Development of a Monoclonal Antibody Based Enzyme Immunoassay Method for Analysis of Maleic Hydrazide

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Two monoclonal antibodies specific for the synthetic plant growth regulator maleic hydrazide (MH) have been used to develop an enzyme immunoassay (EIA) for MH. Splenic lymphocytes from mice immunized with synthesized MH–protein conjugates were used to make hybridomas. Two resultant subclass IgG_1 monoclonal antibodies (1.9×10^5 L/mol affinity for MH) were purified by ammonium sulfate precipitation and affinity chromatography. The purified antibodies recognized only MH and its derivatives and did not cross-react with the natural compounds tested. Detection limits ranged from 0.11 to 1.3 ppm MH and were repeatable within less than 2-fold on consecutive days. Heterologous assays were more sensitive than homologous assays to both MH and nonspecific interferences. These assay systems demonstrate the ability to measure MH by EIA at levels comparable to those typically found in tobacco, potatoes, and onions.

Maleic hydrazide (MH) is a synthetic plant growth regulator that is widely used in the United States and Europe despite uncertainty about its chronic toxicity to nontarget organisms (Hoffman and Parups, 1964; Haley, 1977; Swietlinska and Zuk, 1978; USDA, 1979; Ponnampalam et al., 1983). After more than three decades of study, conflicting results still prevent widespread agreement on the chronic toxicity of MH. In response to a rebuttable presumption against registration (RPAR), the U.S. Environmental Protection Agency has evaluated the available data on toxic effects of MH, human exposure, and its toxicological significance, concluding that the presence or absence of adverse effects from MH cannot be established (Johnson, 1983). Estimates of actual residue levels are 10–40 ppm for fresh potatoes, 80 ppm for potato chips, and 5–7 ppm for onions, while respective tolerances are set at 50, 160, and 15 ppm (USDA, 1979). Residue levels in domestic tobacco generally fall between 50 and 200 ppm on a dry-weight basis, but values over 400 ppm have been reported (USDA, 1979). Dietary intake via treated potatoes and onions has been estimated at 20 μg/kg per day for the average American adult (USDA, 1979), and intake via smoke from treated tobacco is also a significant concern (Haeberer and Chortyk, 1974).

Gas and liquid chromatographic methods for MH have been developed (Haeberer and Chortyk, 1974; Newsome, 1980a; King, 1983), but a modified colorimetric method (Wood, 1953; Ihnat et al., 1973) remains the official and most widely used method (AOAC, 1980). These methods are all laborious and expensive, and analysis for MH is not routinely performed on food crops despite the existence of legal tolerances. Knowledge of human exposure to MH is restricted significantly by the laboriousness of analysis. A faster and easier method of MH residue analysis should contribute to our understanding of the hazard of its continued use. The development of simple and rapid immunoassay methods for a range of synthetic pesticides (Langone and Van Vunakis, 1975; Wing et al., 1978; Newsome and Shields, 1981; Wie and Hammock, 1984; Brimfield et al., 1985; Ercegovich et al., 1981; Kelley et al., 1985) suggests this may be a viable alternative analytical method. We describe here the production and characterization of two hybridoma cell lines that produce monoclonal antibodies specific for maleic hydrazide. We also describe the use of these antibodies in an enzyme-linked immunosorbent assay and the effects of some important variables on the assay.

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MATERIALS AND METHODS

All chemicals were reagent grade or better and were supplied by Aldrich Chemical Co. unless noted otherwise. MH derivatives were analyzed for purity by thin-layer chromatography on silica gel (Baker) using solvent systems of either ethyl acetate–acetonitrile–acetic acid (5:1:1), chloroform–methanol–acetic acid (10:10:1), ether–methanol (9:1), methanol–acetonitrile (2:1), or 1-butanol–acetic acid (4:1).

Synthesis. *3-Hydroxy-6(1H)-oxopyridazineacetic Acid (1).* MH and $ClCH_2COOH$ were reacted according to the method of Schönbeck (1959). 1 was recrystallized from water by acidification, giving white crystals, mp 246–248 °C. 1 was identified on the basis of its IR (Beckman Acculab 4), ^1H NMR (Varian EM-360), and MS (HP 5985B): IR (KBr, cm^{-1}) vs (1515, 1275), s (3070, 1790, 1405, 1195), m (2640, 1665, 1440, 945, 865, 855, 830); ^1H NMR (DMSO-d_6) δ 4.5 (s, 2, acidic), 6.9 (q, 2, aromatic, J = 9 Hz), 12 (s, 1, carboxylic); MS (70 eV) m/e (rel intens) 54 (22), 55 (25), 69 (5), 80 (100), 82 (18), 98 (64), 112 (3), 125 (93), 126 (65), 170 (M^+, 36); neutralization equivalent, 86.3 (n = 1); UV, λ_{max} (CH_3OH) 313 nm, ϵ_M 2841 ± 126 (n = 4).

