Preparation and Chemical Cleavage of *S*-Phenacyl-L-methioninesulfonium Bromide

Samuel H. Lipton* and C. E. Bodwell

The loss of nutritional availability of methionine in heat-damaged proteins may be due to chemical alteration of the methylthio group of the side chain of methionyl residues to form either oxidation products or sulfonium derivatives. The synthesis and study of the chemical cleavage of the model compound *S*-phenacyl-L-methioninesulfonium bromide (SPM) were carried out to provide information on the chemical behavior of sulfonium derivatives of methionine. Under conditions used for protein hydrolysis, SPM was cleaved by 6 *N* HCl into a complex mixture of products. SPM was more simply cleaved by treatment with dimethyl sulfoxide (Me_2SO) under relatively mild conditions to form homoserine lactone and methyl phenacyl sulfide. Applications of Me_2SO and other reagents which cleave sulfonium derivatives of methionine are discussed in relation to chemical studies of nutritionally available methionine.

Reduced nutritional availability of methionine has for some time been a recognized consequence of heat damage to proteins (Clandinin et al., 1947; Evans and Butts, 1949; Miller et al., 1965). Nevertheless, the chemical changes which account for these losses have not been precisely defined. Generally, they are assumed (see Ford, 1973, and citations therein) to involve interactions within and between protein and other food constituents (particularly carbohydrates). Since chemical alteration of the methylthio functional group of the side chain of methionyl residues might form either oxidation products or sulfonium derivatives, a knowledge of the chemical behavior of these derivatives might contribute to an understanding of methionine availability. A model sulfonium derivative, *S*-phenacyl-L-methioninesulfonium bromide (SPM), was therefore synthesized and its chemical cleavage was studied. This work and a discussion of its possible application to studies of methionine availability are the subject of this paper (this work has been described in part in a preliminary report; see Lipton et al., 1974).

EXPERIMENTAL SECTION

Materials and Methods. L-Methionine, *S*-methylmethioninesulfonium chloride (SMM), *S*-adenosylmethionine (SAM), L-homoserine, DL-homocystine, mercaptoethanol, phenacyl bromide, Eastman reagent grade dimethyl sulfoxide (Me_2SO), and other chemicals were purchased from commercial sources. (Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.) Spectra were obtained on the Cary Model 14 recording spectrophotometer (uv) and the Varian Model A-60 spectrometer (NMR).

A Phoenix Biolyzer, Model 3000 instrument, was used as previously described (Lipton and Bodwell, 1973) for amino acid analyses. A 17-cm basic column was used with 0.35 *M* sodium citrate buffer (pH 5.28) at a flow rate of 80 ml/hr. When available, authentic compounds were used for chromatographic comparison with amino acid cleavage products. The observed elution times for products from the basic column were as follows: *S*-phenacylhomocysteine, 47 min; SMM, 67 min; ammonia, 67 min; homoserine lactone, 82 min; SPM, ca. 175 min. Yields of cleavage products were calculated from measurements of peak areas with the assumption that all products had the same peak area per mole. Molar distributions of products were thus approximations since homoserine lactone has been estimated to yield 82% of the color given by an equimolar amount of leucine (Neumann et al., 1962).

Cleavage of Sulfonium Derivatives. For studies of the cleavage of SPM and SMM by various reagents, a 5-mg sample of sulfonium salt was treated in a 12-ml conical centrifuge tube with 1 ml of reagent. Observations on SAM were made by using 1-mg samples and the same relative volume of reagent. For reaction, the tubes were incubated in a water bath from 5 min to 21 hr at either 25 or 100°C. Aliquots of 10-μl volume of a given reaction solution were removed for immediate analyses on the 60-cm (for acidic and neutral amino acids) and the 17-cm (for basic amino acids) columns of the amino acid analyzer. With these low sample volumes, no pH adjustments of the samples were necessary.

