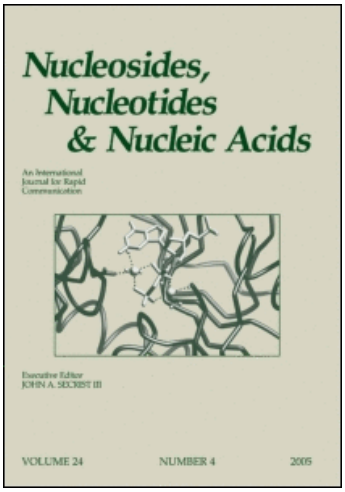

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Novel Modification of 5-Formyluracil by Cysteine Derivatives in Aqueous Solution

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NOVEL MODIFICATION OF 5-FORMYLURACIL BY CYSTEINE DERIVATIVES IN AQUEOUS SOLUTION‡

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ABSTRACT: Reactivities of 5-formyl-2'-deoxyuridine (fdU) and its 5'-monophosphate (fdUMP) to amino acids, amines and thiol compounds in neutral aqueous solution have been studied to elucidate the postmodification of the 5-formyluracil (fU) moiety in cells. fdU and fdUMP specifically reacted with cysteine and its analogs to form thiazolidine derivatives. The reaction involved condensation of the formyl group of fU with both α-NH_2 (or NH_2 at the equevalent position) and SH groups of cysteine derivatives.

5-Formyluracil (fU) is an oxidative thymine damage formed by ionizing radiation[1-3] as well as photochemical sensitization[4,5]. Similar to the other thymine damages[6-10], fU or its deoxyribonucleoside (5-formyl-2'-deoxyuridine, fdU) exhibits cytotoxic and mutagenic effects on cells[2,11-14]. The mechanism of the former effect appears to involve inhibition of thymidylate synthetase and thymidine kinase responsible for intracellular metabolism of thymidine 5'-monophosphate[12-14]. The mutagenic mechanism of exogenously added fdU is not fully clarified[2], but recent *in vitro* studies indicate that mispairing between fU and guanine during DNA replication accounts for the mutagenic effect[15,16]. In this case, fU can be either in a dNTP form or a templating base. Furthermore, it has been also shown that the ionized or enolate form of fU participates in the misparing with guanine[16,17].

In addition to the mechanisms described above, it has been suggested that the formyl group in fU reacts with cellular molecules generating secondary damages such as crosslinks, which are also assumed to be responsible for the cytotoxic and/or mutagenic effects of this damage[2,15]. However, so far no systematic studies have been performed on

‡This paper is dedicated for the late Professor Tsujiaki Hata.

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the reactions between fU and cellular molecules in aqueous solution. In this study, reactions of fdU and its 5'-monophosphate (fdUMP) with amino acids, amines, and thiol compounds were investigated to obtain a defined picture of the possible postmodification of the fU moiety by cellular molecules.

MATERIALS AND METHODS

5-formyluracil (fU), 5-formyl-2'-deoxyuridine (fdU), and 5-formyl-2'-deoxyuridine 5'-monophosphate (fdUMP) were synthesized according to the reported methods[16,18,19], and their structures were confirmed by ^{1}H-, ^{13}C-NMR and UV spectra. 5-hydroxymethyluracil was purchased from Sigma and 5-hydroxymethyl-2'-deoxyuridine was prepared by $NaBH_4$ reduction of fdU. Other chemicals including amino acids (all in an *L*-form), amines, and thiol compounds were obtained from Wako, TCI, or Sigma.

Chromatographic analysis of reaction mixtures was performed using an HPLC system consisting of Jasco PU-980 pumps, a UV-975 detector, and an 807-IT integrator, equipped with an Inertsil ODS-2 column (4.6 x 150 mm, GL Sciences). Samples were eluted by a gradient of acetonitrile in 50 mM ammonium formate with a flow rate 0.8 ml/min (For more details, see figure legends). Eluents were monitored by UV absorption at 260 nm. NMR spectra were recorded on a JEOL GXS-500 spectrometer at 500 MHz. In all measurements, HDO signals at 4.8 ppm were suppressed by irradiation and chemical shifts were corrected to external TSP-d_4.

