THE DISPOSITION OF METHADONE AND OTHER DRUGS IN MALE REPRODUCTIVE ORGANS

by

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A THESIS

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DEDICATION

This thesis is dedicated to my wife, Paula, whose constant love and prayers have sustained me throughout my doctoral studies.

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LIST OF ABBREVIATIONS

Units of Measurement

g = gram

μg = microgram

ng = nanogram

pg = picogram

L = liter

ml = milliliter

μl = microliter

Routes of Drug Administration

i.v. = intravenous(ly)

i.m. = intramuscular(ly)

i.p. = intraperitoneal(ly)

p.o. = oral(ly)

Pharmacokinetics: Symbols for the Two Compartment Open Model:

α = slope of the initial monoexponential line resulting from drug distribution from plasma into tissues

β = slope of the terminal monoexponential line resulting from first-order elimination processes

$t_{1/2}\alpha$ = half-life of the α slope

$t_{1/2}\beta$ = half-life of the β slope

A = extrapolated y-intercept of the α slope

B = extrapolated y-intercept of the β slope

V_d = dose/β $(\frac{A}{\alpha} + \frac{B}{\beta})$ = apparent volume of distribution

β-phase = period when the processes defined by A and α are insignificant and when the processes defined by B and β are predominant

Chemicals

DPH = diphenylhydantoin (phenytoin)

DPA = dipropylacetate

EDDP = 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine

Fn = floctafenin

(continued)

Abbreviations

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Chemicals (continuation)

FA = floctafenic acid

HB = hexobarbital

OH-HB = hydroxylated hexobarbital

Biologic Fluids and Chemicals

Pl = plasma

Bld = blood

PF = prostatic fluid

SER = smooth endoplasmic reticulum

LH = luteinizing hormone

HCG = human chorionic gonadotropin

FSH = follicle-stimulating hormone

MFO = mixed-function oxidase

Physicochemical Properties and Procedures

GLC or GC = gas-liquid chromatography

GC-MS = combined gas-liquid chromatography and mass spectrometry

U.V. = ultraviolet

Publications Derived from the Thesis

Papers

1. Swanson, B. N., Leger, R. M., Gordon, W. P., Lynn, R. K. and Gerber.: Excretion of phenytoin into semen of rabbits and man: Comparison with plasma levels. In press, Drug Metab. Dispos., 1978.

2. Swanson, B. N., Gordon, W. P., Lynn, R. K. and Gerber, N.: The seminal excretion, vaginal absorption, distribution and whole blood kinetics of d-methadone in the rabbit. Accepted for publication, J. Pharmacol. Exp. Ther., 1978.

3. Swanson, B. N., Harland, R. C., Dickinson, R. G. and Gerber, N .: Excretion of dipropylacetate into semen of rabbits and man. Submitted for publication in Epilepsia, 1978.

Abstracts

1. Swanson, B. N. and Lynn, R. K.: Drug metabolism in the testis: N-demethylation of d-, l- and racemic methadone. Fed. Proc. 36: 971, 1977.

2. Swanson, B. N. and Gerber, N.: The seminal excretion, vaginal absorption and half-life of d-methadone in the rabbit. Proc. West. Pharmacol. Soc. 20: 477-482, 1977.

GENERAL INTRODUCTION

Andrology is only now emerging as an independent medical specialty (1). Among the many recent advances in the understanding and diagnosis of male fertility problems, has been the observation that numerous pharmacologic agents can influence the function of male reproductive organs. The effects of androgenic and antiandrogenic agents are widely recognized. Excessive amounts of androgens cause atrophy of the testis and hypertrophy and hypersecretion of accessory sex organs (2-4). Administration of antiandrogens results in atrophy and hypofunction of accessory sex organs (5-7). Disturbances in reproductive function are frequently associated with psychotropic and autonomic drugs (8). Guanethidine, various phenothiazines and Rauwolfia alkaloids can impair ejaculation (9-11). In contrast, amphetamines are known to stimulate and prolong ejaculation (12). The secretory activity of accessory sex organs is increased by cholinergic drugs such as pilocarpine and decreased by anticholinergics such as atropine (13, 14). Phenelzine (a monoamine oxidase inhibitor) improves semen analyses in a significant percentage of infertile men (15). Chronic administration of phenothiazines can result in testicular atrophy and loss of sexual potency (16). A syndrome of hypogonadism and gynecomastia has been linked to the abuse of ethanol (17-19). Extensive use of marihuana can induce gynecomastia and diminish sex-drive (20-22). Men who are addicted to narcotics frequently complain of diminished libido and potency (23, 24). Narcotics cause atrophy of accessory sex glands in rodents (25, 26) and decrease ejaculate volumes in men. The mechanisms of drug effects on male reproductive function are diverse and include: indirect effects (e.g. suppression or stimulation

of the pituitary and hypothalamus; peripheral nerve blockade; induction or inhibition of hepatic metabolism of sex steroids) and direct effects (e.g. antagonism of androgens and gonadotropins peripherally; inhibition of enzymes in the testis or accessory sex organs; carcinogenesis; mutagenesis) (27-31).

Despite the apparent burgeoning of information on the pharmacology and toxicology of male reproductive organs, there are several obvious deficits in this area of investigation. Particularly limited are data on the distribution and metabolism of drugs in sex organs; on the concentration of drugs in seminal fluids; and on drug effects on sperm motility. Such information would be invaluable, not only in the interpretation of toxic drug effects on male sex organs, but also in the design and development of contraceptives for men. Drugs may exert selective, deleterious effects on reproductive organs by virtue of their extensive cumulation in sexual tissues and secretions. Many chemicals can be converted enzymatically into carcinogens, cytotoxins and mutagens (32). Such biotransformation processes in the testis, if they occur, could have devastating consequences for fertility and subsequent offspring. Therefore, topics for future investigations in reproductive pharmacology should include: the quantification of foreign compounds in male sex glands and seminal fluids; descriptions of the drug-metabolizing capability of the testis; and assessment of drug effects on sperm motility.

I. EXCRETION OF DRUGS INTO SEMEN

A. Introduction

1. Secretions of Male Reproductive Organs

Human semen is composed of secretions from a number of reproductive organs (33). Fluids from the prostate and seminal vesicles constitute the majority of the ejaculate--13 to 33% from the prostate and 46 to 80% from the seminal vesicles (34). Contents of the ampullary glands, vas deferens and epididymis (which include the spermatozoa) represent less than 10% of the semen volume, while secretions from the bulbourethral (Cowper's) glands and glands of Littré comprise an even smaller fraction of the ejaculate. Prostatic secretions are characterized by high concentrations of zinc, citrate and acid phosphatase and by an acidic pH of 6.5 to 6.8 (33). Fluids from the seminal vesicles are reported to be alkaline (pH 7.4 to 8.0) and rich in fructose, protein, prostaglandins and phosphorylcholine (33). The mixture of seminal secretions (i.e., semen) coagulates immediately after ejaculation; proteolysins then liquify the semen within 5 to 20 minutes. The pH of human semen is 7.05 to 7.4 (35). Constituents of seminal plasma aid in sperm transport by initiating and maintaining sperm motility and by increasing smooth muscle activity in female reproductive organs (36).

Secretions from the various accessory sex organs are not ejaculated simultaneously, but are emitted in a particular sequence. Thus, secretions from the glands of Cowper and Littré appear first. These are followed by prostatic secretions, then by sperm-laden fluids of the ampulla and vas deferens, and finally by seminal vesicular fluids (34). The ejaculate is delivered as a series of pulses corresponding to

contractions of the perineal musculature (9). The anatomic origin of a chemical in seminal plasma can be determined by collecting the individual pulses (usually 4 to 6) of an ejaculate and measuring the concentration of the chemical in each fraction. If there are high concentrations in early fractions and low concentrations in late fractions, then the chemical is probably secreted or excreted by the prostate rather than by the seminal vesicles. As would be predicted, this concentration pattern is observed for zinc and acid phosphatase (37).

2. Significance of Chemicals in Semen

There is currently little information on the extent to which xenobiotics equilibrate into seminal fluids. To date, less than 10 publications have described the concentration of foreign compounds in semen, and perhaps 30 more articles have reported the concentrations of xenobiotics (particularly antibiotics) in prostatic fluid alone. Further knowledge in this area would be helpful in evaluating:

1) the relationship between carcinogens and genitourinary tract malignancies;
2) allergic reactions of women to sexual intercourse;
3) toxic drug effects on fertility and fetal development;
4) the feasibility of oral contraceptives for males; and
5) chemotherapy of genital organ infections.

3. Prostatic and Cervical Cancer

Cancer of the prostate is the third most prevalent cancer in men (38). The long-term prognosis for this common malignancy is poor due to metastases. Optimum proliferation of prostatic neoplasms is frequently androgen dependent. Thus, orchiectomy often causes temporary

regression of prostatic carcinomas, whereas drugs with androgenic activity can enhance tumor growth (39). It is possible that other foreign compounds can initiate or promote prostatic cancer. For example, occupational exposure to cadmium oxide is associated with a significant increase in the incidence of prostatic carcinoma (40). Benzpyrene and methylcholanthrene can induce squamous cell carcinomas and adenocarcinomas in prostates of rats and mice (41, 42). The potential of chemicals to cause cancer of the prostate can be evaluated in part by ascertaining the concentrations such substances achieve in the prostate and prostatic secretions of experimental animals. To date, only two known carcinogens, N-hydroxyurethane and 2-acetylaminofluorene, have been quantified in prostatic fluid (43, 44). No attempt has been made to measure levels of carcinogens in the ejaculate. This is somewhat surprising, since there is evidence that some component of semen is important in the etiology of cervical carcinoma (45, 46). The incidence of cervical tumors has a high correlation with the frequency of coitus, the number of sex partners, and the onset of first coitus (47).

4. Allergic Reactions to Chemicals in Semen

Some women experience intense vaginal urticaria or generalized allergic phenomena (hives, urticaria, rhinitis, swollen facies, sneezing, anaphylaxis, wheezing, etc.) following coitus (48-54). The true incidence of post-coital allergic reactions is not known. The majority of these reactions would be attributed to upper respiratory infections or hypersensitivity to air-born allergens, and therefore escape diagnosis. Even the more bothersome symptoms of allergy might evade the attention of the medical community due to the reluctance of patients to discuss problems

related to sexuality. Post-coital rhinitis and anaphylaxis have been linked on occasion to proteins in semen (54). Foreign compounds that diffuse into seminal fluids are also likely causes of post-coital allergy. Most drugs are probably excreted into semen in only minute quantities. However, microgram amounts of chemicals are known to elicit local and/or systemic allergic reactions. Furthermore, the vaginal mucosa is at least as sensitive as skin to irritants and allergens (55, 56). For example, one woman experienced vaginal discomfort after sexual intercourse when her husband was receiving vinblastine chemotherapy for Hodgkin's disease, but not when he wore a condom or was no longer receiving medication (57). Her reaction was apparently a form of contact dermatitis, since she exhibited a positive skin test for the periwinkle plant (the natural source of vinblastine).

5. Absorption Through the Vaginal Mucosa

Systemic allergic reactions to drugs excreted into semen should also be anticipated since there is evidence that a variety of chemicals can be rapidly absorbed through the vaginal mucosa (58). In 1918, Macht (59) demonstrated that aconitine, morphine, pilocarpine, apomorphine, cocaine, phenol, mercuric chloride and potassium cyanide could each initiate pharmacologic responses in dogs after vaginal administration. Robinson (60) proved by surgically isolating the vagina from the urethra and uterus in the dog that this absorption process had to occur through the vaginal mucosa. Besides substantiating that a number of alkaloids can be absorbed from the lumen of the vagina, Robinson discovered that peptides such as oxytocin and insulin could cause systemic effects when administered per vaginum. Other workers have later shown

that large proteins can penetrate the vaginal mucosa (61). Robinson described one patient who developed an extensive erythematous rash each time quinine (a spermicide) was introduced into the vagina. It is clear from these observations, that drugs present in the ejaculate are likely to be absorbed into the female's circulation.

The vagina has been used occasionally as a route of administration for systemic drugs, notably, antibiotics and steroid contraceptives. As early as 1947, sulfanilamide, sulfathiazole and penicillin G were found to be absorbed across the vaginal mucosa (62, 63). Cephalosporins, sulfonamides and penicillins are still administered as intravaginal suppositories. Recently, silicone rings containing sex steroids have been placed in the vaginal fornix and proven to be effective contraceptives (64, 65). The employment of these drug preparations would seem to justify studies on the pharmacokinetics of vaginal absorption. However, such research was not attempted until 1975 (66-68) and has emphasized small alcohols and unbranched hydrocarbons.

6. Effect of Drugs on Male Fertility

There is now a vast literature on the chemical regulation of fertility in women and on the teratogenic effects of drugs consumed during pregnancy (69, 70). On the other hand, little progress has been made toward the development of chemical contraceptives for men, and almost nothing is known concerning the teratogenic effects of drugs after consumption by the male (71-74). The acquisition of oral contraceptives for men will require lengthy research dealing with drug effects on sperm development, motility, capacitation, and fertilizing capacity. Therapeutic efficacy of these drugs may require that certain drug

concentrations be maintained in seminal fluids. Therefore, experiments that describe the pharmacokinetics of drug excretion into seminal fluids will be an integral part of the research effort. Further information on drug concentrations in semen may also afford new insight into the etiology of congenital birth defects and male infertility. Lutwak-Mann, Schmid and Keberle (75, 76) have suggested that drugs in seminal fluids may become concentrated in spermatozoa and subsequently act as teratogens. They found thalidomide and/or thalidomide metabolites in rabbit semen after administering ^{14}C-thalidomide to male rabbits. Thalidomide that was added to semen in vitro became bound to spermatozoa. Furthermore, male rabbits that were pretreated with thalidomide and mated with drug-naive females, produced small litters. The young exhibited low birth weights, poor neonatal survival, and a high incidence of congenital malformations (including spina bifida, absence of the tail, cranial blister, absence of both kidneys, paralysis of hind limbs with lack of ossification centers, and hemangiomas of the forelimbs). Similarly, male rats that are given methadone will generate offspring with low birth weights and diminished neonatal survival (77, 78). The effects are dose-dependent and are maximal if the narcotic is administered within 24 hours prior to mating.

7. Excretion of Drugs into Prostatic Fluid

Chronic infections of the prostate have been notoriously difficult to treat, despite the availability of numerous antimicrobial agents. The bacteria responsible for genitourinary tract infections in men are usually Gram negative rods and sensitive to antibiotics in vitro (79). The perplexing resistance of these bacteria to chemotherapy in

vivo could be explained if antibiotics were unable to diffuse into the prostate and its secretions. Indeed, experiments from 1947 to 1963 showed that many antibiotics do not readily partition into prostatic fluids (80-85). The penicillins, sulfonamides, aminoglycosides and cephalosporins were particularly poor in their ability to achieve therapeutic concentrations in prostatic fluid. In 1967, Dunn and Stamey found that a dog preparation could provide detailed information on the equilibration of drugs into prostatic fluid (86, 87). Since the dog does not possess seminal vesicles or bulbourethral glands, essentially pure prostatic fluid can be collected from this species. The possibility of urine contamination can be eliminated by simply ligating the urethra at a point proximal to the prostate and shunting the urine through an abdominal fistula. The resting state production of prostatic fluid in the dog is only 0.2 ml/hr but this flow rate can be increased to as much as 50 ml/hr by administering a cholinergic agent--usually pilocarpine. The dog animal model permitted systematic investigations of large numbers of antibiotics (87-99). Winningham, Nemoy and Stamey (97) were the first to postulate that ion-trapping is important in the equilibration of antibiotics into prostatic secretions. Prostatic fluid is acidic (pH 6.5 to 6.8); therefore, weak organic bases should cumulate in secretions of the prostate whereas weak organic acids should be nearly excluded (100). With few exceptions this ion-trapping hypothesis adequately explains and predicts the behavior of drugs in prostatic fluids of the dog as well as of man. Winningham and Stamey (98) and Robb et al. (93) have shown in the dog that the prostatic fluid/plasma drug concentration ratio (PF/Pl) for sulfonamides is directly proportional to the pKa of

the drug. For example, sulfanilamide had the largest pKa (10.4) and the largest PF/P1, whereas sulfisoxazole had the smallest pKa (5.0) and the smallest PF/P1. Winningham et al. (97) noted that some weak bases, such as polymixin B and kanamycin, did not cumulate in dog prostatic fluid. This was explained by the poor lipid solubility of these drugs at the pH of plasma; they do not readily penetrate the lipophilic membranes of prostatic tissue and are in large part confined to the vascular compartment. Winningham and his co-workers concluded that the ideal drug for treating bacterial prostatitis should be lipid soluble and a weak base with a pKa of 8.6 or greater. The macrolides, erythromycin and oleandomycin, fit these criteria, but are unfortunately ineffective against Gram negative organisms and are least potent at acidic pH values. Reeves and Ghilchik (92) demonstrated in dogs that trimethoprim, a weak base (pKa = 7.3) with a high lipid-solubility, achieves high concentrations in prostatic fluid relative to concentrations in plasma. The same results were later obtained in man (101). Numerous papers on the quantification of antibiotics in seminal fluids are cited in Table 1.

Although probably correct in their conclusions, the above experiments reporting antibiotic concentrations in prostatic fluid warrant some criticism. The mainstay of any drug disposition study is a sound, reliable drug assay. The specificity of a drug assay is particularly important; every precaution must be taken to assure that one, and only one, substance is contributing to the parameter (U.V. absorbance, antimicrobial activity, etc.) being measured. Most bioassays for antibiotics, which include minimum inhibitory concentration (serial

dilution) and plate diffusion methods, cannot distinguish between parent drugs and active drug metabolites. The specificity of these assays can be established only after the introduction of extraction or chromatographic methods. None of the manuscripts reviewed above provided evidence that metabolites were not being measured along with the parent drug. Similarly, some of the workers used the Bratton-Marshall reaction (102) for quantifying sulfonamides without first separating the antibiotics from their metabolites. Thus, all sulfonamide metabolites that retained a primary amine group contributed to the total drug value. Furthermore, several of the investigators employed saline or serum in place of prostatic fluid for preparing drug standards used in plate diffusion bioassays. Prostatic secretions are known to have mild bacteriostatic properties and failure to check the antimicrobial activity of prostatic fluid with respect to bioassay organisms could result in serious assay errors. Finally, one should be cautious in drawing conclusions about excretory processes in man on the basis of data obtained in the dog. Prostatic fluid was collected from dogs during maximal stimulation of the prostate with pilocarpine. This hyperstimulation causes obvious alterations in the concentrations of normal constituents of prostatic fluid (103) and, likewise, may cause antibiotic levels to deviate considerably from "resting state" concentrations. Data acquired in man is far less consistent than data acquired in dogs. Differences between antibiotic concentrations in prostatic fluid and concentrations in blood are more dramatic and reproducible in dogs than in man. Some of the inconsistencies in the human data may be an artifact of the methodology employed; small amounts of urine containing large quantities of drug

could easily contaminate the prostatic secretions during the collection process.

8. Excretion of Drugs into Semen

In contrast to the studies on drug concentrations in prostatic fluid, papers reporting drug concentrations in semen are much fewer in number (about 6) and give contradictory results. A paper by Armstrong, Cook and Robinson (104) reported drug levels in semen for 15 antibiotics. However, the credibility of this work cannot be evaluated because almost no details on drug assays were provided. Furthermore, some of the results conflicted diametrically with data obtained by other workers (37, 81, 105, 106). Gerber and Lynn (107) were apparently the only workers to employ a drug assay that was specific for the drug being quantified. They used a gas-liquid chromatographic assay to measure methadone in the semen and blood of narcotic addicts, and found that the drug concentration in semen was nearly two times the concentration in blood.

9. Purpose of the Investigation

The toxicologic or therapeutic effects of drugs in semen cannot be properly assessed until more information is available on drug concentrations in the ejaculate. The factors that influence drug equilibration into the seminal fluid compartments (e.g., active transport, drug lipid solubility and pKa, fluid pH, plasma protein binding) need further consideration. The purpose of the following investigation was to develop and apply precise, sensitive and drug-specific assays for quantifying drugs in semen. An appropriate animal model was sought for studying drug excretion into semen. It was hoped that the time course

and extent of drug equilibration from blood into seminal fluids could be described in both man and the animal model. A critical comparison could then be made between the two species, and a judgement ventured as to the suitability of the animal model for predicting excretory phenomena in man. In addition, experiments that quantified levels of drugs in reproductive organs relative to other tissues and that estimated the rate of drug absorption through the vaginal mucosa could provide information that is peripheral, but, nonetheless, pertinent to the seminal excretion of drugs.

B. Materials and Methods

1. The Rabbit as an Animal Model for Semen Studies

The domestic rabbit has been used as an animal model for studying the physiology of semen (108). Other small animals, such as the rat, guinea-pig, cat and monkey, must be subjected to a painful electro-ejaculation procedure (33). Rabbits, on the other hand, can be trained to mount a rabbit skin sleeve worn on the arm of the researcher and ejaculate within 1 to 5 seconds into a hand-held artificial vagina. Most rabbit bucks are able to mount and ejaculate every 15-30 minutes throughout the day. Lagomorph semen is easily manipulated in the laboratory because it does not coagulate and does not require microtechniques; primary ejaculates average about 0.7 ml. In contrast, rodent semen is scanty and coagulated.

Rabbit accessory sex organs include a prostate, ampullary glands, paraprostates, bulbourethral glands and a seminal vesicle. The seminal vesicle (glandula vesicularis) is unique in that it is unpaired and secretes, in addition to fluids, a clear gel (109). The gel is not

the product of coagulation processes subsequent to ejaculation, but, rather, exists as a semisolid gel within the seminal vesicle in vivo. This was determined by dissection of reproductive organs in anesthetized animals. Only about 30% of the rabbits produced the gel. Furthermore, the ejaculates of rabbits known to produce the gel do not always possess a gel component. Because of the erratic disposition of the gel portion of rabbit semen, drugs are probably best quantified in the fluid part alone in order to standardize the data.

The artificial vagina used for semen collection (Figure 1) was constructed from 2-inch diameter Penrose tubing and a plastic cylinder, 4 cm diameter x 8 cm long. The surgical tubing was pulled through the cylinder and retracted over the ends of the cylinder to form an enclosed space between the rubber and plastic. This space was filled with hot water through a hole in the side of the cylinder. A test tube was placed in one end of the artificial vagina just prior to filling the reservoir so that the expanding rubber formed a tight seal around the tube's orifice. The water-inflated vagina was then lubricated with a small amount of water-soluble, surgical jelly (Lubafax) and presented to the male rabbit.

To prepare a rabbit skin sleeve, cuts were made through the skin around the neck, tail and paws of a freshly killed rabbit. The skin was then pulled entirely free from the carcass, and all muscle, fat and membranes were carefully scraped or pulled off the hide. A concentrated ethanolic solution of tannic acid was rubbed into the skin, and the rabbit skin was ready for use 24 hours later. For best experimental results, the fur was periodically rubbed on the hind quarters of a

living female rabbit so as to maintain the fur's pheromone scent (110). The latter procedure increased mounting behavior by males.

A rabbit was trained for semen studies by permitting him to mount but not penetrate a living doe, withdrawing the female, and immediately presenting to the buck an artificial vagina. This process was repeated until the male successfully mounted the rabbit skin sleeve and ejaculated into the artificial vagina. Subsequently, the male rabbit would freely ejaculate into the artificial vagina with only occasional reintroduction of a living female.

Multiple blood samples were drawn from each rabbit by placing an indwelling cannula (Argyle, Medicut, 20 G x 2 in, Sherwood Medical Laboratories, St. Louis, Mo.) in the central artery of an ear. Patency of the cannula was maintained by flushing it periodically with heparinized saline (10 units Panheprin/ml).

Some rabbits contaminated their semen with urine. This was evidenced by an ejaculate with an inordinately large volume, urinous odor, yellow color, and dead spermatozoa. High concentrations of drug metabolites in the ejaculate also indicated urine-contamination. Animals that urinated during ejaculation were excluded from all semen studies.

The rabbits used in the following experiments were New Zealand whites (males, 3.3 to 4.5 kg, and females, 3.3 to 3.9 kg). They were maintained on standard rabbit chow (either Albers or Oregon State University brands) in a well-ventilated room, temperature 22 to 26° C.

2. Selection of Drugs To Be Studied

d-Methadone, phenytoin and dipropylacetate were selected for the semen studies (see Figure 2). The three drugs differ greatly in their lipid solubility and pKa values; therefore, experiments using these drugs could provide valuable information on the chemical properties favoring or retarding drug cumulation in semen. In addition, all three drugs may exert deleterious effects on reproductive function; examination of their ability to enter seminal fluids could help define the mechanism(s) of these toxic effects.

Methadone is a potent narcotic analgesic and exhibits pharmacologic properties similar to those of morphine. It differs from morphine in that it is highly effective after oral administration. Methadone is a weak base with a pKa of 8.6 and is extremely lipid soluble (111). As was mentioned previously, administration of methadone to male rats prior to mating results in offspring of low birth weight and diminished survival rate (77, 78). These adverse effects are not due to alterations in spermatogenesis, since the transit time of sperm in the epididymis is at least a week (112) and maximal drug effects occurred if animals were mated within 24 hours prior to drug administration. It is conceivable that methadone cumulates in male sex organs, and subsequently disrupts reproduction function. Methadone reportedly cumulates in human semen above the concurrent levels in blood (107). The drug can alter metabolism in the testis (113) and the prostate (114) in vitro. There is currently no information on the concentrations that methadone achieves in male reproductive organs. Methadone exists as two optical isomers. Both isomers exhibit pharmacologic properties common to narcotics (e.g.,

analgesia, respiratory depression, pupillary miosis, hypnosis). However, the (1-) isomer is reported to be considerably more potent than d-methadone in several mammalian species (115, 116). In order to avoid adverse drug effects on rabbit libido, d-methadone was selected for the following studies.

Phenytoin (diphenylhydantoin, DPH) is widely used as an anticonvulsant and is beneficial in the treatment of some cardiac arrhythmias. DPH is a weak acid (pKa = 8.33), poorly soluble in aqueous solutions below pH 9, and highly soluble in most organic solvents (117, 118). The incidence of birth defects is reportedly increased among children of epileptic women receiving DPH (119). DPH is likewise teratogenic when given to pregnant experimental animals (120). Recently, anticonvulsant therapy has been reported to diminish sexual potency and fertility in young, epileptic men (121, 122). Abnormal sperm morphology and reduced sperm motility were prominent features in semen analyses in 45 of 47 patients.

Dipropylacetate (valproic acid, DPA) has been used extensively in Europe for the treatment of epilepsy and is currently undergoing clinical testing in the U.S.A. (123, 124). It has a pKa of 4.95. Preliminary findings in another laboratory show that chronic administration of DPA can cause testicular atrophy in rabbits (personal communication, Abbott Labs, North Chicago, Ill.).

3. Pharmacokinetics

The two-compartment open model was employed to describe the drug concentration-time curves that were obtained in rabbits (125). The

concentration of drug, C, at any time, t, can be derived from two mono-exponential lines having y-intercepts A and B and slopes α and β, respectively: $C = Ae^{-\alpha t} + Be^{-\beta t}$. β was estimated by the least squares method. The line $Ae^{-\alpha t}$ was acquired by subtracting $Be^{-\beta t}$ from the experimentally determined curve. The apparent volume of distribution was calculated using the equation: $V_d = \text{Dose}/\beta(\frac{B}{\beta} + \frac{A}{\alpha})$.

4. Methadone

Methadone Assay. d-Methadone was quantified by a gas-liquid chromatographic (GLC) assay. Blood (3.0 ml), tissue homogenate (1.0 ml brought to 3.0 ml with water), semen (brought to 3.0 ml with water) or dialysis bag contents (3.0 ml of a $\geq$ 1:2000 dilution) were extracted in conical, glass-stoppered centrifuge tubes containing 3.0 ml 0.4 M carbonate-bicarbonate buffer (pH 9.6) and 30 ml 1-chlorobutane. Twenty-five milliliters of the 1-chlorobutane extract were equilibrated with 2.0 ml 1 N sulphuric acid. The 1-chlorobutane layer was removed with an aspirator; and 1.8 ml of the acid were transferred to a Reacti-vial (Pierce, 5 ml), alkalinized to pH 9.6 with 1.0 ml 13.5% ammonium hydroxide and extracted with 50 µl chloroform. The chloroform contained tetracosane as an internal standard. Chloroform extracts were analyzed in a Hewlett-Packard Model 5830 A gas chromatograph equipped with a flame ionization detector and a peak integrator for determination of peak areas. The column was 1.7 m x 2 mm I.D. glass packed with 1.5% OV-101 on Gas-Chrom Q 100-200 mesh (Varian, Sunnyvale, Calif.). Injector and detector temperatures were 250° C and 275° C, respectively. The oven temperature was programmed to rise 10° C/min from 170° to 250° C. Flow-rates of

nitrogen, hydrogen and air were 30, 30 and 250 ml/min, respectively. Figure 3 shows a typical gas chromatogram for an extract of rabbit blood following a dose of d-methadone HCl. Natural constituents of biologic material that were extracted by the above procedures did not interfere with the methadone and tetracosane GLC peaks.

During the first extraction step, 97% of the d-methadone in a 3.0 ml sample of rabbit blood is recovered in the 1-chlorobutane. Lynn et al. (126) have shown that the recovery in subsequent steps is greater than 99%. In contrast, the recovery of d-methadone from tissue homogenates in the first extraction procedure can be as low as 70% depending on the tissue. Therefore, tissue homogenates (still at pH 9.6) were extracted a second time with 30 ml of fresh 1-chlorobutane. The amount of d-methadone in a homogenate sample was determined by adding together the quantity of drug measured in both 1-chlorobutane extracts plus the calculated amount of drug remaining in the homogenate sample after two extractions. The latter calculation was based on the assumption that each extraction removes the same percentage of the remaining drug.

Standard curves for d-methadone were prepared daily by subjecting known amounts of d-methadone HCl in water to the above extraction and chromatographic procedures. The ratio between d-methadone and tetracosane peak areas was plotted versus the amount of d-methadone added to the centrifuge tubes (Figure 4). d-Methadone HCl was a gift from the Eli Lilly Company (Indianapolis, Ind., U.S.A.). Chloroform (nanograde) was purchased from Mallinckrodt (St. Louis, Mo., U.S.A.), and 1-chlorobutane was acquired from Burdick and Jackson (Muskegon, Mich.). Tetracosane was obtained from Applied Sciences Laboratories, Inc. (State College, Pa., U.S.A.).