Synthesis of *S*-Phenacyl-L-methioninesulfonium Bromide (SPM). One-tenth mole quantities of L-methionine (14.9 g, dissolved in 275 ml of water) and phenacyl bromide (19.9 g, dissolved in 275 ml of ethanol) were mixed during vigorous magnetic stirring at room temperature. The milky mixture became clear after about 1 hr but stirring was continued for 6 more hr. During overnight storage in the refrigerator, the clear solution was transformed into a crystalline mass. By filtering on a sintered glass funnel, washing with ethanol, and drying in vacuo, 20.3 g (57% yield) of product was obtained. After recrystallization from warm water, dried waxy white crystals were obtained. Amino acid analysis showed the product to be free of ninhydrin-reactive impurities, with a single peak in the far basic region of the ion-exchange chromatogram (the sulfonium isomers were not resolved): $\lambda_{max}^{H_2O}$ (pH 1.0) 254 nm (ϵ 14100), min 221 nm (ϵ 470); NMR spectrum (δ) from external tetramethylsilane (deuterium oxide) 7.9 (5 H, m), 3.97 (1 H, t), 3.7 (2 H, m), 3.13 (3 H, s), 2.5 (2 H, m); (dimethyl-d_6 sulfoxide) 2.15 (3 H, s). Anal. Calcd for $C_{13}H_{18}NO_3BrS \cdot 0.5H_2O$: C, 43.70; H, 5.36; N, 3.91; S, 8.91. Found: C, 43.93; H, 5.60; N, 3.92; S, 8.73.

Synthesis of Methyl Phenacyl Sulfide. Preparation of this sulfide was by the reaction of phenacyl chloride with sodium methyl mercaptide in methanol solution (Prelog et al., 1944). The redistilled oily product had bp 113°C at 1.5 mm pressure. This product was used for comparison with the ether-soluble product cleaved from SPM (see discussion of SPM structure).

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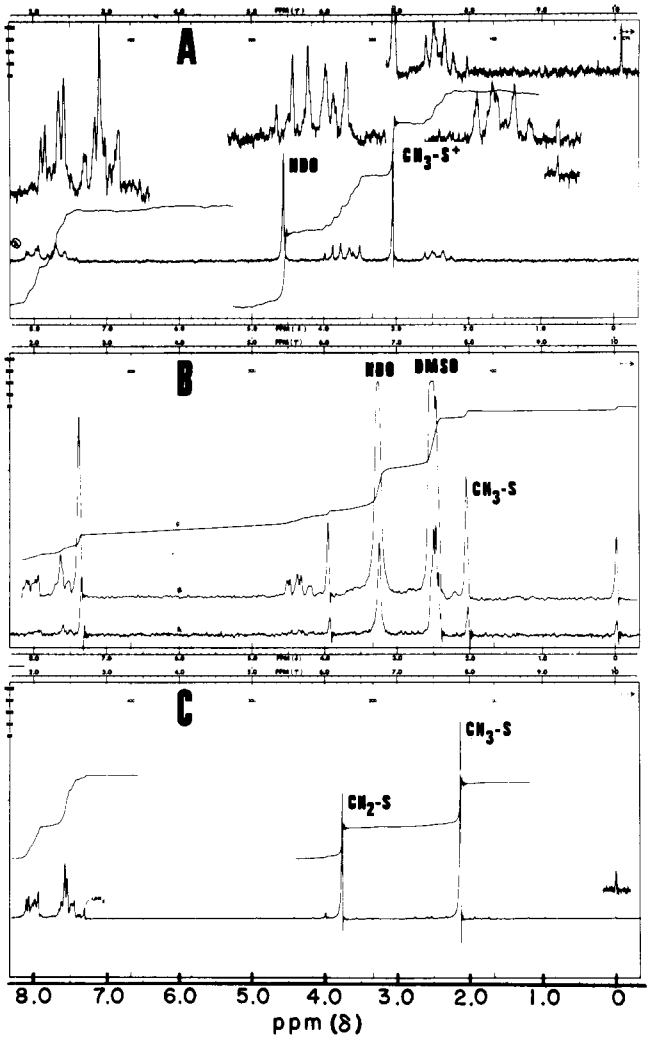


Figure 1. NMR spectra: (A) *S*-phenacyl-L-methioninesulfonium bromide (SPM) in D_2O solution; (B) SPM in Me_2SO-d_6; (C) methyl phenacyl sulfide in $CDCl_3$ solution.

