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X-RAY SPECTROCHEMICAL STUDIES[1]

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I. Introduction

The constancy of "le milieu intérieur" of Claude Bernard, amplified further as "homeostasis" by Cannon is a widely cherished concept in physiology. Yet at this late date there exist large voids in our knowledge of the homeostatic mechanism(s) with respect to many simple chemical entities. Outstanding among such entities are the chemical *elements* themselves. In 1960 the Handbook entitled "Blood and Biological Fluids" provided data for only nineteen chemical elements in the blood of man, and even this list includes such common elements as sodium, potassium, calcium, chlorine, sulfur, phosphorus, iron, copper, and zinc among them (1). Some values have recently appeared for a few additional chemical elements (2, 3). Analyses have been reported for a much larger number of elements in certain tissues and organs of the body (4, 5). Both for blood and for tissues, what data have appeared are extremely sporadic, representing often a value for a single case or a few cases of

[1] This work was supported by the U.S. Atomic Energy Commission.

[2] The original researches included in this discussion are those of a research group including J. W. Gofman, O. F. deLalla, G. Johnson, E. L. Kovich, O. Lowe, D. L. Piluso, R. K. Tandy, and F. Upham.

a particular pathological entity. Thus with respect to the elemental homeostasis of the human body in health, as a function of sex or age, and with respect to deviations therefrom in common pathological states, the biological information available is, at the best, highly fragmentary.

From numerous other areas of biochemical and physiological investigation, a vast body of information has developed demonstrating that biological systems are profoundly influenced by chemical elements throughout the periodic table and by the balance between pairs of chemical elements in many cases. Such studies have led to the formulation of numerous attractive hypotheses which must remain hypotheses in the absence of the requisite data concerning levels of the various chemical elements in the fluid and fixed tissues of such mammalian organisms as man. There exists, therefore, an urgent need for systematic development of a body of biological data concerning the distribution of the chemical elements in the fixed and fluid tissues of man, if the newer biochemical and pharmacological concepts are to find application in the study of health and disease.

As a start in the direction of this over-all endeavor, it has appeared worth while to study one tissue that is so often involved in regulatory mechanisms of homeostasis, namely, the fluid portion of the blood, plasma (or serum). Such systematic studies should ultimately be extended to a variety of cellular tissues, including red cells, leucocytes, and the various fixed tissues. Which elements deserve evaluation? *In vitro* studies have shown that almost all of the chemical elements *can* produce biochemical effects. Industrial and environmental toxicology have provided ample evidence that some of the less common elements have produced serious derangements of health upon exposure (including nickel, chromium, silver, cobalt, uranium, thallium, bismuth, mercury, osmium, selenium, beryllium, arsenic, and others). In view of these findings, the foregoing question may best be answered by stating that almost any chemical element *can* be important for human pathophysiology if man comes into contact with the element in one of a variety of ways. And if man has as yet rarely encountered certain elements, the rapidly developing technologies of nuclear, electronic, and space science have brought many once exotic elements into commonplace utilization. Such elements as niobium, tantalum, zirconium, hafnium, germanium, and beryllium are now handled daily in pound and ton lots in numerous technological applications. Many such uncommon elements will ultimately gain access to the human organism and their behavior will have to be understood.

Certain elements may be reasonably subclassified and eliminated from the proposed study. For example, hydrogen, oxygen, carbon, and nitrogen

biologically occur primarily in the form of such compounds as water, small organic molecules such as sugars and amino acids, and as large organic molecules such as proteins, nucleic acids, and lipoproteins. It appears reasonable, therefore, to exclude these elements from study in terms of level of the element itself. Even this exclusion is made with some reservations, of course, since the hydrogen ion as such is of profound biological significance. Indeed, chemically there is logic in the classification of hydrogen ion with the metallic ions. Elements 43 (technetium) and 61 (promethium) are essentially nonexistent in stable isotopic form on earth and hence will not be anticipated to occur in biological systems. The noble gases, helium, neon, argon, krypton, and xenon, though not without potential biological effect, present such special problems of containment in samples for measurement that they must be deferred in present considerations. Elements 84, 85, 86, 87, 88, 89, 91 and all the transuranic elements are part of a field of their own, where biological effects of their radioactivity will take precedence over chemical effects of the elements themselves. Left for evaluation as chemical entities of potential biological importance, after all these exclusions, are seventy-three elements. If the biology of such elements in the blood and tissues of man is to be studied in health and disease, it becomes necessary to consider the practical problems of analytical methods capable of encompassing these seventy-three elements. Two of the broad requirements of such analytical methods are (*a*) reasonable speed per analysis and (*b*) adequate sensitivity for the measurement of those elements occurring at low concentration.