To study reactions of fdU and fdUMP with amino acids, amines, and thiol compounds, fdU or fdUMP (1 mM) was incubated with an additive (5 mM) in phosphate buffer (20 mM, pH 7.0) at room temperature. After appropriate incubation periods, the reaction mixture was subjected to HPLC and UV analyses. For NMR measurements, fdU and an additive (33 mM each) were incubated in D_2O containing phosphate buffer (100 mM, pD 7.0) for 30 min-8 h and subjected to NMR measurements as described above.

RESULTS AND DISCUSSION

Effects of Amino Acids, Amines and Thiol Compounds on fdU Conversion.

fdU was incubated with or without the additives listed in TABLE 1. The time course of fdU conversion and reaction products were analyzed by HPLC for up to 20 days of incubation. Typical time courses of fdU conversion in the presence and absence of the additives are shown in FIG. 1. In the absence of the additives, fdU underwent spontaneous slow decomposition with a half-life of 17 days. HPLC analysis of the reaction products using authentic markers revealed that a major product was 5-formyluracil derived from hydrolysis of the N-glycosidic bond. This product accounted for nearly 80%

TABLE 1. Effects of amino acids, amines and thiol compounds on the conversion of fdU.

Additives		fdU Conversion*
amino acid	arginine	-
	cysteine	+++
	cysteine methyl ester	+++
	N-acetylcysteine	-
	glutathione	-
	methionine	-
	lysine	-
	N^{α}-acetyllysine	-
	serine	-
amine	aminoethanol	-
	spermidine	+
SH compound	mercaptoethanol	+
	dithiothreitol	+
	cysteamine	+++

*Effects on the decomposition rate of fdU were sorted into three groups: strong enhancement with an fdU half-life ca. 2 h (+++), slight enhancement with a half-life ca. 13 days (+), no or inhibitory effects with a half-life more than 20 days (-).

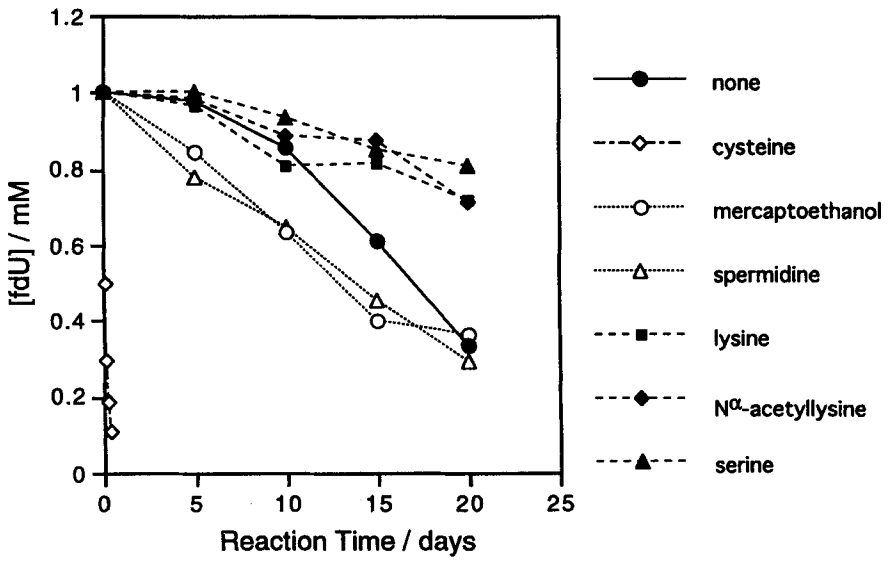

FIG. 1. Time courses of fdU conversion in the presence and absence of amino acids, amines and SH compounds. fdU (1 mM) and an additive (5 mM) were incubated in phosphate buffer (pH 7.0) and the conversion of fdU was determined by HPLC.

of converted fdU. Under these conditions, depyrimidination of thymidine was virtually negligible, indicating that substitution of the electron-withdrawing formyl group promotes hydrolysis of the N-glycosidic bond. The amino acids, amines and thiol compounds added in the reaction mixture differentially affected the decomposition rate of fdU. These additives were able to be sorted into three groups according to their reactivities to fdU. The first group including cysteine showed an extremely high reactivity to fdU so that the half-life of fdU was reduced to 1/200 (ca. 2 h) relative to that without additives (17 days, FIG. 1). The second group including mercaptoethanol and spermidine also promoted the decomposition of fdU, but the reduction factor of the half-life was only 0.76-fold (half-life ca. 13 days). The third group including lysine, N^{α}-acetyllysine, and serine exhibited no or even inhibitory effects on the decomposition of fdU. The effects of all additives tested in this study were divided into the three groups and are summarized in TABLE 1.