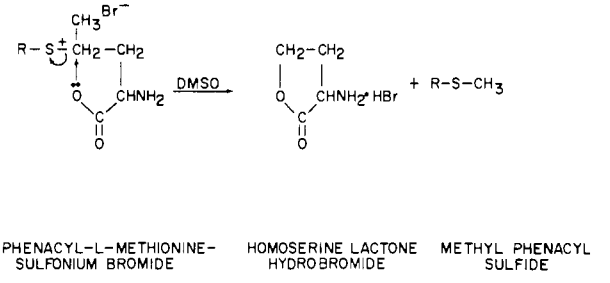
RESULTS AND DISCUSSION

The synthesis of SPM for its intended use as a model compound in a study of sulfonium derivatives of methionine was an outgrowth of previous work on the nonenzymic browning reaction of foods. In that investigation, *N*-furacyl-L-methionine (Lipton and Dutky, 1972a) and *N*-phenacylglycine (Lipton and Dutky, 1972b) had been synthesized. A rearrangement of *N*-furacyl-L-methionine by an internal N to S migration of the furacyl group could be expected to form the *S*-furacyl-L-methioninesulfonium salt. SPM was selected for study, rather than the *S*-furacyl derivative, because of the similar chemical stabilities of phenacyl and furacyl derivatives and the commercial availability only of phenacyl bromide.

Structure of SPM. In previous work (Stein and Moore, 1946), reaction of mustard gas [bis(β-chloroethyl) sulfide] with free methionine yielded the corresponding methioninesulfonium derivative. Similarly, in the synthesis of SPM by direct reaction of phenacyl bromide and free L-methionine, the thioether group was the preferred point of attack, even though the amino and carboxyl groups were unblocked. The SPM structure was supported by analyses. The NMR spectrum in D_2O solution had (see Figure 1A) a singlet at δ 3.13 that corresponded to the CH_3S^+ group. The absence of phenacyl methylene protons was due to their rapid exchange in D_2O solution; the NMR spectrum of SPM was therefore run in Me_2SO-d_6 solution since this solvent had been used to obtain NMR spectra of other sulfonium salts (Chamberlain and Reed, 1971). The instability of SPM in Me_2SO was apparent from the drastic spectral changes, especially the upfield shift of the singlet from δ 3.13 to 2.15 (Figure 1B).

This surprising lability of SPM in Me_2SO solution was confirmed by observations on the amino acid analyzer and subsequent chemical studies. Although intact SPM was eluted from the basic column at ca. 175 min, the SPM sample used for the observation of the NMR spectrum in Me_2SO solution was found to be eluted as a new peak at 82 min in the basic amino acid chromatogram. The elution position of SPM depended on the timer setting for automatic regeneration of the column. Apparently, SPM was eluted just before break-through of the 0.4 *N* NaOH regenerant. This new peak was identified as homoserine lactone by chromatographic comparison with an authentic sample of homoserine which had been lactonized by heating at pH 1. Cleavage of SPM to give homoserine lactone plus free homoserine was obtained by boiling in water solution. The solution became milky white due to the separation of a water-insoluble neutral oil. After separation by extraction with ether, this sulfide oil was found to have an NMR spectrum which was identical with that of synthetic methyl phenacyl sulfide (Figure 1C). This same neutral sulfide product was obtained from the cleavage of SPM by heating for 5 min at 100°C in Me_2SO solution.

