THE ABSORPTION OF ULTRA-VIOLET RADIATION BY CRYSTALLINE PEPSIN

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In a previous paper (Gates, 1933–34) the absorption spectrum of Northrop's crystalline pepsin was shown to have a characteristic shape similar to that of certain amino acids (Gates, 1928), corresponding to the essential protein nature of the enzyme (*cf.* Northrop, 1929–30, 1932–33*a*; Sumner, 1933).

As a possible step toward more definite knowledge of enzyme structure it is of interest to know whether the destruction of pepsin activity varies in the same way with different wave-lengths as does the absorption of energy in the different parts of the spectrum.

The changes produced in pure crystalline pepsin by ultra-violet radiation were studied in two principal ways: (1) by direct determination of the absorption spectrum of pepsin inactivated by ultra-violet energy, and (2) by measuring the activity of pepsin solutions after irradiation with different bands of wave-lengths of the mercury vapor arc spectrum. As in the previous work (Gates, 1933–34), all of the pepsin preparations used were obtained through the kindness of Dr. John H. Northrop.

I

The Absorption Spectrum of Crystalline Pepsin Inactivated by Ultra-Violet Radiation

Early absorption tests made upon different preparations of pepsin after irradiation with the total radiation from a quartz mercury vapor arc and with single wave-lengths obtained by means of a crystal quartz monochromator (Gates, 1929–30), indicated that definite

* This paper is one of several in which are presented results of work completed by Dr. Frederick L. Gates before his death on June 17, 1933. The manuscripts have been prepared by Professor W. J. Crozier and Dr. R. H. Oster.

changes occurred in the absorption curve of pepsin solutions exposed to high incident energies. These changes were especially marked in the region of the spectrum between 2400 and 2750 Å.u.

Numerous tests carried out on different preparations of pepsin (of varying stages of purification) produced essentially similar curves of absorption at corresponding incident energies. Only tests made on a highly purified preparation of crystalline pepsin (Lot 4) are considered here.

EXPERIMENTAL

Highly purified crystalline pepsin (Lot 4) having the characteristics: 2× crystalline pepsin in M/10 sodium acetate, pH 5.0, protein nitrogen—21.4 mg. per ml., $(P.U.)^{Hb}_{ml.}$—4.4, was diluted to 1 in 25 with M/10 sodium acetate, pH 5.0 (by quinhydrone electrode). Two 25 ml. samples of this solution were pipetted into 9 cm. Petri plates covered with cellophane; the control was also covered with a plate of soda glass (1.55 mm. thick). Exposure was at 30 cm. from an air-cooled horizontal quartz mercury vapor arc operated at 67 volts and 5.5 amperes. During the exposures the temperature of the specimens was maintained between 20 and 22°C. by a bath of ice water.

At intervals of 20, 60, 180, and 360 minutes 5 ml. samples were removed for tests of the pepsin activity by the hemoglobin method of Anson and Mirsky (1932–33), and for absorption tests. The latter tests were made as previously described by Gates (1930–31, 1933–34), using a quartz sector photometer (Judd-Lewis, 1919, 1921) and a large quartz spectrograph. The pepsin solution, as irradiated, along with a control solution of the solvent (M/10 sodium acetate buffer, pH = 5.0) was placed in micro-Baly photometer tubes in the path of twin beams of light from the tungsten-iron spark source. Variation of the length of the solution in conjunction with the sector vanes of the photometer permitted a wide range in the intensity of the transmitted energies.

From the spectrograms thus obtained the absorption curves were plotted in terms of the molecular extinction coefficients as a function of wave-length (Fig. 1).

The curves of Fig. 1 show a progressive change in the S shape of the absorption curve with increasing periods of exposure. For analysis the curves may be divided into four regions: (1) the slope upward between 3100 and 2850 Å.u., (2) the peak at 2775 Å.u., (3) the valley at 2500 Å.u., (4) the upward slope from 2475 to 2300 Å.u. Irradiation did not greatly affect the peak at 2775 Å.u., or the final slope between 2475 and 2300 Å.u., but the absorption in (1) broadened into the near ultra-violet and (3) filled up.