The above assay is similar to the one published by Lynn et al. (126) except that, 1) tetracosane was used as the internal standard in place of 2-dimethylamino-4,4-diphenyl-5-nonanone, and 2) the isooctane wash step was eliminated.

2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine (EDDP). The pyrrolidine metabolite of methadone, EDDP (see Figure 5), can be quantified in biologic material using the same extraction and GLC procedures as employed for methadone (126). The GLC hydrocarbon numbers for EDDP and methadone are 20.1 and 21.3 respectively. No attempt was made to quantify EDDP in these studies. Despite the lack of absolute, quantitative information, one can still use the GLC peak areas for EDDP, to describe the kinetics of EDDP in body fluids and to compare EDDP concentrations in various tissues.

Pharmacokinetics of d-Methadone. In order to determine the β phase half-life independently of drug absorption processes, the kinetics of d-methadone in blood were studied in male rabbits after an intravenous (i.v.) infusion of 40 mg d-methadone HCl. The drug (dissolved in 1.0 ml distilled water) was injected over a one-minute period into a marginal vein of the ear. Timed 3.2 ml blood samples were collected from the opposite ear using a heparinized plastic cannula situated in the central artery of the ear. The concentration of methadone in the blood samples was determined by the previously described method.

Semen Experiments. Male rabbits were injected intramuscularly with 40 mg d-methadone HCl in 1.0 ml distilled water. The intramuscular route was employed in order to avoid toxic drug effects associated with the i.v. infusion. Ejaculates and 3.2 ml blood samples were obtained at

various times after drug administration and analyzed for methadone. A total of five animals was used for these studies. Each animal participated in at least four experiments, but was never used more than once in a two week period to avoid drug effects on microsomal enzymes and reproductive organ function.

Vaginal Absorption Experiments. Preliminary experiments in which a solution of d-methadone HCl was introduced directly into the vagina of a rabbit proved to be unsatisfactory because volumes as small as 0.1 ml were rapidly expelled through the vaginal orifice. Restraint of the animal on its back with vertical orientation of the hindquarters did not alleviate the problem, nor did the use of highly viscous drug vehicles such as methyl cellulose or tragacanth gum. Therefore, small dialysis bags (1.5 cm long) were constructed from size #8 dialysis tubing (Van Waters & Rogers, San Francisco, Calif.) and used for drug delivery. This latter method prevents drug loss through the vaginal orifice while permitting rapid conveyance of the drug to mucous membranes. The use of dialysis bags may have one shortcoming in that, for some drugs, the rate-limiting step for vaginal absorption might be diffusion across the dialysis membrane.

In the definitive experiments, a dialysis bag containing 40 mg d-methadone HCl in 0.5 ml distilled water was inserted into the vagina of a rabbit; and timed 3.2 ml blood samples were drawn from a cannula in the central artery of the ear. Each sample was then analyzed for d-methadone. Premature extrusion of the bag was prevented by placing a small metal clip over the vaginal orifice. The dialysis bag was removed 90 minutes after implantation, and the amount of residual drug in the bag was quantified.

Tissue Distribution Studies. Each of five male rabbits was injected i.m. with 40 mg d-methadone HCl. Arterial blood samples were drawn 130 minutes after the injection, and the animals were killed with 60 ml, intravenous air emboli. Tissues were rapidly removed, weighed, frozen in liquid nitrogen and stored at -20° C. Thawed tissue was homogenized in twice its weight of 0.1 N HCl. The amount of d-methadone in 1.0 ml homogenate was then determined.

Mass Spectrometry. Stock solutions of d-, l- and racemic methadone and biologic samples from rabbits given d-methadone, i.m., were extracted in the previously described manner. A portion of the final chloroform extract was chromatographed as before, and mass spectra were obtained for various GLC peaks using a Dupont Model 21-491 B mass spectrometer equipped with a jet separator (accelerating potential, 1.8 kV; ionizing potential, 70eV; trap current, 300 μamp; source temperature, 200° C).

5. Phenytoin

Human Studies. Human volunteers were recruited locally by the Epilepsy Center of Oregon. Individuals receiving oral DPH (phenytoin sodium, Dilantin) and no other drugs were used for the study. Patient ages ranged from 21 to 49 years with a mean of 33. Consent forms describing the purpose of the project and risks involved were signed by all participants. Each subject was instructed in advance to abstain from sexual activity for two days prior to the study, and to consume his usual dose of DPH (Dilantin) on the morning of the study. Semen was obtained by masturbation, and blood samples were drawn about 10 minutes after ejaculation. The concentration of DPH in blood plasma and semen was

determined by the colorimetric method of Dill, et al. (127). Semen and plasma samples from males using no drugs were employed for blank determinations. Figure 6 shows a typical standard curve for DPH extracted from human plasma. Semen blanks did not contain substances that interfered with the assay.

Animal Studies. For each experiment, 12 μCi ^{14}C-DPH (New England Nuclear, specific activity 200 μCi/mg) were dissolved in 150 μl Dilantin ready-mixed solution (Parke-Davis, 50 mg/ml phenytoin sodium). One hundred μl of this solution were injected over a 45 second time period into an ear vein. Timed, 6.0 ml blood samples were collected from the opposite ear using a heparinized cannula situated in the central artery of the ear. Ejaculates were obtained at various times after the intravenous injection. The DPH in plasma and semen was quantified, with minor modications, by the radiolabel assay of Gerber et al.; this assay distinguishes between DPH and its major metabolites (128). The volume of the fluid portion of the semen was measured, and adjusted to 3.0 ml with water. Three ml plasma (or 3.0 ml semen diluted with water) were extracted in stoppered tubes containing 1.0 ml 2 M phosphate buffer, pH 6.8, and 40 ml 1-chlorobutane. The 40 ml of 1-chlorobutane extract were washed once with 4.0 ml 1 M phospate buffer, pH 6.8. The amount of radioactivity in a portion of the 1-chlorobutane was then determined. No animal was used more than once in a two week period to avoid enzyme induction by DPH.

6. Dipropylacetate

Assay for Dipropylacetate (DPA). DPA in biologic samples was quantified with a new gas-liquid chromatographic assay. A 1.0 ml sample

was added to 1.0 ml 1 M HCl and 20 ml 1-chlorobutane (nanograde, Burdick and Jackson) in a centrifuge tube, mechanically shaken (15 min) and centrifuged (10 min at 1500 RPM in an IEC Model K centrifuge). Fifteen ml of the organic phase were then added to 3.0 ml of 0.5 M NaOH in a centrifuge tube, and shaken and centrifuged as before. Two ml of the aqueous phase were then transferred to a Reactivial (Pierce, 5 ml) containing 1.5 ml 4 M NaCl--1 M HCl and nonanoic acid (50 µg) in chloroform (200 µl). The mixture was shaken vigorously for one minute and centrifuged at 2000 RPM for 5 minutes. Four µl of the chloroform extract were injected into a Hewlett-Packard Model 5830 A gas chromatograph equipped with a flame ionization detector and automatic integrator for determination of peak areas. The column used was 1.7 m x 2 mm i.d. glass packed with 5% Free Fatty Acid Phase on 80/100 Gas-Chrom Q (Applied Science Laboratories, State College, Penn.) with operating conditions: oven temperature, 175°; injector temperature, 185° C; detector temperature, 250° C; nitrogen carrier gas flow, 40 ml/min. Standard curves were prepared by extracting known amounts of DPA from semen or plasma and then plotting the ratios between DPA and nonanoic acid peak areas versus the amount of DPA (see Figure 7). The extraction efficiency for DPA is greater than 97% at each step. Metabolites of DPA and natural constituents of plasma and semen do not interfere with the above assay (see GLC tracing, Figure 8).

Rabbit Studies. For each experiment, sodium DPA (a gift of Abbott Laboratories, North Chicago, Ill.) in 0.9% saline was infused over a one minute period into an ear vein at a dose of 50 mg/kg. Timed, 2.0 ml blood samples were drawn from the opposite ear via an indwelling,

arterial cannula and then centrifuged in heparinized tubes. An ejaculate was also obtained after each infusion. One ml samples of blood plasma and semen were extracted and assayed for DPA immediately after collection. Ejaculates having volumes less than 1.0 ml were each diluted to 1.0 ml total volume with distilled water prior to extraction. Standard curves were prepared from rabbit plasma or semen.

Some semen and plasma samples (obtained 12 hrs after 50 mg/kg sodium DPA) were subjected to two 1-chlorobutane extractions to remove more than 99.8% of the DPA. Each 1 ml sample was then heated to 80° C in the presence of 1.0 ml 3 M NaOH for 1 hour. This treatment has been shown to hydrolyze DPA conjugates in rat urine (personal communication from R. Dickinson, Department of Pharmacology, University of Oregon Health Sciences Center). One ml of 4 M HCl was then added, and the acidified sample was extracted and analyzed for DPA as previously described.

Human Studies. One volunteer (age 28 years) consumed two 250 mg capsules of dipropylacetic acid (Abbott Labs) on four separate occasions; at least one week elapsed between successive doses. Another volunteer (age 32 years) consumed 500 mg of the drug only once. Ejaculates were obtained either by masturbation or by coitus interruptus. Care was taken to collect the complete ejaculate. Blood samples were drawn within 10 minutes of each ejaculation and stored for up to 12 hours at 4° C in citrate-fluoride Vacutainer tubes. One ml aliquots of blood plasma and semen were analyzed for DPA by the previously described methods. Standard curves were prepared from plasma acquired from the local blood bank.

C. Results

1. Methadone

Intravenous d-Methadone and Pharmacokinetics. Infusions of 40 mg d-methadone HCl had significant pharmacologic effects on male rabbits (4.0 ± 0.5 kg). Pronounced respiratory depression (as evidenced by dark arterial blood samples during the first 30 minutes after drug administration) and sedation were prominent features in all rabbits. Two of six animals experienced lethal convulsions within five minutes of the infusion period. Scott, Robbins and Chen (115) have noted in dogs and mice that the toxic effects of d-methadone are different than the toxic effects of l-methadone. Deaths due to d-methadone always occurred soon after drug administration as a result of convulsions, whereas deaths due to l-methadone were a result of prolonged respiratory depression. The current study reveals that d-methadone is also a convulsant in rabbits.

Table 2 shows the concentrations of d-methadone in blood at various times after i.v. infusions to four animals. The mean values for these data are plotted in Figure 9. The two-compartment open model has been used to describe the concentration-time curve (Table 3). The β phase half-life of d-methadone in the rabbit was 106 ± 21 minutes. The apparent volume of distribution was 10.6 liters/kg on the basis of whole blood concentrations. The volume of distribution was even larger when calculated on the basis of drug concentrations in plasma, since methadone cumulates in rabbit blood cells (plasma/blood drug concentration ratio = 0.69 ± 0.02). Due to pronounced vasoconstriction immediately following the infusion period, arterial blood samples were not obtainable via the cannula during the first 5 minutes. The concentration of d-

methadone in blood during that time may have been higher than is predicted by the calculated kinetic parameters. Therefore, the reported A and α values may be deceptively low. The reported V_d is still reasonably accurate, since a similar figure (13.7 L/kg) can be obtained by another widely employed formula: V_d = Dose/B.

Semen Experiments. The concentration of d-methadone in rabbit blood and semen (excluding the gel) after an intramuscular injection of 40 mg d-methadone HCl is shown in Figure 10. The blood concentration-time curve represents data from four experiments (four different animals) while the semen concentration-time curve includes data from 19 experiments (five animals). The absorption of d-methadone after i.m. administration appears to be rapid and complete. The peak drug concentration in blood occurred 10 minutes after the injection. The β phase half-life in blood was 121 minutes. The d-methadone concentration in ejaculates obtained soon after drug administration was lower than the concentration in concurrent blood samples. However, d-methadone continued to cumulate in seminal fluids after the drug concentration in blood began to decline. Thus, the drug concentration in semen peaked between 90 to 120 minutes after the injection. At 120 minutes the d-methadone concentration in semen was 6 to 7 times the concentration in blood. The concentration of d-methadone in the infrequently-ejaculated gel component of rabbit semen was lower than the level in the fluid portion. The fluid/gel drug concentration ratio ranged from 1.5 to 4.7.

Absorption of d-Methadone from the Vagina. d-Methadone is rapidly absorbed through the vaginal mucosa of the rabbit (Figure 11). When 40 mg d-methadone HCl was administered in a dialysis bag per vaginum,

the peak drug concentration in blood occurred 60 minutes later. Drug absorption was incomplete 90 minutes after implantation, since dialysis bags removed at that time contained about 20% of the initial drug load. This was also evidenced by the abrupt decline in drug levels in blood immediately following removal of the implants.

For one experiment, a female rabbit was mated 4 weeks prior to the vaginal absorption study. The levels of drug quantified in this animal's blood after a 40 mg intravaginal dose of d-methadone HCl were, at all times, 45 to 50% less than the levels found in non-pregnant animals. The pregnant rabbit later delivered 10 normal offspring.

Distribution of d-Methadone in the Rabbit. The concentration of d-methadone in various rabbit tissues 130 minutes after an intramuscular injection of 40 mg d-methadone HCl is shown in Table 4. Every tissue that was analyzed for methadone exhibited a drug concentration exceeding the concurrent level in blood. The d-methadone concentration was particularly high in lung, kidney and spleen. The drug levels in gel removed from the seminal vesicles of two animals were 0.316 and 0.230 μg/g, respectively.

EDDP. EDDP was apparent in extracts of rabbit blood and semen acquired after i.m. d-methadone. On the basis of GLC peak areas for EDDP, it was observed that maximal concentrations of EDDP in blood occurred 90 to 120 minutes after an i.m. injection of 40 mg d-methadone HCl and 10 minutes or sooner after an equivalent i.v. dose. High levels of EDDP were evident in kidney and lung, but not in testis, ampullary gland, seminal vesicle, spleen and heart. EDDP concentrations in semen were always much lower than concurrent levels in the blood.

Mass Spectrometry. Alleged EDDP and methadone in biologic material exhibited GLC retention times and mass spectra (Figure 12) similar if not identical to chemical standards (EDDP, Applied Science Laboratories, State College, Pa., and d-methadone HCl, Eli Lilly and Company, Indianapolis, Ind.). However, identification of EDDP in samples obtained from animals which had received methadone is not absolute by GC-MS because EDDP was found to be a minor impurity (about 0.2 to 0.3%) in d-, l- and racemic methadone. The precursor in the chemical synthesis of methadone, 4-dimethylamino-2,2-diphenylvaleronitrile, was similarly identified by GC-MS as a contaminant in all samples of methadone (see Figure 13). These latter observations were subsequently confirmed by other workers (Eli Lilly and Company Laboratories, Indianapolis, Ind.). The valeronitrile compound constitutes up to 3% of Dolophine HCl (marketed methadone) (see Figure 14). The presence of this impurity may be of clinical importance, since we have observed that the valeronitrile compound is neurotoxic when given to mice intraperitoneally. Only trace amounts of the valeronitrile were found in the samples of d- and l-methadone HCl.

2. Phenytoin

DPH in Human Plasma and Semen. Data obtained from nine epileptic subjects receiving oral Dilantin conclusively demonstrated that DPH is excreted into human semen (Table 5). However, the concentration of DPH in semen is much lower than the concurrent concentration in plasma, as indicated by an average semen/plasma concentration ratio of 0.17.

Five of the nine human subjects had ejaculate volumes distinctly below the normal 3.2 ml (129).

DPH in Rabbit Plasma and Semen. Figure 15 shows the decline of DPH in rabbit plasma and semen after a single intravenous dose (8 μCi, specific activity 1.73 μCi/mg). The concentration-time curve for DPH in semen is parallel to the concentration-time curve for DPH in plasma. The correlation coefficient (least-squares method) between semen and plasma DPH concentrations is 0.964 (see Figure 16). Sixty minutes after drug administration, both curves conform to first order elimination kinetics. The semen/plasma drug concentration ratio approximates 0.2 throughout the experiment; this is about the same ratio observed for DPH in man. Ejaculation of an animal early in an experiment did not significantly alter the DPH concentration in a second ejaculate collected two or more hours later. For example, one animal ejaculated twice during an experiment, at 180 and 300 minutes after drug administration, resulting in DPH semen concentrations of 119 and 63 ng/ml respectively. In a subsequent experiment using the same animal, an initial ejaculate at 300 minutes had a drug concentration of 74 ng/ml.

Table 6 presents the actual drug concentrations measured in rabbit plasma after intravenous DPH. The two-compartment open model was used to interpret the data for each rabbit (Table 7). Variation in the $t_{1/2}{}^{\alpha}$ may in part reflect fluctuations in the rate of dispersion of the viscous ready-mix Dilantin (40% propylene glycol, 10% ethanol, 50% water, pH 12). Variation in the $t_{1/2}{}^{\beta}$ represents true biologic differences between rabbits, since the $t_{1/2}{}^{\beta}$ for an individual animal remained the same in subsequent experiments.

3. Dipropylacetate

Rabbit Studies. Figure 17 shows the concentration of DPA in rabbit semen and plasma at various times after an i.v. infusion of sodium DPA (50 mg/kg). Data for individual animals are presented in Table 8. The distribution or α phase in plasma was brief and ill-defined. The mean β phase half-life and volume of distribution for 4 rabbits were 56 ± 6 min and 0.21 ± 0.05 L/kg, respectively. DPA concentrations in plasma were clearly much greater than concurrent drug concentrations in semen. The concentration-time curve for DPA in semen was approximately parallel to the concentration-time curve for DPA in plasma, indicating that drug levels in semen were proportional to drug levels in plasma and that equilibration of DPA between the plasma and semen compartments was rapid.

Alkaline hydrolysis of DPA semen and plasma samples did not liberate free DPA, indicating that DPA conjugates were absent or present in very low concentrations.

Human Studies. DPA achieved concentrations in human semen that were much lower than concurrent levels in plasma (Table 9). The semen/plasma drug concentration ratio for both subjects ranged from 0.058 to 0.091. The mean ratio for all the paired samples was 0.072. The time course of drug absorption and elimination appeared to be similar in the two subjects.

D. Discussion

1. Improved Drug Assays for Semen Studies

Most of the previous studies on the excretion of drugs into seminal fluids have employed drug assays which cannot distinguish between drugs and their metabolites. This problem has now been circumvented through the use of extraction and chromatographic procedures that separate drugs from their metabolites as well as from interfering biologic substances. In the case of DPH, the colorimetric assay of Dill *et al.* (127) and the radiolabel assay of Gerber *et al.* (128) were used; both of these assays are of proven specificity. New assays were developed for quantifying methadone and DPA; the specificity of these assays is established by virtue of gas-liquid chromatographic separation of the parent drugs from their respective metabolites.

The extinction coefficient of methadone is too low to permit its routine measurement by U.V. spectrophotometry. Oxidation of the drug to a benzophenone with barium peroxide or cerium sulphate increases the degree of U.V. absorption (130); however, the sensitivity of this method is still insufficient to allow quantification of therapeutic levels of methadone in blood. Gas chromatography with flame ionization detection is now the most frequently used method for determining methadone concentrations in biologic material (126, 131-134). Assay sensitivity can be increased by using an electron capture detector (130). Mass fragmentography (135) and radioimmunoassay (136) are also utilized for measuring methadone concentrations. The GLC method that is introduced at this time is more rapid than most of the previous assays and is sensitive to approximately 10 ng on the GLC column. It incorporates the

extraction methods of Lynn et al. (126), but in contrast, employs an internal standard that is easier to obtain.

A variety of gas chromatographic assays have been used for quantifying DPA in biologic samples (137-139). Only the technique of Loscher is more rapid than the one introduced at this time. The one-step extraction procedure of Löscher would not be acceptable for measuring DPA in tissue homogenates because of excessive column contamination.

2. Comparison Between Rabbits and Man

Besides employing better assays for quantifying drugs in semen, the current investigation has introduced an animal model suitable for studying drug excretion into semen. Three drugs (d-methadone, DPH and DPA) have been quantified in blood samples and ejaculates from rabbits and human subjects. The data obtained in rabbits correlates well with the data acquired in men. For example, the mean semen/plasma drug concentration ratio (S/P) for DPH was 0.20 in the rabbit and 0.17 in man. The same ratio for another drug, DPA, was 0.04 in the rabbit and 0.07 in man. The degree of agreement between the two species was less striking for methadone. The mean semen/blood drug concentration ratio (S/B) for methadone in man was reportedly 1.8 (107). This ratio in the rabbit was time dependent and ranged between 6 to 10 at later times. The apparent species difference may be an artifact of the time in which the human samples were collected. The ejaculates and blood samples were obtained from men at a time when levels in blood would be maximal (about 2 to 3 hours after an oral dose). However, the data from rabbits clearly shows that d-methadone concentrations in seminal fluids continue to rise after drug levels in blood begin to decline. Thus, the

S/B for methadone in rabbits continues to increase after the peak drug concentration in blood has occurred. The observed S/B for methadone in man might well have been greater, if ejaculates and blood samples had been acquired from men at later times. The overall conclusion is that experimental data obtained in rabbits can be used to predict with fair to good accuracy the concentrations that drugs will achieve in human semen.

3. Ion-Trapping of Drugs in Seminal Fluids

The persuasive evidence for ion-trapping of weak bases in prostatic fluids has been reviewed earlier (See Introduction). This ion-trapping hypothesis does not necessarily predict drug levels in the ejaculate because prostatic fluid is highly diluted during ejaculation by secretions from several organs of reproduction. For example, if weak bases are nearly excluded from seminal vesicular or bulbourethral gland secretions, then extensive drug cumulation in prostatic fluid might not be readily apparent in the ejaculate. Nonetheless, the data for d-methadone, DPH and DPA in both the rabbit and man now indicate that ion-trapping may be important in the regulation of drug levels in semen. The weak base, methadone, cumulates in seminal fluids to levels that exceed concurrent concentrations in blood. The weak acids, DPH and DPA, achieve much lower concentrations in semen than in plasma. If seminal fluids are treated as a single compartment with a pH equal to that of semen, then a formula derived from the Henderson-Hasselbalch equation can be used to describe the relative drug concentrations in the vascular and seminal fluid compartments. Thus, the drug concentration ratio for two compartments (Sides 1 and 2) separated by a lipid membrane

equals $\frac{1 + 10^{pK_a - pH_1}}{1 + 10^{pK_a - pH_2}}$ for weak bases

and equals $\frac{1 + 10^{pH_1 - pK_a}}{1 + 10^{pH_2 - pK_a}}$ for weak acids.

The pH of rabbit semen is 6.6, and the pH of human semen is about 7.1. The predicted S/B for methadone is 6.0 in the rabbit and 1.9 in man, while the actual values were 6 to 10 in the rabbit and 1.8 in man. The predicted S/P for DPH in the rabbit and man are 0.91 and 0.95, respectively. The actual S/P values were 0.20 in the rabbit and 0.17 in man. For DPA, the predicted S/P values are 0.16 in the rabbit and 0.50 in man. The actual S/P values for DPA were, respectively, 0.04 and 0.07. The ion-trapping model accurately predicts differences between the two species. Furthermore, the correlation between the actual and predicted ratios is excellent for methadone. The two weak acids achieve levels in semen that are lower than anticipated. The explanation for this discrepancy may possibly be found in the results of experiments in which antibiotics were measured in human split ejaculates (37, 105). Zinc and antibiotic concentrations were determined for each ejaculate fraction, and a judgement was made as to which reproductive organ(s) was responsible for excreting the drug (see Introduction). Weak acids (e.g., ampicillin and sulfamethoxazole) appear to achieve only low concentrations in prostatic fluid and are nearly excluded from seminal vesicular fluid. Thus, the concentrations of weak acids in the ejaculate are extremely low because small quantities of weak acids excreted by the prostate are highly diluted by the relatively drug-free secretions of the seminal vesicles. In contrast, weak bases (e.g., erythromycin and trimethoprim)

appear to reach high concentrations in human prostatic fluid and are also excreted by the seminal vesicles. The concentrations of weak bases are high in the ejaculate because the drug-rich prostatic fluid is diluted with seminal vesicular secretions that already contain appreciable levels of drug. Extrapolating to the current data, small amounts of DPH and DPA from the prostate may experience considerable dilution by drug-free secretions from other accessory sex organs. The weak base, methadone may be excreted into both prostatic and seminal vesicular fluids--a hypothesis that is supported by data obtained in the rabbit. d-Methadone achieves high concentrations in the glandula vesicularis as well as in the prostate. Also, the narcotic was found to enter the seminal vesicular gel prior to ejaculation.

4. Influence of Lipid Solubility and Tissue Binding of Drugs

Winningham and his co-workers (97) were the first to suggest that drug lipid solubility was important for movement of drugs into dog prostatic fluids. They later found that sulfonamide cumulation in these fluids was more dependent on drug pKa values than on lipid solubility of the unionized drugs (98). This is not surprising since the lipid solubility of an unionized weak base or weak acid would probably influence the rate of equilibration between two body compartments more than the final equilibrium. The unionized form of DPH has good lipid solubility, and DPH is only about 10% ionized at the pH of plasma. Therefore, DPH should move rapidly out of plasma into reproductive organs and establish an equilibrium between plasma and seminal fluids soon after drug administration. This concept is corroborated by data obtained in the rabbit (Figure 15). In contrast, the equilibration of methadone into seminal

fluids is likely to be delayed because methadone is nearly 94% ionized at the pH of plasma. Furthermore, relatively more methadone must enter the seminal fluids before equilibrium is established, since the S/B for methadone is large while the S/P for DPH is small. Again, the actual data acquired in rabbits supports this theory. The binding of drugs to plasma proteins reportedly inhibits the diffusion of drugs out of the vascular compartment. This particular factor does not influence the above comparison between DPH and methadone because both drugs are comparably bound to plasma proteins (140, 141).

The possible importance of hydrogen ion gradients in the regulation of drug equilibration between plasma and seminal fluid compartments has been discussed already. However, semen and blood plasma exhibit many other differences that could account for fluctuations in semen/plasma drug concentration ratios. The total protein concentration in blood plasma is 73 mg/ml, while the protein concentration in seminal plasma is 42 mg/ml (1). Over 90% of the protein in blood plasma is non-dialysable; of the non-dialysable proteins, albumin is the most abundant (concentration = 42 mg/ml). In contrast, only 40% of the protein in seminal plasma is non-dialysable, and albumin constitutes only 20% of the non-dialysable, seminal protein (1, 33). Drugs that are highly bound to plasma proteins (and to albumin in particular) may tend to achieve higher concentrations in blood plasma than in semen. Methadone appears to be an exception to this hypothesis. However, distribution studies for methadone clearly demonstrate that any tendency of this drug to be sequestered in blood plasma by binding to plasma proteins is insignificant compared to non-specific binding to chemicals in or on cells. Thus, methadone reaches high concentrations in many tissues relative to concentrations in blood

or plasma (Table 4; references 146-149). A plasma/blood drug concentration ratio of 0.69 for d-methadone in the rabbit indicates that d-methadone binds to or cumulates in blood cells. The extensive cumulation of methadone in many tissues and secretions (including semen) may be due to non-specific drug-binding to proteins or lipids that are more abundant outside than inside the plasma compartment. It is also conceivable that specific opiate receptors exist in semen and that methadone associates with these receptors. In addition, methadone may bind extensively to spermatozoa. For example, thalidomide reportedly binds to rabbit spermatozoa (76).

5. Active Transport into Seminal Fluids?

Several epithelia are noted for their ability to actively transport small molecules. Current evidence argues against the active transport of drugs into seminal fluids. Furthermore, foreign compounds probably enter prostatic and seminal vesicular fluids by simple diffusion without prior packaging in secretory vacuoles. These issues were initially considered by Stamey, Bushby and Bragonje (96). They demonstrated in dogs that the prostatic fluid/serum drug concentration ratio for trimethoprim remained nearly constant throughout individual experiments, despite large fluctuations in drug concentrations in serum. Trimethoprim appeared to move freely both into and out of prostatic fluids; this phenomenon seems incompatible with unidirectional secretory processes. One characteristic of an active transport mechanism is its saturability. The movement of trimethoprim into dog prostatic fluid was unencumbered even at drug concentrations as high as 1.56 mg/ml. The data on DPH and DPA in the rabbit similarly refutes the possibility of active or secretory transport of drugs into seminal fluids. Figures 15 and 17 show that DPH and DPA concentrations in semen decline at approximately the same rate

as drug concentrations in plasma. The seminal fluid and plasma compartments are therefore in bidirectional equilibrium. The situation for d-methadone is less clear (Figure 9) because d-methadone does not immediately equilibrate between blood and seminal fluids. However, the d-methadone concentration curves in semen and blood are parallel enough after 120 minutes to suggest that d-methadone is capable in vivo of diffusing out of seminal fluids. Furthermore, the difference between the drug concentrations in seminal fluids and the drug concentration in blood can be adequately explained on the basis of passive processes alone.

6. Drug Entry Into Rete Testis Fluid

The drugs quantified in semen are probably derived, in part, from the testis. The entry of foreign compounds into seminiferous tubule fluid is important to consider because of possible toxic effects on spermatozoa. Several investigators have quantified xenobiotics in rete testis fluid (142-144). The inability of certain dyes to enter rete testis fluid suggested the existence of a blood-testis barrier (145). Thus, spermatozoa in the seminiferous tubules and rete testis are afforded some degree of protection from toxic foreign chemicals. However, many lipid soluble drugs can readily penetrate the blood-testis barrier (143).

7. Fate of Methadone Excreted Into Semen

Methadone that is excreted into semen is probably absorbed through the vaginal mucosa after coitus. This follows from Figure 11, which illustrates the rapid appearance of d-methadone in arterial blood of rabbits after intravaginal drug administration. Experiments by previous workers on the vaginal absorption of drugs have been primarily

qualitative and only vaguely oriented toward the time course of absorption. In contrast, the present study on d-methadone in rabbits permited certain useful observations on the kinetics of vaginal absorption processes. For example, the rate of d-methadone absorption after intravaginal administration was only slightly slower than after an i.m. injection (Figure 10). The rate of absorption through the vaginal mucosa appeared to decrease substantially during pregnancy, although this phenomenon was not rigorously established.

