X-ray Fluorescence Analysis in Biology

Both standard and special x-ray methods can help in the difficult study of low-concentration elements.

Theodore Hall

Only a few chemical elements are abundant in living materials. The nucleic acids consist mainly of carbon, nitrogen, oxygen, hydrogen, and phosphorus; sulfur is needed for proteins, and calcium for bone. All the other chemical elements found in living matter have been called "minor constituents." Although the "minor" elements are omitted from the crude diagrams of many important molecules and often appear in tissues only in very low concentrations, we know that they are tremendously important.

Today we have a variety of instruments for measuring the concentrations of the minor elements. These measurements must be made if we are to understand the many roles of the minor elements in life. Many desirable assays are still technically beyond us. This article describes the place of one measuring tool, x-ray fluorescence analysis, in the exploration of the low-concentration elements in biology.

Before discussing the contribution that this method can make, let us briefly review the foundations of the technique.

Primer

The analysis of chemical elements by x-ray fluorescence is analogous to emission spectroscopy. In both cases, elements are excited to emit their characteristic radiations, and the instrument distinguishes between the wavelengths associated with the different elements. In emission spectroscopy (as the term is usually used) the excitation affects the outer electrons of the various atoms and the radiation is in the visible or near-visible region. In x-ray analysis, inner electrons are ionized and the wavelengths are much shorter, approximately 0.2 to 10 angstroms.

The term *fluorescence* refers to the characteristic radiation emitted by a material when it is exposed to radiation of shorter wavelengths. Most x-ray analysis of elements is "fluorescence" analysis, in the strictest sense of the word, because the characteristic x-rays are usually excited by exposing the specimen to other x-rays. Alternatively, one can use an electron beam for excitation, a method which has become popular recently and is considered briefly at the end of this article. In this case the characteristic x-rays are not "fluorescent" in the strict sense of the word, and the method is more properly placed under the general heading of x-ray emission spectroscopy.

Any x-ray fluorescence analysis system includes an x-ray source to excite the specimens and an analytical system to discriminate between the radiations from the different elements in a specimen and to measure the intensity of each element's contribution. The x-ray detector is generally either a gas-filled tube (a Geiger or proportional counter) or a scintillation crystal coupled to a photomultiplier. With any of these detectors, each incident x-ray quantum may be converted to a discrete electrical pulse. The pulses are counted or recorded by scalers or counting rate meters.

In commercial instruments (see Fig. 1) a diffracting crystal is mounted between specimen and detector (as shown in Fig. 2). At any moment, ideally, x-rays from only one element enter the detector. The diffracting crystal generally discriminates quite cleanly against all the other elements in the specimen (*1*, *2*).

X-ray analysis has some major advantages.

1) X-ray spectra correspond to the simple pattern of energy levels of inner atomic orbits; this results in relatively uniform sensitivity over a wide range of atomic numbers.

2) Because the energies of the inner electron orbits are affected only negligibly by chemical binding, element analysis is independent of chemical state even in unprocessed specimens.

3) Preparation of the specimen is usually simple. Often the specimen can be placed directly in the x-ray beam without preparation.

4) X-ray analysis is essentially nondestructive in that constituents of the specimen are not displaced (molecular bonds may be ruptured).

X-ray analysis also has some major disadvantages.

1) Absorption and self-excitation effects in the specimen ("matrix effects") necesssitate elaborate calibration curves for quantitative work, except in the case of thin specimens.

2) In the region of atomic numbers 12 to 22, helium or vacuum specimen chambers are desirable to avoid excessive attenuation of the "soft" characteristic x-rays. For atomic numbers below 12, attenuation makes x-ray analysis impractical for most applications.

In mineralogy and metallurgy, where x-ray fluorescence has its widest use, the sensitivity limits for standard instruments are considered to be one or a few micrograms of an element of interest per specimen, in minimum concentrations of around 10^{-4} (*3*). However, as petroleum analysts have known for some time (*4*), sensitivity is higher with organic specimens because of the much longer mean free paths for background scattering and for absorption in material of low atomic number. Natelson considers the measurement of 0.1 microgram of iodine to be feasible in organic specimens (*5*). As shown in a subsequent section, various modifications of the standard arrangement of Fig. 2 can extend the performance much further in certain biological studies. But first I shall review the practicable biological applications of standard x-ray analysis units.

The author is affiliated with the Sloan-Kettering Institute for Cancer Research, New York, N.Y., in the division of biophysics.

Application of Standard Instruments in Biology

For reasons of speed, simplicity, and reliability, x-ray analysis is a preferred method wherever its sensitivity is high enough for handling specimens after mere drying. Examples of such applications are clinical assays for iron, calcium, potassium, chlorine, sulfur, and phosphorus in blood (*5*, *6*), and for bromine in blood and urine in cases of bromide poisoning (*7*).

