kylation of lawsone with the ethereal solution of peroxide was carried out in warm acetic acid solution.[27] The quinone was obtained as brilliant yellow needles (41% yield) from dilute methanol, m. p. 128–129°.

2-γ-Bromopropyl-3-bromo-1,4-naphthoquinone (V).—The quinone IV (1 g.) was dissolved in 5 cc. of warm glacial acetic acid. Benzoyl peroxide (*ca.* 10 mg.) was added and then gaseous hydrogen bromide was bubbled into the solution for one minute. During this addition the solution became warm. After cooling, the flask was stoppered and allowed to remain at room temperature overnight. The solution was cooled in an ice-bath and 25 cc. of 48% hydrobromic acid was added. The dark black precipitate that formed was filtered and washed with water. Recrystallization of this material (0.35 g.) from ligroin (b. p. 90–100°) and a small amount of benzene (Norit) gave 0.25 g. of beautiful yellow needles, m. p. 130–131°. This compound gives a pale orange color with alcoholic alkali that slowly changes to deep red on boiling for one minute.

Anal.[29] Calcd. for $C_{13}H_{10}O_2Br_2$: C, 43.61; H, 2.82. Found: C, 43.70; H, 2.96.

2-(10′-Phenylmercaptodecyl)-3-hydroxy-1,4-naphthoquinone (II, $n = 10$, X = C_6H_5). **Procedure C.**—A solution of 2.3 g. (0.0059 mole) of II ($n = 10$, X = Br), 0.98 g. (0.0089 mole) of thiophenol (Eastman Kodak Co. White Label) and 0.66 g. (0.0165 mole) of sodium hydroxide in 25 cc. of 95% methanol was refluxed under nitrogen for four hours. The red solution was acidified with concentrated hydrochloric acid, and the yellow precipitate was extracted with ether. The ethereal solution was dried over "Drierite," and the ether was removed in an air blast. The yellow residue was taken up in 100 cc. of hot methanol. On cooling, 1.9 g. (76%) of beautiful yellow prisms, m. p. 80–81°, crystallized.

2,3-(5,6-Dihydropyrano-2′,3′)-1,4-naphthoquinone (VII).—In an attempt to prepare a thioether from the quinone II ($n = 3$, X = Cl), 1 g. (0.004 mole) of the quinone, 0.75 g. of *p*-chlorothiophenol and 0.35 g. of sodium hydroxide in 50 cc. of 75% ethanol solution was refluxed in a nitrogen atmosphere for three hours. Upon acidification there was obtained an oil that was taken up in ether, the ether evaporated, and the residue dissolved in hot ligroin (b. p. 90–120°). On cooling, 350 mg. of yellow needles, m. p. 216–218° was obtained. Recrystallization from ligroin gave 280 mg. of yellow needles (31%), m. p. 220–221°. This compound is not immediately soluble in dilute alkali, but on shaking for two or three minutes the quinone does dissolve slowly to produce a red solution.

Anal. Calcd. for $C_{13}H_{10}O_3$: C, 72.89; H, 4.71. Found: C, 72.66; H, 4.83.

2-*p*-Chlorophenylmercaptomethyl-3-hydroxy-1,4-naphthoquinone (II, $n = 1$, X = C_6H_4Cl-*p*). **Procedure D.**—A mixture of 2.5 g. (0.14 mole) of lawsone and 1.9 g. of *p*-chlorothiophenol was dissolved in 50 cc. of warm dioxane; 2 drops of 36% hydrochloric acid and 4 g. of 37% formalin solution were added, and the dark solution was refluxed under nitrogen for an hour. The mixture was poured into 250 cc. of water, and the tarry precipitate that formed was taken up in ether. Evaporation of the ether left a gummy residue that was dissolved in warm methanol. On cooling 3.8 g. of brown needles, m. p. 162–168° were deposited. One recrystallization from ethanol–benzene (Norit) gave 3.0 g. (65%) of long orange needles, m. p. 172–173°. This compound gives an orange–red color in alcoholic alkali.

