X-RAY INACTIVATION OF ENZYMES

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1960

Submitted to the Faculty of the Graduate College of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
May, 1966

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ACKNOWLEDGMENTS

I want to express my gratitude to Professor George Gorin for his advice and supervision throughout my studies. I also want to thank the National Institutes of Health for financial help and support in the form of a research assistantship.

My gratitude is extended to Dr. Quintiliani for his kind help to my experiments.

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CHAPTER I

INTRODUCTION

Enzymes are inactivated by exposure to ionizing radiation, and this inactivation doubtlessly plays a role in the development of radiation injury in living organisms (1). Different enzymes exhibit varying sensitivities (2) but as yet little is known about the relationship between radiosensitivity and other properties of the enzyme molecules.

This thesis attempts to make a contribution toward the solution of this problem. Two enzymes have been chosen for study, urease and lysozyme. These enzymes are very different, and the choice was made deliberately, as it might facilitate the identification of the characteristics that are important in determining radiosensitivity. Urease is an -SH enzyme of molecular weight 483,000, while lysozyme is basic protein of low molecular weight, whose structure has recently been determined by X-ray diffraction.

In this thesis, quantitative studies have been made of the inactivation-dose relationship. It will be seen that this relationship is exponential for urease, linear for lysozyme. The inactivation yield, G, expressed as the number of molecules inactivated per 100 ev. of radiation energy absorbed, has been determined at various enzyme concentrations.

The principal part of this thesis is Chapter III, in which the more important findings have been described and discussed in a form which would be suitable for publication in a Journal.

Chapter IV reports additional experimental details, which were not included in Chapter III for reasons of brevity, as well as some experimental results, of lesser importance, which do not merit publication at this time.

Chapter II contains a survey of the pertinent literature.

CHAPTER II

LITERATURE SURVEY

The literature which deals with radiation and with enzymes is so vast no attempt can be made here at a complete coverage of it. Only some special aspects of the problem will be considered, and only to a circumscribed extent. These aspects are: (a) a brief survey of the effect of ionizing radiations on water; (b) chemical dosimetry, as it pertains to the experiments performed in this thesis; (c) a discussion of the nature of the inactivation-dose relationship in enzymes; (d) a bibliography of Chemical Abstracts references to enzyme inactivation by x- and γ- radiation for the period 1965 to 1961; (e) a brief description of urease and a bibliography of references pertaining to its inactivation by radiation; (f) a brief description of lysozyme and a bibliography of references pertaining to its inactivation by radiation.

The Effect of Ionizing Radiations on Water

From the theoretical work of Weiss (3) and subsequent experimental testing by numerous workers, it is now generally concluded (4,5) that the important activated products in irradiated water are $H^{\bullet}$ and $OH^{\bullet}$ radicals and the hydrated electron, HOH^{-}. When a water molecule interacts with a quantum of x- or γ-radiation an electron may be ejected, giving $OH^{\bullet}$:

$$HOH \xrightarrow{\text{Ionizing radiation}} HOH^{+} + e \qquad (1)$$

$$HOH^+ \longrightarrow H^+ + OH^\cdot \qquad (2)$$

In neutral and alkaline solution the hydrated electron, HOH^-, is probably the major reducing species:

$$HOH + e \longrightarrow HOH^- \qquad (3)$$

A small number of hydrogen atoms are formed by reactions (4) and (5).

$$HOH^- \longrightarrow OH^- + H^\cdot \qquad (4)$$

$$HOH^- + HOH \longrightarrow HOH + H^\cdot + OH^- \qquad (5)$$

In acid solution the HOH^- may be converted to hydrogen atoms by reaction with hydrogen ions:

$$HOH^- + H^+ \longrightarrow HOH + H^\cdot \qquad (6)$$

This reaction is much faster than reaction (5).

As these products diffuse in the solution, they may recombine to form HOH, H_2 or H_2O_2. The latter are the principal products of water radiolysis, in the absence of dissolved oxygen.

Oxygen is a very efficient scavenger for hydrogen atoms, combining with them to give perhydroxyl radicals, $HO_2^\cdot$:

$$H^\cdot + O_2 \longrightarrow HO_2^\cdot \qquad (7)$$

The $HO_2^\cdot$ radical is a strong oxidizing agent and reacts with hydroxyl radicals and other $HO_2^\cdot$ radicals to give oxygen.

$$HO_2^\cdot + OH^\cdot \longrightarrow O_2 + H_2O \qquad (8)$$

$$HO_2^\cdot + HO_2^\cdot \longrightarrow O_2 + H_2O_2 \qquad (9)$$

The perhydroxyl radical can also be formed by reaction of a hydroxyl radical with hydrogen peroxide.

$$OH^\cdot + H_2O_2 \longrightarrow HO_2^\cdot + H_2O \qquad (10)$$

Chemical Dosimetry

The ferrous sulphate dosimeter was pioneered by Miller (6) and has been very extensively used. It is one of the most reliable and convenient methods of chemical dosimetry. It is based on the fact that the radiolysis products of water oxidize ferrous to ferric ions. The reaction is usually carried out in aerated solution at a pH below 1.5.

Fricke (7) chose 0.8N sulfuric acid as the solvent; in this medium, the response to x-rays is then the same as that of standard air ionization chambers over a range of x-ray energies. Weiss, Allen and Schwarz (8) have recommended a dosimeter solution containing 10^{-3}M ferrous ammonium sulfate, 10^{-3}M sodium chloride and 0.8N sulfuric acid. Chloride is added to the dosimeter solution to inhibit the oxidation of ferrous ions by organic impurities (9). The water should be very pure (triply distilled). In aerated acid solutions Fe^{++} is oxidized by $OH^{\cdot}$, by $HO_2^{\cdot}$, and by H_2O_2, so that Fe^{3+} is obtained instead of any of the other products of water radiolysis.

$$OH^{\cdot} + Fe^{2+} \longrightarrow Fe^{3+} + OH^{-} \qquad (11)$$

$$H^{\cdot} + O_2 \longrightarrow HO_2^{\cdot} \qquad (12)$$

$$HO_2^{\cdot} + Fe^{2+} \longrightarrow Fe^{3+} + HO_2^{-} \qquad (13)$$

$$HO_2^{-} + H^{+} \longrightarrow H_2O_2 \qquad (14)$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + HO^{\cdot} + OH^{-} \qquad (15)$$

The most common method of measuring ferric ion formed is by spectrophotometry at 304 mμ, the wavelength at which ferric ions show maximum absorption. The mean absorbed dose D in the volume occupied by the dosimeter solution is derived as follows (4,5). For any chemical system, given that:

G (products) = molecules of product formed/100 ev. of energy absorbed

100 ev. = 1.602×10^{-10} ergs

the energy E, in ergs, absorbed in the dosimeter solution:

$E = 1.602 \times 10^{-10}$ (ergs/100 ev.) P/G (molecules of product/100 ev.)

where P is the number of molecules of products formed. Given further that:

1 rad = energy absorption of 100 ergs/g

the dose D, in rads, is given by

$$D = E/W = 1.602 \times 10^{-12} \, P/GVd$$

where W is the weight of the dosimeter solution, and d the density.

In the ferrous sulfate dosimeter, the concentration of ferric ions is given by:

$$C_{Fe(III)}(\text{moles}/l) = (A_i - A_b)/\epsilon l$$

where A_i and A_b are the absorbances of the irradiated and unirradiated (blank) dosimeter, respectively, ϵ is the molar absorbancy and l the thickness of sample in cm.

P, the ions of Fe^{3+} formed, is given by:

$$P = (A_i - A_b)/\epsilon l \ (\text{moles}/l) \times 6.023 \times 10^{23} (\text{molecules/mole}) \times V \ (\text{liter})$$

and

$$D = 1.602 \times 10^{-12} \ (\text{g rads}/100 \text{ ev}) \times 6.023 \times 10^{23} \times (A_i - A_b)V/\epsilon l \ (\text{molecules}) \times \frac{1}{G \ (\text{molecules}/100 \text{ ev}) V d \ (\text{g/ml})}$$

For the dosimeter solution, $\epsilon = 2.174 \times 10^{6}$ ($\text{mole}^{-1} \text{ cm}^2$), $l = 1$ (cm), $G = 13.9$, and $d = 1.024$ (g/ml):

$$D = 0.32095 \times 10^{5} \ (A_i - A_b).$$

Inactivation-Dose Relationship in Enzymes

In several instances, it has been found that the enzymatic activity decreases exponentially with the dose (1,10). This relation is described by the equations;

$$A = A_o e^{-KD} \qquad \alpha = A/A_o = e^{-KD} \qquad \ln \alpha = -KD \tag{16}$$

in which A_o is the initial enzymatic activity, A the activity remaining after dose D, and K a constant that can be determined from the experimental data. To determine K one may plot ($\ln \alpha$) or ($\log \alpha$) vs. D and determine the slope of the line, which equals $-K$ or ($-0.4243K$), respectively; alternatively, one may determine the dose needed to produce 63% inactivation, D_{37}, which equals ($1/K$) (actually, 63.2% inactivation should be produced, since $-1 = \ln 0.368$). The inactivation yield, G, in molecules inactivated per 100 ev of energy absorbed, is related to K or D_{37} by a simple proportion:

$$G = E_o/D_{37} = E_o K \tag{17}$$

When E_o is expressed in moles liter^{-1} and D_{37} in rads, the proportionality constant κ is given by:

$$\kappa = 6.02 \times 10^{23} \text{ (molecules/mole) } 10^{-3} \text{ (liter/g) } 10^{-2} \text{ (rad-g/erg)}$$
$$1.6 \times 10^{-10} \text{ (erg/100 ev)}$$
$$= 9.632 \times 10^{8} \text{ (molecules-liter-rads/mole-100 ev)}$$

The simplest rationalization of K in terms of a physico-chemical mechanism is that the enzyme reacts with an active species X. The concentration of X is proportional to the dose rate (dD/dt) (with proportionality constant κ') and X disappears only by reaction with the enzyme, whether it be active or inactivated, with a specific rate constant k_e.

