Properties of Photoaddition Products of Thymine and Cysteine†

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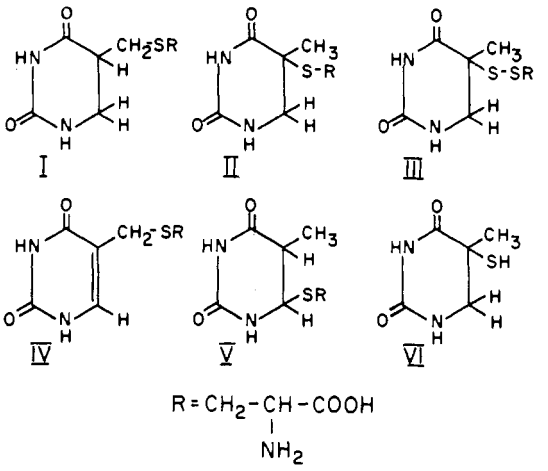
ABSTRACT: Photochemical changes of thymine irradiated (λ >230 nm) in aqueous solution in the presence of cysteine have been studied. The results show that the presence of cysteine enhances the photochemical reactivity of thymine. Addition of cysteine to thymine is the major reaction. The major photoproducts have been identified as 5-*S*-cysteinylmethyl-5,6-dihydrouracil (I), 5-*S*-cysteinyl-5,6-dihydrothymine (II), 5-*S*,*S*-cysteinyl-5,6-dihydrothymine (III), 5-*S*-cysteinylmethyluracil (IV), and 5,6-dihydrothyminyl-5-thiol (VI). There is evidence that a heat- and acid-reversible product, probably 6-*S*-cysteinyl-5,6-dihydrothymine (V), is also formed. Adducts I–IV are stable in acidic solutions but only IV is stable in alkaline solutions. When irradiated (λ 265 nm), II is converted to thymine and cysteine and III to VI and cysteine. I and IV are stable to ultraviolet light. The presence of a disulfide group in III probably indicates a two-step reaction for its formation.

Irradiation of biological systems with ultraviolet light results in the production of different types of products (Setlow, 1967; Smith and Hanawalt, 1969). One type of lesion that is receiving much attention recently is the one responsible for cross-linking of nucleic acid and protein. In a series of papers (Smith, 1967, 1968, 1970; Smith and Meun, 1968), Smith has presented evidence that photochemical addition of pyrimidine bases and amino acids is responsible for this type of lesion. Photoaddition of a number of amino acids to uracil has been demonstrated (Smith, 1969). Similarly, photoaddition of cysteine to uracil, thymine, polythymidylic acid, polycytidylic acid, DNA, and RNA has also been shown to occur (Smith and Meun, 1968). One class of photoaddition products of cysteine and pyrimidines has been identified as II, 5-*S*-cysteinyl-5,6-dihydropyrimidines (Smith and Aplin, 1966; Smith, 1970). However, the properties of these photoproducts are not clearly understood. Moreover, there is evidence (Smith, 1970; Fisher and Johns, 1971) that cysteine and pyrimidines form more than one type of photoaddition products. A thorough knowledge of the chemistry of these products is a prerequisite to understand their relative importance in ultraviolet-induced DNA–protein cross-linking.

We have studied the photoaddition of thymine and cysteine and have found that 5-*S*-cysteinylmethyl-5,6-dihydrouracil (I), 5-*S*-cysteinyl-5,6-dihydrothymine (II), 5-*S*,*S*-cysteinyl-5,6-dihydrothymine (III), 5-*S*-cysteinylmethyluracil (IV), and 5,6-dihydrothyminyl-5-thiol (VI) are formed when thymine is irradiated (λ >230 nm) in the presence of cysteine. There is indirect evidence that 6-*S*-cysteinyl-5,6-dihydrothymine (V) is also formed.

Experimental Section

Materials. Thymine, uracil, dihydrothymine, L-cysteine hydrochloride, and cysteine dihydrochloride were from Sigma Chemicals. Thymine-*2*-^{14}C (62 mCi/mmol) and L-cysteine-$^{14}C(U)$ hydrochloride (25 mCi/mmol) were obtained from Amersham/Searle and thymine-*methyl*-^{14}C (56 mCi/mmol) was from New England Nuclear Corp. For chromatography, Baker Analyzed reagents were used.



