Development of a Single ELISA for Detection of Sulfonamides

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A sulfathiazole derivative was chemically linked to proteins in such a way that the aromatic amino group, common to all sulfonamides, was distal to the protein. A subset of the antibodies developed against this immunization conjugate could be used competitively with different sulfonamide haptens, linking methods and proteins to develop ELISA methods that had a broad spectrum of sulfonamide recognition. By use of the best ELISA protocol, nine structurally different sulfonamides decreased absorbance values 50% at concentrations of less than 2 nM per assay. The sulfonamides recognized by the competitive ELISA had similar steric characteristics but considerable variation in electronic configuration.

INTRODUCTION

Sulfonamides are bacteriostatic compounds widely used in animal husbandry and as feed additives (Long et al., 1990). As a result, foods derived from animals treated with sulfonamides may be contaminated with these drugs (Barnes et al., 1990). At least 10 sulfonamides are currently in use for human medicine (Mandell and Sande, 1990), and there is cross-sensitivity between different sulfonamides (Lloyd and Mercer, 1984). Approximately 5% of the patients receiving sulfonamide therapy experience some type of untoward effect (Bevill, 1984). Although it is very rare for sulfonamides to be found in foods at levels that are toxic or cause allergic reactions, there are concerns that extensive use of sulfonamides for animals could result in resistance in pathogenic organisms which could then affect human health (Guerineau et al., 1990). To safeguard the public, limits of 0.1 μg of sulfa-drug/g of food have been established by agencies such as the U.S. Department of Agriculture Food Safety and Inspection Service (Parks, 1982).

Immunoassay screening methods for sulfonamides in foods and related materials have been reported in the literature (Fleeker and Lovett, 1985; Dixon-Holland and Katz, 1988; Singh et al., 1989; McCaughey et al., 1990; Sheth et al., 1990; Sheth and Sporns, 1990). Using similar methodology, immunoassay kits for the detection of sulfonamide residues have been developed by many commercial companies. The antibodies used for all of these methods are only specific for individual sulfonamides, while the list of sulfonamides of concern in food continues to grow (Long et al., 1989; Matusik et al., 1990; Takatsuki and Kikuchi, 1990). Since immunoassays act as rapid screening methods for sulfonamides and require further confirmation by other more definitive methods, it would be more efficient if an immunoassay using antibodies that could detect a number of different commercial sulfonamides was developed.

EXPERIMENTAL PROCEDURES

Materials. Water was prepared by using a Millipore Milli-Q system. For flash chromatography 230–400-mesh silica gel (E. Merck, Darmstadt, Germany) was used. Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were obtained from Difco Laboratories (Detroit, MI). 2-Amino-4-thiazoleacetic acid, *N*-acetylsulfanilyl chloride, *p*-nitroaniline, *N*-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide, and *N*,*N*-dimethylformamide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Eleven sulfonamides (sulfapyridine, sulfamethizole, sulfadiazine, sulfamethoxazole, sulfamoxole, sulfadimethoxine, sulfathiazole, sulfacetamide, sulfanilamide, sulfamerazine, sulfamethazine), thimerosal, Tween 20, bovine serum albumin (BSA), *Limulus polyphemus hemolymph* (LPH), and ovalbumin (OV) were purchased from Sigma Chemical Co. (St. Louis, MO). Platinum dioxide was supplied by BDH Inc. (Edmonton, AB). Urea peroxide, *o*-phenylenediamine tablets, goat anti-rabbit peroxidase conjugated antibodies, and citric acid (monohydrate) were obtained from Calbiochem Co. (San Diego, CA). Immobilized papain and protein A were supplied by Pierce (Rockford, IL). Dynatech Immulon 1 microtiter plates and Spectrapor dialysis tubing (12 000–14 000 MW cutoff) were purchased from Fisher Scientific (Edmonton, AB). Thin-layer chromatography (TLC) plates were 20 × 20 cm PE SIL G/UV with polyester backing obtained from Whatman Ltd. (Maidstone, England). Filter papers GVWP-025 were purchased from Millipore Corp. (Bedford, MA). All other chemicals were of reagent grade or better.

