X-Ray Methods in Histochemistry

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The aim of histo- and cytochemical studies is to identify and eventually to quantitate substances on a microscopic level. This necessitates isolating extremely small areas for analysis, preferably in the intact biological tissue. Physical methods have proven to be of value in this respect, in particular the radiant energy micro-absorption techniques (Fitzgerald & Engström, 1952).

The field of histochemistry is rapidly developing. In this summary, X-ray methods that have found use in histochemistry and histophysiology will be briefly discussed. Critical reviews (Glick, Engström, & Malmström, 1951; Fitzgerald & Engström, 1952) have recently been published.

### A. FORMATION OF X-RAY IMAGES WITH HIGH RESOLVING POWER

The usual principle that has been adopted to obtain X-ray images of high definition is to place the object in close contact with a fine-grained photographic emulsion and make the exposure with X-rays as parallel as possible. The best emulsions, Eastman Kodak Spectroscopic Plate 548 or 649, Kodak Maximum Resolution Plate, Lippman emulsion (Gevaert), have a resolving power of about 1 μ. It is easy to arrange the geometry of the X-ray beam in such a way that the geometrical unsharpness (penumbra) is far below the resolving power of the emulsion. This is possible by having a relatively small focal spot in the X-ray tube, a thin sample in close contact with the photographic emulsion, and a relatively long distance between the focal spot and the sample-film. The method of microradiography described above can thus give a resolution of about 1 μ and has been used to image a variety of tissues and cells by X-rays, for example, bone tissue (Amprino, 1952; Amprino & Engström, 1952), gastric mucosa cells (Engström & Malmström, 1952), nerve-fibre (Engström & Lüthy, 1950), giant salivary gland chromosomes (Engström & Ruch, 1951), &c.

The method of primary magnification introduced by von Ardenne (1940) has recently been further developed by Cosslett & Nixon (1951). This method depends upon making an enlarged shadow micrograph of the sample and necessitates a very fine focus. Intensity limitations make it doubtful if this method will give a resolving power much better than the simple microradiography, at least when it concerns biological objects which require very soft X-rays for contrast. The

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shadow X-ray microscope has recently been greatly improved by Cosslett and Nixon. The greatest hopes for an increased resolving power in the X-ray image formation are on the real X-ray microscope (Kirkpatrick et al., 1948, 1950). Thus, using grazing incidence on polished, curved surfaces, a real enlarging system has been constructed. The resolving power of the X-ray microscope is expected to fall somewhere between that of the light and electron microscopes (1950).

B. DETERMINATION OF THE DRY WEIGHT OF HISTOLOGICAL AND CYTOLOGICAL STRUCTURES

A microradiogram registered with 8 to 12 Å X-rays can be evaluated in terms of the dry weight of the different structures in a dried smear or section of a soft biological tissue (Engström & Lindström, 1950). The procedure involves the photometric evaluation of the X-ray absorption in the various biological structures, a procedure which can cause difficulties. In most cases a reference system is printed on the fine-grained emulsion simultaneously with the microradiogram and with the same X-radiation. Improved methods have been described for manufacturing such small reference systems (Brattgard, 1952; Brattgard & Hallén, 1952; Brattgard & Hydén, 1952). As very small areas are measured photometrically it must be ascertained that a sufficient number of silver halide grains are within the measurement area in order to reduce the pure statistical error in the photographic process. The method of weighing biological structure will be greatly improved when the X-ray microscope (Kirkpatrick & Baez, 1948; Kirkpatrick, 1950) is available.

The accuracy of the cytochemical X-ray weighing procedure is not high compared to standards in analytical chemistry. There are several factors that contribute the relative low analytical accuracy (Fitzgerald & Engström, 1952; Glick, Engström, & Malmström, 1951). Most of these factors are inherent in the objects themselves. Factors like the inhomogeneous distribution of the absorbing material, artifacts introduced by fixing, sectioning, and drying the biological sample, errors in determining the length of the absorbing path, are all difficult to master (Glick, Engström, & Malmström, 1951). The methodological errors such as defects in the fine-grained photographic emulsions, systematic errors (generally not exceeding 5 per cent.) introduced by the varying composition of the biological material, and errors in photometry must also be carefully considered.

