Arch. Pharm. Res. 11(2), 114-121(1988)

Studies on Reaction of Formaldehyde with Naturally Occurring Thiol Compounds and Ascorbic Acid

Lajos Trezl,* Young Bong Cho, Peter DiMaria,*** Sangduk Kim,**** Prabhakar D. Lotlikar****, and Woon Ki Paik****§**

**Department of Organic Technology, Technical University, Budapest, Hungary*
***Department of Environmental Science, College of Health Science, Yonsei University 162 Ilsan-Dong, Wonju, Kangwon-Do 220-701, Korea*
****Department of Biology, SUNNY College of Fredonia, Fredonia, NY 14063 and*
*****Fels Research Institute, Temple University School of Medicine, Philadelphia, PA 19140, USA*
(Received March 24, 1988)

Abstract☐ To gain insight into possible cellular protective mechanisms against the insult of formaldehyde, we have investigated this molecule's reactivity with both naturally occurring thiol compounds including glutathione and L-ascorbic acid. By UV measurements, formaldehyde was found to rapidly react with glutathione forming an S-hydroxymethyl covalent adduct. The adduct which was confirmed by NMR is transiently stable. Formaldehyde is also significantly reactive with L-ascorbic acid in a reaction which was observed to be dissimilar to its reaction with dimedone. The reaction of formaldehyde with glutathione was reduced by 40% in the presence of an excess amount of L-ascorbic acid, due to the trapping of formaldehyde by L-ascorbic acid. The data suggest that L-ascorbic acid may have a possible *in vivo* role in the metabolism of formaldehyde, thereby protecting cellular glutathione from possible depletion.

Keywords☐ formaldehyde, L-ascorbic acid, glutathione, metabolism, reactivity

Formaldehyde is an extremely reactive molecule which can covalently modify and hence damage numerous cellular macromolecules including DNA, RNA and proteins. Formaldehyde is liberated during the *in vivo* decomposition of various xenobiotics including dimethylnitrosamine,[1-4] N-nitrosomethyl-N-ethylamine,[5] N,N'-dimethylhydrazine, aflatoxin B_1, *p*-nitroanisole,[6] hexamethyl-triazine, methylenedimorpholine,[7] and others. According to recent studies,[5] approximately 60-65% of the [^{14}C]methyl groups from methyl[^{14}C]-nitrosamine injected into rats was exhaled in the form of CO_2. This CO_2 was formed from the liberated formaldehyde. Another source of the formaldehyde is from the reaction of methyl cations with water, thereby forming methanol[2] which is converted to formaldehyde *in vivo*.

Among other cellular targets, formaldehyde has been shown to react very rapidly with glutathione (GSH) in both *in vitro* and *in vivo* conditions.[8-11] *In vivo* GSH could be significantly depleted as a result of reaction with formaldehyde, thereby compromising other cellular processes in which GSH is necessarily involved.

The exact chemical nature of the reaction of GSH with formaldehyde is poorly understood, and the influence of other compounds, particularly those which are naturally occurring, is also not known. It is possible that *in vivo* other compounds may trap formaldehyde, thereby protecting the cellular GSH from depletion. Previous work by one of us[12] has shown that L-ascorbic acid can inhibit the spontaneous methylation and formylation reactions which occur in the reaction of formaldehyde with lysine. In light of this, we have investigated the chemical nature of the reactions of formaldehyde with L-ascorbic acid and thiol compounds including GSH and, in addition, have examined the influence of L-ascorbic acid on the reaction of formaldehyde with GSH.

MATERIALS AND METHODS

Materials

L-Cystein, DL-homocysteine, L-methionine, reduced and oxidized glutathione, S-methylglu-

*Correspondence should be adrressed.

tathione and thioproline were obtained from Sigma Chemical Co. S-Methyl-L-cysteine was obtained from Nutritional Biochemical Co. 5,5-Dimethyl-1,3-cyclohexanedione (dimedone) and L-ascorbic acid were purchased from Eastman Kodak Co., and formaldehyde and semicarbazide hydrochloride from Fisher Chemcial Co. [^{14}C]Formaldehyde (specific activity, 60mCi/mmol) was purchased from New England nuclear.