Instrumentation. Absorbance values at 280 and 545 nm were recorded with a Hewlett-Packard Model 8451 A diode array spectrophotometer (Hewlett-Packard [Canada] Ltd., Mississauga, ON). Microtiter plate optical densities were measured with a Model EL 309 ELISA reader (Bio-Tek Instruments, Inc., Burlington, VT). A Buchi Rotavapor R (Fisher Scientific) was used for the removal of solvents from samples. Centrifugation was performed with a Damon/IES Division Model HN-S II centrifuge from International Equipment Co. (Needham Heights, MA). A Virtis freeze dryer (The Virtis Co. Inc., Gardiner, NY) was used to dry protein conjugates and immunoglobulin G (IgG). TLC was used to monitor the progress of chemical reactions. Two solvent systems were used: methanol–chloroform (1:3) and acetone–*n*-heptane–methanol–ammonia–*n*-butanol (72:21:9:10:10; Bregha-Morris, 1979). Spots were visualized by their ultraviolet (UV) absorbance and by using Bratton-Marshall reagents (Parks, 1982).

Nuclear magnetic resonance (NMR) analysis was carried out by the high-field NMR Laboratory of Chemistry Services at the University of Alberta. NMR spectra were measured on Bruker WH-200, AM-300, or WH-400 instruments. Mass spectrometry (MS) analyses were carried out by the MS Laboratory of Chemistry Services at the University of Alberta and were obtained using a Kratos AEI MS-50 (high resolution, electron impact ionization) for exact mass determination.

2-Amino-4-thiazoleacetic Acid Methyl Ester. Synthesis 1. Methanol (100 mL), previously dried over anhydrous sodium sulfate, was added to a 250-mL three-necked flask in an ice bath. Hydrochloric acid gas was bubbled into the flask, while the methanol was being stirred, until the weight of the solution had increased by about 60 g. 2-Amino-4-thiazoleacetic acid (2.1 g) was slowly added to the flask, and the solution was removed from the ice bath and stirred for another 10 min. The contents of the flask were then refluxed for 6 h, giving a slightly yellow

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0021-8561/91/1439-1696$02.50/0

solution. All solvent was removed on a flash evaporator, dry methanol (25 mL) was added, and this solvent was also removed. The resulting solid was recrystallized (melting point 169–171 °C) from ethyl acetate and methanol to give 2-amino-4-thiazoleacetic acid methyl ester as slightly yellow crystals (1.9 g, 83% yield): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.30 (br s, 2 H, NH), 6.71 (s, 1 H), 3.76 (s, 2 H, CH_2), 3.65 (s, 3 H, CH_3). Exact mass M^+, m/z 172 (87.29%) calcd for $C_6H_8N_2O_2S$: 172.0306 (measured 172.0308).

p-Aminobenzoic Acid Methyl Ester. *p*-Aminobenzoic acid methyl ester was synthesized from 10.0 g of *p*-aminobenzoic acid according to synthesis 1 (yield 9.41 g, 85%; melting point 196–198 °C): ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.46 (br s, 2 H, NH), 7.83 (d, 2 H, 7.6, Ar H), 7.05 (d, 2 H, 7.6, Ar H), 3.79 (s, 3 H, CH_3). Exact mass M^+, m/z 151 (58.01%) calcd for $C_8H_9NO_2$: 151.0633 (measured 151.0632).

