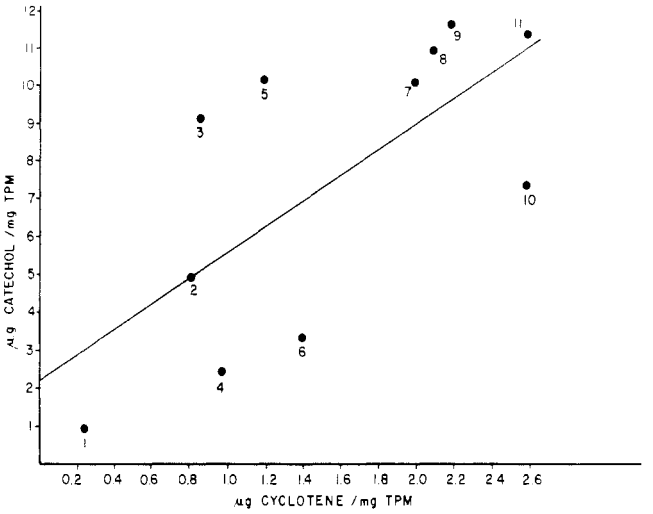

Figure 3. Correlation between levels of catechol and 2-hydroxy-3-methyl-2-cyclopenten-1-one in the smoke of 11 nonfilter cigarettes. Code: (1) Burley, bottom stalk position; (2) Burley, top stalk position; (3) reference IRI (Table III, entry 1); (4) SEB IV, extracted (Table III, entry 6); (5) cigarette B (Table I, entry 2); (6) Burley cigarette (Table III, entry 3); (7) Oriental cigarette (Table III, entry 4); (8) cigarette C without filter (Table II, entry 1); (9) cigarette A (Table I, entry 1); (10) SEB IV (Table III, entry 5); (11) Bright cigarette (Table III, entry 2).

modified cellulose cigarette was only 3.7 μg/cigarette.

The results of these analyses were parallel in certain respects to results obtained when cigarette smoke was analyzed for catechol. Bright tobacco gave smoke with relatively high levels of catechol (Kallianos et al., 1968; Matsushima et al., 1979). Levels of catechol were higher in smoke from the top leaves of Burley tobacco compared to the bottom leaves (Hoffmann et al., 1980), and the concentration of catechol was markedly reduced in the smoke of hexane–ethanol-extracted cigarettes (Brunnemann et al., 1976). We investigated further the possible relationship of smoke catechol and cyclotene levels for several cigarettes as shown in Figure 3. A least-squares regression analysis showed that the correlation between catechol and cyclotene levels was 0.694 ($P < 0.05$). The regression line fit for the data is

$$\text{catechol} = 2.19 + 3.40(\text{cyclotene})$$

These data strongly suggest that catechol and 1 have similar leaf precursors. Further studies are necessary to determine the nature of these precursors.

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Oxidation of Glutathione by Hydrogen Peroxide and Other Oxidizing Agents

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Reduced glutathione (GSH) in aqueous solution was oxidized with hydrogen peroxide, benzoyl peroxide, potassium bromate, and linoleic acid hydroperoxide. The resulting oxidized compounds were measured by amino acid analysis or by ^{1}H nuclear magnetic resonance. Glutathione was oxidized at various pH values, and the ratios of the oxidation products were measured. The various hydrolysis products observed after acid hydrolysis and amino acid analysis are discussed and related to the results with ^{1}H NMR on intact glutathione.

Food proteins are often exposed to oxidants or oxidizing conditions during processing and storage. Oxidizing agents such as benzoyl peroxide and potassium bromate, for example, are added to bread doughs to improve baking quality, and hydrogen peroxide is used to control microorganisms and improve the color of various food products during processing. Although not added deliberately, lipid hydroperoxides, well-known for causing off-flavors and odors in food products, are probably the most common oxidizing agents in foods. They are formed from unsaturated fatty acids by either enzyme or free

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metal ion catalysis. The protein quality of food products can be adversely affected by the presence of either deliberately added oxidants or lipid hydroperoxides. The extent of the adverse effect of the oxidative changes can be significantly affected by subsequent processing or storage conditions.

