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A Mixed Photoproduct of Uracil and Cysteine (5-*S*-Cysteine-6-hydrouracil). A Possible Model for the *in Vivo* Cross-Linking of Deoxyribonucleic Acid and Protein by Ultraviolet Light*

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ABSTRACT: A heterodimer of uracil and cysteine (5-*S*-cysteine-6-hydrouracil) has been produced by irradiating a solution of uracil-2-^{14}C (2.8×10^{-3} M) and cysteine-HCl (10^{-2} M) with ultraviolet light (2537 A). A simple technique for the bulk isolation of this photoproduct using ion-exchange chromatography and cuprous oxide precipitation of free cysteine is described. The heterodimer is not ultraviolet absorbing, is ninhydrin positive, and contains sulfur as judged from experiments using cysteine-^{35}S. The structure of this photoproduct was determined using ultraviolet, infrared, nuclear magnetic resonance, and mass spectroscopy. Treating the photoproduct with deuterated Raney nickel yields 5-monodeuteriodihydrouracil, thus confirming the point of attachment of the cysteine. Raney nickel treatment also yields alanine. 5-*S*-Cysteine-6-hydrouracil–HCl is stable to heat (100°) in water solution and is stable to 6 N HCl at room temperature but is not stable to the heat and acid conditions used for the hydrolysis of deoxyribonucleic acid (DNA). It is quite unstable to alkali. R_F values for this heterodimer in several solvents are tabulated.

5-*S*-Cysteine-6-hydrouracil may serve as a model for the mechanism by which DNA and protein are cross-linked *in vivo* by ultraviolet irradiation. The photochemical addition of cysteine-^{35}S to polyuridylic acid, polycytidylic acid, and DNA lends support to this hypothesis.

Since the discovery of the cross-linking of DNA and protein by ultraviolet light (Smith, 1962; Alexander and Moroson, 1962), we have been searching for the chemical mechanism by which this interaction takes place. Originally, we had tried to form heterodimers (terminology suggested by Wang, 1965) between thymine and the several aromatic amino acids by irradiation of these mixtures in frozen solution. These attempts have thus far proved unsuccessful. More recently, we have reasoned that the cross-linking of deoxyribonucleic acid (DNA) and protein could be accomplished by the addition of the OH groups of serine, tyrosine, etc., or the sulfhydryl (SH) group of cysteine to cytosine (or uracil), analogous to the photochemcial addition of the OH group of water to the 6 position of uracil (Sinsheimer and Hastings, 1949; Moore, 1958). Consistent with this postulate is the fact that if uracil is irradiated in anhydrous alcohol, the alcohol adds to the 5–6 double bond of uracil (K. C. Smith, unpublished data) and to dimethyluracil (Moore and Thomson, 1956; Wang, 1961). The water addition product of uracil is labile to heat and changes in pH, but it would be expected that if a protein were joined to

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a DNA molecule through several of these hydration bridges a rather firm binding might result.

We have tested this hypothesis by irradiating uracil-2-^{14}C in solution with ultraviolet light (2537 A) in the presence of several OH and SH amino acids and looking for the formation of heterodimers. Thus far, our experiments with the OH amino acids have been negative but those with SH derivatives have been successful. This paper describes the formation and properties of a heterodimer of uracil and cysteine. The amino acid, however, adds to the 5 position of uracil rather than to the 6 position with the formation of 5-*S*-cysteine-6-hydrouracil. Cysteine-^{35}S also adds photochemically to polyuridylic acid (poly U), polycytidylic acid (poly C), and to DNA, thus adding credence to our postulate that a heterodimer formed between a pyrimidine and a sulfur (or hydroxy) amino acid may constitute a mechanism for the photochemical cross-linking of DNA and protein *in vivo* (Smith, 1962, 1964b; Smith *et al.*, 1966), and possibly the cross-linking of ribonucleic acid (RNA) and protein in ultraviolet-irradiated tobacco mosaic virus (TMV) (Goddard *et al.*, 1966).

