY


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**Abstract.** The interior of a 1 micrometer diameter intracellular structure, the zymogen granule, isolated from the acinar cell of the rat pancreas, whole and otherwise unaltered in preparation for microscopic viewing, was observed with a scanning transmission soft x-ray microscope. Contrast in this microscope is based primarily on differences in element and wavelength dependent x-ray absorption. For the zymogen granule examined at 3.2 nm wavelength, absorption is dominated by carbon content. The data suggest that proteinaceous material is not uniformly arranged, that there is preference for placement away from the granule center, with further non-uniformity evidenced by patchy areas of absorption. These characteristics are different from conclusions based on conventional electron microscopy, which suggest uniform concentration, particularly on the larger scale. Estimates of overall granule protein concentration, based on quantitative measures of x-ray absorption, are comparable to direct chemical measurements.

We have been able to observe the interior of the approximately 1 μm diameter pancreatic zymogen granule that contains some 20 or so digestive enzymes, proteins that bear primary responsibility for the digestion of food. The object, isolated from the cell, was whole, suspended in and containing water, unaltered by chemicals normally added to fix contents in place and provide imaging contrast.

The ordinary light microscope can resolve objects of a feature size roughly equivalent to the wavelength of visible light, in common use about 0.5-1.0 micrometers in diameter. This limitation made the existence, no less the character, of much of the internal structure of biological cells (below the resolving power of the light microscope) one of substantial

uncertainty and ambiguity before the development of the electron microscope. High resolution imaging with the electron microscope uncovered a diverse and complex subcellular structure, upon which theories for a variety of cell functions have been built. However, because cells and their constituents have to be altered severely for viewing in the electron microscope as used in common practice, requiring fixation, staining, dehydration and sectioning, questions of the fidelity of the viewed object to the natural one often remain (l). Beyond questions of structural fidelity, examination of dynamics in an individual cell or component is beyond the capability of electron microscopy.

Soft x-ray microscopy offers a promising avenue to overcome many of these limitations, as well as simply providing a complimentary method for viewing structure at sub-l00 nm resolution, that while lacking the high spatial resolution of electron microscopy permits imaging in the natural state. Using focused x-rays, it is possible to image biological material without preparative procedures, such as those generally required for electron microscopy. For the present observations <u>all</u> such procedures--fixation, staining, dehydration, and sectioning--were avoided.

Spatially resolved variations in the absorption of x-rays by the object provides contrast naturally, through elemental differences as a function of wavelength, and even sensitivity to chemical environment. At wavelengths between the K absorption edges of carbon and oxygen, the so-called "water window," the difference in absorption between organic molecules and that of water provides contrast based on variations in their relative concentration along the direction of

observation. For the present observations, x-ray absorption is primarily due to the presence of carbon atoms, and thus protein, within the granule.

Zymogen granules, and structures of a similar size scale, are well suited to today's advances in soft x-ray microscopy. Soft x-ray penetration is well matched to object thickness (microns) and image formation is possible on a reasonable time scale with high brightness soft x-ray sources, such as the soft x-ray undulator at Brookhaven National Laboratory's National Synchrotron Light Source (NSLS). Furthermore, resolution obtainable with the finest (Fresnel zone plate) x-ray lenses permits observation of internal features ($\leq$ 0.1 micron) considerably smaller than the size of the granule, and smaller than that resolvable with optical microscopes.

The scanning x-ray microscope. Soft x-rays of 3.2 nm wavelength were focused on the object by a 60 $\mu$m diameter Fresnel zone plate lens. The zone plate had an outer zone width ($\Delta r$) of 70 nm (figure 1), with a measured resolution of approximately 90 nm. Transmitted x-rays were collected by a proportional counter as the sample was scanned mechanically through the focal region of the small x-ray probe. Dwell time per pixel was approximately 20 msec. Scanning the whole 80x80 pixel field of view, that included approximately 15 x 15 pixel granules, took a few minutes. Details of the zone plate lens (2) and the microscope (3) are presented elsewhere.