2-(3′-*p*-Chlorophenylmercapto)-propyl-3-hydroxy-1,4-naphthoquinone (II, $n = 3$, X = C_6H_4Cl-*p*). **Procedure E.**—A mixture of 1.6 g. (0.0075 mole) of IV and 1.2 g. of *p*-chlorothiophenol was dissolved in 30 cc. of warm glacial acetic acid. A pinch of benzoyl peroxide was added, and the yellow solution was gently heated on the steam-bath for 2.5 hours, after which time the solution was red. The solution was poured into water, and the mixture was twice extracted with 30-cc. portions of ether. The ethereal extracts were combined and the ether evaporated. The yellow residue was crystallized from ethanol, and 1.1 g. of product, m. p. 146–148°, was obtained. One recrystallization from ethanol gave 0.9 g. (33%) of beautiful yellow needles, m. p. 150–151°. This compound gives a red color when a drop of alkali is added to a warm methanolic solution.

(29) We are indebted to Mrs. M. Reese and Miss S. Katz for the microanalyses reported in this paper.

Summary

Forty 2-aryl-(alkyl)-mercaptoalkyl-3-hydroxy-1,4-naphthoquinones have been synthesized as potential antimalarial drugs. Some of these quinones showed activity in *in vitro* assay, but it is probable that these compounds have a low therapeutic index.

CAMBRIDGE 38, MASS. RECEIVED APRIL 6, 1950

[CONTRIBUTION FROM THE INSTITUTE OF POLYMER RESEARCH, POLYTECHNIC INSTITUTE OF BROOKLYN]

Photochemistry of Proteins. X. The Influence of Reagents and Conditions on the Quantum Yield for the Inactivation-denaturation of Chymotrypsin

BY A. D. MCLAREN AND PAUL FINKELSTEIN[1]

Recently quantum yields for the inactivation of enzymes, proteins and viruses have appeared in the literature.[2,3] The apparent relationship between quantum yields (Φ) and molecular weights (M) is given roughly by

$$\Phi = QM^{-2/3} \qquad (1)$$

where Q is a constant.[4] In this laboratory we have begun investigating factors which may influence the value of Φ for a given protein.[5] The quantum yield has been found to be dependent on hydrogen ion activity and wave length. As previously reported for chymotrypsin,[6] Φ is independent of intensity, the presence or absence of oxygen, and concentration in the range previously investigated (2×10^{-8} to 8×10^{-9} mole per ml; the molecular weight has been taken as 41,000). Since the quantum yield for inactivation is not dependent on the presence or absence of oxygen

(1) Fellow of the Monsanto Chemical Company, 1948–1949.

(2) Landen, THIS JOURNAL, **62**, 2465 (1940).

(3) Mandl and McLaren, *Arch. Biochem.*, **21**, 408 (1949).

(4) McLaren, *Acta Chim. Scand.*, **4**, 386 (1950).

(5) The literature up to 1949 has been summarized and reviewed by McLaren in "Advances in Enzymology," Interscience Publishers, Inc., New York, N. Y., 1949, Vol. IX, p. 75. The photochemistry of amino acids and peptides is also reviewed here.

(6) Finkelstein and McLaren, *J. Polymer Sci.*, **4**, 573 (1949). We wish to report a printer's error in this paper: the graph on p. 578 is Fig. 2 and that on p. 577 is Fig. 3; *ibid.*, **5**, 267 (1950).

we have concluded that the primary photochemical process[7] leading to the loss of enzymatic activity is not a photooxidation.[8] Subsequent protein degradation is, however, definitely different if under oxygen instead of nitrogen. The nature of these secondary reactions involves aromatic groups for the most part. Quantum yields for cleavage of the peptide bond are not markedly influenced by oxygen.[9]

In this paper we report the results of exploratory inactivation studies under conditions of variation of ionic strength, nature of the buffer, initial enzyme concentration (chymotrypsin) and temperature. Changes in the identity of the enzyme during irradiation at wave length 2537 Å. have been followed by activity determinations, absorption spectrophotometry, the ultracentrifuge and solubility in ammonium sulfate solutions. The kinetics of these changes have been calculated.