3-(Carboxymethoxy)-6(1H)-pyridazinone (2). The above reaction was repeated, except that the starting pH was adjusted to 8.0. Acidification and cooling as for 1 yielded a white precipitate that was recrystallized as above to give white crystals, mp 242–245 °C. 2 was identified on the basis of its IR, ^1H NMR, and MS: IR (KBr, cm^{-1}) vs (1655, 1600, 1205), s (2960, 1740, 1555, 1460), m (1225, 1055, 1010, 860, 800); ^1H NMR (DMSO-d_6) δ 4.6 (s, 2, acidic), 7.0 (q, 2, aromatic, J = 9 Hz), 12 (s, 1, carboxylic); MS (70 eV) m/e (rel intens) 54 (37), 55 (34), 67 (8), 80 (22), 82 (62), 97 (35), 112 (67), 113 (16), 125 (46), 170 (M^+, 100); neutralization equivalent, 163.5 ± 0.1 (n = 2); UV, λ_{max} (CH_3OH) 303 nm, ϵ_M 2296 ± 194 (n = 5).

3-Hydroxy-6(1H)-oxo-4-pyridazineacetic Acid (3). A solution of 870.5 mg (5 mmol) of *cis*-aconitic acid (Sigma) and 0.142 mL (4.5 mmol) of anhydrous hydrazine (Baker) in 100 mL of methanol was refluxed for 16 h. After the mixture was cooled to 20 °C, impurities were precipitated by the addition of ether and removed by filtration. The filtrate was decolorized twice with activated charcoal and concentrated by evaporation, and crystallized product was collected by filtration. In an alternative method, 156 mg (1 mmol) of *cis*-aconitic anhydride (Sigma) was dissolved in 25 mL of methanol and the resultant mixture cooled in a dry ice–ethanol bath. Anhydrous hydrazine (0.032 mL, 1 mmol; Baker) in 25 mL of methanol was dripped slowly (over 4.5 h) into the cooled solution. Partial evaporation precipitated impurities, which were

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removed by filtration. The filtrate was concentrated by evaporation, and crystallized product was collected by filtration. **3** from both methods was recrystallized from water by acidification to give white crystals, mp 225–227 °C. **3** was identified on the basis of its IR, ^{1}H NMR, and MS: IR (KBr, cm^{-1}) vs (1690, 1405), s (1580, 1545, 1275, 1255, 1215), m (3040, 1345, 950, 925, 820); ^{1}H NMR (DMSO-d_6) δ 3.4 (s, 2, acidic), 6.9 (s, 1, aromatic), 11.6 (s, 1, carboxylic); MS (70 eV) m/e (rel intens) 39 (32), 44 (15), 58 (27), 67 (23), 68 (16), 69 (14), 84 (5), 96 (35), 97 (13), 112 (3), 126 (100), 152 (16), 170 (M^+, 13); neutralization equivalent, 89.4 ± 0.7 (n = 3); UV, λ_{max} (CH_3OH) 307 nm, ϵ_M 2230 (n = 1).

Maleic Hydrazide Glucoside (*4*). In a modification of the method of Newsome (1980a), acetobromo-α-D-glucose (8.22 g, 0.02 mol) was dissolved in 50 mL of dry CH_3CN, to which were added oven-dried MH (4.48 g, 0.04 mol), oven-dried $CaSO_4$ (5.50 g, 0.04 mol), and Ag_2CO_3 (5.52 g, 0.02 mol). The mixture was stirred at 20 °C for 20 h in the dark and filtered (Whatman No. 1), and the filtrate was evaporated in vacuo to give a light brown syrup, which was dissolved in a minimum volume of methanol. Light brown crystals (mp 172–175 °C) precipitated from methanol–ether were identified as MH-glucoside tetraacetate based on UV and IR spectra. **4** was prepared by deacetylation of MH-glucoside tetraacetate with methanolic NH_3 (Dorough et al., 1974). Deacetylation was quantitative as shown by TLC, and the residue after evaporation at 77 °C in vacuo (mp 184–187 °C) was identified as **4** based on UV and IR: UV (CH_3OH) λ_{max} 303 nm, ϵ_M 1912 ± 23 (n = 3); IR (KBr, cm^{-1}) vs (3410, 3340, 3240, 1595, 1070), s (1675, 1650, 1280, 995), m (1435, 1315, 1100, 835). No titratable protons were observed.

Coupling of Haptens to Proteins. Acetic acid derivatives of MH were coupled to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) via *N*-hydroxysuccinimide-activated esters by the method of Langone and Van Vunakis (1975). Conjugates of **4** to BSA were made by the periodate method of Stollar (1980). Conjugates of **4** and glucose to BSA and **4** to KLH were made by the 4-(dimethylamino)pyridine-catalyzed esterification method of Hung et al. (1980).

Characterization of Hapten–Protein Conjugates. Conjugates of **1**, **2**, and **4** were analyzed to determine hapten densities by recording ultraviolet spectra for unconjugated proteins and conjugates from 250 to 350 nm. Absorbance due to the conjugated derivative was determined by subtraction of the appropriate control absorbance, the molar hapten concentration was calculated from the molar extinction coefficient, and the molar hapten to protein ratio was calculated. The protein molecular weights used were as provided by the suppliers, 66 000 for BSA and 3×10^6 for KLH.