Zymogen granules. The granules were extracted from the pancreas of male Sprague-Dawley rats (4) according to a modification (5) of the method of Ermak and Rothman (6) and suspended in 0.3 M sucrose. A small volume of the granule suspension was placed in a wet cell chamber

constructed from two rectangular silicon wafers (roughly the size and shape of a microscope slide) each containing an x-ray transmitting 200 x 200 μm silicon nitride window approximately 80 nm thick (7).

The appearance of granule contents in the x-ray microscope. The observations reported here are our first results, and for limited samples, and thus, the interpretations that follow must be viewed as preliminary. Furthermore, the image of the interior of the zymogen granule is a two-dimensional projection of the whole object, not a thin section through it, and thus the appearance of connections between regions, may simply reflect overlapping, but unconnected, material at different depths.

Two false color images are shown of the same object (figures 3a and 3b). The images were formed in sequence about 20 minutes apart. The general form of the granule, the non-uniform nature, and the feature in the lower right hand corner are clearly present in the two images. Smaller features, near the limit of spatial resolution, while showing some similarities, are not identical in shape or location. For each image, the object was exposed to approximately I Mrad radiation dose.

In electron microscopic images, zymogen granules generally appear as uniform circular, electron-opaque disks (figure 2). This appearance is observed whether the object is stained or unstained (usually glutaraldehyde fixed, and when stained, osmium tetroxide or uranyl acetate stained thin sections). If material were distributed evenly throughout a sphere, as electron micrographs suggest for the zymogen granule, we would see increasing absorption from edge to

center in a two-dimensional projection of the whole object (figure 4). X-ray images do not have such a uniform appearance (figures 3a and b), and the data more closely fit an object in which the absorbing material is preferentially located away from the center, as in a relatively thick shell (figure 4).

Assuming a spherical shape, equal absorption observed at the periphery and in the center of an object, as seen in figures 3a and 3b, implies that the center is considerably more transparent. Thus, the central area of the granule appears to be relatively carbon poor (thus protein poor; see below), and hence water rich, as compared to more peripheral loci. Moreover, relatively strongly absorbing patchy regions are seen away from the center (figures 3a and b), indicating localized variations and further preference for the placement of proteinaceous material away from the center.

<u>Quantitative estimates.</u> The zymogen granule's contents are known to be almost entirely protein, associated carbohydrates and water (8). The proteins are thought to be in great part the secreted digestive enzymes themselves (9). The 3.2 nm x-rays used as the probe are within the soft x-ray "water window," where water is relatively transparent to x-rays, and on the non-absorptive side of the nitrogen K edge. Therefore, absorption within the granule is primarily due to the presence of carbon atoms. If the carbon content of the granule is in major part in its proteins, and carbon is the main absorber at 3.2 nm wavelength, then we can estimate the spatial distribution of protein from the absorption.

Such an estimate, calculated from measured x-ray absorbance and an average mass absorption

coefficient for some of the major proteins contained within the granule (viz., $1.3 \times 10^4$ $cm^2/g$ for trypsinogen, chymotrypsinogen, ribonuclease, and carboxypeptidase), permits an accurate measure of protein mass on a pixel by pixel (picture element) basis within the image. The mass associated with a typical pixel is less than one femtogram, a very sensitive measure of protein mass. The total protein mass of the granule was 240 femtograms, and was unchanged between exposures (figures 3a and 3b).

To compare these x-ray measures of protein content with published chemical values (10), it is necessary to express them in units of density. To do so requires a volumetric model with inherent uncertainties with respect to both shape and dimension, and in particular a strong sensitivity, due to the cubic dependence, on linear measures. Nonetheless, assuming a spherical shape which matches our observed two-dimensional projection, we obtain a protein concentration of approximately 260 mg/ml for the granule in both figures 3a and 3b. This is comparable to published values obtained by direct chemical measurement, for example, 269 mg/ml $\pm$ 35 SE for rabbit granules, and earlier estimates of 240-320 mg/ml for rat granules (10). The average protein concentration in the cell from which the granules were extracted (the acinar cell), exclusive of the granules themselves, is about 150 mg/ml (10).