Points plotted from data secured on unexposed control solutions and on specimen solutions exposed for 6 hours under soda glass are in close agreement with those of solution A_1 exposed for 20 minutes. This indicates that relatively long exposures to radiation of shorter wave-

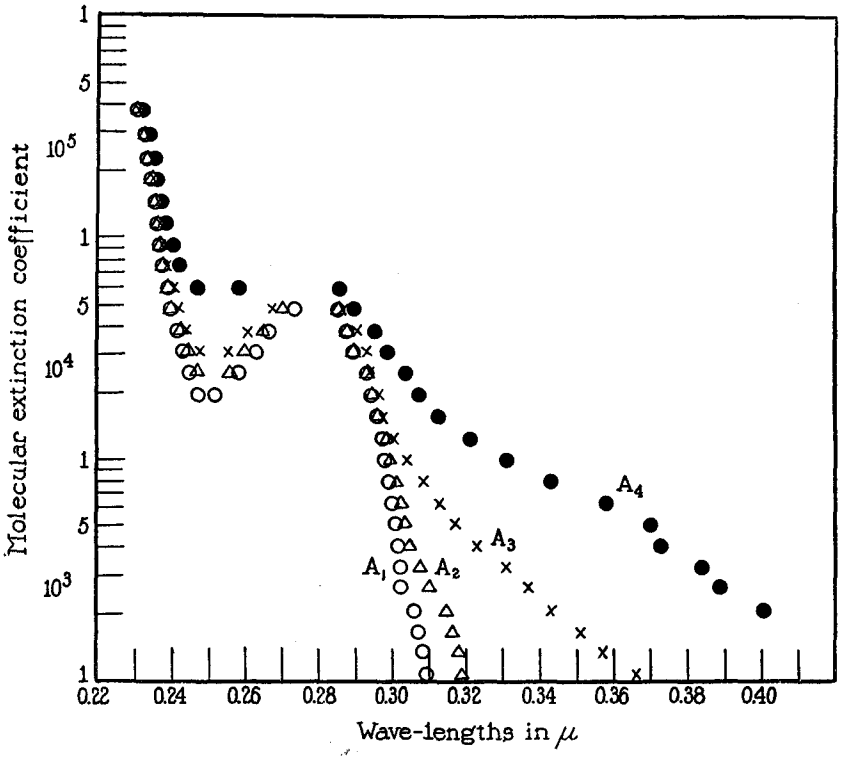

FIG. 1. Curves showing the absorption spectra of solutions of pure crystalline pepsin (5.65 mg. per ml.) in N/10 sodium acetate buffer, pH 5.0, irradiated for different periods of time at 30 cm. from an air-cooled horizontal quartz mercury vapor arc operated at 67 volts and 5.5 amperes. Clear circles represent points obtained on solutions irradiated 20 minutes; triangles, 60 minutes; crosses, 180 minutes; and solid circles, 360 minutes.

lengths than those transmitted by soda glass are required to change appreciably the absorption spectrum of the pure enzyme.

Absorption tests on solutions of pepsin (at the same dilution and pH as the above) with higher energies gave results similar to those of

Fig. 1 with a further rise in the curve at 2500 Å.u. and with a further broadening out of the absorption into the near ultra-violet and blue region of the spectrum.

Crystalline Pepsin Inactivated by Heat

To test for any change in the absorption spectrum of pepsin due to heat, a pure preparation of crystalline pepsin (21.4 mg. protein nitro-

