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Reaction of Reducing Sugars with Sulfathiazole and Importance of This Reaction to Sulfonamide Residue Analysis Using Chromatographic, Colorimetric, Microbiological, or ELISA Methods

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Studies with sulfathiazole (ST) as a representative sulfonamide demonstrated that the N^4 aromatic amino group of sulfonamides can react with reducing sugars to form a variety of different sugar-bound compounds. ST was not destroyed in this reaction, and free ST could be produced from some of these sugar-bound compounds by aqueous dilution and, especially, by acidification. Different effects of these sugar-bound ST compounds, especially the N^4-linked ST Amadori compound, were noted on various methods of sulfonamide residue analysis. The sugar-bound ST compounds exhibited chromatographic behavior different from that of free ST, did not visualize with a common TLC Bratton–Marshall spray specific for sulfonamides, did not inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* strains susceptible to free ST, but did inhibit binding of antisera in an indirect competitive enzyme-linked immunosorbent assay (ELISA) as much as 33 times greater, on a molar basis, than free ST.

The widespread use of antibiotics for animal husbandry and as feed additives has led to concern that foods derived from animals could be contaminated with antibiotics (Moates, 1986). Antibiotics in food could result in allergic or toxic reactions in sensitive consumers. Also, there are general concerns that the widespread use of antibiotics could contribute to antibiotic resistance in pathogenic organisms. A variety of sensitive methods have been developed to detect, quantitate, and therefore control antibiotic residues in food.

The sulfonamides (Anand, 1975) are a group of synthetic antibiotics widely used in agriculture and human therapy. Using a colorimetric method to detect sulfathiazole (ST) in honey, Low et al. (1989) noted that this sulfonamide disappeared by reaction with reducing-sugar solutions at elevated temperatures. This apparent loss of ST was surprising, since ST was reported to be remarkably stable in heated acidic aqueous solutions (Pawelczyk and Zajac, 1976).

The formation of a sugar–sulfonamide reaction product, N^4-glucopyranosylsulfamethazine, has been reported by Paulson et al. (1981), Giera et al. (1982), and Parks (1984) for sulfamethazine (also known as sulphadimidine in the literature) residue analysis of swine tissue. Parks suggested that analytical procedures for sulfonamides should account for this type of derivative; however, this suggestion has been ignored in some more recent analytical procedures for sulfonamide residues.

The following study was undertaken to investigate further the reaction between ST and reducing sugars and the effect that this reaction would have on various analytical procedures commonly used for sulfonamide analysis.

EXPERIMENTAL SECTION

Instrumentation and Materials. UV absorbances were recorded with a Hewlett-Packard Model 8451A diode array spectrophotometer (Hewlett-Packard (Canada) Ltd., Mississauga, ON). Absorbance values in microtiter plates were measured with a Model EL 309 ELISA reader (Bio-Tek Instruments, Inc., Burlington, VT). Solvents were removed from samples with a Büchi Rotavapor-R (Fisher Scientific, Edmonton, AB) with a bath temperature maintained ≤40 °C. Thin-layer chromatography (TLC) plates were 20 × 20 cm PE SIL G/UV with polyester backing purchased from Whatman Ltd., Maidstone, England. A Master-Mite Model 10008 heat gun (Master Appliance Corp., Racine, WI) was used to evaporate solvent and for charring of TLC plates. A Model UVS-54 lamp (254-nm light emission produced by Ultra Violet Products, Inc., San Gabriel, CA) was used to check for TLC UV absorbance. Centrifuga-

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tion was performed with a Damon/IEC Division Model HN-S II centrifuge from International Equipment Co., Needham Heights, MA. Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), tryptic soy agar, and Mueller Hinton (MH) broth were obtained from Difco Laboratories, Detroit, MI. MH agar was obtained from Oxoid Ltd., Busingstoke, England. All sulfonamides, thimerosal, Tween 20, bovine serum albumin (BSA), and *Limulus polyphemus* hemolymph (LPH) were obtained from Sigma Chemical Co., St. Louis, MO. Urea peroxide, *o*-phenylenediamine tablets, and goat anti-rabbit peroxidase conjugated antibodies were purchased from Calbiochem Co., San Diego, CA. Dynatech Immulon 1 microtiter plates and petri dishes were purchased from Fisher Scientific, Edmonton, AB. Spectrapor dialysis tubing was obtained from Spectrum Medical Industries Ltd., Los Angeles, CA.