Methods. IRRADIATION. Monochromatic irradiations were carried out in our ultraviolet light monochromator (Johns and Rauth, 1965). The intensity was monitored and exposures were calculated as described by Johns (1971). Solutions for irradiations usually had 2.5 ml with an initial absorbance of 1.5 at 264 nm.

For bulk irradiations, a water-cooled high-pressure mercury lamp (PEK AH6-1B-6440) enclosed in a Vycor jacket was used as the radiation source. This type of irradiation excludes wavelengths lower than 230 nm. The lamp was placed in the solution to be irradiated, which was kept cold by running water. The solutions were degassed prior to irradiation by bubbling nitrogen for 1 hr and the bubbling was continued throughout the irradiation period. Unless otherwise stated, portions (750 ml) containing 2 mmol of thymine, 8 mmol of L-cysteine hydrochloride, and 10 μCi of thymine-*2*-^{14}C were irradiated for a period of 6 hr. About 20% of thymine was converted to products during this period.

CHROMATOGRAPHY. The irradiated solution was concentrated on a rotary evaporator at 40° to about 25 ml and filtered. The filtrate was applied on a column (50 × 4 cm) of Dowex 50W-X12 (H^+, 100–200 mesh). The column was

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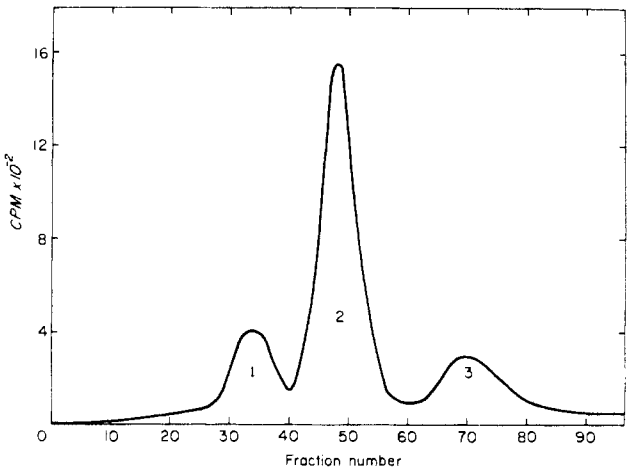


FIGURE 1: Fractionation of thymine–cysteine addition products by column chromatography. The irradiated resolution was applied on the column and was eluted with water until the eluate had neither ultraviolet absorbance above 210 nm nor radioactivity. Subsequently, the column was eluted with a hydrochloric acid gradient as described in the text. The radioactive peaks are designated as 1, 2, and 3.

eluted with water until the eluate had neither radioactivity nor ultraviolet absorbance above 230 nm. Subsequently, the column was eluted with an HCl gradient (1800 ml of water and 200 ml of concentrated HCl in the mixing chamber, and 1600 ml of water and 400 ml of concentrated HCl in the reservoir). Fractions of 30 ml each were collected and aliquots from each fraction were counted for radioactivity. Fractions containing the desired products were pooled, evaporated on a rotary evaporator at 35–40° to dryness, and analyzed as required.

Paper chromatography was carried out by the descending technique, using Whatman No. 3MM sheets. An aqueous solution of the sample was streaked on the paper. The solvent systems used were: S_1, *n*-butyl alcohol–acetic acid–water (80:12:30); S_2, *tert*-butyl alcohol–methyl ethyl ketone–ammonium hydroxide–water (40:30:10:20); S_3, *n*-propyl alcohol–water (70:30); and S_4, *sec*-butyl alcohol saturated with water. From the chromatograms the desired regions were cut out and eluted with 0.1 N HCl. The extract was evaporated to dryness and the residue was analyzed as desired.

