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Radioimmunoassay for Psychotropic Drugs I: Synthesis and Properties of Haptens for Chlorpromazine

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Abstract □ For the development of radioimmunoassay procedures for chlorpromazine and its active metabolites, three chlorpromazine haptens, 7-(or 8-)(3-carboxypropionyl)chlorpromazine, *N*-(3-carboxypropionyl)desmethylchlorpromazine, and *N*-(2-carboxyethyl)desmethylchlorpromazine, were synthesized and characterized by GLC–mass spectrometry, PMR spectrometry, and IR spectrophotometry. Each hapten was coupled to bovine serum albumin, and the number of hapten residues per mole of bovine serum albumin was calculated by UV spectrophotometric methods. Antibodies to each hapten–protein conjugate were obtained in rabbits, and titers of the antiserums were checked by evaluating their binding characteristics to tritiated chlorpromazine.

Keyphrases □ Chlorpromazine—three haptens synthesized and characterized, antibodies developed in rabbits, antiserum titers determined □ Haptens, chlorpromazine—three synthesized and characterized, antibodies developed in rabbits, antiserum titers determined □ Antibodies—to three chlorpromazine haptens developed in rabbits □ Tranquilizers—chlorpromazine, three haptens synthesized and characterized, antibodies developed in rabbits, antiserum titers determined

Chlorpromazine is widely used in the treatment of certain psychiatric disorders. It is extensively metabolized in humans, and several of its metabolites are pharmacologically active. For thorough study of the metabolism, pharmacokinetics, and clinical monitoring of this drug, sensitive specific procedures are required for the determination of chlorpromazine levels in biological fluids.

BACKGROUND

Various current techniques with adequate sensitivity to determine plasma chlorpromazine concentrations are GLC with electron-capture detection (1), labeled derivative formation (2), fluorescent labeling with dansyl chloride (3), TLC of a quaternary ammonium derivative (with 9-bromomethylacridine) followed by UV photolysis and subsequent spectrofluorometric determination (4), and GLC with mass spectrometric detection (5). But these methods are tedious, require extraction of the drug from biological fluids and/or its derivatization, and are not easily amenable for routine clinical monitoring.

Radioimmunoassay also may be applicable for determining plasma chlorpromazine concentrations. This technique is simple, requires no extraction, and is readily applied to routine analysis. A reported radioimmunoassay procedure for chlorpromazine (6), in which the protein (bovine serum albumin) was presumably coupled through the side chain of the drug, is not sufficiently sensitive (*cf.*, 7) and does not differentiate between chlorpromazine and its didemethylated metabolite at any level or among chlorpromazine, 7-hydroxychlorpromazine, and chlorpromazine sulfoxide at the 20-ng level.

Another published procedure (7), in which the bovine serum albumin was coupled to the ring system of chlorpromazine through a carboxyphenylazo bridge, was claimed to distinguish to some extent between chlorpromazine and several of its ring-modified and side-chain-modified metabolites, but it has not been widely evaluated or applied.

The present approach toward a sensitive, specific radioimmunoassay for chlorpromazine and other tricyclic antidepressants was to synthesize three chlorpromazine–protein conjugates in which the drug was coupled through a modified side chain as well as through a ring-modified chlorpromazine hapten. These conjugates will subsequently be evaluated to determine which had the specificity and sensitivity to measure the concentration of unchanged chlorpromazine and the total concentration of chlorpromazine and its active metabolites such as monodesmethyl- and didesmethylchlorpromazine.

This paper describes the synthesis and characterization of three new drug–protein conjugates, the production of antibodies to these conjugates in rabbits, and the titers of the antiserums.

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EXPERIMENTAL

GLC—The gas chromatograph[1] was equipped with a flame-ionization detector. The columns[2] were of coiled glass tubing, 1.83 m (6 ft) long × 2 mm i.d., packed with 5% OV-7[2] (Column 1) or 2% OV-25[2] (Column 2) on acid-washed dimethylchlorosilane-treated high performance 100–120-mesh Chromosorb W. The columns were conditioned by maintaining the oven at 310° for 18 hr with a low nitrogen flow.

Operating temperatures were: injection port, 325°; detector, 325°; and ovens, 300° (Column 1) and 270° (Column 2). The nitrogen flow rates for Columns 1 and 2 were 60 and 65 ml/min, respectively. Hydrogen and compressed air flow rates were adjusted to give maximum response.

GLC–Mass Spectrometry—GLC–mass spectrometry was carried out on a mass spectrometer[3] coupled to a gas chromatograph[4] through a two-stage jet separator. The ionization potential was 70 ev. The GLC column and conditions were the same as for direct GLC. For accurate mass measurement[5], the sample of reacted product was collected from the detector at its appropriate retention time after the flame was extinguished.

PMR—All PMR spectra were recorded on a spectrometer[6] operating at 60 mHz at ambient temperature with deuterated chloroform as the solvent and tetramethylsilane as the internal standard.

IR—All IR spectra were recorded on a spectrophotometer[7] at ambient temperature.

Synthesis of 7-(or 8-)(3-Methoxycarbonylpropionyl)chlorpromazine (III)—A solution of 3-methoxycarbonylpropionyl chloride[8] (2.03 g, 13.5 mmoles) in methylene dichloride[9] (30 ml) was added to a solution of anhydrous aluminum chloride[10] (3.60 g, 27 mmoles) dissolved in methylene dichloride (100 ml). The mixture was stirred at room temperature for 30 min, after which a solution of 3.19 g (10 mmoles) of chlorpromazine[11] free base (I) in methylene dichloride (30 ml) was added all at once. The dark-red reaction mixture was stirred at room temperature in the dark for 72 hr, and then the stirring was stopped to allow the solids to settle. The solvent was decanted and discarded since it was shown by GLC to contain unreacted I (1.25 g, 3.98 mmoles after evaporation).

Ice water (50 ml) was cautiously added to the solid residue, followed by 100 ml of 1.0 *N* NaOH. The greenish-yellow aqueous suspension was then extracted by ether[12] (6 × 100 ml). The combined ethereal extracts were washed with water (100 ml) and saturated brine (2 × 100 ml) and dried over anhydrous magnesium sulfate[8]. Evaporation[13] under reduced pressure afforded III as a yellow oil (2.6 g, 60%).

GLC showed one major peak (90% by area) with a retention time (T_R) of 5.2 min (Column 1); IR (chloroform): ν 1738 (ester C=O) and 1680 (aromatic keto C=O) cm^{-1}; PMR [after purification by preparative high-performance liquid chromatography (HPLC)[14]] (deuterated chloroform): δ 1.96 (m, 2H), 2.28 (s, 6H), 2.57 (m, 4H), 3.17 (m, 2H), 3.70 (s, 3H), 3.97 (m, 2H), 6.93 (m, 4H), and 7.80 (m, 2H) ppm (Fig. 1B). GLC–mass spectrometry gave a molecular ion at *m/e* 432/434 and other diagnostic ions (Fig. 2).

