Immunochemical Approach to the Detection of Aminotriazoles Using Selective Amino Group Protection by Chromophores

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A selective approach for amino masking and hapten design for aminotriazole conjugates is described. Haptenic 1,2,4-triazole and 3-amino-1,2,4-triazole compounds were conjugated to proteins through spacer arms on the C5 ring carbon, containing hydroxyl or mercapto groups. The amino groups present in several haptens were protected via sulfenylation, using a chromophoric protecting reagent, 2-nitrophenylsulfenyl chloride. Protection of haptens, conjugation to proteins, and deprotection on the proteins were followed by UV spectroscopy, monitoring the specific absorbance of the protecting group at 349 nm. Homo- and heterobifunctional spacers allowing spacer, hapten, and multiple heterology were used for the protein conjugates for immunizing and coating. Antisera raised against aminotriazole conjugates in New Zealand white rabbits and Swiss Webster mice were found to be specific for amitrole. Inhibition tests resulted in 60% inhibition by amitrole, indicating low-affinity antibodies. Antibody binding to the various antigens synthesized was characterized in a qualitative immunoassay.

INTRODUCTION

Amitrole (3-amino-1,2,4-triazole), the major triazole herbicide, is a highly water soluble compound causing possible leaching problems (Zandwoort et al., 1981) and mutagenicity (Polya, 1955; Sakiyama et al., 1964; Tschudy and Collins, 1957; Steinhoff et al., 1983). Besides amitrole, various triazole derivatives are used on large scale, not only as agricultural chemicals but also as synthetic precursors of polymers, pharmaceuticals, photographic chemicals, and dyestuffs (Potts, 1961). In spite of their broad industrial use as synthetic precursors, there has been no report for specific detection of the triazole ring system.

Analysis of amitrole in soil and crops has utilized colorimetric methods (Kröller, 1961; Storherr and Burke, 1961; Galoux et al., 1982), as well as HPLC (Lokke, 1980) and GC (Barrette, 1973; Jacques, 1984). Recently, Van der Poll et al. (1988) described capillary gas chromatographic detection of amitrole in water, with a limit of detection of 5 μg/L. However, these conventional methods do not specifically detect free amitrole but rely on derivatization.

Immunochemical detection of aminotriazoles offers a rapid and cost-effective analytical method of improved sensitivity (Hammock et al., 1987). The design of an immunoassay for aminotriazole raises many questions, such as choice of a hapten which best mimics the target molecule, detection of conjugation to the protein, and handling of the two reactive groups in the hapten. Due to their low molecular size and electronic structure, aminotriazoles are likely to be of low immunogenicity. In addition, Newsome (1986) found no cross-reactivity with 1,2,4-triazole in an immunoassay against the triazole fungicide triadimefon. Therefore, we feel that aminotriazole haptens should contain no other immunogenic moieties, and anti-amitrole antibodies are expected to be of low avidity.

The present study addresses two major aspects in immunoassay development for small molecules. First, it describes a route of synthesis of aminotriazole haptens with amino group protection and selective removal of the protecting group after conjugation. The second aspect is related to the low molecular size (84.1) which indicates that the whole molecule will be presented to the antibody binding site as a single epitope. To answer the question if a spacer would enhance immunogenicity or compete with the epitope of interest, several homo- and heterobifunctional spacers were tested. Evaluation of the avidity of the interaction between aminotriazole derivatives and anti-aminotriazole antibodies is discussed. The antibody characterization described in this paper illustrates some of the major problems in the design of an immunoassay for small haptens. Examples of some of the potential pitfalls and the negative results in the process of assay development will also assist future workers in this area.

MATERIALS AND METHODS

Chemicals. Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and ICN ImmunoBiologicals (Lisle, IL). Triazoleacetic acid (4) and 2-(4-chlorophenyl)-2-(1,2,4-triazol-1-yl)methylhexanoic acid were provided by Rohm and Haas Co. (Spring House, PA). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI).

The purity and structures of haptens and intermediate compounds were confirmed by melting points, analytical TLC, UV and/or IR spectroscopy, and ^{1}H NMR. Analytical TLC was performed on 250-μm silica gel F_{254} plates (Aldrich), while preparative TLC was carried out on 2000-μm silica gel F_{254} plates (Analtech, Newark, DE) by using chloroform/methanol (7:3). Compounds were detected by UV light (254 nm) and/or iodine vapor. Melting points are uncorrected.

Instruments. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Varian EM-390 (Varian, Sunnyvale, CA) in dilute solutions in DMSO-d_6 with or without D_2O saturation and using tetramethylsilane as internal standard. Ultraviolet–visible spectra (UV) were run in methanol/water (1:1) solutions on a Beckman DU-6 spectrophotometer (Beckman Instruments, Palo Alto, CA). Infrared spectra (IR) were obtained on a Perkin-Elmer 521 instrument (Perkin-Elmer, Norwalk, CT). Enzyme-linked immunosorbent assays (ELISA) were carried out in high-capacity 96-well microplates (Nunc, Roskilde, DK, 442404) and read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA).

Hapten Synthesis and Verification. *5-(3-Hydroxypropyl)-3-amino-2H-1,2,4-triazole* (**1a**) is commercially available through

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0021-8561/91/1439-0129$02.50/0

the Alfred Bader Library of Rare Chemicals (Milwaukee, WI) or can be prepared from *N*-aminoguanidine and γ-butyrolactone by the method of Ried and Valentin (1968). The amino groups in haptens were protected via sulfenylation with 2-nitrobenzenesulfenyl chloride as described below.