Without specimen preparation more elaborate than mere drying, the minor elements generally cannot be measured in tissues (zinc and iron may be exceptions). Hence, "as-is" analyses are limited mainly to the cases above and to special situations, such as measurements of iodine in the thyroid gland, of pathological accumulations, or of the metallic constituent of an extracted metalloenzyme.

With preconcentration techniques the group of feasible analyses is significantly enlarged. Examples are the assay of strontium in bone and in serum after ashing (*8*) and the assay of bromine in a centrifuged liver fraction from a bromine-intoxicated individual (*7*). The attractiveness of x-ray analysis after element concentration depends partly on the merits of competing techniques, represented in Table 1. (The reader must recognize that there are sweeping approximations in such a compressed tabulation.)

General reviews of x-ray analytical applications (*9*) list few items in the field of biology. This is partly because biological analysts are more familiar with other techniques. Most of the methods of Table 1 require considerable preparation of specimens; with similar effort devoted to chemical concentration, x-ray analysis could be applied relatively conveniently to many biological specimens. However, for the purpose of scanning a wide variety of tissues for a wide variety of minor elements [as Tipton (*10*) has done, for example], one must use techniques with which lower element concentrations and smaller amounts can be measured, such as emission spectroscopy and neutron activation.

In sum, a commercial x-ray fluorescence analysis unit is very desirable for routine, mass-production assays of certain minor elements, including some of the most important clinical assays. The instrument cannot be the major resource of a laboratory devoted to general research on the minor elements in biological tissues, but it can be quite useful in such a laboratory as one of a group of analytical tools.

Table 1. Capabilities of some methods for assay of chemical elements.

Minimum amount (μg)	Minimum concentration*		Range of elements†	Independent of chemical state?	Nondestructive?	Reference
	ppm	μg/ml				
			Emission spectroscopy			
10^{-4}–0.01		0.002–0.2	*N*, 41 *Z*, 3–92	Yes	No	(26)
0.01–0.3	3–100		*N*, 12			(27)
			Flame photometry			
		0.002–0.1	Li, Na, K, Ca, Mn, Cu, Rb, Sr	Yes	No	(26)
1–15			Ca, K. Na			(28)
			Atomic absorption spectroscopy			
		0.03–2	*N*, 13 *Z*, 11–79	Yes	No	(29)
			Colorimetry			
		0.001–0.2	*N*, 31 *Z*, 4–82	Yes	No	(26)
0.4–5			B, Fe, Mg, P, S			(28)
			Neutron activation			
10^{-6}–0.1			*N*, 61 *Z*, 11–92	Yes	No	(30)
			Mass spectroscopy			
$\sim 10^{-6}$	$\sim 10^{-6}$		*N*, 68	Yes	No	(31)
			Electron spin resonance			
$\sim 10^{-3}$–10^{-4}			Transition elements	No	Conceivably	(32)
			X-ray fluorescence (commercial)			
0.1–1	1–10		*Z*, 12–92	Yes	Yes	(33)

* Concentration in the specimen fed to the device. The concentration in the original specimen may be much lower if preconcentration is used. †*N* = number of elements; *Z* = atomic numbers. Elements outside the indicated range may be measurable, but with sensitivities poorer than shown.

Histological Localization

It may be far from easy to determine the role of a minor element in a biological issue. For example (this is discussed at greater length in a subsequent section), no one knows whether zinc plays any role in the prostate gland even though the extraordinary concentration of the element there has been investigated by many scientists since its discovery 40 years ago (*11*). Element assay in a gross piece of tissue can be provocative but is not enlightening. To gain understanding, we must localize elements in some way—localize them, for example, to a biochemical fraction such as an enzyme, or to a separated cellular component such as the mitochondrion, or histologically to a cell type such as the islet cells of the pancreas.

Today's instruments are least satisfactory for histological localization. It is very desirable to analyze individual tissue sections and to inspect either the identical or the adjacent sections microscopically, in order to discover any correlations between element concentrations, cell types, and pathological conditions. Since an element is often studied in just those tissues where its concentration is strikingly high, the technical challenge may not involve assays at very low concentrations, but it does involve very small amounts. A conventional tissue section, perhaps 0.1 square centimeter in area and about 10 microns thick, weighs only about 0.1 milligram. Even at, let us say, the relatively high minor-element concentration of 10^{-5}, there will be only about 10^{-9} gram of an element of interest per section. None of the techniques listed in Table 1 provides convenient measurements of such small amounts of elements in such small specimens, especially if one prefers not to destroy the sections (*12*).

For the histological localization of minor elements, the most popular methods are radioautography and histochemical staining. In the most favorable cases each of these methods can provide localization with a resolution approaching that of the microscope. Other methods are still needed because

these two are not quantitative and are often found to be inapplicable. Furthermore, it is quite difficult to establish the specificity and uniformity of a histochemical stain in a chemically complex tissue.