The chemical cleavage of SPM into homoserine lactone hydrobromide and methyl phenacyl sulfide is shown in the following reaction:



S-PHENACYL-L-METHIONINE-SULFONIUM BROMIDE | HOMOSERINE LACTONE HYDROBROMIDE | METHYL PHENACYL SULFIDE

(R = $C_6H_5COCH_2$-)

This internal nucleophilic displacement reaction is apparently accelerated by Me_2SO. The acceleration by Me_2SO of many other nucleophilic reactions has been described (Parker, 1965), but Me_2SO has not previously been reported to cleave sulfonium derivatives of methionine. The cleavage of sulfonium derivatives of methionine into homoserine lactone and a sulfide has been known for some time (Toennies and Kolb, 1945; McRorie et al., 1954; Gundlach et al., 1959); in fact it has been used for the specific cleavage of methionyl peptides (Lawson et al., 1961). The selective cleavage of proteins at methionyl residues by cyanogen bromide (Gross, 1967) involved an *S*-cyanosulfonium intermediate, which split in a similar manner. (These cleavages in aqueous solution gave both free homoserine and its lactone, while only the lactone was obtained in Me_2SO.) The gas chromatographic measurement of methyl thiocyanate (Inglis and Edman, 1970) has been used to quantitate the cyanogen bromide cleavage reaction. The same cleavage has been used for determination of methyl-labeled radioactive methionine by counting the radioactivity of the methyl thiocyanate (Clark et al., 1974).

Cleavage of SPM by Various Reagents. The products of hydrolysis of SPM by 6 *N* HCl were of primary

Table I. Cleavage of *S*-Phenacyl-L-methioninesulfonium Bromide (SPM) by Various Reagents

Reagent	Conditions	Major amino acid products[a]
6 *N* HCl	16 hr at 100°C	*S*-Phenacylhomocysteine (50%) Methionine (25%) Homoserine (4%) Homoserine lactone (5%) *S*-Methylmethionine (SMM) (8%) SPM (5%)
48% HBr	75 min at 100°C	Methionine (64%)
88% formic acid	5 min at 100°C	*S*-Methylmethionine (SMM) (47%)
0.5% mercaptoethanol	15 min at 25°C in 5% $NaHCO_3$ soln	Methionine (ca. 100%)
Dimethyl sulfoxide (Me_2SO)	5 min at 100°C or 18 hr at 25°C	Homoserine lactone (>80%)

[a] For reagents other than 6 *N* HCl, only the main ninhydrin-reactive product is listed; percent values are on a molar basis and were calculated from amino acid analyses as described in the text.

interest since this reagent is commonly used for hydrolysis of samples before amino acid analysis. As summarized in Table I, SPM was cleaved into a complex mixture of amino acid products by 6 *N* HCl. The main product (ca. 50% yield), which was eluted from the basic column at 47 min, was *S*-phenacylhomocysteine. Next in abundance was methionine (ca. 25% yield), with lesser amounts of homoserine, homoserine lactone, SMM, and apparently residual SPM. This unexpected formation of SMM from SPM may have resulted from an intermolecular disproportionation reaction. An exchange of a methyl and a phenacyl group between two molecules of SPM would form SMM and another sulfonium compound, *S*,*S*-diphenacylhomocysteine. The latter compound was not identified, but it probably was not eluted from the column during chromatography. The SMM product occupied the same 67-min elution position as ammonia, but it was definitely identified by isolation and subsequent comparison of its NMR spectrum with that of an authentic sample of SMM.

The isolation of SMM in pure form was carried out from the syrupy vacuum concentrate obtained from the HCl hydrolysate of 10.5 g of SPM. By adding an equal volume of ether to an ethanol solution of the concentrate, a precipitate was obtained which, following recrystallization from ethyl alcohol, was exclusively the "67-min component" (SMM). The main ingredient of the syrupy concentrate (the "47-min component") was thus freed of much of the contaminating SMM, but it was still not chromatographically pure. Its designation as *S*-phenacylhomocysteine was supported by its susceptibility to performic acid oxidation according to the procedure of Moore (1963) (SMM, in contrast, was not displaced from its 67-min position in the chromatogram by this performic acid treatment). The elution of this neutral amino acid derivative in the vicinity of the basic amino acids of the chromatogram may be attributed to an attraction of the phenacyl ring to the nonpolar region of the polystyrene ion-exchange resin.