Synthesis of 7-(or 8-)(3-Carboxypropionyl)chlorpromazine (IV)—Sodium hydroxide (5 ml, 1.0 *N*) was added to a rapidly stirred solution of III (1.0 g, 2.3 mmoles) in 100 ml of water containing 2.3 ml of 1.0 *N* HCl. A fine oily precipitate appeared and was kept in suspension by continual rapid stirring as the mixture was heated to a gentle reflux. After 3 hr, the precipitate had all dissolved, and the solution was allowed to cool to room temperature. It was washed (at pH 11.5) with ether (5 × 100 ml) to remove any unreacted I or III. The pH was then adjusted to 6.2 with 1.0 *N* HCl.

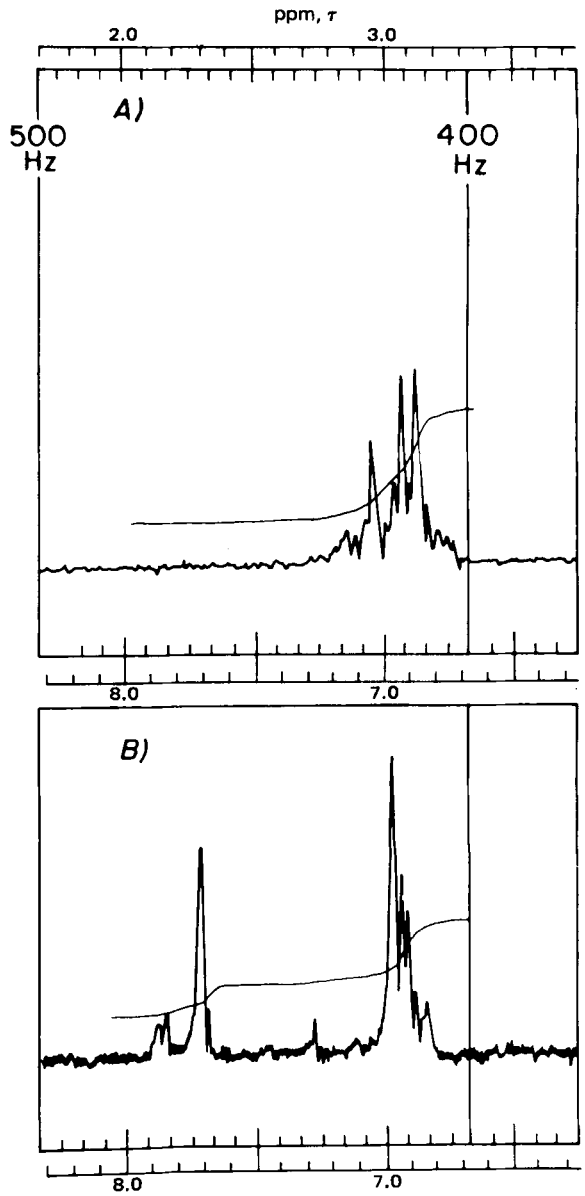


Figure 1—*PMR spectra of aromatic protons of I (A) and III (B) in deuterated chloroform*

The product was recovered by continuous extraction[15] (56 hr) with ether (500 ml) to yield IV as a yellow oily solid (0.9 g); IR (chloroform): ν 1710–1720 (carboxylic acid C=O) and 1680 (keto C=O) cm^{-1}. GLC analysis of this material showed no peak corresponding to III. However, on treatment with diazomethane[16] and subsequent GLC (Column 1), a single peak corresponding to III (T_R 5.2 min) was obtained. GLC–mass spectrometry of the diazomethane-treated material was identical to that of III (Fig. 2), as were the IR and PMR spectra. Accurate mass determi-

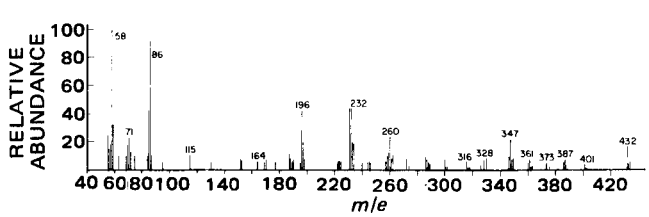
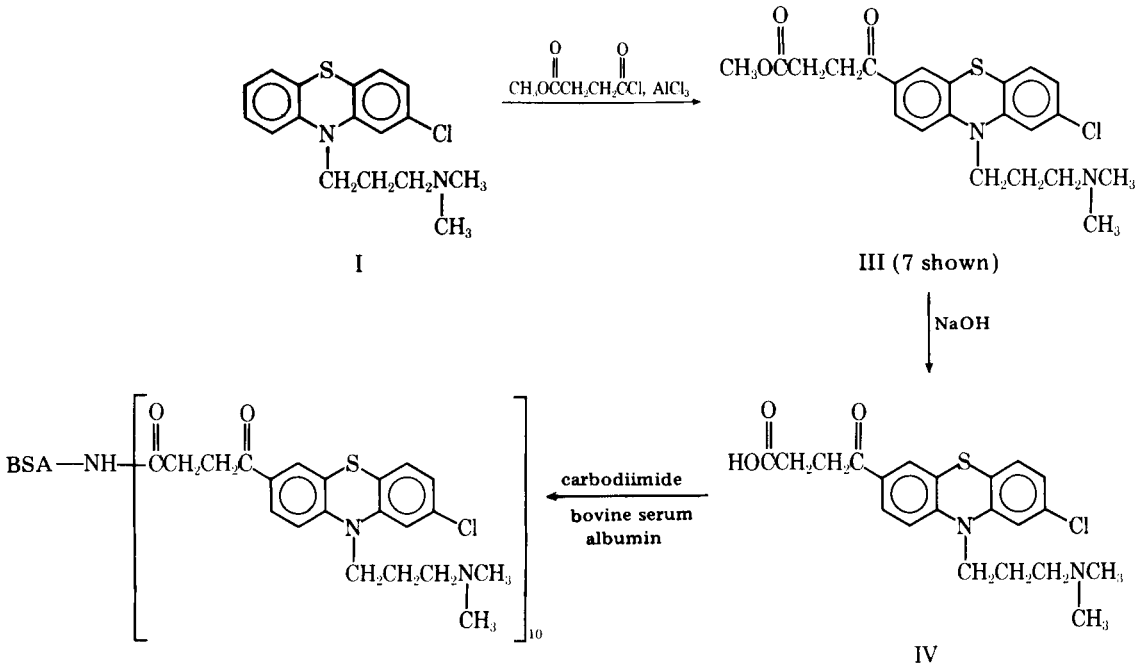


Figure 2—*GLC–mass spectrum (normalized) of III.*

[1] Model 3920, Perkin-Elmer, Montreal, Quebec, Canada.
[2] Chromatographic Specialties, Brockville, Ontario, Canada.
[3] Hitachi Perkin-Elmer, model RMU 6L.
[4] Model 990, Perkin-Elmer, Montreal, Quebec, Canada.
[5] Varian MAT 311, Varian of Canada, Georgetown, Ontario, Canada.
[6] Varian A-60A, Varian of Canada, Georgetown, Ontario, Canada.
[7] Unicam SP-1000, Canlab, Montreal, Quebec, Canada.
[8] Aldrich Chemical Co., Milwaukee, Wis.
[9] Caledon Laboratories, Georgetown, Ontario, Canada.
[10] Baker Analyzed Reagent, J. T. Baker Chemical Co., Phillipsburg, N.J.
[11] Poulenc Laboratories, Montreal, Quebec, Canada.
[12] Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[13] Flash-Evaporator, Buchler Instruments, Fort Lee, N.J.
[14] Carried out on a Lichrosorb Si 60 column (230 × 4.6 mm i.d.) with a mobile phase of 15% 2-propanol–15% methylene dichloride–15% methanol–0.1% triethylamine in *n*-hexane. Under these conditions, κ' values for chlorpromazine and the desired product were 2.7 and 8.7, respectively. A. G. Butterfield, Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario, Canada, personal communication.
[15] Kontes Glass Co., Vineland, N.J.
[16] Prepared from Diazald, Aldrich Chemical Co., Milwaukee, Wis.



