fide formation with protein mercaptan groups is not responsible for the change in absorption spectrum is indicated by the failure of the mercaptans MEA and MEG to cause a similar change.

However, it is again possible that salt formation has taken place with catalase since the over-all shape of the sum of the absorption curves has not been appreciably altered in the mixture, and the average difference in absorbance, 0.040, is not greatly different from those of the previous cases where salt formation is suspected. Moreover, it appears that complex formation of enzymes may be demonstrated by this method of absorbance differences in absorption spectra in certain cases at least.

CONCLUSIONS

Spectrophotometric evidence has been found that a known antiradiation agent, sodium diethyldithiocarbamate, undergoes complex formation with lactic dehydrogenase and either salt or weak field complex formation with catalase. No definite evidence for complex formation of these enzymes with either 2-mercaptoethylamine or 2-mercaptoethylguanidine was found, although salt or weak field complex formation is possible. Apparently, any complexation by the latter agents is more readily dissociable than that by the dithiocarbamate.

No spectrophotometric evidence of complex formation between salicylate and either lactic dehydrogenase or catalase was found, although both enzymes are known to be inhibited by salicylate. It is possible that any complex formed has dissociated too rapidly to be observed by this method.

Measurement of absorbance differences between the sum of the absorption spectra of the individual components and the spectrum of the mixture appears to be a possible method of demonstrating complex formation of enzymes in select cases. In these cases the binding is either irreversible or of sufficient strength to exist for a measurable period of time and thus be observable by the stationary, or nonflow, technique employed here.

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Assay of Iodine Content of Thyroid Extract by X-Ray Absorption Edge Analysis

By HARRY A. ROSE and DONALD E. FLICK

The assay of thyroid extract for iodine content by the X-ray absorption edge technique has been investigated. The method has been found to be applicable to the crude, U.S.P. powder, and uncoated Enseals. Excellent precision has been found for the assay of crude thyroid extract. It is also shown that because of the nature of the method the determination is specific for iodine. The instrumentation used is the same as that used for X-ray crystallography by the diffractometer method.

THE ASSAY of the iodine content of thyroid extract by X-ray absorption edge analysis was developed as a possible replacement for the chemical assay for this element.

The chemical assay that is used is prescribed by the "United States Pharmacopeia." The present procedure has undergone only minor improvements since the U.S.P. IX of 1916. Essentially, it is a potassium carbonate fusion to liberate the iodine from the organic matrix. After several intermediate steps, the determination ends with a sodium thiosulfate titration. It is about a 4-hour procedure with many opportunities for loss of sample.

X-ray absorption edge analysis has been applied in the analysis of molybdenum and zinc in hydrocarbons by Barieau (1). A recent review of the field has been given by Liebhafsky *et al.* (2). Because of the completeness of the description of the method given by Barieau, only a brief description will be given here.

X-ray absorption edge analysis is a very powerful tool because it is free from matrix effects and is specific for the element to be determined. This is so because the absorption of X-rays by a

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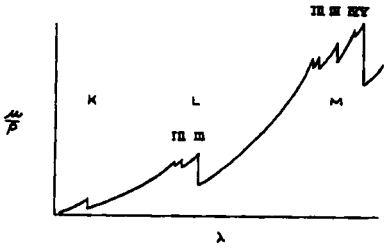

Fig. 1.—A plot of X ray absorption against wavelength. The ordinate shows the mass absorption coefficient which is the absorption from a beam of unit cross section by 1 Gm. of sample.

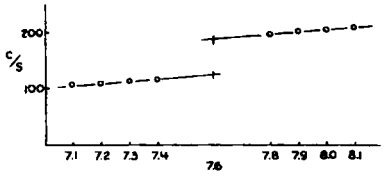

Fig. 2.—A graph showing the relation between transmitted X-ray intensity (ordinate) in terms of counts per second and the angular position of the detector (abscissa). The absorption edge of iodine is at 7.6° for the conditions used.

sample occurs by two mechanisms: (*a*) X-ray photons may be scattered by the sample; and (*b*) X-ray photons may be absorbed by using their energy to eject electrons from the atoms of the sample, that is, by ionizing the atoms.

