

SELECTIVE TOXICITY AND COMPARATIVE PHARMACOLOGY
OF TRICAINE METHANESULFONATE

by

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A THESIS

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DEDICATION

To my parents who have always selflessly given of themselves to the happiness and fulfillment of their children.

To my daughter, Kary, who is the light of my life and to whom I hope to be what my parents are to me.

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LIST OF PUBLICATIONS FROM THESIS

- Abstracts: Metabolism as a Basis of the Selective Toxicity of Tricaine Methanesulfonate. K.A. Wayson, H. Downes and N. Gerber. Fed. Proc. 24: (3) 772, 1975.
- Papers: Synthesis of Ethyl m-Aminobenzoate (Carboxyl-¹⁴C) Methanesulfonate. R.K. Lynn, K.A. Wayson, H. Downes and N. Gerber. Nat. Aux. Publications Service #02844, 1976.
- Anesthetic Effects and Elimination of Tricaine Methanesulfonate in Terrestrial Vertebrates. K.A. Wayson, H. Downes, R.K. Lynn and N. Gerber. Comp. Biochem. Physiol. 55C: 37-41, 1976.
- Studies on the Comparative Pharmacology and Selective Toxicity of Tricaine Methanesulfonate: Metabolism as a Basis of the Selective Toxicity in Poikilotherms. K.A. Wayson, R.K. Lynn, H. Downes and N. Gerber. J. Pharm. and Exp. Ther. 198: 695-708, 1976.

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Tricaine is the meta-isomer of the local anesthetic, benzocaine. The drug, administered as the methanesulfonic acid salt at a dose of 150-300 mg/kg i.p., produces loss of the righting reflex and flaccid paralysis in amphibian, reptilian and avian species. In contrast, similar doses (mg/kg) administered i.p. produce no apparent pharmacological response in mammals. The metabolism of tricaine was studied in vivo and in vitro in the various species. Tricaine was separated from its more polar metabolites in tissue homogenates by extraction with 1-chlorobutane and then measured in 1N HCl by UV analysis at 228 nm and by colorimetric analysis at 545 nm after development of the azo dye with the Bratton-Marshall reaction. The metabolites of tricaine, left in the residual homogenate, were also quantified by the Bratton-Marshall colorimetric assay. The biological half-life of tricaine in frogs (Rana pipiens) is about 70 min at both 23° C (room temperature) and 37.5° C. The drug is metabolized so rapidly in mice that none of the unchanged drug could be recovered from the animals 5 min after i.p. administration of a 250 mg/kg dose of the drug. Incubations of tricaine with sera from bullfrogs, mice and humans indicated that the drug is

hydrolyzed quantitatively to m-aminobenzoic acid (MABA). The $V_{\max}$ for incubations of the drug with bullfrog and human sera is 40 nmol/min/ml of serum, whereas, with mouse serum it is 93 nmol/min/ml of serum. In vitro studies with tissue homogenates showed that the liver is the major site of tricaine metabolism in mammals. The selective toxicity of tricaine for poikilotherms is a consequence of their low capacity to hydrolyse tricaine in the liver.

The primary reaction in the biotransformation of tricaine is hydrolysis of the ester linkage of the drug with the resultant production of MABA and ethanol. Metabolite identification studies in mice involved investigation of both urine samples and tissue homogenates. The metabolites were isolated by means of countercurrent distributions (CCD) and identified with gas chromatography/mass spectrometry after various derivatization reactions. The major urinary metabolites of tricaine in the mouse were acetyl, glycine and glucuronic acid conjugates of MABA.

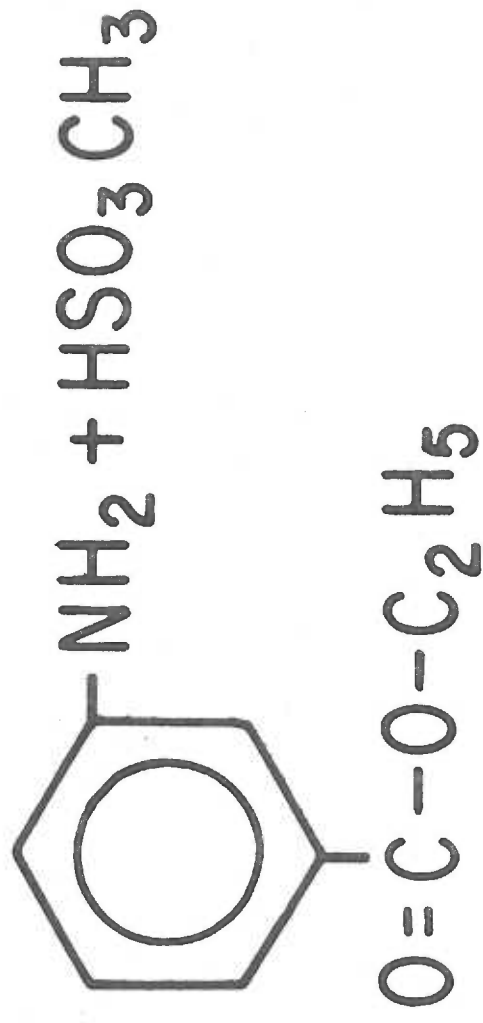
In vitro tissue studies demonstrated the uniformly depressant effects of tricaine on cardiac muscle, smooth muscle and nerve tissue preparations. These studies also showed that tricaine is equipotent with benzocaine and more potent than procaine in its actions. The lethal event of tricaine toxicity in frogs (Rana pipiens) is cardiac arrest. In vitro studies with cardiac tissue indicated that guinea-pig atria are more sensitive to the depressant effects of tricaine than are comparative preparations from bullfrogs (Rana catesbiana). Antiarrhythmic actions of tricaine were demonstrated on both in vitro aconitine-induced arrhythmias and in vivo ouabain-induced arrhythmias.

INTRODUCTION

Tricaine methanesulfonate is the methanesulfonic acid salt of ethyl m-aminobenzoate (Fig. 1). The ethyl ester of m-aminobenzoic acid, which was synthesized many years ago by Muller (1886) and Bauer and Einhorn (1901), is a close analogue of the local anesthetic, benzocaine (ethyl p-aminobenzoate). The local anesthetic and other pharmacological properties of tricaine methanesulfonate were described by Sandoz (1920) during his investigations to develop a synthetic substitute for cocaine. The drug was originally used as a local anesthetic in humans and its general anesthetic activity in poikilotherms was recognized soon thereafter (Christensen 1931, Baudin 1932, 1934).

The potential usefulness of tricaine as a general anesthetic for cold-blooded animals was not widely appreciated until Wood (1956) and Ball and Cowen (1959) independently reported on the carcinogenic properties of urethane, which was the agent commonly used for anesthesia of fish and amphibia at the time. An editorial comment accompanying Wood's report indicated that tricaine might be a suitable substitute for urethane. Since then, the anesthetization of fish and other poikilotherms with tricaine methanesulfonate to facilitate their handling has become routine (Bove 1962, Schoettger et al. 1967). Anesthesia is induced by placing the fish directly into a solution of tricaine methanesulfonate with an appropriate concentration for the desired depth and duration of anesthesia (Schoettger and Julin 1967). When anesthesia is no longer desired, the fish are placed in tricaine-free water. Both the induction of and recovery from anesthesia are rapid due to rapid diffusion of the drug across the gill membranes

TRICAINE METHANESULPHONATE



Ethyl m-aminobenzoate
methanesulphonate

Figure 1: The Structure of Tricaine Methanesulfonate

There is a considerable variety of names, both common and chemical, applied to tricaine methanesulfonate in the literature. They include:

MS 222	Tricaine - Sandoz	Methane sulfonic acid salt of <u>meta</u> -amino ethyl benzoate
MS-222 Sandoz	Tricaine methanesulfonate	
M.S.-222 Sandoz	Metacaine	Methanesulphonate of <u>meta</u> -aminobenzoic acid
TS-222	Metacaine methanesulphonate	
TS-222 Sandoz	<u>M</u> -amino ethyl benzoate	Methanesulphonate <u>meta</u> -aminobenzoic acid ethyl ester
Tricaine	Ethyl <u>m</u> -aminobenzoate	

In this thesis, the terms tricaine and ethyl m-aminobenzoate have been used to denote the free base form of the compound and the term, tricaine methanesulfonate, has been used to denote the methanesulfonic acid salt.

(Hunn and Allen 1974). This same method, immersion in a solution of tricaine methanesulfonate, is also the recommended procedure for anesthetizing amphibians.

Marking (1967) determined the effect of tricaine in eight different species of fish and showed that the toxicity varies with the species, temperature, pH and duration of exposure. Concentrations of tricaine methanesulfonate of 1:10,000 in water cause rapid induction of anesthesia in fish. Duration of exposure appears to be the single most important factor in determining the toxicity of tricaine in fish. The drug has a low toxicity in mammals (Spector 1956). The oral LD₅₀ in Sprague-Dawley rats is between 5 and 10 gm/kg (Walker and Schoettger 1967a).

Maren and coworkers (1968b) studied the excretion of tricaine by the spiny dogfish, Squalus acanthias, and observed that passage across the gill membrane is the major route of elimination of the drug. An acetylated metabolite of the drug, acetylated tricaine, is also excreted across the gill membranes. Allen et al. (1972) and Luning (1973a) have recently shown that fish also eliminate the drug by hydrolyzing tricaine to m-aminobenzoic acid (MABA). The more polar metabolites, MABA and its acetylated derivative, are excreted by the kidneys (Maren et al. 1968a). Other investigators have studied the disposition of tricaine in fish (Schoettger 1967, Walker and Schoettger 1967b, Stenger and Maren 1968, Allen et al. 1970, Hunn 1970, Luning 1973b). The influence of tricaine on the skeletal muscle and the neuromuscular junction of frogs (Rana pipiens) has been examined by Maeno (1966) and Friedman et al. (1974) and the methanesulfonate moiety has

been shown to have no depressant effect on neuromuscular transmission in the dose range used for anesthesia in poikilotherms (Friedman et al. 1974).

STATEMENT OF THE PROBLEM

The major purpose of the investigations described in this thesis was to elucidate the basis of the selective toxicity of tricaine methanesulfonate for poikilotherms. In addition, this drug is widely used as a general anesthetic, particularly in the fisheries industry, without detailed knowledge of its general pharmacology in any vertebrate species. Therefore, I also examined the pharmacokinetics of tricaine in both warm and cold-blooded animals, the comparative effects of the drug on various organ systems, and the metabolic disposition of tricaine including both metabolite identification and determination of rates of biotransformation. The need for studies of the effects and the disposition of tricaine methanesulfonate in mammals arises from the possible future use of this drug for medicinal purposes and from the possible exposure of mammals to the drug through environmental pollution, e.g., presence of the drug in the food chain or in the water supply.

MATERIALS

A. Animals

Male Swiss-Webster mice, 20-30 gm, and Sprague-Dawley rats, 200-300 gm, were purchased from Simonsen Laboratories Inc., Gilroy, Calif. Male guinea pigs, 350-400 gm, were purchased from Lab Associates Inc., Kirkland, Wa. Bullfrogs (Rana catesbiana), 200-500 gm, were obtained

from College Biological Supply Co., Bothwell, Wa. Frogs (Rana pipiens), 20-30 gm; salamanders (Taricha rivularis), 7-13 gm; snakes (Thamnophis sirtalis), 20-40 gm; turtles (Pseudemys scripta elegans), 525-850 gm; lizards (Anolis carolinensis), 2-5 gm; parakeets (Melopsittacus undulatus), 30-40 gm; domestic cats, 3.0-3.5 kg; and domestic rabbits, 2.5-3.5 kg, were obtained from local sources (See Appendix I). All animals were housed in well-ventilated rooms maintained at 21°C. Care was taken to avoid exposure of the animals to compounds known to influence drug-metabolizing enzyme activity, such as chlorinated insecticides (Hart and Fouts 1963).

B. Tissue Specimens

Liver specimens of various marine mammals which had washed ashore along the Oregon coast were acquired from Richard K. Stroud, D.V.M. of the Veterinary Diagnostic Laboratory at Oregon State University, Corvallis, Oregon. Bovine Serum Albumin (crystalline) was purchased from Calbiochem, San Diego, Ca.

C. Equipment

Countercurrent distributions (CCD) were carried out in a ten tube manual machine (Spectrum Medical Industries Inc., New York, N.Y.). Ultraviolet and colorimetric analyses were performed on a Cary 15 recording spectrophotometer with quartz cuvettes having a 1 cm light path. Gas chromatography was carried out using a Varian 2700 instrument equipped with a 5 ft x 1/8 inch stainless steel column packed with 1.5% OV-101 on Gas Chrom Q, 100/200 mesh. The mass spectra were obtained

on a Dupont, Model 21-491 B, gas chromatograph/mass spectrometer under the following conditions: accelerating potential, 1.8 kV; ionizing potential, 70 eV; trap current, 300 μ A; source temperature, 200°C. A 6 ft x 2 mm (internal diameter) glass column, packed as described above, was used for the gas chromatography. Radioactivity was measured on a Packard Tricarb Liquid Scintillation counter. pH readings were made on a Corning Digital 110 pH meter. Physiological measurements were recorded on either a Grass Model 7 polygraph or a Harvard Apparatus 10 speed chart mover. Isolated tissues were homogenized in a Potter-Elvehjem homogenizer and whole animals were homogenized in a Waring Blendor. A Buchi/Brinkman Rotovapor Model R rotary evaporator was used to dry solvent extracts at room temperature. Incubations were maintained at 37.5°C in a Dubnoff Metabolic Shaking Incubator (120 cycles/min). Samples were shaken on an Eberbach shaker (120 cycles/min) and centrifuged on a ICE Model K centrifuge. Chromatographic paper (Chromar^R) was purchased from Mallinckrodt Chemical Works, St. Louis, Mo. (Code 2182).

D. Chemicals

The solvents used were 1-chlorobutane, methylene chloride, ethyl acetate and 2,2,4-trimethylpentane (isooctane) (Burdick and Jackson Laboratories, Muskegon, Mi.); methanol, butyl alcohol and nanograde chloroform (Mallinckrodt Chemical Works, St. Louis, Mo.) and ethanol (Commercial Solvents Corp., Agnew, Ca.).

Tricaine methanesulfonate (Finquel^R) was a gift from Ayerst Laboratories Inc., New York, N.Y. The para-, meta- and ortho- isomers

of ethyl aminobenzoate and of aminobenzoic acid were purchased from Eastman Kodak Co., Rochester, N.Y. M-aminobenzoic acid (MABA) and methanesulfonic acid (98%) were acquired from Aldrich Chemical Co., Milwaukee, Wi. Benzoic acid (carboxyl- ^{14}C) was purchased from New England Nuclear, Boston, Ma. Procaine hydrochloride was obtained from Sigma Chemical Co., St. Louis, Mo. Ten per cent palladium on charcoal, N-1-naphthylethylenediamine dihydrochloride and ammonium sulfamate were purchased from Matheson, Coleman and Bell Inc., Norwood, Oh. and sodium nitrite was acquired from Mallinckrodt Chemical Works, St. Louis, Mo. The tricaine methanesulfonate, the three isomers of ethyl aminobenzoate and the MABA were shown to be 99+% chemically pure by CCD, ultraviolet (UV) spectrophotometry and gas chromatography (see below).

METHODS

A. General Chemical Assay Procedures (See Figure 2)

1. Ultraviolet Assay for Tricaine

Preliminary experiments demonstrated that tricaine has absorption maxima at 274 nm and 228 nm (A_{274} , A_{228}) in 1N HCl. Tricaine can be selectively extracted from 0.2 M phosphate buffer, pH 7.4, with 1-chlorobutane. The partition coefficient, C_o (See Appendix III), of tricaine in the 1-chlorobutane:phosphate buffer, pH 7.4, system is greater than 33. In the usual experiment, tissue homogenate was extracted with 1-chlorobutane. An aliquot of the 1-chlorobutane was extracted with 1N HCl. The partition coefficient of tricaine in the 1-chlorobutane:HCl system is less than 0.0008. The tricaine was quantified by scanning the absorption spectrum of the 1N HCl extract from 360 nm to 190 nm, using 1N HCl as the reference and measuring the A_{228} . The molar extinction coefficient,

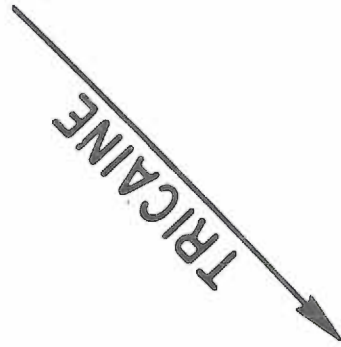
$$\text{extinction coefficient} = \frac{(\text{absorbance})}{(\text{path length})(\text{concentration})},$$

of tricaine in 1N HCl is 1.16×10^4 at 228 nm. The absorption spectra and standard curve of this assay for tricaine in whole mouse homogenate are shown in Figure 3.

2. Colorimetric Assay for Tricaine

Following the quantification of tricaine in the HCl extract by the UV analysis, the Bratton-Marshall reaction (See Appendix II) was used as a second and independent assay for tricaine. Two ml of 15 gm% TCA (trichloroacetic acid) and 0.5 ml of 0.1 gm% sodium nitrite were added to 1 ml of the HCl extract. The sample was allowed to stand for 15 min and then 0.5 ml

BUFFERED HOMOGENATE

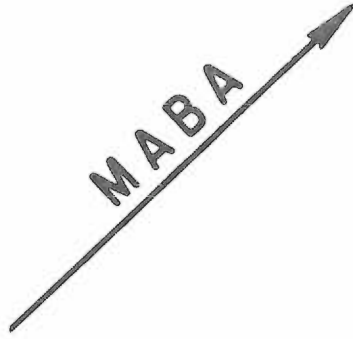


I-CHLOROBUTANE

IN HCL

UV ANALYSIS

B-M ANALYSIS



RESIDUAL HOMOGENATE

B-M ANALYSIS

Figure 2: Methodology for Quantification of Tricaine Methanesulfonate

The addition of the ethyl group on m-aminobenzoic acid (MABA) results in a compound (tricaine) with low polarity and high lipid solubility. The high lipid solubility allows tricaine to be separated from its metabolites by extraction of buffered tissue homogenate ($\text{pH} = 7.4$) with 1-chlorobutane. Since tricaine is a base, it was possible to extract the drug from 1-chlorobutane into 1N HCl. The HCl was then analyzed both spectrophotometrically and colorimetrically. The metabolites of tricaine remained in the lower aqueous layer after the 1-chlorobutane extraction and were analyzed colorimetrically and quantified in terms of MABA.

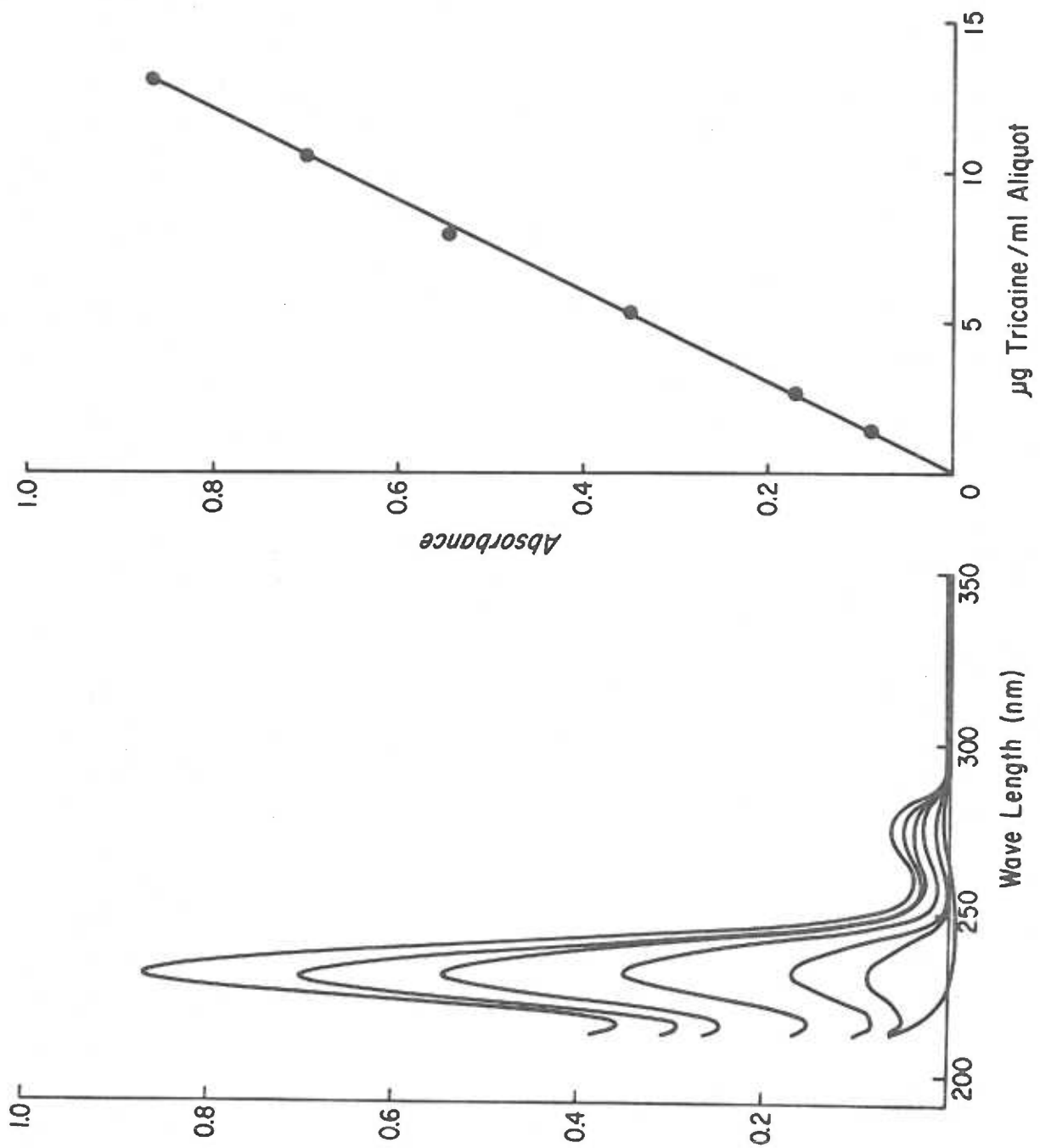


Figure 3: Absorption Spectra and Standard Curve of the Ultraviolet Assay for Quantification of Tricaine

The absorption maxima in the ultraviolet (UV) spectrum of tricaine in 1N HCl are at 274 nm and 228 nm. The absorption maximum at 228 nm was used to quantify tricaine and, at this wavelength, the molar extinction coefficient of tricaine methanesulfonate is 1.16×10^4 .

of 0.5 gm% ammonium sulfamate, 1 ml of methanol and 0.5 ml of 0.1 gm% N-1-naphthylethylenediamine dihydrochloride were added. After the addition of each reagent, the sample was mixed on a Vari-Whirl mixer (VWR Scientific, Portland, Or.) for 5 sec. The reaction produced an azo dye with an absorption maximum at 545 nm (A_{545}). The tricaine-azo dye was quantified 30 min after addition of the last reagent by scanning the spectrum of the dye from 700 nm to 400 nm using 1 N HCl as the reference and measuring the A_{545} . The molar extinction coefficient of the dye, expressed in terms of tricaine, is 4.88×10^4 at 545 nm.

Tricaine was measured by both the UV and colorimetric procedures in all studies and the results were in agreement. The colorimetric assay depends upon the presence of the primary aromatic amine group on the tricaine molecule. A discrepancy between the two assays would have indicated the presence, in the HCl extract, of a metabolite which was either substituted on the amine group (e.g., an acetylated compound) or deaminated. The specificity of both assays was also checked by CCD (see below). Moreover, it was noted that in timed kinetic studies in whole animals and tissue incubations, the concentration of tricaine approached zero, if metabolism was allowed to proceed long enough, thus proving that the assays were specific for tricaine and that metabolites were not interfering with either assay.

3. Colorimetric Assay for Tricaine Metabolites

The metabolites of tricaine were quantified in the tissue homogenate by the Bratton-Marshall reaction (see Appendix II) after tricaine had been extracted from the homogenate with 1-chlorobutane. Although MABA was the major metabolite in both whole animal studies and tissue

incubations of tricaine, the Bratton-Marshall assay for metabolites of tricaine was not specific for MABA and measured all metabolites which retained the primary aromatic amine portion of the drug molecule. The Bratton-Marshall reacting metabolites (BMRM) were quantified in terms of MABA equivalents. After the 1-chlorobutane extraction, the tissue homogenate was diluted 1:3 with 15 gm% TCA, shaken for five min and centrifuged at 1500 rpm for 10 min; 0.5 ml of 0.1 gm% sodium nitrite was added to 3 ml of the supernatant and this solution was allowed to stand for 15 min; 0.5 ml of 0.5 gm% ammonium sulfamate and 0.5 ml of 0.1 gm% N-1-naphthylethylenediamine dihydrochloride were then added. The sample was thoroughly mixed after each addition. (Methanol was not included because it decreased the solubility of the azo dye produced). The resulting dye, with an absorption maximum at 545 nm (A_{545}), was used to quantify the tricaine metabolites 30 min after the addition of the last reagent. The molar extinction coefficient of the dye, expressed in terms of MABA, is 4.47×10^4 at 545 nm. The absorption spectra and standard curve of MABA measured by this assay in whole homogenate are shown in Figure 4.

B. Specificity of the Assays

1. Countercurrent Distribution (CCD) of Tricaine

The specificity of the UV and colorimetric assays was checked by countercurrent distribution (CCD) as described by Bush and Densen (1948) and Bush and Post (1969) (See Appendix III). A ten tube CCD machine was used. Figure 5 illustrates the CCD of 3 mg of tricaine methanesulfonate in a CCD solvent system of isooctane (10 ml) and 0.2 M

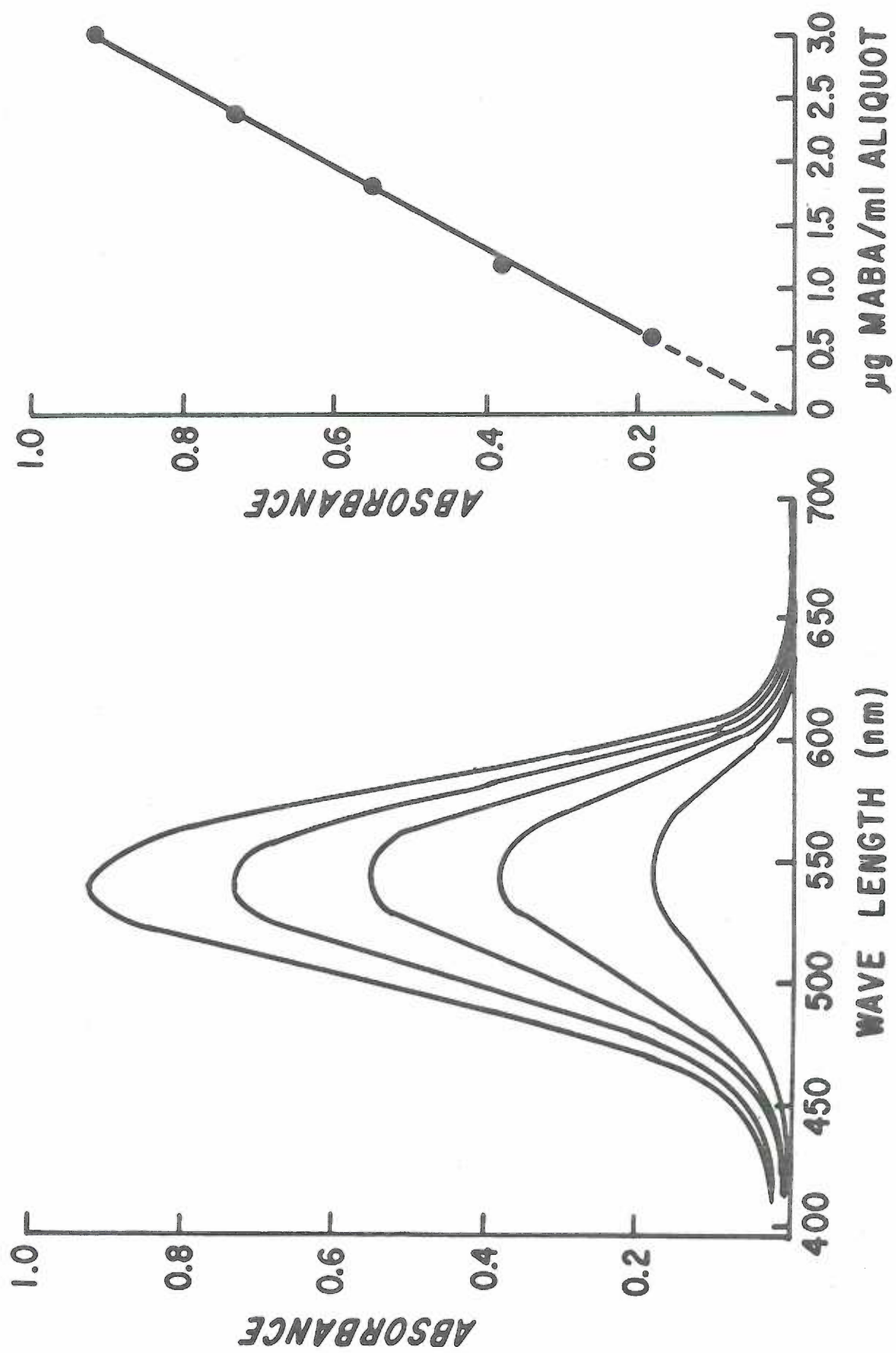


Figure 4: Absorption Spectra and Standard Curve of the Bratton-Marshall Colorimetric Assay for Quantification of Metabolites of Tricaine

The absorption maximum of the azo dye produced by the Bratton-Marshall reaction is at 545 nm and, at this wavelength, the molar extinction coefficient of the dye, expressed in terms of MABA, is 4.47×10^4 . The standard curve of the Bratton-Marshall assay for quantification of tricaine is very similar to Figure 4 except for the diluting effect of the methanol on the overall sensitivity. The absorption maximum of the azo dye produced from tricaine by the Bratton-Marshall reaction is also at 545 nm and, at this wavelength, the molar extinction coefficient of the dye, expressed in terms of tricaine, is 4.88×10^4 .

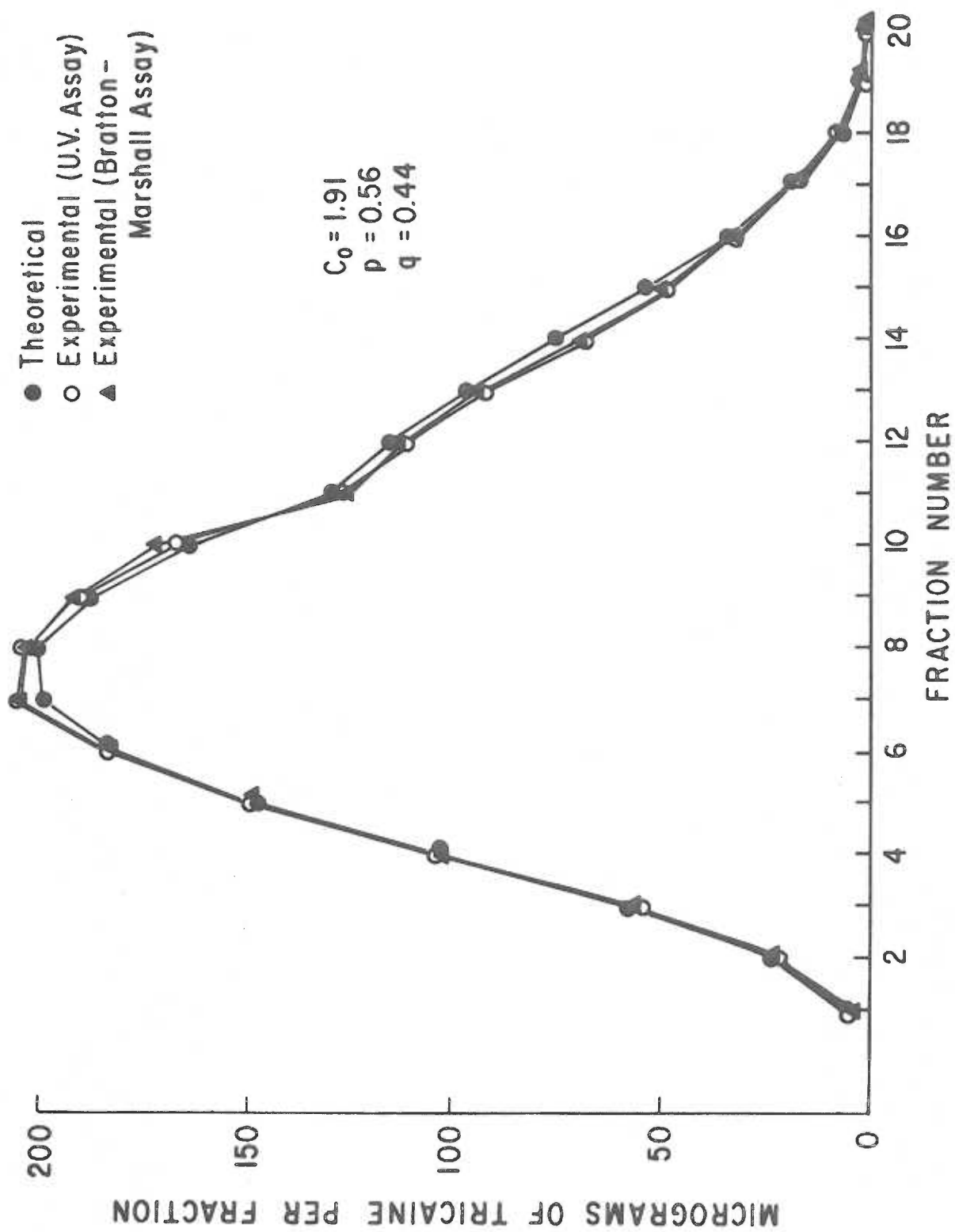


Figure 5: Countercurrent Distribution (CCD) of 3 mg of Tricaine Methanesulfonate

The ten tube CCD was performed between isooctane (10 ml) and 0.2 M phosphate, pH 7.4 (15 ml). Tricaine distributes with a p value of 0.56. The p value for tricaine is calculated from the partition coefficient (C_o) by the equation: $p = [C_o V_x / (C_o V_x + V_y)]$ where V_x is the volume of the organic layer and V_y is the volume of the aqueous layer. Fractions 1-10 are isooctane and 11-20 are the aqueous fractions, left in the machine, and numbered in reverse order: the first 20, the second 19, etc.

phosphate buffer, pH 7.4 (15 ml). The two solvents were equilibrated by shaking 300 ml of each together for 30 min before the CCD procedure was begun. Fifteen ml of the phosphate buffer were placed in each tube of the CCD apparatus (aqueous fractions A_1 - A_{10}) and 3 mg of the drug were dissolved in the phosphate buffer fraction in the first tube of the apparatus (A_1). Ten ml aliquots of isooctane (organic fractions) were then added to the first three tubes of the CCD apparatus. The aliquot of isooctane in the first tube represented the first (O_1) of ten organic fractions to be used in the CCD. The aliquots of isooctane in the second and third tubes served as blanks and as isooctane "rinses" to wet the glass tubes of the CCD machine. Without the blanks to dampen the tubes, a significant quantity of the O_1 fraction would have remained on the walls of the ten tubes of the CCD apparatus and, thereby, would have altered the final distribution of tricaine. The three isooctane fractions (O_1 and the two blanks) were equilibrated with the aqueous fractions in the first three tubes of the CCD machine (A_1 - A_3 , respectively). Equilibration involved rocking the CCD machine for three min to disperse one phase within the other. The machine was then left undisturbed for three min to allow the phases to re-separate. The three isooctane fractions (O_1 and the two blanks) were transferred to the succeeding tubes (tubes 2-4 of the apparatus) and 10 ml of isooctane (O_2) were introduced into the first tube of the machine. The four organic fractions (O_2 , O_1 and the two blanks) were equilibrated with the aqueous fraction (A_1 - A_4 , respectively) and allowed to re-separate. Each of the four organic fractions was then transferred to the succeeding tube and another 10 ml aliquot of isooctane (O_3) was added to the first

tube. The process of (1) equilibrating the organic fractions with the aqueous fractions, (2) allowing the two phases in each tube to re-separate, (3) transferring the organic fractions to succeeding tubes, and (4) adding another aliquot of isooctane to the first tube, was repeated until the O_1 fraction had been transferred to the tenth tube; the O_2 fraction had been transferred to the ninth tube; the O_3 fraction had been transferred to the eighth tube, etc. (the "blank" aliquots of isooctane had been equilibrated with each of the aqueous fractions in succession and then transferred from the CCD machine to capped centrifuge tubes). At this stage of the CCD procedure, each of the tubes (1-10) of the CCD apparatus contained the respective aqueous fraction (A_1 - A_{10}) and an organic fraction that had been successively equilibrated with each of the preceding aqueous fractions. The process of equilibration, separation, and transferral was then continued without additional aliquots of isooctane being introduced into the first tube. Each organic fraction, after equilibration with and separation from the aqueous fraction in the tenth tube (A_{10}), was transferred from the CCD apparatus to a capped centrifuge tube.

The tricaine in the isooctane (organic) fractions was quantified by three separate assays. (1) Tricaine in isooctane has absorption maxima at 314 nm (A_{314}), 240 nm (A_{240}) and 219 nm (A_{219}), and the respective extinction coefficients for tricaine are 2.67×10^3 , 8.62×10^3 , and 3.04×10^4 . Tricaine was quantified directly in the isooctane using isooctane as the reference and measuring the A_{219} ; (2) Nine ml of each organic fraction were extracted with 5 ml of 1N HCl. The tricaine concentrations were quantified in the HCl extracts using

HCl as the reference and measuring the A_{228} . (3) The tricaine in the HCl extracts was also quantified with the Bratton-Marshall reaction.

The drug in each of the phosphate buffer (aqueous) fractions was measured by three different assays. (1) Tricaine in phosphate buffer has absorption maxima at 314 nm (A_{314}) and 219 nm (A_{219}), and the respective extraction coefficients for tricaine are 2.09×10^3 and 2.61×10^4 . Tricaine was quantified directly in the phosphate buffer using the buffer as reference and measuring the A_{219} ; (2) Nine ml of each buffer fraction were extracted with 30 ml of 1-chlorobutane and 25 ml of the latter were extracted with 5 ml of 1N HCl for quantification by the UV assay; (3) The tricaine in the HCl extracts was also quantified with the Bratton-Marshall reaction.

The three separate assays for tricaine used on each of the fractions (organic and aqueous) of the CCD produced similar results (Figure 5) and the C_o of tricaine in the isooctane:phosphate buffer, pH 7.4, system is 1.91.

2. Assays for Tricaine

An incubation consisting of 3 ml of human serum, 3 ml of 0.2 M phosphate buffer, pH 7.4, and 3 ml of a solution of tricaine methane-sulfonate in phosphate buffer (3 mg/ml) was prepared. The serum and phosphate buffer were placed in a 50 ml Erlenmeyer flask containing eight 6 mm glass beads and warmed in the incubator at 37.5° C for 15 min. The tricaine solution was also warmed. After the addition of the tricaine solution to the incubation flask (0 time), 1 ml samples were removed from the flask at 0, 105 and 210 min. Each of these samples was extracted with 30 ml of 1-chlorobutane; 15 ml of the latter were

extracted with 15 ml of 1N HCl and the HCl extracts were analyzed for tricaine content; $A_{228} = 1.427$ (0 min); $A_{228} = 1.042$ (105 min); $A_{228} = 0.669$ (210 min). Immediately after the removal of the 210 min sample from the flask, the remaining 6 ml of the incubation were extracted with 180 ml of 1-chlorobutane. Five ml of the 1-chlorobutane extract was extracted with 5 ml of 1N HCl ($A_{228} = 0.679$). One hundred and seventy ml of the 1-chlorobutane extract were evaporated to dryness in vacuo in the rotary evaporator at room temperature. The residue in the rotary-evaporator flask after evaporation was then transferred to the first tube of the CCD machine by two washings with isooctane (5 ml/wash), followed by three washings with 0.2 M phosphate buffer, pH 7.4 (5 ml/wash). The CCD was performed, as described above, between isooctane (10 ml) and 0.2 M phosphate buffer, pH 7.4 (15 ml).

The tricaine content of the organic and aqueous fractions of the CCD were quantified as described above and Figure 6 shows the distribution of a single substance with $C_0 = 1.91$ in this CCD. The UV spectrum of the material in each of the 20 CCD fractions conformed exactly to that of tricaine. A comparison of Figures 5 and 6 indicated that the substance which was extracted from the incubation was tricaine and that there were no substances extracted with the 1-chlorobutane which interfered with the tricaine assays (UV or colorimetric). The identity of the material in the CCD was also checked by gas chromatography and mass spectrometry. The conditions were as follows: injector temperature 250°C , detector temperature 250°C , carrier gas (N_2) flow rate 30 ml/min, temperature programming from 90°C at $10^{\circ}/\text{min}$. The hydrocarbon number (15.0) and the mass spectrum (Figure 7) of the material

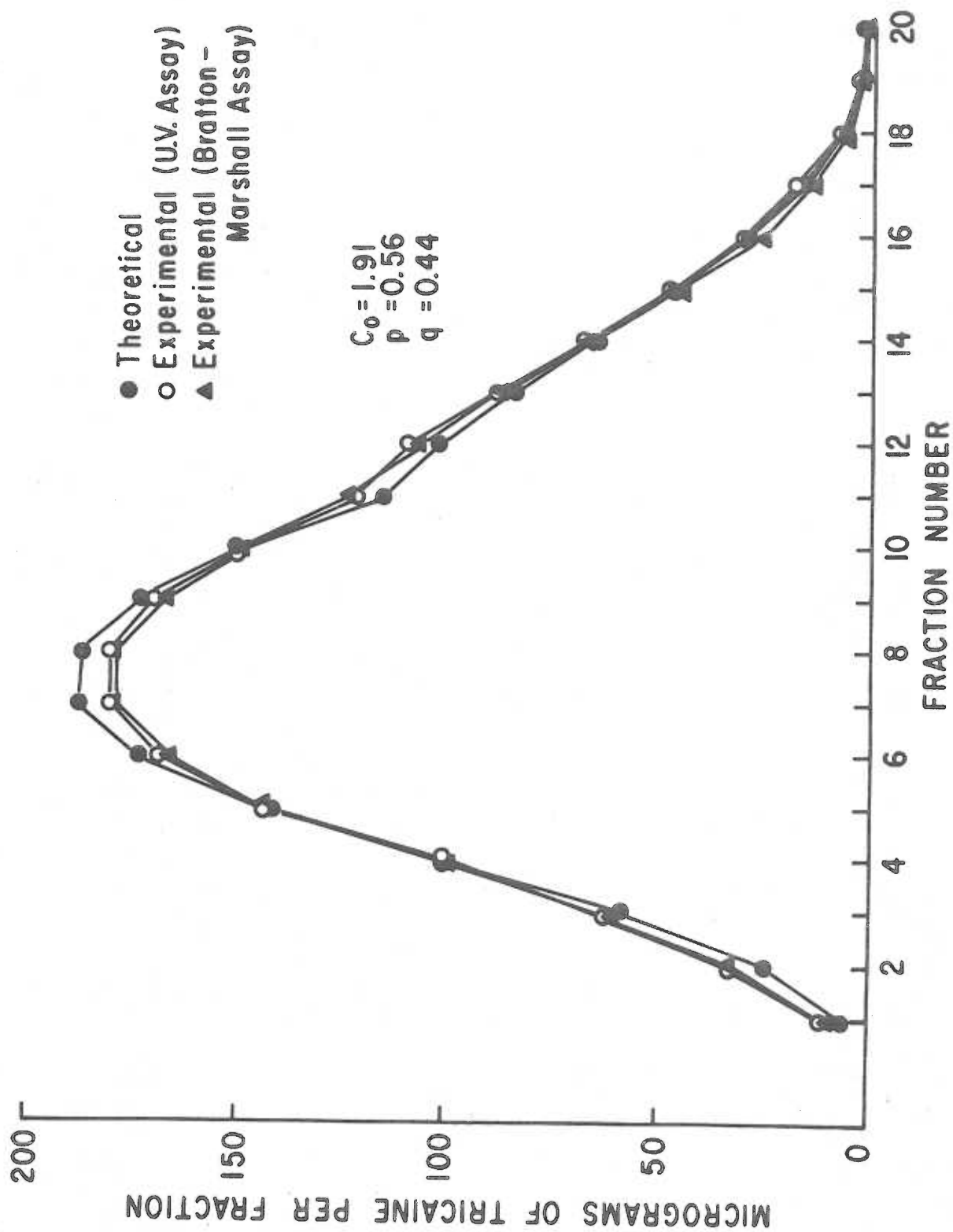


Figure 6: CCD of the 1-Chlorobutane Extract of a Serum Incubation

An incubation of tricaine with human serum was extracted with 1-chlorobutane 210 min after the addition of the drug solution to the incubation flask. A CCD was then performed on the 1-chlorobutane extract residue between isooctane (10 ml) and 0.2 M phosphate buffer, pH 7.4 (15 ml). The compound extracted from the incubation by 1-chlorobutane distributes in this CCD system with the same C_0 as does tricaine. The identity of this substance was further verified by UV spectral, gas chromatographic and mass spectral analyses.

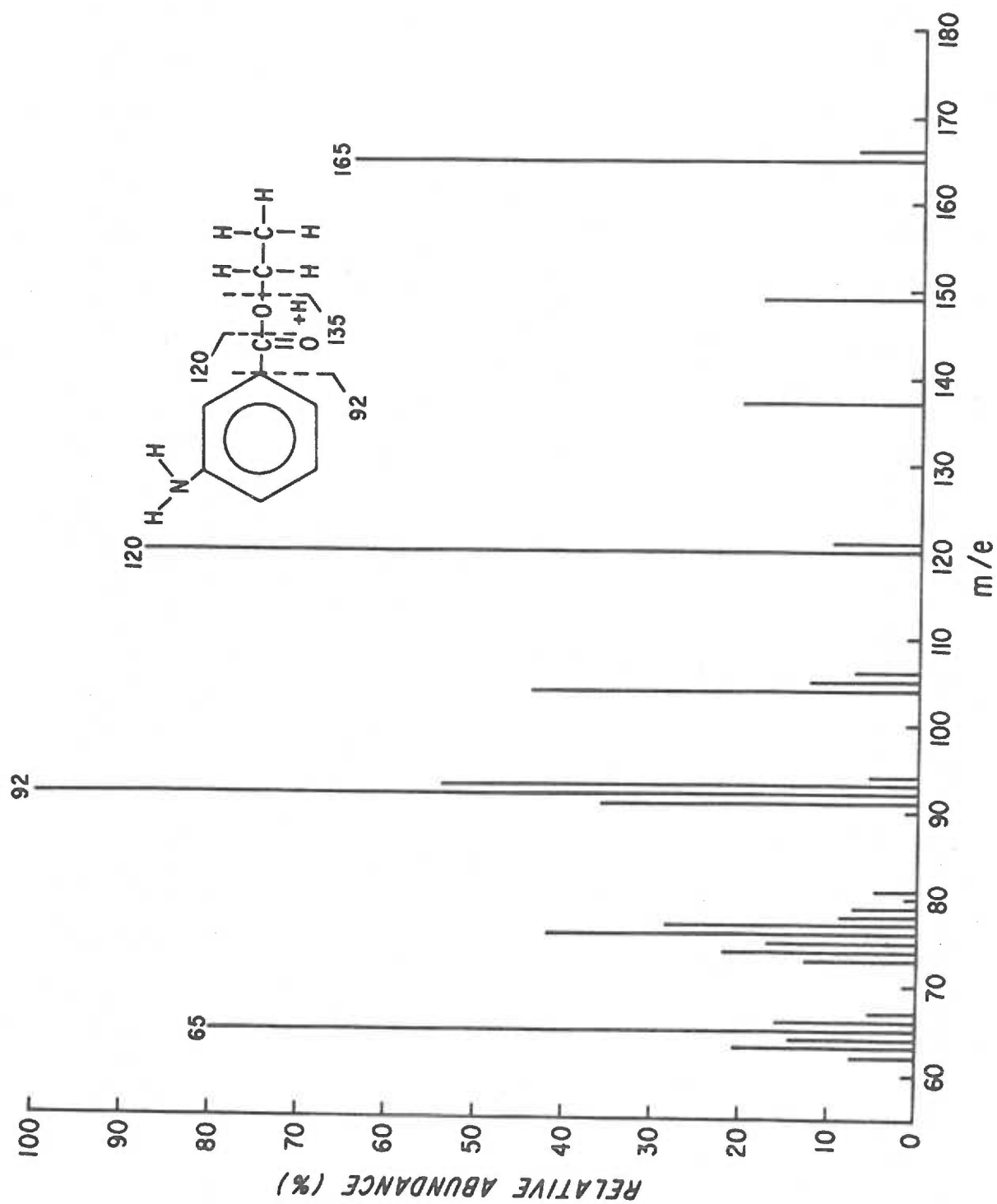


Figure 7: Mass Spectrum of the Compound in the 1-Chlorobutane Extract of a Serum Incubation of

Tricaine

This compound, tricaine, was recovered from a serum incubation of tricaine methanesulfonate by extraction with 1-chlorobutane and a CCD of that extract. The above spectrum is that of the material contained in fraction #8 of Figure 6.

in the CCD were identical to those of tricaine (Figure 8).

The remaining 5 ml of the 1-chlorobutane were aspirated off the 6 ml of incubation mix and the latter was re-extracted with 40 ml of 1-chlorobutane; the 1-chlorobutane extract was extracted with 5 ml of 1N HCl for UV analysis ($A_{228} = 0.284$). This indicated that 99+% of the tricaine had been extracted with the first 1-chlorobutane wash (180 ml).

3. Assay for Tricaine Metabolites in Serum Incubations

After the extraction of the above incubation with 1-chlorobutane, 24 ml of 0.2 M phosphate buffer, pH 4.3, and 1 ml of 1N HCl were added to the incubation to bring the pH to 4.4; 1 ml of this solution was diluted 1:29 with 15 gm% TCA and analyzed by the Bratton-Marshall assay for metabolites ($A_{545} = 0.380$). The remainder of the incubation mix (30 ml) was extracted three times with 180 ml of ethyl acetate; 530 ml of the ethyl acetate were recovered. A 25 ml aliquot of the ethyl acetate extract was extracted with 0.2 M phosphate buffer, pH 7.4, for colorimetric analysis ($A_{545} = 0.887$). The remainder of the ethyl acetate extract (505 ml) was evaporated to dryness in vacuo in the rotary evaporator at room temperature. The residue in the rotary-evaporator flask after evaporation was then transferred to the first tube of the CCD apparatus by three washings with ethyl acetate (10 ml/wash), followed by three washings with 0.2 M phosphate buffer, pH 5.5 (5 ml/wash). The CCD was performed between ethyl acetate (30 ml) and 0.2 M phosphate buffer, pH 5.5 (15 ml). Twenty five ml of each organic fraction (ethyl acetate) were extracted with 5 ml of 0.2 M phosphate buffer, pH 7.4. The Bratton-Marshall assay for tricaine

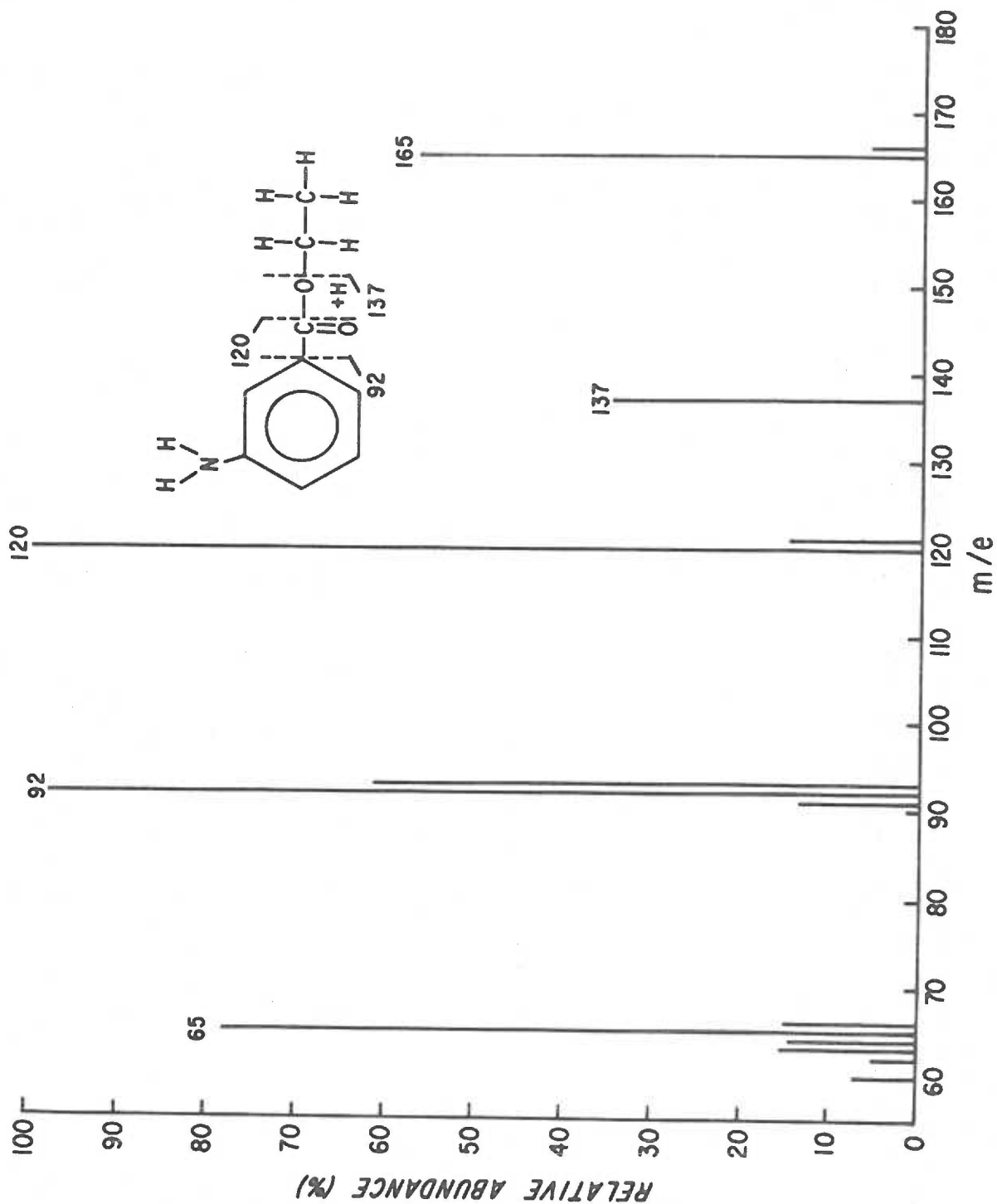


Figure 8: Mass Spectrum of Tricaine

A solution of tricaine methanesulfonate in distilled water was extracted with 1-chlorobutane. The 1-chlorobutane extract was then evaporated by impingement with N_2 and the residue redissolved in chloroform for gas chromatograph-mass spectral analysis.

metabolites was performed on the buffer extract of each organic fraction and directly on each aqueous fraction of the CCD. Figure 9 shows the distribution of a single substance with a C_o of 0.89 in this CCD. A similar ten tube CCD of 1.5 mg of MABA between ethyl acetate (30 ml) and 0.2 M phosphate buffer, pH 5.5 (15 ml) is shown in Figure 10; the C_o of MABA in this system is 0.89. Comparison of Figures 9 and 10 indicated that MABA was the only metabolite of tricaine in the serum incubation with tricaine.

The incubation, after extraction with 1-chlorobutane and ethyl acetate, was analyzed for any remaining Bratton-Marshall reacting metabolites (BMRM) and less than 1% of the original 3 mg of tricaine methanesulfonate was recovered.

4. Assay for Tricaine Metabolites in Whole Mouse Homogenates

The specificity of the colorimetric assay for tricaine metabolites in the whole mouse was studied as follows: 5 min after i.p. injection of 5 mg of the drug, a 22 gm mouse was homogenized in 300 ml of absolute ethanol for 3 min. The homogenate was filtered through glass filter paper under suction. The homogenate residue and the filter paper were re-homogenized in 300 ml of 95% ethanol and filtered under suction through glass filter paper. The re-homogenation process was repeated 4 more times to give a final volume of 2030 ml of ethanol extract and water (including the volume of ethanol used to rinse the blender and glassware). A 10 ml aliquot of the ethanol was evaporated to dryness by impingement with N_2 and then brought to a volume of 5 ml with phosphate buffer for quantification by the Bratton-Marshall assay for tricaine metabolites ($A_{545} = 0.199$). The remaining ethanol

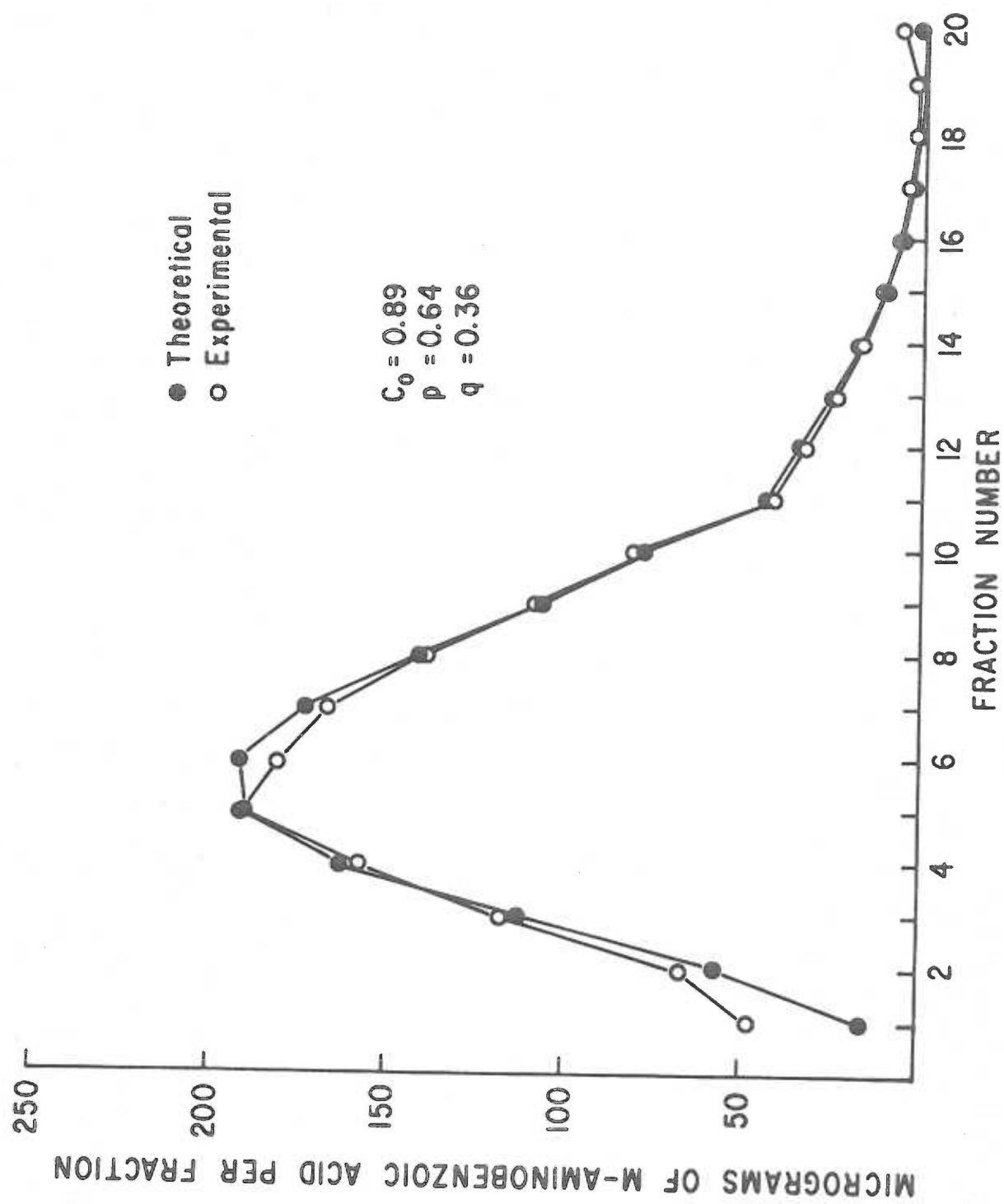


Figure 9: Ten Tube CCD of the Ethyl Acetate Extract of a Serum Incubation of Tricaine Methanesulfonate

The serum incubation of tricaine methanesulfonate, which had previously been extracted with 1-chlorobutane, was extracted with ethyl acetate. The ethyl acetate extract was evaporated and a CCD of the residue was performed between ethyl acetate (30 ml) and 0.2 M phosphate, pH 5.5 (15 ml). MABA, the only metabolite of tricaine present in the serum incubation, distributes with a C_o of 0.89.