Experimental[10]

Irradiation methods and reagents have been described previously,[6,11] except for irradiation at low temperature. For the low temperature studies the mercury resonance lamp, filter cell, reaction cell and stirrer mechanism were placed in a constant temperature box maintained at 3.5 ± 0.5° and allowed to come to temperature equilibrium for one hour before irradiation. The intensity of the source was measured with a uranyl oxalate actinometer by assuming that the temperature coefficient of the actinometer was unity.[12] It was also found that the temperature dependence of light absorption by protein was negligible. (Any error made here does not influence the comparative results of Table III: lower absorption at low temperature could only mean a still higher Φ at the lower temperature; higher absorption could not reduce Φ to the value at room temperature; see below.) The *p*H of the irradiated solution (4.50) was checked after exposure to ultraviolet light; it had not changed (*M*/15 phosphate buffer). Since the *p*H chosen is near the minimum of the *p*H-Φ curve for this enzyme,[6] small changes in *p*H could not account for a significant fraction of the temperature dependence of Φ observed. After exposure the enzyme was incubated with casein at 0° and the loss in activity was determined from a calibration curve of the action of chymotrypsin on casein at 0° (prepared as described in earlier work[6] at 35.5°). The method consisted essentially in determining the optical density at 2800 Å. of the supernatant liquid from the precipitation, by trichloroacetic acid, of the enzyme–casein digestion mixture.

It was found by trial that 0.555 saturated ammonium sulfate would precipitate denatured chymotrypsin from a 0.5% protein solution in acetate buffer of ionic strength 0.05 at *p*H 5.50. At the time of the work this *p*H was thought to be the isoelectric point of chymotrypsin.[13] The isoelectric point has since been reported as 8.1–8.6 depending on ionic strength.[14] Doubtless a lower concentration of ammonium sulfate would have sufficed at the true isoelectric point, but this does not influence the validity of our results. The enzyme was irradiated for various times and samples were withdrawn. One ml. of each sample was then placed in a 40° water-bath for one hour. At this temperature and for this time heat denaturation was found to be negligible. No precipitate was observed either after the irradiation or the heating. To each sample was added 1 ml. of saturated ammonium sulfate and 82.5 mg. of solid ammonium sulfate. A precipitate was formed which was allowed to stand for one hour and then centrifuged down. The activity of the supernatant liquid was determined as was the activity before the addition of any sulfate. It was thereby found that a certain amount of active enzyme was adsorbed on the precipitate. It was not found possible to quantitatively separate active enzyme from the precipitate by washing the precipitate with acetate buffer–ammonium sulfate solution. The extent of absorption was therefore estimated from a calibration curve prepared as follows. A 0.5% enzyme solution was irradiated to a residual activity of 11.8%. Portions of this solution were then mixed with 0.5% solutions of unirradiated enzyme. The activity of each of these solutions was calculated. The solutions were heated for one hour at 40° and precipitated with ammonium sulfate as before. The activity of the supernatant solutions was compared with the previously calculated values. The difference was due to adsorption. The data were used to correct the activities of supernatant solutions after precipitation in subsequent experiments. In a separate experiment no inhibition of active enzyme by inactive enzyme was found. There is also a negligible amount of digestion of denatured enzyme by active enzyme at this *p*H (5.5)[13] as found by trial. Concentrations of native enzyme were determined by the method of Kunitz.[15]

For observations with the ultracentrifuge, 0.5 or 1% solutions of salt-free chymotrypsin (Worthington Biochemical Laboratory) in *M*/15 phosphate, *p*H 4.50, were irradiated. As irradiation proceeded a precipitate was formed which increased in quantity with increased irradiation. It first became visible after about a 10% loss in activity.[16] The irradiated solutions were centrifuged to remove the precipitate and the supernatant solution was then spun in the ultracentrifuge (air driven type with diagonal blade cylindrical lens optics) at about 165,000 times gravity.

