

Methods of BIOCHEMICAL ANALYSIS

VOLUME

12

METHODS OF BIOCHEMICAL ANALYSIS

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VOLUME XII

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PREFACE TO THE SERIES

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The current volume is another in this series which is designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analyses.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the editor to make this series of volumes as useful as possible and to this end suggestions will always be welcome.

DAVID GLICK

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Determination of Elements by X-Ray Emission Spectrometry*

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I. INTRODUCTION

1. Principles of the Technique

When an element is stimulated by some energy source, one or more electrons move to higher energy levels. On returning to their original orbits, energy is emitted. Emission spectrometry is concerned with the measurement of the energy so emitted.

The means of excitation of the element usually determines the designation of the type of emission spectrometry. The metal or carbon arc, the gas flame, and the plasma jet have been used to excite the elements to produce emission in the visible or ultraviolet region. If higher energy sources, such as the electron or x-ray beam, are used for excitation, emission is in the x-ray region. This type of emission spectrometry is the one with which we are concerned. This technique has been variously called x-ray fluorescence, x-ray spectroscopy, or x-ray emission spectrometry. The last term seems to be preferred generally at the present time, and for this reason we have chosen this term in the title.

Figure 1 illustrates the basic principle of the technique. The primary x-ray source is located in close proximity to the specimen and its x-ray beam is directed so that it impinges directly onto the surface of the specimen. The emitted characteristic x-ray spectrum of all the elements present in the specimen is collimated by means of parallel nickel blades closely spaced. The collimated beam is now directed to the surface of the analyzing crystal. The analyzing crystal serves to disperse the spectra so as to produce a pattern consisting of lines characteristic of the element excited. The reflected energy is now collimated and directed to an x-ray detector. In the present equipment, the detector consists of counting equipment such as that used in work with radioactive materials. By placing the crystal and counter at the proper angle for a characteristic line of a particular

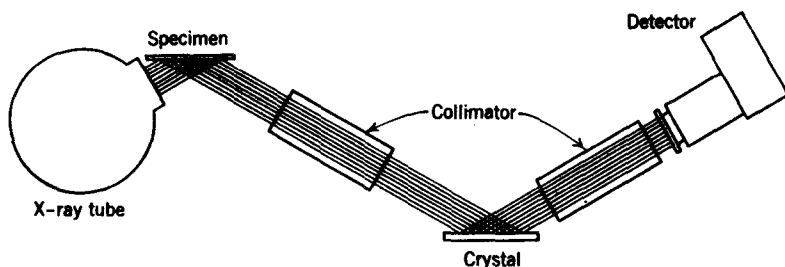


Fig. 1. Illustrating the technique of x-ray emission spectrometry.

element, the element may be detected and the quantity analyzed on a *ratemeter* or by measuring the time required to reach a fixed number of counts.

When examining a specimen of which little is known, it is often required that one scan the different lines for purposes of identification of elements that might be present. This is done by rotating the crystal and simultaneously moving the counter in an arc at a fixed distance from the crystal. The response may then be drawn on a chart by using an integrating counting circuit.

In the crystal, the atoms are arranged in layers. The wavelength of the x-ray beam is of the order of the distance between successive layers. Thus, a beam of monochromatic x rays reflected from the topmost layer will be reinforced by a beam from the next lower layer, provided the two beams are in phase. If they are not in phase, interference will occur. This is essentially the same principle as the *diffraction grating*, except that in the crystal we are dealing with lengths of the order of x rays, while with the diffraction grating we are dealing with lengths of the order of visible or ultraviolet light.

Rotation of the crystal changes the incident angle, which in turn changes the path difference between two photons striking successive layers. Thus, we can put the crystal in tune with a particular wavelength depending upon the angle the crystal is turned. This is defined mathematically by the well-known Bragg equation:

$$n\lambda = 2d \sin \theta$$

where n represents the integers 1, 2, 3, etc.; d is the distance between planes of the atoms in the crystal; and θ is the angle of incidence of the beam. Thus, neglecting the reflections of different orders (n).

