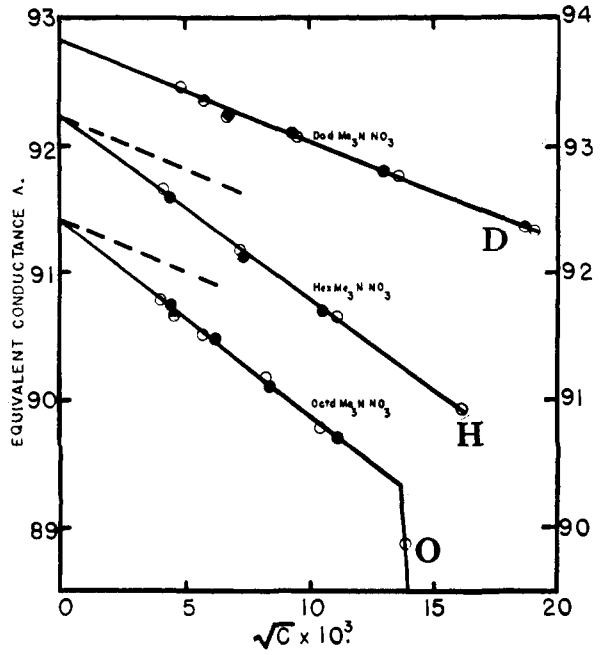

Fig. 1.—Λ-$\sqrt{C}$ plots for salts in water (broken lines, Onsager slope).

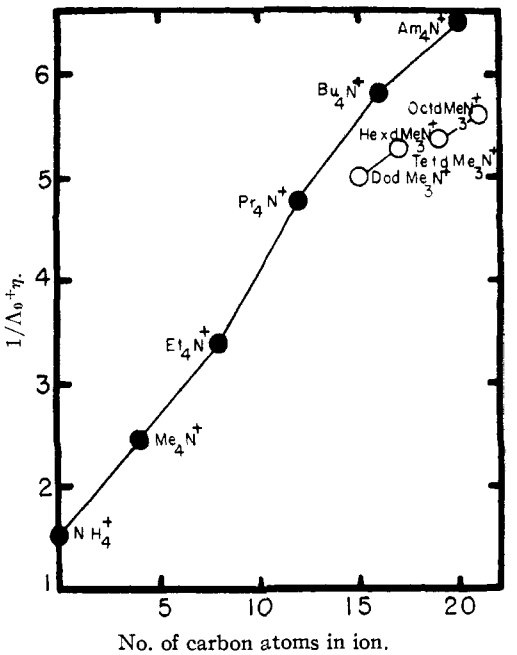

Fig. 2.—Resistance plots for quaternary ammonium ions in water.

est. In Fig. 2 are plotted values of the reciprocal of the ion resistance–viscosity product, $1/\Lambda_0{}^+\eta$, as a function of the number of carbon atoms in the ions. On the same plot are shown resistance values for the symmetrical quaternary ammonium ions as determined by Daggett.[4] For the same number of carbon atoms, the resistance of the long chain ions is markedly lower than that of corresponding symmetrical quaternary ammonium ions. It will be noted, too, that there is a break in the resistance curve between the tetradecyl- and the hexadecyltrimethylammonium ions. Such a break is not unexpected in view of the differences in the slopes which appear in the Λ-$\sqrt{C}$ plots for the same salts.

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

Effect of Ultraviolet Light on the Specific Activity of Chymotrypsin and Trypsin[1]

BY HARRY GOLDENBERG[2,3] AND A. D. MCLAREN

Introduction

It has been shown that the quantum yield for inactivation of pepsin at 2537 Å. is the same with either casein or hemoglobin as a substrate for assay of enzymatic activity.[4] Verbrugge[5] reported, however, that trypsin gave a higher quantum yield with Anson's colorimetric hemoglobin method[6] for estimating tryptic activity than was obtained with titrimetric methods for following the digestion of hemoglobin, casein and benzoyl-L-argininamide (BAA). This finding has been questioned for reasons cited elsewhere.[7] Consequently we have repeated the work of Verbrugge to redetermine the effect of ultraviolet light (2537 Å.) on the specific activity of trypsin. In addition we have determined the quantum yield for the inactivation of chymotrypsin as calculated from its loss in ability to hydrolyze casein, L-phenylalaninamide (PA), glycyl-L-phenylalaninamide (GPA), L-phenyl alanine ethyl ester (PEE) and chloroacetyl-DL-phenylalanine ethyl ester (CAPEE).

Experimental

Enzymes.—Three samples of crystalline chymotrypsin were used: six-times crystallized enzyme, salt free, supplied

(1) Photochemistry of Proteins. XIII.