I now discuss a modified x-ray fluorescence system by which measurements may conveniently be made of the average concentration of an interesting element in a histologist's tissue section (although the sensitivity is not high enough for making measurements on microareas within the section).

Nondispersive X-ray Analysis and Histology

When a standard x-ray instrument is set to assay for a particular element, the diffracting crystal (Fig. 2) rejects not only x-ray quanta from other elements but also, unfortunately, most of the quanta from the element of interest. Only the fraction that strikes the crystal face within a certain small range of angles is passed on to the detector. Sensitivity is lost through this waste of good quanta.

An alternative arrangement, with no diffracting crystal (Fig, 3), is called "nondispersive" because x-rays of different wavelengths are not spacially dispersed. X-rays of all wavelengths reach the detector, which then has the job of distinguishing between them. Nondispersive instruments may have only one detector; the reason for the second detector in Fig. 3 is explained later.

At full power dispersive x-ray instruments yield "absolute sensitivities" of 10^7 to 10^8 counts per second per gram of element of interest. The absolute sensitivity of a given instrument for a given element can be increased by a factor of the order of 100 (a conservative figure) by the act of omitting the diffractor and rearranging the components nondispersively. Nevertheless, nondispersive instruments are rare, for the good reason that only diffractors can resolve the complex spectra involved in most applications.

For several reasons the x-ray spectra excited in histological specimens are relatively simple. The low average atomic number results in relatively weak background radiation from the bulk of the specimen. In studying certain interesting elements like zinc and iron, one encounters few interfering elements of comparable concentration. Finally, in

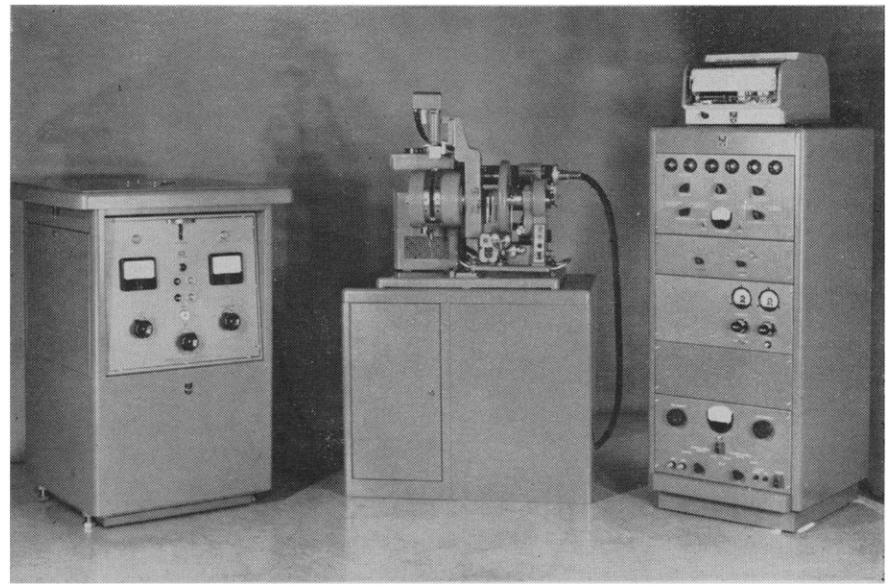

Fig. 1. A standard x-ray fluorescence analyzer. [Philips Electronic Instruments]

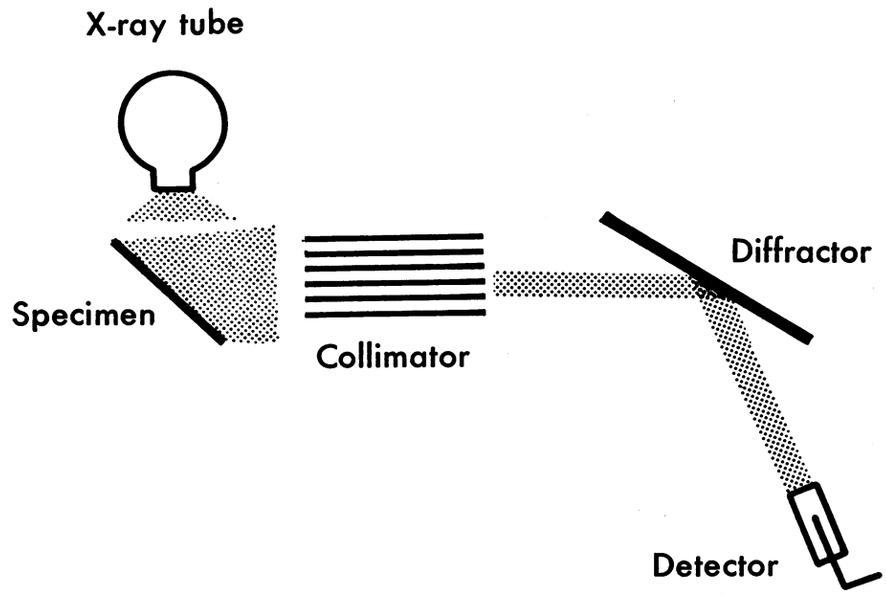

Fig. 2. A standard arrangement for x-ray fluorescence analysis.