Despite the relative large errors (Lindström, 1953a, 1953b) in the weighing procedure there are several problems in histo- and cytochemistry to which the method of X-ray weighing can be applied.

C. HISTOCHEMICAL ELEMENTARY ANALYSIS BY ABSORPTION

When the amount of an element is determined by X-ray absorption measurements, the characteristic absorption discontinuities are used (Glocker & Frohn-
For histochemical elementary analysis the absorption of monochromatic X-rays is measured on either side of the absorption edge (Engström, 1946). The procedure thus involves the transmission measurements of at least two wavelengths. There is a required minimum surface density of the element to be analysed (Engström, 1946). Therefore it is not possible to make histochemical elementary analysis of thin sections by X-ray absorption analysis.

The X-ray absorption method for histochemical elementary analysis has the advantage that very small samples (the area of an ordinary mammalian cell) can be measured, but in that sample the elements must have a high concentration. For that reason the X-ray absorption histochemical analysis can at present only be applied to elements in relatively high concentrations (for example, carbon, oxygen, nitrogen, sulphur; calcium and phosphorus in bone tissue).

**D. HISTOCHEMICAL ELEMENTARY ANALYSIS BY X-RAY FLUORESCENCE**

Chemical analysis by X-ray fluorescence was early used in metallurgy and geochemical research (Hevesy, 1932; Hevesy & Alexander, 1933). In principle the sample is irradiated by X-rays of a suitable wavelength and the excited fluorescence radiation (secondary X-rays) is analysed spectroscopically. The method is suitable both for qualitative and quantitative analysis of elements with relative high atomic numbers (Hevesy, 1932). Recently there has been rapid technical development of the X-ray fluorescence methods, especially for industrial purposes.

The fact that the biological material consists of elements with low atomic numbers makes this method less applicable to studies in biology. However, it has been used in certain instances to determine, for example, zinc and iron. The structural resolution is poor.

The curved crystal image X-ray spectrograph introduced by von Hamos (1938, 1953) offers a possibility of analysing the fluorescence X-rays emitted from areas as small as 10µ by 10µ. The quantitative aspects of this method have been carefully considered (Hamos, 1953).

**E. STEREOSCOPIC MICRO-ANGIOGRAPHY**

Studying the function of the smallest blood-vessels is of considerable interest for the understanding of the function of organs and tissues. Barclay and his colleagues (1951) used a radiological technique to demonstrate the finest blood-vessels. They called the procedure micro-arteriography. This method has recently been critically examined (Bellman, 1952, 1953; Bellman & Engström, 1952). The radiological background for micro-angiography (Bellman & Engström, 1952) is simple straightforward microradiography, as described earlier in this survey. The great problem, however, is to fill all the fine blood-vessels with X-ray contrast. It can be said that the problem of micro-angiography is that of introducing the contrast (Bellman, 1952, 1953).
The application of stereoscopic microradiography (Berthold, 1941; Engström, 1951) to the problem of micro-angiography has added much to the value of the method (Bellman, 1953). A three-dimensional representation of the capillary net in an organ can now be obtained. Micro-angiography has been performed in living animals (Bellman, 1953), and by this technique it is possible to study the physiology of the finest blood-vessels.

F. MICRO X-RAY DIFFRACTION

The great importance of X-ray diffraction methods in studying biological systems is well illustrated by the results obtained from essentially homogeneous macroscopic samples. Information concerning molecular orientation, particle and molecular sizes, and in certain cases detailed molecular structure has been obtained.

Micro-diffraction (wide angle) has been used to study different problems of cellular organization (bone tissue by Amprino & Engström, 1952; starch grains by Kreger, 1946, &c.). Various types of microcamera have been designed (Jeffery, 1952) including such using a fine glass capillary as collimator. The use of very fine focus X-ray tubes (Ehrenberg & Spear, 1951; Ehrenberg & Franks, 1952) is of great importance in micro-diffraction, especially when studying long spacings (Ehrenberg & Franks, 1952) (low scattering angles). It can be expected that micro X-ray diffraction will find a wide use in histochemistry, especially for studying large spacings and for particle size determination from the low-angle continuous scatter (Guinier, 1952).

REFERENCES