5-(3-Hydroxypropyl)-3-(2-nitrophenylsulfenyl)amino-2H-1,2,4-triazole (**1b**). 3-Amino-5-(3-hydroxypropyl)-2*H*-1,2,4-triazole (**1a**) (0.426 g, 3 mmol) was dissolved in 20 mL of methanol, and 0.405 g (4 mmol) of triethylamine was added following the addition of 0.569 g (3 mmol) of 2-nitrobenzenesulfenyl chloride. The reaction mixture was stirred overnight. After the solvent was evaporated, the resulting solid was washed extensively with hot cyclohexane and recrystallized three times from methanol to give 0.189 g (0.64 mmol) of **1b** at 21.3% yield: mp 151–2 °C; TLC R_f = 0.73 (chloroform/methanol 7:3); IR (KBr, cm^{-1}) 3400 (m, ν_{str} NH), 3200–3000 (vs, ν OH), 1680 (s, ν triazole), 1600, 1570, 1550 (s, ν_{str} arom), 1530 (s, ν_{sym} NO_2), 1320, 1340 (s, ν_{asym} NO_2, NH); NMR (DMSO-d_6) δ 1.75 (2 H, CH_2, m), 2.45 (2 H, CH_2, t), 3.45 (2 H, CH_2OH, t), 4.10 (OH, s), 6.45 (1 H, aromatic, d), 6.80 (NH–S, s), 7.45 (1 H, aromatic, t), 7.70 (1 H, aromatic, t), 8.45 (1 H, aromatic, d); UV λ_{max} (CH_3OH–H_2O, nm) 275 (triazole), 349 (NO_2PhS); ϵ_M(349) 3337 + 122.

3-Amino-5-[(3-succinyloxy)propyl]-2H-1,2,4-triazole (**1c**) was prepared from **1b** by the procedure of Zervas et al. (1963) with the following modifications: 2,2,2-Trichloroethyl hemisuccinate (0.299 g, 1.2 mmol), prepared from 2,2,2-trichloroethanol and succinic anhydride by the method of Okabayashi et al. (1977), was dissolved in 1.309 g (11 mmol) of thionyl chloride and stirred at 65 °C for 1 h, and the solution was concentrated under vacuum (2 Torr). **1b** (0.354 g, 1.2 mmol) and 0.132 g (1.3 mmol) of triethylamine dissolved in 1.8 mL of *N,N*-dimethylacetamide were added, and the mixture was stirred overnight. The solvent was evaporated in vacuo (4 Torr), the residue suspended in methanol, the resulting solid, 2-nitrophenyl disulfide, filtered out, and the filtrate evaporated to give 0.437 g of the crude product. The crude product was purified by preparative TLC, using chloroform/methanol (8:1), and gave 0.231 g (0.62 mmol) of 3-amino-5-[[3-((2,2,2-trichloroethyl)succinyl)oxy]propyl]-2*H*-1,2,4-triazole at 51.5% yield: R_f = 0.38; NMR (DMSO-d_6) δ 1.65 (2 H, CH_2, m), 2.10 (2 H, CH_2, t), 2.55 (4 H, succinyl CH_2, t), 2.95 (2 H, CH_2O, t), 4.80 (2 H, CCl_3CH_2, s). The product was dissolved in 1 mL of DMF; 0.195 g (2.8 mmol) of zinc dust and 0.28 mL of glacial acetic acid were added, and the mixture was stirred overnight. Any remaining zinc was filtered out, and the solvent was removed under vacuum (5 Torr). The resulting viscous liquid was suspended in 0.30 mL of acetone, and the residual solid was filtered out. The filtrate, after concentration, was purified by preparative TLC, using a chloroform/methanol (7:3) solvent system, to give 0.142 g (0.59 mmol) of **1c** at a total yield of 48.4%: R_f = 0.33; IR (KBr, cm^{-1} 3750–3650 (m, ν_{str}, NH), 1650 (vs, ν triazole), 1300 (vs, ν NH); NMR (DMSO-d_6) δ 1.90 (2 H, CH_2, m), 2.10 (2 H, CH_2, t), 2.50 (4 H, succinyl CH_2, t), 2.85 (2 H, CH_2O, t), 3.40 (NH_2), 6.20 (COOH); UV λ_{max} (CH_3OH–H_2O, nm) 282 (triazole).

3-[(2-Nitrophenylsulfenyl)amino]-2H-1,2,4-triazole-5-thiol (**2b**) was prepared in the same fashion as **1b** starting from 3-amino-2*H*-1,2,4-triazole-5-thiol (**2a**) with a yield of 51.1%: mp 185–6 °C; R_f = 0.92 (chloroform/methanol 7:3); IR (Kbr, cm^{-1}) 3850–3650 (m, ν_{str} NH), 2350 (vs, ν SH), 1680 (s, ν triazole), 1700, 1650, 1500 (s, ν_{str} arom), 1550 (s, ν_{sym} NO_2), 1340 (m, ν_{asym} NO_2, NH); NMR (DMSO-d_6) δ 6.50 (1 H, aromatic, d), 6.60 (1 H, NH–S, s), 7.55 (1 H, aromatic, t), 7.80 (1 H, aromatic, t), 8.35 (1 H, aromatic, d); UV λ_{max} (CH_3OH–H_2O, nm) 276 (triazole), 347 (NO_2PhS); ϵ_M(347) 4080 + 63.