All water was prepared with a Millipore Milli-Q system. For column chromatography silica gel (Kieselgel 60, 70–230 mesh size; Terochem Laboratories Ltd., Edmonton, AB) was used. For flash chromatography 400–230-mesh silica gel 60 from E. Merck, Darmstadt, was used. All other chemicals used were reagent grade or better.

IR and ^{1}H NMR spectra were recorded on a Perkin-Elmer 257 grating infrared spectrophotometer and a 60-MHz Varian Model EM-360, respectively. A Kratos MS-9 was used for fast atom bombardment with xenon (POSFAB MS). Elemental analysis was carried out by the Microanalytical Laboratory of Chemistry Services at the University of Alberta.

Concentrated Sugar–Sulfathiazole Reactions. Two solutions were prepared, one using glucose and one using fructose. The respective reducing sugar (78.8 g, 0.437 mol) and 37.2 g (0.134 mol) of sodium sulfathiazole were added to a 250-mL volumetric flask with water and dissolved, and the flask was made up to volume with water. A magnetic stir bar was added to each flask, and each solution was stirred at room temperature protected from light with aluminum foil.

Total free and bound ST, or isolated free and isolated bound ST, were estimated by dissolving samples in 3.5 M hydrochloric acid and reading the absorbance at 280 nm (extinction coefficient 11 700 M^{-1} cm^{-1}).

Free and bound ST could be separated by silica gel chromatography. The sugar–ST solution (0.5 mL) was added to 20 mL of water and 1 g of silica gel. The water was removed with a flash evaporator. This dried material was then carefully applied to the top of a 15-g silica gel column (2.2-cm i.d. × 30-cm height) and free ST was eluted with ca. 200 mL of 1:4 methanol–chloroform until no further UV-absorbing material could be detected by thin-layer chromatography (TLC). The sugar-bound ST was eluted with 1:1 methanol–chloroform (ca. 150 mL). Pooled samples of each material were evaporated to dryness for use in other experiments.

TLC Procedure. TLC plates (10 cm high) were developed in 4:1 chloroform–methanol. The developed plates were examined by using a UV lamp or a Bratton–Marshall spray reagent (Parks, 1982). Charring of the plates was carried out by using the Bratton–Marshall (B–M) spray without the final *N*-(1-naphthyl)ethylenediamine spray or 10% sulfuric acid and heating the plates for 2 min with a heat gun set on the hot setting.

Microbial Growth Inhibition Test. All agars and broths were sterilized before use at 121 °C for 15 min in an autoclave. The test organisms, from the American Type culture collection, with either *Escherichia coli* (ATCC 11775) or *Staphylococcus aureus* (ATCC 25923). They were maintained on tryptic soy agar slant at 4 °C and subcultured twice in MH broth at 35 °C for 18–24 h for use in the experiments.

Petri dishes (150-mm diameter) were prepared by adding 70 mL of MH agar and drying for 30 min in a laminar flow hood. A "soft" MH overlayer (21 g/L MH broth and 7.5 g/L MH agar) was sterilized and tempered to 45 °C. MH broth containing one of the organisms was added to the "soft" MH overlay agar (10 μL/mL agar) and mixed gently. The prepared "soft" MH overlayer was added to the petri dishes (17 mL/dish) and dried for 30 min at room temperature in a safety hood.

Solutions (10 μL) of known amounts of ST, succinyl-ST, and ST Amadori in water or isolated glucose-bound ST and fructose-bound ST in methanol (10 μL of methanol alone did not inhibit microbial growth) were spotted with a micropipet onto the surface of the MH overlayer. The spots were separated by at least 4 cm. The petri dishes were dried until the spots disappeared in a safety hood. The petri dishes were inverted, placed into a plastic bag, and incubated for 18–24 h at 35 °C. Clear zones of inhibition, if present, were observed to determine minimum inhibitory concentrations of compounds.

