# Microanalysis of Individual Mitochondrial Granules with Diameters Less Than 1000 Angstroms

Abstract. *A scanning electron microscope was converted to an electron microprobe with high spatial resolution by the addition of a transmitted electron detector and a solid-state x-ray detector. Spectra obtained from mitochondrial granules of chondrocytes in situ confirm the suspected presence of calcium and phosphorus. Contamination during analysis can lead to false indications of silicon in living tissue.*

It has been hypothesized that granules found in the mitochondria of cells from calcifying tissues play a role in the transport and deposition of Ca and P (*1*). The evidence for this hypothesis is quite circumstantial. Microincineration studies have shown that the granules seen in electron microscope preparations have a high ash content, but the Os used to fix the tissues could have contributed to the ash content of the granules (*2*). Isolated liver mitochondria form granules when incubated with $Ca^{2+}$, $HPO_4^{2-}$, and adenosine triphosphate. These granules have been shown by chemical analysis to contain Ca and P, and after microincineration they were found to give an electron diffraction pattern similar to that of hydroxyapatite (*3*). The appearance of these granules in electron micrographs (*4*) is similar to that of granules seen in mitochondria of bone cells and chondrocytes (*5*). Although the data presented thus far suggest that the mitochondrial granule is involved in calcification, the presence of Ca and P in the granules of bone cells and chondrocytes has never been demonstrated. Using a scanning electron microscope and a solid-state x-ray detector we have developed microanalysis techniques which make possible the direct analysis of structures having dimensions of the order of a few hundred angstroms. Applying these techniques we have obtained characteristic x-ray spectra from mitochondrial granules of hypertrophic chondrocytes from the costal epiphyseal plates of mice and have thus shown by direct analysis the presence of Ca and P in the granules.

The rationale behind the development of the special microprobe has been discussed elsewhere (*6*). Briefly, the key to obtaining a high degree of spatial resolution is the analysis of thin sections (approximately 2000 Å) of the tissue. This technique prevents the spreading of the beam in the specimen, which in bulk specimens is responsible for the poor spatial resolution of microprobe analysis. A special stage was built for the scanning electron microscope (Cambridge Stereoscan) which held the thin tissue sections supported on 75-mesh Ni grids. By operating the microscope in the scanning transmission mode, images similar to those produced by the conventional electron microscope were obtained. The transmitted electron detector was an acrylic light pipe bent so that the plastic scintillator (Pilot B sheet) bonded to one end was perpendicular to the path of the electron beam and located directly below the specimen. The magnification range was 20 to 100,000 with a resolution of 200 Å. Both bright-field and dark-field images could be obtained. Probe placement was accomplished by electrostatically positioning a static beam while observing an image of the specimen at 20,000 to 100,000 magnifications. Since the size of the source of characteristic x-rays is very small, the x-ray signal is weak and therefore very efficient x-ray detection is required. Nondispersive spectrometers are more efficient than crystal spectrometers because the solid angle of photon interception is larger and because they measure intensities throughout the whole spectrum simultaneously. We used a lithium-drifted Si detector 10 mm in diameter (Ortec) with an energy resolution of 215 ev at a photon energy of 5.9 kev mounted on the side of the specimen chamber. The x-ray source was 50 mm from the detector, and the x-ray take-off angle was 39°. Spectra were stored in a multichannel analyzer (1024 channels) calibrated to 29.7 ev per channel. One-half of the memory was used to accumulate a spectrum. A reference spectrum was stored in the remaining half of the memory; thus rapid comparison of spectra was possible.

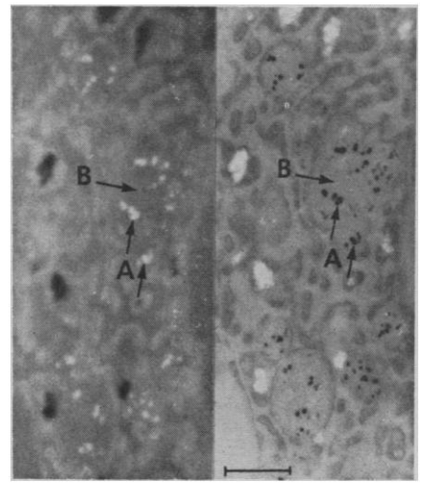


Fig. 1. Scanning transmission micrograph (dark field, left) of a chondrocyte from the costal epiphyseal plate of a normal mouse compared with a conventional electron micrograph (right) of the same area. Arrows labeled *A* point to mitochondrial granules, those labeled *B* to the mitochondrial matrix. The marker is 1 μm. The magnifications are approximately equal vertically, but there is foreshortening horizontally in the scanning micrograph because of a 30° tilt of the specimen. The tissue was fixed in a glutaraldehyde-$RuO_4$ mixture in cacodylate buffer; no staining was used.