8. Distribution of Methadone in vivo

Previous studies on the distribution of methadone in mammalian species have not described drug concentrations in the seminal vesicles, prostate or testis (133, 146-149). The current investigation in rabbits demonstrates that d-methadone achieves high concentrations in reproductive organs relative to levels in the blood (Table 4). Narcotic analgesics are known to disrupt reproductive function in males (23); elevated levels of methadone in male sex organs may have a role in the etiology of these adverse effects. For example, methadone interferes with carbohydrate metabolism in the prostate (114) and steroidogenesis in the testis (113) in vitro. Other investigators have found uniformly high concentrations of methadone in spleen, lung, kidney, and liver of rats, dogs and humans after both peroral and parenteral doses. The present distribution study in rabbits is consistent with these observations except that the drug level in rabbit liver is low. Some of the earlier workers used radiolabeled methadone and did not distinguish between methadone and methadone metabolites. The contribution of metabolites to the total radioactivity could have been significant in some tissues. However, this possibility was ruled out in the case of rabbit liver by

administering ^{14}C-d,l-methadone to rabbits and measuring radioactivity in various tissues. Total counts per weight of liver were far below the counts per weight of tissue for spleen, kidney and lung.

Since the current distribution study reports drug concentrations at only one point in time (130 minutes after an i.m. injection), it is not possible to speculate on the actual time course of drug equilibration into and out of tissues. It is likely that rates of d-methadone cumulation and release differ considerably among the various tissues and that relative drug concentrations in the tissues change over time.

9. Summary

In summary, a study was undertaken to describe the time-course and extent of drug cumulation in seminal fluids. New gas-liquid chromatographic assays were devised for DPA and methadone. DPA, DPH and d-methadone were administered to rabbits and then quantified in semen and in plasma or blood. The β phase half-lives of DPA, DPH and d-methadone were found to be 56, 171 and 106 minutes, respectively. d-Methadone (a weak base) achieved high concentrations in semen relative to blood, whereas DPA and DPH (weak acids) reached much lower levels in semen than in plasma. DPA and DPH were also quantified in semen and plasma from human subjects who had consumed the drugs. Again, the concentrations of DPA and DPH in semen were low relative to the drug concentrations in plasma. These data, together with information from previous workers, provide evidence that ion-trapping may be important in the cumulation of drugs in seminal fluids. In view of the good correlation between the data obtained in rabbits and the data acquired in man, the rabbit appears to be a suitable animal model for studying drug excretion into semen and for predicting drug concentrations in human ejaculates. d-Methadone was

shown to be rapidly absorbed through the vaginal mucosa of the rabbit. Therefore, d-methadone that is excreted into semen is likely to enter the circulation of the female after coitus. d-Methadone was quantified in various tissues of the rabbit 130 minutes after an intramuscular dose; the drug was highly concentrated in spleen, lung and kidney and moderately concentrated in the testis, prostate and seminal vesicles.

II. EFFECT OF DRUGS ON SPERM MOTILITY

A. Introduction

1. Sperm Motility

The mammalian spermatozoan is propelled by undulating, sinusoidal movements of its tail (150). The tail consists of a mitochondrial sheath and a flagellum. Transverse sections of the sperm flagellum exhibit a pattern of microtubules and matrix components which is identical to that observed for cilia found in the lung, nasal passages and ductuli efferentes (151). Thus, there are 9 microtubule doublets evenly spaced around a central pair of single microtubules. Human spermatozoa undergo a gradual maturation process during their 1 to 3 week journey through the epididymis (152). Sperm obtained from the head of the epididymis are incapable of self propulsion whereas sperm obtained from the more distal segments have acquired primitive patterns of motility (153). Sperm do not exhibit vigorous motility until after ejaculation when they are exposed to components of seminal plasma (154). Additional increases in sperm activity are evoked by follicular fluid and secretions of the female reproductive tract (155).

2. Drug Effects on Sperm Motility

The possibility that drugs excreted into semen can alter sperm motility has not been investigated. However, a variety of physiologic

ions and foreign compounds have been shown to influence sperm motility *in vitro* (150). Nitrofurazone and nitrofurantoin can immobilize sperm, but only at extremely high concentrations (156). Chelating agents can disrupt sperm motility at much lower concentrations (157, 158). Cyclic nucleotides and drugs that increase the intracellular concentration of cyclic nucleotides (e.g., caffeine and theophylline) enhance and prolong sperm activity (159, 160). Peterson and Freund (161-163) have shown that several secondary and tertiary amines can diminish sperm motility *in vitro*. These included propranolol, lidocaine, chlorpromazine, atropine, diphenhydramine, phentolamine, and phenoxybenzamine. Chlorpromazine was one of the more potent inhibitors and could abolish sperm motility within 15 minutes at a concentration of 0.2 mM. Quinine and ouabain are also known to decrease sperm motility (162). It is curious that the amine, chloroquine, can stimulate respiration and motility of spermatozoa (164).

3. Statement of the Problem

Dipropylacetate, phenytoin and methadone are excreted into the seminal fluids of rabbits and man (see section on Excretion of Drugs into Semen). It was possible that these drugs could diminish sperm motility at concentrations achieved in the human ejaculate. Therefore, the effects of these drugs on sperm motility were evaluated *in vitro*. In the case of methadone, it was interesting to compare the (d-) and (l-) isomers and to ascertain if any effects on sperm could be prevented by simultaneous treatment with a narcotic antagonist. In order to demonstrate that the methodology in these experiments could generate results comparable to those of Peterson and Freund, a class of drugs already included in their studies (i.e. phenothiazines) was investigated.

B. Material and Methods

1. Drug Solutions

All drugs were dissolved in distilled water, except for sodium phenytoin which required the addition of a small amount of NaOH for dissolution. The drugs included: d-, l-, and racemic methadone HCl (Eli Lilly, Indianapolis, Ind.), sodium phenytoin (Parke, Davis and Company, Detroit, Mich.), sodium dipropylacetate (Abbott Laboratories, North Chicago, Ill.), chlorpromazine HCl (Smith, Kline and French, Philadelphia, Pa.), trifluoperazine di-HCl (Smith, Kline and French, Philadelphia, Pa.), desipramine HCl (Geigy Pharmaceuticals, Ardsley, N.Y.), and naloxone HCl (Endo Laboratories, Garden City, N.Y.).

2. Semen Incubations

Rabbit ejaculates were obtained as previously described. The semen was diluted 10-fold in Baker's buffered glucose solution (15 g glucose, 1.77 g Na_2HPO_4, 1.0 g NaCl, 0.15 g KH_2PO_4 in 500 ml distilled water, pH 7.0) and maintained at 37° C in a water bath. For each drug, 200 µl of diluted semen and 50 µl of drug solution were mixed together and incubated at 37° C. Tubes containing 200 µl diluted semen and 50 µl distilled water served as the control incubations. Each drug-treated tube was paired with a simultaneously-prepared control tube. After 30 minutes, a matched pair of tubes was agitated briefly, and samples from the drug-treated and control incubations were placed in opposite chambers of a Levy hemocytometer equipped with double Neubauer ruling (Hausser Scientific). The number of motile and nonmotile sperm in 3 groups of 16 squares was determined for each sample. Motility was defined as movement of the tail, regardless of the degree of forward progression. The results were expressed as a percentage (to the nearest 20%) of control values.

C. Results

The effects of various drugs on sperm motility are summarized in Table 10. The two phenothiazine antipsychotics, chlorpromazine and trifluoperazine, completely immobilized rabbit spermatozoa within 30 minutes at a concentration of 1.0 mM. A lower concentration (0.01 mM) had little or no effect. These results are in agreement with those of Peterson and Freund, who found that 0.2 mM chlorpromazine inactivated sperm (161). The tricyclic antidepressant, desipramine, could diminish sperm motility but was less potent in this regard than the two phenothiazines. High concentrations of DPA (2.0 mM) and of DPH (0.2 mM) had no effect on sperm motility. Both isomers of methadone could abolish sperm motility, and the effect of l-methadone was not antagonized by naloxone. Naloxone by itself had no effect on sperm motility. A dose-response curve for sperm-immobilization by l-methadone is shown in Figure 18.

D. Discussion

The quantities of DPH, methadone and DPA that are excreted into human semen are insufficient to diminish sperm motility in the ejaculate. The maximum concentration of DPH measured in semen from epileptic males was 6 µg/ml (see Table 5). This is too low to adversely affect sperm motility, since a concentration as high as 50 µg/ml did not diminish sperm motility *in vitro*. The highest concentration of methadone found in semen from individuals in the methadone maintenance program was 0.42 µg/ml (107). This concentration is far below the sperm-immobilizing levels (> 35 µg/ml) determined *in vitro*. DPA at a concentration of 288 µg/ml was ineffective in altering sperm motility.

To achieve this concentration of DPA in the ejaculate, the plasma level of DPA would have to be increased to over 4000 µg/ml (see Table 9). Such drug concentrations are unlikely to be found in the plasma of epileptic subjects, since the therapeutic plasma level of the drug is 50 to 80 µg/ml.

The adverse effect of methadone on sperm motility does not appear to be a specific narcotic action (165). The (l-) isomer of methadone is usually several times more potent than the (d-) isomer (115, 116). However, both isomers of methadone were able to abolish sperm motility at or near a drug concentration of 1.0 mM. True narcotic actions are also typically reversed or prevented by narcotic antagonists. An equimolar concentration of naloxone failed to block the sperm-immobilizing effect of l-methadone.

Most of the drugs that decreased sperm motility exhibit chemical properties which would favor their cumulation in seminal fluids. Thus, the phenothiazines, tricyclic antidepressants and narcotics are all weak bases with high lipid solubility. Chlorpromazine is particularly noteworthy because it is administered in large doses (up to 1 or more g/day), achieves high concentrations in many organs (especially the lung), and is retained in body fat for several months after cessation of drug therapy (166). Chlorpromazine levels in ejaculates of men receiving the drug may well exceed the threshold for sperm-immobilizing activity.

III. DRUG METABOLISM IN THE TESTIS

A. Introduction

1. Location of Steroidogenic Enzymes in the Testis

The testis is comprised of primarily two anatomical components: the seminiferous tubules and the interstitial tissue. The interstitial tissue can be further divided into Leydig cells and general stroma. The tubules are the predominant structures in all mammalian testes, although there are considerable species differences in the fraction of testicular volume occupied by the tubules. At one extreme is the boar testis, in which the tubules and Leydig cells make up, respectively, 60% and 37% of the testicular volume (167). Rat testes are, by weight, nearly 90% seminiferous tubules and only 6% Leydig cells (168). Human testes consist, by volume, of 66% seminiferous tubules and 3% Leydig cells (169). Steroidogenesis in the testis occurs chiefly in the Leydig cells. In the rodent, selective destruction of testicular germ cells with radiation leaves the androgen status of the animal intact. Histochemical methods have proven that certain oxidative enzymes involved in steroid synthesis are localized principally in Leydig cells (170). Seminiferous tubules in the rat can be cleanly separated from the interstitial tissue (168); *in vitro* incubations of the dissected tissues have demonstrated that most of the steroidogenic activity is situated in the interstitial tissue rather than in the seminiferous tubules (168, 171).

2. Effect of Gonadotropins on Steroidogenesis

The rate of steroid synthesis in the testis is regulated by pituitary gonadotropins, primarily luteinizing hormone (LH, interstitial cell stimulating hormone) (167, 172). The level of circulating LH is in

turn controlled by a feed-back inhibition mechanism in which androgens and estrogens suppress the release of gonadotropin from the pituitary (173). Hypophysectomy of adult rats causes a marked decrease in testicular cytochrome P-450 and in steroidogenic enzymes (174). Administration of human chorionic gonadotropin (HCG) can prevent these changes in testicular biochemistry (175-177). Furthermore, steroid-synthesizing enzymes can be induced in immature rats by chronic administration of HCG (178, 179). In adult rats with intact pituitaries, HCG can increase testicular protein synthesis, cytochrome P-450 and the activities of several enzymes, including: 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase, steroid 17,20-lyase, 5α-steroid reductase and β-hydroxybutyrate dehydrogenase (170, 174, 175, 180, 181).

3. Effect of Sex Steroids on the Testis

Androgens and estrogens both decrease steroidogenesis in the testis. This effect is due, at least in part, to the suppression of pituitary gonadotropin secretion (167, 173). In the rat, small doses of testosterone cause testicular atrophy, whereas large doses do not (3). Gonadotropin secretion is inhibited by relatively low as well as high doses of testosterone, but at high doses the stimulatory effect of testosterone on seminiferous tubule development and maintenance is predominant (182). The integrity of Leydig cells is maintained by LH, and, therefore, both high and low doses of testosterone cause regression of these cells. Thus, hypophysectomized rats can continue to produce spermatozoa despite atrophy of Leydig cells if they are supplemented with large amounts of androgen (183). Histochemical studies have shown that testosterone administration decreases the activity of some

steroidogenic enzymes in the testis (170). Chronic testosterone administration dramatically decreases cytochrome P-450 levels in the rat testis (184). Estrogens can cause testicular atrophy in several mammalian species, including man (185-187). Low doses of estrogen decrease the activity of numerous testicular enzymes, for example, 17β-hydroxysteroid dehydrogenase, steroid 17,20-lyase, and 17α-hydroxylase (188, 189). These effects can result independently of estrogen-pituitary interactions, since the depression in enzyme activity occurs in hypophysectomized rats that are maintained on constant doses of gonadotropins.

4. Drug Metabolism by the Testis?

The liver is recognized as the chief drug-metabolizing organ in the body (190). Mixed-function oxidases in hepatocytes can metabolize a wide variety of drug substrates. Leydig cells of the testis exhibit several ultrastructural and biochemical features that also are prominent in hepatocytes. Both cell types possess an abundant smooth endoplasmic reticulum (SER) (190, 191). Microsomes derived from this SER are rich in enzymatic activity. The hepatic microsomal enzymes metabolize steroids and foreign compounds, including drugs (192); the testicular microsomal enzymes are essential for steroidogenesis (167). The activity of many microsomal enzymes from liver and testis is dependent on cytochrome P-450, NADPH, molecular oxygen and magnesium ion; are inducible; and are inhibited by carbon monoxide (167, 174, 190, 193-197). In view of these similarites between Leydig cells and hepatocytes, it is somewhat surprising that no previous investigations have focused on the drug-metabolizing capability of the testis. Only two groups of workers have noted microsomal metabolism of foreign compounds by the testis. In

1962, Wattenberg and Leong provided histochemical evidence that testicular interstitial cells of the rat can metabolize perylene (a polycyclic hydrocarbon), but only after pretreatment of the rats with methylcholanthrene (198). Heinze et al. later showed that microsomes from boar testis can N-hydroxylate aniline and N-deethylate N-ethylaniline (199).

5. Enzymatic Activation of Toxins

Enzymes are important in the generation of many potent cytotoxins, mutagens and carcinogens (200-203). Toxic metabolites of foreign compounds are often highly reactive, unstable compounds that exert their adverse effects at or near their site of formation. Therefore, organs that possess substantial quantities of SER and mixed-function oxidases become victims of their own metabolism. Many lipid-soluble compounds cumulate in the liver and subsequently initiate hepatic necrosis. The possibility that chemical-induced, testicular atrophy may result from the conversion of chemicals into cytotoxins, has been overlooked (16, 204). For example, carbon tetrachloride causes extensive damage to both the liver (205) and the testis (206, 207). The hepatic necrosis is unquestionably related to the formation of toxic metabolites, whereas the testicular atrophy is attributed, perhaps mistakenly, to the suppression of gonadotropin release from the pituitary.

6. Esterases of the Testis

There are prolific amounts of non-specific carboxylesterases in the mammalian testis. Their physiologic role is, presumably, to hydrolyze cholesterol esters, thus providing cholesterol for steroidogenesis. Testicular esterases have been characterized according to

distribution within the testis, response to hypophysectomy, susceptibility to enzyme inhibitors, and substrate specificity. Holmes and Masters (208) have found 10 different carboxylesterases in the 100,000 X g supernatant of rat testis homogenates. The 100,000 X g pellet (i.e., the microsomes) also contains substantial quantities of esterases (209). Using p-nitrophenyl propionate as a substrate, Huggins and Moulton (210) showed that the testis ranked 4th or 5th in total esterase activity (units/g tissue) among 22 rat tissues. They noted that testicular esterases decreased after hypophysectomy and that administration of gonadotropins blocked this diminution in esterase activity. Also, the development of peak esterase activity in the rat testis coincided chronologically with the onset of sexual maturation. Huggins and Moulton postulated that testicular esterase activity paralleled the degree of steroid synthesis in the testis. This hypothesis was supported by subsequent experiments which showed that both the esterases and the steroid-synthesizing enzymes develop prematurely in rat testes after systemic doses of HCG (179, 211). Histochemical studies have shown that testicular esterases are located primarily in the Leydig cells, although a lesser amount can be found in the Sertoli cells of the seminiferous tubules (212). Esterases in the Leydig cells are of the B-type esterases of Aldridge (213) (i.e., sensitive to the inhibitor E 600) and are diminished by hypophysectomy (214). Esterases of the Sertoli cells are of the C-type and are unaffected or increased by hypophysectomy. Estrogen administration decreases testicular esterase activity (215). The role of testicular esterases in the biotransformation of drugs has not been studied.

7. Effect of Drugs on Testicular Enzymes

Drugs can potentially influence metabolic processes in the testis via several mechanisms. Alteration of gonadotropin secretion is a not uncommon side effect of drugs that act upon the central nervous system (e.g., barbiturates, phenothiazine antipsychotics, ethanol and opiates) (16, 28, 216, 217). Clomiphene, an estrogen antagonist, blocks feedback inhibition of gonadotropin release, elevates plasma levels of LH and FSH, causes hyperplasia and hypertrophy of Leydig cells and increases synthesis of testosterone (218). On the other hand, cyproterone, an androgen antagonist, diminishes circulating LH and decreases steroidogenesis in the testis (219). Direct effects of drugs on testicular enzymes have been seldomly investigated. Morphine can inhibit 3β-ol-dehydrogenase activity in testicular homogenates (113). Clomiphene can stimulate synthesis of steroids when added to testis incubations (220). The fact that spironolactone has adverse effects on reproductive organs of rats, led Menard, Stripp and Gillette to investigate the effect of this drug on testicular cytochrome P-450 and associated enzymes (184). In rats, a single injection of spironolactone caused a 70 to 80% decline in cytochrome P-450 concentration after 16 hours and a substantial decrease in 17α-hydroxylase activity. Similar effects were noted in four other species. For various reasons, the effects of spironolactone on cytochrome P-450 and steroidogenesis in the testis were thought to occur independently of the hypothalamus-pituitary-testis axis. Direct drug effects on protein synthesis and/or carbohydrate metabolism in the testis have been demonstrated for chlorpromazine, clomiphene and cannabinoids (31, 221, 222).

8. Effect of Narcotics on Reproductive Organs

There is a high incidence of sexual dysfunction among men who are addicted to narcotic analgesics (23, 24). This disturbance is not simply a result of poor diet, narcosis or neuroses, but stems from pharmacologic effects on the reproductive organs. In 1961, Hohlweg et al. administered morphine to rats for 3 weeks and found a pronounced, selective diminution in the size of the prostate and seminal vesicles but not the testis (25). It was later reported that human ejaculate volumes and libido are reduced by narcotics. These adverse effects on male accessory sex organs and sex-drive are apparently a result of decreased steroidogenesis in the testis. Narcotic addicts have subnormal plasma concentrations of testosterone (223, 224). Chronic and acute doses of methadone cause a reduction in serum testosterone levels in rats (225). There is evidence for both direct and indirect narcotic effects on testicular steroidogenesis. In vitro, methadone can inhibit steroid-synthesizing enzymes from the testis (113) and the adrenal (226, 227). Narcotics also interfere with hepatic microsomal metabolism (228, 229). It is possible that narcotics serve as alternative substrates for steroid-metabolizing enzymes, and act as competitive inhibitors of steroidogenesis. An indirect effect of narcotics on testosterone synthesis is strongly suggested by experiments that have shown diminution of LH levels in plasma in response to narcotic administration (28, 230, 231). Direct narcotic effects on the accessory sex glands are also a possibility. Dombrosky et al. reported that morphine can alter carbohydrate metabolism in mouse prostate (114). La Bella has noted that opiates and steroid hormones are similar in many respects (23). They

"are both derivatives of perhydrophenanthrene and may produce euphoria, nausea, vomiting, analgesia, sedation, hypo- or hyperthermia, convulsions, respiratory depression, inhibition of the hypothalamus-pituitary axis and permanent effects on sexual development and behavior". La Bella demonstrated that narcotics can specifically displace testosterone from hepatic microsomes. Although not proven, narcotics may displace androgens from receptors in male accessory sex organs, thereby functioning as an anti-androgen.

9. Biotransformation of Methadone

In 1956, Axelrod found that formaldehyde was liberated from incubations containing liver microsomes and methadone (233). This suggested that methadone undergoes enzymatic N-demethylation. However, N-desmethylmethadone has never been isolated, despite rigorous attempts to do so (234). Efforts to synthesize N-desmethylmethadone have never yielded the desired product, and instead, frequently resulted in the formation of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (235). In 1951, Way et al. (236) had isolated an unidentified basic metabolite of methadone from rat bile. The infrared spectrum of this metabolite was found to be similar, if not identical, to the spectrum of EDDP (237). Beckett et al. (238) and later Pohland, Boaz and Sullivan (239) have provided evidence that EDDP is in fact a major metabolite of methadone. It is likely that N-desmethylmethadone is highly unstable and spontaneously cyclyzes to form EDDP (see Figure 5). Besides N-demethylation, methadone can undergo hydroxylation, keto-reduction and C-deethylation (235). EDDP can be further N-demethylated or hydroxylated.

10. SKF 525-A

In the early 1950's, SKF 525-A (diethylaminoethyl diphenylvalerate HCl) was found to potentiate the pharmacologic actions of narcotic analgesics (240), anticonvulsants (241), hypnotics (242) and central nervous system stimulants (243). Since then, SKF 525-A has been shown to inhibit a variety of drug-metabolizing enzymes, including mixed function oxidases, glucuronyl transferase, nitroreductase and esterases (244-246). Thus, SKF 525-A is known to cause both competitive and noncompetitive inhibition of drug metabolism in vitro (246, 247). Possible mechanisms for the inhibition include: 1) uncoupling of the electron transport system in microsomes, 2) blockade of the entry of drug substrates into the microsomes, 3) occupation of substrate-binding sites on cytochrome P-450 resulting in competitive inhibition, and 4) alteration of the enzyme protein resulting in noncompetitive inhibition. The "alternate substrate" hypothesis (#3 above) is certainly feasible since SKF 525-A is rapidly N-deethylated by hepatic microsomes (248, 249). The secondary amine metabolite (SKF 8742-A) can undergo further N-dealkylation to form the primary amine compound. SKF 525-A is also deesterified in the liver (249). The ability of SKF 525-A to bind tightly to microsomes (250) and to inhibit a variety of metabolic reactions, indicates that this chemical may alter steroidogenesis in the testis. Chronic administration of SKF 525-A to rats causes a selective increase in the size of the testes (245). Furthermore, the synthesis of cholesterol (an important precursor of steroids) is inhibited by SKF 525-A (251).

11. Floctafenin

The analgesic and anti-inflammatory properties of 2,3-dihydroxypropyl-N-(8-trifluoromethyl-4-quinolyl) anthranilate (floctafenin, Fn) have been established by acid-induced writhing and inflamed paw tests in animals and by double-blind clinical trials (252-254). The plasma kinetics and metabolism of ^{14}C-Fn has been described in men, mice, rats and dogs (255). Enzymatic hydrolysis of Fn yielded floctafenic acid (FA) and glycerol. FA was found to be the major metabolite in all species. After an oral dose of Fn, plasma levels of Fn are always considerably lower than the plasma concentrations of FA, indicating that hydrolysis of Fn occurs rapidly in vivo. Other prominent metabolites of Fn included: hydroxy-Fn, hydroxy-FA and glucuronide conjugates (see Figure 19). There have been no reliable, physicochemical assays suitable for the routine quantification of Fn and FA in biologic samples.

12. Hexobarbital

Hexobarbital (HB) is a short-acting sedative-hypnotic. The major metabolites of HB are 3-ketohexobarbital, 3-ketonorhexobarbital and 3-hydroxyhexobarbital (OH-HB) (256). The duration of HB-induced sleep in mice is frequently employed to detect induction of hepatic microsomal enzymes by drugs. Thus, chronic administration of enzyme inducers will often diminish HB sleep times in mice.

13. Tricaine

The methanesulfonate salt of tricaine is currently used as a general anesthetic for cold-blooded animals. Massive doses of tricaine are required to induce hyponosis in homeotherms because the drug is rapidly converted to less active metabolites (ethanol and m-aminobenzoic

acid) by esterases (257). There has been recent interest in utilizing tricaine as a local anesthetic and smooth muscle relaxant during bronchoscopy in man.

14. Statement of the Problem

Mixed-function oxidases (MFO) in hepatic microsomes frequently exhibit poor substrate specificity. The steroidogenic, MFO system in the testis may similarly accept foreign chemicals as substrates in place of endogenous compounds. This could result in competitive inhibition of testosterone synthesis and in systemic androgen deficiency. Furthermore, many mutagens, carcinogens and cytotoxins are known to be enzymatically activated. Such metabolic activity in the testis could culminate in birth defects, testicular carcinoma and infertility. An appraisal of these possibilities cannot be ventured at this time because there is currently little information on the metabolism of foreign substances in the testis. Therefore, a study which conclusively demonstrates biotransformation of xenobiotics by testicular enzymes could help elucidate the etiology of several disorders in male reproductive function.

The present study investigated the possible N-dealkylation of methadone and SKF 525-A, oxidation of hexobarbital and hydrolysis of floctafenin and tricaine by testicular enzymes. In order to monitor the metabolism of floctafenin and SKF 525-A, new assays for their respective metabolites had to be developed.

B. Materials and Methods

1. General

Sprague-Dawley white rats (Simonsen Labs, Gilroy, California) were used in all the experiments. The method of Lowry et al. (see

Appendix IV) was employed for determining the protein concentration in the incubation mixes and in tissue homogenates.

Data were interpreted with the aid of analysis of variance and Duncan's new multiple range test. Differences between mean values were considered statistically significant if a p less than 0.05 was established. Unless otherwise indicated, each mean value is expressed as a plus or minus one standard deviation.

2. Methadone: Testis Incubations

Testes were removed from freshly decapitated rats (280-340 g) and homogenized on ice in 1/3 their weight of 0.2 M sodium phosphate buffer, pH 7.4, using a Potter-Elvehjem glass homogenizer and a motor-driven teflon pestle. The homogenates were centrifuged for 20 min. at 9000 X g in polycarbonate tubes in a refrigerated (4° C) Sorvall RC 2-B centrifuge (SS-34 rotor). The incubations consisted of 5.5 ml 9000 X g supernatant, 0.1 ml cofactor solution (glucose-6-phosphate, 30 μmol; nicotinamide, 74 μmol; NADP, 5.1 μmol; and magnesium chloride, 63 μmol, in water) and 0.4 ml aqueous solution of d- or l-methadone HCl in a 25 ml Erlenmeyer flask. The flask was swirled, briefly flushed with 95% O_2-5% CO_2, stoppered and then incubated in a Dubnoff shaking water bath set at 37° C and 120 cycles/min. One ml samples were removed periodically from the incubation flask and analyzed for EDDP by gas-liquid chromatography. The extraction and chromatographic procedures were identical to those used in the assay for methadone (see Materials and Methods under Excretion of Drugs into Semen), except that the initial volume ratio, 1-chlorobutane/aqueous phase, was 10 (126). Figure 20 shows a typical gas-chromatogram for a sample taken from a testicular incubation containing

d-methadone (0.193 μmol/ml). A standard curve for EDDP was prepared by extracting known amounts of synthetic EDDP (EDDP hydroiodide, Applied Science, State College, Pa.) from 9000 X g supernatant of rat testis homogenate (see Figure 21). In the above experiments, testes from four animals were pooled together to prepare each incubation flask. Thus, each flask contained enzymes from four animals, but no two flasks contained enzymes from the same group of rats.

The identity of the GLC peak designated EDDP was established by: 1) comparing the GLC retention time of the substance in the incubation with the retention time of synthetic EDDP; 2) incubating testicular enzymes in the absence of methadone; and 3) subjecting extracts of the methadone-testis incubations to GC-MS.

3. SKF 525-A

Assay for SKF 8742-A. The N-desethyl metabolite of SKF 525-A (SKF 8742-A; ethylaminoethyl diphenylvalerate) was quantified using a GLC assay that was nearly identical to the one employed for methadone and EDDP. A 1-ml sample containing SKF 8742-A was mixed with 1.0 ml 1 M sodium bicarbonate-carbonate buffer, pH 10, and extracted with 40 ml 1-chlorobutane. Thirty ml of the organic phase were further extracted with 2.0 ml 1 N sulphuric acid. A portion of the acid extract (1.8 ml) was alkalinized with 1.0 ml 15% ammonium hydroxide and extracted with 75 μl chloroform. Tetracosane in the chloroform served as an internal standard. The GLC column and gas chromatograph operating conditions were the same as employed for the methadone assay. A standard curve for SKF 8742-A (Smith, Kline and French, Philadelphia, Pa.) extracted from 9000 X g supernatant of rat testis is shown in Figure 22. A gas-liquid

chromatogram for a standard curve sample containing both SKF 525-A and SKF 8742-A is presented in Figure 23.

Testis Incubations. Testes were homogenized and centrifuged at 9000 X g as previously described for the methadone experiments. Each incubation flask received 5.0 ml 9000 X g supernatant, 0.5 ml cofactor solution (nicotinamide, 90 μmol; glucose-6-phosphate, 30 μmol; NADP, 2.4 μmol; and magnesium chloride, 72 μmol), and 0.5 ml drug solution (SKF 525-A in water). The flasks were flushed with 95% oxygen-5% carbon dioxide, stoppered and maintained at 37° C in a Dubnoff shaking water bath. Timed, 0.7 ml samples were removed from the flasks and analyzed for SKF 8742-A. Initial incubations had a final SKF 525-A concentration of 667 μg/ml. The substrate concentration was reduced in subsequent incubations to 20 μg/ml in order to permit more complete separation of the SKF 525-A and tetracosane GC peaks.