N^4-Acetyl-N^1-[4-[(methoxycarbonyl)methyl]-2-thiazolyl]-sulfanilamide. Synthesis 2. *N*-Acetylsulfanilyl chloride (2.65 g) was added to a 50-mL round-bottom flask, and 5 mL of dry pyridine was added with stirring. 2-Amino-4-thiazoleacetic acid methyl ester (1.50 g) was slowly added to the pyridine solution while a temperature of not more than 40 °C was maintained. The brownish solution was refluxed for 1.5 h and allowed to cool to room temperature. While the solution was stirred vigorously, warm water (30 mL) was added and the mixture was extracted with (3 × 25 mL) methylene chloride. The methylene chloride layer was dried over sodium sulfate and filtered and most of the solvent removed on a flash evaporator. About 5 g of silica gel was added and the remaining solvent removed to leave a yellowish powder which was applied to a 50-g silica column (50 × 2.4 cm). The column was eluted with 1:4 methanol–chloroform (150 mL). Removal of these solvents gave the N^4-acetyl-N^1-[4-[(methoxycarbonyl)methyl]-2-thiazolyl]sulfanilamide (1.6 g, 50% yield): ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.35 (br s, 1 H, NH), 10.25 (s, 1 H, NH), 7.70 (s, 4 H, Ar H), 6.60 (s, 1 H), 3.65 (s, 2 H, CH_2), 3.61 (s, 3 H, CH_3), 2.05 (s, 3 H, CH_3). Exact mass $(M - SO_2)^+$, m/z 305 (10.32%) calcd for $C_{14}H_{15}N_3O_3S$: 305.0834 (measured 305.0835).

N^1-[4-(Carboxymethyl)-2-thiazolyl]sulfanilamide (TS). Synthesis 3. Sodium hydroxide solution (25 mL, 2 M) was added to 0.95 g of purified N^4-acetyl-N^1-[4-[(methoxycarbonyl)methyl]-2-thiazolyl]sulfanilamide. The resulting mixture was kept under reflux conditions for 2 h and cooled to room temperature. The pH was then adjusted to 4.0 with 6 N hydrochloric acid. The turbid solution obtained in this manner was extracted with (3 × 25 mL) ethyl acetate. The ethyl acetate extract was dried over sodium sulfate and filtered and the solvent removed to yield a brown solid (0.26 g, 32% yield): ^{1}NMR (300 MHz, DMSO-d_6) δ 12.20 (br s, 1 H, COOH), 7.5 (very br s, 1 H, NH), 7.40 (d, 2 H, 7.8, Ar H), 6.54 (d, 2 H, 7.8, Ar H), 6.46 (s, 1 H), 5.82 (br s, 2 H, NH), 3.48 (s, 2 H, CH_2). Exact mass $(M - CO_2)^+$, m/z 269 (13.60%) calcd for $C_{10}H_{11}N_3O_2S_2$: 269.0293 (measured 269.0291).

N^1-(4-Carboxyphenyl)sulfanilamide (CS). N^4-Acetyl-N^1-[4-(methoxycarbonyl)phenyl]sulfanilamide was synthesized from 4.0 g of *p*-aminobenzoic acid methyl ester and 7.5 g of *N*-acetylsulfanilyl chloride according to synthesis 2, and then the acetyl and methyl ester groups were hydrolyzed according to synthesis 3 (yield 0.21 g, 23%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.68 (br s, 1 H, COOH), 10.44 (s, 1 H, NH), 7.90 (s, 2 H, NH), 7.78 (d, 2 H, 7.0, Ar H), 7.46 (d, 2 H, 7.0, Ar H), 7.17 (d, 2 H, 7.0, Ar H), 6.56 (d, 2 H, 7.0, Ar H). Exact mass M^+, m/z 292 (8.92%) calcd for $C_{13}H_{12}N_2O_4S$: 292.0518 (measured 292.0520).

Phosphate-Buffered Saline (PBS) Solution. In 3.4 L of water were dissolved 31.5 g of sodium chloride, 3.88 g of disodium hydrogen phosphate, 1.05 g of potassium dihydrogen phosphate, and 350 mg of thimerosal. 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 phosphate-buffered saline containing Tween (PBS-T).