Results and Discussion

Photochemical Reaction of Uracil in the Presence and Absence of Cysteine. In the presence of cysteine *ca.* 25% of the uracil-2-^{14}C was converted to a material having an R_F of 0.01 in butyl alcohol–water (86:14) (Table I). That this material might be a heterodimer of uracil and cysteine was first demonstrated by the fact that it was retained by a Dowex 50 column (indicating that the uracil skeleton must now contain a basic group). Cysteine, of course, was also retained by this column. The heterodimer of uracil and cysteine could be recovered by eluting the column with 2 N HCl (see below).

The heterodimer gave a positive reaction with ninhydrin and exhibited bands in its infrared spectrum (KBr disk) at 2900–2500 (acid OH) and 1740 cm^{-1} (C=O) typical of a free carboxyl group in an α-amino acid hydrochloride, suggesting that the carboxyl and amino groups of the cysteine residue were free. This implied that the linkage to the uracil skeleton was through the sulfur bond. Direct proof that sulfur was present in the photoproduct came from experiments with cysteine-^{35}S.

Using cysteine-^{35}S (Volk Radiochemical Co.) and unlabeled uracil, only one radioactive photoproduct was produced and this had the same R_F (0.1) in butyl alcohol–acetic acid–water (80:12:30) as the cysteine–uracil heterodimer produced using uracil-2-^{14}C and unlabeled cysteine (Table II). Their identity was further confirmed by eluting and resubmitting to chromatography the ^{14}C- and ^{35}S-labeled photoproducts both separately and as a mixture. In all cases, the R_F values were identical. The presence of sulfur, a free

TABLE I: Photochemical Interaction of Uracil-2-^{14}C and Cysteine.[a]

Identity of Products	R_F in Butyl Alcohol–Water (86:14)	
	Uracil Only	Uracil + Cysteine
Uracil–cysteine heterodimer	...	0.01
Uracil dimer	0.03	...
?	...	0.08
?	...	0.17
Water addn product of uracil	0.23	0.24
Dihydrouracil	...	0.29
Uracil	0.35	0.36
?	...	0.51
?	...	0.61

[a] Uracil-2-^{14}C (Calbiochem; 5 μc/ml and 0.513 mg/ml) was mixed with an equal volume (0.1 ml) of water or of cysteine–HCl (Eastman; 0.02 M) and was irradiated with ultraviolet light (2537 A) for 60 min at *ca.* 5 cm from a Mineralight Lamp (Ultraviolet Products, Inc.). The total samples were spotted on 1.5-in. strips of Whatman No. 1 paper and chromatographed (descending) in *n*-butyl alcohol–water (86:14) for *ca.* 18 hr. The strips were photographed with ultraviolet light (Smith and Allen, 1953), and the distribution of radioactivity determined using a 4-π strip scanner and automatic data system (Vanguard Instruments, Inc.).

TABLE II: Photochemical Interaction of Uracil-2-^{14}C and Cysteine-^{35}S.[a]

Identity of Products	R_F in Butyl Alcohol–Acetic Acid–Water (80:12:30)			
	Uracil-2-^{14}C + Cysteine		Cysteine-^{35}S + Uracil	
	No Ultraviolet	+ Ultraviolet	No Ultraviolet	+ Ultraviolet
Impurity	...	...	0.06	0.06
Uracil–cysteine heterodimer	...	0.11	...	0.11
Impurity	0.15	0.15	...	...
Cysteine	...	...	0.29	0.29
?	...	0.33		
Water addn product of uracil	...	0.38		
Uracil and dihydrouracil	0.50	0.51		

[a] Cysteine-^{35}S (3.4 mg/ml of water; Volk Radiochemical Co.; 1 mc/17 mg) was used as indicated; otherwise the conditions were the same as used in Table I.

carboxyl group, and a positive ninhydrin reaction suggested that the entire cysteine molecule was combined with the uracil. This fact was further confirmed by nuclear magnetic resonance and mass spectral data (see below).

