1967

A Photo-Catalytic Enzymatic Hydrolysis

Michael Mosby Daly
Eastern Illinois University

A PHOTO-CATALYTIC

ENZYMATIC HYDROLYSIS

(TITLE)

BY

MICHAEL MOSBY DALY

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE IN EDUCATION

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1967
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
THIS PART OF THE GRADUATE DEGREE CITED ABOVE

May 15, 1967
DATE

May 15, 1967
DATE

ACKNOWLEDGEMENTS

Grateful acknowledgement is made to the Eastern Illinois University Research Committee for the support of this research.

I wish to express my sincere appreciation to the Department of Chemistry and Chemical Engineering of the University of Illinois for providing the spectropolarimetry instrumentation so vital to this research.

I also wish to thank Dr. Robert Woody of the Department of Chemistry and Chemical Engineering, University of Illinois, for technical aid in obtaining rotatory dispersion data.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

Enzymatic starch hydrolyses and the effect that light has upon them have been under investigation for a number of years. The papers published by Baly and Semmens (1,2) and Semmens (3,4) were the first of a number dealing with the effect of plane-polarized light on biological processes. In these papers plane-polarized light was described as having pronounced effects in accelerating the hydrolysis of starch by enzymes. Subsequently, two sets of divergent results appeared in the literature. Macht, et al (5) confirm the results of Baly and Semmens while Jones (6), Bunker and Anderson (7), and Navez and Rubenstein (8) were unable to support the earlier findings.

In the earlier papers concerned with photocatalytic starch hydrolyses little attention was paid to experimental design and technique. Later work, such as that of Bunker and Anderson, and Navez and Rubenstein is much more articulate and the results more reliable.

While these later papers have demonstrated that no measurable difference in photolytic influence can be ascribed to plane-polarized radiation over that of heterogenous (non-polarized) radiation, it was demonstrated that light, whether heterogenous or plane-polarized, does accelerate the rate of diastase starch

hydrolysis. Moreover, it is stated in the work of Navez and Rubenstein that irradiation of the enzyme alone will result in a pronounced hydrolytic rate difference.

The fact that any rate differences were observed at all is thought to be curious. It implies the existence of an absorption band in the near-visible or visible spectral regions for materials that are colorless.

The period during which these works were published afforded little opportunity for the investigators to extend these findings in an effort to satisfactorily explain the observed phenomena. Consequently, it has been deemed desirable to re-demonstrate, if possible, the effects of light upon diastase starch hydrolysis and to extend these earlier investigations by means of more recent techniques and instrumentation.

In addition, one may wonder whether or not the implied band is optically active (i.e., whether or not the absorption phenomenon is associated with some center of asymmetry). The substrate and the enzyme are both dissymmetric materials and are thereby necessarily circularly dichroic. It follows that if such a system, or its components, were illuminated with dissymmetric electromagnetic radiation in the spectral region of an optically active absorption band, one could expect preferential absorption of right or left circularly polarized radiation. If such an interaction occurred in a photochemically active system it would be reasonable to expect differential hydrolytic velocities.

The investigation reported herein involves the study of the phenomena associated with enzymatic hydrolyses which have been

influenced with heterogenous and dissymmetric electromagnetic radiation.

The specific system investigated was that of starch and Taka-diastase. Similar aliquots of an identical preparation of starch and diastase were simultaneously exposed to heterogenous and circularly-polarized radiation of equal intensities, all other conditions being equal. The hydrolysis rates were experimentally measured by quantitative determinations of the hydrolytic products (i.e., by measuring the concentration of reducing sugar at various times throughout the hydrolysis) utilizing photometric techniques.

The comparison of hydrolytic rates of samples irradiated with heterogenous and circularly-polarized light would then disclose the dissymmetrical nature of the photo-catalysis.

In addition, optical rotatory dispersion (ORD) data were obtained and used to theoretically confirm the effect of heterogenous and dissymmetric radiation on the system.

CHAPTER II

METHODS AND MATERIALS

Method of Illumination

The hydrolytic studies included the following investigations:

(1) Hydrolyses conducted in such a way that the reaction itself was illuminated during the entire extent of hydrolysis with either heterogenous or dissymmetric light. Hereafter this will be referred to as "system-illumination."

(2) Hydrolyses conducted in the dark subsequent to substrate illumination with either heterogenous or dissymmetric light.

(3) Hydrolyses conducted in the dark subsequent to enzyme illumination with either heterogenous or dissymmetric light.

All of the hydrolyses were conducted within the confines of a laboratory darkroom. In these quarters it was possible to rigidly control illumination during the course of reactant preparation and hydrolysis. In addition, the quarters were so situated that minimal temperature fluctuations occurred. This facilitated the employment of temperature-constancy controls.

The Illumination Bath

The bath construction.--The illumination bath was so constructed that it housed two completely separate optical systems. Each system consisted of a source of radiation, an optical compartment, a front-surfaced metallic mirror, and a cell containing the reacting solutions (see Fig. 1.). The bath was constructed using ¼ and ½ inch plywood board. It was then doubly coated with flat-black

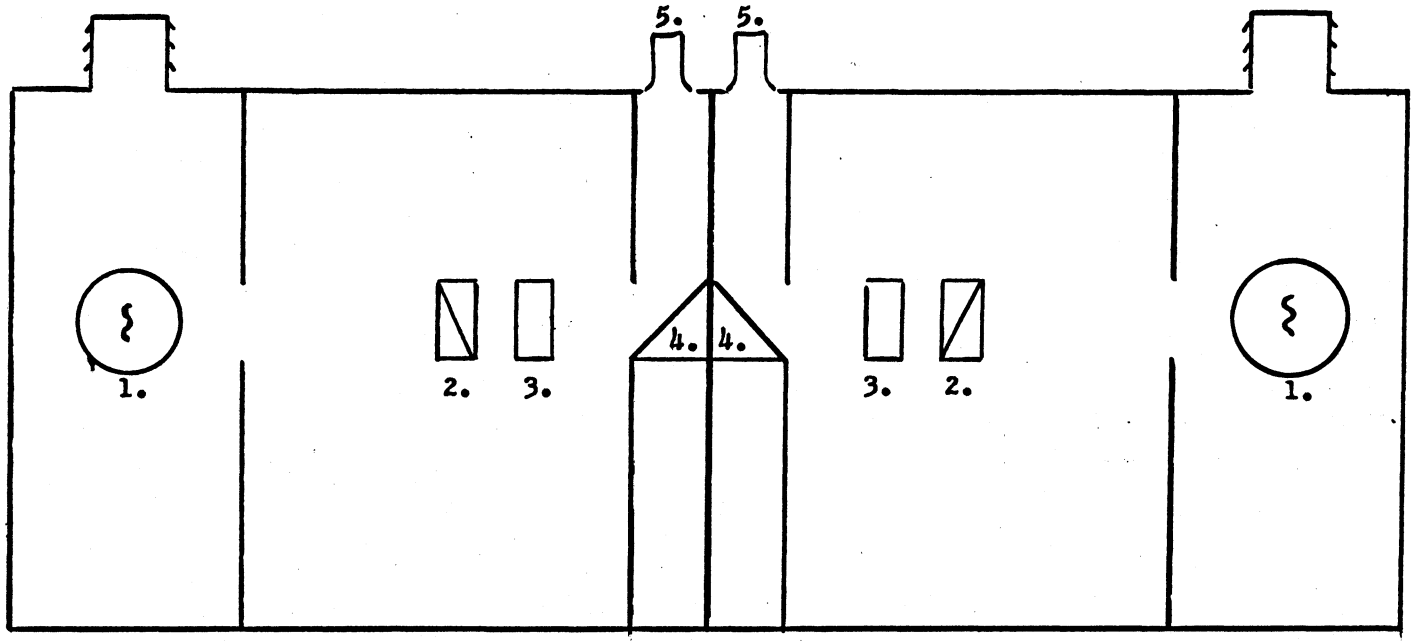

Fig. 1.--Diagram of the illumination bath which houses the incandescent lamps (1), the polarizing filters (2), the retardation plates (3), the front-surface metallic mirrors (4), and the reaction cells (5).

paint. The compartments housing the 300-watt Westinghouse lamps were made of the heavier board and were insulated with ¼ inch asbestos panels. Each lamp compartment was furnished with a heat vent so constructed that heat could be effectively dissipated without allowing stray light to fall on the reaction cells. In addition, provisions were made so that each compartment could be cooled with compressed air. Convection-cooling proved to be quite adequate. The compartment housing the optical components was provided with facilities that allowed the mounting of plane-polarizers and retardation-plates when it was necessary to generate dissymmetric radiation. The polarizers used were Leybold polarizing filters. They consist of dichroic filters embedded between glass plates. The degree of polarization achieved is more than 99% and within the visible range of the spectrum is almost independent of wavelength. Leybold quarter-wave and Lambrecht three-quarter-wave retardation plates were utilized in generating circularly polarized light. Great care was taken to protect both the polarizers and the retardation-plates from excessive heat.

The light emerging from the optical compartment of the bath was then reflected 90°, by a front-surfaced metallic mirror, into the reaction cells.

Illumination-intensity Control.--Each lamp's intensity was controlled by individual rheostats. Power for the lamps came from the same A. C. source so that both would be subject to the same current fluctuations, if any. The intensity measurements were made at the cell position by means of an illuminometer. Such measurements preceded each of the hydrolyses that were conducted.

Reaction cells.--Glass cylinders 118 mm. long and of 44 mm. inside diameter to which optical-quality glass windows (the windows were prepared from dense flint glass, their faces parallel to within 300 mμ.) had been cemented served as the cells in which the hydrolyses were conducted. Cell volume was in excess of 70 ml. Each cell was equipped with a rubber stopper into which a calibrated Wilkens-Anderson thermometer had been inserted. The reaction cells were supported by the illumination bath, but were insulated from it by styrofoam panels containing optical-path apertures.

Composition of Solutions

The phosphate buffer.--A buffer solution of pH 5 was used as the solvent for both the substrate and the enzyme. This pH had been shown to be optimal for diastatic action by Sherman, et al (9). The buffer solution employed was prepared by mixing two stock buffers. One part of a 0.33M Na_2HPO_4 + 0.25M NaCl solution was mixed with 99 parts of a 0.33M NaH_2PO_4 + 0.25M NaCl solution. The resulting solution was then diluted to four times its initial volume. The mixed buffer was prepared as needed and its hydrogen-ion concentration was checked with a pH meter.

The substrate solution.--Fischer's Reagent soluble starch served as the substrate. Stock 0.6% (w/v) solutions were prepared in buffer by boiling for three minutes and re-establishing the initial volume with boiled distilled water. These solutions were stored under refigeration and kept for no longer than one week.