Scheme I

nation for $C_{22}H_{25}ClN_2O_3S$: calculated for $C_{22}H_{25}{}^{35}ClN_2O_3S$, 432.1274; found, 432.1270; calculated for $C_{22}H_{25}{}^{37}ClN_2O_3S$, 434.1245; found, 434.1259.

Coupling of IV to Bovine Serum Albumin—Compound IV was coupled to bovine serum albumin[17] by the carbodiimide method (8–12). A solution of bovine serum albumin (0.07 g, 0.001 mmole) in 5 ml of distilled water was added to a solution of IV (0.042 g, 0.1 mmole) in 10 ml of water. A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride[17] (0.057 g, 0.3 mmole) in 1.0 ml of water was added, and the solution was adjusted to pH 4.5 by the dropwise addition of 0.01 *N* HCl.

The mixture was then allowed to stand in the dark at room temperature for 24 hr before dialysis[18] against running distilled water (pH 5.5) for 24 hr. After lyophilization, the conjugate was obtained as a yellow crystalline solid (0.06 g). A blank was prepared in the same manner but without IV. The number of hapten residues per mole of conjugate was calculated by the UV method (13, 14) to be 10.

Synthesis of *N*-(3-Carboxypropionyl)desmethylchlorpromazine (V)—Desmethylchlorpromazine[19] free base (II) (0.342 g, 1.12 mmoles) and succinic anhydride[20] (0.112 g, 1.12 mmoles) were dissolved in ethanol (50 ml) and stirred at room temperature in the dark for 48 hr. The ethanol was evaporated[13], and the oily residue was dissolved in 20 ml of 1.0 *N* NaOH. Then the solution was washed with ether (3 × 20 ml). On acidification with 1.0 *N* HCl, an oily precipitate separated at pH 7.5.

This precipitate was extracted with ether (2 × 20 ml). The combined ether extracts were dried over molecular sieves[21] and evaporated[13] to give V as a colorless oil (0.43 g); IR (chloroform): ν 1715–1730 (carboxylic acid C=O) and 1640 (amide C=O) cm^{-1}; PMR (deuterated chloroform): δ 2.02 (bm, 2H), 2.40 (m, 2H), 2.58 (m, 2H), 2.88 (m, 3H), 3.47 (bm, 2H), 3.88 (bm, 2H), 7.03 (bm, 7H), and 7.67 (bs, 1H) ppm. The single proton signal at δ 7.67 ppm exchanged with deuterium oxide.

Compound V was converted into its methyl ester with diazomethane. The ester gave only one peak (T_R 8.4 min) on GLC analysis (Column 1). GLC–mass spectrometry gave a molecular ion at 418/420 and other diagnostic ions (Fig. 3). Accurate mass determination for $C_{21}H_{23}ClN_2O_3S$: calculated for $C_{21}H_{23}{}^{35}ClN_2O_3S$, 418.1118; found 418.1101; calculated for $C_{21}H_{23}{}^{37}ClN_2O_3S$, 420.1088; found 420.1078. In the PMR spectrum of the methyl ester, the broad singlet at δ 7.67 ppm in the spectrum of V was replaced by a 3H singlet at δ 3.68 ppm; IR (chloroform): ν 1735 (ester C=O) and 1640 (amide C=O) cm^{-1}.

Coupling of V to Bovine Serum Albumin—A solution of V (0.04 g, 0.1 mmole) in 10 ml of 0.1 *N* NaOH was adjusted to pH 8.0 with 0.1 *N* HCl. The bovine serum albumin (0.07 g, 0.001 mmole) in 5 ml of water was added, followed by a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in 1.0 ml of water. The solution (final pH 7.0) was mixed by swirling and allowed to stand at room temperature in the dark for 24 hr. It was then dialyzed against bicarbonate buffer containing 0.2% sodium azide[22] (0.042 *M*, pH 8, 6 × 500 ml) and then against running distilled water (pH 6.2) for 12 hr. After lyophilization, the conjugate was obtained as a white crystalline solid (0.063 g).

A blank was prepared in the same manner but without V. The number of hapten residues per mole of bovine serum albumin was calculated by the UV method (13, 14) to be 50.

Synthesis of *N*-(2-Methoxycarbonylethyl)desmethylchlorpromazine (VI)—Methyl acrylate[22] (0.353 g, 4.1 mmoles) was added all at once to a solution of II (1.19 g, 3.9 mmoles) in 50 ml of methanol. The mixture was maintained at a gentle reflux for 48 hr, and the solvent was then evaporated[13] to yield a pale-yellow oil (1.35 g). GLC analysis of this material (Column 2) showed two peaks. The major peak (69% by area) had a retention time of 5.2 min. GLC–mass spectrometry gave a molecular ion at *m/e* 390/392 and other diagnostic ions (Fig. 4).

