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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Received September 26, 1977, from the **Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada,* and the ‡*Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario, Canada.* Accepted for publication March 21, 1978.

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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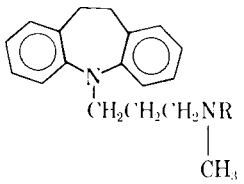
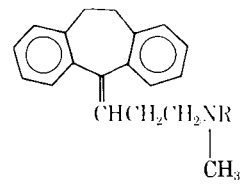
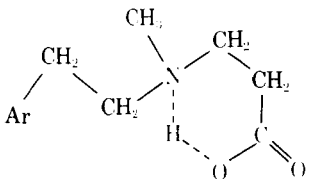


I: R = H
IIa: R = $COCH_2CH_2COOH$
IIb: R = $COCH_2CH_2COOCH_3$
IIIa: R = CH_2CH_2COOH
IIIb: R = $CH_2CH_2COOCH_3$



IV: R = H
Va: R = $COCH_2CH_2COOH$
Vb: R = $COCH_2CH_2COOCH_3$
VIa: R = CH_2CH_2COOH
VIb: R = $CH_2CH_2COOCH_3$



VII

(39), in which the protein (bovine serum albumin) is coupled to the ring system through a carboxyphenylazo bridge, lacks specificity and does not distinguish between a drug and its metabolites. The other radioimmunoassay (40) procedure for amitriptyline–nortriptyline, in which a linkage occurs through the alkylamine side chain to the bovine serum albumin, is not sensitive enough to monitor plasma levels following single or therapeutic dosage.

In the present study, two drug–protein conjugates were prepared for each of the two amitriptyline–nortriptyline and imipramine–desipramine groups. Prior to coupling to bovine serum albumin, the structural integrity of the four haptens was established by GLC–mass spectrometry, PMR spectrometry, and IR spectroscopy to establish a basis for an understanding of the antigenic determinants. The production of antibodies to these conjugates in rabbits and the titers of the antiserums are reported.

EXPERIMENTAL

GLC—The gas chromatograph[1] was equipped with a flame-ionization detector. The column[2] was coiled glass tubing, 1.83 m (6 ft) long × 2 mm i.d., packed with 5% OV-25[2] on acid-washed dimethylchlorosilane-treated high-performance Chromosorb W[2] support (100–120 mesh). The column was conditioned by maintaining the oven at 310° for 18 hr with a low nitrogen flow. Operating conditions were: injection port temperature, 325°; detector temperature, 325°; oven temperature, 300°; and nitrogen flow rate, 60 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

GLC–Mass Spectrometry—The mass spectrometer[3] was 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.

PMR—All PMR spectra were recorded on a spectrometer[5] operating at 60 MHz at ambient temperature with deuterated chloroform as the solvent and tetramethylsilane as the internal standard.

[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 A-60A, Varian of Canada, Georgetown, Ontario, Canada.

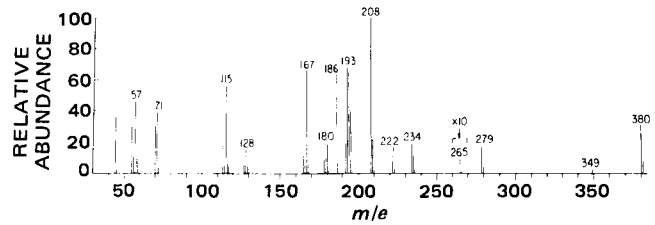


Figure 1—*GLC–mass spectrum (normalized) of IIb.*

IR—All IR spectra were recorded on a spectrophotometer[6] at ambient temperature.

Synthesis of *N*-(3-Carboxypropionyl) Derivative of Desipramine (IIa)—Succinic anhydride[7] (0.45 g, 4.5 mmoles) was added to a solution of desipramine[8] free base (I) (1.16 g, 4.34 mmoles) in ethanol[9] (50 ml). The mixture was stirred at room temperature for 48 hr, and the ethanol was then evaporated[10].

The pale-yellow oil thus obtained was dissolved in aqueous sodium hydroxide (50 ml, 1.0 *N*) and washed with ether[11] (3 × 50 ml) to remove any unreacted I. The aqueous solution pH was then adjusted to 7.5 with aqueous hydrochloric acid (1.0 *N*), whereupon IIa separated as fine white crystals. These crystals were filtered by suction, washed with distilled water (20 ml), and dried in a vacuum desiccator (1.52 g yield, 94%); IR (chloroform): 1715–1740 (carboxylic acid ν C=O) and 1635 (amide ν C=O) cm^{-1}; PMR (deuterated chloroform): δ 1.5–2.0(b, 2H), 2.38(m, 2H), 2.61 (m, 2H), 2.82 (s, 3H), 3.19 (s, 4H), 3.29 (m, 2H), 3.74 (m, 2H), 7.08 (m, 8H), and 9.36 (bs, 1H) ppm. The proton giving rise to the signal at 9.36 ppm exchanged with deuterium oxide.

