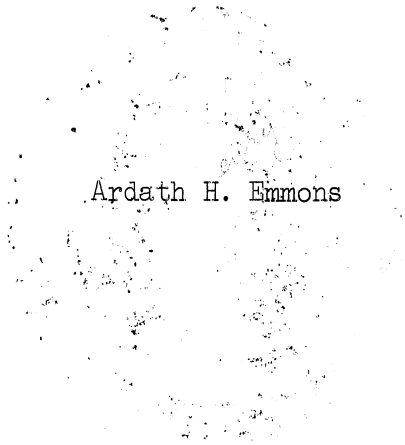
THE UNIVERSITY OF MICHIGAN

INDUSTRY PROGRAM OF THE COLLEGE OF ENGINEERING

RESONANCE RADIATION EFFECTS OF LOW ENERGY MONOCHROMATIC X-RAYS ON CATALASE



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I. INTRODUCTION

A. Definition of Problem

Existing theories of the biological effects of radiation treat these effects as arising from two types of interaction. The absorption of energy in a molecule giving rise to damage in that specific entity is called a direct action of the ionizing radiation. An indirect action is one in which the ionizing radiation produces ions and radicals in the solvent. These species, in turn, interact with the solute molecules producing biological damage. In both cases the spectrum of damage is usually considered to be similar to that of the absorption of the radiation. In the biological studies reported in the literature to date, this assumption appears to be quite valid. The work reported here seeks to determine if, in addition to these two interaction modes, there is another type of interaction which might be termed a resonance interaction. The intent of the investigation reported here was to look for the existence of resonance phenomena in a particular biochemical system.

Varying from the established pattern of approach to the study of the biological effects of radiation (in which one equates the effect produced to the amount of energy absorbed), this study seeks to determine if a discrete energy x-ray is capable of producing damage in excess of that produced at x-ray energies slightly higher and slightly lower. This type of interaction phenomenon will be referred to as a resonance effect. It is possible to postulate two cases of absorption of radiation from which a resonance effect might result.

Case I In which damage is produced by the absorption of the radiation in a heavy atom of high atomic cross section, producing energy concentration at a particular point in the molecule.

Case II In which damage is produced by that absorption of the radiation in a particular atom to produce concentrated biological damage because the atom is located in a vulnerable spot in the molecule. By precise control of the energy of the impinging radiation, more or less energy per 100 ev totally absorbed is absorbed at this spot.

It has been suggested that the biological (or chemical) effects of an excitation process should closely approximate those of a normal ionization event. Platzman[89] suggests that, as a consequence of the refinement of the meaning of excitation and ionization events, "there may exist unknown dependencies on energy or character of radiation."

This thesis is an attempt to seek out heretofore unknown responses in catalase to radiation in a limited energy region centered about the k-energy levels of iron.

Figure 1 is a simplified energy-level diagram for an atom.[29] It is simplified in that the L, M, and N levels are considered as single levels, whereas they are actually groups of closely spaced levels. The diagram illustrates that an amount of work, W_k, must be done to remove a K electron. The atom is then said to be in the K energy state. If an impinging x-ray quantum has energy less than W_k, where:

$$W_k = h\nu_k = \frac{hc}{\lambda_k}$$

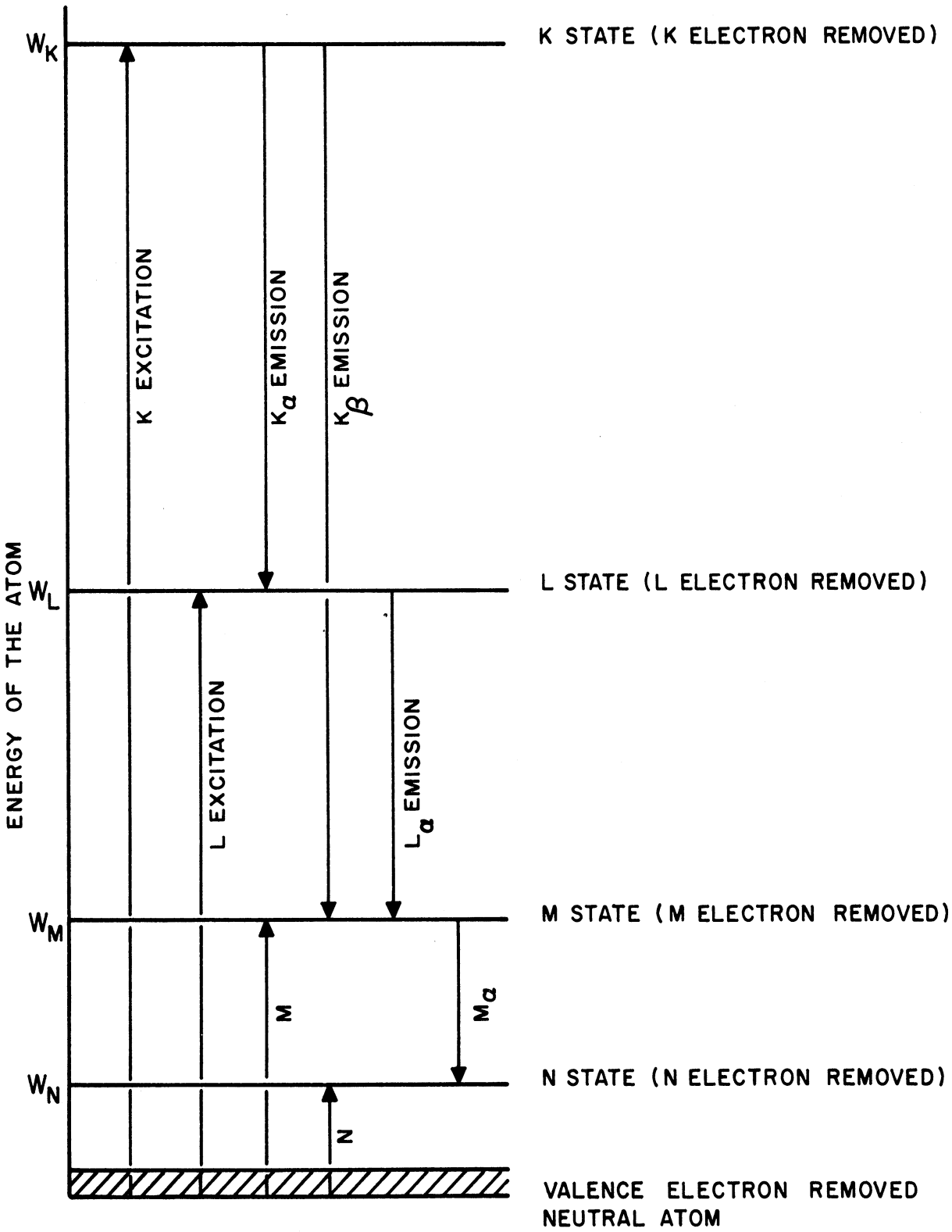


Figure 1. Simplified Energy Level Diagram of an Atom.[29]

and h = Planck's constant

ν_k = frequency

c = velocity of light

λ_k = wave length

this quantum is not capable of raising the atom to the K energy state, but may raise it to any lower energy state. The K, L, and M energy states for iron are known and identified as the absorption edges. The K absorption edge for iron occurs at 7.113 Kev, the three L edges L_1, L_2, and L_3, are at 849, 722 and 709 ev respectively, while the M edge occurs at 93.7 ev.

If one plots the x-ray absorption coefficient for iron as a function of energy, one observes a sharp fluctuation at precisely 7.11 Kev corresponding to the iron K-edge. If, on the other hand, one sums (with appropriate weighting) the absorption coefficients of the elements constituting a large complex organic molecule containing a trace of iron (as is done for catalase in Section II of this thesis) and plots these data, the x-ray absorption curve is a smooth, slowly varying function of energy with no sharp fluctuations. If the radiation effect observed in this region is a similar smooth curve, the existence of resonance effects will be ruled out for the energy region under consideration.

B. The Molecular System Tested

The biochemical system selected for this study was the enzyme catalase. The enzyme has a molecular weight of 225,000 with an iron content of 4 iron atoms or approximately 0.09 percent iron by weight. It functions in the catalytic destruction of hydrogen peroxide. The

destruction of the hydrogen peroxide is attributed to the iron content of this enzyme.[19,38,52]

A large number of studies of the effects of radiation on catalase have been reported in the literature. The radiation effects reported may be classified either as a direct effect on the dry catalase molecule or an indirect effect arising from the production of free radicals in the solvent (water) system.

The enzyme is not an easy one with which to work. It is heat sensitive and loses its catalytic function with time at elevated temperatures. It also displays a high sensitivity to ultra-violet radiation, losing its enzymatic capability very rapidly when subjected to high intensity ultra-violet light.

A technique has been developed which permits one to measure the reaction rate between catalase and hydrogen peroxide over the first one minute of interaction and, in turn, permits the evaluation of the catalase concentration as a function of the monomolecular reaction rate.

C. Review of Pertinent Literature

The literature survey which follows is limited to references on (a) the effects of irradiation of enzymes and (b) the existence of a wave length (energy) dependence. A comprehensive bibliography constitutes the last entry of this report. This bibliography is arranged alphabetically, therefore reference numbers will appear in a non-ordered sequence.

In June, 1927, Fricke and Peterson [48] published an account of the study of the transformation of oxyhemoglobin to methemoglobin

resulting from the action of x-radiation of varying wave length. They found that from 0.527 to 0.754 angstroms (23 Kev to 16.5 Kev) the amount of chemical action was independent of the wave length of the radiation. Fricke and Morris[49] reported in November of 1927, on a study of the oxidation of ferrous sulphate to ferric sulphate. Again they found that the chemical effect produced, the oxidation of iron per unit energy absorbed, was independent of wave length from 0.204 to 0.765 angstroms (62 Kev to 16 Kev). (It was as a result of this latter investigation that Fricke proposed the use of the ferrous-ferric dosimetry technique.) As a result of these studies they postulated that the oxidation of the ferrous iron was a secondary effect produced by the action of radiation on the water solvent and dependent on the dissolved oxygen in the solution.

Hussey and Thompson [65, 66, 67, 68, 69], during the period from 1923 to 1926 report a series of experiments on the effects of x-rays on the enzymes trypsin, pepsin and invertase. They found that all three of these enzymes were inactivated in solution form. Rothstein [103] also reported in 1927 on the inactivation of trypsin by x-rays. Clark and Northrop[23] reported a series of experiments on the same enzyme in September, 1925. All of these results indicated inactivation of the enzyme in solution. None of these authors studied the wave length dependence of this inactivation.

In 1930, Wyckoff [125] reported a study on the "Killing of Colon Bacilli by X-rays of Different Wave Lengths." He irradiated bacilli at wave lengths of 0.564, 0.710, 1.537, 2.29 and 3.98 angstroms (corresponding to energies of 22 Kev, 17.5 Kev, 8.1 Kev, 5.4 Kev, and 2.5 Kev). In each

case he obtained a straight line on semi-log paper when plotting the log fraction surviving versus dose. He reported that it required more quanta at low energies to attain the same inactivation.

In 1941, Lea[79] published a paper titled "The Dependence of the Biological Effect of Radiation on Intensity and Wave Length," in which he compared the wave length dependence of gamma and x-irradiation. He found a ratio of effectiveness, as measured by chick embryo death, of 1 to 0.60 to 0.55 for energies of 0.014 angstroms, 0.15 angstroms and 0.363 angstroms respectively. For chick embryo death, he found the lethal dose to be dependent on the dose rate up to a certain point, and then rate independent after this point. He presents a theory postulating a recovery time for the biological material which is based on the accumulation and destruction of a chemical poison.

In 1944, Lea, Smith, Holmes and Markham[83] described direct and indirect actions of radiation on viruses and enzymes. They irradiated dry preparations of myosin and ribonuclease with x-rays of wave length 1.5 angstroms (8.3 kev). They found that it required a dose of 3.4×10^7 roentgens to reduce the activity of the ribonuclease by a factor e^{-1} (i.e. 37 percent of its initial value) and for the enzyme myosin, the 37 percent dose was 5.5×10^6 roentgens. Their results indicated that the inactivation of the enzyme was a one-hit phenomenon. In this paper, arguments are presented which allow calculation of the molecular weight of a virus or an enzyme from the 37 percent dose of x or gamma radiation.

In 1940, Dale[33] studied the effect of x-rays on enzymes. He irradiated the two enzymes carboxypeptidase and polyphenoloxidase with x-rays. His experiments were performed using comparatively high energy x-radiation. About half of the experiments used a 450 kev x-ray tube

producing radiation of half value layer 6 millimeters copper equivalent. The remainder of the radiations were performed with a 250 Kev machine producing radiation of half value layer 1.7 millimeters of copper. He used dose rates of approximately 1000 r per hour and all of his irradiations were done on solutions of the enzymes. He discusses the biological significance of his experimental results and concludes that the effects of radiation on enzymes in a cellular system may be of much more importance than indicated in earlier publications. He arrives at this conclusion in light of his determination that the dose required to inactivate an enzyme is low for low concentrations of the enzyme.

Dale (31), in a paper published in June, 1943, makes a fairly comprehensive summary of "The Effect of X-rays on Aqueous Solutions of Biologically Active Compounds." In this paper, Dale points out that a dose of 50 r inactivated about 30 percent of a very dilute solution of the enzyme carboxypeptidase, whereas a dose of 100,000 r was required to produce the same relative effect on a solution 345 times more concentrated. He concludes that this dilution phenomena can be understood by one assumption only; that the radiation acts primarily on the water which in turn produces the effect on the enzyme. He states that this fact has been insufficiently appreciated in the past and therefore enzymatic reactions have been labeled as radio-resistant because solutions of too high a concentration have been examined.

In 1945, Forssberg (42) reported on the effect of x-rays on catalase. His investigations were performed with pure crystalline catalase in buffered water solutions using a 155 Kev machine filtered by 1 millimeter aluminum and 1 millimeter glass. The results of these investigations

may be summarized as follows: 1) he found greater enzymatic activity after irradiation in vivo; 2) he found the inactivation of catalase solutions to be dose rate dependent; 3) he found a certain amount of enzymatic activity recovery at pH greater than 6; 4) he found that all proteins afforded the enzyme some protection; 5) he found that oxidizing agents dissolved in solution with the enzyme accelerated inactivation. This is an excellent paper, presenting a wealth of information on the radiation effects on solutions of catalase.

In 1946, Forssberg[44] published a study of the action of roentgen rays on the enzyme catalase, and in 1947, he [43] presented a paper titled, "The Mechanism of the Action of X-rays on Enzymes in Water Solution." For this study he used crystalline catalase dissolved in water to a concentration of 3×10^{-6} grams per ml at pH 7.4. He added various substances at concentrations of 0.1 to 0.2 millimoles and measured the inactivation of the enzyme solution produced by a dose of 12,440 roentgens at a dose rate of 275 r per minute. He evaluated the protection afforded by various substances added to the enzyme solution. He found an inactivation of 54 percent for the enzyme alone and various lesser inactivation values when the enzyme was protected by any of a number of materials.

In 1949, Dale, Gray and Meridith[35] reported inactivation of carboxypeptidase in aqueous solution by x and alpha radiation. They concluded that the inactivation occurs as an indirect effect. Their yield was 0.18 enzyme molecule inactivated per ion pair independent of the enzyme concentration from 10^{-4} to 10^{-1} grams per ml. They could not

produce the inactivation with hydrogen peroxide concentrations of up to 0.1 molar.

Feinstein, et al.[40] in 1950, reported on the effect of whole body x-radiation and of intra-peritoneal hydrogen peroxide on mouse liver catalase. They demonstrated a sharp reduction of liver catalase in mice subject to 800 r at a dose rate of 17 r per minute. They also demonstrated a loss of liver catalase from hydrogen peroxide injection. They suggest the possibility of hydrogen peroxide toxicity.

In 1953, Setlow and Doyle[110] published a paper on "The Effect of Temperature on the Direct Action of Ionizing Radiation on Catalase." Setlow and Doyle studied the inactivation of catalase by deuteron irradiations at temperatures of 90 to 380 degrees Kelvin. They found 3 sensitivity plateaus as a function of temperature. The catalase was more sensitive to deuteron radiation at room temperatures. The plateaus in their results are attributed to the inactivation of sub-units of catalase.

Barron[8], in 1954, published a review paper on "The Role of Free Radicals and Oxygen in Reactions Produced by Ionizing Radiations." He confines his consideration to reactions of biological interest produced by ionizing radiations of moderate intensity in aqueous solutions. In his paper he refers to the yields of enzymes inactivated by x-irradiation.

Lasnitzki and Lea[75] published a paper in 1940 titled "The Variation with Wave Length of the Biological Effect of Radiation." They report on the measurements of the inhibition of division in tissue cultures

produced by gamma, hard, and soft x-radiation. They compared the relative effectiveness of x-rays and gamma rays. The x-rays were of effective wave length of 0.107, 0.150, and 0.363 angstrom units. (116, 83 and 34 Kev.) They found that the biological effect produced per roentgen was not significantly different for these three x-ray energies. Gamma radiation was found to be less effective than x-radiation of the given energies. The gamma radiation they used was that produced by radium.

Sutton[115,116] published on the effects of radiation on catalase solutions. He demonstrates: (1) that the inactivation of dilute aqueous solutions of catalase proceeds at a rate proportional to a power (n) of dose rate, where n is less than one, (2) that this dose rate dependence results from protection of the catalase by hydrogen peroxide generated by the radiation, (3) that dilute catalase solutions are protected against x-ray inactivation when the iron centers are complexed with hydrogen cyanide, (4) that the protein portion of the catalase may be damaged by radiation without loss of catalytic activity, and (5) that the major inactivating effect of x-rays on catalase solutions is localized on the iron centers.

Three references most pertinent to the problem under consideration are an article published in 1952 by Guild[56], another published by Manoilov[86], and a thesis by Garsou.[50] Guild studied the inactivation of bacteriophage and catalase by soft x-rays. His investigation attempted to determine if the x-ray action spectrum differed from that of the absorption spectrum. He irradiated the mentioned materials with a bremsstrahlung spectrum produced by electrons with energy less than,

equal to, and greater than the absorption edge energy of phosphorus, which is at 2.14 Kev. He concludes that "the absorption of a photon by a phosphorus atom in nucleic acid is no more effective for inactivation of T-1 bacteriophage then absorption by any other atom."

Manoilov irradiated frog hearts, isolated by the Straub method, with x-rays generated in x-ray tubes with anodes of iron, copper, nickel, and cobalt. The irradiation conditions for all tubes were: 30 kv, 10 ma, 360 r per minute for 30 minutes. The frog hearts were isolated immediately after irradiation and placed in a barochamber which was evacuated to 80 mm Hg. The unirradiated hearts, and those irradiated with the iron and cobalt anode x-ray tubes, functioned for 20 to 30 minutes. The hearts irradiated with copper and with nickel anode x-ray tubes ceased to function, in 70 to 86 percent of the cases, in a time period of 3 to 15 minutes after being placed in the low pressure chamber. It is the author's conclusion that the direct action of x-rays on the cytochrome molecules produces this effect. He postulates that the x-rays from nickel and copper eject a k-electron from the iron atom of the cytochrome molecule and produce damage, however no specific tests to check this hypothesis were made. This study by Manoilov is directly related to the content of this thesis.

The first tests in which monochromatic photons were used in which the energy of the photon corresponded to the energy region of the k-absorption edge of a particular atom in a molecule were made in this laboratory by J. Garsou. Garsou[50] in his doctoral dissertation, reported on the effects of monochromatic x-rays on some simple halogenated hydrocarbons. His investigations preceded and accompanied the studies reported in this thesis.

Garsou deduces from his data that the organic compounds he investigated contain a target atom; the halogen. He found (1) that greater damage (decomposition) was produced at x-ray energies slightly higher than the k-absorption edge energy of the target atom, and (2) that a peak response to radiation occurred when the energy of the impinging x-rays was in the vicinity of the k_{α_1} and k_{α_2} emission line energies of the target atom. The first indications of these effects initiated this thesis investigation.

Garsou studied compounds in which the target atom constituted more than 90 percent of the molecular weight. This thesis reports on the studies of a much more complex organic compound (catalase) in which the target atom (iron) makes up less than 0.1 percent of the molecular weight.

There are a number of references in the literature on the application of radiation to the determination of molecular characteristics of complex organic entities.[95,96,97,98,106,107] These authors have employed deuteron (or electron) beams to determine molecular weight and physical size of viruses, enzymes, and antibodies.

D. Irradiation Techniques Available

This brief literature survey cites a number of references to studies of the wave length dependence of various chemical and biological effects. In most instances the studies reported were conducted in a search for response changes of magnitude two to one or, at most, three to one. These studies usually covered from two to five different energy values separated by thousands of electron volts.

The usual method of specifying x-ray energy has been in terms of half-value layers, pkv, or average energy. In no instance do these precisely define the x-ray energy. Manoilov,[86] in the study of frog heart response, utilized the emission lines of differing anode materials. This technique yields a series of intense, well defined lines characteristic of the tube anode material, but this characteristic spectrum is a minor part of the total x-ray output of the tube. The major portion of the x-rays emitted by the tube come from the continuous (or Bremsstrahlung) spectrum.

There are only two (readily apparent) techniques for attaining x-rays of precise energy. One of these involves the use of a crystal spectrometer. It is possible in this way to select with good precision a very narrow band of x-ray energies for irradiation purposes. The disadvantage is the low x-ray yield due to extremely low diffraction efficiencies. This leads to long irradiation times in an experiment. The second technique is to employ a high-output beryllium-window x-ray tube to obtain line spectra by fluorescence in selected target materials. The advantages of this latter technique are twofold; firstly, there is no continuous spectrum, and second, the available energy (photon flux) is thousands of times greater than that from a crystal spectrometer. Each of these methods has been employed in the studies reported in this thesis.

II. EXPERIMENTAL PROCEDURES

A. Radiation

1. Radiation Sources

a) Monochromatic X-rays

The x-ray equipment utilized for this study consisted of two General Electric, Model XRD-5, x-ray diffraction units. With these units it is possible to select monochromatic x radiation by utilizing a crystal as a diffracting medium.

The x-ray diffraction unit provides excellent energy selection. It has a number of limitations when used in biological investigations; the primary one is its inability to give high dose rates. If a high dose rate is desired, one is forced to increase the tube current to its maximum value. Additional increases in the exciting potential applied to the x-ray tube then gives decided increases in the number of photons diffracted at the desired energy. The difficulty with this procedure is that when the exciting potential exceeds the selected x-ray energy by a factor of two, one gets second order diffraction from the crystal as a contaminant. The intensity of this contamination is a function of the exciting potential (pkv) of the x-ray tube.

The diffraction of x-rays is described by the Bragg equation:

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

where n is the order of the diffraction line, θ is the incident angle on the diffracting crystal, λ is the wave length of the diffracted radiation, and d is the lattice (or grating) spacing of the crystal used for diffraction.

In all the studies reported in this thesis, the crystal used in the diffraction unit has been sodium chloride.

Figure 2 is a photograph of the General Electric XRD-5 diffraction unit, showing, on the left, the scaler, rate meter, timer, and recorder assembly; in the center, the goniometer assembly with the tube mounted and a proportional counter detector unit in position. On the right is the high voltage power supply for the x-ray tube.

Figure 3 is a photograph of the diffraction crystal-spectrometer assembly (goniometer table) with a lucite sample holder in position just in front of the housing of the proportional counter. This shows the x-ray tube mounted in such a manner that the direct x-ray beam impinges upon the crystal from which it is diffracted to the sample.

Figure 4 is a photograph of the x-ray unit with an emission target holder mounted in such a way that the beam from the x-ray tube impinges upon the target material contained in the sample holder box. The target material emits its characteristic emission lines isotropically. A portion of these characteristic x-rays moves through the beam tunnel to strike the diffracting crystal. The diffracted x-rays pass through the collimator assembly into the proportional counter.

A second technique for the attainment of very nearly monochromatic x radiation is to utilize the fluorescent emission spectrum from various target materials. The General Electric Model XRD-5 diffraction unit may be utilized for study of these emission spectra. In this technique, the x-ray tube is turned 90° to the crystal (as shown in Figure 4), a sample holder box is mounted below the x-ray tube, and the full intensity (maximum pkv and maximum ma) of the x-ray emission from the tube is permitted

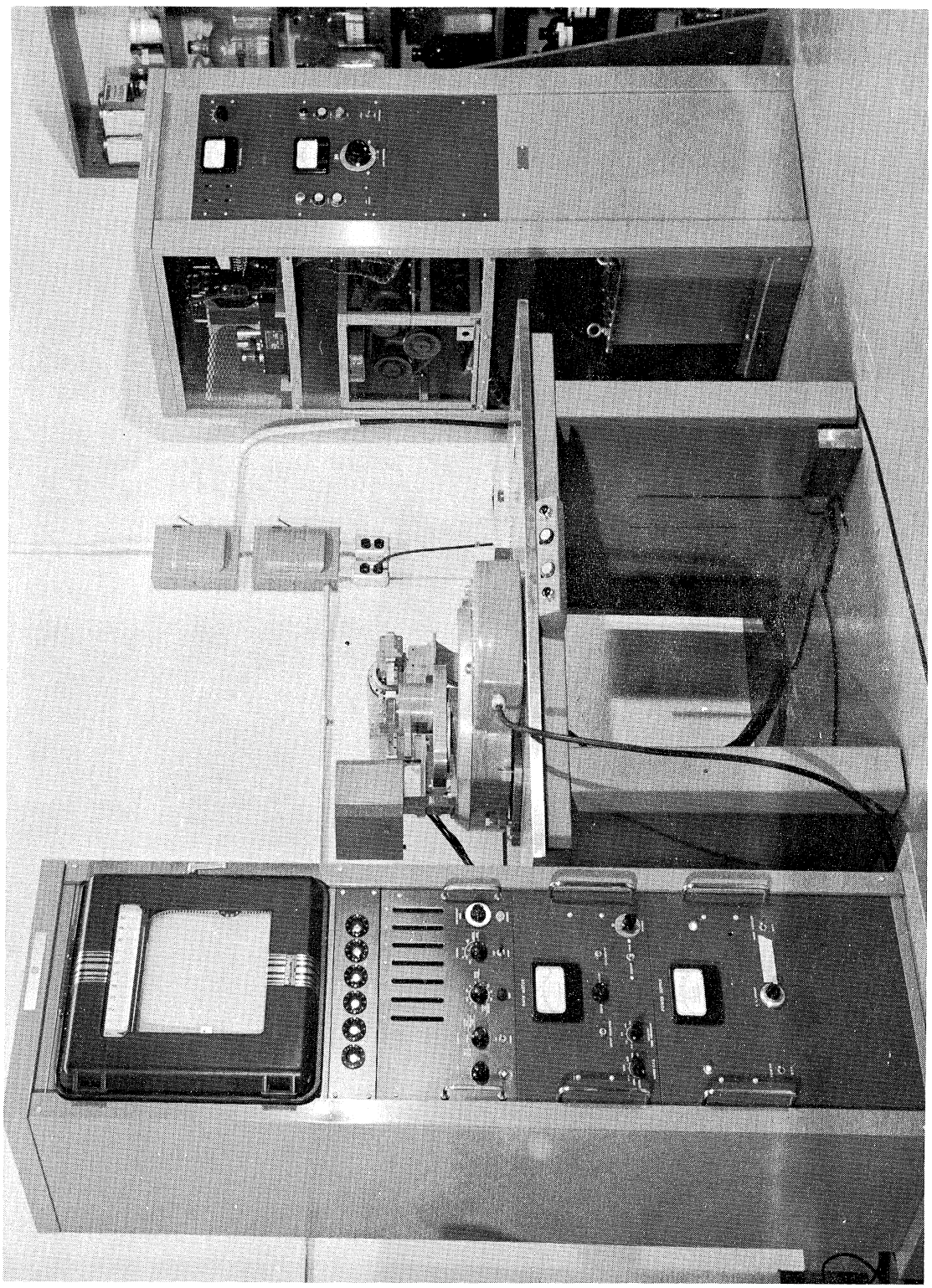


Figure 2. Photograph of General Electric XRD-5 X-ray Diffraction Unit.