Preparation of TS-Conjugated Proteins. Synthesis 4. TS (62.5 mg), *N*-hydroxysuccinimide (34.5 mg), and 1,3-dicyclohexylcarbodiimide (45.3 mg) were reacted in 1.5 mL of dry *N*,*N*-dimethylformamide at 4 °C for 18 h. Dicyclohexylurea precipitate produced in the reaction was removed by filtration. The filtrate was added to 65 mg of LPH in 2 mL of PBS adjusted to pH 7.6 with sodium hydroxide. The mixture was stirred at 4 °C for 24 h. There was no evidence of polymerization of the sulfonamide by TLC, nor was any expected, because of the low basicity of the free aromatic aminosulfonamide group (for example, the pK_a for sulfathiazole is 2.36; Bell and Roblin, 1942), especially in comparison to the ϵ-amino group of lysine (pK_a of 10.28; *The Merck Index*, 1989) which is probably the primary amino acid in each protein involved in amide formation.

After 24 h, the reaction mixture was transferred to dialysis tubing and dialyzed against 1 L of 8 M urea for 12 h. The dialyzing solvent was then changed to 4 L of 50 mM ammonium bicarbonate followed by 4 L of 25 mM ammonium bicarbonate. Dialysis with each ammonium bicarbonate solution was carried out for a minimum of 8 h. The contents of the dialysis bag were freeze-dried. TS-conjugated BSA was produced by using the same ratios (as above) of TS, *N*-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide, and BSA instead of LPH.

Preparation of N^1-(4-Nitrophenyl)sulfanilamide (NS) and N^1-(4-Nitrophenyl)sulfanilamide-Conjugated OV (NS-Conjugated OV). Sulfanitran [N^4-acetyl-N^1-(4-nitrophenyl)-sulfanilamide] was synthesized from 7.0 g of *p*-nitroaniline and 20 g of *N*-acetylsulfanilyl chloride according to synthesis 2. Besides synthesis, authentic sulfanitran was later obtained 85% pure from Sigma. N^1-(4-Nitrophenyl)sulfanilamide (NS) was synthesized from N^4-acetyl-N^1-(4-nitrophenyl)sulfanilamide according to synthesis 3 (yield 9.30 g, 55%).

To synthesize the conjugate, sulfanitran (50 mg) was dissolved in 25 mL of methanol and platinum oxide (50 mg) added. Hydrogen gas was then injected into the stirred sulfonamide catalyst mixture. After 24 h, platinum oxide was removed by filtration, and removal of methanol gave N^4-acetyl-N^1-(4-aminophenyl)sulfanilamide. This reduced material (100 mg) was dissolved in 2 mL of 3.5 N hydrochloric acid in a 50-mL round-bottom flask, and 4.5 mL of 1% sodium nitrite was added. The solution was stirred for 10 min at room temperature. Ammonium sulfamate (4.7 mL, 0.8%) was added, and the reaction was stirred for another 10 min. This reaction solution was added to OV (200 mg) dissolved in 2.5 mL of PBS in a stirred 25-mL flask and the pH of the reaction adjusted to 9.0. The reaction mixture was kept at 4 °C overnight with stirring. The next day the reaction mixture was transferred to dialysis tubing and dialyzed as described above for the TS conjugates. The contents of the dialysis bag were freeze-dried. This freeze-dried protein conjugate was taken into a 100-mL round-bottom flask and 10 mL of 2 M sodium hydroxide added and refluxed for 1 h. After base hydrolysis, the protein conjugate was dialyzed as above and freeze-dried, and the epitope density (moles of base-hydrolyzed sulfanitran per mole of protein) in the conjugate was determined (see below).

Preparation of CS-Conjugated OV. CS-conjugated OV was prepared according to synthesis 4. After dialysis, dialyzed material was centrifuged at 8000 rpm for 1 h to separate insoluble particles. The supernatant was freeze-dried and the epitope density determined.