TEST FOR PRODUCTS. (1) Radioactivity was determined by counting aliquots (1 ml) of aqueous solutions of the sample with 10 ml of PCS solubilizer (Amersham/Searle). Paper chromatograms were cut into 1-cm strips and eluted with water, and aliquots were counted as mentioned above. (2) The development of a yellow color when the chromatograms were sprayed with Ehrlich's reagent after pretreatment with alkali revealed the presence of dihydropyrimidines (Cline and Fink, 1956). (3) Amino acids were detected on paper by spraying ninhydrin reagent. (4) The development of a magenta color when paper chromatograms were sprayed with an alkaline solution of sodium nitroprusside indicated the presence of thiols (Greenstein and Winitz, 1961). (5) To test the nature of the linkage between the thymine and cysteine, Raney nickel was used (Smith and Aplin, 1966). The products under test were stirred with a 20 times excess of Raney nickel (W. R. Grace & Co.) for 3 hr, filtered, and rechromatographed on the column as described earlier. The products of this procedure were then analyzed. (6) A general method for detection and estimation of disulfides involves reduction to thiols in the presence of zinc dust in dilute acetic acid (Hubbard *et al.*, 1958; Karchmer and Walker, 1958). The reduction procedure was the same as that described by Hubbard *et al.*, 1958. After 1 hr, the suspension was filtered and the filtrate was subjected to column chromatographic separations as described under Methods.

Results and Discussion

The photochemical reactivity of thymine in water solution is enhanced by a factor of 10 in the presence of 10^{-2} M cysteine. The reaction was followed by measuring the loss of the characteristic absorbance of thymine at 264 nm. When about 20% of the absorbance had been lost 10% of it could be recovered by heating the irradiated solution, suggesting the formation of a heat-reversible photoproduct.

Changes in cysteine concentrations in the range four- to tenfold did not produce significant changes in the reaction rates; hence, in our bulk irradiations, a fourfold excess of cysteine was used. Upon irradiation, the solution became gradually yellow (due to colloidal sulfur) and there was evolution of hydrogen sulfide. These reactions were predominant in irradiations involving cystine dihydrochloride instead of cysteine hydrochloride, or higher concentrations of cysteine, and those in which the Vycor jacket was removed from the irradiator, suggesting that photolysis of cysteine or cystine is responsible for the evolution of H_2S and the formation of sulfur. Results of our studies on the identity of thymine-derived products in the irradiated solution are summarized in Table I.

Thymine Dimer, Dihydrothymine, and V. Initial separation of products was achieved by column chromatography. When the column was eluted with water as described under Methods, the unreacted thymine and products which do not contain an amino acid group were eluted. By paper chromatographic analysis, thymine, cyclobutane-type thymine dimer, dihydrothymine, and 5,6-dihydrothyminyl-5-thiol were identified in the water eluate. In experiments where radioactive cysteine was used (and no radioactive thymine) less than 1% of the cysteine activity was found in the water eluate. This suggests that photochemical deamination is a very minor reaction under these conditions.

Thymine–Cysteine Adducts (I–IV). The pattern of radioactivity in the fractions eluted from the column with hydrochloric acid is shown in Figure 1. Three peaks were observed. The ratio of activity in peaks 1, 2, and 3 was 1:3.5:0.7.

Paper chromatographic analysis in different solvents suggested that the radioactivity in peak 1 was due to a single compound. After repeated paper chromatography the purified product was crystallized from alcohol–water (1:1) and was identified as I.

Paper chromatogram (solvent S_1) of peak 2 showed only one radioactive region which appeared as a dark band when viewed under an ultraviolet lamp. However, by repeated chromatography using solvent systems S_1, S_3, and S_4 we were able to isolate these products which were identified as II, III, and IV. Of these only IV survived paper chromatography in the basic system S_2. In fact, paper chromatography of the mixture of II, III, and IV in S_2 is a good way to obtain IV in a relatively pure form.

Proton Magnetic Resonance Spectra. Proton magnetic resonance (pmr) spectra[1] in D_2O at 220 MHz, with tetramethylsilane as the external standard, provided valuable information concerning the structures of the various products. The spec-

[1] Taken at the 220-MHz Nuclear Magnetic Resonance Center Ontario Research Foundation, Sheridan Park, Ontario, Canada.

TABLE I: Products of Thymine Irradiated ($\lambda > 230$ nm) in the Presence of Cysteine.