The enzyme solution.--A modified form of Merck's Taka-Diastase, dissolved in buffer, served as the enzyme. The preparation procedure followed was that of Naves and Rubenstein's (8). The entire

modification was performed in the dark (as specified by Navez, et al). One gram of Taka-diastase was dissolved in 100 ml. of a 1% (w/v) NaF solution. The subsequent solution was maintained in an ice bath at 0°C. for two hours with intermittent stirring. It was then centrifuged for five minutes at 1800 R. P. M. The sediment was then discarded and the supernatant liquid cooled to 0°C. A volume of 95% ethanol sufficient to make the alcoholic titer 50% was then added to precipitate the first enzyme fraction. Following thorough mixing the solution was centrifuged for five minutes and the precipitate (i.e., the first enzyme fraction) was discarded. The alcoholic titer of the supernatant liquid was then increased to 65% and the solution re-centrifuged for ten minutes. The supernatant liquid was then discarded and the precipitate dried in an opaque dessicator for 18 hours at 5°C. The product yield was approximately 45%. Subsequently 0.400g. of the white material was dissolved in 100 ml. of the phosphate buffer. The solution was stored, under refrigeration, in a taped bottle. The enzyme proved to be quite stable under these conditions.

IRRADIATION TECHNIQUES

Innoculation and Irradiation

When the enzyme-substrate system was to be irradiated during the course of hydrolysis (i.e., "system-illumination") a 100 ml. volume of the starch solution was placed in a stoppered flask. The enzyme solution was then withdrawn from refrigeration. Both solutions were allowed to come to room temperature. Subsequent to this the starch solution was innoculated with 9.4 ml. of the enzyme

solution. (Previous studies had shown this enzyme-substrate ratio was necessary for the attainment of complete hydrolysis 60 minutes after innoculation.) Innoculation was assumed to initiate hydrolysis. The resulting solution was then mixed and divided equally. One volume served as the irradiated hydrolytic solution, the other as the dark-control. In the case of the dissymmetrically-irradiated reactions, one volume was irradiated with right circularly-polarized light and the other with left circularly-polarized light.

When hydrolysis occurred subsequent to irradiation of the substrate only or the enzyme only, a different innoculation method was used. The case of enzyme irradiation will be described as representative of the method. Two 50 ml. volumes of the substrate were pipetted into taped flasks. These flasks were placed in a constant-temperature bath and allowed to attain thermal equilibrium. During this time two 10 ml. volumes of enzyme solution were placed in the reaction cells and cooled to 0°C. One cell was then irradiated with either heterogenous or dissymmetric light while the other cell served as a control, either in the dark or by means of being irradiated with the other form of dissymmetric light. The length of irradiation time was varied from 5 to 30 minutes. Following irradiation the substrate solutions were innoculated with 4.65 ml. of the appropriate enzyme solution. The hydrolyses were then carried out in the dark and were individually timed.

Hydrolysis duration.--The duration of hydrolysis was one hour, in all cases. This time-length of hydrolysis was chosen

because it afforded ample time for sampling and temperature control without permitting heat build-up to occur to the extent of damaging the optical components of the illumination bath.

Analytical Technique

The hydrolyses.--During the course of the hydrolyses, 2 ml. aliquot samples of reaction-mixture were withdrawn from both cells. Initially such samples were withdrawn at 6 minute intervals. In later studies more than 50% of the samples to be analyzed were withdrawn in the first 15 minute segment of the overall one hour hydrolysis.

The Folin (10) modification of the Folin-Wu reducing-sugar analysis was employed in analyzing the withdrawn aliquots for the amount of reducing-sugar formed during the course of hydrolysis. (It is important to note that while the final hydrolytic products of the reaction are maltose molecules, the reducing-sugar formed in the course of this reaction may consist of dimers or polymers of maltose units.) The first step of the Folin procedure specifies the discharge of a 2 ml. sample into a flask containing 16 ml. of N/12 sulfuric acid and 2 ml. of 10% (w/v) sodium tungstate. This solution deproteinates the reaction-mixture. Completion of this discharge procedure was assumed to terminate hydrolysis for each aliquot. Samples treated in this way were then stored in their corresponding flasks until the one hour monitoring procedure was complete. All samples, including appropriate starch-in-buffer and reagent blanks, were then simultaneously subjected to the remaining phases of the photometric analysis. Starch solutions "stored" in the manner described above disclosed no detectable acid-catalyzed

hydrolysis.

Photometric measurements of the resulting molybdenum blue samples were made at 800 mμ. The absorption spectra of the blue Folin-Wu product (see Fig. 2.) indicates the absorption band is of significantly greater amplitude at this wavelength than at 580 mμ.

The data accumlated from these procedures consisted of sample-absorbancy as a function of time. In order to convert this data to that of reducing-sugar formed as a function of time it was necessary to prepare a maltose calibration curve (i.e., absorbancy as a function of maltose concentration). The Bunker, et al (7), method of sample preparation was utilized. Stock standards of 400, 267, 178, 119, and 79 mg. of maltose per 100 ml. (each of these is 2/3 the concentration of the next higher and 1½ times that of the next lower) were prepared and utilized in the dilution scheme described below.

1	2	3	4	5	6	7	8
400	267	178	119	79	52.7	24	0
385	257	171	114	76.2	50.7	24	3
374	249	166	111	73.8	49.2	24	6
363	242	162	108	71.8	47.8	24	9
349	233	155	103	68.9	45.9	24	15
338	225	150	100	66.7	44.5	24	21
333	222	148	98.7	65.8	43.8	24	24
320	213	142	94.8	63.2	42.2	16	24
311	207	138	92.3	61.6	41.0	12	24
300	200	133	89.0	59.3	39.5	8	24
286	190	127	84.7	56.4	37.7	4	24

Table 1.--Showing proportions in which standards are to be mixed in order to make samples of desired maltose concentration.

The desired concentration of a maltose sample (C), or a close approximation to it, is found in Columns 1 to 6 of Table 1. On the same line in Column 7 will be found the amount to be taken

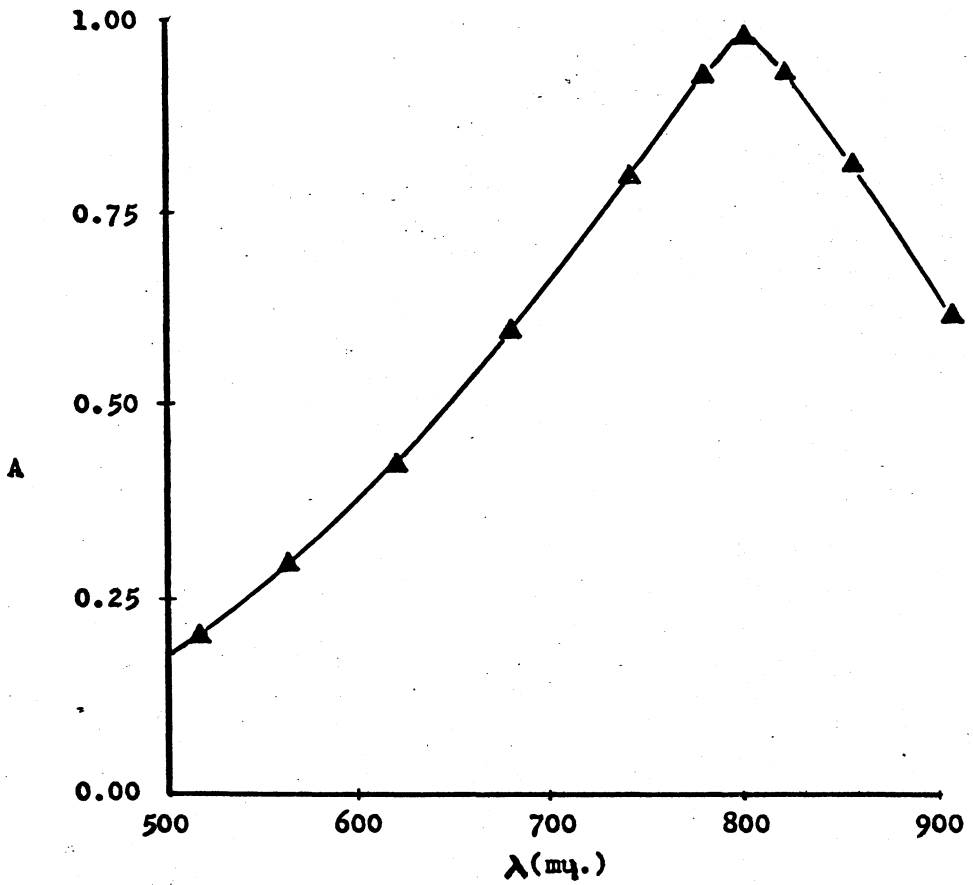

Fig. 2.--Graph showing the absorption spectra of the Folin-Wu product.

of the stock standard whose value heads the column in which the C value was found. In Column 8 on the same line is the amount of the next lower stock standard to be mixed with it to furnish a useful working standard.

The samples of known maltose concentration were then subjected to the colorimetric analysis. From the resultant data a calibration or standard curve was constructed.

Ultra-violet absorption spectra.--Cary 15 and Perkin-Elmer Hitachi 139 UV-VIS Spectrophotometers were utilized in obtaining spectra of irradiated (heterogenously) and dark-control enzyme samples. The solvent and concentration of the samples were identical to those used in the hydrolyses.

Optical rotatory dispersion.--A JASCO ORD/UV-5 spectropolarimeter was used in obtaining the rotatory dispersion curves of irradiated and dark-control enzyme samples. The samples were identical to those utilized in the ultra-violet spectral analyses.

Phenomena observed in the course of the ultra-violet absorption studies indicated the necessity of investigating a particular enzymatic absorption band existing at 320 mμ. The band is not generally characteristic of proteins and the phenomena associated with the band were first studied by varying the pH of the enzyme solutions and obtaining absorption spectra. The pH variation was accomplished by the introduction of small volumes of concentrated NaOH or H_2SO_4 into relatively large (300 ml.) volumes of enzyme solution. A drop of the resulting solution was then placed upon a glass slide and mixed with universal indicator to determine the solution's pH. In each range spectra were taken.

CHAPTER III

RESULTS

The System-Illumination Hydrolyses

The influence of heterogeneous light.--The starch-diastase system was found to be photochemically active. The rate of starch hydrolysis was accelerated in the presence of heterogenous light over that of the corresponding dark-control. A graphical representation of data from this study may be found in Fig. 3.

Slope differences between the two curves indicate the presence of differential hydrolytic rates. In addition, both curves possess a sigmoidal character corresponding to a "threshold time" of reaction.

The influence of circularly-polarized light.--Duplicate hydrolyses, one volume being irradiated with right circularly-polarized light and the other with left circularly-polarized light, demonstrated no differential hydrolytic rates (see Fig. 4.). Apparently no preferential absorption of dissymmetric radiation in this spectral region can be ascribed to the starch-diastase system.