Immunization. Immunogens (KLH conjugates) were emulsified with Freund's adjuvant for injection into adult male or female Balb/C mice (Jackson Laboratories) according to the following schedule: day 0, 0.2 mg of immunogen/mouse, in Freund's complete adjuvant, subcutaneously; day 14, 0.05 mg/mouse, in Freund's incomplete adjuvant, intraperitoneally; day 28, 0.02 or 0.05 mg/mouse, given as for day 14. Later boosts of 0.01–0.015 mg/mouse were given as for day 14. Blood samples were taken by tail snip 3–4 days after the last injection and centrifuged in heparinized tubes. Serum was frozen for later use or stored at 4 °C after dilution to 1:50 with phosphate-buffered saline, pH 7.4, +0.05% Tween 20 (PBST) for immediate use.

Enzyme Immunoassay and Competitive Inhibition Enzyme Immunoassay. Serum samples were screened by the indirect EIA method of Hunter and Lenz (1982) using plate coating antigens (BSA conjugates) applied to 96-well plates at 1–10 μg/mL (100 μL/well) in 0.1 M carbonate–bicarbonate coating buffer, pH 9.6. Plates (Dynatech or Nunc) were sealed with adhesive plate sealers to prevent evaporation and incubated 14–20 h at 4 °C to adsorb the target antigen to the wells. All subsequent incubations and washes were done at room temperature in buffer containing 0.05% Tween 20 (except substrate buffer). Assay response was measured by reading the absorbance at 405 nm with an MR 600 EIA plate reader (Dynatech). Results were recorded and analyzed on an Apple II+ computer and the Immunosoft 1.4 plate-reading program (Dynatech). The ability of sera to detect MH was evaluated by competitive inhibition enzyme immunoassay according to Hunter and Lenz (1982) and Hunter et al. (1986). Inhibitor dilutions in PBST were incubated with serum dilutions for 30–60 min at 22 °C before being applied to the antigen-coated EIA plate. Inhibition curves were analyzed by an Apple II+ computer using a weighted four-parameter logistic curve-fitting procedure (Rodbard, 1983) which calculated IC_{50} values (molar inhibitor concentration giving 50% inhibition) and confidence limits.

Hybridoma Production and Evaluation of Fusion Products. Spleens were taken from immunized mice 3–4 days after the last booster injection and fused with P3-X63-AG8.653 plasmacytoma cells (Kearney et al., 1979) according to Hunter et al. (1982). Rapidly growing cultures 7–18 days old were sampled to analyze by EIA for antibodies binding to **1**–BSA or **2**–BSA. Positive cultures were tested by CIEIA for inhibition of antibody with 15 mM MH. Cultures more than 50% inhibited compared to a mock inhibited control were grown up further for cloning and freezing. Specificity for MH was verified by CIEIA using MH derivatives and natural pyrimidines and purines.

Cloning of Hybridomas. Cultures were cloned by dilution to a calculated density of 0.5 cell/well in 96-well microtiter plates. Diluted cells (0.1 mL/well) were placed onto feeder layers of thymocytes (0.1 mL/well) prepared from thymus glands of 3-month-old Balb/C mice by the same procedure as for spleens. Wells containing single colonies were identified and screened for antibody production by EIA and CIEIA when colony size reached approximately one-fourth of the well diameter. Two positive clones, designated IH9 and IIC7, were grown up further for freezing and ascitic tumor production.

Monoclonal Antibody Production and Purification. Six-month-old male or female Balb/C (Jackson Laboratories) mice were primed by intraperitoneal injection of 0.5 mL of 2,6,10,14-tetramethylpentadecane (pristane; Aldrich) 14–30 days before injection of (5–13) × 10^6 IH9 or IIC7 hybridoma cells per mouse. Ascitic fluids were clarified by centrifugation for 15 min at 2000g and stored frozen. Cells recovered by centrifugation of the first ascitic fluids harvested were injected into an equal number of new mice for a second passage. This strategy increases the amount of antibody produced without the need to culture large number of cells again for initiation of ascitic tumors. Mice were tapped three to four times each over 6–8 days beginning 7 days after injection. MH-specific monoclonal antibodies were purified from ascitic fluids by ammonium sulfate precipitation (Williams and Chase, 1976) followed by affinity chromatography on columns of **1**–BSA coupled to an activated silica matrix (Boehringer-Mannheim). Bound antibodies were eluted with 0.2 M glycine, pH 2.5, and the eluate was adjusted immediately to pH 7.6, dialyzed against PBS, and lyophilized for long-term storage.

Monoclonal Antibody Isotype and Affinity Determination. Affinity-purified antibodies were tested by a modified EIA, using affinity-purified goat anti-mouse isotype antibodies conjugated to alkaline phosphatase (Southern Biotechnology Associates) and plates coated with **1**–BSA. Affinity constants were measured for both MH-specific affinity-purified antibodies by equilibrium dialysis according to Weir (1973), using [^{14}C]MH (ICN Biochemicals, 5.05 Ci/mol). Initial protein concentration was 160 μg/mL (1 μM) for all antibody and IgG control half-cells. Radioactivity measurements were made on a Packard 4550 liquid scintillation counter with efficiency correction made by the automatic external standard. Affinity constants were determined graphically with Sips plots.