As revealed by HPLC analysis, reactions of fdU with the additives in the second and third groups resulted in complex mixtures of products. The yield of released 5-formyluracil was much lower than that observed for fdU alone. These results suggest that in the presence of the second and third groups of additives, decomposition of fdU proceeds in multiple pathways including simple hydrolysis of the N-glycosidic bond and other reactions that presumably involve interactions between the additives and the base moiety. It is likely that these interactions promote or retard further decomposition of fdU depending on the nature of the interaction.

Reactions of fdU and fdUMP with Cysteine Derivatives.

Since cysteine showed an exceptionally high reactivity to fdU, more detailed studies were carried out to elucidate the reaction mechanism. FIG. 2A and 2C show the HPLC chromatogram and UV spectrum of the fdU-cysteine mixture reacted for 8 h, respectively. As seen in FIG. 2A, a single major product (peak 2) was formed in this reaction. Comparison of the HPLC retention times of this product and authentic markers revealed that the product was none of 5-formyluracil, 5-hydroxymethyluracil, or 5-hydroxymethyl-2'-deoxyuridine produced by hydrolysis of the N-glycosidic bond or reduction of the formyl group. The time courses of the fdU conversion and formation of the major product determined by HPLC are shown in FIG. 2D. The UV spectrum of the reaction solution (FIG. 2C) indicated that the product had an absorption maximum around 268 nm with $\varepsilon_{268} \approx 8000$, assuming an almost quantitative conversion of fdU to the product. These UV spectral features are quite different from those of fdU having a conjugated exocyclic chromophore (λ_{max} = 280 nm, ε_{280} = 13400) and resemble those of thymidine (λ_{max} = 267 nm, ε_{280} = 8400).