<u>Conclusions.</u> These preliminary studies with soft x-ray microscopy of zymogen granules, suggest that granule content is not uniform. There is a prefered placement of protein away from the center, and substantial local variations in protein concentration within the object. This is consistent with other evidence that the protein contents of the granule are in great part not in aqueous solution or suspension, and that there is internal structure (11). More generally, we

report here a demonstration of the ability of focused soft x-rays to visualize at high spatial resolution the interior of a small, whole biological object without fixation, staining, dehydration or sectioning, and allowing estimation of its protein content. We view the present results as a significant step towards our goals of natural imaging and chemical mapping with soft x-rays (13).

**References:**


1. The character and stringency of these alterations is in great part due to the need to provide contrast between features (stain), the constraint imposed by the short free mean path of electrons (sectioning), the requirements for image formation in vacuo, as well as the need to attempt to "fix" the cellular material in place initially to prevent mass loss and distortion during preparation.
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4. After an overnight fast (water ad lib), the pancreas was removed, fat and connective tissue dissected away, the tissue minced finely, homogenized in 0.3 M sucrose, 10:1 (v/w), pH 5.5, at 4 C, and zymogen granules collected by differential centrifugation.
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7. N. Iskander, Senior Honors Thesis, Department of Physics, University of California, Berkeley, 1987. The windows were located towards one end of the silicon wafer. A small drop of the granule suspension was added to one wafer away from the window and spread towards the window with the other wafer. The windows (etched side out) were first aligned visually and then the chamber placed in a mounting device under the light microscope to complete

alignment and expel excess water by the application of a graded pressure. When only a single layer of granules could be discerned, the edges were sealed with nail polish to prevent evaporation. A region was selected for study and a large granule-containing area identified. From this population, individual granules were chosen for examination in detail.

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12. The image is formed from the numerical data correcting for incident flux, applying a line-by-line pedestal normalization, a 2x2 pixel matrix averaging (which reduces feature resolution to no better than 120 nm), a 5 step linear interpolation across each pixel, and finally a quantitiative color map for feature recognition, as indicated.

13. We acknowledge the valuable assistance of P. Batson, D. Ciarlo, K. Conkling, D. Joel, A. Renema, D. Shu, D. Sweeney, and A. Thompson. We also acknowledge support of the Air Force

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Figure Legends:

**Fig. I.** Fresnel zone plate lens. Scanning electron micrograph of a 62-μm diameter zone plate showing concentric rings of opaque gold on a transparent silicon nitride substrate. The resolving power of the zone plate lens is roughly equal to the width of the outermost transparent zone (70 nm for this zone plate). [Reprinted, with permission, from Y. Vladimirsky et al (2)].

**Fig. 2.** Electron micrograph of a zymogen granule pellet isolated (as described in references 5 and 6) from rat pancreas. The material was fixed in glutaraldehyde, stained with $OsO_4$ and uranyl acetate, dehydrated in ethanol and propylene oxide, embedded in plastic, and then sectioned thinly. After this treatment, the granules appear as circular profiles with relatively dense homogeneous content (see text). [Reprinted, with permission, from Ermak and Rothman (6)].

**Fig. 3.** A l-μm diameter pancreatic secretion of zymogen granule, imaged with a zone plate microscope and undulator radiation at 3.2 nm. Red represents high carbon content, blue low content, and yellow intermediate values. The granule in Figure 3a is the same as the one in Figure 3b, imaged 20 minutes earlier (12).

**Fig. 4.** Simple models are illustrated for a uniformly absorbing sphere (top) and a not so thin absorbing shell (bottom), with the transmitted x-ray intensity of each to the right. The data described herein for the zymogen granule does not fit the absorption profile of the uniform

sphere. Being somewhat flat in radial absortion profile, it is closer to that of a model which has relatively weak absorption - and thus carbon content - near the granule center. In addition, the data indicate marked digression from radial symmetry, suggesting localized regions of high absorption.