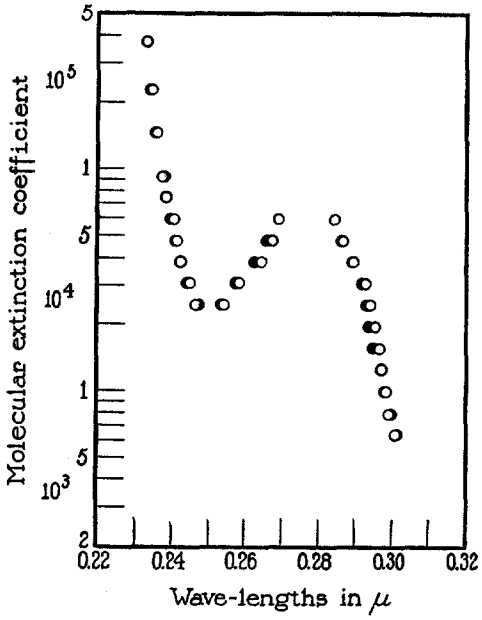

FIG. 2. The absorption spectrum of pepsin inactivated by heating to 65°C. for 5 minutes, compared with the normal absorption spectrum. Clear circles represent points on the ultra-violet absorption curve of pure crystalline pepsin (0.1412 mg. per ml., pH 4.9) at 100 per cent activity. Solid circles represent points on the absorption curve of the same pepsin solution inactivated by heat; activity, 43.5 per cent.

gen per ml. in N/10 sodium acetate buffer, pH 5.0) was diluted 1 in 25 with N/100 HCl.[1] A 5 ml. sample of this solution was heated for 5 minutes at 65°C. while an equivalent sample was held as a control.

[1] Later tests of the pH of the diluted pepsin solution indicated that the pH at the time of the absorption tests was probably between 4.9 and 5.0. The N/100 HCl had probably deteriorated.

Both samples were then diluted 1 in 40 with N/100 HCl and their activity was tested by the hemoglobin method and read against the tyrosine standard set at 20. On the basis of the undiluted pepsin the heated sample showed an activity of (P.U.) = 1.18, or a 56.5 per cent loss in activity in the heated sample.

Both samples were studied spectrographically in the ultra-violet with the Judd-Lewis photometer and micro-Baly tubes as described previously. On plotting the absorption curves (Fig. 2) it is evident that there is not enough difference between the two to be significant.

II

The Inactivation of Crystalline Pepsin by Ultra-Violet Radiation[2]

It is essential that a correlation be made between the changes in the absorption spectrum of crystalline pepsin as a result of irradiation and changes in the activity of the enzyme. To obtain such data tests of the proteolytic activity of pepsin after irradiation were made.

Early tests of the activity of pepsin solutions by viscosity measurements (Northrop, 1932–33*a*) on the rate of digestion of edestin after irradiation of the enzyme solutions, showed that exposure to narrow bands of wave-lengths for 1 and 2 hours was not sufficient to produce any appreciable inactivation, but that 35 minute exposure at 30 cm. to the total radiation from an air-cooled horizontal quartz mercury vapor arc was sufficient to inactivate the pepsin at dilutions of 0.002 mg. protein nitrogen per ml.

Tests of the activity of the highly purified pepsin solutions used in obtaining the absorption curves given in Fig. 1 were made by the hemoglobin method of Anson and Mirsky (1932–33). For the activity determinations the pepsin solutions (as irradiated; see above) were further diluted to 1 in 40 with M/100 HCl to give a pH of 2.7.

[2] The results of a number of tests on the activity and the absorption spectra of a number of preparations of crystalline pepsin of varying purities, at different concentrations, and at different levels of pH, agree essentially with those reported here and are therefore omitted to avoid undue repetition. In these tests the activity of the irradiated pepsin solutions was tested by several methods: the dissolving action on gelatin (Northrop and Hussey, 1922–23; Northrop, 1929–30) and on gelatin films (Gates, 1927, 1930), and the rate of digestion of casein and of 5 per cent edestin as measured by the viscosimetric method (Northrop, 1932–33*b*).

1 ml. of this 1/1000 dilution was used in each test (Table I); blank tests on hemoglobin using 1 ml. of M/100 HCl in place of pepsin yielded no coloration with the phenol reagent (Folin and Ciocalteau, 1927).

All irradiated and control specimens were clear in color, excepting the 3 and 6 hour exposure specimens (A_3 and A_4) which showed a yellow coloration. A_4 had the odor of fresh urine or stale straw. The results of these tests, with the calculated pepsin activity, $(P.U.)^{Hb}_{ml.}$, are given in Table I.

TABLE I

Pepsin Activity Measured by Titration with Hemoglobin (Anson and Mirsky, 1932–33)

Samples irradiated with the total radiation from a horizontal quartz mercury vapor arc at 30 cm., operated at 67 volts and 5.5 amperes.