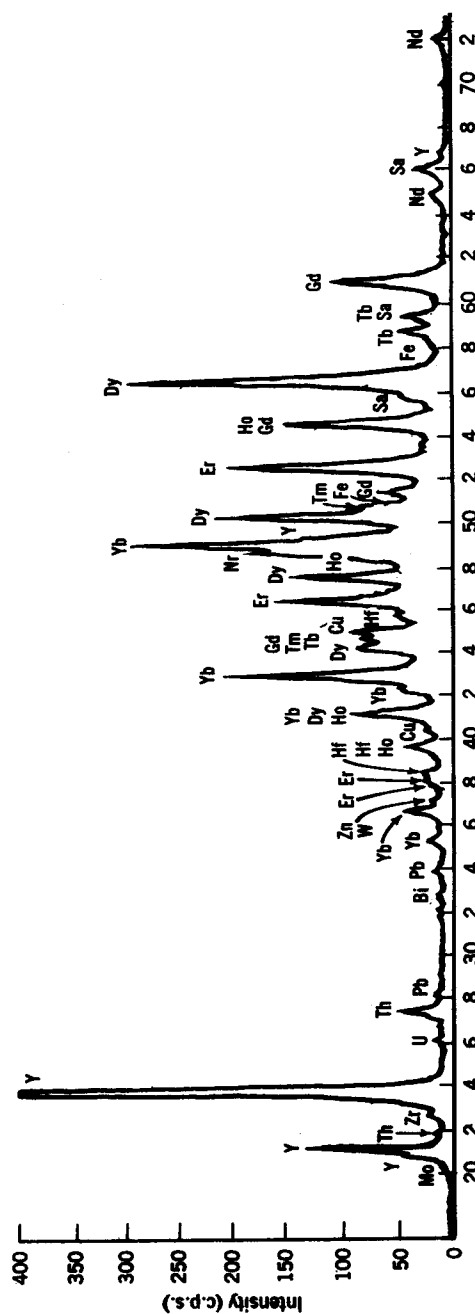


Fig. 2. Tracing of a scan of an ore of the rare earths with the x-ray emission spectrometer. Eighteen elements are identified at low sensitivity. Peaks may be raised for specific elements.

reflection for a given wavelength will occur at only one sharply defined angular setting of the crystal. The crystal may then be rotated at a constant speed to scan the desired angular region.

As can be seen in a mirror reflecting a beam of light, if the mirror is turned, say, 10° , the reflected light will be turned 20° . The same is true in the case of x rays reflected off a crystal. Thus, while the crystal rotates an angle θ , the reflected beam will have moved 2θ . In order to be in a position to pick up the reflected beam, therefore, the

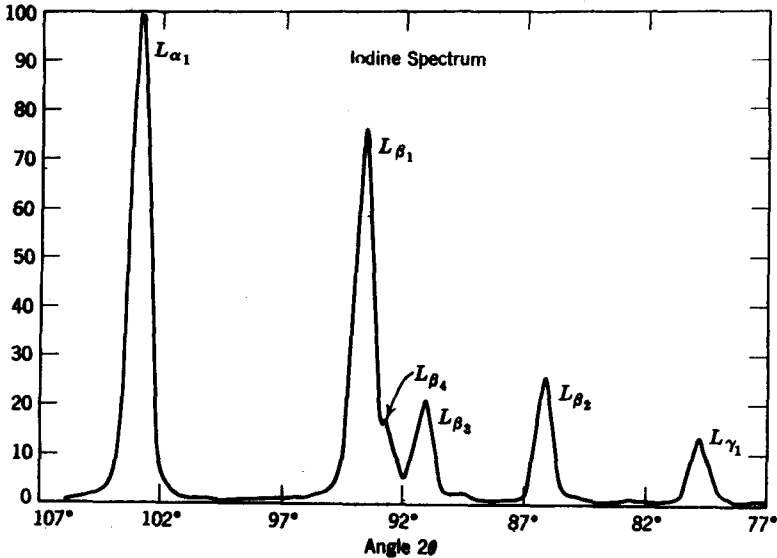


Fig. 3. The characteristic lines for iodine x-ray emission.

detector (e.g., proportional gas flow counter) must be moved at exactly twice the angular velocity at which the crystal is being turned.

A typical chart is shown in Figure 2 for a mixture of elements. Note the relatively few peaks as compared to the confusion of lines one would obtain in an arc emission spectrograph. The peak height for each element is a measure of concentration when compared to a standard. The senior author has used this technique to examine the element content of tissue (e.g., lung tissue as in silicosis, kidney in heavy metal poisoning) and also to examine the trace element content of enzyme preparations.

Another application of this technique is for studying the spectrum of a particular element. This enables one to choose a particular line at an angle where emission from a known contaminant will not interfere. Figure 3 is the spectrum obtained for iodine. Again note the small number of peaks obtained.

The paucity of lines obtained by this technique is due to the fact that, generally, we are dealing with transitions involving the inner-most electrons, that is, emission involving the *K*, *L*, and sometimes the *M* shells, only. The instrument is "blind" to the myriad ultra-violet and visible lines which are due to transitions involving the outer electron shells.

The use of this instrument for the assay of a particular element, by setting the counter at the angle for that element and comparing the intensity of emission with standards, is a most important application of this instrument.