A steady state is assumed to exist throughout the irradiation:

$$(d\underline{X}/d\underline{t}) = 0 = \nu'(d\underline{D}/d\underline{t}) - \underline{k}_e\ \underline{E}_o\ \underline{X}$$

$$\underline{X} = \nu'(d\underline{D}/d\underline{t})/\underline{k}_e\ \underline{E}_o \qquad (18)$$

The inactivating reaction of $\underline{X}$ with active enzyme proceeds with rate constant $\underline{k}_i$:

$$-(d\underline{E}/d\underline{t}) = \underline{k}_i\ \underline{XE}$$

On substituting the value for $\underline{X}$ given by equation (18) one obtains:

$$d\underline{E}/\underline{E} = -(\nu'\ \underline{k}_i/\underline{k}_e\ \underline{E}_o)d\underline{D}$$

which may be integrated between the limits $\underline{E}_o$ and $\underline{E}$ to give:

$$\ln\ \underline{E}/\underline{E}_o = -(\nu'\ \underline{k}_i/\underline{k}_e\ \underline{E}_o)\underline{D}$$

Comparison of this result with equation (16) shows that:

$$\underline{K} = \nu'\ \underline{k}_i/\underline{k}_e\ \underline{E}_o$$

and:

$$\underline{G} = (\nu\ \nu')\ (\underline{k}_i/\underline{k}_e) \qquad (19)$$

In other words, according to this simplified mechanism, the yield $\underline{G}$ measures the ratio of the rate constants for inactivation of the enzyme by $\underline{X}$ to that for inactivation of $\underline{X}$ by (active and inactivated) enzyme, times a proportionality factor which relates to the concentration of $\underline{X}$ produced by the radiation dose in unit time.

Equation (19) implies that $\underline{G}$ should be independent of $\underline{E}_o$. This has been found to be approximately true in one or two cases (11), but more often it has been found that $\underline{G}$ increases regularly with dose. Hutchinson has concerned himself with this problem and pointed out that at low enzyme concentrations one may expect an appreciable fraction of $\underline{X}$ to be consumed by reactions not involving the enzyme. In such circumstances, the apparent yield measured by inactivation of the enzyme would be lower than

the "true" yield. Hutchinson and Ross (12) have proposed an experimental procedure and a method of treating the data by which this effect may be determined and corrected for. In this treatment, it is assumed that the "scavenging ability of the medium" may be regarded as a constant, $\underline{S}$. This term is added to equation (17), which leads to the relation:

$$\underline{D}_{37} = \varkappa \underline{E}_o/\underline{G} + \underline{S} \qquad (20)$$

$\underline{D}_{37}$ is measured at decreasing concentrations, preferably small, and the results are plotted $\underline{vs}$. $\underline{E}_o$. According to equation (20), a straight line should be obtained, the intercept of which is $\underline{S}$ at $\underline{E}_o = 0$, while the slope is $(\varkappa/G)$.

In the preceding discussion, the possibility has not been considered that the activity-dose relationship might not in fact conform to equation (16) as in the case of lysozyme. However, other instances have been reported in the literature (13). Sanner and Pihl have considered this problem in a recent paper (14). They point out that equation (16) depends on the assumption that active and inactivated enzyme react with $\underline{X}$ at the same rate ($\underline{k}_e$); if this condition is not fulfilled, deviations from (16) should occur, and the plot of α will tend to become linear with $\underline{D}$ if $\underline{k}_i \gg \underline{k}_e$. The line that corresponds to the limiting slope of the exponential equation (16) is described by the equation:

$$\alpha = -\underline{KD} + 1; \qquad (21)$$

this is the activity-dose relationship at the beginning of the irradiation, before any enzyme has been inactivated. If the inactivated enzyme did not consume radicals, α would continue to decrease linearly, while we have already seen that equation (16) results if inactive and active enzymes react at equal rates. Clearly, an intermediate situation, i.e. one in which the inactivated enzyme consumed radicals, but not so rapidly

as the native enzyme, would give rise to a curve of intermediate shape.

Bibliography on Inactivation of Enzymes by Ionizing Radiations (1961-65)

For the purpose of this literature survey, the indices to Chemical Abstracts were consulted. The articles are arranged according to year of publication and cover the five year period 1965 to 1961 inclusive. The survey in this section excludes lysozyme and urease, which are discussed separately in other sections. Some articles which had not been indexed by Chemical Abstracts have been found by cross reference; they are denoted by an asterisk(*).

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CA, 63, 18605e: The action of ionizing radiation on dilute aqueous solutions; Boag, J. W., Phys. Med. Biol. 10(4), 457, (1965)--A review with 44 references.

16740g: Influence of dimethylsulfoxide on the radiation sensitivity of catalase; Lohmann, W., Moss, A. J., and Perkins, W. H., J. Nucl. Med. 6(7), 519 (1965)--With increasing Me_2SO concn. the protective effect increases to a max. (at 1.39×10^{-3} M) and then declines slowly.

13675g: Radiation and chemical means of protection: sulfur compounds; George, V., Chim. Chronika 30(4), 54 (1965)--The mechanism of action of radiation and test for the protective action of various S compds. is reviewed.

13672g: Changes of alkaline and acid phosphatase activities by Co^{60} γ-radiation of their solutions; Nikol'skaya, E. B., and Proko'eva, E. G., Radiobiologiya 5(4), 618 (1965)--γ-Radiation inactivates the soln. of acid phosphatase much more than that of alk. phosphatase of the same specific activity.

7316g: Influence of temperature on the activity of alkaline phosphatase, irradiated with the γ-rays of cobalt-60; Nikol'skaya, E. B., Den'ga, V. V., Ivanenko, E. A., and Popova, I. V., Radiobiologiya 5(3), 464 (1965)--Temps. above 25° exert an appreciable inactivation.

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16602e: Effect of metal ions on the radiation sensitivity of catalase; Lohmann, W., Moss, A. J., Jr., and Perkins, W. H., Radiation Res. 24(1), 9 (1965)--Fe^{3+}, Cu^{2+}, and Mn^{2+}, exert a protective effect of catalase. The effect is explained by a radical scavenging mechanism.

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1964

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3279f: The relative roles of ionization and excitation processes in the radiation inactivation of enzymes; Augenstein, L. G., Brustad, T., and Mason, R., Advan. Radiation Biol. 1, 227 (1964)--A review with many references.

CA,62,12126h: Inactivation of β-amylase by γ-radiation; Scoppa, P., and Tafuri, F., Intern. J. Radiation Biol. 8(5), 415 (1964)--The inactivating action of γ-radiation on β-amylase from barley in aq. soln. is mainly of the indirect type and resembles that of strong oxidizing agents.

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4672a: Effect of X-rays on adenosinetriphosphatase (ATPase) activity of myosin; Szabolcs, M., Zsindely, A., Damjanovich, S., Arch. Biochem. Biophys. 105(2), 447 (1964)--The increase in ATPase activity of myosin solns. is produced by damage of SH groups sensitive to x-rays.

3393a: Effect of γ-radiation on ribonuclease; Hayden, G. A., and Friedberg, F., Radiation Res. 22, 130 (1964)--The enzyme solution is resistant to chemical covalent changes at low doses.

2156e: Pancreatic ribonuclease. IV. A novel artificial ribonuclease obtained by γ-ray irradiation of ribonuclease A in the presence of cytidine 2'(+3')-phosphate; Ukita, C., and Waku, K., J. Biochem. (Tokyo) 55, 420 (1964)--Denaturation of ribonuclease A on irradiation in aqueous solution by γ-rays is prevented by uridine or cytidine 2'(+3')-phosphate.

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CA,60,13514f: Factors affecting the in vitro inactivation of aldolase by x-rays; Quintiliani, M., and Boccacci, M., Intern. J. Radiation Biol. 7(3), 255 (1963)--Low concentration of urea protect aldolase from inactivation by x-ray, but higher concentrations (2M) increase the degree of inactivation.