With this system we could visualize the tissue sections, select a structure of interest, and excite it to emit x-rays by positioning the static electron beam on the structure. The position of the probe was checked periodically during the analysis by interrupting the measurement, returning to the visual mode of operation and repositioning the beam if necessary. This procedure assured us that the selected structure remained under the probe for the entire counting period, usually 500 seconds.

The most difficult problem to overcome in the development of the microanalysis technique was the buildup of a heavy contamination deposit at the point of impact of the electron beam and the specimen. This deposit presented three obstacles to successful microanalysis: (i) the deposit quickly obscured the structure being analyzed, making probe positioning impossible; (ii) being much more electron dense than the tissue section, the deposit produced the greatest proportion of the x-ray signal; thus, the composition of the granule was also obscured; and (iii) the deposit contained Si and an anomalous Si x-ray peak was often the most prominent peak in the spectrum.

With respect to the composition of the contamination, others have found that C and O are the major (greater than 97 percent) constituents of contamination in microprobes (*7*). Our detector window was too thick (0.025

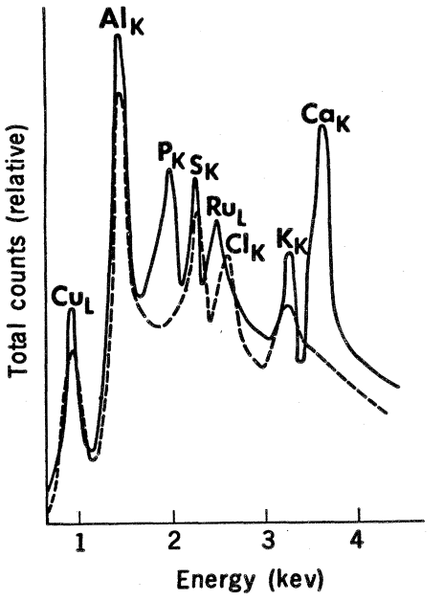


Fig. 2. A tracing through the spectra obtained from a mitochondrial granule (solid line) and from the mitochondrial matrix (dashed line). The system was operating without Si contamination; Ca and P were detected in the granule but not in the matrix. Peaks of Al and Cu are from the instrument, and those of S, Cl, and K may be due to embedding material. The accelerating voltage was 20 kv and the counting time was 500 seconds.

mm) and the electronic noise level (190 ev) too high to permit us to detect these elements. Of the elements that we were able to detect (those with an atomic number greater than that of Na), only Si could be definitely related to contamination. During one analysis in which contamination was prominent, the strength of the Si signal increased with time while that of the Ca signal decreased as a result of the absorption of both x-rays and electrons by the contaminants being deposited.

By cleaning the vacuum system, using vacuum pump fluids with very low vapor pressure, and playing a jet of inert gas on the surfaces of the tissue section, we were able to reduce contamination to an acceptable, often negligible, level. The sources of Si were Si fluids and greases used in the microscope vacuum system and semiconductor devices studied in the scanning microscope. Changing to organic fluids and greases eliminated the major source of Si contamination. Silicon contamination also occurs in scanning electron microscopes in which Si vacuum fluids and greases have never been used (*8*). We noticed that in our instrument an increase in the Si x-ray intensity occurred after the microscope had been used to study integrated circuits. We believed that Si from these devices contaminated the vacuum system. When vacuum system fluids were clean, contamination was reduced to a negligible level by allowing a jet of inert gas, for example, Ar, to impinge upon the surfaces of the specimen during the analysis. However, Si contamination did eventually recur if integrated circuits were studied after the vacuum system was cleaned. We found that, if contamination occurred, the spectra from granules showed the effects of contamination buildup more than spectra from the mitochondrial matrix.