In the latter experiments, some of the rats were injected subcutaneously with 1 mg testosterone propionate (in 0.2 ml vegetable oil) daily for 10 days. The rats were killed 24 hours after the last injection, and a testis incubation was prepared. Control incubations were prepared from testes of rats which received injections of vegetable oil alone.

In order to determine the intracellular location of N-dealkylating enzymes in the testis, SKF 525-A was also incubated with purified testicular microsomes and microsome-free supernatants. Homogenates of rat testes were prepared as before and centrifuged at 10,000 X g for 20 min. at 4° C in a Sorvall RC2-B centrifuge (SS-34 rotor). The 10,000 X g supernatant was recentrifuged at 100,000 X g for 90 min.

at 4° C in a Sorvall OTD-2 ultracentrifuge (Beckman type 65 rotor). The 100,000 X g supernatant was transferred to a separate container and kept on ice. The 100,000 X g pellet (microsomes) was rinsed 3 times with ice-cold phosphate buffer and then resuspended in 3.0 ml 0.2 M sodium phosphate buffer, pH 7.4, using a Downes homogenizer and type B pestle. Cofactors for the regeneration of reduced NADP (nicotinamide, 240 μmol; glucose-6-phosphate, 75 μmol; magnesium chloride, 180 μmol; NADP, 3.1 μmol; and glucose-6-phosphate dehydrogenase, 4 units) were dissolved in 5 ml 0.2 M sodium phosphate buffer, pH 7.4. One incubation flask received 0.5 ml SKF 525-A solution (30 μg/ml in water), 1.0 ml cofactor solution, and 1.5 ml microsome suspension. A second flask was prepared in an identical fashion, except that the SKF 525-A solution was 8 times more concentrated. A third flask received 1.0 ml SKF 525-A solution (30 μg/ml), 2.0 ml cofactor solution and 3.0 ml 100,000 X g supernatant. The flasks were briefly swirled, and a 0.5 ml sample was removed from each flask. The flasks were then flushed with 95% oxygen-5% carbon dioxide, stoppered and incubated at 37° C in a Dubnoff shaking water bath. After 60 min., another 0.5 ml sample was removed from each flask. The samples were all analyzed for the presence of SKF 8742-A. In another experiment, rats were injected intraperitoneally with sodium phenobarbital, 100 mg/kg/day, for 4 days. Forty-eight hours after the last injection, the testes were removed from the rats, and an incubation was prepared from washed testis microsomes using the previously described methods. The control incubation was made from testis microsomes from rats which received saline in place of the phenobarbital. The above dosage schedule for phenobarbital is known to induce numerous drug-metabolizing enzymes in the rat liver.

Liver Incubations. Rat livers were rapidly removed from freshly decapitated rats, rinsed briefly in ice-cold phosphate buffer, and homogenized in 2 times their weight of 0.2 M sodium phosphate buffer, pH 7.4, using a Potter-Elvehjem glass homogenizer and a motor-driven teflon pestle. The homogenates were centrifuged at 9000 X g for 20 min. at 4° C in a Sorvall RC2-B centrifuge (SS-34 rotor). A portion of the supernatant was incubated with SKF 525-A (final concentration = 667 µg/ml) and cofactors (same concentrations as described for testicular 9000 X g supernatant incubations) at 37° C in a Dubnoff shaking water bath. Aliquots of the incubation were removed at various times and analyzed for SKF 525-A and its metabolites.

Mass Spectrometry. Chloroform extracts of SKF 525-A testis and liver incubations were subjected to GC-MS using a Finnigan model 3200 GC-MS equipped with a model 6100 MS data system. The GC column was packed with 1.5% OV-101 on Gas Chrom Q (100-120 mesh), and the MS ionizing potential was 70 eV.

4. Hexobarbital (HB)

A six ml incubation containing 9000 X g supernatant of rat testis homogenate and enzyme cofactors was prepared as previously described for the methadone-testis incubations, except that 0.5 ml of HB solution (1.036 µCi ^{14}C-HB/0.9 ml 2 X 10^{-5} M NaOH; specific activity = 3.7 µCi/mg) was used in place of the methadone solution. The incubation flask was maintained in a Dubnoff shaking water bath at 37° C. Timed, 1.0 ml samples were removed and analyzed for HB and HB metabolites according to the radiolabel method of Gerber et al. (256). This method separates HB from its metabolites, but does not distinguish between 3-hydroxyhexobarbital and 3-ketohexobarbital.

5. Floctafenin

Spectrophotometric Assays for Floctafenin and Floctafenic Acid. The ultraviolet (U.V.) absorption spectra for Fn and FA exhibit a prominent peak at 348 nm. U.V. assays that are specific for each compound require extraction procedures which separate Fn from FA. This was accomplished by adding 8 ml 0.2 M sodium carbonate-bicarbonate buffer, pH 9.2, to biologic samples (final aqueous volume = 10 ml) and extracting with 30 ml 1-chlorobutane (nanograde, Burdick and Jackson). The 1-chlorobutane removed more than 90% of the Fn, while extracting little or no FA. The pH of this extraction step was critical, since lower pH values resulted in a less efficient extraction of Fn and increased extraction of FA, and higher pH values resulted in excessive alkaline hydrolysis of Fn. When solutions of Fn were mixed with 0.4 M sodium carbonate (final pH approximately 11), more than 50% of the Fn was hydrolyzed within an hour (see Table 11). The rate of hydrolysis was slow at pH 9.2 and negligible at neutral and acidic pH values. Therefore, the first extraction procedure was executed as expeditiously as possible. After 10 minutes of mechanical shaking and 10 minutes of centrifugation (1000 RPM in an International model K centrifuge), 25 ml of the 1-chlorobutane were transferred immediately to a second tube and equilibrated with 3.0 ml 1 N HCl. The remaining 5 ml 1-chlorobutane (above the pH 9.2 aqueous phase) was discarded. The aqueous phase was extracted a second time with fresh 1-chlorobutane (20 ml) in order to remove most of the remaining Fn. This second 1-chlorobutane extract was discarded. Eight ml of the alkaline aqueous phase were adjusted to pH 4.0 with 1.2 ml 1 M citric acid, and then extracted with 30 ml ethyl ether (anhydrous,

Mallinckrodt). Twenty-five ml of the ether extract were transferred to a clean tube and equilibrated with 2.0 ml 6 N HCl. Fn in the 1 N HCl was quantified by determining the absorbance of the acid extract at 348 nm in a Cary 15 recording spectrophotometer with 1 N HCl as the reference. Similarly, FA in the 6 N HCl was measured by determining the absorbance of the acid extract at 348 nm using 6 N HCl as the reference. Standard curves were prepared by extracting known amounts of Fn and FA from tissue homogenates (see Figure 24). Proof that the above assays distinguish between Fn and FA was provided by other workers in the laboratory using countercurrent distribution.

Testis and Liver Incubations. Sprague-Dawley rats (280-300 g) were decapitated, and the testes and livers were rapidly removed and placed (separately) into ice-cold 0.2 M sodium phosphate buffer, pH 7.4. Each tissue was homogenized in five times its weight of fresh phosphate buffer in a glass homogenizer equipped with a motor-driven teflon pestle. Four ml of ice-cold homogenate and five 1 cm diameter glass beads were added to each 25-ml incubation flask. Tissue from only one animal was used per flask. Each flask was warmed for 1 minute at 37° C before receiving 2.0 ml Fn solution (10 mg dissolved in 1.5 ml 0.1 N HCl, then diluted to 50 ml with water). Each flask was then maintained at 37° C in a Dubnoff shaking water bath (120 cycles/min.). One milliter samples from the incubations were removed at various times and assayed for Fn and FA.

6. Tricaine

Testis Incubations. Testes from freshly killed rats were homogenized in five times their weight of ice-cold 0.1 M sodium phosphate

buffer, pH 7.4, using a Potter-Elvehjem glass homogenizer and a motor-driven teflon pestle. Two ml of the homogenate were pipetted into a 15 ml Erlenmeyer flask and warmed for 3 minutes at 37° C; each flask received tissue from only one animal. Three glass beads (1 cm diameter) and 4.0 ml tricaine methanesulfonate solution (45 mg Finquel in 10 ml 0.1 M sodium phosphate buffer, pH 7.4) were then added. The flask was swirled, and a 0.5 ml aliquot of the incubation mix was immediately removed and extracted with 1-chlorobutane. The flask was then stoppered and incubated in a Dubnoff shaking water bath set at 100 cycles/min. Further aliquots of the incubation mix were periodically removed and immediately analyzed for tricaine and m-aminobenzoic acid (MABA).

Three adult rats (282 ± 11 g) were used in one experiment, while 9 immature rats (age, 34 days; 136 ± 11 g) were used in another. Five of the immature rats received subcutaneous injections of morphine sulfate twice daily for 12 days prior to the testis incubations. The dose of morphine sulfate was increased 10 mg/kg/day every 3 days, so that the initial 6 injections each delivered 30 mg/kg and the final 6 injections each delivered 45 mg/kg. Approximately 20 hours transpired between the last dose of morphine and the removal of the testes. Control animals received injections of saline instead of morphine.

Assays for Tricaine and m-Aminobenzoic Acid. Ultraviolet spectrophotometry and colorimetry have been employed recently for quantifying tricaine and its primary metabolite MABA (257). These methods have now been used to monitor the disappearance of tricaine and appearance of MABA in testis incubations. Each one-half milliliter aliquot of incubation mix was immediately extracted with 30 ml 1-chlorobutane. Ten

milliliters of the organic phase were further extracted with 15 ml 1 N HCl. Tricaine was quantified by measuring the absorbance of the acid layer at 228 nm in a Cary 15 recording spectrophotometer using 1 N HCl as the reference. To quantify the MABA, the first aqueous phase was diluted with 15 ml 15% trichloroacetic acid (TCA) and centrifuged at 1000 X g for 10 minutes. Three ml of the supernatant were mixed with 0.5 ml 0.1% sodium nitrite. The mixture was allowed to stand for 15 minutes. Then, 0.5 ml 0.5% ammonium sulfamate was added. This was followed by thorough mixing and the further addition of 0.5 ml 0.1% N-naphthyl-ethylenediamine dihydrochloride. The final reaction mix was agitated briefly and allowed to stand 20 minutes. The optical absorbance of this mixture was then determined at 550 nm in the Cary spectrophotometer. The reference was composed of 15% TCA solution and the three Bratton-Marshall reagents mixed in the same manner as described above. Standard curves for tricaine and MABA were linear and were prepared for each experiment.

C. Results

1. Methadone

Testicular oxidases were found to N-demethylate both isomers of methadone. Figure 25 shows the appearance of the methadone metabolite EDDP in incubations containing 9000 X g supernatant of rat testis homogenate (50 mg protein/ml incubation mix), cofactors for the regeneration of reduced NADP, and d-methadone HCl (66.7 µg/ml) as a substrate. If the initial concentration of methadone was reduced in the incubations, then less EDDP was formed, indicating that relatively high substrate concentrations were required to saturate the enzymes. The presence of

EDDP in the incubations was substantiated by GC-MS. However, this information did not conclusively demonstrate that EDDP was generated in the incubations, since EDDP was found to be a minor contaminant in all available samples of methadone. Nonetheless, there was good evidence that EDDP was in fact formed in the incubations via metabolism of methadone. The substance extracted from testis incubations and designated EDDP had a GLC retention time identical to that of synthetic EDDP (Applied Science Labs, State College, Pa.) and increased significantly ($p < .05$) in concentration during the incubation. Formation of the alleged EDDP did not occur in the absence of methadone or the absence of tissue.

2. SKF 525-A

Metabolites of SKF 525-A. Several metabolites of SKF 525-A were generated in hepatic and testicular incubations (see Figures 26 and 27). Three hepatic metabolites of SKF 525-A were identified by GC-MS. These were: 2-ethylaminoethyl 2,2-diphenylvalerate (M-I, SKF 8742-A); aminoethyl 2,2-diphenylvalerate (M-II); and hydroxylated SKF 525-A (M-III). The mass spectra for these metabolites are shown in Figures 28 and 29. Positive identification of M-III was made by comparing the mass spectrum of this metabolite with the mass spectrum of SKF 525-A (see Figure 30 and Table 12). Another metabolite (M-IV) was present, but was not identified. M-I and M-II were also formed in the testis incubations. A small peak corresponding to M-III was noted in gas chromatograms of testis incubation extracts. However, the identity of this material was not established by GC-MS. The metabolic pathways for SKF 525-A are illustrated in Figure 31.

Testis Incubations. Significant quantities of M-I (SKF 8742-A) were generated from SKF 525-A in testis incubations (see Table 13). A gas-liquid chromatogram from one experiment is presented in Figure 32. Pre-treatment of animals with testosterone propionate appeared to reduce the rate of M-I formation. The testosterone injections did not change the amount of protein per weight of testis. Thus, there were 37 mg protein/ml incubation mix in each flask. However, the accessory sex organs of the testosterone pre-treated rats were about three times larger than the accessory sex organs of the control animals. The mean body weight for the testosterone pre-treated rats (317 g ± 6) was not significantly different than the corresponding weight for control rats (329 g ± 19).

As indicated in Table 14, the enzymes of N-dealkylation in testis homogenate were located in the microsomes (100,000 X g pellet) and not in the 100,000 X g supernatant. Relatively high concentrations of SKF 525-A were required to saturate the microsomal enzymes, since the yield of metabolite (SKF 8742-A) per amount of protein was increased 5-fold by increasing the substrate concentration from 5 to 40 μg/ml. Pre-treatment of rats with phenobarbital did not appear to alter the rate of N-deethylation of SKF 525-A by testicular microsomes.

3. Hexobarbital

There was no change in HB concentration during the 60-minute incubation period. A small amount of radioactivity was found in the aqueous layers after the HB had been removed with 1-chlorobutane. This radioactivity probably represented small amounts of impurities in the HB rather than HB metabolites, since the amount of radiation (after removal

of HB) per sample remained about the same throughout the incubation period.

4. Floctafenin

Figure 33 shows the disappearance of Fn and production of FA in incubations containing homogenates of liver and testis. Both tissues were capable of hydrolyzing Fn, and most of the metabolized drug could be accounted for as FA. The fact that small amounts of FA were found at the beginning of the incubation indicates that some Fn was hydrolyzed during the few seconds required to mix the drug with the tissue homogenate. Since all the incubations contained equivalent concentrations of tissue (167 mg/ml), it is readily apparent that the liver was significantly more active than the testis in hydrolyzing Fn. Thus, the mean Fn concentration in liver incubations was significantly ($p < .05$) less than the mean Fn concentration in testis incubations at 10, 20, 40 and 60 minutes, respectively (but not at time 0). The mean FA concentration in liver incubations was significantly ($p < .05$) greater than the mean FA concentration in testis incubations at respective times after 20 minutes.

The esterases may not have been saturated at the substrate concentration that was employed. This was suggested by the decreasing rate of hydrolysis during the incubations. Subsequent efforts to achieve zero-order hydrolysis of Fn were not successful. Increases in Fn concentration resulted in precipitation of the drug at pH 7.4. When the enzyme concentration was reduced, then the amount of FA generated was insufficient for accurate measurement of this metabolite.

5. Tricaine

Figure 34 illustrates the rapid disappearance of tricaine and appearance of MABA in incubations containing tricaine methanesulfonate, and homogenate of testes from sexually mature rats (282 ± 11 g). Essentially all of the metabolized tricaine can be accounted for as the metabolite MABA. The rate of tricaine hydrolysis was approximately 0.224 μmol/min. As shown in Figure 35, the esterase activity of testes from immature rats (136 ± 11 g; 33 days of age) was only slightly less (0.189 μmol/min.). Figure 35 also shows that chronic administration of morphine to young rats diminished the esterase activity in the testes by 63%.

D. Discussion

1. Microsomal Metabolism of Drugs in the Testis

Evidence has been presented which conclusively demonstrates the presence of drug-metabolizing oxidases in the testis. Methadone and SKF 525-A were both N-dealkylated *in vitro* by testicular enzymes. Although aromatic hydroxylation of hexobarbital was not detected, small quantities of hydroxy-SKF 525-A were probably generated from SKF 525-A in testis incubations. The N-dealkylation enzymes in the testis were insoluble and located in the microsomes. Pretreatment of rats with phenobarbital did not cause an obvious change in N-dealkylation activity in the testis. However, the ability of phenobarbital to diffuse into cells of the testis has not been established. Furthermore, the incubations that were employed to assess differences in enzyme activity were not executed when the rate of N-dealkylation was independent of substrate concentrations. The ability of the methodology to detect small changes

in enzyme activity is therefore questionable. On the other hand, it was demonstrated that pre-treatment of rats with testosterone propionate caused a significant reduction in N-dealkylation activity in the testis. This latter observation is not surprising, since the microsomal, drug-metabolizing enzymes are presumably located primarily in the Leydig cells, and these cells atrophy in response to chronic systemic doses of testosterone. However, part of the inhibitory effects of testosterone pre-treatment could be due to competition between SKF 525-A and testosterone (or testosterone metabolites) for enzyme cofactors, cytochrome P-450 or active sites on enzymes.

2. Possible Mechanism for SKF 525-A-Induced Enzyme Inhibition

The enzymatic hydroxylation of SKF 525-A has not been described in previous works. The current study has shown that hydroxy-SKF 525-A (M-III) is a major metabolite for SKF 525-A in hepatic incubations. Hepatic microsomes reportedly generate, in the presence of SKF 525-A, a stable, oxygenated complex that contains cytochrome P-450 (245). This complex may be responsible for the inhibition of drug metabolism by SKF 525-A. It was postulated that an unidentified metabolite of SKF 525-A becomes strongly bound to cytochrome P-450 and stabilizes the hemoprotein in a reduced form that complexes with molecular oxygen. Thus, the cytochrome P-450 is no longer available for catalyzing the biotransformation of drug substrates. It is possible that M-III is in fact the metabolite that stabilizes the oxygen-cytochrome P-450 complex.

3. Hydrolysis of Floctafenin by Esterases

The ability of homogenates of various rat tissues (excluding the testes) to hydrolyze floctafenin was investigated by Pottier, Busigny

and Raynaud (255). Their data were expressed in units of Fn metabolized per weight of tissue. The greatest esterase activity with respect to Fn was located in the liver. Using the same basis of comparison, the current investigation indicates that the testis is about 60% as active as the liver in hydrolyzing Fn. Therefore, the testis ranks third behind liver and kidney and ahead of 8 other tissues in esterase activity. If the comparison between testis and liver is made on the basis of protein (i.e. μmol Fn hydrolyzed/mg protein), then there is no significant difference between the two tissues. Thus, rat livers were found to have 215 ± 15 mg protein/g tissue, and rat testes had 90 ± 4 mg protein/g tissue.

4. Effect of Morphine on Testicular Esterases

Huggins and Moulton (210) have shown, using p-nitrophenyl propionate as a substrate, that testicular esterase activity (units/weight of tissue) in rats is extremely low prior to 25 days of age. Thereafter, the esterase activity increases abruptly until a level equivalent to 80% of the values in adult testes is achieved at 34 days of age. These data are in agreement with the current studies using tricaine as a substrate for testicular esterases. The esterase activity in testes from 33-day-old rats was 86% of that in testes from adult rats. Esterase activity in the testis is maintained by pituitary gonadotropins (179, 210). Recently, opiates were shown to diminish circulating levels of LH (28, 230). It follows that narcotic analgesics should cause a reduction in testicular esterases. This latter possibility was investigated by injecting morphine into young rats during the period when testicular esterase activity develops most rapidly. As indicated in Figure 35,

chronic administration of morphine causes degeneration and/or retards development of testicular esterases in the rat testis. It is unlikely that residual amounts of morphine at the time of homogenization could directly influence esterase activity in vitro. The β phase half-life of morphine in young Sprague-Dawley rats is reportedly less than 2 hours (258). Therefore, less than 0.01% of the peak drug load would be present at the time of the incubations (20-24 hours after the last drug injection). The activity of serum esterases in vitro is reportedly increased by low concentrations of morphine and decreased by high concentrations (10^{-3} M) of morphine (259). If tissue esterases respond to morphine in a manner similar to that of serum esterases, then a slight increase, not a decrease, in enzyme activity should have been observed.

IV. SUMMARY STATEMENT

This thesis has presented new data on the disposition of foreign compounds in reproductive organs. The areas of investigation included: excretion of drugs into semen; absorption of drugs through the vaginal mucosa; drug effects on sperm motility; and drug metabolism in the testis. The rabbit proved to be an excellent animal model for studying the excretion of drugs into semen. Information acquired in the rabbit was used to describe the rapidity and extent of drug cumulation in seminal fluids. Furthermore, data obtained in the rabbit correlated well with data obtained in man. In both species, methadone (a weak base) became concentrated in reproductive organ secretions, whereas phenytoin and dipropylacetate (both weak acids) achieved low concentrations in seminal fluids relative to drug levels in plasma. It was postulated that ion-trapping may influence the equilibration of drugs into and out of seminal fluids. Drugs that are excreted into semen are probably absorbed through the vaginal mucosa. This was suggested by experiments that demonstrated rapid absorption of intravaginally administered d-methadone in rabbits.

The effects of drugs on sperm motility were investigated in vitro. Methadone (both isomers), trifluoperazine and desipramine were found to immobilize spermatozoa, whereas phenytoin, dipropylacetate and naloxone were observed to have no effect on sperm motility. The immobilizing effect of l-methadone was not antagonized by naloxone.

Finally, the ability of the testis to metabolize several drug substrates was demonstrated in vitro. Microsomal enzymes in the rat testis were shown to N-dealkylate methadone and SKF 525-A. The abundant

esterases of the rat testis were found to rapidly hydrolyze tricaine and floctafenin.

The data and concepts discussed in this thesis will hopefully lead to a better understanding of interactions between drugs and male reproductive organs. Continued efforts in this area of research will help elucidate toxic drug effects on reproductive function, and possibly generate improved methods of fertility regulation.

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Table 1: Antibiotics quantified in seminal fluids

Drug	Chemical characteristics	Drug concentrations in seminal fluids versus concentrations in blood		First Author	
		Relationship	Species		
Ampicillin	Weak acid pKa = 2.5 Poor lipid solubility	P.F. << Bld.	Dog	Wolf	(99)
		P.F. $\leq$ Bld.	Man	"	(99)
		P.F. << Plasma	Dog	Winningham	(97)
		1st Part Ejac. < Plasma	Man	Malmborg	(37)
Bacitracin	Amphoteric Poor lipid solubility	P.F. = Bld.	Man	Borski	(81)
Cephalothin	Weak acid pKa = 2.5 Poor lipid solubility	P.F. << Plasma	Dog	Winningham	(97)
Chloramphenicol	Weak base Lipid soluble	P.F. < Bld.	Man	Borski	(81)
		P.F. $\leq$ Serum	Man	Atkinson	(80)
Chlortetracycline	Amphoteric Poor lipid solubility	P.F. << Bld.	Man	Borski	(81)
		P.F. $\leq$ Bld.	Man	Schatten	(84)
		P.F. $\leq$ Serum	Man	Atkinson	(80)

Table 1 (Continued)

Drug	Chemical Characteristics	Drug concentrations in seminal fluids versus concentrations in blood		First Author	
		Relationship	Species		
Erythromycin	Weak base pKa = 8.8 Lipid soluble	P.F. $\leq$ Bld.	Man	Borski	(81)
		P.F. > Plasma	Dog	Winningham	(97)
		1st Part Ejac. = Plasma	Man	Malmborg	(37)
Kanamycin	Weak base pKa = 7.2 Poor lipid solubility	P.F. << Plasma	Dog	Winningham	(97)
Nalidixic acid	Weak acid pKa = 6.0 Lipid soluble	P.F. << Plasma	Dog	Winningham	(97)
Nitrofurantoin	Weak acid pKa = 7.2 Lipid soluble	P.F. < Plasma	Dog	Dunn	(87)
		P.F. < Bld.	Dog	Scott	(95)
		P.F. but no bld. data	Dog and Man	Madsen	(91)
Oleandomycin	Weak base pKa = 8.5 Lipid soluble	P.F. >> Plasma	Dog	Winningham	(97)
Penicillin G	Weak acid pKa = 2.7 Poor lipid solubility	P.F. << Plasma	Man	Borski	(81)
		P.F. << Plasma	Dog	Winningham	(97)

Table 1 (Continued)

Drug	Chemical Characteristics	Drug concentrations in seminal fluids versus concentrations in blood		First Author	
		Relationship	Species		
Oxytetracycline	Amphoteric pKa = 3.3, 7.3, 9.1 Poor lipid solubility	P.F. < Bld.	Man	Borski	(81)
		Semen < Bld.	Man	Borski	(81)
		P.F. < Serum	Man	Atkinson	(80)
		P.F. < Plasma	Dog	Winningham	(97)
		P.F. < Plasma	Dog	Hessl	(90)
Polymixin B	Weak base pKa = 8 Poor lipid solubility	P.F. << Plasma	Dog	Winningham	(97)
Sulfadiazine	Weak acid pKa = 6.8 Lipid soluble	Semen < Bld.	Man	Osenkop	(82)
Streptomycin	Weak base Poor lipid solubility	P.F. << Serum	Man	Pulaski	(83)
		P.F. << Bld.	Man	Borski	(81)
Sulfonamides (general)	Weak acids Variable pKa Variable lipid solubility	P.F. < Plasma for all sulfonamides. The larger the pKa, the larger the P.F./plasma drug concentration ratio.		Winningham	(98)
				Robb	(93)
			Dog	Robb	(94)

Table 1 (Continued)

Drug	Chemical Charateristics	Drug concentrations in seminal fluids versus concentrations in blood		First Author
		Relationship	Species	
Sulphamethoxazole	Weak acid pKa = 6.1 Poor lipid solubility	1st Part Ejac. < Plasma	Man	Eliasson (105)
Tetracycline	Amphoteric pKa = 3.3, 7.7, 9.7 Lipid soluble	P.F. < Bld.	Man	Borski (81)
		P.F. $\leq$ Serum	Man	Pulaski (83)
		P.F. << Bld.	Dog	Hessl (90)
Trimethoprim	Weak base pKa = 7.3 Lipid soluble	P.F. > Serum	Man	Nielsen (101)
		P.F. > Serum	Dog	Stamey (96)
		1st Part Ejac. > Plasma	Man	Eliasson (105)
		P.F. > Bld.	Dog	Reeves (92)
		P.F. > Plasma	Dog	Robb (93)

KEY: P.F., Bld., Serum, Plasma, and 1st Part Ejac. indicate drug concentrations in prostatic fluid, blood, serum, plasma and the first part of a split ejaculate, respectively.
a = b, a $\leq$ b, a < b, a << b, and a > b mean that, respectively, a is 0.75 to 1.25 times b; a is 0.5 to 1.0 times b; a is 0.2 to 0.5 times b; a is less than 0.2 times b; and a is greater than 2 times b.

Table 2: Concentration of d-methadone base in rabbit blood (µg/ml) after 40 mg d-methadone HCl i.v.

Time (Min.)	Animal #1	Animal #2	Animal #3	Animal #4	Mean ± S.D.
5	-	-	1.86	2.10	-
10	1.53	2.26	1.41	1.33	1.63 (0.43)
20	1.03	1.66	1.03	0.80	1.13 (0.37)
40	0.65	1.05	0.59	0.49	0.70 (0.25)
60	0.39	0.70	0.43	0.40	0.48 (0.15)
90	0.32	0.60	0.41	0.26	0.40 (0.15)
120	0.25	0.55	0.30	0.22	0.33 (0.15)
180	0.19	0.34	0.24	0.13	0.23 (0.09)
240	-	0.24	0.12	0.11	-
270	0.10	0.20	0.08	0.09	0.12 (0.06)
360	0.05	0.15	0.04	0.04	0.07 (0.05)
480	0.03	-	-	-	-

Table 3: Pharmacokinetics of d-methadone in the rabbit. Parameters are calculated from drug concentrations in whole blood after 40 mg d-methadone HCl i.v.

Animal	Body Weight	α ($min.^{-1}$)	t 1/2 α (min.)	A (µg/ml)	β ($min.^{-1}$)	t 1/2 β (min.)	B (µg/ml)	Vd (liter/kg)
1	4.5	0.05372	13	1.59	0.00604	115	0.52	11.4
2	3.3	0.05193	13	2.29	0.00543	128	0.95	9.2
3	4.0	0.09356	7	1.43	0.00873	79	0.96	8.2
4	3.9	0.05626	12	1.54	0.00671	103	0.49	13.6
Mean	3.9		11			106		10.6
± S.D.	± 0.5		± 3			± 21		± 2.4

Table 4: Distribution of d-methadone base in rabbit tissues 130 minutes after 40 mg d-methadone HCl i.m.