Compound IIa was converted to its methyl ester IIb by reaction with an ethereal solution of diazomethane[12]. The ester gave only one peak (T_r 11.26 min) on GLC analysis. GLC–mass spectrometry gave a molecular ion at *m/e* 380 and other diagnostic ions (Fig. 1); IR (chloroform): 1730 (ester ν C=O) and 1635 (amide ν C=O) cm^{-1}. In the PMR spectrum of IIb, the singlet at δ 9.36 (1H) ppm was absent and a singlet appeared at δ 3.69 (3H) ppm.

Coupling of IIa to Bovine Serum Albumin—A solution of IIa (0.037 g, 0.1 mmole) in sodium hydroxide (10 ml, 0.1 *N*) was adjusted to pH 8.0 with aqueous hydrochloric acid (0.1 *N*). The bovine serum albumin[13] (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 (0.057 g, 0.3 mmole) in 1.0 ml of water. The solution was mixed by swirling (pH 6.8) and allowed to stand at room temperature for 24 hr.

The mixture was then dialyzed[14] against bicarbonate buffer containing 0.2% sodium azide[15] (0.042 *M*, pH 8.0, 6 × 500 ml) and then against acetate buffer (0.012 *M*, pH 4.0, 6 × 500 ml, with 0.2% sodium azide[15]). After lyophilization[16], the conjugate was obtained as a white crystalline solid (0.064 g). A blank was prepared in the same manner without IIa. The number of hapten residues per mole of conjugate was calculated by the UV method (41, 42) to be 23.

Synthesis of *N*-(2-Carboxyethyl) Derivative of Desipramine (IIIa)—Methyl acrylate[15] (0.39 g, 4.5 mmoles) was added all at once to a solution of I (1.56 g, 4.34 mmoles) in 50 ml of methanol[9]. The mixture was maintained at a gentle reflux for 48 hr, after which the solvent was evaporated[10] to yield a pale-yellow oil (1.33 g). This material was dissolved in hydrochloric acid (4.0 ml, 1.0 *N*) and diluted to 100 ml with distilled water. Aqueous sodium hydroxide (10 ml, 1.0 *N*) was then added, giving a fine oily precipitate.

This precipitate was kept in suspension by continuous rapid stirring as the mixture was heated to a gentle reflux. After 1 hr, the precipitate had all dissolved, and the solution was allowed to cool to room temperature. It was then washed with ether[11] (3 × 100 ml) to remove any unreacted I. The pH was then adjusted to 7.5 with aqueous hydrochloric acid (1.0 *N*), and the mixture was allowed to stand overnight at 4°.

Fine white crystals were collected, washed with distilled water (30 ml), and dried in a vacuum desiccator (1.46 g, 89.4%); IR (chloroform):

[6] Unicam SP-1000, Canlab, Montreal, Quebec, Canada.
[7] Eastman Kodak Co., Rochester, N.Y.
[8] Ciba-Geigy Ltd., Basel, Switzerland (supplied as the hydrochloride).
[9] Burdick & Jackson Laboratories, Muskegon, Mich.
[10] Flash-Evaporator, Buchler Instruments, Fort Lee, N.J.
[11] Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[12] Prepared from Diazald, Aldrich Chemical Co., Milwaukee, Wis.
[13] Sigma Chemical Co., St. Louis, Mo.
[14] Fisher dialyzer tubing (size C), Fisher Scientific Co., Pittsburgh, Pa.
[15] British Drug Houses, Poole, England.
[16] Virtis, Fisher Scientific Co., Montreal, Quebec, Canada.

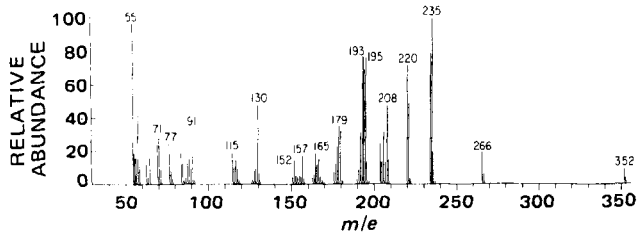


Figure 2—*GLC–mass spectrum (normalized) of IIIb.*

1710–1725 (carboxylic acid ν C=O) cm^{-1}; PMR (deuterated chloroform): δ 1.83 (b, 2H), 2.29 (s) and 2.36 (m) overlapping (5H), 2.67 (m, 4H), 3.16 (s, 4H), 3.78 (m, 2H), 7.08 (m, 8H), and 11.27 (bs, 1H) ppm. The proton giving rise to the broad singlet at δ 11.27 ppm exchanged with deuterium oxide.

Compound IIIa was converted into its methyl ester IIIb by reaction with an ethereal solution of diazomethane[12]. GLC analysis of this material showed only one peak (T_r 2.5 min). GLC–mass spectrometry gave a molecular ion at *m/e* 352 and other diagnostic ions (Fig. 2). In the PMR spectrum (deuterated chloroform) of IIIb, the broad singlet (1H) at δ 11.27 ppm in the spectrum of IIIa was absent and a singlet (3H) appeared at δ 3.63 ppm; IR (chloroform): 1735 (ester ν C=O) cm^{-1}.