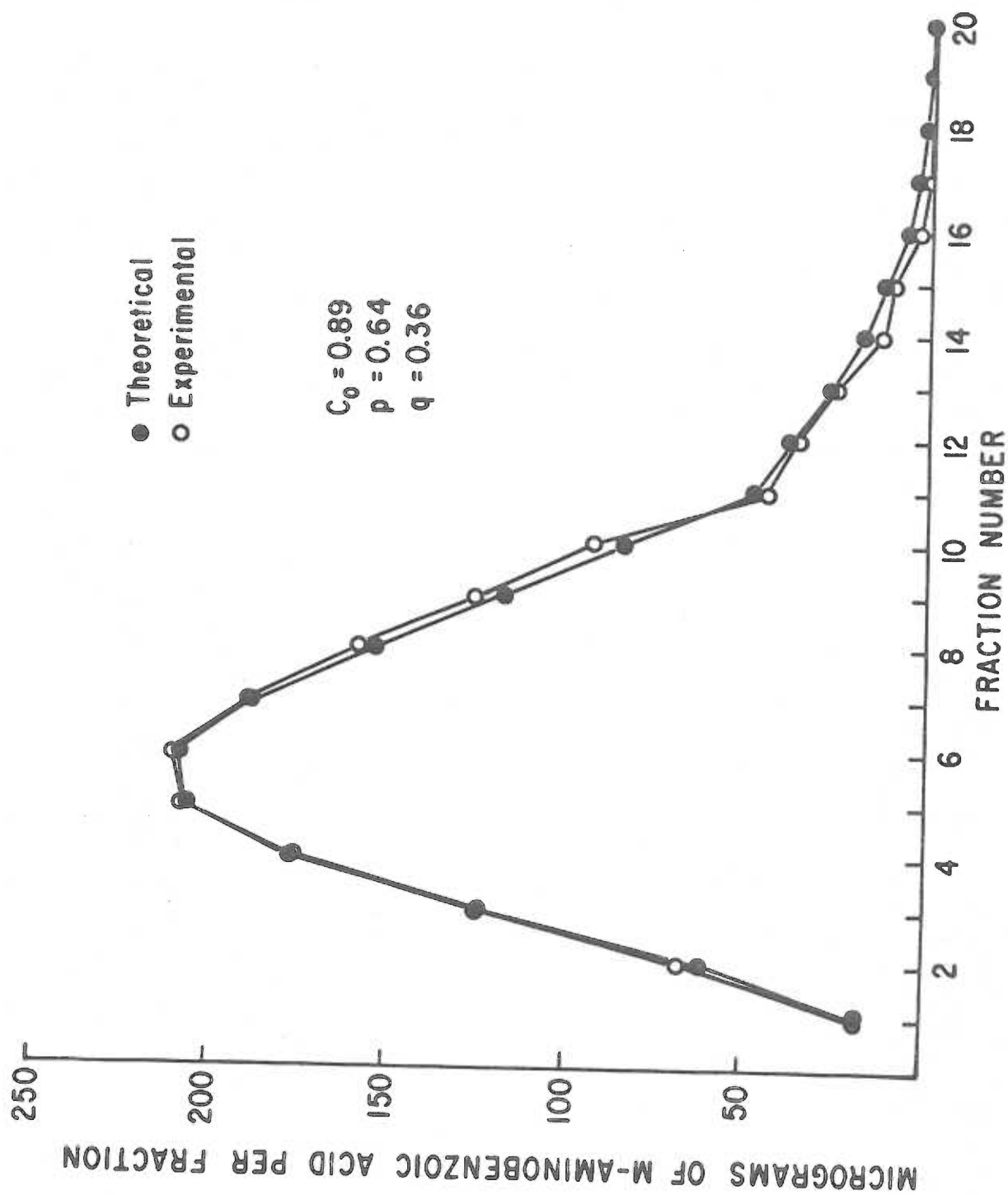


Figure 10: Ten Tube CCD of 1.5 mg of MABA

The CCD was performed between ethyl acetate (30 ml) and 0.2 M phosphate, pH 5.5 (15 ml), as in Figure 9. The C_0 of synthetic MABA, 0.89, was the same as that for the biologically derived MABA in Figure 9.

(2020 ml) was evaporated to dryness on a rotary evaporator in vacuo at room temperature using co-distillation with toluene to remove the residual water. The contents of the rotary-evaporator flask after evaporation were transferred to the first tube of the ten tube CCD apparatus by three washings with ethyl acetate (10 ml/wash), followed by three washes at 0.2 M phosphate buffer, pH 5.5 (5 ml/wash). The CCD was performed between ethyl acetate (30 ml) and 0.2 M phosphate buffer, pH 5.5 (15 ml). A 25 ml aliquot of each organic fraction (ethyl acetate) was extracted with 5 ml of 0.2 M phosphate buffer, pH 7.4. The Bratton-Marshall assay for tricaine metabolites was performed on the phosphate buffer extracts of each organic fraction and directly on each aqueous fraction of the CCD. Figure 11 shows the resulting distribution; there were two distinct peaks in the CCD, one confined primarily to the organic fractions (1-10) which exhibited a constant C_o of 0.89. (Comparison with Figure 10 indicates that this material was MABA). The second peak did not exhibit a constant C_o value (See Appendix III) which indicated the presence of, at least two, more polar, water soluble metabolites of tricaine. Figure 11 shows that the tricaine had almost completely disappeared from the mouse in 5 min and had appeared, quantitatively, as BMRM (Bratton-Marshall reacting metabolites).

The residual mouse homogenate and glass filter paper remaining after the final extraction with 95% ethanol were homogenized in 150 ml of distilled water. The homogenate was then centrifuged and the supernatant was analyzed by the Bratton-Marshall assay for tricaine metabolites. Less than 1% of the original 5 mg of tricaine methanesulfonate

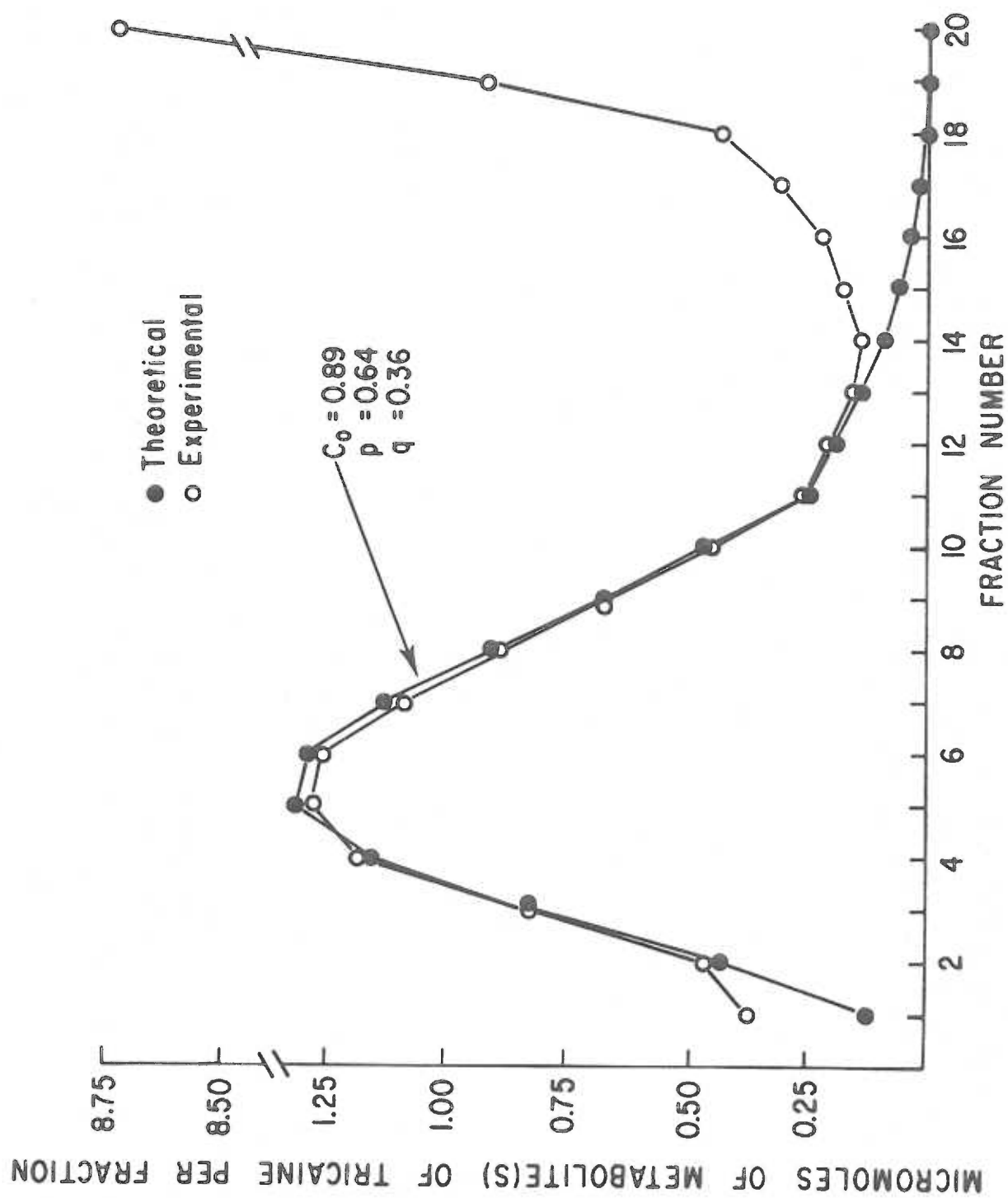


Figure 11: CCD of the Ethanol Extract of a Mouse Sacrificed 5 Min after I.P. Injection of 5 mg of Tricaine Methanesulfonate

Ten tube CCD was performed between ethyl acetate (30 ml) and 0.2 M phosphate, pH 5.5 (15 ml), as in Figure 10. MABA distributes with a C_o value of 0.89 and at least two other polar metabolites, measured as equivalents of MABA, are present in aqueous fractions 14-20.

was recovered.

C. Purity of the Ayerst Product: Tricaine Methanesulfonate (Finquel^R)

The CCD of tricaine methanesulfonate (Figure 5) suggested that the Ayerst product was 99+% chemically pure. Tricaine methanesulfonate (Ayerst) and ethyl m-aminobenzoate (Eastman) had identical UV spectra and very similar molar extinction coefficients (1.16×10^4 and 1.15×10^4 , respectively). Neither the CCD technique nor the UV comparison exclude the presence of contaminants with UV spectra and C_o 's in the isooctane:phosphate buffer CCD system which are similar to those of tricaine, e.g., ethyl p-aminobenzoate.

Gas chromatography is capable of separating the three isomers of ethyl aminobenzoate. A mixture of approximately equal amounts of the para-, meta- and ortho- isomers of ethyl aminobenzoate in chloroform was analyzed gas chromatographically with the following conditions: injector temperature 250° C, detector temperature 350° C, carrier gas (N₂) flow 30 ml/min, temperature programming from 90° C at 10°/min. The resulting gas chromatographic tracing (Figure 12) shows the separation of the three isomers; the hydrocarbon numbers are: ortho- 13.8, meta- 15.0, and para- 15.6. Tricaine methanesulfonate (Finquel^R - Ayerst) was dissolved in distilled water and extracted with 1-chlorobutane; the 1-chlorobutane was evaporated by impingement with N₂. The extract residue was re-dissolved in chloroform and analyzed by gas chromatography. Figure 13 shows the single peak of tricaine (hydrocarbon number 15.0), indicating that the purity of the Ayerst product was greater than 99.9%. Similar results were obtained with the free base

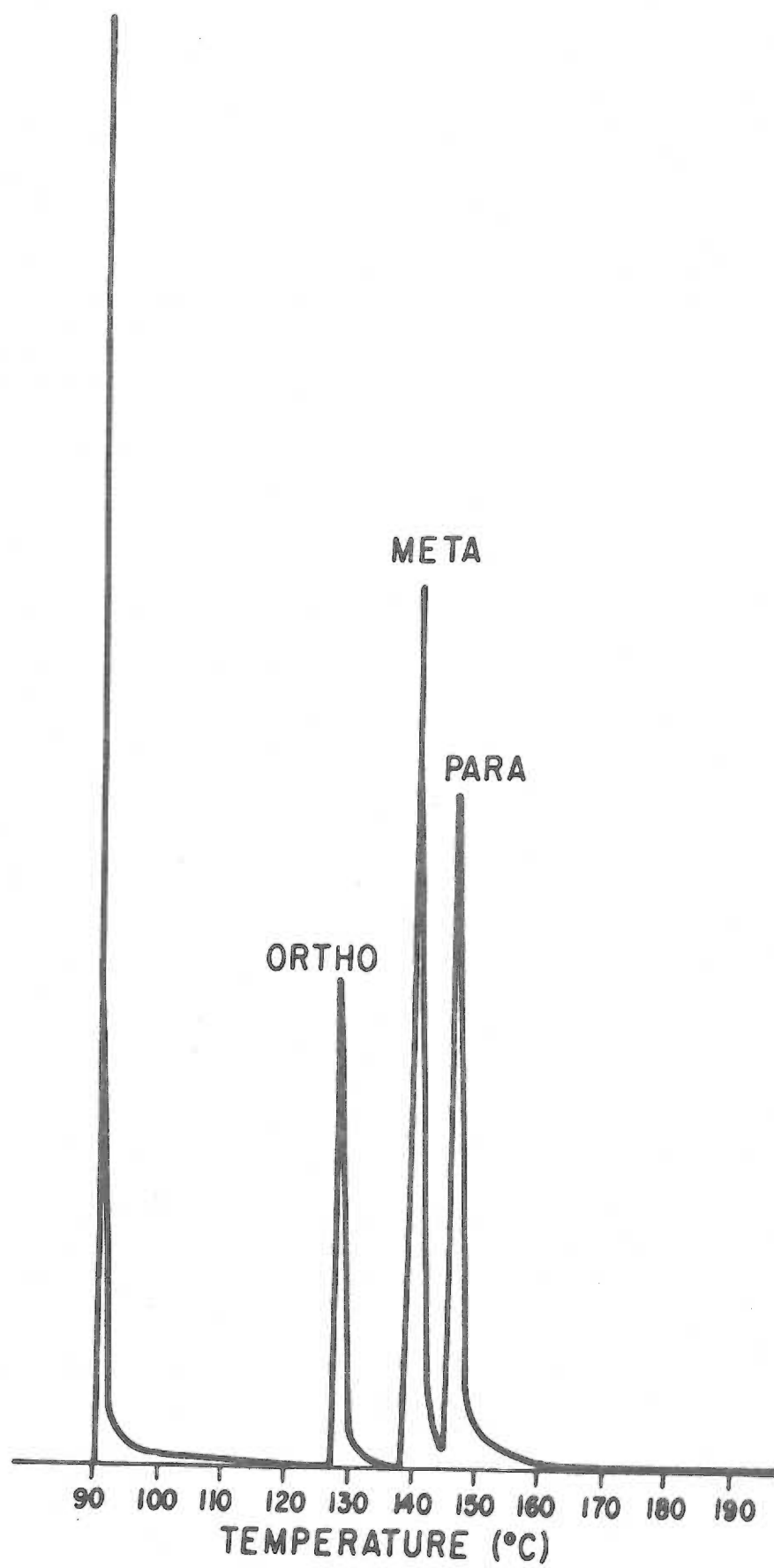


Figure 12: Gas Chromatographic Separation of the Three Isomers of Ethyl Aminobenzoate

The tracing shows the separation of the three isomers of ethyl aminobenzoate, para-, meta- and ortho-. The three compounds, in their free base forms, were dissolved in a common aliquot of chloroform. The hydrocarbon numbers of the three isomers were: ortho- 13.8 (127°), meta- 15.0 (140°), and para- 15.6 (146°). The numbers in parentheses denote the approximate temperature of the oven at time of detection.

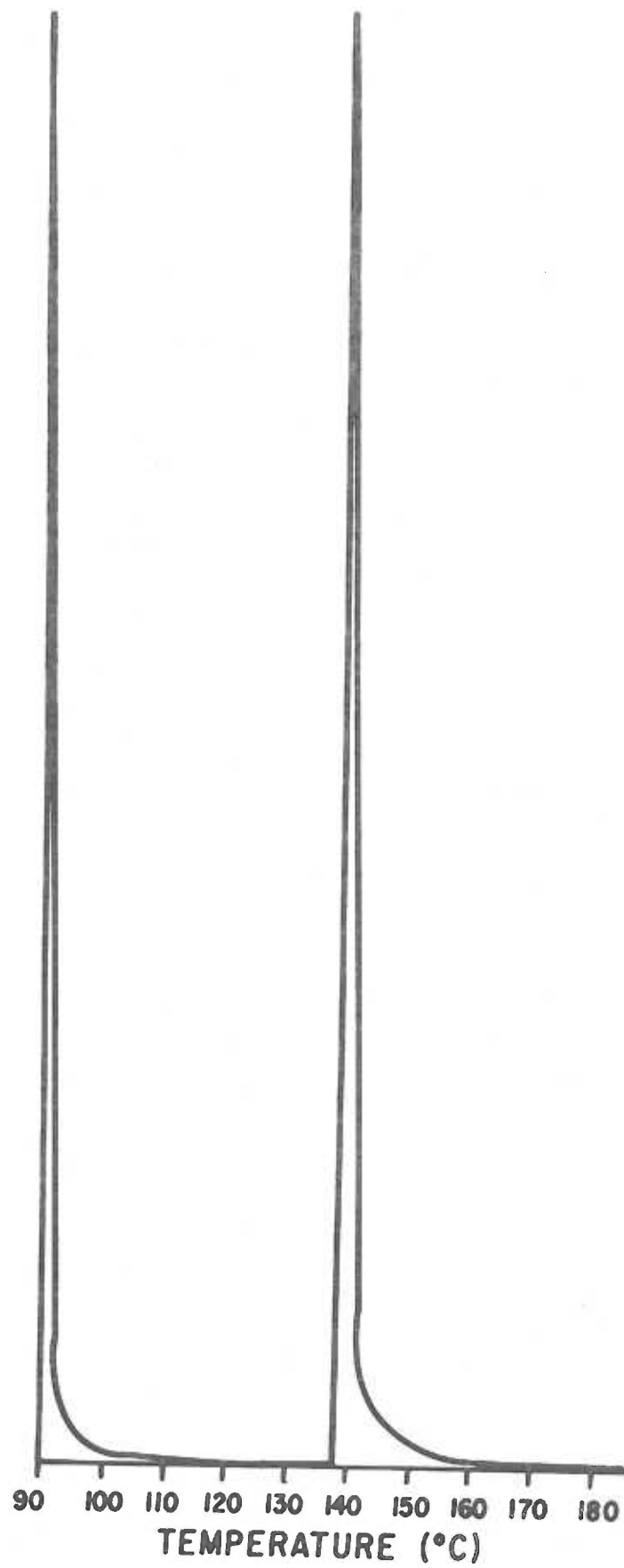


Figure 13: Gas Chromatographic Analysis of the Tricaine Methanesulfonate Product from Ayerst Labs (Finquel^R)

A solution of tricaine methanesulfonate (Ayerst) was extracted with 1-chlorobutane. The 1-chlorobutane was evaporated by impingement with N₂ and the residue redissolved in chloroform and analyzed. The presence of the single substance, tricaine (hydrocarbon number 15.0), indicated that the Ayerst product, Finquel^R, was chemically pure (greater than 99.9%).

forms of the three isomers obtained from Eastman Kodak Corp.

D. Stability of Tricaine in HCl

The stability of tricaine in 1N HCl was measured to determine the rate of acid hydrolysis of the drug. Tricaine methanesulfonate (400 mg) was dissolved in 100 ml of 1N HCl and stored in a clear glass flask on a windowsill at room temperature. Approximately every three days this stock solution was assayed for tricaine in the following manner: 0.1 ml of the stock solution was added to 4.9 ml of 0.2 M dibasic sodium phosphate, pH 4.3, and the 5 ml sample (pH approximately 7.4) was extracted with 1-chlorobutane (30 ml); 15 ml of the latter were extracted with 5 ml of 1N HCl for quantification of tricaine content by the UV assay. Figure 14 shows the graphic plot of the acid hydrolysis of tricaine with time. In all experiments described in this thesis, the quantification of tricaine was completed within 4 hr of extraction of the drug into 1N HCl. Therefore, the effect of acid hydrolysis on the determination was negligible.

E. pKa of Tricaine

The pKa value for tricaine was determined at 24° C by titration with phosphoric acid. Ten mg of tricaine methanesulfonate were dissolved in 100 ml of 0.1 M phosphate buffer, pH 6.74. This solution was titrated down to a low pH (1.95) in successive small increments with concentrated phosphoric acid. The neutral or nonionized form of tricaine (free base form) has absorption maxima at 313 nm, 240 nm and 219 nm, in contrast to the absorption maxima at 274 nm and 228 nm of the ionized form (Figure 15).

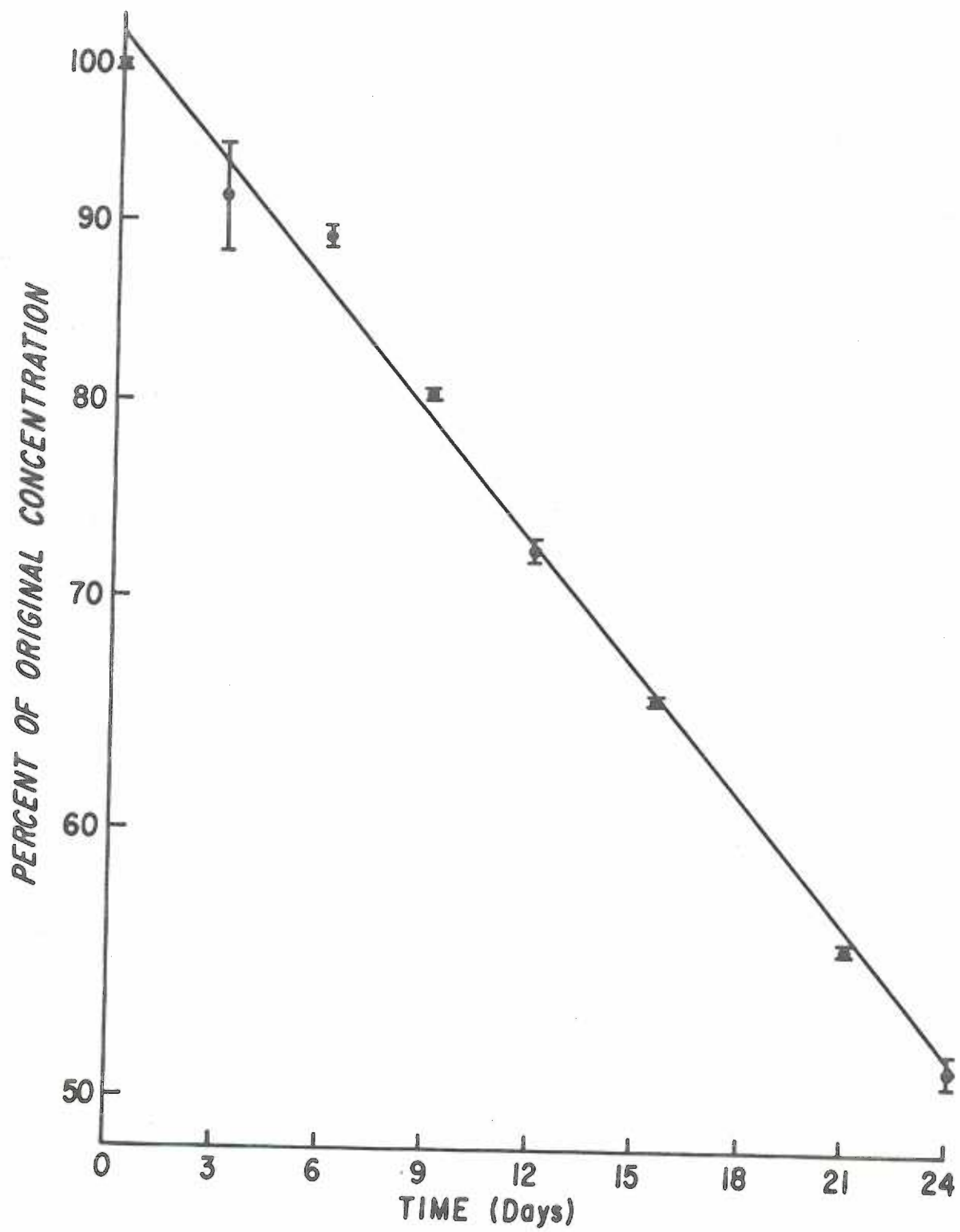


Figure 14: Stability of Tricaine in 1N HCl

Tricaine methanesulfonate was dissolved in 1N HCl and stored in a clear glass flask on a windowsill. This solution was analyzed periodically to monitor the amount of acid hydrolysis of the drug which took place with time. Each point represents the mean $\pm$ S.D. of four determinations of tricaine content in the stock solution.

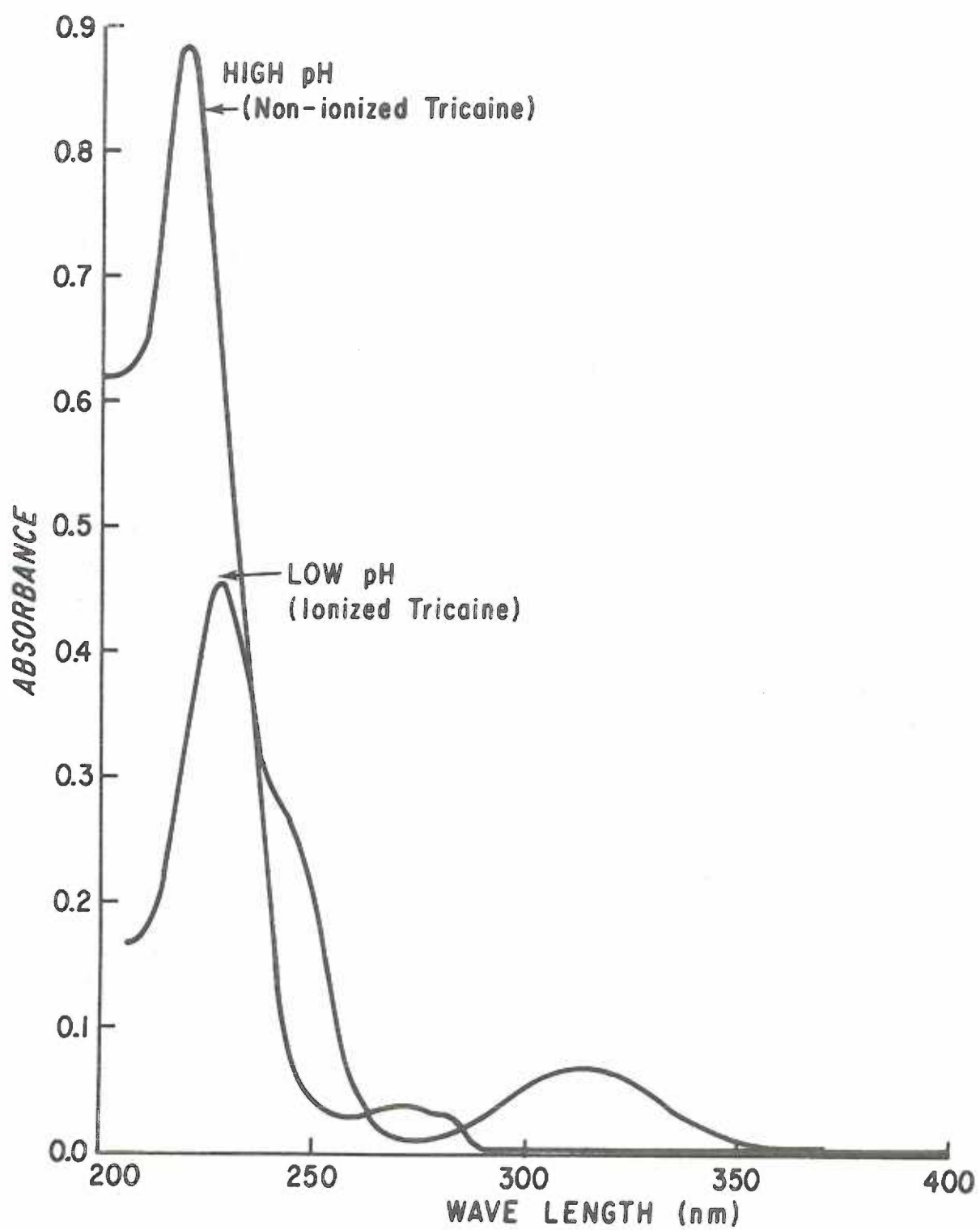


Figure 15: Ultraviolet Spectra of Tricaine in Solutions Having Low
and High pH

The pH of the solution in which tricaine is scanned spectrophotometrically greatly influences the spectral pattern of tricaine. The two, markedly different spectra, particularly at the 313 nm wavelength, allows for easy determination of the pKa of the drug (3.8 ± 0.1).

Therefore, with each titration step, the sample was analyzed at the single wavelength, 313 nm, and the optical density recorded. The pKa was then calculated from the relationship,

$$\text{pKa} = \text{pH} + \log \frac{(D_1 - D)}{(D - D_2)},$$

where D_1 is the optical density of the anion, D is the optical density at the pH under investigation, and D_2 is the optical density of the neutral molecule (Butler *et al.* 1955). The pKa of tricaine was found to be 3.8 ± 0.1 (S.D.) and agreed with the value (3.5) reported by Maren *et al.* (1968a).

F. Synthesis of Ethyl M-Aminobenzoate (Carboxyl- ^{14}C) Methanesulfonate
(See Figure 16)

Benzoic acid (carboxyl- ^{14}C) (33.4 mCi/mM) was mixed with unlabelled benzoic acid (Eastman). A mixture of sulfuric acid (98%, 0.5 ml) and nitric acid (70%, 0.5 ml) was added dropwise in 6 min to a stirred solution of ^{14}C -benzoic acid (0.4998 gm, 1 mCi) in 2 ml of sulfuric acid (98%) maintained at 10-15° C (Gerland 1854, Varma and Kulharni 1925). After the addition was completed, the solution was stirred for an additional 15 min at 10-15° C and then poured onto crushed ice. The precipitate was collected and washed with 2 ml of ice water and dried at 60° C. This crude precipitate was dissolved in absolute ethanol containing sulfuric acid (98%, 0.25 ml) and refluxed for 90 min (Chancel 1849). Six ml of methylene chloride was added to the cooled reaction mixture and the organic layer (methylene chloride) equilibrated

SYNTHESIS OF LABELLED TRICAINE METHANESULPHONATE

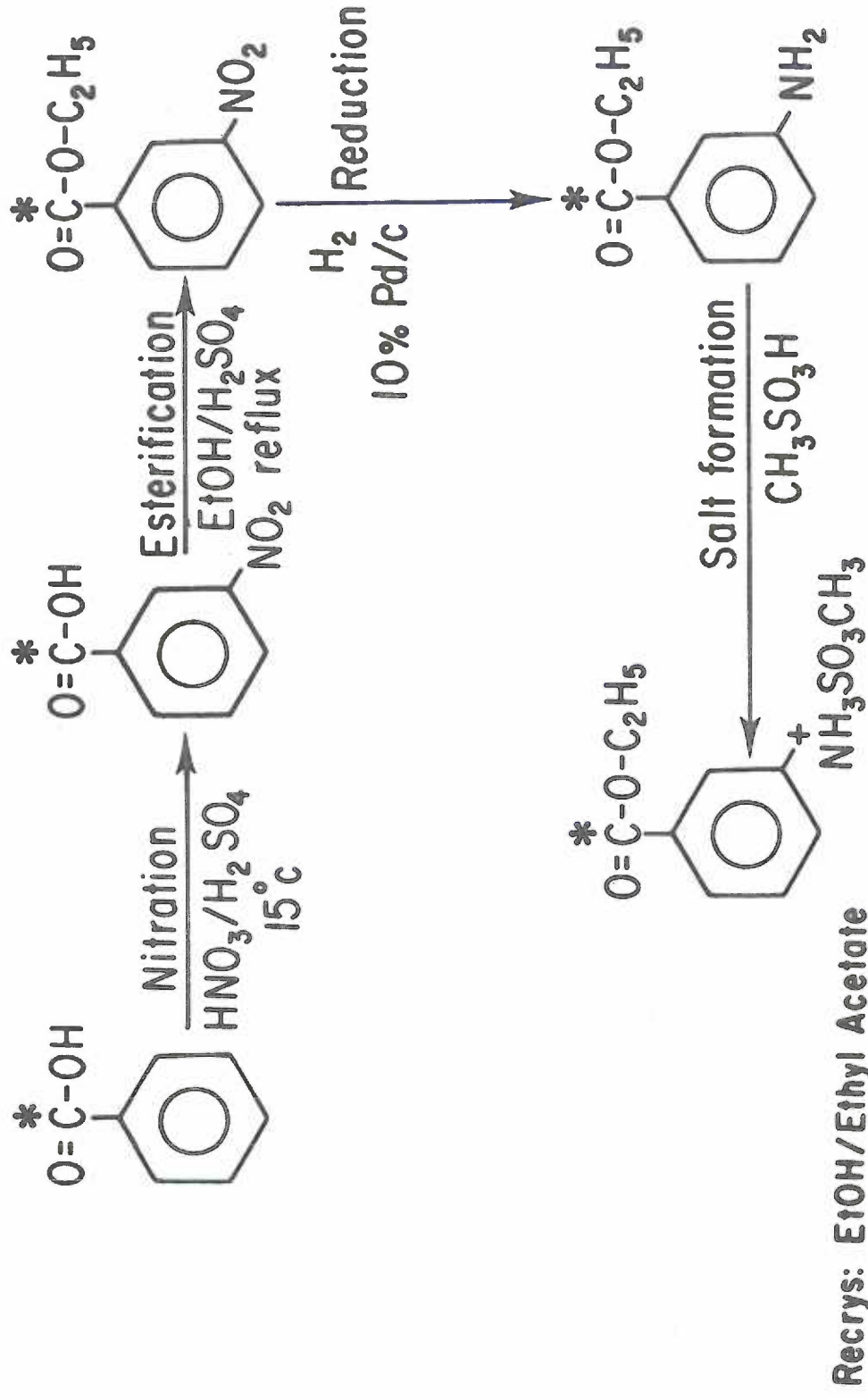


Figure 16: Methodology for the Synthesis of Radioactively Labelled Tricaine Methanesulfonate.

with water (2 ml x 2), with sodium carbonate (0.4 M, 2 ml x 2) and again with water (2 ml x 2). The organic layer was dried over anhydrous sodium sulfate and the methylene chloride distilled. The residue from the distillation was hydrogenated (Billeter *et al.* 1928) in absolute ethanol (5 ml) over 0.05 gm of 10% palladium on charcoal. The theoretical volume (135 ml) of hydrogen was consumed within 1 hr. Filtration of the reaction mixture and distillation of the ethanol yielded crude ethyl *m*-aminobenzoate (a liquid).

This crude product (approximately 1.1 ml), methanesulfonic acid (0.3 ml) and absolute ethanol (0.4 ml) were warmed on a steam bath for one min. Ethyl acetate (3.8 ml) was immediately added and the solution filtered. Refrigeration of the filtrate yielded a precipitate which was collected and washed with cold ethyl acetate (2 ml). This product (315 mg) was dissolved in boiling ethanol (0.4 ml), a double volume (0.8 ml) of ethyl acetate was added and the solution was filtered. Refrigeration of the filtrate precipitated a salt which was collected and dried to yield ^{14}C -labelled tricaine methanesulfonate. The identity of the product was confirmed by UV spectrophotometry (absorption maximum - 228 nm, extinction coefficient - 1.16×10^4), and gas chromatography (hydrocarbon number - 15). The purity of the product was determined to be greater than 99.9% by gas chromatography. The overall yield of ethyl *m*-aminobenzoate (carboxyl- ^{14}C) methanesulfonate was 19% (202 mg) with specific activity of 0.23 mCi/mM.

G. Pharmacokinetic Studies of Tricaine Methanesulfonate

1. Tricaine Paralysis Time

Frogs (Rana pipiens). Frogs (20-30 gm) became paralyzed about 30 sec after i.p. administration of a 150 mg/kg dose of tricaine methanesulfonate. The animals (10) were placed on their backs and left undisturbed until they spontaneously assumed the prone position. The time required to regain the righting reflex was noted.

Mice. When mice (male, 20-30 gm) were injected with a dose of 150 mg/kg of tricaine methanesulfonate i.p., there was no apparent pharmacological response. A 1500 mg/kg dose of the drug (i.p.) produced a brief loss of the righting reflex. The mice (10) were placed on their backs and left undisturbed until they spontaneously assumed the prone position. The duration of this loss of the righting reflex was noted.

2. The Biological Half-Life of Tricaine Methanesulfonate

Frogs (Rana pipiens). At timed intervals after i.p. administration of 1 mg of tricaine methanesulfonate to 16 frogs, groups of 4 frogs were sacrificed. Each frog was weighed, placed in a blender, and killed by starting the blender. Sufficient 0.2 M phosphate buffer, pH 7.4, was added to bring the volume to 150 ml and the frog was homogenized for 3 min. The homogenate was filtered through cheesecloth and a 10 ml aliquot of the filtered homogenate was placed in a tube containing 30 ml of 1-chlorobutane. The tube was shaken immediately by hand and subsequently centrifuged to separate the two phases; 25 ml of the 1-chlorobutane extract were extracted with 5 ml of 1N HCl. The tricaine in the HCl extract and the metabolites of tricaine which remained in

the aqueous lower layer after the extraction with 1-chlorobutane were quantified as described above. Standard curves were prepared in each experiment by adding tricaine methanesulfonate or MABA to the homogenate of an untreated animal. Tissue blanks never exceeded 0.010. Zero time measurements were obtained by sacrificing animals (4) in the blender and adding 1 mg of the drug directly to the blender immediately prior to homogenization.

To examine the effect of temperature on the half-life of tricaine in the frog, the same experiments were performed with animals maintained at 7° C, 23° C and 37° C for at least 24 hrs before the experiments. All manipulations of these animals (dosing, homogenizing, etc.) were carried out at the respective temperatures.

To assess the loss of tricaine by metabolism and by passage across the skin in frogs, 20 frogs were injected i.p. with 1 mg (893 nanocuries) of ^{14}C -labelled tricaine methanesulfonate and immediately placed in a 21 liter water bath. Tap water (23° C) flowed into the bottom of the bath at a rate of 37.5 liters/min and drained by overflow. Other frogs (16) were injected i.p. with 1 mg of either ^{14}C -labelled or non-labelled tricaine methanesulfonate and returned to their cages. Groups of frogs were sacrificed at timed intervals and each frog was homogenized in a total volume of 200 ml (as above). Zero time measurements were obtained by sacrificing animals (4) in the blender and then adding 1 mg of radioactive drug directly to the blender immediately prior to homogenization. The homogenates of frogs injected with nonlabelled tricaine methanesulfonate were extracted as above and analyzed by the UV and colorimetric assays. The homogenates (10 ml) of frogs injected

with ^{14}C -labelled drug were extracted with 30 ml of 1-chlorobutane; 10 ml of the latter were added to scintillation counting vials (glass) with 5 ml of dioxane scintillator (300 gm of naphthalene, and 15 gm of 2,5-diphenyloxazole per liter of dioxane). Fifteen ml of the 1-chlorobutane extract were extracted with 5 ml of 1N HCl; 0.5 ml of this HCl extract was added to glass counting vials with 2 ml of water and 15 ml of toluene solution (1 liter of toluene, 5 gm of 2,5-diphenyloxazole, 500 ml of Beckman Bio-Solv 3). After the extraction with 1-chlorobutane, the lower aqueous layer of the homogenate sample was diluted 1:3 with 15 gm% TCA (trichloroacetic acid) and centrifuged; 2 ml of the supernatant were added to counting vials with 15 ml of toluene scintillator. The radioactivity in all vials was quantified by triplicate counts in the scintillation counter. The efficiencies were then determined by adding a known amount of radioactivity (^{14}C -toluene standard - New England Nuclear, Boston, Ma.) to each vial and recounting the vials in triplicate. The efficiencies were as follows: 88% for 1-chlorobutane samples, 82% for HCl samples and 83% for aqueous samples. The radioactivity of the tricaine methanesulfonate solution used for the injections was checked by adding 0.2 ml of the solution to glass vials containing 15 ml of toluene scintillator.

Mice. Mice (4) were injected with a dose of 5 mg i.p. of tricaine methanesulfonate and immediately placed in a blender. Each animal was sacrificed 5 min after the injection by starting the blender. Sufficient 0.2 M phosphate buffer, pH 7.4, was added to bring the volume to 150 ml and the mouse was homogenized for 3 min. The homogenate was filtered through cheesecloth and a 10 ml aliquot of the filtered

homogenate was extracted with 30 ml of 1-chlorobutane; 15 ml of the latter were extracted with 5 ml of 1N HCl. Tricaine and its metabolites were quantified as above.

3. Tricaine Paralysis Times in Mammalian and Nonmammalian Species

The doses of tricaine methanesulfonate that produced general anesthesia in 7 different species (frogs, salamanders, snakes, turtles, lizards, parakeets, mice) were determined on the basis of preliminary experiments. For each species, the dose was adequate to cause temporary loss of the righting reflex in all animals. All injections of the drug were intraperitoneal (i.p.). The length of time to loss of the righting reflex (induction time) and the duration of the loss of the righting reflex (paralysis time) were noted. Turtles were injected by introducing the needle into the coelomic cavity through the soft tissue immediately adjacent to the hind leg. Since turtles are nearly incapable of righting themselves in the control state when placed on their backs, the righting reflex was not used in these animals as a test of anesthesia. Instead, the turtles were left on their ventral surface and their heads were passively extended and allowed to drop onto the surface of the table. Recovery from anesthesia was indicated by resistance of the reptiles to remain in this position for a period of more than 1 min.

Immediately upon righting, each animal (except the turtles) was placed in a blender and sacrificed by starting the blender. Sufficient 0.2 M phosphate buffer, pH 7.4, was added to bring the volume to 200-400 ml (volume constant for a given species and determined by the

average weight) and the animal was homogenized for 3 min. The homogenate was filtered through cheesecloth and a 10 ml aliquot of the filtered homogenate was extracted with 30 ml of 1-chlorobutane. A 15-25 ml aliquot of the latter was extracted with 10 ml of 1N HCl. Tricaine and its metabolites were quantified as above.

With each species, standard curves were prepared by homogenizing an untreated animal in the appropriate volume of phosphate buffer and filtering the homogenate. Aliquots (10 ml) of the homogenate were placed to tubes containing 30 ml of 1-chlorobutane and known amounts of tricaine or MABA were then added. These tubes were handled in a manner identical to that described above. Tissue blanks for each species were prepared and their absorbance never exceeded 0.010.

4. In Vitro Hydrolysis of Tricaine by Liver Homogenates

Frogs (*Rana pipiens*). Frogs (4) were pithed and their livers removed immediately. The gall bladders were dissected off and discarded. The livers were washed in ice-cold saline, blotted dry, weighed and placed in ice-chilled homogenizers. All subsequent manipulations were performed at 0-4° C. Each liver was homogenized in 20 times its weight of ice-cold 0.2 M phosphate buffer, pH 7.4, for 3 min. Three ml of the homogenate were added to a 50 ml Erlenmeyer flask containing eight 6 mm glass beads. The flask was warmed for 15 min in the metabolic incubator at 37.5° C and then 6 ml of a warmed (37.5° C) solution of tricaine methanesulfonate (4.5 mg/ml in phosphate buffer) were added. The flask was immediately swirled and a 1 ml sample was removed (0 time). Samples (1 ml) were also taken at 5, 10, 15, 20 and 25 min after drug addition. The 1 ml samples were

immediately extracted with 30 ml of 1-chlorobutane; 15 ml of the latter were extracted with 10 ml of 1N HCl. The HCl extract was diluted 1:4 with 1N HCl and analyzed for tricaine content as described above. The aqueous lower layer, after the extraction with 1-chlorobutane, was diluted 1:99 with 15 gm% TCA (trichloroacetic acid) and analyzed by the Bratton-Marshall assay for tricaine metabolites. Standard curves were prepared by the addition of known amounts of tricaine methanesulfonate or MABA to the liver homogenate and assaying for the compounds as above.

Mice. Four mice were sacrificed by cervical dislocation and their livers were removed and treated in a manner identical to that described for the frogs. Incubations with tricaine were prepared and assayed as above. In a separate series of 4 incubations, 1.5 ml of the mouse liver homogenate were combined with 1.5 ml of the frog liver homogenate, incubated with tricaine and assayed for tricaine metabolism as described above.

To determine if the tricaine methanesulfonate concentration in the mouse-liver incubations (3.0 mg/ml) was sufficient to produce zero-order kinetics, 4 incubations were prepared as follows: 0.5, 1.0, 2.0 and 3.0 ml aliquots of a mouse-liver homogenate were each placed in separate Erlenmeyer flasks (each with eight 6 mm glass beads) containing 2.5, 2.0, 1.0 and 0.0 ml of 0.2 M phosphate buffer, pH 7.4, respectively. The 4 flasks were then incubated and assayed for tricaine metabolism as described above for the frog livers.

The deterioration of the "tricaine-esterase" activity of the liver homogenates during the 15 min "warming" period was assessed by preparing a mouse-liver incubation in a manner identical to that described above

for the frog livers except that the 15 min warming period was deleted from the experimental protocol. Instead, the 6 ml of drug solution were immediately added to the incubation; the incubation was placed in the metabolic incubator (37.5° C) and samples (1 ml) were removed every 3 min for 15 min. The samples were assayed as above.

Other Terrestrial Vertebrates (bullfrog, salamander, snake, lizard, cat, rat, guinea pig, rabbit). The animals were sacrificed and their livers (or parts thereof) were removed, washed in ice-cold saline, blotted dry, weighed and homogenized in 20 times their weight of 0.2 M phosphate buffer, pH 7.4. Incubations were prepared and treated in a manner identical to that described above for the frog livers.

Marine Vertebrates. Frozen liver specimens from a dolphin, a seal and 2 sea lions were obtained from Dr. Stroud and stored in a walk-in freezer (-10° C). Fresh specimens were not available. The livers of the seal and sea lions had partially decomposed before being frozen and did not metabolize tricaine when incubated with the drug. The dolphin liver (as were those of the seal and sea lions) was removed from the freezer 12 hr before use and allowed to thaw at room temperature. It was then washed in normal saline. A 1 gm piece of tissue was dissected from the middle of a lobe (centrilobar) and homogenized in 20 times its weight of 0.2 M phosphate buffer, pH 7.4. An incubation of the homogenate with tricaine methanesulfonate was prepared and analyzed for tricaine metabolism as described above for the frog livers.

Effect of Freezing on In Vitro Liver Metabolism of Tricaine. Since the dolphin liver was frozen before being analyzed for tricaine metabolism, the effect of freezing on the in vitro hydrolytic activity

of the liver was studied. Four mice were sacrificed by cervical dislocation and their livers were removed and immediately placed in a walk-in freezer (-10°C). Two weeks later, the livers were removed from the freezer and allowed to thaw for 12 hrs at room temperature. They were then homogenized and incubated with tricaine in a manner identical to that described above for the frog livers.

5. In Vitro Hydrolysis of Tricaine in Serum Incubations

Inhibition of Serum Hydrolysis of Tricaine. Blood was drawn from a male human (antecubital vein) and allowed to stand for 10 min in glass tubes which were inverted several times to facilitate clotting. The clots were "rimmed" and the tubes centrifuged at 2000 rpm for 10 min. The serum was separated after the centrifugation and combined in a single flask. Three incubations were prepared as follows:

(i) 10 ml of the serum were added to a 50 ml Erlenmeyer flask containing eight 6 mm glass beads and 11 ml of 0.2 M phosphate buffer, pH 7.4. Nine ml of a tricaine methanesulfonate solution (250 $\mu\text{g}/\text{ml}$) were added to the flask. The flask was vigorously swirled and a 1 ml sample was removed immediately (0 time). The flask was then incubated at 37.5°C . Samples (1 ml) were removed from the incubation every 15 min for 5 hr after drug addition. The 1 ml samples were immediately extracted with 30 ml of 1-chlorobutane; 25 ml of the 1-chlorobutane extract were extracted with 2 ml of 1N HCl. The tricaine in the HCl extract and the metabolites of tricaine that remained in the aqueous lower layer after the extraction with 1-chlorobutane were quantified as described above.

(ii) 10 ml of the serum were added to a 50 ml Erlenmeyer

flask containing eight 6 mm glass beads, 2 ml of 0.2 M phosphate buffer, pH 7.4, and 9 ml of a neostigmine bromide solution (4×10^{-3} M) made up in the phosphate buffer. Nine ml of the tricaine methanesulfonate solution (250 μ g/ml buffer) were added to the flask. The incubation was vigorously swirled and a sample (1 ml) was removed (0 time). The flask was incubated at 37.5° C and samples were removed every hr for 5 hr after drug addition. The samples were immediately extracted and analyzed as above.

(iii) 10 ml of the serum were added to a tube containing 11 ml of 0.2 M phosphate buffer, pH 7.4. Since this solution retained a significant amount of its hydrolytic activity after being heated in a water bath (90-95° C), it was boiled over an open flame for 5 min. The solution was then cooled under tap water and homogenized for 2 min. Sufficient distilled water was added to bring the volume back to the original 21 ml. Nine ml of the tricaine methanesulfonate solution (250 μ g/ml) were added to the homogenizer and the 0 time (drug addition) noted. This solution was re-homogenized to insure homogeneity and then transferred to a 50 ml Erlenmeyer flask and incubated at 37.5° C in the metabolic incubator. Samples (1 ml) were removed every hr for 5 hr after drug addition, immediately extracted, and analyzed as above.

Standard curves were prepared by placing a known amount of tricaine or MABA in (a) phosphate buffer, and (b) a 50:50 solution of serum and phosphate buffer. These samples were then extracted and analyzed in a manner identical to that detailed above.

Variation in the "Tricaine-Esterase" Activity of Human Serum.

Blood was drawn from 3 male humans (antecubital vein) and 1 bullfrog,

Rana catesbiana (cardiac puncture). The 4 samples of sera were prepared as above. A 2.5 ml aliquot of each of the serum samples was added to separate 50 ml Erlenmeyer flasks containing eight 6 mm glass beads and 1.5 ml of 0.05 M tris buffer, pH 7.4. One ml of a tricaine methane-sulfonate solution (250 µg/ml tris buffer) was added to the 4 flasks. Each incubation was immediately swirled and a 0.5 ml sample was removed (0 time). The flasks were then incubated at 37.5° C and 0.5 ml samples were removed every 30 min for 4 hrs after drug addition. The samples (0.5 ml) were immediately extracted with 30 ml of 1-chlorobutane; 25 ml of the latter were extracted with 2 ml of 1N HCl. Tricaine and its metabolites were quantified as above. Separate standard curves were prepared with the serum from one of the human donors and from the bullfrog. The protein content of each serum sample was measured by the method of Lowry et al. (1951). (See Appendix IV for detailed methodology.)

The tris buffer was found to be undesirable due to 1) the appreciable solubility of tris buffer in organic solvents (Good and Izawa, 1972); 2) the ability of 1N HCl to extract tris buffer from 1-chlorobutane, and 3) the UV spectrum of tris buffer in 1N HCl which interferes with that of tricaine. Therefore, the tris buffer was not used in subsequent experiments.

Hydrolysis of Tricaine by Serum Albumin. Since albumin is the major component of serum protein, the tricaine-hydrolysis activity of serum albumin was measured. An incubation consisting of 2 ml of a bovine serum-albumin solution (8 gm/100 ml) and 2 ml of 0.2 M phosphate buffer, pH 7.4, was prepared in a 50 ml Erlenmeyer flask containing

eight 6 mm glass beads. The flask was warmed in the metabolic incubator (37.5° C). After a 15 min warming period, 2 ml of a warmed (37.5° C) tricaine methanesulfonate solution (2 mg/ml buffer) were added to the flask. The incubation was vigorously swirled and a 1 ml sample was removed (0 time). Samples (1 ml) were also removed at 30, 60 and 120 min after drug addition. All samples (1 ml) were immediately extracted with 30 ml of 1-chlorobutane; 15 ml of the latter were extracted with 10 ml of 1N HCl. The tricaine content of the HCl extracts was quantified as described above.

Kinetic Studies of the Hydrolysis of Tricaine in Serum Incubations. Whole blood was obtained by direct aortic puncture from bullfrogs (Rana catesbiana), from mice by retro-orbital venous puncture and from humans by venipuncture of the antecubital vein. For each experiment, the blood of ten bullfrogs was pooled, as was the blood of 30 mice. The human blood was obtained from a single male individual. The sera were prepared as described above and from each serum sample, 6 incubations consisting of 1-2 ml of serum and 2-3 ml of 0.2 M phosphate buffer, pH 7.4, were prepared in 50 ml Erlenmeyer flasks containing eight 6 mm glass beads. The flasks were warmed at 37.5° C in the metabolic incubator. Solutions of tricaine methanesulfonate, 300, 600, 1200, 1500 and 2250 µg/ml buffer, were also warmed at 37.5° C. After a 15 min warming period, 2 ml aliquots of the tricaine solutions were added to the respective flasks, thus giving final incubation volumes of 6 ml. One ml samples of the incubations were removed at the zero time (drug addition) and 30 min after drug addition. The samples were immediately extracted with 30 ml of 1-chlorobutane; 15 ml of the latter

were extracted with 10 ml of 1N HCl. The HCl extracts were assayed as described above. The aqueous lower layers, after extraction with 1-chlorobutane, was diluted 1:5 with 15 gm% TCA and analyzed by the Bratton-Marshall assay for tricaine metabolites. Standard curves were prepared for tricaine and MABA by the addition of 0.5 ml of the varying concentrations of tricaine or MABA to tubes containing 0.5 ml of the respective serum and 30 ml of 1-chlorobutane. These samples were then assayed as described above. The protein content of each serum sample was measured by the Lowry method for protein determinations (See Appendix IV).

6. In Vitro Hydrolysis of Tricaine in Saliva Incubations

Saliva was collected from 5 humans (4 males and 1 female) and incubated with tricaine methanesulfonate as follows: 9 ml of each saliva sample were added to separate flasks (50 ml Erlenmeyer) containing eight 6 mm glass beads. The flasks were incubated for 15 min at 37.5° C and then 1 ml of a warm (37.5° C) solution of tricaine methanesulfonate (4.0 mg/ml buffer) was added to each flask. The flasks were vigorously swirled and a 1 ml sample was removed (0 time). Samples (1 ml) were removed at 30, 60 and 120 min after drug addition. All samples (1 ml) were immediately extracted with 30 ml of 1-chlorobutane; 15 ml of the latter were extracted with 5 ml of 1N HCl. Tricaine and its metabolites were quantified as described above. Standard curves were prepared with a mixture of all 5 saliva samples.

7. Tissue Distribution of the Tricaine-Hydrolysis Activity in the Mouse

A 22 gm mouse was sacrificed by cervical dislocation and 2 ml

of blood were collected by direct aortic puncture. One ml of serum was prepared by the previously described method and placed in a 50 ml Erlenmeyer flask containing 2 ml of 0.2 M phosphate buffer, pH 7.4, and eight 6 mm glass beads. One kidney, one lung, one gastrocnemius muscle, the heart and the brain were removed, washed in ice-cold normal saline, blotted dry, and weighed. Each was homogenized in 10 times its weight of ice-cold 0.2 M phosphate buffer, pH 7.4, for 3 min. The liver was treated in a similar manner except that it was homogenized in 20 times its weight of phosphate buffer. 1-3 ml of the homogenates were added to 50 ml Erlenmeyer flasks containing eight 6 mm glass beads. The flasks were warmed for 15 min at 37.5° C and then 2-6 ml of a warmed (37.5° C) tricaine methanesulfonate solution (4.5 mg/ml phosphate buffer) were added to the flasks. Each incubation was vigorously swirled and a 1 ml sample was removed (0 time). Samples (1 ml) were taken at 10, 20 and 30 min after the drug addition. All samples were immediately extracted with 30 ml of 1-chlorobutane; 15 ml of the latter were extracted with 10 ml of 1N HCl. The HCl extract was diluted 1:4 with 1N HCl and analyzed for tricaine content as described above. Tricaine metabolites in the aqueous lower layer were quantified as described above for frog livers. Standard curves were prepared from the liver, kidney and muscle homogenates.

8. Tissue Distribution of Tricaine and Its Metabolites in the Bullfrog

Four bullfrogs (Rana catesbiana) were injected i.p. with a 200 mg/kg dose of tricaine methanesulfonate. Twenty min after the injection, the ventral wall of the coelomic cavity was removed and 1 ml

of blood was collected via aortic puncture. The sample (1 ml) of whole blood was immediately extracted with 30 ml of 1-chlorobutane. The heart, one lung, a centrilobar piece of liver, the brain, one of the gastrocnemius muscles and the abdominal fat body were removed, washed in ice-cold normal saline, weighed and stored at -70°C . The frozen tissues were individually homogenized in small volumes of 0.2 M phosphate buffer, pH 7.4, (heart 6 ml, lung 6 ml, liver 15 ml, brain 1 ml, muscle 21 ml, fat 7 ml). One ml of the brain homogenate and 5 ml of the homogenates of the other tissues were extracted with 30 ml of 1-chlorobutane; 15 ml of the latter were extracted with 10 ml of 1N HCl. The HCl extracts from the brain and muscle were assayed directly for tricaine. The HCl extracts from the heart, lung and blood were diluted 1:4 with 1N HCl and those from the liver and fat were diluted 1:49 before being assayed for tricaine. The aqueous lower layers from all the homogenates were assayed for metabolites of tricaine as described above. The respective tissues from a nontreated bullfrog were used in the preparation of the appropriate standard curves.

H. Studies of the Metabolic Fate of Tricaine in the Mouse

Mice were injected i.p. with 5 mg of ^{14}C -labelled tricaine methane-sulfonate (diluted 1:4 with nonlabelled drug). The animals were immediately placed in a blender to collect all excreta and were sacrificed at 5 or 70 min after the injection of the drug. After sacrifice of the animal, 300 ml of absolute ethanol were added to the blender and the mouse was homogenized for 3 min. The homogenate was filtered through glass filter paper under suction. The homogenate

residue and the glass filter paper were re-homogenized in 300 ml of 95% ethanol for 3 min and filtered again under suction through glass filter paper. This process was repeated 4 more times and the final volume of ethanol filtrate was brought to 2000 ml with absolute ethanol. The mouse extract was divided into two 1000 ml samples and each was evaporated to dryness on the rotary evaporator in vacuo at room temperature. Co-distillation with toluene was used to remove the residual water.

1. Chromatographic Separation of Tricaine Metabolites

The residue in one of the rotary-evaporator flasks after evaporation was dissolved in 50% methanol (20 ml) and 2 ml of this solution was applied to an "origin" site on a 43 cm x 8 cm piece of chromatographic paper (ChromAR^R). The sample (2 ml) was applied with a syringe in small aliquots which were dried with a conventional hair dryer. The ChromAR^R sheet was then placed in a chromatography tank which had previously been equilibrated with 95% butyl alcohol. The solvent was allowed to descend approximately 30 cm beyond the origin site. The chromatographic sheet was then air dried and a 43 cm x 2 cm strip was analyzed for radioactivity as follows: the strip was divided into eighty-six 0.5 cm x 2 cm pieces and each piece was placed in a separate glass counting vial containing 2 ml of distilled water and 15 ml of toluene scintillator (see page 39). The vials were allowed to stand for 24 hr and then they were vigorously shaken. The shaking resulted in the complete disintegration of the chromatographic material. Blank samples were prepared in a manner identical to the one described above. The remainder of the chromatographic sheet was frozen (-11° C). The radioactivity in each of the vials was quantified by triplicate counts

in the scintillation counter. The amount of "quenching" in each vial (efficiency) was determined by adding a known amount of radioactivity (^{14}C -toluene standard) to each vial and recounting the vials in triplicate. The efficiency was 86%.

2. CCD of Tricaine Metabolites

The other sample of mouse extract, after being evaporated to dryness, was transferred to the first tube of the CCD apparatus by 3 washings with ethyl acetate (10 ml/wash), followed by 2 washings with distilled water (3.5 and 4.0 ml, respectively). 7.5 ml of 4 M phosphate buffer, pH 4.3 were then added to the first tube of the CCD apparatus. The CCD was performed between ethyl acetate (30 ml) and 2M phosphate buffer, pH 4.3 (15 ml). Fifteen ml of each ethyl acetate fraction were added to separate glass scintillation vials containing 5 ml of dioxane scintillator (see page 39). 0.2 ml of each aqueous fraction of the CCD was added to separate scintillation vials containing 2 ml of distilled water and 15 ml of toluene scintillator. Blanks of both ethyl acetate and 2 M phosphate buffer were prepared as above. The quantification of radioactivity and the efficiency were determined as above. The efficiency was 82%.

After the final extraction with 95% ethanol and filtration through the glass paper, the mouse homogenate and filter paper were re-homogenized in 200 ml of distilled water; 2 ml of this homogenate were added to scintillation vials containing 15 ml of toluene scintillator. The radioactivity of the tricaine methanesulfonate solution used for the injections was also checked by adding 0.2 ml of the solution to vials containing 15 ml of toluene scintillator.

3. Derivatization of Tricaine Metabolites

Derivatization for metabolite identification was performed on 2 different samples.

(i) The bands of radioactivity on the chromatographic sheet (frozen portion) were extracted repeatedly with methanol and the methanol extracts were evaporated to dryness by impingement with N_2 .

(ii) Urine from 4 mice was collected in an ice-chilled flask for 24 hr after the mice had each been injected i.p. with 1 gm of the free base form of tricaine. Aliquots (1 ml) of the urine were then evaporated to dryness by impingement with N_2 .