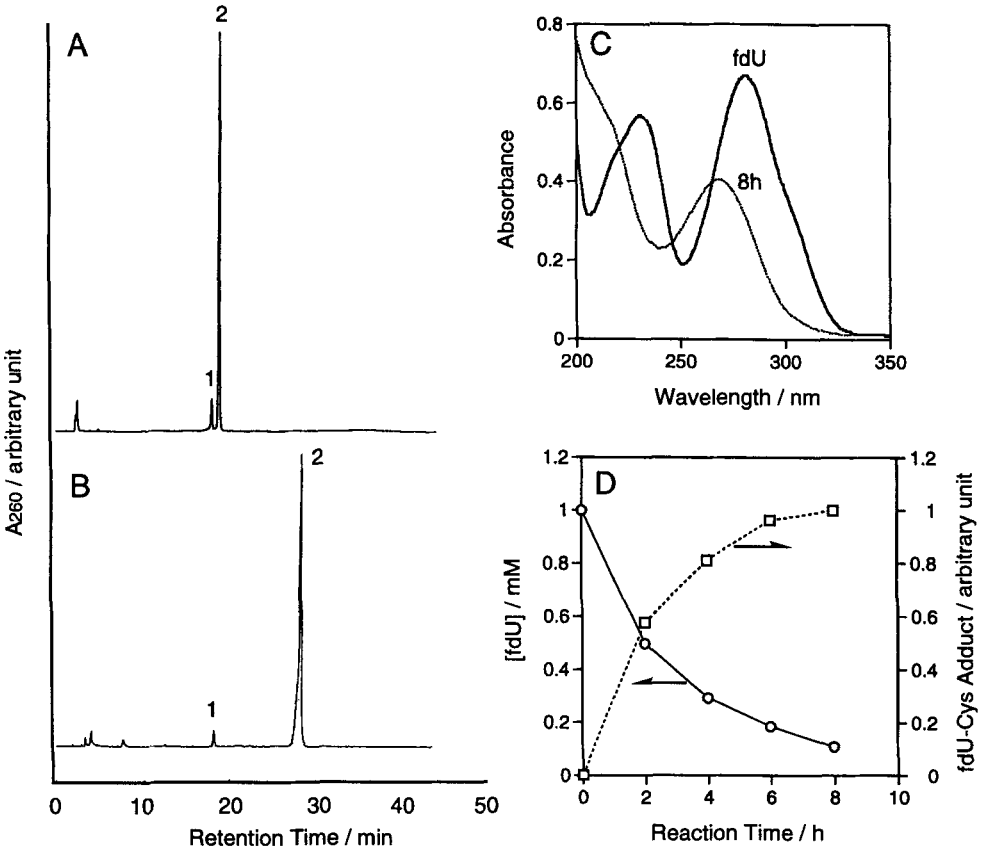

FIG. 2. Reaction of fdU with cysteine and cysteamine. (A) HPLC profile of an fdU-cysteine mixture incubated for 8 h. Peaks 1 (17.9 min) and 2 (18.8 min) were fdU and the major reaction product, respectively. The following gradient of acetonitrile in 50 mM ammonium formate was used: 0-8 % (0-30 min), 8-0 % (30-45 min). Authentic markers were eluted at 5.0 min (5-hydroxymethyluracil), 6.0 min (5-formyluracil), and 13.5 min (5-hydroxymethyl-2'-deoxyuridine) under these conditions. (B) HPLC profile of an fdU-cysteamine mixture incubated for 8 h. Peaks 1 (17.9 min) and 2 (27.8 min) corresponded to fdU and the major reaction product, respectively. (C) UV spectra of fdU and an fdU-cysteine mixture. Samples containing fdU (1 mM) or fdU (1 mM)-cysteine (5 mM) incubated for 8 h were diluted 20-fold by phosphate buffer (20 mM, pH 7.0) and UV spectra were measured. (D) Time courses of fdU conversion and formation of the major product (fdU-Cys adduct) in the presence of cysteine.

To identify the functional group of cysteine involved in the reaction with fdU, several cysteine derivatives such as cysteine methyl ester, N-acetylcysteine, and cysteamine were tested for their reactivities to fdU. Cysteine methyl ester and cysteamine lacking a free carboxyl group reacted with fdU as rapidly as cysteine, and yielded a single major product, respectively. A typical HPLC chromatogram for cysteamine is shown in FIG. 2B. The time courses of fdU conversion, formation of the products, and UV spectral changes were essentially similar to those with cysteine shown in FIG. 2 (data not shown). In contrast, no reaction proceeded between N-acetylcysteine and fdU under the same conditions, indicating that the α-amino group is essential for the reaction. Considering that rapid conversion of fdU did not occur with other amino acids lacking SH group, or compounds having an SH or NH_2 group alone, it was concluded that both SH and α-NH_2 groups were simultaneously involved in the reaction between fdU and cysteine. It is also noted that these two functional groups need to be present in the same molecule at a certain spacing since fdU conversion was not promoted by the coexistence of mercaptoethanol and aminoethanol, or by glutathione containing distant α-NH_2 and SH groups.

Similar to fdU, fdUMP reacted with cysteine, cysteine methyl ester, and cysteamine, giving rise to a single major product in each reaction. A typical HPLC profile for cysteine methyl ester is shown in FIG. 3. The reactions proceeded faster than fdU and completed in 1 h. UV spectra of the reaction mixtures after 1 h incubation displayed characteristics similar to those with fdU (data not shown). These results show that the 5'-phosphate group or the negative charges on this group do not alter the reaction mode and somehow facilitate the reaction.