Coupling of IIIa to Bovine Serum Albumin—*Carbodiimide Method (IIIa–Bovine Serum Albumin A)*—A solution of IIIa (0.034 g, 0.1 mmole) in sodium hydroxide (1.0 ml, 0.1 *N*) was added dropwise to the stirred mixture of bovine serum albumin[13] (0.070 g, 0.001 mmole) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride[13] (0.057 g, 0.3 mmole) in 1 ml of distilled water. The solution pH was 9.0 and was then adjusted to 7.2 with aqueous hydrochloric acid (0.1 *N*); then the mixture was allowed to stand at room temperature overnight.

The solution was then dialyzed against bicarbonate and acetate buffers as described for IIa–bovine serum albumin. After lyophilization[16], the conjugate was obtained as a white crystalline solid (0.065 g). A blank was prepared in the same manner without IIIa. The number of hapten molecules per mole of conjugate was calculated by the UV method (41, 42) to be 1.0.

Mixed Anhydride Method (IIIa–Bovine Serum Albumin B)—Isobutyl chloroformate[17] (0.017 g, 0.13 mmole) and IIIa (0.044 g, 0.13 mmole) were dissolved in freshly distilled dioxane[15] (2.5 ml). The solution was cooled to 13° and allowed to react for 20 min. The mixture was then added in 0.5-ml aliquots to a stirred, cooled (4°) solution of bovine serum albumin (0.182 g, 0.0026 mmole) in 50% dioxane–water (25 ml), previously adjusted to pH 9.3 with aqueous sodium hydroxide (1.0 *N*). Throughout the addition, the pH was maintained between 9.0 and 9.5 with aqueous sodium hydroxide (1.0 *N*). After the addition was complete, the clear solution was allowed to react for 12 hr at 4° with continuous stirring.

The solution was dialyzed as described for IIa–bovine serum albumin. During the dialysis procedure with the acetate buffer, the protein slowly precipitated. This material was separated by centrifugation[18] (0.5 hr at 2500 rpm) and redissolved in bicarbonate buffer (0.05 *M*, pH 9.3) before lyophilization. The conjugate, a white crystalline solid (0.167 g), was found to contain eight hapten residues per mole by the UV method (41, 42).

Synthesis of *N*-(3-Carboxypropionyl) Derivative of Nortriptyline (Va)—Succinic anhydride[7] (0.10 g, 1.0 mmole) and nortriptyline[19] (IV) (0.24 g, 0.91 mmole) were reacted as described for IIa. The residue was dissolved with aqueous sodium hydroxide, washed with ether, and adjusted to pH 7.5 with aqueous hydrochloric acid (1.0 *N*). The product separated as a colorless oil and was extracted with ether[11] (3 × 50 ml).

The combined ether extract was washed with distilled water (2 × 75 ml) and saturated brine (2 × 75 ml), dried over molecular sieves[20] (3Å), and evaporated[10] to give Va as a colorless oil (0.30 g, 82%); IR (chloroform): 1715–1740 (carboxylic acid ν C=O) and 1635 (amide ν C=O) cm^{-1}; PMR (deuterated chloroform): δ 2.20–3.68 (overlapping multiplets, 15H), 5.81 (t, J = 7 Hz, 1H), 7.17 (m, 8H), and 8.87 (bs, 1H) ppm. The proton giving rise to the singlet at δ 8.87 ppm exchanged with deuterium oxide.

Compound Va was converted to its methyl ester Vb by treatment with diazomethane[12]. The ester gave only one peak on GLC analysis (T_r 5.9 min); when scanned by GLC–mass spectrometry, it gave a molecular ion at *m/e* 377 and other diagnostic ions (Fig. 3). The PMR spectrum of Vb showed that the signal (1H) at 8.87 in the spectrum of Va was absent and a singlet (3H) appeared at δ 3.67 ppm; IR (chloroform): 1730 (ester ν C=O) and 1635 (amide ν C=O) cm^{-1}.

Coupling of Va to Bovine Serum Albumin (Va–Bovine Serum Albumin)—Compound Va was coupled to bovine serum albumin[13] using the carbodiimide method and dialysis procedure described for IIa–bovine serum albumin. The conjugate was obtained as a white crystalline solid (0.67 g), which was shown by the UV method (41, 42) to contain 18 moles of hapten/mole of protein.

Synthesis of *N*-(3-Carboxyethyl) Derivative of Nortriptyline (VIa)—Nortriptyline[19] (0.27 g, 1.02 mmoles) was reacted with methyl acrylate[15] (0.088 g, 1.02 mmoles) under the conditions described for IIIa. After the aqueous medium pH was adjusted to 7.5, the product separated as a colorless oil and was extracted with ether[11] (3 × 50 ml).

The combined ether extract was washed with water (2 × 75 ml) and saturated brine (2 × 75 ml), dried over molecular sieves[20] (3Å), and evaporated[10] to yield VIa as a colorless oil (0.275 g, 82%); IR (chloroform): 1715–1720 (carboxylic acid ν C=O) cm^{-1}; PMR (deuterated chloroform): δ 2.10–3.35 (overlapping multiplets, 15H), 5.78 (t, J = 7 Hz, 1H), 7.18 (m, 8H), and 10.45 (s, 1H) ppm. The proton giving rise to the signal at δ 10.45 ppm exchanged with deuterium oxide.