Methylation. The residues of the N_2 -dried metabolite samples were permethylated by the method of Leclercq and Desiderio (1971), as modified by Thompson *et al.* 1973. Each sample was dissolved in 100 μ l of DMSO (dimethyl sulfoxide, distilled over calcium hydride) and the reaction vial flushed with N_2 to eliminate moisture and oxygen. Each vial was immediately capped with a rubber septum and the following reagents were added to the vial by piercing the septum. Twenty-five μ l of DMSO anion (sodium salt) were added, followed, after 15 min, by 10 μ l of CH_3I or CD_3I . The reaction was stopped after 1 hr by the addition of 1 ml of water. The permethylated product mixture was extracted with 1 ml of chloroform and the chloroform extract was washed twice with distilled water. After the chloroform extract was evaporated under a stream of N_2 , it was redissolved in 20 μ l of chloroform for gas chromatographic and mass spectrometric analyses.

Other metabolite samples were methylated with diazomethane in ether. The ethereal alcoholic solution of diazomethane was prepared

as follows: ethanol (95%, 25 ml) was added to a solution of KOH (5 gm) in water (8 ml) in a 100 ml distilling flask. Two receiving flasks in series were connected to the distilling flask via a condenser and were cooled to 0°C in an ice bath. The distilling flask was heated in a water bath (65° C) and a solution of 21.5 gm of Diazald (Aldrich Chemical Co., Milwaukee, Wi.) in 200 ml of ether was added through a dropping funnel into the distillation flask in about 25 min. The rate of distillation was approximately equal to the rate of addition. When the dropping funnel was empty, another 40 ml of ether was slowly added and the distillation was continued until the distilling ether was colorless. The combined ethereal distillate contained about 3 gm of diazomethane.

The residue of a N₂-dried metabolite sample was dissolved in 1 ml of the diazomethane-ether solution, allowed to stand for 30 min, and then evaporated to dryness via impingement with N₂. This residue was redissolved in 20 µl of chloroform for gas chromatographic and mass spectrometric analyses.

Silylation. Samples were silylated with the use of TRI-SIL (Pierce Chemical Co., Rockford, Il.) The residue of a N₂-dried metabolite sample was dissolved in 1 ml of TRI-SIL and shaken vigorously to assist the dissolution. The sample was allowed to stand for 30 min and then the mixture was ready for gas chromatographic and mass spectrometric analyses.

Samples which had previously been methylated via the diazomethane-ether solution were also silylated as above and analyzed.

Acetylation. Samples which had previously been methylated

via the diazomethane-ether solution were acetylated with trifluoroacetic acid anhydride (Pierce Chemical Co., Rockford, Il.). The N_2 -dried, methylated residue was dissolved in 100 μ l of pyridine and 100 μ l of the trifluoroacetic acid anhydride. The vial was tightly closed with a teflon stopper and heated for 2 hr at 60° C. This mixture was then used for the gas chromatographic and mass spectrometric analyses.

The gas chromatography/mass spectrometry analyses were performed using the Dupont machine described above under the following conditions: injector temperature 250° C, detector temperature 300° C, oven temperature programmed from 90° C to 320° C at 10°/min, carrier gas (N_2) flow rate 30 ml/min.

I. Studies of the Comparative Effects of Tricaine and Other Local Anesthetics on Various Organ Systems of Mammalian and Nonmammalian Species

1. Isolated Tissue Studies

Isolated Atria. A comparison was made between the effects of tricaine on the atria isolated from bullfrogs and guinea pigs. The influence of the methanesulfonate moiety was also studied by comparing the effects of tricaine methanesulfonate and tricaine hydrochloride on the two preparations. The hydrochloride salt of tricaine was prepared by dissolving 165.19 mg of tricaine (free base form - Eastman) in 2 ml of 1N HCl and then bringing this solution to 100 ml with the appropriate physiological solution (see below).

Bullfrogs (*Rana catesbiana*). Bullfrogs were pithed and the ventral wall of their thorax was removed to expose the heart. A suture

was attached to the left branch of the truncus arteriosus and the heart was excised. This suture was tied to the lever arm of a Harvard 356 isotonic transducer. A second suture was tied to the margin of the sinus venosus and affixed to a stationary mount in the muscle bath. Before mounting the atria in the bath chamber, most of the ventricular tissue was trimmed away, leaving only a ring underlying the atria and giving rise to the bulbus and truncus arteriosus. To improve circulation of the bath solution to and from the atria, the atrio-ventricular valves were ruptured. Care was taken not to occlude the opening of the sinus venosus. The atria were mounted under 1 gm of tension in 60 ml water-jacketed muscle bath chambers. The Krebs-type bath solution used in these experiments contained the following chemicals in mM concentrations: NaCl, 89.0; NaHCO₃, 20.0; KH₂PO₄, 2.5; CaCl₂, 2.0; MgSO₄·7H₂O, 1.5; glucose 10.0. This solution was aerated with 98.7% O₂ and 1.3% CO₂, producing a pH of 7.8. The ionic composition (Fern 1936, Conway 1957) and the pH (Howell et al. 1970) of this bath solution were similar to values found in the plasma of intact bullfrogs.

Guinea Pigs. Guinea pigs were sacrificed by a blow to the back of the head and their hearts excised. These atria were mounted in a fashion similar to that described for the bullfrog atria by sutures attached to each auricular appendage. Again, most of the ventricular tissue was cut away before mounting the preparation in the muscle bath and the atrio-ventricular valves were ruptured. The Krebs-type bath solution used with these preparations contained the following chemicals in mM concentrations: NaCl, 118.9; NaHCO₃, 25.0; KH₂PO₄, 1.2; CaCl₂, 2.5; MgSO₄·7H₂O, 0.6; glucose, 11.1; KCl, 4.7. The bath

solution was aerated with 95% O₂ and 5% CO₂, producing a pH of 7.5.

Both the bullfrog and the guinea-pig atria were maintained at a constant 30° C with a Lauda K-2/R circulator and thermoregulator. The preparations were allowed to equilibrate for one hr after being mounted, during which time the bath solution was changed 4 times, at 5, 15, 30 and 45 min after mounting. Tricaine, as the hydrochloric or methane-sulfonic acid salt, was dissolved in the appropriate bath solution and added directly to the muscle bath so as to give final concentrations in the bath of 10⁻⁵ M to 3 x 10⁻³ M. The spontaneous contractions of the preparations were recorded on the Harvard polygraph and the response of the atria to each concentration of drug was monitored until there was a plateau in the response. At this point (plateau of response), the drug was washed out with 2-3 changes of the bath solution and the preparation was allowed to re-equilibrate for 10-15 min before the introduction of the next concentration of the drug.

The drug was added in increasing concentrations until a concentration was reached which caused cessation of the contractile activity of the atrial preparation for more than 1 min. This effect was then reversed by repeated washings with fresh bath solution. After the return and stabilization of vigorous contractile activity, the effects of MABA on the preparation were assessed. A concentration of MABA equivalent to that of tricaine which had "arrested" the preparation was added to the bath. Ten min after the addition of MABA, the response of the preparation to the presence of both MABA and tricaine was investigated. Tricaine methanesulfonate (3 x 10⁻⁴ M) was added to the MABA solution bathing the atrial preparation. This was done to elucidate

any competitive, additive or synergistic effects MABA might have had on the preparation's response to tricaine. The 3×10^{-4} M concentration of tricaine was used because it was the most effective concentration of the drug that did not arrest either the bullfrog or the guinea-pig atrial preparations. Therefore, both a decrease or an increase in the effect of tricaine could have been observed.

The pH of the bath was monitored throughout each experiment. The higher concentrations of tricaine hydrochloride caused momentary shifts in the pH of the bath to as low as 7.0. Therefore, after each experiment, the muscle was washed and allowed to re-equilibrate. Sufficient HCl was then added to the bath to produce a decline in the pH of the bath similar to that produced by tricaine hydrochloride. The pH shift never resulted in any persistent alteration in contractility of the preparations.

The effects of benzocaine (ethyl p-aminobenzoate) hydrochloride and procaine hydrochloride on the isolated atrial preparations were assessed using the same experimental protocol as that used with the tricaine salts. Benzocaine hydrochloride was prepared in a manner identical to that for tricaine hydrochloride. (Procaine hydrochloride was obtained commercially.)

Isolated Lung. Frogs (Rana pipiens) were pithed and their lungs removed. Each lung was split longitudinally so as to provide 2 preparations. These lung strips were mounted under 2 gm tension in the water-jacketed 60 ml muscle bath chambers, using the Harvard 356 isotonic transducers. The composition of the Krebs-type bath solution and the aerating gas were the same as that used for the isolated bullfrog

atria (see above). In order to reduce the frequency and amplitude of spontaneous contractions of these preparations, the bath solution was maintained at 15° C.

The lung strips were allowed to equilibrate for at least 3 hr before exposure to either tricaine methanesulfonate or procaine hydrochloride. During the first 2 hr, most of the lung strips underwent a slow, progressive contraction. When a muscle strip had attained a stable resting length, the drug was added to the bath in increasing, cumulative concentrations (no changes in the bath solution between drug additions). The preparation was allowed to re-stabilize before the addition of the next higher drug concentration. The concentration of drug in the bath was progressively increased until the maximum response of the preparation to a given drug had been elicited. At the end of each experiment, the preparation was washed (change of bath solution) several times and allowed to re-equilibrate. The tissue was then contracted by progressively increasing the K^+ concentration from a normal of 2.5 mM to 26, 44 and 59 mM. Higher K^+ concentrations resulted in a depression of the contraction. The maximal K^+ -induced contraction (achieved at 44 or 59 mM K^+) was invariably higher than that produced by other compounds which stimulated contraction and was therefore used as the standard of comparison.

Isolated Stomach. Frogs (Rana pipiens) were decerebrated and their stomachs were removed. Each stomach was split longitudinally with a spiralling incision from the pylorus to the esophageal-gastric junction, thereby producing a single flat sheet of gastric muscle. The mucosa was removed by blunt dissection and two, 3 mm wide strips

were longitudinally cut from the middle of sheet of gastric muscle. The muscle strips were mounted under 1 gm of tension in the water-jacketed 60 ml muscle bath chambers. Contractions were recorded by Harvard 356 isotonic transducers. The composition of the Krebs-type bath solution and the aerating gas was the same as that used for the isolated bullfrog atria (see above). In order to reduce the frequency and amplitude of spontaneous contractions and to stabilize the preparations, the bath solution was maintained at 15° C. The experimental protocol for testing the effects of the drug on this preparation was the same as that described for the isolated frog lung except that these preparations were not challenged at the end of each experiment with increasing concentrations of K^+ .

Isolated Trachea. Guinea pigs were sacrificed by a blow to the back of the head and their tracheas were removed from larynx to carina. Tracheal chains were prepared as described by Castillo and de Beer (1947), as modified by a pairing procedure similar to that of Foster (1960). In the pairing procedure, the tracheas were removed from 2 guinea pigs and rings, 2-3 mm wide, were cut from each trachea, beginning at the laryngeal end. The rings were cut alternately from each trachea and placed successively in 2 or 3 petri dishes containing a Krebs-type solution identical to that used with the guinea-pig atria. These rings were then assembled into 2 or 3 matched chains, each of 8 rings in length and composed of tracheal rings from both animals.

The chains, each composed of 8 rings tied together with 5-0 silk, were mounted under 300 mg tension in the water-jacketed 60 ml muscle bath chambers. The composition of the bath solution and the aerating

gas was the same as that used for the isolated guinea-pig atria (see above). Contractions were recorded by Harvard 356 isotonic transducers. The bath solution was maintained at 37° C.

The tracheal chains were allowed to equilibrate for at least 1 hr and until a stable resting length had been reached before exposure to the drug. Tricaine methanesulfonate or lidocaine was added to the bath in progressively increasing, cumulative concentrations (without changes in the bath solution between drug additions). Sufficient time was allowed after each addition of drug to obtain the maximal effect (a plateau in response) of each drug concentration. At the end of each experiment, the chains were washed repeatedly with fresh bath solution and allowed to re-stabilize at the original resting length. The preparations were then relaxed with increasing concentrations of isoproterenol (10^{-9} to 10^{-6} M) and/or contracted with increasing concentrations of acetylcholine (10^{-6} to 10^{-3} M). The effect of the different concentrations of tricaine methanesulfonate were expressed as per cent of the maximal relaxation produced by isoproterenol (10^{-6} M) or of the maximal contracture elicited by acetylcholine (10^{-3} M).

Nervous Tissue Preparations. Frogs (Rana pipiens) were decerebrated, thus preserving the spinal cord intact. One of the sciatic nerves was isolated along its course through the thigh and that thigh was ligated with suture ties which passed under the isolated sciatic nerve. Therefore, the nerve was excluded from the ligation (Sollman 1918). Each frog was suspended from a hook passed through its lower jaw. Both of the frog's feet were simultaneously immersed in 0.1 N HCl and a brisk reflex withdrawal was observed. The feet

were then immediately immersed in 0.1 N NaOH several times, followed by several washes with distilled water. A dose of 200 mg/kg of tricaine methanesulfonate or an equal volume of normal saline (0.3 ml) was injected i.p. into the frog. At 1 min intervals after the injection of the drug, the simultaneous immersion of both feet in 0.1 N HCl was repeated and the withdrawal time for each foot was noted. An arbitrary time limit of 10 sec of acid exposure was imposed to avoid damage to the exposed tissue. After each acid exposure the feet were immediately washed with 0.1 N NaOH and water as above.

Other frogs were decerebrated as above and eviscerated, taking care to avoid traumatizing the sciatic nerves. The animals were suspended as above and the reflex withdrawal from acid observed (0.5 N HCl). Small pledgets of filter paper (0.5 cm x 1 cm), which had been soaked in either normal saline or one of several tricaine methanesulfonate solution, 0.1 gm%, 0.3 gm%, 1 gm% and 3 gm%, were placed on the exposed portion of one of the sciatic nerves. The reflex withdrawal from 0.5 N HCl was then monitored at 2 min intervals. Again, an arbitrary time limit of 10 sec of acid exposure was imposed. The withdrawal reflex was monitored until neither leg was withdrawn from acid within 10 sec. After each acid exposure, the feet were washed with 0.5 N NaOH and water as above. Each frog was used to test a single drug concentration once and then disposed of.

2. Intact Animal Studies

Cats (4) were anesthetized by i.p. injection of 1 ml/kg of a chloralose-urethane solution (0.9 gm of chloralose + 3.5 gm of urethane/10 ml of 50% ethanol). A tracheotomy was performed and a plastic

endotracheal (E.T.) tube was inserted into the tracheal opening. The E.T. tube was secured in position with suture ties above and below its insertion into the trachea and was connected to a Harvard small animal respiration pump. The animals were mildly hyperventilated (20 respirations/min; 40-50 ml tidal volume) to suppress spontaneous respirations. An indwelling venous catheter (heparinized) was placed in the cephalic vein for drug administration. A second indwelling catheter (heparinized) was placed in the femoral artery and was connected to a Statham blood pressure transducer. A continuous electrocardiogram was recorded by placing leads on the right foreleg and left hindleg with a ground lead on the right hindleg. All readings, ECG and blood pressure, were continuous and were recorded on a Grass polygraph.

The animals were allowed to stabilize for 1 hr after surgery. Tricaine methanesulfonate, dissolved in normal saline (10^0 to 10^{-3} M concentrations) was injected intravenously (i.v.) in increasing doses (0.15 to 200.0 mg/kg) at 20 min intervals. Each i.v. injection (2 ml of drug solution/20 sec) was immediately followed by a 1 ml injection of heparinized normal saline to clear the dead space of the catheter tubing of drug. Controls for the volume effect of the drug diluent were obtained by injecting equal volumes of normal saline.

The injected tricaine methanesulfonate dose was sequentially increased until cardiac arrest occurred. In several experiments, the cardiac arresting dose was excluded from the protocol and these animals were allowed to stabilize for 30-60 min after the last tricaine methanesulfonate injection. An i.v. infusion of ouabain (infusion rate = 0.5 ml/min; dosage = 5 μ g/kg/min) was then started. After an infusion

period of approximately 20 min, the ECG tracing demonstrated evidence of arrhythmias, usually atrial-ventricular dissociation and premature ventricular contractions. With the onset of the arrhythmia, the ouabain infusion was stopped and attempts were made to abolish the arrhythmia (return the heart to normal sinus rhythm) with i.v. injection of various doses of tricaine methanesulfonate.

RESULTS

A. Pharmacokinetic Studies of Tricaine Methanesulfonate

1. Tricaine Paralysis Time

Frogs (Rana pipiens). In ten frogs kept at room temperature (23°C), each of which received 150 mg/kg of tricaine methanesulfonate i.p., the paralysis time, as indicated by the loss of the righting reflex, was 133 ± 28 (S.D.) min. The onset of paralysis occurred about 30 sec after the injection.

Mice. Ten mice, each of which received a dose of 250 mg/kg tricaine methanesulfonate i.p., did not lose their righting reflex and did not exhibit any overt signs of drug action. When mice (10) were injected i.p. with a dose of 1500 mg/kg of the drug, there was a brief loss of the righting reflex for 5 ± 1 (S.D.) min. The onset of paralysis occurred within 1 min of the drug injection.

2. The Biological Half-Life of Tricaine Methanesulfonate.

Frogs (Rana pipiens). The biological half-life of tricaine (Figure 17), as measured in the whole bodies of frogs which received 1 mg of tricaine methanesulfonate i.p., was 68 min at room temperature (23°C), 73 min at 37.5°C and 309 min at 7°C . At all three temperatures, the disappearance of the drug approached a first-order process.

Figure 18 compares the elimination of tricaine from frogs kept in running water (23°C) and from frogs kept in a dry environment (23°C). In a dry environment, the drug is primarily eliminated by biotransformation (see below) and follows first-order kinetics. In running water, however, the whole-body tricaine concentration declined more rapidly, but with a biphasic or triphasic slope.

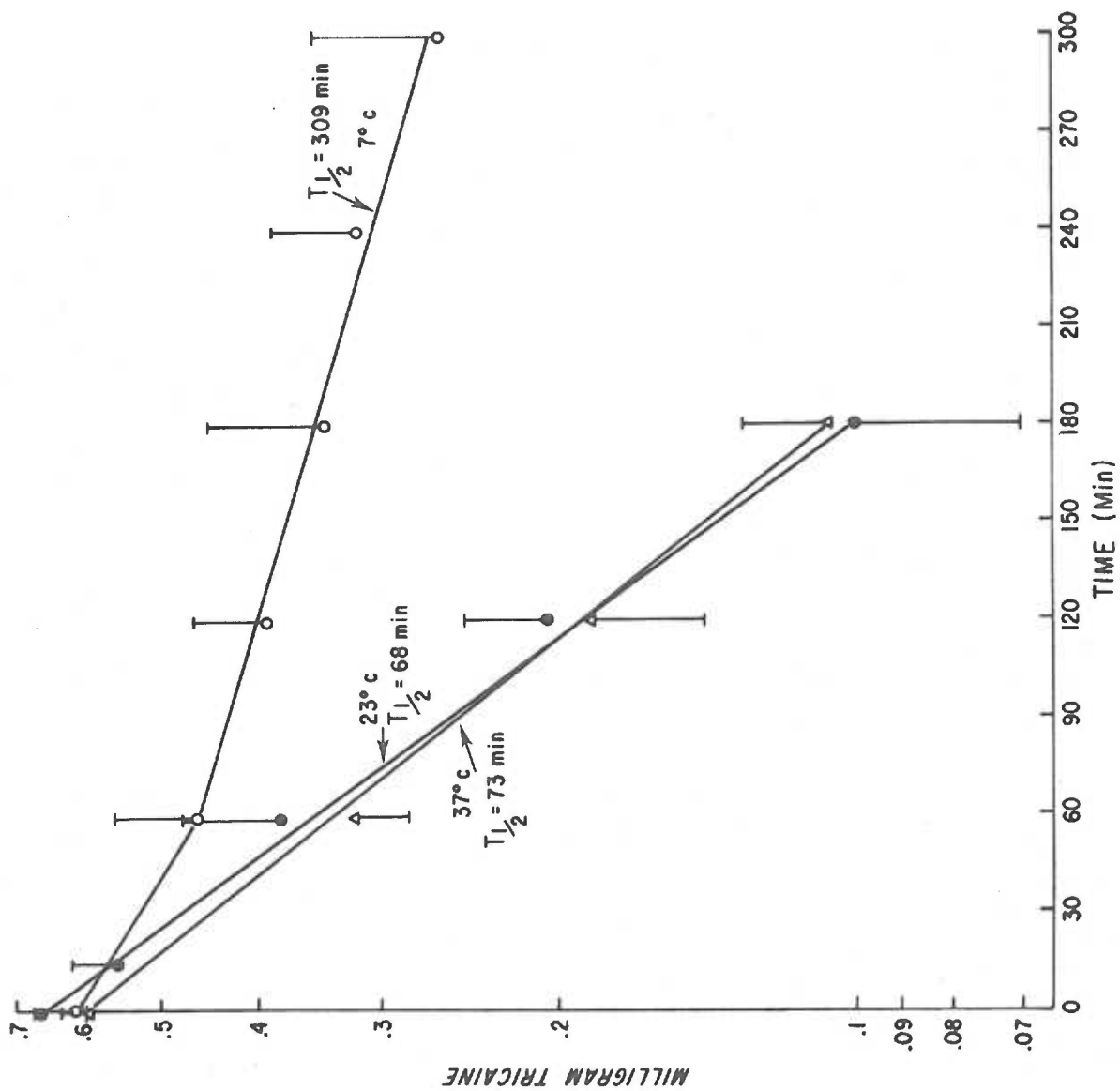


Figure 17: The Biologic Half-Life of Tricaine in Frogs and the Effect of Body Temperature on the

Rate of Tricaine Disappearance

The whole-body half-life ($t_{1/2}$) of 1 mg of tricaine methanesulfonate was 68 min in frogs kept at room temperature (23° C), 73 min in frogs kept at 37° C, and 309 min in frogs kept at 7° C. The rate of tricaine metabolism in the frogs at all 3 temperatures approximated first-order kinetics. Each point represents the mean $\pm$ S.D. of four animals.

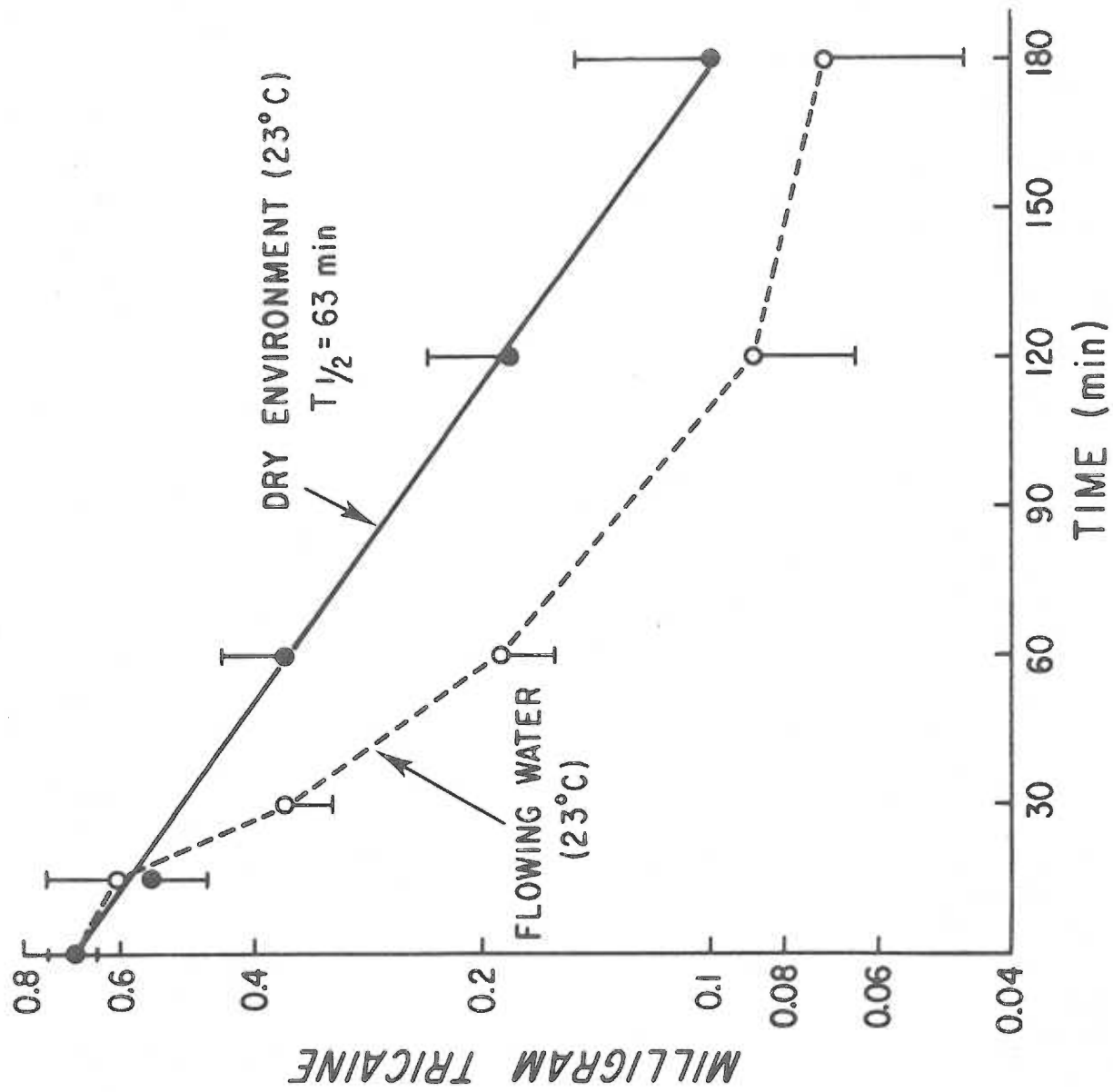


Figure 18: Comparison of the Biologic Half-Life of Tricaine in Frogs Kept in a Wet and a Dry Environment

The whole-body half-life of a 1 mg dose of tricaine methanesulfonate in frogs that were in flowing water (23° C) was markedly different from that of frogs in a dry environment. The rate of tricaine disappearance in frogs that were in flowing water did not approximate a first-order kinetic process; rather, it exhibited a biphasic or triphasic slope. Each point represents the mean $\pm$ S.D. of four animals.

Mice. Tricaine was metabolized so rapidly in the mouse that after an i.p. dose of 250 mg/kg tricaine methanesulfonate none of the unchanged drug could be recovered from the animals 5 min after the injection. It was therefore not possible to obtain an accurate value for the half-life of tricaine in the mouse (see below for estimate).

3. Tricaine Paralysis Times in Mammalian and Nonmammalian Species.

Table I shows i.p. anesthetic doses of tricaine methanesulfonate for seven different species and gives the resulting paralysis times. The doses were selected on the basis of preliminary experiments and were adequate to cause a temporary loss of the righting reflex in all animals. At the peak of the anesthetic effect, the animals were flaccid and their responses to nociceptive stimuli (toe pinch) were markedly reduced or absent. Loss of the righting reflex occurred within 1 min of drug injection in frogs, parakeets and mice; within 5 min in salamanders and lizards; and within 15 min in snakes and turtles.

Table I shows that i.p. doses of 150 to 300 mg/kg of tricaine methanesulfonate were adequate to produce loss of the righting reflex in all non-mammalian species. However, a dose of 250 mg/kg of the drug did not produce loss of the righting reflex or any other noticeable signs of drug action in mice, rats, guinea pigs, or cats. In mice, a sixfold increase in dose (1500 mg/kg) produced a loss of the righting reflex (anesthesia) for 5 min. Although these mice all recovered their righting reflex, they showed continued lethargy and died within 72 hr. The deaths were most likely the result of the injection of a large amount of an acidic salt, rather than of tricaine itself, since the injection of an equimolar

Species	N	Dose	Paralysis (min.)	Total Body Concentration (Upon Righting)	(1) % Recovery of Consumed Tricaine (Bratton-Marshall Assay)	(2) Estimated T _{1/2} (min.)
Frog (<i>Rana pipiens</i>)	(9)	150 mg/Kg	113±28	46.61±14.77 mg/Kg	54.2±7.5%	62.0±10.7
Salamander (<i>Taricha rivularis</i>)	(10)	150 mg/Kg	22±9	51.11±10.48 mg/Kg	96.2±10.2%	16.7±3.0
Snake (<i>Thamnophis sirtalis</i>)	(7)	200 mg/Kg	80±22	120.22±36.43 mg/Kg	41.3±10.6%	111.8±37.6
Turtle (<i>Pseudemys scripta elegans</i>)	(8)	250 mg/Kg	70±44	—	—	—
Lizard (<i>Anolis carolinensis</i>)	(9)	250 mg/Kg	23±7	30.84±6.08 mg/Kg	92.4±9.4%	7.9±2.5
Parakeet (<i>Melopsittacus undulatus</i>)	(6)	175 mg/Kg	2±1	51.43±5.54 mg/Kg	92.1±5.6%	2.6±0.8
Parakeet (<i>Melopsittacus undulatus</i>)	(4)	300 mg/Kg	6±2	58.50±24.25 mg/Kg		
Mouse (<i>Mus musculus</i>)	(8)	1500 mg/Kg	5±1	93.67±31.57 mg/Kg	107.7±6.3%	1.5±0.3

Table I: Comparative Pharmacokinetics of Tricaine Methanesulfonate

1. Measured in terms of tricaine methanesulfonate.
2. Measured in terms of equivalents of MABA.

Each value represents the mean $\pm$ S.D. of "N" animals.

dose of methanesulfonic or hydrochloric acid produced the same delayed death. An equimolar dose of tricaine (950 mg/kg) was injected i.p. into other mice (10) in the form of the free base, a viscous liquid. The free base form of tricaine produced similar induction and paralysis times but no deaths were observed in the mice during a 2 week period after the drug injection. Tricaine (950 mg/kg of the free base form of the drug) was also injected i.p. into 2 cats. Both animals exhibited a brief period of emesis but no ataxia. The animals were observed for a week after the injection and they did not demonstrate any further effects of the drug.

Table I gives the whole body concentration of tricaine found in the various species upon recovery of their righting reflexes. The half-lives of tricaine elimination (Table I) were estimated by making the assumption that the decline in the total body tricaine concentration followed first-order kinetics from time zero (drug injection) to time of sacrifice of the animals (recovery of the righting reflex). The half-life of tricaine in frogs, determined directly by sacrificing groups of animals at set time intervals after drug injection (Figure 17; 23° C), was in good agreement with the half-life of tricaine estimated for frogs in Table I (68 min and 62 min, respectively).

For most species, the total amount of tricaine eliminated from the body could be accounted for as BMRM (Bratton-Marshall Reacting Metabolites). This was not true for either the frogs or the snakes. Only 40-55% of the disappearance of the parent drug could be accounted for by the appearance of BMRM in these 2 species. To determine the basis of this low recovery, frogs were injected with 150 mg/kg of tricaine methanesulfonate i.p., immediately placed in a blender at room temperature (23°) to collect any

excreted substances and sacrificed 70 min after drug injection. The percent recovery of eliminated tricaine as BMRM was unchanged [$59 \pm 11\%$ (S.D.)], indicating that in frogs kept in a dry environment the discrepancy between the disappearance of tricaine and the appearance of BMRM was not the result of excretion. However, the percent recovery of eliminated tricaine as BMRM in frogs kept in running water (23°C) was significantly lowered [$29 \pm 3\%$ (S.D.); 60 min after drug injection].

4. In Vitro Hydrolysis of Tricaine by Liver Homogenates

Frogs (*Rana pipiens*) and Mice. Figure 19 shows a comparison of the metabolism of tricaine in incubations of the drug with liver homogenates obtained from frogs and mice. Twenty-five min after the addition of drug to the incubations, 491 μmoles of tricaine had been metabolized per gm of mouse liver, whereas no measureable metabolism had occurred in the incubations with frog-liver homogenate. Attempts to increase the amount of hydrolysis of tricaine by increasing the concentration of liver in the incubations were unsuccessful. Drug addition under these conditions precipitated the protein in incubations from both species. Sandoz (1920), in his original studies of tricaine methanesulfonate, noted that the drug precipitated the protein in serum.

In incubations that contained the liver homogenates of both species (mouse and frog), the rate of metabolism of tricaine was approximately one-half of that observed in incubations of the drug with mouse-liver homogenate alone (32.0 μmoles metabolized/25 min and 70.2 μmoles metabolized/25 min, respectively). However, the rate of metabolism per gm of mouse liver was approximately the same, with or without the presence of the frog-liver homogenate [19.6 ± 1.7 (S.D.) μmoles of tricaine/gm of mouse liver/min].

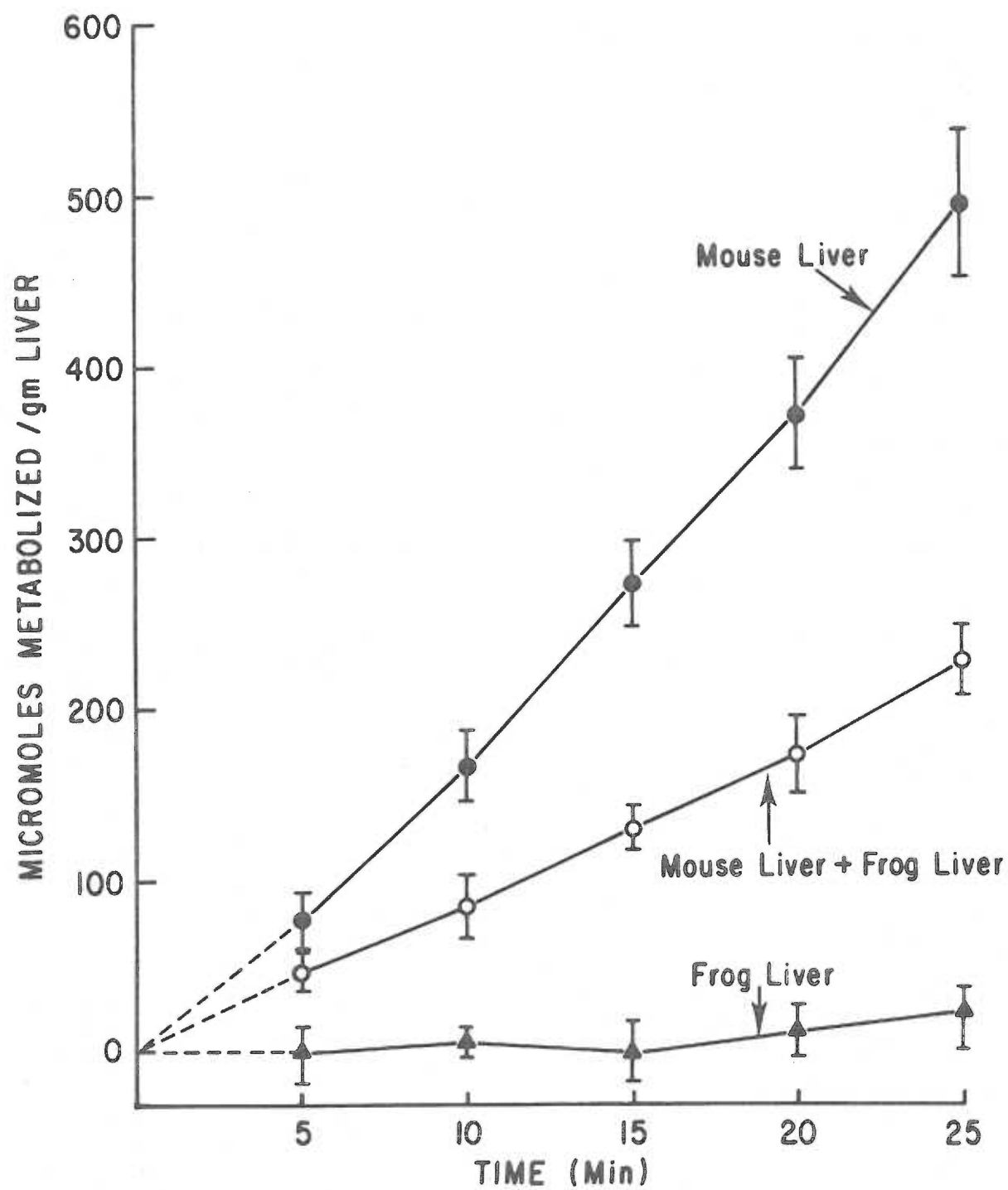


Figure 19: Tricaine Metabolism in In Vitro Liver Incubations

Tricaine was metabolized in mouse-liver incubations at a rate of 19.6 ± 1.7 (S.D.) μ moles of tricaine/gm of liver/min and in combined liver incubations (mouse and frog) at a rate of 8.94 μ moles of drug/gm of liver/min. There was no measureable metabolism in the frog-liver incubations. Each point represents the mean $\pm$ S.D. of four incubations.

The linearity of tricaine disappearance and appearance of BMRM with time in mouse-liver incubations, suggested that the drug concentration (3 mg/ml incubation) was sufficient to saturate the tricaine-esterase system of the incubations, and that, therefore, the rate of tricaine hydrolysis was a measure of the maximum rate ($V_{\max}$) of tricaine metabolism in the in vitro mouse liver. Other incubations (Figure 20) showed that the rate of tricaine metabolism varied directly with the mouse-liver concentration.

Both the mouse-liver and the frog-liver homogenates were assayed to determine if either preparation deteriorated during the 15 min warming period. Figure 21 shows the rate of tricaine metabolism in a mouse-liver incubation that was not warmed (20.3 μ moles of tricaine/gm of liver/min). The rate of drug metabolism was approximately the same as that found in "pre-warmed" incubations. The slower rate in the initial 3 min was most likely the result of the low temperature (4° C) of the incubation at zero time (drug addition). There was no measureable metabolism of tricaine in frog-liver incubations during the 15 min warming period.

Other Terrestrial Vertebrates. Figure 22 illustrates the marked difference in hepatic tricaine-esterase activities of poikilothermic and mammalian species. In vitro liver homogenates from frogs, bullfrogs, salamanders, snakes and lizards all failed to exhibit any measureable hydrolysis of tricaine during the 25 min incubation period. In contrast, liver incubations of all mammalian species showed substantial tricaine-hydrolysis activities. The rate of tricaine metabolism by mammalian

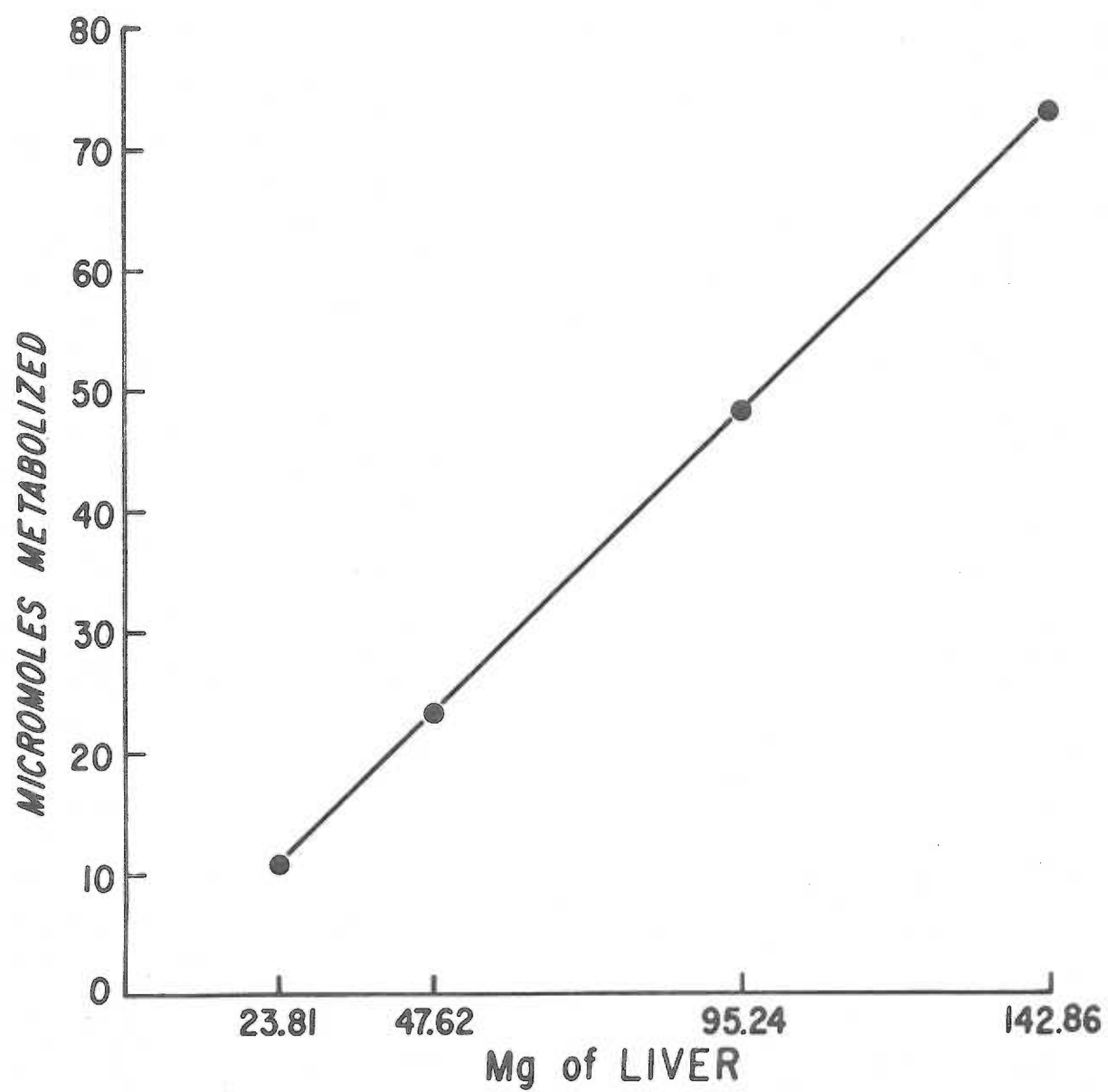


Figure 20: The Relationship Between the Rate of Tricaine Metabolism and the Concentration of Mouse Liver in an Incubation

The rate of metabolism of tricaine was directly proportional to the concentration of mouse liver in the incubation throughout the 25 min incubation period. The ordinate shows the total micromoles of tricaine metabolized in 25 min and the abscissa shows the total amount of mouse liver in the 9 ml incubations. Each point represents a single incubation analyzed 25 min after the addition of drug to that incubation.

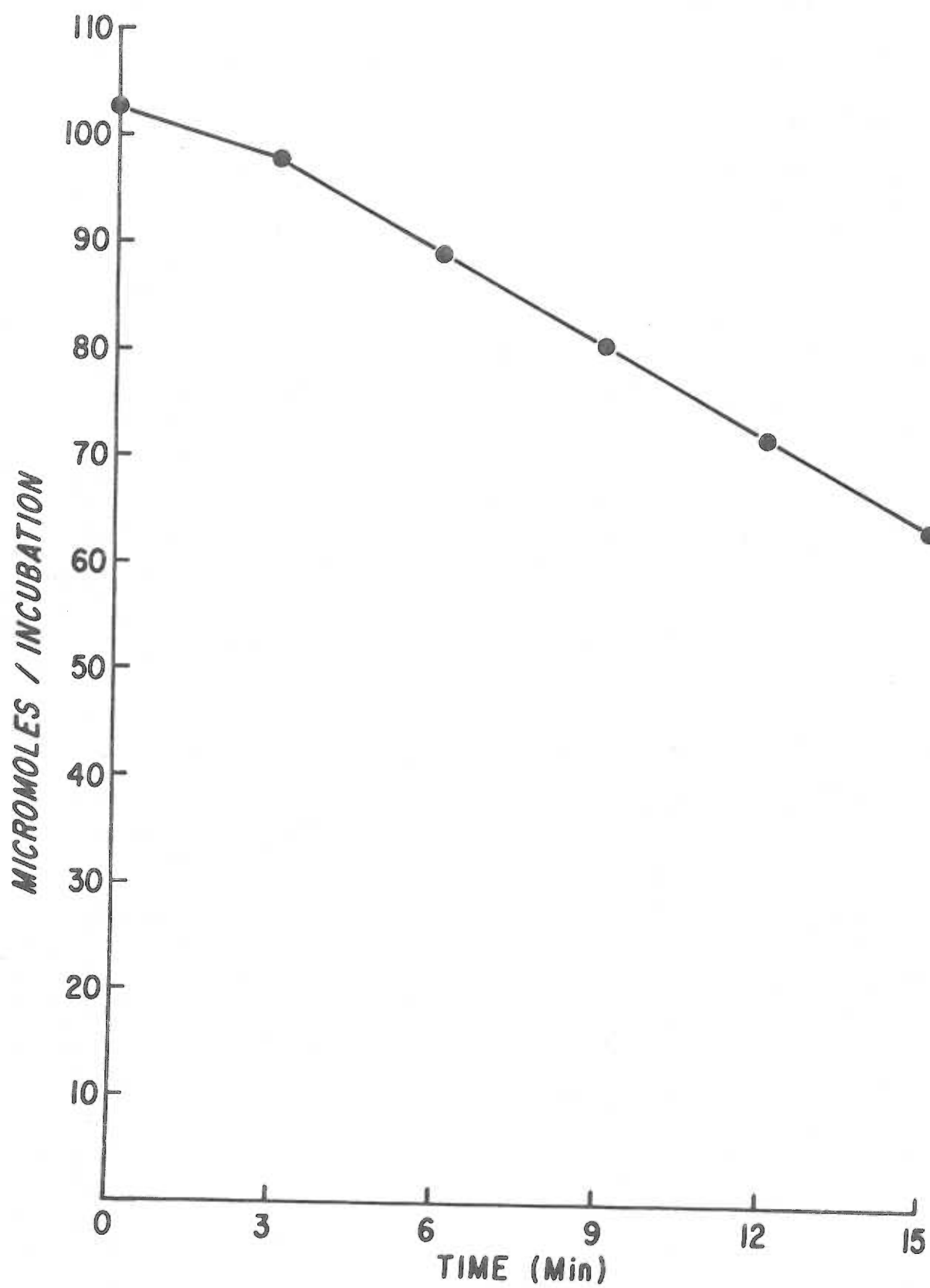


Figure 21: The Integrity of the In Vitro Mouse-Liver Homogenate During the 15 Min Pre-Incubation Warming Period

The disappearance of tricaine from the mouse-liver incubation was linear throughout most of the 15 min warming period. The slowed rate of tricaine metabolism in the initial 3 min was most likely the result of the low temperature (4° C) of the incubation at time zero. No tricaine metabolism was detectable in frog-liver incubations during the 15 warming period. Each point represents a single sample from an incubation of tricaine methanesulfonate with mouse-liver homogenate.

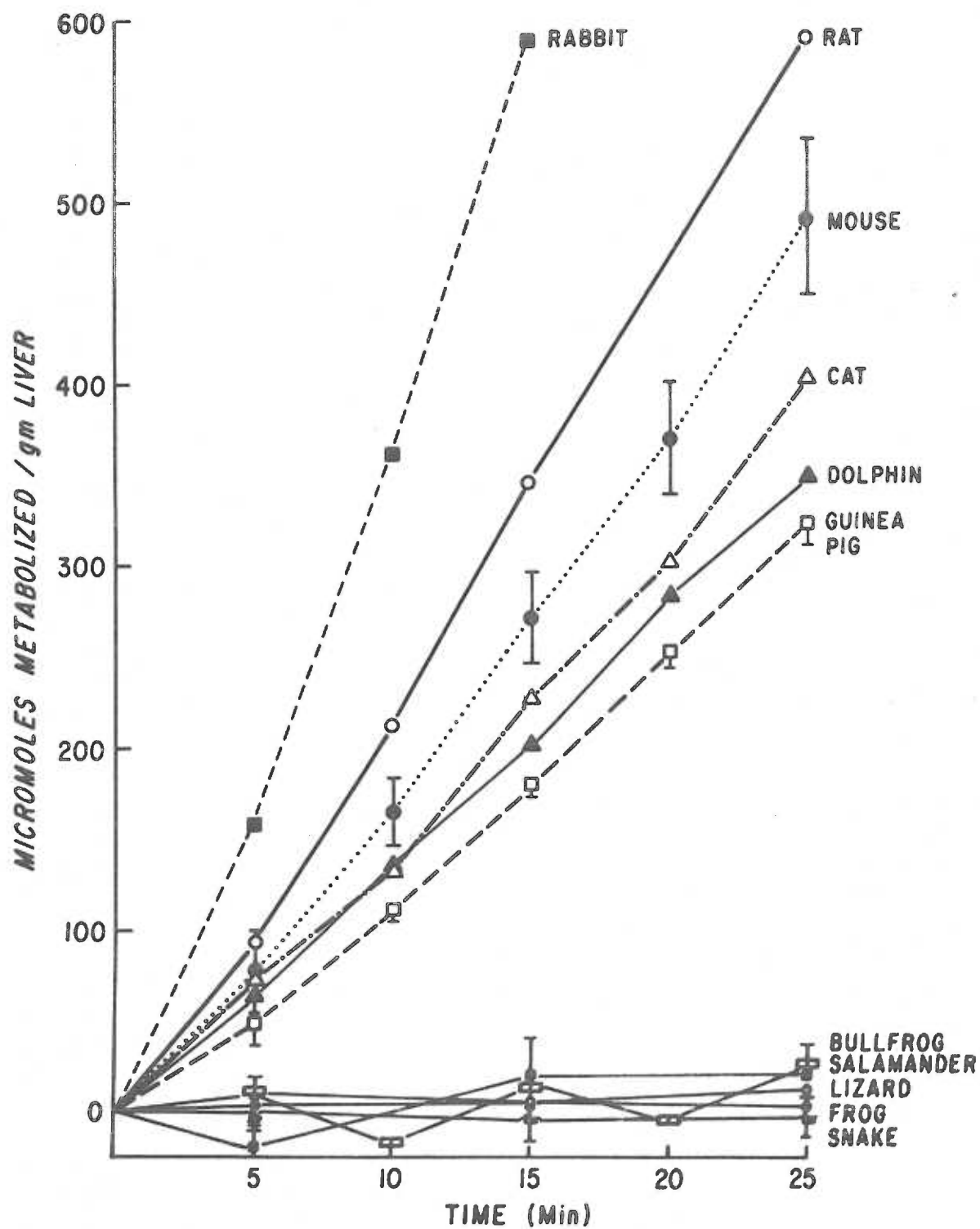


Figure 22: Comparison of the Metabolism of Tricaine in In Vitro Liver Incubations from Amphibians, Reptiles and Mammals

The in vitro liver homogenates of poikilothermic species failed to metabolize any significant amount of tricaine when incubated with the drug for 25 min. In contrast, substantial quantities of tricaine were metabolized in all in vitro mammalian-liver incubations. The rate of tricaine metabolism ($\mu\text{moles/gm of tissue/min}$) was 39.3 for rabbits, 23.6 for rats, 19.6 for mice, 16.0 for cats, 13.9 for dolphins and 12.8 for guinea pig.

Each point with brackets represents the mean $\pm$ S.D. of four incubations, each from a different animal. All other points represent single measurements made on single incubations.

livers varied from 13 μ moles/gm of liver/min for the guinea pig to 39 μ moles/gm of liver/min for the rabbit.

Marine Vertebrates. The livers of the sea lions and the seal were partially decomposed before being frozen, as evidenced by their physical appearance, consistency and odor upon thawing. Attempts to acquire fresh specimens were not successful. Homogenates of the sea lion and the seal livers did not demonstrate any measureable metabolism of tricaine. The dolphin's liver, which appeared to be in good condition upon thawing, metabolized tricaine at a rate (13.9 μ moles/gm of liver/min) comparable to that of other mammals.

Effect of Freezing on In Vitro Liver Metabolism of Tricaine.

Figure 23 illustrates that freezing does not adversely affect the in vitro liver metabolism of tricaine. Mouse livers that had been frozen for 2 weeks metabolized tricaine at the same rate as fresh mouse livers.

5. In Vitro Hydrolysis of Tricaine in Serum Incubations

Inhibition of Tricaine Hydrolysis in Serum Incubations. The disappearance of tricaine quantitatively correlated with the appearance of MABA in all serum incubations (see Figure 9). Figure 24 shows the time course of tricaine hydrolysis in incubations of the drug with human sera. The sera were (1) incubated alone (untreated) with tricaine methanesulfonate, (2) incubated with the drug in the presence of neostigmine (1.2×10^{-3} M), and (3) boiled before being incubated with tricaine methanesulfonate. Nearly all of the tricaine incubated with untreated human sera was metabolized in 5 hr [$97 \pm 2\%$ (S.D.)], whereas less than half [$42 \pm 3\%$ (S.D.)] of the drug incubated with human sera in the presence of neostigmine was metabolized in the same time period. There was

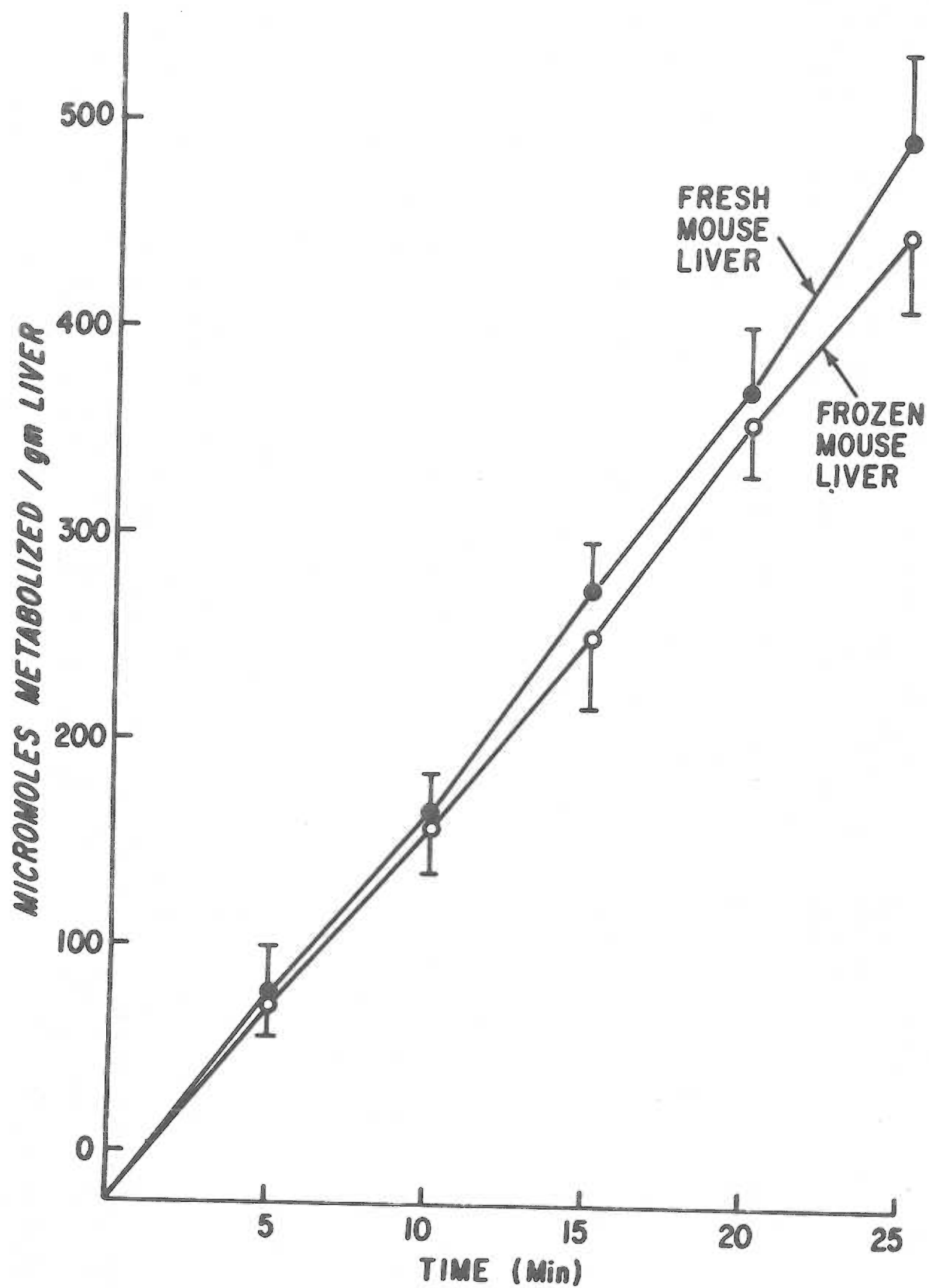


Figure 23: The Effect of Freezing on the In Vitro Mouse Liver

Mouse livers that were frozen (-11°C) for 2 weeks, metabolized tricaine in in vitro incubations at the same rate as fresh mouse livers. Each point represents the mean $\pm$ S.D. of four incubations, each from a different animal.

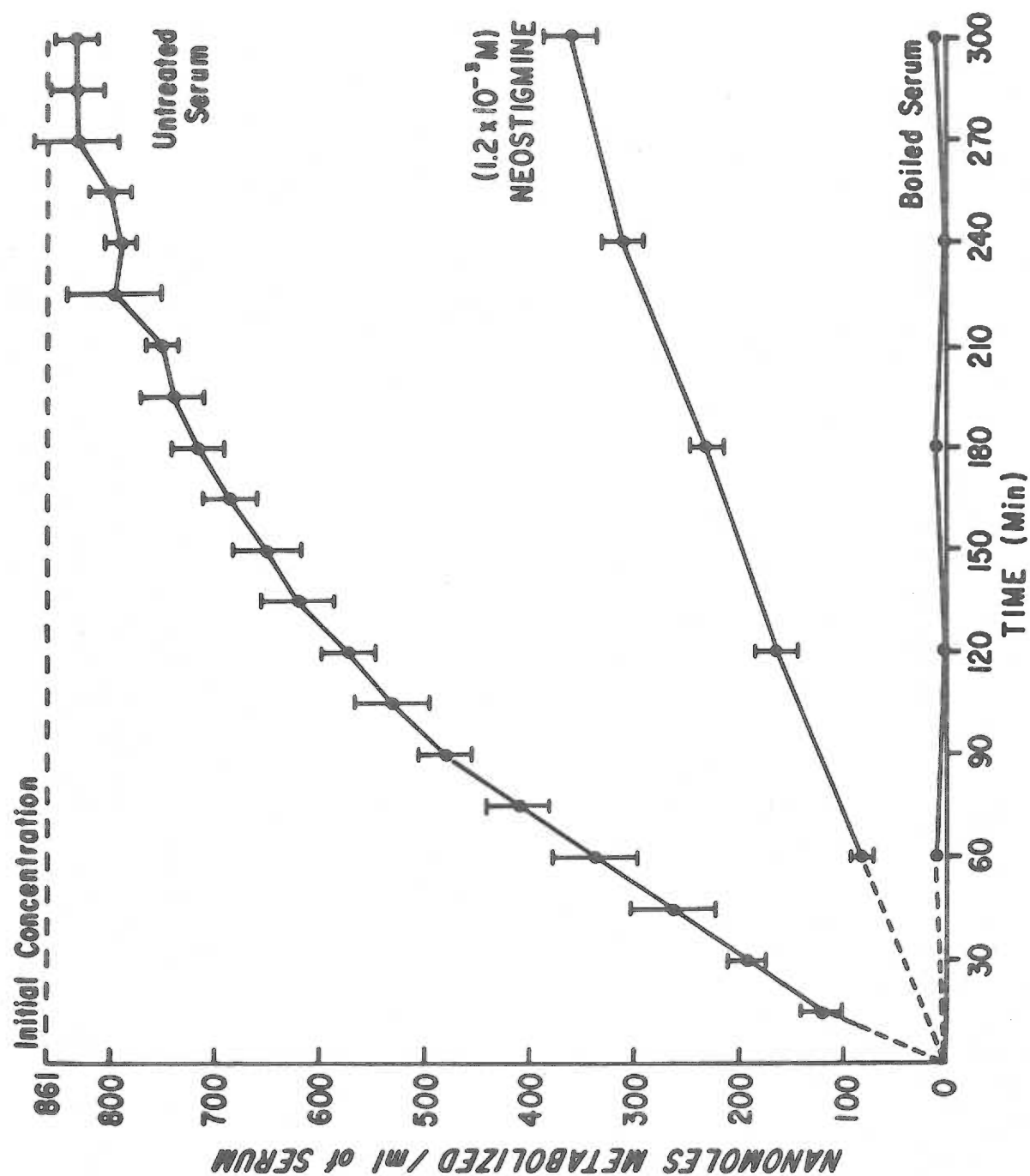


Figure 24: Inhibition of Tricaine Hydrolysis in Serum Incubations

The incubations were prepared with 10 ml of human sera and 20 ml of phosphate buffer. The initial tricaine concentration was 2.87×10^{-4} M. The human sera incubated alone (untreated) with tricaine, metabolized 97% of the drug in 5 hr. The same serum incubated with tricaine in the presence of neostigmine metabolized only 42% of the drug in the same period. Boiling the serum before the incubation destroyed the tricaine hydrolysis activity. Each point with brackets represents the mean $\pm$ S.D. of four incubations, each from a different serum sample from the same individual. The incubation of tricaine with boiled serum was only prepared once and, therefore, each point represents a single measurement from a single incubation.

no metabolism of tricaine in incubations of the drug with boiled sera.

Variation in the "Tricaine-Esterase" Activity of Human Sera.