In light of the present results and the previous studies on the reactions of simple aldehydes with cysteine[20], the major products formed in the reactions of fdU and fdUMP with cysteine were assigned as thiazolidinecarboxylic acid derivatives of fdU and fdUMP (FIG. 4). These products are formed by condensation of the formyl group with both α-NH_2 and SH groups of cysteine. This reaction appeared to be in an equilibrium with the favored formation of the thiazolidine ring. Therefore, attempts to isolate the reaction product of fdU were all unsuccessful. The product gradually reverted to fdU after HPLC purification and subsequent treatments under several conditions tested. However, the thiazolidine ring formation was further supported by the structural data from *in situ* NMR measurement (see below).

NMR Analysis of the Reaction of fdU with Amino Acids and Spermidine.

fdU has been reported to form Schiff bases with amines and amino acids[2,15,21]. However, except for the cysteine derivatives, rapid decomposition of fdU by the addition of these compounds was not observed in this study (TABLE 1). To determine whether or

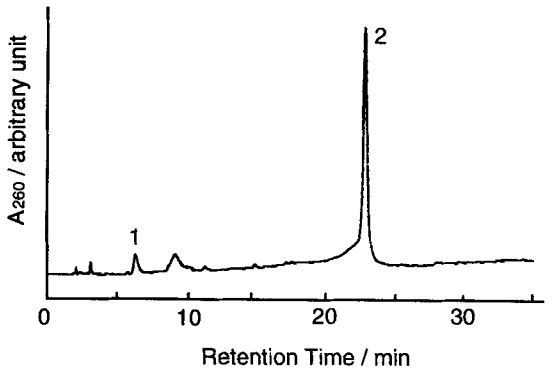

FIG. 3. HPLC profile of a reaction mixture containing fdUMP and cysteine methyl ester. fdUMP (1 mM) and cysteine methyl ester (5 mM) were incubated in phosphate buffer (20 mM, pH 7.0) for 1 h, and the sample was analyzed by HPLC using a isocratic elution of 2 % acetonitrile in 50 mM ammonium formate. Peaks 1 (6.3 min) and 2 (22.9 min) corresponded to fdUMP and the major reaction product, respectively.

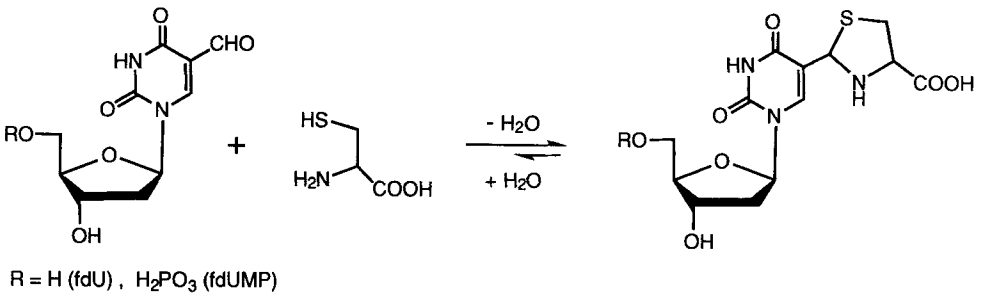

FIG. 4. Reactions of fdU and fdUMP with cysteine to form thiazolidine products.

not the Schiff base formation was relevant in neutral aqueous media, NMR spectra were measured for the mixtures of fdU with amino acids (cysteine, lysine, serine) or spermidine. Except for cysteine, neither changes in the formyl signal (δ = 9.64 ppm) nor development of a new azomethine signal associated with the Schiff base formation was observed (data not shown). These results suggest that the equilibrium constants for the formation of Schiff bases between fdU and the amino acids or spermidine were extremely small in neutral aqueous solution, even if the equilibrium existed. Consistent with these NMR data, the compounds showing no dramatic promotion effects (TABLE 1) did not exhibit a typical Schiff base absorption over 300 nm in the UV spectra[15,22].