Hapten Conjugation and Verification. The hydroxyl group of **1b** was conjugated to carrier proteins, i.e., bovine serum albumin (BSA) and ovalbumin (OVA), via ester bonds. Both proteins were succinylated (Table I) according the method of Hung et al. (1980). The product was extensively dialyzed against water at 4 °C, lyophilized, and stored at −10 °C. Conjugation (II) to the succinylated proteins was carried out under anhydrous conditions. Thus, in two parallel batches, 30 mg (101 μmol) of **1b**, 1.5 mg (9.9 μmol) of 4-(dimethylamino)pyridine (DMAP), and 422 mg (2.2 mmol) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide were added to 72 mg (1.01 μmol) of the succinylated BSA or 52 mg (1.01 μmol) of the succinylated OVA in 15 mL of dry DMSO. The mixture was stirred under nitrogen for 4 h at room temperature and then dialyzed against water at 4 °C. Conjugation was detected by UV spectroscopy performed in a methanol/water solution (1:1) by scanning the sample at wavelengths from 500 to 250 nm. The hapten density on the protein was calculated from the specific absorbance at 349 nm and the molar extinction coefficient of the corresponding hapten, **1b**.

Table I. Summary of Hapten–Protein Conjugates Used

antigen	hapten	spacer	protein[a]
I		succinyl	BSA/OVA
II	**1b**	succinyl	BSA/OVA/HRP
III[b,c]	**1a**	succinyl	BSA/OVA/HRP
IV	**1b**	TDI	BSA/OVA
V[c]	**1a**	TDI	BSA/OVA
VI	**2b**	MBS	BSA/OVA
VII[c]	**2a**	MBS	BSA/OVA
VIII	**2b**	SPDP	BSA/OVA
IX[c]	**2c**	MBS	BSA/OVA
X	**2c**	SPDP	BSA/OVA
XI	**3**	MBS	BSA/OVA
XII	**3**	SPDP	BSA/OVA
XIII[c]	**4**		BSA/OVA/HRP

[a] BSA conjugates were used for immunization and OVA conjugates for the coating antigens. HRP conjugates were applied in the second antibody technique. [b] Antigen (BSA) used for immunization of rabbits. [c] Antigen (BSA) used for immunization of mice.

Compound **1b** was also conjugated to proteins by the method of Schick and Singer (1961) and Modesto and Pesce (1973), using toluene-2,4-diisocyanate (TDI) as a spacer (IV). An equimolar amount of TDI in 3 mL of DMSO was added to a solution of 147 mg (0.5 mmol) of **1b** in 3 mL of DMSO together with a catalytic amount of triethylamine, stirred for 1 h, and cooled on ice. Then, 162 mg (2.5 μmol) of BSA or 110 mg (2.5 μmol) of OVA was suspended in 30 mL of DMSO and added to 0.25 mmol of the hapten–TDI conjugate. The solutions were stirred for 4 h at room temperature and dialyzed extensively against water containing 1.5% activated charcoal to increase efficacy (Tijssen, 1985). UV spectroscopic determination of epitope density was carried out as with conjugate II.

Compound **2b** was conjugated to proteins through their mercapto groups by using the heterobifunctional reagents 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP) (Carlson et al., 1978) or 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) (Kitagawa et al., 1981). An equimolar amount of SPDP or MBS, in 0.2 mL of THF, was added to a solution of 21.6 mg (80 μmol) of **2b** in 1 mL of THF; the mixture was stirred for 2 h at room temperature and added to a solution of 28.4 mg (0.4 μmol) of BSA or 20.4 mg (0.4 μmol) of OVA dissolved in 2.2 mL of PBS. Following an overnight incubation at 4 °C, the hapten conjugates were dialyzed extensively against water (VI, VIII). Hapten conjugation into the protein was detected by UV spectroscopy, with spacer–protein conjugates containing no haptens but treated in the same way as controls.

A similar procedure was followed for the conjugation of 2*H*-1,2,4-triazole-5-thiol (**2c**) and 4-methyl-1,2,4-triazole-3-thiol (**3**) haptens. Lacking chromophores, however, these conjugates could not be detected on the proteins by UV spectroscopy.

Triazoleacetic acid (**4**) was conjugated to proteins by the active ester method (Anderson et al., 1964). Compounds **1c** and **4** were conjugated to horseradish peroxidase (HRP) by the mixed anhydride method of Jung et al. (1989).

Deprotection of the Carrier Proteins. Conjugates II, IV, and VI were deprotected (resulting in conjugates III, V, and VII) by the procedure of Kricheldorf and Fehrle (1974) and Stern et al. (1979). The lyophilized conjugates were dissolved in PBS buffer (pH 7.0) and treated with 1–1000-fold molar excess (in 10-fold increments) of β-mercaptoethanol for 3 min and then extensively dialyzed against the same buffer. After lyophilization, UV spectra were recorded as for II to verify the disappearance of the 2-nitrophenylsulfenyl (NPS) group (349 nm).

Immunization and Serum Collection. *Rabbits.* Five 3-month-old female New Zealand white rabbits were immunized

intradermally with immunogen III. After three injections at 2-week intervals with 0.1 mg of immunogen emulsified in Freund's complete adjuvant (1:1), injections of 0.15 mg of immunogen in Freund's incomplete adjuvant were given at 3-week intervals. The sixth injection was given with the addition of 0.05 mg of the free hapten aminotriazole.