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Urease

Occurence

The enzyme urease was first obtained in crystalline form by Sumner (1926). It has been found in over one hundred species of bacteria, in several species of yeasts, in fungi, and in a large number of higher plants (15). The richest plant source known is the jack bean (16) which contains 0.15% urease (on a dry weight basis).

Properties

The molecular weight of jack bean urease as determined by Sumner is 483,000 (17). The isoelectric point of urease is 5.0 to 5.1 (18). Urease has been analyzed with an automatic amino acid analyzer (19) and found to contain the following amino acids; Lys_{218}, His_{107}, Arg_{166}, Asp_{451}, Thr_{284}, Ser_{222}, Glu_{381}, Pro_{188}, Gly_{371}, Ala_{369}, $(CySH/2)_{85}$, Val_{267}, Met_{114}, $Ileu_{338}$, Leu_{315}, Tyr_{99}, Phe_{105}, $(NH_3)_{519}$, Try_{46}. According to Leslie et al. there are 47 ± 4 SH groups (20) per mole. The experiments of Gorin and Chin (21) with N-methylmaleimide and silver

ion indicate the presence of eight active sites.

Urease is easily inactivated by reaction with metal ions. Their relative effectiveness was shown to be $Ag^{+}>Hg^{++}>Cu^{++}>Cd^{++}>Co^{++}>Ni^{++}>Mn^{++}$ with Pb^{++} and Fe^{++} unassigned but less than Cu^{++} (22). The various "activators" or protectors of urease (proteins, amino acids, gum arabic) function by binding heavy metals, thereby protecting the urease SH groups (23). Bromide ion can reverse the inactivation by the silver ion.

Native urease associates into dimers, trimers or higher polymeric states, probably because of the oxidation of some SH groups into S-S bonds (24). It is dissociated into 6-8 subunits (25) by 6M guanidine hydrochloride.

Sumner (18) has stated "urease is absolutely specific." Hundred of compounds have been tested and shown not to be catalytically hydrolyzed by urease; among these were various substituted ureas and related compounds.

Action of Urease on Its Substrate and Assay of Enzymatic Activity

The hydrolysis of urea which is catalyzed by urease may be represented by the equation

$$H_2N\ CONH_2 + H_2O \longrightarrow 2\ NH_3 + CO_2$$

Since one product is basic and the other acidic, additional reactions may take place depending on the pH of the buffer medium used.

At pH 9,

$$H_2N\ CONH_2 + 2\ H_2O \longrightarrow NH_4^+ + CO_2NH_2^- + H_2O \rightleftharpoons 2NH_4^+ + CO_3^= \rightleftharpoons$$
$$NH_3 + NH_4^+ + HCO_3^-$$

At pH 7,

$$H_2N\ CONH_2 + 2\ H_2O + H_2PO_4^- \longrightarrow 2\ NH_4^+ + HCO_3^- + HPO_4^=$$

In 1966, an alkalimetric assay method was developed by Gorin and Chin (26). The reaction is conducted in Tris buffer, pH 9.0, at 25°C for 2 minutes, then the mixture is treated with an excess of 0.1M HCl and back titrated with 0.05M NaOH. The activity is defined by the number of μmoles of ammonia liberated in one min and is given by the equation:

$$\text{Activity} = 500\ (\underline{V}_b - \underline{V}_a)\ \underline{M}_{OH}$$

where $\underline{V}_b$ is the volume of 0.05M NaOH used in the blank, $\underline{V}_a$ is the volume used in the assay and $\underline{M}_{OH}$ is the molarity of NaOH. This method is more accurate and has a larger range than the method of Sumner (27) and the acidimetric method of Gorin et al. (28).

In Sumner's assay, the enzyme is allowed to react with 3% urea in phosphate buffer, pH 7.0, at 20°. The ammonia liberated is neutralized by H_2SO_4 and its amount is determined by Nesslerization. One difficulty encounted in this assay is the poor reproducibility of Nesslerization. In the acidimetric method (28), the reaction of enzyme and substrate is performed in the same way as in Sumner method, but the ammonia liberated is titrated with 0.1M HCl to pH 4.5.

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Lysozyme

Lysozyme is an enzyme capable of lysing certain bacteria. It was discovered by Fleming, who also isolated a coccus that is very sensitive to the action of lysozyme, Micrococcus lysodeikticus. Lysozyme is widely distributed in nature. It is found in many tissues and secretions of vertebrates, invertebrates, bacteria and even plants. The enzymes from these different sources are probably quite similar but not the same, so one should speak of "lysozymes" in the plural. The most important source of lysozyme is the white of hen's eggs; what follows refers specifically to the enzyme from this source.

Properties and Structure

Lysozyme is a protein of low molecular weight. According to Jollès and to Canfield (29,30) it contains 129 amino acids, as follows: Gly_{12}, Ala_{12}, Ser_{10}, $(Cys-)_8$, Met_2, Thr_7, Val_6, Leu_8, Pro_2, $Ileu_6$, Phe_3, Tyr_3, Try_6, Asp_{21}, Glu_5, Lys_6, Arg_{11}, His_1, $(NH_2-)_{18}$. The molecular weight would then be 14,388. Recent physical methods have given results in close agreement to this value (31). Lysozyme is a basic protein with an isoelectric point between 10.5 and 11.

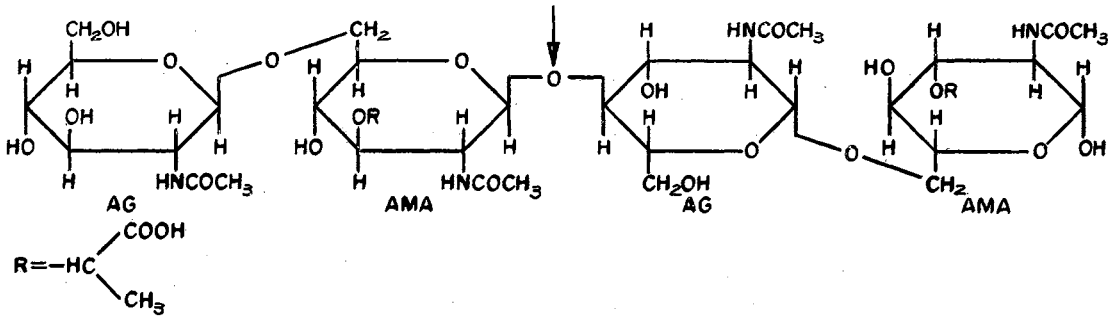
Jollès and Jollès (32) reported the primary structure of egg-white lysozyme in 1961. The protein consists of a single polypeptide chain. The N-terminal amino acid is lysine and the C-terminal leucine. The four S-S bridges occur at positions 6-127, 30-115, 64-80 and 76-94 (33).

The molecular configuration of lysozyme was recently investigated in detail by Blake et al. (33) by means of x-ray crystallography. They studied lysozyme in the tetragonal crystal form, as well as its mercury and palladium derivatives. From these studies, they deduced a three-dimensional electron-density map at 6 Å resolution, which is in agreement with the sequence studies. The polypeptide chain has some helical and some nonhelical portions and is folded at positions 7-14, 25-35, 80-85, 91-99, 108-115, 119-125. Stanford et al. (34) investigated tetragonal lysozyme chloride crystals containing complex ions of niobium and tantalum and Dickerson et al. (35) triclinic lysozyme nitrate; their studies did not, however, lead to a complete electron-density map.

Léonis (36) studied the secondary and tertiary structures. He suggested that hydrophobic bonding is important in stabilizing the molecular configuration of lysozyme.

Enzymatic Action of Lysozyme on Its Substrate

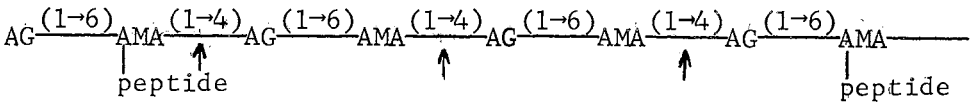
Berger and Weiser (37) have shown that egg-white lysozyme possesses β-glucosaminidase activity. It attacks the cell wall of M. lysodeikticus with liberation of a product, which has been shown by many authors (38) to be the disaccharide of N-acetylglucosamine and N-acetylmuramic acid. It was generally believed that this disaccharide contains a β-1,6 linkage; however, Jeanloz et al. (39) recently have proposed that this disaccharide contains, instead a β-1,4 linkage. In addition to the disaccharide, a tetrasaccharide has been detected, which is a dimer of the disaccharide joined by a β-1,4 linkage. This tetrasaccharide is split by egg-white lysozyme and may be considered to be the basic substrate of lysozyme. The enzyme is, then, a β-1,4-N-acetylglucosaminidase.



Tetrasaccharide released by lysozyme from the cell walls of M. lysodeikticus and used as a lysozyme substrate.
AG = acetylglucosamine; AMA = acetylmuramic acid; the arrow indicates the bond split by lysozyme.

The cell wall of M. lysodeikticus probably has a basic skeleton of alternating N-acetylmuramic acid (AMA) and N-acetylglucosamine (AG) residues with alternating β-1,4 and β-1,6 linkages. Some of the muramic acid residues have peptide substituents. The following figure shows the structure proposed by Salton for the skeleton of cell walls of M. lysodeikticus.

basic skeleton



The bonds marked with arrows are split by lysozyme.