Preparation of ST–Bovine Serum Albumin Conjugate. Sodium sulfathiazole (109 mg) was dissolved in 1.0 mL of 3.5 N hydrochloric acid in a 25-mL flask, and 3.0 mL of 1% sodium nitrite was added. This orange solution was stirred for 10 min at room temperature. Ammonium sulfamate (50 mg) was added, and the reaction was stirred for another 10 min. This reaction mixture was added to bovine serum albumin (BSA, 202 mg) dissolved in 0.5 mL of water and 1.0 mL of 1.0 M sodium carbonate in a stirred 25-mL flask. A further 150 mg of sodium carbonate was added to the combined reaction, and the initial flask was washed with three portions of 0.25 mL of 1.0 M sodium carbonate. The washings were added to the stirring mixture, which was kept overnight at 4 °C.

The next day the entire reaction mixture was transferred to dialysis tubing (6000–8000 MW cutoff) with 5 mL of 8.0 M urea and dialyzed against 1 L of 8.0 M urea, followed by 4 L of 50 mM ammonium bicarbonate and 4 L of 25 mM ammonium bicarbonate. Dialysis with each solution was carried out for a minimum of 8 h. The contents of the dialysis bag were freeze-dried and submitted for elemental sulfur analysis. The BSA–ST conjugate contained 4.51% sulfur, while BSA alone gave a sulfur analysis of 1.68%. From this analysis the calculated ST substitution in the BSA–ST conjugate was 44 mol per mol of BSA (66 000 MW).

Preparation of ST–*Limulus polyphemus* Hemolymph Conjugate. Sodium sulfathiazole (7 mg) was dissolved in 2.1 mL of 3.5 N hydrochloric acid in a 25-mL flask, and 0.2 mL of 1% sodium nitrite was added. The solution was stirred for 10 min at room temperature. Ammonium sulfamate (5 mg) was added, and the reaction was stirred for another 10 min. This reaction mixture was added to *Limulus polyphemus* hemolymph (LPH, 49.4 mg) dissolved in 1.3 mL of 6.0 N sodium hydroxide in a stirred 25-mL flask. A further 9.5 mg of sodium carbonate was added to the combined reaction mixture, and the initial flask was washed with one portion (0.25 mL) of water. The wash water was added to the mixture, which was kept at 4 °C overnight.

The next day the entire reaction mixture was transferred to dialysis tubing and dialyzed as described above for the BSA–ST conjugate. The LPH–ST conjugate contained 3.19% sulfur, while LPH alone gave a sulfur analysis of 1.21%. Therefore the calculated ST substitution in the LPH–ST conjugate was 28 mol per BSA equivalent unit (i.e., using 66 000 MW again so that comparison with the BSA–ST was convenient).

Phosphate-Buffered Saline (PBS) Solution. Sodium chloride (31.5 g), 3.88 g of disodium hydrogen phosphate, 1.05 g of potassium dihydrogen phosphate, and 350 mg of thimerosal (not added for sterilized PBS used in injections) were dissolved in 3.4 L of water. The pH was adjusted to 7.3 with addition of hydrochloric acid or sodium hydroxide as needed. Finally, the solution was made up to 3.5 L with water. Tween 20 (1.75 g, 0.05%) was added before the final step to produce PBST.

Antibody Preparation. Two 11-week-old prebled female Flemish Giant × Dutch Lop Ear cross rabbits were each injected with 0.8 mL of a water-in-oil emulsion of LPH–ST in sterile PBS and FCA (1:1 (v/v)). The rabbits were injected (2 × 0.2 mL) subscapularly and (0.4 mL) into a hip muscle. The rabbits received identical booster injections, with the exception that FIA was used rather than FCA 2 and 4 weeks after the initial injection. The rabbits were bled after 4 weeks from the large artery in the ear (approximately 15 mL from each rabbit) and after 6 weeks by cardiac puncture (70 mL from each rabbit). Collected blood was left for 1.5 h at room temperature, and the serum was decanted from the blood clot. The serum was centrifuged at 1000 rpm for 5 min, and the clear, yellowish serum, free of red blood cells, was collected into 1.5-mL sealed containers and stored at −20 °C. All collected serum had sufficient titer for use in enzyme-linked immunosorbent assay (ELISA) experiments.