Chemical methods can be considered, and in all likelihood could be developed with an enormous expenditure of effort. Evident drawbacks include the necessity for study of interelement interferences in the particular fluid or solid tissue under analysis plus the complications introduced by the possible complexing or chelation of numerous elements by one or more of a variety of organic compounds present. Solution of this latter problem by preliminary ashing of samples introduces the variable of possible selective losses requiring separate evaluation. Adequate sensitivity might require several types of concentration and/or separation steps for single elements or groups thereof. In addition, the prospect for rapidity of analysis encompassing some seventy elements is not encouraging.

Among the potential physical-instrumental methodologies, several merit consideration, including flame spectrophotometry, neutron (or other particle) activation, optical emission spectroscopy, and X-ray emission spectroscopy. Each such methodology has features which are favorable; each suffers from certain severe limitations. It appears at this

time that the ultimate methodology for the problem under consideration will utilize more than one of these approaches.

One of these methods, X-ray emission spectroscopy, is characterized by an advantage absent in all the others, namely, that the sample analyzed emerges from the procedure essentially unchanged in chemical composition and is thus available for any future reanalysis or supplemental analysis by other methods. (This must be qualified only to the extent that the intense X-irradiation to which the sample is subjected may alter the structural physical properties of the sample itself.) Additionally, the X-ray emission spectroscopic technique is characterized by certain important features of *relative* simplicity. First, the method is based upon the excitation of the characteristic K and L spectra of the chemical elements which vary in a regular way as one passes from one element to the next in order of atomic number. The reasonably prominent lines in the K and L spectra are relatively few in number, so that analytical separation of the lines and their measurement free of interference from overlapping lines is a manageable problem, with the application of some diligence. Over the region of the periodic table from atomic number $Z = 11$ (sodium) through $Z = 92$ (uranium), every element is capable of being excited and the characteristic lines can be measured within existing technology. At this time the accessibility for analysis by this technique of elements of atomic number less than 11 is not likely. The elements between atomic number 11 and 14 are difficult, but recent developments in instrumentation will eliminate most of such difficulty in the immediate future. Thus, if a split is made between atomic numbers 10 and 11, it appears possible to consider one general method for all elements of atomic number from 11 through 92, namely, X-ray emission spectroscopy, leaving only lithium, beryllium, boron, and fluorine as elements requiring some other supplemental method of analysis.

This discussion will center around the early experiences in the development of a workable system for the analysis of a large number of chemical elements in a single sample of human blood serum, the limitations and prospects currently characteristic of the method, and the data thus far developed in the study of a population sample of residents of California (northern) including mean values for sixty-six chemical elements (6, 7).

II. Methodology

A. Principles of X-Ray Emission Spectroscopy

Recent technological advances have grossly improved and simplified the analytical applications of X-ray emission spectroscopy, also known

as X-ray spectrochemistry or X-ray fluorescence. The current state of the technology, with critical discussion of the limitations, is well described by Birks (8) and by Liebhafsky and co-workers (9).