The absorption due to scattering increases with increasing wavelength of the incident X-ray. Also, the absorption increases with increasing atomic number leading to the familiar fact that lead is a better X-ray shield than aluminum.

On the other hand, the absorption due to ionization is discontinuous with wavelength. In a generalized plot of absorption against increasing wavelength (Fig. 1), these discontinuities appear as definite, sharp decreases in absorption and are termed "absorption edges." The positions of the edges can be found in handbooks of chemical and physical data. An explanation of the physical basis of this effect including the meaning of the terms *K*, *L*, and *M* and the numerals can be found in any modern physics book—for example, the excellent text by Semat (3).

The over-all absorption of X-rays by a substance is defined by the equation

$$I = I_0 e^{-\mu t} \qquad \text{(Eq. 1)}$$

where I = intensity of transmitted beam, I_0 = intensity of incident beam, t = thickness of sample (X-ray path length), and μ = linear absorption coefficient. The linear absorption coefficient of a sample containing more than one element is the sum of the coefficients of each element times the fraction of the sample made up by that element.

Since the absorption of a given element changes at its absorption edge, the absorption coefficient of the sample containing that element will change at that absorption edge. By measuring the amount of X-rays transmitted by a sample containing a known amount of an element for wavelengths on each side of an absorption edge, the amount of change of the coefficient μ can then be measured. Conversely, if the amount of change for the coefficient is known, the measured change in the transmission can be used to evaluate the amount of the element present.

It is important to realize that the absorption edges of the elements occur at shorter wavelengths as the atomic number increases. Thus, for a sample containing several different elements, only that part of the absorption due to the element to be measured will change at its absorption edges.

Barieau (1) has shown that the relationship between X-ray intensities on each side of an absorption edge and the fraction of the element in question in the sample is given by

$$2.303 \log I''/I' = (\mu'_m - \mu''_m)\, WG \qquad \text{(Eq. 2)}$$

where I'' and I' = transmitted X-ray intensities on each side of an absorption edge, μ'_m and μ''_m = mass absorption coefficients at each side of an absorption edge, W = weight fraction of element in sample and G = pt. or mass thickness of the sample in Gm. cm.^{2}.

The term $(\mu'_m - \mu''_m)$ is characteristic for an element under any conditions. For this assay it was evaluated using solutions of potassium iodide. Reagent grade potassium iodide from Mallinckrodt was used. The test solutions were made up in concentrations to cover the range of iodine concentration expected in thyroid extract. The solutions were placed in a cell made of glass tubing with Mylar windows. By standardizing the method in

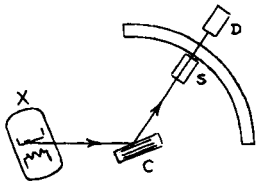

Fig. 3.—Schematic arrangement for X-ray absorption studies. The X-ray tube is at X, the analyzing crystal at C, the sample at S, and the detector at D.

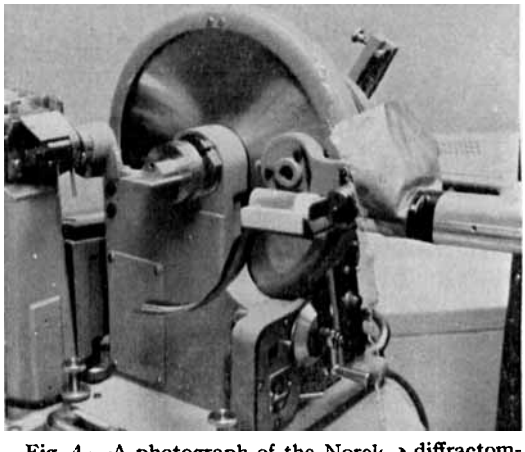

Fig. 4.—A photograph of the Norelco diffractometer set up for X-ray absorption work. (The ray scatter shield has been removed for this photograph. It is normally in place during operation.)

TABLE I.—RESULTS OF REPRODUCIBILITY STUDY OF X-RAY ABSORPTION EDGE ASSAY OF THYROID EXTRACT CRUDE[a]

Sample No.	Result, %	Deviation from Av.
1	0.79	−0.04
2	0.79	−0.04
3	0.82	−0.01
4	0.84	+0.01
5	0.87	+0.04
6	0.84	+0.01
7	0.84	+0.01
8	0.82	−0.01
9	0.82	−0.01
10	0.82	−0.01

[a] Same lot used throughout.

this fashion, the method becomes independent of the chemical assay.