Substrate-Illumination Hydrolysis

When the substrate was irradiated with heterogenous light (see Fig. 5) for periods up to 30 minutes and the subsequent hydrolysis conducted entirely in the dark, no differential rates

were demonstrated. The reactions proceeded as if they had never been illuminated. As a consequence of this result, no dissymmetric irradiation was employed.

Enzyme-Illumination Hydrolyses

The influence of heterogenous light.--When the enzyme alone was irradiated with heterogenous light (see Fig. 6.) for periods greater than 10 minutes with a subsequent dark hydrolysis, it was found that differential hydrolytic rates were occurring. Irradiation of the enzyme for longer than 15 minutes produced no corresponding rate increases. The rate differences (i.e., the slope differences) observed in this manner were only slightly less pronounced than those of the system-illumination type. In particular, it was noticed that the plotted data for the irradiated hydrolysis lacked a sigmoidal character as pronounced as in the system-illumination.

The influence of circularly-polarized light.--When the enzyme was irradiated with dissymmetric light (i.e., one sample with right and the other sample with left circularly-polarized light) and the subsequent hydrolysis conducted in the dark, no detectable rate differences occurred. This corroborates the evidence secured in the dissymmetric system-illumination hydrolysis. The graphical representation of the data from this study may be found in Fig. 7.

Maltose Standard Curve

The calibration curve, prepared as described on pages 11 and 12, results in a plot as shown in Fig. 8. The Beer-Lambert behavior

that was demonstrated facilitated conversion of the data derived from the Folin-Wu procedure into reducing-sugar formed as a function of time. The precision obtainable with this method is on the order of ±5%. Since the qualitative differences between irradiated samples and dark-control samples were always consistently demonstrated and were always greater than the precision, the results seem to be reliable.

Ultra-Violet Absorption Spectra

Comparison of the ultra-violet absorption spectra of the irradiated enzyme with that of the dark-control enzyme disclosed that two absorption bands were present in each. The spectra are represented in Fig. 9. In addition to the classical protein absorption band at 280 mμ. (due to a $\pi \longrightarrow \pi^*$ transition in the aromatic amino acid residues), there appeared to be an "absorptive shoulder" or small-amplitude band at 320 mμ. The latter band is not generally characteristic of proteins. The presence of this band proved to be reproducible.

Optical Rotatory Dispersion

Comparison of the optical rotatory dispersion curves for the irradiated and the dark-control enzyme solutions (see Fig. 10.) disclosed very striking differences. The wavelength of the Cotton effect inversion point for the irradiated sample (λ_o^*) is greater than that of the dark-control (λ_o). Since the amplitude of the Cotton effect for the irradiated sample is greater than that of the dark-control, it follows that b_o^* is greater than b_o (where b_o is a helix constant defined in Chapter IV and is directly proportional to Cotton effect amplitude). The two rotatory dispersion

curves are similar in that no Cotton effect is associated with the 320 mμ. spectral region.

pH Dependency of Absorption

The methods used in investigating the pH dependency of the 280 and 320 mμ. absorption bands proved satisfactory (see Fig. 11). The 280 mμ. band was, as expected, found to be variable with respect to changing hydrogen-ion concentrations. A shift toward shorter wavelengths of the absorption band was noted in more basic conditions. This shift in the 280 mμ. band is due to the dissociation of the phenolic group of tyrosine to the corresponding phenolate ion (11). No changes were evident in the absorption occurring at 320 mμ. throughout the pH ranges investigated (2-9).

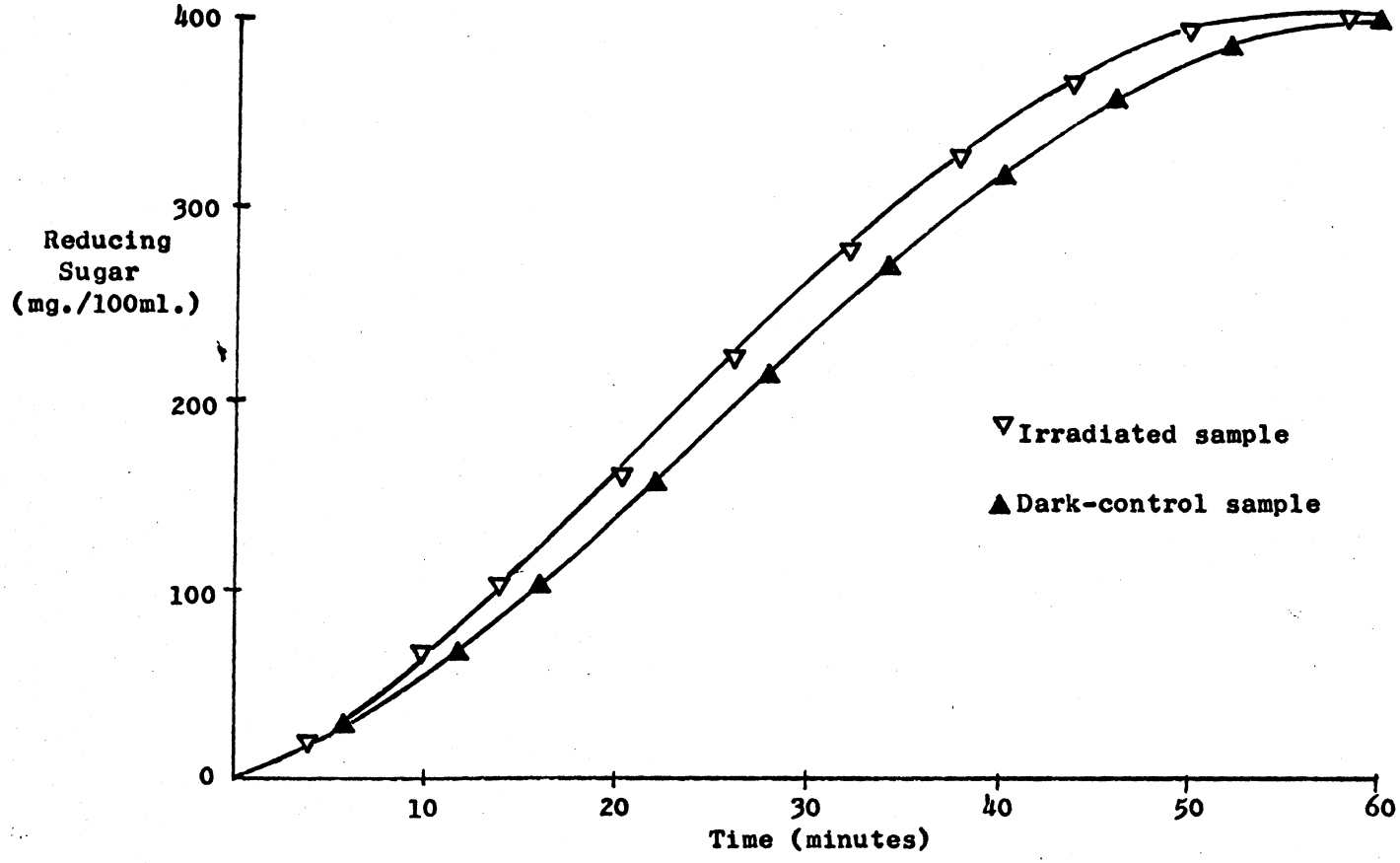

Fig. 3.--Graph showing the concentration of reducing sugar formed as a function of time for the system-illumination with heterogenous radiation.

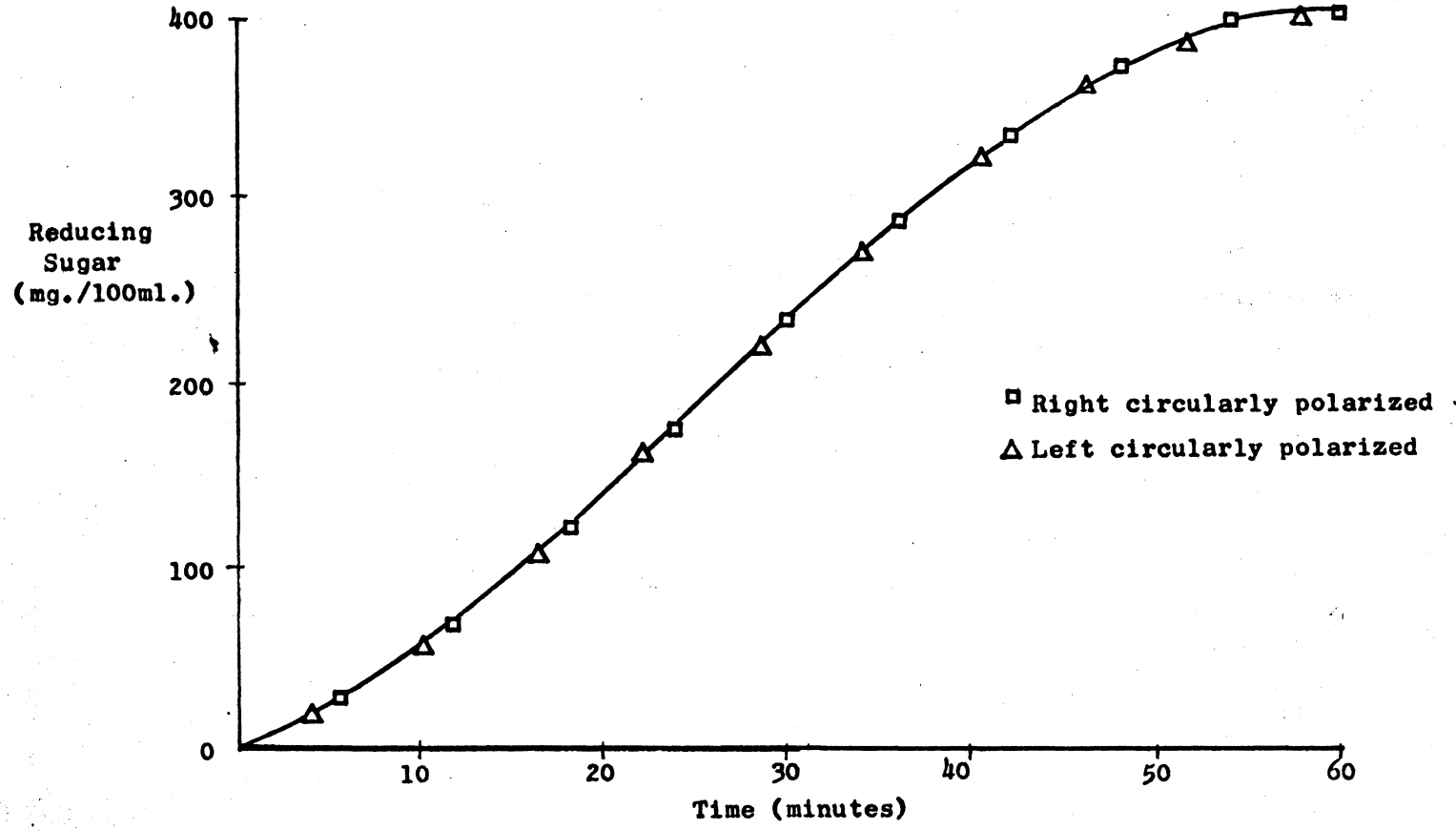

Fig. 4.--Graph showing the concentration of reducing sugar formed as a function of time for the hydrolyses irradiated with right and left circularly-polarized radiation.