UV Spectroscopic Analysis

The UV spectroscopic analytical method was found to be the most convenient among the various analytical methods examined to follow the reaction of formaldehyde with L-ascorbic acid, GSH, cysteine, dimedone, and homocysteine. The change in UV absorbance in the reaction mixtures were monitored versus time. The reactions were carried out in 3.0m*l* cuvettes in 0.1M sodium phosphate buffer, pH 7.4, at 25°C.

Proton NMR Spectroscopy

The reactions between the various compounds and formaldehyde were assessed by NMR spectroscopy utilizing a Bruker 300 MHz instrument. The proton NMR spectra of standard compounds were determined after first dissolving the compounds in 99.8% D_2O, lyophilizing, and subsequently dissolving the compounds in 100% D_2O (internal standard: Shift 4.7 ppm for DOH). For NMR spectroscopy of reaction mixtures, the reactions were terminated by freezing and the mixtures were then lyophilized. The freeze-dried samples were dissolved in 0.5m*l* of 100% D_2O and the NMR spectra were taken immediately (due to the instability of S-hydroxymethyl groups).

TLC Analysis

Separation of reaction products by thin-layer chromatography (TLC) was carried out on Avicel cellulose plates (250 μm thick; Analtech Inc., Newark, DE). The chromatographic solvent was 1-butanol/glacial acetic acid/water (65:10:25 by v/v/v). Before analysis on TLC, reaction mixtures containing [^{14}C]formaldehyde were stopped by freezing and they were lyophilized and dissolved in a minimal volume. Non-labeled marker compounds were added to the samples as needed. After completion of the chromatographic separation, the compounds were visualized by spraying the plate with ninhydrin solution. The colored spots were scraped and radioactivity determined by Prias scintillation counter Model 240 CL/D with dpm converter.

RESULTS

Reaction of Formaldehyde with L-Ascorbic Acid and Dimedone

We first examined the reaction of formaldehyde with L-ascorbic acid at pH 7.4. As mentioned earlier under Methods, the UV spectroscopic method was found to be the most convenient means to follow the reaction. L-Ascorbic acid has two characteristic absorption maxima at 266 and 281 nm (Fig. 1A), while only one is seen at 266 nm at lower concentration (Fig. 1C). The enediol structure in L-ascorbic acid is responsible for this characteristic absorbance. Upon reaction with formaldehyde, the absorbances are seen to decrease with time (Fig. 1C). Although this does not allow us to make a definitive conclusion as to the nature of the chemical reaction which took place, the disappearance of the characteristic UV spectrum indicates that the reaction is not involving the alcoholic functional groups but the double bond in L-ascorbic acid.

In order to gain further insight, we compared the formaldehyde reaction with L-ascorbic acid and that of dimedone which has been well characterized.[13] As shown in Fig. 1B, dimedone has structural characteristics similar to L-ascorbic aicd and a similar UV spectra (maxima at 266 and 281 nm, and similar shape of spectrum). However, dissimilar to L-ascorbic acid, one peak at 281 nm was observed at lower concentration (Fig. 1D). In the reaction of dimedone with formaldehyde, the characteristic absorbances were seen to decrease, however no further decrease is seen after 2 minutes. The formed product, hydroxymethyldimedone, has been shown to react with another dimedone molecule in a condensation reaction.[13]

Reaction of Formaldehyde with GSH and Cysteine

Next, we examined the addition reaction of formaldehyde with various naturally occuring thiol compounds. In Fig. 2, the UV spectra of GSH (lower panel) and L-cysteine (upper panel) are observed to change in the presence of formaldehyde. Both GSH and L-cysteine have the characteristic UV absorption maxima at 252.5nm, and these maxima decrease very rapidly in the early time period of the reaction. However, at later times, in contrast to cysteine, the absorbance in GSH reaction recovers and is actually higher than the original absorbance after 90 minutes. The results suggest that the reaction product with GSH may be unstable while the product with cysteine is stable (no