Treatment of proteins with oxidizing agents such as hydrogen peroxide has been reported to significantly decrease the nutritional value of the protein (Ellinger and Palmer, 1969; Rasekh et al., 1972; Slump and Schreuder, 1973; Anderson et al., 1975; Sjöberg and Bostrom, 1977). The emphasis in the early work was centered on the oxidation of the nutritionally essential methionine residues in the proteins. Rasekh et al. (1972), however, found a lower PER for hydrogen peroxide treated fish protein concentrate than for controls, and suggested that losses of both methionine and cysteine were responsible. Slump and Schreuder (1973), studying casein and fish meal, reached a similar conclusion. They suggested that cysteine was unavailable because it was oxidized to cysteic acid and methionine was oxidized to methionine sulfone. The interrelationship of methionine oxidation products and cysteine was discussed by Snow et al. (1975) in a study of the kinetics of the reduction of methionine sulfoxide by cysteine. Earlier, Bennett (1937, 1939) investigated the nutritional availability of various cysteine oxidation products. She observed that cysteine disulfoxide was capable of supporting growth, but the cysteinesulfinic acid was not. Kuzmicky et al. (1970) reported that methionine sulfoxide was only partially available as methionine and that cysteic acid was not available. Much work has been done in recent years on the oxidation of methionine and cysteine in food systems. Little and O'Brien (1967) studied the oxidation of protein thiol groups after reaction with various oxidizing agents. It was concluded that weak oxidants such as cupric sulfate or potassium ferricyanide caused oxidation to the disulfide while strong oxidants such as hydrogen peroxide and lipid hydroperoxide(s) caused formation of monomeric oxidation products (cysteinesulfinic acid and cysteic acid). Calam and Waley (1962) reported synthesis and separation of several glutathione oxidation products. Lewis and Wills (1962) discussed in great detail the oxidation of sulfhydryl groups by oxidizing linoleic acid and suggested that cystine is oxidized to a variety of products. Karel et al. (1975) and Schaich and Karel (1976) used ESR to study the interactions of amino acids including cysteine with peroxidizing lipids. The reactivity of lipid hydroperoxides with proteins and amino acids has been comprehensively reviewed (Jocelyn, 1972; Friedman, 1973; Gardner, 1979). In the excellent review by Gardner, the formation of cystine oxides and cystine addition products is discussed.

The purpose of this work is to study the oxidation of cysteine by using glutathione as a model for cysteine in proteins, under various conditions of pH and time of exposure to oxidizing agents that may be encountered in food processing.

MATERIALS AND METHODS

Glutathione [(γ-glutamylcysteinyl)glycine] (Sigma Chemical Co., St. Louis, MO) was dissolved in deionized water or deuterium oxide, adjusted to the desired pH, and diluted to yield a 0.25 M reduced glutathione (GSH) solution. The reported pH values were corrected for deuterium effect in deuterium oxide. All oxidants were added from highly concentrated solutions to minimize dilution of the glutathione solutions. In general, 0.4 mL of GSH solution was reacted in an 5-mm NMR tube or similarly sized test tube. Benzoyl peroxide was added as a solid and suspended by using a vortex mixer.

Table I. Amino Acid Analyzer Study of the Oxidation of GSH with Hydrogen Peroxide

	mole ratios of oxidation products, hydrogen peroxide added/mol of GSH				
	0	1	2.2	4.4	6.6
Product Hydrolysis					
GSH	1.0	0.3	0.2		–
GSSG		0.6	0.6		–
GSOG		TR	0.2	0.5	0.3
GSO_2G			TR	0.3	0.2
GSO_2H		TR		0.2	0.4
GSO_3H				TR	0.1
After Acid Hydrolysis					
glycine	1.0		1.0	1.0	1.0
glutamic acid	1.0		1.0	1.0	1.0
cysteine cystine	1.0		0.9	0.2	TR
cysteinesulfinic acid or cysteic acid		TR	0.1	0.8	0.9

Cystine monoxide was prepared according to the procedure of Savige et al. (1964). Cystine dioxide was synthesized according to Toennies and Lavine (1936). Cysteinesulfinic acid and cysteic acid were purchased from Sigma Chemical Co. Glutathione analogues for standards were prepared according to Calam and Waley (1962).

Amino acids were determined by the method of Lipton (1978) using a Phoenix Biolyzer Model 3000. NMR studies were carried out on a Varian 390 NMR spectrometer, using the standards described above.

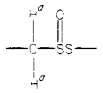
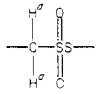
Linoleic acid hydroperoxide was prepared by dissolving 500 mg of linoleic acid (Applied Science Labs, State College, PA) in a few milliliters of 50% ethanol and adding the solution to 750 mL of oxygen-saturated 0.01 M borate buffer at pH 9.0. Aliquots (3–5 mL) of a 50 mg/15 mL solution of soy lipoxygenase (Worthington Biochemical Co., Freehold, NJ) were then added over a 1-h period, after which sodium chloride was added to near saturation and the solution brought to pH 4.0 with concentrated HCl. The solution was extracted 3 times with diethyl ether, the extract dried over anhydrous sodium sulfate, and solvent removed in vacuo. The sample was streaked on two Kontes 1000-μm silica TLC plates and developed in 50:50:1 Skelly F–ethyl acetate–acetic acid. The hydroperoxide (R_f 0.5) was visualized by dipping the plates for ~10 s in iodine vapor. The hydroperoxide was scraped from the plates and eluted from the gel with redistilled diethyl ether and evaporated to dryness. The yield was ~300 mg of hydroperoxide based on UV absorbance at 234 nm (ϵ = 25 000 L mol^{-1} cm^{-1}) in 95% ethanol.