Competitive Indirect ELISA Procedure. The 96 well microtiter plates were coated with 200 μL of 20 μg/mL BSA–ST in PBS per well. The plates were stored at 4 °C overnight in a plastic bag containing a wet paper towel. The next day the solution was shaken from the plate and each well was coated with 200 μL of 1% BSA in PBS per well. The plates were again placed into the same plastic bag and held at room temperature for 1 h. The coating solution was shaken from the plate, and the plate was washed 3 times with PBST and blotted with force on paper tissues. Sulfonamides at various concentrations in water were added to wells (100 μL/well). Each plate was prepared with some wells containing ST (100 μL/well) in aqueous concentrations of 0, 10, 100, 200, 300, 400, 500, and 600 ppb. A few wells used as controls had no solutions added at this point. Serum diluted 32 000 times with 0.05% BSA in PBST was now added to all wells (100 μL/well) containing aqueous solutions. The plate was held in the plastic bag at room temperature for 2 h. The solution was removed from the plate, and the plate was washed 3 times with PBST as before. Goat anti-rabbit peroxidase conjugated antibodies diluted 3000 times with PBST was added to each well (200 μL/well), including the controls, and the plate was stored in the plastic bag at room temperature for 2 h. The plate was washed 3 times with PBST as before. Substrates, *o*-phenylenediamine (0.4 mg/mL), and urea peroxide (1.0 mg/mL) in 0.1 M citrate buffer at pH 4.75 were added to each well (200 μL/well). After exactly 30 min at room temperature, the absorbance was measured with the ELISA reader set for 450 and 660 nm. Absorbance values for each well were recorded as the absorbance found at 450 nm minus the absorbance found at 660 nm.

Synthesis of 1-(N^4-Sulfathiazole)-1-deoxy-D-fructose (Sulfathiazole Amadori). D-Glucose (1.0 g, 5.5 mmol) and ST (1.4 g, 5.5 mmol) in 20 mL of dry methanol were refluxed for 20 h. The solvent was evaporated, and the residue was dissolved in a minimum amount of methanol and flash chromatographed (Still et al., 1978) on a column (3 cm × 15 cm) of silica gel, using acetonitrile as solvent. Twelve fractions of 10 mL each were collected, and those (fractions 8 and 9) showing a positive test for both carbohydrate and Amadori product (Yaylayan and Sporns, 1987) by TLC, using acetonitrile solvent, were combined. Evaporation of the solvent yielded 400 mg (16% yield) of the title compound as a yellow solid, mp 155–160 °C.

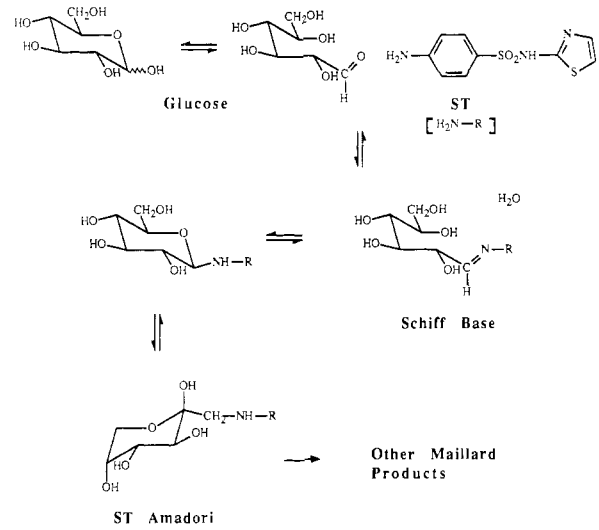
^{1}H NMR (D_2O): δ 1.6 (2 H, CH_2N), 3.0–3.6 (5 H, fructose), 6.1–7.5 (6 H, aromatic). IR (mineral oil): ν_{max} 1720, 1580, 1555, 1500 cm^{-1}. POSFAB MS (3-nitrobenzyl alcohol): m/z 418 (MH^+).

RESULTS AND DISCUSSION

Effect of Reducing Sugars Reacting with ST on Chromatographic and Colorimetric Analysis Methods. To study the reaction between ST and individual reducing sugars, experiments were carried out with large concentrated quantities of the sulfonamide and an approximately threefold excess of the reducing sugars glucose and fructose. The presence of the ST in any reaction material could be detected by UV absorbance after TLC analysis with plates containing a fluorescent indicator, since the monosaccharides had no UV absorbance.