Assay

The enzymatic activity of lysozyme has been assayed by methods that are based upon different principles and conditions. All of them leave something to be desired and it is difficult to evaluate their advantages and limitations.

Fleming (40), as soon as he found lysozyme, assayed it by measuring its ability to lyse M. lysodeikticus colonies on an agar plate. Goldsworth and Florey (41) in 1930 let the enzyme and the substrate incubate at 30°C for 1 hour and measured the least amount of lysozyme needed to produce complete lysis. Boasson (42) in 1938 developed a technique involving the measurement of optical turbidity. Hartsell (43) conducted the incubation at 52°C in pH 6.2 phosphate buffer and observed the clearing of the suspension visually. In 1952 Dickman and Proctor (44), and in 1955 Smith et al. (45) used Sarcina lutea cells as the substrate and a wavelength of 440 mμ to follow the cell lysis. Shugar and Smolelis et al. (46,47) incubated M. lysodeikticus cells with lysozyme at 20°C for 1 min in M/15 phosphate buffer, pH 7.1, and measured the absorbance at 450 mμ. The enzyme concentration was determined from a calibration curve. Other investigators (48) increased the incubation time to 15 min or longer.

Dickman and Proctor, and Kerby and Eadie (44,49) proposed methods in which the change in absorbance of the substrate was measured as a function of time. The results conformed to first-order kinetic, i.e.,

to the equation:

$$\log(S_0/S_t) = \underline{KEt} \qquad (22)$$

where $\underline{S}_0$ is initial substrate concentration; $\underline{S}_t$ the final substrate concentration; $\underline{K}$ the reaction velocity constant; $\underline{E}$ the effective enzyme concentration in the incubation mix; and $\underline{t}$ the incubation time in min. The concentration limits of $\underline{K}$ were established with known solutions and the lysozyme concentration of experimental samples could then be calculated by solving equation (22) for $\underline{E}$.

On the other hand, Smith et al. and Parsad and Litwack (50) suggested that the disappearance of substrate follows second-order kinetics and that the enzyme concentration within limits is proportional to their second-order velocity constant.

Meyer and Hahnel (51) in 1946 developed a method for measuring the mucolytic activity of lysozyme that is based on an entirely different principle. The assay is based on the depolymerization of mucopolysaccharide, which causes a change in the viscosity of the substrate-enzyme mixture.

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CHAPTER III

X-RAY INACTIVATION OF LYSOZYME AND UREASE

This chapter comprises the principal subject of the present thesis, an experimental investigation of the x-ray inactivation of lysozyme and urease. It is hoped that the results will be published and this chapter consists of a paper which has been written for publication. This paper contains some data obtained by Drs. Harold Kolenbrander and Marcello Quintiliani, who are coauthors of the paper. The references cited in the paper are numbered in a separate series.

Papers written for publication must be brief, and this precludes reporting the results in full detail; such details are given in Chapter IV.

Manuscript for Publication

Inactivation of Enzymes by Ionizing Radiation

III. Lysozyme and Urease

G. Gorin, L. W. Tai, H. Kolenbrander, M. Quintiliani

Introduction

The present work is part of a systematic survey of the radiosensitivity of enzymes. It is hoped that the results of such an investigation will lead to useful generalizations relating the radiosensitivity to other physicochemical properties. In this context, it seemed of interest

to examine two enzymes that are very different from the physicochemical standpoint. Lysozyme is a small molecule, of molecular weight 14,388 (1), of very compact structure which is comparatively stable. Urease, on the other hand, is a very large enzyme, of molecular weight 483,000 (2); it is rather unstable, although the cause(s) of its lability is not very well understood. A point of contrast of special importance is the fact that urease contains many mercapto groups, some of which seem to be essential to activity (3), whereas lysozyme contains no mercapto groups.

These enzymes have been investigated by others, but unfortunately there is not very good agreement between the results heretofore reported. It was one of the purposes of this work to try to resolve the existing discrepancies. The results obtained by other investigators will be considered in some detail in the discussion section, alongside the results obtained in the present investigation.

Materials and Methods

Chemicals

All chemicals were of A.C.S. reagent grade. The water used to prepare all solutions was distilled, passed through a mixed-bed ion-exchange column, and redistilled in an all-Pyrex still. The compositions of the buffers were as follows (quantities to make 1 liter of solution): phosphate (0.02M), pH 7.0, 1.657 g Na_2HPO_4 and 1.150 g $NaH_2PO_4 \cdot H_2O$; phosphate (0.05M), pH 7.0, 4.31 g Na_2HPO_4 and 2.69 g $NaH_2PO_4 \cdot H_2O$; phosphate (M/15, pH 6.2 ± 0.1), 1.89 g Na_2HPO_4 and 7.36 g $NaH_2PO_4 \cdot H_2O$; Tris (0.10M, pH 9.0 ± 0.1), 12.114 g Tris(hydroxymethyl)aminomethane and 57 ml of 0.2M HCl; some solutions also contained disodium ethylenedinitrilotetraacetate (EDTA) as indicated.

Urease preparation and assay

Highly purified crystalline urease was prepared as described by Mamiya and Gorin by extracting jack-bean meal with aqueous acetone containing 0.014M 2-mercaptoethanol (4). The urease was crystallized four times. The crystals obtained after the last crystallization were stored in the mother liquor at 4^{o}. Stock solutions of the enzyme, 0.5 - 0.6%, were prepared by centrifuging the suspension, discarding the centrifugate, dissolving the residue in 0.02M phosphate buffer, with or without 10^{-4}M EDTA, centrifuging the solution at 27,000 g for 30 min and discarding any insoluble residue. The solutions containing 10^{-4}M EDTA could be stored at 4^{o} for as long as 2-3 weeks with little loss of activity, but most experiments were done with solutions less than 1 week old; the solutions that did not contain EDTA were considerably less stable and were used within a few days from the time of preparation. The concentration of enzyme in the stock solution was determined by measuring the absorbance after appropriate dilution; specific absorbance index was taken to be 0.640 (1 mg of enzyme ml^{-1}, 1 cm thickness).

The urease solutions were assayed at 25^{o} by the alkalimetric method of Gorin and Chin (5); to attain the concentration suitable for assay the samples had to be diluted 7-50 times and it should be noted that this was done, in all cases, with 0.02M phosphate-10^{-3}M EDTA. In the aforementioned reference, an activity unit has been defined, U_a^{25}, which is in accordance with the recommendations of the International Union of Biochemistry; this unit is related to the Sumner unit that was almost universally used in earlier work by the relation: 1 Sumner unit $\simeq$ 11.0 U_a^{25}. The purest preparations of urease had a specific activity of 1650 $\pm$ 30 U_a^{25} mg^{-1}. The

samples used in the present work had the following specific activities, $\underline{U}_a^{25}$ mg^{-1}: I, 1680; II, 1600; III, 1660; and IV, 1620.

Lysozyme Preparations and Assay

Crystalline egg-white lysozyme was purchased from Calbiochem, Los Angeles. The acetone powder of Micrococcus lysodeikticus was obtained from Difco Laboratories, Detroit. Solutions of lysozyme were prepared in 0.05M phosphate, pH 7, in the same way as described above for urease; the concentration was determined from the absorbance at 280 mμ taking the specific absorbancy index as 0.473 (6).

Assay were conducted by the method of Shugar (6). The substrate was prepared by mixing 60 mg of M. lysodeikticus powder with 20 ml of 1/15M phosphate buffer (pH 6.2) in a hand homogenizer (A. H. Thomas Co., Philadelphia, No. C 12422) for 5 min and then diluting with more buffer to 200 ml. The stock solution could be stored at 4°, but no longer than 4 days. The lysozyme sample was diluted to within the range 1-4 μg of enzyme ml^{-1} with 1/15M phosphate. A 1-ml aliquot of this solution was then mixed with 2 ml of substrate at 25° and after 5 min the absorbance of the mixture at 570 mμ was measured with a Beckman DU spectrophotometer, against water as reference. The absorbance of a blank (2 ml substrate + 1 ml of buffer) was measured at about the same time (its absorbance was in the range 0.56 - 0.59).

A calibration curve was constructed, on the same day as the irradiation experiments, by adding samples of lysozyme, containing 1-4 μg/ml, to the substrate, in the manner described above. A plot of the difference between the absorbance of the control and that of the samples, plotted as a function of enzyme concentration, gave a straight line. The concentration of enzyme remaining in the irradiated samples was estimated from this line.

Dosimetry and Irradiation Procedure

The x-ray source and procedure were the same as described elsewhere (7). The dose rate was 1550 rads min^{-1}.

Results

Four samples of urease were studied in this work. The most precise measurements were made on samples II-IV, but several measurements were also made on sample I; some of the results obtained with this sample will be reported, though not in much detail, for purposes of comparison.