Estimation of Epitope Density for Conjugates. The presence of the free aromatic amino and group epitope density was measured by the modified Bratton-Marshall test of Low et al. (1989). Several sulfonamides (sulfadiazine, sulfapyridine, sulfathiazole, sulfamoxole) gave similar results with this test on a mole basis and were used to determine the calibration curve for the free aromatic amino group (increasing color for increasing free aromatic amino group). LPH, BSA, and OV gave no color in the Bratton-Marshall test. TS-conjugated LPH and TS-conjugated BSA used in immunization were insoluble under the modified Bratton-Marshall conditions (in large measure because of the number of groups attached), but the insoluble protein particles developed an intense purple color, indicating the presence of a considerable number of free aromatic amine groups.

All conjugates used for ELISA coating were soluble in the above test, and the number of moles of sulfonamide per mole of protein could be estimated. TS-conjugated OV had about 10 mol of hapten/mol of protein; NS-conjugated OV had about 30 mol of hapten/mol of protein, and CS-conjugated OV had about 5 mol of hapten/mol of protein.

Antibody Preparation. Four 10-week-old prebled female Flemish Giant × Dutch Lop Ear rabbits were each injected

with 0.8 mL of a water in oil emulsion of conjugate in sterile PBS and FCA (1:1 v/v). One pair of rabbits was injected with TS-conjugated LPH while the other pair received TS-conjugated BSA as immunogen. The rabbits were injected with 0.5 mg of conjugate in 0.8 mL of water in oil emulsion (2 × 0.2 mL) subscapularly and (0.4 mL) gluteal. The rabbits received identical booster injections, with the exception that FIA was used rather than FCA for injections at 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 (about 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.

Isolation of IgG from Rabbit Serum. A 0.6-mL protein A slurry was loaded into a 10-mL disposable polypropylene column. The column was then washed with 10 mL of PBS containing 0.02% sodium azide. Filtered rabbit serum (0.5 mL) was slowly added on the top of the protein A column and allowed to sit for 15 min. Approximately 15 mL of PBS containing azide was required to elute serum proteins other than IgG at the rate of 1 mL/3 min. Serum proteins were collected as their presence was noted by absorbance at 278 nm. Next, acetic acid (1 M) was used to elute IgG (about 5 mL) and the eluate transferred into dialysis tubing. The dialysis was carried out against 4 L of 25 mM ammonium bicarbonate for 24 h with changing of solution every 4 h. The contents of the dialysis bag were freeze-dried and stored at −20 °C. Recovery of IgG from 0.5 mL of rabbit serum was 2 mg.

Generation of Immunoglobulin Fragment with Antigen Binding (Fab). The manufacturer's methodology was followed (Pierce, 1989). Immobilized papain was gently mixed, and 0.5 mL was added to a glass test tube. Digestion buffer [2.76 g of sodium dihydrogen phosphate, ethylenediaminetetraacetate tetrasodium salt (3.80 g), and 3.51 g of cysteine hydrochloride adjusted to a pH of 7.0 with 1 M sodium hydroxide made up to 1 L, used 4 mL] was then added to the test tube, and enzyme was separated from buffer by centrifugation. This procedure of washing enzyme with digestion buffer was repeated once and immobilized papain was resuspended in 0.5 mL of digestion buffer.

Lyophilized IgG (4 mg) was dissolved in 2 mL of fresh digestion buffer and added to the test tube containing prepared papain. The enzyme–IgG mixture was incubated for 5 h in a shaker water bath at 37 °C. After 5 h of incubation, immobilized papain was separated from the digest by centrifugation. Prior to centrifugation, 1.5 mL of 10 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.5, was added to the IgG–enzyme digest. The Fab fragments were separated from unhydrolyzed IgG and immunoglobulin crystallizable fragments (Fc) by using a protein A column. The mixture of Fab fragments, unhydrolyzed IgG, and Fc fragment was loaded on top of the protein A slurry, and the column was washed with 10 mL of PBS at the rate of 1 mL/3 min. The first milliliter was discarded, and the next 3 mL collected was transferred into dialysis tubing and dialyzed against 4 L of 25 mM ammonium bicarbonate for 24 h with changing of solution every 4 h. The contents of the dialysis bag were freeze-dried and stored at −20 °C. Recovery of Fab from 0.5 mL of rabbit serum was 1.1 mg (Fab purity was confirmed by gel electrophoresis).