Specimen	Period of irradiation	Colorimeter readings (standard set at 20)				$(P.U.)^{Hb}_{ml.} \times 100$	Activity
					Average		
Control	*min.*						*per cent*
	0	11.8	11.8	11.8	11.8	1.5	
A_1	20	11.2	11.2	11.2	11.2	1.6	100*
B_1	20	11.2	11.4	11.2	11.2	1.58	
A_2	60	14.0	14.0	14.1	14.0	1.24	79.5
B_2	60	11.4	11.5	11.4	11.4	1.56	
A_3	180	16.2	16.3	16.4	16.3	1.04	68.5
B_3	180	11.8	11.6	11.6	11.7	1.52	
A_4	360	31.4	31.3	31.3	31.3	0.478	32.4
B_4	360	11.9	11.9	11.9	11.9	1.49	

* Since A_1 and its control exposed under glass, B_1, are close together and B_2, B_3, and B_4 fall off gradually from the first figures and are all above or equal to the unexposed control, it is advisable to regard 1.59 as representing 100 per cent and to calculate the per cent loss in activity on this basis.

On plotting the percentage pepsin activity semilogarithmically against the period of irradiation a straight line is obtained, indicating that the pepsin is inactivated in a first order reaction with a one-quantum relationship, assuming the energy flux to be constant. Northrop (1933–34) found a similar relationship between the energy

and the rate of inactivation for pepsin solutions at different pH values (see also: Collier and Wasteneys, 1932; Hussey and Thompson, 1923–24).

Since the activity tests show a mass reaction (one quantum) curve, presumably a single reaction takes place, and the successive curves ($A_1 - A_4$) represent mixtures of the pre- and post-irradiated substance. On this assumption the reciprocals of the valley points at 2500 Å.u. should bear some relation to the change in activity of the pepsin. The successive values are given in Table II with the respective pepsin activities for comparison.

TABLE II

Pepsin Activity Estimated from the Reciprocals of the Ordinates of the Absorption Curves of Fig. 1 at Wave-Length 2500 Å.u. for Pepsin Solutions Irradiated for Varying Periods of Time

Absorption curve	Curve ordinate at 2500 Å.u.	Reciprocals of ordinates	Unchanged pepsin	Pepsin activity
			per cent	*per cent*
A_1	1.9	0.526	100*	100
A_2	2.34	0.425	80.8	79.5
A_3	2.85	0.351	66.7	68.5
A_4	5.68	0.176	33.4	32.4

* On the assumption that the reciprocal of the ordinate in Curve A_1 represents unchanged pepsin at 100 per cent activity, the other activities are in the ratios given in Column 4 of Table II.

Since the actual ratios of pepsin activity found by the hemoglobin method are given in Column 5 of Table II, the agreement with the values obtained from the reciprocals of the absorption ordinates (Column 3) suggests that the destruction spectrum of pure crystalline pepsin coincides with its absorption spectrum, at least in this region of the ultra-violet. If we assume the final substance to have a straight line absorption between 2850 and 2350 Å.u. the curve would cross the 2500 Å.u. line at about 20.0. This point, considered as above, corresponds to an activity of 10 per cent.

Although the agreement between the values of Columns 4 and 5 of Table II is close, certain relations between the pepsin concentration and the absorption coefficient should be kept in mind:

If we let C = the concentration of pepsin at any time t
and C_o = the original concentration of pepsin
then $C_o - C$ = the reaction products at time t.
Now letting β = the extinction coefficient of pepsin,
γ = the extinction coefficient of the reaction products,
μ_x = the absorption coefficient of the mixture at time t,
and μ_o = the initial absorption coefficient,

then

$$\mu_x = C\beta + (C_o - C)\gamma \tag{1}$$

from which we get

$$C = \frac{C_o\gamma - \mu_x}{\gamma - \beta} \text{ (since } \gamma > \beta) \tag{2}$$

and

$$C = C_o\left(\frac{\gamma}{\gamma - \beta}\right) - \left(\frac{1}{\gamma - \beta}\right)\mu_x \tag{3}$$

or

$$C_o - C = k(\mu_x - \mu_o). \tag{4}$$

Equation (4) implies a linear relationship between the amount of inactivation and the change in the absorption coefficient, and hence is not in accordance with the data of Column 5, Table II. Two explanations may be advanced to explain this divergence: (1) error in the determination of the ordinates from the curves of Fig. 1 at 2500 Å.u., and (2), a further change in the reaction products resulting in an increased absorption with increasing periods of exposure.