Organ	Testis	Prostate	Seminal Vesicle	Lung	Liver	Kidney	Spleen	Heart	Blood
Mean Concentration (µg/g tissue)	4.31	4.58	3.52	33.5	1.54	15.2	30.6	5.75	0.42 (µg/ml)
S.E.M.	0.78	1.25	1.10	5.37	0.31	2.85	6.33	0.98	0.04
Number of Animals	5	4	4	5	5	5	5	5	5

Table 5: DPH concentration in chronologically paired semen and plasma samples from epileptic subjects maintained on chronic, oral Dilantin

Subject	Age (Years)	Daily Dose phenytoin sodium, Dilantin (mgs)	Semen Volume (mls)	DPH Concentration (µg/ml) Semen	Plasma	Semen/Plasma
1	34	500	7.3	1.34	7.62	0.18
2	37	400	4.4	2.32	11.40	0.20
3	21	300	2.8	2.91	17.73	0.16
4	29	200	1.9	0.97	4.59	0.21
5	44	400	1.0	0.33	9.77	0.03
6	49	500	1.0	1.18	17.76	0.07
7	23	600	3.9	2.97	18.51	0.16
8	21	400	1.5	6.03	19.11	0.32
9	38	400	2.0	2.76	17.35	0.16
MEAN $\pm$ STANDARD DEVIATION	33 ± 10	411 ± 117	2.9 ± 2.0	2.31 ± 1.69	13.76 ± 5.47	0.17 ± 0.08

Table 6: DPH in rabbit plasma (ng/ml) after an intravenous infusion of 4.64 mg

Time (Min.)	Rabbit #1	Rabbit #2	Rabbit #3	Rabbit #4	Mean ± S.D.
5	2213	1750	2306	2730	2250 ± 402
15	-	-	-	2011	-
30	1452	1386	1234	1527	1400 ± 125
60	1190	1108	925	1273	1124 ± 149
75	861	-	-	-	-
120	692	695	687	954	757 ± 131
180	481	490	516	771	565 ± 138
240	-	-	-	656	-
300	234	321	269	505	332 ± 121
420	207	171	140	338	214 ± 87
540	117	109	82	221	132 ± 61

Table 7: Pharmacokinetics of DPH in the rabbit after an intravenous infusion of 4.64 mg

Rabbit	Body Weight (kg)	α ($min.^{-1}$)	$t_{1/2}\alpha$ (min.)	A (ng/ml)	β ($min.^{-1}$)	$t_{1/2}\beta$ (min.)	B (ng/ml)	V_d (L/kg)
1	4.0	0.01991	34.8	1377	0.00364	190.6	846	1.06
2	4.1	0.02379	29.1	875	0.00428	161.9	1087	0.91
3	4.4	0.08035	8.6	1593	0.00514	134.8	1272	0.77
4	3.6	0.05007	13.8	1318	0.00351	197.3	1475	0.82
MEAN ± S.D.	4.0 ± 0.3		21.6 ± 12.4			171.2 ± 28.7		0.89 ± 0.13

α = slope of the initial monoexponential line resulting from drug distribution from plasma into tissues

β = slope of the terminal monoexponential line resulting from first order elimination processes

$t_{1/2}\alpha$ = half-life of the α slope; $t_{1/2}\beta$ = terminal plasma half-life

A = extrapolated Y intercept of the α slope; B = extrapolated Y intercept of the β slope; Vd = Dose/β $(\frac{A}{\alpha} + \frac{B}{\beta})$ = apparent volume of distribution

Table 8: DPA concentration in rabbit plasma (µg/ml) after 50 mg/kg sodium dipropylacetate i.v.

Time (min.)	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Mean ± S.D.
5	259.22	290.42	265.91	312.95	282.13 (24.54)
10	242.38	253.00	241.97	253.42	247.69 (6.38)
15	230.01	-	207.05	205.82	-
20	202.29	214.44	190.98	186.38	198.52 (12.54)
40	151.56	167.52	146.53	158.78	156.10 (9.13)
60	131.27	133.14	113.68	108.35	121.61 (12.45)
90	74.00	96.46	73.06	90.58	83.53 (11.79)
120	50.29	70.44	45.91	66.55	58.30 (12.02)
180	28.18	27.21	17.41	28.20	25.25 (5.25)
240	14.66	13.62	6.75	11.59	11.66 (3.51)
300	7.58	7.17	4.25	5.42	6.11 (1.55)
360	3.85	3.47	1.96	2.75	3.01 (0.84)
$t_{1/2}^{\beta}$ (min.)	64.07	55.99	51.87	50.56	55.62 (6.09)

Table 9: Concentration of dipropylacetic acid in human plasma and semen after 500 mg oral doses of the carboxylic acid

Subject	Experiment Number	Time after dose (hours)	DPA Concentration (µg/ml) Semen	Plasma	Semen/Plasma
A	1	2.0	2.92	50.51	0.058
	2	3.0	2.71	35.25	0.077
	3	4.3	3.26	35.69	0.091
	2	6.8	2.15	28.13	0.076
	3	8.7	2.28	29.22	0.078
	4	14.5	1.15	19.02	0.061
	4	28.7	0.67	11.06	0.061
B	1	5.5	2.72	35.83	0.076
	1	13.0	2.35	--	--
	1	14.5	--	21.32	--
	1	29.0	0.81	10.73	0.076
	1	39.0	0.53	8.74	0.061

Table 10: Effect of drugs on sperm motility after 30-minute incubations at 37° C

Drug	Drug Concentration (mM)	Sperm Motility (Nearest 20% of Control Value)
Chlorpromazine HCl	2.0	0
	1.0	0
	0.01	100
Trifluoperazine HCl	1.0	0
	0.01	100
Desipramine HCl	1.0	40
	0.01	100
Sodium Phenytoin	0.2	100
Sodium Dipropylacetate	2.0	100
d,l-methadone HCl	2.0	0
	0.02	100
d-methadone HCl	1.0	0
l-methadone HCl	2.0	0
	0.02	100
Naloxone HCl	1.0	100
Naloxone HCl + l-methadone HCl	1.0 + 1.0	0

Table 11: Hydrolysis of Floctafenin in Alkaline Solution (pH 11.0) at 25° C.

Floctafenin (µg/ml)	Optical Density at 348 nm Time 0	Optical Density at 348 nm after 60 minutes
6.67	.290	.113
13.33	.574	.225
26.67	1.157	.490
33.33	1.471	.688

Floctafenin was added in various amounts to a series of tubes. Sodium carbonate solution was added such that the total aqueous volume in each tube was 10 ml and the pH was 11.0. Some of the tubes were extracted immediately with 15 ml ethyl ether, while other tubes were extracted after 60 minutes. Ten ml of each ether extract was further equilibrated with 2 ml 0.1 N HCl. The u.v. absorbance of the acid extract was determined at 348 nm using 0.1 N HCl as the reference. The concentration of floctafenin is expressed in the table as the theoretical drug concentration in the cuvette if no hydrolysis had occurred.

Table 12: Identification of Hydroxylated SKF 525-A

Ions in the Mass Spectra for SKF 525-A	Mass Difference	Ions in the Mass Spectra for Hydroxy-SKF 525-A
353 (Molecular Ion)	16	369 (Molecular Ion)
338	16	354
281	16	297
238	16	254
209	16	225
167	16	183
166	16	182
165	16	181
152		152
115		115
100		100
99		99
86 (Mass Ion)		86 (Mass Ion)

Table 13: Generation of metabolite-I (SKF 8742-A) from SKF 525-A by 9000 X g supernatant of rat testis homogenate: Effect of testosterone pretreatment

Each flask contained equivalent amounts of protein (37 ± 3 mg/ml incubation mix), SKF 525-A (initial concentration of 20 µg/ml) and cofactors for the regeneration of NADPH. Testosterone propionate was administered subcutaneously at a dose of 1 mg per day (2.9 to 3.9 mg/kg/day) for 10 days, while control animals received injections of drug vehicle alone. Animals were killed about 24 hours after the last injection.

Metabolite-I was not generated in additional control flasks that contained testicular enzymes and cofactors, but no drug, or in flasks that contained SKF 525-A (20 µg/ml) and cofactors, but no enzymes.

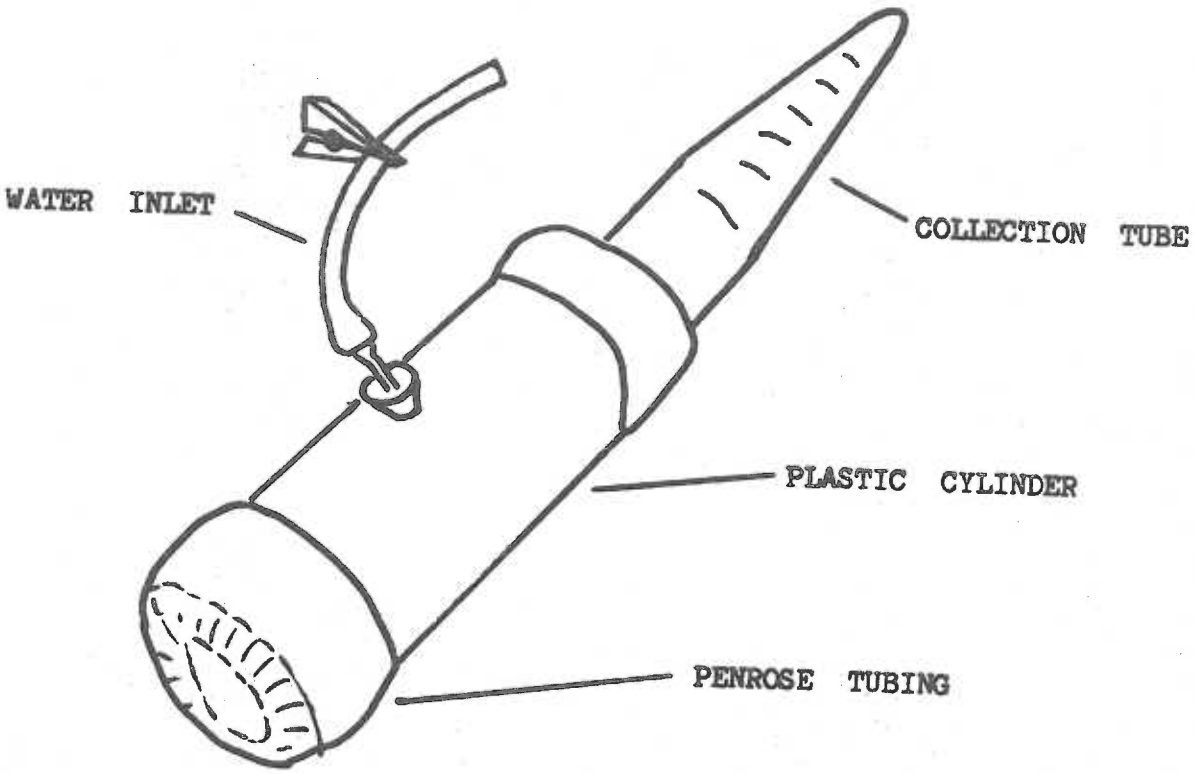
CONCENTRATION OF METABOLITE-I (nmol/ml)

Time (min)	Control				Testosterone - Pretreated	
	Flask #1	Flask #2	Flask #3	Flask #4	Flask #1	Flask #2
0	0	0	0	0	0	0
10	0.59	0.59	0.44	0.41	0	0
20	1.05	0.79	0.80	0.87	0	0.12
40	1.72	1.26	1.11	0.89	0	0.51
60	1.66	1.48	1.24	1.03	0.16	0.85

Table 14: Formation of SKF 8742-A from SKF 525-A in Incubations Containing Enzymes from Rat Testes

Source of Enzymes	Initial Concentration of SKF 525-A (μg/ml)	Concentration of SKF 8742-A after 60 minutes (μg/ml)	(nmol/ml)	Concentration of Protein in Incubation Mix (mg/ml)
100,000 x g pellet of 10,000 x g supernatant	40	6.06	16.7	6
100,000 x g pellet of 10,000 x g supernatant	5	1.19	3.3	6
100,000 x g supernatant of 10,000 x g supernatant	5	0	0	16
100,000 x g pellet of 10,000 x g supernatant				
Control	5	1.04	2.9	7
Phenobarbital pretreated	5	1.05	2.9	7

Figure 1: Artificial vagina used for the collection of ejaculate from rabbits.


WATER INLET
COLLECTION TUBE
PLASTIC CYLINDER
PENROSE TUBING

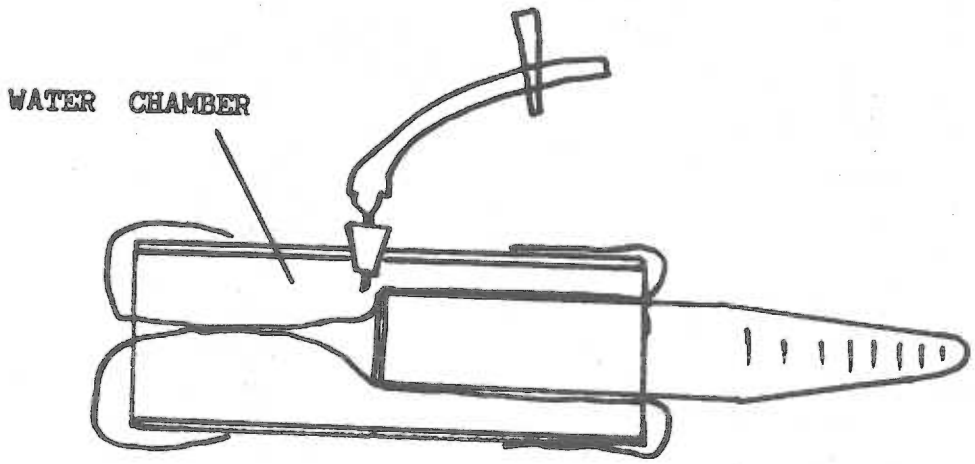
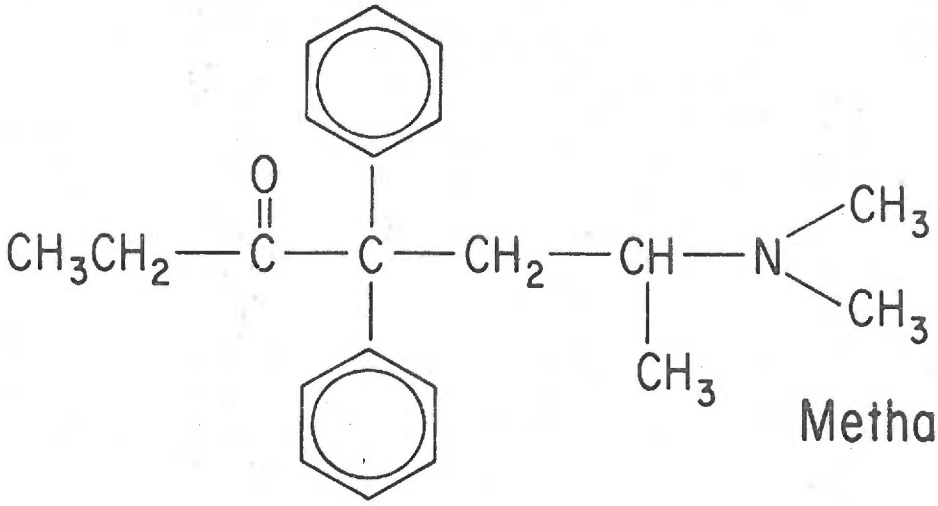
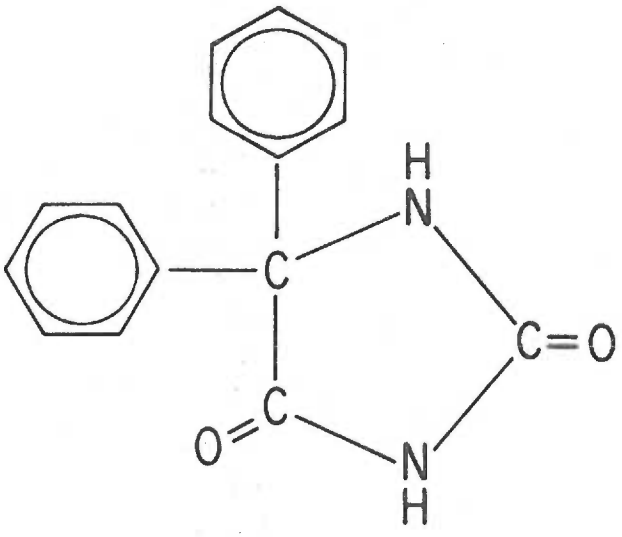

WATER CHAMBER

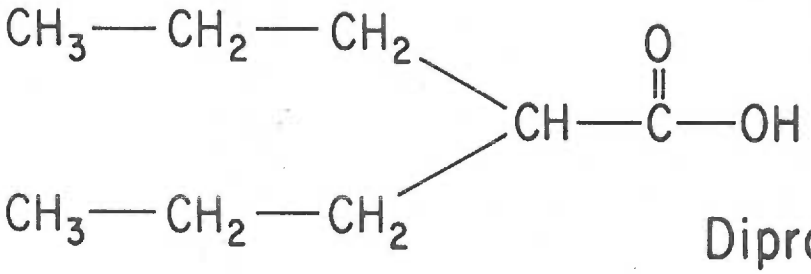
Figure 2: Chemical structures of methadone, phenytoin and dipropylacetate.



Methadone



Phenytoin



Dipropylacetic Acid

Figure 3: Gas-liquid chromatogram of an extract of rabbit blood obtained 40 minutes after an intramuscular dose of 40 mg d-methadone HCl. Tetracosane was added during the last extraction step and subsequently employed as the internal standard. Two metabolites of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl pyrroline (EMDP), are also apparent in the chromatogram.

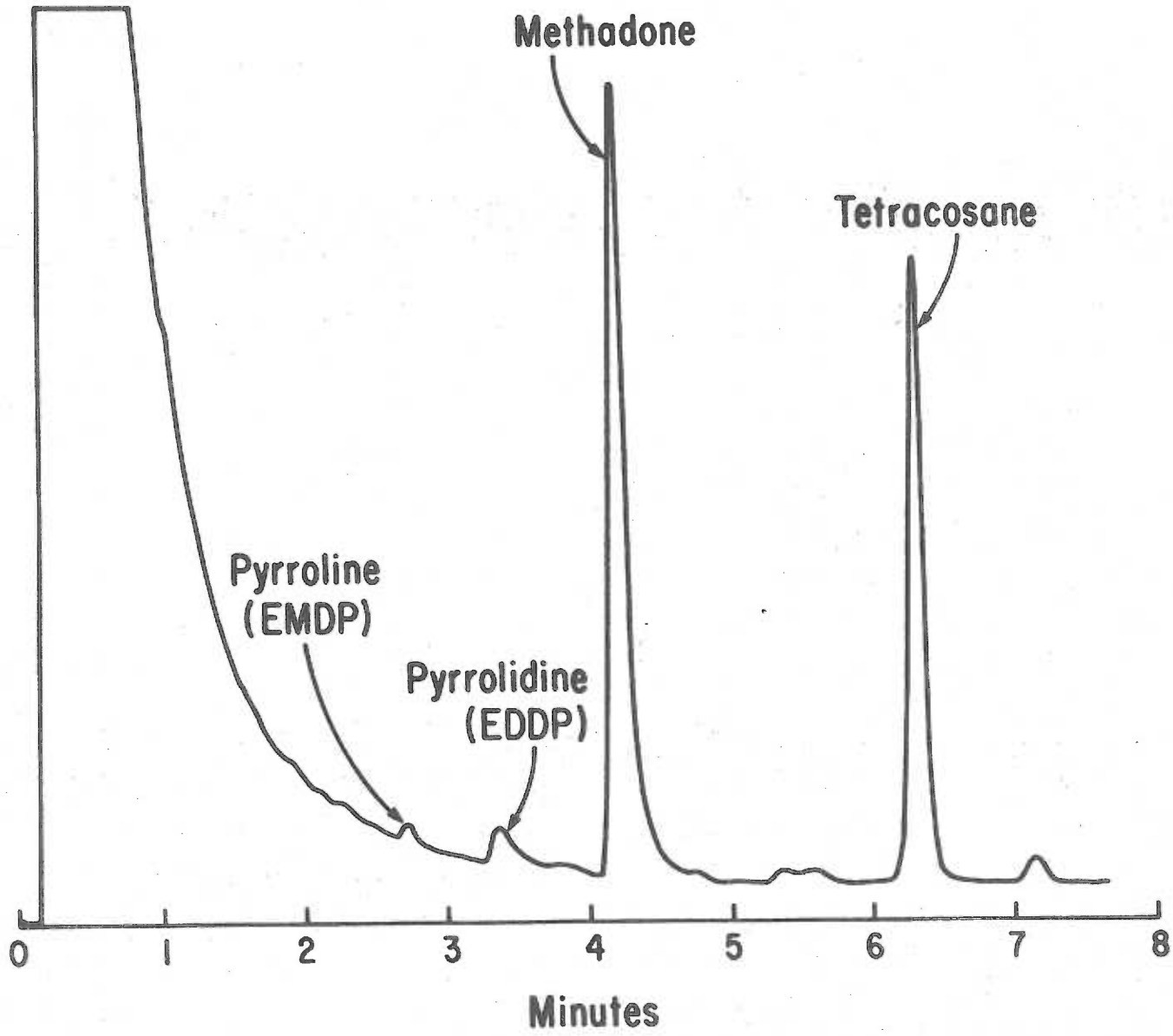

Methadone
Tetracosane
Pyrroline
(EMDP)
Pyrrolidine
(EDDP)
0
1
2
3
4
5
6
7
8
Minutes

Figure 4: Standard curve for d-methadone using gas-liquid chromatography.

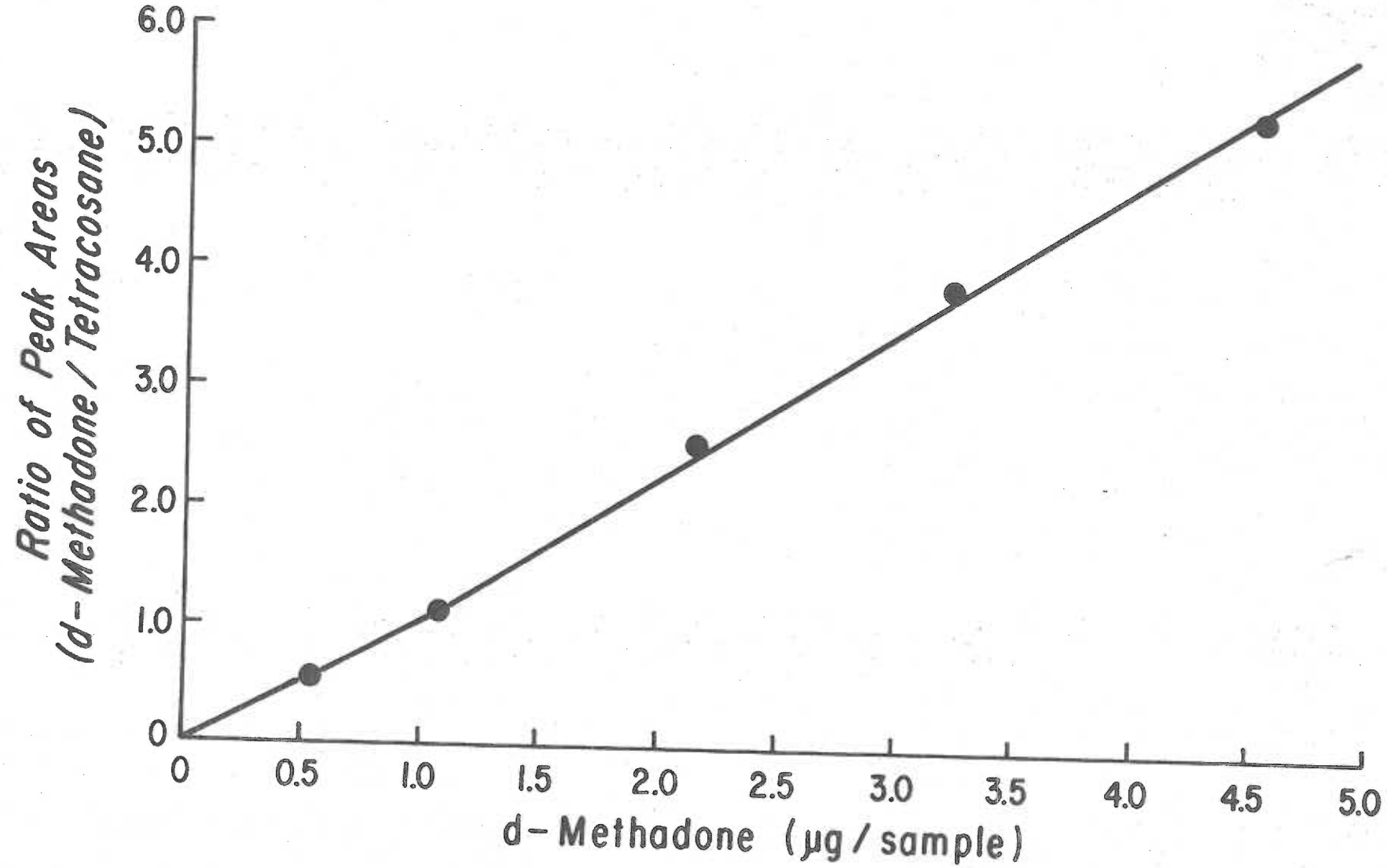

Ratio of Peak Areas
(d-Methadone / Tetracosane)
6.0
5.0
4.0
3.0
2.0
1.0
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
d-Methadone (µg / sample)

Figure 5: Biotransformation of methadone.

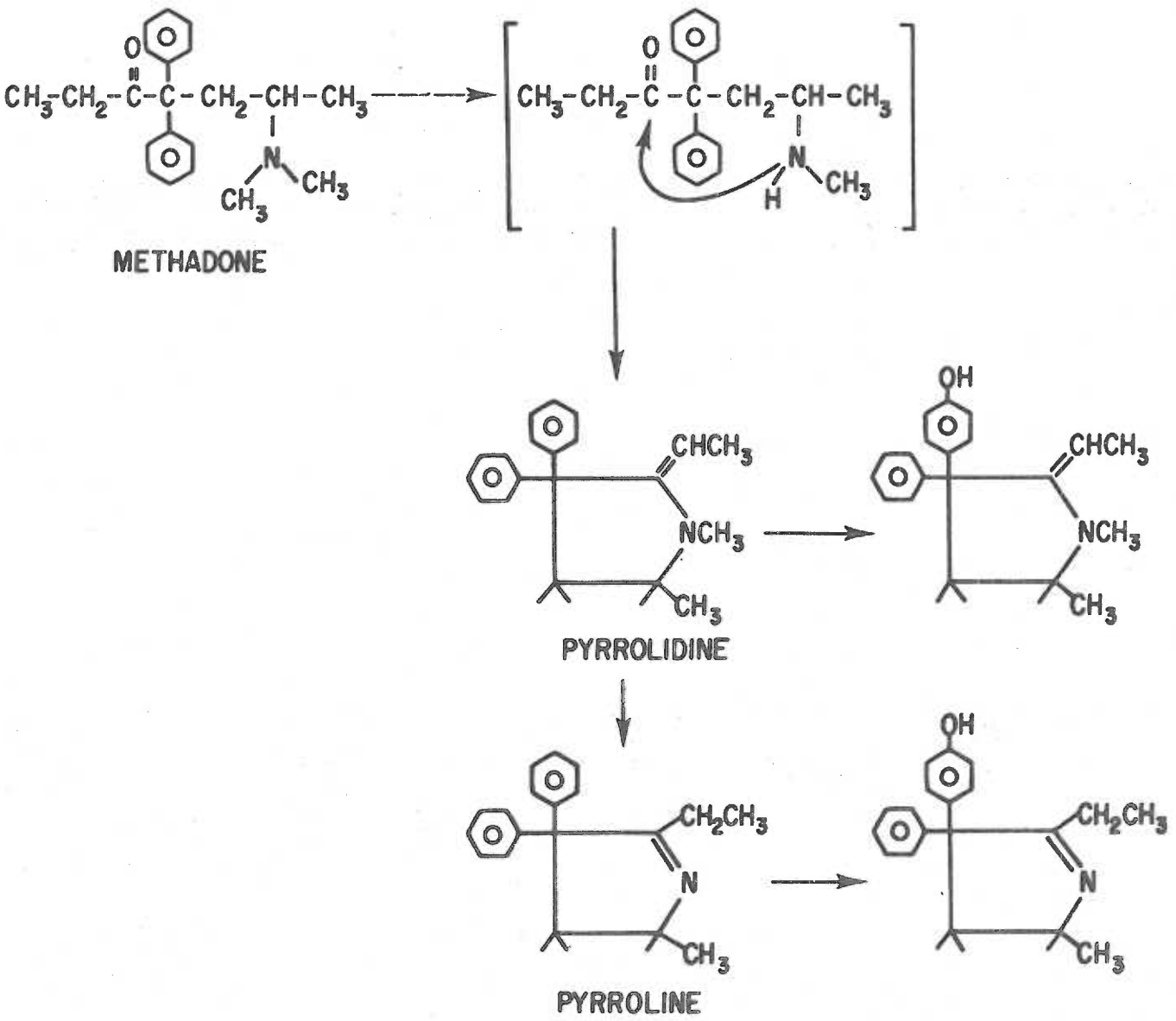

METHADONE
PYRROLIDINE
PYRROLINE

Figure 6: Standard curve for phenytoin using colorimetry. Aliquots of a stock solution of phenytoin (40 µg/ml in ethanol) were evaporated to dryness in a series of extraction tubes. Five ml human plasma were added to each tube. The method of Dill et al. (J. Pharmacol. Exp. Ther. 118: 270-279, 1956) was then employed. The reported standard curve indicates the amount of drug added to each tube prior to extraction.

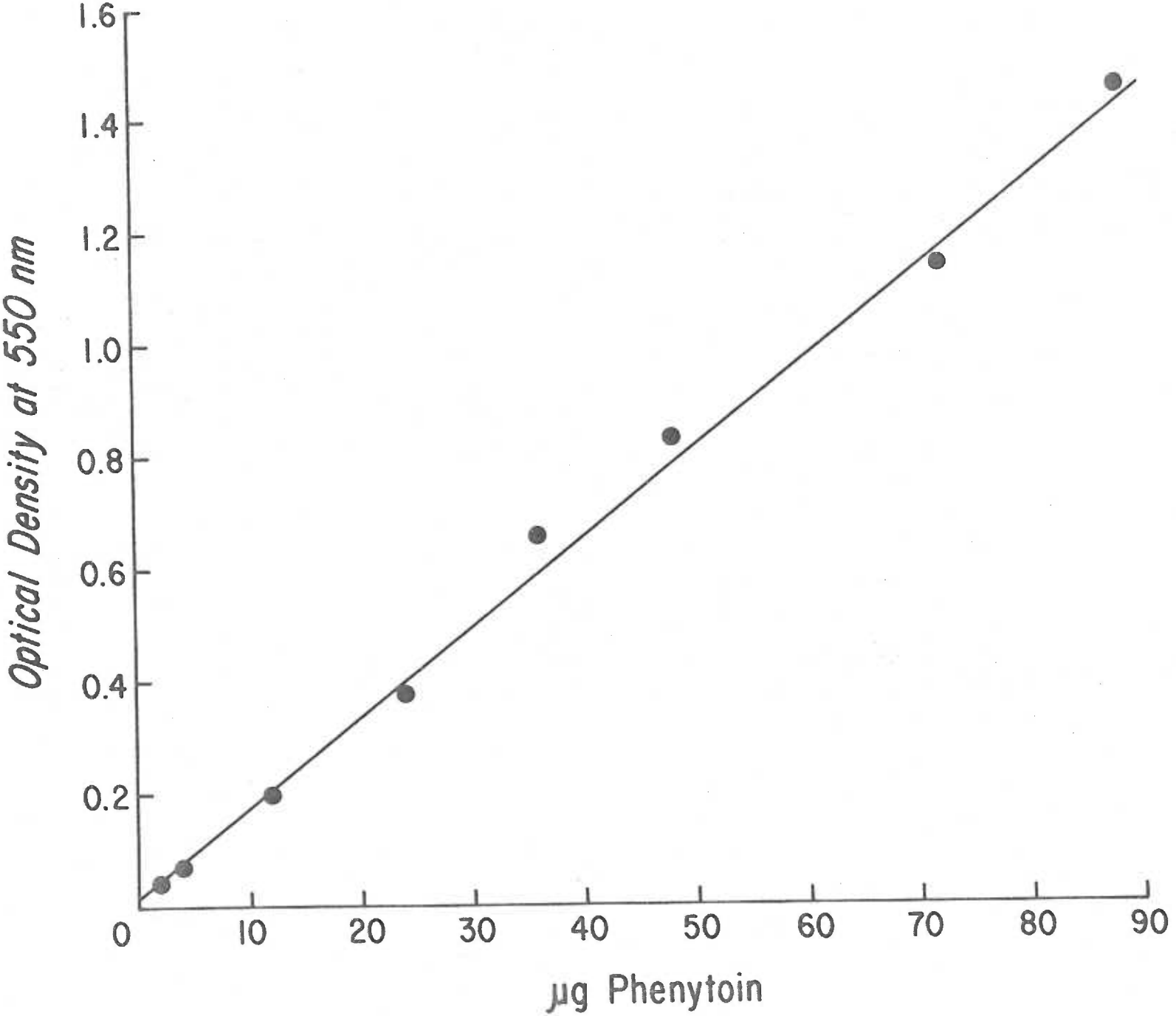

Optical Density at 550 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
70
80
90
µg Phenytoin

Figure 7: Standard curve for dipropylacetate using gas-liquid chromatography. Known amounts of sodium dipropylacetate were extracted from human plasma and chromatographed as described in the text. The amount of drug is reported as the quantity of dipropylacetic acid in each tube prior to extraction.