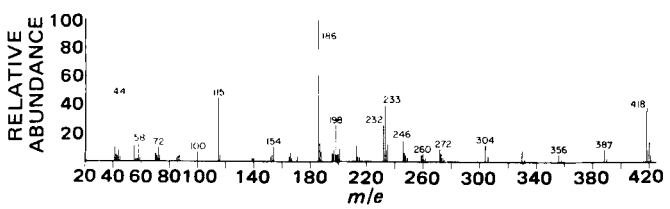


Figure 3—*GLC–mass spectrum (normalized) of methyl ester of V.*

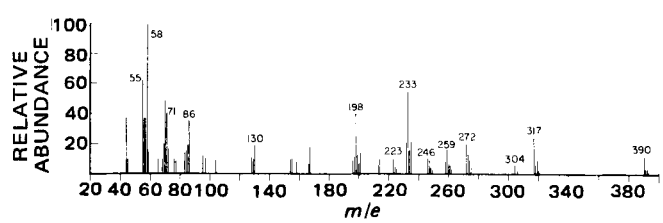
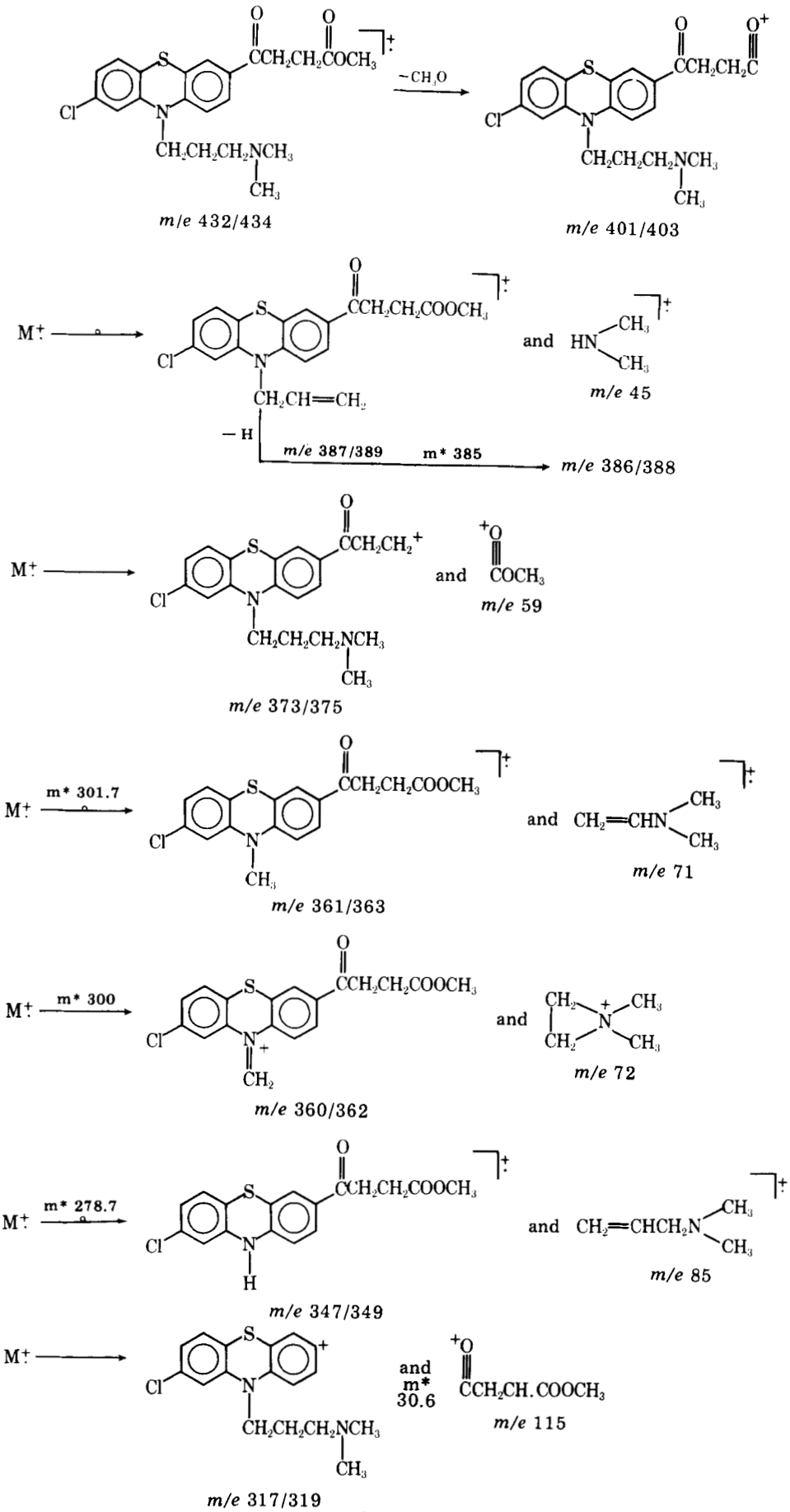
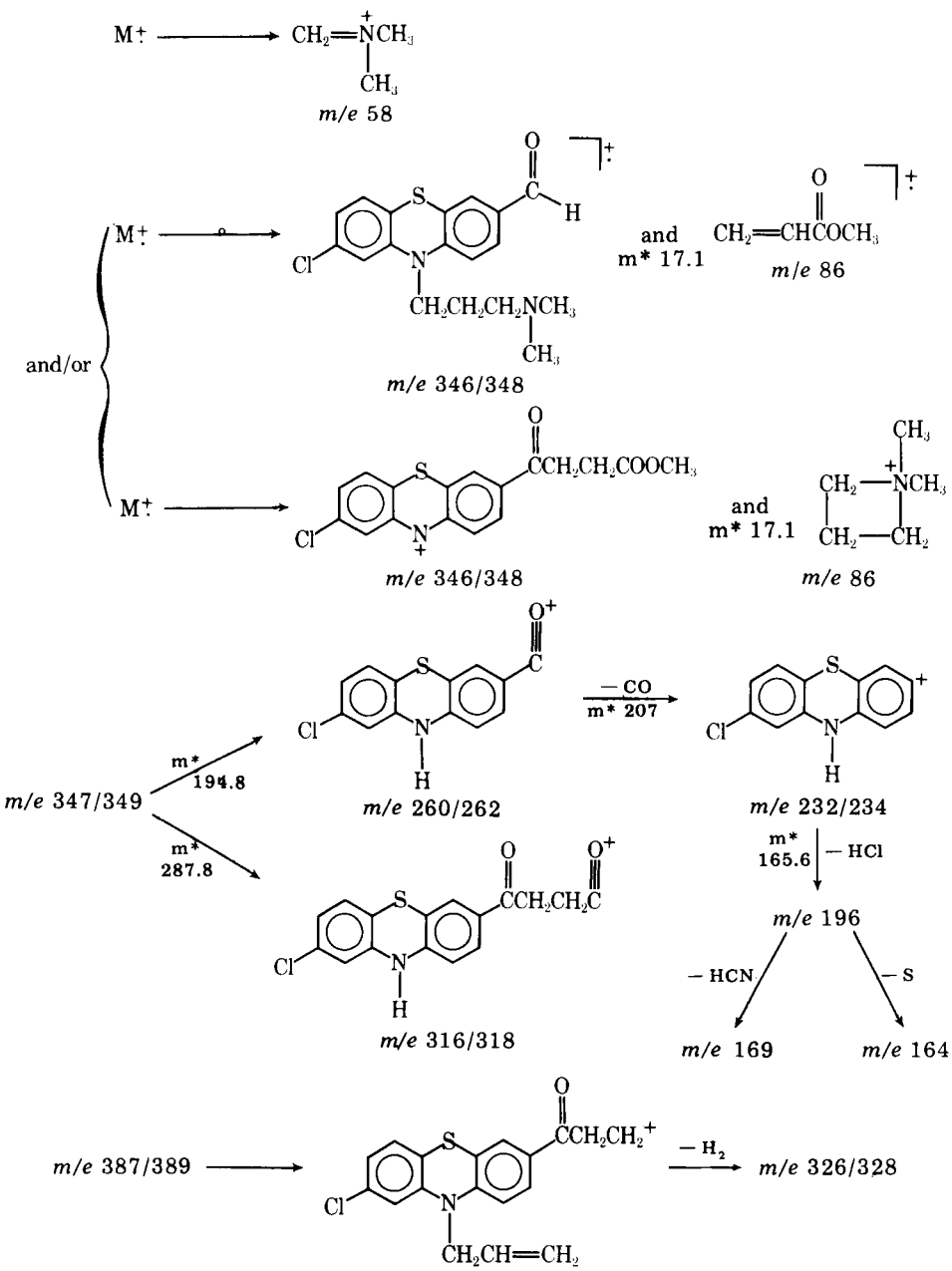