RESULTS AND DISCUSSION

Characterization of Conjugates. UV spectra exhibited qualitative differences between conjugates and carrier proteins in the region of hapten absorption maxima (in water, 295–307 nm), indicating that conjugation had occurred. Hapten densities (and conjugation efficiencies) were calculated to be 6.4 (26%) for **1**–BSA, 6.2 (25%) for **2**–BSA, 1580 (29%) for **1**–KLH, and 1830 (34%) for **2**–KLH. Hapten densities were calculated to be 5.5 for **4**–BSA and 440 for **4**–KLH. Conjugation of **3** to proteins via NHS esters was unsuccessful.

Production of Monoclonal Antibodies. A summary of spleens fused and hybridomas produced is given in Table I. Only antibodies from hybridomas made from **1**–KLH-immunized mice were effectively inhibited by MH.

Table I. Summary of Hybridomas Derived from Mice Immunized with KLH Conjugates of MH Derivatives

hapten	spleens fused	no. of wells: EIA screened pos/total	no. of wells: inhib by homologous hapten	no. of wells: inhib by MH
1	3	7/450	6	6
2	9	8/800	6	0[a]
4	3	2/500	0	0

[a] Inhibition by MH observed for three wells at 15 mM only.

The clones IH9 and IIC7 were selected for ascitic tumor amplification based on stability, growth, and preliminary specificity studies. Pooled ascitic fluid volumes were 120 mL for IH9 and 150 mL for IIC7 (seven to eight mice per cell line per passage). Ascitic fluid titers were 10^5–10^6 as tested by homologous EIA. Solubilized ammonium sulfate precipitated antibodies were further purified by affinity chromatography, yielding 0.5–2.0 mg of purified antibody per column run from 20–200 mg of starting material. Both IH9 and IIC7 antibodies were stable to lyophilization and were unaffected by exposure for 60 min to the 0.2 M glycine, pH 2.5, column eluent.

Monoclonal Antibody Isotype and Affinity. Modified EIA using heavy- and light-chain-specific antibodies showed both IH9 and IIC7 antibodies to be class IgG_1 with κ light chains. Equilibrium dialysis using 1 μM of purified antibody indicated affinity values of (1.9–2.0) $\times$ 10^5 L/mol for both IH9 and IIC7.

Specificity of Antibodies Raised against 1-KLH. The binding of purified IH9 and IIC7 antibodies to BSA–MH conjugates and a BSA control was tested by EIA to verify their specificity for immobilized MH. Both antibodies exhibited low binding to BSA and recognized 1–BSA and 2–BSA conjugates (IH9; Figure 1). Specificity of purified IH9 and IIC7 antibodies was tested by homologous CIEIA using MH, MH derivatives, and several related compounds. Molar values giving 50% inhibition (IC_{50}'s) and percent cross-reactivity values were calculated (Table II). Both antibodies recognized MH and its derivatives in the same relative order of 1, MH, 2, 4, and 3 (low to high IC_{50}), in agreement with the order of binding to MH–protein conjugates already described. Neither antibody recognized any purine or pyrimidine (inhibition of 50% was not observed for any of these compounds). Inhibition greater than 50% was observed for 3, barbituric acid, daminozide, and nicotine only at the highest inhibitor concentration of 15 mM (approaching the limit of MH solubility in PBST). These results were not corrected for pH effects, which may be significant at these inhibitor concentrations, causing elevated estimates of true cross-reactivity. Since the only significant cross-reactivity observed for IH9 and IIC7 antibodies is for synthetic derivatives of MH, these antibodies are potentially useful for detecting free MH in the presence of other organic compounds. These specificity results closely parallel results obtained for the other four anti-1–KLH hybridomas of Table I and sera from nine mice immunized with 1–KLH (data not shown).

Specificity of Antibodies Raised against 2-KLH and 4-KLH. Antibodies raised against 2–KLH and 4–KLH were tested by EIA for binding to BSA–MH conjugates and a BSA control as for Figure 1. Hybridomas (Table I) and 14 sera (data not shown) from mice immunized with 2–KLH bound poorly to all antigens tested except 2–BSA. These antibodies were inhibited by 2 in CIEIA (IC_{50} values below 1.5 mM), but none exhibited significant inhibition by MH, 1, 3, or 4 at the same concentration. These anti-2 hybridomas and sera were also inhibited in CIEIA by uracil and thymine, but not cytosine (data not shown). Hybridomas (Table I) and five sera from mice immunized with 4–KLH bound poorly to all antigens tested except glucose–BSA and 4–BSA (Hung et al., 1980). Nearly all of the EIA activity of these antibodies was blocked by glucose–BSA, indicating strong spacer recognition. Inhibition in CIEIA was not achieved by MH or any related compound.

