X-RAY SCANNING MICROANALYSIS OF ELEMENTAL IRON LOCALIZED IN TESTICULAR NUTRITIVE CELLS OF *CIPANGOPALUDINA MALLEATA* REEVE

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With the advancement of descriptive cytology brought about by electron microscopy, there has arisen a need for cytochemical methods at high resolution. The method of x-ray scanning microanalysis has already found numerous applications in the field of metallography (1–3), and may also be extended to the study of biological specimens. Recently, Yasuzumi *et al.* (4) have succeeded in showing an Fe K_α spectrum in human erythrocytes, and Boyde *et al.* (5) in taking images formed by Ca K_α and Fe K_α radiations in dental tissue. The method is based upon the principle of x-ray emission spectrography. A very finely focused beam of electrons is projected onto the surface of a specimen, at the point where it is desired to examine its chemical composition. The minute volume of the specimen which is thus irradiated emits a complex x-ray spectrum consisting principally of the characteristic radiations of the elements present in the volume. Measurement of the wavelength and intensity of each component of this spectrum thus affords a simple method of determining the chemical identity and concentration of these elements.

The apparatus itself consists of three main parts: an electron optical system, an x-ray spectrograph, and an optical microscope. The specimen is prepared in the usual way as for optical microscopy. The x-radiation, emitted at the focal spot where the electrons strike the specimen, is analyzed with a curved crystal, Geiger-Müller counter, and vacuum spectrograph.

The equipment described here, called hereafter a microanalyzer, enables one to determine all the elements of atomic number greater than 12. In the previous (6) and present studies, organic iron elements have been demonstrated in testicular nutritive cells of the pond snail *Cipangopaludina malleata* Reeve. The present study has been made in order to determine whether this apparatus can also be usefully applied to the elemental iron localized in the cytoplasm.

MATERIALS AND METHODS

PROCEDURE FOR ORGANIC IRON COMPOUNDS: The testis of the pond snail was fixed for 24 hours in 95 per cent alcohol, and then washed in iron-free distilled water for a few minutes. After the tissue had been placed for 2 hours in 5 per cent gelatin solution at 40°C, it was transferred to 5 per cent formalin for 30 minutes at 18°C. Frozen sections were soaked in an organic iron conversion solution for 4 days at 38°C. They were washed in 90 per cent alcohol, followed by iron-free distilled water, and then were placed in Turnbull's blue reagent for 5 minutes, and finally stained with eosin.

PROCEDURE FOR INORGANIC IRON COMPOUNDS: The material mentioned above was stained with Turnbull's blue reagent without treatment with the organic iron conversion solution.

PROCEDURE FOR X-RAY SCANNING MICROANALYSIS: The same material was fixed for 30 minutes in 1 per cent osmium tetroxide buffered to pH 7.3 with the Michaelis veronal-acetate buffer. It was then embedded in a mixture of *n*-butyl and methyl methacrylates according to the established method. Sections 1 μ or more thick were cut and mounted on copper grids covered with formvar. They were stained, without removing the plastic, with the periodic acid–Schiff reagent, because the PAS-reaction makes it possible to detect the location of granules containing elemental iron, which appear in nutritive cells (6). The preparations were covered with a carbon film. The specimen was observed with an Akashi x-ray scanning microanalyzer, model Tronalyzer-TRA type, with an accelerating voltage of 22 kv and a probe current of 10 μA.

RESULTS

An ordinary light micrograph of seminiferous tubules stained with Turnbull's blue reagent for organic iron compounds shows dark blue granules

which are localized at the peripheral portion of the tubules filled with numerous spermatozoa (Fig. 1). It has been already shown that such granules appear in nutritive cells (6). The inorganic iron test was negative in nutritive cells and also in germ cells. The PAS-positive granules have been found in a cell to which numerous spermatozoa are closely attached (Fig. 2).

A fine electron-probe of about 1 μ in diameter is directed at the granule (marked by the arrow in Fig. 2) with the aid of an optical microscope, and the resulting characteristic x-ray emission is collected by a counter of an x-ray spectrometer. The output pulses from the counter modulate the brightness of a beam scanning an oscilloscope screen synchronized with the electron-probe scanning the specimen. By means of a crystal spectrometer it is possible to select one particular wavelength of x-ray and collect in the counter the emission of the defined wavelength only. An image is thus formed in which the pulse density at any point is proportional to the amount of the emitting element present at the corresponding point on the surface of the specimen (Fig. 3). The image formed by the Fe K_α emission demonstrates that the granules contain iron elements larger in amount than those of the surrounding cytoplasmic matrix. Scanning can then be stopped and the electron-probe placed on any desired feature of the specimen. The x-ray spectral analysis is then carried out by driving the spectrometer with a synchronous motor and recording the intensity of the emission on a pen recorder. Fig. 4 clearly shows an Fe K_α spectrum obtained from a granule. In the specimen studied, a quantitative analysis of iron was not made because the amount of the element was too small to analyze.