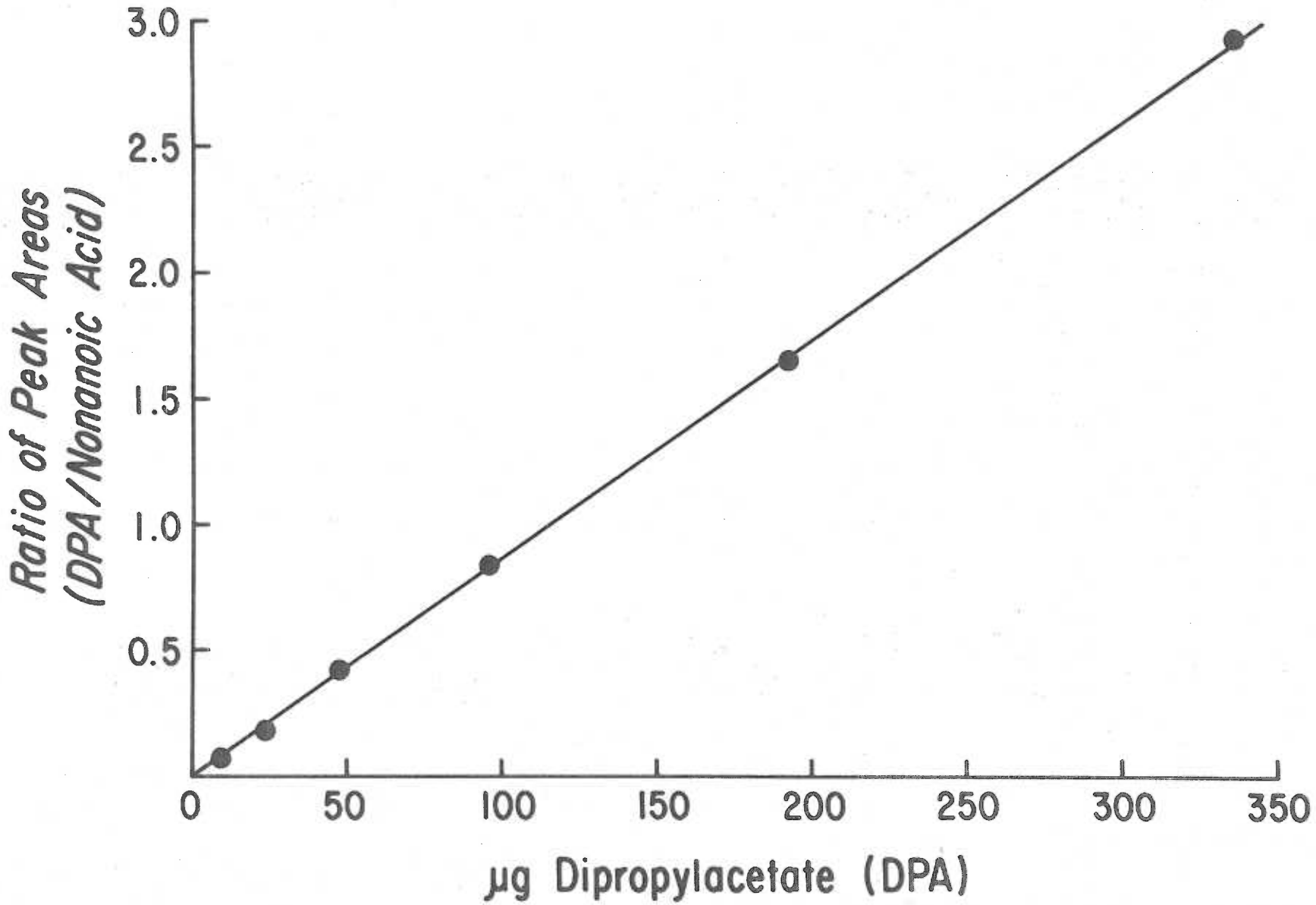

Ratio of Peak Areas
(DPA/Nonanoic Acid)
3.0
2.5
2.0
1.5
1.0
0.5
0
50
100
150
200
250
300
350
µg Dipropylacetate (DPA)

Figure 8: Gas-liquid chromatogram of an extract of rabbit plasma containing dipropylacetate and its metabolites. Nonanoic acid was added during the last extraction step and subsequently used as the internal standard.

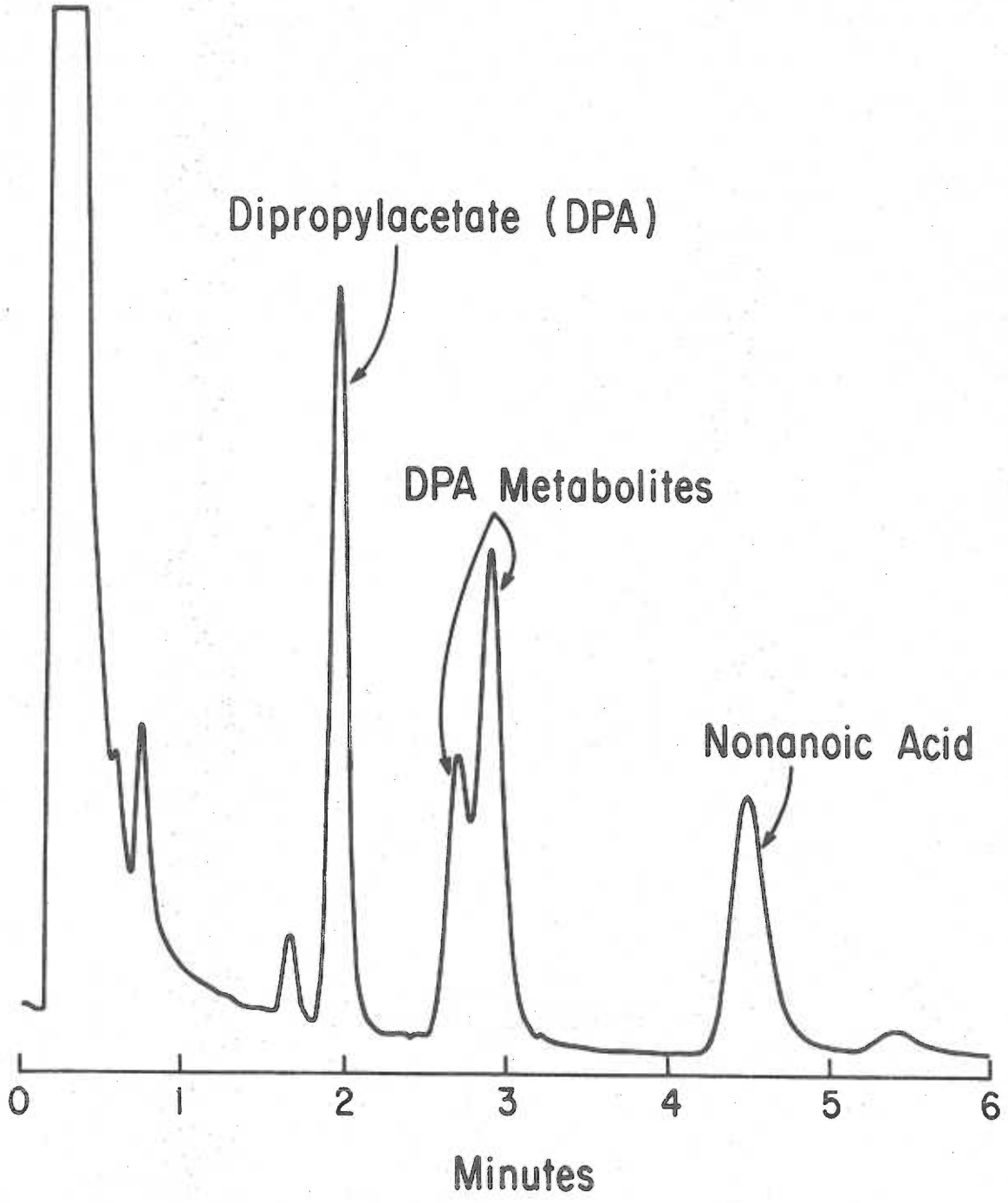

Dipropylacetate (DPA)
DPA Metabolites
Nonanoic Acid
0
1
2
3
4
5
6
Minutes

Figure 9: Concentration of d-methadone in rabbit arterial blood after a one-minute intravenous infusion of 40 mg d-methadone HCl. Points and bars represent the mean values and standard deviations, respectively, for four animals.

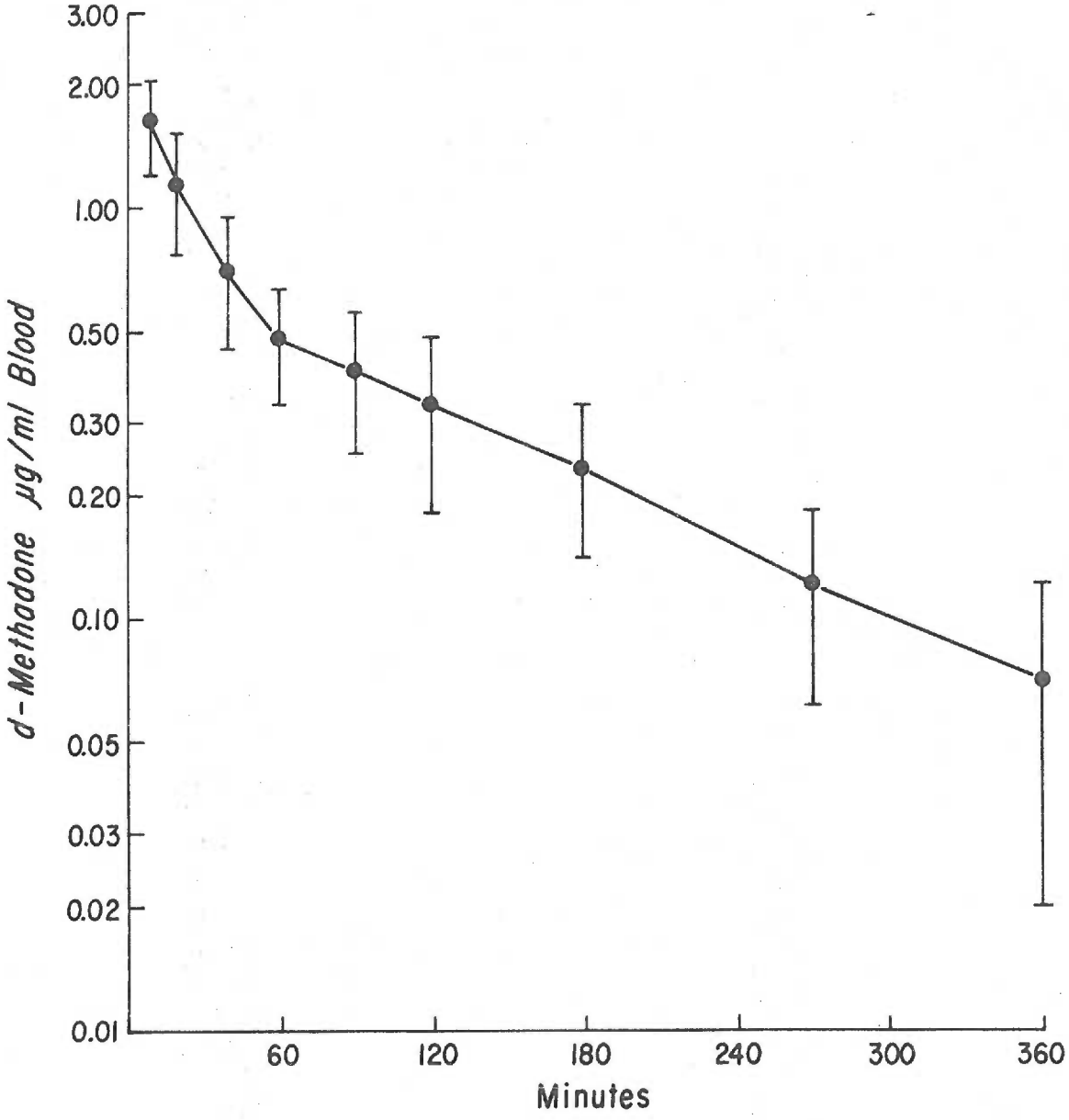

3.00
2.00
1.00
0.50
0.30
0.20
0.10
0.05
0.03
0.02
0.01
d-Methadone µg/ml Blood
60
120
180
240
300
360
Minutes

Figure 10: Concentration of d-methadone in arterial blood and semen from rabbits after an intramuscular injection of 40 mg d-methadone HCl. Points and bars represent mean values and standard deviations, respectively. Four animals were used for the concentration-time curve for plasma. The number of drug determinations for each point in the concentration-time curve for semen is indicated in parentheses.

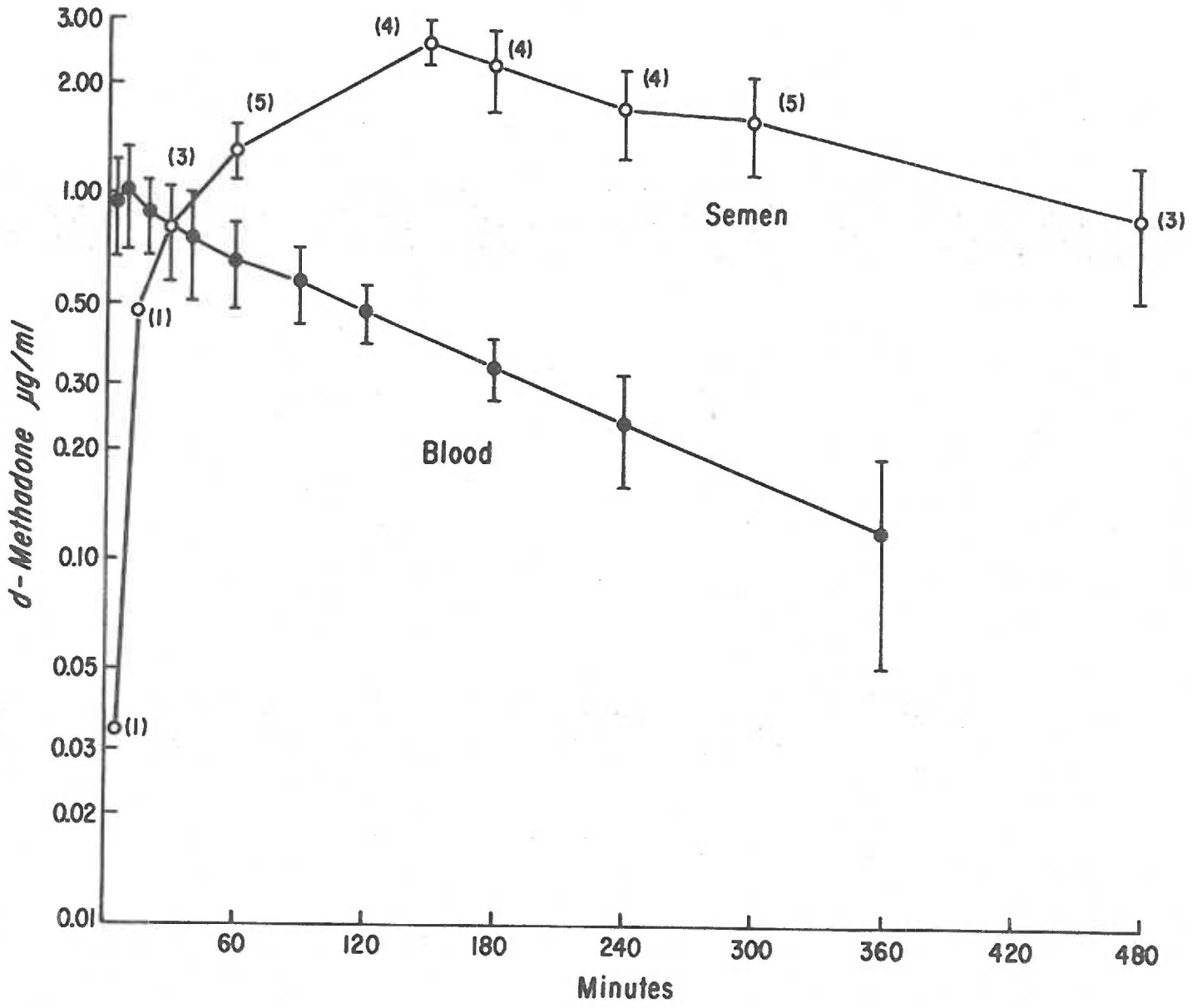

3.00
2.00
1.00
0.50
0.30
0.20
0.10
0.05
0.03
0.02
0.01
d-Methadone µg/ml
(4)
(4)
(4)
(5)
(5)
(3)
(3)
(1)
(1)
Semen
Blood
60
120
180
240
300
360
420
480
Minutes

Figure 11: Concentration of d-methadone in rabbit arterial blood after intravaginal administration of 40 mg d-methadone HCl. Points and bars represent the mean values and standard deviations, respectively, for four animals. A dialysis bag containing the drug was maintained in the vaginal lumen for a period of 90 minutes.

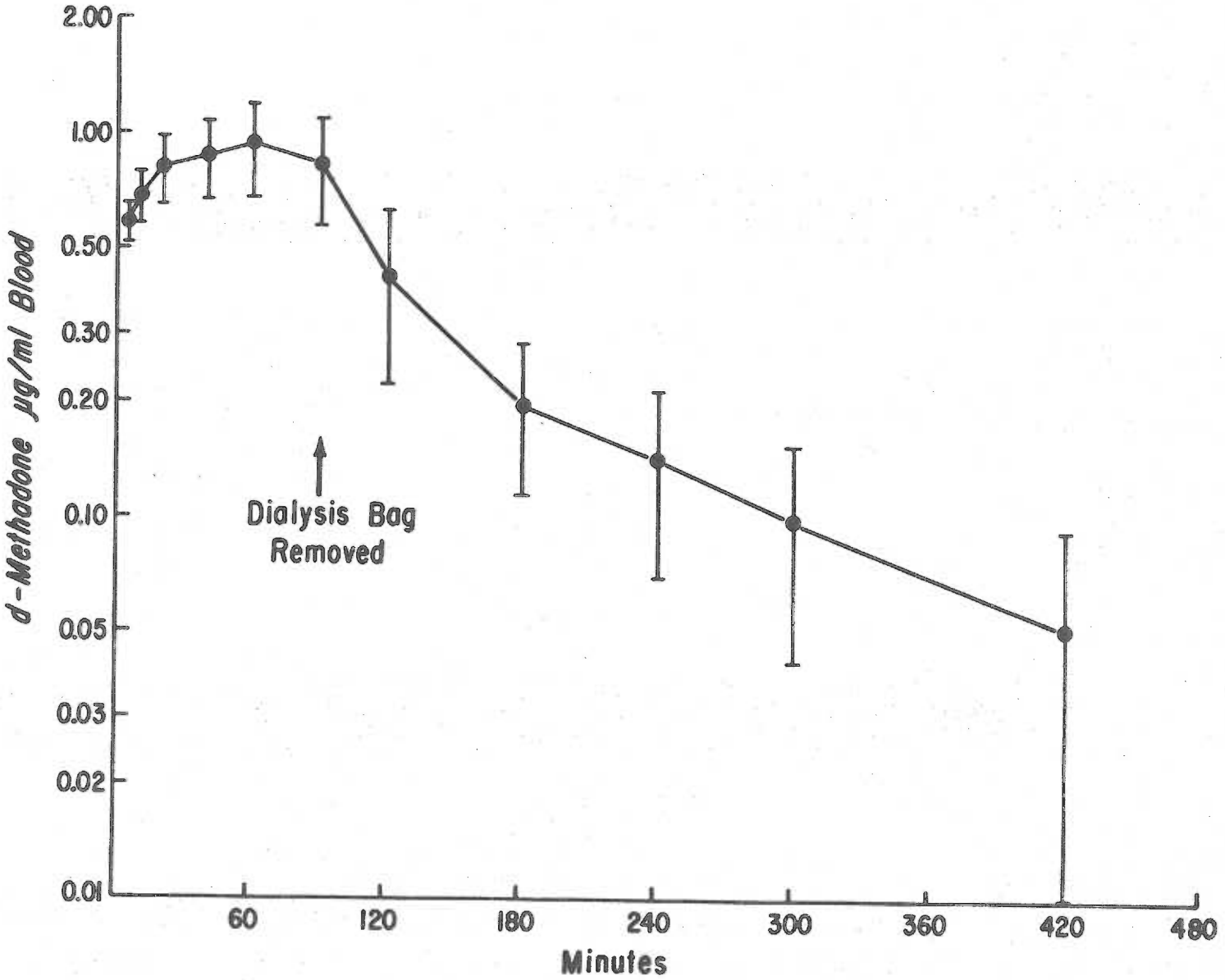

2.00
1.00
0.50
0.30
0.20
0.10
0.05
0.03
0.02
0.01
d-Methadone µg/ml Blood
Dialysis Bag
Removed
60
120
180
240
300
360
420
480
Minutes

Figure 12: Mass spectra of synthetic 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine (EDDP) (upper spectrum) and of EDDP extracted from rabbit blood after a dose of d-methadone (lower spectrum). The substance that was extracted from blood had a mass spectrum and GLC retention time similar, if not identical, to that of synthetic EDDP.

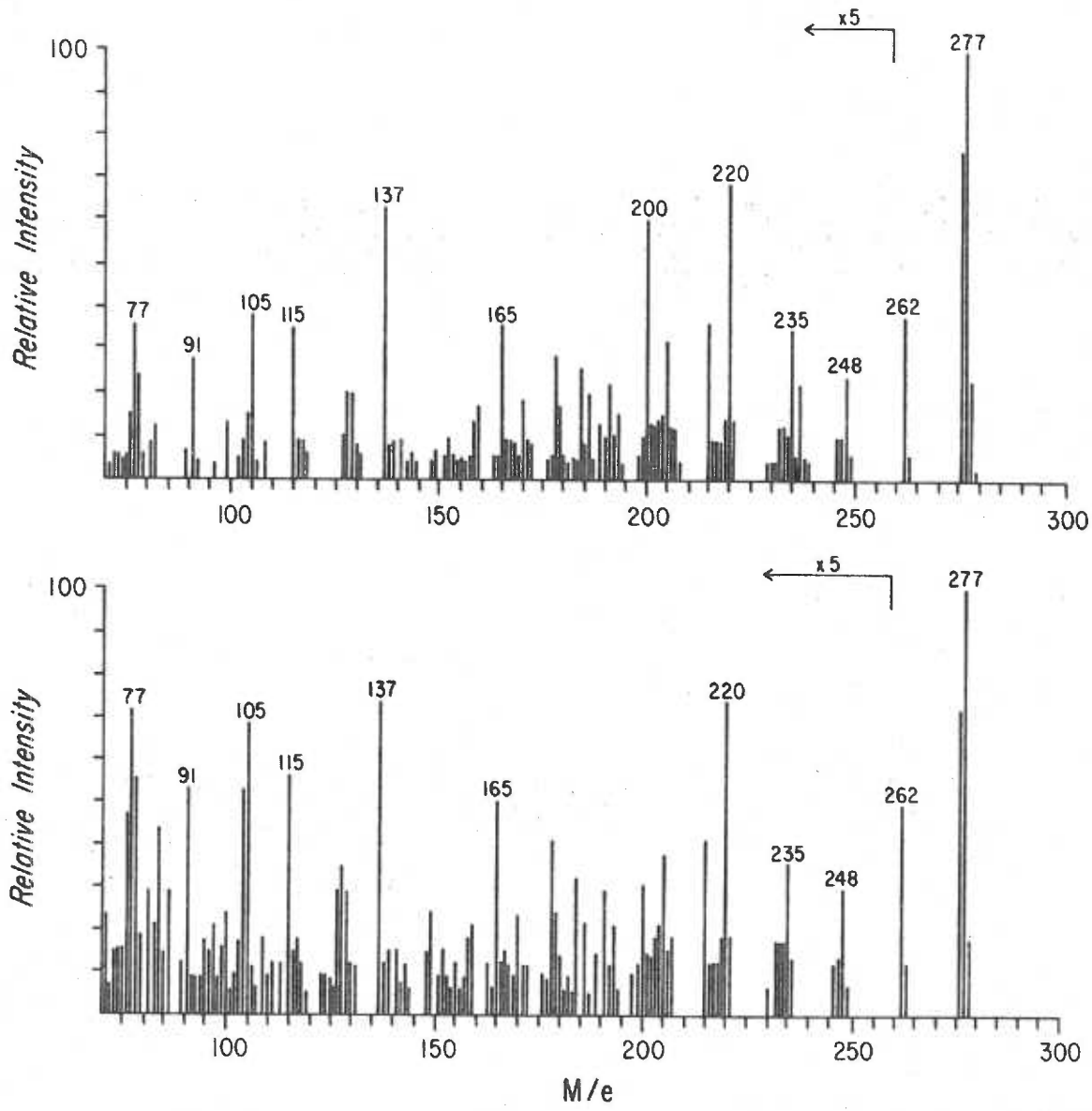

x5
100
277
220
137
200
77
105
115
165
235
262
91
248
Relative Intensity
100
150
200
250
300
x5
100
277
77
137
220
105
91
115
165
262
235
248
Relative Intensity
100
150
200
250
300
M/e

Figure 13: Mass spectra of synthetic 4-dimethyl-2,2,-diphenyl valeronitrile (upper spectrum) and of the impurity (lower spectrum) extracted from Dolophine (d,l-methadone HCl, Eli Lilly). The similarity of the two spectra indicates that the valeronitrile compound is present in marketed methadone.

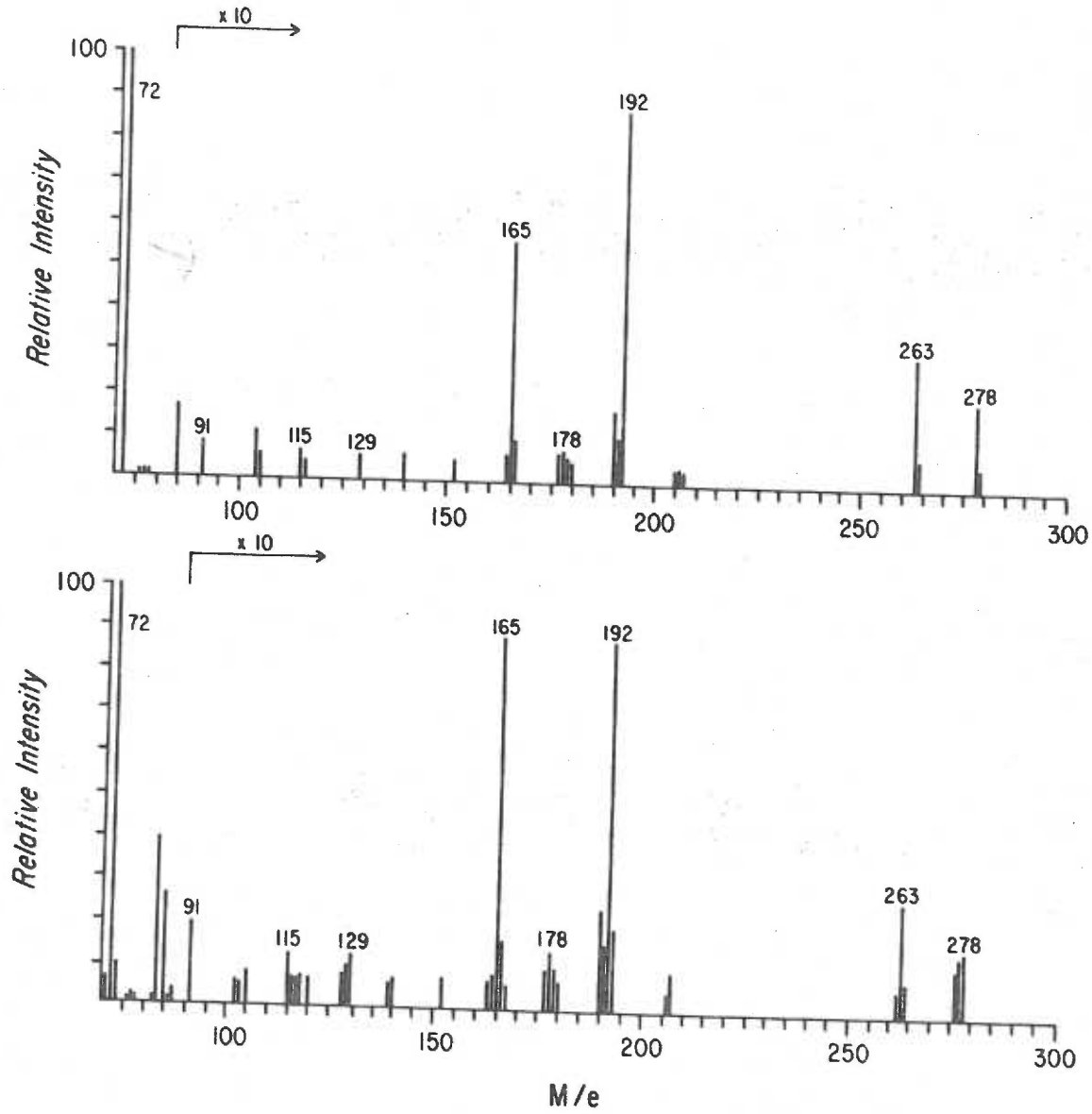

x 10
100
72
192
165
263
278
91
115
129
178
Relative Intensity
100
150
200
250
300
x 10
100
72
165
192
91
263
115
129
178
278
Relative Intensity
100
150
200
250
300
M/e

Figure 14: Gas-liquid chromatogram of an extract of Dolophine (d,l-methadone HCl, Eli Lilly) showing the presence of EDDP and the valeronitrile precursor of methadone.