In essence, the method rests upon the removal of an electron from the K or L shell of an atom either by electrons or X-rays (other excitation, such as by α-particles or deuterons is also possible), to be followed by the emission of X-rays when the vacancy created by electron ejection is filled by an electron from a higher-energy level, with certain electron transitions being more probable than others. Whether or not ejection of an electron from a particular atom is from the K shell or the L shell depends upon the energy of the incident radiation. Thus, for a particular element, a minimum energy is required to remove an electron from the K shell, the minimum energy corresponding to what is known as the K absorption edge. If photons of energy lower than this minimum value impinge upon the element even in great intensity, no ejection of K electrons will occur, and hence no characteristic K radiation will be emitted. However, the energy required to eject an electron from one of the L shells is very much lower than for the K shells, so that incident radiation incapable of ejecting K electrons can still eject L electrons and give rise to characteristic lines of the L spectrum, which lines can be used in analytical work. In practice, the most common source of exciting radiation for X-ray spectrochemistry is a thin window X-ray tube operated at voltages up to 60 kv. Efficiency of excitation is such that K-shell excitation is used for analysis up to atomic numbers in the 55–60 range, and L-shell excitation is used for all elements of higher atomic number. Tungsten, molybdenum, or chromium anodes are most commonly in use, with the result that the available spectrum of radiation is a combination of polychromatic (white) radiation of all energies up to the maximum voltage applied to the X-ray tube plus characteristic line spectra of the particular anode employed in the tube. Thus for a tungsten target tube operated at 60 kv., the exciting radiation consists of the various L lines ($L\alpha_1$, $L\alpha_2$, $L\beta_1$, $L\beta_2$, plus several weaker lines) and the white, or continuous, spectrum of X-rays of all energies up to the energy corresponding to the peak voltage of the tube. The properties of the exciting radiation are extremely important, in the ultimate determination of the sensitivity of analysis for any particular element.

It is a well-known physical principle that the most efficient radiation for ejecting electrons from the K shell of an element is that energy which just exceeds the K absorption edge and that the probability of K electron ejection is nearly inversely proportional to the cube of the energy of the exciting radiation. Therefore, it is clear that the ideal source of excitation for an element whose K absorption edge is X kv. would be monochromatic radiation of energy just greater than X kv.

Similar considerations would apply to every element that is to be analyzed. It is self-evident, therefore, that the mixture of polychromatic continuous radiation plus the characteristic line spectrum from an X-ray tube with a particular anode, e.g., tungsten, could hardly constitute an ideal excitation source. Several approximate efforts can be made to approach the direction of ideality. First, one can and does make use of several different X-ray tubes, each having a different element as target (anode) material, including tungsten, molybdenum, chromium, silver, gold, copper, and platinum. There is no theoretical reason why a number of additional elements could not be used as anode materials and in this way make available characteristic spectra of energies suitable for the analysis of certain elements. Practically, however, there are problems in that the target element must be one that can be suitably bonded to the water-cooled base of the target so as to allow a reasonable wattage input to the X-ray tube. Hall (10) has approached this problem in a productive fashion by making use of a secondary radiator. In his system the primary polychromatic X-radiation is used to excite a material whose characteristic spectrum is ideal for the element under analysis, the secondary characteristic radiation being used to excite the analytical sample. It is to be hoped that further developments along the lines of Hall's work will become available more generally.

The question might be raised as to why the polychromatic beam, as from a tungsten anode tube, is not perfectly suitable, since within the polychromatic continuous spectrum there do exist X-rays of the energy desired to excite a particular element. Indeed, this is what is done in practice, but the polychromatic beam brings up problems that relate critically to the over-all ability to analyze for constituents at low concentration in the sample. These problems have to do with what is called the "background" radiation, bearing a similar relationship to the desired radiation that is commonly encountered in the field of radioisotope analysis. Any material that is under analysis will scatter X-rays of various wavelengths by two processes, coherent and incoherent (Compton) processes. As a result there will exist in the radiation under analysis not only the excited characteristic radiation of a particular element but also some scattered radiation from the tube spectrum of precisely this same wavelength, or energy. No analytical method, except for one based upon polarization of X-rays, will distinguish scattered X-rays of a particular wavelength from the excited X-rays of the same wavelength from the element being analyzed. An unavoidable background radiation from this source is thus mixed with the characteristic radiation from the element under analysis. The background problem is complicated still further in the usual analytical procedures by the

presence of scattered X-rays of two, three, four, or more times the energy of the characteristic radiation from the element under analysis. Such higher-energy radiation interferes in the usual analytical procedure because it is common to utilize crystal dispersion of the X-rays as part of the measuring system. When a crystal is used to monochromatize X-rays, the Bragg law operates as follows:

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

where λ is the wavelength of the X-radiation; d, the interplanar spacing of the analyzing crystal; and θ, the angle of incidence of the radiation upon the crystal.