All absorption spectra were taken with a Beckman Spectrophotometer. For studies in solution of varying ionic strength and buffer identity, crystalline salt-free enzyme was used. The *p*H did not change (4.47) in 0.1 *M* phosphate during irradiation. In 0.01 *M* phosphate the *p*H was 4.21 before irradiation and 4.30 after ten minutes exposure (*ca.* 1.6×10^{-5} einsteins per cc. per hour falling on the solution). In 0.001 *M* phosphate the *p*H increased from 4.07 to 4.25 during a ten min. exposure. It was not practical to work at lower buffer concentration because of the *p*H change during the exposure, in view of the *p*H dependence of quantum yield. To look for a specific buffer effect, acetate buffer, *p*H 5.5, ionic strength 0.05 was also used.

(7) By primary process as used here we mean the act of absorption of a quantum by a protein molecule together with the events which may happen to the energy rich molecule up to and including dissociation. Compare Noyes and Leighton, "The Photochemistry of Gases," Reinhold Publ. Corp., New York, N. Y., 1941, pp. 88 and 153.

(8) The quantum yield is also independent of the substrate, whether synthetic ester, amide, or casein (Goldenberg and McLaren, paper in preparation).

(9) Mandl and McLaren, *Nature*, **164**, 749 (1949); Mandl, Levy and McLaren, THIS JOURNAL, **72**, 1790 (1950) (the previous paper in this series).

(10) Data from the Ph.D. Thesis of P. Finkelstein, Polytechnic Institute of Brooklyn, May, 1949.

(11) Oster and McLaren, *J. Gen. Physiol.*, **33**, 215 (1950).

(12) It is only 1.03; Leighton and Forbes, THIS JOURNAL, **52**, 3139 (1930).

(13) Northrop, Kunitz and Herriott, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948.

(14) Anderson and Alberty, *J. Phys. Colloid Chem.*, **52**, 1345 (1948). By use of the amino acid analyses for chymotrypsin and chymotrypsinogen (ref. 13) and the usually accepted values for pK of the ionizing groups present, we have calculated the isoionic point (isoelectric point in the absence of salts) of this protein and obtained results in agreement with those of Anderson and Alberty and different from those of Northrop and Kunitz (*cf.* McLaren and Lewis, *J. Polymer Sci.*, **5**, 379 (1950)).

(15) Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947), p. 309.

(16) At *p*H 3.0 a 0.1% solution showed turbidity at about 85% inactivation. At *p*H 5.5 no precipitate was formed in 0.5% solutions unless ammonium sulfate was added.

Results

Inactivation of Chymotrypsin and Loss of Solubility.—The results of experiments on change of solubility during irradiations at room temperature are reported in Table I. The activities of the supernatant solutions following irradiation and precipitation with ammonium sulfate are given in column one. In the second column are reported the activities corrected for adsorption. The third column shows the activities of the same solutions before precipitation. There is fair correspondence between the last two columns indicating that loss in activity parallels loss in solubility of chymotrypsin in ammonium sulfate solutions.

TABLE I

INFLUENCE OF IRRADIATION AT 2537 Å. ON ACTIVITY AND SOLUBILITY OF CHYMOTRYPSIN

% Activity after precipitation	% Activity corrected after precipitation	% Activity before precipitation
86.6	86.7	89.5
58.5	74.8	73.5
55.5	70.4	64.7
45.2	66.5	63.5
34.5	54.0	57.9
29.4	49.8	51.2
25.2	48.5	37.6
17.1	34.6	32.5

Rate of Inactivation of Chymotrypsin.—Loss of activity during irradiation in $M/15$ phosphate buffer was studied as a function of concentration at pH 4.50 and 3.00. The results at pH 4.50 are summarized in Table II. Those at pH 3.00 were similar. The quantum yield is seen to be a constant except in the most dilute solutions.

TABLE II

QUANTUM YIELD AS A FUNCTION OF CONCENTRATION OF CHYMOTRYPSIN AT pH 4.50 AND 20–25°

E_0, moles/ml. $\times 10^8$	% Light absorption	K[a], hr.$^{-1}$	$\Phi \times 10^3$
0.0985	7.7	5.9	4.8
.197	16.7	4.2	3.2
.394	28.2	3.3	2.9
.789	47.1	2.8	2.9
1.940	81.2	2.0	3.0
12.50	~100	0.36	2.9
25.00	~100	0.19	3.0

[a] $K = \Phi I_{abs}/E_0$; Φ is taken as 3×10^{-3} and I_0 is taken as 0.16×10^{-4} einstein/ml./hr. falling on the solution.