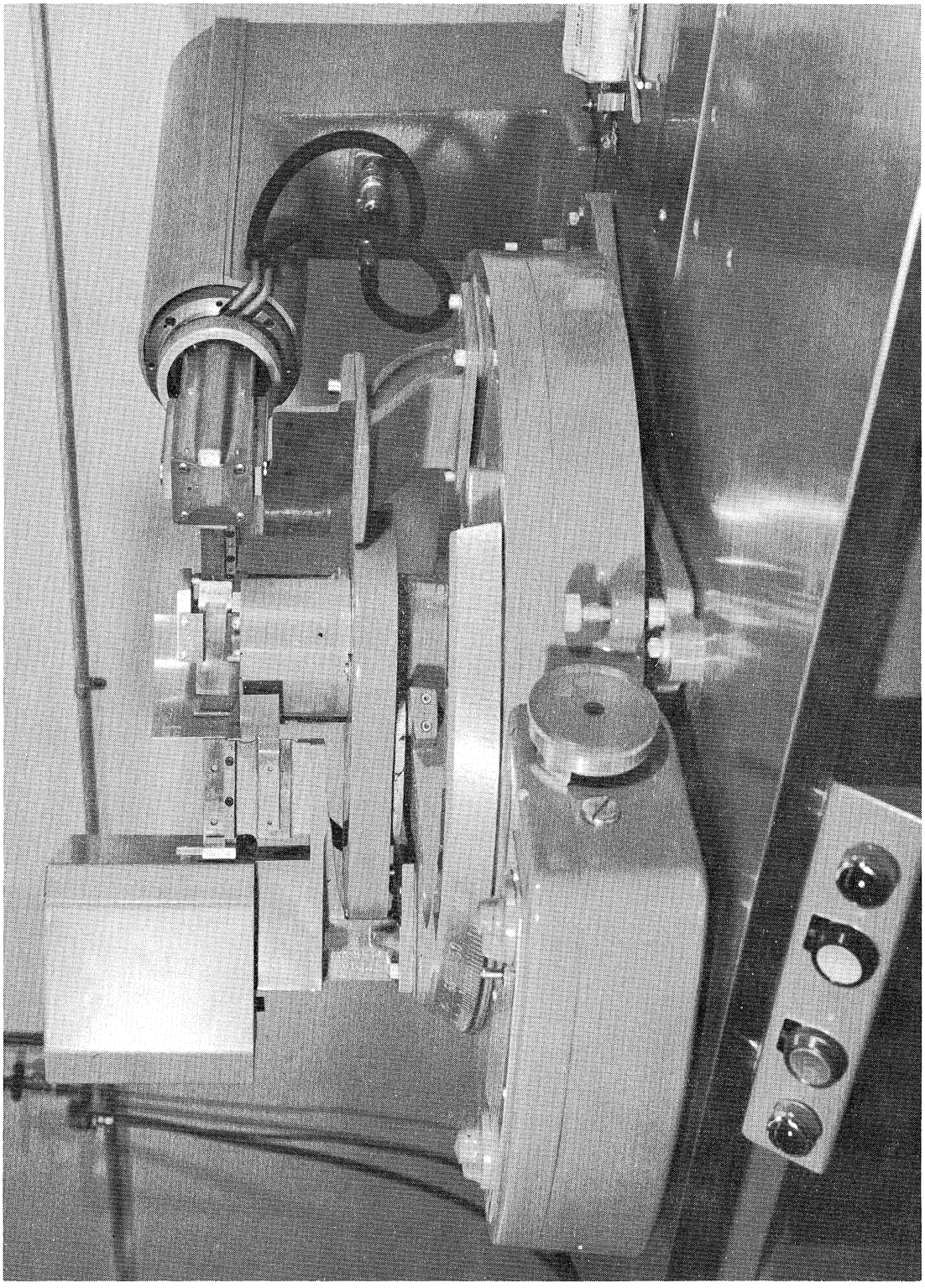


Figure 3. Photograph of Diffraction Crystal Spectrometer Assembly with Sample in Position.

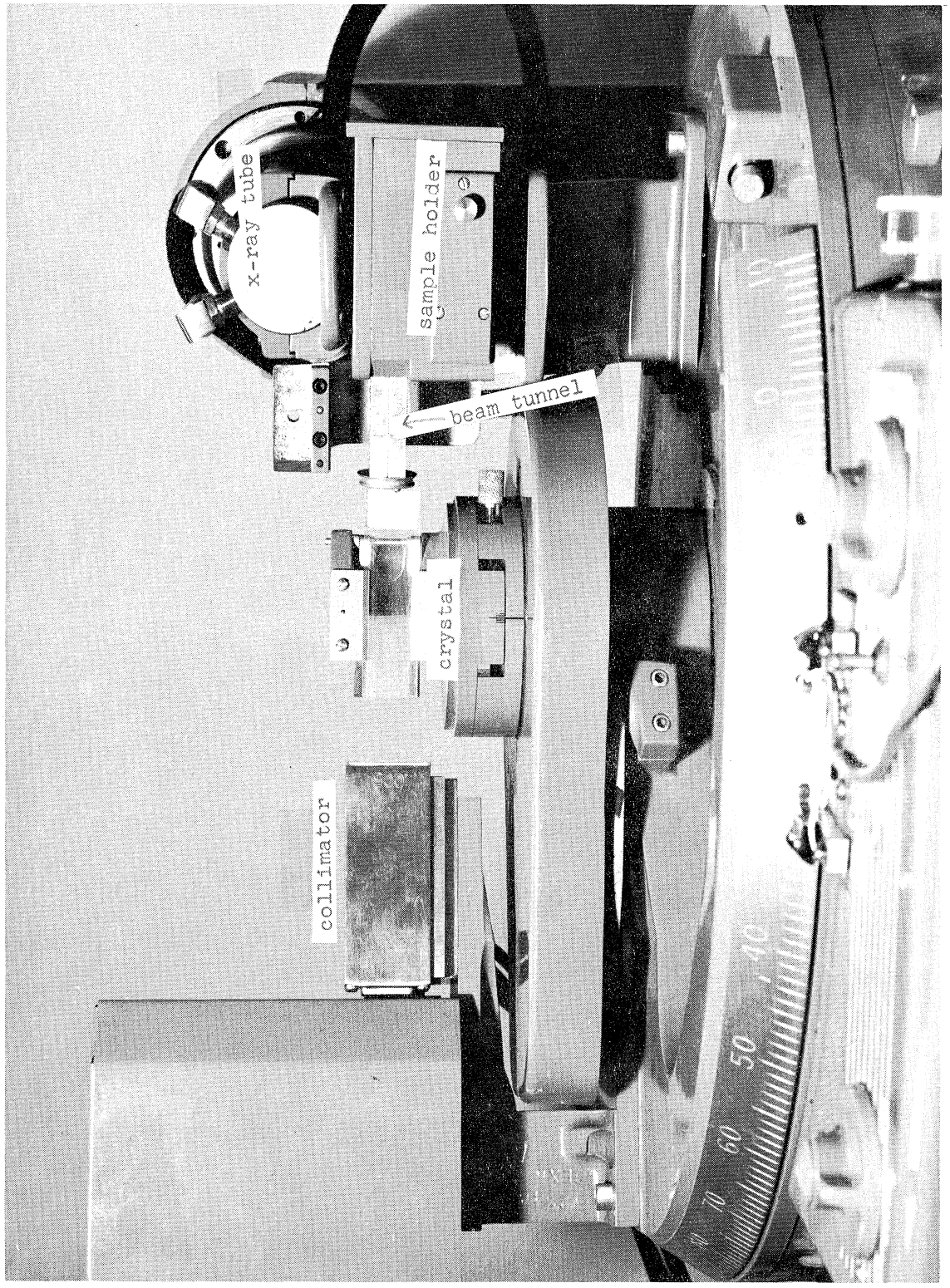


Figure 4. Photograph of X-ray Unit with Emission Target, Crystal, and Collimators for Spectrum Evaluation.

to fall on the target in the sample holder. This target then emits predominantly its characteristic x radiation emission lines. These emission lines consist primarily of the k-alpha emission characteristic of a given target material. Curves showing measured emission spectra from the various materials utilized as targets in the studies reported in this thesis are presented in Figures 5, 6, 7 and 8. These spectral distributions were measured using the arrangement shown in Figure 4.

A sample of the material to be irradiated may be substituted for the beam tunnel which is attached to the sample holder on the XRD-5 unit. Figure 9 is a photograph illustrating this arrangement. All emission line irradiations of catalase were performed in this manner.

b) Cobalt-60 Unit

In addition to the x-ray studies, a number of studies were performed with a 5000 curie Cobalt-60 gamma source to ascertain the general effects of radiation on catalase and catalase solutions. The source was calibrated using the Fricke[47] dosimetry technique. The energy of the emitted gamma rays is 1.17 and 1.33 mev. The dose rate is great enough to permit measurement of the G value* of dry catalase, as well as to allow investigations of the indirect radiation effect on catalase solutions. Appendix B contains results of these studies.

The source consists of a cylindrical configuration of cobalt rods 11" high with an inside diameter of 4", and an outside diameter of 5". In the inside well of this cylinder dose rates up to a maximum of 1.1×10^6 rep per hour are produced. The cobalt is utilized as an in-air source.

* G value = the number of molecules produced, destroyed or made to react per 100 ev of absorbed energy.

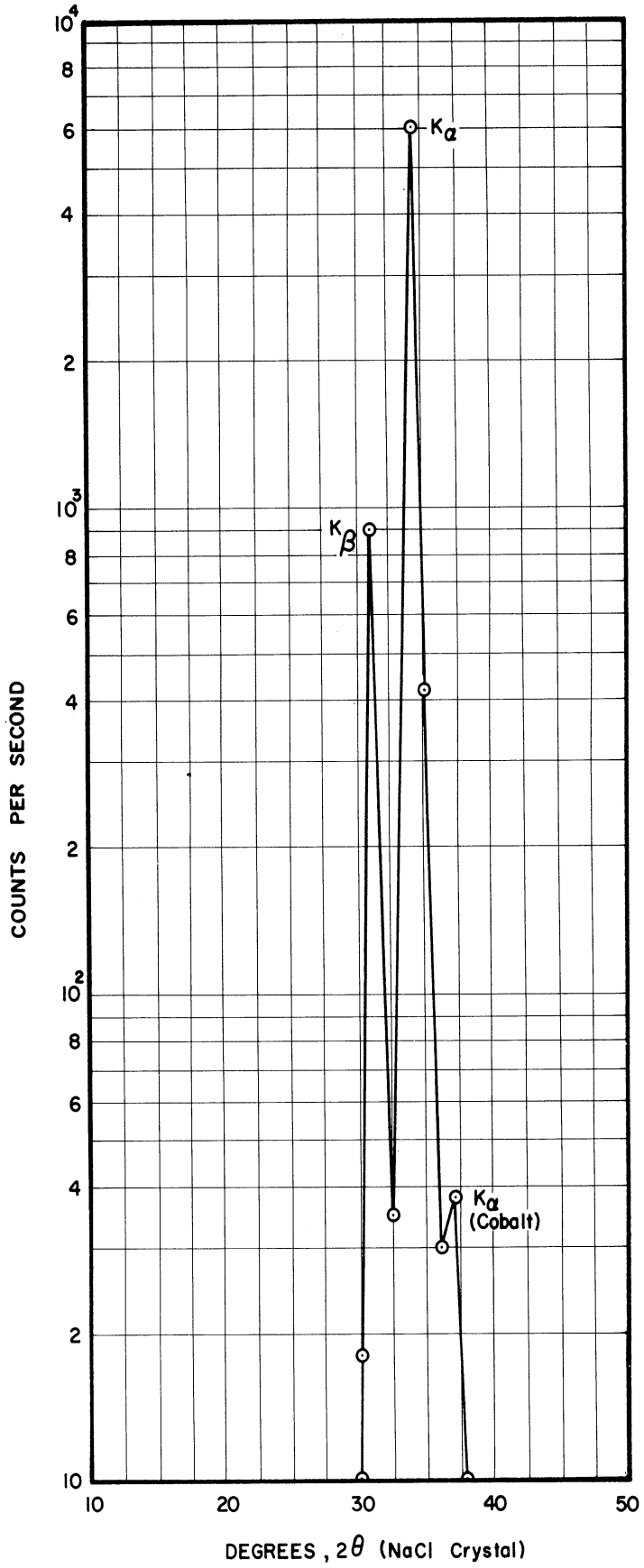


Figure 5. Measured Emission Spectrum from the Nickel Target.

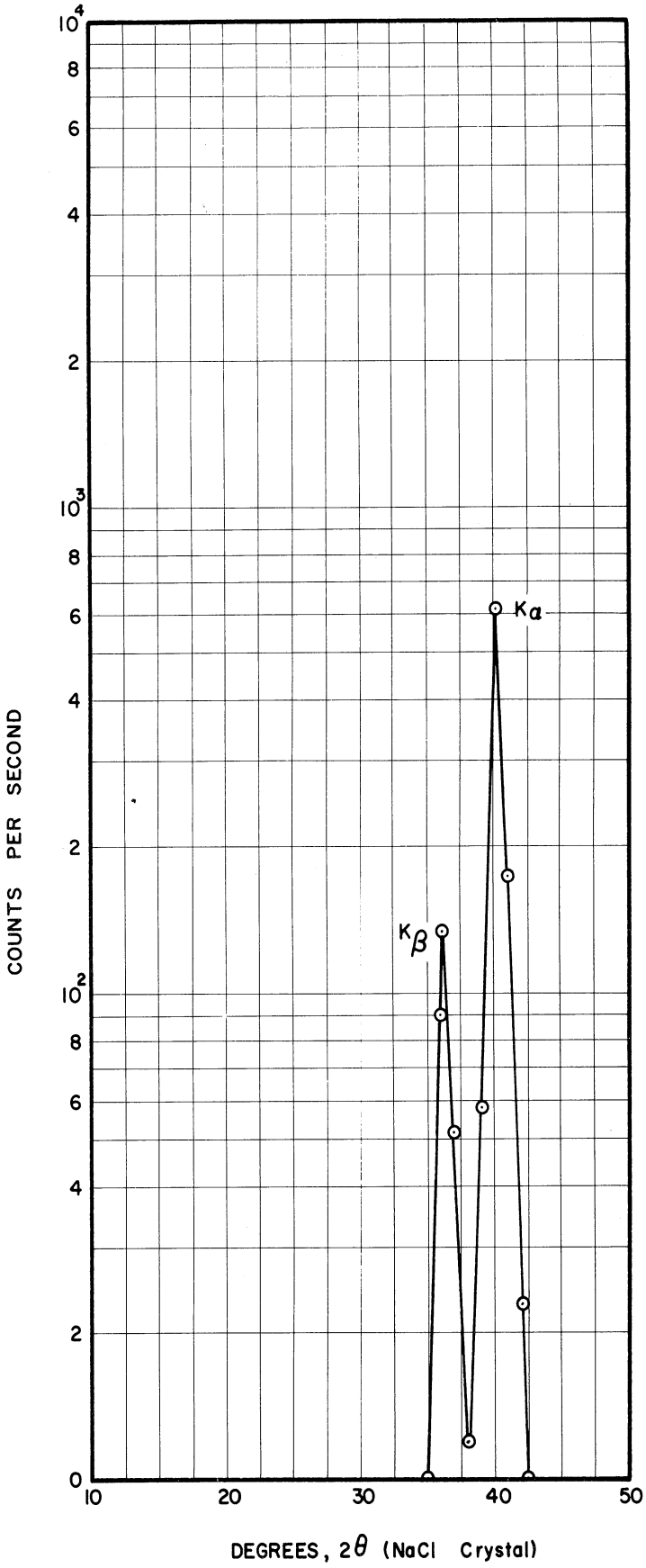


Figure 6. Measured Emission Spectrum from the Iron Target.

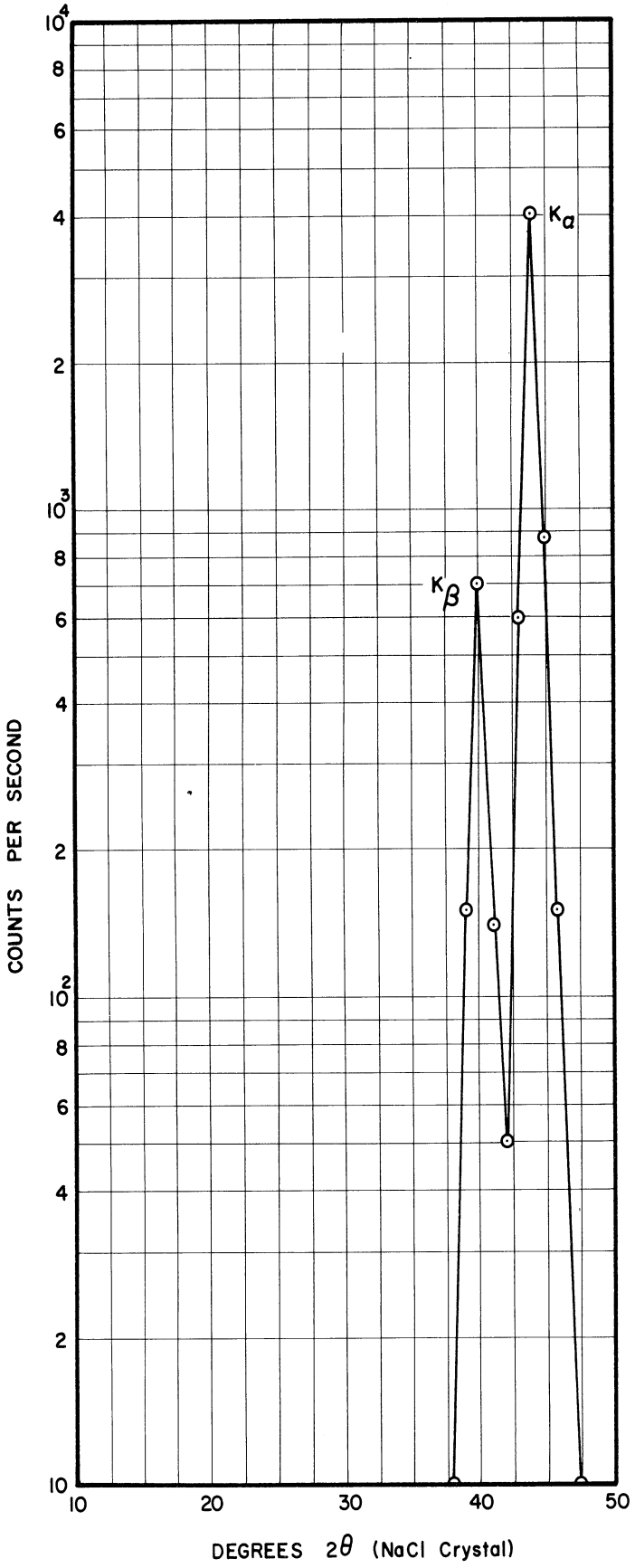


Figure 7. Measured Emission Spectrum from the Manganese Target.

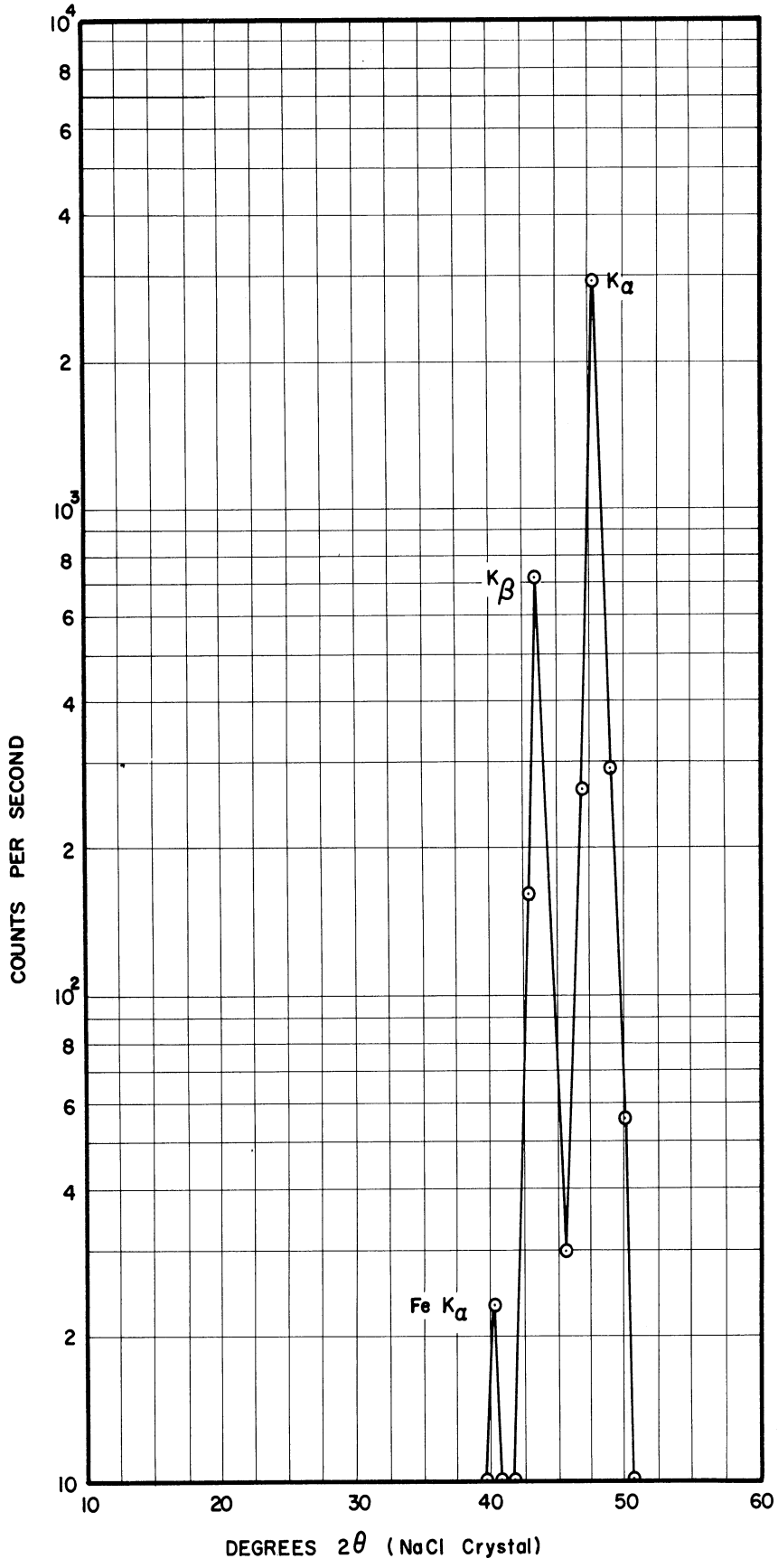


Figure 8. Measured Emission Spectrum from the Chromium Target.

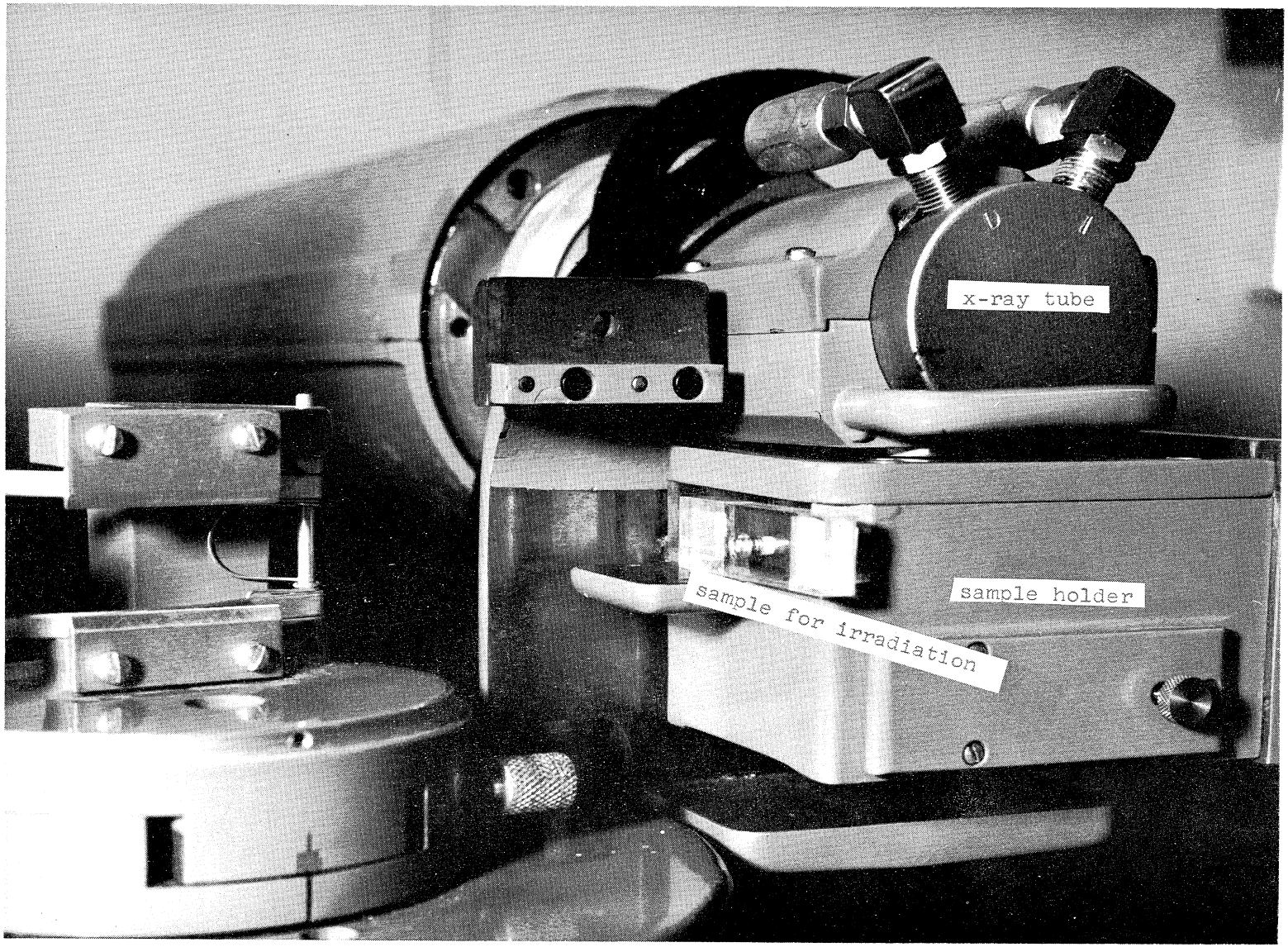


Figure 9. Photograph of X-ray Unit set up for Emission Target Irradiation of Dry Catalase Sample in Lucite Holder.