Fig. 1 represents graphically the data obtained with samples II-IV. At least two sets of measurements were made on each sample, and the data, i.e., six or more values, were averaged to give each point on this graph. The average deviation of the mean was ± 4%. It can be seen that the inactivation-dose relationship conforms to the equation;

$$A/A_0 = \alpha = e^{-k_1 D} \qquad (1)$$

In such case, the constant k_1, which measures the intrinsic radiosensitivity of the enzyme, is numerically equal to the reciprocal of D_{37}, the dose required to reduce the activity to 37% of its original value. This value quantity was calculated for each line by least-square analysis, with the results given in the legend.

Earlier measurements on the x-ray inactivation of urease (see Discussion) were done on very impure samples, and is of some interest to see how these compare with pure sample. Table I gives the results of these experiments.

The inactivation of lysozyme was measured both in 0.05M phosphate buffer and in distilled water. The results obtained were the same within

Fig. 1 Inactivation of urease, samples II-IV. Upper graph, in 0.05M phosphate: A, 500 μg urease ml^{-1}, D_{37} 76.7 krads; B, 200 μg ml^{-1}, D_{37} 32.6; C, 50 μg ml^{-1}, D_{37} 21.1 Lower graph, in 0.05M phosphate-10^{-4}M EDTA, urease concentrations as above: A, D_{37} 91.3; B, D_{37} 54.0; C, D_{37} 31.5.

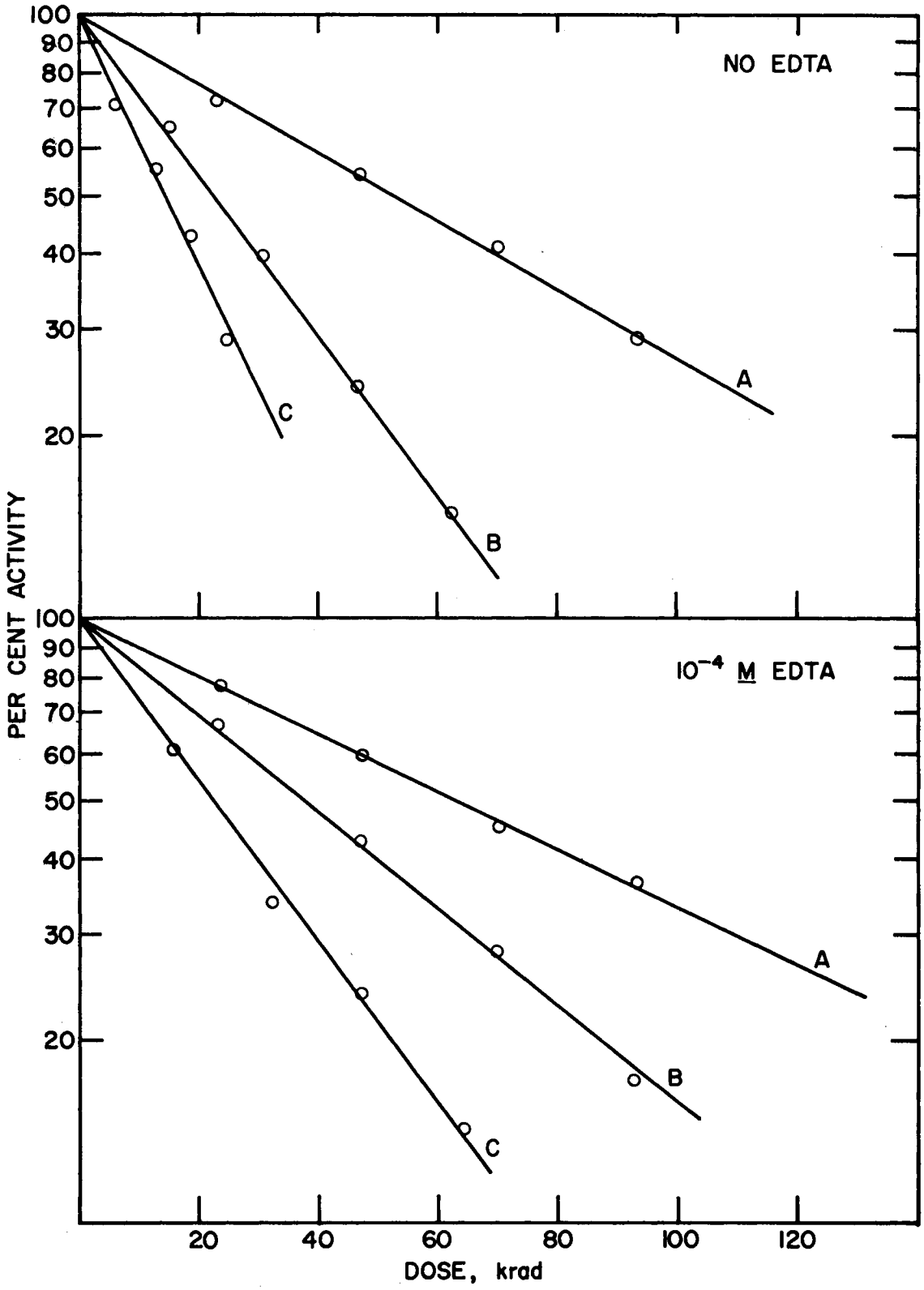
NO EDTA
A
B
C
10^{-4} M EDTA
A
B
C
PER CENT ACTIVITY
100
90
80
70
60
50
40
30
20
20
40
60
80
100
120
DOSE, krad

TABLE I

INACTIVATION OF PURE AND IMPURE UREASE

Description of sample	Concentration of protein (mg ml^{-1})	Percent of active enzyme	Inactivation by 64 krads dose
Sample II	0.5	94	55%
Sample III	0.5	100	51%
Extract A	5.0	0.07	2%
Extract B	0.3	4.60	3%

experimental error, and for this reason the results were averaged (at least two measurements in buffer and two measurements in water) to give the points in Fig. 2; the average deviation of the mean was ± 4%. As can be seen, the data fit the equation:

$$\underline{A}/\underline{A}_0 = \alpha = -\underline{k}_0\underline{D} + 1 \qquad (2)$$

Table II reports some representative experimental results from single experiments, which show that doubling the dose doubled the extent of inactivation, in conformance with equation (2)..

The values of $\underline{k}_0$ from equation (2) was calculated for each line by least-squre analysis and the results are reported in Table III.

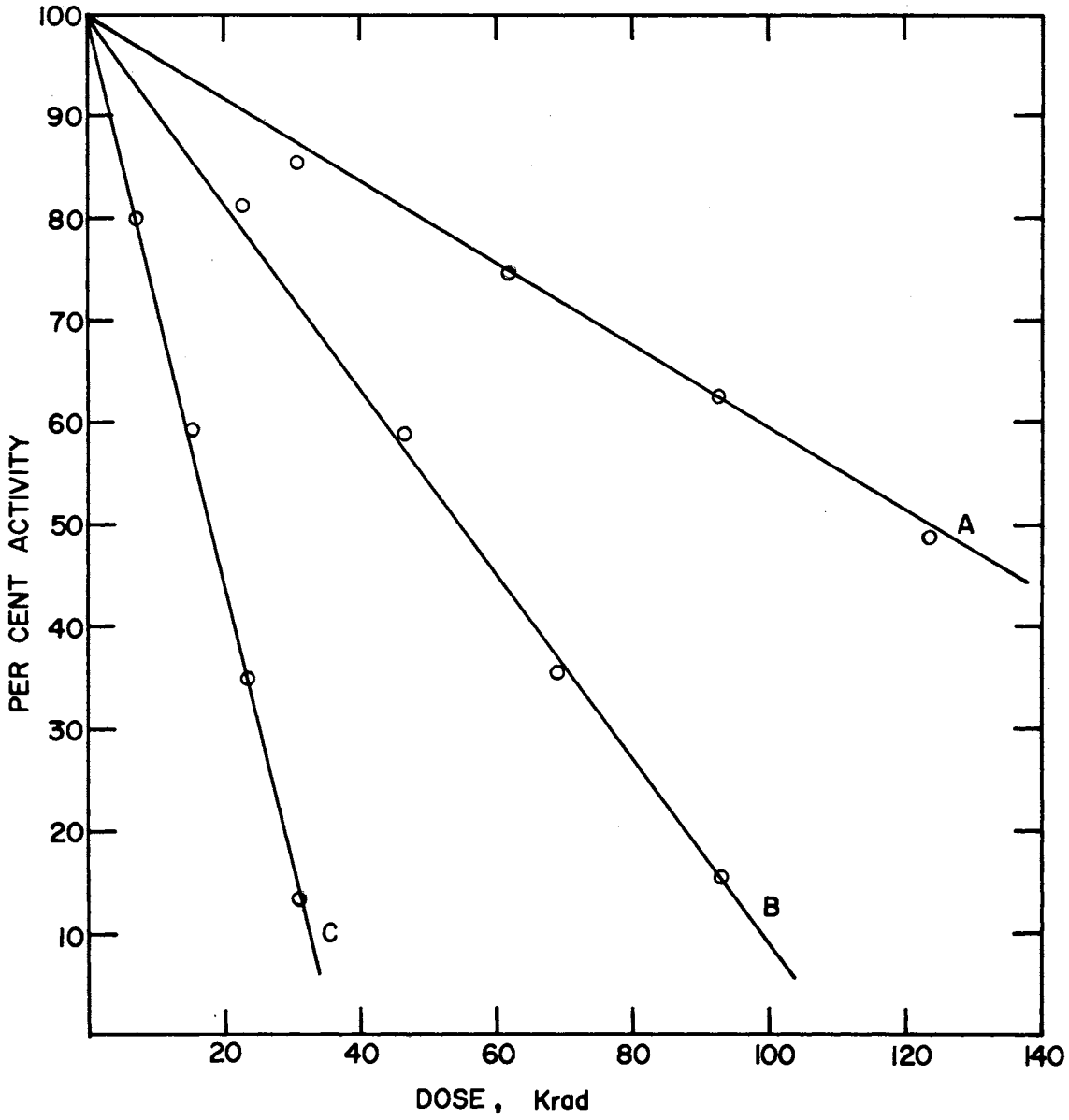

Fig. 2 Inactivation of lysozyme in 0.05M phosphate buffer or water. A, 500 μg lysozyme ml^{-1}, D_0 258 krads; B, 200 μg ml^{-1}, D_0, 113; C, 50 μg ml^{-1}, D_0, 36.9.