The variation in tricaine-esterase activities of human sera, was studied and compared with that of bullfrog sera. Figure 25 shows the time course of the hydrolysis of tricaine by human sera from 3 individuals and by bullfrog sera. Human sera metabolized an average of 87.3% of the drug in 4 hr; the range was 78.9% to 91.9%. The frog sera metabolized 82.5% of the drug in the same time period.

Hydrolysis of Tricaine by Serum Albumin. The protein concentration of the 3 human sera (above) ranged from 7.8 to 8.4 gm% and the rate of tricaine hydrolysis of a given human serum sample was inversely related to its protein concentration. The bullfrog sera had approximately 59% of the protein content of human sera (4.7 gm%) but exhibited a tricaine-hydrolysis activity similar to that of human sera. The lack of a direct correlation between protein concentration and tricaine-hydrolysis activity is explained by the fact that, although albumin is the major component of serum protein, it does not exhibit any tricaine-esterase activity.

Kinetic Studies of the Hydrolysis of Tricaine in Serum Incubations. Figure 26 shows a comparison between the 3 independent assays for tricaine hydrolysis in sera. There were no substances in the incubations which interfered with the assays. The disappearance of tricaine in all serum incubations was quantitatively accounted for by the appearance of MABA; MABA was the only metabolite of tricaine in serum incubations (See Figure 9).

The rate of hydrolysis of tricaine in incubations of the drug with sera from bullfrogs, mice and humans is shown in Figure 27. The sera

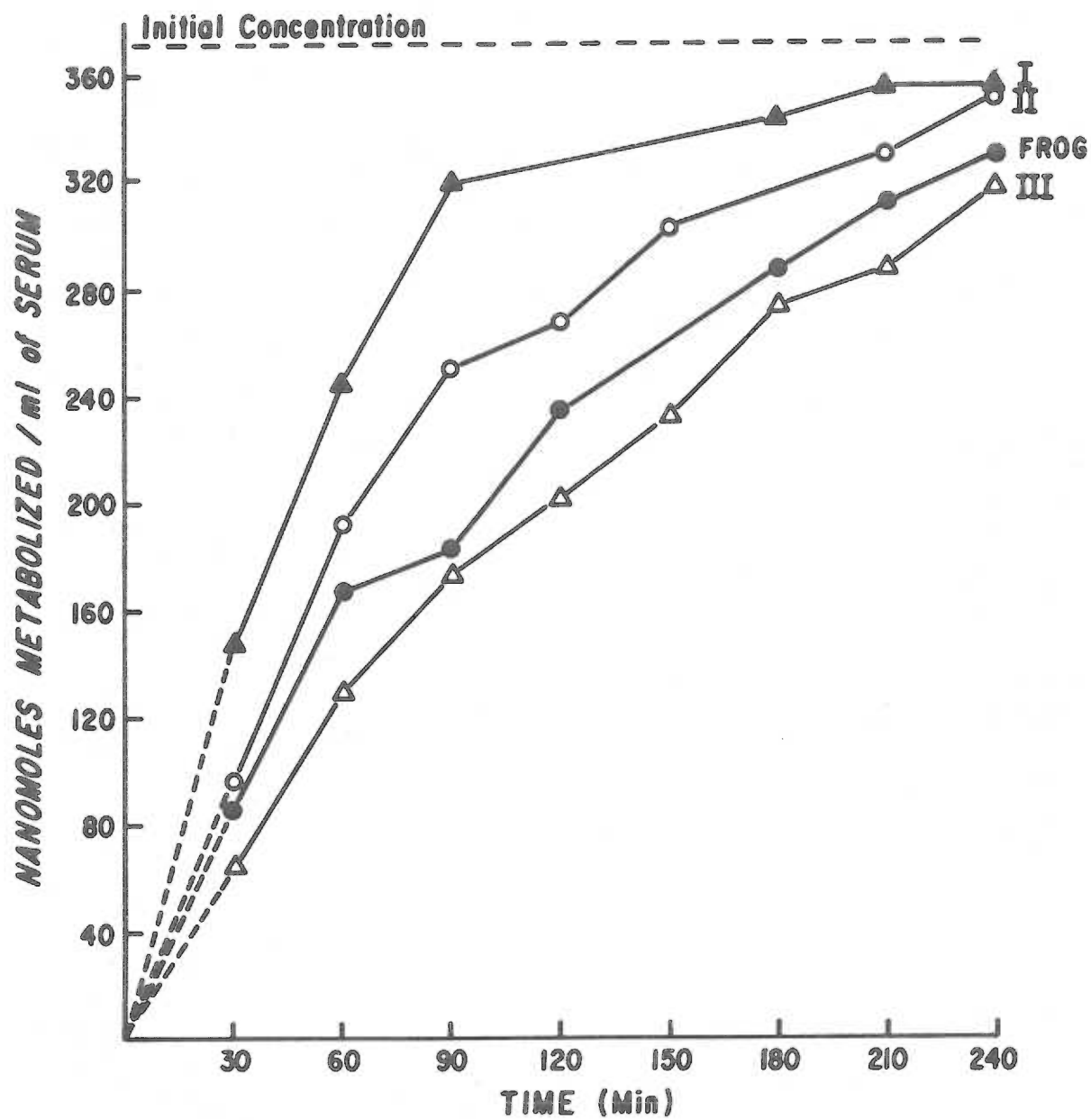


Figure 25: Variation in the "Tricaine-Esterase" Activity of Human Serum

The incubations were prepared with 2.5 ml of serum and 2.5 ml of phosphate buffer. The initial tricaine concentration was 1.91×10^{-4} M. The human sera (I-III) metabolized an average of 87.3% of the drug in 4 hr. The human sera samples (I-III) had an average protein concentration of 8.0 gm% [7.8 gm% ($\blacktriangle$), 7.9 gm% (O) and 8.4 gm% (Δ), respectively]. The bullfrog sera ($\bullet$) had a protein content of 4.7 gm% and metabolized 82.5% of the tricaine in the incubation in 4 hr. Each point represents a sample from a single incubation.

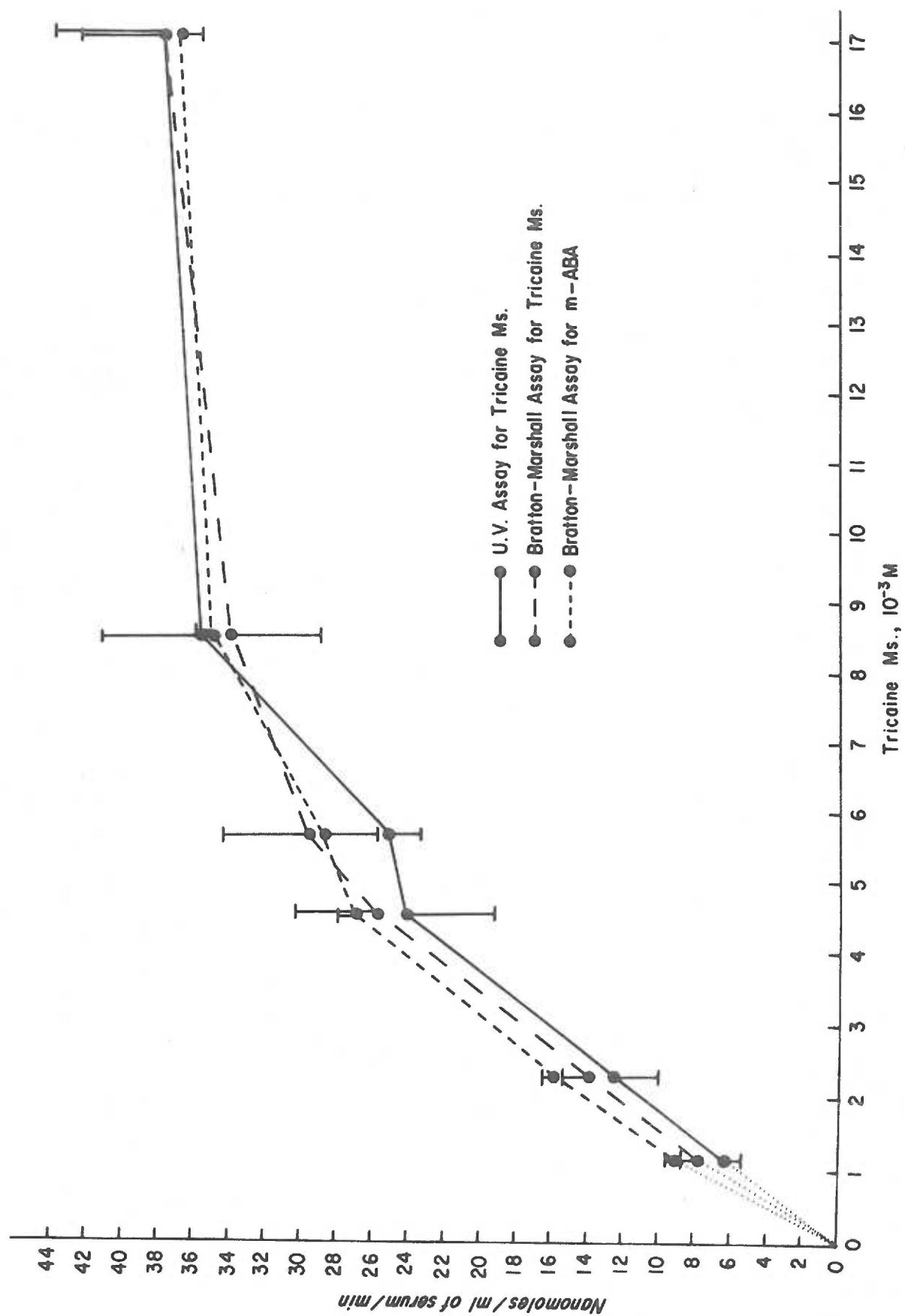


Figure 26: Comparison of the 3 Assays for Tricaine Hydrolysis in Human Serum

The 3 separate assays for tricaine metabolism, two of which independently measured the disappearance of the parent drug and one which measured the appearance of metabolites, were in close agreement in this and all other studies. Therefore, the results of the 3 assays were averaged for each sample and that average listed as a single determination in all other experiments. Each point represents the mean $\pm$ S.D. of four serum incubations, each from a different serum sample from the same individual. Each incubation was assayed 30 min after drug addition.

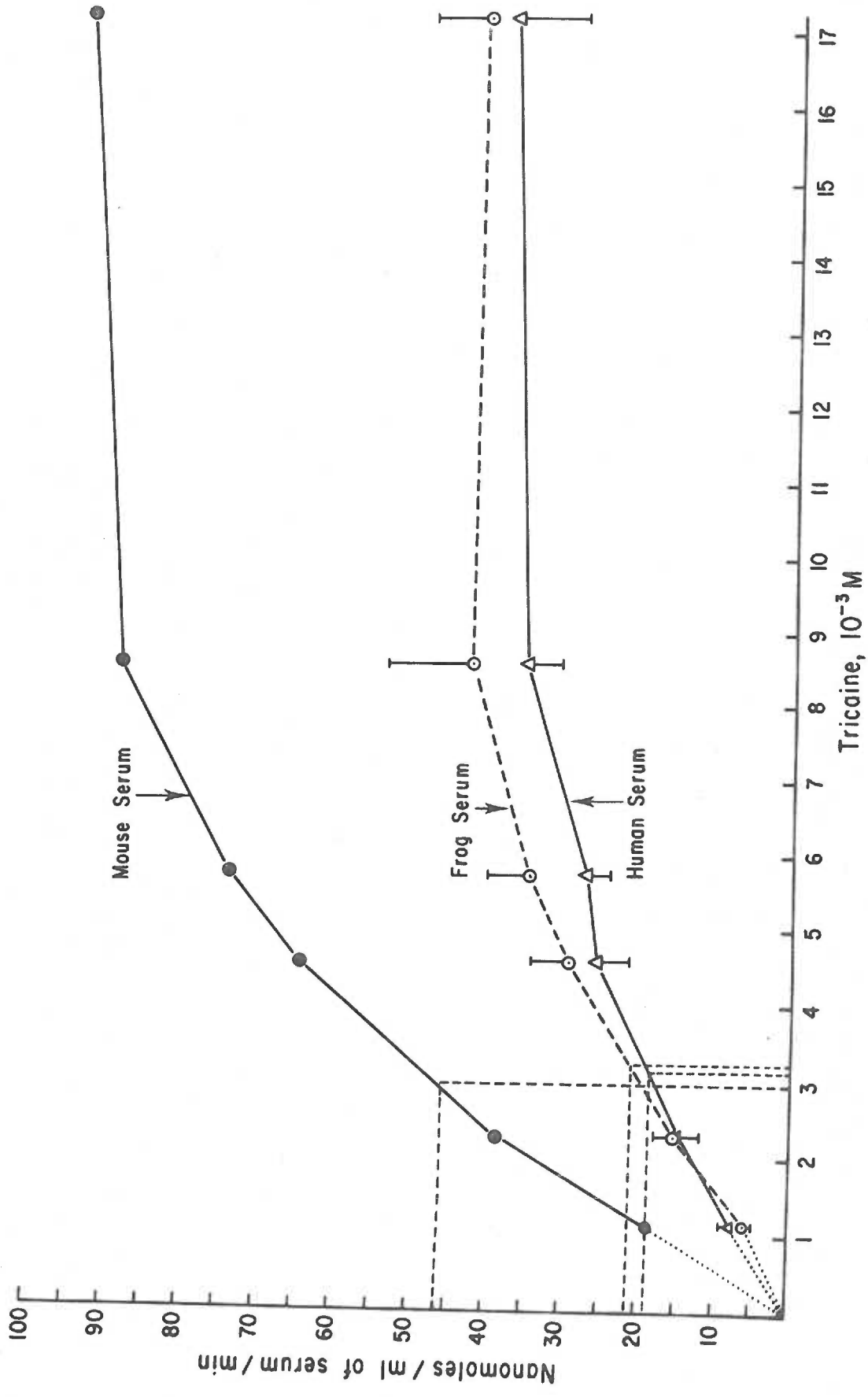


Figure 27: Kinetics of Serum Hydrolysis of Tricaine

A greater than twofold increase in the rate of disappearance of tricaine (and the appearance of MABA) was seen with mouse serum as compared with bullfrog and human sera. Apparent K_m values of approximately 3×10^{-3} M were estimated for the sera of all 3 species from their apparent V_{max} 's. Each point of the frog serum and human serum represents the mean $\pm$ S.D. of four incubations, each from a different serum sample, whereas each point of the mouse serum represents that of a single incubation.

of bullfrogs and humans hydrolyzed the drug at approximately the same rate, and the apparent maximum rate of hydrolysis for both sera was about 40 nanomoles/ml of serum/min. The rate of tricaine metabolism in the mouse sera was about double that of human and bullfrog sera. The hydrolysis of tricaine in the mammalian sera appeared to conform to Michaelis-Menten kinetics while that of the bullfrog sera did not. Figure 28 shows the Lineweaver-Burk linear transformation of the Michaelis-Menten

equation,
$$v = \frac{V_{\max} \cdot S}{K_m + S}$$
 (Christensen and Palmer 1967), using the serum-hydrolysis data of Figure 27. In this equation, "v" is the velocity of the enzymatic reaction, "S" is the substrate concentration, "K_m" is the substrate concentration which results in a half-maximum velocity, and "V_{max}" is the maximum velocity that can be attained by elevating S. For enzyme reactions which follow Michaelis-Menten kinetics, the transformation results in straight lines with an ordinate intercept of 1/V_{max}, an abscissa intercept of -1/K_m, and a slope of K_m/V_{max}. The resultant kinetic values for tricaine metabolism by human sera from the transformation were K_m = 9.1 x 10⁻³ M and V_{max} = 69.0 nanomoles of tricaine/ml of serum/min. The discrepancy between these values and those approximated from Figure 27 (K_m = 3 x 10⁻³ M, V_{max} = 40 nanomoles of tricaine/ml of serum/min) is inherent in the construction of the Lineweaver-Burk transformation. In this transformation, the low substrate concentrations determine the slope of the line to a much greater extent than do the higher substrate concentrations; the slope of the line is "weighted" in favor of the lower concentrations. The Woolfian linear transformation (Figure 29) corrects this anomaly (weighting). In this transformation, the ordinate intercept is K_m/V_{max}, the abscissa intercept is -K_m and the

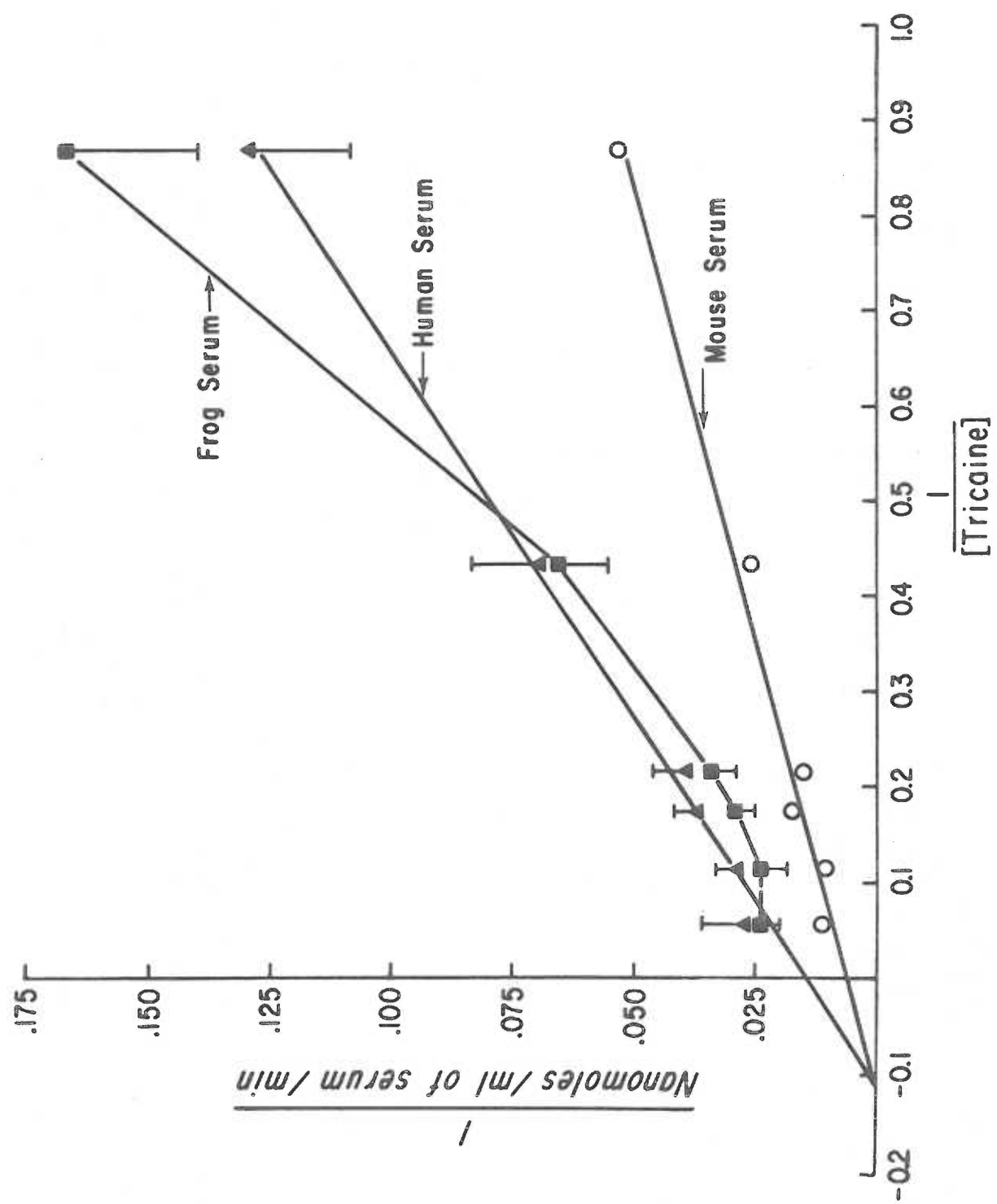


Figure 28: Lineweaver-Burk Plot of Serum Hydrolysis of Tricaine

The hydrolysis of tricaine by human and mouse sera followed Michaelis-Menten kinetics, whereas, the hydrolysis by bullfrog sera deviated from those kinetics. The K_m value for the hydrolysis of tricaine was the same for human and mouse serum (9.1×10^{-3} M). Each point of the frog serum and human serum represents the mean $\pm$ S.D. of four incubations, each from a different sample of serum, whereas each point of the mouse serum represents that of a single incubation.

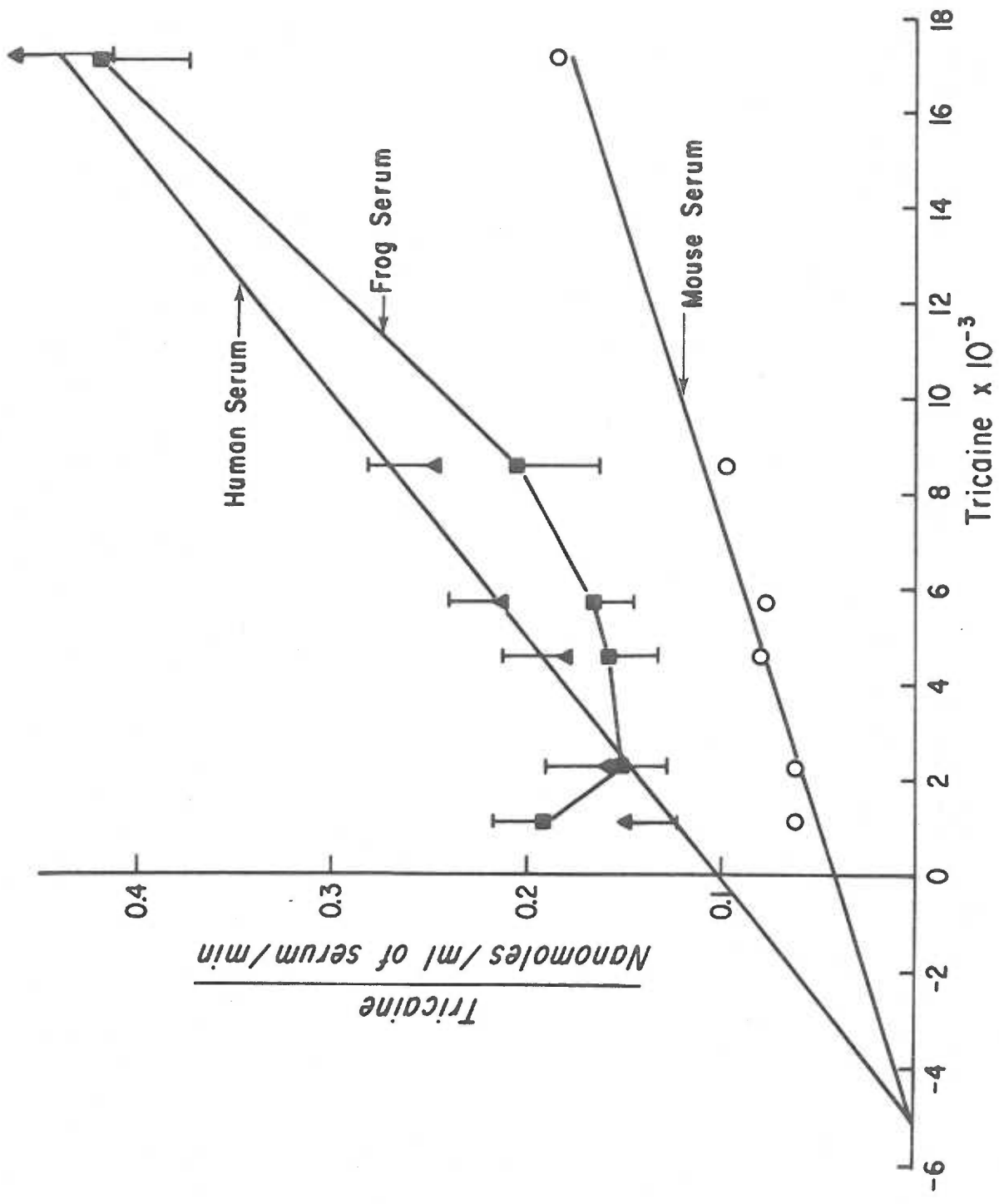


Figure 29: Woolfian Plot of Serum Hydrolysis of Tricaine

The data from mouse and human sera both conformed to straight lines in this transformation and this indicates that the hydrolysis of tricaine by these sera followed Michaelis-Menten kinetics. The data from frog serum described a curve on this plot and, therefore, the hydrolysis of tricaine in frog serum did not appear to follow Michaelis-Menten kinetics. The K_m values for the hydrolysis in the two mammalian sera was the same, 5×10^{-3} M. Each point of the frog serum and human serum represents the mean $\pm$ S.D. of four incubations, each from a different sample serum, whereas each point of the mouse serum represents that of a single incubation.

slope is $1/V_{\max}$. The resultant kinetic values for tricaine metabolism in human sera from this transformation were $K_m = 5 \times 10^{-3}$ M and $V_{\max} = 49.0$ nanomoles of tricaine/ml of serum/min. Both of these kinetic values agree closely with the values estimated from Figure 27 ($K_m = 3 \times 10^{-3}$ M; $V_{\max} = 40$ nanomoles of tricaine/ml of serum/min). Both mammalian sera appeared to have the identical K_m values for their hydrolysis of tricaine.

6. In Vitro Hydrolysis of Tricaine in Saliva Incubations

There was no detectable metabolism of tricaine in any of the 5 human saliva incubations. In other experiments, tricaine was dissolved directly in saliva and, again, there was no measureable hydrolysis over a 60 min period.

7. Tissue Distribution of the Tricaine-Hydrolysis Activity in the Mouse (Table II)

The liver of the mouse displayed the highest in vitro rate of tricaine metabolism of all tissues tested. The lung and kidney exhibited substantial activities, but due to their low percentage of the body weight, they are responsible for little of the total whole-body tricaine-hydrolysis activity.

8. Tissue Distribution of Tricaine and Its Metabolites in the Bullfrog

Four bullfrogs, with an average weight of 540 ± 69 gm (S.D.), were injected i.p. with a 200 mg/kg dose of tricaine methanesulfonate and various tissues were analyzed for tricaine and its metabolites 20 min after the drug injection. At the time of tissue sampling, all 4 bullfrogs were flaccid, did not respond to nociceptive stimuli (toe pinch), and had not regained their righting reflex. Table III shows the various

TISSUE	PERCENT BODY WEIGHT	MAXIMUM VELOCITY OF TRICaine HYDROLYSIS (μ moles of tricine/gm of tissue/min)
<i>Liver</i>	6.41	18.2
<i>Kidney</i>	1.90	6.8
<i>Lung</i>	0.66	6.3
<i>Muscle</i>	---	1.6
<i>Heart</i>	0.55	0.5
<i>Brain</i>	1.41	0.1
<i>Serum</i>	---	0.1

Table II: Tissue Distribution of Tricaine-Hydrolysis Activity in the Mouse

Each determination in this table is from a single 22 gm mouse.

TISSUE	TRICAINE METHANESULFONATE ($\mu\text{g/gm}$ of tissue)	TRICAINE METABOLITES ¹ ($\mu\text{g/gm}$ of tissue)	METABOLITES (moles/gm) TRICAINE (moles/gm)
<i>Muscle</i>	57.06 $\pm$ 12.25	6.38 $\pm$ 2.57	21.30%
<i>Brain</i>	154.15 $\pm$ 39.99	15.45 $\pm$ 4.69	19.10%
<i>Lungs</i>	187.69 $\pm$ 75.75	18.02 $\pm$ 3.64	18.29%
<i>Heart</i>	191.57 $\pm$ 17.68	22.90 $\pm$ 2.21	22.78%
<i>Blood</i>	236.04 $\pm$ 5.28	23.38 $\pm$ 5.41	18.87%
<i>Liver</i>	1026.81 $\pm$ 771.67	137.31 $\pm$ 47.80	25.48%
<i>Fat</i>	1372.33 $\pm$ 1185.51	23.44 $\pm$ 20.81	3.25%

¹ Metabolites measured in terms of MABA

Table III: Tissue Distribution of Tricaine and Its Metabolites in the Bullfrog 20 Min after
I.P. Injection

The weights of the various tissue specimens were as follows: (1) muscle - 8.88 ± 1.98 gm;
(2) brain - 0.24 ± 0.08 gm; (3) lungs - 2.00 ± 0.77 gm; (4) heart - 1.69 ± 0.24 gm; (5) liver -
 5.25 ± 0.81 gm; and (6) abdominal fat - 6.70 ± 1.85 gm. All values (both above and in table
itself) are means $\pm$ S.D. of tissues of 4 different bullfrogs.

1. Measured in terms of equivalents of MABA.

tissue concentrations of tricaine. The tricaine concentration in the brains was approximately 98 $\mu\text{g}/\text{gm}$ of tissue (free base form of tricaine) and this brain level was apparently sufficient to cause anestheisa (paralysis) of the bullfrogs. The high drug concentrations in the abdominal fat and the liver were probably artificially high as a result of the intraperitoneal route of drug administration. The tissue distribution of tricaine metabolites was approximately the same as that of the parent drug. The amount of the metabolites per gm of tissue was approximately 20% of the tricaine tissue concentration for all tissues except the abdominal fat body.

The half-life ($t_{1/2}$) of tricaine in frogs in a dry environment (23°C) is 68 min and the elimination approaches a first-order (exponential) process (Figure 17). Since $t_{1/2} = 0.693/k_e$ (k_e = elimination constant) in first-order eliminations, the k_e for frogs in a dry environment (23°C) is 10.2×10^{-3} . By assuming that the k_e (elimination constant) is similar in bullfrogs, a theoretical ratio of metabolite * /tricaine * can be calculated from the equation: $\text{Log } \frac{x^*}{x_0} = -k_e T^*/2.3$, where x^* represents the drug content at time T^* and x_0 represents the initial drug level. The theoretical metabolite/tricaine ratio for bullfrogs 20 min after drug injection is 22.70%. This closely agrees with the experimental ratio of $20.97 \pm 2.79\%$ (S.D.) (the mean ratio of all the tissues analyzed, excluding the abdominal fat body) and suggests that tricaine was metabolized in situ in the respective tissues.

B. Studies of the Metabolic Fate of Tricaine in the Mouse

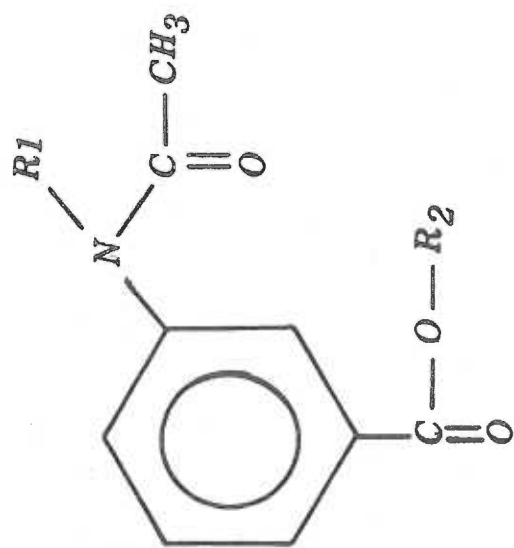
Previous experiments with mice showed that tricaine (250 mg/kg of

the methanesulfonic acid salt) was completely metabolized 5 min after i.p. injection, that the appearance of BMRM (Bratton-Marshall Reacting Metabolites) quantitatively accounted for the disappearance of tricaine, and that the metabolites of tricaine included MABA, ethanol and several, more polar, water-soluble compounds (See Figure 11).

1. Urinary Metabolites of Tricaine

Table IV-VI show the major ion fragments in the mass spectra of three urinary metabolites of tricaine recovered from mice that had received 1 gm of the free base form of tricaine. (See Appendix V for the complete mass spectra.) Table IV shows the major ion fragments in the mass spectra of the two methyl derivatives of acetylated MABA, found after methylation with methyl iodide, and their respective deuterio-methylated counterparts. Each 3 atomic mass unit increase seen with the deuterio-methyl compounds indicates the presence of a methyl group that was added during the methylation process. The base ion (the most abundant fragment in a spectrum) in the spectra of all 4 compounds was the demethylated fragment. Other major fragmentations of the compounds included loss of the methoxy radical of the carbonyl group and loss of the entire carbonyl group.

Table V shows the fragmentation patterns in the mass spectra of 3 distinct species of the methylated glycine conjugate of MABA and the respective deuterio-methylated compounds. The major fragmentation seen in the spectra was the loss of the methylated glycine group. Two distinct tri-methyl glycine conjugate compounds were found that fragmented with the loss of 88 and 102 atomic mass units to form their base ions respectively. The respective deuterio-methyl compounds lost 91 and 108 atomic



Acetylated MABA

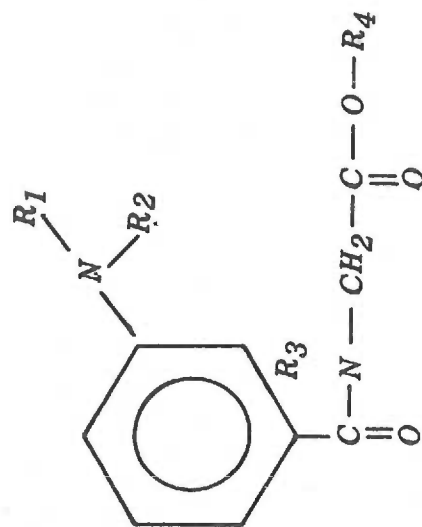
	R_1	R_2
(a)	H	CH ₃
(b)	H	CD ₃
(c)	CH ₃	CH ₃
(d)	CD ₃	CD ₃

Substituents

(a)	193* (38.5)	162 (16.4)	152 (10.0)	151 —	120 (55.3)	93 (10.0)	92 (21.3)	65 (12.3)
(b)	196* (36.0)	162 (13.0)	155 (10.3)	154 —	153 (11.2)	120 (29.6)	92 (18.3)	65 (11.4)
(c)	207* (34.5)	176 (12.1)	166 (10.3)	165 —	164 (25.0)	134 (17.2)	107 (11.2)	106 (16.7)
(d)	213* (35.1)	179 (10.7)	172 (10.1)	171 —	169 (14.3)	137 (19.1)	109 (14.9)	104 (12.1)

Table IV: Major Ions in the Mass Spectra of Methyl and Deutero-Methyl Derivatives of a Mouse Urinary Metabolite of Tricaine: Acetylated MABA

Both the mono and di-methyl derivatives of acetylated MABA and the respective deutero-methyl compounds were identified in derivatized samples of mouse urine. The urine was collected over 24 hr after the injection of 4 mice with 1 gm of the free base form of tricaine intraperitoneally. The numbers in parentheses indicate the relative abundance of the particular ion fragment in relation to the base ion (underlined). The * denotes the molecular ion. See Appendix V for complete mass spectra.



Glycine Conjugate of MABA

	R_1	R_2	R_3	R_4
(a)	CH_3	CH_3	H	CH_3
(b)	CD_3	CD_3	H	CD_3
(c)	H_3	CH_3	CH_3	CH_3
(d)	H_3	CD_3	CD_3	CD_3
(e)	CH_3	CH_3	CH_3	CH_3
(f)	CD_3	CD_3	CD_3	CD_3

Substituents

(a)	236* (78.2)	149 (20.2)	148 —	121 (31.2)	120 (55.1)	79 (16.5)	77 (26.6)
(b)	246 (18.5)	245* (80.0)	155 (20.0)	154 —	127 (28.6)	126 (42.9)	
(c)	236* (45.9)	222 (31.7)	148 (15.7)	135 (26.6)	134 —	120 (24.3)	107 (15.0)
(d)	245* (41.0)	228 (15.2)	154 (33.3)	138 (21.9)	137 —	109 (35.3)	
(e)	250* (63.7)	149 (17.3)	148 —	134 (25.0)	121 (26.7)	120 (47.1)	77 (22.8)
(f)	262* (62.9)	155 (20.0)	154 —	137 (20.0)	127 (28.5)	126 (42.9)	109 (18.9)

Table V: Major Ions in the Mass Spectra of Methyl and Deutero-Methyl Derivatives of a Mouse Urinary Metabolite of Tricaine: The Glycine Conjugate of MABA

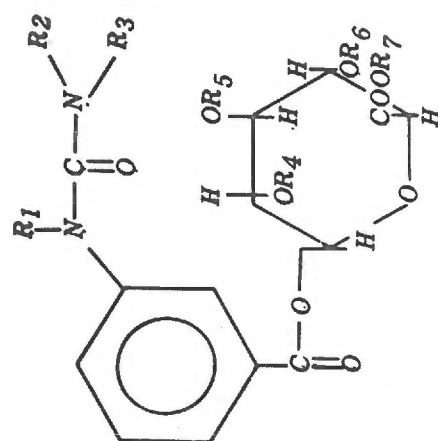
The tri-, and tetra-methyl derivatives of the glycine conjugate of MABA and the respective deutero-methyl compounds were identified in the urine samples described in Table IV. The numbers in parentheses indicate the relative abundance of a particular ion fragment in relation to the base ion (underlined). The * denotes the molecular ion. See Appendix V for complete mass spectra.

mass units. Therefore, the compound that fragmented to its base ion with the loss of 88 atomic mass units had a single methyl group on the glycine moiety and 2 methyl groups on the aromatic amine. The compound which lost 102 atomic mass units to form its base ion had 2 methyl groups on the glycine moiety and a single methyl group on the aromatic amine. The fragmentation pattern of the tetra-methyl compound was similar to the latter tri-methyl compound; 102 atomic mass units were lost in the formation of the base ion. The other major fragmentation, seen with all 6 compounds, was the loss of the entire carbonyl group.

Table VI shows the mass spectral fragmentation patterns of 2 methylated and 2 deuterio-methylated species of the ureido-glucuronide of MABA. The fragments at 75 atomic mass units ($\text{CH}_3\text{O}^+\text{=CH-OCH}_3$), 88 atomic mass units ($\text{CH}_3\text{O}^+\text{=CH-CH-OCH}_3$) and 101 atomic mass units ($\text{CH}_3\text{O}^+\text{=CH-CH=CH-OCH}_3$) are characteristic of the fragmentation of methylated glucuronides and methylated glucuronic acid (Thompson *et al.* 1973). The deuterio-methyl compounds had respective fragments at 81, 94 and 107 mass units, indicating that each of the 3 fragments had 2 methyl groups, as had been predicted. (Thompson *et al.* 1973).

2. Whole Body Metabolites of Tricaine

Mice Sacrificed 5 Min after Drug Injection. Figures 30 and 31 show the CCD and the descending chromatograph, respectively, of the ethanol extract of one of the 4 mice sacrificed 5 min after i.p. injection of 5 mg (860 nanocuries) of tricaine methanesulfonate. There were 2 distinct peaks of radioactivity in the CCD, one of which was in the organic fractions (1-9) and exhibited a constant C_0 of 3.35 ($p = 0.87$). The other peak (fractions 13-20) did not exhibit a constant C_0 and, therefore, included



Ureido-Glucuronide Conjugate of MABA

Substituents

	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇
(a)	H	CH ₃	CH ₃	CH ₃	CH ₃	CH ₃	CH ₃
(b)	H	CD ₃	CD ₃	CD ₃	CD ₃	CD ₃	CD ₃
(c)	CH ₃	CH ₃	CH ₃	CH ₃	CH ₃	CH ₃	CH ₃
(d)	CD ₃	CD ₃	CD ₃	CD ₃	CD ₃	CD ₃	CD ₃

(a)	440*	338	302	278	265	251	249	222	207	200	158	148
	(3.9)	(2.5)	(1.6)	(2.8)	(1.8)	(3.0)	(3.0)	(2.6)	(2.4)	(1.9)	(12.0)	(14.5)
	127	101	88	75								
	(14.0)	(57.0)		(43.2)								
(b)	459	458*	350	287	274	260	258	257	228	167	148	133
	(1.8)	(5.0)	(2.9)	(3.1)	(2.2)	(1.6)	(1.9)	(3.0)	(2.7)	(15.0)	(16.5)	(22.2)
	107	95	94	81								
	(42.8)	(14.0)		(18.2)								
(c)	455	454*	423	352	348	333	292	279	265	249	236	207
	(1.9)	(7.3)	(5.0)	(3.3)	(2.2)	(1.6)	(5.0)	(3.3)	(8.9)	(6.1)	(2.4)	(1.9)
	205	162	159	134	132	127	111	101	89	88	75	73
	(3.8)	(37.9)	(17.1)	(11.4)	(10.0)	(27.1)	(10.0)	(48.6)	(13.5)		(20.7)	(20.7)
(d)	476	475*	441	367	304	291	274	258	211	168	165	133
	(1.9)	(5.9)	(3.6)	(2.5)	(3.8)	(2.9)	(6.0)	(2.0)	(2.2)	(13.5)	(30.8)	(23.5)
	114	107	95	94	81							
	(10.8)	(45.0)	(12.3)		(17.5)							

Table VI: Major Ions in the Mass Spectra of Methyl and Deutero-Methyl Derivatives of a Mouse Urinary Metabolite of Tricaine: The Ureido-Glucuronide of MABA

The hexa- and hepta-methyl derivatives of the ureido-glucuronide of MABA and the respective deutero-methyl compounds were identified in the urine samples described in Table IV. The numbers in parentheses indicate the relative abundance of a particular ion fragment in relation to the base ion (underlined). The * denotes the molecular ion. See Appendix V for complete mass spectra.

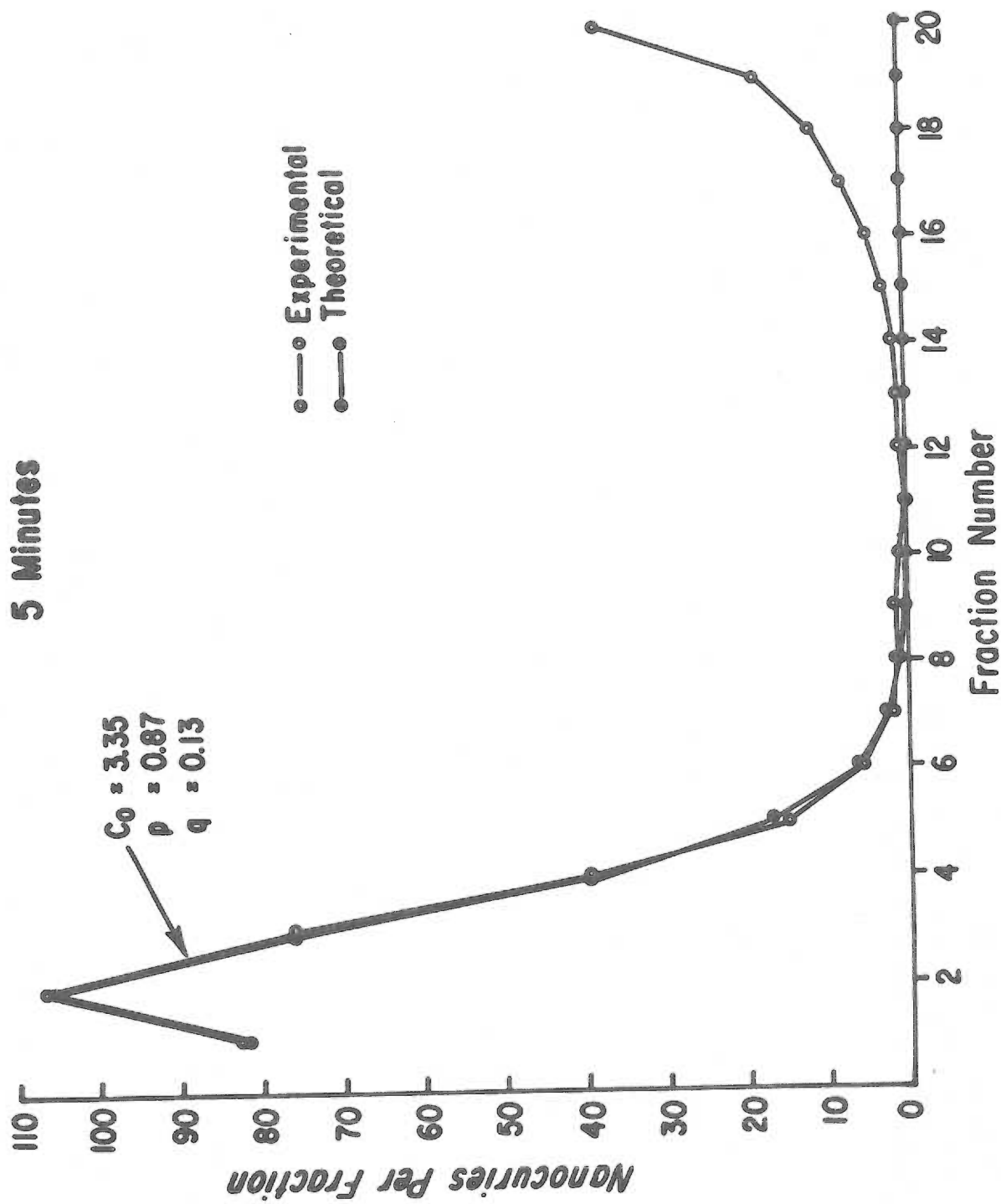


Figure 30: Ten Tube CCD of the Ethanol Extract of the Whole Body Homogenate of a Mouse
Sacrificed 5 Min after I.P. Injection of Tricaine Methanesulfonate

The CCD was performed between ethyl acetate (30 ml) and 2 M phosphate buffer, pH 4.3 (15 ml). The mouse had been injected i.p. with 5 mg (860 nanocuries) of radioactively labelled tricaine methanesulfonate. A single substance with a C_o of 3.35 was present in the organic fractions (1-9); two or more substances were present in the aqueous fractions (14-20). The single substance (MABA) in the organic fractions (1-9) accounted for 76% of the total radioactivity in the CCD.

5 MINUTES

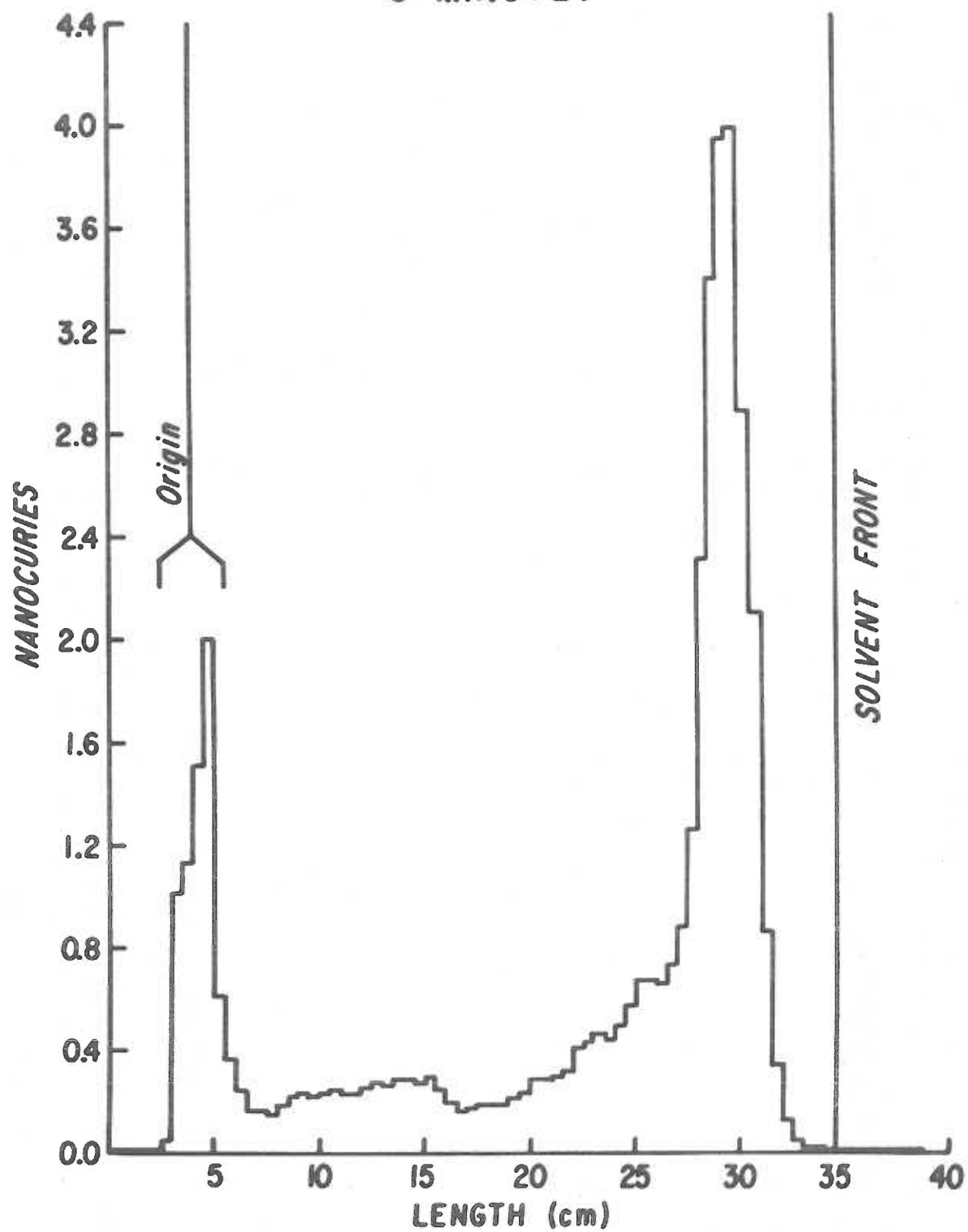


Figure 31: Descending Chromatograph of the Ethanol Extract of the Whole
Body Homogenate of a Mouse Sacrificed 5 Min after I.P.
Injection of Tricaine Methanesulfonate

The ethanol extract used for this chromatograph was the same as that used for the CCD pictured in Figure 30. There were 3 distinct peaks of radioactivity in the chromatograph; $R_F = 0$, 10 and 26 cm. The peak at 31 cm ($R_F = 26$ cm) accounted for 71% of the total radioactivity present in the chromatograph. The other peaks, $R_F = 0$ and 10 cm, accounted for 18% and 11% of the total radioactivity, respectively. The "origin" was the site of sample application to the chromatographic sheet; the "solvent front" indicates the maximum progression of the solvent system down the chromatograph. The ordinate shows the nanocuries present/0.5 cm of the chromatograph; the abscissa shows the length of the chromatograph with the origin ($R_F = 0$) at 5 cm.

at least 2, more polar, water-soluble metabolites. Comparison of the first peak ($C_0 = 3.35$) with a similar ten tube CCD of 2.0 mg of MABA (Figure 32) confirmed that the material in the organic fractions of the mouse-extract CCD was MABA. In the 4 separate CCD's, MABA accounted for a mean of $64 \pm 9\%$ (S.D.) of the total radioactivity in the CCD's. Figure 31, the descending chromatograph, shows 3 separate peaks of radioactivity with RF values of 0, 10, and 26 cm. The 3 peaks were the only ones observed in the descending chromatographs of the 4 mice. The mean percentage of the total radioactivity in each of the 3 peaks was: $27 \pm 7\%$ (S.D.) in the first peak (RF = 0 cm), $13 \pm 2\%$ (S.D.) in the second peak (RF = 10 cm), and $60 \pm 9\%$ (S.D.) in the third peak (RF = 26 cm).

Attempts to identify the components in the first 2 peaks (RF = 0 and 10 cm) of the chromatograph were unsuccessful. Permethylation, with either methyl iodide or diazomethane, of the metabolite in the third peak (RF = 26 cm) did permit its identification. Table VII shows the major fragments in the mass spectra of 3 distinct methylated species of the metabolite in the third peak (RF = 26 cm). Comparison of these spectra with the mass spectra of synthetic MABA (Table VIII), confirmed the identity of the compound in the third peak (RF = 26 cm) of the chromatograph as MABA. Ethanol, the other primary metabolite of tricaine, had been used to extract the mouse homogenates and, therefore, biologically derived ethanol could not be differentiated from the commercial ethanol used for extractions.

The residual radioactivity which remained in the mouse homogenate after the repeated extractions with ethanol was less than 2% ($1.5 \pm 0.3\%$, S.D.) of the total radioactivity injected into the mice. The overall recovery of the radioactivity from the 4 mice was $98.3 \pm 2.5\%$ (S.D.).

Synthetic MABA

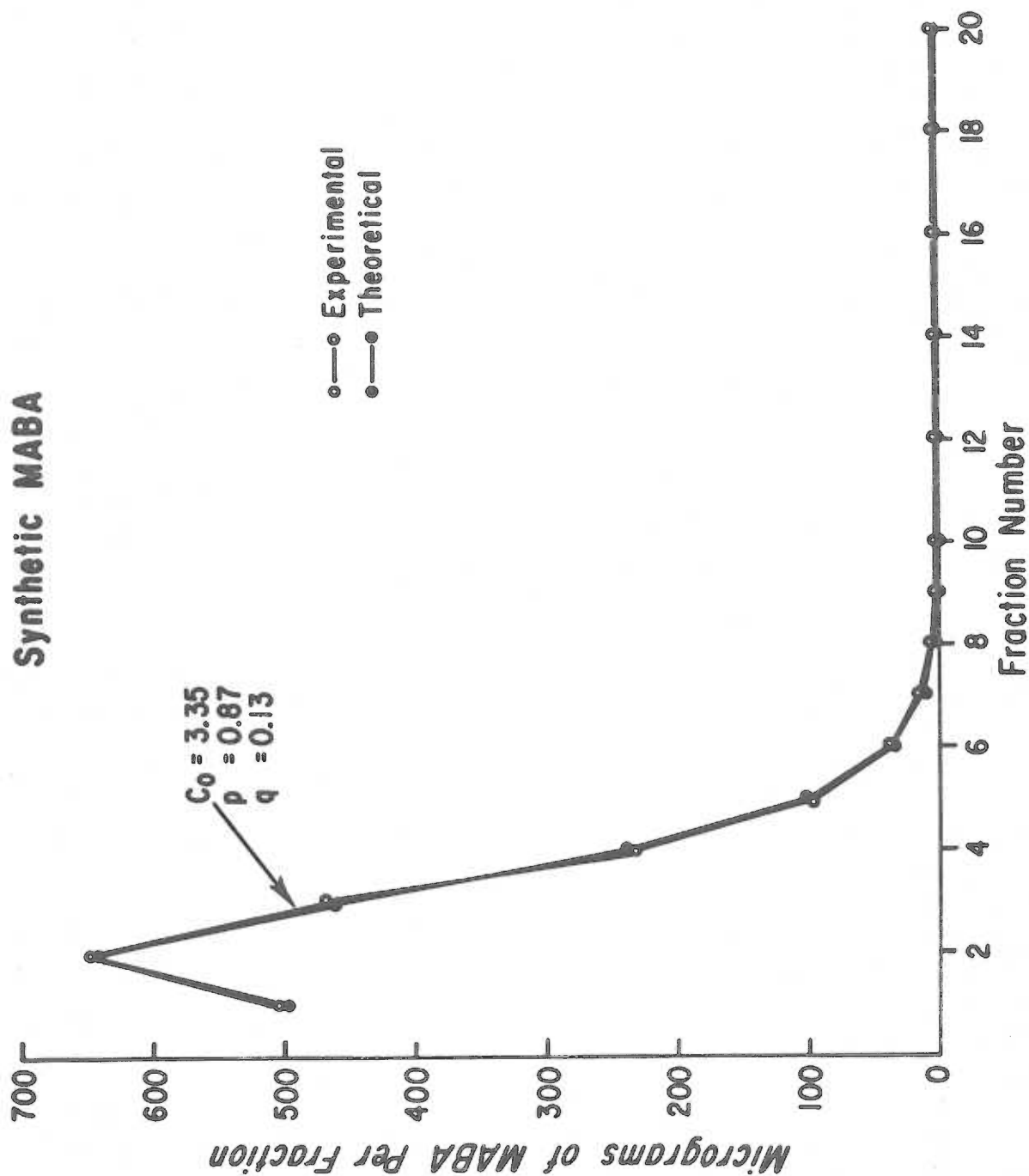
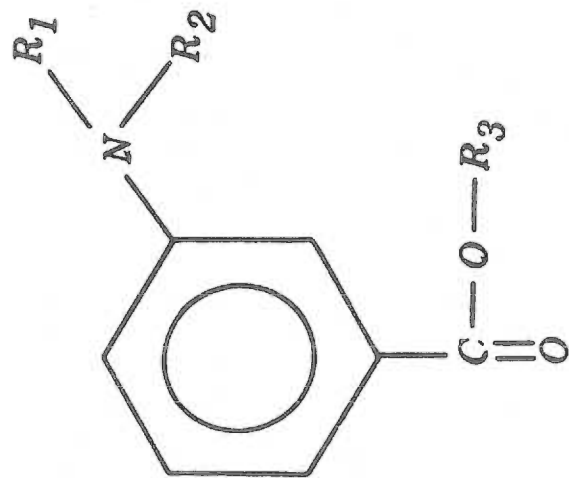


Figure 32: Ten Tube CCD of 2.0 mg of MABA

The CCD was performed between ethyl acetate (30 ml) and 2 M phosphate buffer, pH 4.3 (15 ml). MABA distributes with a C_o of 3.35.



MABA

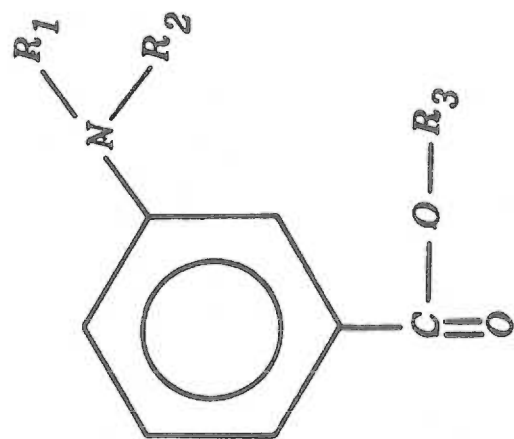
	R_1	R_2	R_3
(a)	H	H	CH ₃
(b)	H	CH ₃	CH ₃
(c)	CH ₃	CH ₃	CH ₃

Substituents

(a)	152 (10.5)	<u>151*</u>	121 (15.5)	120 (86.8)	93 (21.1)	92 (71.1)	65 (36.8)	63 (13.2)	
(b)	166 (10.4)	<u>165*</u>	164 (34.6)	134 (35.7)	107 (15.7)	106 (41.4)	77 (15.7)	65 (10.0)	
(c)	180 (12.5)	179*	<u>178</u>	148 (16.7)	120 (22.2)	105 (11.1)	104 (13.9)	77 (16.7)	73 (12.5)

Table VII: Major Ions in the Mass Spectra of Methyl Derivatives of a Mouse Whole-Body Metabolite of Tricaine: MABA

The mass spectra are of the compound in the third peak ($RF = 26$ cm) of the descending chromatograph (Figure 31). The 3 methyl derivatives by MABA were identified in the sample eluted off the descending chromatography sheet. The numbers in parentheses indicate the relative abundance of a particular ion fragment in relation to the base ion (underlined). The * denotes the molecular ion. See Appendix VI for complete mass spectra.



MABA

Substituents

	R_1	R_2	R_2
(a)	H	H	CH ₃
(b)	H	CH ₃	CH ₃
(c)	CH ₃	CH ₃	CH ₃

(a)	152 (17.0)	151* —	121 (15.8)	120 (91.7)	93 (45.0)	92 (93.3)	91 (13.5)	66 (13.3)	65 (90.8)
	64 (15.3)	63 (19.3)	60 (10.8)						
(c)	180 (10.7)	179* (94.9)	178 (96.6)	165 —	164 (32.9)	148 (11.9)	134 (30.5)	120 (15.3)	107 (11.0)
	106 (33.1)	105 (10.2)	104 (12.2)	79 (10.2)	78 (10.2)	77 (20.3)	73 (16.3)		

Table VIII: Major Ions in the Mass Spectra of Methyl Derivatives of Synthetic MABA

The ion fragments listed for substituent (c) include those of 2 compounds, the di- and tri-methyl derivatives. The combination results from very similar retention times of the 2 compounds in the gas chromatograph. The numbers in parentheses indicate the relative abundance of a particular ion fragment in relation to the base ion (underlined). The * denotes the molecular ion. See Appendix VII for complete mass spectra.

Mice Sacrificed 70 Min after Drug Injection. Figures 33 and 34 are the CCD and descending chromatograph of the ethanol extract of a mouse sacrificed 70 min after the i.p. injection of 5 mg (860 nanocuries) of tricaine methanesulfonate. There were 2 distinct peaks of radioactivity in the CCD, one of which was in the organic fractions (1-5) and exhibited a constant C_0 at 5.75. The other peak (fractions 11-20) did not exhibit a constant C_0 . Therefore, MABA as well as tricaine had completely disappeared from the mouse in 70 min. The compound in the organic fractions (1-5) was not MABA for it had a C_0 in this CCD system of 5.75, whereas MABA had a C_0 of 3.35 (Figure 32).