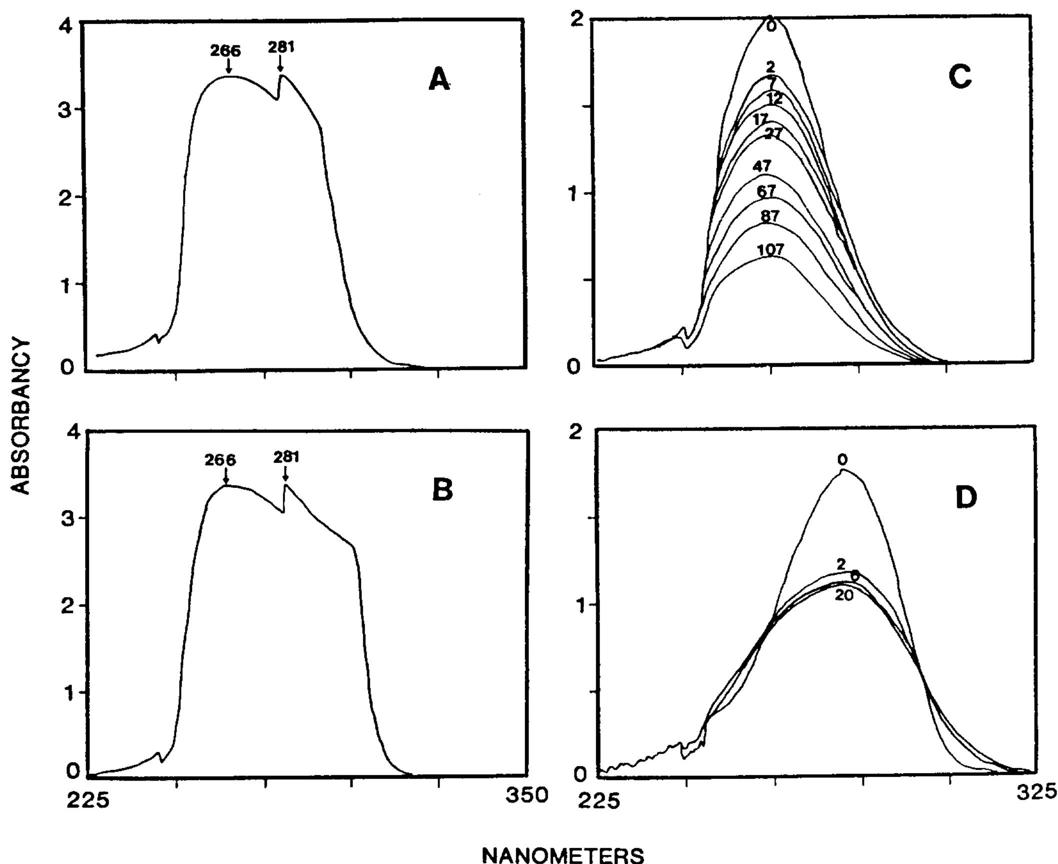

Fig. 1. UV spectra of L-ascorbic acid and dimedone, and their adducts with formaldehyde

A: Three m*l* of solution contained 3.4 umoles of L-ascorbic acid in 0.1M phosphate buffer, pH 7.4. Light path was 1.0cm in Gilford Response™ UV-VIS spectrophotometer.

B: Three m*l* solution contained 2.0 umoles of dimedone in the buffer.

C: Three m*l* solution contained 0.4 umoles of L-ascorbic acid and 336 umoles of formaldehyde in 0.1M phosphate buffer, pH 7.4. Reaction was recorded after 2 to 107 minutes.

D: Three m*l* solution contained 0.21 umoles of dimedone and 30 umoles of formaldehyde in 0.1 M phosphate buffer at pH 7.4. Reaction was recorded after 2 minutes and 20 minutes.

All the analysis was carried out at room temperature (25 °C).

recovery of absorbance even after 24hr). As will be shown below, cysteine traps the formaldehyde in a stable bond in a very fast reaction, forming a cyclic compound thioproline (thiazolidine-4-carboxylic acid). Homocysteine showed reaction characteristics similar to cysteine as well.

Interrelationship between the Amounts of GSH and Formaldehyde, and Period of Reaction Time

We further investigated the UV-spectra changes in the GSH-formaldehyde reactions as a function of formaldehyde concentration and varying period of incubation time. In Fig. 3, it can be seen that at very low concentrations of formaldehyde (the ratio of formaldehyde: GSH at 2) the maxima of absorbance decreased very rapidly (within 2 minutes). The nearly same level of decrease was observed with higher amounts of formaldehyde, however, marked differences in the recovery of absorbance was observed as a function of formaldehyde concentrations. At low formaldehyde concentrations, the rate of absorbance recovery is very slow whereas the recovery with the higher formaldehyde concentrations is much faster, and moreover increases over the initial level. The higher UV absorbances indicate that more than a simple reversal of the reac-