III

The Destruction Spectrum of Crystalline Pepsin

To determine the relation existing between radiant energy absorbed by the pepsin and the amount of inactivation, a series of tests was made in which pure crystalline pepsin from the same preparation already described was diluted to 1 in 500 with N/100 HCl, at pH 2.06, and exposed in two cells (5 × 5 × 23 mm.) cut in a glass block and faced by a 2 mm. quartz plate, by means of a large quartz monochromator (Gates, 1929–30). Specimens were irradiated for different periods at four different calibrated scale settings of the monochromator. At

each of these settings the exit slit of the monochromator was so adjusted that a band of known wave-lengths was incident on the exposure cell. To obtain the energy absorbed by the solution the intensity of the incident and transmitted radiation was read with a sensitive four-junction thermopile and a sensitive Leeds and Northrup galvanometer. During the exposure the control cell was cut off from direct radiation by the exit slit of the monochromator. Due to the very small free surface there was very little evaporation from the test

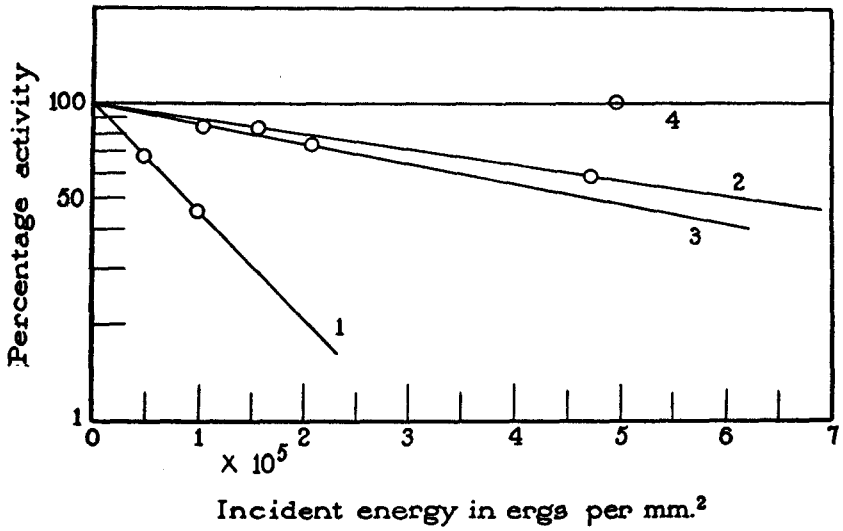

FIG. 3. The inactivation of pure crystalline pepsin, pH 2.06, by ultra-violet radiation at different parts of the spectrum; Curve 1, the band of wave-lengths between 2300 and 2400 Å.u.; Curve 2, 2425 to 2570 Å.u.; Curve 3, 2640 to 2820 Å.u.; Curve 4, 2860 to 3131 Å.u.

samples. If evaporation could be detected the specimen was made up to the correct volume (0.575 ml.) by addition of N/100 HCl.

After irradiation specimens were removed from the exposure cell with capillary pipettes, placed in conical centrifuge tubes, measured with 1 ml. pipettes, and an equal quantity of N/100 HCl was added to give a final dilution of 1/1000 of the original pepsin solution.

The exposed, control, and stock specimens were tested for activity by hemoglobin proteolysis, using one-half quantities throughout.

After mixing, the specimen was kept at 35.5°C. for 5 minutes and then read against a carefully prepared tyrosine standard obtained from Dr. P. A. Levene. During the exposures the temperature of the specimens varied between 25° and 27°C.