The distribution of the products from the 6 *N* HCl cleavage of SPM closely resembled that which had been obtained from methioninecarboxymethylsulfonium iodide (Gundlach et al., 1959). In this earlier study, *S*-carboxymethylhomocysteine and methionine, respectively, accounted for 50 and 20% of the cleaved sulfonium compound, with small yields of homoserine and its lactone (similar to the yields reported here for SPM). Due to its greatly delayed elution position, homocystine was not determined in our amino acid analyses. Gundlach et al. (1959) found only 2% of homocystine in their 6 *N* HCl hydrolysate; a similar small yield of homocystine may have been present in the SPM hydrolysate. (Since these authors observed sizable peaks in their acid hydrolysates which they designated as ammonia, it would be of interest to reexamine the HCl hydrolysate of methioninecarboxymethylsulfonium iodide in order to determine whether the presence of SMM was missed.)

The cleavage of SPM by other reagents gave much different distributions of products than was obtained with 6 *N* HCl. As shown in Table I, the main product from 48% HBr treatment was methionine, which was obtained in about 64% yield after 75 min at 100°C. Methionine was most effectively regenerated from SPM by mercaptoethanol, which previously had been used to remove phenacyl groups from methionyl residues (Naider and Bohak, 1972). With this thiol, the phenacyl group was presumably removed as the hydroxyethyl phenacyl sulfide, since no other amino acid cleavage products were obtained. (By detection of a hydroxyethyl sulfide after the treatment of a heat-damaged protein with mercaptoethanol, it might be possible to identify alkyl groups derived from sulfonium derivatives which may have been formed in the heat treatment.)

Cleavage of SPM by Me_2SO (Table I) gave homoserine as the principal product (ca. 80%) when SPM (in Me_2SO solution) was heated for 5 min at 100°C or allowed to stand overnight at room temperature. This way of desulfurizing methionine was suggested (Lipton et al., 1974) as the basis of one possible approach for developing a chemical method for estimating nutritionally available methionine, since the neutral methyl phenacyl sulfide could be measured either by spectrophotometry or by gas chromatography. However, this desulfurization by Me_2SO has not yet been evaluated for the methionyl residues of proteins. Possibly it would suffer the same disadvantage as previously observed for the methioninecarboxymethylsulfonium derivative. Gundlach et al. (1959) were unable to obtain quantitative desulfurization of methionyl residues of proteins, despite the fact that the free sulfonium derivative was readily desulfurized by boiling at pH 6.6. Since cyanogen bromide (Gross, 1967) did efficiently desulfurize methionyl residues of proteins, this reagent deserves further study as a reagent for detection of heat damage to methionine.

SMM was a major product (47% yield) obtained by heating SPM in 88% formic acid for 5 min at 100°C (Table I). The strongly protic solvent formic acid was selected for this use because its properties contrasted with those of the dipolar aprotic solvent Me_2SO. (When the methioninecarboxymethylsulfonium derivative was heated in 88% formic acid in the same manner as above, SMM was not a major product of the decomposition.)

Cleavage of SMM and SAM by Various Reagents. The data in Table II summarize observations on the cleavage of SMM by various reagents. The regeneration of free methionine from SMM by 6 *N* HCl at 100°C was not rapid (21% at 6 hr and 53% at 21 hr). Thus, SMM was much more stable to 6 *N* HCl than was SPM. [A half-life of 25 hr was reported (Ramirez et al., 1973) for SMM at 90°C in 1 *M* aqueous solution at pH 1 (HCl), vs.

Table II. Cleavage of S-Methylmethioninesulfonium Chloride (SMM)

Reagent	Conditions	Major amino acid products[a]
6 *N* HCl	6 hr at 100°C	Methionine (21%)
6 *N* HCl	21 hr at 100°C	Methionine (53%)
90% Me_2SO	5 min at 100°C	Homoserine lactone (90%)
H_2O	5 min at 100°C	Homoserine lactone (3%)
0.2 *N* NaOH	15 min at 100°C	Homoserine (25%)
0.5% mercapto-ethanol	15 min at 25°C in 5% $NaHCO_3$ soln	None

[a] Only the main ninhydrin-reactive product is listed; percent values were calculated on a molar basis from amino acid analyses as described in the text.

a half-life of 50 min at pH 7.] The acceleration by Me_2SO of the cleavage of SMM to form homoserine lactone was evident from the 90% yield of this product formed in 90% Me_2SO after only 5 min at 100°C vs. the mere 3% yield given by boiling water solution for 5 min. The failure of mercaptoethanol to regenerate free methionine from SMM confirms a previously described dependence (Naider and Bohak, 1972) of the reaction of this sulfur nucleophile with sulfonium derivatives on the nature of the sulfonium groups. [An electrochemical cleavage of SMM to dimethyl sulfide and homoserine lactone has been reported (Iwasaki et al., 1973).]