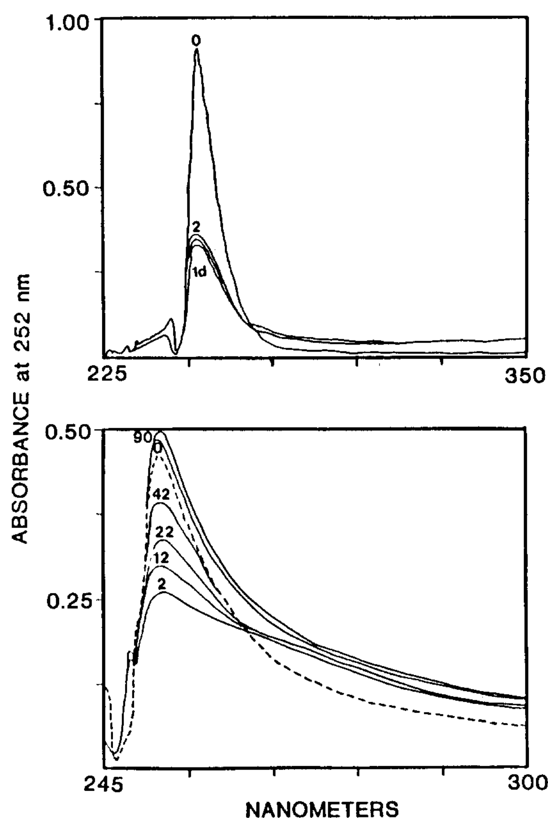

Fig. 2. UV spectra of cysteine and GSH, and their rate of adduct formation with formaldehyde

Upper panel: 3m*l* sample contained 24.75 umoles of L-cysteine and 72 umoles of formaldehyde in 0.1M phosphate buffer at pH 7.4. Lower panel: 19.5 umoles of GSH and 72 umoles of formaldehyde are present in 3.0m*l* sample in 0.1M phosphate buffer, pH 7.4.

Numbers in the figures indicate the time elapsed.

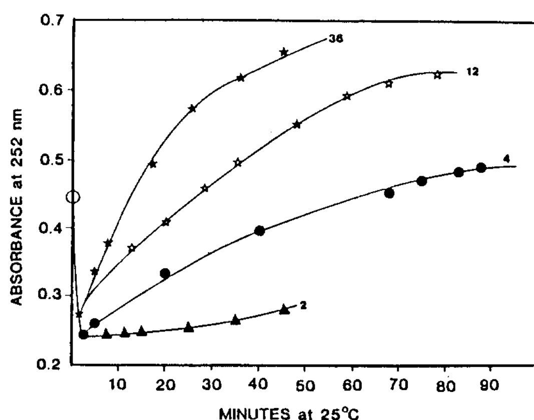

Fig. 3. Dependency of absorbance intensity of GSH-formaldehyde adduct on time

The numbers in the figure indicate the ratio of the amount of formaldehyde to that of GSH. 19.0 umoles of GSH and increasing amounts of formaldehyde were present in 3.0m*l* sample volume containing 0.1M phosphate buffer at pH 7.4.

tion is occurring: compound(s) with higher absorbances than GSH are probably formed. As described below, proton NMR spectroscopy confirmed the formation of S-hydroxymethyl-GSH (S-CH_2OH) in the rapid reaction of formaldehyde with GSH.

NMR Spectroscopy

The main goal of the NMR studies was to prove the formation and existence of labile (redissociable) S-hydroxymethyl-GSH. Other investigators[8-11] have earlier proposed the existence of such labile groups, however, no evidence for their existence has been put forth.

As shown in Fig. 4, we have proven the existence of the labile group from the NMR spectra. In comparing the spectra of standard GSH (lower panel of Fig. 4) with that of the reaction product (middle panel), one can see an appearance of a peak at position B and this peak B disappeared on standing at 25 °C for 150 minutes (upper panel). This peak corresponds to S-hydroxymethyl. As peak B disappears, the peak E (corresponding to SH-CH_2-CH_2) chemical shifted from a double peak at 4.2ppm to a broad multiplet peak with a lower chemical shift (compare upper and middle panels). This result indicates that the attack of formaldehyde was on the SH-CH_2- group of GSH, and that this group compound is transformed into the peak of E to form multiplet peak at 2.7ppm. Since the UV absorbance of reaction product of formaldehyde with GSH first decreased and increased on standing at room temperature (lower panel of Fig. 2), the decrease of the absorbance within 2 minutes of reaction might be corresponding to the formation of S-hydroxymethyl GSH, and the increase of absorbance to conversion of S-hydroxymethyl to another "new unknown" derivative.