TABLE III

Protocol of Tests of the Relation between Absorbed Energy and the Amount of Inactivation of Pepsin (pH 2.06) at Different Bands in the Ultra-Violet

Point wave-length	Specimen sample	Energy		Exposure time	Hb (P.U./ml. for original No. 4	Pepsin activity	Energy required to inactivate 50 per cent of pepsin		α
		Incident	Absorbed				Incident	Absorbed	
Å.u.		*ergs/mm.²*	*ergs/mm.²*	*min.*		*per cent*	*ergs/mm.²*	*ergs/mm.²*	
2357	A_1	4.96×10^4	4.26×10^4	45	2.14	68.6			
(2300–	B_1				3.12				
2400)	A_2	9.92×10^4	8.52×10^4	90	1.43	45.6	87,000	77,500	14×10^4
	B_2				3.12				
2509	C_1	15.7×10^4	7.5×10^4	60	2.58	82.7			
(2425–	D_1				3.12				
2570)	C_2	47.3×10^4	22.5×10^4	180	1.84	59.0	617,000	305,000	1.97×10^4
	D_2				3.12				
2719	F_1	10.4×10^4	5.3×10^4	60	2.18	84.4			
(2640–	G_1				2.58				
2820)	C_0*				2.58		468,000	230,000	
	F_2	20.8×10^4	10.6×10^4	120	2.00	74.0			2.62×10^4
	G_2				2.70				
2930	H_1	49.9×10^4	11.8×10^5	120	2.60	98.5			
(2860–	I_1				2.64				
3131)	H_2	49.9×10^4	11.8×10^5	120	3.12	100.0			
	I_2				3.12				

* Flask control.

From calculations of the relative energies of the wave-lengths included in each of the four bands of the spectrum used, the relative absorption of the pepsin solutions at these wave-lengths, the relative transmission of energy, and the relative efficiency of each wave-

length in contributing absorbed energy, it was found that the light acted practically as though concentrated at the following points:

Monochromator setting.....	11.0	9.5	7.8	6.5
Point wave-length, *in Å.u.*.....	2357	2509	2719	2930

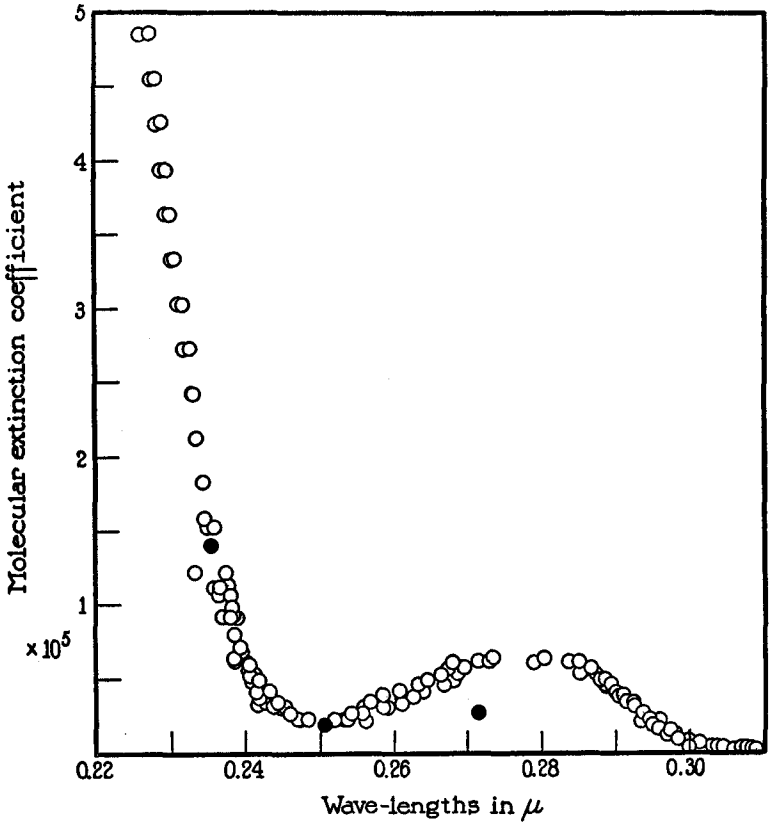

FIG. 4. Comparison of the destruction spectrum of Northrop's crystalline pepsin with its absorption spectrum. Clear circles represent points obtained with a pure crystalline pepsin preparation (0.2376 mg. per ml.) in N/100 HCl, pH 2.54, on the normal absorption curve. Solid circles represent the reciprocals of the energy/100 required to inactivate 50 per cent of the pepsin (0.1412 mg. pepsin per ml.) in M/100 HCl, pH 2.06, plotted against the point wave-length. (See text.)