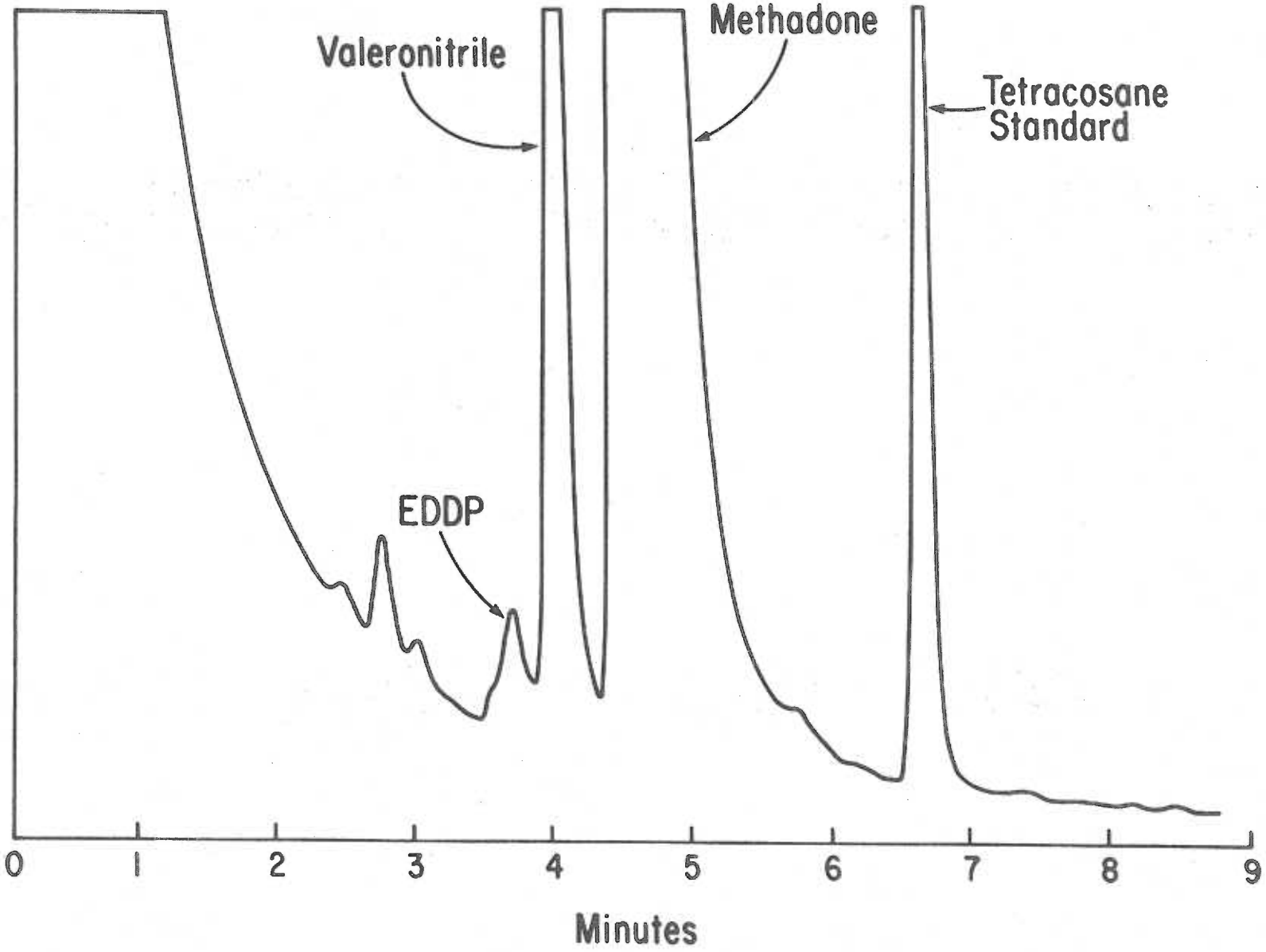

Valeronitrile
Methadone
Tetracosane
Standard
EDDP
0
1
2
3
4
5
6
7
8
9
Minutes

Figure 15: Concentration of phenytoin in plasma and semen of rabbits after a 45-second, intravenous infusion of 4.64 mg. Points and bars in the concentration-time curve for plasma represent mean values and standard deviations, respectively, for four animals. The least-squares method was used to determine a line for the drug concentrations in semen at 180 minutes and later.

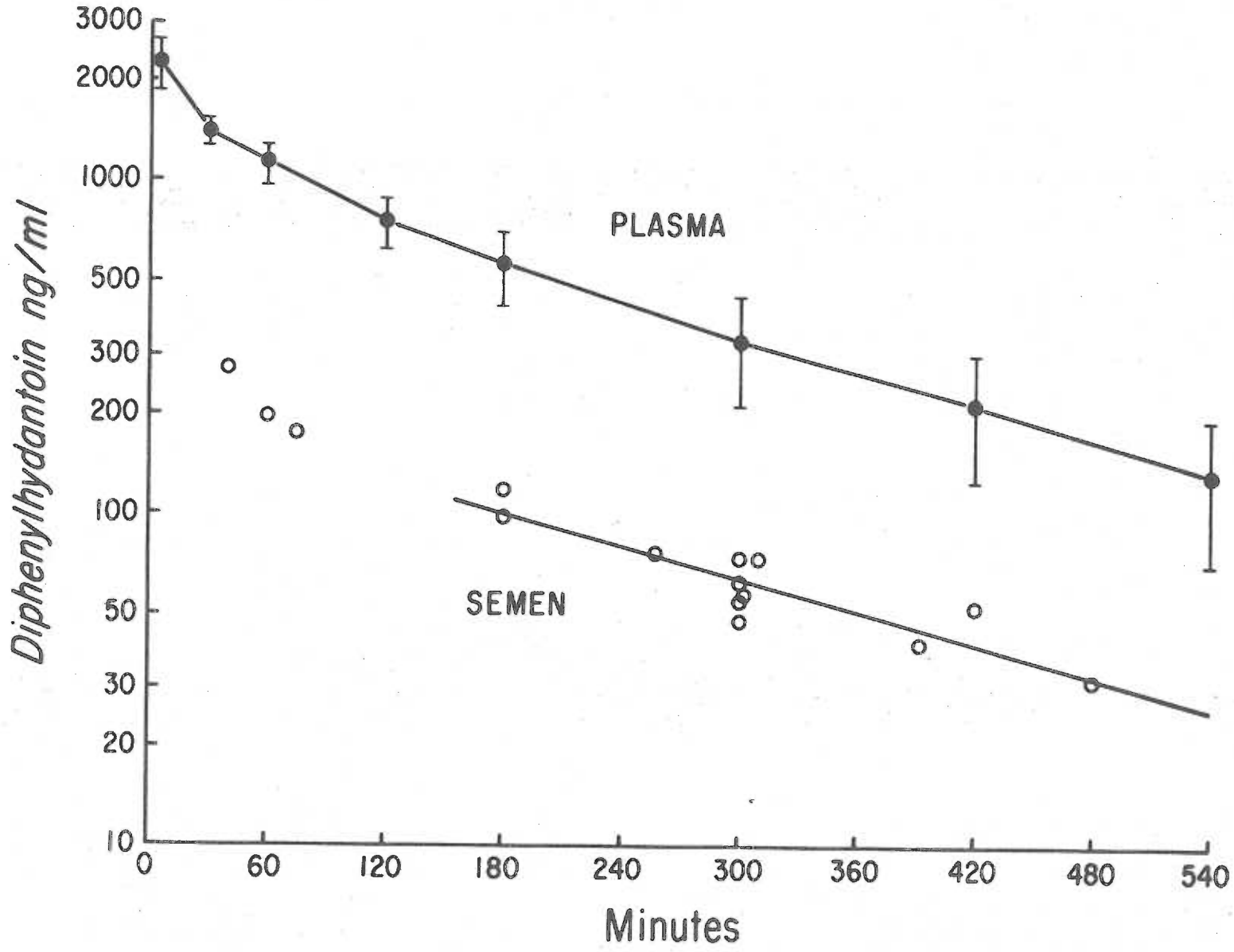

3000
2000
1000
500
300
200
100
50
30
20
10
Diphenylhydantoin ng/ml
PLASMA
SEMEN
0
60
120
180
240
300
360
420
480
540
Minutes

Figure 16: The linear relationship between the phenytoin concentration in semen and the phenytoin concentration in plasma. A best-fit line was determined by the least-squares method.

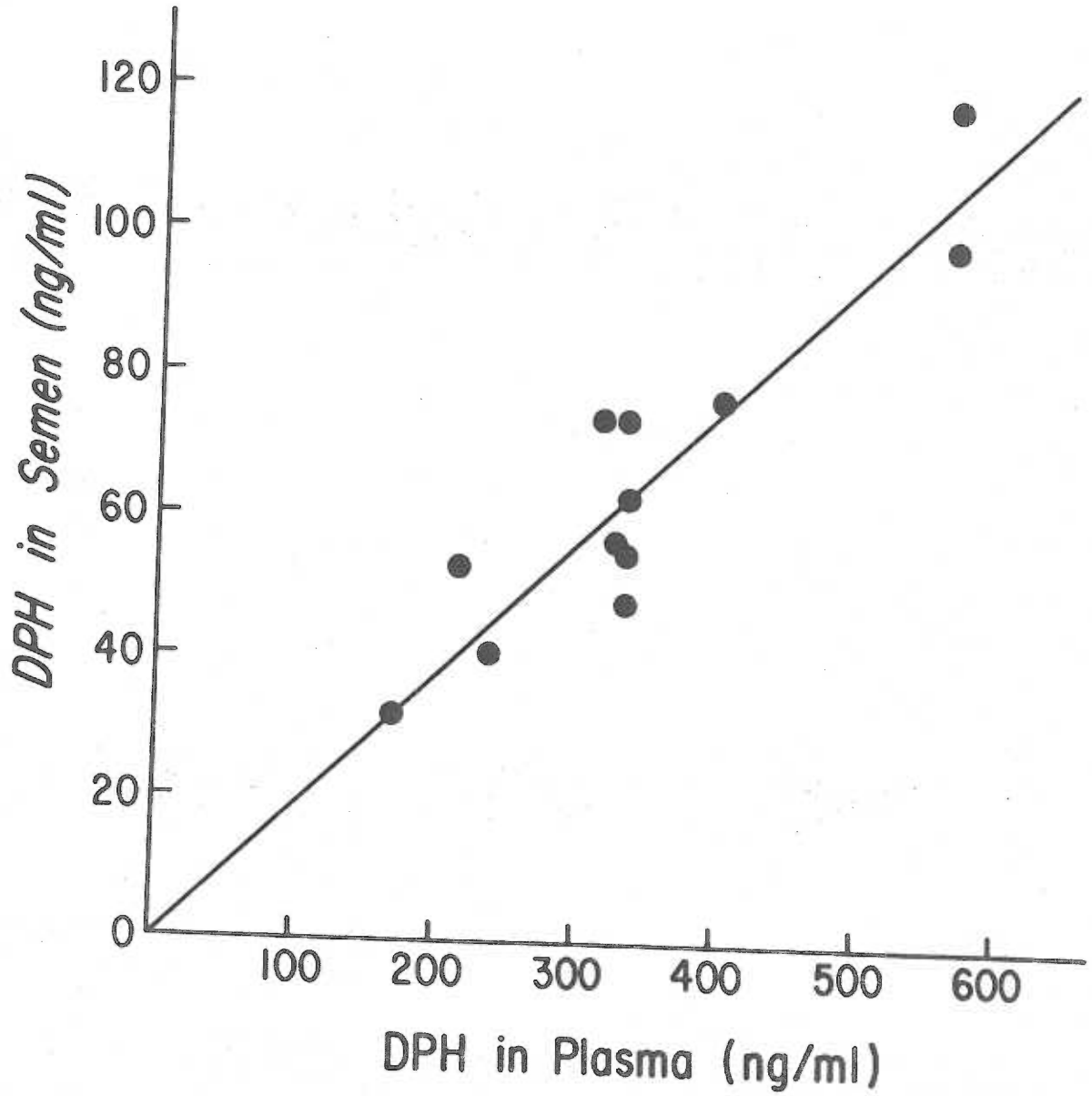

DPH in Semen (ng/ml)
120
100
80
60
40
20
0
100
200
300
400
500
600
DPH in Plasma (ng/ml)

Figure 17: Concentration of dipropylacetate in plasma and semen of rabbits after a one-minute, intravenous infusion of sodium dipropylacetate (50 mg/kg). Points and bars represent mean values and standard deviations, respectively. Data from four animals were used for each point in the concentration-time curve for plasma. The number of determinations for each point in the concentration-time curve for semen is indicated in parentheses.

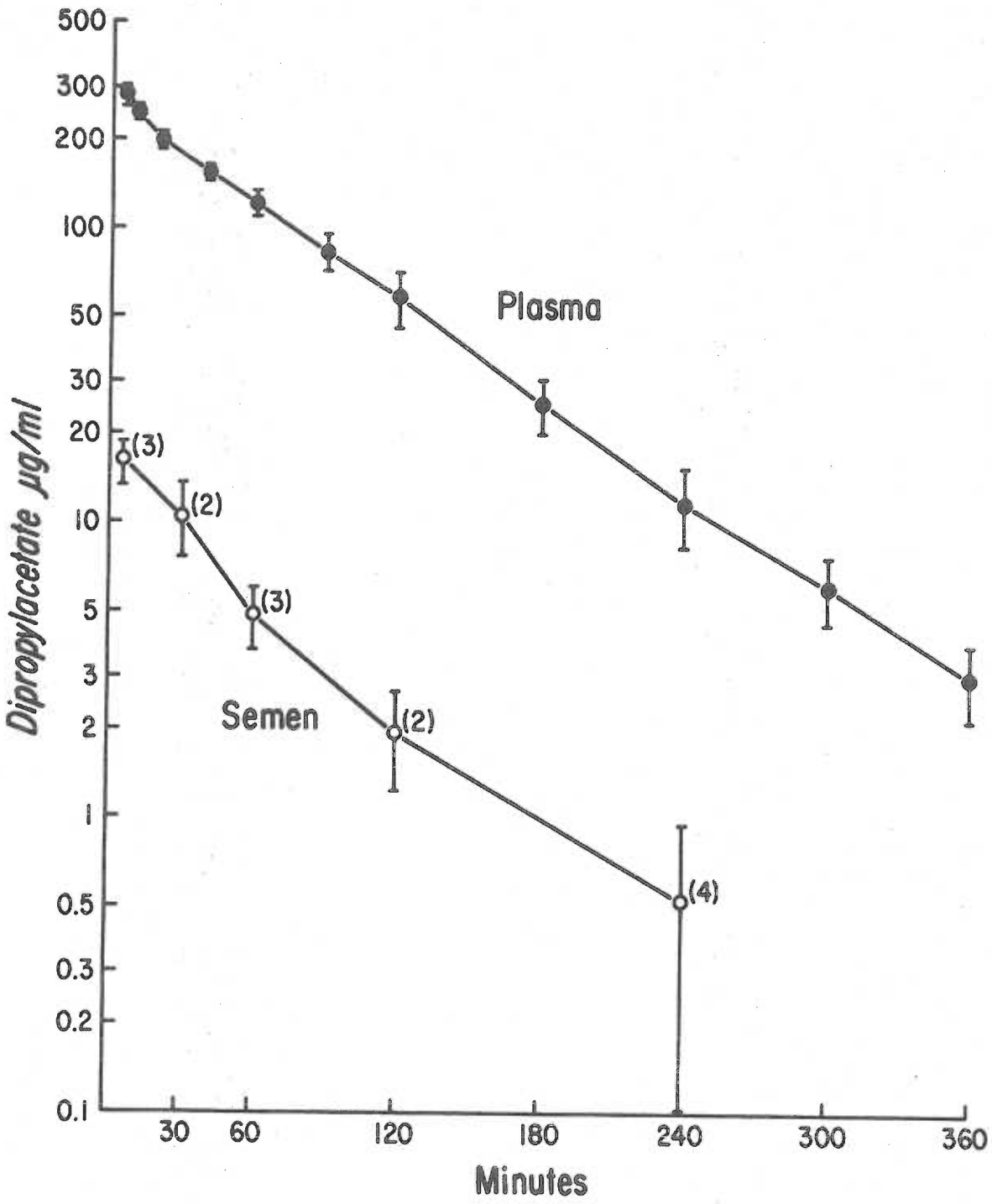

500
300
200
100
50
30
20
10
5
3
2
1
0.5
0.3
0.2
0.1
Dipropylacetate µg/ml
Plasma
Semen
(3)
(2)
(3)
(2)
(4)
30
60
120
180
240
300
360
Minutes

Figure 18: Effect of various concentrations of l-methadone on spermatozoan motility after a 30-minute incubation period.

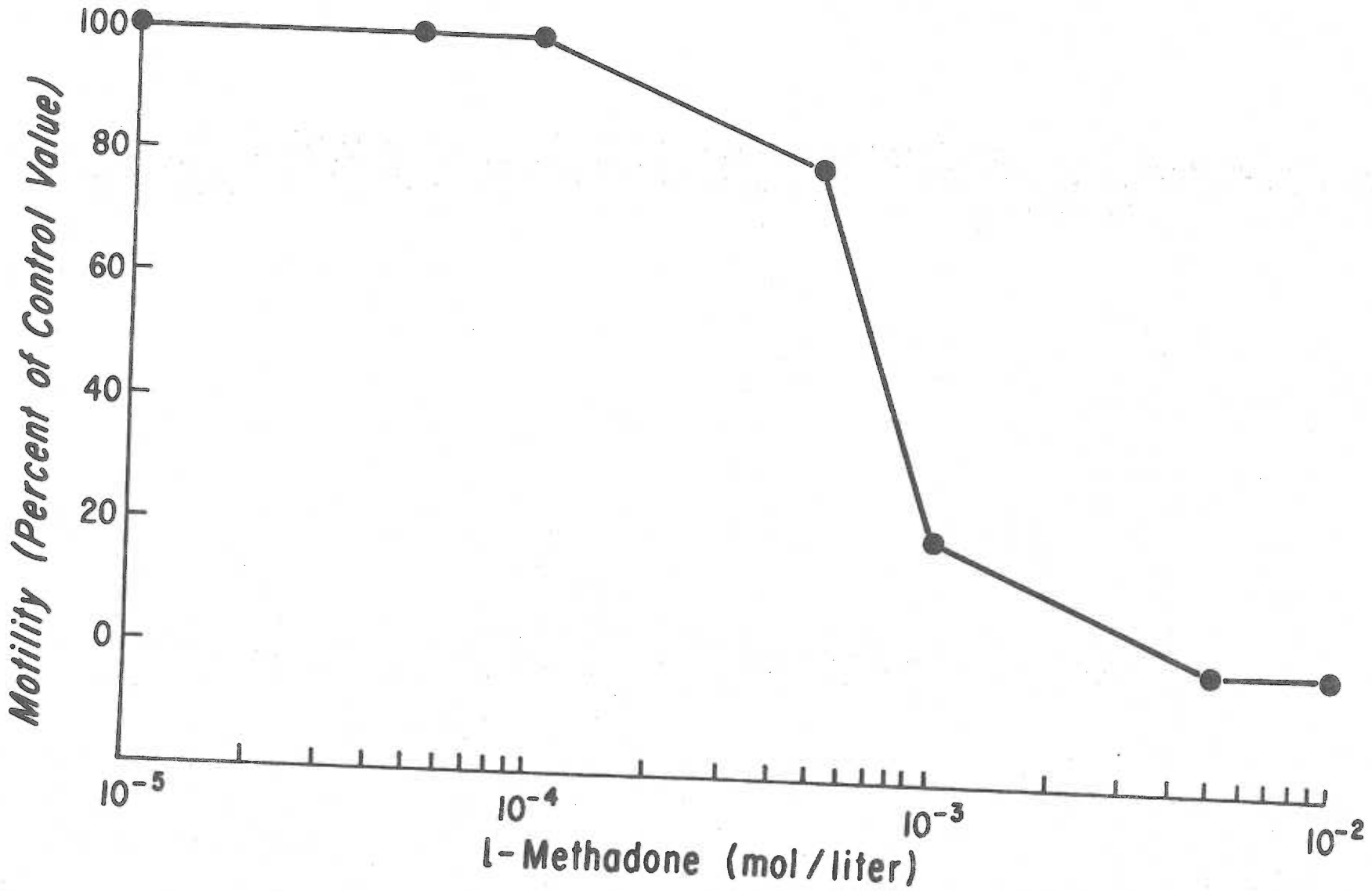
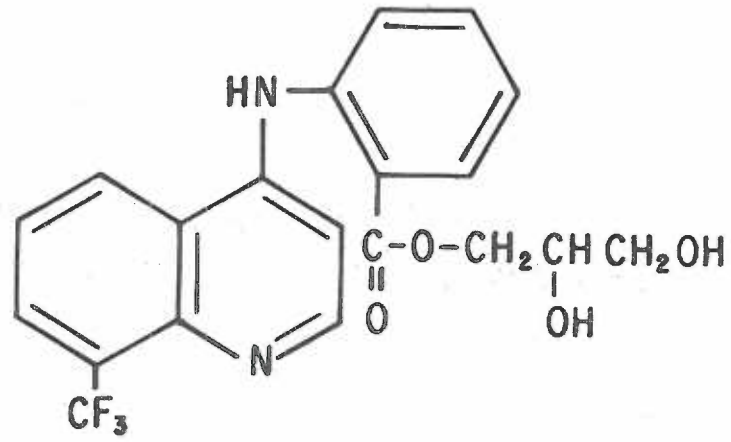
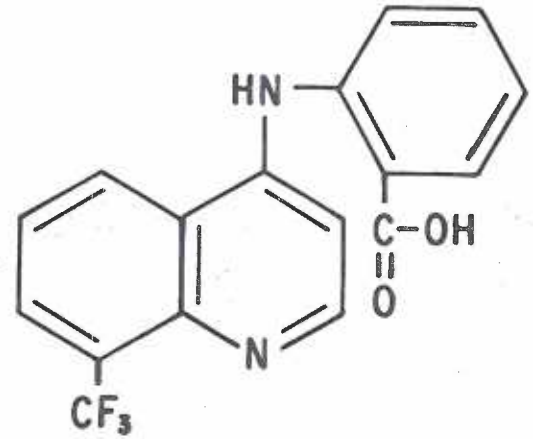

Motility (Percent of Control Value)
100
80
60
40
20
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
l-Methadone (mol/liter)

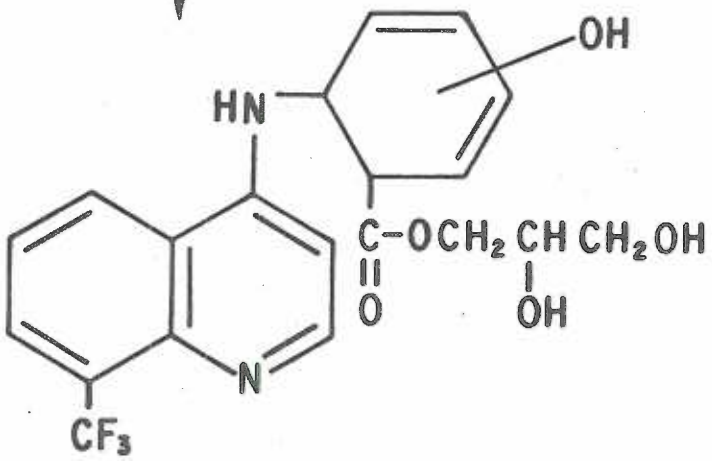
Figure 19: Biotransformation of floctafenin.



Floctafenin



Floctafenic Acid



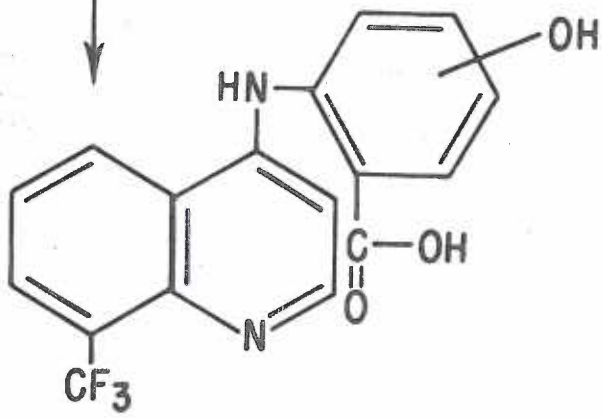


Figure 20: Gas-liquid chromatogram showing EDDP and d-methadone extracted from a testis incubation.

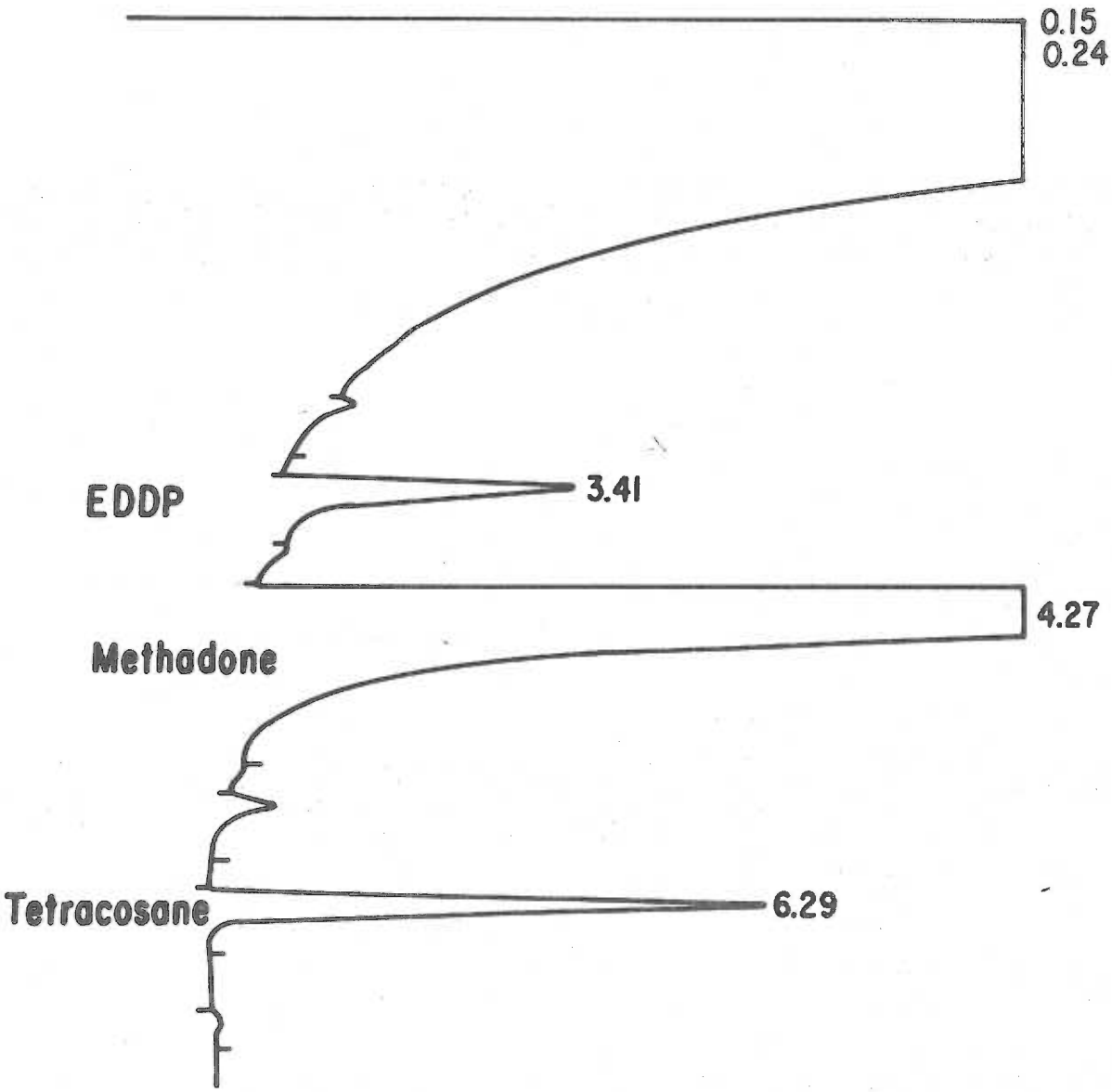

0.15
0.24
EDDP
3.41
4.27
Methadone
Tetracosane
6.29

Figure 21: Standard curve for 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine (EDDP) using gas-liquid chromatography. Known amounts of EDDP hydroiodide were extracted from tubes containing 9000 x g supernatant of rat testis homogenate.