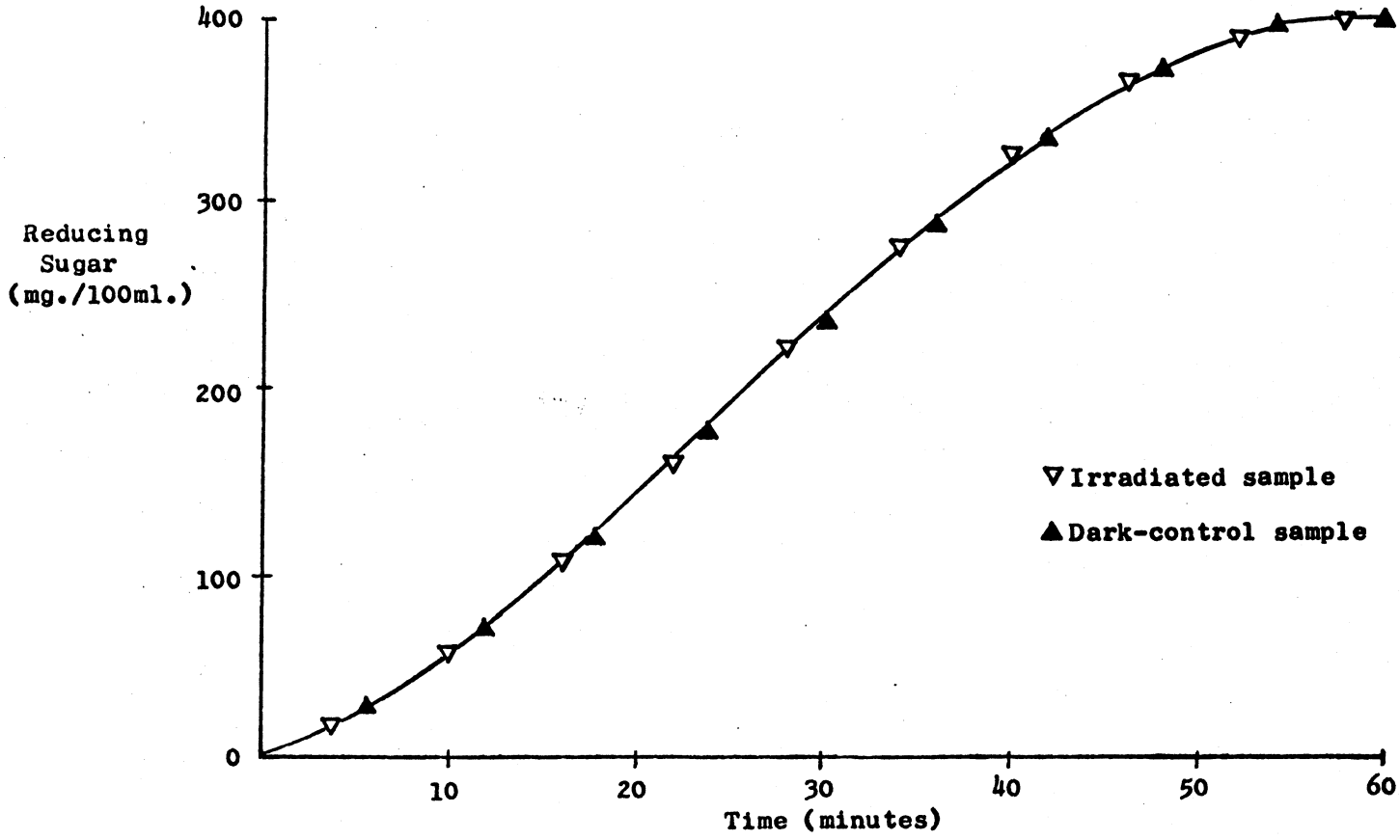

Fig. 5.--Graph showing the concentration of reducing sugar formed as a function of time for the heterogenous irradiation of the substrate solution.

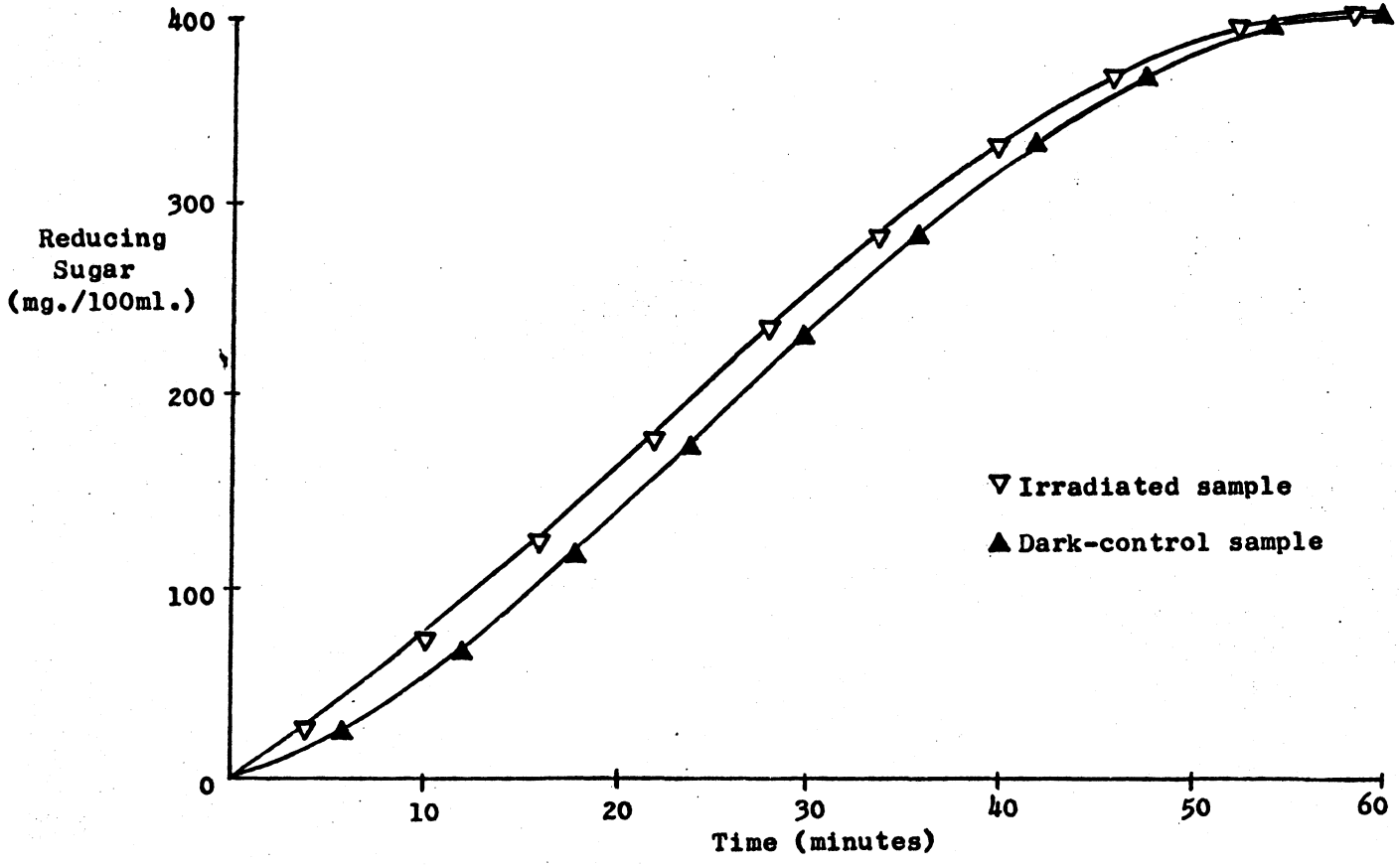

Fig. 6.--Graph showing the concentration of reducing sugar formed as a function of time for the heterogenous irradiation of the enzyme solution.