Variation of the molarity of the phosphate buffer at constant pH, *ca.* 4.5, did not influence the value of the quantum yield. The quantum yield in acetate buffer at pH 5.5 was 3.5×10^{-3}; in phosphate buffer at the same pH it was only 2.4×10^{-3}. It is interesting to note that similar specific buffer effects are known to occur in thermal denaturation of enzymes.[17]

Results of irradiation at 3.5° are tabulated in Table III. These data indicate that irradiation at a low temperature results in higher quantum yields than irradiation at room temperature.

TABLE III

QUANTUM YIELDS AT 3.5° BEFORE AND AFTER WARMING TO ROOM TEMPERATURE AT pH 4.5

Solution	E (moles/ml.) $\times 10^8$	I_0(einsteins/ml. hr.) $\times 10^4$	$\Phi \times 10^3$
Unwarmed	1.97	0.0204	5.6
Unwarmed	1.97	.0250	5.7
Warmed	1.97	.0204	8.9
Warmed	1.97	.0250	8.9

The quantum yield is 5.7×10^{-3} at 3.5° (as measured at 3.5°) and 3.0×10^{-3} at room temperature. For a solution that is irradiated at low temperature and for which the activity is determined (at 35.5°) after the irradiated solution has been allowed to warm up for two hours, the quantum yield is still higher, namely, 8.9×10^{-3}.

Change in Sedimentation Constant Accompanying the Irradiation of Chymotrypsin.—A series of 1.0% solutions of chymotrypsin, in $M/15$ phosphate buffer at pH 4.52, were irradiated and the sedimentation constants were determined from the sedimentation rates of the major peaks of the schlieren pictures.[18] The sedimentation constants, in solvent corrected to 20°, fell exponentially from 3.31 to 2.61 svedberg units as the activity fell from 100 to 32%.

Discussion

It has been shown many times that for enzymes the rate law of inactivation is given by an equation of the form

$$K = kI_0 = (1/t) \ln (E_0/E) \qquad (2)$$

where I_0 is the incident intensity, E_0 is the initial concentration in moles/ml. and E is the concentration of active enzyme after irradiation time t.[5] With proteins, although the molar concentration cannot be high, the absorption of light on a molar basis is very high indeed and first order kinetics are observed. As has been shown elsewhere,[3,11] as well as in Table II, the rate constant K is generally inversely proportional to E_0, however.

If a reactant goes over to a product with no further change in the product, we may write[5]

$$E_0 - E = \frac{1}{\epsilon_p} (d_t - d_E) \qquad (3)$$

where ϵ_p is the molar extinction coefficient of the product, d_t is the optical density of the mixture at time t of irradiation and d_E is the density of the native enzyme in the mixture. From our data we have prepared Fig. 1. The data extrapolate to a value of 0.845 for d_p, the optical density for the initial reaction product formed from active chymotrypsin. Further photochemical changes

(17) *E. g.*, J. Steinhardt, *Kgl. Danske Videnskab. Selskab, Math.-fys. Medd.*, **14**, No. 11, 1 (1937).

(18) The authors are indebted to Mr. E. Sheppard for help in obtaining these results.

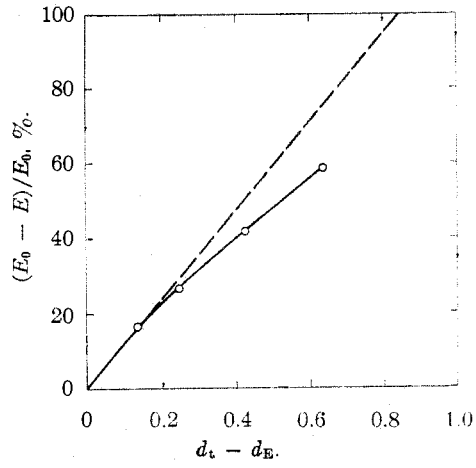

Fig. 1.—Changes in the absorption of chymotrypsin solution irradiated in air at *p*H 7.50, for an initial, active enzyme concentration (E_0) of 2.03×10^{-8} mole/ml. *cf.* equation (3).

are of course responsible for an early departure from the simple relationship, equation (3).[5] Nevertheless, equation (2) holds experimentally up to at least 80% inactivation.[6]

Summary

1. The kinetics of photochemical inactivation of chymotrypsin at 2537 Å. have been investigated. The pseudo-first order rate constant is a function of the initial enzyme concentration. The quantum yield for inactivation is virtually independent of initial enzyme concentration over a very wide range; a small change is evident at very low concentrations.