(2) From the Ph.D. Thesis of H. Goldenberg, Polytechnic Institute of Brooklyn, 1950. Predoctoral Fellow of the National Institutes of Health, 1947–1949.

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(4) A. D. McLaren and S. Pearson, *J. Polymer Sci.*, **4**, 45 (1949).

(5) F. Verbrugge, *J. Biol. Chem.*, **149**, 405 (1943).

(6) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

(7) A. D. McLaren, *Advances in Enzymology*, **9**, 75 (1949).

by Worthington Biochemical Laboratory, Freehold, N. J.; once-crystallized Armour[8] chymotrypsin, containing 50% ammonium sulfate; and once-crystallized chymotrypsin (CB 1) containing 37% ammonium sulfate plus moisture, which was prepared in this Laboratory by recrystallizing Plaut[9] chymotrypsinogen nine times and converting it to the active enzyme according to the procedure of Kunitz and Northrop.[10]

The ultraviolet absorption spectrum of lot CB 1 in distilled water, *p*H 4.6, showed a minimum at 2500 Å. and a maximum at 2820–2830 Å., as determined with a Beckman spectrophotometer. A detailed study of the spectrum has been published elsewhere.[11] Armour chymotrypsin gave virtually identical results.

Two batches of crystalline trypsin were employed for the irradiation studies: once-crystallized trypsin, with a magnesium sulfate content of about 50%, generously furnished by Dr. Frank Verbrugge; and twice-crystallized enzyme (Worthington) containing 50% magnesium sulfate. Both preparations when dissolved in *M*/15 KH_2PO_4, *p*H 4.55, gave ultraviolet absorption minima at 2500 Å. and maxima at 2780–2800 Å.

Substrates.—Hemoglobin was prepared by the directions of Anson.[6] The casein was a product of General Biochemicals, Inc., Chagrin Falls, Ohio.

Benzoyl-L-argininamide hydrochloride (BAA) was synthesized according to Bergmann, Fruton and Pollok.[12]

L-Phenylalanine ethyl ester hydrochloride (PEE) was prepared in the usual manner; m.p. 147.5–148.5° (uncor.). The corresponding amide (PA) was derived by dissolving the free ester (a colorless oil) in cold anhydrous methanol saturated with ammonia, which was permitted to remain in the ice-box for two days, and then removing the solvent by vacuum distillation. Recrystallization from ethyl acetate gave the desired compound with a melting point of 92–93° (uncor.). *Anal.* Calcd. for $C_9H_{12}ON_2$: N, 17.06. Found: N, 16.84. Unlike DL-phenylalaninamide, prepared in the same manner (m.p. 137–137.5° (uncor.)), this compound is extremely soluble in methanol and water.

The synthesis of glycyl-L-phenylalaninamide acetate (GPA) has been described by Fruton and Bergmann.[13]

The facile condensation of chloroacetyl chloride and DL-phenylalanine ethyl ester to give chloroacetyl-DL-phenylalanine ethyl ester (CAPEE) is based on the Fischer technique for making the analogous tyrosine derivative.[14] Sodium hydroxide (1 *N*, 41 ml., 0.041 mole) was added to a mixture of 9.40 g. (0.041 mole) of DL-phenylalanine ethyl ester hydrochloride and 100 ml. of chloroform kept at ice temperature. Then 5.00 g. (0.0443 mole) of chloroacetyl chloride was diluted with 50 ml. of chloroform and half of this solution was added to the reaction mixture. The second half of the acid chloride solution was added slowly and concurrently with 20 ml. of potassium carbonate solution, prepared by dissolving 6.1 g. of the anhydrous salt in water to a volume of 20 ml. The underlying chloroform layer was separated, washed with water and dried over magnesium sulfate. On evacuation a white crystalline mass settled out; yield 90%, m.p. 73–74° (uncor.). The melting point was unchanged after recrystallization from aqueous methanol. CAPEE forms beautiful needles when recrystallized from ether-petroleum ether. *Anal.* Calcd. for $C_{13}H_{16}O_3NCl$: C, 57.88; H, 5.98. Found: C, 57.85; H, 5.99.

Methods.—The irradiation methods have been described elsewhere.[4] For each of the substrates employed, a curve was plotted of relative degree of hydrolysis at 25° *vs.* enzyme concentration.[4,5,11] These graphs were then used to determine the per cent. loss in active enzyme as a function of irradiation time.

The action of both chymotrypsin and trypsin on casein was measured by the method of Kunitz.[15]

Hemoglobin digestion was followed by a formol titration of the liberated carboxyl groups,[6] and by measurement of the tyrosine color value or the optical density at 2800 Å. of its trichloroacetic acid-soluble digestion products. The latter two methods are designated in Table III by the names "Anson" and "Kunitz," respectively.