Bulk Isolation of the Heterodimer of Uracil and Cysteine. A solution (1 l.) containing uracil-2-^{14}C (2.8 mM uracil plus 100 μc of uracil-2-^{14}C at 22.7 mc/mM; Calbiochem) and cysteine-HCl (10 mM; Eastman) was irradiated in 100-ml batches for 60 min in a plastic pan (3.25 × 12.75 in.), the bottom of which was *ca.* 0.5 in. from the filters (Corning no. 9863) covering a Chromato-Vue Lamp (Ultraviolet Products, Inc.) containing two 25-w General Electric germicidal lamps. The pooled irradiated solution was poured through a 10-ml column (0.8 cm in diameter) of Dowex 50 (HCl) at a flow rate of *ca.* 0.5 ml/min. All of the ninhydrin-positive material was retained on the column. (The flow-through solution which contained uracil and some photoproducts of uracil could again be made to 0.01 M cysteine and irradiated as above). The column was washed with several volumes of water and then the uracil–cysteine dimer and free cysteine were eluted from the column with *ca.* 50 ml of 2 N HCl. The eluate was taken to dryness in a rotary evaporator, taken up in 0.5 N H_2SO_4, and treated with cuprous oxide (Hopkins, 1929) to precipitate the free cysteine. The resulting supernatant was concentrated under reduced pressure, streaked on sheets of Whatman no. 1 paper, and chromatographed in *n*-butyl alcohol–acetic acid–water (80:12:30; Smith, 1963). The area containing the uracil–cysteine dimer was located by the ultraviolet absorption of an impurity that cochromatographs with the dimer. This area was cut out, eluted from the paper with 0.01 N HCl, and concentrated under vacuum. The photoproduct was dissolved in a small volume of water and treated with H_2S, filtered, evaporated, and again dissolved in a few milliliters of water; *ca.* 20 mg of photoproduct was recovered. The photoproduct could be crystallized by making the solution to 50% methyl alcohol and holding at 4°.

TABLE III: R_F of Two Times Crystallized 5-*S*-Cysteine-6-hydrouracil.

Solvent	R_F
n-Butyl alcohol–water (86:14)	0.01
n-Butyl alcohol–acetic acid–water (80:12:30)	0.09
sec-Butyl alcohol saturated with water	0.11
Isopropyl alcohol–acetic acid–water (60:30:10)	0.20
n-Butyl alcohol–acetic acid–water (40:12:30)	0.41
Methyl alcohol–HCl–water (70:20:10)	0.7

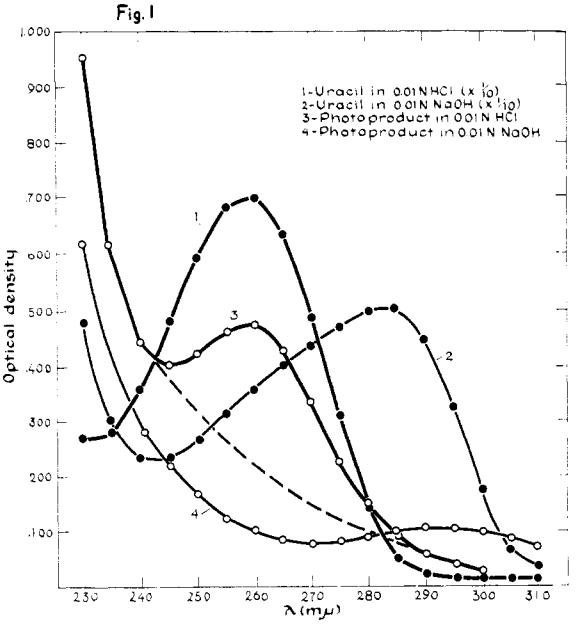


FIGURE 1: Ultraviolet absorption spectra of the heterodimer of uracil and cysteine. Areas containing uracil-2-^{14}C and the photoproduct (labeled with uracil-2-^{14}C) were cut out and eluted from chromatograms that had been developed in butyl alcohol–acetic acid–water (80:12:30). The eluates were treated with H_2S to remove metals, clarified with Norit, filtered, and evaporated under vacuum. The samples were dissolved in water and then made either to 0.01 N HCl or to 0.01 N NaOH and their spectrum determined in a Model DU, Beckman spectrophotometer. The ^{14}C content of the solutions was then determined in a liquid scintillation counter (Nuclear Chicago) and the spectral data for the photoproduct were normalized to that for a solution of uracil containing the same amount of radioactivity.