In the mixture of fdU and cysteine, the formyl signal (9.64 ppm) completely disappeared after 30 min, and some broadening of the 6-H signal (8.78 ppm) occurred

(FIG. 5B). In addition to these changes, new signals attributable to the reaction product appeared (indicated by closed circles in FIG. 5B). These signals, except for that at 9.4 ppm, grew with reaction time and became dominant signals at 8 h (FIG. 5C). The broad signal at 9.4 ppm gradually disappeared as the reaction proceeded. The relative intensities of the 6-H signals of fdU (8.78 ppm) and the product (7.94 and 8.07 ppm, for assignment see below) revealed that approximately 94 % of fdU was converted to the product at 8 h. The NMR signals observed at 8 h were consistent with the formation of the thiazolidine derivative of fdU shown in FIG. 5C, and assigned as follows based on the chemical shifts and signal intensities: 2.42 (2H, 2'-H), 2.96 (1H, β-H_a), 3.30-3.45 (1H, β-H_b), 3.71-3.91 (3H, α-H and 5'-H), 4.04 (1H, 4'-H), 4.48 (1H, 3'-H), 5.36 and 5.68 (1H, 7-H), 6.28 (1H, 1'-H), 7.94 and 8.07 (1H, 6-H). The large upfield shift of the 7-H signal from 9.64 to 5.36 and 5.68 ppm is characteristic to the condensation reaction of the formyl group forming the thiazolidine ring. A similar shift has been reported for the reaction between pyridoxal with cysteine to form a thiazolidine product[23]. The large splitting of 6- and 7-H signals into doublets (7.94 and 8.07 ppm for 6-H, 5.36 and 5.68 ppm for 7-H) is due to the chiral center at the position 7. In addition, the large shift difference between the two nonequivalent β-H protons (2.96 and 3.38 ppm) is also consistent with the presence of the fixed ring structure.

The possibility of a Schiff base as the final product was ruled out because of the absence of the azomethine proton signal in the expected Schiff base, which should appear at 9.2-9.4 ppm[23]. In this regard, the broad signal at 9.4 ppm that was transiently observed during the reaction (FIG. 5B) is attributable to the Schiff base intermediate. Probably the broadening of the signal arises from equilibria between starting materials, the Schiff base intermediate and the final thiazolidine product.

The present study has shown that fdU and fdUMP rapidly react with cysteine, cysteine methyl ester and cysteamine in neutral aqueous solution to form relatively stable thiazolidine derivatives. Both α-NH_2 (or NH_2 at the equivalent position) and SH groups participate in the reaction, and the reaction appear to proceed *via* Schiff base intermediates. Compounds including N-acetylcysteine, glutathione (Glu-Cys-Gly), other amino acids, amines and thiol compounds do not undergo such a reaction since they lack one of the functional groups essential for the reaction or have the functional groups in inadequate positions to form the stable five-membered thiazolidine ring. With respect to the biological relevance of this reaction, 5-formyluracil (fU) in two different environments need to be considered, one in the nucleotide pool and the other in DNA. As demonstrated in this study, the free nucleotide of fU readily reacts with cysteine and its derivatives. However, it remains to be seen whether or not fU in damaged DNA undergoes similar reactions. In addition, it is also necessary to study whether or not cysteine residues in proteins form