Mice. Twenty 4-week-old female Swiss Webster mice were immunized subdermally. Four mice were caged together and ear tagged. Each cage received one of five different immunogens, III, V, VII, IX, and XIII, conjugated to BSA. Injections of 25 μg of immunogen, diluted 1:1 in 100 μL of Ribi adjuvant, were given on days 0, 7, 21, and 26. Serum was collected after 10 days by using capillary pipets. The fifth immunization was carried out by using immunogen containing 20% free hapten. The blood of each mouse was centrifuged at 1500*g* for 10 min in a hematocrit serum separator, diluted 1:10 in PBS, and stored at −10 °C. Mice were boosted in 3–4-week intervals with 50 μg of immunogen.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed in 96-well microplates following the basic solid phase immunoassay principle of Voller (1976). OVA conjugates were used as plate coating antigens. Bound antibodies were exposed to either anti-rabbit or anti-mouse IgG (1:2500 dilution) conjugated to alkaline phosphatase, and enzymatic activity was measured by using *p*-nitrophenyl phosphate as a substrate. Analyte concentrations were measured indirectly by competition with the coating antigen for antibody binding sites. For standard curves, stock solutions of different triazole derivatives in double-distilled water, protected molecules in methanol, or succinic anhydride in acetonitrile (200 mM) were diluted from 2 mM to 0.02 μM in 1:10 dilution steps. Standard curves from the raw data were calculated from a four-parameter (sigmoid) equation (Rodbard, 1981). The detection limit was defined as the lowest concentration of hapten, showing a reduction of 3 standard deviations from the mean blank standard absorbance. The cross-reactivity of several compounds, structurally similar to aminotriazole, was measured along with the immunizing hapten. The solvents used (methanol, DMSO, DMF, acetonitrile) did not show any effect on antibody activity below 10% solvent content.

Sequential tests (Zettner and Duly, 1974) were done by preincubating antibody and hapten in glass tubes at 4 °C and transferring aliquots to coated plates followed by incubation either at 4 °C for 4 h or at room temperature for 2 h. The amount of specific antibody bound was detected as above. In the equilibrium test (Zettner, 1973), the antibody and hapten solutions were added to the coated plate without preincubation.

Due to the low molecular weight of aminotriazoles and their similarities to some natural amino acids, antibodies against them are likely to have low affinity and lack avidity. Therefore, it was crucial to optimize assay conditions to favor antibody hapten association (Tijssen, 1985; Käiväräinen, 1985; Thompson and Hoffmann, 1974). The influence of the following factors was tested in assay buffer (0.2 M sodium phosphate buffer): pH values of 6.4, 7.2, 7.6, and 8.0; ionic strengths of 0 and 0.15 M NaCl; incubation temperatures of 4 °C and room temperature; and addition of 0.05% Tween 20.

RESULTS AND DISCUSSION

Hapten Synthesis. The structures of the haptenic compounds are summarized in Figure 1. The amino groups of aminotriazole haptens were protected prior to conjugation by a 2-nitrophenylsulfenyl (NPS) moiety, a chromophoric amino-protecting group often used in peptide syntheses (Stern et al., 1979). The reagents 2-nitrophenylsulfenyl chloride and 2,4-dinitrophenylsulfenyl chloride selectively react with the amino groups of aminotriazole derivatives even in the presence of hydroxyl or mercapto groups. The resulting sulfenamides are chemically stable during conjugation to proteins but can be readily removed by a treatment with β-mercaptoethanol or 2-pyridinethiol. In addition, their specific absorbance at 349 nm offers a simple and quantitative determination of epitope densities on the proteins by UV spectroscopy. After the chromophoric protecting group is conjugated to **1a**, the

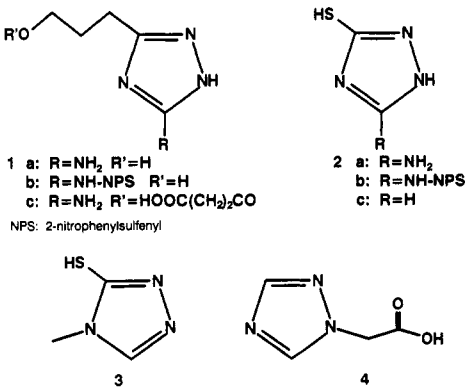

Figure 1. Structure of the triazole haptens.

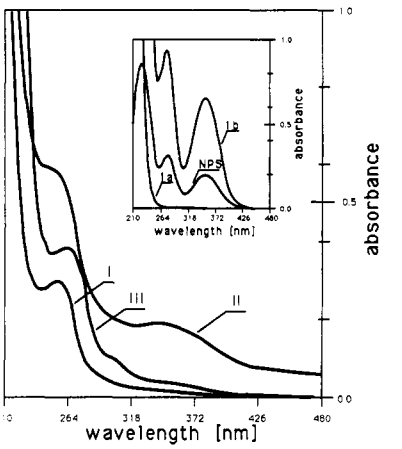

Figure 2. Monitoring protein conjugation by UV spectroscopy. Spectra of protein conjugates I–III are shown. Note the absorbance peak of the chromophore protecting group at 349 nm (II) that disappears during deprotection. Spectra of the corresponding haptens **1a** and **1b** and the NPS protecting group are shown in the insert.

maximal UV absorbance wavelength was 349 nm for the new compound **1b** and the molar extinction coefficient was found to be 3337 + 122 (Figure 2).

Efforts to directly succinylate **1b** under DMAP/base catalysis [according to the procedure of Jonhston (1982)] were unsuccessful. The indirect succinylation of **1b** with 2,2,2-trichloroethyl hemisuccinate, however, removed the sulfenamide moiety, resulting in the succinylated hapten with a free amino group (**1c**). This compound could be protected again and used for conjugation to a reporter enzyme, HRP, or used for affinity purification of aminotriazole antibodies.