The UV spectroscopic analysis described above showed that the reaction between GSH and formaldehyde forming the S-hydroxymethyl group is very fast and is independent of time and the concentration of formaldehyde (Fig. 3). These results were also confirmed by NMR analysis as well: The spectra of the product for a reaction at 5 minutes is similar to that seen for the spectra of a reaction after 60 minutes (data not shown).

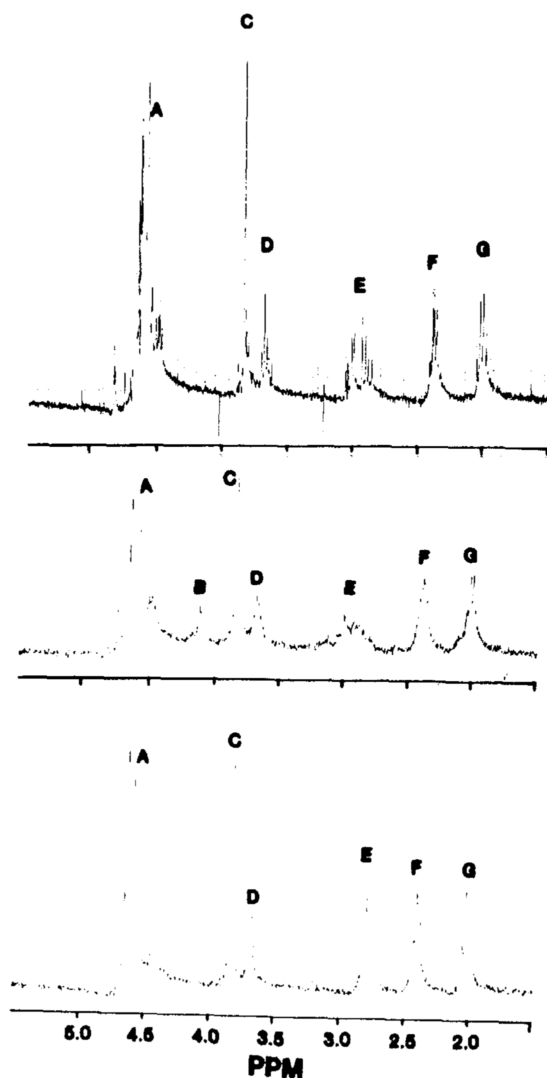

Fig. 4. Proton NMR spectra of GSH-formaldehyde adduct

Lower panel: 15 umoles of GSH In 0.5m*l* of 100% D_2O.

Middle panel: 15 umoles of GSH and 55 umoles of formaldehyde in 0.5m*l* of 0.1M phosphate buffer (pH 7.4) were incubated for 60 minutes at 37 °C, the sample was frozen, lyophilized and was dissolved in 0.5m*l* of 100% D_2O. This panel shows the NMR at 0 time.

Upper panel: Same sample after 150 minutes at room temperature. ^1H NMR chemical shifts are as follows:

Peak	Chemical formula	Multiplicity	δ -value chemical shift
A	HOD	s	4.7
C	HN-C$\underline{H}_2$-COOH	s	3.8
D	HN-C$\underline{H}$-C(=O)-NH (with -CH_2-SH on the CH)	t	3.6
E	-CH-$\underline{CH_2}$-SH	d	2.7
F	-H_2C-C$\underline{H}_2$-C(=O)-	t	2.4
G	CH(-NH_2)-C$\underline{H}_2$-CH_2-C(=O)	m	2.0

We also examined the NMR spectra of the product of the reaction between L-cysteine and formaldehyde which is thioproline (Fig. 5; see also Table I). In this reaction, a cyclic compound rather than a free S-hydroxymethyl group forms because the α-amino group can secondarily condense with the S-hydroxymethyl, thereby forming an -S-CH_2-NH- group. The positions of the protons in this group in the NMR spectra are at 4.3 ppm which is very similar to that seen in the S-hydroxymethyl group (4.2 ppm). The stability of the thioproline was also assessed. After leaving the compound in D_2O for 24 hr, no changes in the NMR spectra were observed. These results are in good agreement with the UV spectroscopic analysis which also showed no change in UV-spectra after 24 hr (upper panel of Fig. 2).