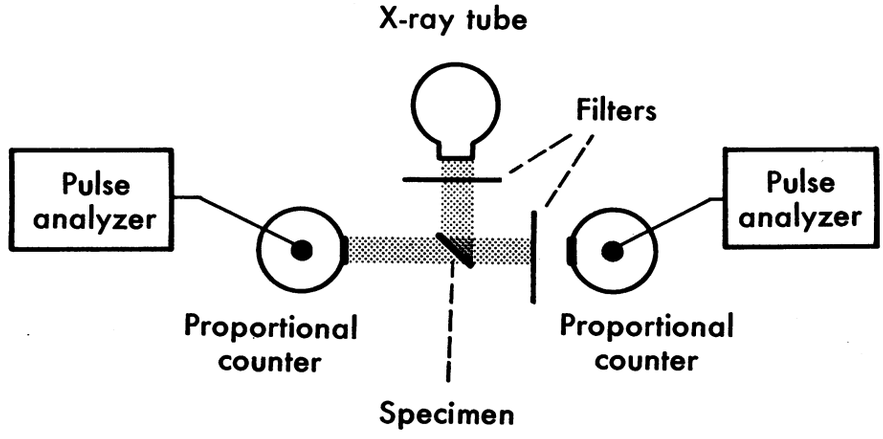

Fig. 3. Arrangement for nondispersive x-ray fluorescence analysis.