Hapten Conjugation. Haptens (1–4) were conjugated to proteins (BSA, OVA, HRP) by homobifunctional (succinic anhydride, TDI) or heterobifunctional (MBS, SPDP) reagents. The protein conjugates synthesized are summarized in Table I and are numbered in Roman numerals (square brackets indicate the proteins). A molar ratio of 100 hapten molecules/mol of protein was used in the conjugation reactions for all conjugates on the basis of the results of Jung et al. (1988).

Figure 2 shows the conjugation of chromophore hapten **1b** to succinylated BSA (I), resulting in II. Epitope densities were calculated by the molar extinction coefficients of the haptens at the absorbance maximum of 349 nm of the conjugates, giving average densities of 8.2 (**1b** in conjugate II), 3.2 (**1b** in conjugate IV), and 1.7 (**2b** in conjugate VI). The loss of the peak absorbance at 349 nm after deprotection indicated the successful cleavage of the NPS protecting group using a 10-fold molar excess of

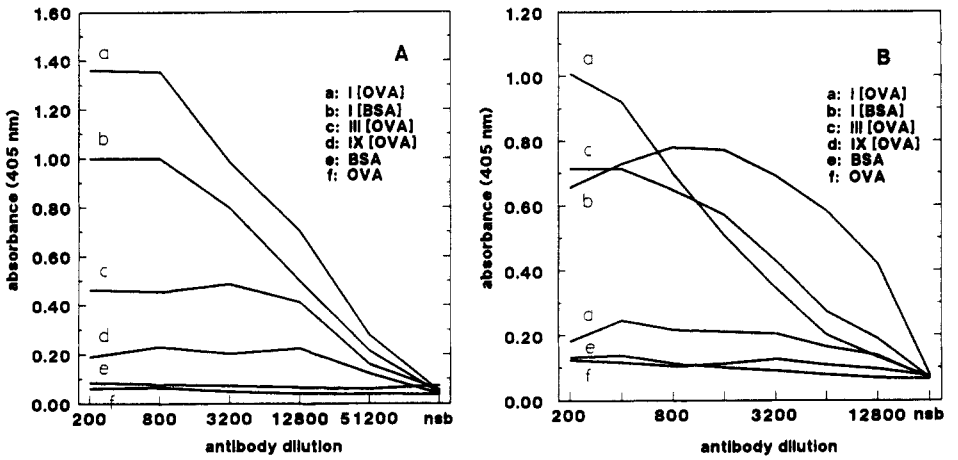

Figure 3. Rabbit antibody specificity to different antigens. Two-dimensional titer of anti-III[BSA] antibody was performed after the second (A) and third (B) bleeds. Note the increased binding to the homologous antigen and decreased recognition of the succinylated proteins in the third bleed.

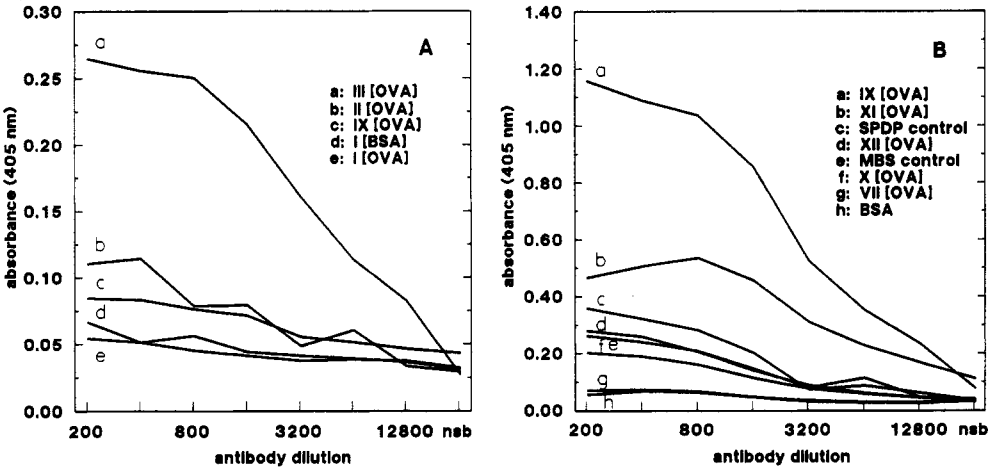

Figure 4. Mouse antibody specificity to different antigens. (A) Binding of anti-III[BSA] antibody (mouse 350, third bleed) to the homologous and heterologous antigens. (B) Binding of anti-IX[BSA] antibody (mouse 368, third bleed) to the homologous and heterologous antigens, as well as the control conjugates with MBS/SPDP linker.

β-mercaptoethanol. Lacking chromophore groups, haptens **2c**, **3**, and **4** could not be detected and quantified by UV spectroscopy.

Five of the protein conjugates were used for immunization of rabbits and mice (Table I). When SPDP was applied as a spacer, the amino-protected thiol hapten (**2b**) in the protein conjugate VIII was cleaved during deprotection, since the reductive treatment (mercaptoethanol, 2-pyridinethiol) used to break the sulfenamide moiety of the protecting group also reduced the disulfide bond attaching the haptenic thiol group to SPDP. Therefore, this conjugate could be used only in the protected form.