An ultramicro adaptation of the Grassmann and Heyde[16] titration of carboxyl groups was used to determine the per cent. hydrolysis of PA and GPA by chymotrypsin. Cleavage of PA to form L-phenylalanine and ammonium ion was estimated alternatively by the Conway[17] method, which involves quantitative diffusion of the ammonia liberated from the basified reaction mixture into a 1% boric acid solution. The boric acid solution is back-titrated with 0.01 *N* hydrochloric acid to a brom cresol green–methyl red endpoint. The amidase activity of trypsin on BAA was also conveniently determined by this procedure.

Both PEE and CAPEE[18] were incubated with chymotrypsin in weakly buffered phosphate solutions which permitted a continuous and non-erratic fall in *p*H with enzyme-induced hydrolysis of the esters. Empirical calibration curves were prepared which related the time (in seconds) required for the *p*H of the enzyme–substrate mixtures to fall through a given interval as a function of enzyme concentration. This method will be referred to as the "*p*H drop-time technique." The esterase activity of chymotrypsin toward PEE was also checked by electrometric titration of the carboxyl groups liberated on hydrolysis.

Results and Discussion

The loss of activity of chymotrypsin solutions on irradiation with ultraviolet light (2537 Å.) as assayed with the various substrates is summarized in Table I.

TABLE I

LOSS OF ACTIVITY OF CHYMOTRYPSIN SOLUTIONS ON IRRADIATION AT CONSTANT INTENSITY

Init. enzyme concn., mg. per ml.	Time of irradiation, min.	Inactivation, %[c] CAPEE	PEE	PA	GPA	Casein
2.42[a]	10	18	19			20
8.56[a]	20	12	13	12	10	12
8.26[b]	40			15	28	16
5.90[b]	35	25		18		24
2.47[a]	10	18	17			17
6.10[a]	40	28	26			25
8.43[a]	30	15	13			14

[a] Crystalline chymotrypsin (CB 1). [b] Crystalline chymotrypsin (Armour). [c] Residual chymotrypsin activity toward CAPEE and PEE and toward GPA and PA measured by *p*H drop-time technique and by Grassmann and Heyde titration, respectively.

From the results in Table I, one can see that within the experimental error the loss of activity of chymotrypsin toward all of the substrates, with time of irradiation, is the same.

It has been shown that inactivation of chymotrypsin proceeds at a first order rate at constant light intensity with casein as a substrate.[11] Similar results were found with PEE (Table II). With the data from the table one calculates, for *p*H 4.59, a quantum yield of 3.1 ($\pm$0.1) $\times 10^{-3}$ for PEE, PA, or casein, in agreement with Finkelstein.[11]

Insofar as trypsin is concerned, Verbrugge concluded that "the quantum yields as measured by benzoylargininamide and casein agree and are independent of the method of determination.

(8) Armour Laboratories, Chicago 9, Ill.

(9) Plaut Research Laboratory, Lehn and Fink Products Corp., Bloomfield, N. J.

(10) M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, **18**, 433 (1935).

(11) P. Finkelstein and A. D. McLaren, *J. Polymer Sci.*, **3**, 223 (1948); *ibid.*, **4**, 573 (1949).

(12) M. Bergmann, J. S. Fruton and H. Pollok, *J. Biol. Chem.*, **127**, 643 (1939).

(13) J. S. Fruton and M. Bergmann, *ibid.*, **145**, 253 (1942).

(14) E. Fischer, *Ber.*, **37**, 2495 (1904).

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

(16) W. Grassmann and W. Heyde, *Z. physiol. Chem.*, **183**, 32 (1929).

(17) E. J. Conway, "Microdiffusion Analysis and Volumetric Error," Crosby Lockwood and Son, Ltd., London, 1947.

(18) The CAPEE—chymotrypsin reaction mixtures were made up to 50 volumes per cent. alcohol, to keep the water-insoluble substrate in solution

TABLE II

EFFECT OF ULTRAVIOLET RADIATION ON CHYMOTRYPSIN[a] ACTIVITY TOWARD PEE, PA AND CASEIN

Irradiation time, min.	Remaining activity, % PEE	Casein	PA
6[b]	85.7	84.9	
14[b]	67.1		
20[b]	57.4	54.2	
25[b]	46.6		
50[c]		38.4	40.4

[a] Six times crystallized enzyme (Worthington). [b] Initial enzyme concentration, 0.756 mg./ml. Per cent. absorption for 1 cm. path length at 2537 Å., 76.0. Incident intensity of light, 1.37×10^{-5} einstein/ml./hr. [c] Initial enzyme concentration, 1.51 mg./ml. Per cent. absorption for 1 cm. path length at 2537 Å., 92.1. Intensity of light, 1.47×10^{-5} einstein/ml./hr.