Figure 4—*GLC–mass spectrum (normalized) of VI.*

[17] Sigma Chemical Co., St. Louis, Mo.
[18] Fisher dialyzer tubing (Size C), Fisher Scientific Co., Pittsburg, Pa.
[19] Rhône-Poulenc, Centre Nicolas Grillet, France (supplied as the maleate salt).
[20] Eastman Kodak Co., Rochester, N.Y.
[21] Davison Chemical Co., Baltimore, Md.
[22] British Drug Houses, Poole, England.


m/e 432/434
−CH2O
m/e 401/403
M+
and
m/e 45
m/e 387/389
m* 385
m/e 386/388
M+
and
m/e 59
m/e 373/375
M+
m* 301.7
and
m/e 71
m/e 361/363
M+
m* 300
and
m/e 72
m/e 360/362
M+
m* 278.7
and
m/e 85
m/e 347/349
M+
and
m* 30.6
m/e 115
m/e 317/319

Scheme II



Scheme III

The second peak (31% by area) had the same retention time (1.8 min) as II. There was no change in the ratio of these peaks if the reaction was allowed to continue beyond 48 hr or if additional methyl acrylate was added. No attempt was made to remove the unreacted II until after the hydrolysis step.

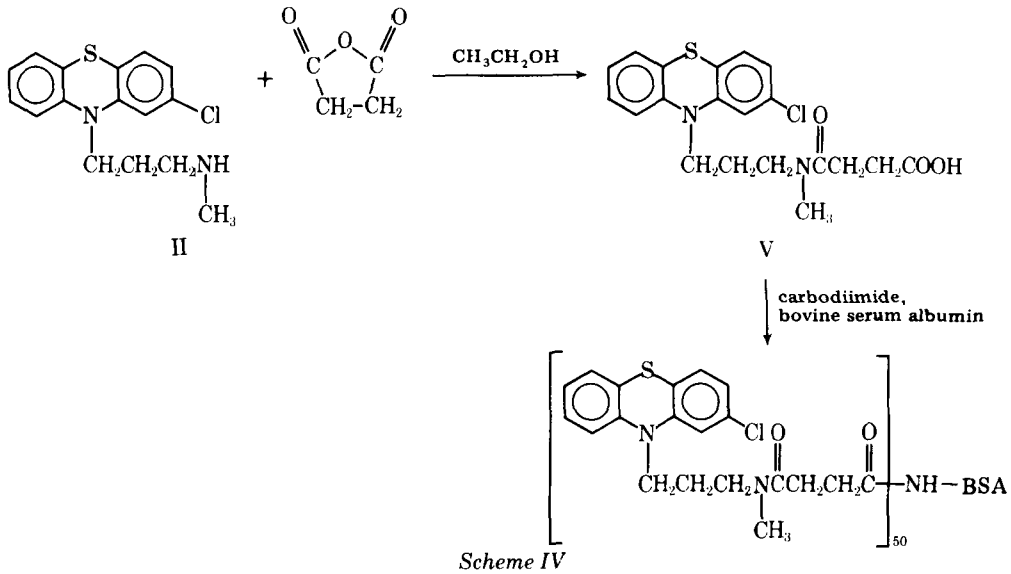
Synthesis of *N*-(2-Carboxyethyl)desmethylchlorpromazine (VII)—The hydrolysis of VI was carried out in a manner similar to that for IV. After the wash step at pH 11.5, the reaction mixture pH was adjusted to 7.5 with 1.0 *N* HCl, and the product separated as a colorless oil. The mixture was allowed to stand at 4° overnight, and the aqueous layer was then decanted and discarded. The oil was taken up in benzene (50 ml) and washed with distilled water (50 ml).

Evaporation of the benzene gave VII in quantitative yield; IR (chloroform): ν 1720 (carboxylic acid C=O) cm^{-1}; PMR (deuterated chloroform): δ 2.00 (m, 2H), 2.33 (m, 5H), 2.75 (m, 4H), 3.92 (m, 2H), 7.00 (m, 7H), and 7.83 (bs, 1H) ppm. The broad singlet at δ 7.83 ppm exchanged with deuterium oxide.

GLC analysis of this material showed no peak corresponding to VI or to unreacted II. However, after treatment with diazomethane[16] and subsequent GLC (Column 2) analysis, a single peak corresponding to VI was obtained (T_R 5.2 min). The GLC–mass spectrum and IR spectrum of this product were identical to those of VI. In the PMR spectrum (deuterated chloroform), the broad singlet at δ 7.83 ppm in the spectrum of VII was replaced by a 3H singlet at δ 3.65 ppm. Accurate mass determination for $C_{20}H_{23}ClN_2O_2S$: calculated for $C_{20}H_{23}{}^{35}ClN_2O_2S$, 390.1169; found 390.1173; calculated for $C_{20}H_{23}{}^{37}ClN_2O_2S$, 392.1139; found 392.1146.

Coupling of VII to Bovine Serum Albumin—Compound VII was coupled to bovine serum albumin by a modified mixed anhydride method (13, 14). Compound VII (0.049 g, 0.13 mmole) and isobutyl chloroformate[10] (0.017 g, 0.13 mmole) were dissolved in freshly distilled dioxane[22] (2.6 ml), and the solution was cooled to 15° and allowed to react for 20 min. The mixture was then added in 0.5-ml aliquots to a stirred, well-cooled (4°) solution of bovine serum albumin (0.182 g, 0.0026 mmole) in 50% dioxane–water (25 ml), which had been adjusted to pH 9.3 with 1.0 *N* NaOH. Throughout the addition, the pH was maintained at 9.0–9.8 with 1.0 *N* NaOH. After the addition was complete, the solution was clear and was allowed to react at 4° for 12 hr with continuous stirring.

The solution was dialyzed[18] against bicarbonate buffer (0.042 *M*, pH 8.0, 6 × 500 ml) and then against acetate buffer (0.012 *M*, pH 4.0, 6 × 500



II V

Scheme IV

ml); both buffer systems contained 0.2% sodium azide[22]. A fine colloidal precipitate appeared during the dialysis procedure and was removed by centrifugation[23] and discarded. After lyophilization, the conjugate was obtained as a white crystalline solid (0.171 g). A blank was prepared in the same manner but without VII. The number of hapten residues per mole of bovine serum albumin was calculated by the UV method (13, 14) to be 17.

Compound VII also was coupled to bovine serum albumin by the carbodiimide procedure as described for V. The number of hapten residues per mole of bovine serum albumin was calculated to be 5.