Chromatography of Crystallized Photoproduct in Several Solvents. Although there was no obvious impurity present when the two times crystallized photoproduct was chromatographed in several solvents (Table III), the tracings of the radioactive areas were never as sharp as that observed for a truly homogeneous compound. The radioactive areas had a very slight amount of ultraviolet absorption (at 2537 A) and were strongly ninhydrin positive.

Ultraviolet Absorption Spectra of the Heterodimer of Uracil and Cysteine. Although the photoproduct (not crystallized) shows a small amount of ultraviolet absorption at 260 mμ (Figure 1), the major amount of the photoproduct material (97%) must be a dihydro derivative of uracil. If one substracts from the absorption curve of the photoproduct (in HCl) the expected absorption curve for a dihydro derivative (dotted line in Figure 1), the remainder of the absorption at 260 mμ is only *ca.* 3% of that shown by the same concentration of uracil.

After Raney nickel treatment of the photoproduct

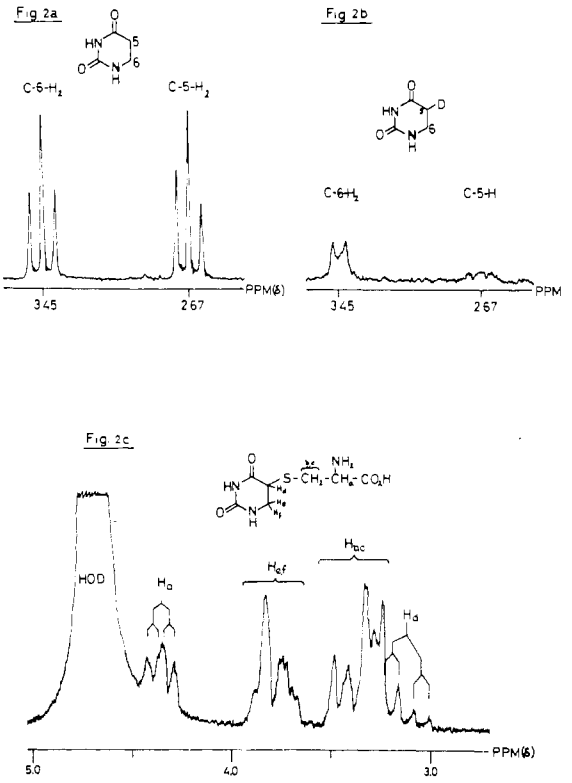
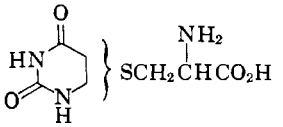


FIGURE 2: Nmr spectra (100 Mc) of dihydrouracil (a), 5-D-dihydrouracil (b), and heterodimer of uracil and cysteine (c). Spectra of D_2O solutions containing a trace of CF_3CO_2H were recorded using a Varian HR-100 nmr spectrometer with dimethylsilapentanesulfonic acid sodium salt as internal reference.

(see below), a small amount of uracil (3%) was recovered. The slight ultraviolet absorption shown by the impure photoproduct may, therefore, be due to the presence of a small amount of a *S*-cysteine derivative of uracil with the 5–6 double bond intact. Such a compound of uracil and cysteamine (5-*S*-cysteamineuracil) was formed when a solution of cystamine and iodouracil was irradiated (Rupp and Prusoff, 1965). 6-*S*-Cysteineuracil, which is strongly ultraviolet absorbing, can be prepared by mixing 6-uracil methyl sulfone (a gift of Dr. Robert E. Handschumacher) with cysteine (Cooper, 1958). This compound has an R_F similar to that of our photoproduct in butyl alcohol–acetic acid–water (80:12:30) (Table III).