SAM, which has been actively studied since it was recognized to participate in many enzymatic methyl transfer reactions (Schlenk, 1965), was also tested for cleavage by Me_2SO. When heated for 5 min at 100°C in 90% Me_2SO solution, SAM was split to homoserine lactone and presumably 5'-methylthioadenosine. Thus, all three sulfonium derivatives were similarly split by Me_2SO. (Amino acid analysis on the basic column disclosed the presence of six ninhydrin-reactive impurities in addition to homoserine lactone in the commercial SAM preparation. Since these impurities were unaffected by this Me_2SO treatment, this complete shift due to Me_2SO of the SAM peak to the homoserine position might be useful as a simple chemical assay for SAM.)

Application to Chemical Studies of Nutritionally Available Methionine. The nutritional availability of an amino acid can be defined as that fraction of the total content of an amino acid in a food or feed which is utilized for growth or other processes of a living organism. Loss of availability of methionine in heat-damaged proteins may be due to chemical alteration of the methylthio group of the side chain of methionyl residues to form either oxidation products or sulfonium derivatives. Thus defined, nutritionally available methionine is methionine which has an unaltered methylthio group which is capable of reacting with a suitable chemical reagent. This definition has been the basis for attempts to develop a chemical method for estimating nutritionally available methionine (Lipton et al., 1974). In one approach (Lipton and Bodwell, 1976), Me_2SO was suggested for estimation of nutritionally available methionine by using Me_2SO as a specific oxidant of methionine to methionine sulfoxide. The observations of this paper on the cleavage of the sulfonium derivatives, SPM, SMM, and SAM, emphasize the possibility of employing the desulfurization of a sulfonium derivative, both for defining the nature of any sulfonium derivative which may have been formed during heat damage, and for estimating unaltered methionyl residues in terms of an alkylated derivative (such as the *S*-phenacyl). Based on earlier studies (Neumann et al., 1962), previously altered methionyl residues would not be expected to undergo either alkylation or oxidation to the sulfoxide.

Sulfonium derivatives of methionine have not been proven to be present in native proteins despite the widespread natural occurrence of SAM and SMM. Although their formation in heat-damaged foods and feeds is a distinct possibility, this has not been definitely established. The complexity of products formed from sulfonium derivatives during 6 *N* HCl hydrolysis complicates their characterization. Some sulfonium derivatives are destroyed during acid hydrolysis; others may be difficult to detect due to their very late elution in the ion-exchange analysis. The coincidence of the SMM and ammonia elution positions indicates that SMM may have been overlooked in some hydrolysates. If SMM is formed from other sulfonium compounds by a disproportionation reaction (like that indicated for SPM), the relatively acid-stable SMM might serve as a general indicator of the presence of sulfonium derivatives of methionine. An elevated SMM level or increased levels of homoserine and homoserine lactone in hydrolysates of heat-damaged proteins would be consistent with sulfonium formation during heat damage. A pretreatment of the protein sample with Me_2SO prior to hydrolysis with HCl would exaggerate the levels of homoserine and homoserine lactone obtained in the hydrolysate and thus help prove the presence of a sulfonium derivative.

Since dye-binding procedures (Lakin, 1973) have been used to assess protein damage during food processing, the effects of alterations of methionine on dye-binding should be considered. Both sulfoxide and sulfonium derivatives of methionine would be expected to bind acid dyes. However, increases of dye binding due to alterations of methionyl residues may not have been detected due to the quantitatively larger decreases in basic groups caused by heat damage to lysine.