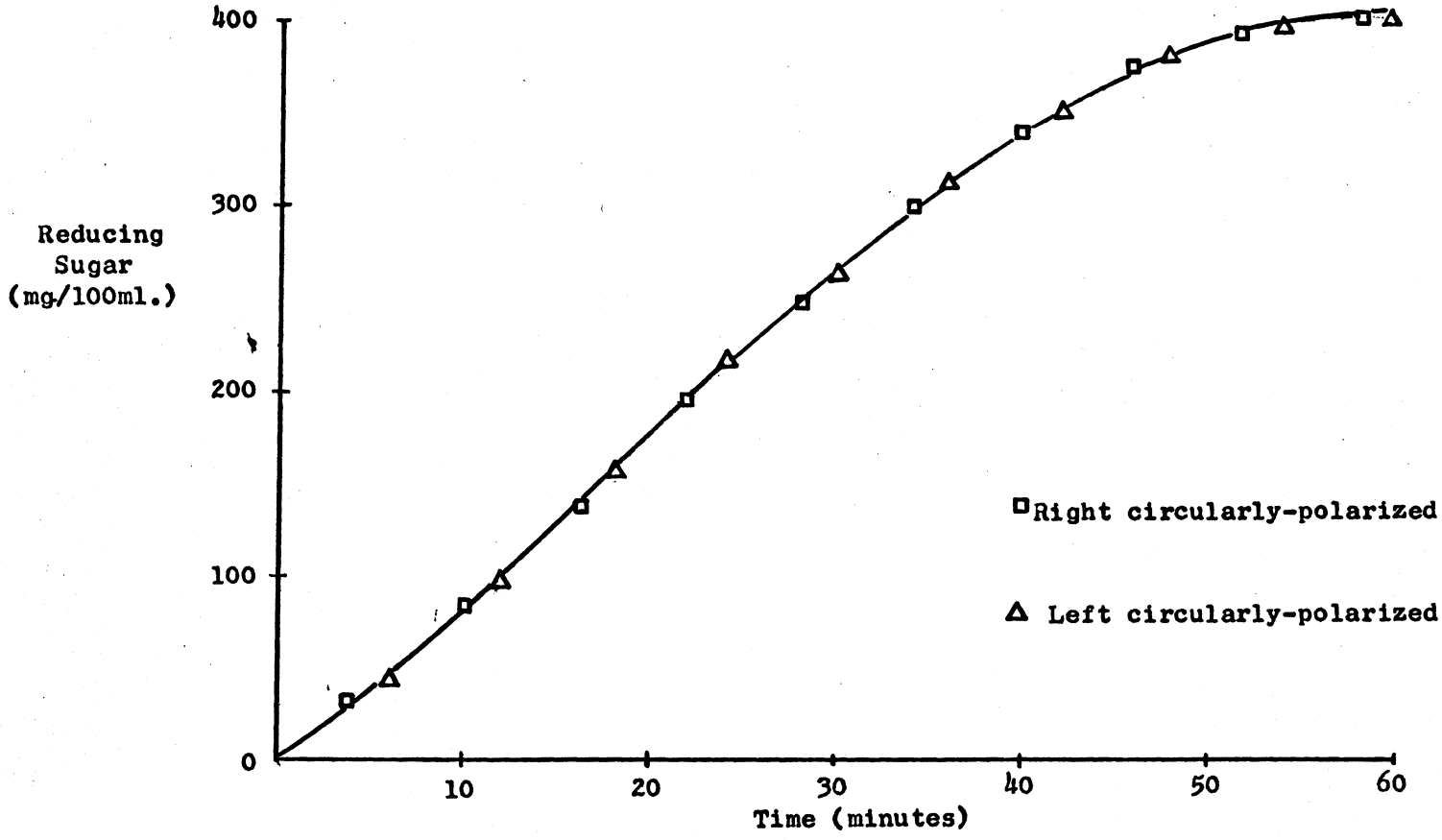

Fig. 7.--Graph showing the concentration of reducing sugar formed as a function of time for enzyme solutions irradiated with right and left circularly-polarized light.

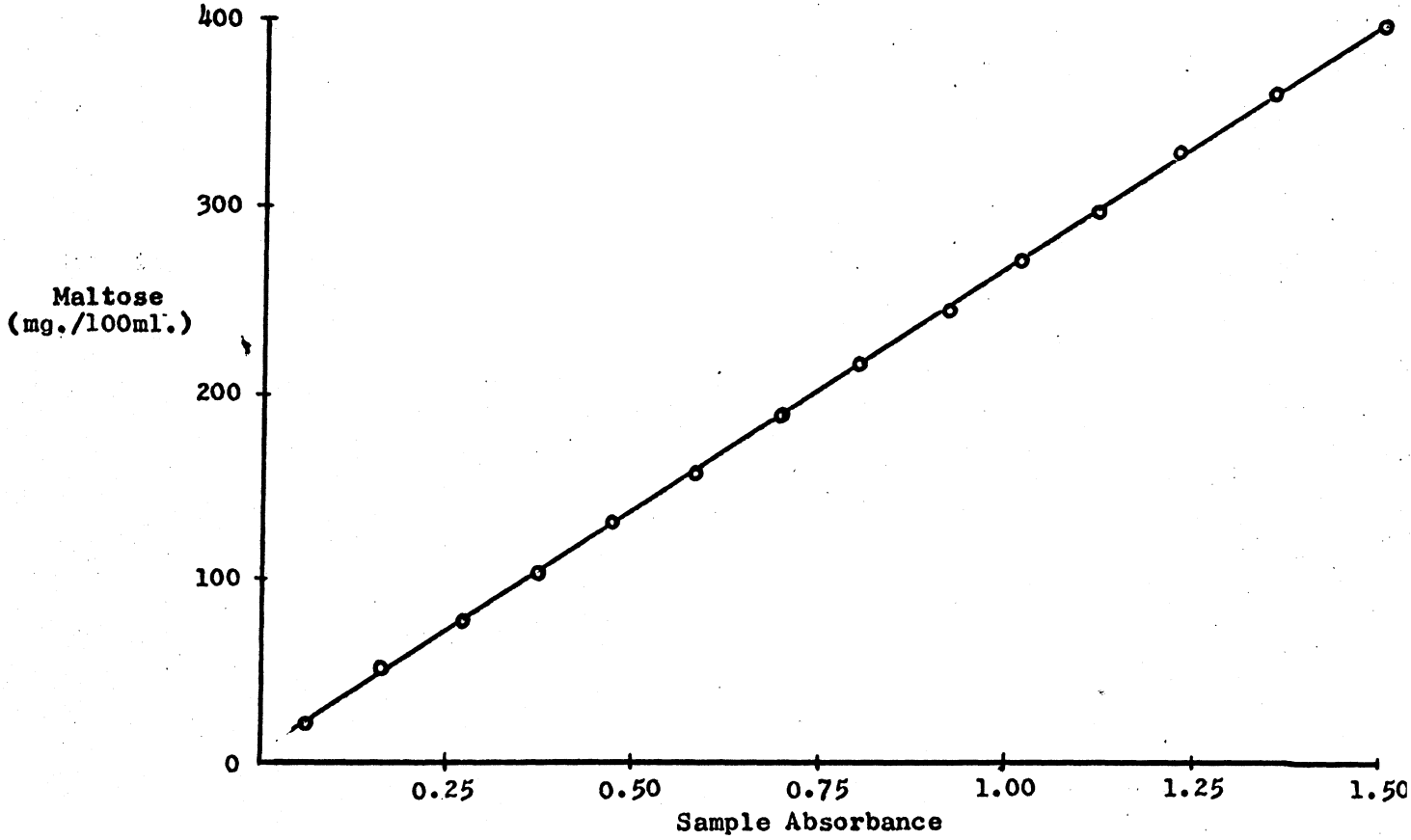

Fig. 8.--Graph showing sample absorbance as a function of maltose concentration

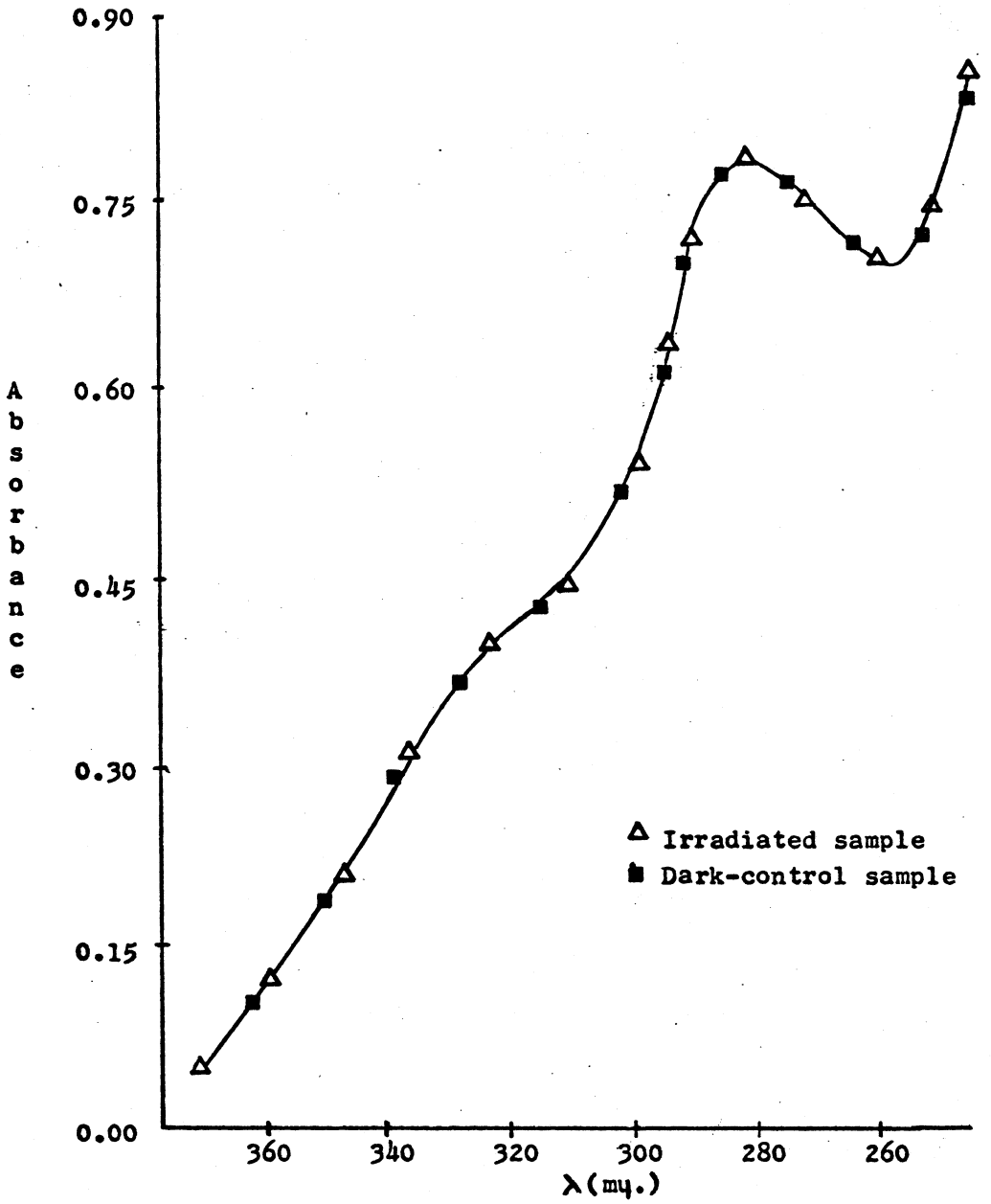

Fig. 9.--Graph showing the ultra-violet absorption spectra of the irradiated and dark-control enzymes.