2. Historical

Although x-ray spectrometry has been finding widespread use in the past 10 or 15 years, it is by no means a new idea. Studies of the chemical elements and their relation to the x-ray spectra were soon to follow the discovery of x rays by Roentgen in 1895. Barkla in 1908-1911 (1) was the first to report that a characteristic radiation was emitted from an element when it was stimulated by x radiation of a shorter wavelength, as demonstrated by the *K* and *L* spectra. (It is interesting to note that the *K* lines were first given the designation *A*, the *L* lines *B*, etc. However, it was felt that lines preceding the *A* lines might exist and therefore one should start from the middle of the alphabet, and hence the *K* designation.)

Moseley, 1913-1914 (2), showed that a relationship existed between the periodicity of the elements and wavelengths of the characteristic x radiation emitted by the element.

In Moseley's experiments, samples of the elements were irradiated with high-speed electrons so as to generate x rays. The x rays so emitted were diffracted by a salt crystal and photographs made of the lines emitted. Moseley then showed that the relationship between these lines could be represented by the formula, $Z \propto \sqrt{1/\lambda}$, where *Z* is the atomic number and λ is the wavelength of the photons emitted. Simply stated, the corresponding line for each element (e.g.,

K_{α} line) will be at a wavelength corresponding to that element's place in the periodic table. This classical experiment is discussed in elementary textbooks of inorganic or physical chemistry.

The next significant stimulus to x-ray spectrometry occurred when Coster and von Hevesy, in 1923 (3), conclusively proved the existence of element 72 (Hf) from the x-ray spectra of Norwegian zircon. In that same year, x-ray spectrometry was firmly established by the appearance of a book on the subject by Siegbahn (4). This was followed by von Hevesy's book on chemical analysis by x rays (5) in 1932 and Compton's book on x rays in theory and practice in 1935 (6).

With the development of efficient counting equipment in the late 30's and 40's (7), the stage was set for the appearance of commercial high-resolution instruments (8) which immediately found application in the industrial fields of metallurgy, mineralogy, and ceramics (9-12).

In the 50's, serious attention was turned to the lighter elements. Developments in this area involved the development of the helium (13) and vacuum x-ray spectrometers, and x-ray tubes using lighter elements (e.g., chromium to aluminum) as the target (14), and more transparent windows on the detector tube (15). More efficient crystals of wider d spacings were introduced. Sensitivity has been increased to the point where quantities of elements in the microgram range can be assayed (16).

At the present writing, analysis of the elements down to magnesium is practical. Sodium is the immediate target in this area, and reports have appeared where this element is determined (17). Under special conditions, detection of still lighter elements has been reported down to beryllium (18).

With the introduction of the electron microscope it became practical to focus on a submicroscopic area and excite x-ray emission of the elements. The development of the curved crystal made possible the gathering of a substantial portion of this emission to a focus for detection (19,20). Thus, the "electron probe" was developed which extends x-ray spectrometry to the examination of the composition of portions of a single cell (21,22).

Of potential interest to the biochemist is the use of radioactive isotopes as an energy source for excitation of the characteristic x-ray emission spectra of the elements. Development of this technique

would eliminate the ponderous equipment associated with the generation of the activating x-ray or electron beam. Thus, the beta emitters strontium-90 and tritium, and the gamma emitter cobalt-60 have potential as practical sources for exciting the emission of the elements directly or indirectly by first acting on a suitable target for generation of the x-ray beam (23). The advantages of this technique would be stability, flexibility as to target, and portability of the equipment. Further, the *K* lines of the heaviest elements are readily excited. It is likely that this technique may become of practical importance for the assay of trace elements in biological systems in the near future.

II. THE X-RAY EMISSION SPECTROMETER AND ITS USE

1. The X-Ray Tubes

When an electron is rapidly decelerated—that is, when it strikes the metal target in the x-ray tube—the energy is converted to x-ray photons, the so-called “white radiation.” The relationship between the wavelength of the light emitted and the voltage driving the electron is given by the well-known equation, $Ve = h\nu$, where V is the voltage, e is the charge on the electron, h is the Planck constant, and ν is the frequency of the light emitted. Taking into account that the frequency is equal to the velocity of light divided by the wavelength, and adjusting for correction from electrostatic to electromagnetic units, we obtain the equation

$$\lambda_{\min} = 12.350/V$$

where V is the voltage in kilovolts and λ is the wavelength in angstrom units. Thus, in the standard tube operated at 50 kv. the shortest wavelength emitted would be 0.247 Å.