Finally, a working equation for per cent iodine in thyroid extract can be derived from (2):

$$\% \text{ Iodine} = \frac{\log I''/I'}{\text{wt. sample}} \times 24.8 \quad \text{(Eq. 3)}$$

The full derivation of this equation is given in the paper by Barieau (1). The constant term 24.8 represents the $\mu'_m - \mu''_m$ term, a factor to convert logarithms from the natural base to the base 10, and a factor to convert to per cent of iodine.

The measurement of I'' and I' cannot be made directly at the absorption edge. It is necessary to measure intensities at some distance from the edge and extrapolate to the edge. This leads to a graph as shown in Fig. 2. In Fig. 2 the intensity of the X-rays is measured in counts per second (c/s) with a Geiger counter. The distance from the absorption edge is measured in degrees 2θ which is related to the wavelength of the X-rays by the familiar Bragg equation used in X-ray diffraction work.

INSTRUMENTATION

A Norelco X-ray diffractometer with a standard copper target X-ray tube was used for this work. The X-ray generator was operated at 45 kv. and 20 ma. This method requires the use of the "white radiation" which all X-ray tubes emit along with their characteristic radiation. A schematic diagram of the apparatus is given in Fig. 3. A photograph of the apparatus is shown in Fig. 4. The X-ray tube is at X. The diffracting crystal is at C. For this work a crystal of sodium chloride taken from a broken infrared absorption cell was used. The crystal was trimmed so that it fit in a holder of the type ordinarily used to hold the powder sample for crystallographic studies. This crystal "analyzes" the X-ray beam from the X-ray tube and provides a relatively monochromatic X-ray beam of the proper wavelength to pass through the sample. The sample, to be described below, is at S. The radiation detector is at D. In this work a Geiger tube was used. While a Geiger tube cannot count as fast as scintillation counters or flow counters, it has sufficient speed for the work presented here.

The essential point of the instrumentation is that it interferes as little as possible with the crystallographic use of the apparatus. The slit system used to define the X-ray beam for absorption studies is the same as that used for crystallographic work so that there is no time lost in changing from one mode of operation to the other.

The samples used in this work were disks of thyroid extract made by pressing the powder in a 0.75-in. die at about 10,000 p.s.i. The disks were weighed to determine the weight of sample. It was found that a 5-Gm. sample was adequate for the crude material; a 15-Gm. sample was used for the U.S.P. powder. Fortunately, neither preparation gives much trouble by sticking to the die. Because the samples used for determination of the crude were much smaller than those used for the U.S.P. powder, it was advisable to reduce the over-all transmitted intensity by placing a block of Lucite in the beam while running samples of crude. This does not change the absorption due to the iodine content of the sample.

The radiation counting was done on a fixed count basis. At wavelengths shorter than the absorption edge 32,000 counts were taken for each point, while at longer wavelengths 64,000 counts were taken.

RESULTS

The X-ray absorption edge assay has a peculiar advantage over other types of instrumental assays because it can be set up based on standards not of the substance itself but on elemental standards. That is, for the assay of iodine it would be possible to evaluate the $(\mu'_m - \mu''_m)$ term of Eq. 2 not on thyroid extract itself but on elemental iodine. In this work potassium iodide solutions were chosen for ease of handling. Because of this choice, the assay results of the X-ray absorption technique are independent of any systematic error which may be present in the chemical assay and which would be included if the X-ray absorption method had been based on a standard thyroid extract sample.