Tests of the pepsin activity translated into percentage of activity remaining give straight lines when plotted semilogarithmically against the incident energy (Fig. 3), indicating the same one-quantum rela-

tionship already suggested. Protocols of these experiments are summarized in Table III.

If we assume that the inactivation efficiency of the various wave-lengths is proportional to their respective absorption coefficients, the ordinates of the destruction curve may be calculated from the relation:

$$\frac{\alpha_2}{\alpha_1} = \frac{(\ln C_o/C)_2}{(\ln C_o/C)_1} \cdot \frac{(i\,t)_1}{(i\,t)_2}$$

where α_1 represents the extinction coefficient determined by the destruction at a reference wave-length λ_1 and α_2 represents the coefficient at any other wave-length λ_2. C_o and C represent the concentration of active pepsin at the beginning and after a period of exposure t, and i is the intensity of the incident radiation (*cf.* Kubowitz and Haas, 1933).

Calculation of the number of quanta absorbed (2×10^{14}) and the number of molecules of pepsin inactivated (5.6×10^{14}) at the point wave-length 2357 Å.u., indicates that the one-quantum relation holds at this wave-length under the conditions of the experiment. Since most of the incident energy is absorbed at 2357 Å.u., the extinction coefficient has been substituted for α_1 as the point of reference in calculating the other values of α by the above equation (Column 10, Table III).

These values when plotted as a function of wave-length (points represented by solid circles in Fig. 4) give the approximate curve of the destruction spectrum of pepsin.

IV

DISCUSSION

Comparison of the destruction spectrum of pure crystalline pepsin with the absorption spectrum (Gates, 1933–34) reveals an essential agreement in the location of the maxima and minima of the two curves for the several bands of wave-lengths tested. However, it should be noted that an exact agreement does not exist between the two curves at 2719 Å.u., with the present method of comparison. Furthermore, a comparison of the incident and absorbed energies required to inactivate 50 per cent of the pepsin shows that considerably more energy must be absorbed at the wave-lengths 2509 Å.u. and 2719 Å.u. to

inactivate a given amount of pepsin than would be required if a quantum yield of one (as indicated at 2357 Å.u.) holds for each of these wave-lengths. From the logarithmic rate of inactivation (Fig. 3), and from the change in the absorption spectrum as the reaction proceeds with increased exposures to the radiation (Fig. 1), it is probable that the differences observed may be due to varying absorption of energy by the products of the reaction.

Kubowitz and Haas (1933) describe a similar correspondence between the destruction spectrum and the absorption spectrum of urease (Sumner, 1926) (with the exception of a discrepancy in the values of the absorption coefficients at the wave-length 254 mμ).

As previously noted for the absorption spectra (Gates, 1933–34), there is considerable agreement between the destruction spectrum of pepsin at this level of pH (2.06) which Northrop (1933–34) has shown to be in the range of optimum inactivation by ultra-violet radiation, and the destruction spectrum of urease.

In connection with the protein nature of pepsin it is of interest to note the parallelism of the observed increase in the total absorption of energy by pepsin inactivated by ultra-violet radiation with the increase in total absorption and loss of the specific absorption band near 265 mμ by tuberculin after ultra-violet irradiation (Spiegel-Adolf and Seibert, 1933).

SUMMARY

Determination of the absorption spectra of pure preparations of Northrop's crystalline pepsin inactivated by irradiation with ultra-violet light shows that the total absorption in the ultra-violet region of the spectrum increases with the degree of inactivation. This increase is especially marked between 2400 and 2750 Å.u. The rate of photoinactivation is shown to be sensitive to changes in pH, increasing with lower values, and evidently bears a one-quantum relationship to the energy flux. Tests of the rate of inactivation of pepsin exposed to several different bands of the ultra-violet spectrum, in relation to the absorbed energy, indicate that the destruction spectrum of the enzyme agrees essentially with its absorption spectrum and is similar to that of urease.

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