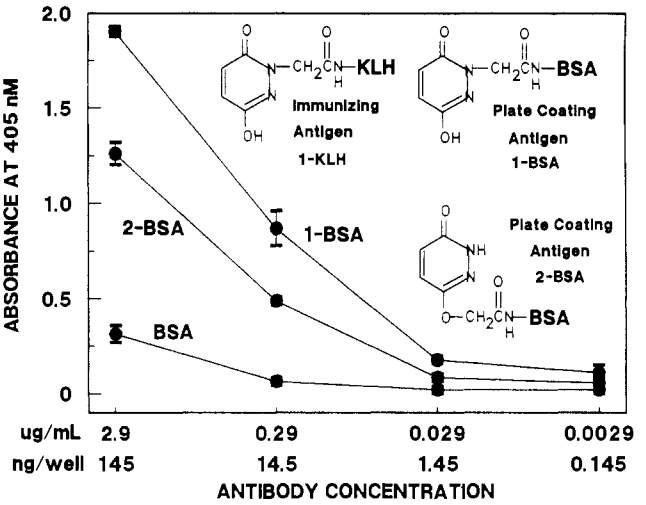


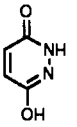
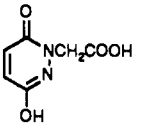
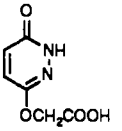
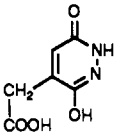
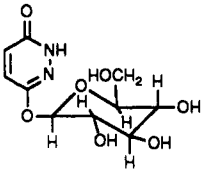
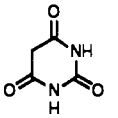
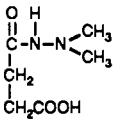
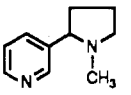
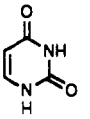
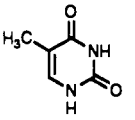
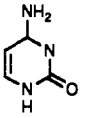
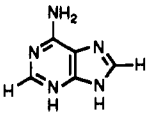
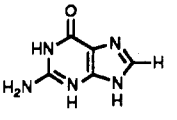
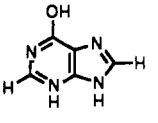
Figure 1. EIA titration of purified IH9 antibody (raised against 1–KLH) on three plate-coating antigens, 1–BSA, 2–BSA, and BSA. Concentration of all coating antigens was 1 μg/mL (100 ng/well).

Basis of Specificity Differences between Haptens 1 and 2. Results of specificity tests of sera and hybridomas against 1–KLH and 2–KLH using heterologous haptens and BSA conjugates demonstrate a striking nonreciprocal cross-reactivity. Anti-1–KLH antibodies recognize MH and homologous and heterologous haptens and conjugates (Figure 1; Table II), while anti-2–KLH antibodies recognize only the homologous hapten and conjugate. It is logical to ask why MH-specific antibodies have been produced against only one of these conjugates when heterologous EIA results of the anti-1–KLH antibodies (Figure 1) indicate that 2–protein conjugates retain the MH structure.

Titration data indicate that both 1 and 2 have a titratable proton due to the acetic acid substitution, but only 1 also exhibits the titratable phenolic proton of MH. This suggests differences in hydrogen-bonding possibilities between the two haptens due to the change in charge and may be the cause of differences in specificity of the resulting antibodies. This observation may also explain the cross-reactivity of all tested anti-2 antibodies with uracil and thymine (data not shown), while all anti-1 antibodies were MH specific. The locking of the unsubstituted oxygen of 2 into a carbonyl structure causes the distal end of the conjugated MH ring to retain a uracil-like structure (CH=CHC=ONH), which could account for the observed cross-reactivity. Dependence on the phenolic proton of MH and 1 for binding of anti-1 antibodies would account for their observed lack of cross-reactivity with uracil and thymine. The MH structure is retained in conjugates of 2, but the specific antibodies produced in response to these conjugates bind poorly to MH. The absence in 2 of the phenolic proton and the MH enol structure may be the critical factor in preventing MH-specific antibody production.

These observations emphasize the importance of knowledge of the physical chemistry of a system in understanding antibody–hapten interactions. Because the complexity of such interactions should decrease with

Table II. Molar IC_{50} Values and Cross-Reactivity Values of 14 Inhibitors for Purified IH9 and IIC7 Antibodies As Determined by Homologous CIEIA

compd		IH9 molar IC_{50} (SD)	IH9 % CR[a]	IIC7 molar IC_{50} (SD)	IIC7 % CR[a]
maleic hydrazide		$2.2\ (0.6) \times 10^{-5}$	100	$2.4\ (0.7) \times 10^{-4}$	100
1		$1.3\ (1.0) \times 10^{-5}$	165	$6.5\ (2.4) \times 10^{-5}$	370
2		$1.1\ (0.8) \times 10^{-4}$	19	$2.1\ (0.2) \times 10^{-3}$	12
3		$3.1\ (0.3) \times 10^{-3}$	0.7	$1.2\ (0.1) \times 10^{-2}$	2
4		$8.2\ (0.6) \times 10^{-4}$	3	$3.8\ (0.3) \times 10^{-2}$	6
barbituric acid		$4.5\ (0.4) \times 10^{-3}$	0.5	$1.2\ (0.2) \times 10^{-2}$	2
daminozide		$4.4\ (0.6) \times 10^{-3}$	0.5	$1.2\ (0.2) \times 10^{-2}$	2
nicotine		$8.8\ (1.9) \times 10^{-3}$	0.3	$4.6\ (0.2) \times 10^{-2}$	0.5
uracil		$>1.5 \times 10^{-2}$	<0.14	$>1.5 \times 10^{-2}$	<1.6
thymine		$>1.5 \times 10^{-2}$	<0.14	$>1.5 \times 10^{-2}$	<1.6
cytosine		$>1.5 \times 10^{-2}$	<0.14	$>1.5 \times 10^{-2}$	<1.6
adenine		$>1.5 \times 10^{-2}$	<0.14	$>1.5 \times 10^{-2}$	<1.6
guanine		$>1.5 \times 10^{-2}$	<0.14	$>1.5 \times 10^{-2}$	<1.6
hypoxanthine		$>1.5 \times 10^{-2}$	<0.14	$>1.5 \times 10^{-2}$	<1.6