2. The quantum yield for inactivation is not dependent on phosphate buffer concentration but is different in acetate buffer.

3. Loss of activity of chymotrypsin at room temperature is parallel with loss of solubility in ammonium sulfate solution.

4. Irradiation at low temperature followed by raising the temperature of the solution to 25° gives a higher quantum yield than irradiation at 25°. Irradiation at low temperature gives a lower quantum yield for inactivation, as measured at low temperature, than is found after bringing the irradiated solution to 25°, as measured at 25°. Thus active intermediates exist at low temperature which are inactivated on warming of the solutions irradiated at low temperature.

BROOKLYN, NEW YORK RECEIVED MARCH 11, 1950

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Effect of Structure on the Rate of Spontaneous Thermal Decomposition of Substituted Benzoyl Peroxides

BY C. GARDNER SWAIN, WALTER H. STOCKMAYER AND JOHN T. CLARKE

The effect of structure on the rate of non-radical reactions has received considerable study. This has led to such extensive and successful quantitative relationships as the Hammett equation,[1] which by 1940 served to correlate the rates of 1763 non-radical reactions of *m*- and *p*-substituted benzene derivatives in terms of the electron-repelling or electron-attracting power of the substituent. With the 332 of these reactions which had been measured experimentally up to that time, the median deviation between calculated and observed rates was only ±15%, in spite of the fact that the variation in rate on changing from a *p*-methoxy to a *p*-nitro substituent averaged thirty-fold and exceeded a thousand-fold in several series.

In contrast, the effect of structure on the rate of free radical reactions has thus far received relatively little quantitative investigation. Nevertheless, pioneer work has been done on the "alternating tendency" in copolymerization,[2,3,4] and orientation effects in both aliphatic chlorination[5] and aromatic substitution.[6] Here also, the electron-repelling or electron-attracting power of the substituent appears to be most important in determining the rate of the reaction. To predict the effect of structural changes on the rate, it is necessary to consider the stability of the transition state. One must consider the contribution in the transition state of resonance forms involving transfer of one electron from one reactant ("donor" molecule or radical) to the other reactant ("acceptor" molecule or radical).[2,4,5] This results in a high rate when the transfer is strongly favored as a result of good electron-repelling groups in the donor or good electron-attracting groups in the acceptor. This concept is in accord with the only published application of the Hammett equation to a free radical reaction (copolymerization),[7] and it appears to be widely applicable to rates of *bimolecular* free radical reactions whether they are of the initiation, propagation, transfer or termination type.

(1) Hammett, *Chem. Revs.*, **17**, 125 (1935); "Physical Organic Chemistry," McGraw-Hill Book Co., New York, N. Y., 1940, Chapter VII.

(2) Bartlett and Nozaki, THIS JOURNAL, **68**, 1495 (1946).

(3) Alfrey and Price, *J. Polymer Sci.*, **2**, 101 (1947); Price, *ibid.*, **3**, 772 (1948).

(4) Mayo, Lewis and Walling, THIS JOURNAL, **70**, 1529 (1948); Walling, *ibid.*, **71**, 1930 (1949); Mayo, Abstracts of A. C. S. Organic Symposium, Madison, Wis., June 22, 1949.

(5) Ash and H. C. Brown, *Record of Chemical Progress*, **9**, 81 (1948).

(6) Hey and Waters, *Chem. Revs.*, **21**, 179 (1937).

(7) Walling, Briggs, Wolfstirn and Mayo, THIS JOURNAL, **70**, 1537 (1948); Walling, Seymour and Wolfstirn, *ibid.*, **70**, 1544 (1948).