All cobalt radiations reported in this study were performed in this source facility. The facility has been in constant use since 1955, and repeated calibrations have been performed.

2. Calibration of Sources and Measurement of Radiation

Three techniques were available for calibrating the x-ray sources. The three techniques were: 1) Fricke dosimeter, 2) photon (x-ray) counter, and 3) ion chamber. The Fricke dosimeter was selected as the basis of all calibration work. The photon counter and the ion chamber were calibrated by comparison to the Fricke dosimeter.

a) Fricke Dosimeter

The Fricke[47] dosimeter, utilizing the oxidation of ferrous sulfate by primary ionic species generated in a radiated solvent, has gained wide acceptance in the last decade. It has proven to be reliable and of high precision. In application, a solution of ferrous sulfate in sulfuric acid is irradiated and the ferric-ion yield is measured spectro-photometrically at 305 millimicrons.

There are numerous evaluations of the yield (G value) reported in the literature. It appears that this yield is a slowly varying function of the linear energy transfer (LET) achieved in the water solvent.[87] The mean value of measured G values from cobalt-60 gamma radiation appears to be 15.4 ferrous ions oxidized per 100 ev absorbed. A recent measurement of the yield at 8 and 10 kev has been made by Cottin and LeForte.[25] They report a yield of 13.4 and 13.6 respectively.

A "stock" ferrous sulfate solution was prepared as follows:

3.9 gm. $FeSO_4 (NH_4)_2 (SO_4) \cdot 6H_2O$

0.12 gm. NaCl

100 ml. Triple-distilled H_2O

2.2 ml. Concentrated $H_2 SO_4$

A one milliliter portion of the stock was then diluted as follows for actual use as a dosimeter solution:

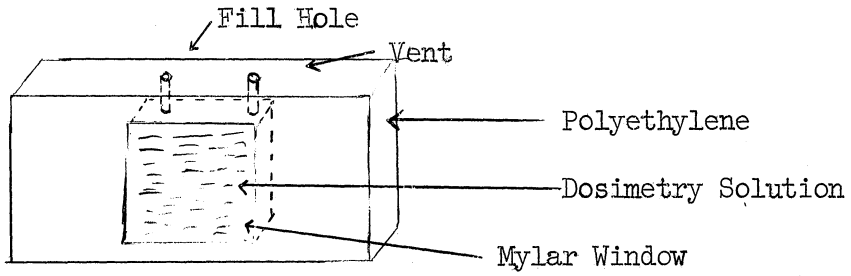
200 ml. Aerated triple-distilled H_2O

4.4 ml. Concentrated H_2SO_4

1 ml. of stock solution

This dilution of ferrous sulfate was used to measure energy outputs of the various emission targets utilized in a portion of this study.

An irradiation cell was constructed of polyethylene 0.7 cm thick. The cell was equipped with a mylar window on front and back. The cell volume was 1.95 cm^3 of ferrous sulfate solution and the window area was 2.28 cm^2. This cell, containing the dosimetry solution, was positioned to intercept the emission target x-ray beam. A sketch of the cell follows:



The cell thickness of 0.7 cm was practically black to x-rays of energy less than 10 kev since:

$$I = I_0 e^{-\mu x}$$

where for 10 kev, $\mu = 5.3\ cm^{-1}$, then for a thickness of X = 0.7 cm $I/I_0 = 0.02$. Thus at 10 kev 98% of the impinging x-rays are absorbed in the cell.

After irradiation for a measured length of time, the cell was removed and the contents transferred to a quartz cuvette. The optical density change was compared with that of a control sample, using the Beckman DU at a wave length of 305 millimicrons and a slit width of 0.5 mm. A number of runs of different duration were made for each emission target. The data were plotted as optical density change versus time and a line of best fit, as determined by visual inspection, drawn through the data. The value of optical density change after one hour of irradiation was read from the graph for each of the targets. This information, together with a value for the molecular extinction coefficient and the G value, permit one to calculate the total energy input into the ferrous dosimeter. The G value selected for these calculations is that given by Cottin and LeForte.[25] The value of 2174 liter $mole^{-1}$ cm^{-1} at 24.5°C is used as the best value for the molecular extinction coefficient.[25,62,87] The calculations are as follows:

An optical density change of one unit is a change in the ferric ion concentration of $1/\epsilon \times 6.02 \times 10^{23}$ molecules/liter

$$= 1/2.174 \times 10^{3} \times 6.02 \times 10^{23}$$

$$= 2.77 \times 10^{20} \text{ ferrous ions oxidized per liter, or}$$

$$= 2.77 \times 10^{17} \text{ ferrous ions oxidized per } cm^{3}.$$

Then the energy absorbed per cm^3 to produce an optical density change of 1 unit is:

$$\frac{2.77 \times 10^{17} \text{ ions}/cm^{3}}{13.4 \text{ molecules}/100 \text{ ev}}$$

$$= 2.07 \times 10^{18} \text{ ev}/cm^{3}$$

$$\text{or} = 2.07 \times 10^{18} \text{ ev}/cm^{3} \times 1.6 \times 10^{-12} \text{ erg/ev}$$

$$= 3.31 \times 10^{6} \text{ ergs}/cm^{3}.$$

The values of O.D. change per hour were in the neighborhood of 0.1, the total volume for energy absorption was 1.95 cm^3, and the window area was 2.28 cm^2. One may compute the beam energy per square centimeter assuming total energy absorption.

$$0.1 \ \ O.D. = 3.31 \times 10^5 \text{ ergs/cm}^3$$

and 3.31×10^5 ergs/cm^3 x 1.95 cm^3/ 2.28 cm^2

then 0.1 O.D. change = 2.84×10^5 ergs/cm^2

$$= 1.77 \times 10^{17} \text{ ev/cm}^2$$

This technique permits one to measure the beam energy of the various emission targets. Further, if one assumes complete absorption of the x-ray photons by photoelectric conversion, it is possible to compute the number of photons per cm^2 per unit time making up the beam. These data are presented in Table I.

b) Photon Counter

The General Electric XRD-5 diffraction unit is equipped with a model SPG No. 1 x-ray counter. This counter is of multicell type construction and is capable of counting rates of up to 10,000 counts per second. The counter is operated in the Geiger region, has a 65 cm Argon gas filling, and a berylium window 0.03 inches thick. In these studies, this counter was utilized for daily evaluations of the x-ray machine stability. It was used, in conjunction with the goniometer, to measure the energy spectra from the various targets (see Section II-A). An attempt was made to use it as a dosimeter. The discussion which follows present the results of calibration studies made with the SPG No. 1 x-ray counter.

TABLE I

FRICKE DOSIMETER MEASUREMENTS OF EMISSION TARGET INTENSITIES

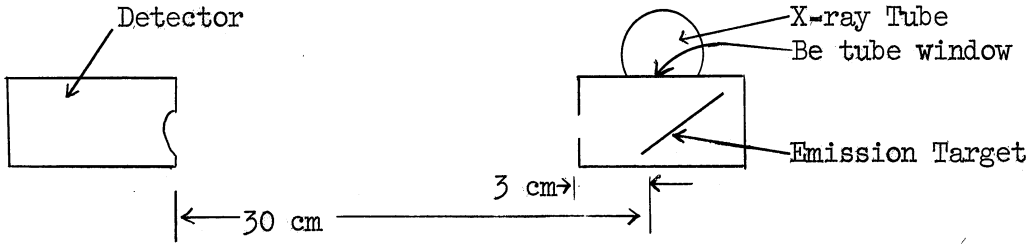
X-ray tube at 50 pkv 40 ma

Target	$E_{K\alpha}$ (Kev)	Δ O.D. Per Hour	Ergs/cm^2-hr*	hν/cm^2-hr**
Nickel	7.47	0.200	5.68×10^5	4.74×10^{13}
Iron	6.40	0.151	4.29×10^5	4.17×10^{13}
Manganese	5.89	0.087	2.47×10^5	2.62×10^{13}
Chromium	5.40	0.045	1.28×10^5	1.47×10^{13}

* Ergs/cm^2-hr = Δ O.D./hr x 2.84×10^6 ergs/cm^2

** $h\nu/cm^2\text{-hr} = \dfrac{ev/cm^2\text{-hr}}{h\nu \text{ energy in ev}}$

The x-ray counter was positioned at ninety degrees to the emergent x-ray beam from the tube and centered with respect to the window in the target holder box beneath the x-ray tube (see sketch).



The distance from the emission target to the window of the x-ray counter was 30 cm. The emission intensities (photon flux) were too high (even

with 1 ma tube current) to be counted in the x-ray counter. In order to decrease the x-ray intensity to a countable level a series of collimators with precisely measured holes were constructed from aluminum. These collimators were positioned in front of the tube window, and the count rate, with the various targets in position, was taken for each of the available aperature sizes. The count rate, as a function of aperature area, was then plotted on linear graph paper and extrapolated to an area of one square centimeter to give a count rate per square cm with the tube positioned at 30 cm from the emission target.

In the irradiations with fluorescent targets the sample of catalase was placed 3 cm from the emission target. The Fricke dosimeter measurements were made at 3 cm. An inverse square correction from the 30 cm counting position to the 3 cm dosimeter and irradiation position was necessary. Next, the counting rate was corrected for air absorption in the 27 cm of path between sample-dosimeter location and that of the x-ray counter. The values used for linear absorption coefficient in the computation of air absorption are those of Victoreen[120] and were as follows: For the nickel fluorescent target 0.0167, for the iron target 0.0239, for the manganese 0.0278, and for chromium 0.0351.

It was also necessary to relate the photon flux at 1 milliampere x-ray tube current to the flux at any tube current, especially a current of 40 ma which was used in the Fricke dosimetry. Data were obtained by the use of limiting aperatures and a series of measurements of the count rate per second as a function of the x-ray tube current. Each of these sets of data was normalized one to the other to permit evaluation of the

total tube current range of 1 to 50 ma. A normalized plot of these data is presented as Figure 10. It is to be noted that the ratio of counts per second at 1 ma to that at 40 ma is 15.35, not 40 as expected. The reason for this discrepancy was not investigated.

The calculations leading to an evaluation of the efficiency of the counter, compared to the Fricke dosimeter, are presented for the fluorescent target of nickel. This computation will serve to illustrate the procedure used with each target material.

The counts per second per cm^2 at 30 cm from the nickel target, obtained by extrapolation of the variable area aperature data, were found to be:

$$5.28 \times 10^5 \text{ c/s/cm}^2 \text{ at 1 ma}$$

then correction for air absorption loss,

$$\frac{5.28 \times 10^5}{e^{-\mu x}} = \frac{5.28 \times 10^5}{0.638} = 8.28 \times 10^5 \text{ c/s/cm}^2$$

inverse square correction,

$$I_1 = \frac{(r_2)^2}{(r_1)^2} I_2$$

$$= \frac{(30)^2}{(3)^2} \times 8.28 \times 10^5 \text{ c/s/cm}^2 \text{ at 1 ma}$$

$$= 8.28 \times 10^7 \text{ c/s/cm}^2 \text{ at 1 ma}$$

correction to 40 ma,

$$8.28 \times 10^7 \times 15.35$$

$$= 1.27 \times 10^9 \text{ c/s/cm}^2 \text{ at 40 ma}$$

and time correction,

$$3.6 \times 10^3 \text{ sec/hr} \times 1.27 \times 10^9 \text{ c/s/cm}^2$$

$$= 4.57 \times 10^{12} \text{ c/hr/cm}^2 \text{ at 40 ma.}$$

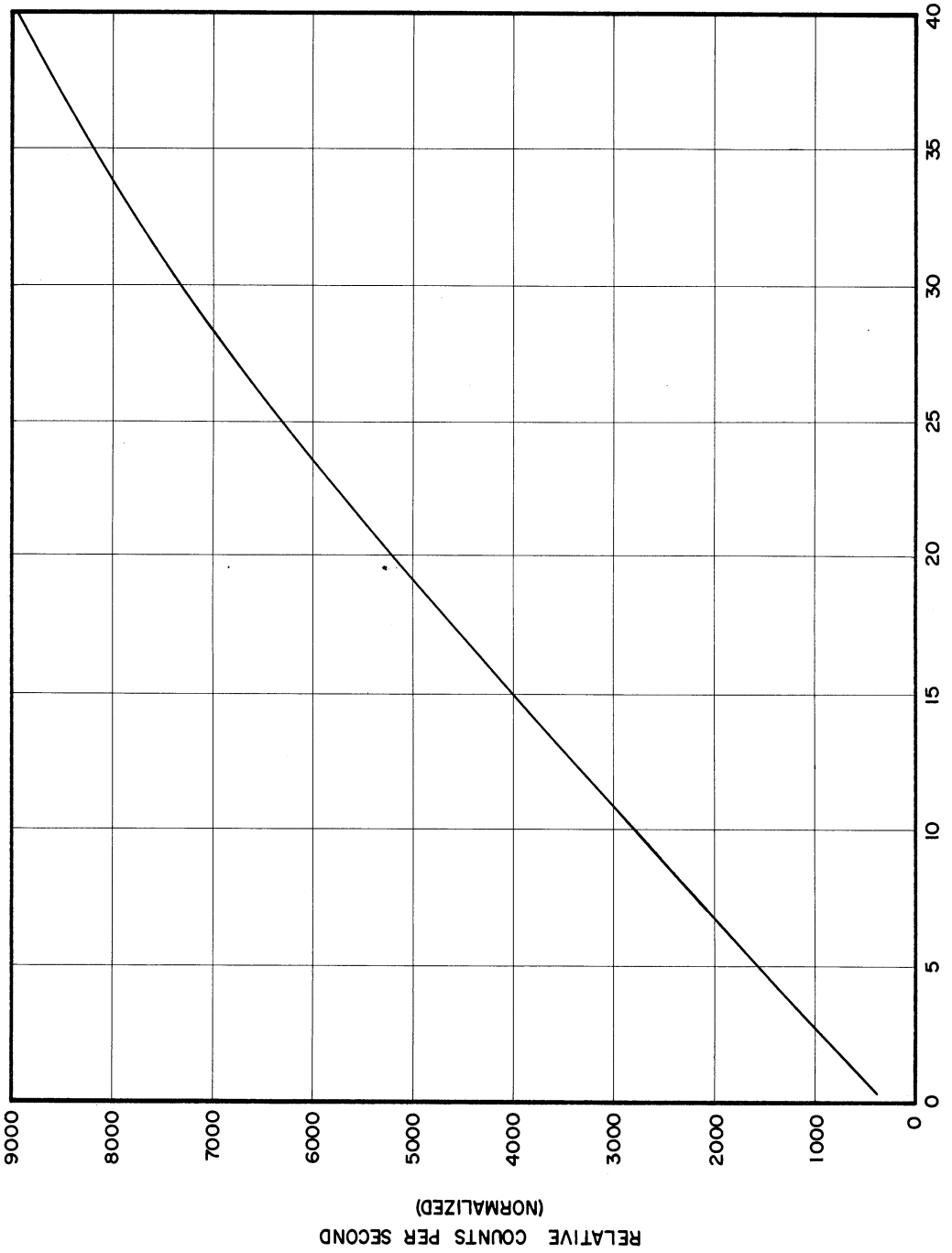


Figure 10. Relationship of Photon Output from the Emission Target to X-ray Tube Current.

Then a photon flux of this magnitude would constitute an energy flux of:

No. of photons x energy of each

$= 4.57 \times 10^{12}$ c/hr/cm^2 x 7.47×10^3 ev/photon

$= 3.42 \times 10^{16}$ ev/cm^{2}hr

or $= 3.42 \times 10^{16}$ ev/cm^{2}hr x 1.6×10^{-12} erg/ev

$= 5.47 \times 10^4$ ergs/cm^{2}hr

and this is to be compared to the energy flux measured with the Fricke dosimeter,

$$\frac{5.47 \times 10^4 \text{ ergs/cm}^2\text{hr}}{5.68 \times 10^5 \text{ ergs/cm}^2\text{hr}}$$

$= 0.096$

= efficiency of the x-ray counter for nickel target emission lines.

One may utilize the calculated photon intensity from each of the target materials to provide an evaluation of the photon counter efficiency based on the Fricke dosimeter as a standard. This is done by converting the photon intensity into ergs per square cm per hour and comparing the energy flux to that measured by the Fricke dosimeter. The results of these calculations are summarized in Table II. It is noted that the photon counter, with targets of nickel, iron, manganese, and chromium, shows a smooth transition in efficiency with no apparent fluctuations at any of the four emission line energies considered.

c) R-Meter

An ion chamber,* together with the standard condenser R-meter, was used in an effort to attain another (third) dosimetric technique

* Victoreen Model 651, The Victoreen Instrument Co., Cleveland, Ohio.

TABLE II

X-RAY COUNTER EFFICIENCY BY COMPARISON
TO FRICKE DOSIMETER

Target	E_{kev}	(A) X-ray Counter ergs/cm^2/hr	(B) Fricke Dosimeter ergs/cm^2/hr	$(\frac{A}{B})$ Counter Efficiency
Ni	7.47	5.47×10^4	5.68×10^5	0.096
Fe	6.40	4.16×10^4	4.29×10^5	0.097
Mn	5.89	2.44×10^4	2.47×10^5	0.099
Cr	5.40	1.33×10^4	1.28×10^5	0.104

applicable to the data. Although the chamber was not designed for use in this very low energy region, because of its ease of application and ready availability to other experimental groups, it was decided that it would be worthwhile to calibrate it against the Fricke dosimeter.

The biochemical system chosen for this investigation is one which contains a trace of iron essential in the catalytic destruction of hydrogen peroxide. The Fricke dosimeter utilizes the oxidation of iron ions as a measurement of free radical production which, in turn is related to the energy absorption. The question logically arises as to the validity of application of an iron containing dosimeter to an investigation of

the effects of radiation on an iron sensitive (or iron containing) system. There is an abundance of information in the literature on the Fricke dosimeter reaction. In every instance the oxidation reaction is attributed to an indirect action of radiation. This thesis seeks to ascertain the existence of a direct action of specific energy x-rays on a macromolecule containing an (essential) iron trace.

To minimize the possibility that an unknown reaction fluctuation might exist at or near the k-edge of iron in the Fricke dosimetric measurements, a large number of fluorescent targets were utilized as x-ray sources and r-meter efficiencies were calculated by comparison to the Fricke dosimeter. The emission energies of these targets ranged from 8.6 to 5.4 kev.

Comparison data between the r-meter and the Fricke dosimeter were obtained as follows. The output from a given target was measured by positioning the r-meter chamber in the same position occupied by the Fricke dosimeter. The output in roentgens per hour was measured at 5 milliamps current and 50 pkv x-ray tube potential. This measurement was corrected to output in r per hour at 40 milliamps by the ratio of x-ray counts at 40 milliamperes to that at 5 milliamperes as given in Figure 10. Using the relationship

$$E(h\nu) = \frac{83.8}{(\mu/\rho)\ \text{air}} \quad \text{ergs/cm}^2$$

where $E(h\nu)$ is the energy flux per roentgen (in $\text{ergs/cm}^2\text{-r}$) and $(\mu/\rho)_{air}$ is the true mass absorption coefficient for air, one may calculate

TABLE III

ENERGY FLUX FOR VARIOUS EMISSION TARGETS
BY THE R-METER

Target	E_{Kev}	$ergs/cm^2 r$	$r/hr (\times 10^3)$	$ergs/cm^2 hr$
Zn	8.64	10.05	23.0	2.31×10^5
Cu	8.05	8.8	26.4	2.32×10^5
Ni	7.47	6.6	36.7	2.42×10^5
Co	6.93	5.6	20.7	1.16×10^5
Fe	6.40	4.5	39.9	2.55×10^5
Mn	5.89	3.5	33.0	1.15×10^5
Cr	5.40	2.9	14.5	4.20×10^4

the $ergs/cm^2$-hr as measured by the r-meter. These calculations are summarized in Table III. The results of these calculations may be compared to the energy flux measurements made with the Fricke dosimeter to yield a value for the efficiency of the r-meter. This information is tabulated in Table IV. The tabulation of r-meter efficiencies, as compared to the Fricke dosimeter (Table IV), indicates that a variation of as much as 25 percent from the average occurs in the r-meter response. Investigation has shown that this chamber is very sensitive to acceptance angle, and

TABLE IV

R-METER EVALUATION BY
COMPARISON TO FRICKE DOSIMETER

Target	E_{Kev}	r-meter ergs/cm^{2}hr	Fricke ergs/cm^{2}hr	Ratio $\frac{\text{r-meter}}{\text{Fricke}}$
Zn	8.64	2.31×10^5	5.67×10^5	0.407
Cu	8.05	2.32×10^5	4.91×10^5	0.472
Ni	7.47	2.42×10^5	5.68×10^5	0.426
Co	6.93	1.16×10^5	3.03×10^5	0.383
Fe	6.40	2.55×10^5	4.29×10^5	0.595
Mn	5.89	1.15×10^5	2.47×10^5	0.466
Cr	5.40	4.20×10^4	1.28×10^5	0.328

consequently, to positioning in the x-ray beam. These results indicate that a fluctuation in response of the iron containing Fricke dosimeter in this energy region could not exceed 25 percent, and the earlier studies with the x-ray counter indicate the energy dependence in the energy region of interest to be negligible.

The radiation studies reported in this thesis are based on dosimetric measurements as made with the Fricke dosimeter. Studies are presently in

progress (at this University) on the applications of a calorimetric device for measuring beam intensities in the region of 5 to 25 kev. As these measurements become available, it will be possible to compare the Fricke dosimetric data to that attained with a calorimeter type device. It is felt that the utilization of the Fricke dosimeter provides a standard which permits comparison with other work and allows appropriate adjustments as the dosimetric techniques in this energy region become more reliable.

B. Test System - Catalase

1. Determination of Radiation Absorption Coefficient

a) Calculation from Atomic Composition

The intent of this investigation was to ascertain if the selected biological test system displayed damage phenomena out of proportion to that expected from energy absorption considerations. If a rapid fluctuation existed in the absorption curve, as a function of photon energy, then the action spectrum (damage) would be expected to follow this fluctuation. The test system - catalase - contains 0.09 percent iron, which would, if present in greater quantity, display its characteristic k-edge discontinuity. However, at the concentration of less than 1/10th of one percent iron the k-edge does not add an appreciable (or even measurable) amount to the mass absorption coefficient of catalase.

The total mass absorption coefficient of catalase was computed on the assumption that its composition is very similar to that of other pure enzymes[113]. The assumed weight percent composition is as follows:

Carbon	49.9 percent
Hydrogen	7.0 percent
Oxygen	25 percent
Nitrogen	16 percent
Sulfur	1 percent
Phosphorus	1 percent
Iron	0.1 percent

The values used for the mass absorption coefficient for each element, over the energy range investigated in this study, are given in Table V.[24] These values were used to compute the weighted mass absorption coefficient for the elements making up the catalase molecules. A summary of the computation is presented in Table VI. These values of the coefficients were then summed at each energy and the result is presented in Table VII. The information in these latter two tables (VI and VII) is graphed in Figure 11.

The computed values of the mass absorption coefficients for catalase do not display any rapid fluctuations in the energy region of interest. It remained to ascertain experimentally that this was true.

b) Experimental Measurement

Two sample holders were prepared of 0.3 cm thickness lucite. Each with a hole 1 cm by 0.3 cm milled completely through in such a location that it aligned with the opening in the sample holder device on the General Electric x-ray diffraction unit. One holder was covered with a 5 mil mylar window on each side and used as the "blank." The other was covered with 5 mil mylar windows and 11.8 mg (0.038 g/cm^2) of dry catalse was placed between the windows.

TABLE V

MASS ABSORPTION COEFFICIENTS FOR THE ELEMENTS IN CATALASE[24]

ENERGY (KEV)	ABSORPTION COEFFICIENT FOR THE ELEMENT						
	C	H	O	N	S	P	Fe
4	30	0.58	68	46	660	480	265
5	15.6	0.55	38	25	345	255	140
6	9.8	0.52	23.5	15.5	205	158	87
7	6.6	0.51	15.3	10.3	130	100	58
8	4.6	0.48	10.8	7.1	88	71	315
9	3.25	0.48	7.7	5.2	63	49	225
10	2.48	0.47	5.7	3.9	47	37	170
11	1.88	0.46	4.5	3.0	36	29	134
12	1.48	0.45	3.5	2.4	28	22.6	104
13	1.16	0.44	2.8	1.9	22	18	84
14	0.98	0.44	2.3	1.6	18	14.5	68

TABLE VI

WEIGHTED MASS ABSORPTION COEFFICIENTS
FOR THE CATALASE ELEMENTS

ENERGY (KEV)	ABSORPTION COEFFICIENT FOR THE ELEMENT OF FRACTION ABUNDANCE						
	C (0.499)	H (0.07)	O (0.25)	N (0.16)	S (0.01)	P (0.01)	Fe (0.001)
4	14.970	0.041	17.000	7.360	6.60	4.80	0.265
5	7.784	0.039	9.500	4.000	3.45	2.55	0.140
6	4.890	0.036	5.875	2.480	2.05	1.58	0.087
7	3.293	0.036	3.825	1.648	1.30	1.00	0.058
8	2.295	0.034	2.700	1.136	0.88	0.71	0.315
9	1.622	0.034	1.925	0.832	0.63	0.49	0.225
10	1.238	0.033	1.425	0.624	0.47	0.37	0.170
11	0.938	0.032	1.125	0.480	0.36	0.29	0.134
12	0.739	0.032	0.875	0.384	0.28	0.226	0.104
13	0.579	0.031	0.700	0.304	0.22	0.18	0.084
14	0.489	0.031	0.575	0.256	0.18	0.145	0.068

TABLE VII

TOTAL MASS ABSORPTION COEFFICIENT OF CATALASE

ENERGY (KEV)	ABSORPTION COEFFICIENT (cm^2/g)
4	51.036
5	27.463
6	16.998
7	11.160
8	8.070
9	5.758
10	4.330
11	3.359
12	2.640
13	2.098
14	1.744

An exciting potential of 11 pkv and a current of 1 ma was used on an AEG-50 tungsten target x-ray tube. Goniometer angle settings were selected corresponding to a particular diffracted x-ray energy. The x-rays were counted with the SPG #1 x-ray counter. The number of x-rays passing through the sample (I) was compared to the number through the blank (I_o) to arrive at an evaluation of sample transmittance, and, in turn, an evaluation of the percent absorbed in the sample. The results

of this study are presented in Table VIII and Figure 12. The absorption of monochromatic x-rays in catalase is a smooth function over the energy region studied.