Antibody Characterization. The titer, defined as the antiserum dilution that binds 50% of the tracer, was determined after the third immunization and resulted in an average dilution of 6400–9000 for the rabbits and 500–800 for the mice. Although the antibody titer of both species increased with repeated injections, the titer in mice was always 2 orders of magnitude lower than in rabbits. Since the amount of mouse antisera was low, optimization of assay conditions was done with rabbit antisera. Fortunately, limited experiments indicated that the optimized conditions could be applied to assays on mouse antisera. The characteristic binding properties of 10 mouse and rabbit antisera are summarized in Figures 3 and 4 and Tables II and III. Although the conjugation ratios (epitope densities) were obviously highly variable for different haptens and spacers, which alter relative binding, the structure of different coating antigens that bind the antibody provided additional information about antibody specificity. Further parameters examined were the difference in antibody binding to the SPDP, TDI, and MBS control conjugates without hapten and the protecting group, as well as the native and denatured protein. Figure 3A shows that all antibodies raised against III[BSA] lost specificity

Table II. Relative Binding of Rabbit and Mouse Anti-III[BSA] Antibodies to Homologous and Heterologous Antigens[a]

immunogen	III[BSA]					
coating antigen [OVA]	III	IX	VII	V	X	XII
hapten	1a	2c	2a	1a	2c	3
spacer	succ[b]	MBS	MBS	TDI	SPDP	SPDP
type of heterology[c]	none	H/B/S	H	B	H/B	H/B
rabbit 1277	1	0.17	0.10	0.02	0.03	0.08
rabbit 1280	1	0.48	0.04	0.04	0.00	0.00
rabbit 1281	1	0.20	0.12	0.03	0.03	0.04
rabbit 1290	1	0.43	0.12	0.01	0.03	0.08
rabbit 1291	1	0.55	0.14	0.02	0.04	0.08
mouse 350	1	0.10	0.00	0.00	0.04	0.44
mouse 351	1	0.27	0.00	0.00	0.02	0.10
mouse 352	1	0.09	0.00	0.00	0.03	0.18
mouse 353	1	0.54	0.00	0.00	0.05	0.28

[a] Relative binding was normalized to binding to the homologous antigen. [b] Succinyl handle. [c] H, hapten heterology; B, spacer heterology; S, site heterology.

Table III. Relative Binding of Mouse Anti-IX[BSA] Antibodies to Homologous and Heterologous Antigens[a]

immunogen	IX[BSA]				
coating antigen [OVA]	IX	XI	VII	XII	X
hapten	2c	3	2a	3	2c
spacer	MBS	MBS	MBS	SPDP	SPDP
type of heterology[b]	none	H	H	H/B	B
mouse 365	1	0.37	0.04	0.36	0.36
mouse 366	1	0.24	0.04	0.10	0.21
mouse 367	1	0.42	0.03	0.12	0.38
mouse 368	1	0.37	0.01	0.23	0.15

[a] Relative binding was normalized to binding to the homologous antigen. [b] H, hapten heterology; B, spacer heterology; S, site heterology.

for native BSA. In addition, antibodies raised in rabbits and in mice did not bind to mercaptoethanol-denatured proteins, with the exception of one mouse, injected with VII[BSA]. Mouse anti-III antibody did not bind to succinylated OVA, while the corresponding rabbit antibodies showed high recognition for the succinate spacer.

The results in Table II indicate a high specificity for the immunogen. When conjugates with single heterology were used as coating antigens, anti-III[BSA] sera from rabbits and mice bound poorly, probably due to the low epitope density of the antigen conjugate and the low antibody affinity. Coating antigens with multiple heterology (IX), in which hapten, site, and spacer heterology are combined, gave as much as 50% binding, indicating triazole ring specificity, and—as compared to VII[OVA]—limited spacer specificity. In contrast, V[OVA] did not show significant binding.

Table III summarizes the results of antibodies raised in mice against immunogen IX[BSA]. A similar hapten with the same spacer, XI[OVA], resulted in significant antibody binding, indicating that the MBS spacer, due to the larger molecular size, was a crucial determinant in antibody recognition. Interestingly, a site heterologous hapten (**2a** in conjugate VII) gave almost no binding, despite the fact that the same MBS spacer was used. This is likely due to the low hapten density on this carrier. Specificity of mouse antibodies for the homologous antigens is depicted in Figure 4A. Curves d and e of this figure indicate no binding to the succinylated proteins, curve b shows no binding to the protecting group, and curve c illustrates the low binding of the mouse antibodies to the heterologous antigen, IX[OVA]. In addition to Table III, Figure 4B clearly shows the high binding of the antibody to the spacers (curves b, c, and e). Immunogen IX[BSA] (mouse 368) resulted in a high titer for the homologous conjugate, while the heterologous conjugate using a different hapten, XI (curve b), showed about 37% recognition compared to the immunizing antigen, indicating affinity to the spacer. Nevertheless, the spacer heterologous system (X, curve f), and the spacer and hapten heterologous system (XII, curve d), also bound to the antibody in a range similar to that of the MBS and SPDP control conjugates (curves c and e). In common with other mice but in contrast to rabbits, no binding to the immunizing protein is seen in curve h.

On repeated immunizations, the binding of anti-III antibody (rabbit 1291) to hapten increased, while binding to the succinylated carrier protein decreased (Figure 3). This is probably due to antibody maturation, even though the increase of antibody titer seems to be mainly due to a hapten-specific activation of B-cells. Similar phenomena of antibody maturation were described by Eisen and Siskind (1964).