For hemoglobin the titration method gives results which agree with those with the other substrates; the colorimetric method gives values which are considerably higher. These results indicate that the Anson colorimetric technique is not generally applicable to irradiated enzymes."

If the quantum yield for inactivation of trypsin relative to hemoglobin (colorimetric method) is more than four times as great as the yield relative to casein or BAA,[5] one would anticipate that such a difference should be readily detected by cross-assay. Accordingly, several solutions of Verbrugge's trypsin were irradiated in $M/15$ potassium dihydrogen phosphate and assayed against casein, BAA and hemoglobin. The hemoglobin digestion was followed colorimetrically and by formol titration. The results are presented in Table III.

TABLE III

LOSS OF ACTIVITY OF TRYPSIN SOLUTIONS ON IRRADIATION WITH ULTRAVIOLET LIGHT (2537Å.) AT *p*H 4.55

Init. enzyme concn., mg. per ml.	Time of irradiation, min.	Inactivation, % Hemoglobin Kunitz	Anson	Casein	BAA
1.80[a]	10	33		34	31
1.97	13	48		45	47
1.97	25	59			61
1.92	6	32	34		
1.92	12	48	49		
1.90	6	31		29	30
1.90	12	43		44	44
2.01	10	37		37	36
2.01	20	50		51	48
0.750[b]	2				15.3
	6		37.0		35.6
	10				52.0
	14				63.5
	20				75.9
		Formol			
0.889[c]	5	37.0			
	12	62.5			63.7
	20	83.1			

[a] Verbrugge trypsin. [b] Worthington trypsin. Per cent. absorption, 69.1; intensity of light, 1.37×10^{-5} einstein/ml./hr. [c] Worthington trypsin. Per cent. absorption, 74.3; intensity of light, 1.58×10^{-5} einstein/ml./hr.

Contrary to the findings of Verbrugge, the inactivation (or quantum yield) of trypsin based on the hemoglobin colorimetric methods is no greater than with casein and BAA as substrates[19] or with a formol titration of hemoglobin digests.

From the fact that the activity of chymotrypsin (and trypsin) is reduced equally and simultaneously toward all substrates by ultraviolet light, it may be concluded (a) that only one kind of active catalytic center exists and/or (b) that the entire molecule is denatured. It has been shown concurrently with this investigation that loss of activity of chymotrypsin parallels loss of solubility in ammonium sulfate solution upon irradiation.[20] Since the latter is a common criterion of denaturation, our results are consistent with the physical evidence. Recent work of Balls, *et al.*, indicates, incidentally, that chymotrypsin has probably but one active center per enzyme molecule, as indicated by diisopropyl fluorophosphate inhibition.[21]

Verbrugge reported the quantum yield for inactivation of trypsin at 2537 Å. to be twice that at 2804 Å., contrary to the results of Uber and McLaren,[22] who had found that the values were about the same. He attributed this to the high molecular extinction coefficient of the Uber–McLaren trypsin in the region of minimum absorption, *viz.*, 29,000 at 2500 A., as compared to his own markedly lower value of 12,000. Using a Beckman spectrophotometer, we have found that the absorption spectra of the Verbrugge, Uber–McLaren and Worthington trypsin preparations are almost identical, with molecular extinction coefficients of 22,600, 22,800 and 22,200, respectively, at 2500 Å., and an extinction value of 62,400 at 2800 Å. The latter figure is the ratio of the molecular weight of trypsin used here, 36,500, to the Kunitz factor,[15] 0.585. It follows, therefore, that the quantum yield at 2537 Å. is probably somewhat larger than the quantum yield at 2804 Å. but considerably less than twice its value.

Summary

The quantum yields for inactivation of chymotrypsin or trypsin by ultraviolet light (2537 Å.) are independent of the substrates, natural or synthetic, which are used to follow loss of enzyme activity.

BROOKLYN 2, N. Y. RECEIVED JULY 5, 1950

(19) With the data of Table III one can calculate a quantum yield of *ca.* 0.010. This is approximately one-half that reported previously.[5,22] The difference may be due to discrepancies in the measurement of light intensity and/or optical absorption measurements. In the present series of papers, the intensities of the mercury arc were determined with a uranyl oxalate actinometer,[4] while the optical absorption at 2537 Å. of the enzyme solutions was measured with a Beckman spectrophotometer. Spectra of the three samples nearly coincided throughout the ultraviolet region (data may be found in reference 2). The molecular weight used here, as earlier, is 36,500. [The value is probably nearer to 20,000 (A. D. McLaren, *Compt. rend., Lab. Carlsberg*, in press).]

(20) P. Finkelstein and A. D. McLaren, THIS JOURNAL, **72**, 5423 (1950).

(21) E. F. Jansen, M.-D. F. Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949).

(22) F. M. Uber and A. D. McLaren, *ibid.*, **141**, 231 (1941).