It is possible to compute the value for the mass absorption coefficient from the data in Table VIII (or plotted in Figure 12). This is done using the following relationship:

$$\mu/\rho = 2.303 \times \frac{\text{Area}}{\text{Weight}} \times \log_{10} \frac{I_0}{I}$$

$$\mu/\rho = 60.5 \log_{10} \frac{I_0}{I}$$

where $$\frac{I}{I_0} = 1 - \frac{\text{Percent absorbed}}{100}$$

Figure 13 presents the computed and experimental values for the mass absorption coefficient of catalase at various energies. There is not a discernible fluctuation in the experimentally measured absorption of monochromatic x-rays in the energy range of 5.5 to 8.5 kev. The absorption coefficient is a smooth curve and the measured value is within six percent of the computed value.

Since a portion of this study had to do with the effects of monochromatic x-irradiation of catalase in a phosphate buffer-water solution, an evaluation of the x-ray absorption of such a solution was performed. This solution was 10^{-7} molar catalase and 1/15th molar phosphate buffer. The absorption measurements were made in a manner identical to those described for dry catalase. No fluctuations were observed in these data.

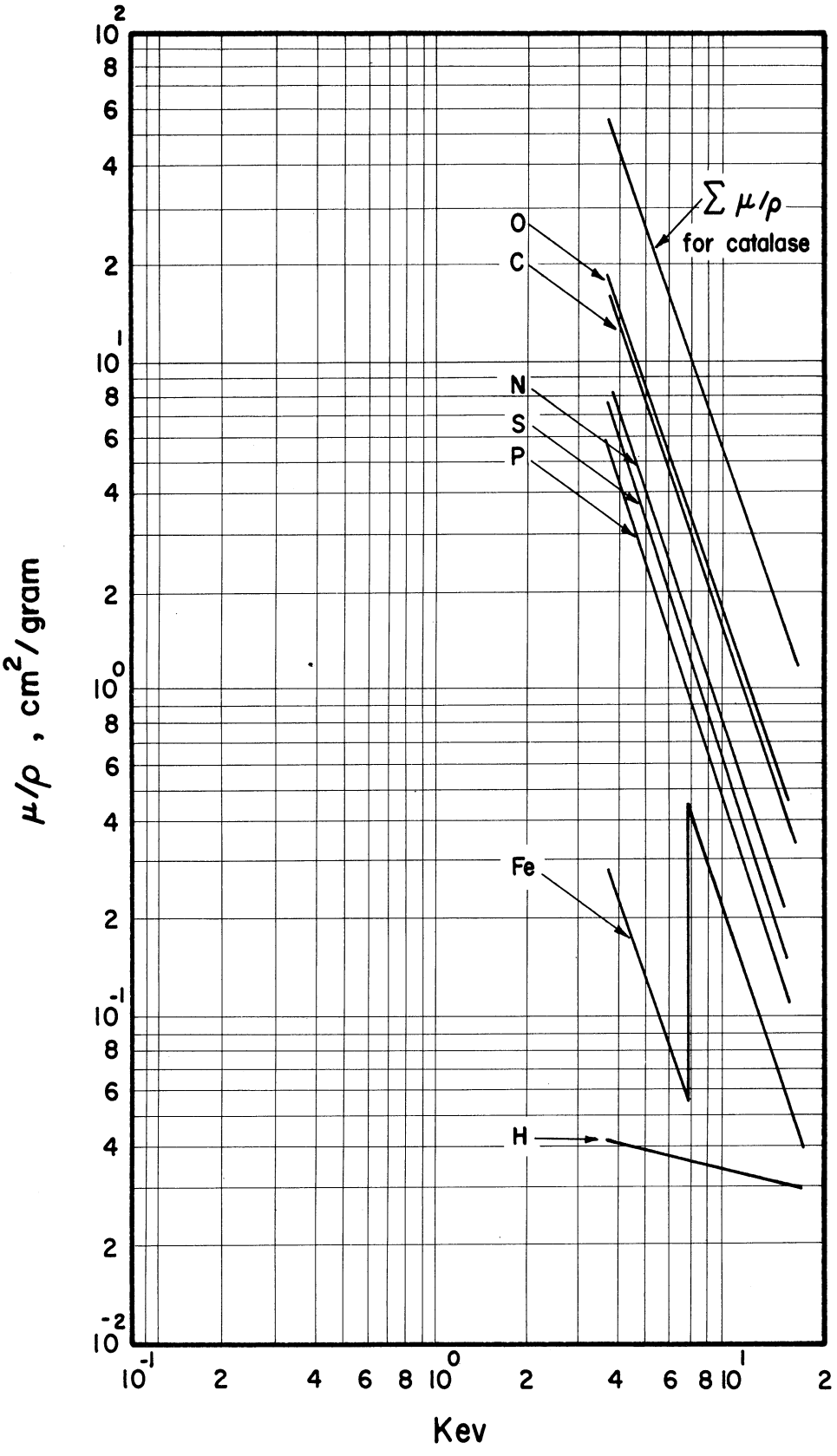


Figure 11. Weighted and Total μ/ρ Values for Catalase.

TABLE VIII

THE ABSORPTION OF MONOCHROMATIC X-RAYS IN DRY CATALASE

Sample thickness = 0.3 cm

Sample weight = 11.8 mg

Sample area = 0.31 cm^2

Diffraction Crystal - NaCl

11 pKv, 1 ma

Energy in Kev	Percent Absorbed	Energy in Kev	Percent Absorbed
5.50	54.9	7.15	31.2
5.65	51.3	7.33	29.5
5.80	48.8	7.43	29.0
5.95	46.5	7.60	27.2
6.11	43.4	7.77	25.8
6.26	41.4	7.93	25.0
6.42	39.2	8.06	23.7
6.56	37.0	8.21	22.0
6.69	36.2	8.34	21.8
6.85	33.7	8.49	21.2
7.00	33.0		

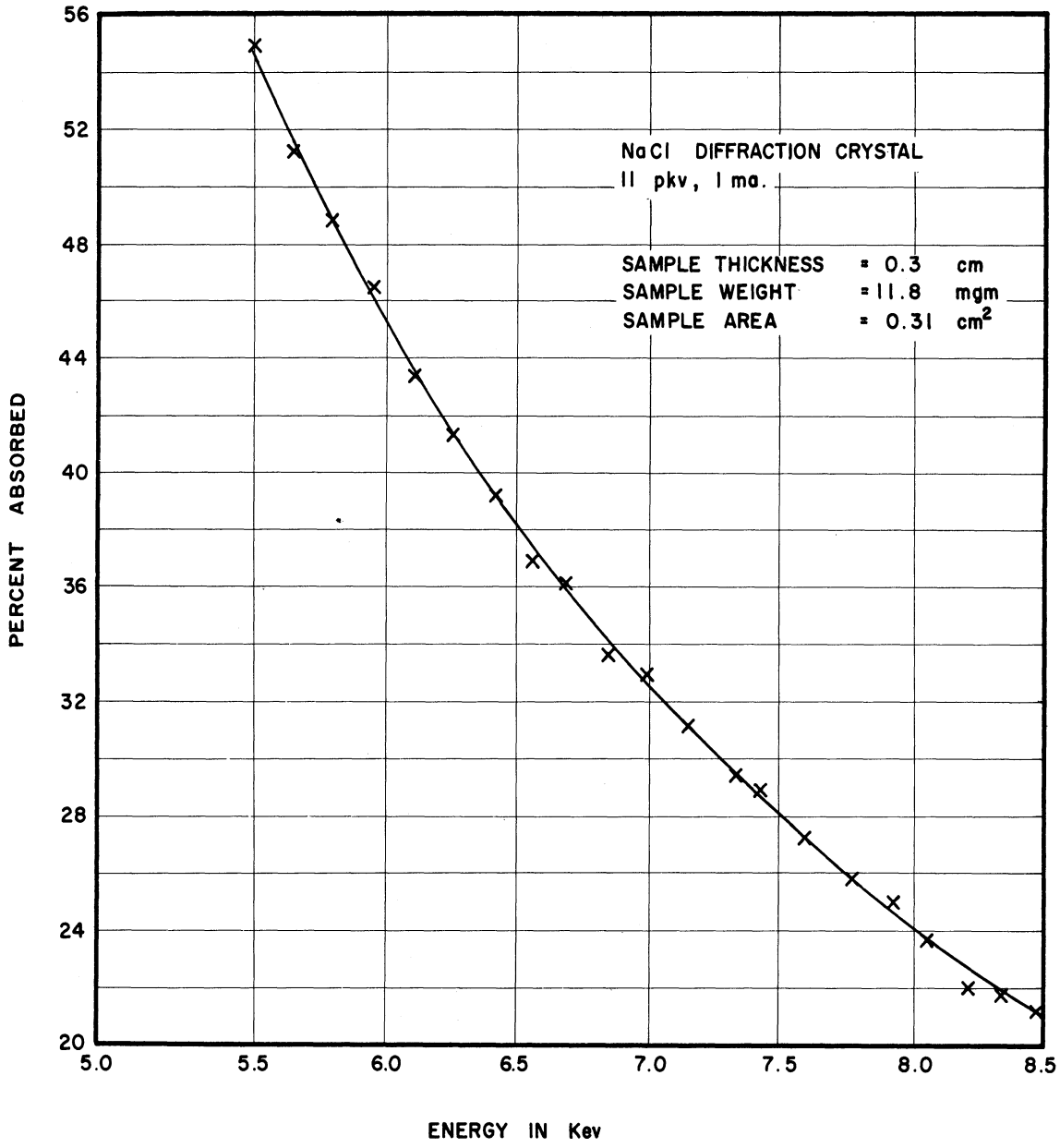


Figure 12. The Absorption of Monochromatic X-rays in Dry Catalase.

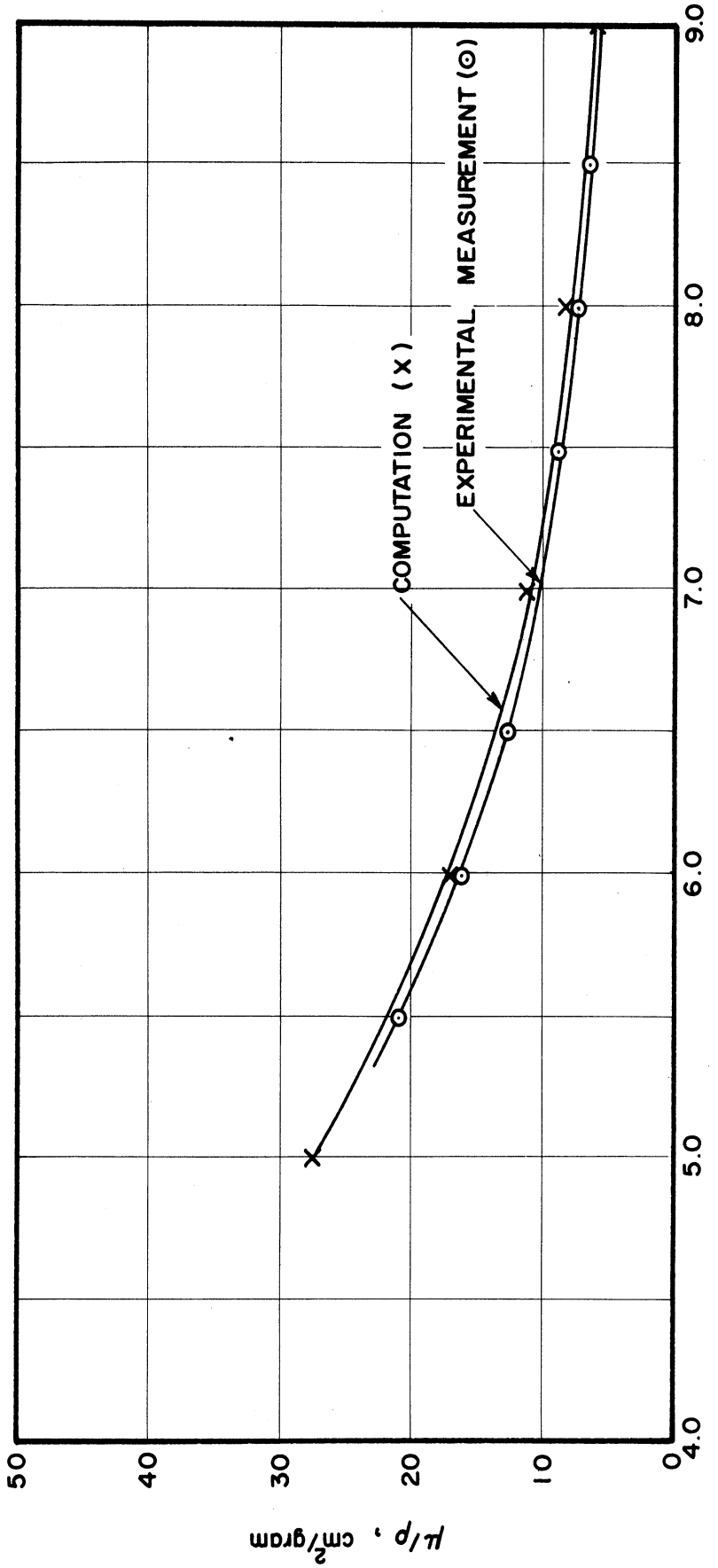


Figure 13. Computed and Experimentally Measured μ/ρ Values for Dry Catalase.

In all the x-ray irradiations reported in this study the catalase (dry or in solution) was placed in a lucite holder which was sealed with scotch tape. The scotch tape has been found to be of uniform thickness. A series of measurements were made to evaluate the percent of a given energy x-ray beam which was transmitted through a scotch tape window. These measurements were made under the same conditions as those reported for the dry catalase transmittance experiment. The percent transmittance of monoenergetic x-rays through scotch tape as a function of x-ray energy is plotted on Figure 14.

2. Methods of Determining Catalytic Ability

The catalase used in these studies was procured from Nutritional Biochemical Corporation of Cleveland, Ohio as the lyophilized catalase. All samples of catalase were dissolved in 1/15th molar sodium phosphate buffer of pH 6.8.

The hydrogen peroxide substrate for the evaluations of the reaction rate was prepared by the addition of 1 cc of 3 percent hydrogen peroxide to 99 cc of 1/15th molar phosphate buffer. The hydrogen peroxide must be kept under refrigeration and in the dark to minimize its decomposition by photolysis. In the majority of the weighings, for sample preparation, it was necessary to employ a microbalance to procure the accuracy required in the catalase determinations. This was a necessity because of the high sensitivity of detection of low concentrations of catalase.

A number of samples were weighed on the microbalance in the weight range of from 0.2 to 4 milligrams of weight. Each of these

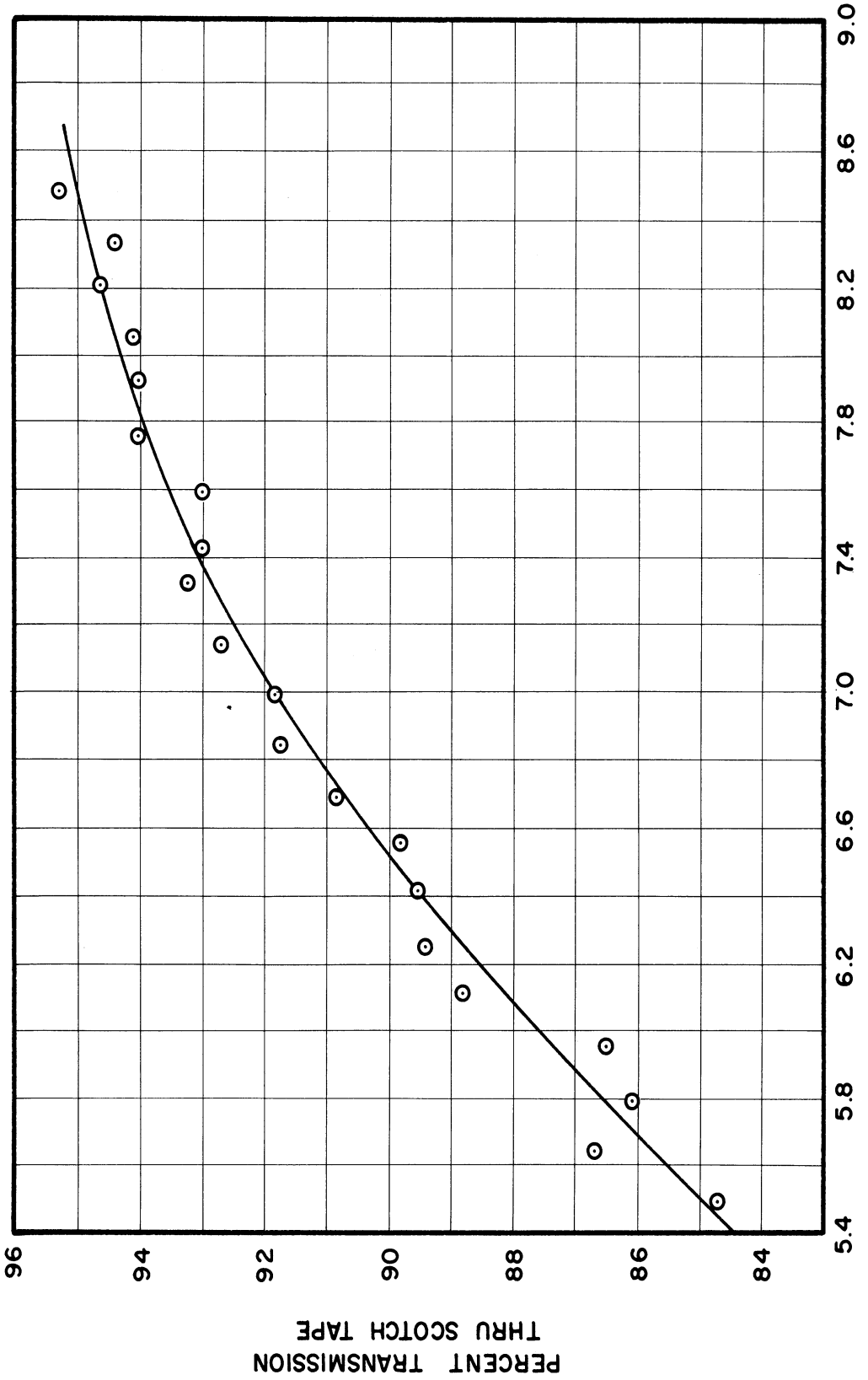


Figure 14. Scotch Tape Transmittance of X-rays as a Function of Energy

TABLE IX

EXPERIMENTALLY MEASURED MASS ABSORPTION COEFFICIENTS FOR DRY CATALASE

Kev	Percent Absorption	μ/ρ
5.5	54.9	20.9
6.0	45.4	15.8
6.5	37.9	12.5
7.0	32.9	10.5
7.5	28.2	8.71
8.0	24.1	7.20
8.5	21.2	6.23

weighed samples was then dissolved in 1/15th molar phosphate buffer and made up to 50 cc volumes in volumetric flasks. Then 1 cc of each of these standard solutions was placed in a quartz cuvette. A sample of buffer was placed in the number one position in the sample housing of the DU Beckman Spectrophotometer. The spectrophotometer recorder unit was zeroed on the dark current, then with the shutter open, the recorder was adjusted to read 100 percent transmittance (no hydrogen peroxide) by means of the zero adjustment on the spectral recording attachment. Then to a 1 cc sample of catalase solution, in position 2, 3, or 4 of the spectrophotometer, was added 2 cc of the standard hydrogen peroxide substrate. Immediately the cuvette housing was closed, the shutter was

opened and a plot of percent transmittance (T) as a function of time was recorded. As the hydrogen peroxide substrate is destroyed by the catalase the percent transmittance tends to approach that of the buffer.

The reaction was followed for a period exceeding one minute. A number of runs were made on each concentration solution during the course of one investigation. These data were then replotted, plotting (100-T) on a log scale versus time on a linear scale. The initial portion of this graph is a straight line indicative of the first order reaction. This initial portion was extrapolated from 0 to 1 minute time and the slope of the line determined from the relationship:

$$\log_e \frac{(100-T_1)}{(100-T_o)} \div 60 = k_1 \text{ sec}^{-1}$$

where T_o is the percent transmittance at time zero, T_1 is the percent transmittance at the end of one minute and k_1 is the reaction velocity rate.

Figure 15 is a reproduction of the data recorded in one of the catalase runs. The recorder plots in a linear coordinate system. To better display (and linearly average) the exponential character of the reaction the data is replotted on semi-log paper (Figure 16). From this plot one can evaluate the slope and, in turn, the catalase concentration. The relationship between the reaction rate and catalase concentration is shown in Figure 17. These data represent three runs on each of four different concentrations of catalase weighed on the microbalance, put in solution, and evaluated on the spectrophotometer.

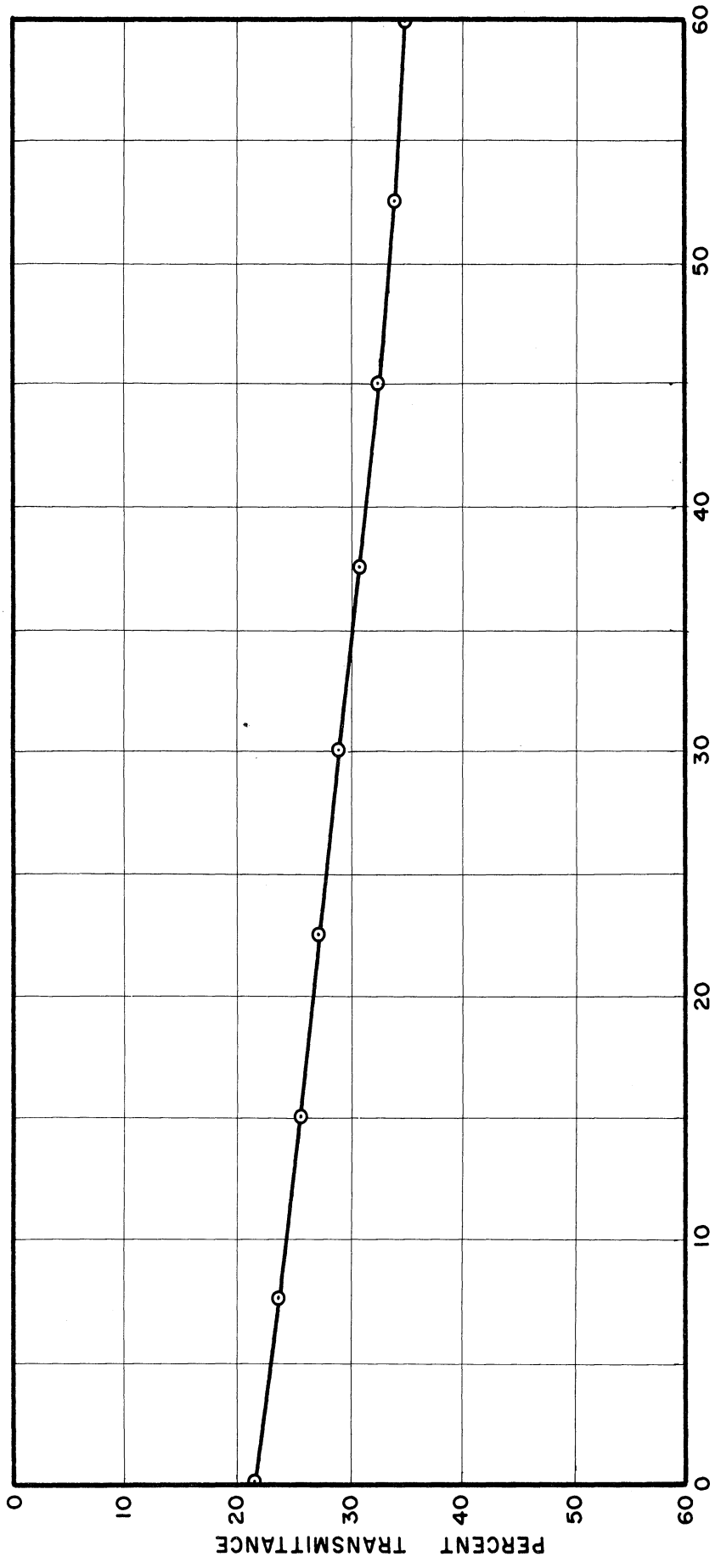


Figure 15. Reproduction of Recorded Data from a Catalase Measurement.