Compound VIa was converted to its methyl ester VIb by reaction with diazomethane[12]. GLC analysis of the ester showed only one peak (T_r 2.1 min); when scanned by GLC–mass spectrometry, it gave a molecular ion at *m/e* 349 and other diagnostic ions (Fig. 4). The PMR spectrum of the methyl ester showed that the signal (1H) at δ 10.45 ppm in the spectrum of VIa was absent and a singlet (3H) appeared at δ 3.62 ppm; IR (chloroform): 1735 (ester ν C=O) cm^{-1}.

Coupling of VIa to Bovine Serum Albumin—*Carbodiimide Method (VIa–Bovine Serum Albumin A)*—Compound VIa was coupled to bovine serum albumin[13] using the carbodiimide method escribed for IIIa–bovine serum albumin A and the dialysis procedure described for IIa–bovine serum albumin. The conjugate was obtained as a white crystalline solid (0.066 mg) and was shown by the UV method (41, 42) to contain 0.5 hapten residues/mole.

Mixed Anhydride Method (VIa–Bovine Serum Albumin B)—Compound VIa was coupled to bovine serum albumin[13] by the mixed anhydride technique described for IIIa–bovine serum albumin B and the dialysis procedure described for IIa–bovine serum albumin. The conjugate, a white crystalline solid (0.176 g), was shown by the UV method (41, 42) to contain 10 hapten molecules/mole.

Immunization—Each hapten–protein conjugate (4 mg) was dissolved in normal saline (2.0 ml) and emulsified with complete Freund's adjuvant[21] (2.0 ml). Each conjugate was administered to four New Zealand White female rabbits[22] by injection of 0.5 ml of the emulsion into each flank. At 2-week intervals thereafter, the injections were repeated with the emulsion prepared as 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.

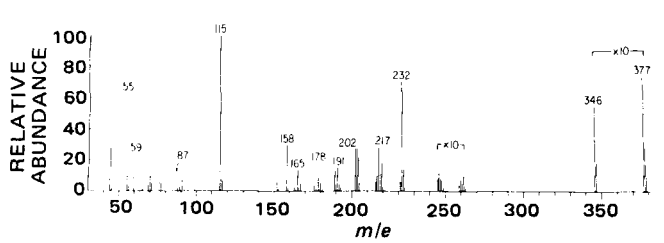


Figure 3—*GLC–mass spectrum (normalized) of Vb.*

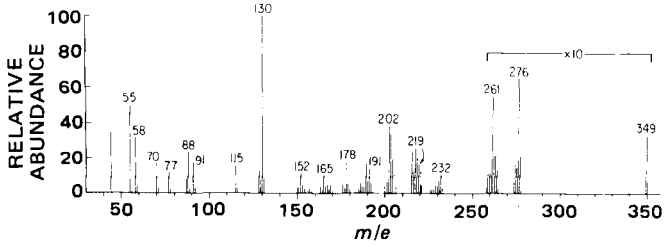
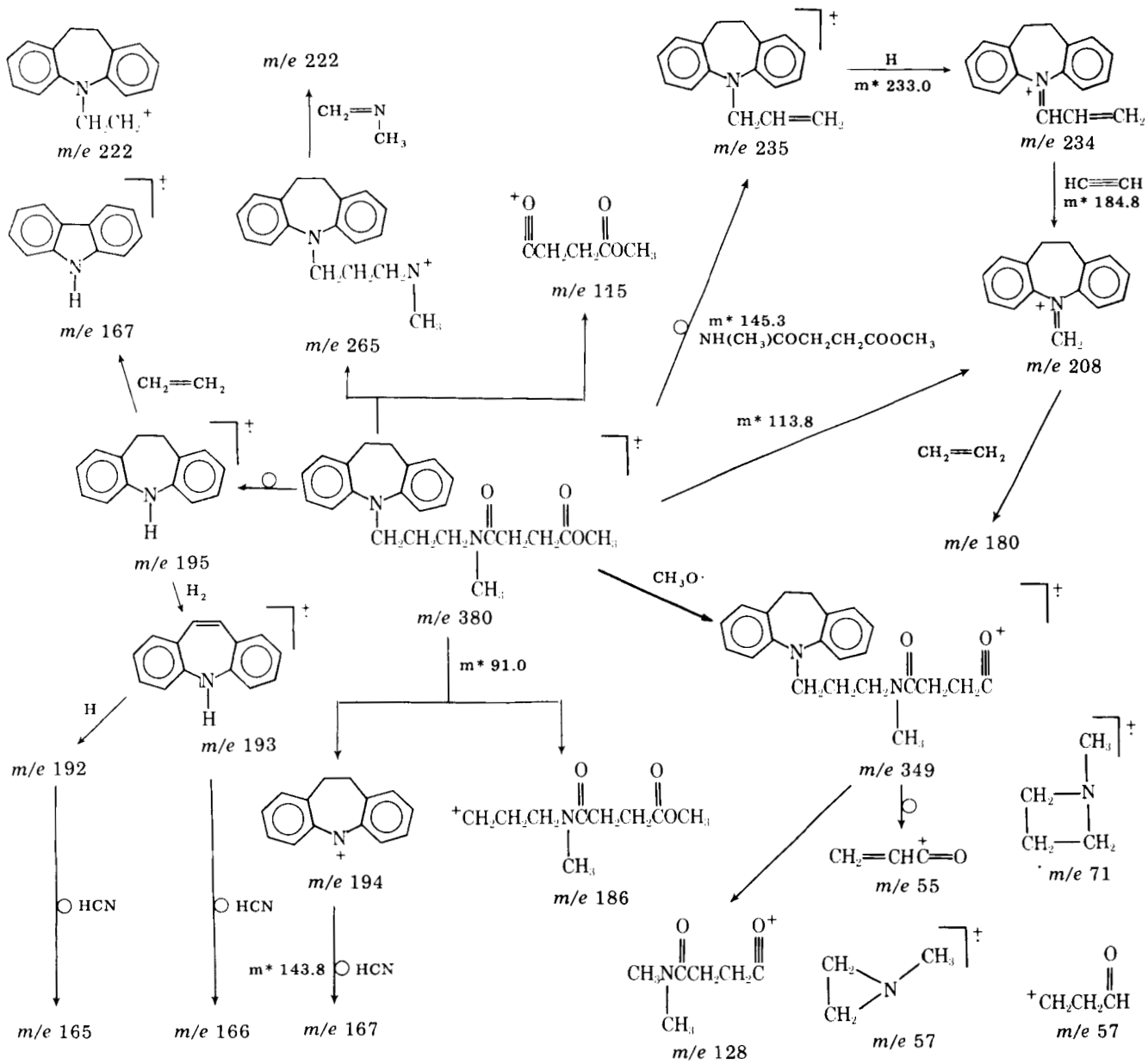