RESULTS AND DISCUSSION

Glutathione was chosen as a model for the study of oxidation of cysteine residues because of its high purity and the fact that its oxidized forms are more soluble than free cystine and its oxidation products. Glutathione was oxidized with hydrogen peroxide to maintain the simplest possible system for the initial studies. After oxidation at pH 5.0 for 60 min, the glutathione samples were injected directly on the amino acid analyzer and the mole ratios of the products compared (Table I).

Table I also shows the results on the same samples after acid hydrolysis. It can be seen from the data that as the level of oxidant increases, the extent of oxidation of the cysteine portion of the glutathione also increases. The results are consistent with those discussed by Gardner (1979) concerning the products formed during the oxidation of cysteine with lipid hydroperoxide.

Table II. Approximate Proton NMR Shifts for Protons Adjacent to Oxidized Sulfur Groups

amino acid	shift, ppm
	3.0
	3.3
	3.8
	3.9
	2.8
	3.5-3.56

[a] Shifts are for these protons which are adjacent to oxidized sulfur groups.

Little and O'Brien (1967) reported that high levels of monomeric oxidation products (cysteic acid and alaninesulfinic acid) were formed when cysteine is oxidized with hydrogen peroxide and lipid hydroperoxide. Their results were observed over several hours compared to the short times in our studies. We reasoned, therefore, that if we had allowed the oxidations to continue for longer times, more monomeric oxidation products would have been observed. Samples of the oxidized mixtures were held for several days at room temperature and reanalyzed to test this hypothesis. As expected, considerably more cysteinesulfinic acid and cysteic acid were found, even in the least oxidized samples. When held in the dry state at room temperature, it appeared that cystine monoxide and cystine dioxide disproportionated or hydrolyzed to yield cysteinesulfinic acid. Although the degradation reactions were difficult to quantitate due to the uncontrolled conditions of the experiment, the qualitative effect when disproportionation or hydrolysis occurs is a net increase in monomeric oxidized cysteine products (cysteinesulfinic acid and cysteic acid), thus explaining the differences between our results and those of Little and O'Brien (1967). Our results also differ from those of Lipton et al. (1977), who reported mostly cysteic acid, lanthionine sulfoxide, and lanthionine sulfone when cysteine was oxidized with hydrogen peroxide. It is important to note, however, that our experiments were carried out at room temperature and moderate pH values, while Lipton's were carried out in strong acid solutions and elevated temperatures.

Because we were unable to separate cysteic acid and cysteinesulfinic acid after acid hydrolysis by ion-exchange chromatography, we decided to explore the potential of ^{1}H NMR as a tool to measure the oxidation intermediates and final products as they formed. NMR also minimizes sample handling and is nondestructive, so several determinations can be made on a single sample.

The chemical shift data for the various standards are shown in Table II. With the exception of cystine monoxide and dioxide, the proton on the β-carbon of the various derivatives can be easily differentiated. The first variable to be investigated with the NMR technique was

Table III. Reaction of GSH with Equimolar H_2O_2 at Various pH Values

	% of each derivative after 2 min			
pH	—SH	—SS—	—SS(=O)— + —SS(=O)$_2$—	—SO$_2$H
2.6	91	4	4	0
3.6	90	3	6	
4.6	64	16	18	2
5.6	25	33	36	5
6.6	23	30	40	8
7.6	19	31	45	6

Table IV. Oxidation of GSH with Various Oxidants at pH 6.0

	% of each derivative			
	—SH	—SS—	—SS(=O)— + —SS(=O)$_2$—	—SO$_2$H
bromate				
3 min	22	34	34	9
25 min	17	40	34	10
benzoyl peroxide				
2 min	93	3	3	0
2 h	46	22	24	6
H_2O_2				
2 min	25	32	39	4
60 min	19	34	41	6
hydroperoxide				
2 min	85	1	11	3
10 min	66	10	16	8
60 min	39	14	41	7
500 min	12	25	55	8
Chloramine T	5	92	TR	TR

the oxidation of glutathione by hydrogen perioxide at various pH values. The results in Table III show that the oxidation rate increases with increasing pH, and most of the cysteine in the glutathione is oxidized to the monoxide or dioxide. The formation of sulfinic acid in these short-time oxidations is consistent with the hypothesis of earlier workers (Little and O'Brien, 1967) that some cysteine is oxidized directly to the sulfinic acid. The intermediate oxidation products probably do not disproportionate or hydrolyze at a rate that would account for significant amounts of sulfinic acid.