With our TLC development system, free ST had an R_f of 0.76, while a new streak of UV-absorbing material appeared within 2–3 days at R_f 0.09–0.30 for the glucose solution and at R_f 0.11–0.42 for the fructose solution. The slower moving streak of material was shown to contain carbohydrate by the ease with which this material and the monosaccharides charred (glucose and fructose had R_f's of 0.07 and 0.16, respectively). Unlike free ST, the slower streak of material did not develop a pink color when sprayed with the B–M spray. After the concentrated glucose and ST solution had stirred at room temperature for 40 days, a 0.5-mL portion of the reaction mixture was removed and the glucose-bound ST (material in the slower running streak) was separated from free ST on a silica column. Using UV absorbance at 280 nm in 3.5 N hydrochloric acid solvent, we found that 32% of the recovered ST was bound to glucose, with the remaining ST still in the free form (total recovery 81%). After 57 days the fructose and ST solution treated in a similar manner gave 21% bound ST and a 95% recovery. The glucose and ST solution was checked again after 9 months and was found to contain 85% of the glucose-bound ST with an 82% recovery of ST material. The unseparated ST and glucose solution maintained at room temperature for 9 months showed no change in total UV absorbance at 280 nm (in 3.5 N hydrochloric acid) compared with the original starting absorbance.

Scheme I. Reaction of ST with Glucose



An experiment was carried out with a small amount of ST (100 ppm) and the same concentration of glucose as before. Even though glucose was present in this reaction at almost a 5000-fold molar excess compared with ST, periodic examination by TLC did not indicate significantly faster formation of glucose-bound ST than earlier solutions containing only a threefold excess of glucose.

Further confirmation of the ST–sugar reaction was provided by independent synthesis of the 1-(N^4-sulfathiazolyl)-1-deoxy-D-fructose ST Amadori compound (see Scheme I). The ST Amadori compound had an R_f of 0.21, absorbed UV light, could be easily charred, and did not react with the B–M spray. Therefore, it seemed that at least some of the material formed in the ST and glucose solution and present in the TLC streak was the ST Amadori compound.

If isolated glucose-bound ST containing no free ST was diluted in water, TLC examination indicated the formation of free ST from the glucose-bound material. The ST Amadori also produced some free ST after aqueous dilution but at a considerably slower rate. If either material was diluted in 3.5 N hydrochloric acid, 85–90% of the bound ST was almost immediately converted to the free form as judged by the amount of color development in a B–M reaction (Low et al., 1989). Leaving either material for long periods of time in the acid solution did not increase the amount of color development. TLC examination of either of the bound ST materials after acid treatment also indicated a large increase in free ST. Since quantitation of free ST formed under these acid conditions depended on observation of the amount of color formed in the B–M reaction, it could not be ruled out that part of the reason that such a large proportion of the bound material was converted to free ST was that free ST was removed as it reacted further through the diazonium salt to the colored compound. As free ST

Table I. Temperature Effect on Sulfathiazole in an Aqueous Glucose Solution[a] Monitored by a Bratton-Marshall Colorimetric Assay

temp, °C	time (h) for starting abs (545 nm) to decrease by half
80	104
67	187
62	240
50	245

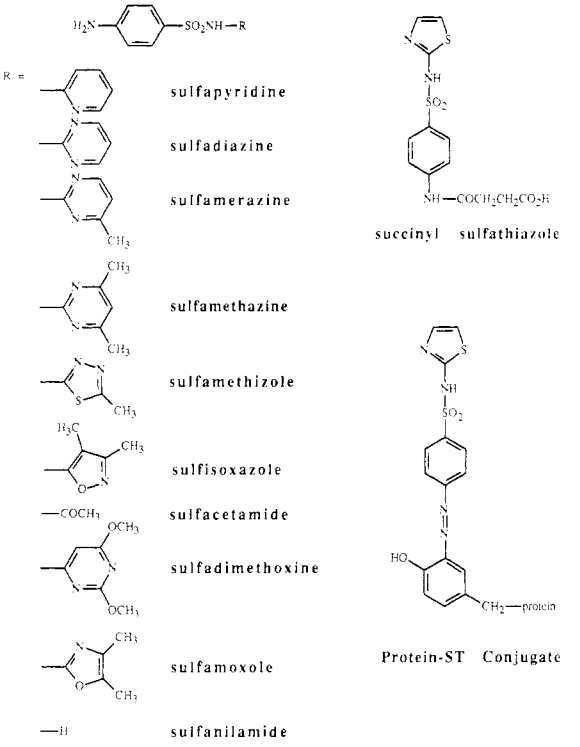
[a] 1000 ppm sulfathiazole in 1.75 M aqueous glucose solution.