The experiments that we report here were designed to show qualitatively the presence or absence of Ca and P. Since solid-state x-ray spectrometers lack the energy resolution of crystal spectrometers, it is more difficult to avoid interferences between elements when solid-state x-ray spectrometers are used. In an effort to minimize the number of elements and hence the number of x-ray energy levels excited, the tissues were prepared with the use of fixation alone with no staining. Osmium, which is commonly used in fixatives, interferes with the detection of P. Ruthenium does not interfere with the detection of either Ca or P, and $RuO_4$ has been used to fix rat liver and kidney (*9*). A method was developed which satisfactorily preserved the mitochondria and granules in hypertrophic chondrocytes. Tissues were fixed for 3 hours in a 1 : 2 (by volume) mixture of 2.5 percent glutaraldehyde in 0.1*M* cacodylate buffer and 1 percent $RuO_4$ in 0.1*M* cacodylate buffer, respectively; the *p*H was 7.4 and the temperature 0°C. The tissue was dehydrated in alcohol solutions ranging from 70 to 100 percent by volume and embedded in Epon. Figure 1 is a composite showing the appearance of mitochondria fixed in $RuO_4$ in the conventional bright-field and scanning transmission dark-field electron micrographs. The scanning micrograph was taken with the same accelerating voltage and lens currents as those used for microanalysis.

Analyses of ten granules in several different tissue sections have been performed over the last few months. In all cases Ca and P have been detected in the granule but not in the matrix of the mitochondrion in which it was located. Figure 2 shows spectra taken from a single granule and from the matrix of the mitochondrion in which it

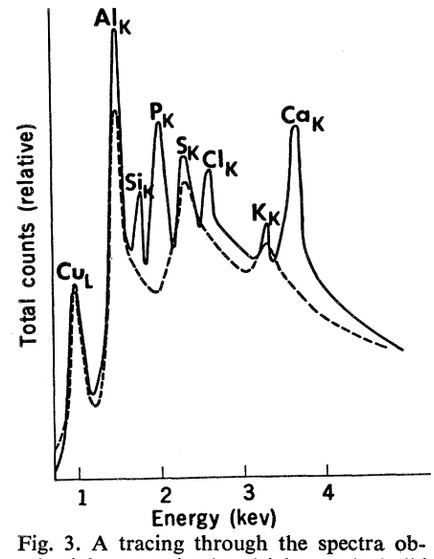


Fig. 3. A tracing through the spectra obtained from a mitochondrial granule (solid line) and the mitochondrial matrix (dashed line) when Si contamination was occurring. Different rates of contamination on different structures may lead to wrong conclusions regarding the Si content. The accelerating voltage was 20 kv and the counting time was 500 seconds.

was located. The localization of Ca and P in the granule is well demonstrated since the two areas being compared were located approximately 2500 Å apart. Both areas are located many micrometers from the calcified matrix, and it is unlikely that the calcifying cartilage is the source of the Ca and P x-rays. Peaks of S, Cl, and K were obtained when the epoxy embedding material alone was analyzed; therefore, one cannot draw conclusions regarding the presence or absence of these elements unless other preparation techniques are used. Contamination was not detected when these spectra were taken. Geometrically, the field of view of our x-ray detector was very wide and radiation from extraneous sources, including characteristic x-ray peaks of Al, Cr, Fe, Ni, Cu, and Zn, was detected. Only the lower energy portion of the spectra is shown for clarity; therefore only the $Al_K$ and $Cu_L$ radiation peaks are included. These peaks tend to mask the presence of peaks from Na and Mg, and nothing can be concluded regarding their presence from these data.

The height of both the $Fe_K$ and $Cu_K$ radiation peaks was about that of the Al peak shown. Since these peaks were the largest in the spectra, these elements accounted for the major fraction of the background radiation on which the Ca and P peaks were superimposed.

A significant improvement in the ratios of peak to background is expected when the field of view of the detector is properly limited. Once extraneous radiation is eliminated, we should be able to obtain semiquantitative results, for example, Ca/P ratios, and detect a larger number of biologically important minerals.

Figure 3 shows spectra obtained from a single granule and from the matrix of a mitochondrion on a day several weeks after those in Fig. 2 were obtained. During that interval the microscope was used extensively to study integrated circuits, and the Si contamination was noticeable. From these spectra, one might suspect the presence of Si in the granule; however, the occurrence of a Si peak is spurious and apparently depends on the nature of the material being analyzed and the history of the instrument. It is possible that contamination of this sort may account for the reported presence of Si in mammalian tissues (*10*). However, multiple wavelength interferences, such as that found between first-order $Si_K$ radiation and fifth-order $Zn_K$ radiation, may also be responsible for the Si reportedly present when crystal spectrometers are used.

These experiments demonstrate that electron probe microanalysis can be used to determine the chemical composition of ultrastructural features of cells. They also demonstrate several pitfalls regarding contamination and sample preparations that must be avoided. The demonstration that Ca and P are present in the granules confirms the opinions of others that these elements are present in granules observed by means of the electron microscope (*1*, *2*). In the absence of data necessary to establish them as features of a living cell and not as artifacts, we feel that a definite role in Ca metabolism cannot be assigned to them at present.