TABLE II.—COMPARATIVE RESULTS OF X-RAY ABSORPTION EDGE ASSAY AND CHEMICAL ASSAY OF THYROID EXTRACT

Lot No.	Product Type	Chemical	X-Ray
761476	Crude	0.86%	0.83%
761478	Crude	0.68%	0.68%
776573	Enseal	0.0728 mg. iodine/Enseal	0.0739 mg. iodine/Enseal
771318	U.S.P. powder	0.24%	0.22%
771320	U.S.P. powder	0.24%	0.21%
763648	U.S.P. powder	0.19%	0.18%
767265	Crude	0.705%	0.720%
773301	Crude	0.825%	0.845%
778288-1	U.S.P. powder	0.203%	0.205%
778288-3	U.S.P. powder	0.209%	0.187%
778288-4	U.S.P. powder	0.206%	0.206%
778288-5	U.S.P. powder	0.207%	0.217%
773302	Crude	0.859%	0.890%

Table I shows the precision of this assay method. The indicated 1Σ standard deviation calculated from these data is ±0.025. This amounts to about ±3% of the amount present.

Table II shows a comparison between the U.S.P. chemical assay and the X-ray absorption edge method. The X-ray values are all single assays, the chemical assays are the final resul. reported by the Lilly control division and are averages of several assays.

CONCLUSION

The X-ray absorption edge assay is a useful method. In the case of the iodine control of thyroid extract the values obtained by X-ray are comparable to those obtained by the chemical method.

The X-ray method requires, in our hands, about 1 hour. This allows about 0.5 hour for measurements and 0.5 hour for preparation and calculations.

It is important to realize that the method may be applied to other elements. Using the relatively simple apparatus described here, elements having an absorption edge in the region from about 0.3 Å. to about 2.0 Å., that is, from cerium to manganese in atomic number can be determined. With a more complicated apparatus, the list of elements can be expanded.

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Kinetics and Mechanism of Isomerization and Hydrolysis of 4,6-Diamino-1-(3,5)-dichlorophenyl-1,2-dihydro-2,2-dimethyl-1,3,5-triazine in Dilute Aqueous Solution

By D. H. SZULCZEWSKI, C. M. SHEARER, and A. J. AGUIAR

Rate measurements, concurrent with reaction product identification, indicate that there are three routes by which inactivation of the title compound can occur: (*a*) unimolecular isomerization of free triazine; (*b*) unimolecular isomerization of protonated triazine; (*c*) acid catalyzed hydrolysis of protonated triazine. The relative importance of these reactions on drug decomposition for various pH values is determined. Likewise, rate studies at critical pH values and several elevated temperatures enable accurate estimation of rate of degradation at any temperature. Equations are derived relating specific rate to both pH and temperature. Identification of reaction products by means of thin-layer chromatography shows that besides the 6-dichloroanilino isomer and 3,5-dichlorophenyl-biguanide hydrolysis product, *N*-3,5-dichlorophenyl-*N'*-guanylurea is formed as a secondary product of degradation.

In recent years a variety of substituted dihydrotriazines has been reported in several areas of chemotherapeutic research (1–3). Although many dihydrotriazines have been prepared and screened, there is little information available concerning their stability in aqueous systems. Likewise, information concerning the likely route and rate of degradation is meager.

In a communication by Carrington *et al.* (3) concerned with isolation, identification, and synthesis of the active metabolite of chlorguanide hydrochloride,[1] isomerization and hydrolysis of 1-phenyl-dihydrotriazines to nonactive products was noted. This suggests that loss of chemotherapeutic activity is possible and that relative rates of destruction need to be known to determine the feasibility of liquid formulations having a suitable shelf life.

The following study, concerned with the kinetics of isomerization and hydrolysis of 4,6-diamino - 1 - (3,5) - dichlorophenyl - 1,2 - dihydro-2,2 - dimethyl - 1,3,5 - triazine[2](I) in aqueous buffered solution, was carried out to determine these rates and to determine the chemistry of the degradative process.

RESULTS AND DISCUSSION

Order and Nature of Degradative Process.—Representative data obtained on the rate of degradation of I in aqueous buffered solution at 45° is shown in Fig. 1. This, together with studies at other temperatures, indicates that in aqueous buffered solution the decomposition of I is first order with respect to I over a broad pH range.

The rate of degradation was not influenced by changes in concentration of buffer (Table I). Likewise, the observed rate remained constant in

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Presented to the Scientific Section, A.Ph.A., Miami Beach meeting, May 1963.

[1] Marketed as Paludrine by Ayerst Laboratories.

[2] Parke, Davis ABT-15, 251.