Immunization—Each hapten–protein conjugate (4 mg) was dissolved in normal saline (2.0 ml) and emulsified with complete Freund's adjuvant[24] (2.0 ml). Each conjugate was administered to four New Zealand White female rabbits[25] by injection of 0.5 ml of the emulsion into each flank. At 2-week intervals thereafter, the injections were repeated with an emulsion prepared as already described, except that complete Freund's adjuvant was replaced by incomplete. The antiserum titer of each rabbit was checked 1 week after the third injection.

Serum was obtained from the marginal ear vein at weekly intervals after the third injection. The antiserum titer of each rabbit was checked by evaluating the binding characteristic of tritiated chlorpromazine[26] to each antiserum. After optimal titer values were achieved, blood was obtained from each rabbit by cardiac puncture. The harvested serum was immediately lyophilized in 1.0-ml samples and stored at −70°.

RESULTS AND DISCUSSION

To couple the ring system of chlorpromazine to bovine serum albumin (BSA), a ring-modified hapten was prepared by the Friedel–Crafts acylation of chlorpromazine with 3-methoxycarbonylpropionyl chloride (Scheme I). GLC analysis of the hapten showed that it contained 90% III (by peak area, T_R 5.2 min). The GLC–mass spectrum of III gave a molecular ion at m/e 432/434 and other diagnostic ions (Fig. 2). Formation of these ions is rationalized in Schemes II and III. In most cases, the fragmentations are supported by the observation of appropriate metastables. Fragments associated with the basic chlorpromazine nucleus are consistent with literature spectra (15).

The minor product (10% by peak area) (T_R 4.2 min) gave essentially the same GLC–mass spectrum as III and was assumed to be a positional isomer of III. Various attempts to hasten the Friedel–Crafts reaction by raising the temperature, changing the molar proportions of the reactants, or changing the order of mixing led to a reduced yield of III and the production of disubstituted chlorpromazine derivatives. Thus, a mixture of III, its positional isomer, and two disubstituted products was obtained when the reaction mixture was heated to reflux under conditions similar to those described by Schmitt *et al.* (16), who prepared some 3-acetylphenothiazine derivatives by a Friedel–Crafts technique.

Attempts to prepare the carboxylic acid IV (Scheme I) directly, by treating I with succinic anhydride in the presence of aluminum chloride, were unsuccessful. Experimental conditions similar to those described for III, or similar to published procedures for the reaction of aromatic compounds with succinic anhydride (17–19), gave either unreacted I or an unidentified compound, the mass spectrum of which bore no resemblance to that of IV. The alkaline hydrolysis of the methyl ester III, however, gave IV in excellent yield.

In the PMR spectrum of III, the aromatic protons gave two distinct signals. The first of these was a 4H multiplet, centered at δ 6.93 (6.80–7.07) ppm (Fig. 1B), comparable to the chemical shift (δ 6.98 ppm) of the aromatic protons of chlorpromazine (deuterated chloroform), δ 6.73–7.23 ppm (m, 7H) (Fig. 1A). The remaining two aromatic protons of III appeared as a 2H multiplet centered at δ 7.8 (7.67–7.92) ppm (Fig. 1B), consistent with a deshielding effect produced by an adjacent keto substituent. These two aromatic protons must be located *ortho* to the keto group of III, which precludes the possibility of the side chain being attached to the 1-, 3-, 4-, 6-, or 9-position of chlorpromazine. The only positions in which the side chain can be situated with two *ortho* protons are, therefore, the 7- and 8-positions.

Kawashima *et al.* (7) suggested that the 3- and 7-positions would be optimum for electrophilic substitution of I because of the participation of the lone pair on the nitrogen. The present PMR evidence, together with the fact that chlorine generally deactivates an aromatic ring to electrophilic attack, suggests that the principal site for electrophilic attack on chlorpromazine is at the 7-position. Full details on the further elucidation of the structure of III will be published elsewhere.

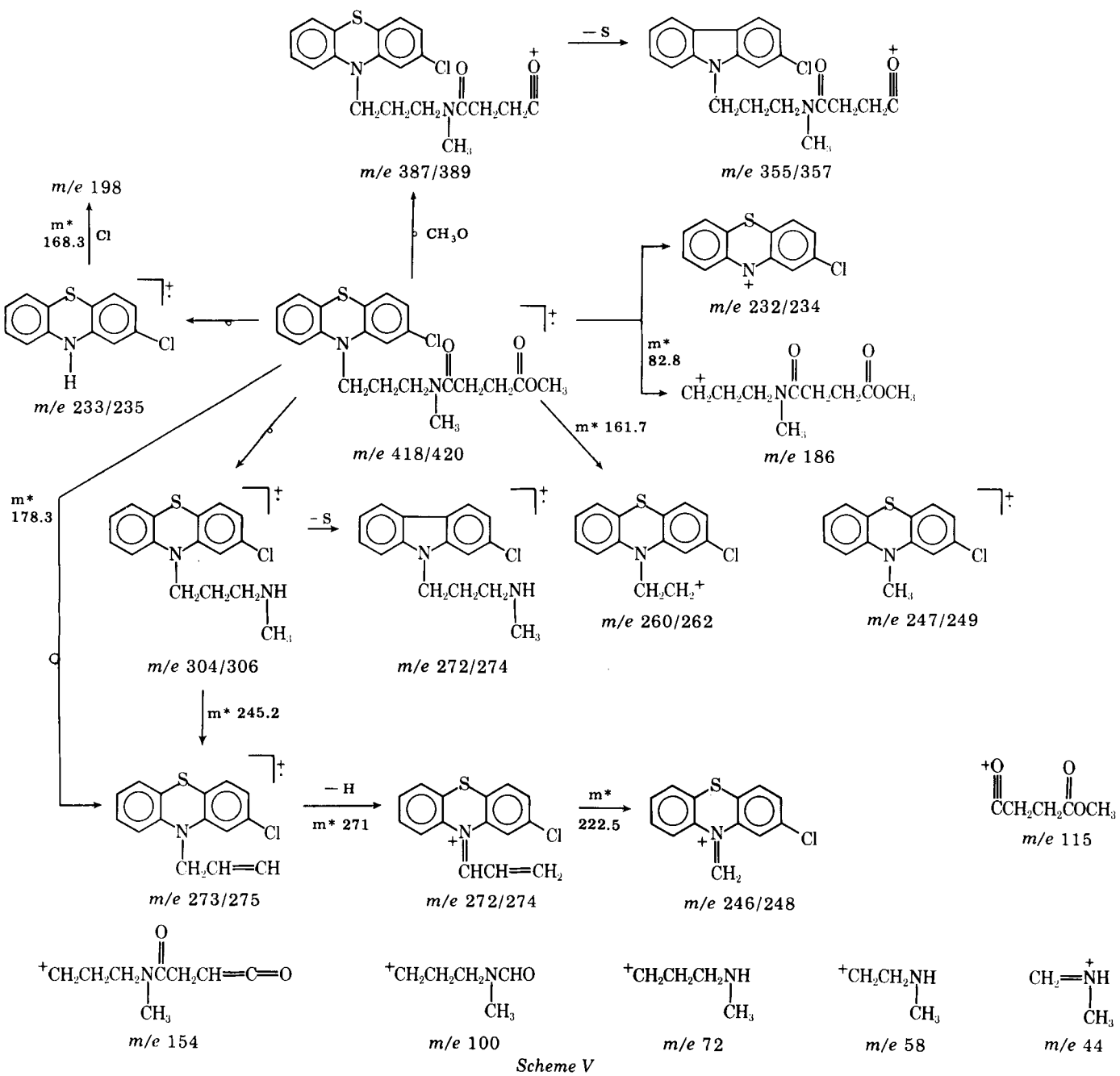
To couple the side chain of chlorpromazine with bovine serum albumin, desmethylchlorpromazine was treated with succinic anhydride to give the amido acid V (Scheme IV) in excellent yield without the addition of