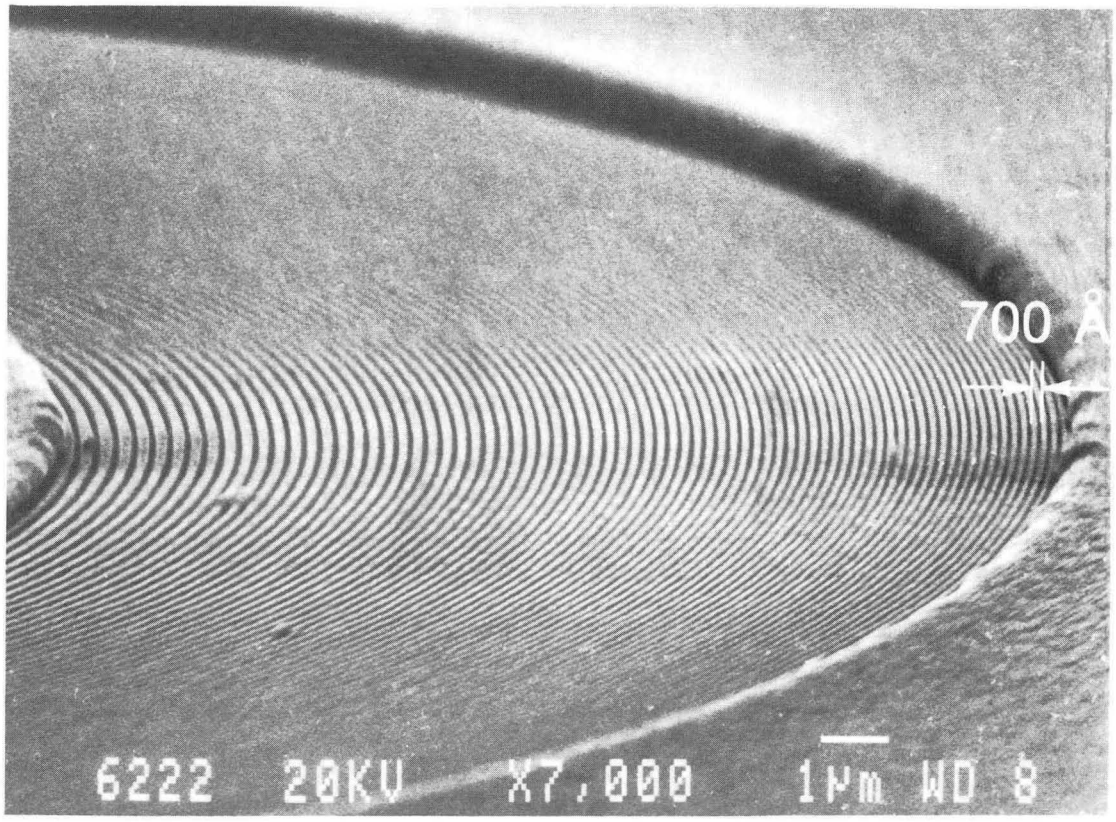


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Figure 1

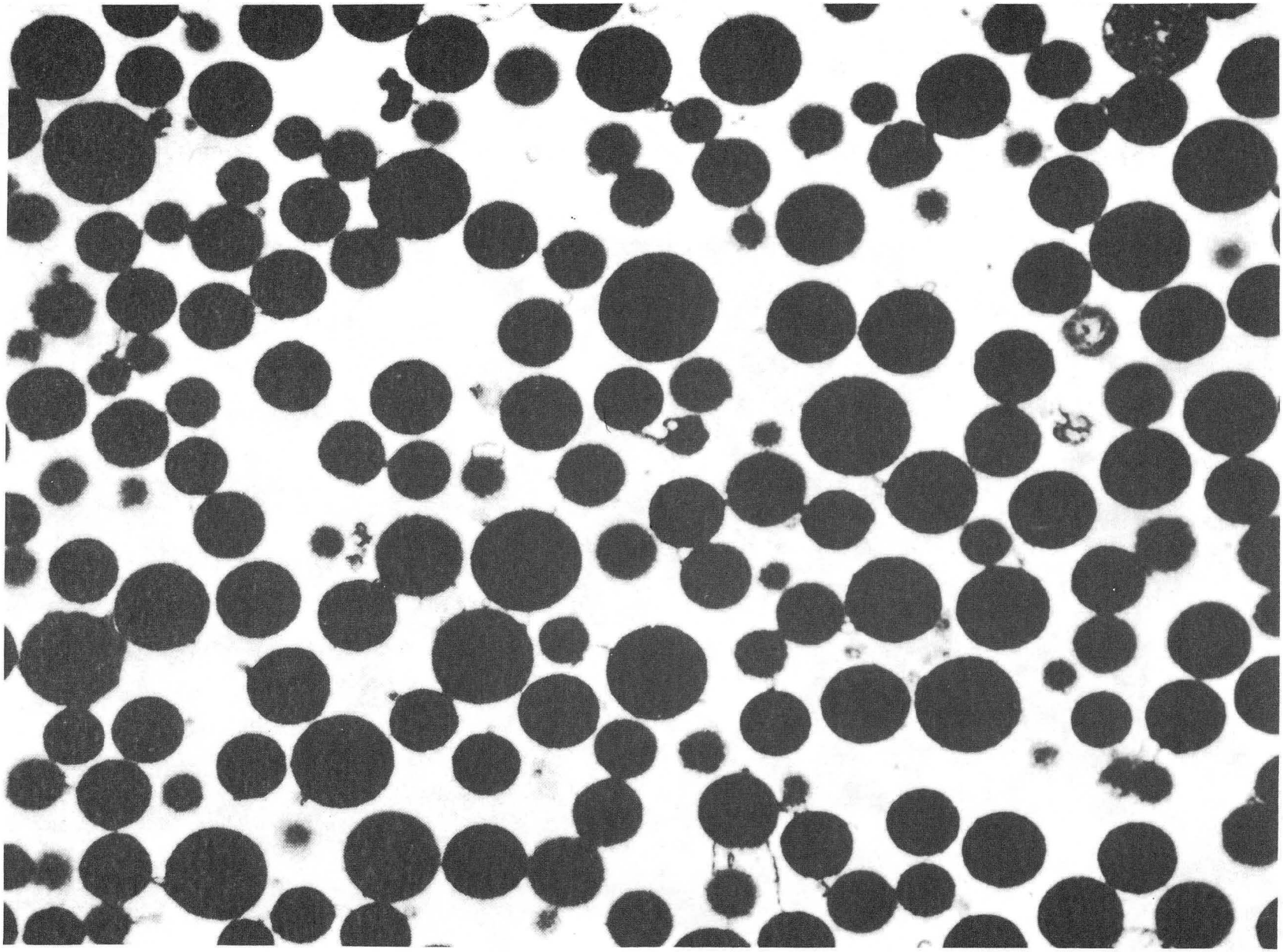

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Figure 2