Raney Nickel Desulfurization. If the cysteine–uracil heterodimer is the 5- (or 6-) *S*-cysteine- 5- (or 6-) hydrouracil (I) as the evidence suggests, then treatment of this compound with Raney nickel should yield dihydrouracil and alanine. A small sample of the uracil-2-^{14}C-labeled heterodimer (not crystallized) was treated with *ca.* 10 times its weight of Raney nickel (W. R. Grace Co.) for 3 hr at room temperature. After removing the Raney nickel by filtration, the sample was taken to dryness under vacuum, spotted on a 1.5-in. strip of Whatman no. 1 paper, and chromatographed in butyl alcohol–acetic acid–water (80:12:30). The ninhydrin-positive spot appeared at R_F 0.22. Alanine has an R_F of 0.21; serine, 0.14; cysteinesulfinic acid, 0.10; cysteic acid, 0.07; and cysteine, 0.28. The major radioactive peak (80%) appeared at R_F 0.45 (a minor peak at 0.58), and gave a positive test for dihydropyrimidines (Fink *et al.*, 1956; Cline and Fink, 1956). Dihydrouracil has an R_F of 0.46 and uracil 0.49.

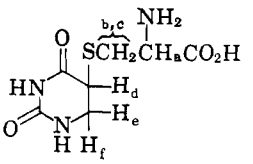


I

These results confirm our expectation of the structure of the heterodimer of uracil and cysteine but do not allow us to assign the position of attachment of the cysteine on the pyrimidine ring. However, repeating this experiment with deuterated Raney nickel should yield dihydrouracil containing one atom of deuterium. The position of the deuterium (and therefore of the sulfur atom in the original photoproduct) should be easily located by nuclear magnetic resonance (nmr) spectroscopy.

Nuclear Magnetic Resonance Spectroscopy. About 10 mg of the photoproduct (not crystallized) was dissolved in D_2O and treated with deuterated Raney nickel (Williams *et al.*, 1963) as described above. The filtrate was chromatographed in butyl alcohol–water (86:14). This solvent gives a better separation of uracil (R_F 0.34) and dihydrouracil (R_F 0.28). The deuterated dihydrouracil area was cut out, eluted with water at 60°, treated with H_2S (no precipitate was obtained), filtered, and taken to dryness under reduced pressure.

Comparison of the nmr spectrum of the deuterated dihydrouracil (Figure 2b) with that of dihydrouracil (Figure 2a) shows that the two-proton triplet (2.67 ppm) due to the C_5 hydrogens (Figure 2a) has collapsed to a one-proton multiplet (triplet of triplets due to germinal coupling with the deuterium) which demonstrates that the deuterium atom is attached to C_5. Furthermore, the low-field two-proton triplet (3.45 ppm) due to the C_6 hydrogens (Figure 2a) has collapsed to a two-proton doublet (3.45 ppm) since the signal is now only coupled to one proton at C_5. These results unambiguously establish the site of the attachment of the sulfur atom to C_5 and the structure of the photoproduct as 5-*S*-cysteine-6-hydrouracil (II).



II

The finding that cysteine adds to the 5 position of uracil was unexpected since we had predicted that it should add to the 6 position analogous to the addition of the OH group of water (Sinsheimer and Hastings,

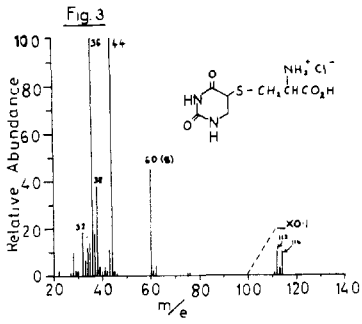


FIGURE 3: Mass spectrum of the heterodimer of uraci and cysteine as the hydrochloride. An Associated Electrical Industries MS9 mass spectrometer was used. The source temperature was 230°.