Figure 4—*GLC–mass spectrum (normalized) of VIb.*

[17] Aldrich Chemical Co., Milwaukee, Wis.
[18] Sorval Inc. RC2-B, Ingram and Bell Ltd., Don Mills, Ontario, Canada.
[19] Eli Lilly Co. Ltd., Toronto, Ontario, Canada.

[20] Davison Chemical Co., Baltimore, Md.
[21] Grand Island Biological Co., Grand Island, N.Y.
[22] Double Rabbit Ranch, Smith Falls, Ontario, Canada.



Scheme I

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 I[23] or tritiated IV[24] to each antiserum. After optimal titers were achieved, blood was obtained from each rabbit by cardiac puncture. The harvested serum in each case was immediately lyophilized in 1-ml samples and stored at −70°.

RESULTS AND DISCUSSION

The four haptens were obtained in good yields as described under *Experimental*. They were characterized by IR and PMR spectral data and by GLC–mass spectrometry of their respective methyl esters, prepared by reacting the carboxylic acids with diazomethane prior to coupling to bovine serum albumin. All IR and PMR spectra of the carboxylic acids and their respective methyl esters were entirely consistent with their proposed structures.

The GLC–mass spectrum of the methyl ester II*b* gave a molecular ion at *m/e* 380 and other diagnostic ions shown in Fig. 1 and rationalized in Scheme I. The base peak at *m/e* 208 apparently arises directly from the molecular ion at *m/e* 380 by cleavage of the bond between C-1 and C-2 of the side chain and also indirectly from the molecular ion through ions at *m/e* 235 and 234 (Scheme I). The observation of appropriate metastable ions (Scheme I) gives support to both pathways.