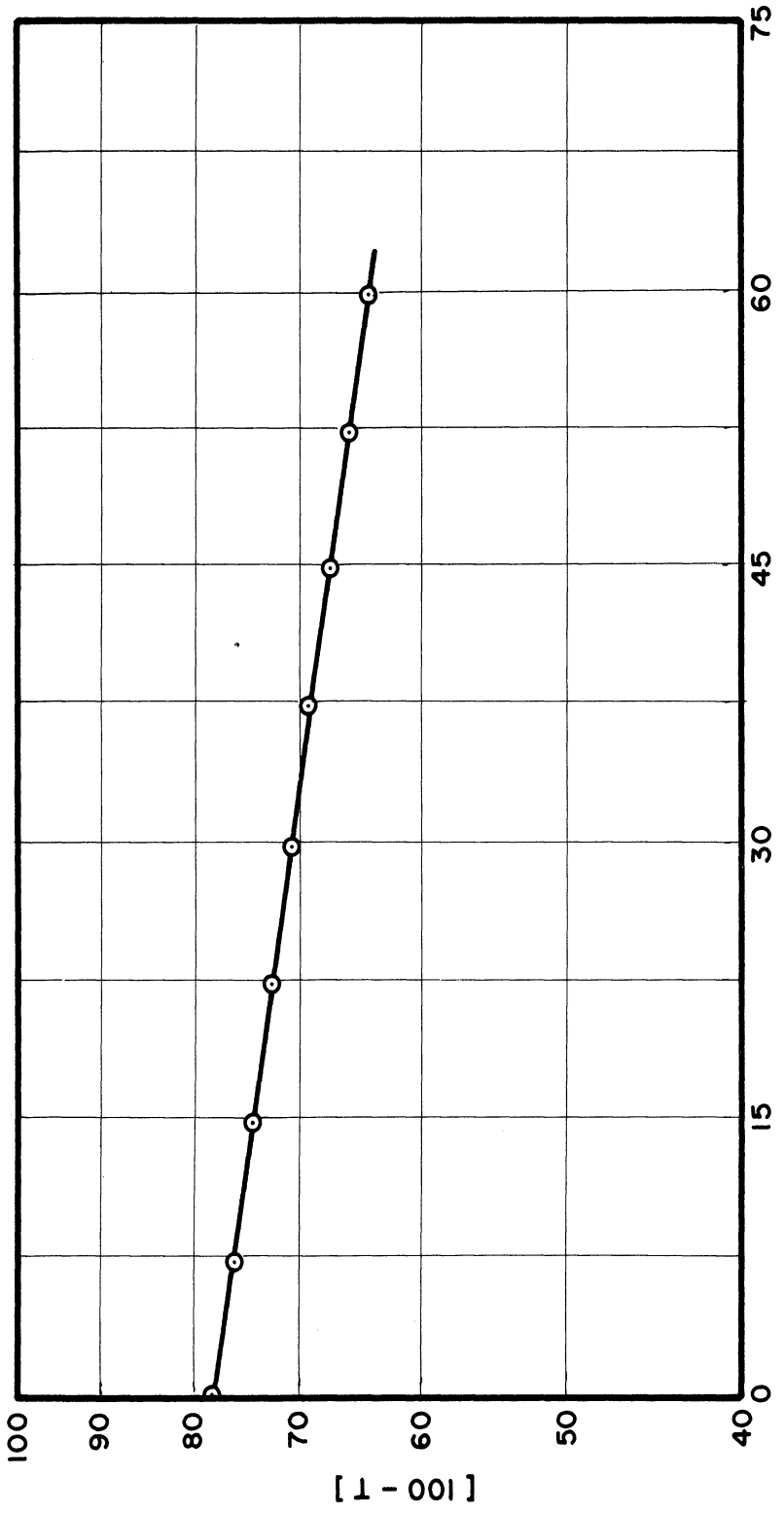


Figure 16. Semi-log Plot of Data of Figure 2.

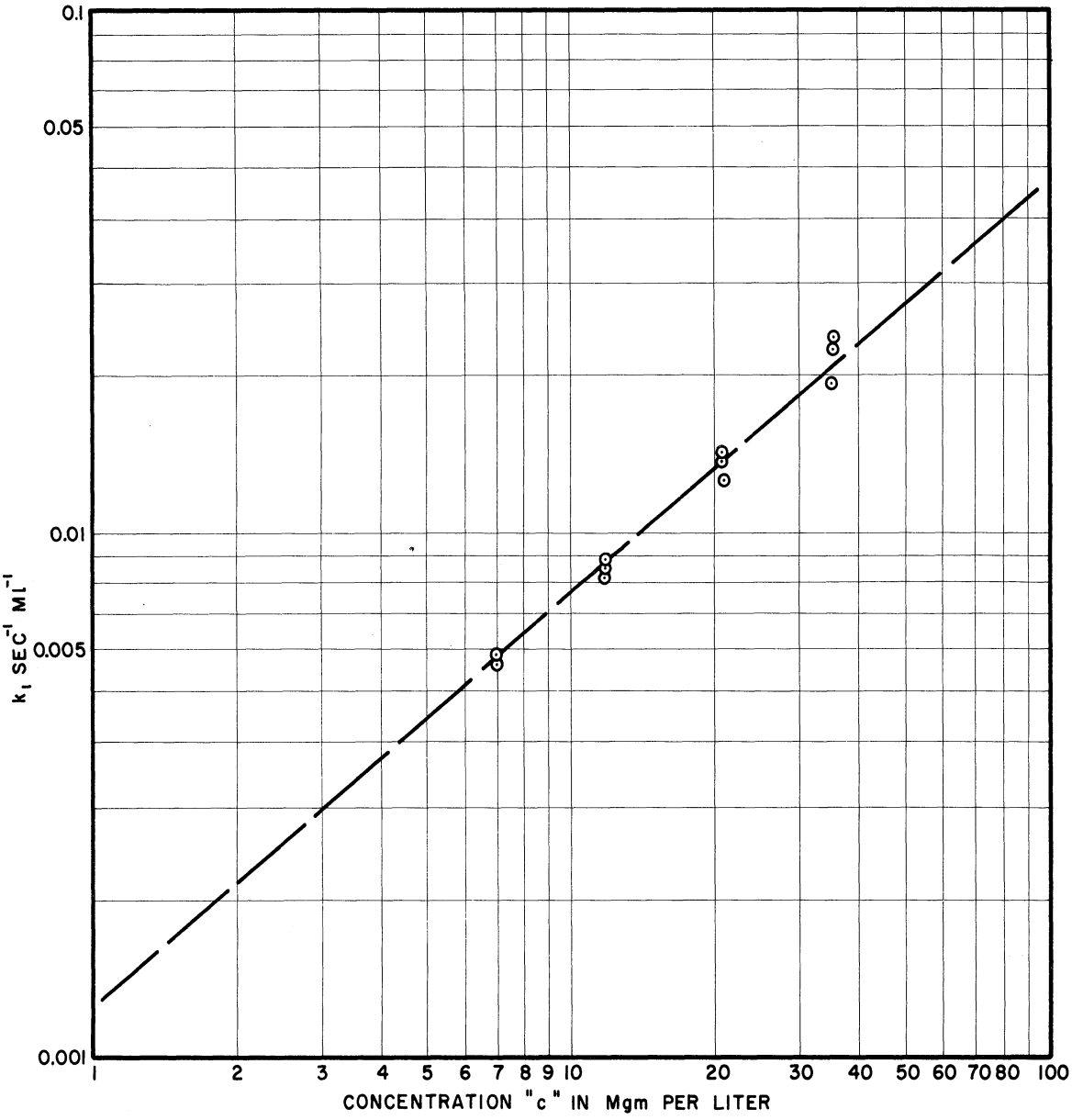


Figure 17. Reaction Rate as a Function of Catalase Concentration.

In the study of an enzyme catalyzed reaction it is of particular importance that one evaluates the initial reaction rate, since the enzyme is of such a labile nature that the reaction products appreciably alter the velocity of the reaction after a short period of time. Dixon and Webb[38] illustrate this very well with data which are reproduced as the two sets of curves in Figure 18A and Figure 18B. In the curves of Figure 18A, they have plotted milligrams of substrate transformed against time for three different concentrations of enzyme. They suggest T_0, T_1, and T_2 as alternative times to measure the velocity (slope of the curve). In Figure 18B, they plot the apparent reaction velocity as a function of enzyme concentration for the three designated study times. It is obvious that only the initial velocity is proportional to the enzyme concentration. The preferred technique for the evaluation of enzymatic activity is thus the one which provides an instantaneous and continuous value of substrate concentration permitting one to determine initial reaction rates.

Beers and Sizer[9] have reported a technique which utilizes the Beckman spectrophotometer for catalase determinations. They begin by adding buffered hydrogen peroxide to a cuvette containing catalase enzyme in buffered solution. They then measure the optical density every ten seconds or, as an alternate technique, they measure the time for the optical density to decrease to one-half of its initial value. The latter method, that is, measurement of the reaction half-time, is not applicable to higher concentrations of catalase. The method of measuring reaction half-time assumes the reaction is of first order for a time equal to the half-time. Such an assumption is not valid for high catalase concentrations.

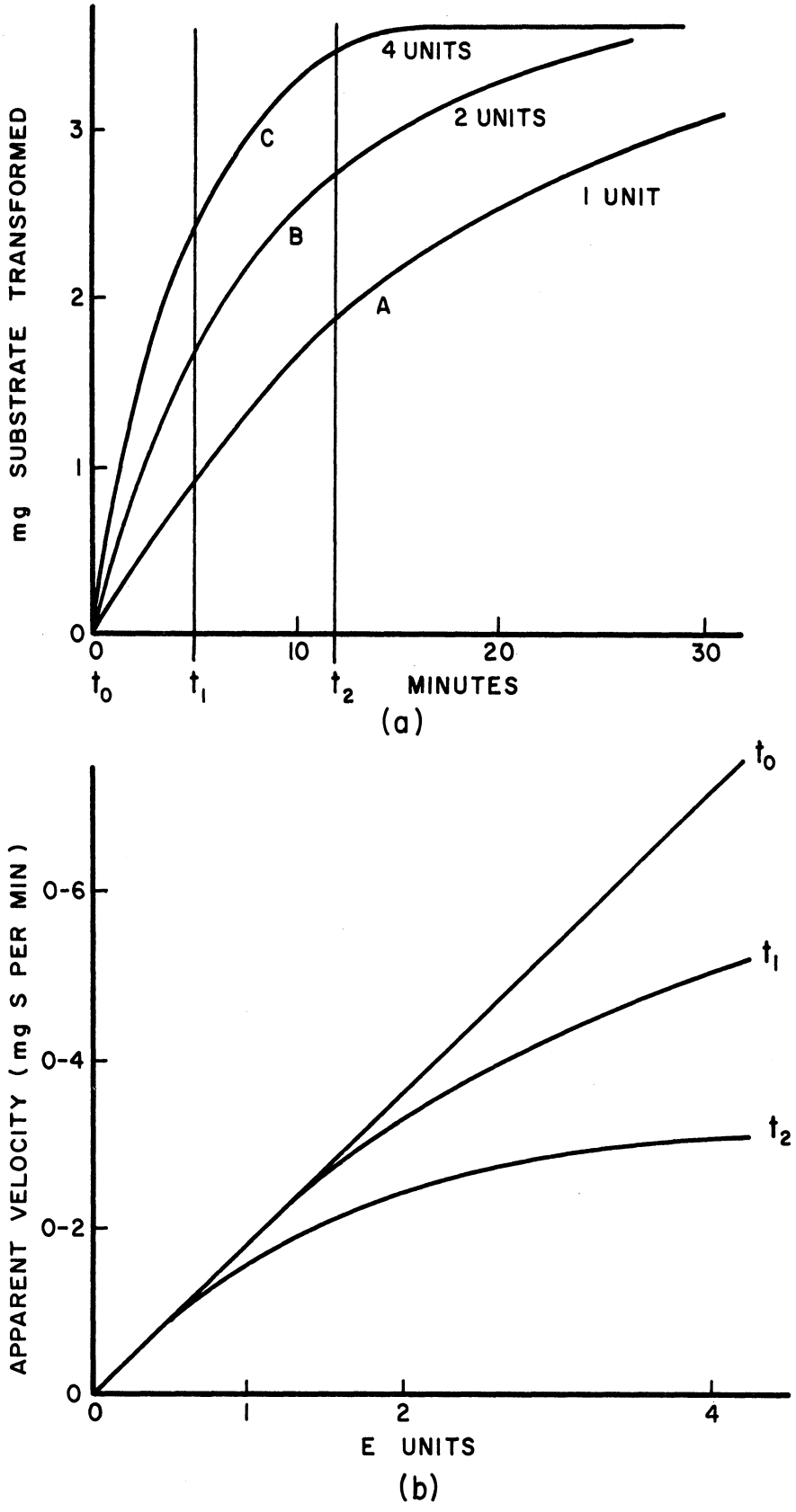


Figure 18. Illustration of the Importance of Taking Initial Reaction Velocities. (a) Progress curves with three different amounts of enzyme. (b) Apparent velocity, derived from points on (a) at three different measuring times, plotted against amount of enzyme.

Figure 19 is a graphical presentation of the data from two actual runs on the hydrogen peroxide-catalase system. These data further illustrate the deviation from a first order reaction which takes place at the higher enzyme concentrations. This graph is a plot of 100 minus the percent transmittance as a function of time after the addition of the hydrogen peroxide substrate to a catalase solution. (The log of percent transmittance is proportional to the concentration of hydrogen peroxide.) These data were taken using the Beers and Sizer technique with a spectrophotometer modified to yield instantaneous and continuous data.

By means of the attachment of a Beckman spectral energy recording attachment and a Varian millivolt recorder to the model DU spectrophotometer, it is possible to plot the percent transmittance as a function of time after the addition of the substrate of hydrogen peroxide to the various catalase solutions. These attachments to the Beckman Model DU Spectrophotometer permit the continuous recording of percent transmittance at a fixed wave length as a function of time. The wave length used in all of these studies was 212 millimicrons at a slit width of 0.03 millimeters.

The basic spectrophotometer is not sensitive enough to permit the measurement of light in this wave length region without the addition of a photomultiplier attachment. This accessory consists of a 1 P 28 photomultiplier tube mounted in a special housing, together with a battery box containing the batteries required to operate the DU and the photomultiplier tube. This attachment permits the operator to vary

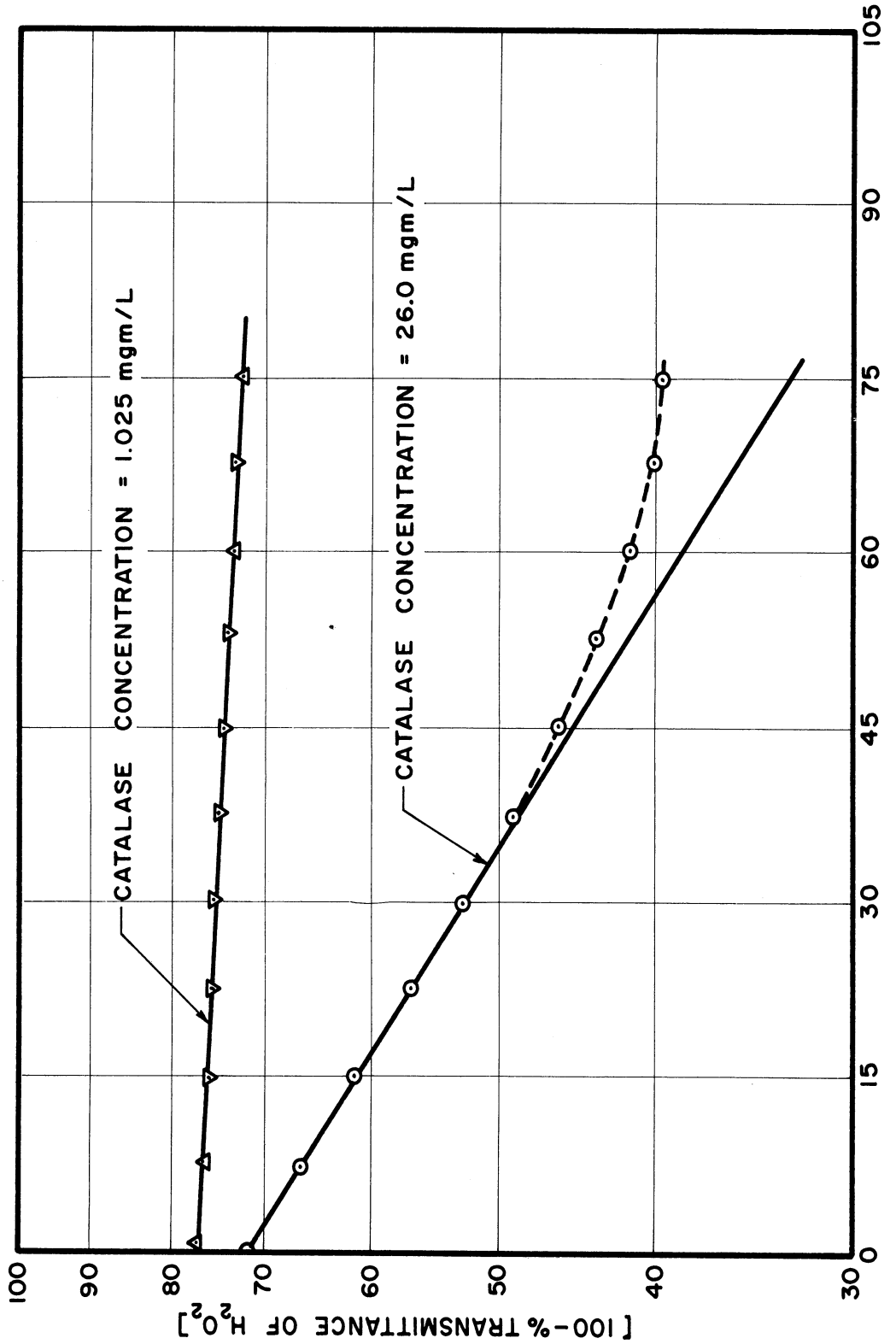


Figure 19. Deviation from First Order Reaction with Concentration.

the sensitivity of the spectrophotometer in the wave length region of 210 to 600 millimicrons. A photograph of the equipment used is presented as Figure 20.

The relation between reaction rate and catalase concentration is not linear. The best visual fit of the line through the points on Figure 17 is described by the function:

$$k_1 = 1.2 \times 10^{-3} \ (\text{concentration})^{0.8}$$

This relationship, between reaction rate and concentration, was used for the calculation of effective catalase concentrations in the various solutions (control and irradiated) throughout this study.

The activity displayed by a catalase solution will change with storage, particularly if the storage is in light and at elevated temperatures. The studies reported in this thesis have to do with the relative effects of radiation at varying wave lengths and, therefore, the author has not concerned himself with the evaluation of the absolute catalytic ability of the enzyme procured for this study. An effort has been made to maintain adequate control of specimens in every instance and to make a precise evaluation of the concentration of catalase in all solution studies. As an aid to investigators who select the system catalase for future study, an appendix is attached dealing with some of the vagaries of catalase and catalase solutions. This appendix can be consulted for information on the loss of activity of catalase under various conditions.

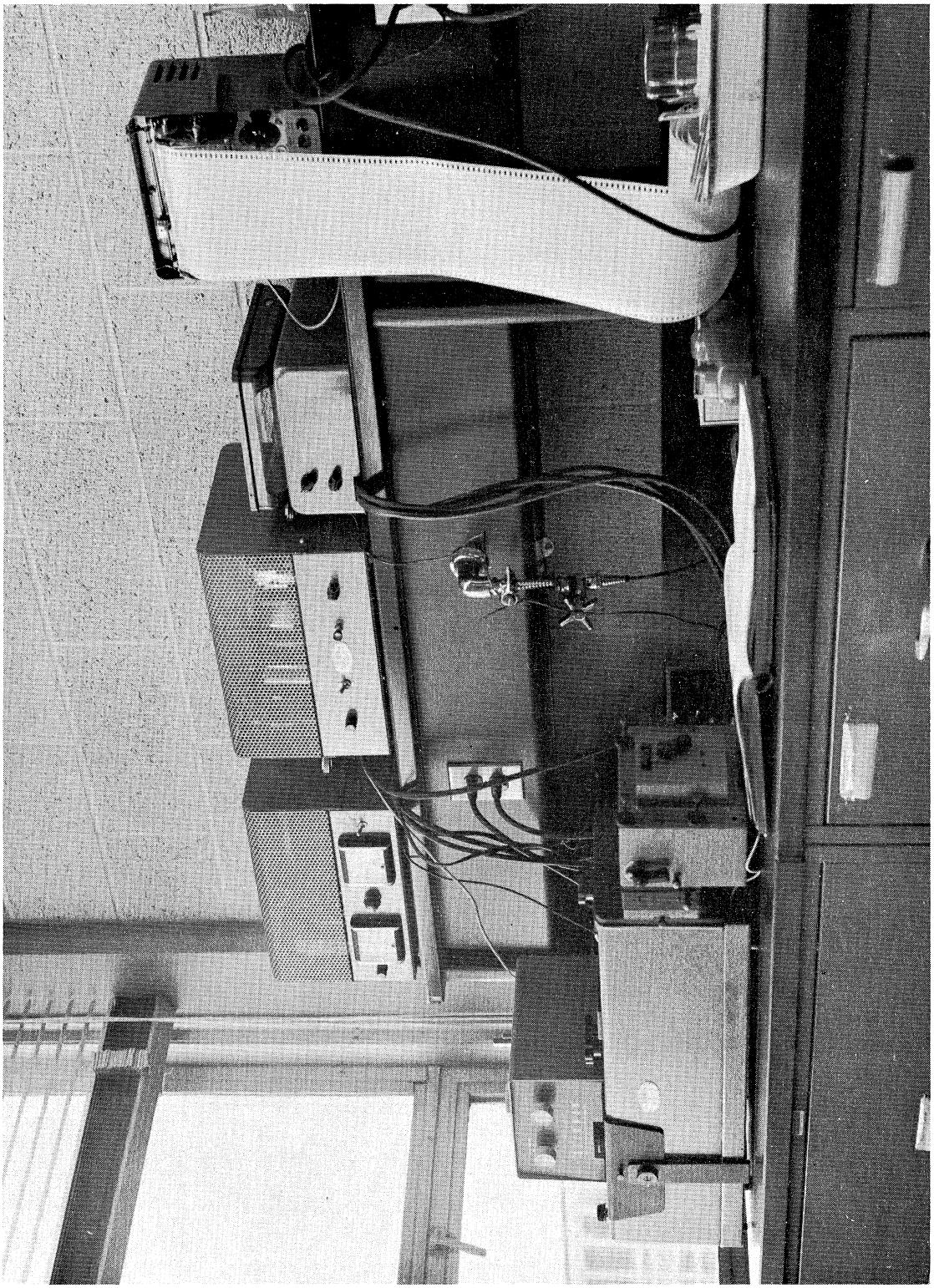


Figure 20. Photograph of D. U. Spectrophotometer-Recorder Unit.

III. EXPERIMENTAL PROGRAM - TESTS AND OBSERVATIONS

A. Studies of Catalase Solutions

1. Monochromatic Irradiations

A stock solution of 10^{-7} molar catalase in 1/15th molar phosphate buffer was prepared. Samples of 0.18 ml were placed in a lucite holder. The holder was constructed with two sample positions, one intercepted the diffracted x-ray beam and the other was shielded from scattered x-rays. The holder was cooled by circulating 5°C water through small holes drilled through the back. A window of scotch tape was placed over the sample and the control. The total assembly was painted black with Krylon spray paint. The cooling and lightproofing were necessary to minimize nonradiation induced enzyme activity loss.

a) Mixed-First and Higher Order Wave Length Irradiations

Each sample was subjected to a selected (by diffraction from a NaCl crystal) x-ray energy to a total absorbed dose of 3.3×10^{11} photons. The correction for loss of photons by absorption in the sample window was made in each instance. The number of photons absorbed was measured by difference in count rate with and without the sample in position in front of the SPG #1 x-ray counter. The dose rate was maintained at $1.7 \pm 0.2 \times 10^{10}$ photons per hour (as measured by the x-ray counter) by adjustment of the x-ray tube current.

The applied potential to the x-ray tube was 30 pkv. This results in 2nd and 3rd order diffraction lines as contaminants in the first order (primary) energy spectrum. The compromise on monochromatic energy was

necessary since a reduction in the pkv exciting potential to a value of less than $\lambda/2$ leaves one with negligible numbers of photons. The relative energy of the x-rays used in this study may be evaluated by inspection of Figure 21. These curves indicate that the extent of 2nd and 3rd order contaminants of the selected energies was excessive at energy settings of less than 10 kev.

After irradiation, three 50 lambda (0.05 ml) aliquots of the irradiated and of the control specimens were evaluated for catalase activity. The three samples were averaged and a calculation was made of the percent catalase destroyed. Figure 22 is a plot of the percent catalase destroyed as a function of the impinging (primary) photon energy. It is apparent that the inactivation of a catalase solution is favored at the iron k-absorption edge. The inactivation curve approximates that of the normalized mass absorption curve for iron, which is sketched on the same figure.

b) True Monochromatic Irradiations

The study described above indicated the presence of a damage sensitivity in the region of the iron k-edge. Since no destruction was apparent at energies of a few tenths kev less than the k-edge energy, it was not possible to evaluate the ratio of damage effectiveness per photon above the edge to that below the edge. A second study was initiated utilizing more nearly monochromatic x-rays. Finer energy resolution was attained by reducing the exciting potential on the x-ray tube to 15 pkv. Measurements of the absorption of these x-rays in aluminum foil are presented as Figure 23. These absorption curves indicate that at 6.9 kev (below the edge) and at 7.3 kev

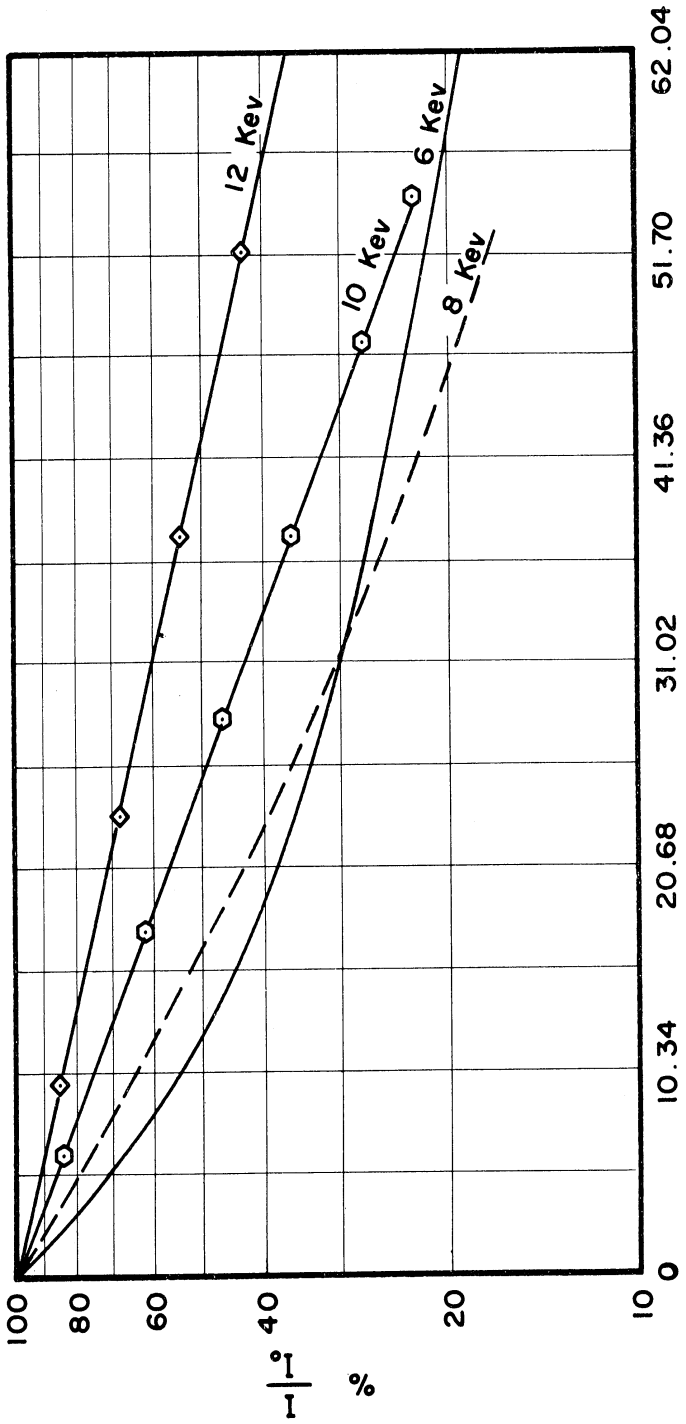


Figure 21. Aluminum Absorption Curves of Diffracted Beam of Various Energies at 30 Peak Kiloelectron Volts.