DISCUSSION

The observations reported here confirm the previous (6) and present results obtained by histochemical techniques and electron microscopy. A microanalysis, such as that illustrated in Fig. 4, can be carried out in a few minutes, while the histochemical technique takes much more time. The spatial resolution of the method of analysis is about 2 to 3 μ^3, and in this volume the sensitivity ranges from one part in 10^4 to one part in 10^3, and the relative accuracy is 1 to 2 per cent. With an electron spot of 1 μ diameter, a beam current of the order of 10^{-6} amp, and an accelerating voltage of 25 kv, an accuracy of 0.01 per cent can be obtained in favorable cases. Thus, certain elements in biological specimens are demonstrable by the microanalyzer with much more precise data than with histochemical methods.

It must be pointed out that the resolving power of the apparatus is limited by the diffusion of electrons in the sample. If very thin specimens are used, the electrons may pass through without scattering and the resolution may thereby be decreased by about 0.5 μ. To be suitable for examination the specimen should be 1 to 2 μ in thickness.

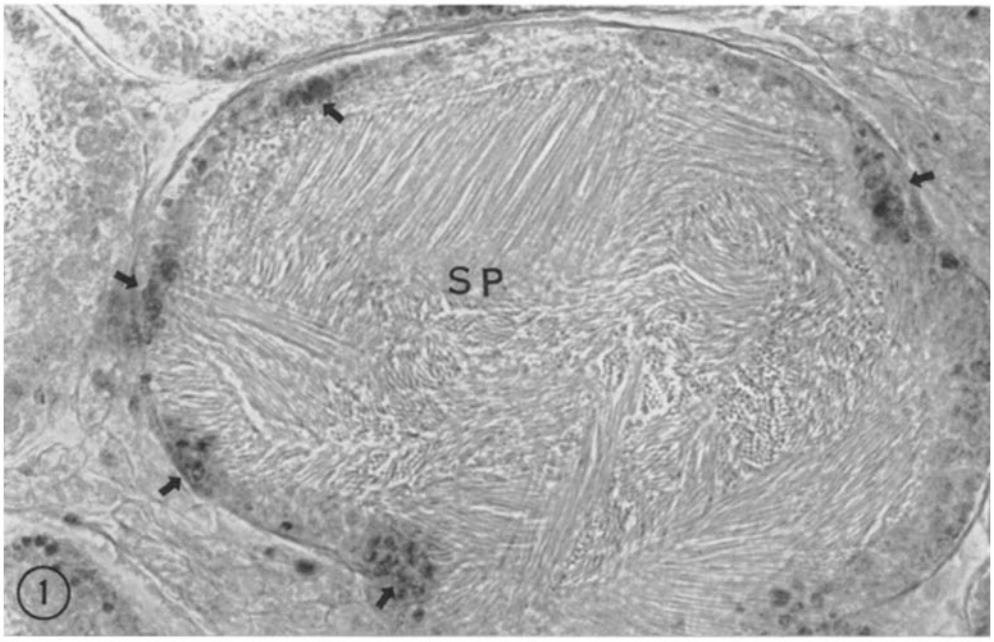
It has already been revealed that the middle piece of the atypical spermatid gives a positive PAS reaction (6, 7). Numerous spermatozoa appearing in Fig. 2, which give a negative PAS-reaction, might be typical spermatozoa. It has also been demonstrated that the PAS-positive granules appear in the nutritive cell, to which typical spermatozoa are closely attached, in a late stage of spermiogenesis of the pond snail (6, 8). On the basis of the results mentioned above, it can be assumed that the cell containing a PAS-positive granule is the nutritive cell (Fig. 2).