Product	Assigned Structure	Method of Isolation	Rel Amounts (%)	Ninhydrin for α-Amino Acid	PDAB[a] Test for Dipyrimidine	Raney Nickel for S–C Linkage	Na Nitroprusside for SH Groups	Zn–HOAc for S–S Linkage	Effect of 254-nm Radiation	Remarks
1	Cyclobutane-type thymine dimer	Paper chromatography of water eluate in S_1 (R_F 0.30)	6	−					Reversed to thymine	
2	Dihydrothymine	Paper chromatography of water eluate in S_2 (R_F 0.65)	4		+					
3	VI	Paper chromatography of water eluate in S_1 (R_F 0.40)	2				+			Structure confirmed by pmr spectra
4	I	Peak 1 (Fig. 1) paper chromatography in S_1, S_3, and S_4	15	+	+	Dihydrothymine and alanine	−	−	−	Structure confirmed by pmr spectra
5	II	Peak 2 (Fig. 1) paper chromatography in S_1, S_2, and S_4	30	+	+	Dihydrothymine and alanine	−	−	Yielded thymine and cysteine	Structure confirmed by pmr spectra
6	III	Peak 2 (Fig. 1), paper chromatography in S_1 S_3, and S_4	15	+	+	Dihydrothymine and alanine	−	VI + cysteine	VI + thymine + cysteine	Structure confirmed by pmr spectra
7	IV	Peak 2 (Fig. 1) reheated chromatography in S_2	5	+	−	Thymine and alanine	−	−	−	Structure confirmed by comparison with synthesized sample
8	V									Evidence is based on heat-reversible absorbance of thymine in the acid eluate
9	5,6-Di-*S*-cysteinyl-5,6-dihydrohydrothymine	Peak 3								Based on chromatographic mobility on column

[a] PDAB = dimethylaminobenzaldehyde.

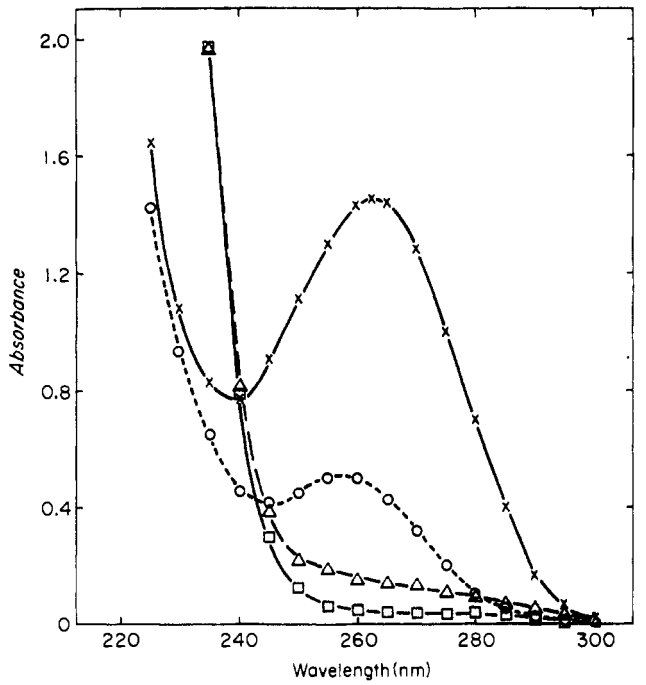


FIGURE 2: Ultraviolet absorption spectra at pH 3 of: a mixture of II and III (○--○); the mixture after exposure to 254-nm radiation (4 × 10^4 J/m²) (×–×); the mixture after treatment with zinc in dilute acetic (□–□); ultraviolet absorption spectrum of II after exposure to 254-nm radiation, 19 × 10^4 J/m² (△—△).

trum of product I showed signals at δ 4.32 (q, 1, HOOC-(H_2N)C*H*CH_2), 3.68, 3.53 (octet, 2, NHC*H*$_2$CH, J_{gem} = 14 Hz, J_{56} = 3, 1.6 Hz), 3.42 (m, 1, CH_2CHCH_2S), 3.32 (octet, 2, CHC*H*$_2$S, J_{gem} = 8 Hz, $J_{\beta 5}$ = 6.5 Hz, $J_{\beta 5}$ = 5.8 Hz), and 3.18 (octet, 2, SC*H*$_2$CH(NH_2)). These signals are consistent with structure I.