TLC Analysis

The UV and NMR analysis proved that in the reaction between thiol compounds and formaldehyde the unstable S-hydroxymethyl or the stable -S-CH_2-NH- group adduct was formed. The possibility of other reaction products formed should also be considered. In particular, it is possible that -S-methyl (-S-CH_3) compound may have

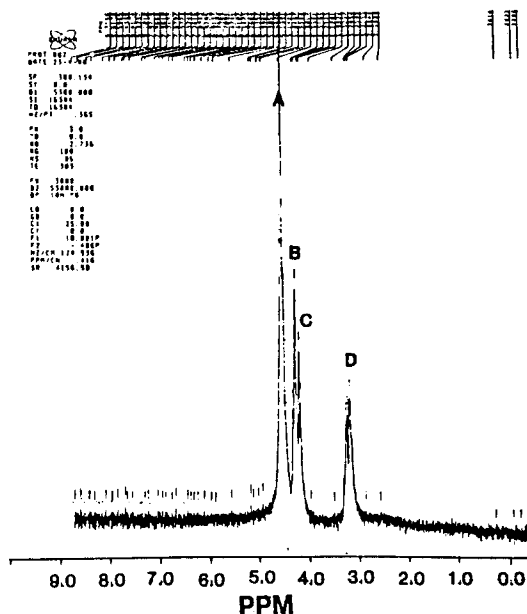

Fig. 5. Proton NMR spectra of thioproline

15 umoles of standard thioproline in 0.5m*l* of 100% D_2O was measured. ^1H NMR chemical shifts are as follows:

Peak	Chemical formula	Multiplicity	δ-value chemical shift
A	HOD	s	4.7
B	| S NH \ / C<u>H</u>$_2$	s	4.3
C	-C<u>H</u>-COOH | NH_2	t	4.2
D	C<u>H</u>$_2$-CH | S \	d	3.2

been formed. A precedent of this is the formation of -N-methyl groups in the reaction between lysine and formaldehyde.[12] Moreover, formaldehyde is generally regarded as a methylating agent.[14] Therefore, the formation of these types of reaction products was examined by TLC. In Table I, it is seen that the reaction product obtained in the reaction between cysteine and formaldehyde included S-methylcysteine and thioproline, and homocysteine and formaldehyde included methionine and cyclic derivative of S-methylhomocysteine. However, the amounts of the S-methyl compounds are much less than the amounts of the -S-CH_2-N-cyclic compounds. Table 1 also shows the rapidity of the reaction in that it is essentially complete after only 5 minutes with essentially no further changes after as much as 60 minutes. Utilizing TLC, we assessed the ability of L-ascorbic acid to influence the reactions incurred by formaldehyde on cysteine and homocysteine. It is seen that L-ascorbic acid can inhibit the reaction as much as 40%. This is consistent with the above described UV studies which showed that L-ascorbic acid can trap formaldehyde in a stable bond (Fig. 1C). The results for the reaction with GSH analyzed on TLC is shown in Table II. TLC analysis showed the formation of S-methyl-GSH and also significant amounts of radioactivity in two other products. One of these is at the same R_f of GSH (0.30) and the other is above S-methyl-GSH (R_f=0.50). The latter product appears to be S-hydroxymethyl-GSH. Specifically, if the lyophilized samples are kept in buffer for 2 hr and then subsequently separated by TLC, the radioactivity of R_f of 0.50 disappeared while those at the other two positions remained. This observation taken together with the NMR spectral results strongly suggest that this spot in fact represents the S-hydroxymethyl-GSH. The identity of the compound comigrating with GSH is unknown and has not been examined further in this study. The effect of L-ascorbic acid on the formaldehyde reaction with GSH was also examined. The L-ascorbic acid was shown to inhibit the reaction by as much as 40% (at a L-ascorbic acid: GSH of 7.6) (data not shown). As in the case of cysteine and homocysteine, this result most likely reflects the ability of L-ascorbic acid to trap formaldehyde in a stable covalent adduct. Similar results were obtained when semicarbazide or dimedone were used instead of L-ascorbic acid (data not shown).