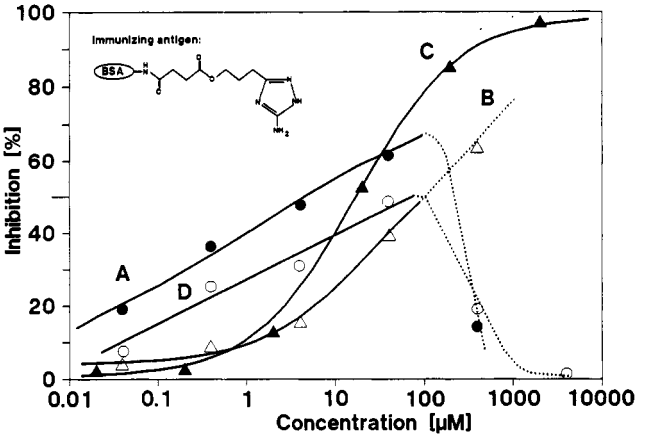

Figure 5. Competitive inhibition of anti-III antibody (rabbit 1291, fifth bleed, 1:10 000 dilution) by aminotriazole haptens. ELISA standard semilogarithmic inhibition curves are shown for amitrole (A, ●) and haptens **1a** (B, △), **1c** (C, ▲), and **2c** (D, ○). ELISA was performed in a sequential test system using 10 μg/mL IX[OVA] as coating antigen. Absorbance values are corrected for nonspecific binding and are the mean of at least three replicates.

Optimization of Assay Conditions. Assay conditions were optimized for temperature, buffer, ionic strength, and pH. For inhibition tests in the chosen ELISA format, it was necessary to optimize parameters that can influence the antibody flexibility and binding to the hapten. Of the five rabbits, the fourth bleed of rabbit 1291 was selected and optimized for binding to the amitrole hapten in a heterologous test system, IX[OVA]. Since the hydrogen bonds between antibody and hapten are more stable at low temperature, immunoreactions were performed at 4 °C. To compete the antibodies off, a sequential assay system was used in which hapten and antibody were preincubated prior to the competition reaction. Assay buffer containing 0.05% Tween 20 did not affect the antibody–hapten interaction. Ionic strength influenced antibody–hapten interaction, as inhibition decreased in the presence of 0.15 M NaCl. In addition, antibody recognition of the succinylated protein can be blocked by using mercaptoethanol-treated succinylated protein in the assay buffer. Interestingly, the inhibition was also pH dependent. No inhibition was seen at pH 6.4, whereas binding increased with pH, having an optimum at 7.6.

Antibody Affinity and Inhibition. Figure 5 illustrates competitive inhibition tests under optimized conditions. The conditions optimized for the rabbit antisera gave the same results on mice antisera. The curves represent means of at least three replicates with a coefficient of variation of less than 7%, and the minimum detectable concentration was 40 μM. As seen on curve C, antibody inhibition by the aminotriazole hapten **1c** resulted in a sigmoid curve with an IC_{50} of 16.8 μM, indicating that the aminotriazole ring and the succinate spacer each contribute significantly to antibody binding site. The lower IC_{50} for aminotriazole, as seen on curve A, indicates the higher specificity of the antibody to this hapten, although the affinity seems to be too low for inhibition greater than 60%. Specificity was directed toward the target analyte by the addition of unconjugated amitrole to the last boost of immunization. This procedure seems to strengthen specific antibody binding. The carbonyl part of the aminotriazolepropanol molecule, as seen in curve B, is not sufficient by itself for structural recognition. Unlike curve C, curves A and D show a rather linear than sigmoid course, and a break is observed at analyte concentrations above 100 μM, as seen on the dotted

Table IV. Cross-Reactivity of Anti-III[BSA] Antibodies (Rabbit 1291) with Various Compounds

compound	IC_{50},[a] ng/mL
3-amino-1,2,4-triazole (amitrole)	119
5-(3-hydroxypropyl)-3-amino-2*H*-1,2,4-triazole (**1a**)	909
5-(3-hydroxypropyl)-3-(2-nitrophenylsulfenyl)amino-2*H*-1,2,4-triazole (**1b**)	(21)[b]
3-amino-5-[(3-succinyloxy)propyl]-2*H*-1,2,4-triazole (**1c**)	4070
3-amino-1,2,4-triazole-5-thiol (**2a**)	(5)
3-(2-nitrophenylsulfenyl)amino-2*H*-1,2,4-triazole-5-thiol (**2b**)	>1 mM[c]
2*H*-1,2,4-triazole-5-thiol (**2c**)	35
4-methyl-1,2,4-triazole-3-thiol (**3**)	>1 mM
(1,2,4-triazol-2-yl)acetic acid (**4**)	>1 mM
1,2,4-triazole	(11)
4-amino-1,2,4-triazole	>1 mM
3-acetamido-1*H*-1,2,4-triazole	>1 mM
3-amino-1,2,4-triazole-5-carboxylic acid hemihydrate	>1 mM
2-(4-chlorophenyl)-2-(1,2,4-triazol-1-yl)-methylhexanoic acid	(13)
succinic acid	8560
imidazole	>1 mM
L-histidine	(15)
L-glutamic acid	>1 mM

[a] IC_{50} values (the concentration which inhibits the assay by 50%, as calculated from a four-parameter fit) were not determined above 1 mM concentration. [b] Values in parentheses indicate percent inhibition at 1 mM concentration. [c] IC_{50} is above solubility limit.

lines. Such a phenomenon is explained by nonspecific binding of the analyte–antibody complex to the solid phase at high concentrations, as has been shown by Peterman (1989). Since these results were consistent during all inhibition tests, we feel they should be reported and considered for further development of routine assays as built-in pitfalls of the detection system.

No cross-reactivity was detected for the structurally related compounds listed in Table IV. These results conclusively show that aminotriazole was poorly recognized by B-lymphocytes. Nonetheless, antibody specificity for the aminotriazole ring can be shown by prudent assay conditions. Although assay sensitivity indicated by this study does not surpass that of some of the conventional methods, it is still promising for a low-cost and less labor intensive qualitative immunoassay.