Table I—Rabbit Antiserum Titers of Chlorpromazine Hapten–Protein Conjugates

Immunizing Antigen	Number of Positives/ Number Tested	Number of Boosters	Titers
IV–bovine serum albumin	4/4	0	1:500
		0	1:1200
		0	1:1500
		0	1:200
V–bovine serum albumin	2/12[a]	—	1:80
		—	1:100
VII–bovine serum albumin	4/4	0	1:500
		1	1:200
		2	1:500
		2	1:1000

[a] Compound V–bovine serum albumin only produced titers after two intravenous boosters.

[23] Sorvall Inc. RC2-B, Ingram and Bell Ltd., Don Mills, Ontario, Canada.
[24] Grand Island Biological Co., Grand Island, N.Y.
[25] Double Rabbit Ranch, Smith Falls, Ontario, Canada.
[26] Radioactive chlorpromazine generally labeled with tritium having a specific activity of 5.5 Ci/mmole; Nuclear Research Center, Negev, Israel. Radiochemical purity was checked by analytical TLC and HPLC.



Scheme V

a catalyst. The IR and PMR spectra of V were entirely consistent with the given structure. To facilitate GLC–mass spectrometry, V was converted to its methyl ester by treatment with diazomethane.

The GLC–mass spectrum of the methyl ester of V gave a molecular ion at *m/e* 418/420 and other diagnostic ions (Fig. 3). A rationale for formation of these ions is given in Scheme V. (Fragmentation pathways were not assigned where unsupported by metastables or corresponding fragments.)

The ion at *m/e* 272/274 could be formed by loss of sulfur (*cf.*, 15) from *m/e* 304/306 and/or by loss of a hydrogen radical from *m/e* 273/275; the latter is supported by the observation of a metastable ion (Scheme V).

With the formation of the amido acid derivative of II, the side-chain nitrogen function is changed from a secondary amine to a carboxamide, which has different physical and chemical properties. To determine if this change has any effect on the sensitivity or specificity of the antibody, II was converted into its *N*-methoxycarbonylethyl derivative VI (Scheme VI). The addition of II to the α,β-unsaturated ester methyl acrylate proceeded smoothly without a catalyst to yield the desired product.

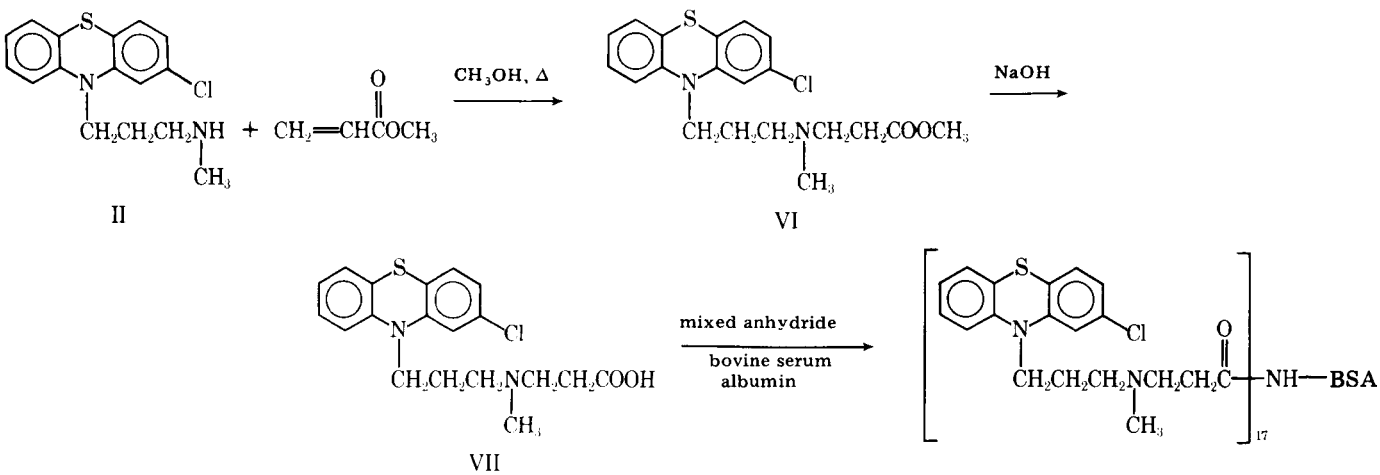
The GLC–mass spectrum of VI (Fig. 4) gave a molecular ion at *m/e* 390/392 and other diagnostic ions as rationalized in Scheme VII. Ions at *m/e* 272/274 and 273/275 are considered to have the same structures as those in Scheme V.

The ions at *m/e* 130 and 260/262 arise from the molecular ion by cleavage of the same carbon–carbon bond. The formation of the ion at *m/e* 130 from the molecular ion is supported by observation of an appropriate metastable ion at *m/e* 43.3.

Similar twin fragment ions at *m/e* 304/306 and 86 from the molecular ion were observed, and the formation of the ion at *m/e* 304/306 is again supported by a metastable ion at *m/e* 237.0. Alternative structures are suggested for the ion or ions giving rise to the base peak at *m/e* 58, both of which appear to arise from daughter ions at *m/e* 317/319 and/or 304/306.

The methyl ester VI was hydrolyzed with alkali to the corresponding carboxylic acid VII. The IR and PMR spectra of VII were entirely consistent with this structure.