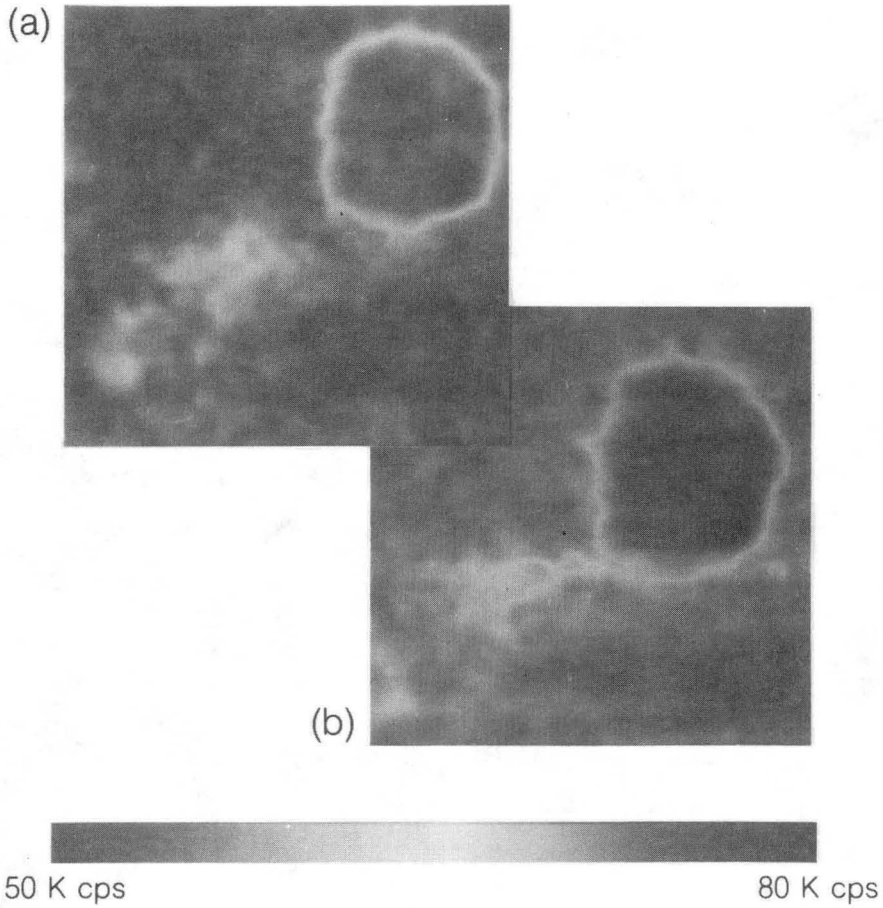


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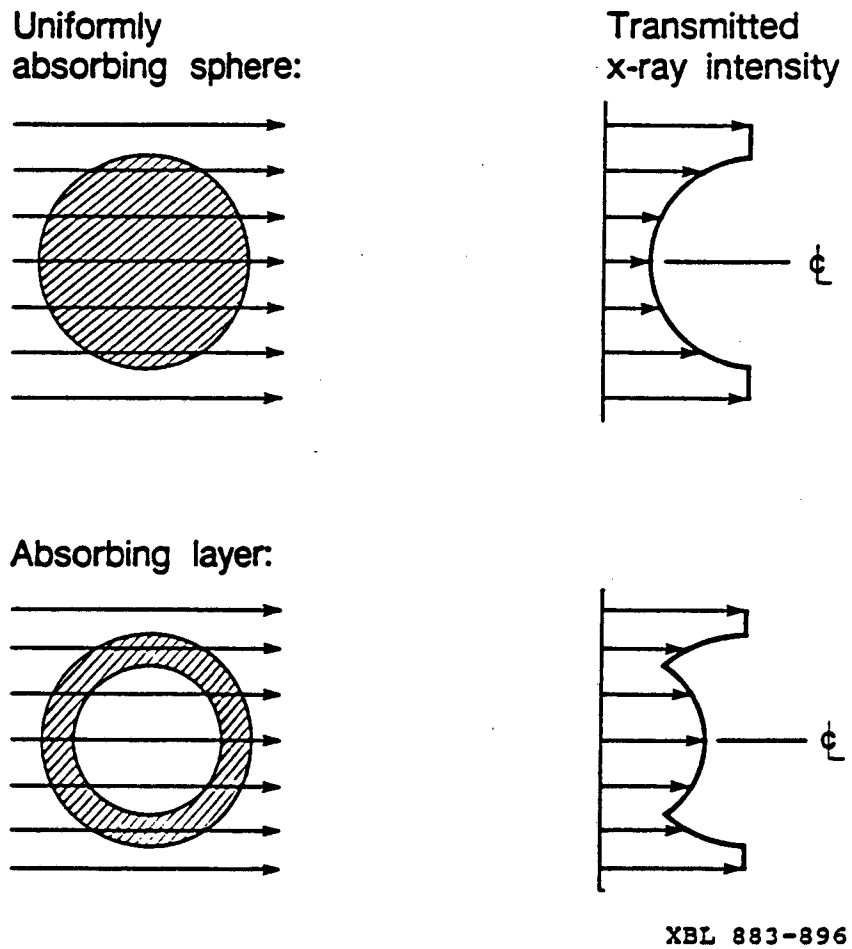
Figure 3


Uniformly
absorbing sphere:
Transmitted
x-ray intensity
℄
Absorbing layer:
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XBL 883-896

Figure 4

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