Enzyme inhibition by phenacyl bromide has been attributed (Stark, 1970) to the alkylation of methionyl residues. (Since SPM has not been synthesized previously, it may also find use in enzyme studies.) Sulfonium derivatives similar to SPM (possibly *S*-furacyl derivatives) may be formed during heat damage to foods and reduce the availability of amino acids by inhibition of digestive enzymes. Alternatively, alkylated methionyl residues as well as oxidized methionyl residues may be nutritionally unavailable due to a failure to be released by digestive enzymes (*Nutrition Reviews*, 1973).

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Quantitative Determination of Saccharin in Food Products by Ultraviolet Spectrophotometry

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A procedure for the determination of saccharin in chewing gum and other food products has been developed. Saccharin is isolated by extracting the acidified sample solution with chloroform. The residue, after evaporation of the chloroform, is dissolved in 1% Na_2CO_3 solution and absorbance is determined at 235 and 244 nm; the latter wavelength absorbance is to correct for any possible interference. Recoveries ranged from 83 to 113%. Analyses of commercial products of various brands gave 90 to 105% of label claim.

Saccharin is the major noncaloric sweetener currently in use on a sucrose sweetness equivalent basis (Walter, 1973). It has been used as a sweetener in a wide variety of food products for more than 60 years. In spite of the limitation on the use of saccharin by the FDA and its removal from the GRAS list its annual consumption in 1972 exceeded 4 million pounds (National Academy of Sciences–National Research Council, 1974).

The literature contains numerous reports on the determination of saccharin in foods and beverages. Various techniques were used; colorimetry (Fernandez-Flores et al., 1973), gas–liquid chromatography (Conacher and O'Brien, 1970; Ratchik and Viswanathen, 1975), infrared spectrophotometry (Coppini and Albasini, 1968), polarography (Lasheen, 1966), gravimetry (Oakley, 1947), and quite recently an ion-selective electrode method (Hazemoto et al., 1974). Basile (1966) reported an ultraviolet procedure for the determination of saccharin in wine, whereby saccharin was isolated as the free acid and interfering materials were eliminated by oxidation with alkaline permanganate solution. The saccharin was extracted from the sample with chloroform, and determined from its absorbance at 278 nm. In this procedure, interfering materials that are extractable with chloroform and possess an aromatic structure after the permanganate treatment will still interfere in the determination. This is true in many food products which contain flavoring materials and benzoates. De Garmo et al. (1952) used a uv procedure to determine the stability of saccharin solution on heating whereby absorbance of saccharin in sodium hydroxide solution was determined at 268 nm.

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The method reported in this paper utilized the solubility of saccharin and its salts in sodium carbonate solution and its unique absorption characteristic in this solution; two distinct absorption maxima are exhibited at 235 and 229 nm in addition to the broad absorption band exhibited in the 265–275-nm range. The double absorption maxima can serve as an identity test for saccharin. High sensitivity is achieved by this method; the molar extinction coefficient of saccharin in 1% Na_2CO_3 solution is 6260 at 235 nm and 8170 at 229 nm, calculated from absorbance of a solution containing 0.03 mg/ml. The coefficient at 268–275 nm in both aqueous and organic matrix is approximately 1550. In addition to the absorption characteristics, the method is simple and accurate requiring minimal preparation to remove interfering substances.

EXPERIMENTAL SECTION

Method. *Reagents and Apparatus.* All reagents used were either ACS reagent grade, U.S.P., or N.F. A UNICAM SP 800 recording spectrophotometer was used, with the following settings: fast scan, energy programme control set at normal energy E, slit width indicator set at 0, the deuterium lamp is on, 190–450-nm wavelength range. Quartz cells, 10 mm thickness, were used.

General Procedure. An aliquot of the specified sample was transferred to a 125-ml separatory funnel. Distilled water was added to make the volume 50 ml. Concentrated HCl, 7.5 ml, was added, and the solution was extracted three times with 35 ml of chloroform. The separatory funnel was shaken 1–2 min each time and the layers were allowed to equilibrate for at least 5 min. The chloroform layer (bottom) was decanted through a funnel fitted with a chloroform-wetted plug of glass wool or cotton (0.5-in. thickness) into a 250-ml beaker. The separatory funnel,