The conjugation of IV, V, and VII to bovine serum albumin by the carbodiimide method was carried out according to established procedures with minor adaptations. In the coupling of VII by the mixed anhydride method, however, the addition of tributylamine (13, 14) was unnecessary since VII contains a tertiary amino residue in it structure. If one equiv-

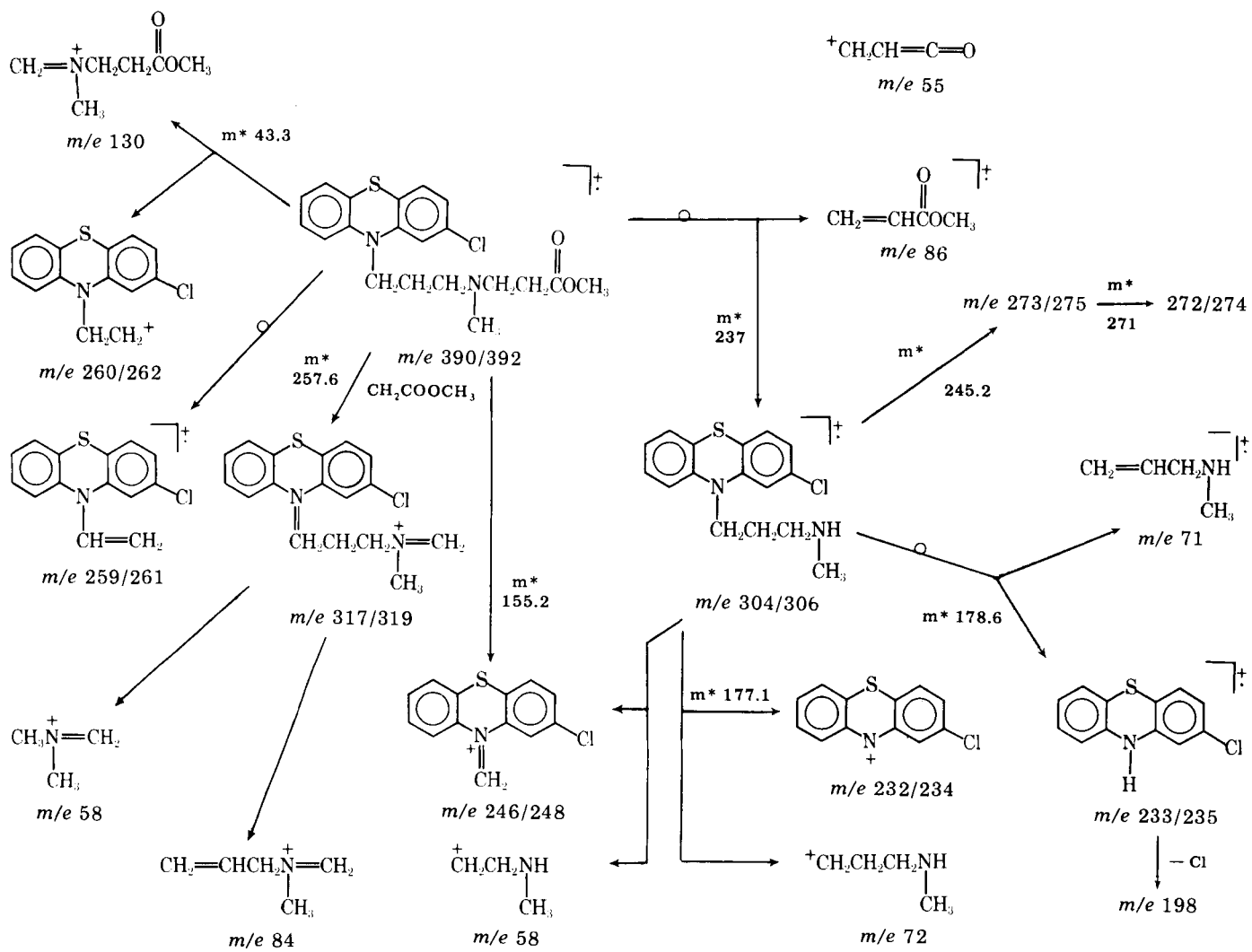


Scheme VI

alent of tributylamine was added to the solution of VII in dioxane, a precipitate was formed on addition of the isobutyl chloroformate. In the absence of tributylamine, the solution remained clear.

If the addition of the mixed anhydride of VII to the bovine serum albumin solution was rapid (13, 14), a precipitate was immediately formed. It did not redissolve when the pH was readjusted to 9.5, even after prolonged stirring. The addition was successfully completed if the mixed anhydride solution was added in small aliquots (0.5 ml) and the pH was maintained at ~9.5 throughout the procedure by dropwise addition of 1.0 *N* NaOH; the initial precipitate redissolved and the final solution was clear.

The preliminary assessment of the antiserums is presented in Table I. All four rabbits immunized with IV–bovine serum albumin (Scheme I) produced operational titers after the fourth injection. Of the four rabbits immunized with VII–bovine serum albumin, one produced an operational titer after the fourth injection, one required a booster shot



Scheme VII

at double the previous dose, and the remaining two each required two booster shots before satisfactory titers were obtained.

Radioimmunoassay sensitive down to the nanogram range was developed using antibodies to IV-bovine serum albumin and VII-bovine serum albumin. The assays were not influenced by plasma and endogenous substances. Details of these procedures will be described in depth in a subsequent publication.

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Radioimmunoassay for Psychotropic Drugs II: Synthesis and Properties of Haptens for Tricyclic Antidepressants

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Abstract □ For the development of radioimmunoassay procedures for tricyclic antidepressants, two drug haptens were synthesized for each of the two amitriptyline–nortriptyline and imipramine–desipramine groups. In one case, nortriptyline or desipramine was treated with succinic anhydride to yield *N*-(3-carboxypropionyl) derivatives; in the other case, the haptens were novel *N*-(2-carboxyethyl) derivatives. The hapten and its corresponding ester were characterized by GLC–mass spectrometry, PMR spectrometry, and IR spectrophotometry. Each hapten was coupled to bovine serum albumin, and the number of hapten residues per mole of bovine serum albumin was determined by UV spectrophotometric methods. Antibodies to each hapten–protein conjugate were developed in rabbits, and titers of the antiserums were checked by evaluating their binding characteristics to tritiated drug.

Keyphrases □ Antidepressants, various tricyclic—haptens synthesized, coupled to bovine serum albumin, antibodies developed in rabbits □ Haptens—of various tricyclic antidepressants, synthesized, coupled to bovine serum albumin, antibodies developed in rabbits □ Antibodies—to hapten–bovine serum albumin conjugates of various tricyclic antidepressants, developed in rabbits

Tricyclic antidepressants are widely used in the treatment of mental disorders, and recent evidence suggests that routine monitoring of plasma levels should provide useful clinical information (1). Some frequently prescribed tricyclic compounds used for the treatment of endogenous depression are imipramine, amitriptyline, and their desmethyl metabolites, desipramine and nortriptyline, respectively.

The determination of the levels of these antidepressant drugs in biological fluids is desirable to study the pharmacokinetics, metabolism, and correlation between blood levels and therapeutic response.

BACKGROUND

Analytical techniques for determining the concentration of the tricyclic antidepressants in biological fluids include spectrophotometry (2, 3), quantitative TLC (4–7), GLC with flame-ionization detection (8–11), GLC with alkali flame (nitrogen specific) detection (12–19), GLC with electron-capture detection (20–23), GLC with mass fragmentographic detection (24–31), GLC with chemical-ionization mass spectrometric detection (32), radioisotope labeling (33–37), and high-pressure liquid chromatography (38). These procedures suffer from a lack of sensitivity or specificity, tedious extraction procedures and/or derivatization techniques, and/or the use of expensive and sophisticated instrumentation. Since they are not easily amenable to routine clinical monitoring, a procedure that may be employed routinely in a standard hospital laboratory is needed.

Radioimmunoassay techniques are simple, usually requiring no extraction, and are readily applied to routine analysis. Of the two published radioimmunoassay procedures, the method for desipramine–imipramine

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