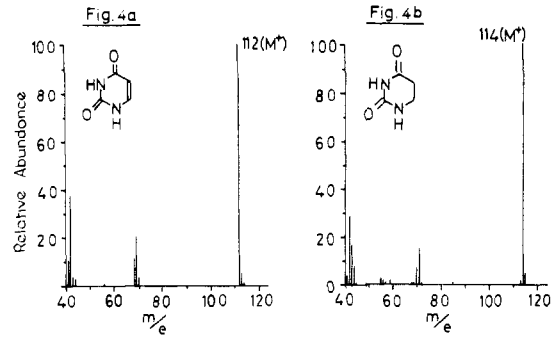
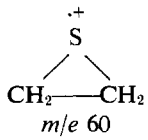


FIGURE 4: Mass spectrum of uracil and dihydrouracil at source temperature of 140°.

1949; Moore, 1958). Uridylic acid has been shown to be selectively reduced to dihydrouridylic acid when irradiated with ultraviolet light in the presence of sodium borohydride (Cerutti *et al.*, 1965). It is, therefore, of interest that the hydrogen of the metal hydride adds to position 5 rather than to position 6 (B. Witkop, personal communication). Dihydrouracil is also produced in significant amounts when uracil is irradiated in the presence of SH compounds (cysteine, cysteamine, and H_2S).

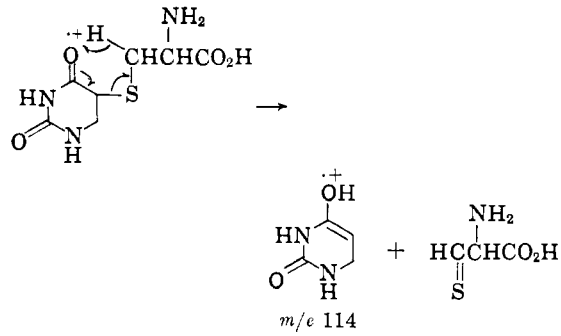
The 100-Mc nmr spectrum of the heterodimer (Figure 2c) shows three groups of signals in the ratio of 1:2:3. These can be assigned to H_a, $H_{e,f}$, H_{b-d} (II) on the basis of their chemical shifts compared with those of the analogous protons in dihydrouracil (Figure 2a) and cysteine (Martin and Mathur, 1965). These assignments were confirmed by nuclear magnetic double resonance studies. H_a is coupled to two protons of the high-field group ($H_{b,c}$), and H_d is coupled to one of the midfield protons either H_e or H_f. The small amount of material available and its low solubility precluded a more detailed interpretation of the nmr spectrum. These results are also in complete agreement with the assigned structure (II) for the heterodimer.

Mass Spectroscopy. The very low volatility and thermal lability of the adduct resulted in a spectrum (Figure 3) lacking a molecular ion. The only peaks of importance in the spectrum, other than those at *m/e* 32 (S), 36 ($H^{35}Cl$), 38 ($H^{37}Cl$), and 44 (CO_2) which arise from the cysteine portion, are at *m/e* 60 (C_2H_4S), 112, and 114. The latter pair correspond to the molecular ions of uracil and dihydrouracil respectively (Figure 4a and b). The appearance of these fragments confirms



m/e 60

the results obtained in the labeling experiments. The *m/e* 60 fragment suggests that the thiol group of cysteine has added to the 5–6 double bond of uracil to give an adduct of the type I. The ion of mass *m/e* 112 is probably formed by thermal decomposition. The formation of the *m/e* 114 fragment can best be rationalized in terms of a 5-*S*-cysteine-6-hydrouracil structure as shown



m/e 114

Effect of Heat, Acid, and Alkali on the Photoproduct. Irradiated mixtures of uracil-2-^{14}C and cysteine (see footnote to Table I) were sealed in melting point capillaries and heated at 70 and 100° for 15 min. After cooling, the mixtures were chromatographed and the amount of heterodimer present was determined. The results in each case were quantitatively identical with those for the unheated control sample. The uracil–cysteine photoproduct is therefore much more stable than the photochemically produced water addition product of uracil (6-hydroxy-5-hydrouracil). It is perhaps pertinent to mention, however, that 5-hydroxy-6-hydrouracil (Fischer and Roeder, 1901) and the corresponding derivative of uridine (Wang, 1962) are not reversed to uracil or uridine by heat.