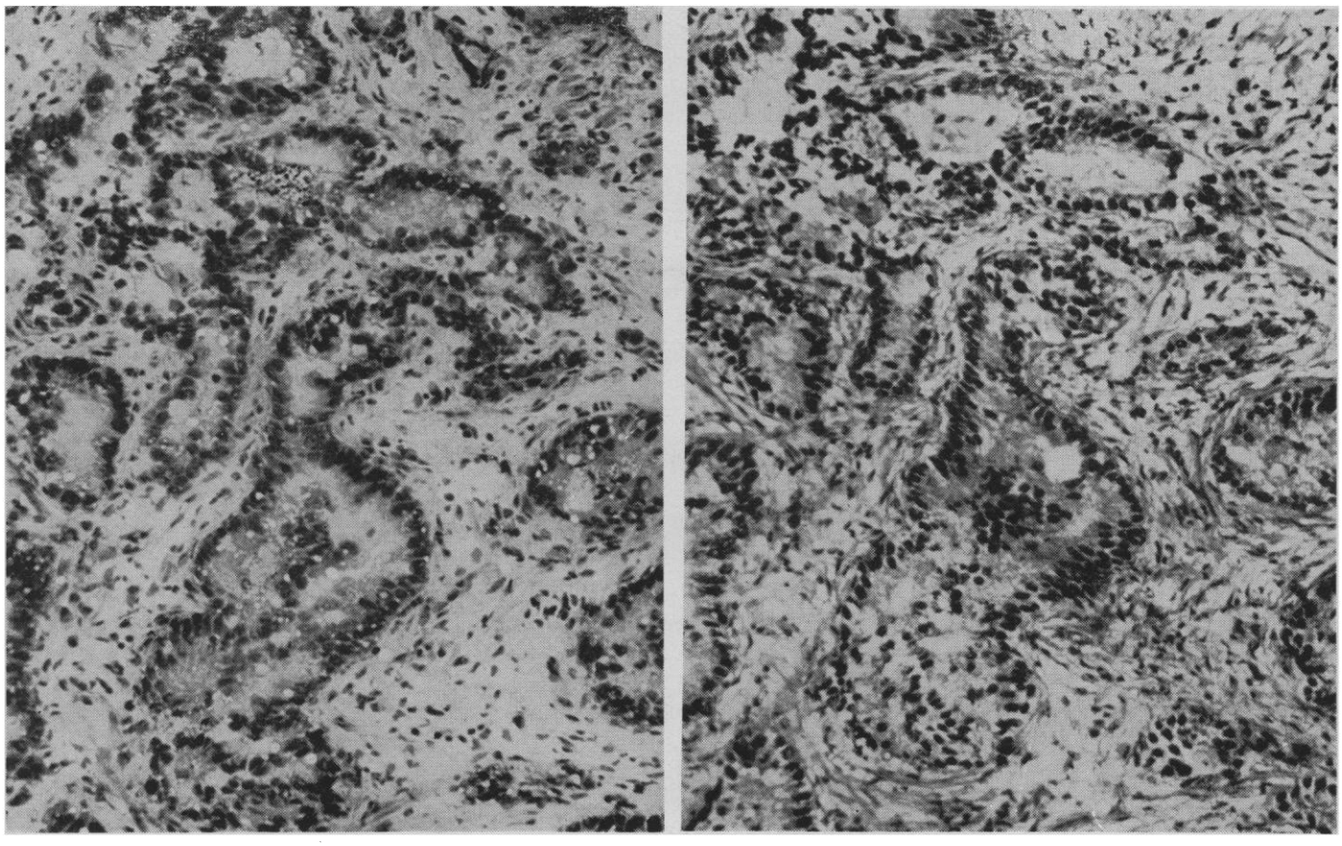

Fig. 4. Sections from a malignant prostate. (Left) Stained after x-ray analysis; (right) stained immediately after sectioning. (× 100)

thin sections, "matrix effects" (that is, attenuation and self-excitation in the specimen) are negligible. Consequently nondispersive x-ray fluorescence analysis is quite practical for a number of histological studies.

For nondispersive work the detector should be a proportional counter coupled to a pulse-height selector. For each element, the proportional counter produces electrical pulses of an average height proportional to the characteristic x-ray quantum energy, so that the selector can be set to respond to one element while discriminating against all others. The rejection is not perfect but is adequate for many biological studies.

The proportional counter is least successful in discriminating between x-rays from elements that are adjacent in the periodic table. Here filters may be especially helpful, since, through a large part of the periodic table, a filter of atomic number $Z-2$ will absorb radiation from element Z much more strongly than it will absorb radiation from $Z-1$. With two counters and one filter (Fig. 3), adjacent elements may be resolved.

It is also necessary to simplify the spectrum of the x-rays incident on the specimens, since the instrumental background is due largely to the electromagnetic scattering of these x-rays into the detector. Such scattering occurs with virtually no change in wavelength. In the biological study described below, the radiation leaving the x-ray tube was filtered to remove almost all intensity at wavelengths near the characteristic lines of the element of interest. Later I discuss briefly an inherently superior method for producing clean exciting spectra, the "secondary radiator" method, application of which has been delayed because of certain technical problems.

Extremely thin and clean specimen supports are essential for histological analyses. Many specimens yield only a few hundred registered quanta from the element of interest during a 10-minute run. Even the widely used ¼-mil Mylar film would produce an intolerable background. In this laboratory, specimens are mounted on "home-made" single-layer nylon films approximately 0.01 mil thick.

The low-concentration limit, both for dispersive and for nondispersive systems, depends on backgrounds arising from scattered quanta (unless element interference intrudes, in the nondispersive case). With pulse-height selection, cleansed exciting spectra and thin specimen supports, background is remarkably reduced. Under such conditions, in a typical nondispersive analysis the counting rates from an element of interest and from background may be equal at a concentration of approximately 20 parts per million in a dried biological specimen, which is equivalent to 4 or 5 parts per million before drying in most soft tissues. This compares quite favorably with the signal-to-background ratio for dispersive systems under their usual operating conditions.

The mediocre resolving power of a nondispersive system makes it poorly suited for scanning for a large number of elements in a given specimen and prohibits certain assays entirely. For example, nondispersive x-ray fluorescence studies of cobalt in unprocessed tissues will probably never be feasible because cobalt occurs in much lower concentration than iron and copper, which are close to it in atomic number. As I hope to illustrate below, a nondispersive system can make a special contribution to an intensive study of a single element, with instrument filtration and excitation appropriately tailored to the element of interest.

Operational Aspects of One Nondispersive Analytical System

Analysts will be interested in certain features of histological x-ray assay, as practiced in this laboratory.

To calibrate, one sets a pulse-height selector to count the pulses produced by an element of interest. A second selector simultaneously monitors the main x-ray line in the exciting radiation. This line is scattered into the detector with an intensity proportional to the mass of the specimen. For thin specimens the ratio

$$\frac{\text{counts from fluorescing element}}{\text{counts from scattered exciting line}}$$

is independent of specimen size. Furthermore, because carbon, nitrogen, oxygen, and all soft biological tissues scatter with virtually the same intensity per unit mass at the wavelengths in question, this ratio is a direct and simple index of concentration. All specimens lacking the element of interest will produce the same (background) ratio, and the increase over this base-line ratio observed in any specimen is linearly proportional to the concentration of the element of interest. Therefore, calibration requires only two samples—a pure organic spot (sucrose, in our studies) and a similar spot with a known enrichment of the element of interest.

Specimens are assayed dry, in a vacuum. Drying reduces the mass of most soft biological materials by a factor of 4 or more, and the corresponding reduction in scattered background is very important for low-concentration work. There is a more compelling reason for assaying in a vacuum: at atmospheric pressure the air mass in the sensitive volume around a thin section would weigh more than the specimen itself, and the mass count would lose meaning.