[a] % CR = ((MH IC_{50}/compound IC_{50}) × 100).

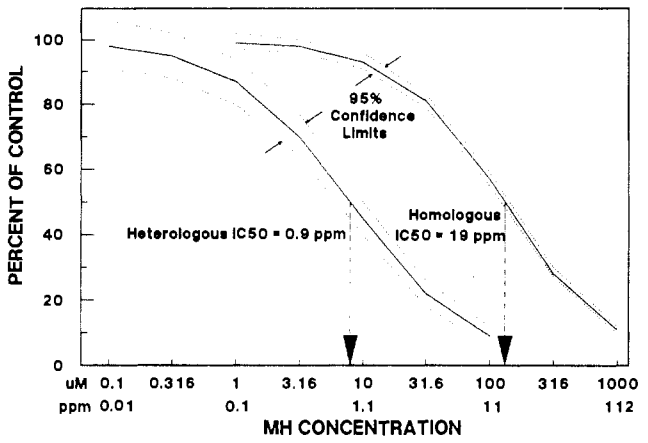


Figure 2. Standard curves of MH in PBST for IIC7 antibody. CIEIA was performed with 0.15 μg/mL IIC7 and 1 μg/mL coating antigens. Limit of detection (LOD) is the MH concentration for the point on the response curve corresponding to the y value of the upper asymptote of the lower confidence limit. Confidence limits are 95%.

hapten size and because MH (112 Da) is among the smallest haptens for which specific antibodies have been described, further analysis of the MH–antibody system (and 2–antibody system) could increase fundamental understanding of the minimum requirements for hapten-specific antibody production.

Assay Optimization. Optimization of homologous and heterologous CIEIA systems was performed according to Hunter and Bosworth (1986) to compare and improve sensitivities. Concentrations of plate-coating antigen, anti-MH antibody, rabbit anti-mouse antibody, and goat anti-rabbit–alkaline phosphatase conjugate were varied in a series of experiments to locate the optimum concentration of each reagent. The criterion for selection of conditions was maximum slope of the inhibition curve at the lowest MH concentrations, assuming an adequate uninhibited control absorbance (over 0.5). Where only minor differences separated curves, conditions were chosen that minimized reagent use. The differences between optimum conditions for homologous and heterologous systems were small. Subsequent experiments used the same plate-coating antigen concentration of 1 μg/mL for both antigens and anti-MH antibody concentrations of 0.75 μg/mL for IH9 and 0.25 μg/mL for IIC7.

Other important improvements in assay performance were made by applying the procedures described in Harrison and Hammock (1988) for plate-reader testing and by EIA testing of plate variability before final plate selection. All subsequent assays were performed on Nunc Immunoplate II plates, with periodic quality control checks on plates and readers. The final optimum concentrations determined for all reagents represented only minor adjustments from the conditions originally chosen but produced an appreciable improvement in sensitivity. The above optimum concentration values require 100 ng of plate-coating antigen and 45 ng of IH9 or 15 ng of IIC7 antibody/well of CIEIA, based on 100 μL/well plate-coating volume and 60 μL/well competitive inhibition volume. These numbers demonstrate the tremendous potential of CIEIA for performing many analyses with small amounts of reagents.

MH Standard Curves. Typical standard curves of MH in PBST using IIC7 antibody are presented in Figure 2. Repeatability of these and IH9 curves is summarized in Table III. Heterologous standard curves are shifted significantly to the left from the homologous curves for both IH9 and IIC7 antibodies, as would be expected from previous similar comparisons (Van Weemen and Schuurs, 1975; Wie and Hammock, 1984; Hinds et al., 1985). Estimates of the limit of detection (LOD) are calculated as the x value (MH concentration) corresponding to the value of the four-parameter logistic function at the estimated upper asymptote of the lower confidence limit (Figure 2). A response below this value must represent a greater than zero concentration value, within the 95% confidence limits of the curve (Rodbard, 1981). The LOD values in homologous and heterologous CIEIA by this estimation method are shown in Table III.

Table III. Repeatability of MH Standard Curves of IH9 and IIC7 (Assays Performed under Identical Conditions on Consecutive Days)

antibody	antigen	n	IC_{50} ± SD, ppm	LOD ± SD,[a] ppm
IH9	1-BSA	6	4.3 ± 0.9	0.30 ± 0.13
	2-BSA	2	0.64 ± 0.07	0.94 ± 0.91
IIC7	1-BSA	6	22.0 ± 4.4	1.3 ± 0.8
	2-BSA	3	0.84 ± 0.10	0.11 ± 0.04

[a] LOD = limit of detection, dose corresponding to absorbance value for estimated upper asymptote of lower confidence limit.