Competitive Indirect ELISA Procedure. The 96-well microtiter phases were coated with 200 μL of 20 μg/mL per well of coating conjugate (TS-conjugated OV, CS-conjugated OV, or NS-conjugated OV) in PBS containing 0.01% thimerosal. 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 thimerosal 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 washed three times with thimerosal PBS-T and blotted with force on paper tissues. Sulfonamides (in triplicate) at 0–25 μg/mL concentrations in thimerosal PBS was added to wells (100 μL/well) followed by the addition of serum appropriately diluted with 0.05% BSA

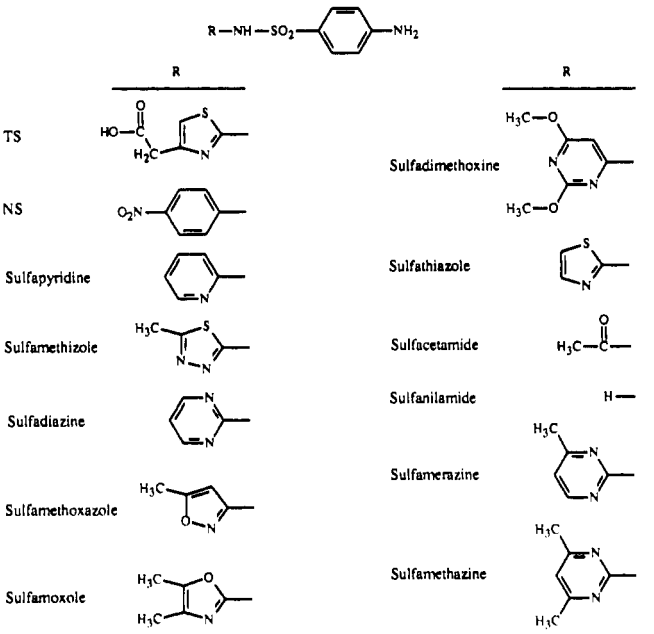

Figure 1. Structures for common sulfonamides.

in thimerosal PBS-T to all wells (100 μL/well). The plate was held in the plastic bag at room temperature for 2 h. The sulfonamide–antibody solution was removed from the plate, and the plate was washed three times with thimerosal PBS-T as before. Goat anti-rabbit peroxidase conjugated antibodies diluted 3000 times with thimerosal PBS-T were 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 three times with thimerosal PBS-T 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 30 min at room temperature, the absorbance was measured with the ELISA reader. Absorbance values for each well were recorded as the absorbance at 450 nm minus the absorbance at 660 nm.

The maximum observed error of the three replicates for all analyses was ±11%.

RESULTS AND DISCUSSION

Sulfonamides are synthetic drugs with bacteriostatic activity, the majority of which have the basic structure shown at the top of Figure 1. The simplest preparation of immunizing conjugate for a specific sulfonamide involves linking through the aromatic amino group common to all sulfonamides. With this linkage the presentation of the hapten is such that the antibodies mainly develop against the R group which is farthest from the protein. Therefore, these antibodies are excellent for the recognition of individual sulfonamides, but since the antibodies are sensitive to minor changes in the R group, they will not recognize other closely related sulfonamides. If an antibody that is cross-reactive with many sulfonamides is desired, the aromatic amino group must be free and the hapten constructed so that attachment to the protein is through the R group.