If for a particular crystal, a diffraction maximum is observed at some angle θ, for a wavelength λ, the equation indicates that diffraction maxima will occur at exactly the same θ value for X-rays of wavelength $\lambda/2$, $\lambda/3$, $\lambda/4$, etc. Thus if such shorter wavelength X-rays (of higher energy) are present in the exciting polychromatic radiation and are scattered from the analytical sample, they will arrive at the detector along with the characteristic radiation excited in the analytical sample. Fortunately, to a large extent, such undesirable higher-order reflections can in large measure be eliminated in modern systems through the use of detectors which discriminate X-rays on the basis of their energies. Among the commonly used detectors of this type are gas-flow and sealed-gas proportional counters or scintillation crystals, either detector being backed up by pulse-height discrimination in the electronic circuitry associated with the detector. One manufacturer provides discrimination physically, using Geiger counters of different path lengths, with the elimination of electronic pulse-height discrimination (11). Such discrimination, as just mentioned, goes a long way to reduce the background introduced by the scattered higher-energy radiation, although *some* of the higher-energy radiation still gets recorded either because of imperfection in the detector response or because of so-called escape peak phenomena (12).

The various sources of background radiation that are the result of the use of polychromatic primary sources are truly a major limitation upon the power of the analytical method. Although the detailed reasons for this are outlined in the following (see Section II,G), it can be stated here that sensitivity is closely related to the relative prominence of the analytical line versus the combined sources of extraneous background radiation. The higher the background radiation is relative to the analytical line, the poorer will be the sensitivity of the analysis. For this reason the use of characteristic, monochromatic radiation as the exciting source is

highly desirable, and the closer one approaches this ideal, the more powerful will the method be. Currently available manufactured equipment falls far short of this goal, and only a few workers, such as Hall, have attempted to provide adequate solutions for this problem.

Some of the undesirable higher-energy X-ray background can be reduced by limiting the voltage applied to the X-ray tube source. This approach is, in general, of limited usefulness for two reasons. First, this may result in setting the maximum in the intensity of the continuous spectrum in an unfavorable position for the element under analysis and thus increase the scattered background of radiation of the same wavelength being utilized as an analytical line. Second, the efficiency of conversion of electrical power to X-rays in the tube varies according to the law:

$$\frac{\text{X-ray energy}}{\text{cathode-ray energy}} = kZV$$

where Z is the atomic number of anode, V is the potential applied to the tube, and k is an empirical constant. Thus, at lower voltages, the total output of X-rays for the energy input is reduced. Such reductions in over-all X-ray intensity themselves lower the sensitivity of the analysis.

There is still another problem associated with the excitation source which limits the sensitivity of the analytical method for many chemical elements. As already discussed, the most efficient radiation for the excitation of characteristic X-rays in a particular element is that of energy just greater than the absorption-edge energy. Many elements of analytical interest have absorption-edge energies below 5 kv. (for magnesium the absorption edge energy is 1.303 kv.). It is, therefore, essential to conserve as much of the output of such low-energy excitation radiation from the source as is possible. Unfortunately the beryllium windows of many X-ray tubes commercially available for this application are much too thick to allow an appreciable fraction of the desired soft X-rays to emerge from the tube. At the same time transmission is allowed of the undesirable higher-energy radiation which provides interfering background radiation. Thinner window tubes are in development, but are as yet not ideal. Ultimately windowless X-ray tubes represent the best solution, that is, where the sample and X-ray tube interior are all part of a single evacuated system. This, of course, means the ability must be at hand to evacuate the system with each sample change sufficiently to provide satisfactory X-ray tube operation (vacuum estimated to be $<0.1\mu$ Hg).