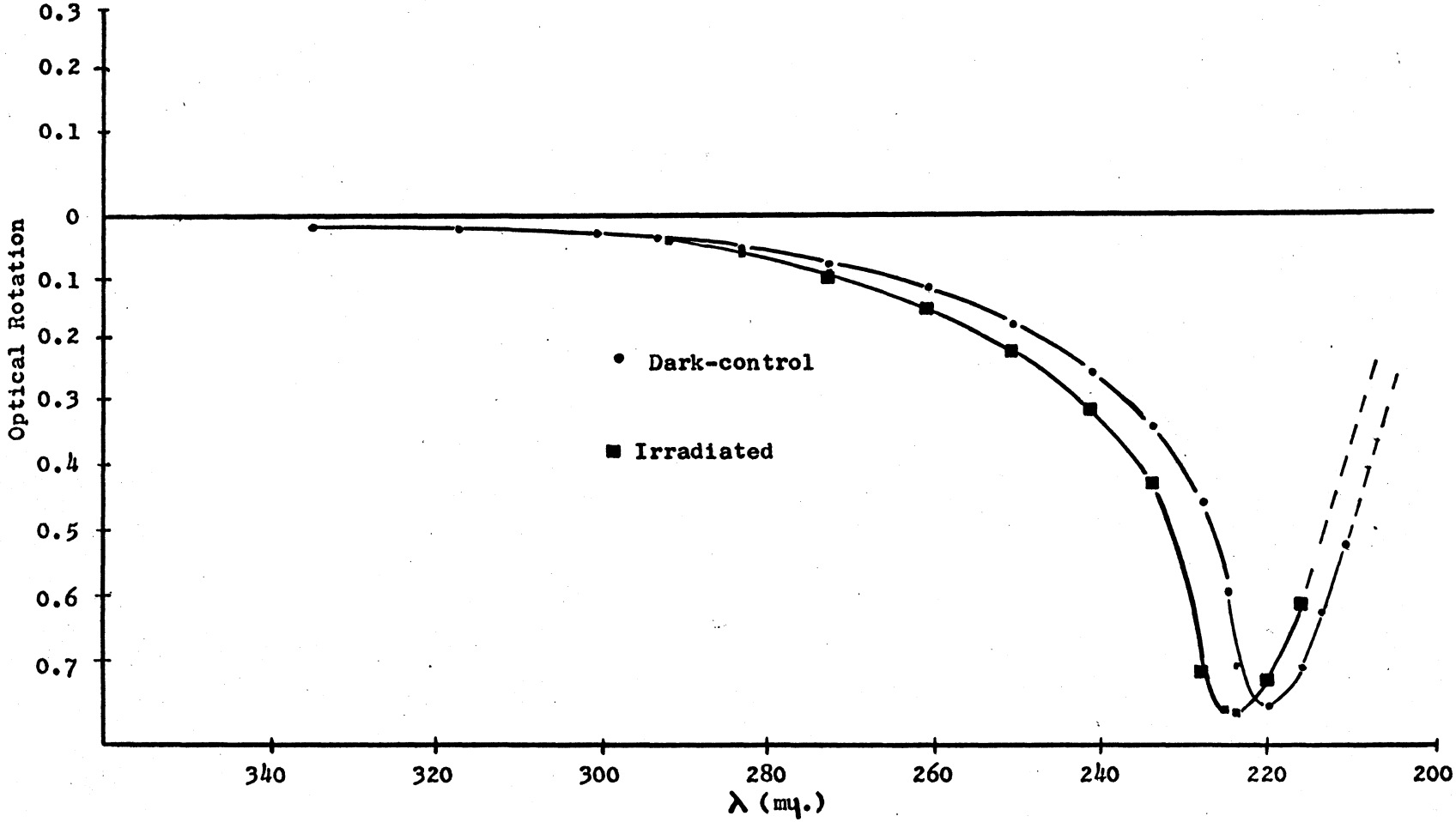

Fig. 10.--Graph showing the optical rotatory dispersion curves of irradiated and dark-control enzyme samples.

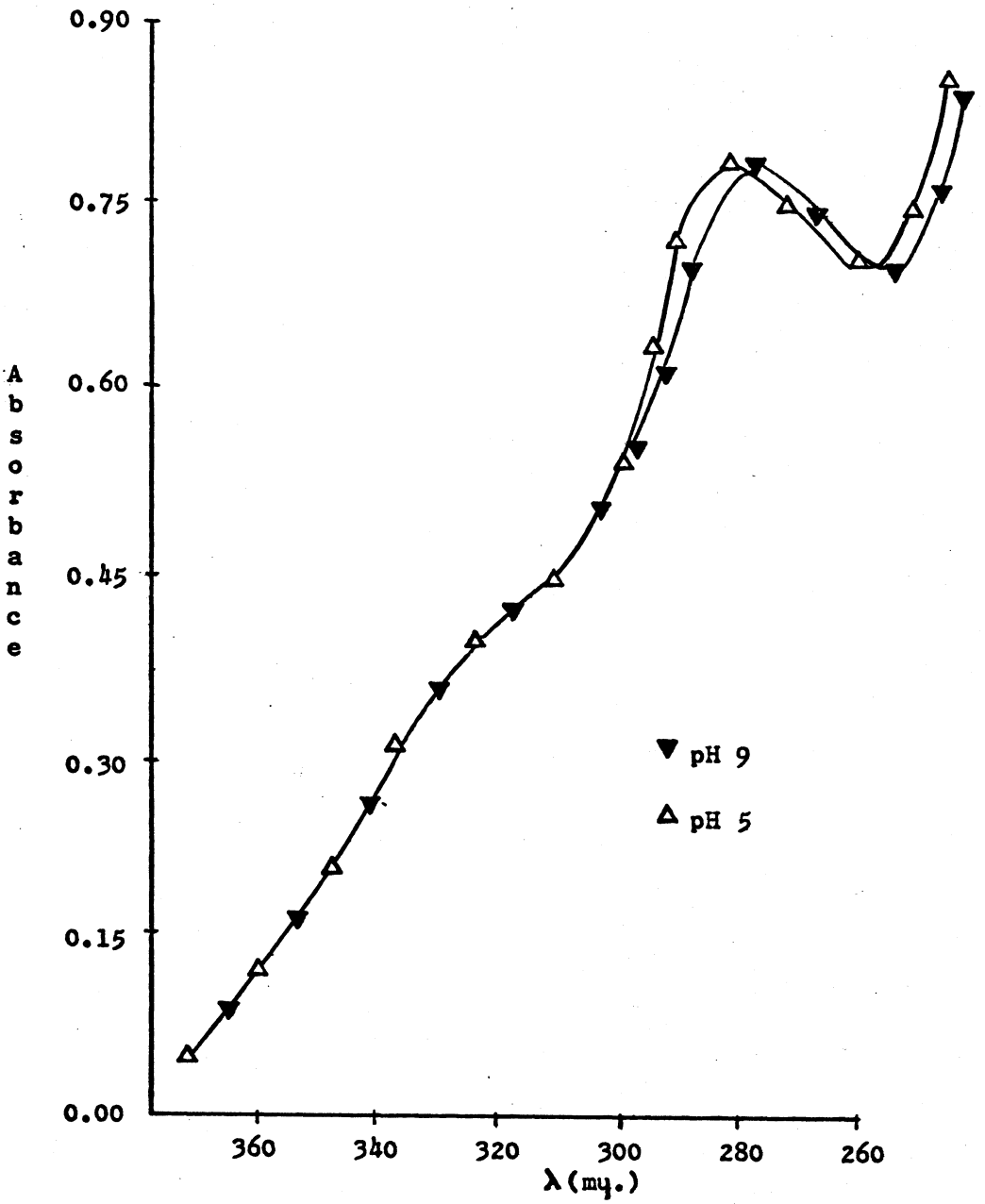

Fig. 11.--Graph showing enzyme absorption spectra under conditions of varying hydrogen-ion concentration.

CHAPTER IV

DISCUSSION

The results obtained from the hydrolytic studies involving system-illumination (p. 14) and enzyme-illumination (p. 15) show clearly that irradiation results in increased hydrolytic activity (i.e., the slope of the irradiated reaction is always greater than or equal to the slope of the dark-control). The fact that the difference in the rates between the dark-control and both types of illuminated samples (i.e., system and enzyme illumination samples) is nearly equal implicates the enzyme as being responsible for the photochemical activity. The substrate-illumination (p. 14) shows no slope difference between irradiated and dark-control samples. This further corroborates the evidence that the enzyme is the functional component of the system during photolysis.

Enzymes

Enzymes are protein materials whose chemical structure is, in principle, reasonably simple. They consist of long chains of amino acid residues linked to each other by secondary amide or peptide bonds. Structural complications arise, however, (1) from the nearly infinite number of linear sequences obtainable from the many different naturally occurring amino acids, (2) from the great length of these chains, which may consist of several hundred amino acids, and (3) from the specific folding of these chains (11).

The specificity of this folding is related to many types of intramolecular association, including hydrogen-bonds, disulfide bridges, dipole induction of polar side chains, and short-range van der Waals forces between non-polar side chains. This folding results in a three-dimensional structural pattern known as protein "conformation."

Protein Conformation

In a series of brilliant theoretical considerations on protein conformation, Pauling, et al (12) predicted and subsequently confirmed, on the basis of X-ray analyses, that certain proteins contain a helix with a nonintegral number of amino acid residues per turn. This helix has been designated as the α-helix.

The conformation of a protein is of great importance for its chemical and physical properties as well as for its biological functions (13). If the natural conformation is destroyed or modified by physical or chemical operations, many of the chemical or physicochemical properties are changed, and the biological functions of the original native protein are altered or lost. In this state the protein is said to be denatured. The general conformational change associated with denaturation of enzymes containing α-helices is the unfolding of the helix (11).

Theory of Optical Activity and Rotatory Dispersion

Optical activity (the rotation of the plane of linearly-polarized light; 14) is classically accounted for as the result of an interaction of linearly polarized electromagnetic radiation with a material possessing an asymmetric orientation of bonding or

chromophore electrons.

Linearly polarized light may be considered the vector sum of two components: a right circularly-polarized component and a left circularly-polarized component (15). This concept of linear-polarization can be more fully understood by considering the electric vector E of a linearly polarized beam (observed along the axis of propagation) as being the vector resultant of the electric vectors of the left and right circularly-polarized components (E_l and E_r). This concept has been experimentally demonstrated (16). It is important to realize that while linearly-polarized radiation is itself symmetric, its two circularly-polarized components are dissymmetric (i.e., they are not superimposable on their mirror images).

Any material which is optically active must also be circularly birefringent (i.e., the material possesses different indices of refraction for the left and right circularly-polarized components). Thus, if linearly-polarized light propagates through such a medium, one of its components will have a different velocity than the other. After passing through the material the beams will recombine with a phase difference and the plane of polarization will be rotated (it should be noted that the observer sees only the resultant of the components).

Optically active materials are associated with the lack of a center or plane of symmetry. A qualitative explanation as to why such materials exhibit optical activity may be achieved by realizing that although the two dissymmetric components of the linearly-polarized light will interact (electromagnetic induction) equally

with a system of symmetrical electron oscillators, they will not interact equally with a system of asymmetric electron oscillators. Any chemical configuration or conformation which lacks a center or plane of symmetry will possess such a system of electrons.

The magnitude of optical activity is frequency dependent, giving rise to rotatory dispersion. Every molecule has certain natural frequencies in accord with the Planck statement $\Delta E = h\nu$. Thus as the frequency of a propagating wave approaches a resonance condition with the natural frequencies of a molecule, the magnitude of absorption, indices of refraction, optical rotation, and other physical parameters become more pronounced.

The dependence of rotatory power on wavelength was first expressed empirically by Drude (17):

$$[\alpha] = \sum_{i=1}^{n} \frac{K_i}{\lambda^2 - \lambda_i^2}$$

where $[\alpha]$ is the magnitude of rotation, K_i is an empirical constant associated with an absorption band at λ_i. It is important to note the relationship between $[\alpha]$ and the natural frequencies of the molecule and the anomaly predicted when $\lambda \longrightarrow \lambda_i$ (see Fig. 12.).