Contamination and displacement hazards prohibit the use of conventional paraffin-sectioning procedures. Sections are cut frozen in a cryostat, then are transferred directly to the nylon support film, pumped dry, and assayed with no further processing. Element concentration can be correlated with the microscopic appearance of the specimen either by staining and inspecting a neighboring section or by staining and inspecting the original section after x-ray assay. Specimens transferred from the nylon support and stained after assay generally appear to be in poorer condition than their serial counterparts, but they can be used for purposes of correlation. (Any damage they show is presumably not from the x-ray beam but from handling and from the delays before drying and staining.) To be able to inspect an assayed section, rather than a neighboring section, microscopically is especially valuable when an assay shows a surprising result and contamination is feared.

In our procedures, contamination hazards from reagents and containers are slight because these are used very little. Special sources of contamination must be identified and removed. A glass tool should be used to transfer sections from microtome knife to nylon film; the more common camel's-hair brush, if touched to the film, can de-

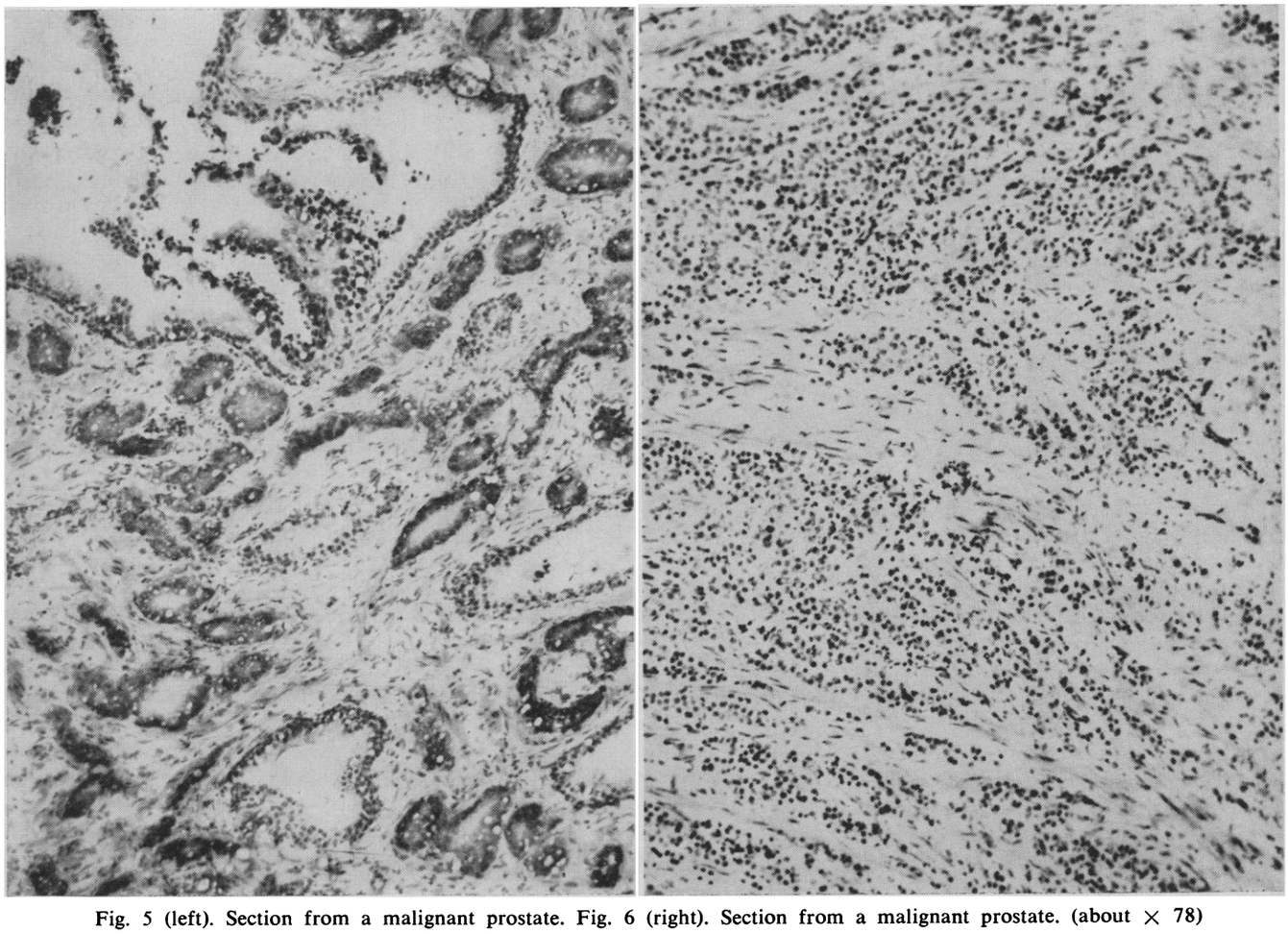

Fig. 5 (left). Section from a malignant prostate. Fig. 6 (right). Section from a malignant prostate. (about × 78)