Figure 34, the descending chromatograph, shows 3 peaks of radioactivity with RF values of 0, 10 and 26 cm. The RF values of these peaks of radioactivity were the same as those of the peaks in the chromatographs of the ethanol extracts of mice sacrificed 5 min after drug administration (Figure 31). The 3 peaks, RF = 0, 10, and 26 cm, were the only peaks observed in the chromatographs of the four "70 min" mice. The mean percentage of the total radioactivity in each of the 3 peaks was: $43 \pm 9\%$ (S.D.) in the first peak (RF = 0 cm), $34 \pm 15\%$ (S.D.) in the second peak (RF = 10 cm), and $23 \pm 5\%$ (S.D.) in the third peak (RF = 26 cm). Attempts to identify the components of the first and third peaks, RF = 0 and 26 cm, were unsuccessful. Permethylation of the methanol extract of the second peak (RF = 10 cm) did permit the identification of the glycine conjugate of MABA. Table IX shows the major fragments in the mass spectra of the same 3 species of the methylated glycine conjugate of MABA that had been found in methylated samples of mouse urine (Table V).

The residual radioactivity which remained in the homogenate after

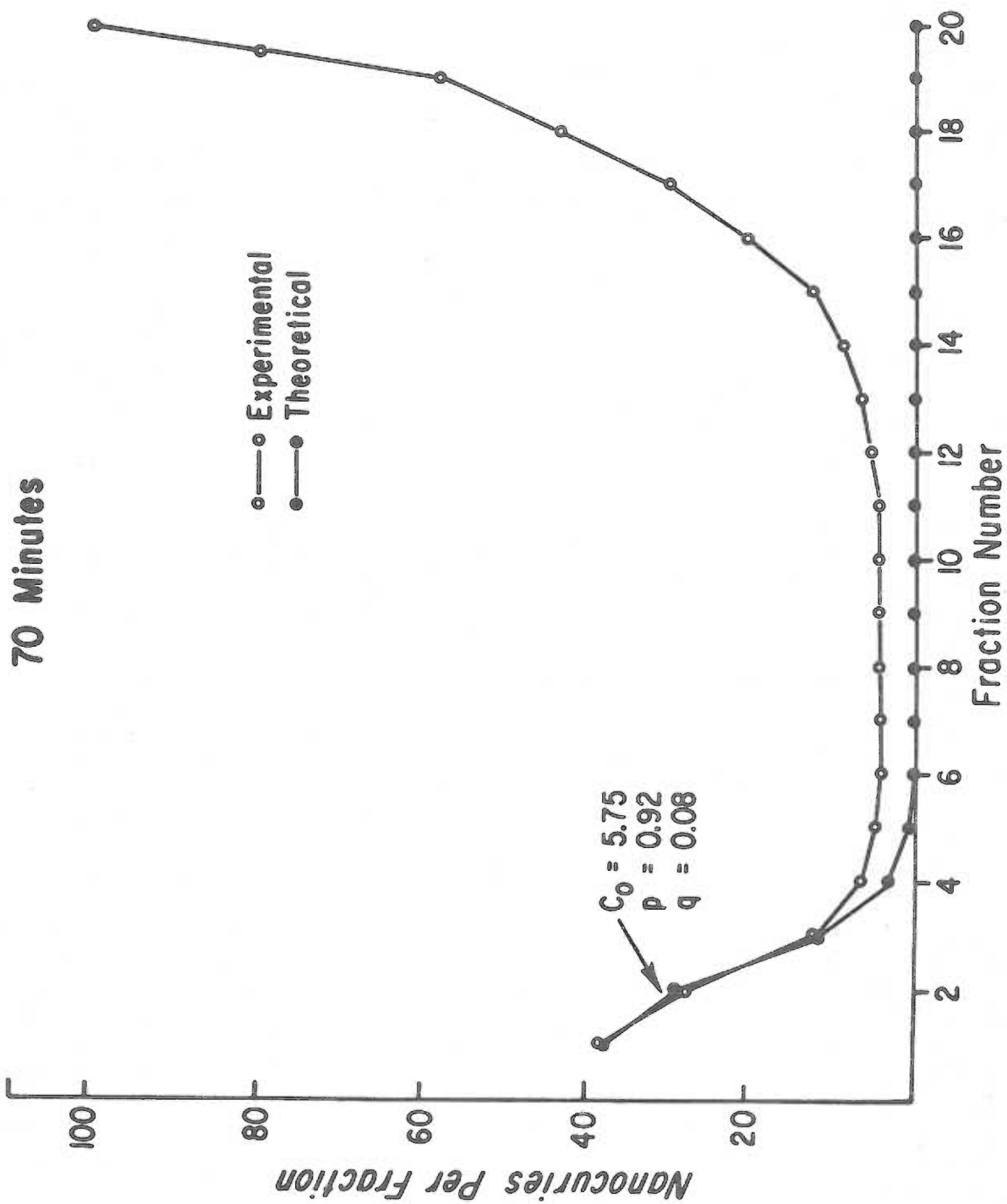


Figure 33: Ten Tube CCD of the Ethanol Extract of the Whole Body Homogenate of a Mouse Sacrificed
70 Min after I.P. Injection of Tricaine Methanesulfonate

The CCD was performed between ethyl acetate (30 ml) and 2 M phosphate buffer, pH 4.3 (15 ml). The mouse had been injected i.p. with 5 mg (860 nanocuries) of radioactively labelled tricaine methanesulfonate. A compound with a C_0 of 5.75 appears in organic fractions 1-5. Two or more substances are present in the other fractions (4-20).

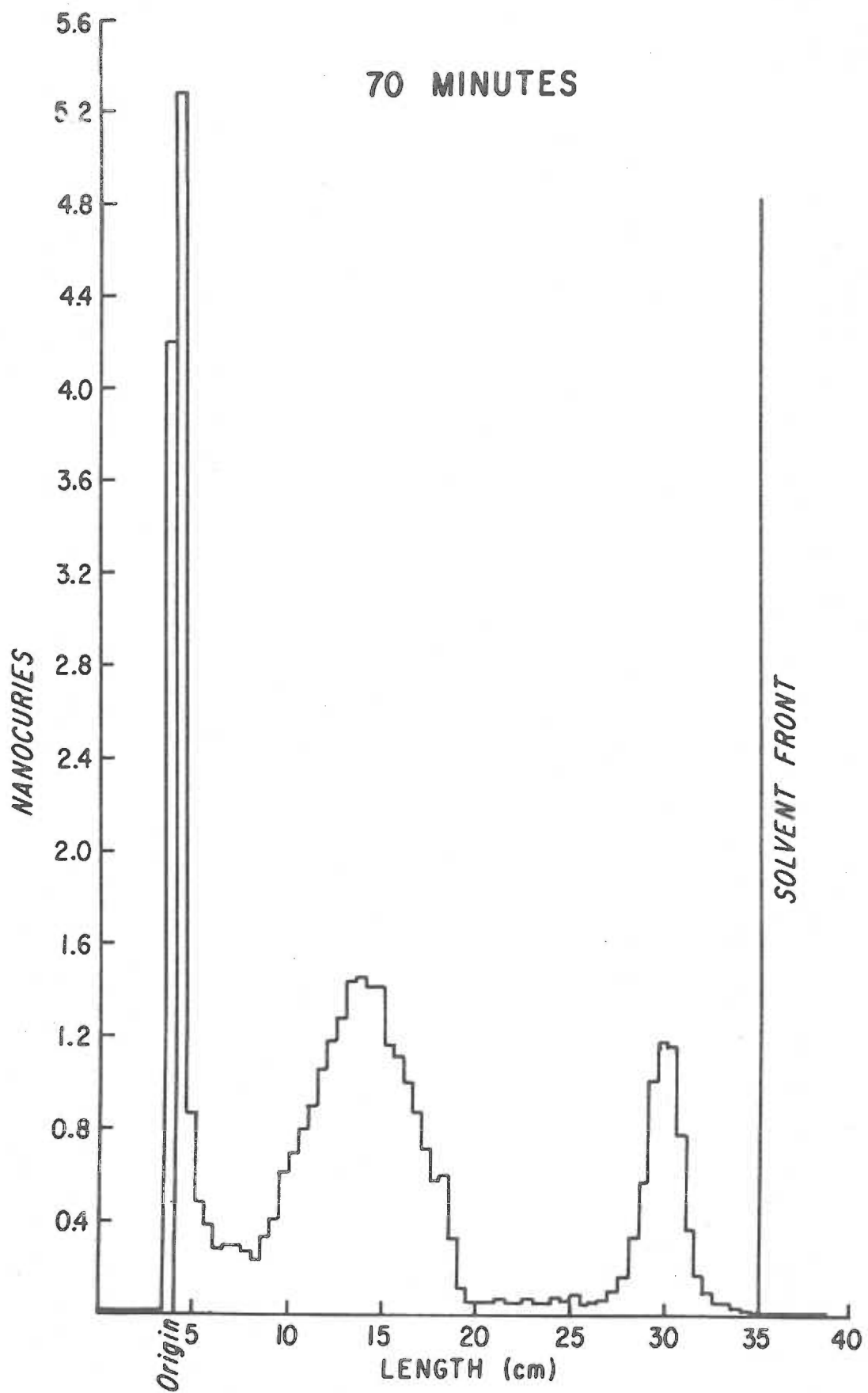
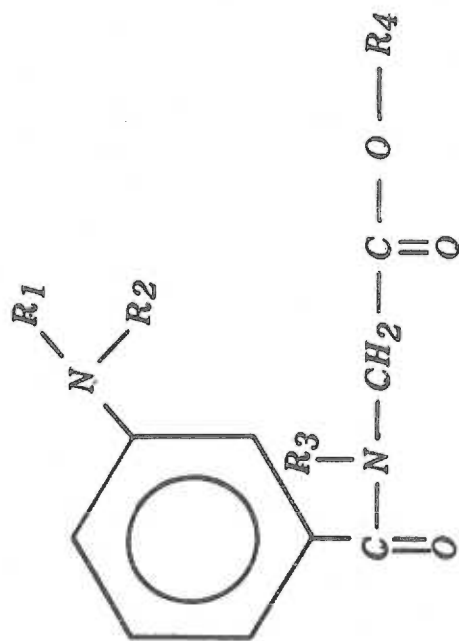


Figure 34: Descending Chromatograph of the Ethanol Extract of the Whole
Body Homogenate of a Mouse Sacrificed 70 Min after I.P.
Injection of Tricaine Methanesulfonate

The ethanol extract used for this chromatograph was the same as that used for the CCD pictured in Figure 33. There were 3 distinct peaks of radioactivity in the chromatograph; $R_F = 0, 10$ and 26 cm. The peaks account for 32%, 51% and 17% of the total radioactivity present in the chromatograph, respectively. The "origin" was the site of sample application to the chromatographic sheet; the "solvent front" indicates the maximum progression of the solvent system down the chromatograph. The ordinate shows the nanocuries present/0.5 cm of the chromatograph; the abscissa shows the length of the chromatograph with the origin ($R_F = 0$) at 5 cm.



Glycine Conjugate of MABA

	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>	<u>R₄</u>
(a)	CH ₃	CH ₃	H	CH ₃
(b)	H	CH ₃	CH ₃	CH ₃
(c)	CH ₃	CH ₃	CH ₃	CH ₃

Substituents

(a)	237	236*	235	149	148	121	120	119	118
	(11.4)	(79.6)	(13.1)	(15.3)	(47.7)	(94.3)	(22.7)	(10.8)	
(b)	105	104	92	91	79	78	77	76	74
	(19.3)	(18.2)	(13.1)	(10.8)	(11.4)	(15.3)	(23.9)	(10.8)	(15.9)
(c)	236*	222	148	135	134	120	107	106	77
	(24.2)	(43.3)	(18.2)	(13.6)	(13.6)	(18.2)	(46.7)	(15.2)	
(c)	250*	149	148	134	121	120	106	105	104
	(61.3)	(16.5)	(21.0)	(29.0)	(50.8)	(11.6)	(12.9)	(11.6)	(18.6)

Table IX: Major Ions in the Mass Spectra of Methyl Derivatives of a Mouse Whole-Body Metabolite of Tricaine: The Glycine Conjugate of MABA

The mass spectra are of the compound in the second peak ($RF = 10$ cm) of the descending chromatograph (Figure 34). The methyl derivatives of the glycine conjugate of MABA were identified in the sample eluted off the descending chromatography sheet. The numbers in parentheses indicate the relative abundance of a particular ion in relation to the base ion (underlined). The * denotes the molecular ion. See Appendix VI for complete mass spectra.

the repeated extractions with ethanol was $2.4 \pm 0.9\%$ (S.D.) of the total amount of radioactivity injected into each of the mice. The overall recovery of radioactivity from each of the 4 mice was $98.4 \pm 4.8\%$ (S.D.).

C. Studies of the Comparative Effects of Tricaine and Other Local Anesthetics on Various Organ Systems of Mammalian and Nonmammalian Species

1. Isolated Tissue Studies

Isolated Atria. Spontaneously beating atria isolated from bullfrogs and guinea pigs were used to study the comparative effects of tricaine on amphibian and mammalian cardiac tissue. The inotropic and chronotropic effects of the drug on the 2 preparations are compared in Figure 35 and 36, respectively. Although the threshold concentration of tricaine necessary to produce a negative chronotropic effect was slightly lower for bullfrog atria, in all other respects the guinea-pig atria were the more sensitive of the 2 preparations to the effects of tricaine. The similarity in the effects of the methanesulfonic and hydrochloric salts of tricaine confirmed the lack of influence of the methanesulfonate moiety on the pharmacological effects of the drug.

The force of contraction was more sensitive to the presence of tricaine than was the rate of contraction. For both species, a significant decrease in the inotropic parameter occurred before any detectable chronotropic depression. In the guinea-pig preparations, particularly, the abrupt drop in rate that occurred with arrest of the atria suggested the complete dissociation of the electrical and mechanical systems of the heart, similar to that described for ouabain by Lullman and Ravins (1973). The rate of

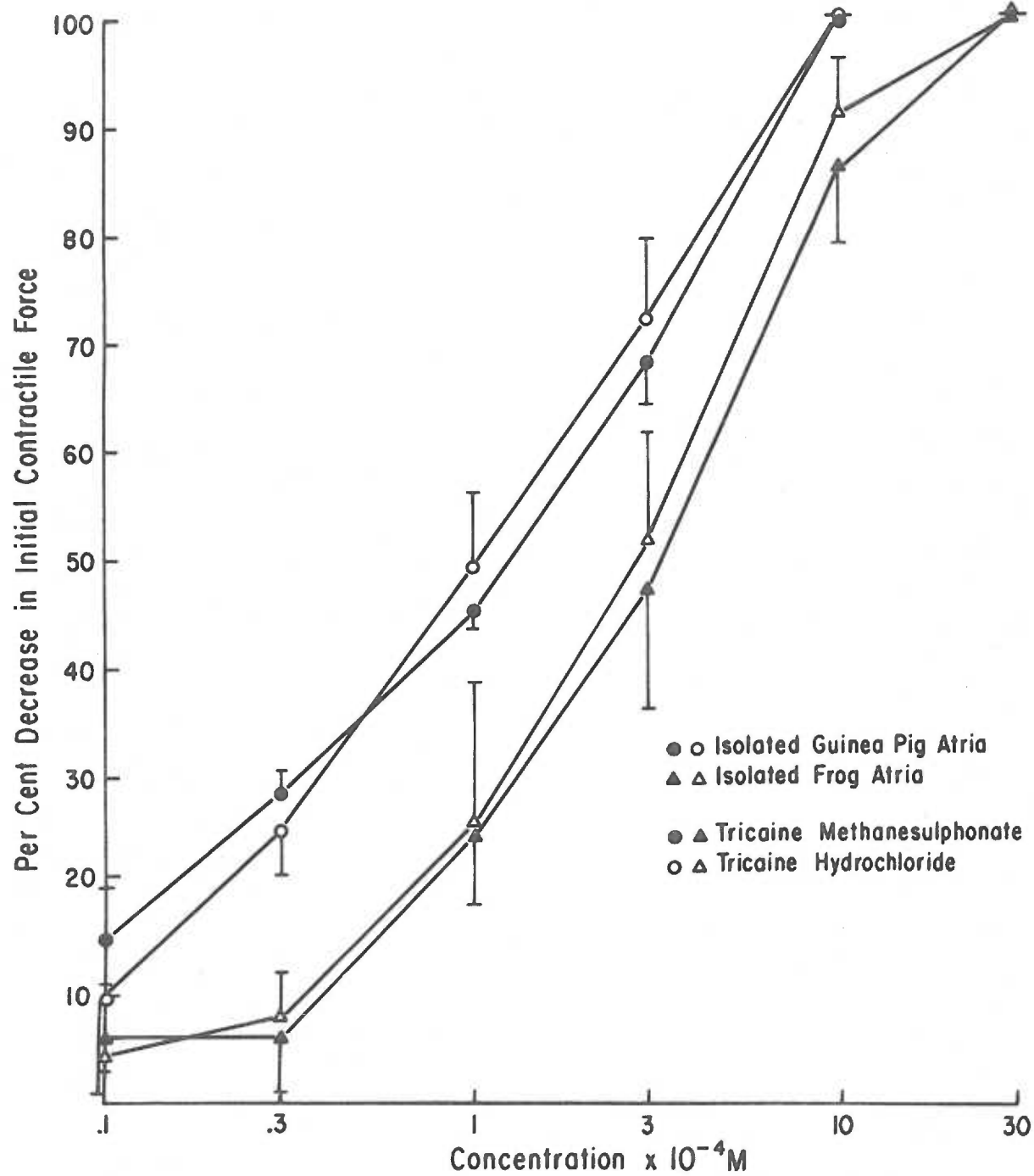


Figure 35: Concentration-Effect Relationships of Tricaine on the Contractile Force of Spontaneously Beating Atria Isolated from Bullfrogs and Guinea Pigs

The similarity of effect of the two tricaine formulations demonstrated a lack of activity of the methanesulfonate moiety. Each point represents the mean $\pm$ S.D. of four preparations.

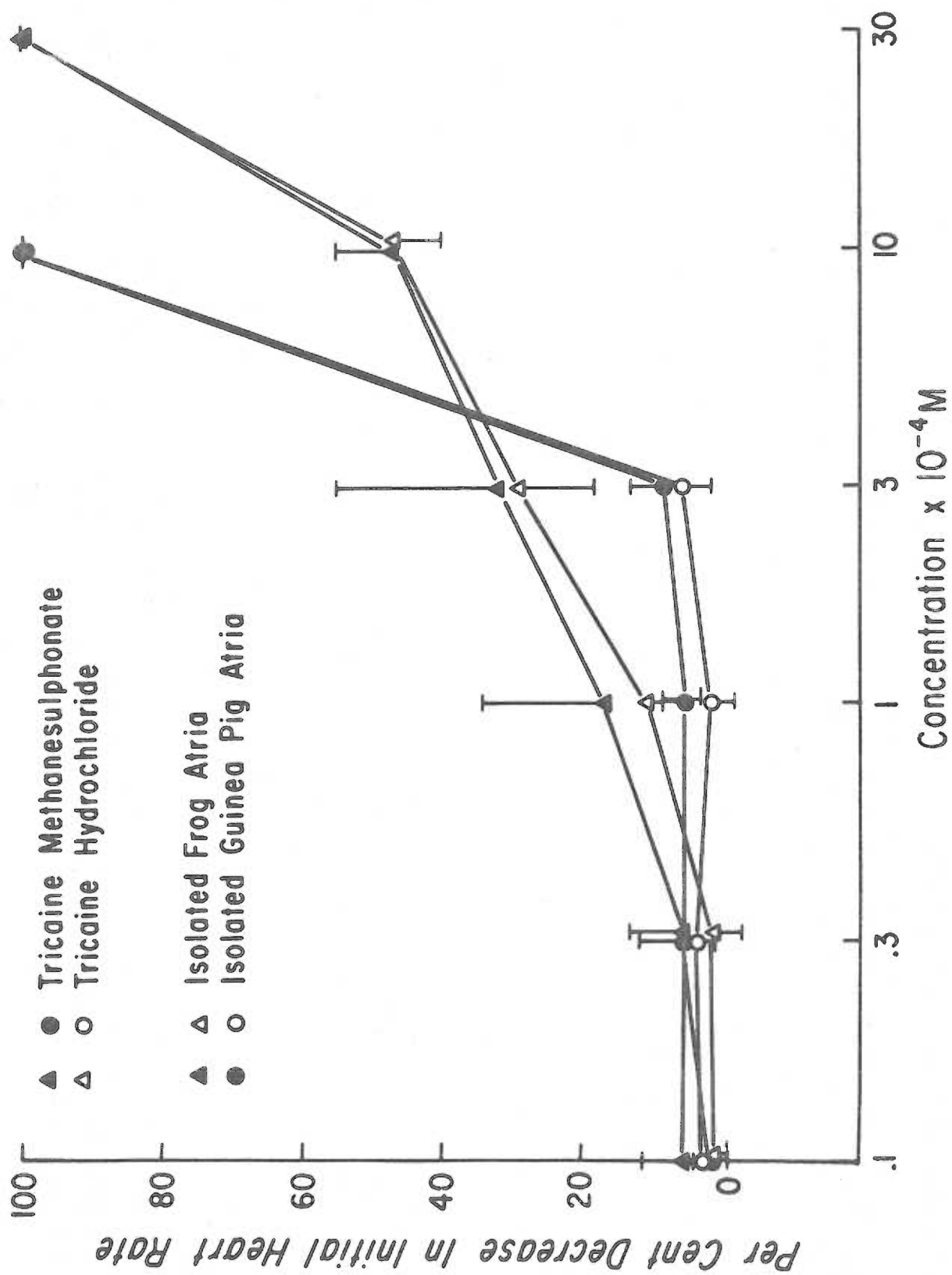


Figure 36: Concentration-Effect Relationships of Tricaine on the Contractile Rate of Spontaneously Beating Atria Isolated from Bullfrogs and Guinea Pigs

The initial rate for the bullfrog preparations was 53 ± 7 beats/min whereas that of the guinea-pig preparations was 147 ± 10 beats/min. The similarity of effect of the two tricaine formulations indicated a lack of effect of the methanesulfonate moiety at these concentrations. Each point represents the mean $\pm$ S.D. of four atrial preparations.

contraction is primarily an electrical phenomenon and is determined by the generation and conduction of electrical depolarizations (electrical systoles) in the heart. However, in our experiments, the rate parameter was calculated from the number of mechanical systoles (contractions) that occurred in a given period of time and was, therefore, directly dependent on the mechanical system of the atria; the lack of mechanical systoles precluded any measurement of electrical systoles. To monitor the rate of contraction independently, recording electrodes were placed in the muscle baths above and below the preparations. In isolated atria from both species, there was always a 1:1 correspondence between mechanical and electrical systoles and concentrations of tricaine that arrested mechanical activity also arrested electrical activity.

MABA did not affect the force or rate of contraction of either preparation and it did not alter the responses of the preparations to tricaine. The momentary pH shifts of 7.4 to 7.0 and 7.8 to 7.0 produced by high concentrations of tricaine hydrochloride (10^{-3} M; 3×10^{-3} M) in the guinea-pig and bullfrog preparations, respectively, had no persistent effects.

The effects of benzocaine hydrochloride and procaine hydrochloride were compared with those of the tricaine salt. In the isolated bullfrog atria, the inotropic effects of low concentrations of procaine differed from those of tricaine and benzocaine (Figure 37); 10^{-4} M procaine caused a slight increase in the contractile force (Figure 38), whereas 10^{-4} M tricaine and 10^{-4} M benzocaine had negative inotropic effects. The chronotropic effects of all 3 drugs were similar (Figure 39) and the 3×10^{-3} M concentration of each drug arrested the bullfrog preparations. Comparison of the 3 drugs on the isolated guinea-pig atrial preparations (Figures 40

Isolated Frog Atria

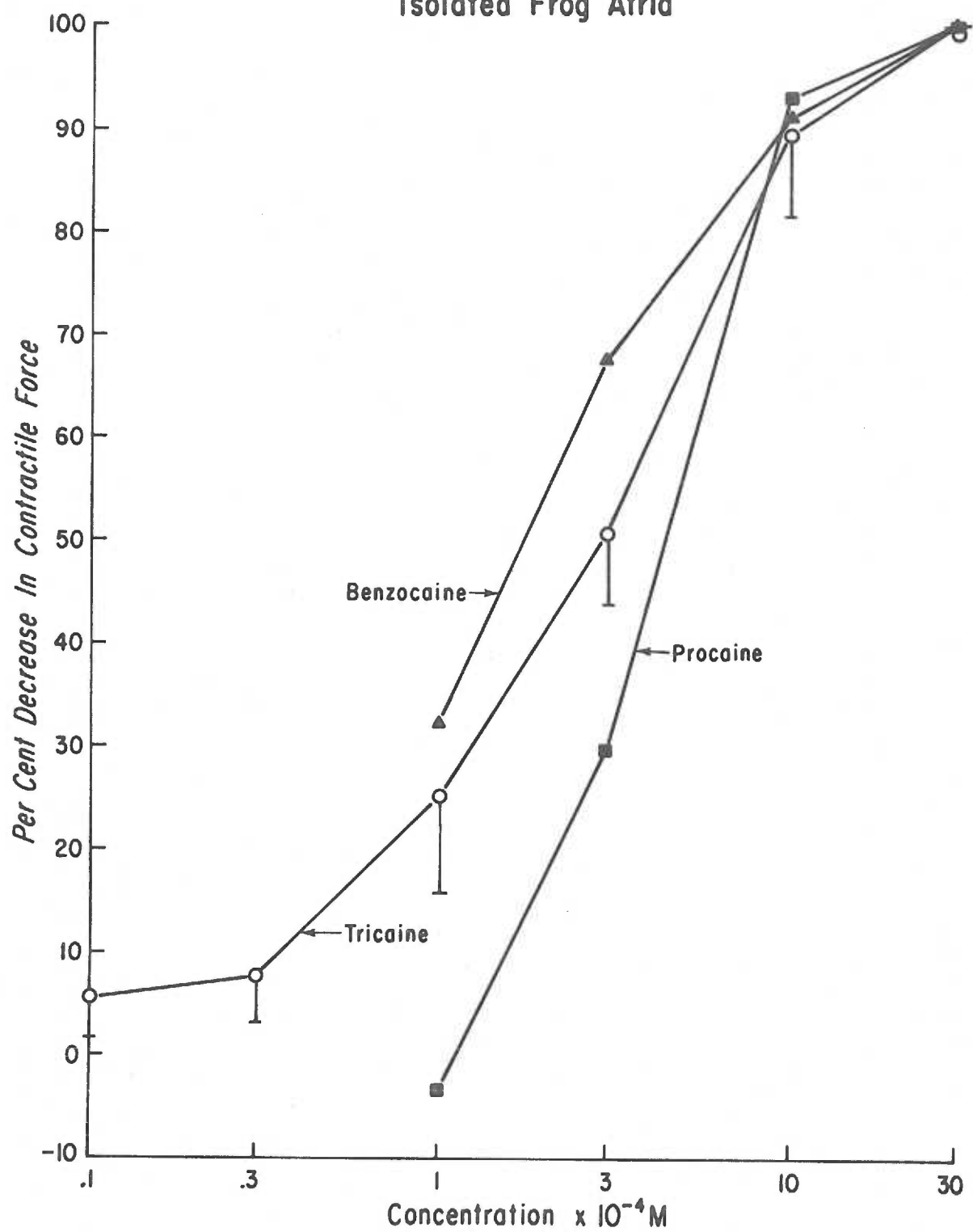
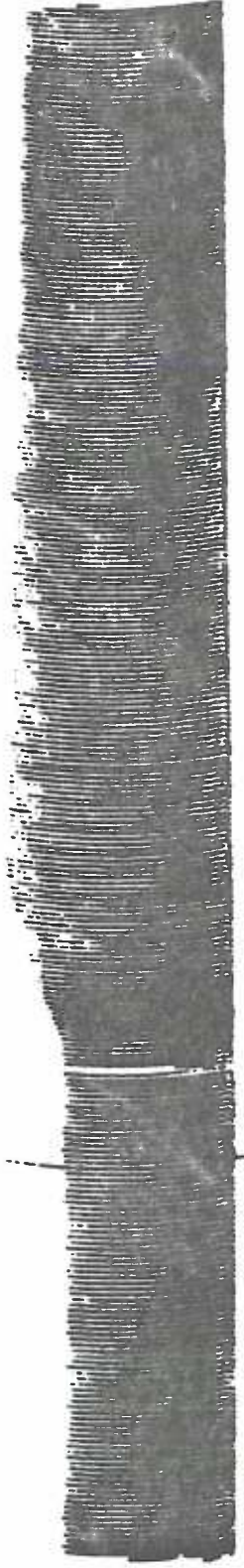


Figure 37: Concentration-Effect Relationships of Tricaine and Other
Local Anesthetics on the Contractile Force of Spontaneously
Beating Atria Isolated from Bullfrogs

The 3 compounds appeared to have similar effects, except for the low concentration of procaine which produced a positive inotropic effect (Figure 38). Each point for tricaine represents the mean $\pm$ S.D. of 4 preparations. Each point for benzocaine and procaine represents the average of two preparations.

10^{-4} M Procaine



10^{-3} M Tricaine

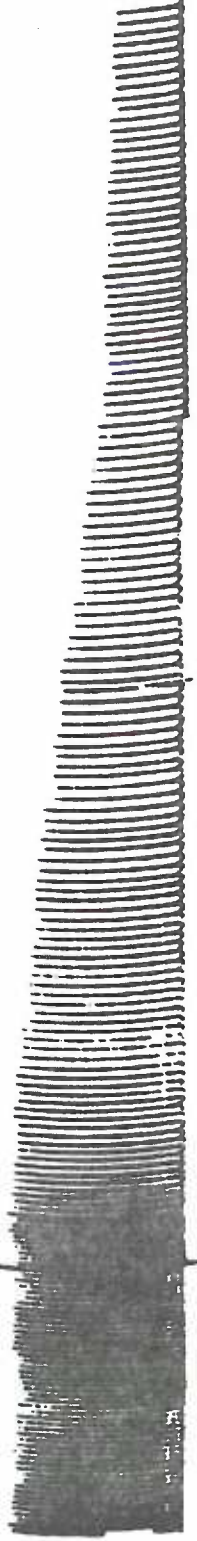


Figure 38: A. Effect of the 10^{-4} M Concentration of Procaine on the Contractile Force and Rate of Spontaneously Beating Atria Isolated from Bullfrogs.

B. Effect of the 10^{-3} M Concentration of Tricaine on the Contractile Force and Rate of Spontaneously Beating Atria Isolated from Bullfrogs.

Isolated Frog Atria

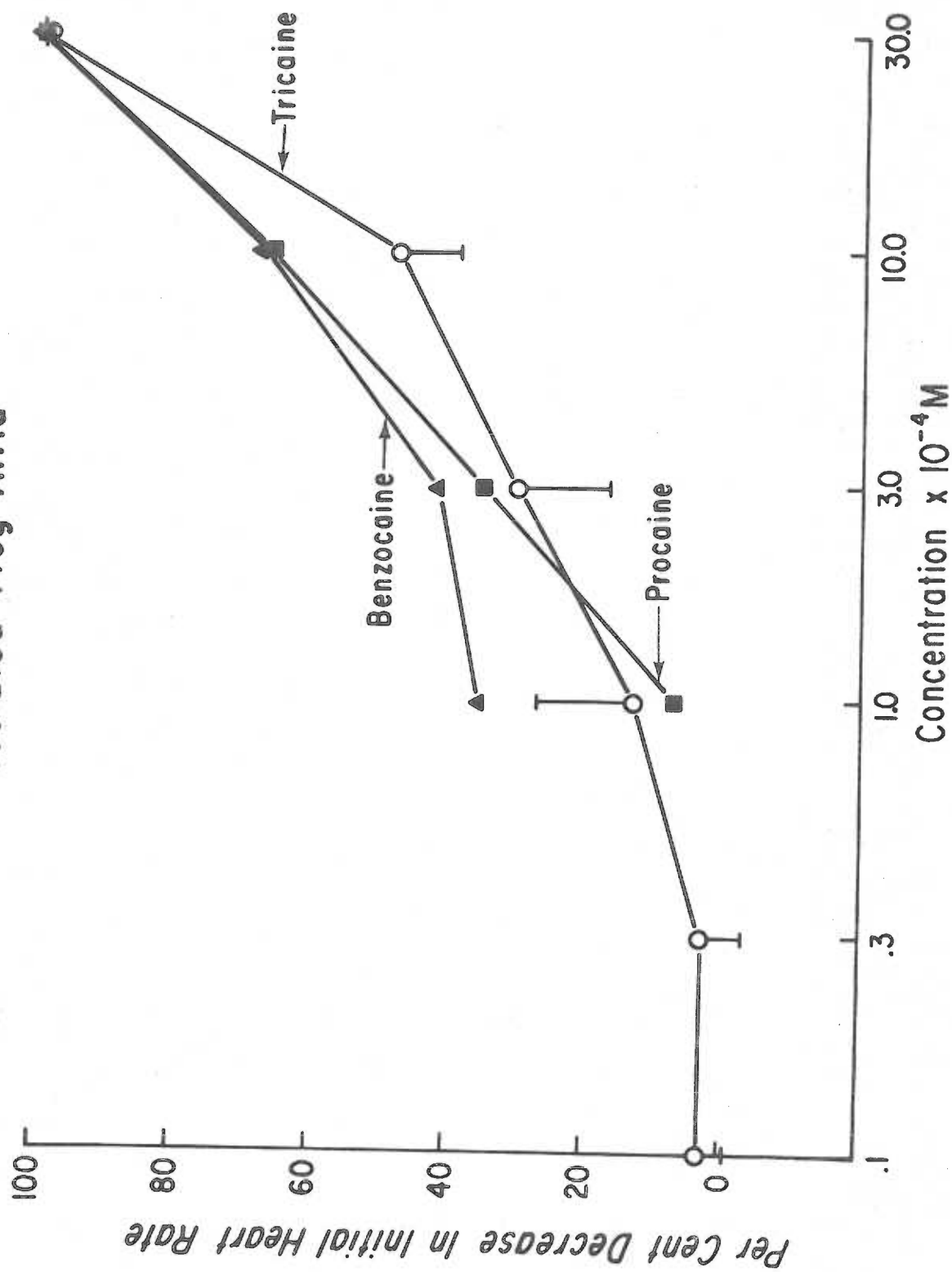


Figure 39: Concentration-Effect Relationships of Tricaine and Other Local Anesthetics on the Contractile Rate of Spontaneously Beating Atria Isolated from Bullfrogs

The 3 compounds had similar effects on the rate of contraction of the preparation. Each point for tricaine represents the mean $\pm$ S.D. of 4 preparations. Each point for benzocaine and procaine represents the average of two preparations.

and 41) showed that benzocaine was equipotent with tricaine and that the inotropic and chronotropic effects of procaine differed markedly from those of tricaine and benzocaine. Low concentrations of procaine (10^{-4} M and 3×10^{-4} M) depressed the rate of contraction, whereas tricaine and benzocaine had no chronotropic effect at low concentrations. High concentrations of procaine appeared less potent than those of tricaine and benzocaine, both in depression of contractile force and depression of contractile rate.

Isolated Lung. Tricaine methanesulfonate relaxed the smooth muscle tone of isolated frog lung at all the concentrations tested (Figure 42). The 10^{-3} M concentration did produce a transient contraction before the relaxation. Procaine hydrochloride was virtually inactive at the 10^{-5} M concentration. Higher concentrations of procaine stimulated contraction of the preparation.

Isolated Frog Stomach. Figure 43 shows the effects of various concentrations of tricaine methanesulfonate and procaine hydrochloride on the gastric muscle preparation. Tricaine depressed the rate and amplitude of the spontaneous contractions. Procaine reduced the rate but increased the amplitude of the contractions.

Isolated Trachea. Figure 44 shows the concentration-effect relationship for tricaine on the smooth muscle tone of the isolated guinea-pig trachea. The 10^{-3} M concentration of tricaine methanesulfonate produced the same degree of relaxation as the maximally effective concentration of isoproterenol (10^{-6} M), the standard drug for comparison. Figure 45 illustrates the divergent effects of tricaine and lidocaine on the preparation. All effective concentrations of tricaine relaxed the

Isolated Guinea Pig Atria

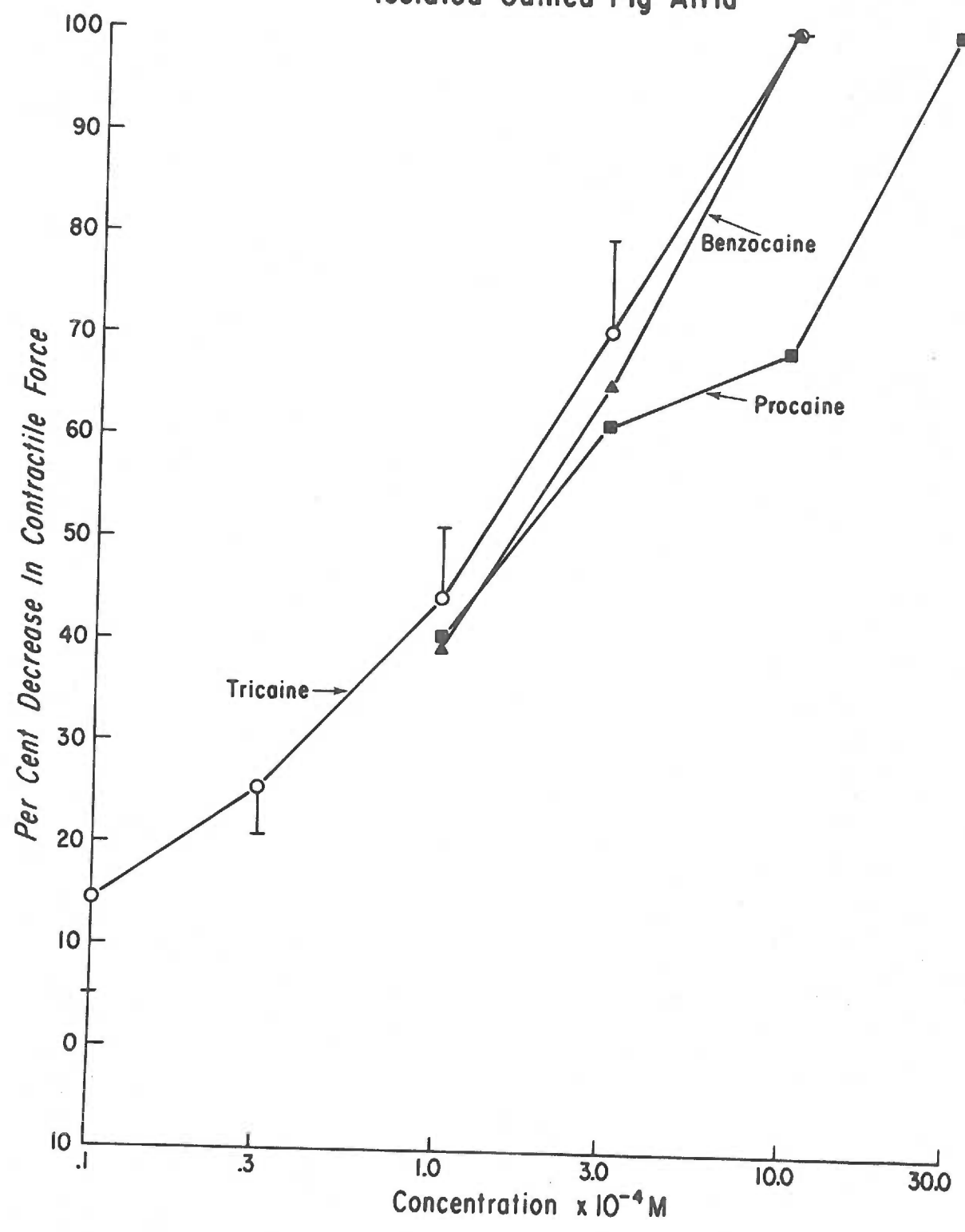


Figure 40: Concentration-Effect Relationships of Tricaine and Other
Local Anesthetics on the Contractile Force of Spontaneously
Beating Atria Isolated from Guinea Pigs

Tricaine and benzocaine had similar effects on the contractile force of the preparation. The high concentrations of procaine were less potent than tricaine and benzocaine. Each point for tricaine represents the mean $\pm$ S.D. of 4 preparations. Each point for benzocaine and procaine represents the average of two preparations.

Isolated Guinea Pig Atria

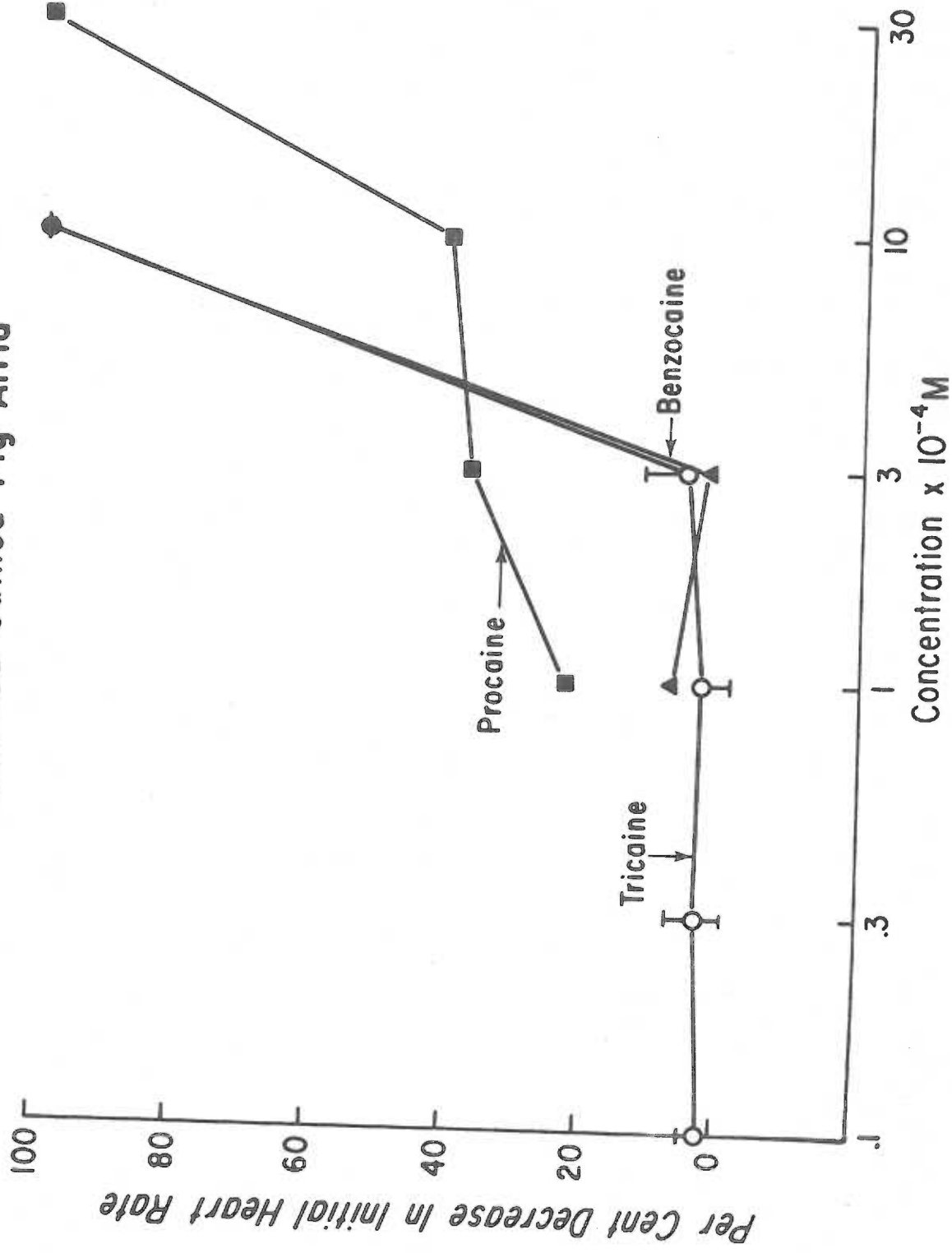
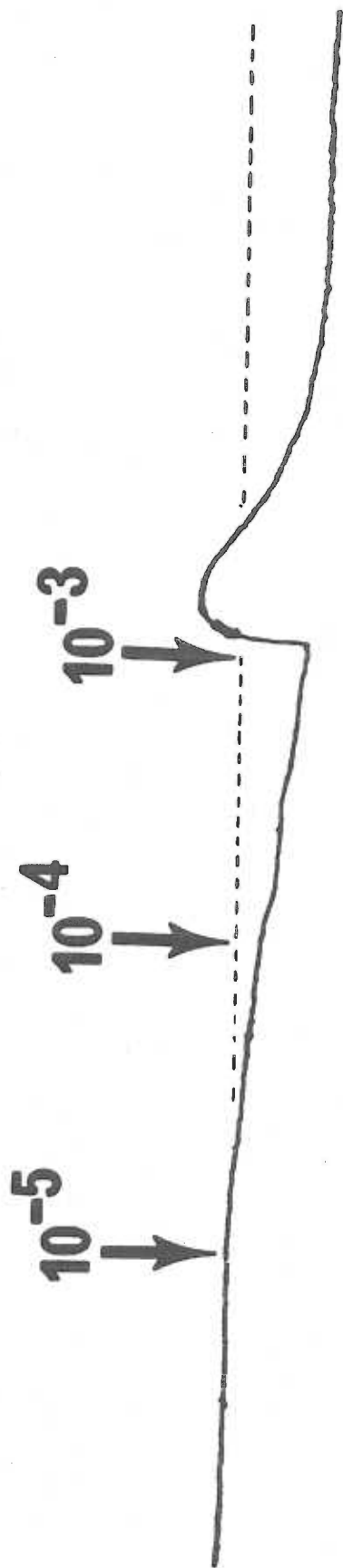


Figure 41: Concentration-Effect Relationships of Tricaine and Other Local Anesthetics on the Contractile Rate of Spontaneously Beating Atria Isolated from Guinea Pigs

Tricaine and benzocaine had identical effects on the contractile rate of the preparation. Procaine differed in its effects, being more potent at low concentrations and less potent at high concentrations than tricaine and benzocaine. Each point for tricaine represents the mean $\pm$ S.D. of 4 preparations. Each point for benzocaine and procaine represents the average of two preparations.

A.

Tricaine (M)



B.

Procaine (M)

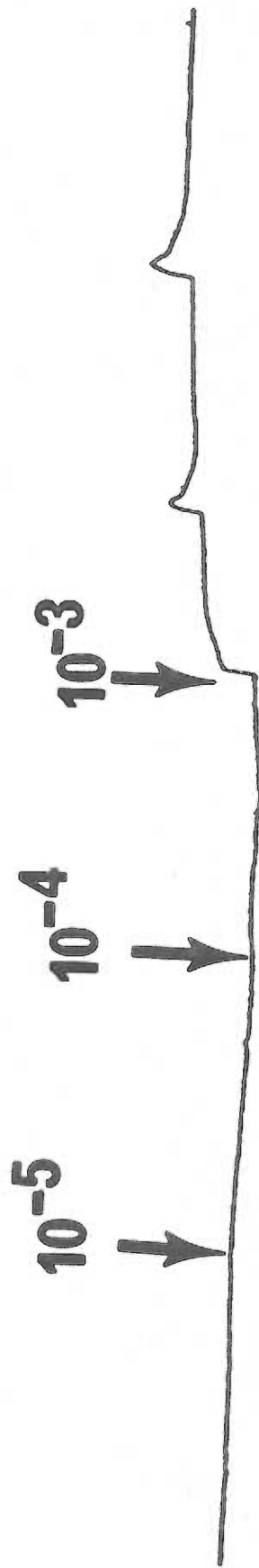


Figure 42: Comparative Effects of Tricaine and Procaine on the Smooth Muscle Tone of Isolated

Frog Lung (Rana pipiens)

Low concentrations (10^{-5} - 10^{-4} M) of both drugs produced relaxation. Higher concentration (10^{-3} M) of tricaine produced a transient contraction and then relaxation; further increases in concentration did not produce any sustained contractions. The high concentration (10^{-3} M) of procaine contracted the preparation and further increases in concentration did not result in any relaxation.

Tricaine (M)

A.

10^{-5} 10^{-4} 10^{-3}



Procaine (M)

B.

10^{-5} 10^{-4} 10^{-3}



Figure 43: Comparative Effects of Various Concentrations of Tricaine and Procaine on the Tone and Spontaneous Contractions of the Isolated Frog Stomach (Rana pipiens)

All effective concentrations of procaine increased the amplitude and decreased the rate of spontaneous contractions of the preparation. All effective concentrations of tricaine decreased the amplitude of the spontaneous contractions. The 10^{-4} M tricaine concentration decreased the rate of the contractions and the 10^{-3} M concentration of tricaine completely abolished the spontaneous contractions and relaxed the tone of the preparation.

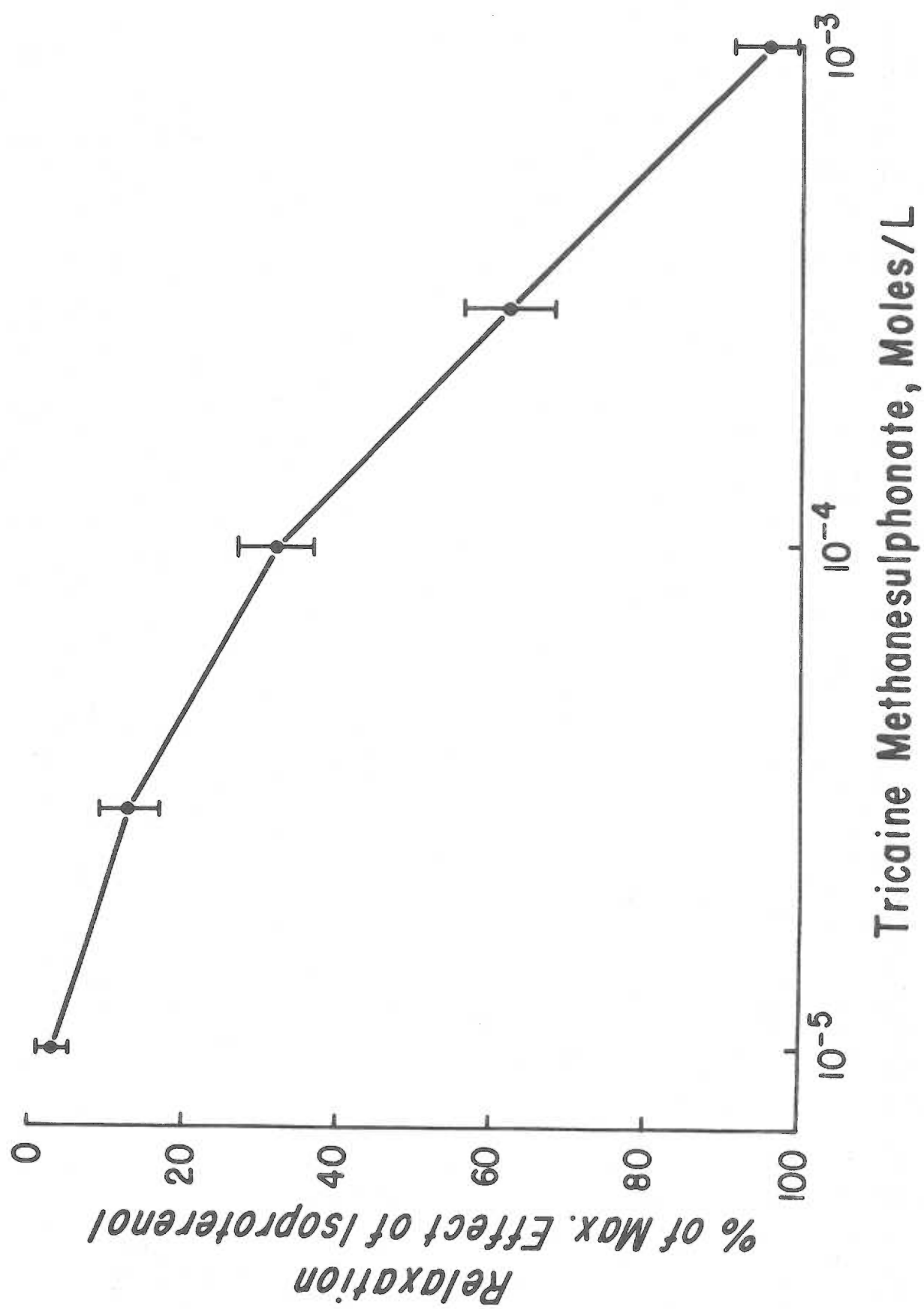


Figure 44: Concentration-Effect Relationship of Tricaine on the Isolated Trachea of Guinea Pigs

Tricaine relaxed the preparation; the high concentration (10^{-3} M) produced the same degree of relaxation as the maximal effective concentration of isoproterenol (10^{-6} M). Each point represents the mean $\pm$ S.D. of four preparations.

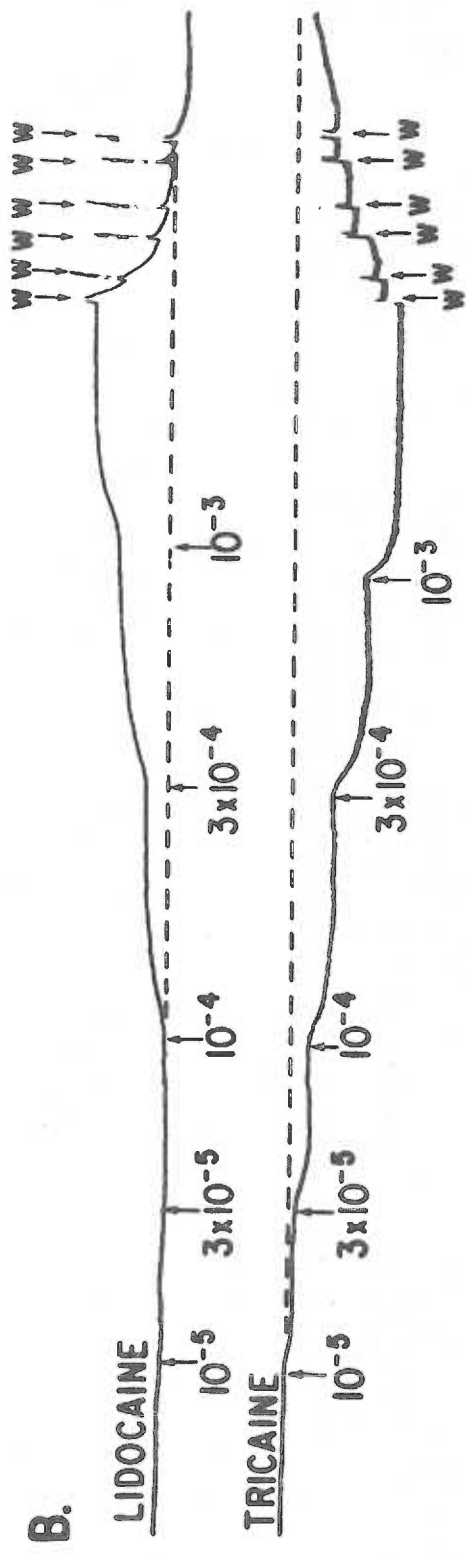
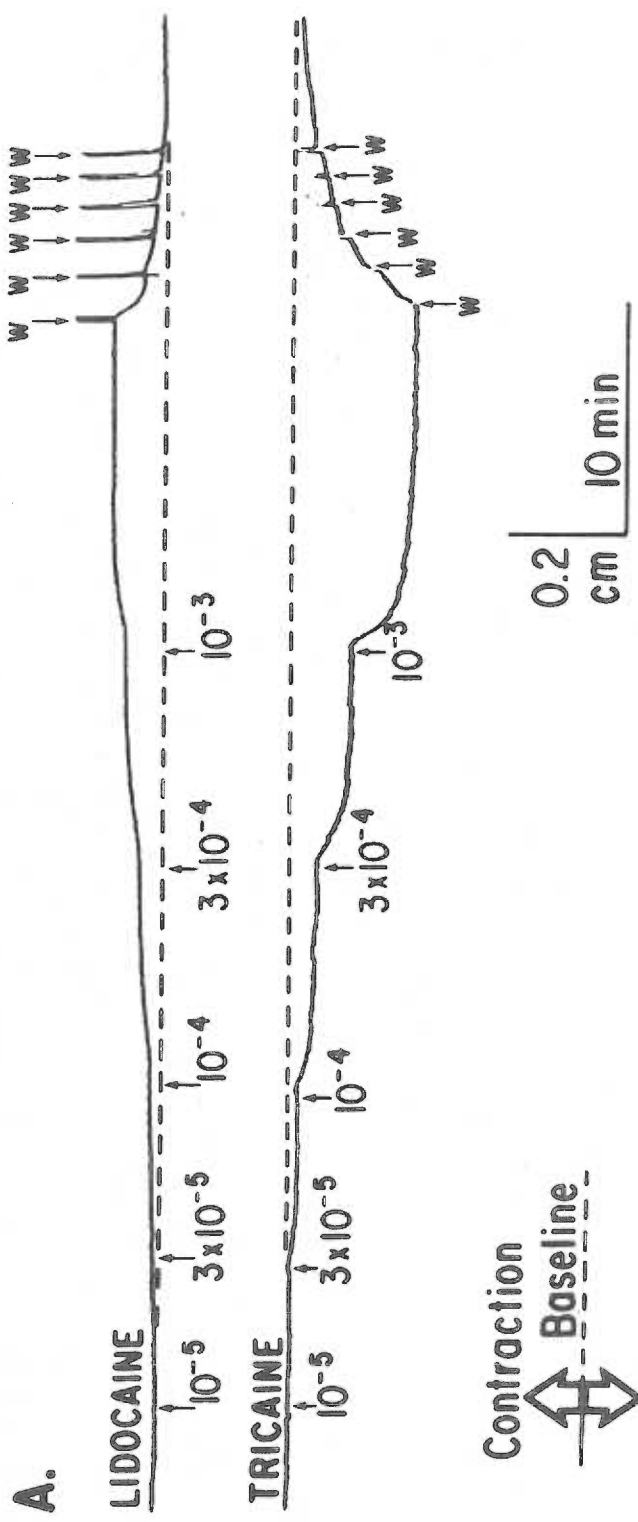


Figure 45: Comparative Effects of Various Concentrations of Tricaine and Lidocaine on the Smooth Muscle Tone of the Isolated Trachea of Guinea Pigs

Low concentrations (10^{-5} to 3×10^{-5} M) of lidocaine produced a slight relaxation of the preparation (seen best in B.). Higher concentrations (10^{-4} to 10^{-3} M) of lidocaine contracted the preparation. With further increases in concentration (not shown), there was a reversal of the contraction. Tricaine, at all effective concentrations, relaxed the preparation. W = wash.

preparation. Lidocaine had a triphasic effect; low concentrations (10^{-5} - 3×10^{-5} M) produced a minor relaxation, intermediate concentrations (10^{-4} - 10^{-3} M) induced a marked contraction and higher concentrations (3×10^{-3} M and higher) produced relaxation (not pictured in Figure 45).

Nervous Tissue Preparations. Decerebrated frogs responded to immersion of their feet in acid with a very brisk withdrawal after a latency of less than one sec. When a ligature was placed around one leg, sparing the sciatic nerve but totally occluding the blood flow, both the ligated and the unligated legs retained the acid withdrawal response. However, the ligated and unligated legs of frogs prepared as described above lost the acid withdrawal response within 1 min of an i.p. injection of a 200 mg/kg dose of tricaine methanesulfonate. Direct electrical stimulation (single pulse - 5 mvolts, 5 msec duration) of the sciatic nerve of the ligated leg elicited a strong contraction of the gastrocnemius muscle throughout the period of time that the leg did not respond to acid exposure. Decerebrated frogs injected i.p. with normal saline and tested once every min exhibited the acid withdrawal response (latency was less than one sec) for 10-15 min after the saline injection. Beyond the 15 min period, the preparations deteriorated rapidly and ceased responding about 20 min after the injection.

In other experiments, decerebrated frogs were eviscerated to expose both sciatic nerves in their intra-coelomic course. Small pledgets of filter paper soaked in saline or a drug solution were placed on one of the 2 sciatic nerves and the opposite nerve served as a control in each experiment. The response of the frogs to immersion of their feet in acid was monitored at 2 min intervals. The higher concentrations of tricaine

methanesulfonate, 1 gm% and 3 gm%, produced a partial block of nerve conduction within 2 min of drug application and complete block of conduction within 6 min (Figure 46).

2. Intact Animal Studies

Effects of Tricaine Methanesulfonate on the Cardiovascular System of the Intact Anesthetized Cat. The effects of i.v. injections of various concentrations of tricaine methanesulfonate on the blood pressure and heart rate of an intact anesthetized cat are shown in Figures 47 and 48, respectively. Each injection consisted of 2 ml of drug solution (drug in normal saline) followed by a 1 ml bolus of normal saline to clear the i.v. line of drug. Injections of normal saline had no significant effect on either the systolic or diastolic blood pressures. The saline injections did cause a slight drop in heart rate, $-2.84 \pm 1.36\%$ (S.D.) of initial. The doses of drug ranged from 0.15 to 200 mg/kg. Tricaine methanesulfonate depressed the blood pressure over the indicated dosage range and the injection of the 200 mg/kg dose of the drug produced cardiac arrest. The effect of tricaine on the heart rate did not differ from that of normal saline, except at the highest dose (200 mg/kg) which arrested the heart both mechanically and electrically.