The uracil-^{14}C–cysteine heterodimer can be dissolved in 6 N HCl at room temperature and taken to dryness in a vacuum desiccator without alteration. Elution from a Dowex 50 column in 2 N HCl and removal of the HCl in a rotory evaporator is one step in the bulk isolation of this product. However, when a sample of the photoproduct was treated with trifluoroacetic acid at 155° for 60 min (Dutta *et al.*, 1956), conditions used to isolate thymine dimers and free bases from DNA (Smith, 1964a), this product was almost completely changed to an unidentified material (R_F 0.2 in butyl

alcohol–acetic acid–water, 80:12:30). There seems little chance therefore of isolating this photoproduct from irradiated cells that have been acid hydrolyzed; however, if the R_F 0.2 material is a unique compound, it might provide secondary evidence for the existence of the primary photoproduct.

Standing overnight at room temperature in 0.01 N NaOH had a slight effect on the photoproduct but treatment with 0.1 N NaOH almost completely destroyed the product with the formation of several minor products and one major one at R_F 0.18 (ninhydrin negative). Treatment of the photoproduct with concentrated NH_4OH overnight almost completely converted it to a material with an R_F of 0.22. This product was ninhydrin negative but did give a positive reaction with *p*-dimethylaminobenzaldehyde, a reagent used to detect dihydropyrimidines and ureido acids (Fink *et al.*, 1956; Cline and Fink, 1956). Although they have similar R_F values, it has not been determined if these several products produced by trifluoroacetic acid, NaOH, and NH_4OH are the same or different.

Preliminary Experiments with Cytosine, Cytidine, Uridine, Thymine, Poly U, Poly C, and DNA. Cytosine appears to be photochemically inert when irradiated in strong acid solution with or without cysteine present. However, uracil was detected when cytosine was irradiated in neutral solution and acidified after irradiation. When cytosine was irradiated in the presence of cysteine at pH 5 a new derivative of cytosine was formed in low yield (R_F 0.09 in butyl alcohol–acetic acid–water, 80:12:30). This product had about the same R_F as 5-*S*-cysteine-6-hydrouracil. At pH 9 (at this pH cysteine does not persist but is rapidly oxidized to cystine) another derivative of cytosine was formed (R_F 0.04).

When uridine and cysteine were irradiated together, using either uridine-^{3}H (New England Nuclear Corp.) or cysteine-^{35}S (Volk Radiochemical Co.), photoproducts were produced which stuck to a Dowex 50 column. These photoproducts could be eluted from the column with HCl and had R_F values in *sec*-butyl alcohol saturated with water of 0.0 and 0.07 regardless of which compound was labeled. These results are suggestive of the formation of heterodimers. Similar R_F values were obtained when cytidine was irradiated in the presence of cysteine-^{35}S. This would suggest that deamination of cytidine had occurred during the formation of the heterodimers.

The presence of cysteine greatly accelerated the photochemical alteration of thymine-2-^{14}C in solution and stimulated the production of new photoproducts; however, the presence of thymine had no apparent effect upon the photochemistry of cysteine-^{35}S. This catalytic action of cysteine on the photochemical reactivity of thymine deserves further investigation. The catalytic action of cytosine on the photochemistry of bromouracil has been described (Smith, 1963).

When poly U, poly C, or DNA were irradiated with ultraviolet light (2537 A) in the presence of 0.01 M cysteine-^{35}S (at pH 5) there was a dose-dependent increase in the amount of radioactivity associated with the polymer. The polymers were separated from the monomer on columns of Bio-Gel P-2 (Bio-Rad Laboratories). These results will be published elsewhere.

Acknowledgments

We wish to thank Dr. Lois J. Durham for running the nmr spectra and to acknowledge the excellent technical assistance of Mr. Dieter H. C. Meun.

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