reacted to form colored compound the ST–sugar reaction equilibrium would adjust to form more free ST. The fact that less than total recovery of free ST occurred under acid conditions could be due to formation of compounds such as N^4-acetyl-ST in reactions similar to those described by Giera et al. (1982) in sulfamethazine residue studies.

It was obvious from earlier work by Low et al. (1989) that when ST was heated in solution in the presence of reducing sugar, an increasing amount of the ST was converted into a sugar-bound form that could not be reversed to free ST in 3.5 N hydrochloric acid. To investigate further the conditions required to produce increasing amounts of irreversibly bound ST–sugar derivatives, four reactions containing 1000 ppm ST were prepared in the same amount of glucose as before (1.75 M). These samples were heated at different temperatures, and the total amounts of free ST and glucose-bound ST that could be reversed by acidic B–M reaction conditions (Low et al., 1989) were quantitated. Aliquots of the reaction were treated with 3.5 N hydrochloric acid for about 30 s followed by quantitation using the B–M reaction (Table I). As heating temperature increased, the rate of formation of irreversibly bound ST–glucose derivatives increased. Maillard browning (Waller and Feather, 1983) was obvious as a result of heating as all solutions turned yellow and later brown. Since none of the solutions was buffered, the reaction was complicated by the fact that acidic products of the reaction lowered the pH as the reaction progressed. Therefore the reaction at 80 °C dropped from pH 8.2 to pH 3.9 in 187 h. Similar, although slower, decreases in pH were observed in the other solutions. The UV absorbance of these reactions at 280 nm in 3.5 N hydrochloric acid was also checked and no decrease in absorbance was seen. After a time (for example, about 48 h with the 80 °C reaction) there was an increase in absorbance at 280 nm due to the formation of UV-absorbing products from the Maillard reaction. When a portion of the concentrated ST and glucose room temperature reaction mixture stored at room temperature for 9 months was tested with the B–M reaction, it was found that about 72% of the ST could be detected. This decrease in B–M colored material occurred despite the fact that all of the ST was detected by using UV absorbance at 280 nm, as noted above.

From these observations, the reaction of ST with glucose could be summarized as given in Scheme I. ST reacted with the reducing sugar glucose to give a Schiff base, which could further react to form the ST Amadori compound. Some of the glucose-bound ST, possibly the Schiff base material, could rapidly form free ST again on aqueous dilution. Other glucose-bound ST material, such as the ST Amadori compound, formed free ST less rapidly under these conditions. Under strongly acidic conditions, the equilibria between all of these compounds favored formation of the free ST due to protonation of the aromatic amino group (ST has a pK_a of 2.4; Bell and Roblin, 1942). Eventually, after a long period of storage at low temperatures, or a shorter time at elevated temperatures, compounds such as the ST Amadori compound formed later Maillard products. These products, which include polymeric brown material, bind ST more strongly, and significant amounts of ST could not be freed by acid treatment.

Chart I. Sulfonamides



The effect of heating sulfamethazine residues in meat was studied by Epstein et al. (1988) and O'Brien et al. (1981); however, it is not clear from these papers whether "losses" of sulfamethazine can be attributed to reaction with reducing sugars.

The significance of ST reacting with reducing sugars in sulfonamide residue analyses is that all of the sugar-bound sulfonamide residues (virtually all sulfonamides have the free reactive aromatic amine group) have different chromatographic mobilities, have different solubilities, and do not show up with a commonly used B–M spray on TLC. Although some of the sugar-bound sulfonamides can be released with acid treatment, later Maillard products cannot. Fortunately, quantitation of acid-released sugar-bound sulfonamides also accounts for the compounds that can act as depots for sulfonamide, releasing free sulfonamide after dilution.

Since a variety of other methods are used for sulfonamide analyses besides chromatographic and colorimetric methods, it would also be useful to understand the effect that sugar-bound sulfonamides have on other methods of analysis.