TABLE II

INACTIVATION-DOSE RELATIONSHIP FOR LYSOZYME

Dose (krads)	Inactivation %		
	Protein concentration:		200 μg/ml
	Expt. 1	2	3
46.5	44	43	42
93.0	82	80	81
	Protein concentration:		50 μg/ml
15.5	41	40	41
31.0	82	83	80

TABLE III

INACTIVATION OF LYSOZYME

Concentration of lysozyme mg ml^{-1}	D_0, (100 ev) in 0.05M phosphate	D_0, (100 ev) in water	G,[a] molecules/100 ev
0.50	1.563×10^{17}	1.365×10^{17}	0.14
0.20	6.946×10^{16}	7.148×10^{16}	0.12
0.05	2.250×10^{16}	2.239×10^{16}	0.09

[a] average of phosphate and water values

Discussion

The inactivation of enzymes in aqueous solution by ionizing radiation likely involves multifarious processes. The interaction of the radiation with water can produce various reactive species, such as $OH^{\bullet}$, $HO_2^{\bullet}$ (in aerated solutions) and the hydrated electron HOH^-, all of which may react with the enzyme. If the reaction causes a chemical alteration of the "active site", inactivation will certainly result, but inactivation may also be induced by reaction at other sites, which disrupt the native structure of the enzyme. While the reaction with radicals will be fast, indirect inactivation may be slower and hence give rise to delayed effects. Finally, reaction can probably occur at some sites on the enzyme, without causing inactivation. When one determines the inactivation of an enzyme as a function of dose, one measured the sum total of these effects, as well as the result of the competition between the remaining active enzyme and that already inactivated. Great caution must accordingly be exercised in the interpretation of results.

At the present state of development in this field, very little is as yet known about the aforementioned processes individually, and one cannot therefore attempt to assess their relative contributions to the overall inactivation process. What one must do, rather, is attempt to explain the results in terms of a simplified scheme that neglects some of these processes, and gradually introduce refinements as the simple scheme proves inadequate.

Sanner and Pihl (8) recently have proposed a scheme, which seems adequate for the interpretation of the present data. In this scheme, a single reactive species X is postulated, which exists in a steady-state

concentration during the irradiation. It is postulated that the species $\underline{X}$ can: (a) react with active enzyme $\underline{E}_a$ to produce inactive enzyme $\underline{E}_i$, with rate k_i; (b) react with active enzyme without inactivating it, with rate $\underline{k}_a$; and (c) react with inactive enzyme, with rate k_o. In addition, it is postulated that $\underline{X}$ disappears by a first-order rate process, of unspecified nature, with rate constant $\underline{k}_d$. The initial enzyme concentration $\underline{E}_0 = \underline{E}_a + \underline{E}_i$. Pihl and Sanner write the steady-state expression:

$$d\underline{X}/d\underline{t} = 0 = \nu\underline{D}/t - \underline{k}_a\underline{E}_a\underline{X} - \underline{k}_0\underline{E}_i\underline{X} - \underline{k}_d\underline{X} \qquad (3)$$

where ν is a proportionality constant relating the concentration of radicals to the dose per unit time; from this, the following expression is derived:

$$\nu\underline{D} = (\underline{k}_e - \underline{k}_0)\,\underline{E}_i/\underline{k}_i + [(\underline{k}_0\underline{E}_0 + \underline{k}_d)/\underline{k}_i]\,\ln \underline{E}_0/\underline{E} \qquad (4)$$

In a number of instances, including the case of urease in the present work, the experimental inactivation-dose relationship is exponential, and an equation of this form may be obtained from equation (4) if it is assumed that $\underline{k}_a = \underline{k}_0$, $(= \underline{k}_e)$, i.e., active and inactivated enzyme react with $\underline{X}$ at the same rate. Then

$$\ln \underline{E}/\underline{E}_0 = \ln \alpha = -\underline{k}_i\underline{D}/\nu(\underline{k}_e\underline{E}_0 + \underline{k}_d) \qquad (5)$$

As Hutchinson and Ross (9) first pointed out, conformance to equation (5) requires that $\underline{D}_{37}$, the dose at which $\ln \underline{E}/\underline{E}_0 = -1$, be a linear function of $\underline{E}_0$. The intercept of the ordinate measures $\underline{k}_d$, which Hutchinson and Ross regard as a measure of the "scavenging ability of the solvent". If this were zero, equation (5) would give a straight line going through the origin. In Fig. 3, the intercept is seen to be 7×10^{17} ev, which means that about one half of the dose is "scavenging the solvent" at 0.005% urease concentration.

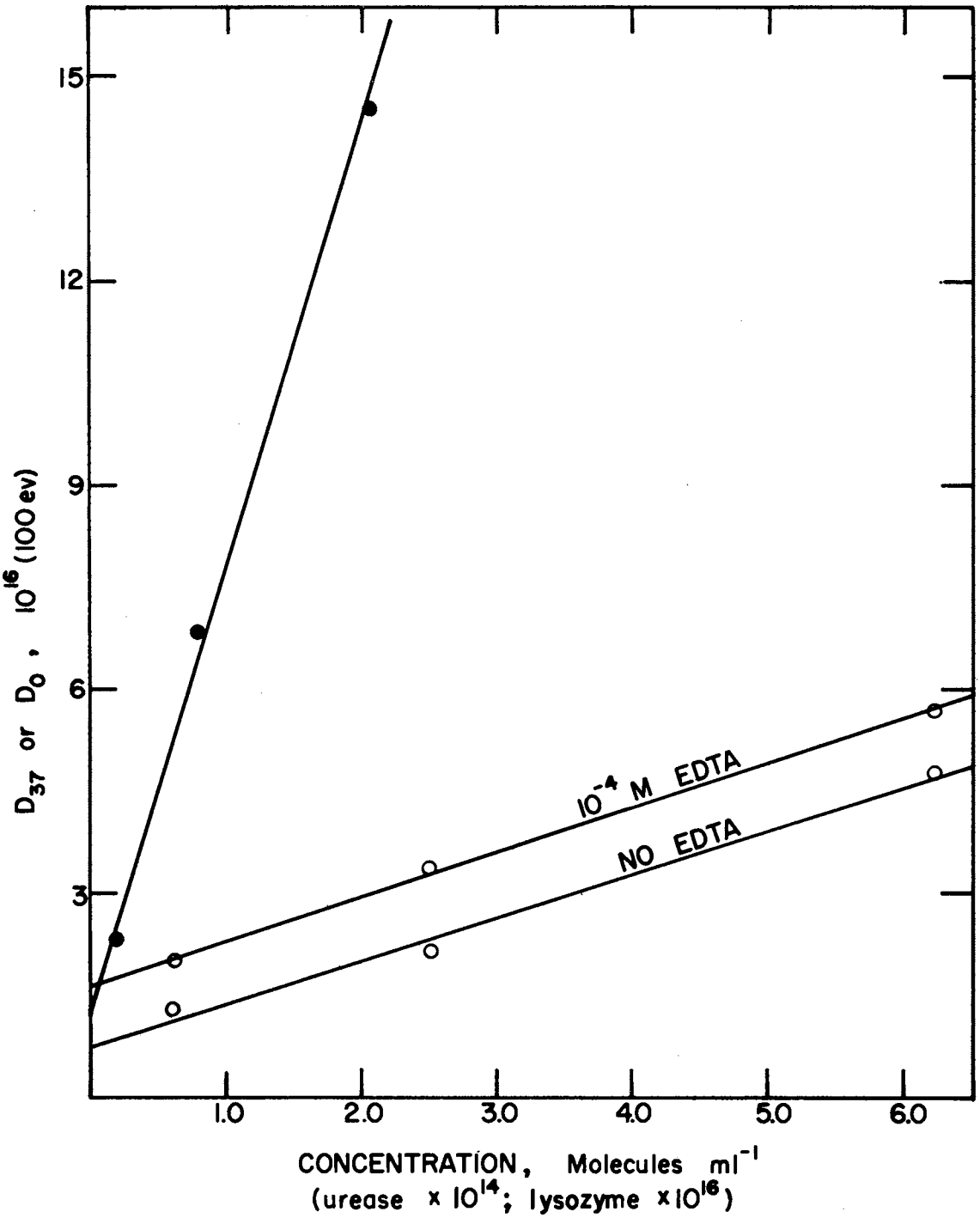

Fig. 3 Plot of D_{37} (in 10^{18} ev) vs. enzyme concentration. O Urease (abscissa x 10^{14} molecules ml^{-1}) in 0.05M phosphate with and without 10^{-4}M EDTA. ● Lysozyme (abscissa x 10^{16} molecules ml^{-1}) average of phosphate and water values.