DISCUSSION

It is well known that formaldehyde is very reactive, toxic, mutagenic and carcinogenic.[2] It is liberated *in vivo* from a number of different exogenous precursor.[6,7,15] This liberated formaldehyde attacks cellular components including DNA, RNA and protein, and can also react rapidly with GSH which is present in the tissues. Although GSH plays a key role in the elimination of formaldehyde through the formation of S-formyl-GSH [catalyzed by formaldehyde dehydrogenase; EC 1.2.1.1.],[8] it is possible that excessive depletion of cellular GSH could occur via the chemical reaction

Table I. Inhibitory effect of L-ascorbic acid on the radioactivity incorporation of [^{14}C] formaldehyde into thiol compounds

Addition	Incubation time (min)	Radioactivity incorporated (%)			
		S-methyl-cysteine[a]	Thioproline	Methionine	Homocysteine cyclic deriv.
None	5	3.1	43.2	1.1	53.1
	60	3.7	49.1	1.5	56.7
L-Ascorbic acid	60	2.1(43.4)[b]	26.5(45.6)	0.8(46.2)	31.2(43.2)

One m*l* incubation mixture contained 8.5 umoles or L-cysteine of L-homocysteine, and 0.165 uCi of [^{14}C] formaldehyde in 0.1M phosphate buffer, pH 7.4, and the mixture was incubated at room temperature (25 °C). The sample was frozen at indicated period of time, lyophilized, resuspended in 1.0 m*l* of water, and 0.010m*l* of aliquot was applied on TLC.
[a]S-Methylcysteine and thioproline are formed by incubating formaldehyde with L-cysteine, and methionine and homocysteine cyclic derivatives are formed from L-homocysteine and formaldehyde. The products were identified on TLC and their R_f values were 0.41, 0.37, 0.52 and 0.43, respectivety.
[b]The numbers in parenthesis indicate percent inhibition of radioactivity incorporation by 3.3×10^{-5}M concentration of L-ascorbic acid.

Table II. Inhibitory effect of L-ascorbic acid on the radioactivity incorporation of [^{14}C] formaldehyde into GSH

Addition	Incubation time (min)	Radioactivity incorporated (%)		
		S-methyl GSH R_f=0.36	Spot with R_f=0.30	Spot with R_f=0.50
None	5	2.2	4.4	15.1
	20	4.8	6.5	17.7
	60	6.5	8.8	20.1
L-Ascorbic acid at 5×10^{-5}M	60	3.5 (46.0)[a]	4.6 (47.1)	11.4 (43.2)

19.50 umoles of GSH and 0.5 uCi formaldehyde[^{14}C] in 3.0m*l* of 0.1M phosphate buffer, pH 7.4, were incubated at room temperature (25 °C), 0.5m*l* aliquot was taken at indicated period of time, and the sample was immediately frozen. The sample was lyophilized, repeated once more freeze and lyophilization, and finally resuspended in 0.5m*l* of water 0.010 m*l* was applied on TLC.
[a]Numbers in parenthesis indicate percent inhibition by L-ascorbic acid at 60 minutes of incubation.

with formaldehyde, and this would compromise other cellular reactions, with which GSH is involved. We therefore have investigated in the present study the rapidity and other characteristics of the reaction of GSH (and other thiol compounds) with formaldehyde and compared them with other biological compounds which would be relevant *in vivo.*

UV spectroscopic analysis revealed that the addition of formaldehyde to GSH, cysteine, and homocysteine is very rapid and takes place in 1-2 minutes. However, L-ascorbic acid which was previously shown by Trezl *et al.*[12] to trap formaldehyde reacts relatively shown (Fig. 1C). Moreover, the rate and the poduct formation of the reaction is dependent on the concentration of formaldehyde (Fig. 3), whereas the reaction with GSH shows no such dependency (data not shown). These characteristics (Fig. 3) make L-ascorbic acid an effective agent to prevent formaldehyde reaction with GSH only when its concentration is relatively high.

Structural studies utilizing NMR spectroscopy have enabled us to characterize the chemical nature of the reactions. In the case of L-ascorbic acid, the formaldehyde reacts with the C-2 atom forming C2-hydroxymethyl-L-ascorbic acid. The reaction of formaldehyde with thiol compounds resulted in the formation of S-hydroxymethyl in the case of GSH and -S-CH_2-NH- in the case of cysteine and homocysteine. The S-hydroxymethyl was found to be unstable. However, we do not know the nature of the compound which results from this instability.