The spectra of II and III were virtually identical and were consistent with structures II and III, showing signals at δ 3.70, 3.56 (q, 1, HOOC(H_2N)C*H*CH_2), 3.70, 3.56 (q, 2, NH-*CH*$_2$C(CH_3)S, J_{gem} = 14 Hz), 3.20 (octet, 2, -(H_2N)CHC*H*$_2$S), 1.46 (s, 3, CC*H*$_3$).

In agreement with structure IV, the spectrum of IV showed signals at δ 7.46 (s, 1, NHC*H*=C), 4.32 (q, 1, HOOC(H_2N)-C*H*CH_2), 3.52 (s, 2, =CC*H*$_2$S), and 3.14 (octet, 2, (H_2N)-CHC*H*$_2$S).

The identity of V was confirmed by nmr spectra which showed signals at δ 1.46 (s, 3, CC*H*$_3$) and at 3.68 and 3.54 (q, 2, J = 14 Hz).

Mass Spectroscopy.[2] The very low volatility of these products resulted in spectra lacking molecular ions. Even though the spectra are complex due to extensive fragmentations and rearrangement of fragments of the amino acid and pyrimidine residues, they provide valuable information in support of the proposed structures. The fragmentation patterns (Table II) are indicative of the characteristic patterns established for pyrimidine derivatives (Rice *et al.*, 1965) and amino acids (Biemann *et al.*, 1961). Peaks at *m/e* 34 (H_2S), 44 (CO_2), 74 ($CHNH_2COOH$), and 88 (CH_2CHNH_2COOH) are observed in all three spectra. The intense peaks at *m/e* 55 and 83 are characteristic fragmentation ions of thymine (Rice *et al.*, 1965). Fragmentation of *m/e* 60, which is not observed in II, could be c-$(CH_2)_2S^+$. The peak at *m/e* 186 would be expected

TABLE II: Mass Spectral Data.

	Relative Intensity		
m/e	I	II	IV
34	23	64	42
44	45	8	14
55	35	54	46
60	14		37
69	5	8	2
74	11	6	3
83	36	47	29
88	8	12	14
112			10
122		7	11
125	8	5	100
126	100	100	30
127	26	31	
158			27
160	7	8	
184	2		7
186	4	3	

to arise from the loss of CO_2 (44 mass units) and NH_3 (17 mass units), from the parent ion in I and II. A similar loss from IV would yield the *m/e* unit 184. α cleavages with or without rearrangements are common fragmentation pathways for sulfides (Levy and Stahl, 1961). The olefinic fragment often carries the charge and gives rise to strong peaks. The intense peaks at *m/e* 126 in the spectra of I and II presumably result from α cleavage without rearrangement. Cleavage of the S–C linkage of the amino acid residue with rearrangement explains the peaks at *m/e* 160 and 158. Thus, the mass spectral data agree with the proposed structures for I, II, and IV. It was not possible to get pure samples of III for mass spectral analysis, but the spectrum of a mixture of II and III was not significantly different from that of II.

Ultraviolet Absorption Spectra. Absence of an absorbance maximum in the 240–280-nm region for pyrimidine derivatives is generally taken as indicative of C_5–C_6 saturation. The absorbance spectrum of I did not have a maximum in that region, consistent with structure I. As expected, II showed only end absorption (see Figure 2). II and III showed a peak at 259 nm; since II showed no peak, the observed peak must be due to III with an extinction of about 1100 at 259 nm at pH 3. This is consistent with the known absorption of mono- and disulfides. For example, monosulfides containing C–S–C linkages are known to absorb at about 215 nm. Disulfides absorb at higher wavelengths (λ ~250 nm). This absorbance is attributed to n → σ* transitions and is sensitive to substitution (Sice, 1960; Balasubramanian, 1963). For example, cysteine does not have a maximum above 220 nm while cystine has a maximum at 248 (ε ~350) (Greenstein and Winitz, 1961). Tetraethyldithiodiamine shows a maximum at 258 with an ϵ_{max} ~4000.