In Fig. 12 the optical rotation increases to a very high positive or negative value as the wavelength of maximal absorption is approached. The rotation then changes sign (i.e., undergoes an inversion) when the wavelength of the absorption band is passed. This type of phenomenon is known as an anomalous Cotton effect (18).

The wavelength at which the inversion occurs is λ_i. The vertical distance between the peak (P) and the trough (T) of the Cotton effect is known as the amplitude (a) of the anomaly.

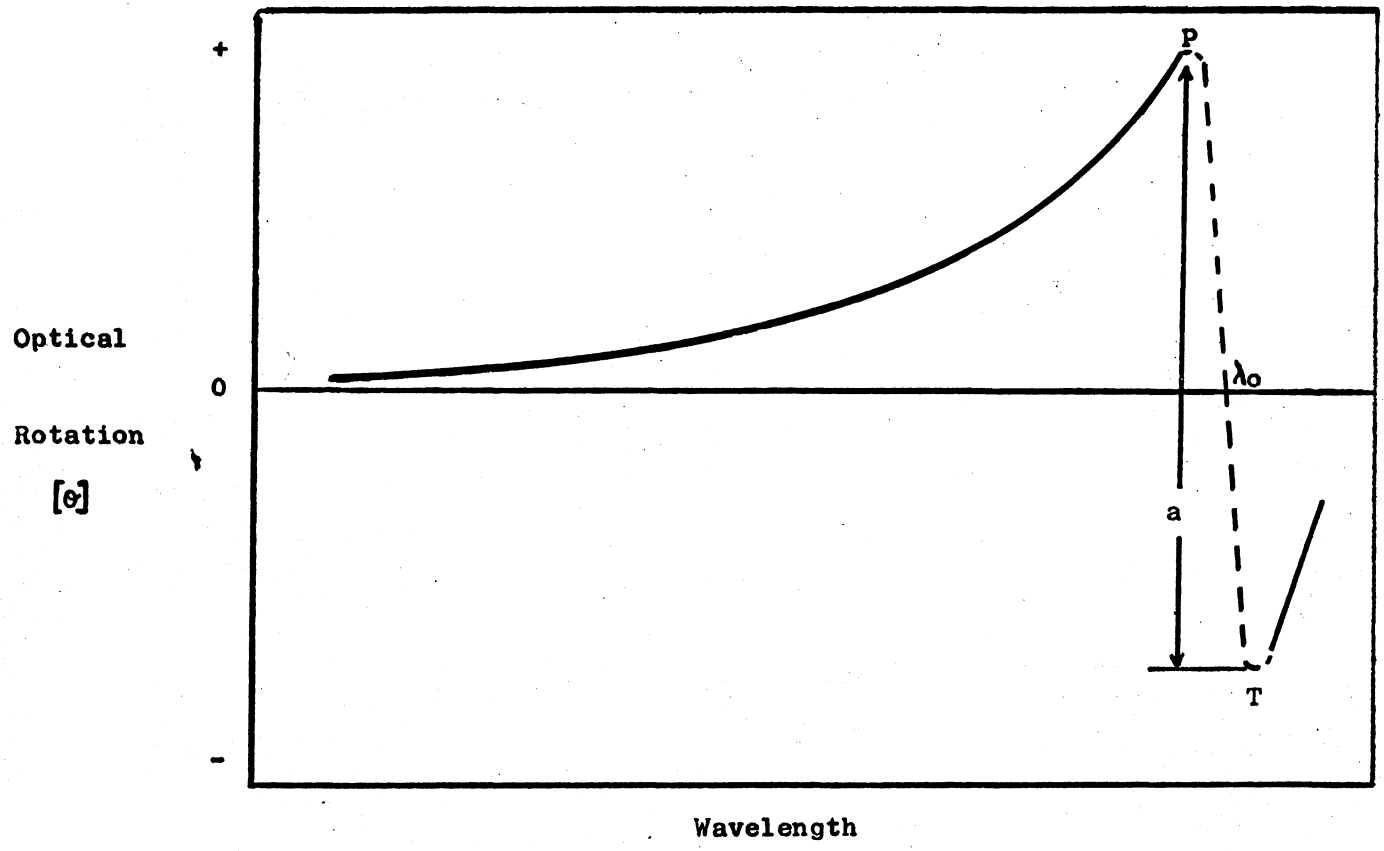

Fig. 12.--Graph of a sample optical rotatory dispersion curve (anomalous). Shown in the graph are the amplitude of the Cotton effect (a), the peak (P), the trough (T), and the wavelength of inversion (λ_o)

Optical Activity and Rotatory Dispersion of Proteins

The amino acids (except for glycine) are all optically active by virtue of their asymmetric carbon atom. In addition, certain amino acids (e.g., threonine) have another asymmetric carbon atom in their side chains. Thus peptides (consisting of amino acids) would be expected to be optically active in virtue of their configuration (i.e., multiple asymmetric carbon atoms). One might, at first glance, expect that the optical activity of a peptide is solely a function of the number of amino acid residues, and hence an additive property described by a one-term Drude equation. Brand, et al (19) found this kind of relationship to be true for molecules having two to six peptide units. However, Goodman (20) has found that as the number of residues in a synthetic peptide becomes larger than five, the rotatory power is no longer simply an additive property and does not obey a single-term Drude equation. It is important to realize that this deviation occurs as the result of a new Cotton effect observed in the region of the peptide bond absorption. From our theoretical considerations, this can only mean that as the number of residues surpasses five, the conformation (and consequently the geometry of electron transitions of the peptide bonds) becomes dissymmetric. It follows then that the rotatory power of a protein is not only an additive property, but also a conformationally dependent property.

The optical rotatory dispersion of proteins may be described by the Moffitt equation (21):

$$[M']_\lambda = \frac{a_o \lambda_o^2}{\lambda^2 - \lambda_o^2} + \frac{b_o \lambda_o^4}{(\lambda^2 - \lambda_o^2)^2}$$

where $[M']_\lambda$ is the effective rotation, λ is the wavelength of the measurement, a_o is a constant characteristic of the helix and residue contribution, and λ_o and b_o are constants characteristic of the helix. The constants relating to the helix (λ_o, b_o) are particularly important to this discussion.

The λ_o constant (a special case of the λ_i described earlier) may be obtained directly or estimated from the experimentally measured Cotton effect.

The b_o constant is an indirect measure of helical content (11, 15). It can be estimated from a Cotton effect since it is directly proportional to amplitude.

In summary, the optical rotatory dispersion of proteins provides not only a tool for detecting a conformational change, but also for describing the qualitative nature of a conformational change.

The Experimental ORD Data

A comparison of the optical rotatory dispersion curves of the irradiated and dark-control enzymes (p. 25) discloses striking differences. The wavelength of inversion (λ_o) for both curves may be estimated by extrapolating the dotted area of the curves to the point of zero-rotation. The wavelength of inversion for the irradiated sample (λ_o^*) has undergone a shift toward a longer wavelength than that of the dark-control sample (λ_o). The amplitudes of the respective Cotton effects should also be noted. Since

the amplitude of the irradiated sample's Cotton effect is larger than that of the dark-control, the b_0 constant for the irradiated enzyme (b_0^*) is larger than that of the dark-control enzyme (b_0). Both of these phenomena are indicative of higher helical content (11, 15, 22). Since denaturation of Taka-diastase, resulting in the loss of enzymatic activity, is accompanied by a reduction in helical content, it is reasonable to conclude that higher helical content will be reflected in increased enzymatic activity. The system-illumination (p. 14) and the enzyme illumination (p. 15) both result in increased enzymatic activity. It would seem that the change in conformation that occurs in system-illumination is a time-dependent process (i.e., the sigmoidal character of the curves may correspond to a "threshold time"). The irradiated sample curve obtained from enzyme-illumination (p. 21) displays a reduced sigmoidal effect. Apparently the influence of light has resulted in a change of the mean conformation of the enzyme that is completed during hydrolysis in the system-illumination studies and that is essentially complete before hydrolysis in the enzyme-illumination studies.

A Postulated Mechanism

The enzyme absorption spectra (p. 24) discloses two regions in which absorption occurs. The 280 mμ. band is characteristic of proteins and is due to the $\pi \longrightarrow \pi^*$ transition in the aromatic groups of tyrosine, tryptophan, and phenylalanine (23). The 320 mμ. band is not generally characteristic of proteins and undergoes no detectable change subsequent to irradiation.

By virtue of the fact that the 280 mμ. band was inaccessible in the radiation experiments (the lower limit of radiation generated was 305 mμ.) the 320 mμ. band would seem responsible for the photochemical activity.

The enzyme, prior to modification (p. 7), existed in aqueous solution in the form of hydrated molecules. The addition of alcohol to the system sets up a competitive hydration process, allowing the protein molecules to intermolecularly associate--and the protein precipitates. It is possible that one such form of association could stem from the complexing of the electron-rich π-clouds of an aromatic amino acid residue with an electron deficient site. Such a charge-transfer complex (24) would delocalise electrons and stabilize the activated state, π^*_c (25). The stabilization of the activated state is shown in Fig. 13.

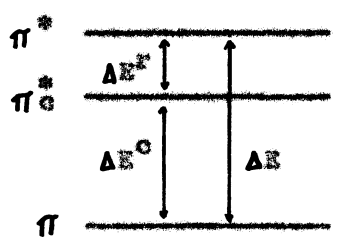

Fig. 13.--Showing energy levels of the ground state (π), excited state (π^*), and the excited state complex (π^*_c).

An absorption shift of the aromatic band at 280 mu. to longer wavelengths for such complexes would then be expected because of this stabilization (i.e., the ΔE of Fig. 13 is reduced by the stability of the excited state, ΔE^r; further, since $\Delta E = hc/\lambda$, a reduction in ΔE must be accompanied by an increase in λ). Thus a charge-transfer complex might account for the 320 mμ. absorption.

Upon re-dissolving the precipitated protein in water, most of these associations are disrupted (i.e., the protein becomes soluble). However, it is possible that some of these charge-transfer complexes are not disrupted. The resulting molecules could then exist in dimeric or polymeric forms. The mean conformation of the protein population could then be designated as C_n and the ratio of complexed sites to non-complexed sites could be given by $\left[\frac{A_{320}}{A_{280}}\right]_n$ (where A is the absorbance of the respective bands). These dimeric or polymeric forms would then constitute a structural restriction. They could prevent the protein molecules from attaining their most thermodynamically stable conformation.