L-Ascorbic acid was seen to markedly inhibit the formaldehyde reaction with GSH at high concentrations. This is intriguing since the reaction of formaldehyde with L-ascorbic acid is actually much slower than with GSH. Nevertheless, this characteristic could have important implications in the pro-

tenction of cellular stores of GSH against depletion by *in vivo* generation of formaldehyde. One could envision instances when the cellular concentrations of L-ascorbic acid would be high and thereby inhibit the reaction with GSH.

ACKNOWLEDGEMENTS

This work was supported by research grants DK09602 from the National Institute of Diabetes, Digestive and Kidney Diseases, CA12226 and CA12227 from the National Cancer Institute, and RG1765-A-1 from the National Multiple Sclerosis Society.

LITERATURE CITED

1. Magee, P.N., and Hultin, T.: Toxic liver injury and carcinogenesis, *Biochem. J.* **83,** 106-114 (1962).
2. Turberville, C., and Craddock, V.M.: Methylation of nuclear proteins by dimethylnitrosamine and methionine in rat *in vivo, Biochem. J.* **124,** 725-739(1971).
3. Jensen, D.E., Lotlikar, P.D., and Magee, P.N.: The *in vitro* methylation of DNA by microsomally activated dimethylnitrosamine and its correlation with formaldehyde formation, *Carcinogenesis,* **2,** 349-354(1981).
4. Kleeberg, V., and Klinger, W.: Age course and inducibility of dimethylnitrosamine N-demethylation in rat liver, *Acta Biol. Med. Germ.* **41,** 675-678(1982).
5. Hofe, E., Grahman, F., Keefer, L.K., Lijinsky, W., Nelson, V., and Kleihues, P.: Methylation versus ethylation of DNA in target and nontarget tissues of Fisher 344 rats treated with N-nitrosomethylethylamine, *Cancer Res.* **46,** 1038-1042 (1986).
6. Sawicki, E., and Sawicki, C.: V. Formaldehyde precursor. In "Aldehyde Photometric Analysis." Academic Press, New York (1978).
7. Ashby, F., and Lefevre, P.A.: Formaldehyde generators, relationship between stability lipophylicity and carcinogenic potency, *Carcinogenesis,* **3,** 1273-1276(1982).
8. Uotila, L., and Koivusalo, M.: Formaldehyde dehydrogenase from human liver, *J. Biol. Chem.* **249,** 7653-7663 (1974).
9. Ayres, P.H., Marshall, T.C., Sun, J.D., Bond, J.A., and Hobbs, C.H.: Interaction of formaldehyde with glutathione in the isolated/ventilated perfused lung and the isolated perfused liver, *J. Toxic Environ. Health,* **15,** 655-662(1985).
10. Guerri, C., Godfrey, W., and Grisolia, S.: Protection against toxic effect of formaldehyde *in vitro* and of methanol or formaldehyde *in vivo* by subsequent administration of SH reagents, *Physiol. Chem. Physics,* **81,** 543-550(1976).
11. Jones, D.P., Thor, H., Andersson, B., and Orrenius, S.: Detoxication reactions in isolated hepatocytes, *J. Biol. Chem.* **253,** 6031-6037 (1978).
12. Trezl, L., Rusznak, I., Tyihak, E., Szervas, T., and Szende, B.: Spontaneous ϵ-N-methylation of ϵ-N-formylation reactions between L-lysine and formaldehyde inhibited by L-ascorbic acid, *Biochem. J.* **214,** 289-292(1983).
13. Spencer, D., and Henshall, T.: The kinetics and mechanism of the reaction of the formaldehyde with dimedone, *J. Am. Chem. Soc.,* **77,** 1943-948(1948).
14. Fieser, L.F., and Fieser, M.: In "Advanced Organic Chemistry" pp. 496-497. Reinhold Publishing Corp(1963).
15. Swenberg, J.A., Kerns, W.D., Mitchell, R.E., Calla, E.J., and Pavkov, K.L.: Nonlinear biological response to formaldehyde and their implications for carcinogenic risk assessment, *Cancer Res.,* **40,** 3398-3402(1980).