Antibody was produced in two pairs of rabbits, which were immunized with TS-conjugated LPH and TS-conjugated BSA (Figure 2). Indirect competitive ELISA tests performed on collected sera using TS-conjugated OV as the coating protein resulted in high titers in each pair of rabbits. Serum dilution of 1/200 000 gave three times background absorbance. However, when the sera were tested in an indirect competitive ELISA with high concentrations (25 ppm for all sulfonamides in Figure 1, 10 ppm for TS) of different sulfonamides with free aromatic

Table I. Concentration of Sulfonamide Required To Reduce the ELISA Absorbance by Half

sulfonamide	TS-conjugated LPH immunogen with NS-conjugated OV in ELISA	TS-conjugated BSA immunogen with CS-conjugated OV in ELISA	TS-conjugated LPH immunogen with CS-conjugated OV in ELISA
TS	0.02 (0.006)[a]	0.44 (0.14)[a]	2.0 (0.64)[a]
NS	0.97 (0.33)	0.82 (0.28)	>25 (>8.5)
sulfapyridine	1.3 (0.50)	3.0 (1.2)	>25 (>10.0)
sulfamethizole	1.3 (0.47)	0.60 (0.22)	1.3 (0.47)
sulfadiazine	1.6 (0.64)	7.0 (2.8)	>25 (>10.0)
sulfamethoxazole	3.3 (1.3)	7.2 (2.8)	>25 (>9.9)
sulfamoxole	3.7 (1.4)	7.0 (2.6)	>25 (>9.4)
sulfadimethoxine	4.7 (1.5)	8.0 (2.6)	>25 (>8.1)
sulfathiazole	5.0 (1.8)	0.40 (0.14)	6.0 (2.2)
sulfacetamide	18.0 (8.4)	>25 (>11.7)	>25 (>11.7)
sulfanilamide	21.6 (12.5)	>25 (>14.5)	>25 (>14.5)
sulfamerazine	>25 (>9.0)	>25 (>9.0)	>25 (>9.0)
sulfamethazine	>25 (>8.7)	>25 (>8.7)	>25 (>8.7)

[a] First number is in parts per million (w/v) and the number in parentheses is in nM/assay.

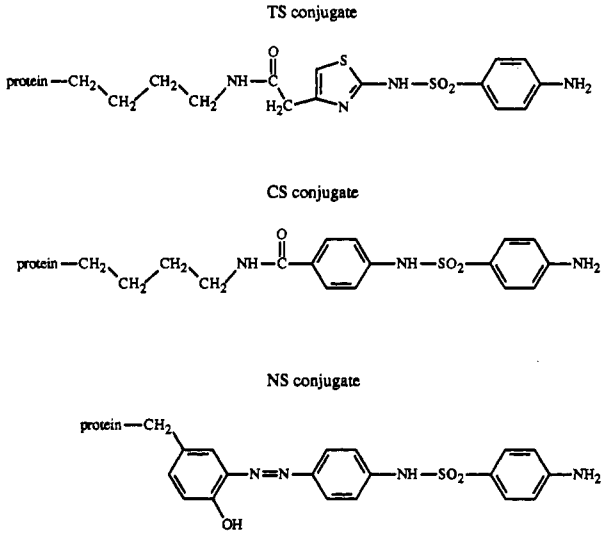

Figure 2. Structures for protein conjugates.

amino groups, no significant decrease in absorbance was noted compared to that of the control. That is, free sulfonamides at high concentrations could not compete with bound hapten TS. To confirm that this lack of competition was not due to avidity of the antibody molecules, Fab fragments were prepared. Use of Fab fragments in the assay resulted in no improvement in competition.

When the TS protein conjugates were prepared, TS was most likely linked to protein through the ϵ-amino group of lysine (Figure 2). From the results, it seemed that during the immune response antibodies were produced against TS and the terminal portion of lysine. Most of these antibodies had greater affinity for this entire structure than for other sulfonamides, even free TS. Another factor was that at the pH of the ELISA experiments the acid group of free TS was negatively charged, unlike the TS conjugates. This phenomenon of linking arm effect has also been noted by other researchers (Vallejo et al., 1982; Wie and Hammock, 1984; Harrison et al., 1991).