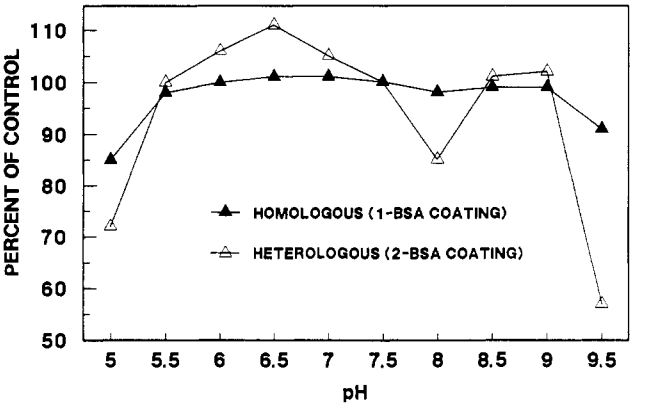


Figure 3. Effect of pH on EIA of IH9 antibody. Plate-coating antigens, 1 μg/mL; IH9 antibody concentration, 0.75 μg/mL. Data points are means of four replicates. Standard deviations averaged 3% (maximum 8%). Control condition is pH 7.5.

Nonspecific Factors Affecting Antibody–Antigen Binding. The binding of IH9 or IIC7 antibody to 1–BSA or 2–BSA in EIA provides a signal that can be modulated by free MH in CIEIA. Practical use of this system for MH measurement would likely require antibody–antigen binding under conditions different from those described up to now. To assess the potential for application of the CIEIA system to measurement of MH in crop samples, the effects of several nonspecific factors on EIA response were studied. The optimum pH values of both IH9 (Figure 3) and IIC7 (data not shown) antibodies for both homologous and heterologous binding were broad, decreasing more sharply at the extremes for heterologous binding. In contrast, variations in ionic strength affected both antibodies significantly (Figure 4, only IIC7 shown), with optima for homologous binding of both antibodies near 1 × PBS (29 mM NaCl, 3 mM sodium/potassium phosphate), and at or below 0.2 × PBS optima for heterologous binding. The effect of Mg^{2+} on homologous binding of both antibodies was negligible at levels below 3 mM for IH9 and 10 mM for IIC7 (data not shown) while 3 mM Ca^{2+} affected both antibodies significantly but was partially blocked by EDTA (Figure 5, only IH9 shown). However, the use of EDTA to block Ca^{2+} effects could be complicated by the effect of ETDA alone on the antibody (Figure 5).

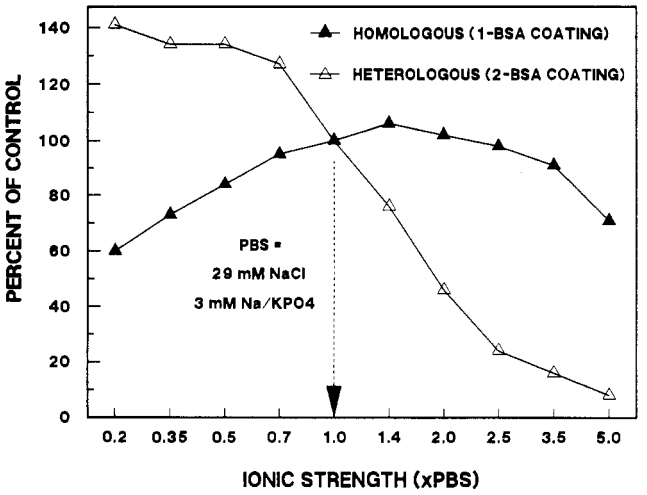


Figure 4. Effect of ionic strength on EIA of IIC7 antibody. Plate-coating antigens, 1 μg/mL; IIC7 antibody concentration, 0.25 μg/mL. Data points are means of four replicates. Standard deviations averaged 4% (maximum 9%). Tween 20 and NaN_3 concentrations were held constant at 0.05%. Control condition is 1.00 × PBS.

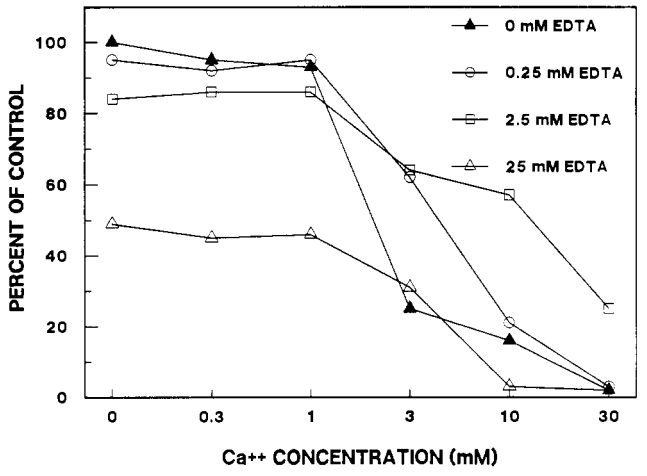


Figure 5. Effect of Ca^{2+} on EIA of IH9 antibody. Plate-coating antigens, 1 μg/mL; IH9 antibody concentration, 0.75 μg/mL. Data points are means of four replicates. Standard deviations averaged 4% (maximum 8%). Control condition is 0 mM EDTA, 0 mM Ca^{2+}.