If these complexes exist in solution, irradiation could result in a $\pi \longrightarrow \pi^*$ (i.e., a bonding to an anti-bonding) transition. In this manner irradiation could provide an excitation-time during which the molecules could assume their most stable conformation (C^*, where * signifies an irradiated population) by changing complex sites. Pauling (12) has shown that high helical content contributes to conformational stability. Denaturation, associated with low helical content, is accompanied by a reduction of enzymatic activity (26). It is reasonable then that high helical content could result in increased enzymatic activity (pp. 14, 15). In all cases of system and enzyme-illumination hydrolytic activities were increased by the influence of light.

The ratios of complexed sites to non-complexed sites before and after irradiation should remain reasonably constant (i.e., the

ratio of $\left[\frac{A_{320}}{A_{280}}\right]_n$ should very nearly equal $\left[\frac{A_{320}}{A_{280}}\right]^*_n$) since the mechanism postulates that only the sites of π-complexing change with irradiation.

It is possible to consider the hypothecated mechanism from the standpoint of a conceptual energy diagram (see Fig. 14.).

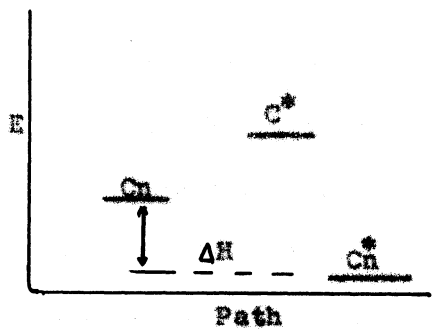

Fig. 14.--Showing conceptual energy diagram for the postulated mechanism.

The relative energy level of the C_n conformational state is shown on the extreme left. The energy required for the attainment of the activated state is that which could be provided by irradiation. The postulation that irradiation enables the enzyme to attain a more stable conformation is indicated in that the energy level for this conformation (C^*_n) is lower than that of the native (i.e., initial) conformation by ΔH. The hypothecated mechanism would then be exothermic. It is well-established that denaturation (i.e., the lowering of helical content) is an endothermic process (27, 28).

A summary of the hypothecated mechanism is diagrammatically presented in Fig. 15.

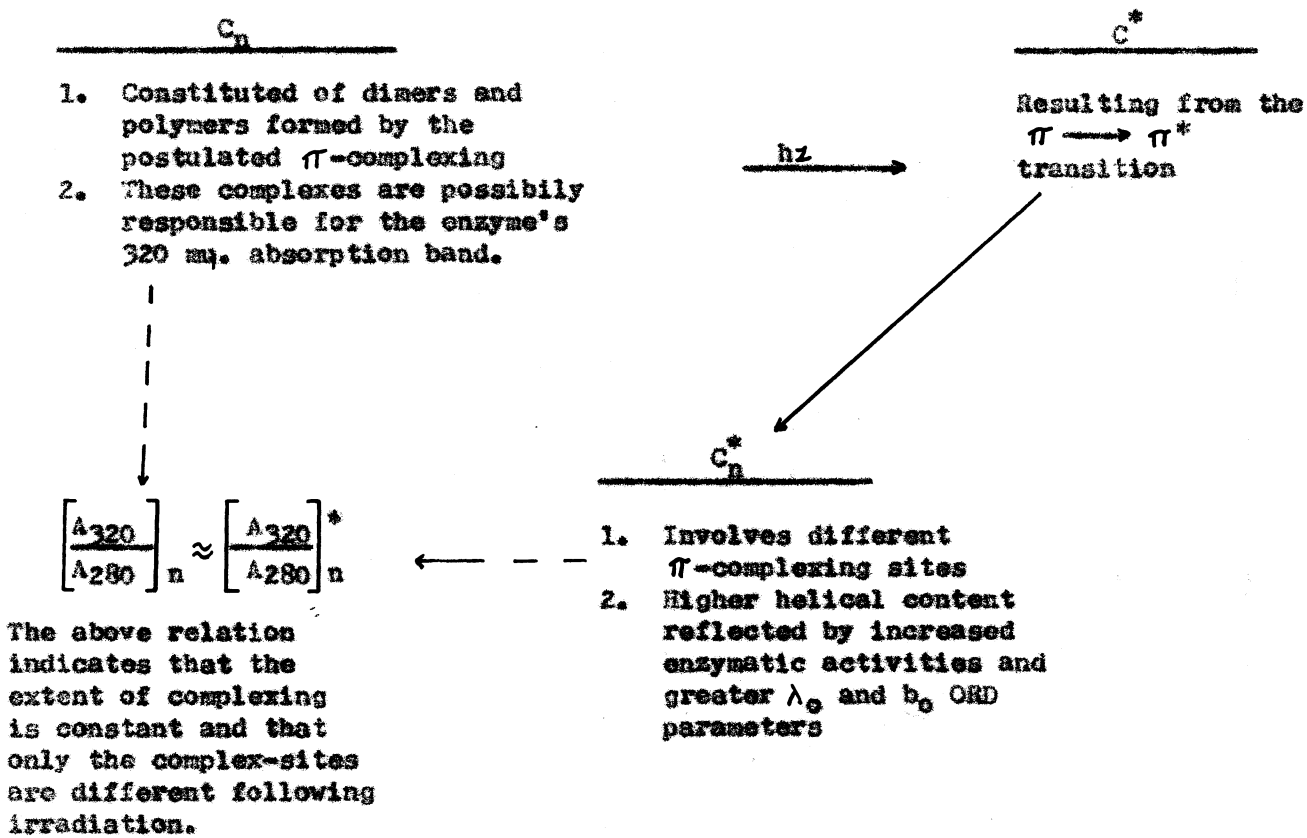

Fig. 15.—A diagrammatic presentation of the postulated photochemical mechanism in Taka-diastase.

The "threshold time" (pp. 14, 18, and 34) associated with the system-illumination can be interpreted, in terms of this mechanism, as being the length of time required for attaining the most stable structure (i.e., the highest helical content) which is reflected in increased enzymatic activity.

If the mechanism involves a charge-transfer complex, the phenomenon is apparently non-dissymmetric. The optical rotatory dispersion curves for the irradiated and dark-control samples (p. 25) show that no Cotton effect is associated with the 320 mμ. band.

Further, photolysis would only enhance attainment of the most stable enzyme conformation. It would seem possible for the enzyme to attain this conformation, over a long period of time, while being kept entirely in the dark.

It is interesting to note, once again, that Navez and Rubenstein (8) reported increased hydrolytic activity upon the irradiation of diastase of malt. Apparently this phenomenon is not restricted to the α-amylase currently being investigated.

The following experimental problems are indicated in extending investigation of this phenomenon:

(1) The investigation of the ultra-violet absorption spectra of malt diastase will disclose whether or not the 320 mμ. band is present in this enzyme.

(2) Dissymmetric irradiation of the Taka-diastase enzyme with electromagnetic radiation whose spectral region includes the optically active 200 mμ. band will disclose the effects of dissymmetric radiation upon a dissymmetric system.

(3) Spectrophotometric experiments should be designed to test the hypothecated mechanism.

(4) Rotatory dispersion studies should be used to determine whether or not the conformational changes brought about by irradiation are reversible.

CONCLUSIONS

(1) Taka-diastase and soluble starch constitute a photochemically active system.

(2) A small-amplitude absorption band existing in the enzyme solution at 320 mμ. is responsible for the phenomenon.

(3) The 320 mμ. absorption band does not involve circular-dichroism (i.e., no Cotton effect is present in the optical rotatory dispersion) and dissymmetric photolysis is not possible in this spectral region.

(4) The absorption at 320 mμ. may be associated with a charge-transfer complex that allows the enzyme to attain higher helical content. This conformational change might account for the increased enzymatic activity observed when the system is irradiated.

LITERATURE CITED

1. E. C. C. Baly, and E. S. Semmens, Proc. Roy. Soc. London, Series B, 25, 250 (1924).

2. E. C. C. Baly, and E. S. Semmens, Nature, 116, 817 (1925).

3. E. S. Semmens, Nature, 111, 49 (1923).

4. E. S. Semmens, J. Soc. Chem. Ind., 42, 76 (1923).

5. D. I. Macht, Proc. Soc. Exp. Biol. and Med., 22, 471 (1925).

6. W. N. Jones, Ann. Bot., 39, 651 (1925).

7. J. W. M. Bunker and E. G. E. Anderson, J. Biol. Chem., 77, 473 (1928).

8. A. E. Navez and B. B. Rubenstein, J. Biol. Chem., 80, 503 (1928).

9. H. C. Sherman, A. W. Thomas, and M. E. Baldwin, J. Am. Chem. Soc., 41, 231 (1919).

10. O. Folin, J. Biol. Chem., 82, 83 (1929).

11. F. Haurowitz, "The Chemistry and Function of Proteins," 2nd. ed., Academic Press, Inc., New York and London, 1963.

12. L. Pauling, R. B. Corey, and H. R. Branson, Proc. Natl. Acad. Sci., U. S., 37, 205 (1951).

13. B. Harrow and A. Mazur, "Textbook of Biochemistry," 9th ed., W. B. Saunders Co., Philadelphia and London, 1966.

14. W. A. Shurcliff, "Polarized Light," Harvard University Press, Cambridge, 1962.

15. C. Djerassi, "Optical Rotatory Dispersion," McGraw-Hill Book Co., Inc., New York, 1960.

16. R. W. Wood, "Physical Optics," 3rd. ed., Macmillan Co., New York, 1934.

17. P. Drude, "Lehrbuch der Optik," 2nd. ed., Hirzel Verlag, Leipzig, 1906.

18. S. Mitchell, "The Cotton Effect," G. Bell and Sons, Ltd., London, 1933.

19. E. Brand and B. J. Erlanger, J. Am. Chem. Soc., 73, 3508, 4025, 4027 (1951)

20. M. Goodman, E. Schmitt, and D. Yphantis, J. Am. Chem. Soc., 84, 1283-96 (1962).

21. W. Moffitt, J. Chem. Phys., 25, 467 (1956).

22. E. H. Fischer, W. N. Summerwill, J. Junge, and E. A. Stein, International Congr. Biochem. Vienna, 8, 124 (1958).

23. P. Doty and E. P. Gaiduscheck, in "Proteins," (H. Neurath and K. Bailey eds.), Vol. 1A, 433, Academic Press, Inc., New York, 1953.

24. S. Weinstein and H. J. Lucas, J. Am. Chem. Soc., 60, 836 (1938).

25. G. E. Wheland, "Resonance in Organic Chemistry," John Wiley and Sons, New York, 1955.

26. A. Oikawa and A. Maeda, J. Biochem. Tokyo, 44, 745 (1958).

27. J. M. Sturtevant, J. Am. Chem. Soc., 74, 1983 (1952).

28. G. Kistiakowsky, J. Am. Chem. Soc., 62, 1895 (1940).