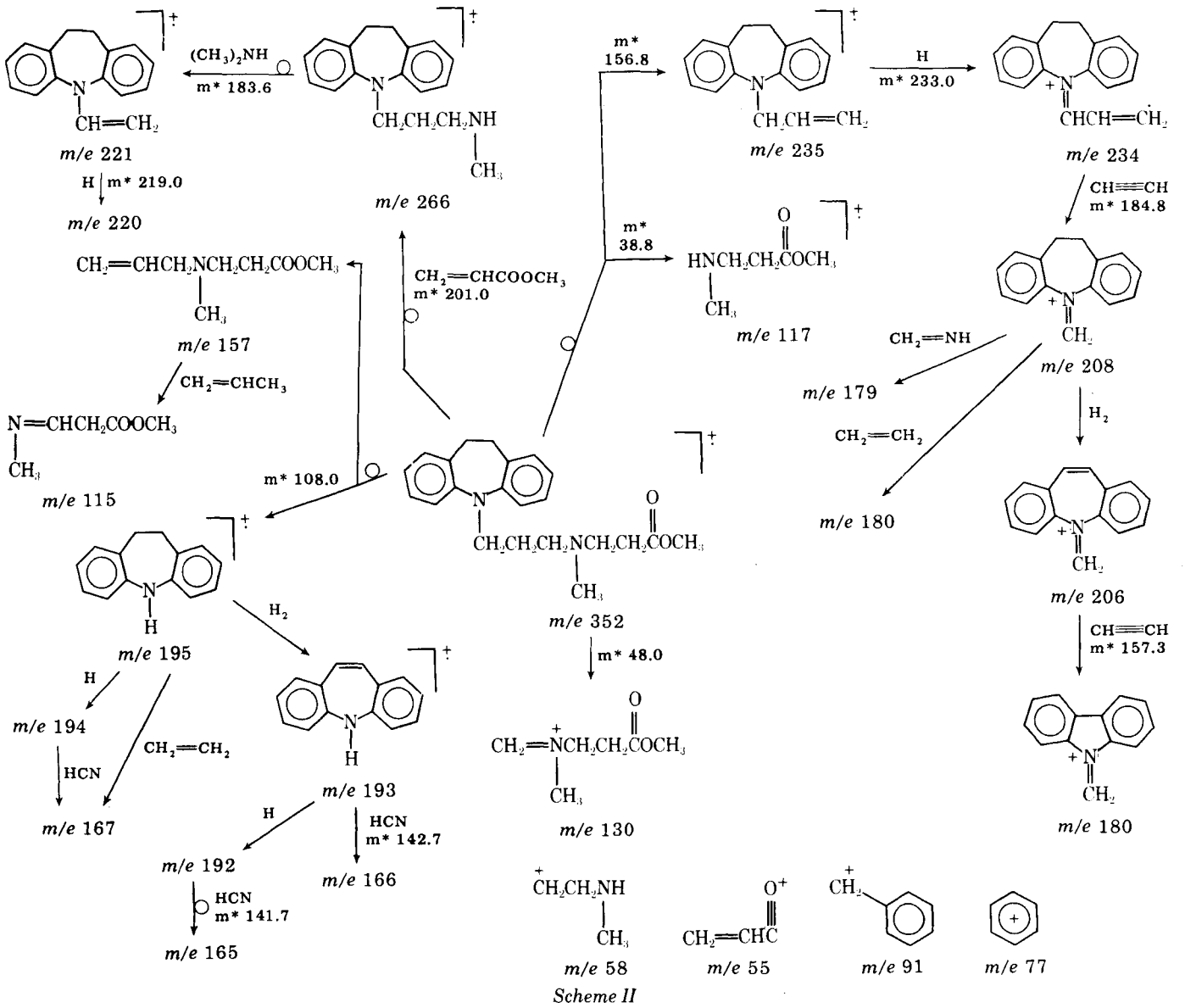
The formation of an ion at *m/e* 186 from the molecular ion, as supported by the appropriate metastable ion at *m/e* 91.0, suggests the loss of the ring system. The presence of an ion at *m/e* 194 (65%) indicates that, on cleavage of the intact side chain, the charge is accommodated to a similar extent by the ring system.

By contrast, in the fragmentation pattern of the methyl ester III*b* (Scheme II), the ion at *m/e* 194 (Fig. 2) seems to arise by a different pathway since no ion corresponds to the intact side chain. In this case, the ion at *m/e* 157 and the corresponding ion at *m/e* 195 (supported by an appropriate metastable ion at *m/e* 108.0) indicate that the molecular ion undergoes cleavage by a rearrangement involving the transfer of a proton from the side chain. The ion at *m/e* 194 seems to arise by loss of a hydrogen radical from the ion at *m/e* 195.

In the mass spectrum of III*b*, the base peak is observed at *m/e* 235 with a corresponding ion at *m/e* 117, with both fragments supported by appropriate metastable ions (Scheme II). This result indicates that the base peak at *m/e* 235 is formed by a rearrangement involving a 1–4 shift. The ion at *m/e* 234 (79%) is formed by loss of a hydrogen radical from the ion

[23] Radioactive imipramine generally labeled with tritium having a specific activity of 20 Ci/mmole; Nuclear Research Center, Negev, Israel. Radiochemical purity was checked by TLC.

[24] Radioactive nortriptyline generally labeled with tritium having a specific activity of 3.2 Ci/mmole; Nuclear Research Center, Negev, Israel. Radiochemical purity was checked by TLC.



Scheme II

at *m/e* 235, as supported by the observation of a metastable ion at *m/e* 233.0. This sequence was observed in the mass spectrum of imipramine (43). Structures for the ions at *m/e* 179, 165, and 152 (Fig. 2) were proposed (44).

In the mass spectrum of the methyl ester V*b* (Fig. 3), the ion at *m/e* 115, which is the base peak (Scheme III), has the same structure as the equivalent ion (56%) in the mass spectrum of the amido ester II*b* of desipramine (Scheme I). The ion at *m/e* 232 (70%) (Scheme III), which arises from the molecular ion at *m/e* 377 with an appropriate metastable ion at *m/e* 142.8, has the same structure as was previously reported in the mass spectrum of *N*-trifluoroacetylnortriptyline (45).