LLOYD V. SUTFIN
MARIJKE E. HOLTROP
*Children's Hospital Medical Center, Boston, Massachusetts 02115*
ROBERT E. OGILVIE
*Massachusetts Institute of Technology, Cambridge 02139*

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11. One of us (L.V.S.) thanks Profs. S. A. Miller and R. S. Harris, present and past directors, respectively, of the Oral Science Training Program at the Massachusetts Institute of Technology for their encouragement and support. This work was supported by NIH training grant DE-105, Office of Naval Research contract NR 105-094, NIH grant AM 06375-09, and the John A. Hartford Foundation. We thank K. D. Altmann for specimen preparation and C. J. MacQuarrie for assistance with the photography.

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## Strontium Induced Rickets: Metabolic Basis

Abstract. *Dietary strontium inhibits both the synthesis of 1,25-dihydroxycholecalciferol and intestinal calcium absorption in vitamin $D_3$–repleted chicks. 1,25-Dihydroxycholecalciferol restores calcium absorption to normal, while 25-hydroxycholecalciferol is without effect in the strontium-fed chick. It is suggested that strontium induces rickets by blocking the biosynthesis of 1,25-dihydroxycholecalciferol, the metabolically active form of vitamin D in the intestine.*

Ingestion of radioactive strontium (for example, $^{90}Sr$) and the biological damage which may result has been of concern for several years. A dietary supplementation of stable strontium is reported to inhibit the intestinal absorption of $^{90}Sr$, thereby serving as a deterrent to ingestion of radioactive strontium (*1*). However, prudence must be used in such treatment since replacement of dietary calcium with strontium results in diminished growth, improper bone mineralization (*2*), and an inhibition of intestinal calcium absorption (*1*). These physiological changes parallel very closely those seen in animals deficient in vitamin D and, therefore, the disease is referred to as "strontium rickets." Treatment with high doses of vitamin D is ineffective in curing strontium rickets (*3*), whereas feeding of a normal calcium diet reverses the lesions (*4*). Strontium is believed to act antagonistically to calcium in the processes of bone mineralization (*4*) and intestinal absorption (*1*).

Cholecalciferol (that is, vitamin $D_3$) is metabolized to hydroxy derivatives prior to its action at the cellular level (*5*). Cholecalciferol is first hydroxylated to 25-hydroxycholecalciferol (25-HCC) in the liver (*6*). The 25-HCC travels via blood to the kidney where it is hydroxylated to 1,25-dihydroxycholecalciferol (1,25-DHCC) and is then sequestered by the intestine, where it effects the assembly of the calcium transport system (*7*, *8*). Not only does 1,25-DHCC act more rapidly than 25-HCC, it stimulates intestinal calcium transport in nephrectomized (*9*) and actinomycin D–treated rats (*10*) whereas 25-HCC does not. This, together with metabolism studies of 1,25-[26,27-$^3$H]-DHCC (*10*), establishes 1,25-DHCC as the metabolically active form of vitamin D in the intestine. Hence, the rachitogenic action of strontium may possibly result from its inhibition of intestinal calcium transport because of a block in the metabolism of vitamin D to its functional hydroxylated derivative. We now show that dietary strontium inhibits 1,25-DHCC production from 25-HCC and that 1,25-DHCC but not 25-HCC can reverse strontium inhibition of intestinal calcium absorption. A concurrent study has also revealed a simi-

Table 1. The effect of dietary strontium on intestinal calcium absorption after cholecalciferol, 25-HCC, or 1,25-DHCC repletion of vitamin D–deficient chicks.

| Treatment | Strontium diet | Daily dose* (pmole) | Calcium absorbed† (micrograms of calcium per 20 minutes) |
|---|---|---|---|
| Cholecalciferol | − | 260 | 47 ± 2‡ |
| Cholecalciferol | + | 260 | 17 ± 2 |
| 25-HCC | − | 130 | 52 ± 4‡ |
| 25-HCC | + | 130 | 14 ± 1 |
| 1,25-DHCC | − | 130 | 61 ± 2‡ |
| 1,25-DHCC | + | 130 | 52 ± 6‡ |
| Ethanol | − | | 14 ± 1 |
| Ethanol | + | | 13 ± 1 |

* Dose was given orally in 0.2 ml of cottonseed oil (Wesson Oil). Chicks were given four doses; experimentation was conducted 24 hours after the last dose. † Values are presented as mean ± standard error of three to six observations. ‡ Significantly different from respective control (ethanol) group ($P < .01$, Student's *t*-test).