Antiarrhythmic Effects of Tricaine Methanesulfonate on Artificially-Induced Arrhythmias. A guinea-pig atrial preparation was exposed to a bathing solution that contained a 0.5 μ g/ml concentration of aconitine nitrate as a preliminary test of the antiarrhythmic actions of tricaine methanesulfonate. Aconitine produces arrhythmias in intact cat hearts (Scherf 1947) and in isolated preparations of cat cardiac tissues (Tanz 1973). In isolated preparations, the tachycardia induced by aconitine

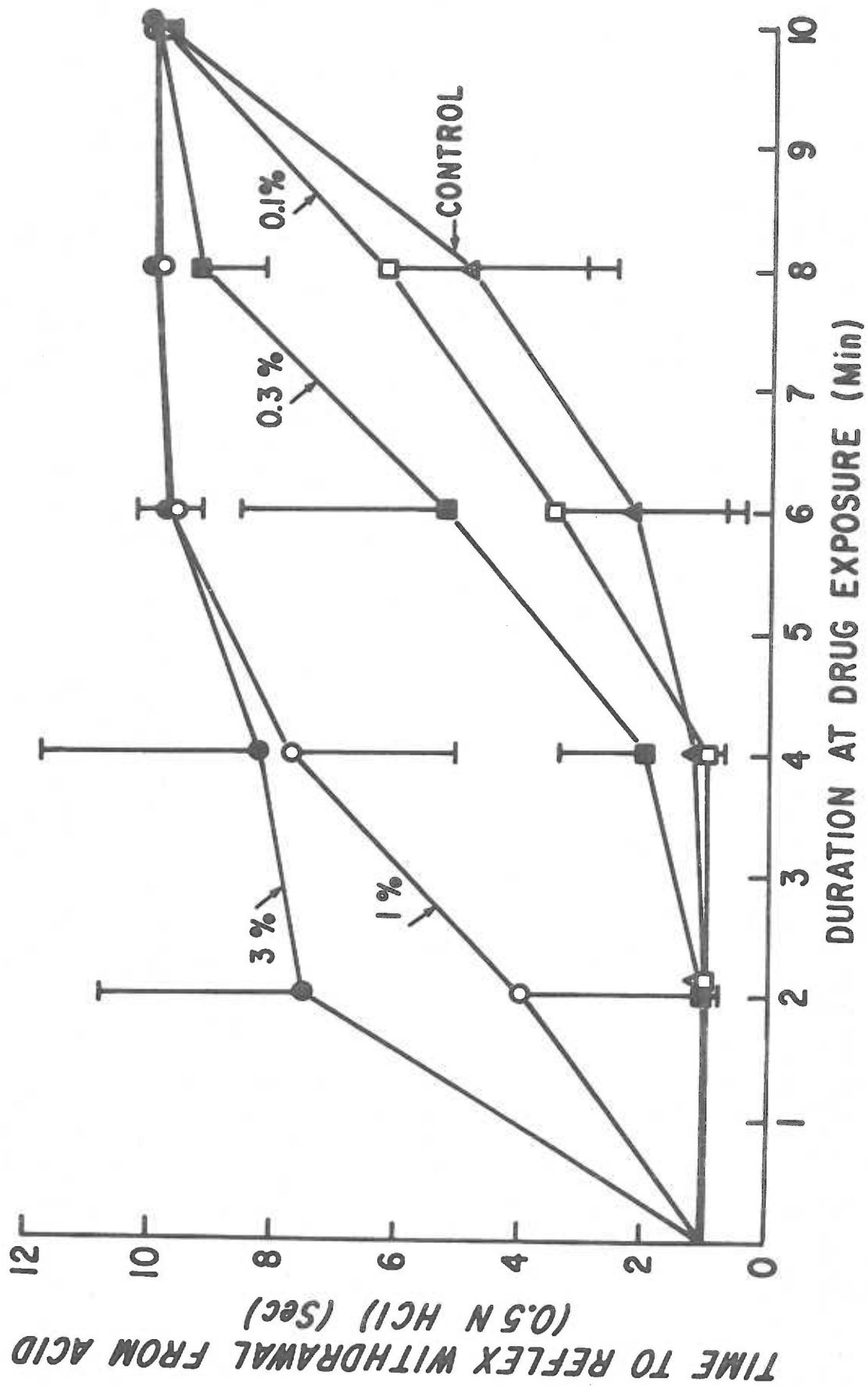


Figure 46: Effect of Various Concentrations of Tricaine Methanesulfonate on a Sensory Motor Reflex Arc in the Frog

The high concentrations (1-3%) of tricaine methanesulfonate significantly delayed the reflex withdrawal of the frog's feet from acid. The temporal pattern of response to the acid exposure did not significantly differ between the saline-treated and untreated nerves. Therefore, these 2 groups were combined into a single control group. Each point represents the mean $\pm$ S.D. of four animals, except for the control group for which $n = 20$ for each point.

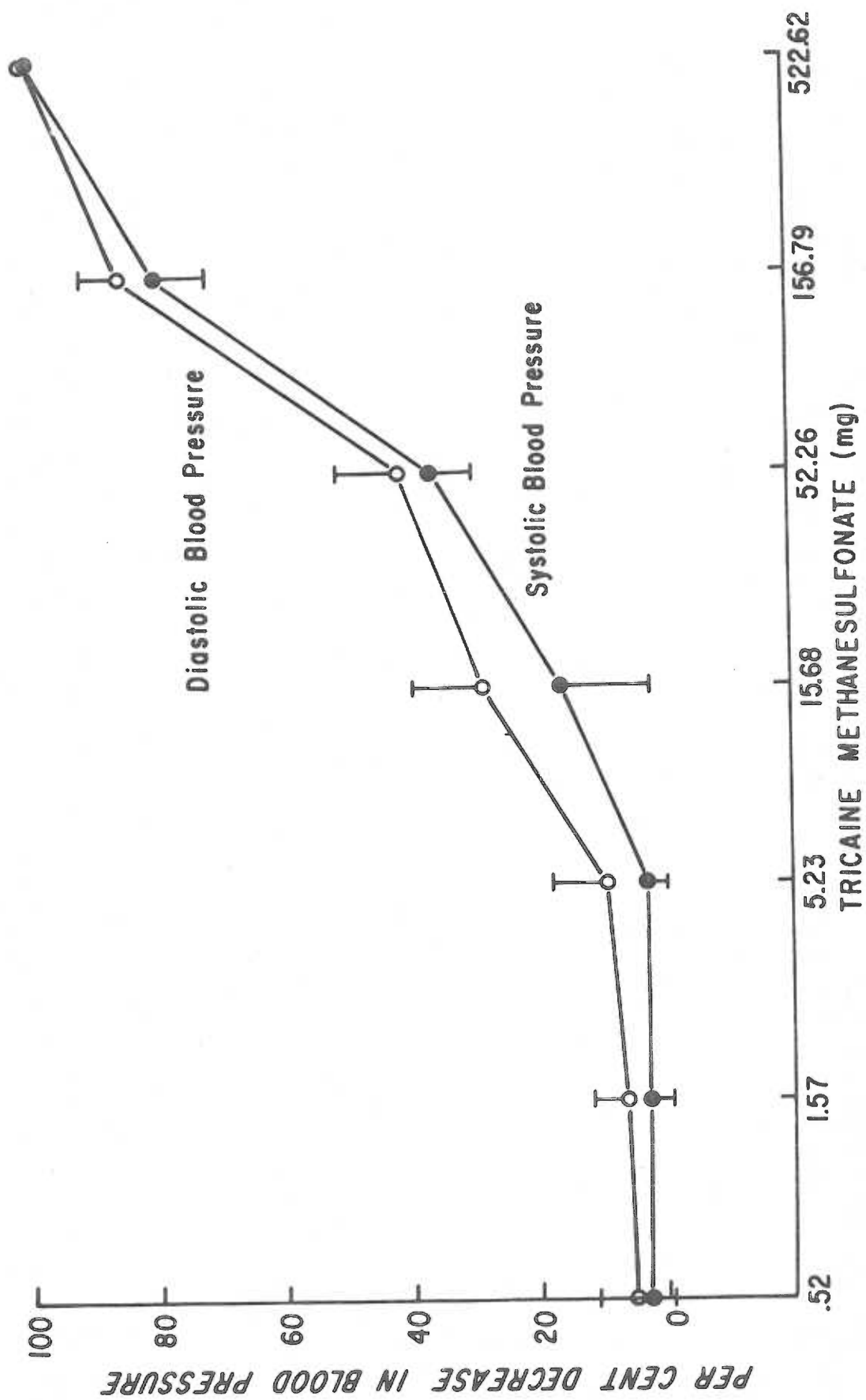


Figure 47: Concentration-Effect Relationship of Tricaine Methanesulfonate on the Blood Pressure of the Intact Anesthetized Cat

Tricaine's depressant effect on diastolic blood pressure was greater, in each preparation, than that on systolic blood pressure. Each point represents the mean $\pm$ S.D. of four animals.

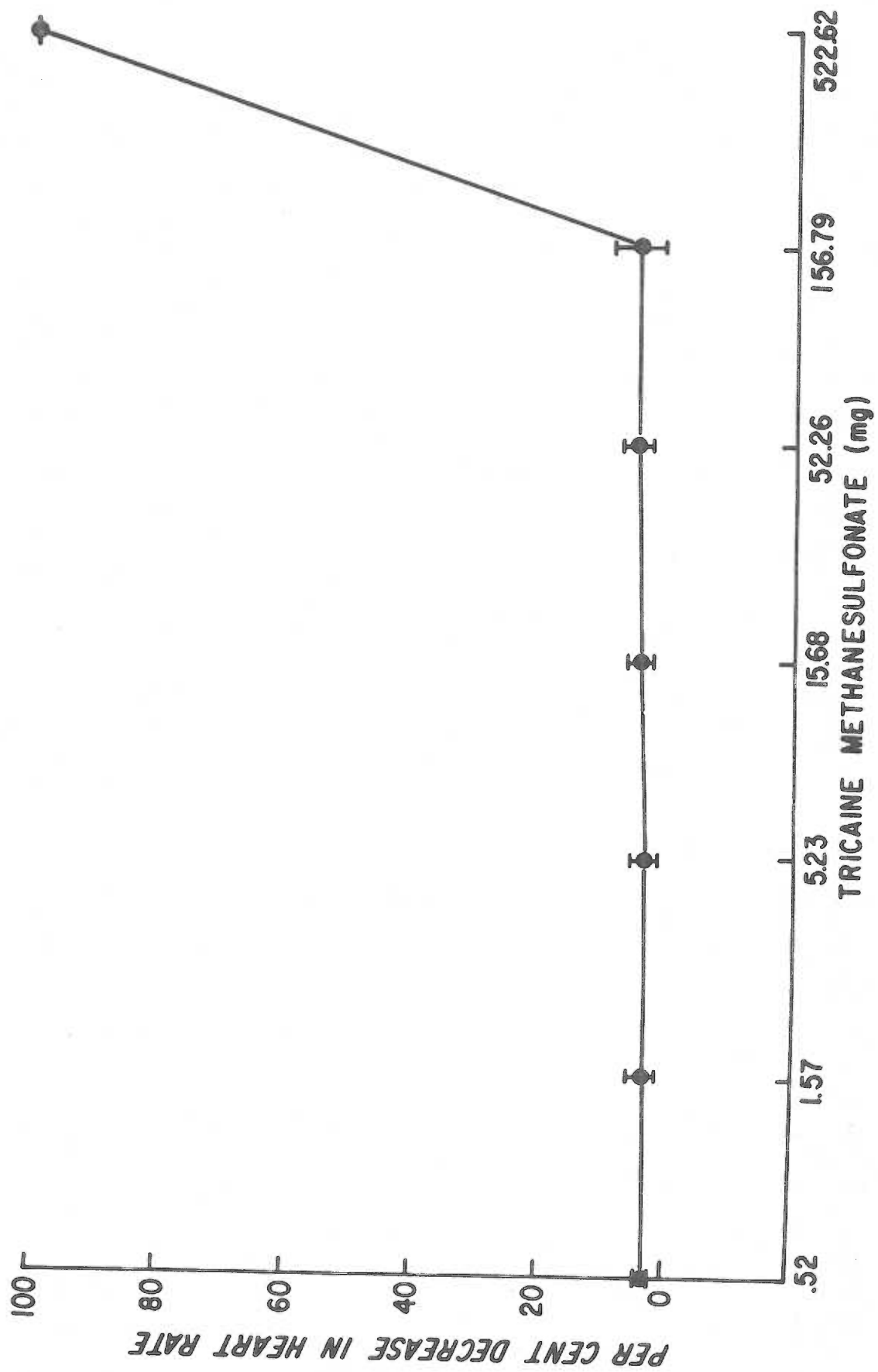


Figure 48: Concentration-Effect Relationship of Tricaine Methanesulfonate on the Heart Rate of the Intact Anesthetized Cat

Tricaine methanesulfonate did not significantly depress the rate of contraction of the heart in the preparation, except for the 200 mg/kg dose of the drug which produced cardiac arrest. Each point represents the mean $\pm$ S.D. of four animals.

can not be reversed by repeatedly washing the preparation with drug-free bathing solution (Tanz 1973). Figure 49 illustrates an aconitine-induced tachycardia in the isolated guinea-pig atria and the response to various concentrations of tricaine methanesulfonate. The tachycardia was partially reversed by 10^{-4} M tricaine methanesulfonate and completely reversed by a 10^{-3} M concentration of the drug.

The antiarrhythmic actions of tricaine on ouabain-induced arrhythmias in the intact anesthetized cat were also investigated. Figure 50A and 50B show the blood pressure and ECG tracings of an intact anesthetized cat before and during a ouabain infusion ($5 \mu\text{g/kg/min}$), respectively. The slowing of the heart rate (from 200 beats/min to 140 beats/min) and the elevation of the S-T segment in the ECG tracing are characteristic effects of ouabain and can be seen in Figure 50B. Figure 50C illustrates ouabain-induced ventricular bigeminy, with an associated pulses alternans-like pattern in the blood pressure recording. The 'P' wave in the ECG tracing, an electrical representation of the depolarization of the atria, appeared at regular intervals, indicating a normal sinus rhythm in the atria. Alternate atrial depolarizations resulted in ventricular "capture", and therefore, in normal ventricular depolarizations (normal QRS complexes). The intervening P waves were followed immediately by abnormal ventricular depolarizations. The abnormal ventricular depolarizations resulted in premature ventricular contractions (PVC's). The characteristics of the PVC's were: (1) the QRS complexes were broadened, reflecting an abnormal conduction pathway within the ventricle; (2) the QRS complexes were increased in amplitude and bizarre in shape; the S-T segment sloped in the direction opposite to the main QRS deflection;

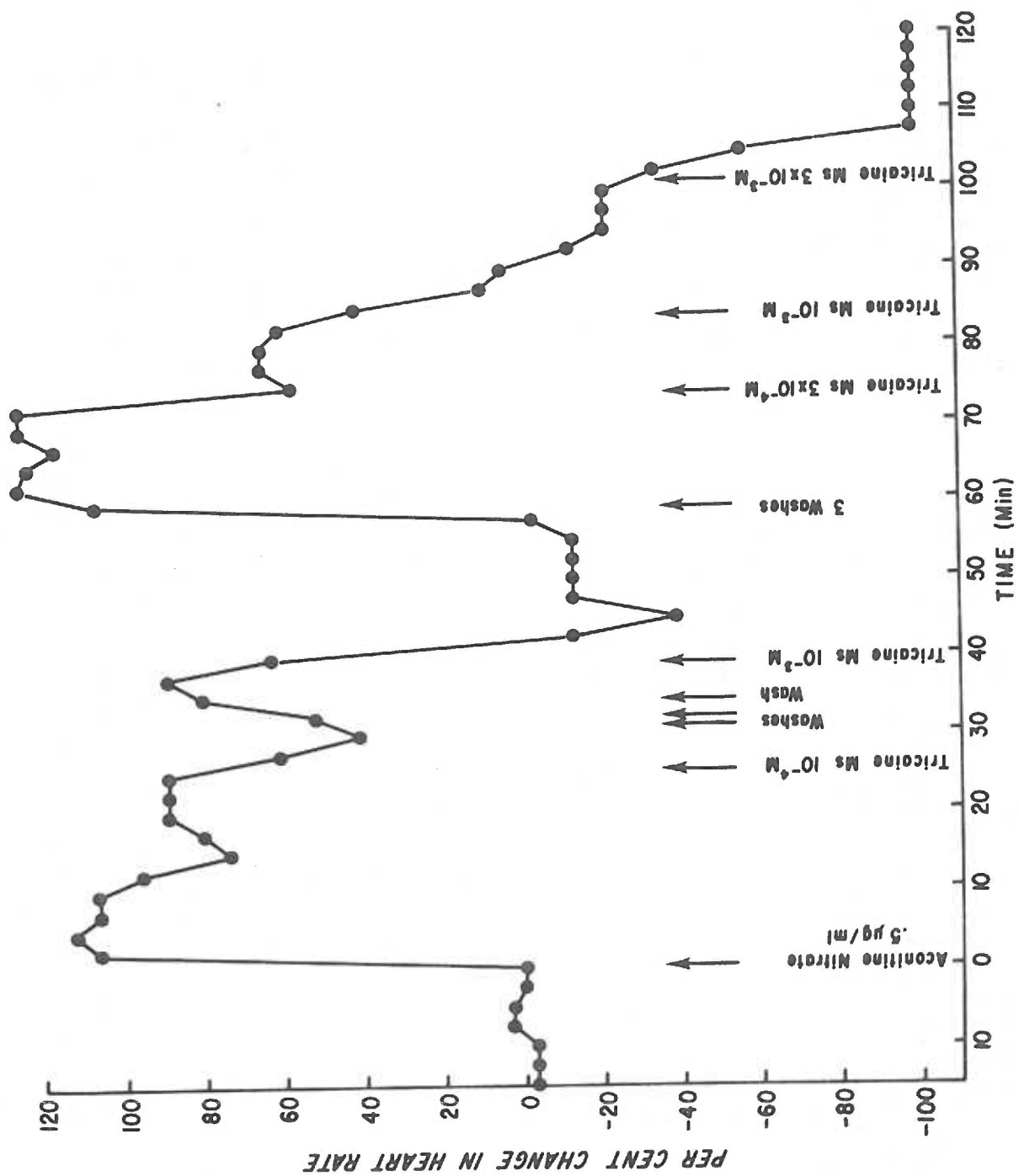
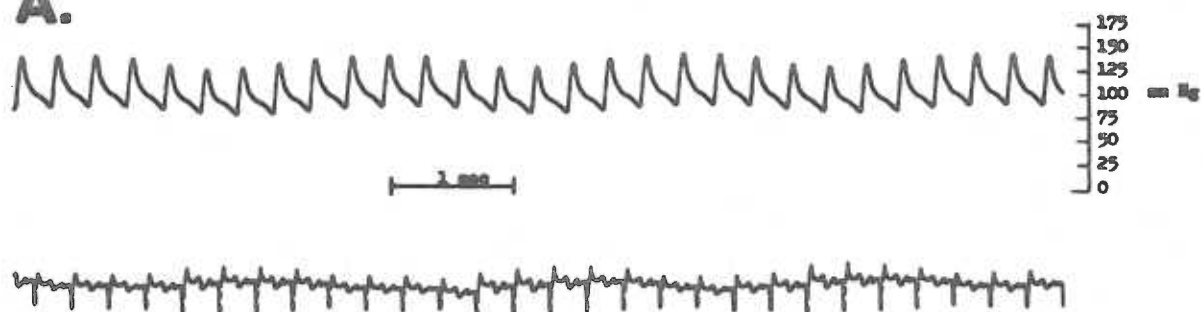


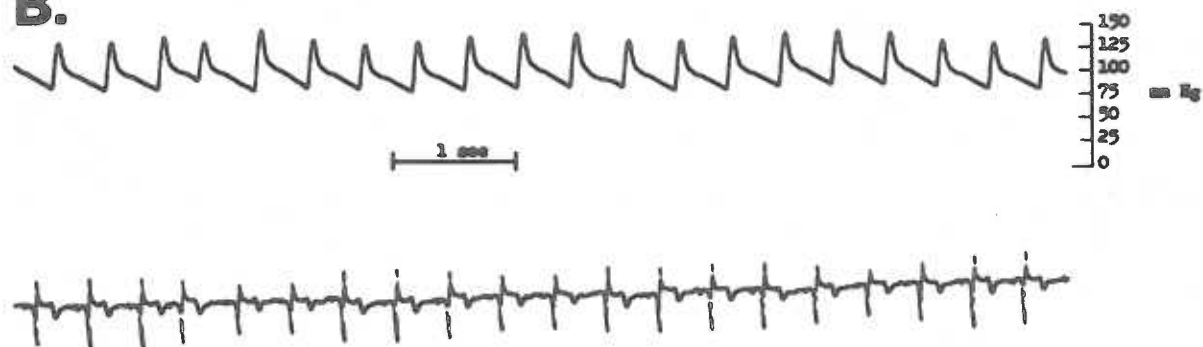
Figure 49: Antiarrhythmic Action of Tricaine on an Aconitine-Induced Arrhythmia in Spontaneously Beating Isolated Guinea-Pig Atria

Aconitine produced a marked tachycardia in the isolated guinea-pig atrial preparation. The 10^{-3} M concentration of tricaine completely reversed the tachycardia. A threefold increase in this concentration (3×10^{-3} M) arrested the preparation. Each point represents a single observation.

A.



B.



C.

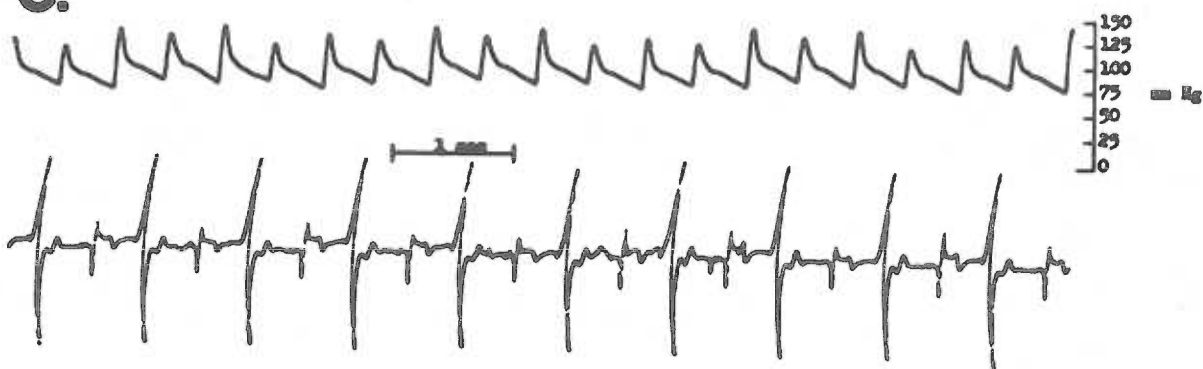


Figure 50: Blood Pressure and ECG Tracings of the Intact Anesthetized Cat

A. Control Period

The intact anesthetized cat was allowed to stabilize for 1 hr before the ouabain infusion ($5 \mu\text{g/kg/min}$) was started to induce arrhythmias. Antiarrhythmic actions of tricaïne were then investigated.

B. Twenty-Three Min After the Start of the Ouabain Infusion

Slowing of the heart rate and elevation of the S-T segment in the ECG tracing are characteristic changes produced by ouabain in in vivo cat hearts.

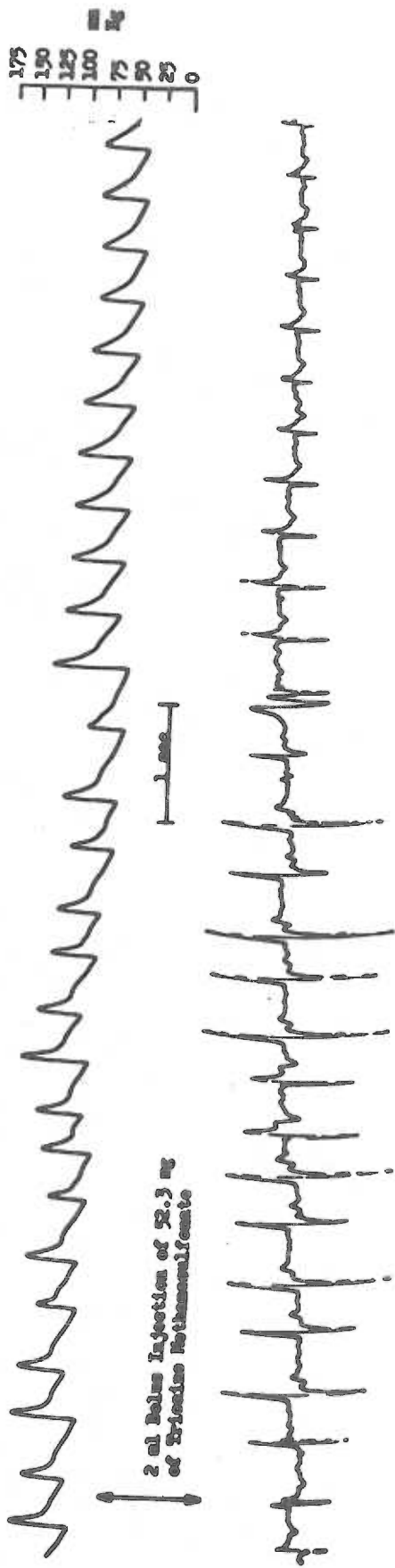
C. Ouabain-Induced Arrhythmia (25 min after start of the Ouabain Infusion)

The ouabain-induced arrhythmia began 24 min after the start of the ouabain infusion. The arrhythmia was initially one of ventricular bigeminy.

(3) the QRS complexes were premature, followed by a compensatory pause. The time between R waves of normal ventricular depolarizations and R waves of the PVC's was 0.40 sec, whereas, that between R waves of PVC's and R waves of the normal ventricular depolarizations was 0.48 sec. The pause that followed each PVC was "fully" compensatory; the interval from the beat preceding the PVC to that following the PVC was exactly two cardiac cycles (0.88 sec). It was not determined whether the abnormal ventricular depolarization represented a fusion of a PVC depolarization wave with a normal ventricular depolarization wave, or whether the abnormal tracings represented only PVC depolarization waves, and the normal depolarization waves of the ventricle were not propagated due to refractoriness of the tissue induced by the PVC's. The contractions associated with PVC's were less efficient than those associated with normal ventricular depolarizations.

Figures 50D and 50E show the conversion of the ouabain-induced arrhythmia to a normal sinus rhythm (rate = 160 beats/min) immediately following a bolus injection of tricaine methanesulfonate (52.3 mg). The normal sinus rhythm was maintained for approximately 90 sec, at which time the heart reverted to the arrhythmia (Figure 53F). A second bolus injection of the drug (52.3 mg of tricaine methanesulfonate), again, converted the heart to a normal sinus rhythm (Figures 53G and 53H). After each tricaine injection, the heart remained in a normal sinus rhythm for 94 ± 13 sec (S.D.) before reverting to the arrhythmia. Eventually the arrhythmia degenerated into one of ventricular fibrillation, and tricaine methanesulfonate injections did not convert the ventricular fibrillation to a normal sinus rhythm.

D.



E.

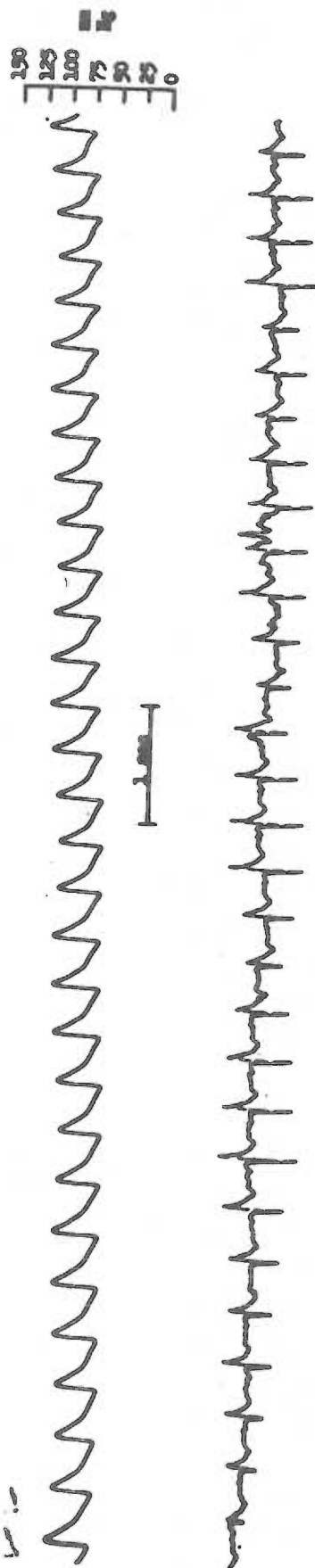


Figure 50: Blood Pressure and ECG Tracings of the Intact Anesthetized Cat

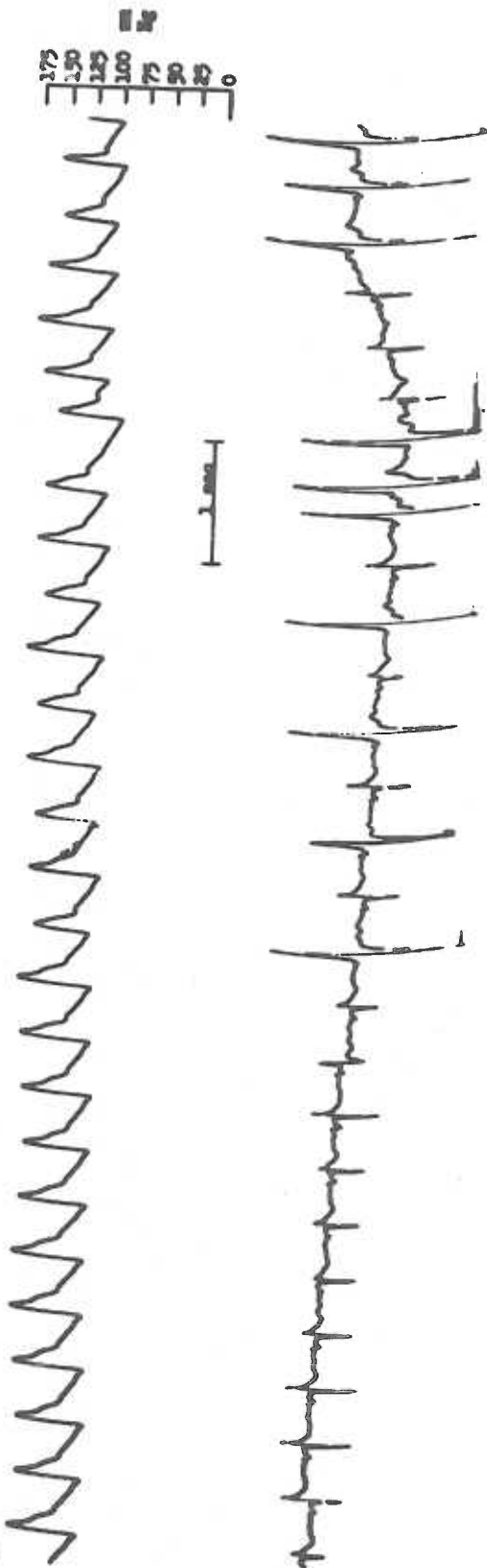
D. Tricaine Reversal of a Ouabain-Induced Arrhythmia

The bolus injection of 5.23 mg/2 ml of tricaine methanesulfonate converted the heart to a normal sinus rhythm within 7 sec. Note the blood pressure response to the tricaine injection. (30 min after start of the ouabain infusion.)

E. Maintenance of Normal Sinus Rhythm

The tracing, which is continuous with the above tracing (D), shows the maintenance of the normal sinus rhythm after the tricaine injection. Note the return of the blood pressure toward the levels observed prior to the tricaine injection. (Ouabain infusion continuing.)

F.



G.

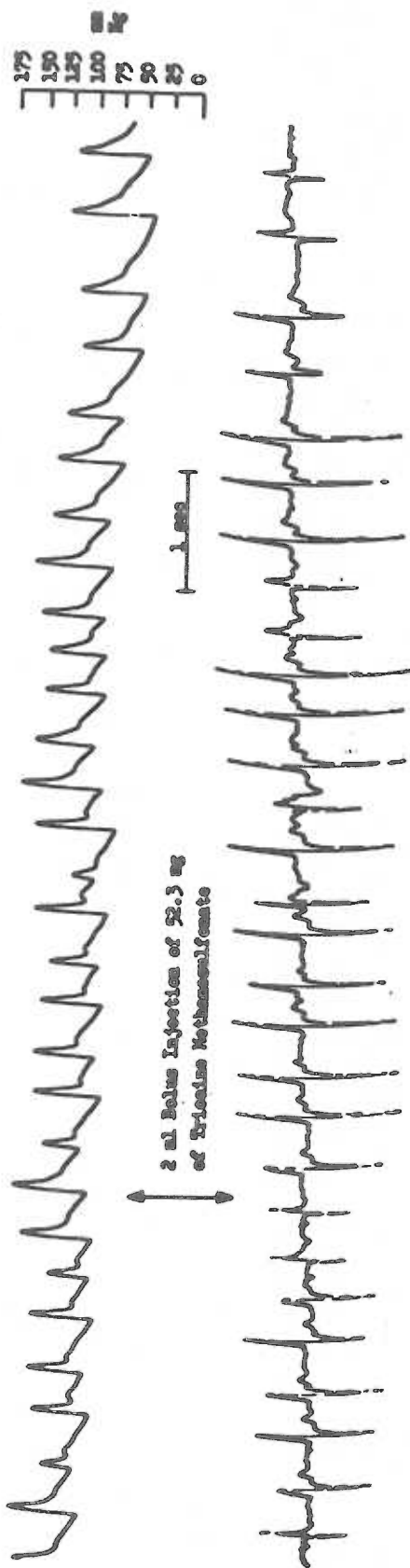


Figure 50: Blood Pressure and ECG Tracings of the Intact Anesthetized Cat

F. Re-Entry of Heart into an Arrhythmia

This tracing was recorded 90 sec after the bolus injection of tricaine. (Ouabain infusion continuing.)

G. Second Bolus Injection of Tricaine Methanesulfonate

The tracing, which is continuous with the above tracing (F), shows the re-establishment of normal sinus rhythm following a second bolus injection of tricaine methanesulfonate (52.3 mg/2 ml). Again, note the response of the blood pressure parameter to the injection of tricaine. (Ouabain infusion continuing.)

H.



1 sec



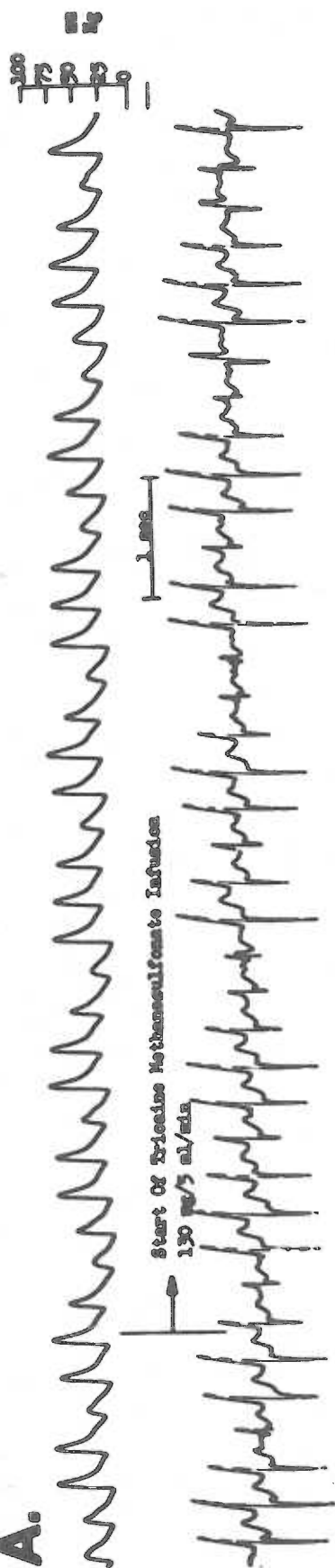
Figure 50: Blood Pressure and ECG Tracing of the Intact Anesthetized Cat

H. Maintenance of Normal Sinus Rhythm After Second Drug Injection

This tracing, which is continuous with the above tracing (G), shows the maintenance of the normal sinus rhythm after the tricaine injection. Again, note the return of the blood pressure parameter toward the levels observed prior to the tricaine injection. (Ouabain infusion continuing.)

Figure 51 shows the blood pressure and ECG tracings from an intact anesthetized cat that was infused with ouabain ($5 \mu\text{g/kg/min}$) for 30 min. The ouabain infusion was terminated with onset of arrhythmias and a tricaine methanesulfonate infusion (130 mg/min) was started. The heart converted to a normal sinus rhythm (Figures 51B and 51C). The tricaine methanesulfonate infusion was continued and, with time, the heart reverted to the arrhythmia (Figure 51D). The heart stopped 4 min after the start of the tricaine infusion (Figure 51E). The tricaine infusion was terminated when the heart arrested and 135 sec later the heart spontaneously started to beat again. The blood pressure returned to pre-infusion levels and the heart remained in a normal sinus rhythm (rate = 200 beats/min) throughout the remainder of the experiment (more than 2 hr).

A.



B.

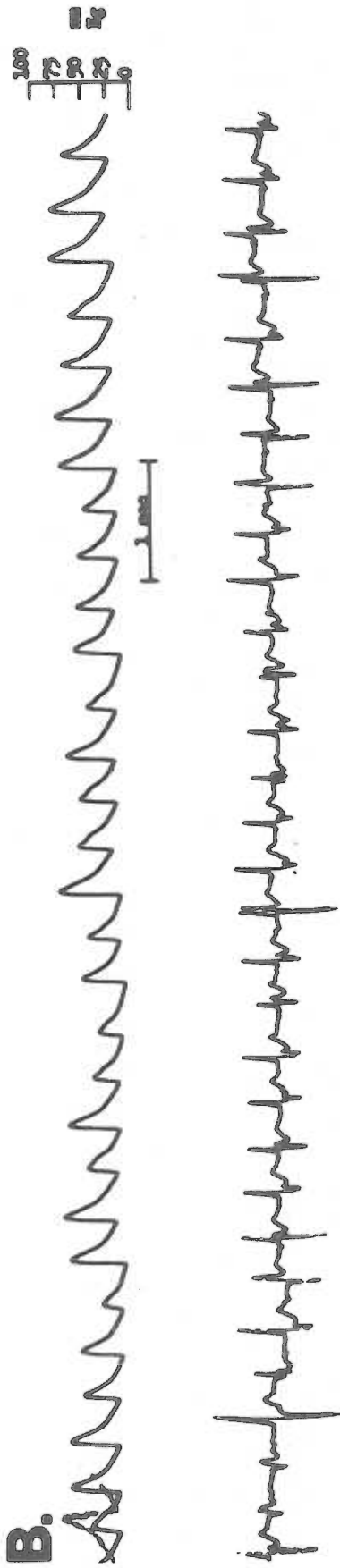


Figure 51: Actions of Tricaine on a Ouabain-Induced Arrhythmia in the Intact Anesthetized Cat

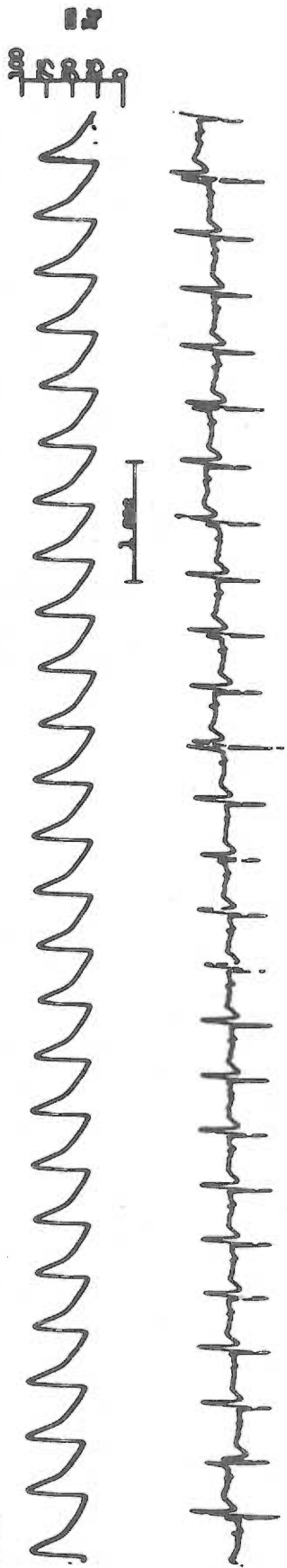
A. Start of the Tricaine Methanesulfonate Infusion

An infusion of tricaine methanesulfonate (130 mg/5 ml/min) was started to reverse the ouabain-induced arrhythmia. Note the response of the blood pressure parameter to the tricaine infusion. The ouabain infusion (5 μ g/kg/min) was stopped immediately before the start of the tricaine infusion.

B. Partial Reversal of Arrhythmia 30 sec After Start of Tricaine Infusion

There was a partial reversal of the ouabain-induced arrhythmia 30 sec after the start of the tricaine infusion. Note the continued depression of the blood pressure parameter in response to the infusion of tricaine. (Tricaine infusion continuing.)

C.



D.

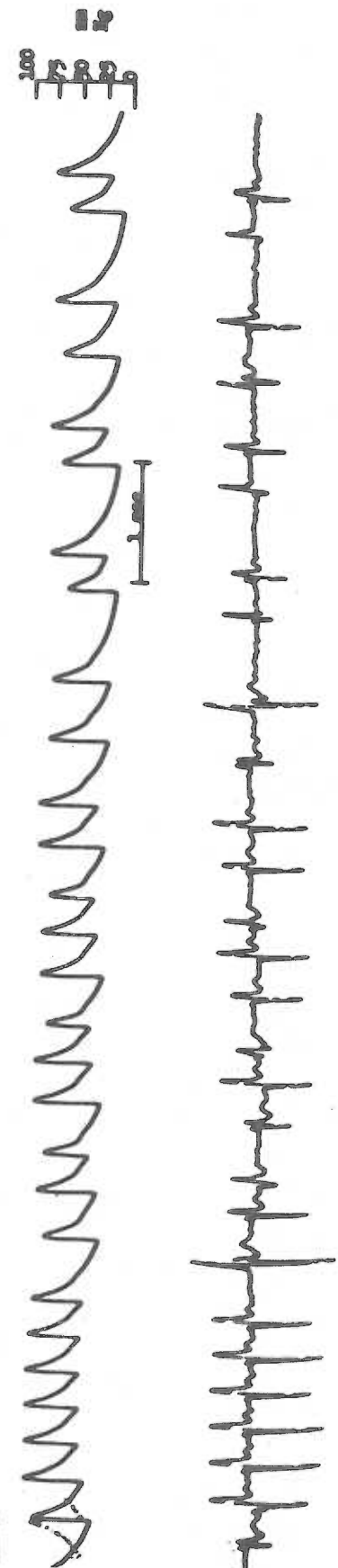


Figure 51: Actions of Tricaine on a Ouabain-Induced Arrhythmia in the Intact Anesthetized Cat

C. Complete Reversal of the Arrhythmia with Tricaine

The tracing is continuous with the above (B). (Tricaine infusion continuing.)

D. Deterioration of Cardiac Function Secondary to the Tricaine Infusion

The continuation of the tricaine methanesulfonate infusion (4 min) resulted in a marked deterioration of the cardiac function of the preparation. (Tricaine infusion continuing.)

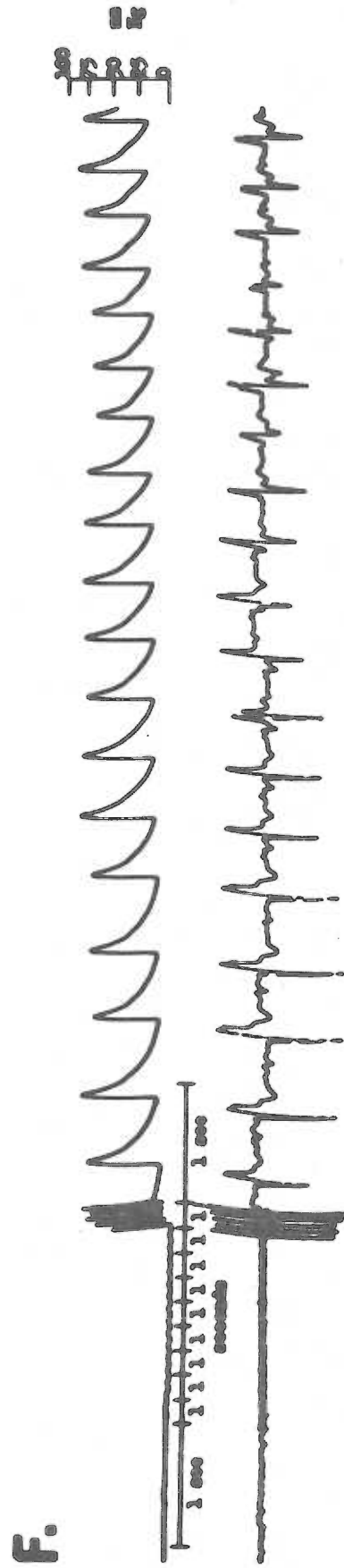
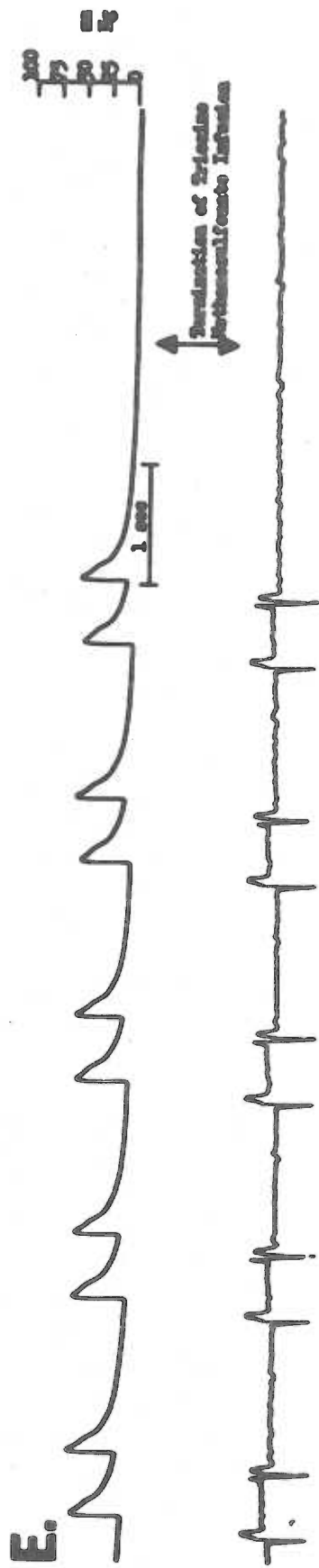


Figure 51: Actions of Tricaine on a Ouabain-Induced Arrhythmia in the Intact Anesthetized Cat

E. Tricaine-Induced Cardiac Arrest

The tracing, which is continuous with the above (D), shows the continuing deterioration of cardiac function and the eventual arrest of the heart. The tricaine infusion was immediately terminated with the onset of cardiac arrest.

F. Spontaneous Reversal of Cardiac Arrest

The spontaneous resumption of cardiac function occurred 135 sec after the onset of cardiac arrest and termination of all drug infusions and injections. Normal cardiac function, including a normal sinus rhythm, continued until the end of observations (more than 2 hr). There were no arrhythmias observed during this period.

DISCUSSION

A. The Basis of the Selective Pharmacological Action of Tricaine Methanesulfonate

1. The Selective Action

Tricaine methanesulfonate exerts a selective pharmacological action on poikilotherms. In frogs, the i.p. injection of 150 mg/kg of the drug produces light surgical anesthesia with loss of the righting reflex and absent or much reduced responses to nociceptive stimuli. Other cold-blooded species (salamanders, snakes, turtles and lizards) are also anesthetized when injected i.p. with doses of tricaine methanesulfonate ranging from 150 to 250 mg/kg. In contrast, an i.p. dose of 250 mg/kg of the tricaine salt produces no apparent pharmacological response in any of the mammalian species which have been tested (mice, rats, guinea pigs and cats). Enormous i.p. doses of the drug (greater than 1000 mg/kg) are required in order to observe even transient effects in these mammalian species. The selective toxicity of tricaine for poikilotherms could be based on a slower rate of drug elimination or a greater target-organ sensitivity in the lower vertebrates.

2. Drug Elimination

Whole Body Half-Lives. Mammals and poikilotherms differ strikingly in their respective rates of tricaine elimination. The mouse eliminates the drug so rapidly that no unchanged drug can be recovered 5 min after i.p. injection of 250 mg/kg of tricaine methanesulfonate. In the frog, the whole body half-life of a similar i.p. dose of tricaine is approximately 70 min. The considerably slower rate of drug elimination in the frog is not the result of a lower body temperature, since

the half-life of tricaine in frogs kept at 37° C is also about 70 min. If, however, the ambient temperature is lowered to 7° C, the half-life of tricaine in frogs is prolonged, i.e., 309 min.

Biotransformation. The rapid disappearance of tricaine in mammals is due to biotransformation. The total dose of tricaine injected i.p. into a mouse, all of which is metabolized within 5 min, can be accounted for as MABA and other, more polar, water-soluble BMRM (Bratton-Marshall Reacting Metabolites). Although proceeding at a much slower rate, biotransformation can also be the major process in the elimination of tricaine from poikilotherms. More than 90% of the tricaine eliminated from salamanders and lizards was recovered as BMRM. A large portion of the eliminated tricaine could not be recovered as BMRM in frogs and snakes (46% and 59%, respectively), but biotransformation appears also to be the primary pathway of elimination in these species. Studies in which frogs were analyzed with and without their collected excreta for tricaine and its metabolites 70 min after an i.p. injection showed that in frogs little if any of the drug or its metabolites were excreted. Therefore, the low recovery of eliminated tricaine as BMRM is not the result of excretion; rather, it is the result of biotransformation of the drug involving the amine group of the drug molecule. The Bratton-Marshall reaction does not detect deaminated MABA or deaminated tricaine, nor does it detect MABA or tricaine conjugated at the amine group, i.e., acetylation (Bratton and Marshall, 1939).

Hepatic Metabolism. Greene (1968) concluded that, in general, esters of acids other than PABA (p-aminobenzoic acid) are more susceptible to hepatic metabolism in man than to serologic hydrolysis. This

is true of the metabolism of tricaine in the mouse, but not in the frog. In vitro studies indicate that mouse-liver homogenates metabolize tricaine at a maximum rate of approximately 19 μ moles/gm of liver/min. In contrast, frog-liver homogenates do not metabolize any measurable amount of the drug. Moreover, the mean weight of the liver of a mouse [1.86 ± 0.18 (S.D.)] is more than 6 times that of a frog of equal body weight [0.30 ± 0.07 gm (S.D.)]. The slow rate of biotransformation in the frog liver homogenates is not due to the presence of any endogenous inhibitors of tricaine metabolism, since incubation of frog liver with mouse liver does not slow the rate of biotransformation of tricaine per gram of mouse liver.

In vitro incubations of tricaine with liver homogenates of other mammalian and nonmammalian species confirm the phylogenetic difference in hepatic esterase activities. The rate of hepatic hydrolysis of tricaine in mammalian in vitro preparations varies from 39 μ moles/gm of liver/min for the rabbit to 13 μ moles/gm of liver/min for the guinea pig. Therefore, the need for massive i.p. doses of tricaine in order to observe even transient effects in mammals is the result of the rapid liver metabolism of tricaine. The mammalian hepatic esterases probably remove most of an i.p. dose of tricaine in the first pass of the drug through the liver before it can reach the systemic circulation. Among poikilotherms, however, in vitro liver incubations show no measurable metabolism of tricaine, even in those species that metabolize the drug comparatively rapidly (salamanders and lizards). The lack of significant hepatic metabolism of the drug in cold-blooded vertebrates allows most or all of the tricaine injected i.p. to be absorbed into the systemic circulation

and to reach its site(s) of action. Therefore, the i.p. anesthetic dose of tricaine is relatively small (150 to 250 mg/kg of tricaine methane-sulfonate) in poikilotherms.

Serum Metabolism. Tricaine is metabolized by the sera of both mammalian and poikilothermic species. The hydrolysis of tricaine to MABA, the sole metabolite of the drug in in vitro serum incubations, appears to follow Michaelis-Menten enzyme kinetics in both human and mouse sera. In Michaelis-Menten kinetics, the K_m value is that concentration of substrate (tricaine) which produces one half of the maximal velocity of an enzyme system (tricaine-esterase) and is, therefore, a measure of the dissociation constant between the particular substrate and the enzyme. The tricaine-esterase activity of the human and mouse sera exhibit the same K_m value for the hydrolysis of tricaine. The similarity of K_m values and, therefore, dissociation constants of the two mammalian sera suggests a common identity for the tricaine-esterase enzyme systems of human and mouse sera. However, in the above studies the sera were incubated with tricaine for 30 min, before being analyzed, to allow for a measurable amount of metabolism to occur in incubations with low initial drug concentrations. The 30 min incubation period is very long compared to that which is used in most other kinetic studies (less than 1 min) and, therefore, the resultant rates of hydrolysis do not represent initial reaction velocities. Since Michaelis-Menten kinetics are based upon initial reaction velocities, the absolute magnitude of the K_m estimates for human and mouse sera hydrolysis of tricaine are artificially inflated.

The maximum velocity or V_{max} of an enzymatic reaction, in Michaelis-

Menten kinetics, is a measure of the total activity of a given amount of enzyme and determination of the $V_{\max}$ is independent of the incubation period. The marked difference in the respective $V_{\max}$'s of tricaine hydrolysis by human and mouse sera may represent an increased amount of tricaine-esterase enzyme in the mouse serum. Voss and Sachsse (1970) showed that the activity of the serum choline esterase, pseudocholinesterase, in mouse serum was twice that in human serum. Pseudocholinesterase is responsible for hydrolysis of procaine in human serum (Huc 1950; Kalow 1952a, b; Augustinsson 1952). The close similarity in the chemical structure of tricaine and procaine suggest that pseudocholinesterase may also be responsible for the tricaine-esterase activity in mammalian sera. However, Richter and Croft (1942) have shown that choline esterases may be differentiated from other serum esterases, e.g., aliesterases and aryleresterases, by their sensitivity to the inhibitory effect of physostigmine and neostigmine. A concentration of approximately 5×10^{-7} M neostigmine is necessary to produce a 50% reduction in the hydrolysis activity of human serum pseudocholinesterase (Blaschko et al. 1949, Burgen and Hobbiger 1951, Foldes et al. 1958, Smith et al. 1953, Funket et al. 1954, Depierre and Funke 1954, Jacob and Funke 1953, Jacob 1955, Copp 1953, Fulton and Mogey 1954, Levin and Jandorf 1955). In contrast, the tricaine-esterase activity in human serum appears very resistant to inhibition by neostigmine. In our experiments, a concentration of about 10^{-3} M neostigmine only produced a 60% reduction in activity. Therefore, the tricaine-esterase enzymatic system in human serum appears much more complex than the single enzyme system of pseudocholinesterase and the resistance to neostigmine inhibition

indicates that choline esterases may account for only a minor part of the total tricaine-esterase activity in mammalian serum.

The hydrolysis of tricaine in frog serum does not follow Michaelis-Menten kinetics, but its maximum rate of tricaine hydrolysis is similar to the $V_{\max}$ of human serum. The rate of serum hydrolysis of tricaine appears to account for a major part of the whole body biotransformation of tricaine in the frog. Thus, serum hydrolysis allows frogs to recover from tricaine-induced anesthesia much more rapidly than from barbiturate-induced anesthesia (Kaplan et al. 1962). Indeed, it is this relatively rapid recovery that makes tricaine particularly useful as a surgical anesthetic in frogs.

Although serum does not play the major role in mammalian metabolism of tricaine, it should be noted that serum hydrolysis of tricaine in mammals proceeds at a very rapid rate, in comparison to most other local anesthetics. Thus, the $V_{\max}$ of tricaine hydrolysis in human serum (40 nmoles/ml/min) is greater than that reported by Kalow (1952a) and Foldes et al. (1956) for procaine hydrolysis in human serum (24.7 nmoles/ml/min and 26.3 nmoles/ml/min, respectively).

Excretion Across Epithelial Membranes. Some poikilothermic species also eliminate tricaine by excretion across the skin or gills. The drug is widely used in the anesthetization of fish because of its rapid movement across the gill membranes (Maren et al. 1968, Hunn et al. 1968, Hunn 1968, 1970, Stenger and Maren 1968). The high membrane permeability of tricaine permits rapid induction of anesthesia when fish are placed in water containing tricaine, and rapid recovery from anesthesia in tricaine-free water. Amphibia are also anesthetized when

placed in tricaine solutions and they eliminate tricaine more rapidly in water (tricaine-free) than out of water. In our experiments, using i.p. injections of tricaine methanesulfonate (50 mg/kg), the $t_{1/2}$ of the drug was approximately 70 min in frogs in a dry environment (23° C). However, when frogs were in running water (23° C), the $t_{1/2}$ was 30 min.

The elimination of tricaine from frogs in a dry environment follows first-order kinetics. In running water, however, the whole-body tricaine concentration declines more rapidly but with a biphasic or triphasic slope. A possible explanation for this deviation from first-order kinetics is as follows: The similar distribution of tricaine and its metabolites in all tissues of the bullfrog, except adipose tissue, suggests that tricaine is hydrolyzed in situ in these tissues. The tricaine-hydrolysis activity has been found in several tissues including muscle (Luhning 1973), lung (Swanson 1976) and serum. Therefore, from the standpoint of drug elimination by biotransformation, the actual location of the drug within the body of a poikilotherm may be relatively unimportant. In contrast, only the drug contained in circulating blood can diffuse across the skin. The biphasic or triphasic curve of tricaine elimination, therefore, could result from sequestration of the drug in poorly perfused tissues. Such sequestration would not necessarily alter the rate of tricaine metabolism but would severely impair loss of the drug across the skin.

Variability in Rates of Tricaine Elimination. The estimated half-lives of tricaine showed marked differences among the poikilothermic species. This variability in rates of tricaine elimination can not be predicted by any simple phylogenetic generalization. Thus, among

reptiles, the estimated half-life of tricaine is 8 min in lizards and 112 min in snakes. Luhning (1973) also noted substantial variations in the rate of tricaine metabolism in different species of fish. Such variations probably reflect the multiplicity of esterases (Holmes et al. 1968) responsible for the hydrolysis of tricaine in different species.

3. Target-Organ Sensitivity

The target-organ sensitivity to tricaine appears to be similar in both mammals and poikilotherms. The whole-body concentrations of tricaine present upon recovery of the righting reflex varies from 1 to 5×10^{-4} M. Considering the gross and sometimes dissimilar criteria used as indices of anesthesia, the whole-body concentrations of tricaine associated with recovery from anesthesia are remarkably similar in all species tested. Also, the cardiotoxicity of tricaine appears to be similar in mammals and poikilotherms. In frogs, respiratory arrest is not an acutely lethal event since extensive gas exchange occurs through the skin (Krogh 1904, 1941). In adequate doses (about 250 mg/kg) tricaine methanesulfonate does produce cardiac arrest in frogs, although equivalent doses are ineffective in mammals. Studies undertaken with the isolated guinea-pig and bullfrog atria indicate that the guinea-pig heart is more vulnerable to the depressant effects of tricaine than is the bullfrog heart. Therefore, at least in cardiac tissues, the resistance of mammalian species to the toxic effects of tricaine does not depend upon target-organ insensitivity.

4. Summary

The selective pharmacological action of tricaine methanesulfonate on poikilotherms, as compared to that on mammals, is the result of a

decreased ability of cold-blooded species to metabolize the drug. The greater total-body metabolism of tricaine observed in mammals is almost entirely a function of the mammalian liver; other tissues exhibit the ability to hydrolyze tricaine but hepatic biotransformation appears to be the major route of drug elimination in mammals. Poikilotherms, some of which are able to excrete the unchanged drug across epithelial membranes when in aqueous environments, also appear to eliminate the drug primarily by way of biotransformation, although hepatic metabolism of the drug is virtually nonexistent in these species. The primary step in the metabolism of tricaine in both warm and cold-blooded animals is hydrolysis of the ester linkage, but the ultimate metabolic fate of the drug appears to be species specific.

The Darwinian concept of evolution demands that each phenotypic characteristic of an organism is present as the result of a continuing selection process that ensures survival of each species. Mammals suckle their young and a major source of energy in mammalian milk is esterified fatty acids (Dittmer 1961, Macy et al. 1953). Therefore, the presence of large amounts of nonspecific esterases in mammalian tissues may be required to utilize the fatty-acid energy source. In contrast, poikilotherms are egg layers and do not suckle their young. Therefore, cold-blooded species may not have experienced the same evolutionary pressures to develop a major metabolic pathway for utilization of esterified energy sources as have mammals.