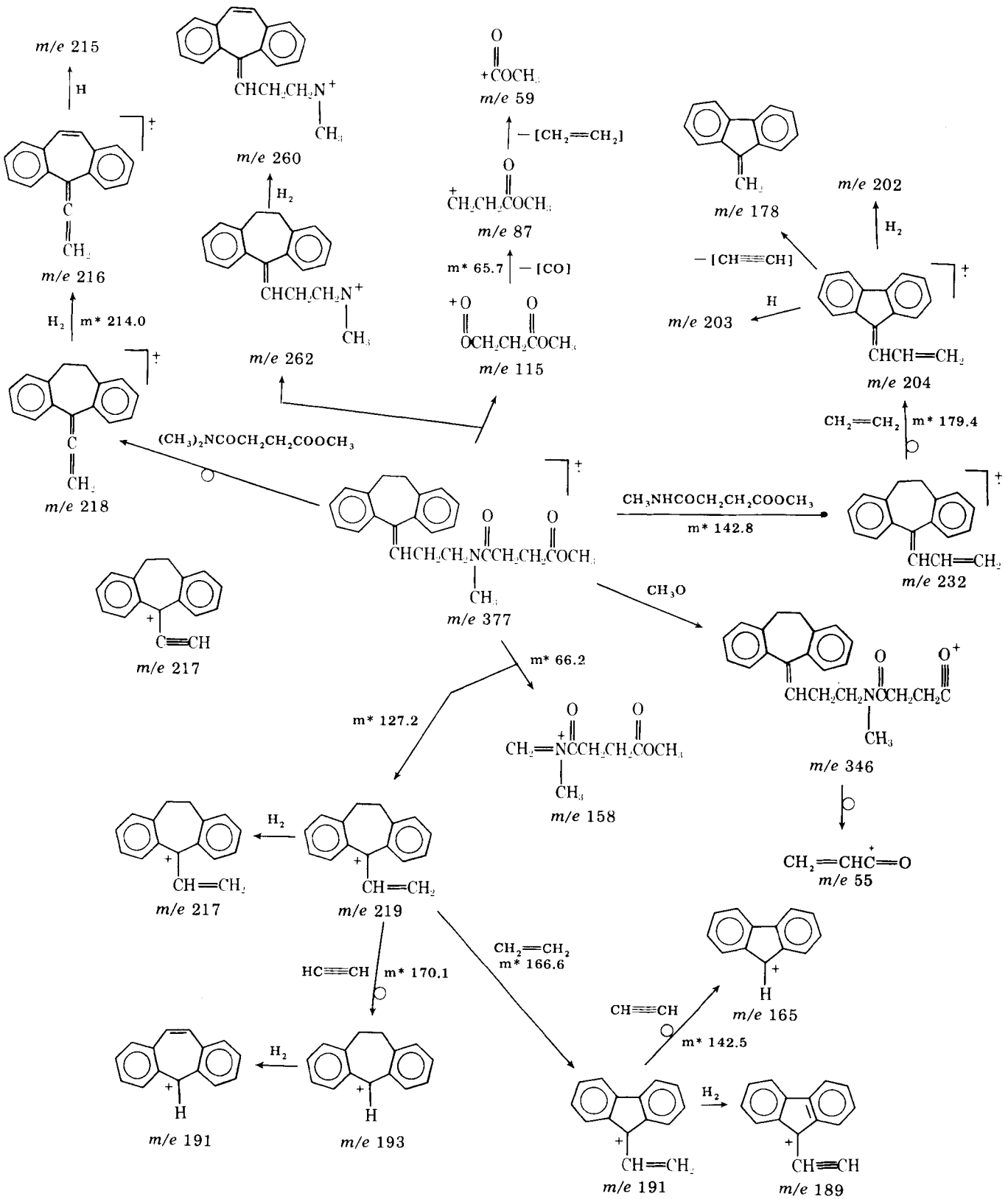
Similarly, in the mass spectrum of the methyl ester VI*b* (Fig. 4 and Scheme IV), the ion at *m/e* 130 is the base peak, in contrast to the mass spectrum of the amino ester III*b* of desipramine (Fig. 2) in which the same ion occurs to a lesser extent (50%). Thus, the relative abundances of the ions at *m/e* 130 and 115 in the amino esters III*b* and VI*b* and in the amido esters II*b* and V*b*, respectively, reflect the influence of the two ring systems on the cleavage of the side chain under electron impact.

The amino acids II*a* and V*a* were coupled to bovine serum albumin by the carbodiimide method, with only minor modifications of established procedures (46–51). The number of moles of hapten coupled per mole of bovine serum albumin was calculated by the UV method (41, 42) to be 23 and 18, respectively. By contrast, the amino acids III*a* and VI*a* proved resistant to coupling by the carbodiimide method, even when the procedure was adapted for the poor solubility of these haptens between pH 3.5 and 9.5. The poor solubility of III*a* and VI*a* may be related to the formation of a stable, intramolecular, hydrogen-bonded, six-membered ring system VII. Aherne *et al.* (40) similarly found that an aminoalkyl derivative of nortriptyline coupled poorly with bovine serum albumin by the carbodiimide procedure.