Potassium bromate, benzoyl peroxide, and lipid hydroperoxide were used at pH 6.0 to oxidize glutathione to expand this observation and gain perspective about other oxidizing agents. Various times of reaction were investigated and the ratios of products compared (Table IV). The results suggest that there are no great differences between the oxidations of glutathione caused by bromate and hydrogen peroxide, although bromate appears to generate more sulfinic acid than the hydrogen peroxide. Benzoyl peroxide does not oxidize glutathione as rapidly as the other oxidants, but the limited solubility of benzoyl peroxide in deuterium oxide could explain those differences. All oxidants were added at 1 mol of oxidant/mol of GSH; however, it is likely that the hydroperoxide continued to form more oxidant as the reaction progressed. Oxidation by lipid hydroperoxide is interesting not only because of its importance in food products but also because the ratio of cystine to cystine monoxide and dioxide is considerably different from that observed with the other oxidants. At all times, when lipid hydroperoxide was the oxidant, the proportion of cystine monoxide and dioxide to the other oxidation products was much greater than when the other oxidizing agents were used, suggesting that lipid hydroperoxides react differently from the other oxidants. Even the additional level of hydroperoxide, however, does not completely explain the slightly higher level

of oxidation. As expected, the rate of oxidation (as expressed by loss in reduced glutathione) by the hydroperoxide is somewhat slower than the rate observed using the more soluble oxidants and faster than the oxidation by the poorly soluble benzoyl peroxide.

In all of the reactions it appears that there is a rapid initial oxidation of reduced glutathione, followed by a slower oxidation and/or reduction of oxidized products by reduced glutathione. The reaction between the sulfhydryl group and sulfinic acid has recently been reported in detail by Finlayson et al. (1979). Snow et al. (1975) earlier reported the reaction between methionine sulfoxide and cysteine. We suggest that a similar interaction takes place with cystine monoxide and dioxide; i.e., cystine monoxide and dioxide can be reduced by cysteine to cystine.

Oxidations such as these, when taking place in food systems, may have great significance due to the reduction in the nutritional quality of the protein. Nutritional losses, however, may only be the tip of the iceberg. The oxidized products discussed here are quite reactive and can lead to further decomposition, losses in protein functionality, undesirable flavor, and potentially toxic compounds. Considerably more investigation in this area is needed, but this work has demonstrated that the oxidations do occur in model systems and that the products can be measured effectively by proton NMR.

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Chemical Aspects of Mutagen Formation by Sorbic Acid–Sodium Nitrite Reaction

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The effect of reaction conditions on the yield of individual products in the reacted mixture of sorbic acid and sodium nitrite was investigated by using TLC and high-performance LC methods. It became clear that mutagenicity of the reaction mixture that reached maximum by the reaction at pH 3.5–4.2 is due to the formation of the product Y (1,4-dinitro-2-methylpyrrole) and ethylnitrolic acid (ENA). The yields of Y and ENA reached maxima at 30 min (at 60 °C), but ENA decreased thereafter. Y and ENA gave maximal yield at 8-fold excess of nitrite to sorbic acid, but their formation was detected even by reaction at 1:0.5 molar ratio. These chemical results well explained the observed pronounced effects of reaction conditions on biological activities. Ascorbic acid and cysteine above certain levels inhibit effectively the mutagen formation in this reaction system.

Induction of mutagenic activity toward bacteria by heating sorbic acid with sodium nitrite in aqueous medium has been reported earlier (Kada, 1974). By subsequent studies we have isolated several products of this reaction and examined their biological activities (Namiki and Kada, 1975; Namiki et al., 1980; Kito et al., 1979). It was shown that the maximum mutagenicity was produced by the reaction at pH 3.5–4.2, by the rec assay (Kada et al., 1972) and by the standard Ames reversion assay without metabolic activation (Ames et al., 1975). The growth-inhibitory activity of the reaction mixture differed from the mutagenic activity in that it was intensified by lower reaction pH. Considering the widespread use of these chemicals as food additives, it seemed to warrant closer examination of the reaction conditions that produce mutagenic activity, as a preparatory study of the possibility of the occurrence of this type of reaction in actual food systems. The present work mainly deals with the reaction of the product dis-

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