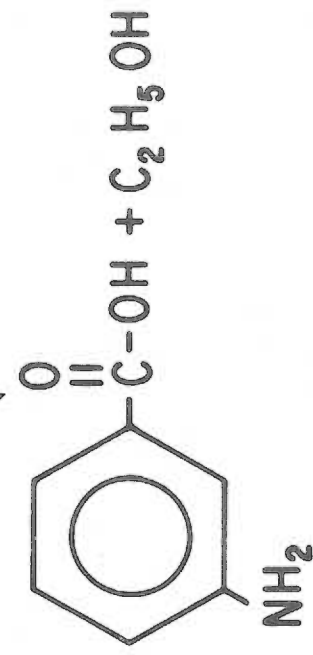
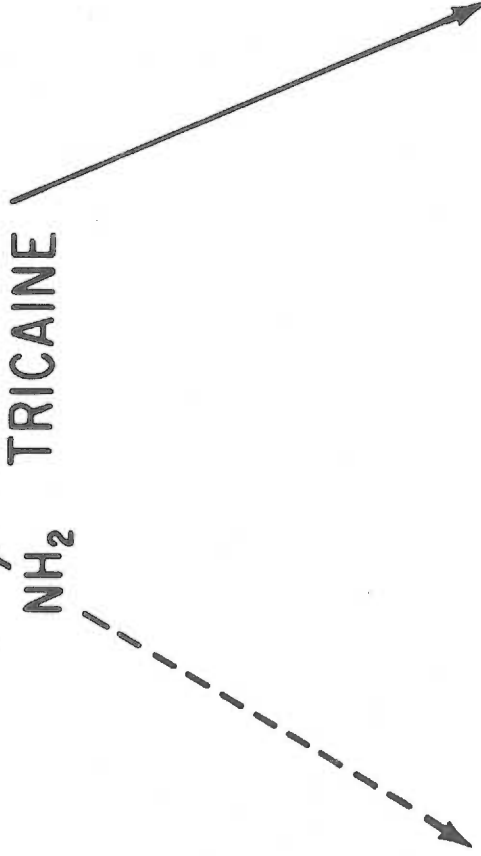
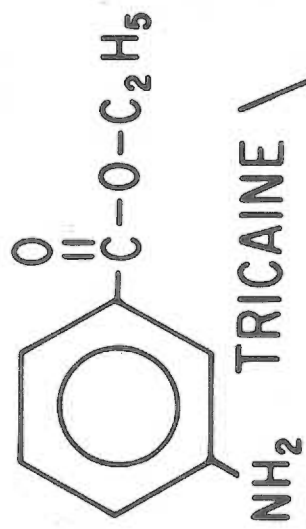
Target-organ sensitivity appears to be of minor importance in the explanation of tricaine's selective effect on poikilotherms and, at least in terms of cardiotoxicity, mammals are more sensitive to the

effects of tricaine than are poikilotherms. In both warm and cold-blooded species, the dose which produces respiratory arrest is lower than that which produces cardiac arrest. Respiratory arrest is a universally fatal event in mammals but in small poikilotherms respiratory exchange across the skin circumvents the lethality of respiratory arrest. Therefore, small poikilotherms are actually protected, to a certain extent, from the lethality of tricaine.

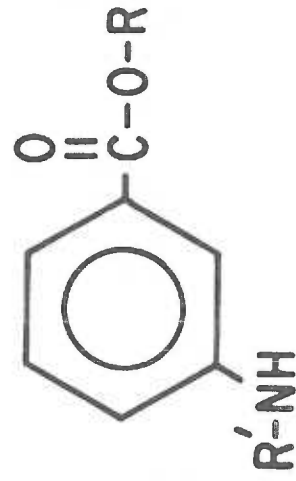
B. The Metabolic Fate of Tricaine

1. Mammals

The main route of biotransformation of tricaine in the mouse appears to be the hydrolysis to MABA and ethanol (Figure 55). This is an effective detoxification mechanism since, at the concentrations produced in vivo (less than 10^{-3} M), both products are virtually inert in terms of pharmacological activity. The evidence for such a biotransformation is as follows: (1) MABA is the primary metabolite (along with ethanol) 5 min after i.p. injection of the drug in a mouse; (2) 70 min after drug injection, MABA has completely disappeared, having been metabolized to more polar water-soluble metabolites; (3) in the mouse, BMRM quantitatively account for all of the tricaine 5 min after drug administration; and (4) the Bratton-Marshall assay is a diazotization reaction which depends upon the presence of the primary aromatic amine group of the tricaine molecule. Thus, BMRM include MABA, carboxyl conjugates of MABA (Figure 55) and possibly hydroxylated or decarboxylated metabolites. Since the reactions of formation of these compounds, excluding hydroxylation, involve the de-esterified carboxyl group of



M-AMINO BENZOIC ACID
(MABA)



CONJUGATES

Figure 52: Proposed Metabolic Pathways of Tricaine

The hydrolysis of tricaine to MABA and ethanol has been confirmed in all species tested. This is based on the appearance of Bratton-Marshall reacting metabolites of tricaine in each of the species. The conjugation at the carbonyl group with glycine, as well as acetylation at the amine group, occurs in the mouse. No evidence has been observed which would indicate that any species conjugates tricaine before or without the hydrolysis of the ester linkage of the compound.

the tricaine molecule, BMRM are all essentially derivatives of MABA. Therefore, the study of the metabolic fate of tricaine, at least in the mouse, is the study of the metabolic fate of MABA and ethanol. The metabolism of ethanol has been extensively studied and very adequately reviewed by Jacobsen (1952) and von Wartburg (1971). Ethanol is oxidized to acetic acid via acetaldehyde; acetate then (1) enters fatty acid and steroid synthesis, (2) is condensed to ketone bodies, or (3) is oxidized to CO₂ and water in the citric acid cycle.

Anthranilic Acid. The main pathways in the metabolism of aminobenzoic acids have been worked out by Bray et al. (1948) in rabbits. These acids can be acetylated at their amine group [not seen in mammals with the ortho-isomer, anthranilic acid (Williams 1959)] and conjugated at their carboxyl group with glucuronic acid or glycine in several species of mammals. There are suggestions that they may also be hydroxylated and decarboxylated (Williams 1959). The main metabolite of large doses of anthranilic acid (o-aminobenzoic acid) in rabbits, dogs, and rats is the glucuronide (Bray et al. 1948, Mitsuba and Ichihara 1927, Quick 1932, Charconnet-Harding et al. 1953). Rats and rabbits also excrete anthranilic acid, o-aminohippuric acid (Bray et al. 1948), 5-hydroxy-anthranilic acid (Kotake and Shirai 1953) and anthraniloylanthranilic acid (Hagihara and Okazaki 1956). These latter 2 metabolites, 5-hydroxy-anthranilic acid and anthraniloylanthranilic acid, are very minor in terms of the total amount of drug injected. There does not appear to be any acetylation of anthranilic acid or any of its metabolites.

PABA. The acetylation of PABA (p-aminobenzoic acid) occurs in man, rabbits, rats, chickens and pigeons but not in dogs (Harrow et al.

increases in the percentage of the original tricaine dose being present in the slower moving peaks (RF = 0 and 10 cm) of the descending chromatographs (Figures 31 and 34). The second peak (RF = 10 cm) does contain m-aminohippuric acid (glycine conjugate) but, due to the broad shape of the peak, the presence of other compounds in this peak can not be excluded. The peak which remains at the origin (RF = 0 cm for both the 5 and 70 min mice) most certainly contains the glucuronyl conjugate, known to exist from the preliminary work done on the urinary metabolites of mice. The third peak (RF = 30 cm) appears, in the chromatographs of extracts of mice sacrificed 70 min after drug injection, to contain acetylated MABA, due to its similarity of movement in the system with that of MABA and the confirmed presence of this compound in the urine of mice treated with tricaine. Again, the lack of quantitative extraction of the metabolites during the derivatization procedures, due to quaternization, may diminish the metabolite concentrations to levels below the sensitivity of the detection system. Therefore, the only metabolites which have been identified, thus far, from the mouse homogenates are MABA and m-aminohippuric acid. The detection of the acetyl and glucuronyl conjugates in the mouse urine, after tricaine treatment, suggests their presence in the mouse homogenates.

2. Poikilotherms

Little literature is available which deals with the biotransformation of drugs in poikilothermic species compared to the voluminous amount of work describing mammalian drug metabolism. To a great extent, the differences in the detoxification mechanisms (e.g., oxidation, reduction, hydrolysis and possibly acetylation) of the various

classes of animals are quantitative in nature. The similarity in drug detoxification mechanisms appears to be the result of the detoxifying reactions being carried out by the normal intermediary metabolic systems in many species (Smith 1964). Synthetic reactions, e.g., conjugations, have a less obvious connection with normal metabolism, and yet, conjugation reactions involving sugars, amino acids and sulfate have been identified in all vertebrate classes, although a given species within a class may lack one or more of these reactions (Failey et al. 1943, Litchfield 1939, Storey and Dutton 1955, Maikel et al. 1958, Brodie and Maikel 1962, Huang and Collins 1962, Smith 1964, O'Brien 1965, Buhler and Rasmusson 1967a, 1967b, Williams 1967, Adamason 1967). The metabolism of the aminobenzoic acids has been studied in several species of reptiles, amphibia and fish (Marshall 1941, Failey et al. 1943, Huang and Collins 1962). Failey and coworkers (1943) found conjugated metabolites of PABA in the blood and urine of 2 species of toad (S. holbrooki and B. valliceps), the turtle (P. elegans) and the tree frog (H. cinera cinera). They were not able to find any evidence of conjugation of PABA in either the leopard frog (R. pipiens) or the bullfrog (R. catesbiana). Huang and Collins (1962) reported that the renal excretory products of PABA and MABA in the flounder (P. americanus), goosefish (L. americanus) and dogfish (S. acanthias) included the free acids and the acetyl, glycine and glucuronyl conjugates for all 3 species of fish. Only the goosefish excreted an acetylated metabolite of anthranilic acid (ortho-isomer). Huang and Collins (1962) also found that acetylation and glycine conjugation took place in the kidney of the fish. Maren et al. (1968) studied the metabolism of tricaine in the dogfish (S. acanthias),

after i.v. injection, and reported that MABA and acetylated MABA were the only urinary metabolites of tricaine while the parent drug and acetylated tricaine were excreted via the gills. Our study of tricaine metabolism by frogs (R. pipiens) has only dealt with the relative abundance of BMRM vs non-BMRM. We find that approximately 50% of the metabolized tricaine, 70 min after i.p. injection, can not be recovered as BMRM. The basis of this low recovery of metabolized tricaine as BMRM in frogs has not been determined, but it remains of interest in light of the findings of Marshall (1941) and Failey et al. (1943) that there was no conjugation of PABA by either frogs (R. pipiens) or bullfrogs (R. catesbiana) and that the total dose of PABA was excreted in the urine as the unchanged free acid by both of these species.

3. Summary

Biotransformation is the major route of elimination of tricaine in both mammals and poikilotherms. The initial step in the metabolism of the drug is hydrolysis of the ester linkage of the tricaine molecule with production of tricaine's primary metabolites, MABA and ethanol. The subsequent metabolism of MABA appears to primarily involve conjugation of the carboxyl group of the molecule with either a glycine or glucuronide moiety. Acetylation of the amine group or deamination can also occur. The relative importance of each of the conjugation reactions is species specific and does not conform to any obvious phylogenetic generalizations. In the mouse, all of the MABA produced in the hydrolysis of tricaine is further metabolized to more polar water-soluble conjugate derivatives and no free MABA is excreted in the urine. The carboxyl glycine conjugate is the major metabolite of MABA in the mouse.

The metabolism of ethanol and that of methanesulfonic acid were not studied.

C. The Effects of Tricaine Methanesulfonate on Various Physiological Parameters

Local anesthetics exert a generalized depressant action on excitable membranes which antagonizes the membrane's ability to propagate action potentials. The inhibition of electrical conduction along excitable membranes by local anesthetics results from interruption of Na^+ and K^+ currents across the cell membrane (Ritchie 1971, Covino 1972). The cardiovascular and nervous systems are particularly susceptible to the depressant action of local anesthetic agents. Tricaine depresses the function of both of these systems.

1. Cardiovascular Parameters

In Vitro. Tricaine methanesulfonate has both a negative inotropic and a negative chronotropic effect on in vitro cardiac tissues. The effects of the drug do not depend on the presence of the methanesulfonate moiety, since tricaine hydrochloride has the same effects as the methanesulfonate salt. The similarity in the effects of tricaine, benzocaine (the para-isomer of tricaine) and procaine on the cardiac tissue of the bullfrog suggests that, in this preparation, the active portion of the local anesthetic agents is the aromatic ring with its ester linkage and primary amine group. This agrees with the contention made by Ritchie and Ritchie (1968) who, after comparing the effects of benzocaine and procaine on the suppression of nerve conduction, postulated that the aromatic lipophilic portion of the local anesthetics was

responsible for the anesthetic (conduction blockade) properties of the agents. The disparity between the effects of tricaine and benzocaine and the effects of procaine on isolated guinea-pig cardiac tissue indicates that the diethyl amine group of the procaine molecule does affect the potency of procaine's depressant action on this preparation. Ritchie and Ritchie (1968) suggested that the charged cationic form of the procaine molecule intensifies the depressant activity. In our experiments with the guinea-pig atrial preparation, both tricaine and benzocaine were significantly more potent than was procaine, except for a negative chronotropic effect of low concentrations of procaine that was not observed with the other two drugs.

Ionization. Any comparison between the effects of tricaine, benzocaine and procaine raises the question of the role of ionization in the relative activities of local anesthetics. Ritchie et al. (1965) suggested that both the uncharged base form and the charged cationic form of local anesthetics were involved in the process of conduction blockade in nerves. The uncharged base form is believed important for the penetration of the local anesthetic through the cell membrane, but, once having re-established the ionization equilibrium intracellularly, the charged cationic form is believed to be ultimately responsible for binding the drug to the receptor and, thereby, suppressing the action potentials being conducted.

All 3 local anesthetic agents, tricaine, benzocaine and procaine, have an aromatic amine group which, although ionizable at low pH's, is completely non-ionized (greater than 99%) at physiological pH's. Of the 3 compounds, only procaine has a second ionizable group, the tertiary

diethyl amine attached to the ethyl ester group. The tertiary amine group of the procaine molecule has a pKa of approximately 8.9 at 30° C (Skou 1954, Ritchie 1971). Therefore, at the physiological pH of frogs, 7.8, approximately 92% of the procaine molecules are in the ionized form. The extent of procaine's ionization indicates that the ionized form of procaine and the non-ionized forms of tricaine and benzocaine are equipotent in their effects on the isolated cardiac tissue of the bullfrog.

Ionization also appears to play a minor role in any explanation of the difference in effects of tricaine, benzocaine and procaine on the isolated guinea-pig atria. Since 97% of the procaine molecules are ionized at the physiological pH of mammals (7.4), there is a 33 fold difference in the molar concentrations of non-ionized procaine and non-ionized tricaine or benzocaine at any given total drug concentration, and an almost infinite difference in the concentrations of the charged cationic forms of these drugs. In contrast, the greatest difference between the effects of procaine and the effects of tricaine or benzocaine on the isolated guinea-pig atria is only about 3 fold. Moreover, as mentioned above, the charged cationic form of local anesthetics (represented by procaine) exhibits less depressant activity than the uncharged base form (represented by tricaine and benzocaine), rather than more intense activity as suggested by Ritchie and Ritchie (1968).

Antagonism of Certain Ca^{++} -Dependent Cellular Processes. The sensitivity of the contractile force of the guinea-pig atrial preparation to the depressant effects of tricaine and benzocaine as compared to that of the contractile rate, may be explained from work done on caffeine contractures. Caffeine contractures develop without significant membrane

depolarization and can be induced: (1) in the absence of extracellular Na^+ , (2) in the presence of marked deficiencies of extracellular Ca^{++} , and (3) in fully depolarized muscles (Schuller 1925, Axelsson and Thesleff 1958, Feinstein 1963, 1966). Caffeine stimulates contraction of in vitro muscle preparations by releasing intracellular stores of Ca^{++} (Bianchi 1961, Feinstein 1963). Local anesthetics prevent the caffeine-induced release of intracellular Ca^{++} and, thereby, abolish caffeine's ability to activate the contractile mechanism (Feinstein 1963, Hertz and Weber 1965, Gruener 1967, Luttgau and Oetliker 1968). A seemingly contradictory type of action of tertiary amine local anesthetic agents is their ability to potentiate caffeine contractures and actually stimulate contractures themselves (Bianchi and Bolton 1967, Feinstein and Paimre 1969). The second action (potentiation) has been attributed to the uncharged base form of the drugs and is associated with the presence of the basic tertiary amine group. The charged cationic form of the tertiary amine compounds is believed responsible for the antagonism of caffeine and the inhibition of Ca^{++} release.

Tricaine and benzocaine, which lack the tertiary amine, do not potentiate or induce contractures, but both drugs block caffeine-induced contractures in a manner similar to that attributed to the charged form of tertiary amine local anesthetics (Bianchi 1968, Friedman et al. 1974). The inhibition of intracellular Ca^{++} release by local anesthetics uncouples the contractile mechanism from the membrane depolarization mechanism (Luttgau and Oetliker 1968, Feinstein and Paimre 1969) and demonstrates a fundamental action of local anesthetics against certain Ca^{++} -dependent cellular processes. The uncoupling of the contractile and membrane

depolarization mechanisms may explain the insignificant effect of tricaine on the contractile rate (a membrane depolarization phenomenon) of the in vitro guinea-pig atrial preparations at concentrations of tricaine which significantly depress the contractile force (a contraction phenomenon). This action, dissociation of contractile force from contractile rate, is not seen in the isolated bullfrog atria. The lack of dissociation of force and rate may indicate a basic physiological difference in the coupling of the contractile and membrane depolarization mechanisms in the bullfrog. Procaine does not exhibit a significantly greater depression of force than of rate in either isolated preparation. Procaine's inability to dissociate the two processes may be based on the following: (1) the ability of a particular local anesthetic to uncouple the 2 processes, contraction and membrane depolarization, varies with the tissue (Feinstein and Paimre 1969, Bianchi 1967); or (2) the conflicting actions of the charged and uncharged forms of tertiary amine local anesthetic agents may mask the ability of an agent to uncouple the 2 processes in a particular tissue (Bianchi and Bolton 1967).

In Vivo. The in vivo cardiovascular responses of the anesthetized cat to tricaine and procaine parallel the effects observed in the in vitro guinea-pig cardiac tissue. Tricaine methanesulfonate depresses the systolic blood pressure, diastolic blood pressure and heart rate of cats anesthetized with chloralose-urethane. The blood pressure parameters in the intact animal mimic the contractile force parameter of the isolated guinea-pig atria; the blood pressure is more sensitive to the depressant action of tricaine than is the contractile rate. Since the effect of tricaine on the blood pressure in an intact animal

is the accumulated response of the cardiac, vascular and nervous systems, the assumption of a direct correlation between blood pressure and cardiac contractile force is not valid. Yet, the disparity between the sensitivities of the blood pressure and heart rate parameters to tricaine in the intact animal is strikingly similar to the dissociation of inotropic and chronotropic responses to tricaine observed in the isolated atria of guinea pigs.

Stewart et al. (1963) attempted to correlate the inotropic response of the heart and the blood pressure response to i.v. injection of a series of local anesthetics in dogs. Using doses of the drugs which would produce a decrease in contractile force of approximately 50%, they observed that blood pressure and contractile force drop simultaneously and that there is a very close correlation between the relative intensity of response of these 2 parameters for all local anesthetics tested, except cocaine. Procaine.HCl (65 mg/kg) produces a 40% decrease in contractile force and a 42% decrease in systemic blood pressure in the intact dog; the values for lidocaine.HCl (17 mg/kg) are 50% and 40%, respectively (not significantly different).

As mentioned above, there is a lack of dissociation between the effects of procaine on the contractile force and the contractile rate in the in vitro guinea-pig atria; the concentration of 10^{-4} M procaine decreases the force of contraction 40% and the rate of contraction about 25%. Stewart et al. (1963) reported the same relative intensities for procaine's actions in the intact animal; a 65 mg/kg dose of procaine.HCl depresses the force of contraction 40% and the rate of contraction 26%. The relative potencies of the local anesthetic agents also appear to be

similar in the in vitro and the in vivo experimental preparations; tricaine is about 3 times as active (potent) as procaine in both of the preparations, the isolated guinea-pig atria and the intact cat.

The duration of action of the anesthetic agents, a factor not calculatable from the isolated tissue preparations, appears to vary greatly. The depressant effect of tricaine on blood pressure in the whole animal completely disappears within 30 sec of its onset, whereas, the depression of blood pressure by procaine lasts for more than 3 min. The discrepancy in durations of action probably results from a marked difference in the relative half-lives of effective blood levels of the drugs; the half-lives of the two drugs are largely determined by rates of serologic and hepatic metabolism.

Antiarrhythmic Effects. Tricaine has antiarrhythmic properties which are demonstrable in both the in vitro and the in vivo cardiac tissue preparations. The isolated guinea-pig atria, when exposed to aconitine (0.5 µg/ml of muscle bath solution), develop a prolonged (hrs) regular tachycardia which is similar to that described in the isolated rat atria by Hollander and Webb (1967). Tricaine antagonizes the arrhythmic effect of aconitine, but, since the effect of aconitine can not be eliminated by repeated washings once the arrhythmia is established (Tanz 1973), the antiarrhythmic action of tricaine is maintained only as long as tricaine is present in the bath. The lower concentrations of tricaine partially antagonize the tachycardia but a 10^{-3} M concentration of the drug is required to immediately (within 5 min) abolish the aconitine-induced tachycardia and return the heart rate to normal (pre-aconitine rate). Tanz (1974) reported that an approximate

concentration of 10^{-5} M lidocaine completely reverses the aconitine-induced tachycardia in the isolated cat Langendorff preparation. Tanz (1974) used a concentration of aconitine of 0.05 $\mu\text{g/ml}$ and found that a period of 150 min was needed for lidocaine to effect the complete reversal of the aconitine arrhythmic action. Therefore, comparison of the above data to determine the relative potencies of tricaine and lidocaine is not valid, but the difference between them is probably closer than the two orders of magnitude indicated above.

Another model which has been used to assay the antiarrhythmic potential of drugs is the ouabain-induced ventricular tachycardia in whole, intact animals. Morrow and Bosomworth (1967) reported that ouabain-induced ventricular tachycardias are converted to a supra-ventricular mechanism, either normal sinus rhythm or nodal rhythm, in dogs by i.v. administration of 1-2 mg/kg of lidocaine.HCl. The duration of the improved cardiac rhythms is between 30 sec and 9 min. Tricaine is also capable of antagonizing the arrhythmic effect of ouabain. A dose of about 17 mg/kg of tricaine methanesulfonate converts ouabain-induced arrhythmias, in intact anesthetized cats, to a normal sinus rhythm and the conversion lasts for about 90 sec. The work of Stewart et al. (1963) indicates that tricaine is equipotent with lidocaine in its cardiac depressant action. Determination of the relative potencies of the antiarrhythmic actions of the two drugs is hindered by a difference in the experimental protocols: Morrow and Bosomworth (1967) induced the arrhythmias in dogs by infusing 36 μg of ouabain/kg/hr, whereas, the cats, in our experiments, were infused with 300 μg of ouabain/kg/hr. Again, the difference in duration of the ouabain antagonism by tricaine

and lidocaine probably results from differences in their relative rates of elimination; the spontaneous resumption of cardiac contractile and electrical activity, after a period (135 sec) of tricaine-induced arrest, is evidence of rapid in situ hydrolysis of tricaine.

2. Axonal Parameters

Tricaine methanesulfonate can depress both the central and peripheral nervous systems in poikilotherms, although the central nervous system appears to be more sensitive. The central nervous system of the frog is depressed (surgical anesthesia) by tricaine methanesulfonate blood levels of approximately 145 $\mu\text{g/ml}$ (9×10^{-4} M), while 1-3% solutions of tricaine methanesulfonate ($4 - 12 \times 10^{-2}$ M) are required to depress conduction along the frog's sciatic nerve trunk. The tricaine methanesulfonate concentrations needed to induce axonal blockade are comparable to those of other local anesthetics (Bennet et al. 1942, Truant and Lanzoni 1952, Knox et al. 1961, de Jong 1970). Therefore, the general anesthetic effect of tricaine, which is of major importance in the anesthetization of cold-blooded animals, appears to result from its central depressant action. In mammals, the effective central nervous system depressant doses of tricaine methanesulfonate are so large that the possible contribution of the methanesulfonate moiety and of the primary metabolite, ethanol, must be considered.

3. Smooth Muscle Parameters

The direct action of local anesthetics on smooth muscle is to inhibit contraction by suppressing the transmembrane movement of calcium or its release from some intracellular storage site (Feinstein 1966, Feinstein and Paimre 1969, Antonio et al. 1970, Quastel and Hackett

1973). As in striated (cardiac) muscle, a dual effect (inhibition and facilitation of contraction) of tertiary amine local anesthetics can be observed in smooth muscle preparations, but inhibition of contraction predominates at higher drug concentrations (Feinstein and Paimre 1969, Quastel and Hackett 1973). The contracture-facilitating action is associated with the basic tertiary amine group and the contracture-inhibiting action is attributed to the protonated amine (Feinstein 1964, Feinstein and Paimre 1969, Sanders 1969, Ritchie 1971). Tricaine methanesulfonate produces a relaxant effect in all the in vitro smooth muscle preparations tested, with the threshold concentration for this effect usually approximating 10^{-5} M. The tertiary amine local anesthetics, procaine and lidocaine, have a triphasic action on the same smooth muscle preparations. Low concentrations (10^{-5} M) cause a slight relaxation which appears comparable to that caused by the lowest effective concentrations of tricaine. The relaxation effect is antagonized by the vigorous contracture-facilitating action of the 10^{-4} - 10^{-3} M concentrations of these 2 drugs. Finally, with still higher concentrations (greater than 10^{-3} M) there is a sudden and precipitous relaxation of the smooth muscle preparations.

Thus, in instances where local anesthetics produce different and often opposing actions, depending on the concentration, the effect of tricaine mimics the action ascribed to the ionized form of the tertiary amine agents and not that associated with the basic, non-ionized form. The similarity between the actions of tricaine and the ionized forms of tertiary amine local anesthetic agents suggests that the lipophilic aromatic portion of the compounds is primarily responsible for the

generalized depressant actions of the drugs on excitable membranes.

The fundamental action of local anesthetics appears to be an antagonism of the action of calcium at the membrane. Calcium and local anesthetic agents both stabilize excitable membranes (Fleckenstein and Hardt 1949; Brink 1954; Shanes 1958a, b; Shanes et al. 1959) and are believed to have the same site of action (Blaustein and Goldman 1966, Ritchie 1971). The drugs compete with calcium for that site of action, thereby altering the conductance of sodium and potassium across the membrane and blocking the conduction of action potentials along the excitable membrane.

4. Summary

Tricaine methanesulfonate has depressant effects on the function of in vitro cardiac tissue. In concentrations of the drug of 10^{-3} M or less, the methanesulfonate moiety does not contribute to the overall action of the drug. Tricaine, benzocaine and procaine are approximately equipotent in their negative inotropic and chronotropic effects on spontaneously contracting atria isolated from bullfrogs. However, in isolated guinea-pig atrial preparations, tricaine and benzocaine are more potent than procaine in depressing both contractile force and contractile rate. The force of contraction of the guinea-pig atria is significantly more sensitive to the depressant effect of tricaine than is the rate of contraction and the marked discrepancy in sensitivities to tricaine suggests that the drug uncouples the contractile mechanism and membrane depolarization.

The isolated guinea-pig atrial preparation is apparently an excellent model for studying the in vivo cardiovascular effects of local

anesthetics. The effects of tricaine and procaine on the cardiovascular parameters of intact animals closely parallel the effects of these drugs on in vitro cardiac tissue. In addition, the uncoupling or dissociation of the contractile and membrane depolarization mechanisms is an effect of tricaine and not procaine in both preparations, the isolated guinea-pig atria and the intact anesthetized cat.

The antiarrhythmic action of tricaine is demonstrable in both in vitro and in vivo preparations. The drug is as effective in converting tachy-arrhythmias to normal sinus rhythm as are procaine or lidocaine, but relative potencies of the three drugs were not determined. The duration of both the general cardiovascular and the antiarrhythmic actions of the three drugs in intact animals depends upon their respective rates of metabolism.

All smooth muscle preparations tested with tricaine methanesulfonate underwent progressive relaxation in response to increasing concentrations of the drug. Comparable studies with lidocaine and procaine demonstrated a triphasic action; low (10^{-5} M) and high (greater than 10^{-3} M) concentrations of the drugs relaxed the preparations, while the intermediate concentrations (10^{-4} - 10^{-3} M) stimulated contraction.

Tricaine methanesulfonate depresses the function of both the central and peripheral nervous systems. The central nervous system is more sensitive to the depressant effect of tricaine and the drug's central action is the basis of its general anesthetic effect in poikilotherms. Tricaine is ideally suited for the role of a local anesthetic due to (1) its high lipid solubility which facilitates penetration of the myelin sheath of peripheral nerves, (2) its depressant action on nerve

conduction, and (3) its rapid metabolism in the systemic circulation.

The liquid state of the free base form of the drug is uniquely suited for injection at either a peripheral site or intrathecally without the use of a carrier or diluent, thereby reducing the overall volume to be injected.

APPENDIX I

Commercial Sources of Animals

<u>Animal</u>	<u>Weight</u>	<u>Source</u>
Mice	20-30 gm	Simonsen Laboratories Inc., Gilroy, California
Guinea Pigs	350-400 gm	Lab. Associates Inc., Kirkland, Washington
Bullfrogs	200-500 gm	College Biological Supply Co., Bothwell, Washington
Frogs	20-30 gm	Carolina Biological Supply Co., Burlington, North Carolina and Gladstone, Oregon
Salamanders	7-13 gm	Carolina Biological Supply Co., Burlington, North Carolina and Gladstone, Oregon
Snakes	20-40 gm	Carolina Biological Supply Co., Burlington, North Carolina and Gladstone, Oregon
Turtles	525-850 gm	Carolina Biological Supply Co., Burlington, North Carolina and Gladstone, Oregon
Lizards	2-5 gm	Carolina Biological Supply Co., Burlington, North Carolina and Gladstone, Oregon
Parakeets	30-40 gm	King Neptune's Pet Store, Portland, Oregon
Cats	3.0-3.5 kg	UOHSC Animal Farm, Cornelius, Oregon
Rabbits	2.5-3.5 kg	UOHSC Animal Farm, Cornelius, Oregon

APPENDIX II

The Bratton-Marshall Reaction

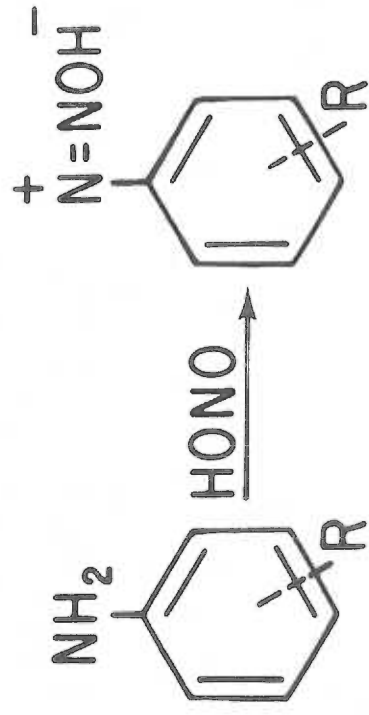
The Bratton-Marshall assay (1939) is based on the diazotization of a primary aromatic amine with nitrous acid and the coupling of the resulting diazo compound with a second substance (the coupler). The overall reaction produces an azo compound which is a dye and can be quantified by colorimetric comparison. Aliphatic amine groups do not interfere with the reaction. Despite the fact that diazotization takes place in an acid solution, the actual chemical species which is attacked is not the salt of the amine, but rather, the small amount of free amine present (March 1968). Therefore, since aliphatic amines are stronger bases than aromatic amines, there is not enough free amine present at pH's below 3 for the former (aliphatic amines) to be diazotized, while the latter (the aromatic amines) still undergo the reaction.

The function of each reagent of the Bratton-Marshall reaction is as follows: (1) 15 gm% trichloroacetic acid (TCA) was used both to establish an appropriate pH for the reaction and to precipitate protein which, if present, would interfere with the reaction. This was unnecessary with the HCl extract containing tricaine and the TCA served merely as a diluting agent. (2) 0.1 gm% sodium nitrite was used as the diazotizing agent. When dissolved in water, sodium nitrite forms nitrous acid (HONO). The actual reagent in the diazotizing step of the procedure is N_2O_3 which acts as a carrier of NO^+ and which forms as follows: $2HONO \rightarrow N_2O_3 + H_2O$. The Bratton-Marshall reaction has been primarily used to quantify compounds with para- or ortho- situated amine groups. Apparently, this reaction proceeds more slowly with a meta- isomer, e.g., tricaine, for

Walker and Schoettger (1967) found that the formation of the diazonium salt of tricaine required a higher concentration (0.2 gm%) of sodium nitrite and had a slower rate of formation than was reported for the other isomeric compounds. Standard curves of tricaine and MABA, in our experiments, exhibited identical absorption levels with both 0.1 gm% and 0.2 gm% solutions of sodium nitrite, but maximal formation of the diazo compound required 15 min. (3) 0.5 gm% ammonium sulfamate was used to neutralize any excess nitrous acid remaining after the diazotization step. (4) Methanol was included to increase the solubility of the azo dye formed with tricaine. Marshall *et al.* (1937) and Bratton and Marshall (1939) found that with certain compounds, the azo dyes formed are not acid-soluble and the addition of an alcohol to the reaction mixture with or just before the addition of the coupling component is necessary to ensure the solubility of the resulting dye. The azo dye formed with tricaine was not highly acid-soluble and in the absence of methanol precipitated at relatively low concentrations. (5) 0.1 gm% N-1-naphthylethylenediamine dihydrochloride was used as the coupling agent due to its rapid coupling, sensitivity and reproducibility. In addition, the rapidity of the coupling of this compound to the diazo component is unaffected by changes in pH from 1 to 2 and the resulting dyes are more soluble in this pH range than that from any other coupler (Bratton and Marshall 1939).

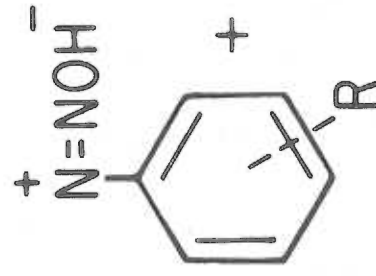
Figure 53 shows the probable flow scheme of the diazotization of tricaine and the coupling reaction with N-1-naphthylethylenediamine (Natelson 1961).

BRATTON-MARSHALL REACTION



Aryl Amine

Aryl diazonium ion



$\text{NHCH}_2\text{CH}_2\text{NH}_2$

Colored Azo Dye

pH 1 to 2

N-1-naphthylethylenediamine

Figure 53: The Bratton-Marshall Reaction (Natelson 1961)

This flow scheme shows the probable mechanism for the diazotization of tricine or its metabolites and the mechanism for the coupling reaction between the diazo compounds and N-1-naphthylethylenediamine. Except for the prolonged formation time of diazo compounds with meta- isomers, this scheme is applicable for all the isomeric types.

APPENDIX III

Countercurrent Distribution (CCD)

CCD or discontinuous multiple fractional extractions (Craig 1943, 1945; Bush and Densen 1948; Bush and Post 1969) is a method developed for separating two or more compounds of a mixture. It involves repeated equilibrations of the mixture between multiple aliquots (fractions) of two immiscible liquid phases. The method is based on the concept that each chemical compound has a partition coefficient (C_o) that defines how the compound (A) will distribute between two immiscible liquid phases, X and Y. At equilibrium,

$$C_o = \frac{\text{amount of A/ cc of X}}{\text{amount of A/ cc of Y}} \quad (1)$$

If the volumes of the two solvents are designated as V_x and V_y , respectively, and p represents the percentage of compound A that is in solvent X at equilibrium ($1 - p$, the remainder of A, is in solvent Y) then,

$$p = \frac{(\text{amount of A/ cc of X})V_x}{(\text{amount of A/ cc of X})V_x + (\text{amount of A/ cc of Y})V_y} \quad (2)$$

and

$$1 - p = \frac{(\text{amount of A/ cc of Y})V_y}{(\text{amount of A/ cc of X})V_x + (\text{amount of A/ cc of Y})V_y} \quad (3)$$

Therefore,

$$\frac{p}{1 - p} = \frac{(\text{amount of A/ cc of X})V_x}{(\text{amount of A/ cc of Y})V_y} = \frac{C_o V_x}{V_y} \quad (4)$$

and

$$C_o = \frac{p}{1 - p} \cdot \frac{V_y}{V_x} \quad (5)$$

Since

$$\frac{p}{1-p} = \frac{C_o V_x}{V_y},$$

then

$$p = \frac{C_o V_x (1-p)}{V_y},$$

and

$$pV_y = C_o V_x - C_o V_x p,$$

and

$$C_o V_x p + pV_y = C_o V_x,$$

and

$$p(C_o V_x + V_y) = C_o V_x.$$

Therefore,

$$p = \frac{C_o V_x}{C_o V_x + V_y}. \quad (6)$$

The calculation of the distribution of compound A is made algebraically with the use of Equation 6. If N is the number of equal portions of each solvent used in a given CCD, then the actual number of extractions performed is $\frac{N^2 + N}{2}$, and the total number of solvent fractions, after the completion of the extractions, is $2N$ (N fractions of solvent X and N fractions of solvent Y). Figure 54 illustrates the CCD process carried through the stage, $N = 4$. The concentration of compound A in each phase (amount of A/solvent fraction) is defined in terms of p and q (where $q = 1 - p$ and $p + q = 1$) and p and q are, in turn, defined for

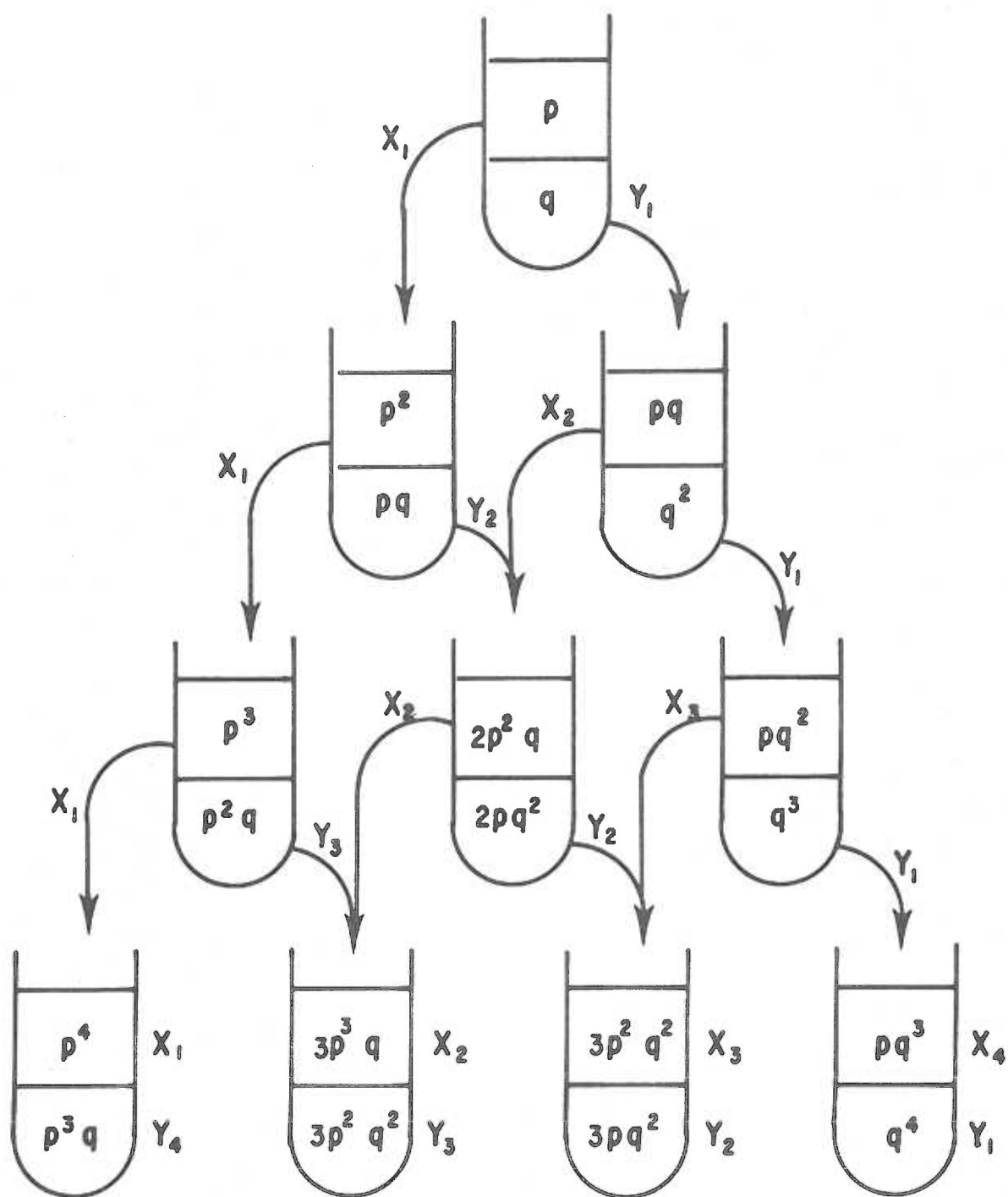


Figure 54: The Distribution of a Solute Between Two Immiscible Phases

A systematic multiple extraction develops from the repeated equilibration of a solute with successive "fractions" of two immiscible liquid phases. Upon the initial separation, the two equilibrated solutions are designated X_1 and Y_1 , where the subscript denotes the different fractions of each liquid phase. Fresh portions of both phases are introduced at each level (X_2 and Y_2 on the second level, X_3 and Y_3 on the third level, etc.), and equilibrated with the initial portion of the opposite phase. The volumes of all fresh portions of solvents X and Y are equal to that of X_1 and Y_1 , respectively. Previously introduced portions of one solvent are systematically equilibrated with those of the other solvent according to the scheme pictured in this figure.

each compound by its C_0 and the solvent ratio (volume of X:volume of Y) of the two phase CCD solvent system. The percentage of the total amount of a single compound in any fraction of either solvent at equilibrium can be determined from the appropriate combination of terms of the binomial expansion of $(p + q)^N$.

In the diamond-shaped arrangement shown in Figure 55, the process has been carried through the "diamond stage"; the total number of solvent fractions remains $2N$ after the completion of all the extractions, whereas, the actual number of extracts increases to N^2 . This "diagonal stage" represents the maximum separation of the compounds of a mixture that can conveniently be performed with N tubes and N fractions of each solvent.

The percentage of the total amount of a given compound A, in a specific fraction of either solvent is determined from calculation of the terms of the N^{th} diagonals (Figure 56). The N^{th} diagonals are calculated from the following equation:

$$\begin{aligned}
 1 = & p^N + \frac{N!}{(N-1)!} p^N q + \frac{(N+1)!}{2!(N-1)!} p^N q^2 + \frac{(N+2)!}{3!(N-1)!} p^N q^3 + \dots + \\
 & \frac{(2N-2)!}{(N-1)!(N-1)!} p^N q^{N-1} + \frac{(2N-2)!}{(N-1)!(N-1)!} p^{N-1} q^N + \dots + \\
 & \frac{(N+2)!}{3!(N-1)!} p^3 q^N + \frac{(N+1)!}{2!(N-1)!} p^2 q^N + \frac{N!}{(N-1)!} p q^N + q^N . \quad (7)
 \end{aligned}$$

Therefore, if the solvent fractions are considered in the following order: $X_1, X_2, X_3, X_4, \dots, X_{N-1}, X_N, Y_N, Y_{N-1}, \dots, Y_4, Y_3, Y_2, Y_1$, then, the respective terms of Equation 7 represent the percentage of the total amount of a given compound A (in the CCD) in a specific solvent fraction. p^N represents the percentage of the total amount of A that is in solvent fraction X_1 , $\frac{N!}{(N-1)!} p^N q$ represents the percentage of the

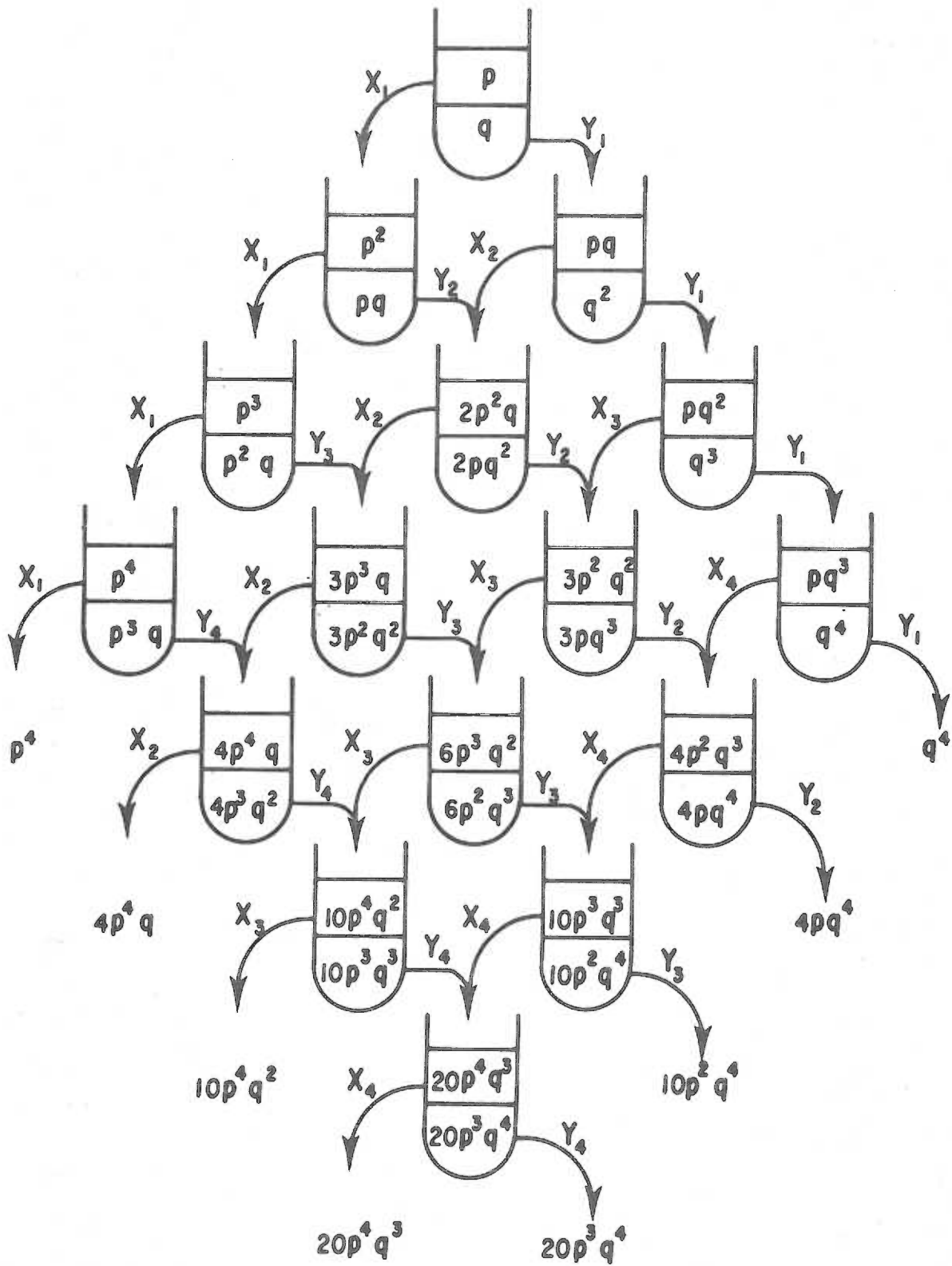


Figure 55: The Expansion of Figure 54 into the "Diagonal Stage"

This expansion allows for the greatest resolution of the distribution of a solute which can be made conveniently without the use of increased quantities of either solvent. This procedure merely represents an extension of that pictured in Figure 54 with each fraction of one solvent being equilibrated with each fraction of the other solvent. While the movement of the fractions of a given solvent is successive, in relation to other fractions of the same solvent, the movement is counter to that of the fractions of the other solvent.

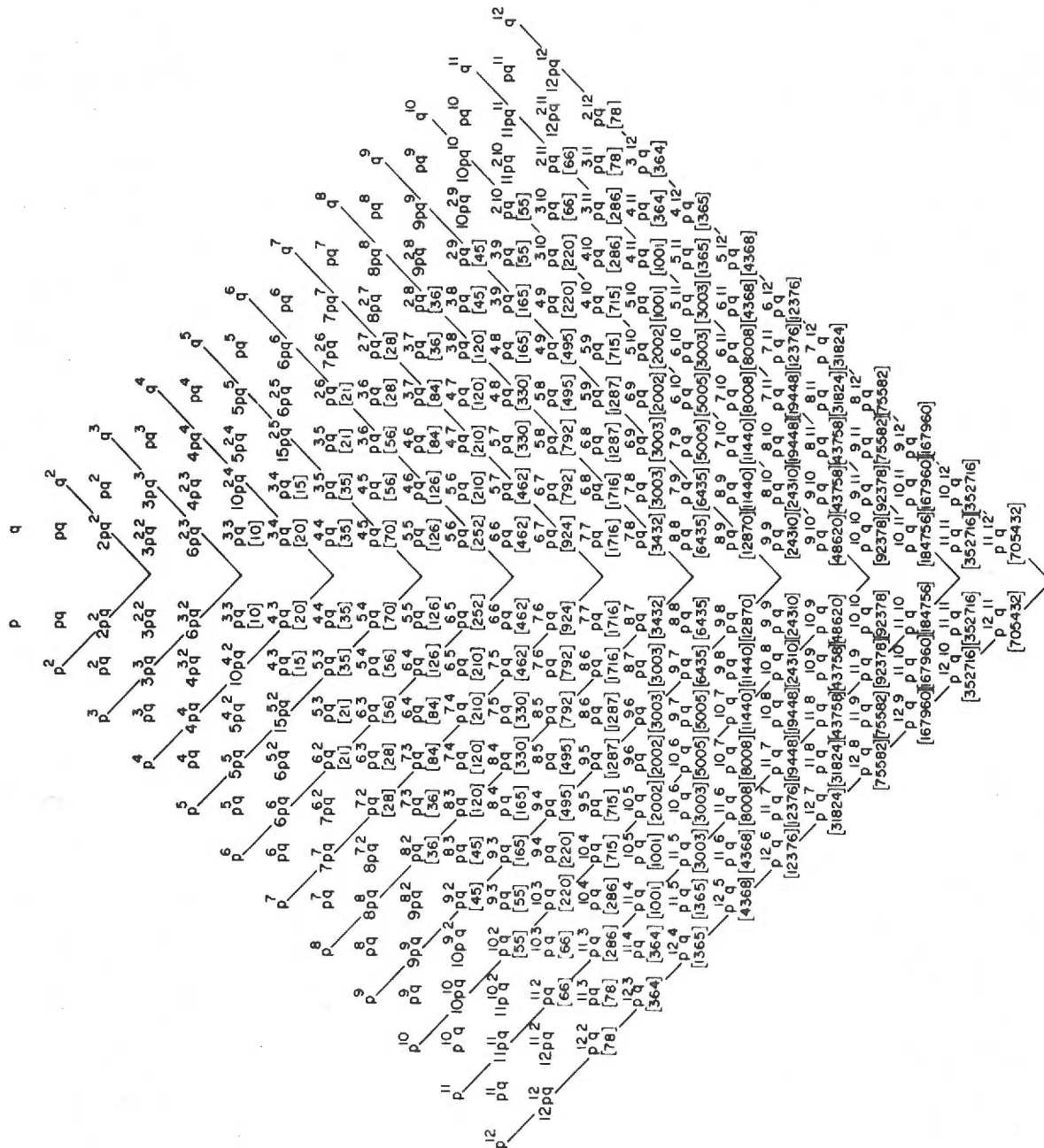


Figure 56: The Binomial Expansion of $(p + q)^N$

The terms of the Nth diagonals are determined for $N = 1$ through 12, and are represented by the connecting lines in the above figure. They can also be calculated by means of Equation 7. Equation 7, while cumbersome to deal with, is excellently suited for computer programming.

total amount A that is in fraction X_2 and so on.

The CCD or multiple fractional extraction technique (diagonal stage) has been used in our experiments to (1) test the chemical purity of a known compound, (2) search for known compounds in unknown mixtures, and (3) examine unknown mixtures.

Chemical Purity. The purity of a compound can be determined by the CCD technique. The conditions of the CCD (pH of the buffered aqueous phase, polarity of the organic solvent, volume ratio of the organic and aqueous phases, etc.) are selected to ensure an adequate distribution of the known compound, A, (p value between 0.2 and 0.8). The quantity of material (compound A and possible contaminants) that is introduced into the CCD apparatus is dependent on two factors: (1) the sensitivity of the assay used for quantification, and (2) the degree of purity to be determined. A known amount of the material is placed in the CCD apparatus and the N^2 extractions are performed, following the scheme in Figure 55. Upon completion of the extractions and quantification of the total amount of the material in each solvent fraction, the p values are calculated from Equation 7 in the following manner: (1) for the p values in solvent X,

$$\frac{\text{amount of substance in } X_2}{\text{amount of substance in } X_1} = \frac{\frac{N!}{(N-1)!} p^{Nq}}{p^N} = Nq ,$$

$$\frac{\text{amount of substance in } X_3}{\text{amount of substance in } X_2} = \frac{\frac{(N+1)!}{2!(N-1)!} p^{Nq^2}}{\frac{N!}{(N-1)!} p^{Nq}} = \frac{(N+1)}{2} q , \dots$$

$$\frac{\text{amount of substance in } X_N}{\text{amount of substance in } X_{N-1}} = \frac{\frac{(2N-2)!}{(N-1)!(N-1)!} p^{Nq^{N-1}}}{\frac{(2N-3)!}{(N-2)!(N-1)!} p^{Nq^{N-2}}} = \frac{(2N-2)}{(N-1)} q .$$

Since N is known in each particular case, q is easily calculated from the above and subtraction of the q values from 1 yields the respective p values. (2) the p values in solvent Y are calculated in a similar fashion.

The calculated p values ($2N - 2$ separate p determinations, if $N \neq 1$) are then listed in the following order of computation:

$$\frac{X_2}{X_1}, \frac{X_3}{X_2}, \frac{X_4}{X_3}, \dots, \frac{X_N}{X_{N-1}}, \frac{Y_N}{Y_{N-1}}, \dots, \frac{Y_4}{Y_3}, \frac{Y_3}{Y_2}, \frac{Y_2}{Y_1}.$$

A consecutive series of 3-4 identical p values (± 0.01) indicates that the fractions from which those particular p values were calculated contain a single substance. If all the p values are identical (± 0.01), the material is pure and is made up entirely of the known compound A . Conversely, if several successive p values deviate significantly from the others (in the same direction, greater or lesser), this indicates the presence of at least one contaminate. The degree of separation of the known compound A and the contaminants depends on the respective C_0 's of those compounds. Polar contaminants (compounds with C_0 's that are less than the C_0 of compound A) distribute into the more polar solvent to a greater extent than compound A . The reverse is true of apolar contaminants (compounds with C_0 's less than that of compound A).

If contaminants are present, the degree of purity of the material can be determined from the solvent fractions which produce p values identical to that of compound A . As stated above, the actual quantity of a compound, A , in a given solvent fraction represents a certain percentage of the total amount of A in the CCD. This percentage is dependent on the p value of compound A and N , the number of aliquots

(fraction) of a solvent used. Since the p value and N are known, the total amount of A in the CCD is calculated by dividing the quantity of A in a "pure" fraction by the respective term of Equation 7. For example, if a significant amount of A is in fractions X_2 , X_3 , X_4 , and X_5 and the p values calculated from these fractions are identical to each other and to the p value of compound A , then these are "pure" fractions and the total amount of A in the CCD is calculated as follows:

$$\text{Total amount of A in CCD} = \frac{\text{amount of A in fraction } X_3}{\frac{(N+1)!}{2!(N-1)!} p^{N_q^2}}$$

The ratio of the total amount of A to the quantity of material (compound A and contaminants) introduced into the CCD apparatus then gives the relative purity of the material.

Known Compound in an Unknown Mixture. The search for a known compound A in an unknown mixture is carried out by selecting a two phase CCD solvent system that results in a reasonable p value (0.2 to 0.8) for compound A . The CCD is performed with an arbitrary N value and, after quantification of the total amount of the mixture in each of the solvent fractions, p values are calculated as described above. A consecutive series of 3-4 identical p values (± 0.01) that correspond to that of A indicates that the respective solvent fractions contain only compound A . The quantity of A in these "pure" fractions is used to calculate the total amount of A introduced into the CCD apparatus. Since the p value of A , the total amount of A in the CCD and the N are all known, the individual CCD of compound A can then be calculated from Equation 7.

The CCD of a mixture is a composite of the distributions of individual component compounds. The distribution of compound A can be

subtracted from that of the mixture. The quantity of A in each fraction is subtracted from the total amount of mixture in the respective fractions. This manipulation results in the composite distribution of the "unknown" component compounds of the mixture. The p values of the "new" distribution are calculated as above and a consecutive series of 3-4 identical p values will identify the CCD of a second component compound. The identification of distributions of component compounds and subtraction of these CCD's from the composite distribution of the mixture is repeated with each consecutive series of identical p values. The lack of identical p values indicates the presence of at least two component compounds of the mixture which are not adequately separated by the CCD solvent system.

Examination of an Unknown Mixture. When previous knowledge of the components of a mixture is not available, selection of conditions in the CCD system is made by means of preliminary studies to give a resultant value of p (average of all components in the mixture) between 0.2 and 0.8. An expanded CCD (N relatively large) will then give a considerable amount of information about the components of the mixture. Subsequent CCD's may then be based on advantageous solvent volume ratios to give more efficient separations. Solvent volume ratios other than 1 are usually more efficient in separating multicomponent mixtures.

APPENDIX IV

The Lowry Method of Protein Determinations

The protein content of various serum samples was measured by the method of Lowry et al. (1951). The following reagents were prepared: A) 2 gm of Na_2CO_3 were dissolved in 100 ml of 0.1 N NaOH; B) 0.05 gm of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was dissolved in 10 ml of distilled water and 0.1 gm of $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ was then added to the 10 ml solution of cupric sulfate; C) 50 ml of reagent A (sodium carbonate in 0.1 N sodium hydroxide) were combined with 1 ml of reagent B (cupric sulfate and sodium and potassium tartrate in distilled water); D) 2 ml of folin phenol reagent were added to 2 ml of distilled water (therefore, a 1 N solution of folin phenol reagent).

The sera were diluted 1:499 with distilled water. One ml of reagent C (see above) was combined with 0.2 ml of each of the diluted sera and the samples were vortexed immediately. After 10 min, 0.1 ml of reagent D was added to each sample and the solutions were vortexed immediately. The samples were allowed to stand for 30 min and then the resulting color in the solutions was quantified spectrophotometrically at a wavelength of 500 nm (A_{500}) using distilled water as the reference.

Standard curves were prepared using a solution of crystallized bovine-albumin (0.05 gm/100 ml water). Standard concentrations of albumin were prepared from the stock solution and varied from 0.05 mg/ml to 0.50 mg/ml.