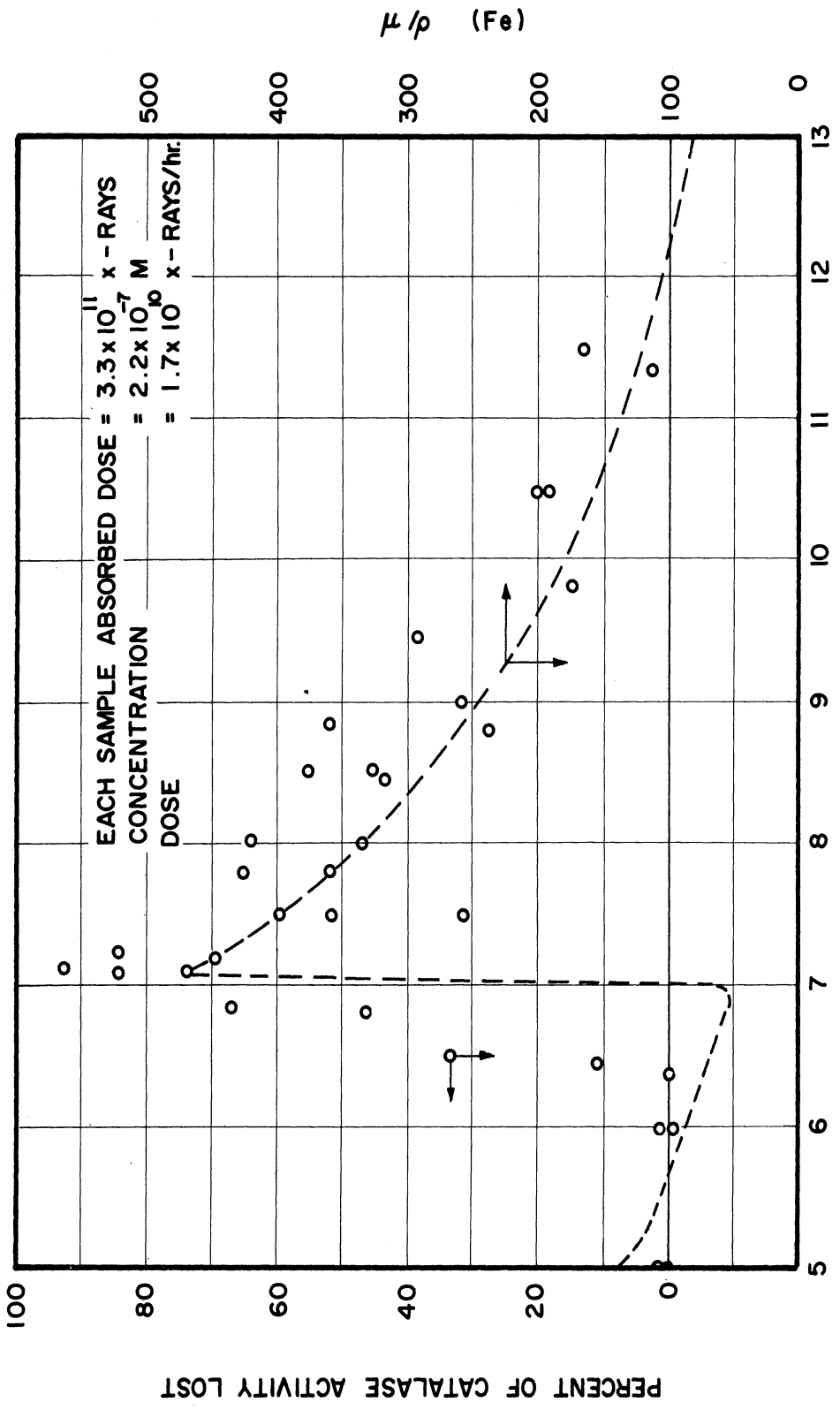


Figure 22. Catalase Solution Loss of Function at Selected X-ray Energies.

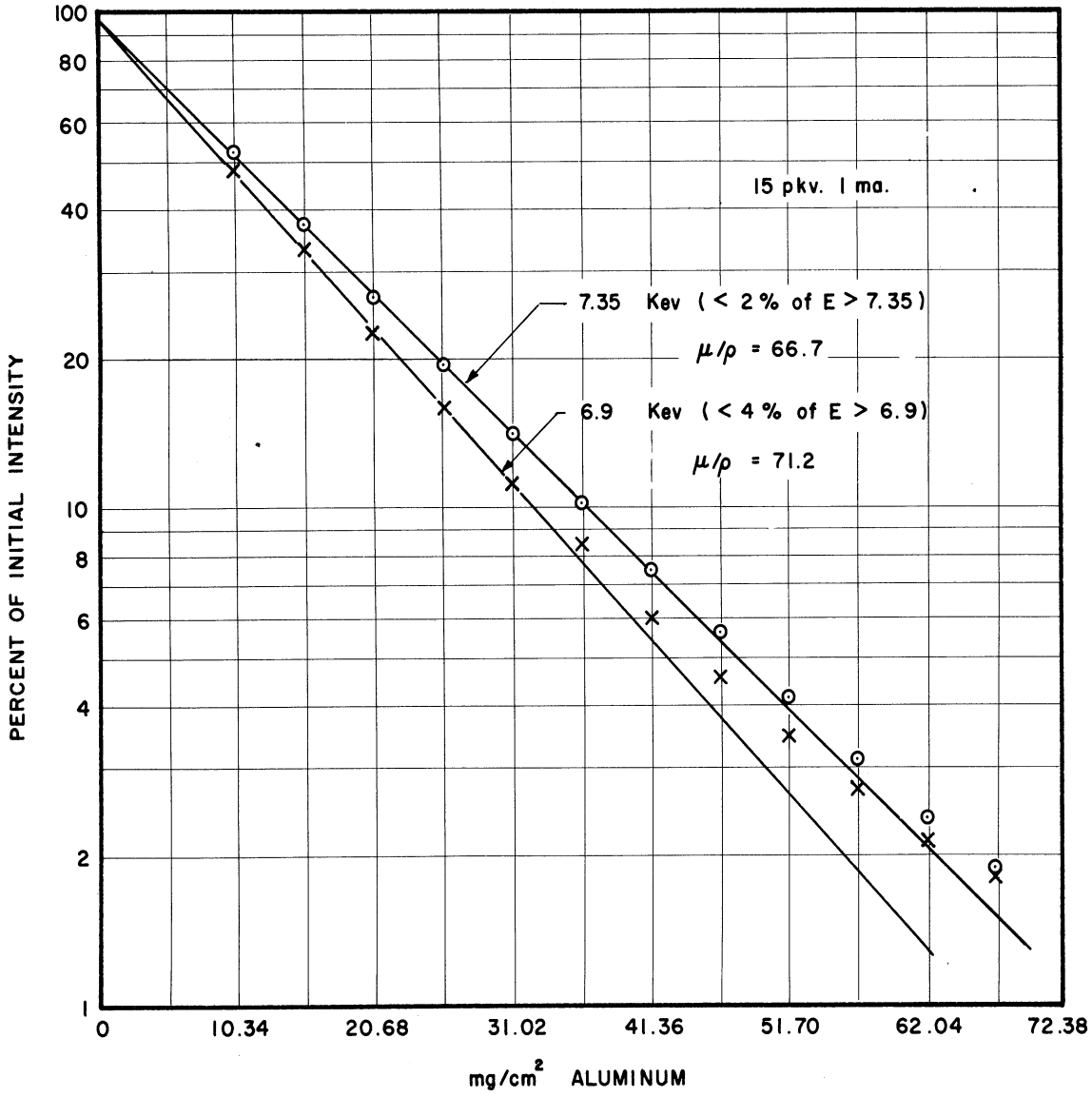


Figure 23. Aluminum Absorption Curves of Diffracted Beam at Energies of 7.35 and 6.9 Kev.

(above the edge) the second order contaminant, as determined by extrapolation and visual inspection, was less than 5 percent of the primary photon beam.

Irradiations of the specimens were alternated so that sample 1 was at 7.3 kev, sample 2 at 6.9 kev, 3 at 7.3 kev, etc. The dose rate was constant at 4.5×10^{9} photons absorbed per hour. The samples were irradiated for various times of from 10 to 130 hours.

The results of this study are presented in Figure 24, which is a graph of the catalase activity remaining after the absorption of a given number of photons. The inactivation curve at 6.9 kev is beginning to indicate catalase destruction at 3.7×10^{11} photons absorbed. It requires 83 hours of irradiation time to deliver this dose of monochromatic x-rays. It may be concluded from this study that (at a given dose rate) it requires three times as many photons to produce 50 percent destruction with photons of 6.9 kev as it does to produce 50 percent destruction at 7.3 kev. If one defines the damage sensitivity as the ratio of the number of the less damaging photons to the number of the more damaging photons, the damage sensitivity at the k-edge is:

$$\frac{5.21 \times 10^{11} \text{ photons}}{1.78 \times 10^{11} \text{ photons}} = 2.9$$

Figure 24 illustrates the characteristic initial increase in catalase activity displayed by irradiated catalase solutions. This peculiarity has been described in earlier studies.[42] This investigator found that he could duplicate this activation of catalase by adding micromole quantities of very dilute hydrogen peroxide to catalase solutions.

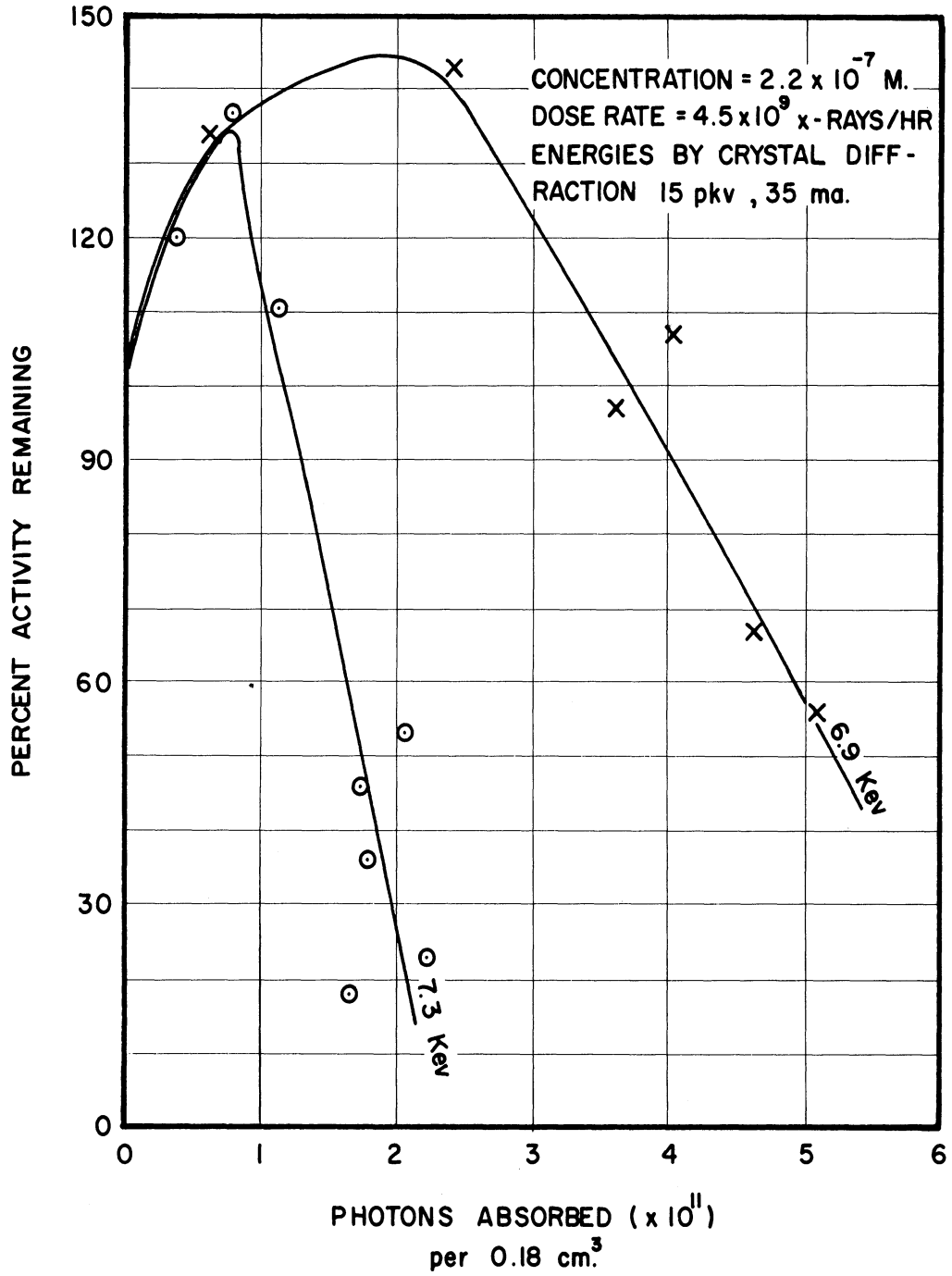


Figure 24. Destruction of a Catalase Solution with Monochromatic X-rays of 6.9 and 7.3 Kev.

Brief mention is made of this phenomena in the appendix on catalase. This activation response does not affect the basic aim of this investigation; it serves only to indicate the complexity of the biological test system - catalase.

In the two studies on catalase solutions, which have been summarized in the preceding paragraphs, the x-rays were obtained by means of diffraction from a NaCl crystal. In the first study, which constituted a gross evaluation of the enzyme solution response, the x-ray tube was operated at 30 pkv. This gives rise to 2nd order energies at selected goniometer settings (x-ray energies) of less than 15 kev. The results of absorption studies of the diffracted x-rays were presented in Figure 22.

In the second study the x-ray tube was operated at 15 pkv which resulted in a more nearly monochromatic beam. The sample was positioned at 14.5 cm from the crystal, where it represented an effective width of 1.1°. This width would correspond to a maximum energy resolution of $\pm$ 0.1 kev at 7 kev, $\pm$ 0.2 kev at 9 kev, and $\pm$ 0.3 kev at 11 kev. Then the x-ray beam energies (with the exception of the small percentage of 2nd order contaminant) utilized in the study plotted as Figure 24, may be defined as 6.9 $\pm$ 0.1 kev and 7.3 $\pm$ 0.1 kev.

2. Irradiations with Fluorescent Target Emission Lines

The utilization of the crystal diffraction technique can provide the experimenter with very nearly monochromatic x-rays. It has the definite disadvantage of very low dose rates. A series of irradiations of catalase solutions were made using fluorescent targets as the x-ray source. The dose rate available from the fluorescent target is 3000 to 10,000 times greater than that from the diffraction assembly.

A lucite sample holder was prepared by machining six slots, each 0.3 cm deep, 0.35 cm^2 area and containing 0.106 cm^3 of volume, into one surface. The slots were positioned in such a manner that five of them intercepted the emission line x-ray beam from the fluorescent target. The volume of the sixth sloth was used as a control. This sample holder was then attached to the sample holder box containing the emission target. All five slots, with their contained samples, were irradiated initially, then a thin lead screen was inserted in front of each slot to terminate the irradiation to that volume. After all five volumes had been irradiated the sample holder was removed and the catalase activity remaining in an 0.05 ml sample was measured in comparison to the unirradiated control.

The x-ray machine set-up used was identical to that picture in Figure 9. The x-ray tube was operated at 50 pkv. The current on the x-ray tube was adjusted down for the higher output targets (Fe and Ni) in an effort to attain approximately equal dose rates.

The dose rate, and subsequently the dose, was measured by means of the Fricke dosimetric technique. The computation of photon absorption was made on the assumption that all photons were photoelectrically converted and the energy of each was totally absorbed.

The data were corrected for 1) x-ray transmission through the scotch tape window, 2) x-ray absorption in the 0.3 cm thick sample, and 3) volume correction to one cm^3. The results are presented as Figure 25. This data illustrates that, not only is the catalase more sensitive to damage by x-rays of energy just greater than the iron k-edge (Nickel K_α

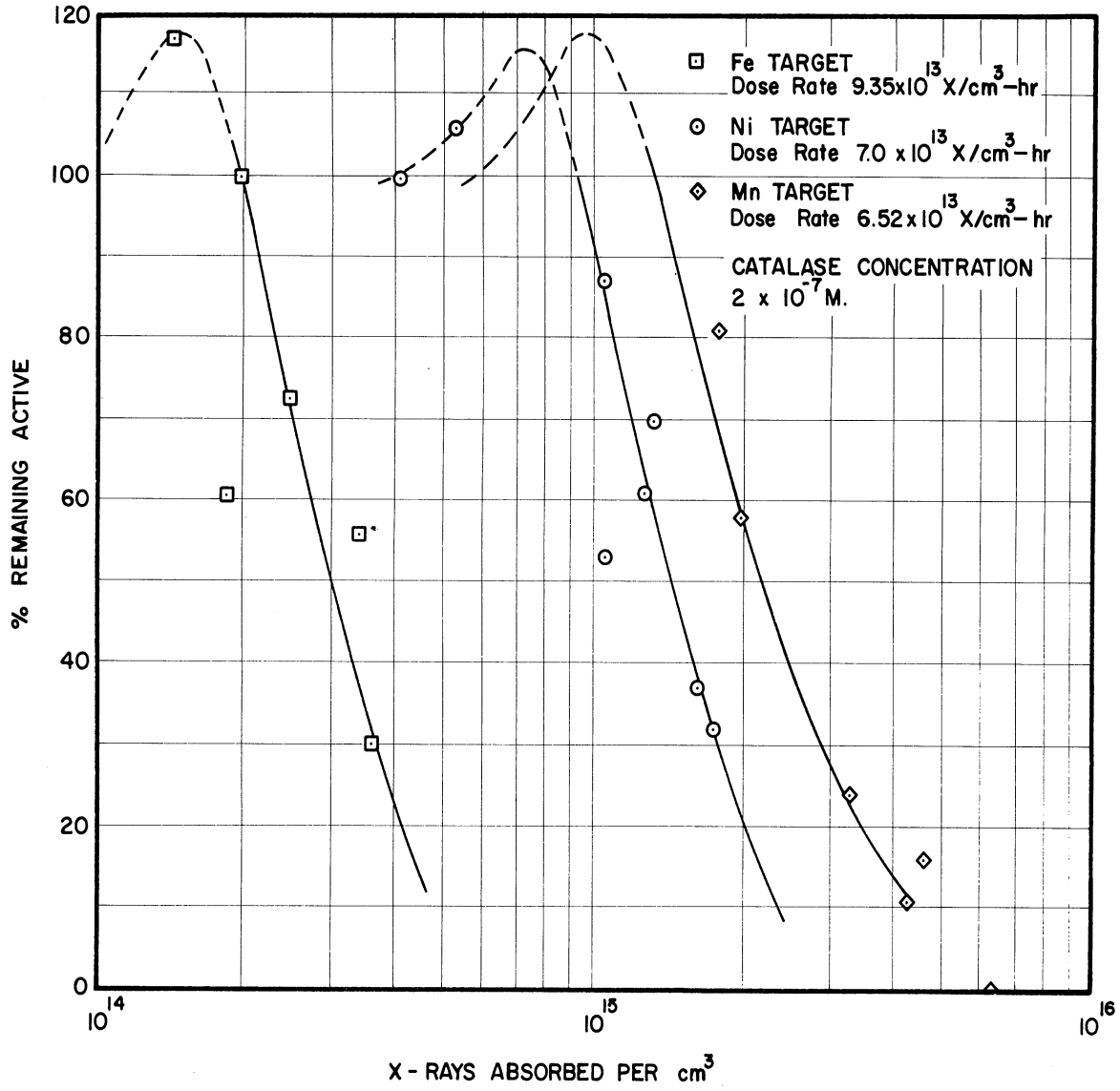


Figure 25. Destruction of Catalase Solutions by X-ray Emission Lines

emission energy of 7.47 kev), but also that the emission line x-rays of the iron target are even more effective in the production of damage. The principle emission line energies from the three target materials used were as follows: manganese K_α of 5.89 kev, iron K_α of 6.4 kev, and nickel K_α of 7.47 kev. The damage sensitivity relative to the photons from the manganese target (K_α = 5.89 kev) for the nickel emission line is:

$$\frac{2.2 \times 10^{15} \text{ photons}}{1.4 \times 10^{15} \text{ photons}} = 1.6,$$

and for the iron target emission line (K_α = 6.4 kev) relative to the manganese emission it is:

$$\frac{2.2 \times 10^{15} \text{ photons}}{3.0 \times 10^{14}} = 7.3 .$$

B. Irradiation of Dry Catalase with Fluorescent Emission Lines

In light of the interesting and unusual results obtained on catalase solutions with the emission line x-rays from various targets, it was decided to investigate the response of dry catalase to this radiation. The target materials used and their pertinent characteristics are summarized in Table X. The measured spectral distributions of these various target emissions are presented graphically in Figures 5, 6, 7 and 8.

1. Technique

In this series of experiments a known weight of dry catalase was pressed into a one by one cm square by 0.3 cm deep depression in a lucite sample holder. The sample was covered with scotch tape. This sample

TABLE X

X-RAY ABSORPTION AND EMISSION ENERGIES*

Element	K_{ab}	K_{β_1}	K_{α_1}	K_{α_2}
Ni	8.331	8.264	7.477	7.460
Fe	7.111	7.057	6.403	6.390
Mn	6.537	6.49	5.898	5.887
Cr	5.988	5.94	5.414	5.405

* from Fine and Hendee[41]

holder was attached to the sample chamber in the position normally occupied by the beam tunnel on the General Electric XRD-5 No. 2 SPG spectrometer assembly (see Figure 9). Studies with dental film packets indicated that the x-ray intensity was uniform over an area greater than the sample area. A control sample of dry catalase was placed on top of the sample chamber in the same heat and light environment but out of the x-ray beam. The x-ray machine was turned on for a number of hours at 50 pkv and 40 ma subjecting the dry catalase to known energy and intensity x-ray exposures.

The samples were removed from the x-ray machine, equal weights of the x-rayed and the control dissolved in 1/15 molar phosphate buffer and made to a volume of 50 ml. The catalase concentration was assayed and

TABLE XI

DRY CATALASE DESTRUCTION WITH
NICKEL TARGET (K_α = 7.47 KEV) EMISSION LINES

Window Transmittance = 0.932
Dose Rate = 5.68×10^5 Ergs/cm^2hr

Run Number	Wt mgm	Hours Irradiated	$1 - e^{-\mu x}$	Ergs Absorbed	Ergs per Gram	Catalase: Percent Remaining
I	40.3	19.6	0.289	2.99×10^6	7.43×10^7	57
II	41.0	68.7	0.293	1.07×10^7	2.60×10^8	18
III	37.3	44.7	0.272	6.42×10^6	1.72×10^8	26
X	39.5	15.7	0.285	2.37×10^6	6.00×10^7	64
XI	34.4	53.8	0.254	7.22×10^6	2.10×10^8	31

that in the x-rayed sample compared to that of the control. Table XI presents a summary of all the runs made with a nickel fluorescent target, Table XII, those made with an iron target, Table XIII, those made with a manganese target, and Table XIV, the runs made with a chromium target. The data presented in these four tables were prepared in an identical manner, then the treatment of a typical nickel run will be utilized as an illustration.

TABLE XII

DRY CATALASE DESTRUCTION WITH
IRON TARGET (K_α = 6.40 KEV) EMISSION LINES

Window Transmittance = 0.891
Dose Rate = 4.29×10^5 Ergs/cm^2 hr

Run Number	wt mgm	Hours Irradiated	$1 - e^{-\mu x}$	Ergs Absorbed	Ergs per gram	Catalase: Percent Remaining
IX	42.6	17.6	0.425	2.85×10^6	6.71×10^7	107
XV	37.1	41.8	0.383	6.12×10^6	1.65×10^8	52
XIV	32.4	18.2	0.343	2.39×10^6	7.36×10^7	91
V	37.4	65.6	0.377	9.45×10^6	2.53×10^8	10
VI	37.0	43.8	0.377	6.31×10^6	1.71×10^8	34
XXI	28.3	7.6	0.308	8.95×10^5	1.61×10^7	103
XXII	26.0	13.1	0.287	1.44×10^6	5.52×10^7	98
XXIII	25.6	30.8	0.283	3.33×10^6	1.30×10^8	72

TABLE XIII

DRY CATALASE DESTRUCTION WITH
MANGANESE TARGET (K_α = 5.89 KEV) EMISSION LINES

Window Transmittance = 0.868
Dose Rate = 2.47×10^5 Ergs/cm^2 hr

Run Number	wt mgm	Hours Irradiated	$1 - e^{-\mu x}$	Ergs Absorbed	Ergs per gram	Catalase: Percent Remaining
XII	32.1	54	0.398	4.61×10^6	1.44×10^8	63
XIII	34.7	106	0.422	9.59×10^6	2.76×10^8	22
VIII	39.9	87.2	0.468	8.75×10^6	2.19×10^8	21
VII	37.4	29.8	0.471	3.01×10^6	8.05×10^7	97
XVI	27.5	112.2	0.374	8.99×10^6	3.27×10^8	7

2. Computation and Data

Run number 1, with the nickel target, was made at a dose rate of 5.68×10^5 ergs/cm^2 hour as measured with the Fricke dosimetric technique. The weight of catalase compressed into the 1 cm square by 0.3 cm deep depression was 40.3 milligrams. The scotch tape window transmitted 0.932 fraction of the impinging x-rays (see Figure 14). The mass absorption coefficient utilized in the calculation of energy absorbed is that measured by the author and plotted as Figure 13.