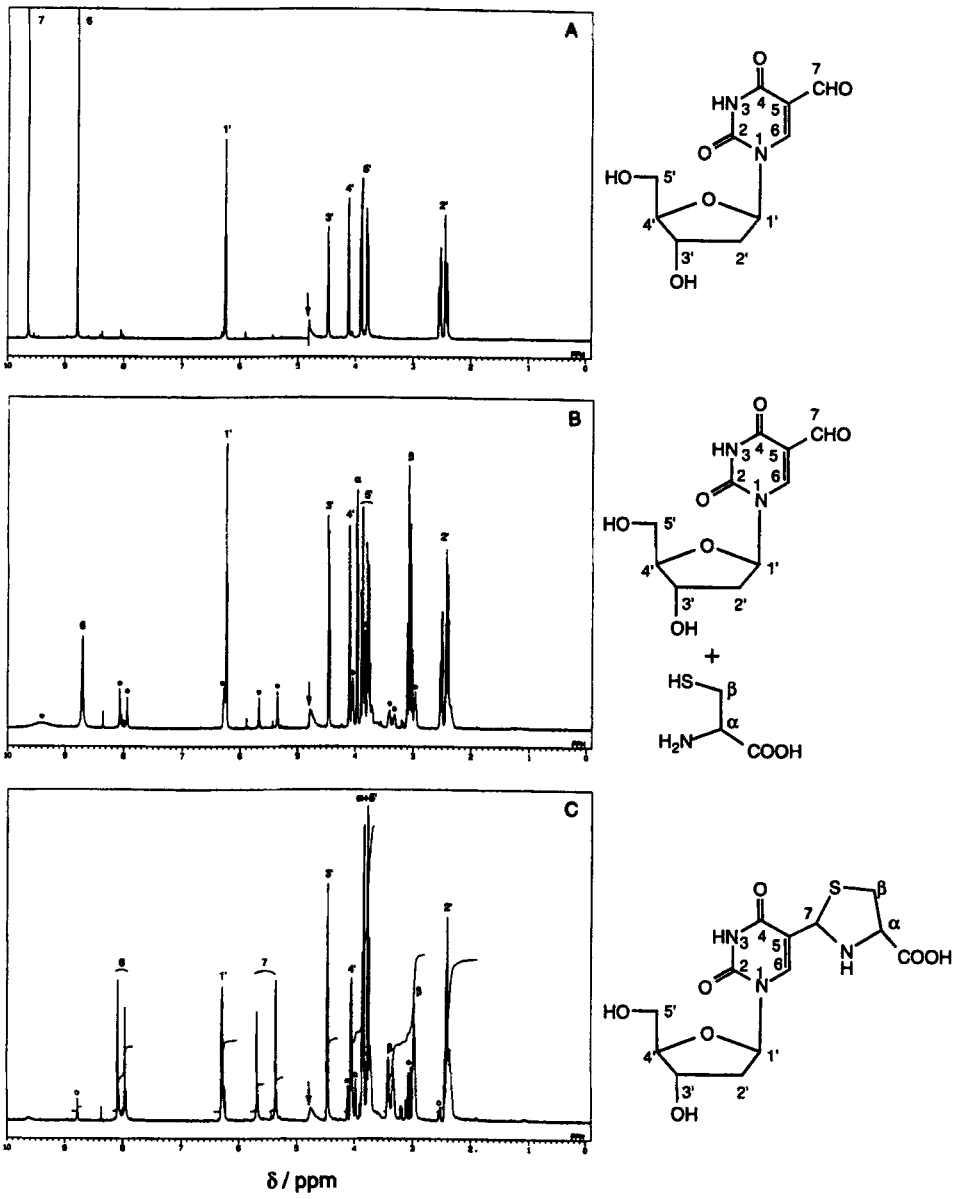

FIG. 5. NMR spectra of fdU (A) and fdU-cysteine mixture incubated for 30 min (B) and 8 h (C) in D_2O. [fdU] = [Cys] = 33 mM. Signals of the thiazolidine product in (B) and remaining fdU in (C) are indicated by closed and open circles on the peaks, respectively. After the incubation for 8 h, signals were mostly (ca. 94 %) due to the thiazolidine product. Integration of the signals are also shown in (C). The arrows at 4.8 ppm indicate the irradiated HDO peaks.

crosslinks with fU in DNA. If it is the case, rare nuclear proteins carrying an N-terminal cysteine residue is expected to be involved in the reaction based on the reaction mechanism shown in FIG. 4. According to the present results, if not at all, the contribution of the Schiff base formation between fU and lysine[2,15] appears to be overestimated in the crosslinking between damaged DNA and proteins since no UV and NMR data indicative of the Schiff base formation were observed in neutral aqueous solution. In aqueous solution, a large excess of amines and basic conditions are generally necessary for noticeable formation of Schiff bases[22].

In conclusion, despite retaining the intact coding region, 5-formyluracil is likely to exert genotoxic effects by several different mechanisms. These include (i) mispairing with guanine due to facilitated ionization[16,17], (ii) susceptibility to the base-catalyzed degradation[15,24], (iii) formation of abasic sites owing to the relatively labile N-glycosidic bond (ref. 25 and this work), and (iv) adduct formation with cellular constituents (this work). In addition, it has been recently shown that fU has a destabilizing effect on oligonucleotide duplexes[26]. Thus, it seems reasonable that cells are equipped with repair enzymes that catalyze the release of fU from DNA thereby avoiding genotoxic effects of fU[25,27,28].

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