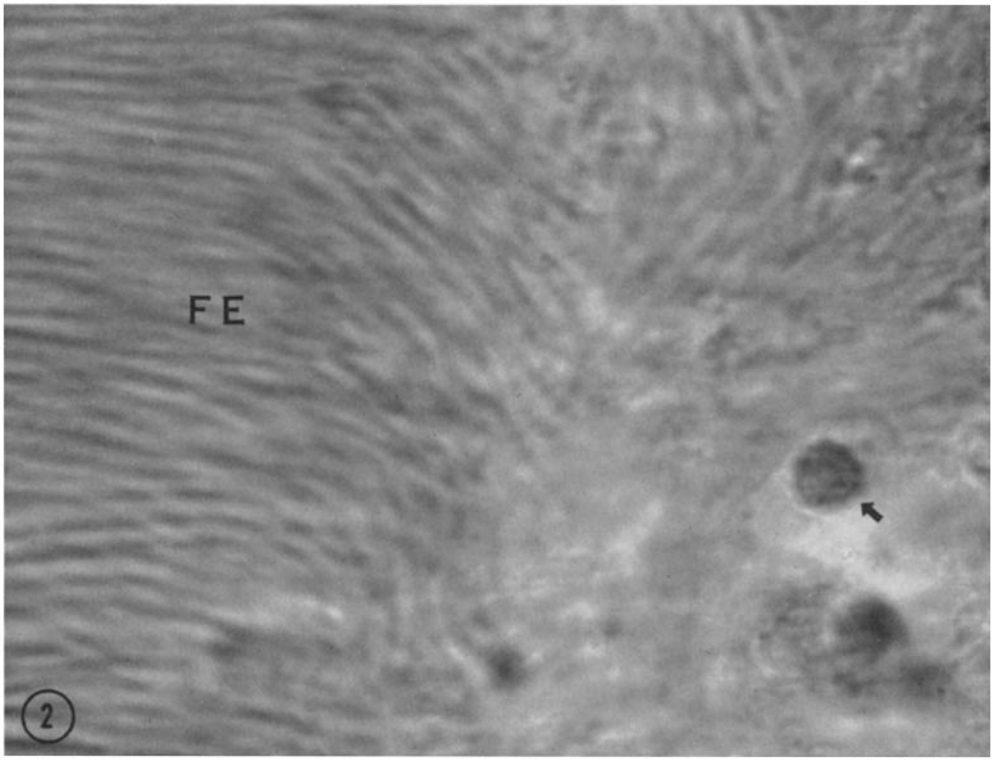
FIGURE 1

A light micrograph of a cross-section of seminiferous tubules of a pond snail. The granules (arrows) in the peripheral layer of the seminiferous tubule (which is filled with numerous spermatozoa (*SP*)) are positively stained for organic iron compound (see text). × 950.

FIGURE 2

A light micrograph of a section of seminiferous tubule of a pond snail, showing a granule (arrow) stained a deep purple red by means of the PAS reaction. Numerous fibrous elements (*FE*) demonstrate spermatozoa. × 1,700.





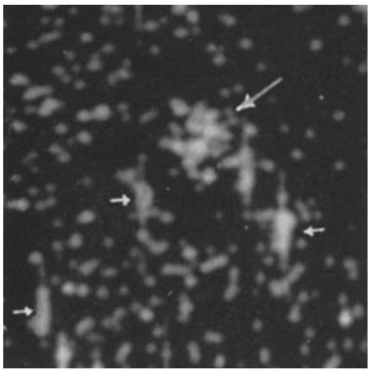


FIGURE 3

Scanning image produced by Fe K_α emission from the granule and its surrounding area as found in Fig. 2. The small arrows show background noise. × 1,700.

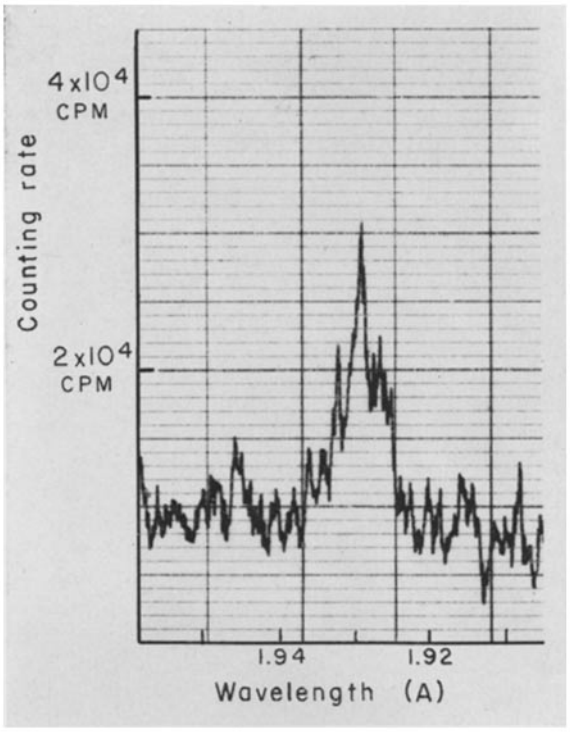


FIGURE 4

Fe K_α spectrum at the point marked by the large arrow in Fig. 3.

CONCLUSION

This short survey of observations made with the x-ray scanning microanalyzer demonstrates the great advantage of its application to biological specimens. With the method, it has been possible to analyze elemental iron localized in the PAS-positive granules appearing in the testicular nutritive cell of the pond snail.

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