TABLE XIV

DRY CATALASE DESTRUCTION WITH
CHROMIUM TARGET (K_α = 5.41 KEV) EMISSION LINES

Window Transmittance = 0.844
Dose Rate = 1.28×10^5 Ergs/cm^2 hr

Run Number	wt mgm	Hours Irradiated	$1 - e^{-\mu x}$	Ergs Absorbed	Ergs per gram	Catalase: Percent Remaining
XVIII	35.6	92.6	0.543	5.43×10^6	1.53×10^8	35
XIX	33.2	87.3	0.518	4.88×10^6	1.47×10^8	40
XX	34.3	53.5	0.530	3.06×10^6	8.93×10^7	43
XXIV	23.2	20.9	0.400	9.03×10^5	3.89×10^7	75
XXV	25.0	9.8	0.423	4.47×10^5	1.79×10^7	84

The calculation of energy absorbed in run number 1 proceeds as follows:

$$5.68 \times 10^5 \text{ ergs/cm}^2 \text{ hr} \times 19.6 \text{ hours} = 1.11 \times 10^7 \text{ ergs/cm}^2$$

then,

$$1.11 \times 10^7 \text{ ergs/cm}^2 \times 0.932 \text{ transmitted} = 1.036 \times 10^7 \text{ ergs/cm}^2$$

then,

$$1.036 \times 10^7 \text{ ergs/cm}^2 \times (1 - e^{-\mu x})$$

$$= 1.036 \times 10^7 \times (1 - e^{-8.5 \times 4.03 \times 10^{-2}})$$

$$= 1.036 \times 10^7 \times (0.289)$$

$$= 2.99 \times 10^6 \text{ ergs absorbed}$$

and,

$$\frac{2.99 \times 10^6 \text{ ergs absorbed}}{4.03 \times 10^{-2} \text{ grams}}$$

$$= 7.43 \times 10^7 \text{ ergs/gram}$$

The absorption of this amount of energy in this sample of catalase decreased its ability to catalyse the destruction of hydrogen peroxide to 57 percent of that of the control sample.

The dose rates available from the various targets were measured with the Fricke dosimeter. The energy of the fluorescent emission was considered to be that of the K_α emission line in the evaluation of scotch tape transmittance and of the mass absorption coefficient.

When the early data from these various runs with different targets were graphed it became obvious that the results followed two distinct trends. These two response trends could not be accounted for in terms of a difference in the catalase, since it all came from the same lot (even the same bottle). Then subsequent runs were made with a different target for each run, rather than a number of runs with one target, followed by a number with a second target, etc. The response pattern persisted, which would eliminate the possibility of a systematic error in a set of runs with a single target.

The data tabulated in the four tables (XI, XII, XIII, XIV) are presented in the form of three figures. The data of Tables XI and XIV have been combined as Figure 26, which illustrates the destruction of dry catalase by the emission line x-rays of nickel (K_α = 7.47 kev) and chromium (K_α = 5.40 kev). These data appear to fit a direct effect, single-hit curve. Figure 27 was prepared from the data of Tables XII and XIII. This figure illustrates the destruction attained with the emission line x-rays of iron (K_α = 6.40 kev) and manganese (K_α = 5.89 kev). Figure 28 is presented to emphasize the difference in the response patterns.

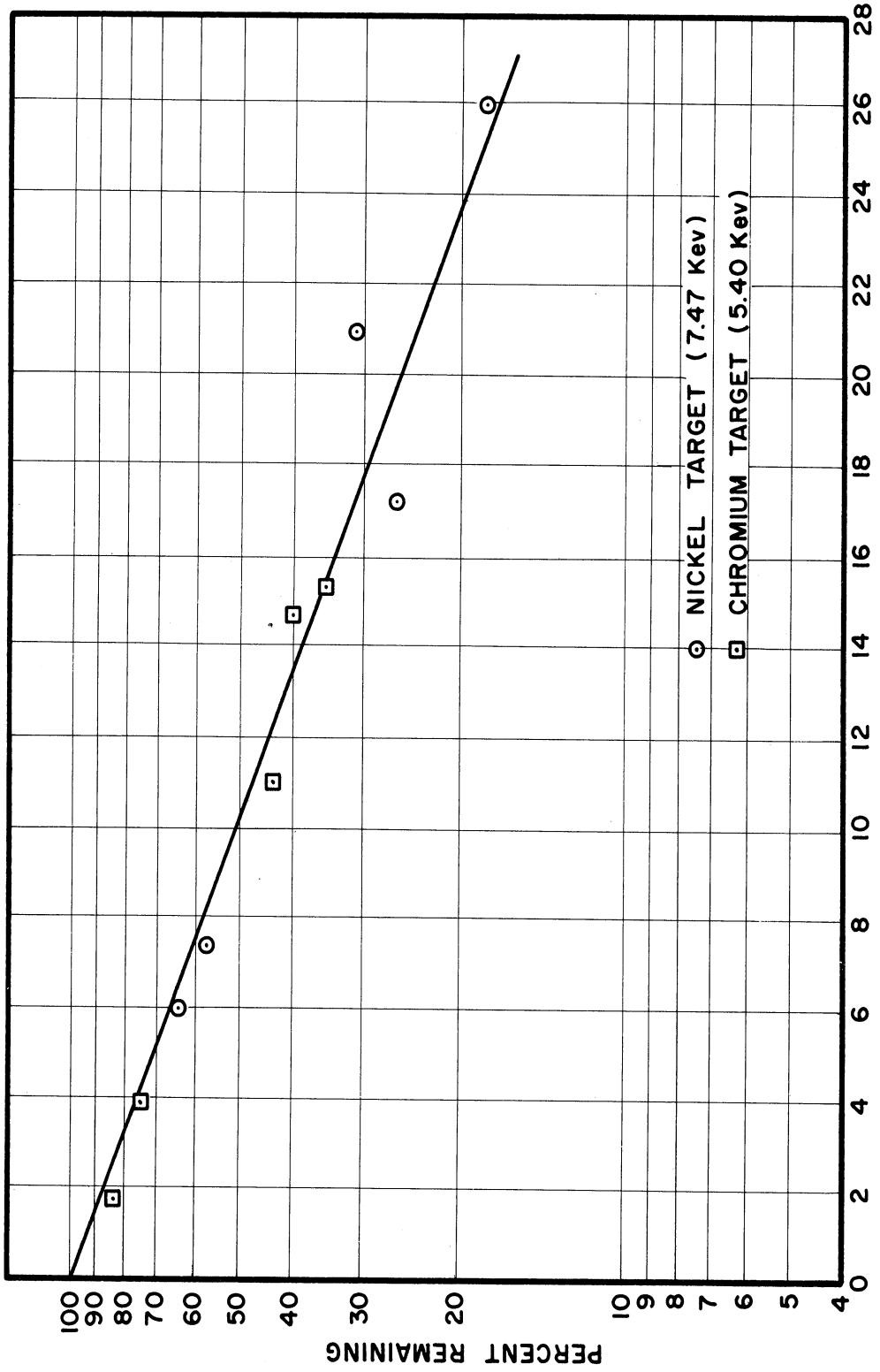


Figure 26. Dry Catalase Destruction by X-rays of 7.47 and 5.40 Kev.

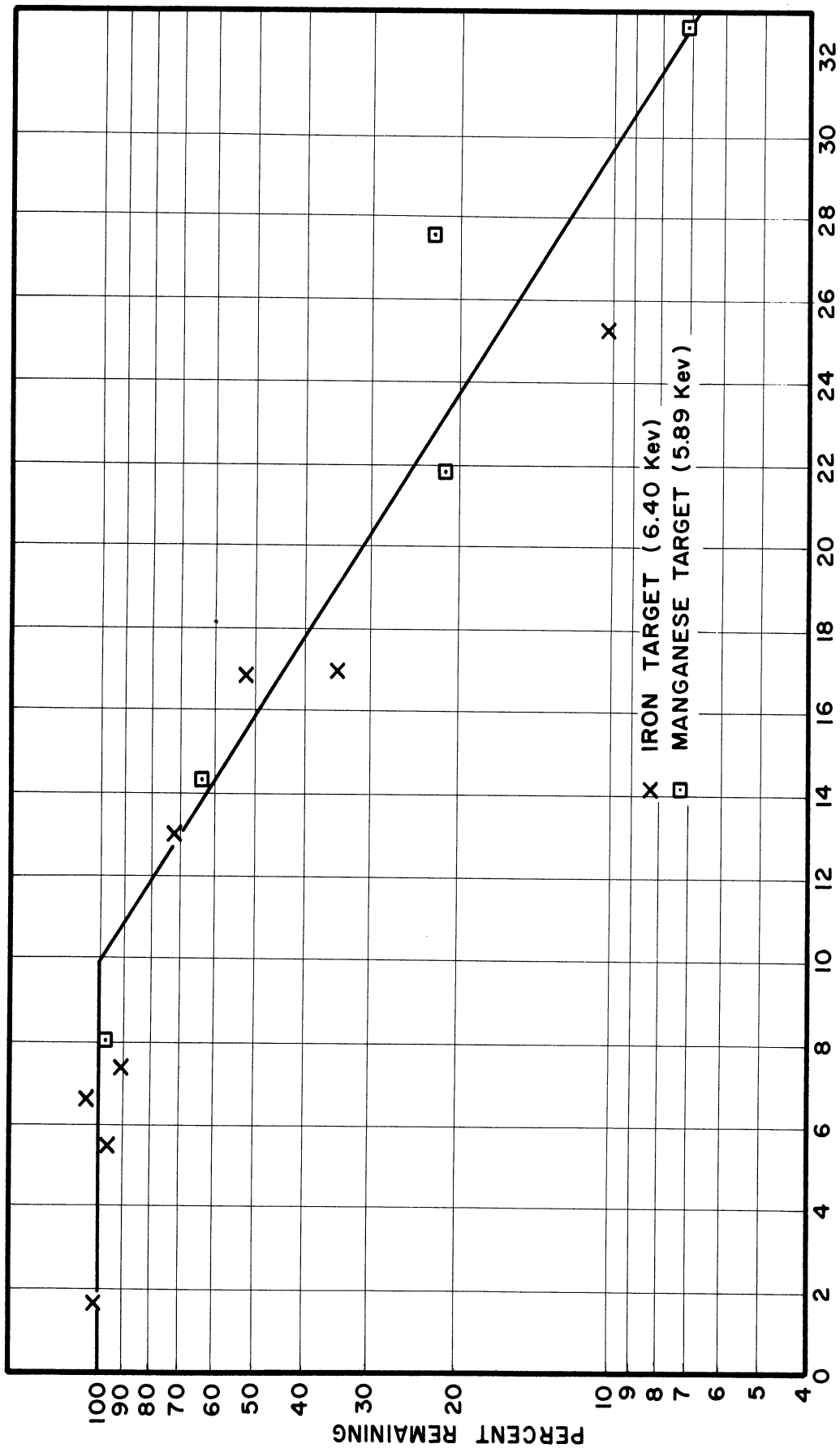


Figure 27. Dry Catalase Destruction by X-rays of 6.40 and 5.89 Kev.

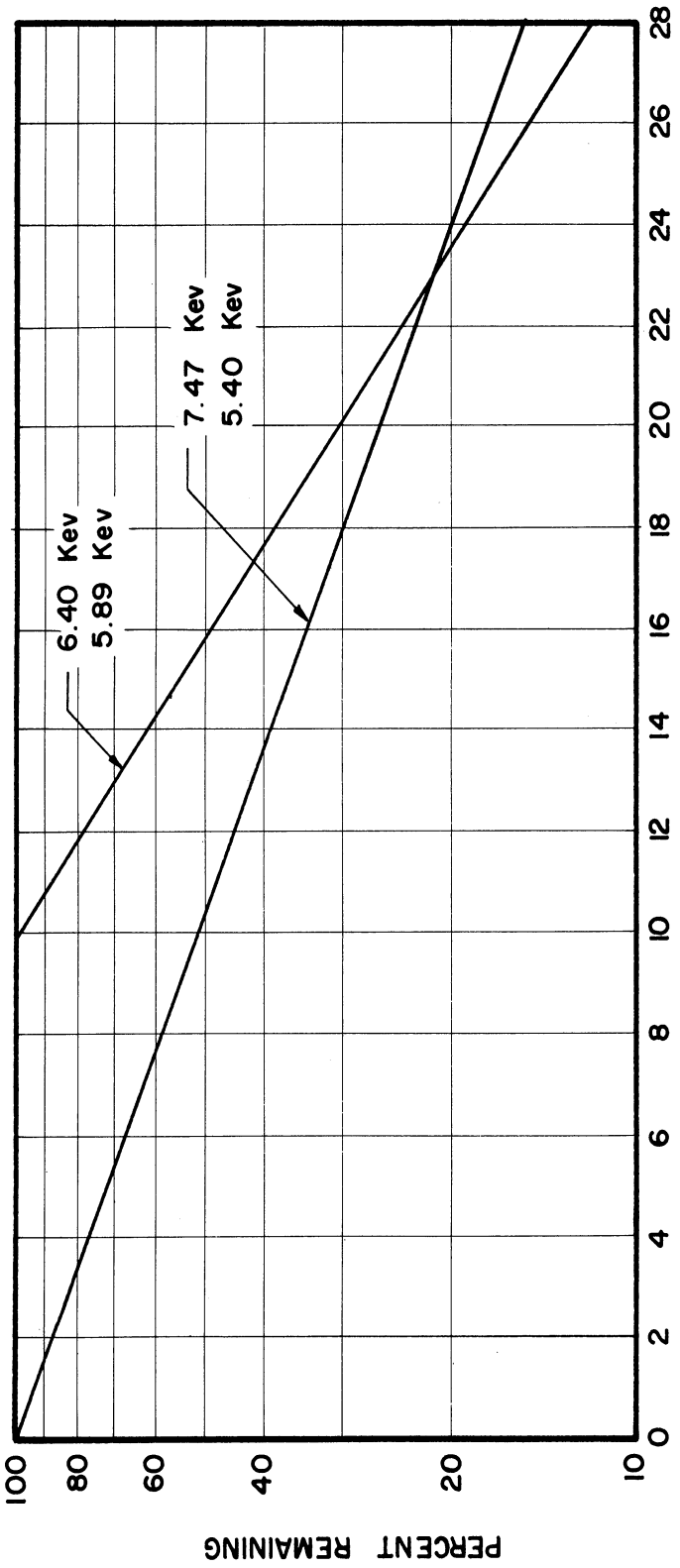


Figure 28. Dry Catalase Destruction by Emission Line X-rays.

In many radiobiological investigations it has been the vogue to present destruction yield in terms of a G factor, where one calculates the molecules destroyed or altered in some fashion as a function of the energy absorbed by the system. It is obvious that such an evaluation of catalase destruction in the instances sighted above would not be meaningful. In one instance, the x-ray effect is not apparent until one delivers to the system approximately 10^8 ergs per gram of energy, at which time a pronounced effect is apparent; note the greater slope (Figure 27). In the other instance, the destruction attained with the 7.4 and 5.4 kev x-rays follows a single-hit type curve with a uniform slope on semi-log paper which permits one to readily evaluate the yield factor. The point to be emphasized is the difference in the type of response to these four different emission x-rays varying only slightly in energy.

C. Consideration of Errors

The primary experimental data consists of dose rate evaluations and chemical rate measurements. The Cobalt-60 source and the fluorescent x-ray sources were calibrated by the Fricke dosimeter technique.[47] A G value of 15.4[87] was used in the dosimetry of the cobalt source (Appendix B). The source has been cross-checked with those of other facilities. The standard error in the measurement of the Cobalt-60 dose should not exceed eight percent.

The x-ray emission dose rates from the various targets were determined by the Fricke dosimeter. A G value of 13.4 was used for the x-ray region of 5 to 10 kev. There is but a single evaluation[25] of the

ferric ion yield in this energy region; therefore, it is difficult to estimate the error in this technique. The author would propose that future calorimetric dosimetry studies, utilizing thermistor detectors, will serve to define precisely the dosimetric relationships in this low energy x-ray region. If a gross error existed in the x-ray dosimetry it is to be expected that this error would not display rapid fluctuations, but would be systematic over the energy range studied (i.e., 5 to 10 kev). If a systematic error existed in these measurements it would alter yield values but would not eliminate the observed differences in damage.

In all measurements with the x-ray counter tube corrections were made for coincidence loss. All counting periods were adjusted in duration to reduce the standard error to less than 5 percent. The efficiency of the x-ray counter, for a given energy of x-ray, was evaluated by means of cross-calibration against the Fricke dosimeter. Then the error in the x-ray data is that associated with the yield of the Fricke dosimeter in this energy range.

The largest error existent in this study is that associated with the evaluation of enzyme activity. This error is the sum of a number of errors which may occur in the weighing, the transfer of aliquots, and in the evaluation of the reaction rate. A series of ten analyses of the reaction rate of a catalase solution was made. In each analysis 0.05 ml of solution was transferred to the cuvette and the hydrogen peroxide added. The reaction rate was measured by the technique described in Section II-B. The reaction rate values, together with the differences, and an estimate of the standard deviation, are given in Table XV. It

TABLE XV

ANALYSIS TECHNIQUE REPRODUCIBILITY EVALUATION

Ten Runs

k_1 Reaction Rate	Δk_1 from median	Δk_1 from average
0.0026	-0.0003	-0.0005
0.0028	-0.0001	-0.0003
0.0028	-0.0001	-0.0003
0.0028	-0.0001	-0.0003
0.0029	Median	-0.0002
0.0030	+0.0001	-0.0001
0.0030	+0.0001	-0.0001
0.0032	+0.0003	+0.0001
0.0035	+0.0006	+0.0004
0.0035	+0.0006	+0.0004

Median = 0.0029 Average = 0.00311

$$\text{Standard Deviation} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{9}}$$

$$= \pm 0.00031$$

- - - - - - - - - - - -

may be concluded that the standard error of an analysis for catalase activity is $\pm$ 10 percent.

The summation of all errors inherent in this study (dosimetry and chemical rate) could not account for the large differences in damage response observed.

IV. CONCLUSIONS AND DISCUSSION

A. Summary of Conclusions

The experimental results enumerated in detail in the preceding pages of this thesis indicate that the catalase system displays response phenomena suggested, but not emphasized, in two earlier publications.[50,86] The experimental evidence for the existence of the first of these response phenomena is presented in that section of this thesis titled "Studies of Catalase Solutions." In this section it is demonstrated that solutions of catalase exhibit a damage sensitivity to x-rays at energies just exceeding the iron k-edge energy. The results indicate that the photon of energy just greater than the iron k-edge energy is three times more effective in the production of damage than that photon of energy just less than the iron k-edge. There are additional experimental data that indicate that the x-ray photon of energy just equal to the fluorescent emission line energies of iron are seven times as effective in the production of damage as are those of slightly lesser energy.

In the section titled "Irradiation of Dry Catalase with Fluorescent Emission Lines," data are presented which indicate that dry catalase, subjected to the emission line x-rays from iron and manganese targets, requires an induction dose before damage is evident. When the dry catalase is irradiated with the emission line x-rays from nickel and chromium a single-hit, exponential decrease in the catalytic effectiveness of the catalase is demonstrated.

B. Discussion

1. Methods of Damage

In every instance the criterion for damage in catalase has been the loss of the enzyme's ability to destroy hydrogen peroxide. A number of instances have been reported in which catalase subjected to x-irradiation in the dry state displayed a reduced solubility in the buffer solutions. This decrease in solubility, as reported by Pollard[96] and others, is attributed to an increase in cross-linkage within the polymerlike enzyme structure. Those samples subjected to large doeses of Cobalt-60 gamma radiation in particular displayed this phenomenon. It is possible that a certain amount of crosslinking could occur within the polymer structure without rendering the enzyme insoluble. In no instance was a sample utilized when it displayed visible insolubility. If a solution were made in which the enzyme was partially cross-linked and yet soluble, it is conceivable that this solution of enzyme might display a lesser ability to catalyze the reaction. This loss of function would be evaluated as radiation damage.

The cross-linking phenomenon is only one of a number of ways that the radiation damage may have occurred. Enzymes are extremely susceptible to the presence of minute concentrations of inhibitors.[38] Some of the inhibitors that have been studied in conjunction with the system catalase-hydrogen peroxide are the cyanide and the azide radical. Concentrations of 5×10^{-6} M in cyanide radical produce up to 50 percent inhibition of catalase by complexing with the iron. A concentration of 2×10^{-5} M of the azide radical is capable of producing 50 percent inhibition of catalase by iron complexing. With the presence of the nitrogen immediately adjacent

to the iron in the active center of the catalase molecule, it is attractive to postulate the possibility of a cyanide poisoning of catalase arising from radiation production of the cyanide radical. The chemical instability of the azide radical (with its three nitrogens in a row) makes the postulation of radiation induced azide inhibition of catalase activity less attractive.

A third type of radiation damage which might be postulated for catalase is a disruption of the structure of the catalase molecule to bring about the loss of function. The ability of catalase to oxidize hydrogen peroxide to free oxygen and water is related to the protoporphyrin group which forms the active center of the enzyme. Evidence indicates that it is this active center which combines with the substrate and is responsible for the catalytic properties of the catalase molecule. This active center determines the specificity and the catalytic activity. It is expected that it will be a structure of some degree of complexity. There is a prevalent theory of enzyme action called the key theory which relates the position of the substrate with respect to the enzyme molecule and its protein parts. This key theory holds that the substrate molecule must fit into close proximity with the active center. The ability to fit into the key would be dependent upon the configuration and intactness of the attached protein molecules. Then the structure of the attached protein is of importance for the enzymic activity. There is evidence that the separation of the adjacent peptide chains in the protein structure results in loss of enzyme activity. It is thus feasible to postulate the possibility of a degradation (breakage) of protein chains attached to the

active center of the catalase molecule and, in turn, the production of loss of activity which is interpreted as radiation effect.

Dale and Russell performed a study[36] of the irradiation of catalase and, as a part of this study, demonstrated some electrophoresis patterns of catalase after varying doses of radiation. For the control (unirradiated) catalase, their electrophoretic pattern showed up as a streak typical of the chromatography pattern of proteins. As the dose of radiation was increased, the length and magnitude of the streak on their chromatogram decreased until, at doses of 10^6 r, (from an electron beam) their samples showed very little migration with solvent flow. This experiment was utilized by Dale and Russell to demonstrate the degree of protection afforded catalase by cysteine and other chemicals. It is possible that this decreased movement of the protein with increasing doses of radiation might be related to the cross-linking of protein structures, resulting in a decreased solvent solubility and, therefore, decreasing motion in a chromatographic study.

This thesis has not been primarily concerned with the determination of the actual mode of destruction of catalase function. It appears that any one or more of the three mentioned inhibitions of catalase activity may be taking place. Additional investigations utilizing spectroscopy, chromatography, and ultracentrifuge techniques might serve to elucidate the basic mechanisms of enzyme inactivation taking place.

2. Resonance Phenomena

When solutions of catalase were irradiated with monochromatic x-irradiation, greater damage sensitivity was displayed at x-ray energies

equal to and slightly greater than the k-edge of iron. Evidence presented in Section II-B of this thesis indicated that the solution of catalase did not show increased absorption of x-rays at energies equal to and greater than the iron k-edge. The absorption of x-rays by catalase solutions exhibited no measurable discontinuities in the k-edge vicinity. In the first study of catalase solutions (Section III-A) it is pertinent that in each case the number of photons absorbed in the solutions was the same, but in those instances where the x-ray energy equaled or slightly exceeded the iron k-edge energy, the effect produced per photon exceeded that produced with x-rays of energy less than the k-edge of iron. This evidence indicates that there is an increased sensitivity to damage per photon absorbed at the iron k-edge in the solutions of catalase.

Solutions of catalase irradiated with the emission line x-rays from iron, nickel, and manganese targets showed widely varying sensitivity to these radiations. It was found that the emission line x-rays from the iron target were approximately seven times more effective (evaluated at 50 percent damage) than were the emission x-rays from the manganese target. The emission line x-rays from the nickel target were approximately 1.6 times more effective than those of manganese. This indicates that catalase is susceptible to radiation damage by x-rays of energy roughly equal to the emission line energies of iron.

It is known that the catalytic ability of catalase is derived from its iron content. Thus the susceptibility to damage by x-rays preferentially absorbed in iron at energies just greater than the k-absorption edge should not be completely unexpected. The greatly

increased damage at energies corresponding to the emission line energies of iron however defies explanation since it does not fit any existent body of theory. Garsou[50] suggested very cautiously that he observed this effect, but no explanation is presented. At no place, in the existent literature, is there any data that would indicate the existence of a preferential interaction or absorption of emission line x-rays in a material of the same identity as the fluorescent target.

A calculation of the number of molecules destroyed per photon at 6.9 kev is as follows:

$$\text{Molecules per liter} = 2.2 \times 10^{-7}\ M \times 6.02 \times 10^{23}$$

$$= 1.33 \times 10^{17}$$

$$\text{Molecules per cm}^3 = 1.33 \times 10^{14}$$

$$\text{Molecules per } 0.18\ \text{cm}^3 = 2.39 \times 10^{13}$$

and 50% destroyed by 5.2×10^{11} photons absorbed or,

$$\frac{1.19 \times 10^{13}\ \text{molecules destroyed}}{5.2 \times 10^{11}\ \text{photons absorbed}}$$

$$= 23\ \text{molecules/photon.}$$

At 7.3 kev, this number increases by a factor of three. Then, in each instance, the damage yield per photon absorbed greatly exceeds one, indicating either an indirect radiation effect arising from the production of free radical and related species in the solvent, or a multiple transfer of energy from molecule to molecule of catalase (chain reaction). The latter argument, that of energy transfer, is of dubious value since the ratio of catalase to water molecules is about one in 10^5. This means the separation between catalase molecules is thousands of angstroms.

If the yields of inactivated molecules were much less than one per photon in each instance, the existence of an indirect effect at energies less than and much greater than the iron k-edge might be postulated, accompanied by a direct effect at, and very slightly above, the iron k-edge. On the basis of the data of this study such a postulate is not warranted since the system displays yields which exceed one molecule per photon.

In the section titled, "Irradiation of Dry Catalase with Fluorescent Emission Lines," a novel response is demonstrated to x-rays whose energies are 6.4 and 5.89 kev. These are the emission line K_α x-rays from iron and manganese fluorescent targets. The response demonstrated is one of extended energy input prior to a loss of catalase function. It is found that the irradiation of dry catalase with the emission lines of nickel and chromium, whose K_α energies are respectively 7.47 and 5.41, produces a single-hit inactivation curve of the type to be expected on the basis of prior work.