Product IV had an absorption maximum typical of pyrimidines: at pH 2, λ_{max} 265 nm (ε 7600); at pH 12, λ_{max} 292 (ε 6900).

To further test the properties of II and III we irradiated II alone and a mixture of II and III at 254 nm. The absorbance at 260 nm increases with dose and after long irradiations a spectrum similar to that of thymine was obtained (see Figure

[2] Mass spectroscopy was carried out at the Ontario Research Foundation, Sheridan Park, Ontario, Canada.

TABLE III: R_F Values of Products.

Compound	Solvent Systems			
	S_1	S_2	S_3	S_4
Thymine	0.60	0.54	0.70	72
Dihydrothymine	0.60	0.77		67
I	0.10		0.11	0.09
II, III	0.12		0.15	0.15
IV	0.12	0.14	0.16	0.14
V	0.45			

2). Chromatography showed that thymine, cysteine, and VI were the major products, consistent with the idea that absorption of a photon leads to the breaking of the C_5–S bond or the S–S bond. We also observed the evolution of H_2S. This finding is of some importance since reversibility is sometimes considered as a test for dimers alone.

Infrared Spectra. The infrared absorption spectra (KBr pellets) of products I and II were quite similar. They showed bands at 1745 (carbonyl), 1730 (amino acid COOH), 1695 (ureidocarbonyl), 1640, 1630, 1495 (NH_3^+), and 1245 cm^{-1}. The infrared absorption of IV was identical with that of a sample which was synthesized following the procedure of Cline *et al.* (1959).

Chromatographic Data. The chromatographic mobilities of thymine–cysteine addition products and other thymine-derived products identified in this study are presented in Table III. Since I, II, and III are unstable in alkali, basic solvents are unsatisfactory for the purification of these products. A pure sample of II could be obtained after the destruction of III and resolution of II and III was not possible in any of the solvent systems. Therefore, the R_F value of III was not determined independently.

Stability in Alkali. Dihydropyrimidines undergo facile ring opening in alkaline solutions, forming urea derivatives. The course of the reaction can be monitored by measuring the loss of absorbance at 240 nm. The rates for alkaline degradation of I, II, and dihydrothymine are presented in Figure 3. It is apparent that all three compounds undergo the same type of reaction with a half-life of less than 6 min. Product IV was stable under these conditions.

Stability in Acid. Thymine–cysteine adducts are stable compounds in acidic solutions. After heating at 100° for 15 min in 6 N HCl, more than 90% of adducts I, II, and IV could be recovered. When heated in trifluoroacetic acid at 155° for 1 hr, less than 25% of these adducts was converted to other products. According to Smith (1970), II is unstable under these conditions. At present we have no explanation for this difference. These observations suggest the possibility of isolating these adducts from ultraviolet-irradiated biological systems. Experiments are in progress in this laboratory to achieve this objective.

Peak 3 (Figure 1). Paper chromatographic analysis (solvent S_1) revealed peak 3 to be a mixture of several compounds. One major (R_F 0.40) and two minor (R_F 0.07 and 0.60) radioactive regions were detected. The major radioactive region developed a yellow color with Erlich's reagent without pretreatment with alkali, and was ninhydrin positive, suggesting that it is probably derived by ring opening of thymine–cysteine adducts. The fact that cystine was also eluted in fractions of peak 3 suggests that the thymine–cysteine adducts of peak 3 are probably derived by the addition of two cysteinyl residues to thymine, analogous to the radiation-induced formation of thymine glycols. Since the amounts were small, chemical characterization was not possible.