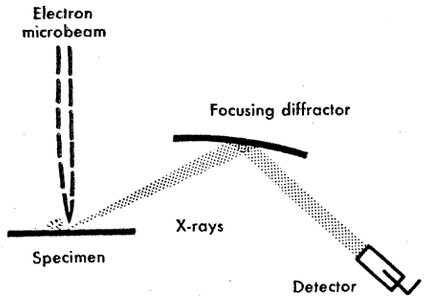

Fig. 7. Arrangement for electron-beam microanalysis.

posit more of an element of interest than is contained in a tissue section. The cryostat operator's sheepskin gloves must be sheathed in plastic to eliminate microscopic shedding. Good technique of a more conventional kind is needed to make adequately clean nylon films. But in general the simplicity of specimen processing makes contamination less of a problem than one might expect with specimens containing 10^{-9} to 10^{-10} gram of an element of interest.

Zinc in Prostatic Tissue

Results obtainable through nondispersive analysis are well illustrated by a study that is now in progress, involving prostatic tissue and the element zinc.

In several mammalian species the concentration of zinc in the prostate gland is about 10 times higher than it is in most soft tissues. Some urologists have wondered if malignancy or benign enlargement of the human prostate gland could be controlled by injecting a zinc-complexing agent. In a recent study (*13*) initiated by the urology section at the Sloan-Kettering Institute it was shown that dramatic selective effects occur in the canine prostate after injection of the zinc-complexing compound diphenylthiocarbazone (dithizone). The effect seems to depend strongly on the concentration of zinc in the tissue. Therefore it became important to determine the zinc levels prevailing in malignant human prostatic tissue, in order to judge the prospects for clinical response to a metal binder.

It has been known for some time that the mean zinc concentration is much lower in malignant prostates than in healthy ones (*14*). This fact does not necessarily imply a lowered zinc level in the malignant epithelial cells themselves, especially since malignant prostates may contain relatively little epithelium. Histochemical staining (*15*) indicates lowered zinc levels in the malignant cells themselves, but this method is not quantitative. A small drop in concentration or even changes in permeability can cause a large loss in staining intensity. The most conclusive data yet published show a correlation between element content and histological and pathological condition in small pieces of tissue (*16*). In collaboration with G. Randolph Schrodt of the pathology division and with the urology section at the Sloan-Kettering Institute, my co-workers and I are trying to sharpen this correlation by a series of zinc assays in individual prostate tissue sections.

Figure 4 shows malignant prostatic biopsy material from a patient who has had conventional estrogen therapy (the two halves of the figure demonstrate the nondestructive nature of the fluorescence analysis). The average zinc concentration in the assayed 10-micron section was 0.17 ± 0.02 milligram per gram of dried tissue, a relatively low value typical for prostatic tissue after estrogen treatment or castration.

Figure 5 shows malignant prostatic biopsy material from an untreated patient. The average zinc concentration in this rather well differentiated 10-micron section was 0.42 ± 0.04 milligram per gram of dried tissue, only about half the average value for the normal human prostate but considerably higher than the average for most (estrogen-treated) prostatic cancers.

Figure 6 shows dedifferentiated malignant, highly epithelial biopsy material from a patient with untreated prostatic cancer. The average zinc concentration for the adjacent (histologically very similar) 10-micron section was 0.43 ± 0.04 milligram per gram of dried tissue. Since our observations and those of others have shown that zinc in prostatic tissue is concentrated in the epithelial cells, and since the section shown in Fig. 6 is much more epithelial than that shown in Fig. 5, it appears that the zinc concentration in the malignant cells is much lower in the tissue of Fig. 6 than in that of Fig. 5.

Figures 4 to 6, selected from a developing series, are shown here solely to exemplify an experimental approach. I draw no biological conclusions at this point. We believe that nondispersive x-ray analysis of tissue sections, as illustrated, will increase our knowledge of the zinc concentrations prevalent in malignant prostatic cells and tissues.

Biological Potential of Methods Related to X-ray Fluorescence

Time has yet to establish the scope of applications in biology of several modern methods of x-ray analysis for chemical elements.