The amino acids III*a* and VI*a* were successfully coupled to bovine serum albumin by a modified mixed anhydride procedure (41, 42, 51),

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

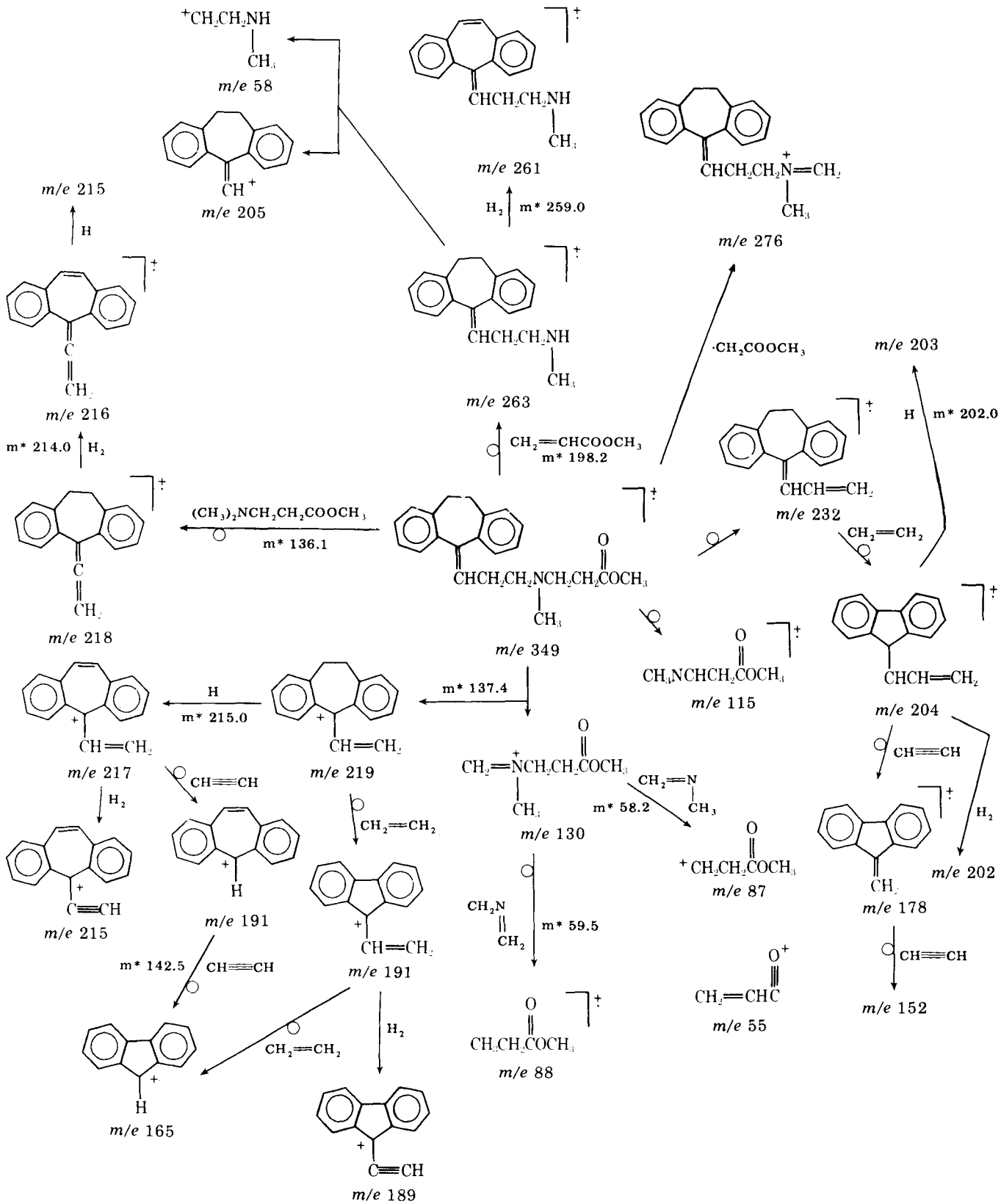
Immunizing Antigen	Number of Positives/ Number Tested	Number of Boosters	Titers
II*a*–bovine serum albumin	3/4	0	1:200
		0	1:400
		0	1:400
		—	—
III*a*–bovine serum albumin	4/4	0	1:1500
		0	1:1600
		0	1:1000
		0	1:1500
V*a*–bovine serum albumin	2/4	1	1:500
		2	1:200
		—	—
VI*a*–bovine serum albumin	4/4	0	1:500
		0	1:500
		0	1:200
		0	1:1000



Scheme III

which gave 8 and 10 moles of hapten/mole of bovine serum albumin, respectively.

The preliminary assessment of the antiserums is presented in Table I. Three out of four rabbits immunized with II*a*–bovine serum albumin produced operational titers after the fourth injection. The fourth rabbit died because of a bacterial lung infection after the first injection. All rabbits immunized with III*a*–bovine serum albumin and VI*a*–bovine serum albumin produced operational titers after the fourth injection. Of the four rabbits immunized with V*a*–bovine serum albumin, two did not produce any titer, one rabbit required one booster shot intravenously at double the previous dose, and the remaining rabbit required two intravenous boosters before satisfactory titers were produced.



Scheme IV

Radioimmunoassays sensitive down to the nanogram range were developed for imipramine and desipramine using antibodies to IIα-bovine serum albumin and IIIα-bovine serum albumin; for amitriptyline and nortriptyline, antibodies to Vα-bovine serum albumin and VIα-bovine serum albumin were used. The assays were not influenced by plasma and endogenous substances. The procedures will be described in depth in a subsequent publication.

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