APPENDIX V

Mass Spectra of the Urinary Metabolites of Tricaine in the Mouse

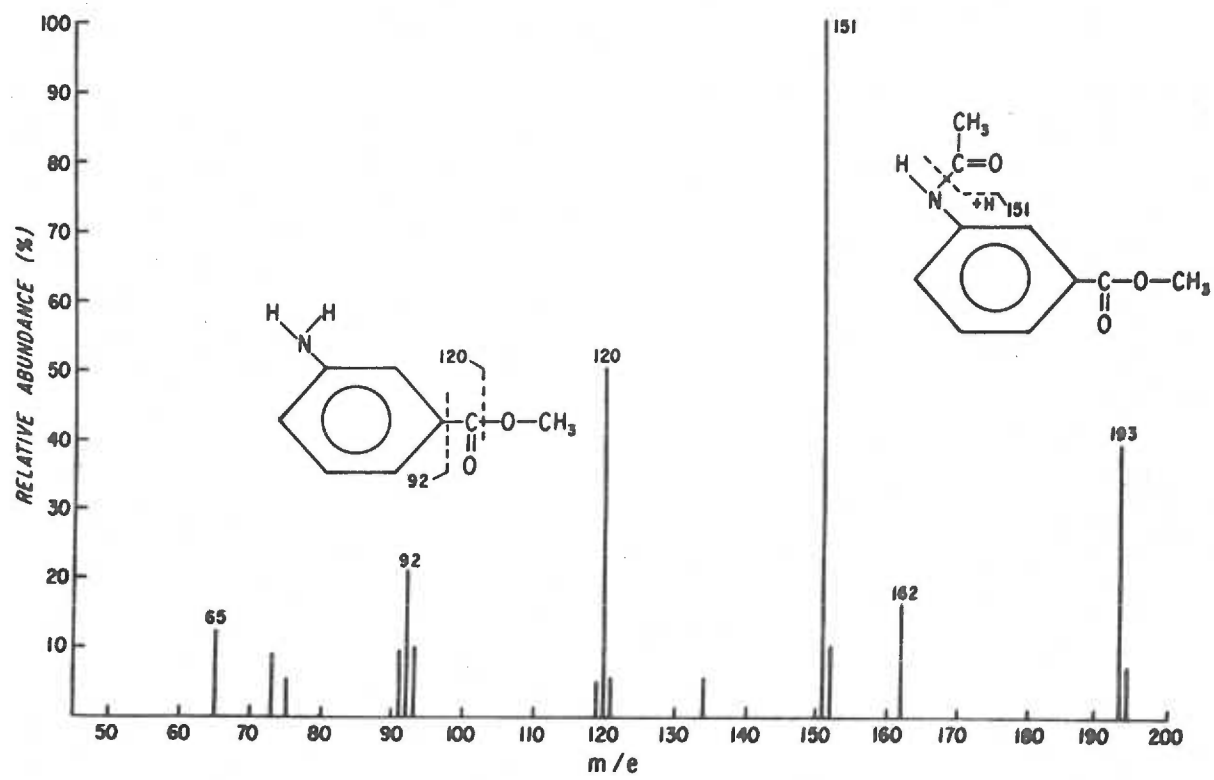
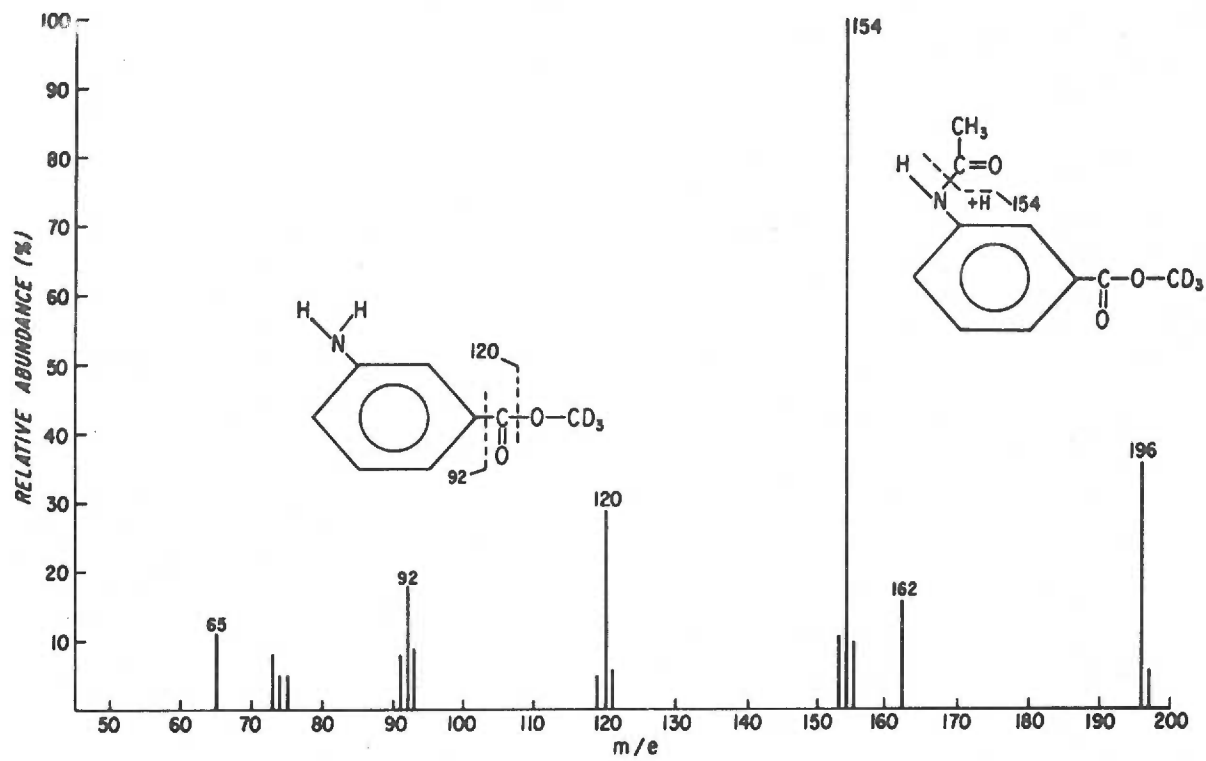


Figure 57: Mass Spectra of the Mono-Methyl and Mono-Deutero-Methyl
Derivatives of Acetylated MABA, a Urinary Metabolite of
Tricaine in the Mouse

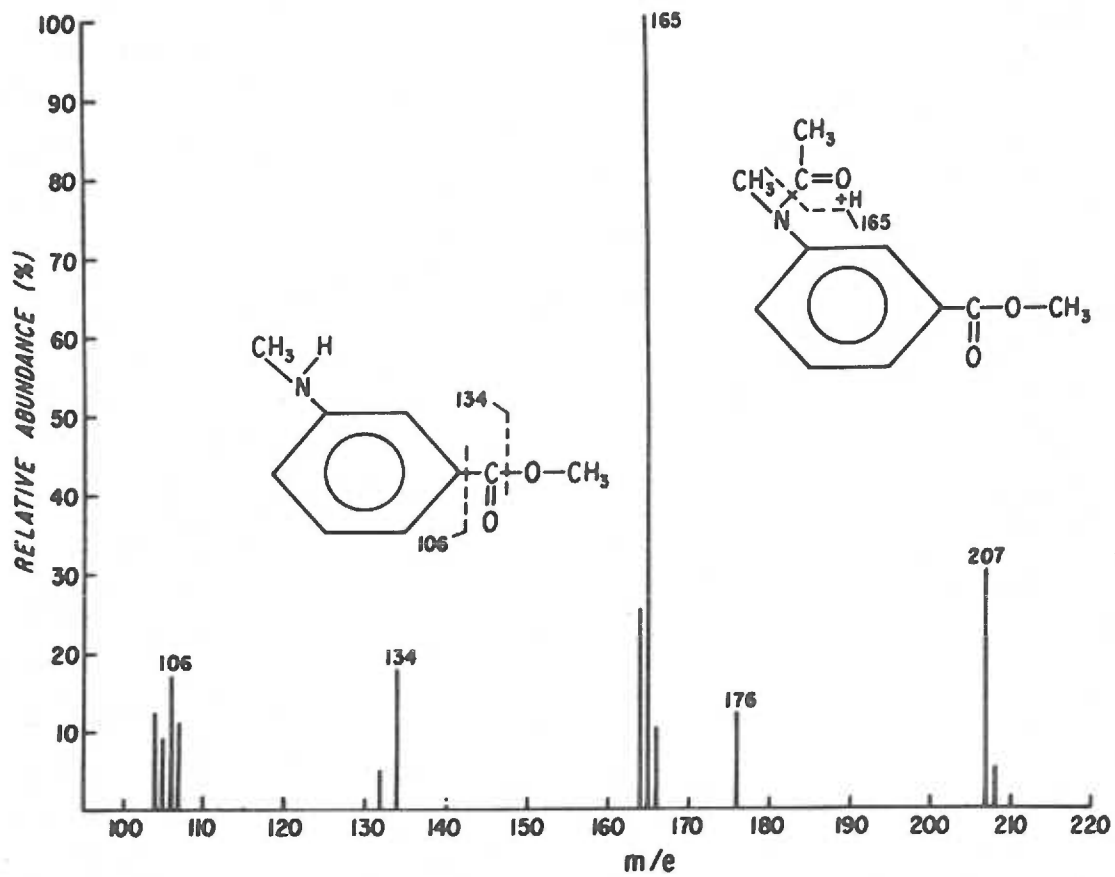
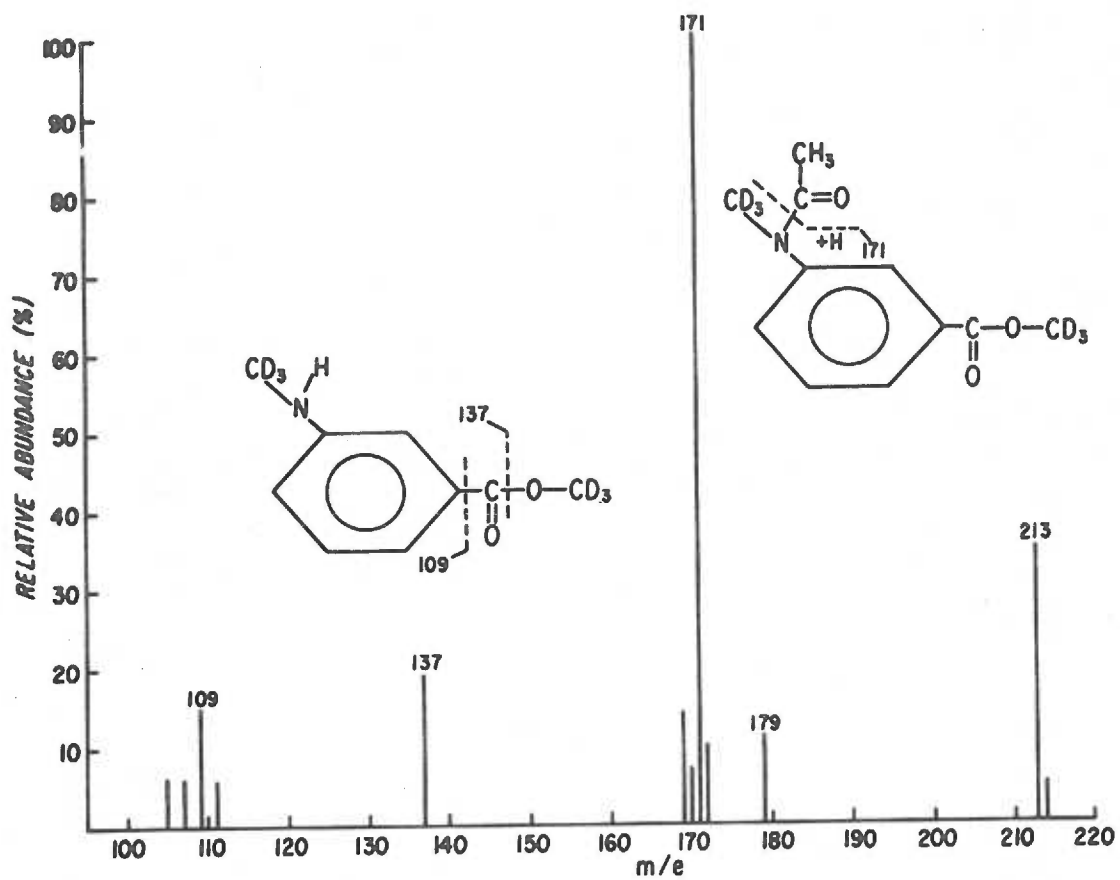


Figure 58: Mass Spectra of the Di-Methyl and Di-Deutero-Methyl
Derivatives of Acetylated MABA, a Urinary Metabolite of
Tricaine in the Mouse

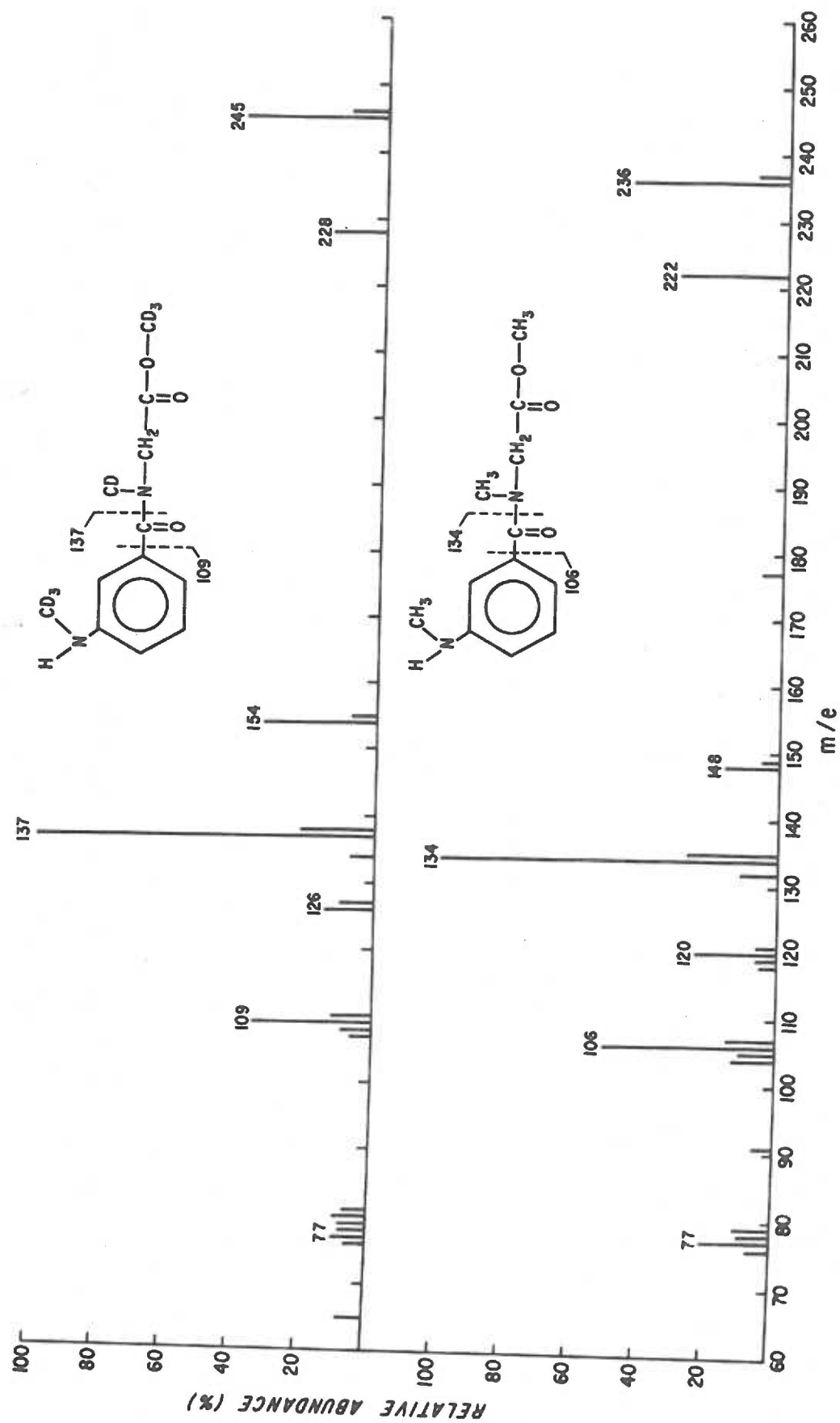


Figure 59: Mass Spectra of the Tri-Methyl and Tri-Deutero-Methyl Derivatives (Mono-Methyl
Amine Compound) of the Glycine Conjugate of MABA, a Urinary Metabolite of Tricaine
in the Mouse

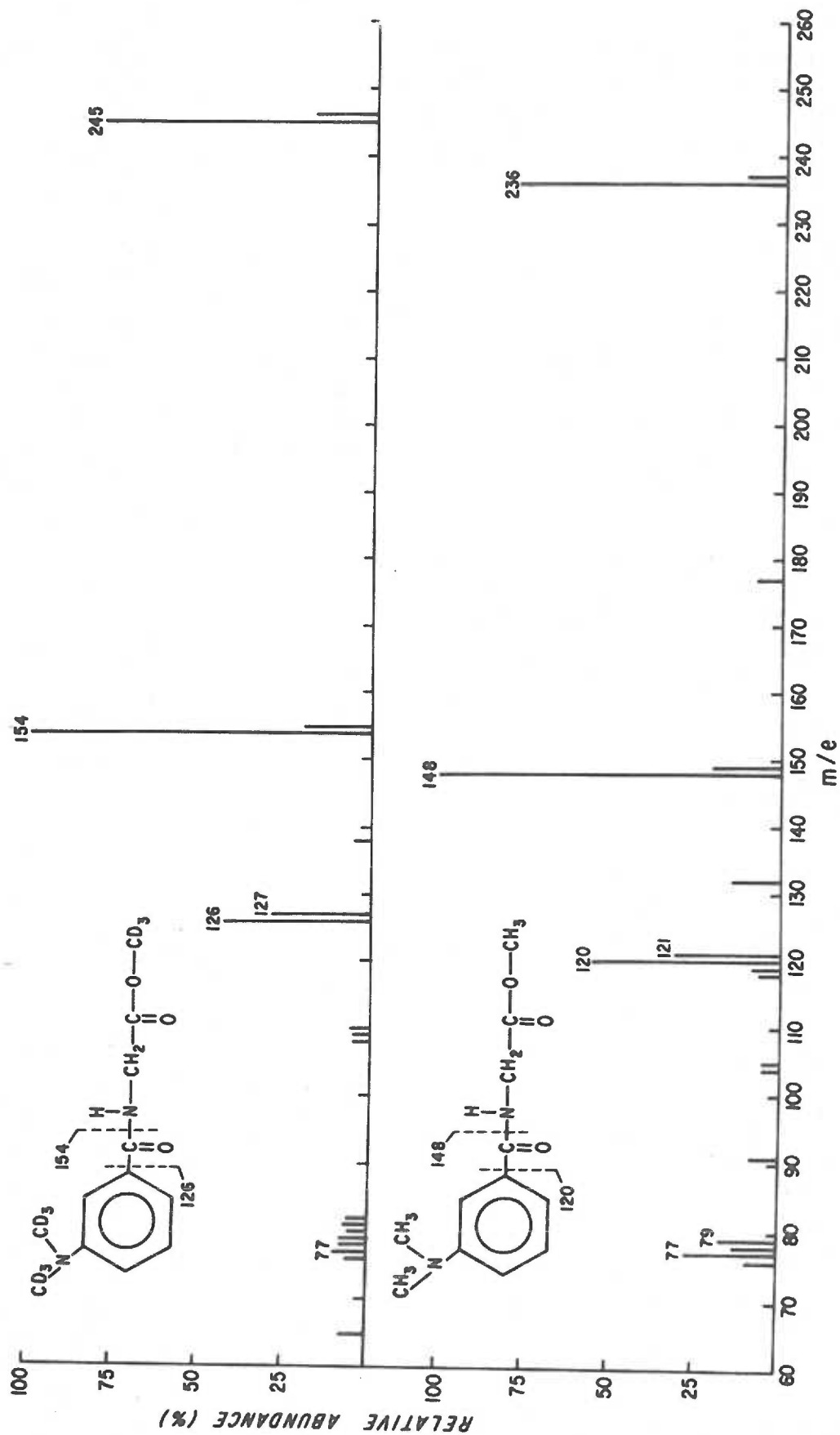


Figure 60: Mass Spectra of the Tri-Methyl and Tri-Deutero-Methyl Derivatives (Di-Methyl Amine Compound) of the Glycine Conjugate of MABA, a Urinary Metabolite of Tricaine in the Mouse.

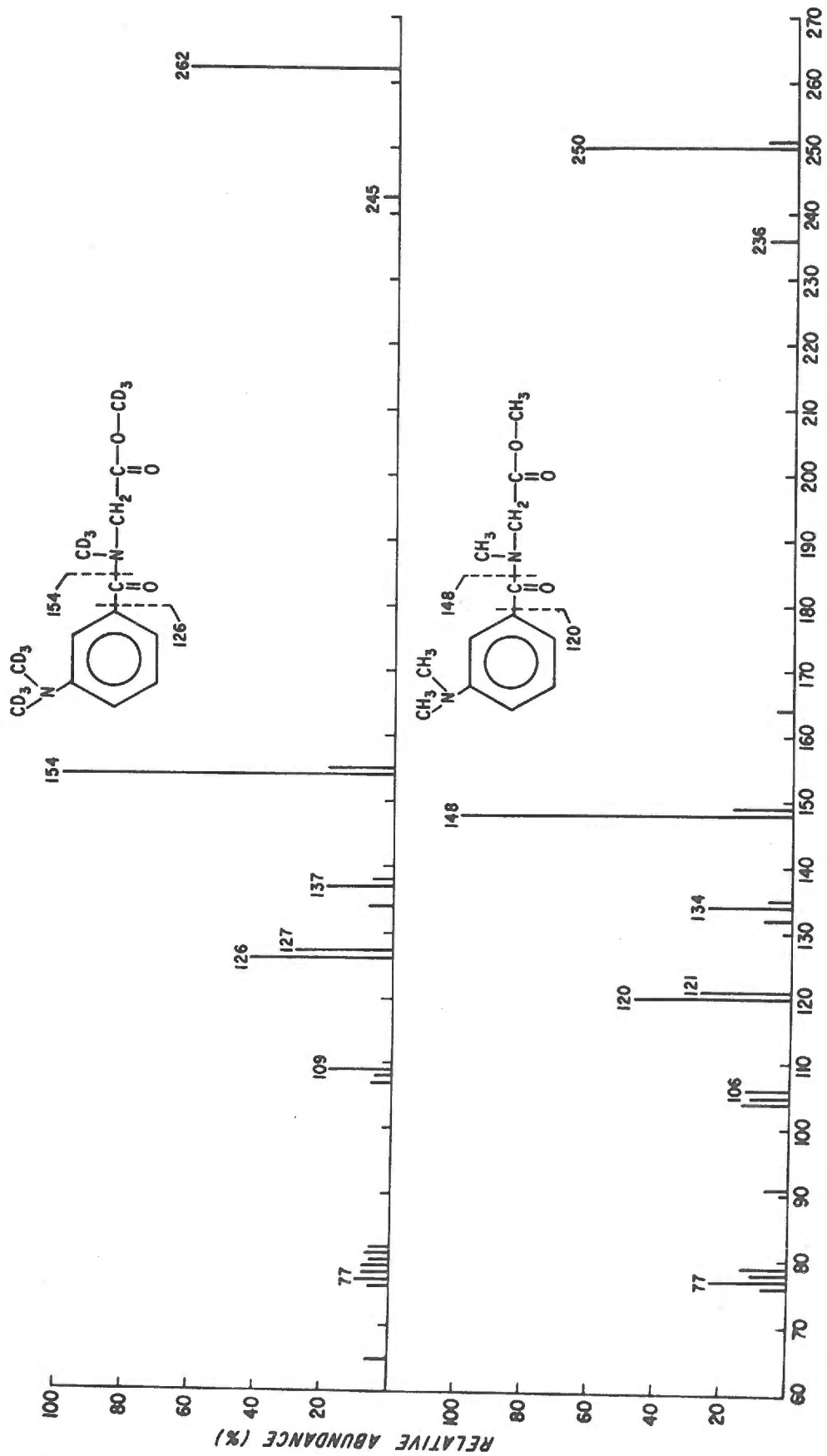


Figure 61: Mass Spectra of the Tetra-Methyl and Tetra-Deutero-Methyl Derivatives of the
Glycine Conjugate of MABA, a Urinary Metabolite of Tricaine in the Mouse

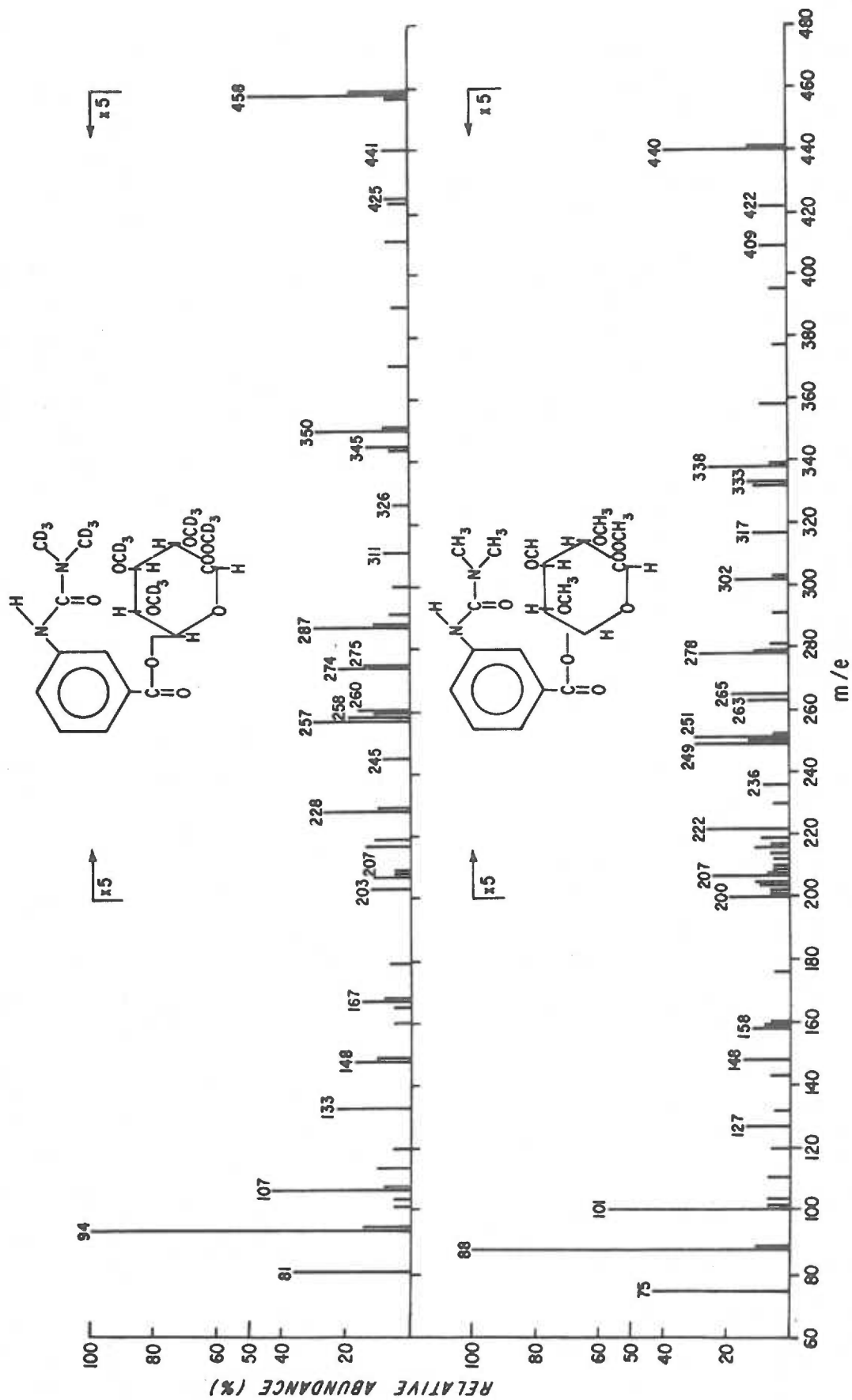


Figure 62: Mass Spectra of the Hexa-Methyl and Hexa-Deutero-Methyl Derivatives of the Ureido-Glucuronide of MABA, a Urinary Metabolite of Tricaine in the Mouse

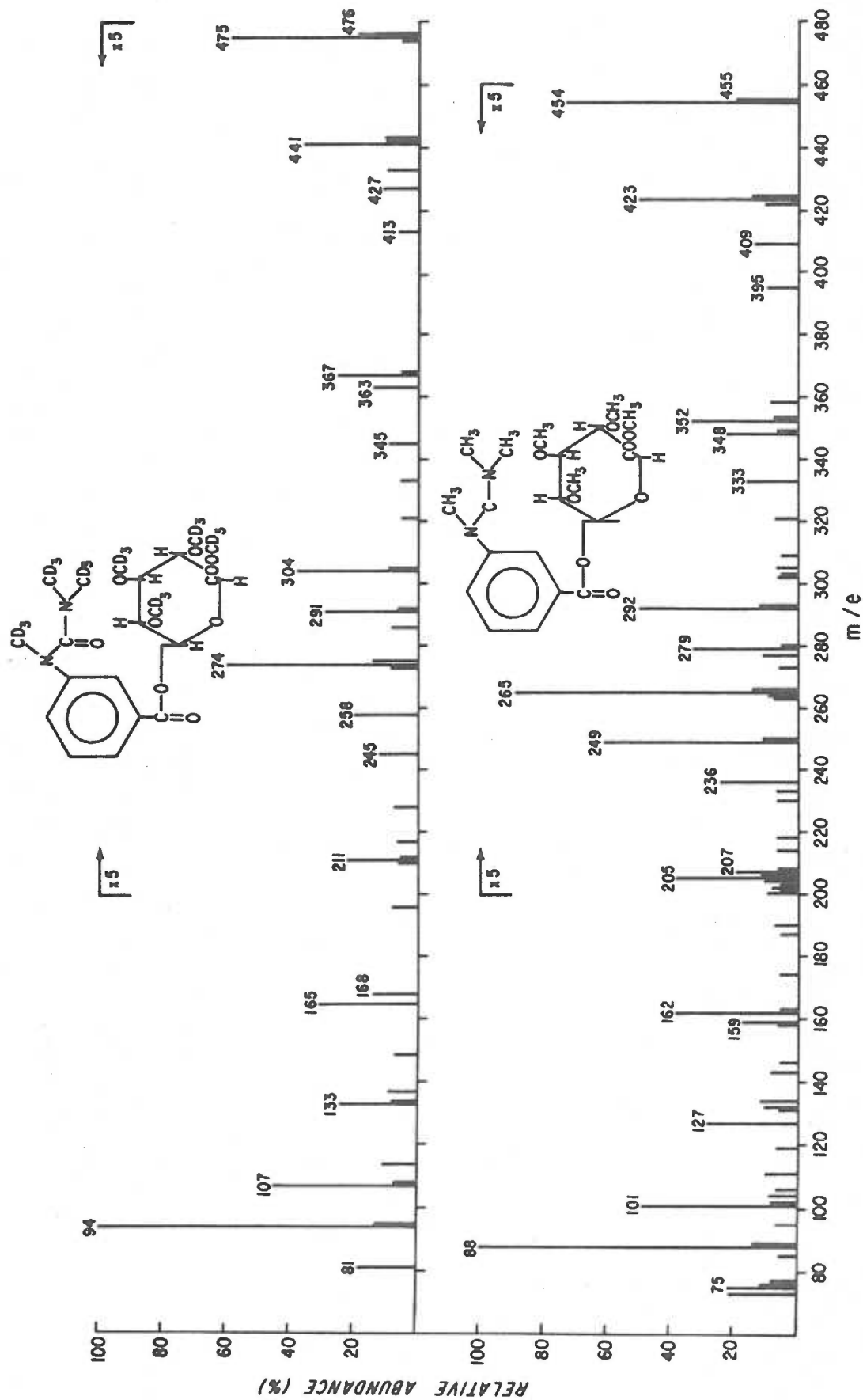


Figure 63: Mass Spectra of the Hepta-Methyl and Hepta-Deutero-Methyl Derivatives of the Ureido-
Glucuronide of MABA, a Urinary Metabolite of Tricaine in the Mouse

APPENDIX VI

Mass Spectra of the Whole-Body Metabolites of Tricaine in the Mouse

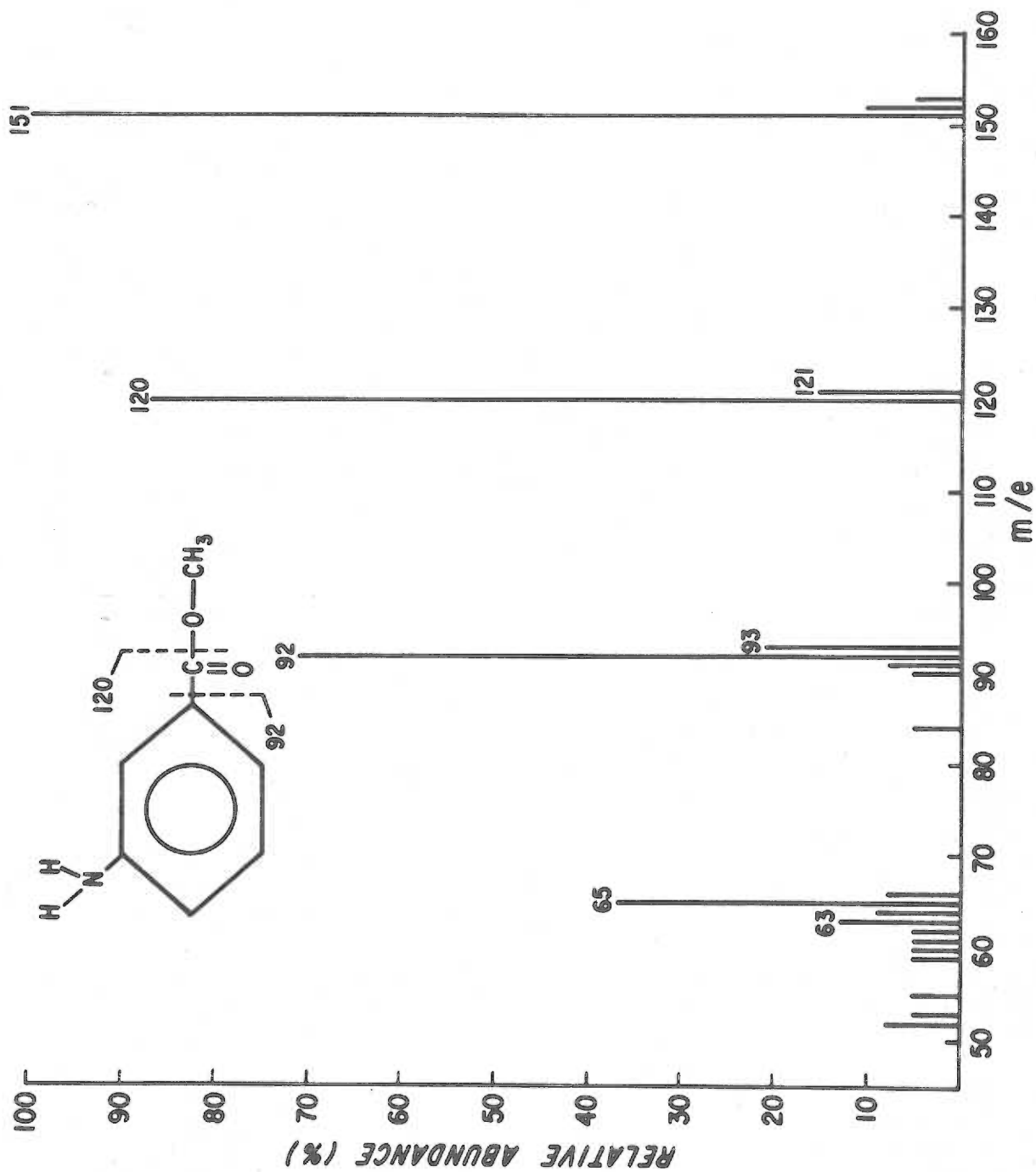


Figure 64: Mass Spectrum of the Mono-Methyl Derivative of MABA, a Whole-Body Metabolite of Tricaine in the Mouse

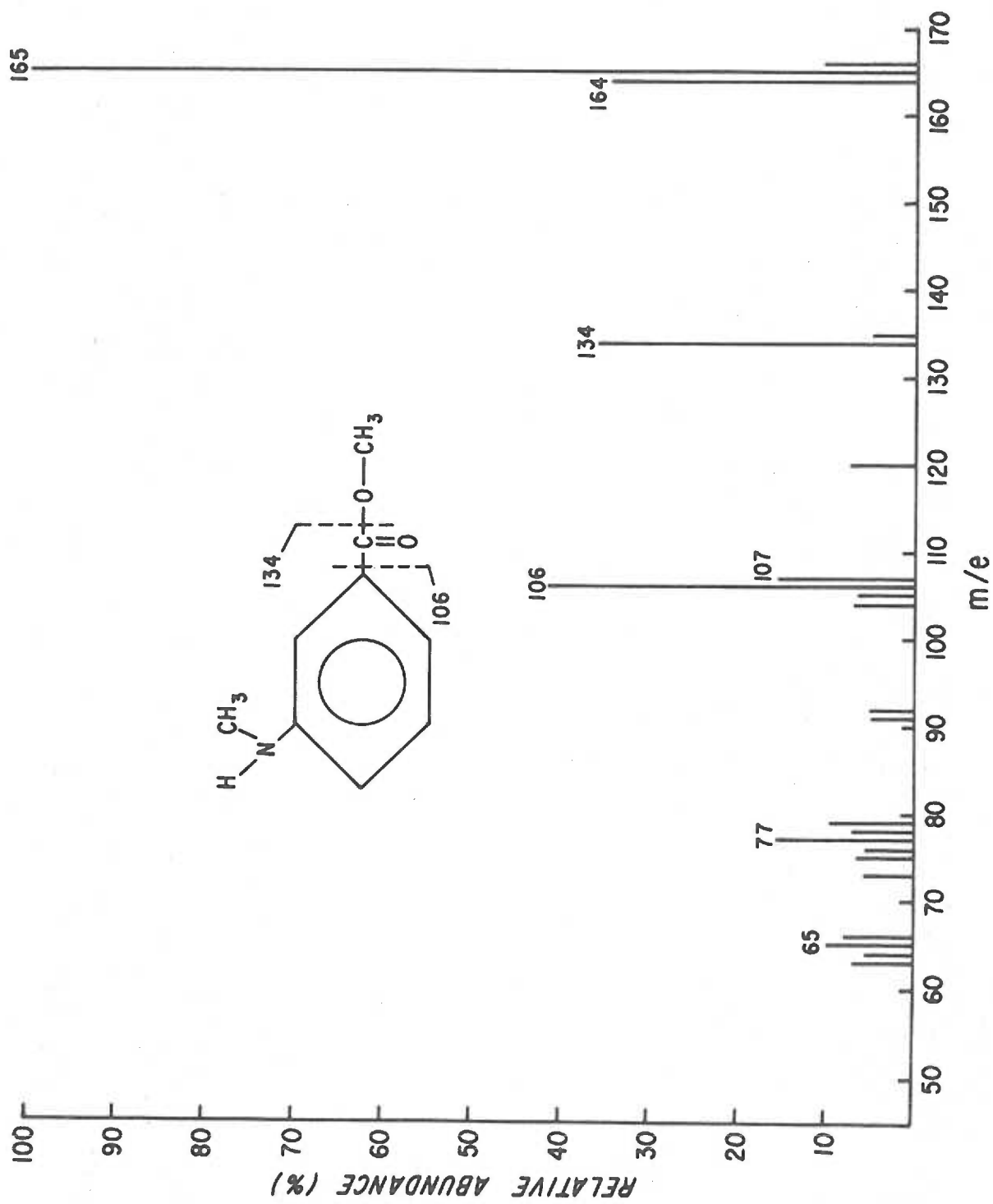


Figure 65: Mass Spectrum of the Di-Methyl Derivative of MABA, a Whole-Body Metabolite of Tricaine in the Mouse

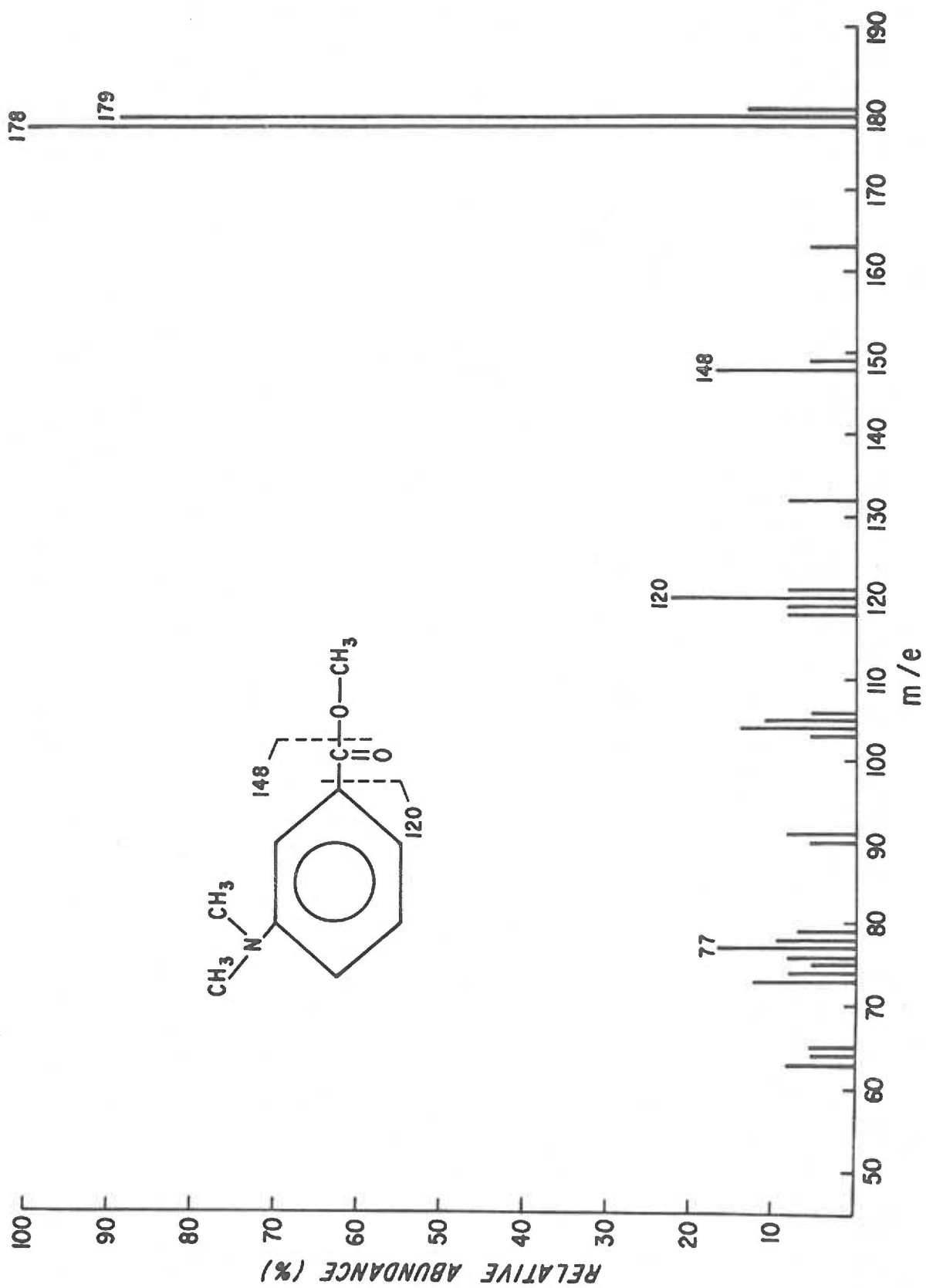


Figure 66: Mass Spectrum of the Tri-Methyl Derivative of MABA, a Whole-Body Metabolite of Tricaine in the Mouse

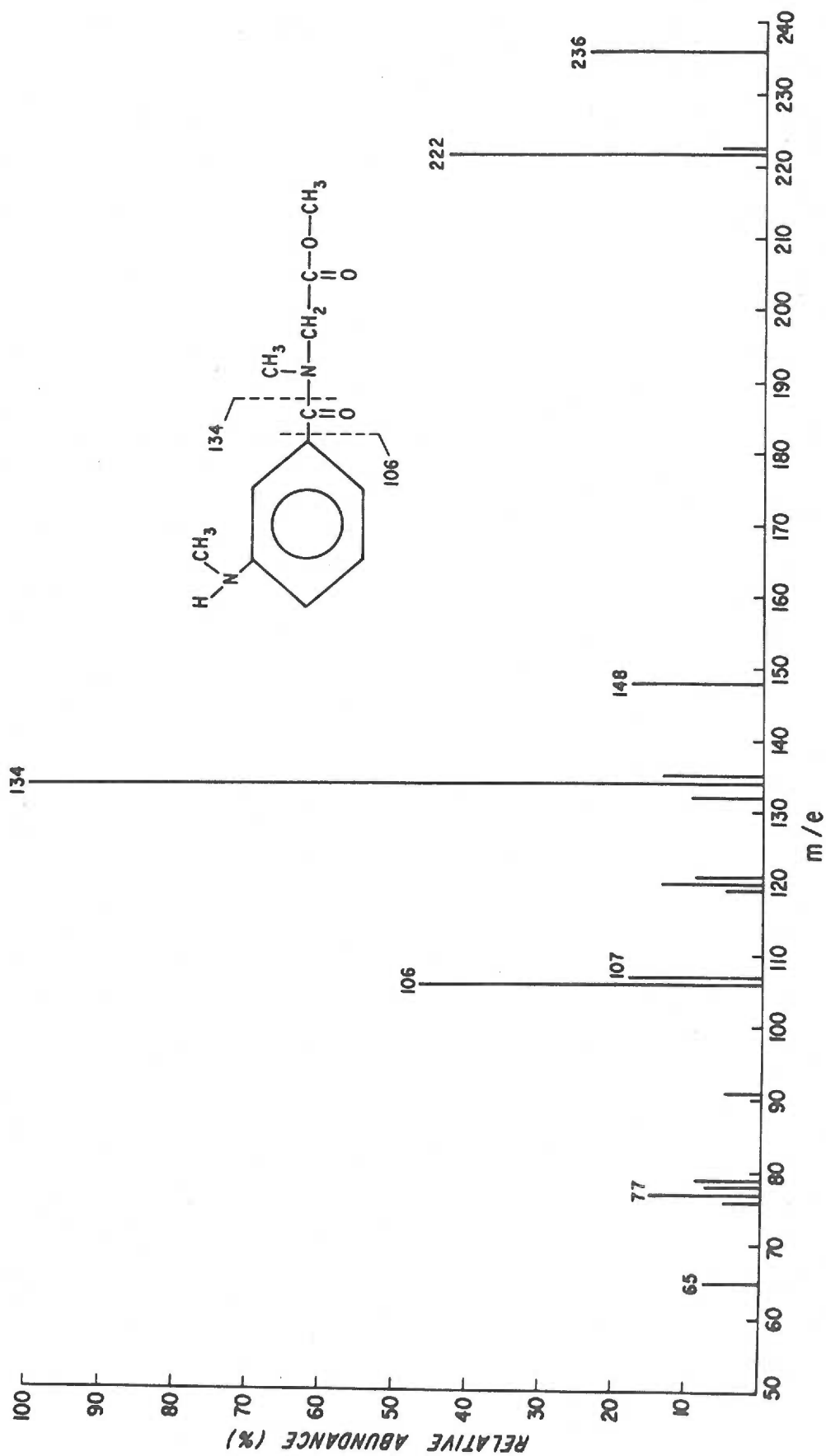


Figure 67: Mass Spectrum of the Tri-Methyl Derivative (Mono-Methyl Amine Compound) of the Glycine Conjugate of MABA, a Whole-Body Metabolite of Tricaine in the Mouse

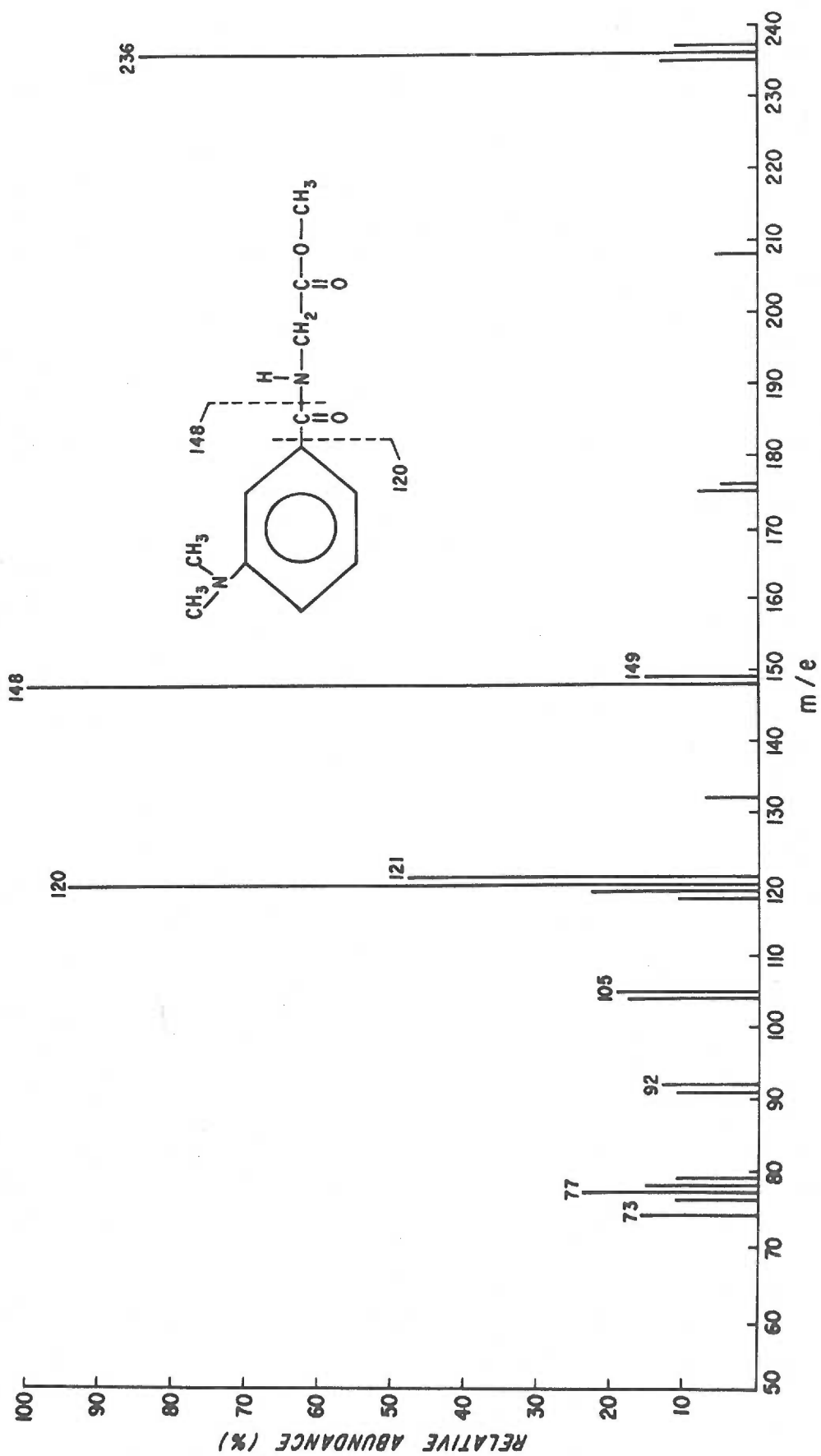


Figure 68: Mass Spectrum of the Tri-Methyl Derivative (Di-Methyl Amine Compound) of the Glycine Conjugate of MABA, a Whole-Body Metabolite of Tricaine in the Mouse

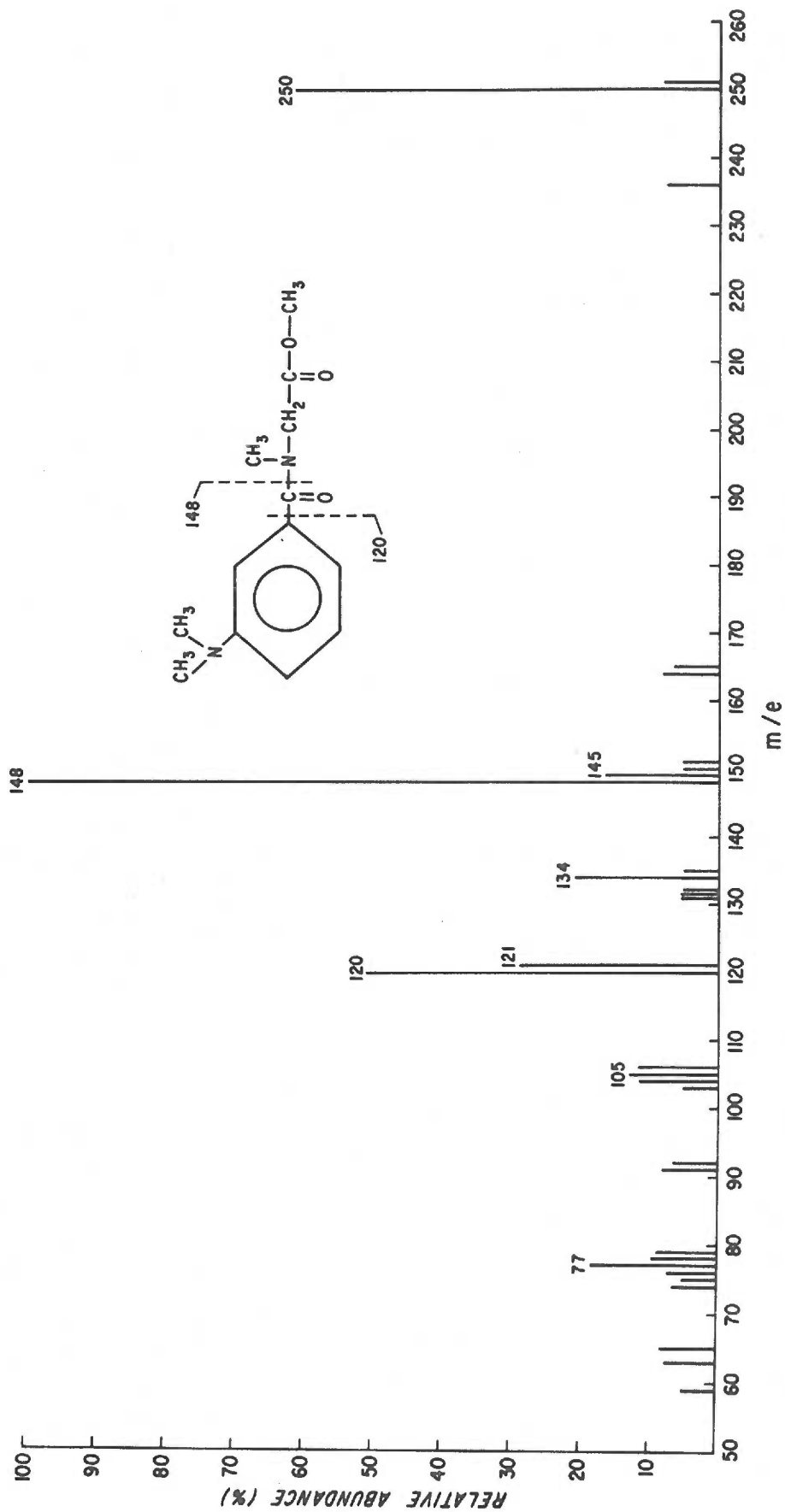


Figure 69: Mass Spectrum of the Tetra-Methyl Derivative of the Glycine Conjugate of MABA, a Whole-Body Metabolite of Tricaine in the Mouse

APPENDIX VII

Mass Spectra of Methyl Derivatives of Synthetic MABA

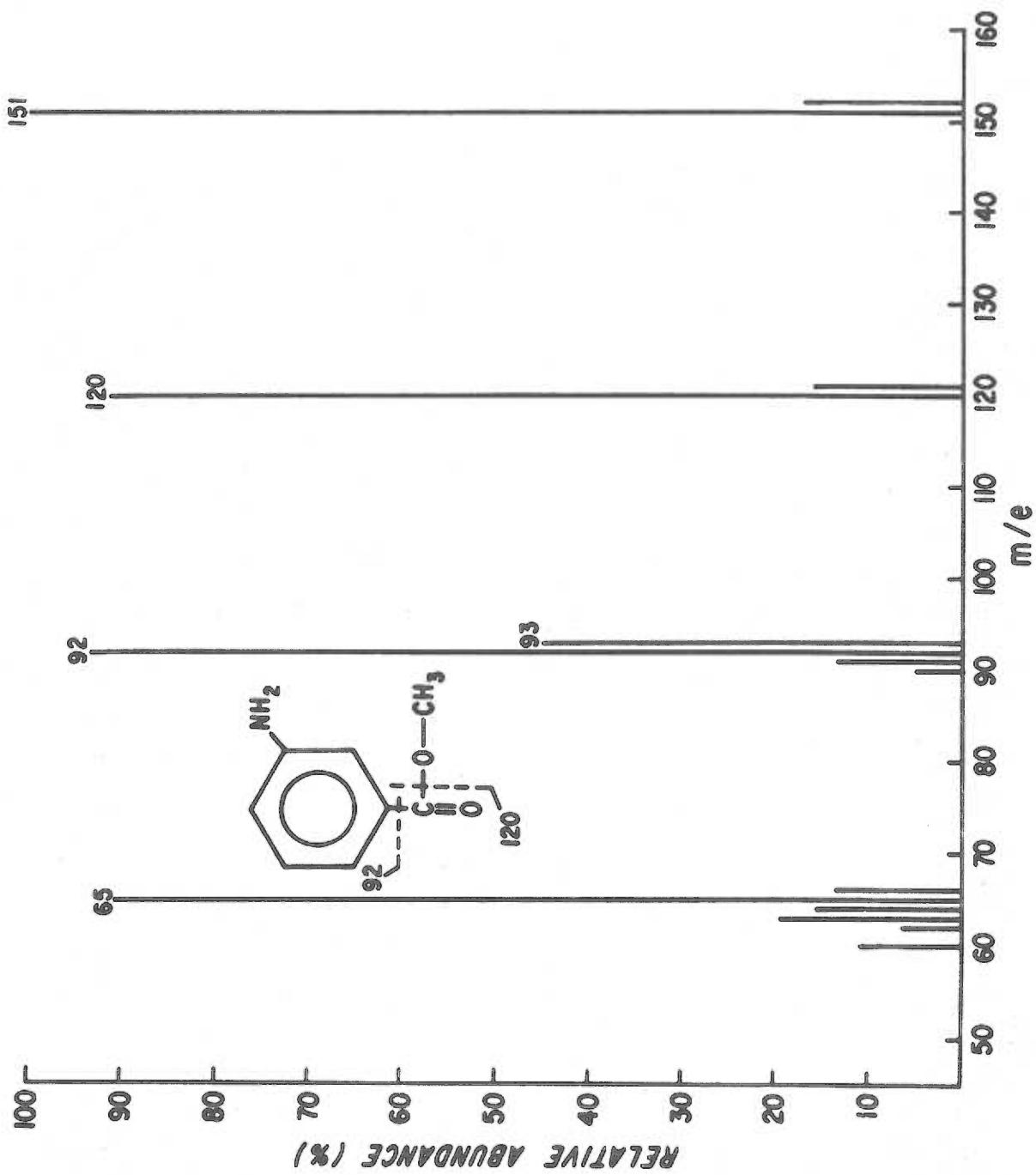


Figure 70: Mass Spectrum of the Mono-Methyl Derivative of Synthetic MABA

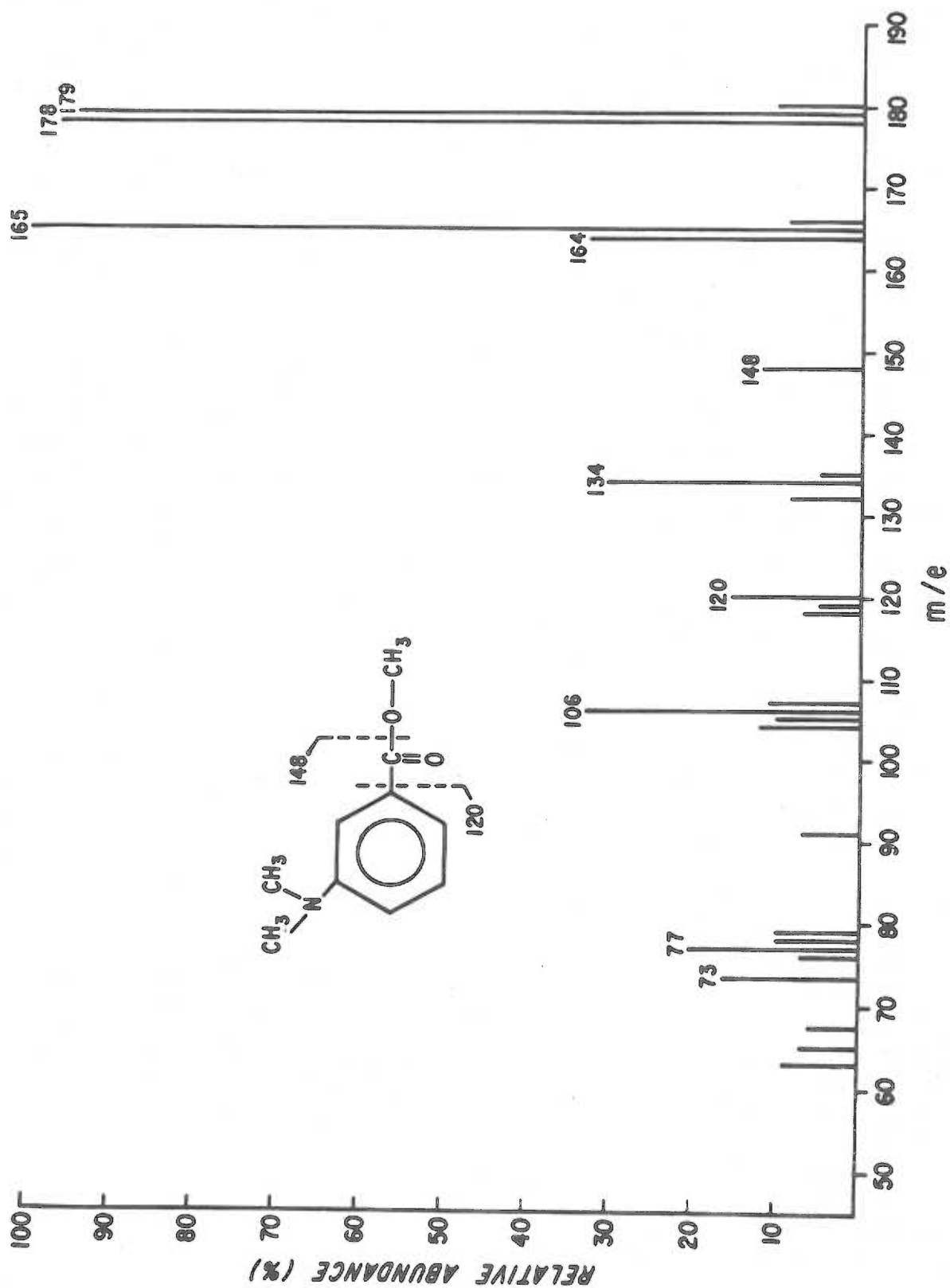


Figure 71: The Combined Mass Spectra of the Di-Methyl and Tri-Methyl Derivatives of Synthetic
MABA

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