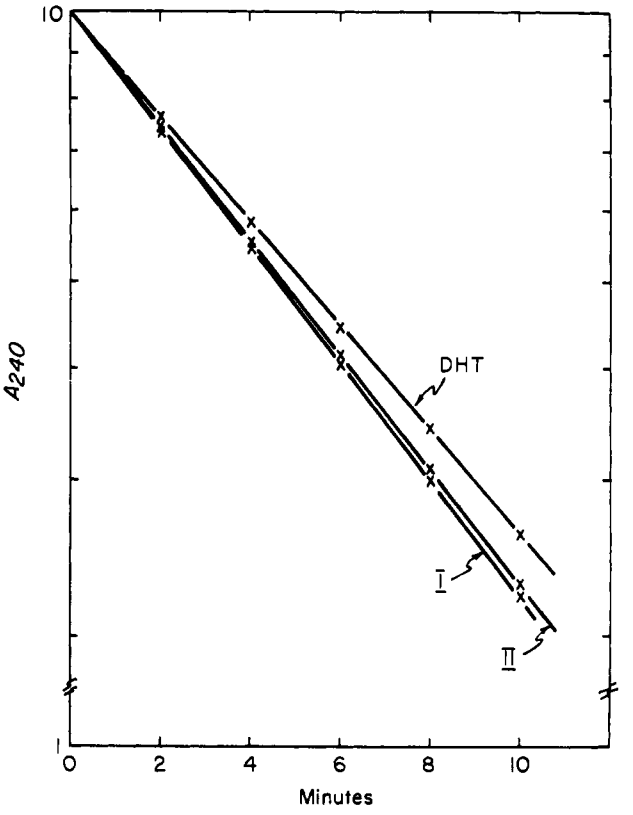
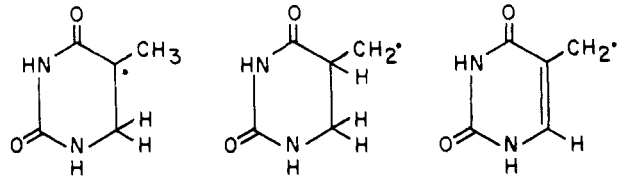


FIGURE 3: Alkaline degradation at pH 12.5 of dihydrothymine, I, and II. The reaction was followed by measuring the loss of absorbance at 240 nm as a function of time. A_{240} represents $A_t - A_\infty$.

6-S-Cysteinyl-5,6-dihydrothymine (VI). Irradiation of thymine in the presence of cysteine results in a large absorbance loss, 10% of which could be recovered by heating, suggesting a heat reversible product. We also have the following evidence for the formation of acid-reversible thymine–cysteine adducts. The irradiated solution was applied on the column and the column was eluted with water until the eluate had neither radioactivity nor absorbance maximum above 230 nm. Subsequently, the column was eluted with 2 N hydrochloric acid and the analysis of this acid eluate showed the presence of thymine, and the radioactivity in the thymine region amounted to about 7% of the total activity on the chromatogram. Since I to IV are not heat or acid reversible, the heat-reversible absorbance changes of the irradiated solution and the presence of thymine in the acid eluate can be attributed to an unstable product. An obvious candidate is the 6-*S*-cysteinyl derivative (VI) which might be expected to be similar in stability to the hydrate. It is known that 5,6-hydroxy derivatives (photohydrates) of thymine and uracil readily regenerate the bases under the influence of acid or heat (for a review see Fisher and Johns, 1973). On the other hand, the corresponding 5,6-dihydro-5-hydroxy derivatives are stable compounds.

Cysteine Photoproducts. In addition to thymine-derived products, a number of compounds derived from photolysis of cysteine were identified. They include sulfur, hydrogen sulfide, cysteine, alanine, lanthionine, and bis(2-amino-2-carboxyethyl) polysulfides (Asquith and Shah, 1971). These results will be discussed in a subsequent paper.

Mechanism. The formation of species VII–IX from thymine appears to be the primary event in the photochemical addition of thymine and cysteine. A detailed discussion on the mechanism will be presented in a separate paper.



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Biological Implications. From Smith's investigations, it is clear that cross-linking of DNA and protein play a role in the killing of bacterial cells by ultraviolet light, and the addition of cysteine to pyrimidine bases is one possible model for the cross-linking. The present study shows that thymine and cysteine form different types of photoaddition products, all apparently having C–S linkages. We have evidence (unpublished data) that the relative amounts of the different products depend to a considerable extent on the irradiation conditions and the presence of other molecules. Recently, Gorelic *et al.* (1972) have shown that primary amines add to 1,3-dimethyluracil, forming photoadducts having C–C and C–N linkages between the pyrimidine and the amine. All these results suggest that the study of the chemical nature of radiation-induced cross-linking of nucleic acids and protein is a complex problem and may involve several types of products.

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