Effect of Reducing Sugars Reacting with ST on Microbiological Analysis Methods. The earliest methods of sulfonamide residue analysis in food included microbial inhibition tests. For example, Read et al. (1971) described a method widely used to detect sulfonamides and other antibiotics in milk. Starting with two bacterial organisms that were susceptible to ST, *Escherichia coli* ATCC 11775 and *Staphylococcus aureus* ATCC 25923, we found that the minimum inhibitory concentrations of ST were 1 and 3 μg (in 10 μL of solvent), respectively. When ST Amadori, purified glucose-bound ST, purified fructose-bound ST, or succinylsulfathiazole (Chart I) was applied at levels up to 100 μg (in 10 μL of solvent), nei-

Table II. Competitive Indirect ELISA of Sulfonamides Using Polyclonal Rabbit Serum

compd	concn of compd (ppb) required to reduce abs of control by half
ST	100
ST Amadori	5
succinylsulfathiazole	100
sulfapyridine	50 000
sulfadiazine	50 000
sulfamerazine	>100 000
sulfamethazine	>100 000
sulfamethizole	>100 000
sulfisoxazole	>100 000
sulfacetamide	>100 000
sulfadimethoxine	>100 000
sulfamoxole	>100 000
sulfanilamide	>100 000

ther organism was inhibited. Not only will traditional microbial inhibition tests not detect sugar-bound ST or any N^4-substituted sulfonamides, but similar tests based on microbial receptors, such as the Charm II test, will almost certainly also suffer from the same problems, since the microbial receptor recognizes the free aromatic amino group on the sulfonamide (Charm and Chi, 1988). Of course, these microbial tests are rarely used for quantitation of sulfonamide residues. If sufficient free sulfonamide or, during workup, sufficient sugar-bound sulfonamide is converted to free sulfonamide, microbial tests would detect sulfonamide residues.

Effect of Reducing Sugars Reacting with ST on ELISA Methods. Recently, researchers such as Fleeker and Lovett (1985), Dixon-Holland and Katz (1988), and Singh et al. (1989) and a number of commercial companies (CITE Sulfamethazine Test Kit, Agritech Systems Inc., 100 Fore Street, Portland, MN 04101; EZ-SCREEN Sulfamethazine Test, Environmental Diagnostics, Inc., P.O. Box 908, 2990 Anthony Road, Burlington, NC 27215; Sulfamethazine Immunoassay Test Kit, Idetek, Inc., 1057 Sneath Lane, San Bruno, CA 94066) have introduced immunoassay-based procedures for sulfamethazine residue analysis. To investigate the effect that sugar-bound sulfathiazole compounds would have on this type of test, an indirect competitive ELISA was prepared for ST. As seen from the data in Table II (structures are given in Chart I), the rabbit serum was very specific for ST and related compounds. A 10% reduction of absorbance was noted with concentrations of only 10 ppb ST. To reduce the absorbance by half required concentrations of about 100 ppb ST. Sulfonamides with any group other than a thiazole R group attached to the sulfonamide residue did not compete effectively for antibody. The best sulfonamide competitors containing different R groups were sulfapyridine and sulfadiazine, which, at 500-fold increase in concentration, reduced the final absorbance by half. Succinylsulfathiazole was expected to compete effectively for antibody and on a weight basis proved to be as effective as ST. Since succinylsulfathiazole has a higher molecular weight than ST, this meant that on a molar basis, succinylsulfathiazole was a better competitor than ST.

Surprisingly, on a weight basis, the ST Amadori compound was 20 times as effective a competitor for antibody as ST. On a molar basis, the ST Amadori compound was about 33 times as effective. When the structure of the LPH–ST conjugate used to obtain the antibodies was considered, however, this increase in sensitivity of the antibodies to ST Amadori compound was explained. The conjugate most likely contained most of the azo ST compound linked to tyrosine residues as shown in Chart I. The antibodies developed with this conjugate obviously bound much better to the more similar Amadori ST compound than to ST itself.