For this reason, the apparent yield calculated from the expression

$$G = E_o/D_{37}$$

does not represent the "true yield". This is given, rather, by the slope of the line, i.e., 0.016 molecules/100 ev (note that this is nearly equal to the apparent yield at 0.05% enzyme concentration, since k_d is now small in relation to the other terms).

According to equation (5), the yield is $(k_i/\nu k_e)$, i.e., the ratio of the rate of inactivating reactions to that of reaction with active and/or inactive enzyme. If we assume that $\nu = 3$, i.e., three X radicals are formed per 100 ev, we have that $k_i \sim 0.05 k_e$, one in twenty reactions with the enzyme is inactivating. This does seem reasonable when one considers that the molecular weight of the enzyme is 60-80,000 per active site.

Reference has been made to the instability of urease. This is not a serious problem at 0.5% concentration -- little loss of activity is observed in such solution over a period of some days' duration. But the enzyme must be assayed in much more dilute solutions, ca. 0.001 - 0.003%, and such dilute solutions are less stable; furthermore, they are easily inhibited by trace of metal ions (10). The studies of Gorin and Chin indicate that this can be avoided, at least to a large extent, by using EDTA; note that 0.02M phosphate-10^{-3}M EDTA was used for diluting the samples for assay.

In some experiments, the urease was irradiated in the presence of 10^{-4}M EDTA, but in this case there is involved an additional effect, i.e., the reaction of radicals with the EDTA. As a result, EDTA "protects" the enzyme. According to the Hutchinson and Ross's simple model, the presence

of EDTA should increase the "scavenging effect of medium", i.e., the intercept, but the slope should remain the same. The results of Fig. 3, are in agreement with this expectation.

A comparison should be made between the result obtained in this work and those reported by other investigations. They are listed in Table IV. It must first of all be noted that all earlier work was done with very crude preparations, one of which contained only 2.5% active enzyme. The significance of these results accordingly depends in large measure upon the relative radiosensitivity of the inactive material.

The results in Table I show that the presence of impurities can have a large effect on the apparent yield. On this basis, it must be suggested that the earlier results should be disregarded.

The aspect of the results obtained with lysozyme that first requires comment is the good conformance of the inactivation-dose relationship to a straight line. According to equation (4), one can see that the inactivation-dose relationship would deviate from the exponential toward the linear form as k_i increases relative to k_e. In other words, the results obtained indicate that, in lysozyme, there is relatively little competition between active and inactivated enzyme.

If the inactivation-dose relationship is linear, the slope of the line is a measure of the radiosensitivity. This is equal to $1/D_0$, the intercept obtained by extrapolating the line to the abcissa. D_0 has the same significance in the case of linear inactivation that D_{37} has in the case of exponential inactivation; both equal to $(d\alpha/dD)_{D=0}$, the limiting slope of the inactivation-dose curve. For this reason, the values of D_0 for lysozyme could be treated in the same way as the values

TABLE IV

CALCULATED YIELDS OF ENZYMES INACTIVATED BY X-RAYS AND Y-RAYS

Investigator	G	Enzyme Purity	Protein Concn.
		Urease	
Tanaka et al.	0.24	4.8×10^{5} I.U.B. g^{-1}	11.2 mg ml^{-1} in 0.05M phosphate
Lewis et al.	0.042	4.3×10^{4} I.U.B. g^{-1}	0.2 mg ml^{-1} in water
Dickens and Shapiro	0.02	2.8×10^{3} I.U.B. g^{-1}	5.0 mg ml^{-1} in 0.05M phosphate
Gorin et al.	0.016	1.65×10^{6} I.U.B. g^{-1}	0.05 to 0.50 mg ml^{-1} in 0.05M phosphate
		Lysozyme	
Dose	1.2×10^{-1}		0.8 mg ml^{-1} in water
Shalek	6.1×10^{-1}		0.464 mg ml^{-1} in water
Gorin et al.	1.4×10^{-1}		0.5 mg ml^{-1} in phosphate or water

of $\underline{D}_{37}$ for urease, and the corresponding plot is also shown in Fig. 3. The "true yield" obtained in this case is 0.14 molecules/100 ev.

A comparison with earlier data shows that our results are quite in agreement with those of Dose (11) (see Table IV), whereas the $\underline{G}$ values obtained by Shalek and Gillespie (12) appear to be somewhat higher. In the reference mentioned, Shalek and Gillespie showed an exponential inactivation curves, but they reported recently (13) that the inactivation of lysozyme by γ-rays follows a linear relationship.

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CHAPTER IV

APPENDIX

This chapter supplements Chapter III by describing some experimental methods in more detail, which could not be included in that chapter for reasons of brevity. It also gives some experimental results that were not included in the paper for publication.

Materials and Methods

Inactivation procedure and dosimetry

The irradiation of enzyme solutions was carried out with Westinghouse industrial x-ray unit. One-half milliliter of the solution to be irradiated was placed in a 10 x 75 mm Pyrex culture tube. The tube was placed in a holder which positioned it in the x-ray beam. The holder was also able to serve as an ice bath. In all irradiations the tubes were placed in an ice-water mixture during the irradiation. The dose rate employed was 1,550 rads min^{-1} determined by chemical dosimetry (4). An ionic yield of 13.9 molecules of ferric ion per 100 e.v. was used as the basis of calculation (5). For each type of sample irradiated and unirradiated control was diluted and assayed in an identical manner.

Urease assay

One milliliter of 3% urea in 0.1M Tris buffer, pH 9, was mixed with 1 ml of urease, 9.0 - 27.0 γ protein ml^{-1}, in 0.02M phosphate buffer at 25°C. After exactly 2 min the reaction was stopped by adding 2 ml of 0.1M HCl with a Folin pipette. The excess HCl was then back titrated with a TTTI automatic titrator (Radiometer, Copenhagen) to pH 4.5 (volume

required in ml = $\underline{V}_s$). A blank sample made up from 1 ml of substrate and 1 ml of 0.02$\underline{M}$ phosphate buffer was treated in the same way (volume required = $\underline{V}_b$). The activity of the sample in I.U.B. units was calculated by the equation

$$\text{Activity} = 500\ (\underline{V}_b - \underline{V}_s)\ \underline{M}_{OH}$$

where $\underline{M}_{OH}$ is the molarity of the NaOH solution.

Lysozyme assay

A representative calibration experiment conducted by adding increasing amounts of lysozyme to the substrate gave the results listed in Table V and represented in Fig. 4. As has been mentioned, a calibration curve was prepared for each series of experiments. The calibration curves differed little if the same stock bacteria suspension was used; different stock solutions gave lines that differed in slope by no more than 10%. All activity measurements were done in duplicate; the average deviation from the mean was ± 2%.

Earlier studies on the assay of lysozyme

The assay of lysozyme based on the lysis of M. lysodeikticus seems to be the best method available at this time, but it falls far short of ideal. Ideally, an enzyme assay should be based on a definite chemical reaction, which, furthermore, fulfills the following requirement: (1) the rate of reaction is negligible in the absence of enzyme; (2) the rate is kinetically of zero-order, i.e., independent of substrate concentration and constant with time; and (3) the rate is directly proportional to the enzyme concentration. These requirements are not fulfilled by the aforementioned method of assay, the results of which must accordingly be interpreted with special care.