To select the subpopulation of antibodies with specificities for the aromatic amino portion of sulfonamides and eliminate antibodies with a high degree of recognition for the linking portion, CS-conjugated OV and NS-conjugated OV were prepared (Figure 2). These conjugates differed from the immunogens in R group and, especially with NS-conjugated OV, in the linking portion. Serum obtained from the TS-conjugated BSA immunization did not have antibody subpopulations which bound to the NS-conjugated OV, judging by ELISA experiments (only background absorbance noted). However, when serum from TS-conjugated LPH immunization was tested with NS-conjugated OV, 11 sulfonamide compounds (Table I) were able to reduce absorbance in the indirect competitive ELISA. Sulfonamides with smaller R groups (sulfacetamide and sulfanilamide) and with larger R groups (sulfamethazine and sulfamerazine) were undetected or poorly detected. However, sulfadimethoxine, which contained a lot of steric bulk in the R group, was detected by the ELISA procedure. It was interesting to note that considerable electronic variation in the R group was tolerated by the antibodies but steric considerations were less tolerated. As expected, the antibodies were most sensitive to TS.

Antibodies from both groups of rabbits immunized with TS-conjugated LPH and TS-conjugated BSA bound to CS-conjugated OV (Table I). In the case of TS-conjugated LPH immunogen, only three sulfonamide structures were competitive, while nine sulfonamide structures were competitive with the TS-conjugated BSA immunogen.

When TS-conjugated OV was used for coating ELISA plates, 1/10 000 dilution of serum gave an absorbance of about 1.0 OD after a 30-min substrate incubation. As expected, fewer antibodies were bound to the modified (NS and CS) coating conjugates and at least 10-fold more concentrated sera (1/500 with NS-conjugated OV and 1/1000 with CS-conjugated OV) was required to achieve similar absorbances. However, from these results it was obvious that by proper choice of immunizing conjugates, and ELISA coating conjugates, methods that had broad sulfonamide group recognition could be developed.

ACKNOWLEDGMENT

This work was supported by Alberta Farming for the Future and Natural Science and Engineering Research Council (NSERC) of Canada grants. The help of Dr. A. Szpacenko in generation of Fab fragments is gratefully acknowledged.

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Received for review February 25, 1991. Revised manuscript received May 23, 1991. Accepted June 3, 1991.

Registry No. Sulfapyridine, 144-83-2; sulfamethizole, 144-82-1; sulfadiazine, 68-35-9; sulfamethoxazoate, 723-46-6; sulfamoxole, 729-99-7; sulfadimethoxine, 122-11-2; sulfathiazole, 72-14-0; sulfacetamide, 144-80-9; sulfanilamide, 63-74-1; sulfamerazine, 127-79-7; sulfamethazine, 57-68-1; 2-amino-4-thiazoleacetic acid, 29676-71-9; methyl 2-amino-4-thiazoleacetate, 64987-16-2; *p*-aminobenzoic acid, 150-13-0; methyl *p*-aminobenzoate, 619-45-4; *N*-acetylsulfanilyl chloride, 121-60-8; N^4-acetyl-N^1[4-[(methoxycarbonyl)methyl]-2-thiazolyl]sulfanilamide, 135285-80-2; N^1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide, 135285-81-3; N^4-acetyl-N^1-[4-(methoxycarbonyl)phenyl]sulfanilamide, 135285-82-4; N^4-acetyl-N^1-[4-(methoxycarbonyl)phenyl]-sulfanilamide, 6336-70-5; *p*-nitroaniline, 100-01-6; N^4-acetyl-N^1-(4-nitrophenyl)sulfanilamide, 122-16-7; N^1-(4-nitrophenyl)-sulfanilamide, 6829-82-9; N^4-acetyl-N^1-(4-aminophenyl)sulfanilamide, 22941-28-2.