The effect of methanol on both antibodies was also examined (Figure 6, only IH9 shown) since MH is methanol soluble and methanol extraction of plant matter has previously been useful in MH residue analysis (Newsome, 1980b). Homologous binding of IH9 was unaffected by 17.5% methanol, while IIC7 response was flat up to only 10%. Heterologous binding of both antibodies was augmented by methanol, with peaks at 25% for IH9 and 10–17.5% for IIC7.

The decreased affinity of heterologous antigen–antibody interaction has previously been exploited to improve the sensitivity of competitive immunoassay systems (Van Weemen and Schuurs, 1975; Wie and Hammock, 1984; Hinds et al., 1985). While such refinements may be valuable in analysis of simple matrices such as water or well-defined complex matrices such as serum, their value in analysis of complex poorly defined matrices has not been demonstrated. The data presented here suggest that heterologous assays for MH may be significantly less tolerant of normal variability in complex sample matrices than their homologous counterparts.

Monoclonal Antibody Identity. Relatedness of IH9 and IIC7 cannot be ruled out on the basis of history be-

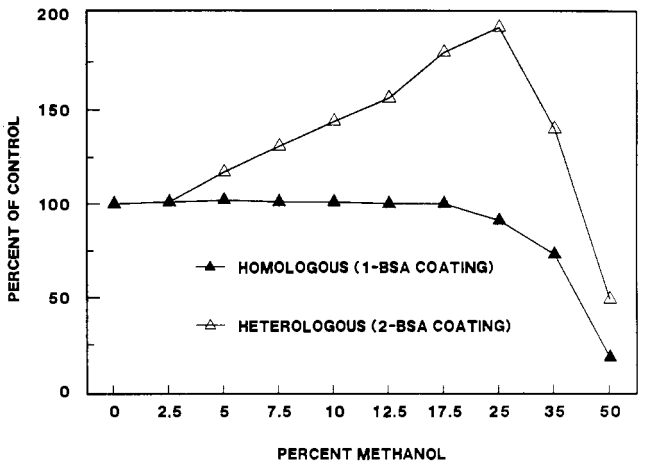


Figure 6. Effect of methanol on EIA of IH9 antibody. Plate-coating antigens, 1 μg/mL; IH9 antibody concentration, 0.75 μg/mL. Data points are means of four replicates. Standard deviations averaged 4% (maximum 7%) for the homologous curve and 9% (maximum 17%) for the heterologous curve. Control condition is 0% methanol.

cause both are derived from the same spleen. Results of isotype and affinity determinations, both potentially definitive in establishing identity, were the same for both antibodies. However, further analysis of purified antibodies by EIA and CIEIA suggests independent lineage, and potentially different usefulness for MH residue analysis. Significant differences were observed in several performance parameters of purified antibodies, including homologous IC_{50} (Table III: 4.3 and 22.0 ppm for IH9 and IIC7, respectively), homologous/heterologous IC_{50} ratios (Table III: 7× and 25×), effects of ionic strength (peaks at 0.5 × PBS and 1.4 × PBS), and respective methanol tolerances of 17.5% and 10%.

The similarity of IH9, IIC7, and all other anti-1–KLH antibodies (monoclonal and polyclonal) leads logically to the question of how much independent hapten-specific monoclonal antibodies could be expected to vary. The binding between hapten and antibody is the sum of van der Waals forces, hydrogen bonding, and other intermolecular interactions of fixed energies. For large haptens such as steroids these interactions should add up to a significantly greater total binding energy than for small haptens, such as MH. Also, the number of possible combinations of individual interactions should be significantly greater for large haptens than for small haptens. Thus, one might reasonably expect lower affinities and less variation in binding characteristics of the hapten-specific antibodies made against small haptens. To our knowledge, the correlation of hapten-specific antibody variability and affinity with hapten size has not been studied systematically. Such an analysis would have practical significance for workers attempting to generate specific antibodies against low molecular weight haptens. The lower limit of molecular size for the development of practical specific antibodies is presently poorly defined, and future work in this area would benefit from clarification of the minimum requirements for hapten-specific antibody production.

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Registry No. 1, 10158-72-2; 2, 34173-61-0; 3, 121073-74-3; 4, 6216-73-5; maleic hydrazide, 123-33-1; chloroacetic acid, 79-11-8;

cis-aconitic acid, 585-84-2; hydrazine, 302-01-2; acetobromo-α-D-glucose, 572-09-8; barbituric acid, 67-52-7; daminozide, 1596-84-5; nicotine, 494-97-3; uracil, 66-22-8; thymine, 65-71-4; cytosine, 71-30-7; adenine, 73-24-5; guanine, 73-40-5; hypoxanthine, 68-94-0.

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