TABLE V

CALIBRATION CURVE FOR LYSOZYME ACTIVITY

Concentration of lysozyme (μg/ml)	Absorbance			Decrease in absorbance[a]
	Expt. 1	2	Avg.	
blank	0.575	0.578	0.577	---
4	0.358	0.356	0.357	0.220
3	0.420	0.415	0.418	0.159
2	0.470	0.468	0.469	0.108
1	0.526	0.526	0.526	0.051

[a]The difference between blank and sample

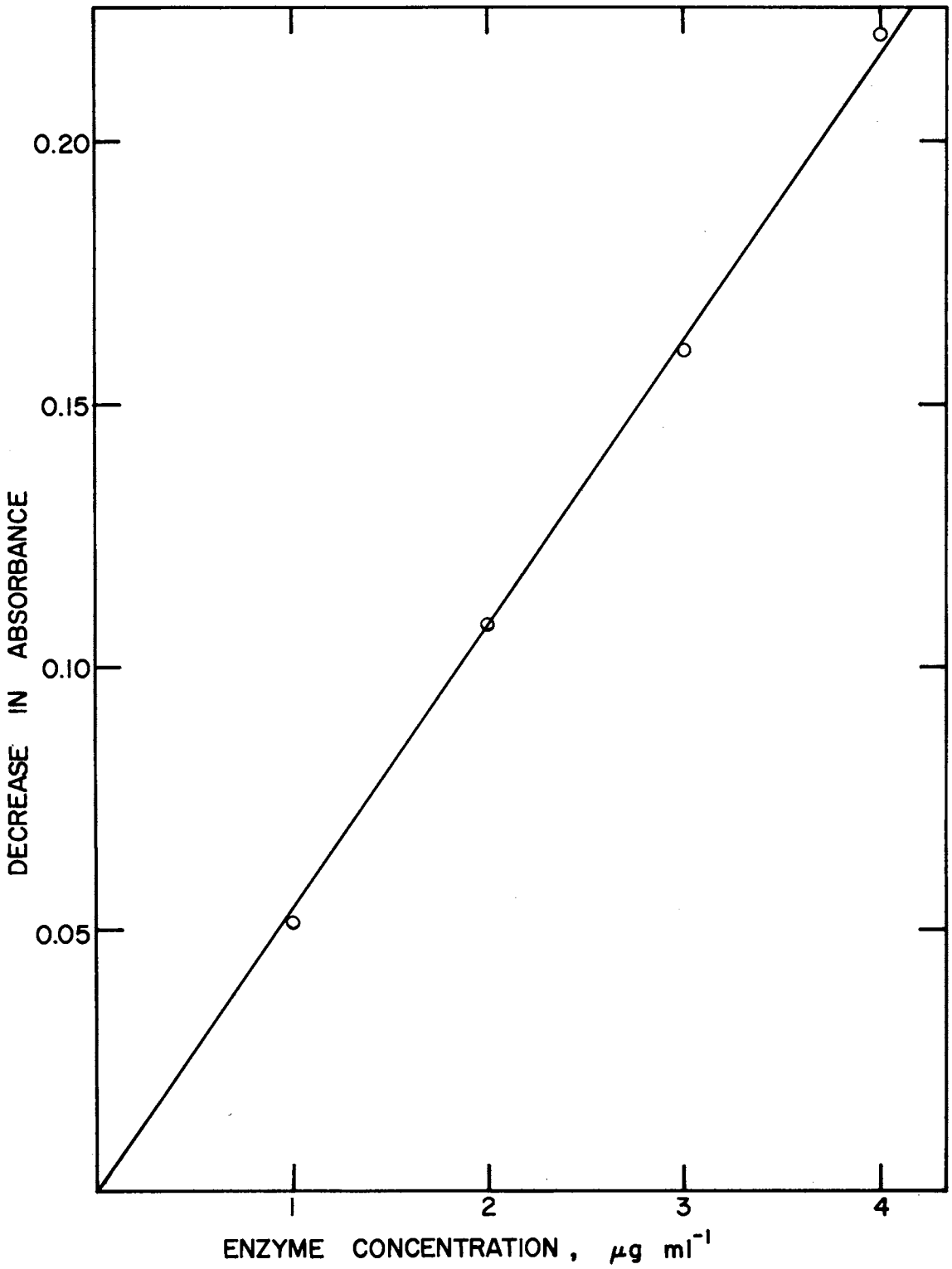

Fig. 4 Calibration curve for lysozyme assay.

From a practical point of view, it is desirable that a plot of the quantity measured in the assay be directly proportional to the enzyme concentration; if this is not so, then at least the curve should not become too flat with respect to the enzyme-concentration axis, since this results in decreasing precision.

The first experiments done in the present research program made use of M. lysodeikticus preparations obtained from Calbiochem. (Los Angeles). A 40-min incubation time was used (52), and the plot of absorbance decrease vs. enzyme concentration showed a marked curvature toward the enzyme-concentration axis; at a concentration beyond 4 μg/ml, the curve became practically horizontal. Shorter incubation times were then tested, down to 5 min; the curves thus obtained had a steeper slope, but the plots still were not linear.

Some experiments were conducted, to test the suggestion (50) that the absorbance due to suspended cells follows second-order kinetics, i.e., a plot of $1/A$ vs. time gives a straight line. This was not found to be the case with the Calbiochem substrate.

When the M. lysodeikticus preparations sold by Difco Laboratories were tested, in essentially the same conditions as used for the Calbiochem preparation, with a 5-min reaction time, the plot of absorbance change vs. enzyme concentration was quite linear to 4 μg ml^{-1} enzyme concentration (see preceding section). This material and procedure were accordingly used in subsequent experiments.

Inactivation measurements on urease

Table VI shows representative experimental data on the inactivation of urease. The protein concentration is 500 μg/ml and 10^{-4} M EDTA is

present in solution. The control and irradiated solutions were assayed at the same dilution and nearly at the same time.

TABLE VI

INACTIVATION-DOSE RELATIONSHIP FOR UREASE

Urease Concentration 500 μg/ml

Description of sample	Volume of M/30 NaOH used (ml)			ΔV^a	Remaining Activity
	Expt. 1	2	Avg.		%
blank	4.37	4.33	4.35	---	---
unirradiated sample	3.46	3.44	3.45	0.90	---
+ 23.3 krads	3.66	3.64	3.65	0.70	78
+ 46.5 krads	3.80	3.82	3.81	0.54	60
+ 69.8 krads	3.96	3.95	3.96	0.39	44
+ 93.0 krads	4.05	4.05	4.05	0.30	33

[a]The difference between blank and sample

The inactivation-dose data for different preparations of urease compared at the same concentration are shown in Table VII.

Table VIII lists the results for the inactivation of urease at different concentrations, with or without EDTA.

Inactivation measurements on lysozyme

The samples of lysozyme to be irradiated were prepared by diluting the stock solution with 0.05M phosphate buffer, pH 7.0 or with triply distilled water. From the calibration curve in Fig. 4, a representative inactivation-dose relationship has been calculated, which is shown in Table IX.

TABLE VII

INACTIVATION-DOSE RELATIONSHIP FOR DIFFERENT PREPARATIONS OF UREASE

Urease concentration: 200 μg/ml

Dose krads	Activity remaining, %		
	Sample II	Sample III	Sample IV
23.3	66	69	---
46.5	44	43	46
69.8	29	28	---
93.0	19	15	18

These data are the averages of two determinations; the average precision was ± 4%.

TABLE VIII

INACTIVATION-DOSE RELATIONSHIP FOR UREASE

Dose krads	Activity remaining, %					
	No EDTA			10^{-4}M EDTA		
	Concn. of urease, mg/ml			Concn. of urease, mg/ml		
	0.05	0.20	0.50	0.05	0.20	0.50
6.2	70.2	--	--	--	--	--
12.4	55.7	--	--	--	--	--
15.5	--	66.7	--	61.0	--	--
18.6	43.7	--	--	--	--	--
23.3	--	--	72.3	--	67.5	78.0
24.8	28.7	--	--	--	--	--
31.0	--	40.4	--	33.5	--	--
46.5	--	24.2	54.0	24.0	43.5	60.0
62.0	--	14.7	--	14.5	--	--
69.8	--	--	41.0	--	28.5	45.0
93.0	--	--	29.0	--	17.0	37.0

These data are the averages for Samples II, III and IV; the average precision was ± 4%.

TABLE IX

INACTIVATION-DOSE RELATIONSHIP FOR LYSOZYME

Lysozyme concentration: 200 μg/ml; diluted 50 fold for assay

Dose krads	Absorbance Expt. 1	2	Avg.	Decrease in absorbance[a]	Remaining Activity, %
0.0	0.354	0.356	0.355	0.222	100
15.5	0.389	0.385	0.387	0.190	88
31.0	0.410	0.410	0.410	0.167	76
46.5	0.436	0.442	0.439	0.138	62
62.0	0.476	0.478	0.477	0.100	45

[a]The difference between blank and sample

Table X reports the average values of the activity of inactivated lysozyme either in phosphate buffer or in triply distilled water.

TABLE X

INACTIVATION-DOSE RELATIONSHIP FOR LYSOZYME IN PHOSPHATE BUFFER OR WATER

Dose krads	Activity remaining, % lysozyme, 0.05 mg/ml Phos. buffer	H_2O	lysozyme, 0.20 mg/ml Phos. buffer	H_2O	lysozyme, 0.50 mg/ml Phos. buffer	H_2O
6.2	85	84	-	-	-	-
12.4	64	61	-	-	-	-
15.5	-	-	85	87	-	-
18.6	44	47	-	-	-	-
23.3	-	-	-	-	90	92
24.8	33	34	-	-	-	-
31.0	-	-	73	76	-	-
46.5	-	-	62	60	81	83
62.0	-	-	43	44	-	-
69.8	-	-	-	-	69	75
93.0	-	-	-	-	57	60

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