As mentioned above, the antibody characterization for anti-IX[BSA] resulted in no workable dose response curves in the heterologous systems tried. Similar results were found also for the V[BSA], VII[BSA], and XIII[BSA] conjugates. However, the XIII[HRP] conjugate allowed tests in a "direct ELISA" format which is described in an earlier paper (Jung et al., 1989). A further conjugate of **1c** to HRP was made by using the carboxyl group of the succinyl spacer; the amino group did not react. Although inhibition tests with this format did not improve the sensitivity for amitrole, they confirmed the results for anti-III[BSA] antibodies.

Protein Succinylation. Immunization of rabbits with haptens conjugated to succinylated proteins resulted in high recognition of the succinyl group. On the basis of the epitope density calculated for III[BSA] and assuming succinylation of all 60 lysine amino residues of BSA, the proportion of succinyl groups having a hapten attached was 13%. Thus, more than 85% of spacer was presented to the lymphocytes as free succinyl groups. To overcome, this problem, direct succinylation of the hapten (**1b**) or activation of the haptenic hydroxyl groups with tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) (Nilsson and Mobasch, 1981) could be suitable alternatives. Succinylation of proteins in nonaqueous media (DMSO) also provides a better control of succinylation and hapten incorporation.

Structural Changes of the Carrier Protein. Carrier proteins can undergo denaturation by a wide variety of agents, including urea, mercaptoethanol, SDS, acid, alkali, organic solvents, and heat. These changes result in a change in conformation and thus may influence immunogenicity. Denatured proteins have been used to increase the specific immunogenicity (Howard and Wild, 1957). None of the disulfide bonds of BSA are accessible to reducing agents in the pH range 5–7, but they become progressively available as the pH is raised or lowered (Katchalski et al., 1957; Peters, 1985). According to Rose et al. (1985), proteins possess two types of antigenic sites, the first consisting of a sequence of residues and the second consisting of the surface topography and the tertiary structure. Mercaptoethanol treatment causes structural changes in the protein, but its effect on the antigenic presentation has not been studied. Further investigations on Raman spectroscopy may give more information about changes in the BSA molecule (Aoki et al., 1982). The mercaptoethanol treatment we describe resulted in antibodies that did not recognize either native or denatured protein. From these experiments, it is conclusive that the structural changes enhance hapten-specific antibodies (Amkraut et al., 1966).

Choice of Species Immunized. The advantage of using mice in screening a large number of antigens is offset by their low total IgG concentration. However, mouse antibodies appeared largely specific for a single epitope, aminotriazole. In contrast, the rabbit antibodies showed recognition also for the succinylated protein. It is unfortunate that the mouse IgG specificity found was not accompanied by a higher affinity to the hapten of interest.

CONCLUSIONS

A series of triazole and aminotriazole haptens were synthesized and conjugated to carrier proteins by using homo- and heterobifunctional reagents. The amino groups of haptenic compounds were successfully protected for conjugation via NPS protection. Of the different haptens used for immunogen synthesis, the anti-3-aminotriazole-propanol antiserum showed considerable selectivity for the parent and compounds closely related to 3-amino-triazoles. Using heterobifunctional spacers for various triazoles failed to produce antibodies for triazoles, but these conjugates were useful as coating antigens.

The hydroxypropyl spacer was the best of those studied, but for further immunizations, this molecule should be conjugated directly to lysine residues (through the activated hydroxyl group), avoiding succinylated protein. The high rabbit antibody binding to succinate, which also represents a small immunogenic entity, is probably a result of the high succinate density on the protein. Consequently, a higher epitope density of small haptens should be emphasized. This assay clearly has a pronounced optimum in ionic strength, pH value, temperature, and equilibrium conditions necessary to obtain inhibition. Preparation of monoclonal antibodies would obviously offer a better selection of antibodies, among hundreds of clones, for the haptens of interest for a quantitative immunoassay. The existing antibodies are of sufficient specificity and affinity to be promising for qualitative assays and for the affinity chromatography of amitrole and related compounds.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; GC, gas chromatography; TLC, thin-layer chromatography;

THF, tetrahydrofuran; NMR, nuclear magnetic resonance spectroscopy; UV, ultraviolet–visible spectroscopy; IR, infrared spectroscopy; ELISA, enzyme-linked immunosorbent immunoassay; BSA, bovine serum albumin; OVA, ovalbumin; DMAP, 4-(dimethylamino)pyridine; DMSO, dimethyl sulfoxide; TDI, toluene-2,4-diisocyanate; SPDP, 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester; MBS, 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; NPS, 2-nitrophenylsulfenyl group; IgG, immunoglobulin G; SDS, sodium dodecyl sulfate; DMF, *N*,*N*-dimethylformamide; Tween 20, polyoxyethylene sorbitan monolaurate.

ACKNOWLEDGMENT

We express our sincere appreciation to Shirley Gee, Robert O. Harrison, and William H. Newsome for their comments and suggestions. This work was supported by EPA Cooperative Research Grant CR-814709-01-0, NIEHS PHS ES04699-01 (Superfund), and the California Department of Food and Agriculture. A.S. is a Fulbright Scholar (Institute of International Education, Program 33917). B.D.H. is a Burroughs Wellcome Scholar in Toxicology.

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Received for review February 22, 1990. Accepted July 19, 1990.

Registry No. **1a**, 18595-97-6; **1b**, 130032-52-9; **1c**, 130032-53-0; **2b**, 130032-54-1; 3-amino-5-[[3-((2,2,2-trichloroethyl)succinyl)oxy]propyl]-2*H*-1,2,4-triazole, 130167-76-9; 2,2,2-trichloroethyl hemisuccinate, 130167-75-8; triethylamine, 121-44-8; 2-nitrobenzenesulfenyl chloride, 1694-92-4.