The existence of an induction dose of radiation prior to the appearance of damage has been noted in other systems and related to a multi-hit effect. The multi-hit theory postulates that a single ionization in a molecular entity is not capable of producing destruction of the entity whereas two or more hits, depending upon the susceptibility of the system, are capable of producing damage. Analysis of the induction curve produced with an iron and with a manganese target indicates that, according to the multi-hit theory, the results produced correspond to approximately 4.6 hits per molecule to produce inactivation. If one accepts this, he

is forced to postulate the existence of excited states within the catalase molecule whose lifetime is sufficiently long that four or more hits can be made upon the molecule before inactivation takes place. The duration of the irradiation time would place the life of this excited species in hours. Such an excitation phenomenon is not presently recognized. On the other hand, enzymes must be capable of energy transfer and a limited amount of energy storage, since the free energy from such chemical reactions as the oxidation of food stuffs is stored and made available for carrying out energy-requiring reactions instead of being lost immediately as heat. It is thus in the realm of possibility that the irradiated catalase is capable of a limited amount of energy storage until such a time as additional energy imparted to the system produces a breakdown of the catalase molecules and subsequent loss of function.

These data indicate that biological sensitivities exist which have not been previously considered in the radiobiological literature. They indicate the need for additional investigation into the mode and site of energy absorption. The data seem to substantiate other evidences that complex biological systems undergo the most complex of interaction phenomena.

The basic question, toward which the investigation has aimed, is whether there existed regions in which damage is sensitive, not only to energy absorbed, but also to the individual photon energy. This question is answered in the affirmative. There is opened a whole spectrum of questions concerning the mechanism, kinetics, and products of damage in these energy regions.

APPENDIX A

CATALASE

The enzymes present in living material have been called the most important tools of the living cell. These enzymes accomplish thousands of chemical transformations which would be impossible except for their presence. They perform chemical reactions of hydrolysis, oxidation, or reduction of substances which might be accomplished in the laboratory by the employment of strong acids, alkalis or oxidizing agents accompanied with high temperature conditions. These temperatures and reagents are incompatible with the living cell. The cells of the living body accomplish these reactions at very high speeds, at an approximately neutral pH, and at body temperatures with the aid of a myriad of enzymes. The enzymes are organic catalysts produced by living organisms. They are all complex colloidal proteins characterized by great catalytic activity, a high degree of specificity, and extreme susceptibility to the influence of pH, temperature and other environmental conditions.

Catalase is a member of a group of enzymes classified as the iron enzymes. The members of this group of enzymes possess catalytic properties which are derived from their iron content. The specific action of catalase has been shown to be the decomposition of hydrogen peroxide. It is called a protective enzyme since it tends to protect living cells by decomposing the hydrogen peroxide formed in certain reactions into water and molecular oxygen. The reaction of catalase on hydrogen peroxide has been formulated as follows:

$$RFe - OH + H_2O_2 \rightarrow RFe - OOH + H_2O$$

$$RFe - OOH + H_2O_2 \rightarrow RFe - OH + H_2O + O_2$$

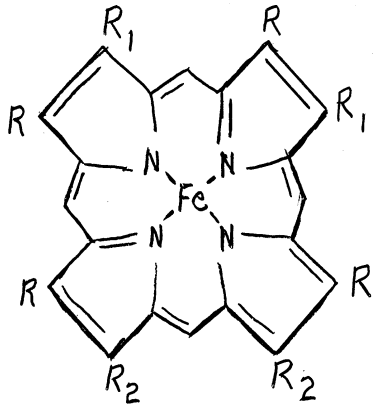
Catalase is found in practically all forms of life. In higher animals such as the human, it is present throughout the body but occurs in particularly large amounts in the liver, kidneys, erythrocytes and in fatty tissues.

The enzymatic action of plant and animal tissues was discussed by Phenard in 1818. He noted that these tissues possessed the property of decomposing hydrogen peroxide. In 1901 Lowe showed that the decompostion of hydrogen peroxide was caused by a specific enzyme which he named catalase. In 1926 Hennichs purified catalase and found that it contained iron. The theory of its action in decomposing hydrogen peroxide was advanced in 1941 by Sumner and Dounce. This theory was further verified and elaborated upon by the work of Chance in 1950.

Setlow[110], in a very interesting study on the radiation sensitivity of catalase as a function of temperature, has pointed out that it is possible to inactivate portions of the total catalase molecule. The catalase molecule contains 4 iron atoms and his data indicate that portions of the total molecule may be inactivated as a function of radiation dose at a given temperature. He plots and presents data on the inactivation cross section of catalase as a function of temperature. These data exhibit three plateaus. He proposes that the existence of these plateaus is indicative of a partial loss of function of the total molecule.

The iron in the catalase molecule is located in the middle of a series of porphyrin rings. The basic structure of the active iron centers

in catalase has been proposed[45] as:



where:

$R = CH_3$

$R_1 = CH\ CH_2$

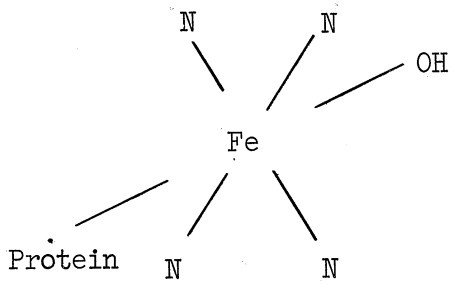
$R_2 = CH_2CH_2COOH$

In spectroscopy of these iron bearing biological compounds this iron center gives rise to a maxima of absorption at 405 millimicrons wave length. This maximum in the wave length curve is known as the soret band. A disappearance or diminution of the soret band of absorption as a result of the irradiation of catalase has been described by Dale.[37]

Forssberg,[43] in 1947, had proposed that the destruction of catalase solutions came about as the result of the generation of hydrogen atoms in water and subsequent destruction of catalase by these hydrogen atoms. Dale and Russell[36], in 1956, reinvestigated this problem in a study of the irradiation of catalase in the presence of cysteine, cystine and glutathione. Their results were indicative of an OH and O_2H radical destruction of catalase solutions. Dale and Russell performed some very interesting electrophoretic and spectrographic studies of the irradiation products. In this same study, Dale and Russell report a change in the absorption spectrum of catalase after irradiation indicative of radiation damage to the iron center group in catalase.

The available evidence on catalase indicates that the reactive group is the iron protoporphyrin whose structure was sketched previously.

This iron protoporphyrin structure forms the active center in four haemoproteins: hemoglobin, myoglobin, peroxidase, and catalase. These four haemoproteins differ only in the protein which is attached to the iron protoporphyrin and in their molecular weights. The protein group is joined to the iron protoporphyrin group by a coordinate bond in the fifth coordination position about the iron atom. The sixth coordination position is occupied by an OH group which completes a stable octahedral coordination unit. This structure would appear thus:



in which each nitrogen (N) is actually in a ring compound (as sketched previously).

Haldane[58] has analyzed data by Zeile and Hallstrom to show that a molecule of catalase at 0°C catalyzes the destruction of about 2×10^5 hydrogen peroxide molecules per second. This would mean that the mean life of an active catalase-hydrogen peroxide molecule is about 10^{-6} seconds.

Nosaka[93] has demonstrated that the reaction between catalase and hydrogen peroxide has a very low temperature dependence. He studied the reaction rate from 0 to 50°C. He found that the rate was 2.9 times greater at 40°C than at 0°C. In light of his findings all measurements of peroxide reaction rate and subsequently, evaluation of catalase concentrations, were performed at room temperature.

Dilute solutions of catalase are both temperature and light sensitive. Figure 29 is a plot of the loss of catalase activity as a function of time for a dilute solution stored in the dark in the refrigerator. The dilute catalase solution loses its catalytic activity even when stored with refrigeration. Figure 30 shows the loss of activity of a dilute catalase solution exposed to sunlight. The catalase solution is extremely sensitive to U. V. radiation. It is recommended that studies of catalase solutions requiring long periods of time be performed under conditions of refrigeration and darkness.

The dilute catalase solutions have demonstrated an initial stimulation or increase in catalase activity from low doses of X or gamma radiation. This activation precedes the destruction of the catalase. In an effort to arrive at a possible explanation for this stimulation (or activation) solutions of catalase were subjected to micromole additions of 0.01 normal H_2O_2. These additions produced a marked increase in enzymatic activity. This stimulation has been reported before. Forssberg[42] reports a similar increase in activity and an investigation of this effect as a function of pH.

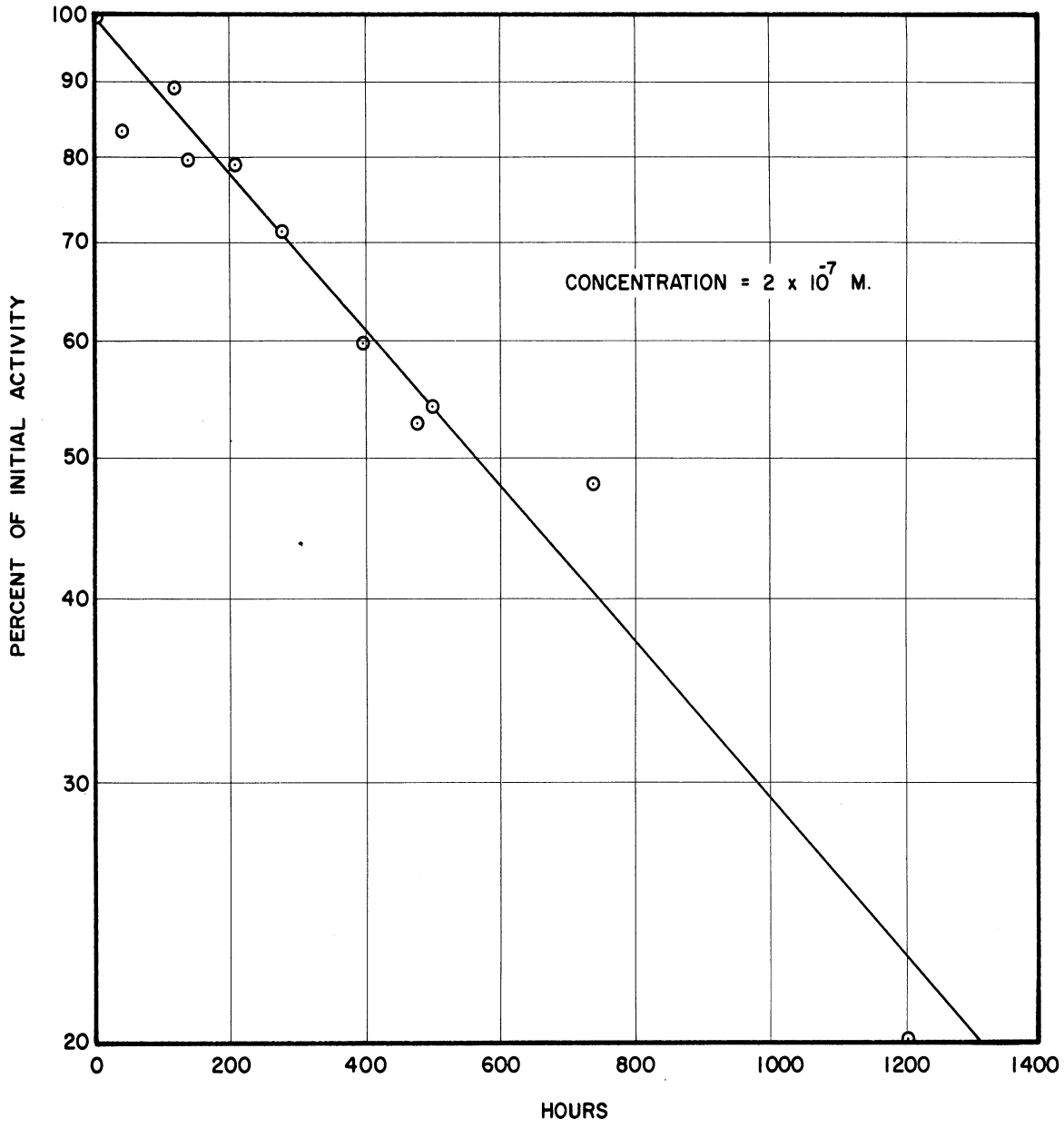


Figure 29. Catalase Solution Loss of Enzymatic Activity at 5°C and in Dark.

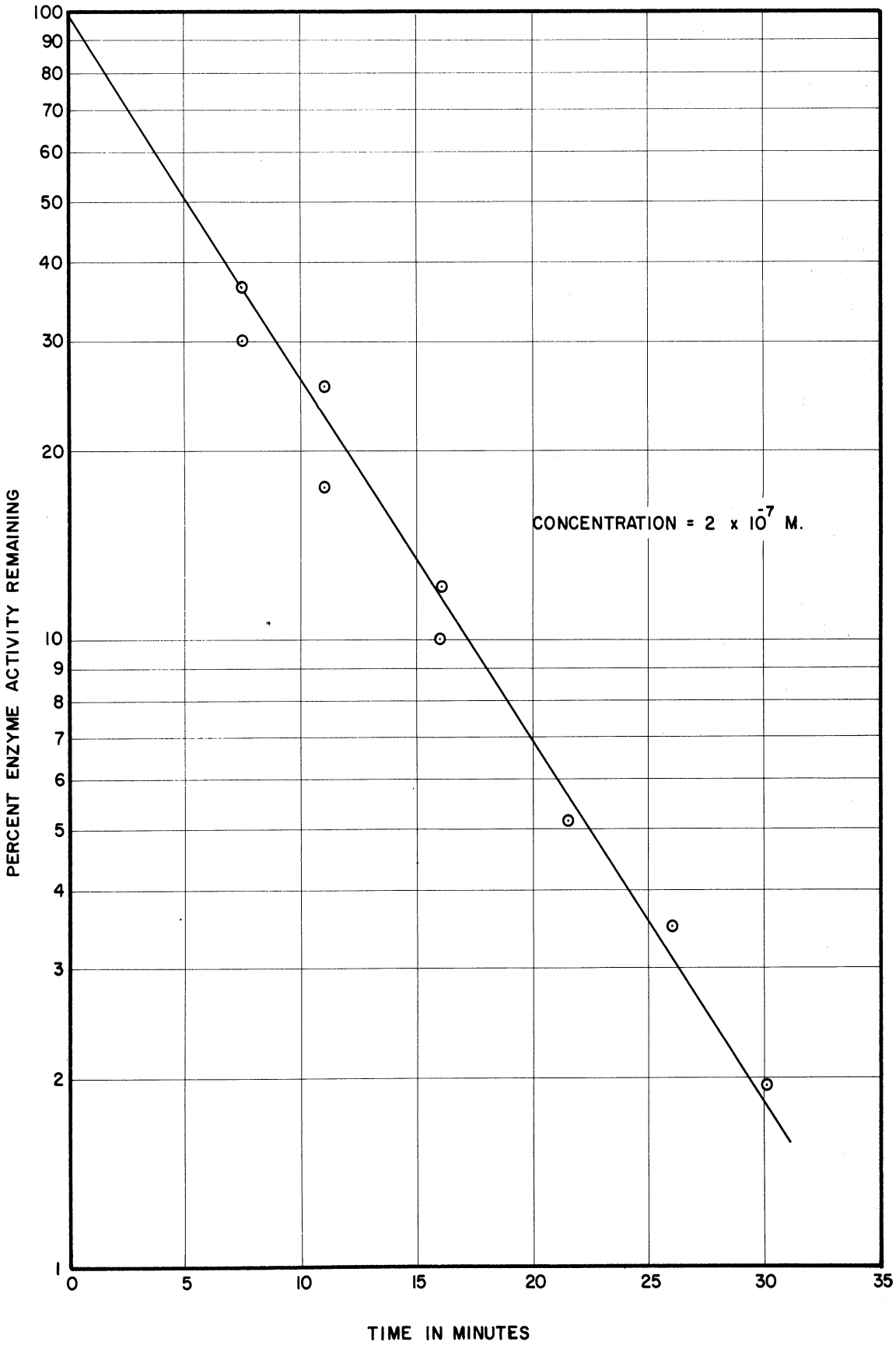


Figure 30. Catalase Solution Loss of Enzymatic Activity at 24° and in Sunlight.

APPENDIX B

SOME NOTES ON CATALASE AND GAMMA RADIATION

Samples of dry catalase in glass bottles were exposed to a Cobalt-60 gamma radiation source. These samples were given radiation doses of from 0 to 14 million rep. After removal from the Cobalt-60 source, they were individually weighed on the microbalance and each sample was dissolved to make 50 ml of buffered catalase solution of known concentration. Aliquots of the prepared solutions were then evaluated with the DU Beckman Spectrophotometer to determine the reaction rate and ultimately the effective concentration of catalase. Figure 31 illustrates the destruction of dry catalase by Cobalt-60 gamma radiation. The line drawn through these data indicates that the inactivation dose to dry catalase from Cobalt-60 gamma radiation is 5×10^6 rep. If one assumes that one rep of gamma radiation in a protein medium is equivalent to 100 ergs of energy dissipation this then yields a 37 percent inactivation dose of 3.12×10^{20} ev per gram. On the basis of the molecular weight of 225,000 this yields a G factor for the direct effect of Cobalt-60 gamma radiation of 0.86.

A number of studies were performed on the destruction of solutions of catalase subjected to Cobalt-60 gamma radiation. These studies were conducted at differing dose rates to confirm the finding of Forssberg[42] who demonstrated a dose rate dependence. In all instances the dose required to destroy 50 percent of the catalase in solution proved to be a function of the dose rate. There are instances in which

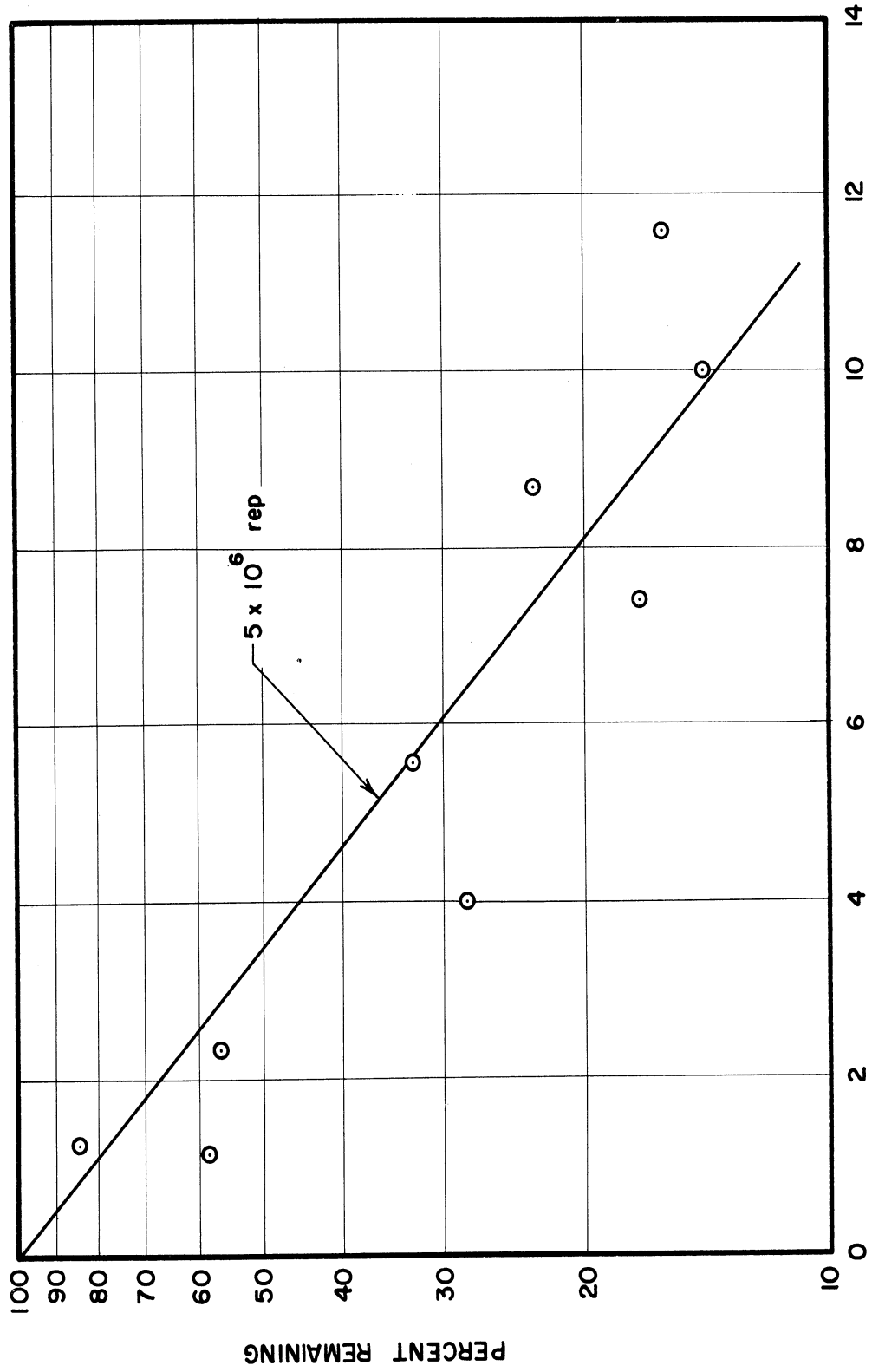


Figure 31. Destruction of Dry Catalase by Cobalt-60 Gamma

prior investigators have indicated that catalase is a radiation resistant enzyme on the basis of a single investigation at an unspecified dose rate or at a dose rate of such a magnitude that they have not reached a break point in the catalase destruction curve.

Table XVI presents the findings of the investigations on various concentrations of catalase solutions subjected to varying dose rates of Cobalt-60 gamma radiation. It is found that over the concentration range studied the destruction is concentration independent and also dose rate dependent. Figure 32 presents a graph of the dose to destroy 50 percent as a function of the dose rate for various concentrations of catalase solutions. The data fit a logarithmic function over a rather wide range of dose rate. In the dose rate range studied, as the rate of energy input increases the destruction yield per unit energy absorbed decreases.

TABLE XVI

DESTRUCTION OF CATALASE SOLUTIONS AS A FUNCTION OF DOSE RATE

(Cobalt-60 Gamma Source)

Dose Rate rep/hr	Concentration mgm/L	Rep Dose to 50% Destruction	Ergs/gram 50% Destruction
650	43.6	1,000	1×10^5
7,800	16.8	7,800	7.8×10^5
86,000	57.6	60,000	6×10^6
131,000	100	85,000	8.5×10^6
131,000	50	75,000	7.5×10^6
1,100,000	18.8	196,000	1.96×10^7

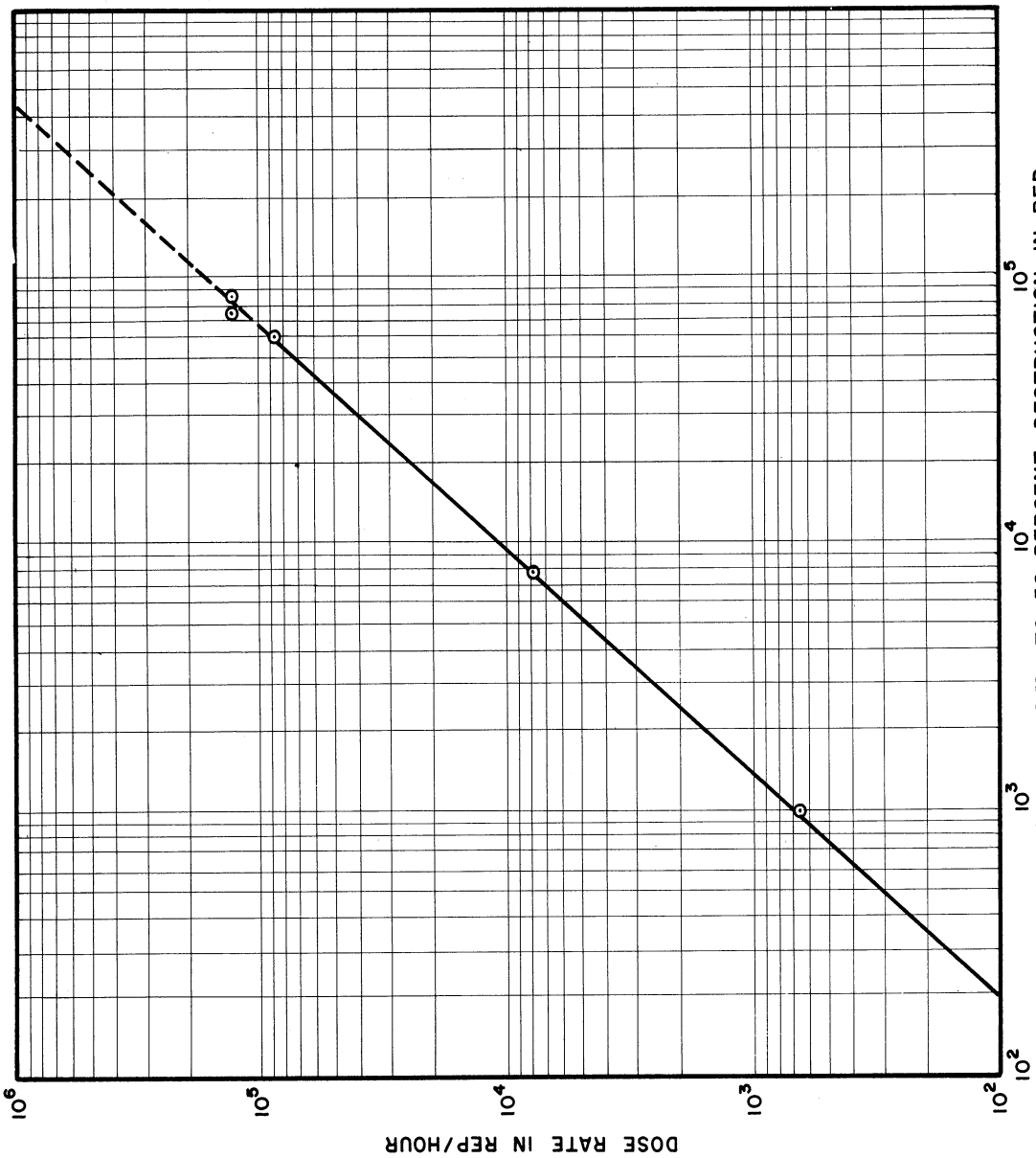


Figure 32. Destruction of Catalase Solutions as a Function Dose Rate - Cobalt 60 Gamma Source.

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