B. The Nature of the Analytical Sample

In the X-ray emission spectroscopic technique, quantitative analysis for elements at low abundance places stringent requirements upon constancy of conditions during all phases of the analytical procedure. The X-ray output discussed in the previous section must be maintained constant or must be monitored if there is significant deviation from constancy. (Our own equipment, Siemens Crystalloflex plus Siemens X-ray tubes, shows 0.2% variation in short-term X-ray beam output.) Probably of even greater importance for analysis at low abundance is rigid control of sample preparation and of geometrical presentation of analytical sample both to the exciting beam and to the analyzing crystal-detector system. This is achieved by having a fixed-geometry system of all parts of the sample drawer coupled with a standard method of preparation of the sample. In the current work human blood serum is the material under analysis, so it was necessary to devise a system for reproducible positioning of the serum sample in the drawer position. It was decided to convert all sera to a dry powder before preparation of the analytical sample. In order to avoid variable concentration of elements in the serum and to avoid any leaching of vessels in techniques such as evaporation, the procedure adopted was that of lyophilization of the serum to a dry powder, followed by pressing of the dry powder into a wafer for analysis. The detailed features of this system will be described elsewhere by deLalla (13). Lyophilization of serum results in a powder, the chief chemical constituents of which are protein, lipoprotein, and sodium chloride, plus relatively small quantities of small organic molecules such as glucose and amino acids. Most of the elements of analytical interest are at very low concentration relative to the protein and sodium chloride, so that in essence each sample has a nearly constant matrix of the protein and sodium chloride. The lyophilization step serves an additional highly important purpose, namely concentration of all the elements present by a factor of approximately twelve. Further concentration could be achieved by ashing all the organic material, leaving a matrix of essentially nothing but sodium chloride. However, the presence of a variety of complexing and chelating agents in blood serum (amino acids, organic acids, etc.) makes it possible that certain elements might be present as compounds of unknown volatility. To verify the safety of ashing with respect to loss of certain elements will be a large undertaking of itself. Therefore, this procedure for additional preanalysis concentration has not been utilized in the current work. The additional concentration factor of six is, however, attractive and will undoubtedly be applied in future work, especially where additional sensitivity is required in the analysis.

Samples for analysis have been prepared by accurate weighing of 400 mg. of the lyophilized powder, followed by the pressing of this powder into a wafer (at 4000 p.s.i.) using a press lined with Teflon. Careful cleaning of the press was performed before each sample was prepared.

Many analysts in this field prepare a sample thick enough to be regarded as of "infinite" thickness with respect to counting rate of the characteristic X-rays emitted from a particular element in the sample. Although this makes sample thickness an unimportant variable (provided it is above some minimum value), it increases the requirement with respect to original material, in this case blood. Since blood is available only in limited quantities, we have utilized the alternative of a constant sample size, reproducibly prepared from case to case. All calibration studies, for every element under analysis, are performed by preparing sera with known additions of a soluble salt of the element in question, lyophilization to dry powder, and preparation of 400 mg. disks for use in the X-ray emission spectrometer. That the use of such noninfinite thickness samples is highly satisfactory will be shown below in representative calibration data. Since the yield of dry powder per unit volume of serum is not identical from sample to sample, each dried sample is weighed immediately after lyophilization, so that appropriate calculations can be made for the true volume of serum represented in a 400-mg. powder sample used in the X-ray spectrometer.

Tests have been made of the linearity of counting rate versus quantity of element present in such 400-mg. disks for analytical elements throughout all regions of the periodic table, with highly satisfactory results. Some representative studies, for manganese, iron, and cobalt are presented in Fig. 1A, B, and C.

Blood serum is a relatively favorable material for analysis in comparison with some that analysts have had to cope with in geological and metallurgical applications. This is so because the *gross* composition of serum is nearly identical from sample to sample. First, all the major elemental constituents of dry serum powder are carbon, hydrogen, nitrogen, oxygen, sodium, and chlorine. All other elements are one or more orders of magnitude below the least abundant of these, except sulfur which is present in approximately one-third the sodium abundance. Further, the relative proportions of C, H, O, and N will be nearly constant from sample to sample. There is some minor variation in the relative abundance of protein versus sodium chloride, but even this is small. For certain refined measurements it may be desirable to ascertain whether matrix effects due to differences in the protein-to-salt ratio will require corrections for some of the analytical values. Such correc-