The effect of sulfonamides bound to reducing sugars in ELISA-type analyses of sulfonamides can therefore have dramatic effects on the detection limit of these tests. Although there are different methods of preparing sulfonamide protein conjugates (Dixon-Holland and Katz, 1988), all antisera developed, including antisera used in commercial sulfamethazine kits, are specific for the R group of the sulfonamide. This means that the sulfonamide has been linked to the protein through the aromatic amine group (N^4 amine group). The aromatic amine group is also the easiest portion of the molecule to attach to a protein. Antisera developed against such antigens would probably bind better to N^4-derivatized sulfonamides. Fleeker and Lovett (1985) did test the sulfamethazine "metabolites", acetylsulfamethazine and glucosylsulfamethazine. They found that the acetylsulfamethazine bound better, but the glucosyl derivative bound less to antisera than sulfamethazine. We did not test either isolated glucose-bound ST or fructose-bound ST (which contained compounds similar to the glucosyl derivative) in our ELISA procedure, since these mixtures rapidly produced free ST under the aqueous ELISA conditions. Fleeker and Lovett (1985) also noted the instability of glycosylsulfamethazine under acidic aqueous conditions. Although their glycosylsulfamethazine was synthesized under nonaqueous conditions, the stability of this compound under the aqueous conditions of their ELISA experiments was not noted. There is, however, the potential for all ELISA-type methods to have considerably lower detection levels for sugar-bound sulfonamides than for free sulfonamides, and even low levels of sugar-bound sulfonamides could dramatically effect quantitation.

Overall then, if samples are to be analyzed for sulfonamide residues, the samples should be analyzed as rapidly as possible, especially if the samples are known to contain reducing sugars. If storage is required, refrigeration is, of course, recommended to limit conversion of free sulfonamide to the sugar-bound form. Some of the sugar-bound material can be made to release sulfonamide again using an acid step in the sample workup. Also, if sulfonamide reaction with reducing sugars in a sample is suspected, the occurrence of this reaction can be confirmed by initially heating a portion of the sample. The heated sample will give lower sulfonamide residue values than an unheated sample in all cases, except for ELISA-type methods, where the reverse can occur.

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An Immunoassay for Ergotamine and Related Alkaloids†

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A competitive inhibition (CI) enzyme-linked immunosorbent assay (ELISA) for ergotamine was developed with use of polyclonal antibodies produced in rabbits by immunization with an ergotamine–albumin conjugate. The assay was found to be specific for the ergot alkaloids having a phenylalanine moiety in the peptide portion of the molecule. These include ergotamine, ergostine, and ergocristine, all of which are produced by the ergot fungi, *Claviceps spp.* The assay was able to detect ergotamine in spiked grain samples at a level of 10 ng/g. Target alkaloids were detected in ergot sclerotia of wheat and tall fescue and in fescue seeds infected with the endophytic fungus, *Acremonium coenophialum* Morgan-Jones and Gams.

Ergotamine is one of the ergot peptide alkaloids produced by fungi of the genus *Claviceps* and some related genera, which were responsible for the classic examples of ergot poisoning in Europe in the middle ages (Lamey et al., 1982; Stoll and Hoffman, 1965). As a human toxicosis, ergotism is no longer prevalent due to prevention of infected grain from entering food channels (Scott and Lawrence, 1980; Lamey et al., 1982). However, sporadic outbreaks of ergotism in livestock due to feeding ergot-infected grain still occur (Lamey et al., 1982; Riet-Correa et al., 1988). These alkaloids exert a number of pharmacological effects, one of the most dramatic being vasoconstriction, which may result in gangrene and loss of extremities (Floss et al., 1973). Other effects include reproductive disorders, such as lowered conception rates, stillbirth, and agalactia (Lamey et al., 1982; Floss et al., 1973). The striking similarity of the latter symptoms to the toxicosis caused by ingestion of tall fescue infected with the endophytic fungus *Acremonium coenophialum* Morgan-Jones and Gams led various investigators to the discovery of ergot peptide alkaloids in tall fescue as well (Porter et al., 1981, 1987; Yates et al., 1985; Yates and Powell, 1988). On a global scale, economic loss caused by ergot alkaloids in endophyte-infected forage grasses may even exceed that from *Claviceps* infestations in grain.

The same pharmacological effects making ergotamine and related alkaloids so toxic in some cases also make them pharmaceutically useful in others. Due to its vasoconstrictive effects, ergotamine is prescribed to control migraine headaches (Barnhart and Huff, 1985) and to

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