In microradiographs of thin tissue

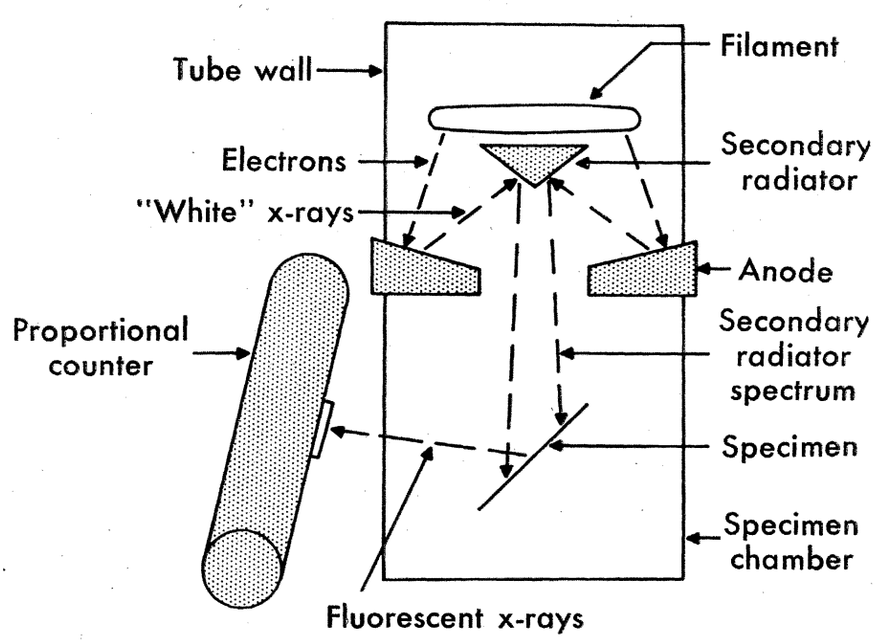

Fig. 8. Arrangement for secondary-radiator x-ray fluorescence analysis. [From Hall (*2*), reproduced by permission of John Wiley and Sons, Inc.]

sections, resolutions of 1 micron or better may be achieved. The distribution of an element within a section may be displayed in contrasting microradiographs, produced successively with x-ray wavelengths on the highly absorbing and the less highly absorbing sides of that element's critical absorption edge (*17*). Only a few elements in a few tissues are concentrated highly enough to be studied in this way (*18*). The technique can be very informative wherever it is applicable.

The outstanding recent development in x-ray analysis for chemical elements is the electron-probe microanalyzer introduced by Castaing and Guinier (*19*). Here the x-ray tube of a fluoresence system is replaced by an electron beam focused onto a specimen spot of the order of 1 micron in diameter (Fig. 7). The microarea under analysis at any moment can be viewed with a built-in microscope. By moving the specimen or scanning with the electron beam, one may map the distribution of an element over the specimen surface. Amounts of elements as low as 10^{-14} gram may be measurable (*20*). This instrumental approach has proved so valuable in metallurgy that several commercial versions are now available, some costing more than $100,000. Biological applications involve special problems: specimen lability, matrix-effect complications, limitations in measurable concentration and in the microscopy of the unstained specimens, and the fact that in the small volumes most efficiently analyzed by the device there may not be even 10^{-14} gram of an element of interest. Hence the role of the electron-probe microanalyzer in biology is still to be established.

Several instruments are based on the principle of the "point" x-ray sources which can be produced with electron microbeams (*20*). Such a source has been used with the absorption-edge method to measure 10^{-10} to 10^{-12} gram of an element (usually calcium) in selected areas of biological specimens, 2 to 10 microns in diameter (*21*). Recently a similar source has been used in a fluorescence arrangement for measuring concentrations down to about 10^{-11} gram in similar microareas (*22*). The range of biological applications for these instruments is not yet defined.

In nondispersive analysis, "secondary radiators" should be very useful (Fig. 8). The output from a secondary radiator consists almost entirely of the few characteristic x-ray lines of the radiator element, with very little continuous-spectrum background. Proper choice of the radiator element can give an exciting spectrum that is both very efficient for a particular element of interest and very low in background near that element's wavelength. The potential of secondary-radiator devices was recognized several years ago (*23*) and explored in theoretical and pilot studies (*1*, *24*). The realization of this potential has been delayed by problems of electron trajectories in the unconventional geometry of a secondary-radiator x-ray tube. It is hoped that a unit now under development in this laboratory will extend the list of measurable elements well beyond the limits imposed by the nondispersive system described here.

Summary

Our efforts to understand the important biological roles of many "minor" elements are limited by the capabilities of the existing instruments. X-ray fluorescence analysis is not the most sensitive method for the assay of minor elements, but it is the instrumental technique of choice both for certain clinical studies and for certain research problems. The technique will be more widely used as it becomes more widely known (*25*).

References and Notes

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31. M. G. Inghram, *Trace Analysis*, J. H. Yoe and H. J. Koch, Jr. Eds. (Wiley, New York, 1957). The listed results are obtainable with the isotope dilution technique. Apparently source problems and unfamiliarity with the method have limited the biological applications of the potent mass-spectrometer method.
32. H. Shields *et al.* [*Nature* **177**, 984 (1956)] present an example of electron spin resonance applied to element detection in biological material.
33. The listed minimum amount is based on results of Natelson and Bender (*5*). For minimum concentrations in specimens of low atomic number, see, for example, (*3*) and the paper by G. V. Dyroff and P. Skiba [*Anal. Chem.* **26**, 1774 (1954)].