This wavelength is a minimum—that is, the photons with the highest energy will be of this wavelength—since one cannot obtain a higher energy level than that of the voltage supplied. However, there will be photons emitted of lower energy also—that is, of longer wavelengths. The reason for this is that when electrons leave a cathode they are not all traveling at the same speed. If you were to plot the speed of these electrons against the number in each group you would get the normal curve of distribution. However, one cannot

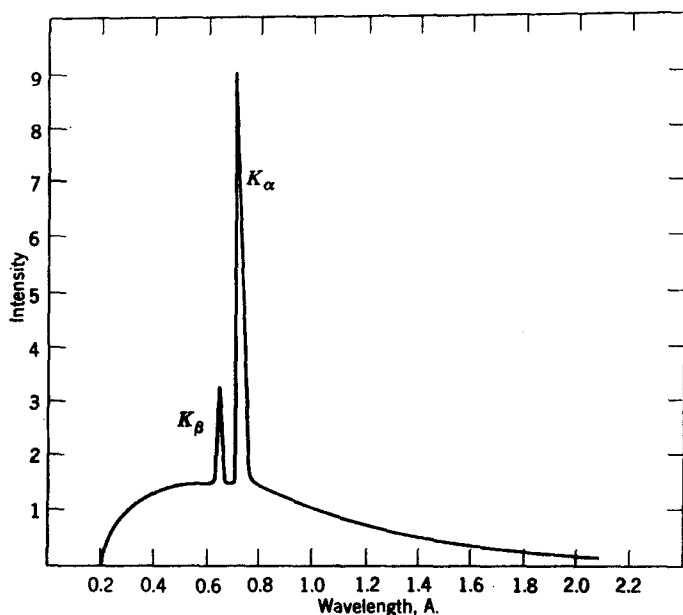


Fig. 4. Intensity distribution of x rays obtained from a molybdenum target. Note the characteristic emission for molybdenum impressed on the "white hump" of the curve.

get more energy than permitted by the maximum voltage. Thus, the curve is chopped off sharply on one side.

The x rays emitted show a similar curve (see Fig. 4). Starting at the minimum wavelength, the curve rises to a maximum and then falls off, following the distribution curve. The peak of the curve is called the "white hump." This hump yields an obligatory high background for the heavier elements. Moving along, one reaches an area of low intensity. This is in the area where the lighter elements have their emission. Thus, for the lighter elements the background is markedly reduced. Since sensitivity is a function of the number of counts obtained for the elements divided by the background counts, it is apparent that this technique affords maximum sensitivity for the lighter elements. Since these elements are of greatest interest to the biochemist this phenomenon is advantageous.

One will notice in Figure 4 that sharp peaks are superimposed on the broad white hump. These are due to excitation of the charac-

teristic emission of the target element. If the target is tungsten, the K lines ($\lambda = 0.21$) will not be excited due to the fact that, as pointed out above, the minimum wavelength excitable is 0.247 Å. at 50 kv. The tungsten L lines appear at 1.47 and 1.48 Å. for L_{α_1} and L_{α_2} , and these are the lines observed. For molybdenum, the $K_{\alpha_{1,2}}$ lines at 0.71 Å. will appear. Thus, the appearance of these sharp peaks

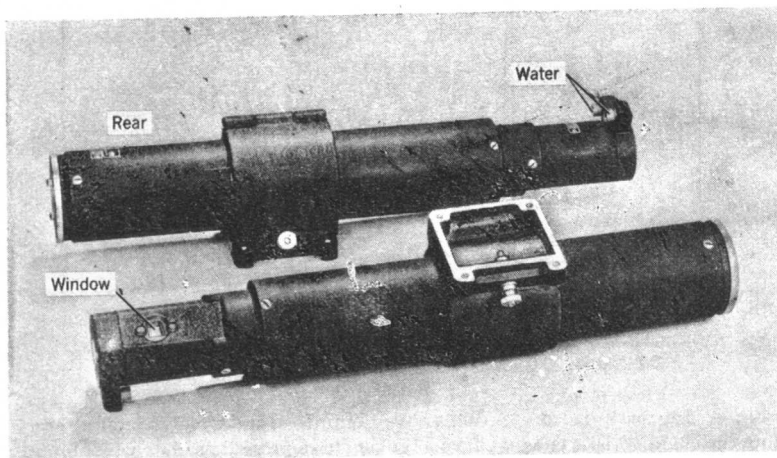


Fig. 5. The x-ray tube used in x-ray spectrometry. Note the lead shield on the window. This controls the shape of the pattern of x rays striking the sample.

superimposed on the white hump radiation will be a function of the target used. If higher voltages are applied, the K lines due to tungsten can be excited. As the voltage is elevated the minimum wavelength becomes smaller and the whole curve shifts to the left.

Figure 5 shows the physical appearance of the x-ray tube used in x-ray spectrometry.

2. Choice of X-Ray Targets

X-ray tubes are available with different metals as targets. Tungsten, molybdenum, copper, and chromium are the metals most commonly used in tubes. Gold and platinum target tubes are also standard equipment. Tubes with targets of nickel, aluminum, titanium, and silver are also available as special tubes. The choice of target is determined by the elements being examined.