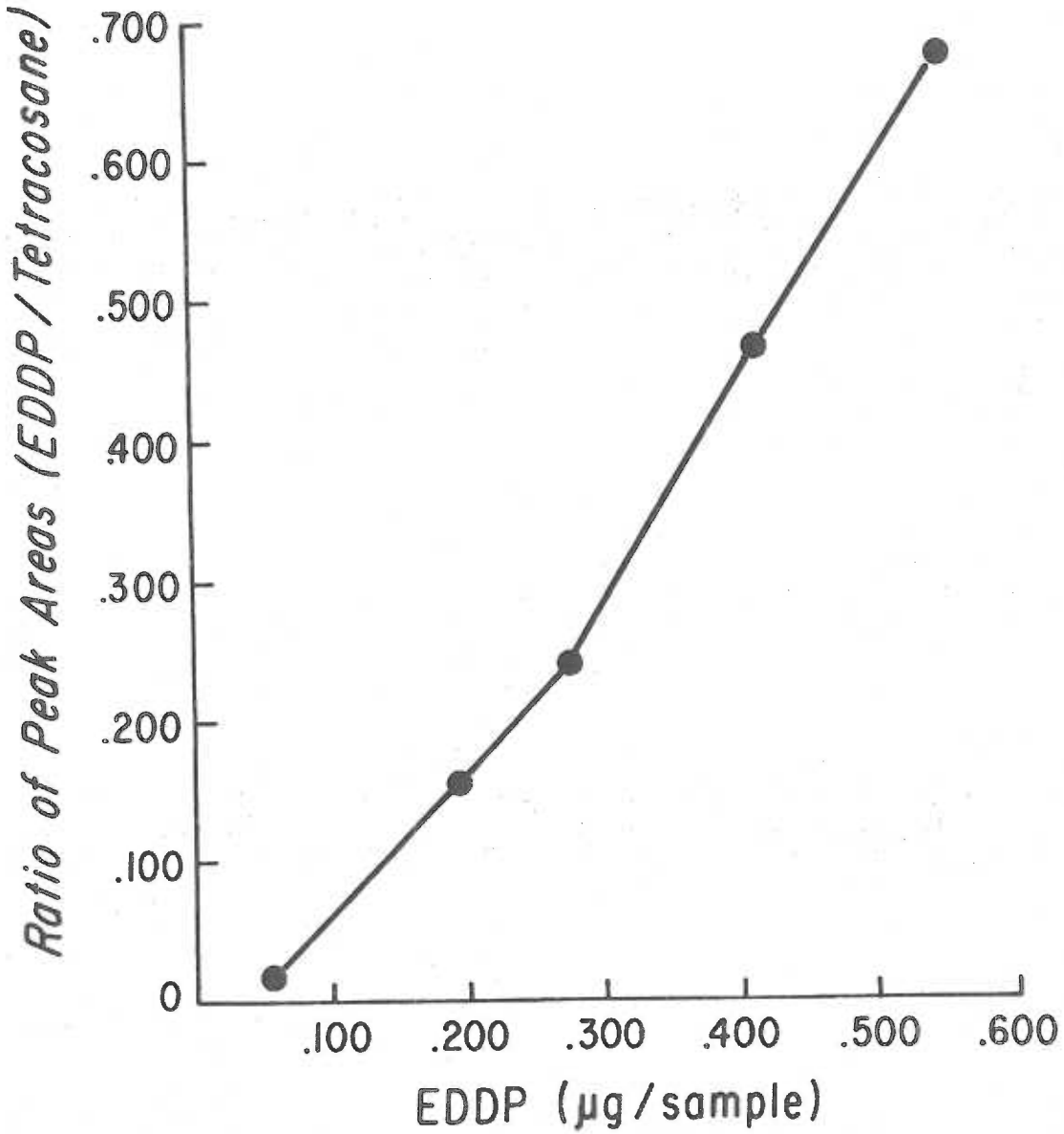

Ratio of Peak Areas (EDDP / Tetracosane)
.700
.600
.500
.400
.300
.200
.100
0
.100
.200
.300
.400
.500
.600
EDDP (µg / sample)

Figure 22: Standard curve for SKF 8742-A using gas-liquid chromatography. Known amounts of SKF 8742-A were extracted from tubes containing 9000 x g supernatant of rat testis homogenate.

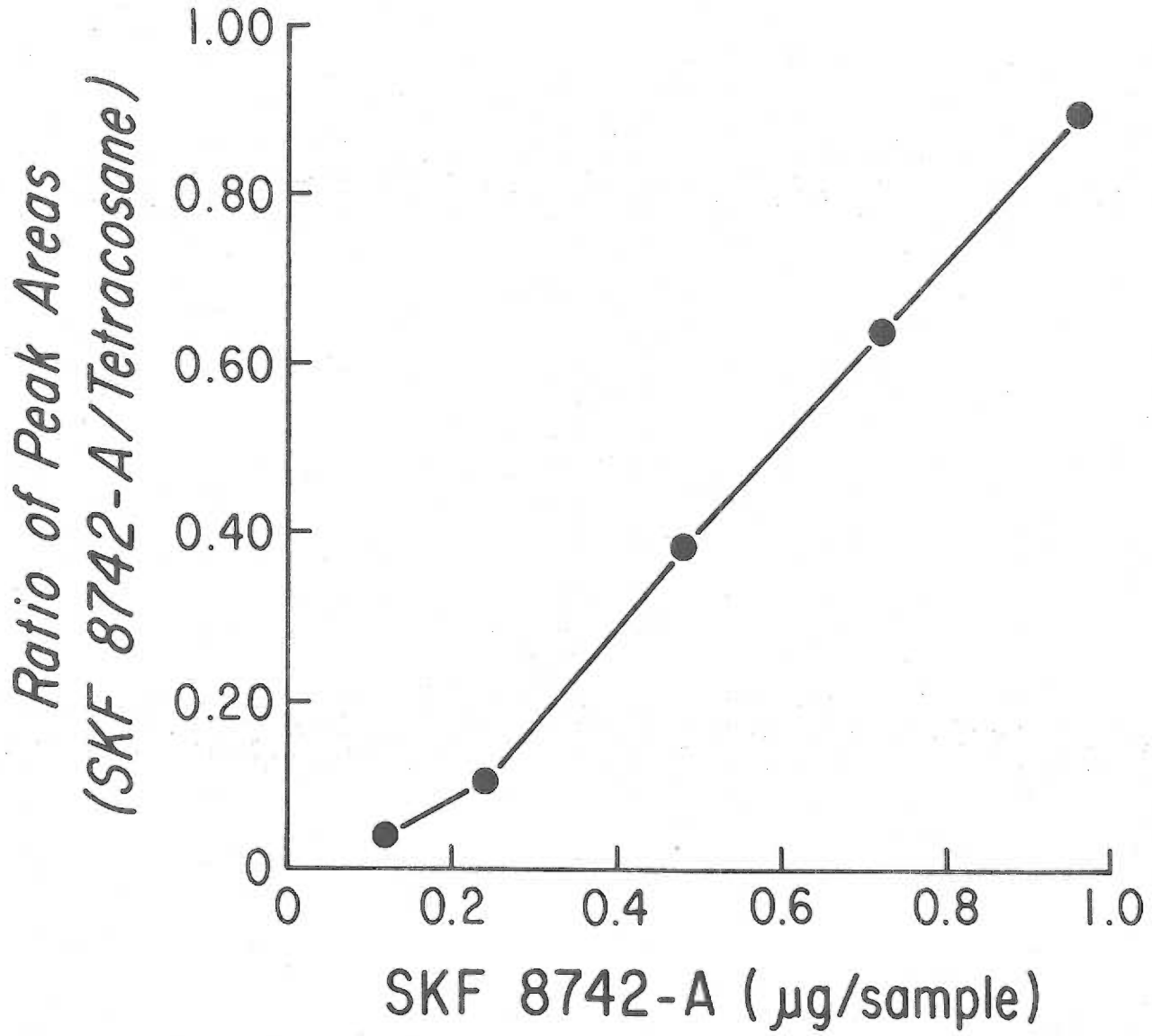

Ratio of Peak Areas
(SKF 8742-A/Tetracosane)
1.00
0.80
0.60
0.40
0.20
0
0
0.2
0.4
0.6
0.8
1.0
SKF 8742-A (µg/sample)

Figure 23: Gas-liquid chromatogram showing SKF 525-A and its metabolite SKF 8742-A. Tetracosane was employed as an internal standard.

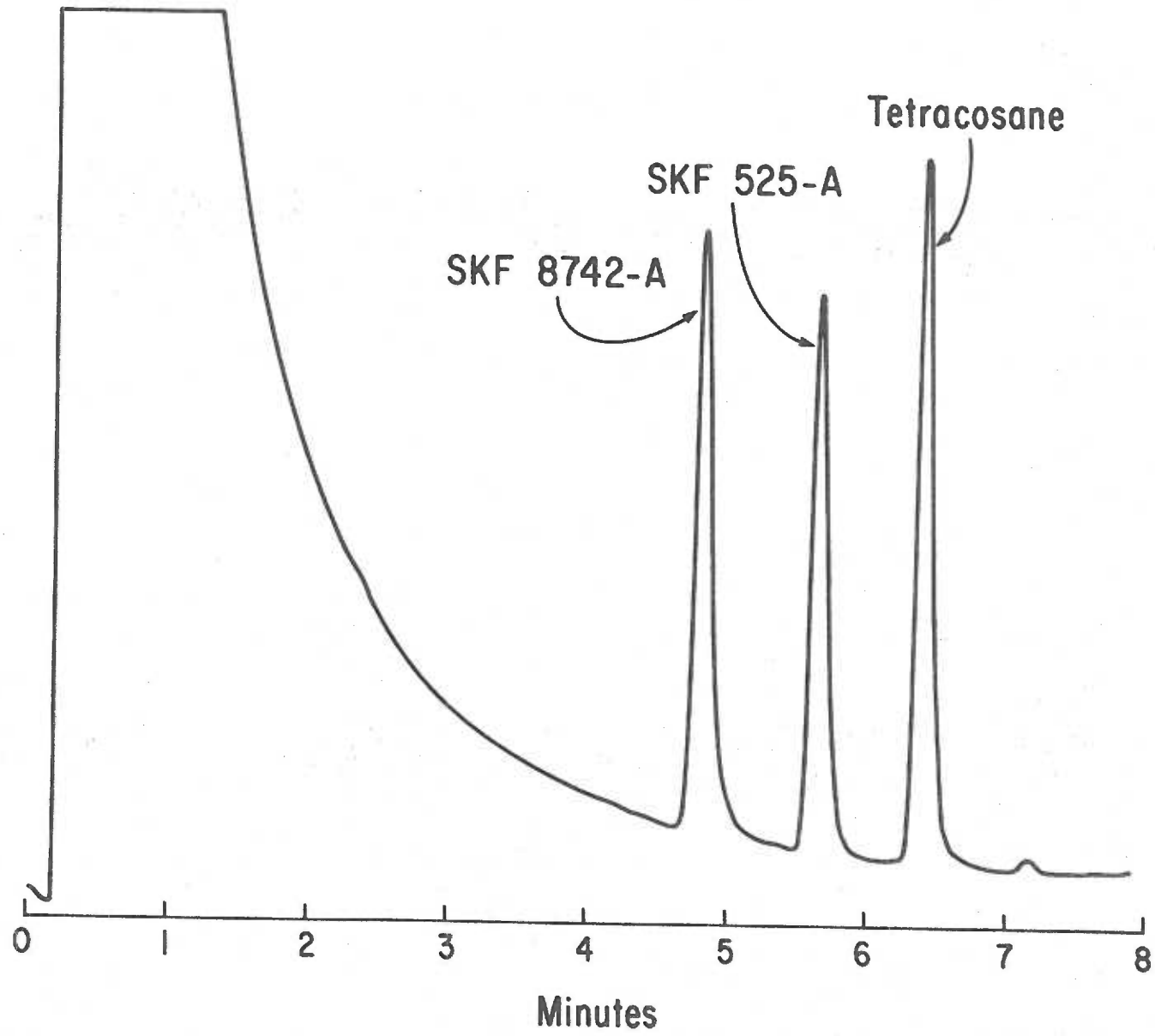

Tetracosane
SKF 525-A
SKF 8742-A
0
1
2
3
4
5
6
7
8
Minutes

Figure 24: Standard curves for floctafenin and floctafenic acid using ultraviolet spectrophotometry. Known amounts of the two compounds were extracted from liver homogenate. Typical absorption spectra for floctafenic acid are shown on the left.

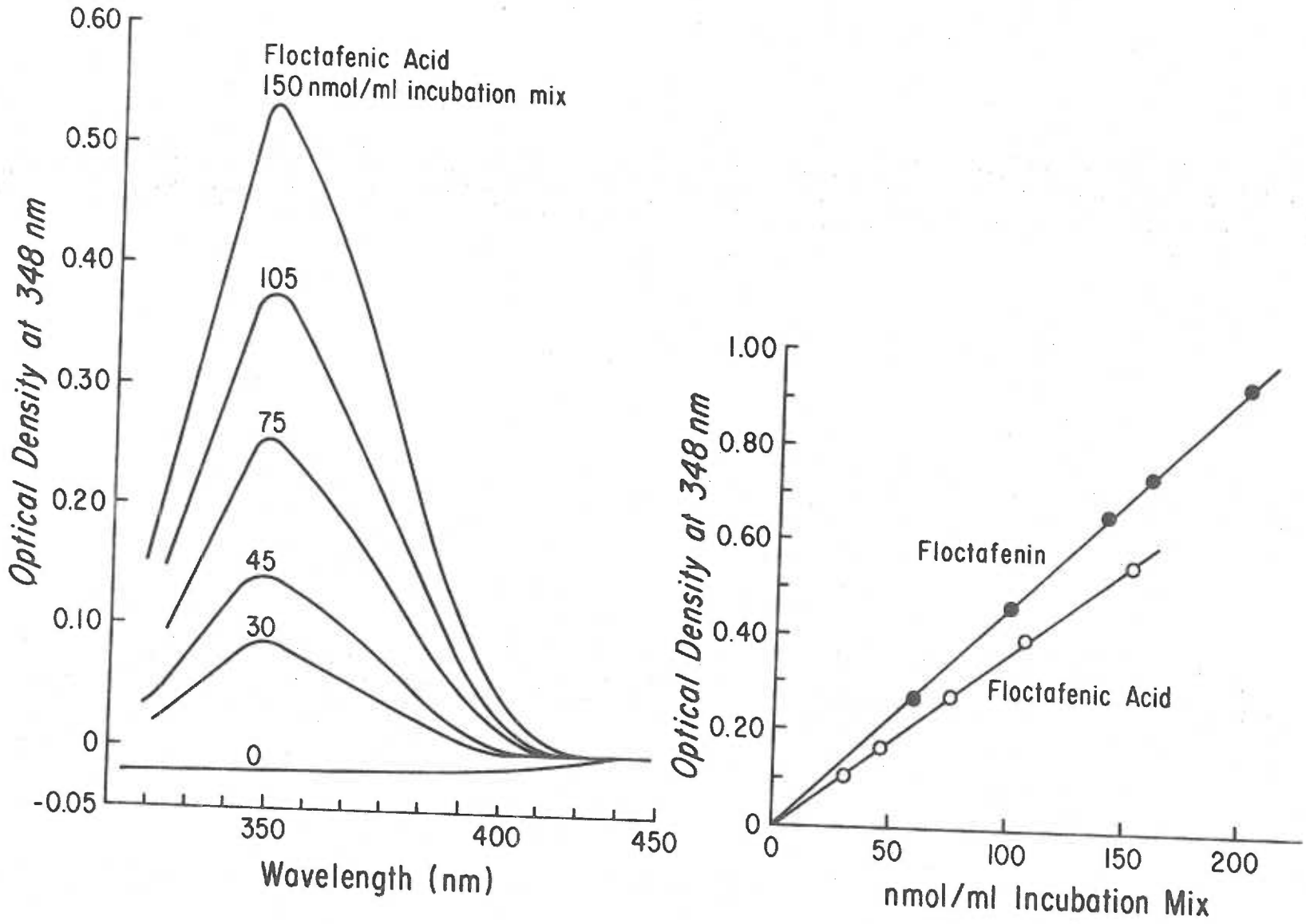

Floctafenic Acid
150 nmol/ml incubation mix
105
75
45
30
0
0.60
0.50
0.40
0.30
0.20
0.10
0
-0.05
350
400
450
Wavelength (nm)
Optical Density at 348 nm
1.00
0.80
0.60
0.40
0.20
0
50
100
150
200
Floctafenin
Floctafenic Acid
nmol/ml Incubation Mix
Optical Density at 348 nm

Figure 25: Formation of EDDP from d-methadone in testis incubations. Since EDDP is a minor contaminant of methadone, the amount of EDDP found at zero time was subtracted from the amount measured at subsequent times. Points and bars represent mean values and standard errors of the mean, respectively, for three determinations.

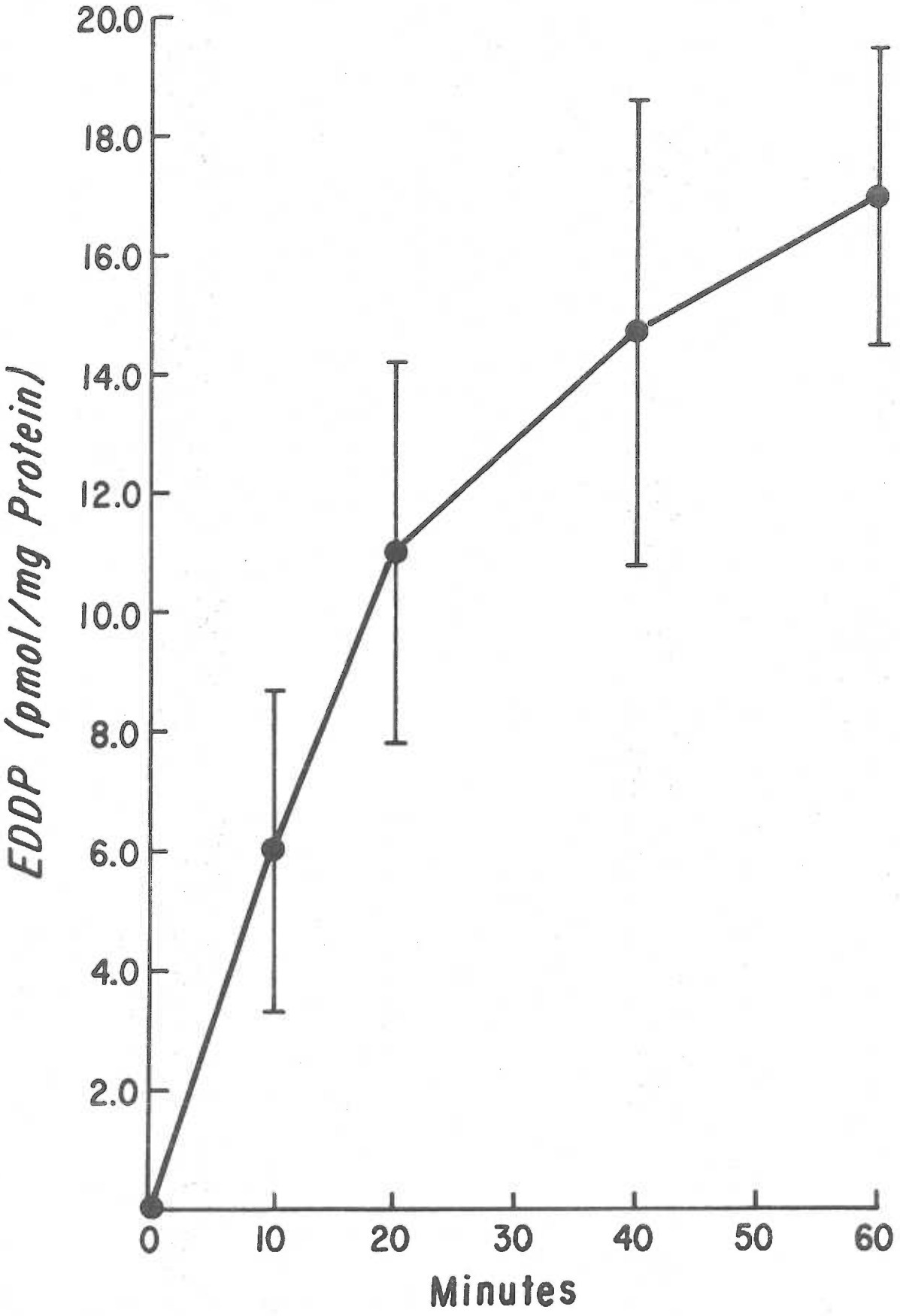
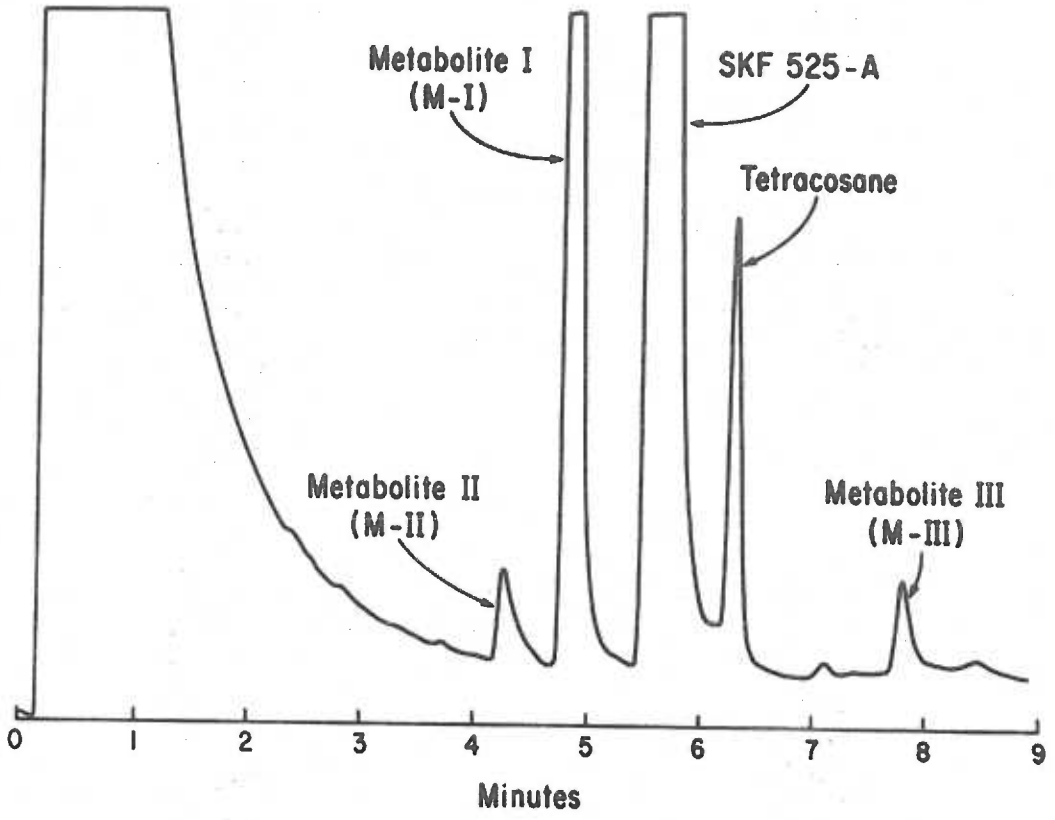

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
EDDP (pmol/mg Protein)
0
10
20
30
40
50
60
Minutes

Figure 26: Gas-liquid chromatograms demonstrating the formation of SKF 525-A metabolites in a liver incubation. The upper chromatogram was derived from an incubation sample that was obtained one minute after SKF 525-A was added. The sample was extracted and chromatographed as described in the text. The lower chromatogram was obtained in an identical fashion except that the sample was acquired 20 minutes after SKF 525-A was added. The SKF 525-A and all the metabolites that are apparent in the chromatograms were presumably metabolized to other, unidentified metabolites, since chromatograms for later samples showed progressive decreases in the GLC peaks (with the exception of the tetracosane peak). The same amount of tetracosane was added in the last extraction step for both samples.


Metabolite I
(M-I)
SKF 525-A
Tetracosane
Metabolite II
(M-II)
Metabolite III
(M-III)
0
1
2
3
4
5
6
7
8
9
Minutes

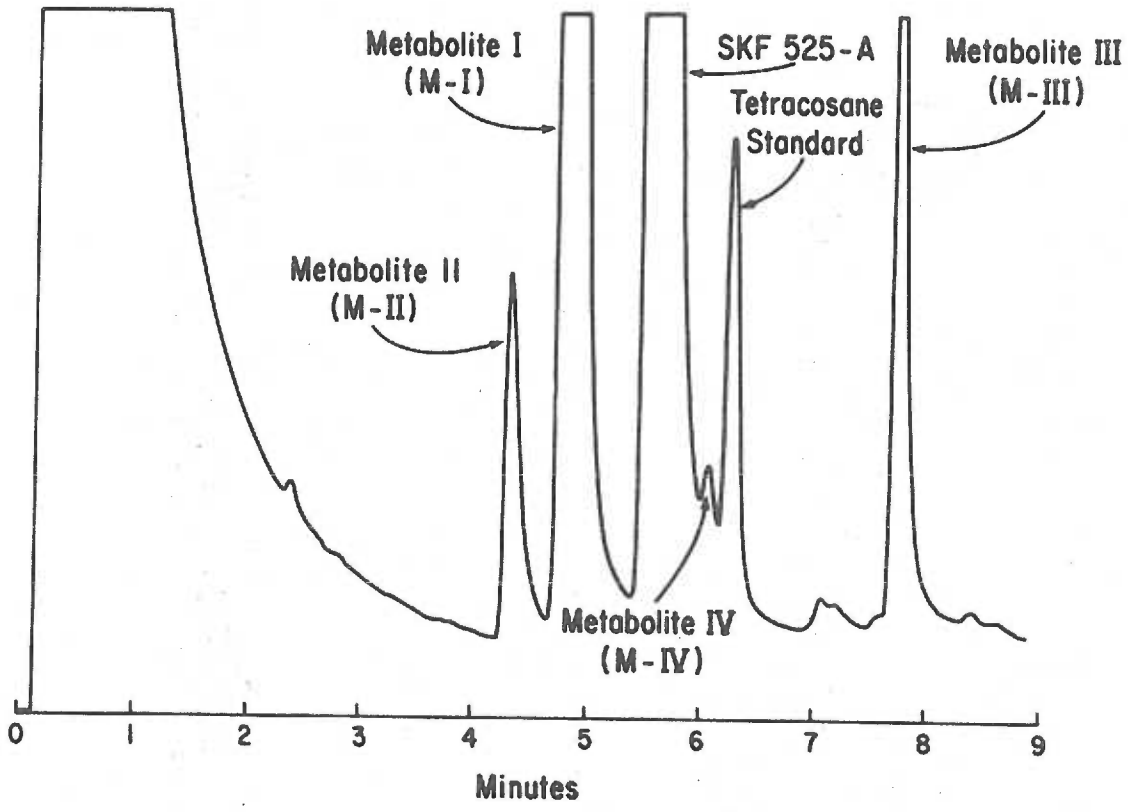
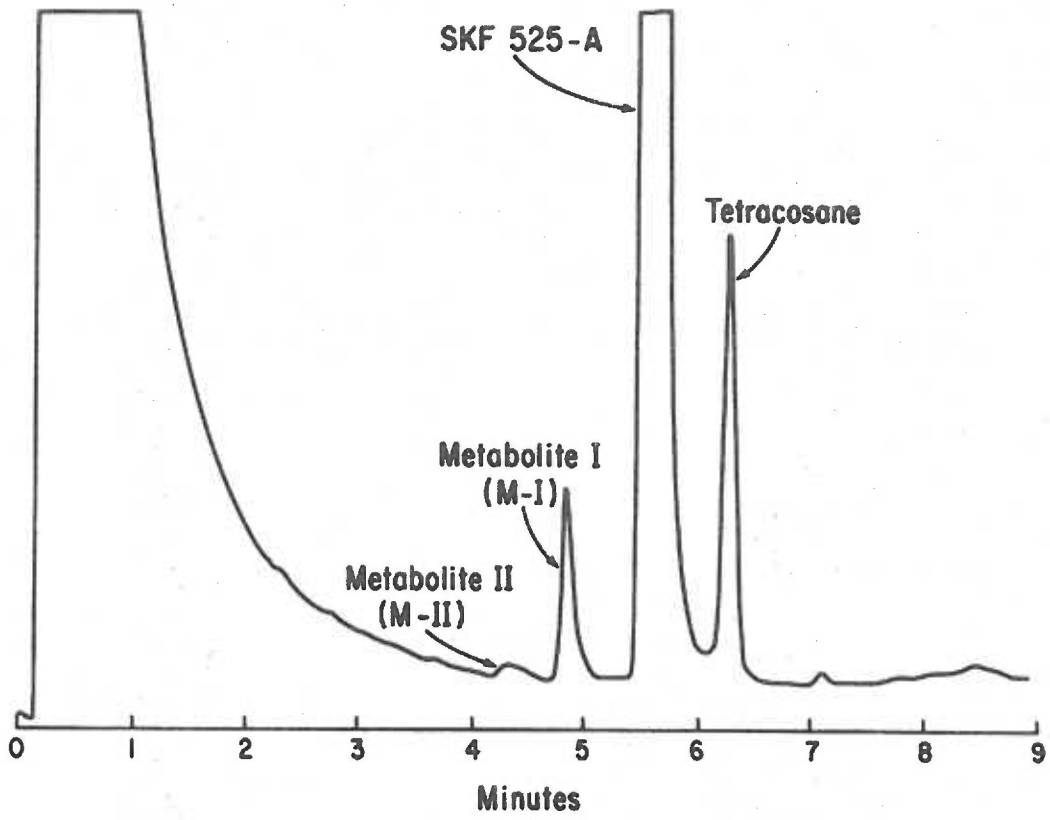

Metabolite I
(M-I)
SKF 525-A
Tetracosane
Standard
Metabolite III
(M-III)
Metabolite II
(M-II)
Metabolite IV
(M-IV)
0
1
2
3
4
5
6
7
8
9
Minutes

Figure 27: Gas-liquid chromatograms demonstrating the formation of SKF 525-A metabolites in a testis incubation. The upper chromatogram was derived from an incubation sample that was obtained one minute after SKF 525-A was added. The sample was extracted and chromatographed as described in the text. The lower chromatogram was obtained in an identical fashion except that the sample was acquired 20 minutes after SKF 525-A was added. The same amount of tetracosane was added in the last extraction step for both samples.


SKF 525-A
Tetracosane
Metabolite I
(M-I)
Metabolite II
(M-II)
0
1
2
3
4
5
6
7
8
9
Minutes

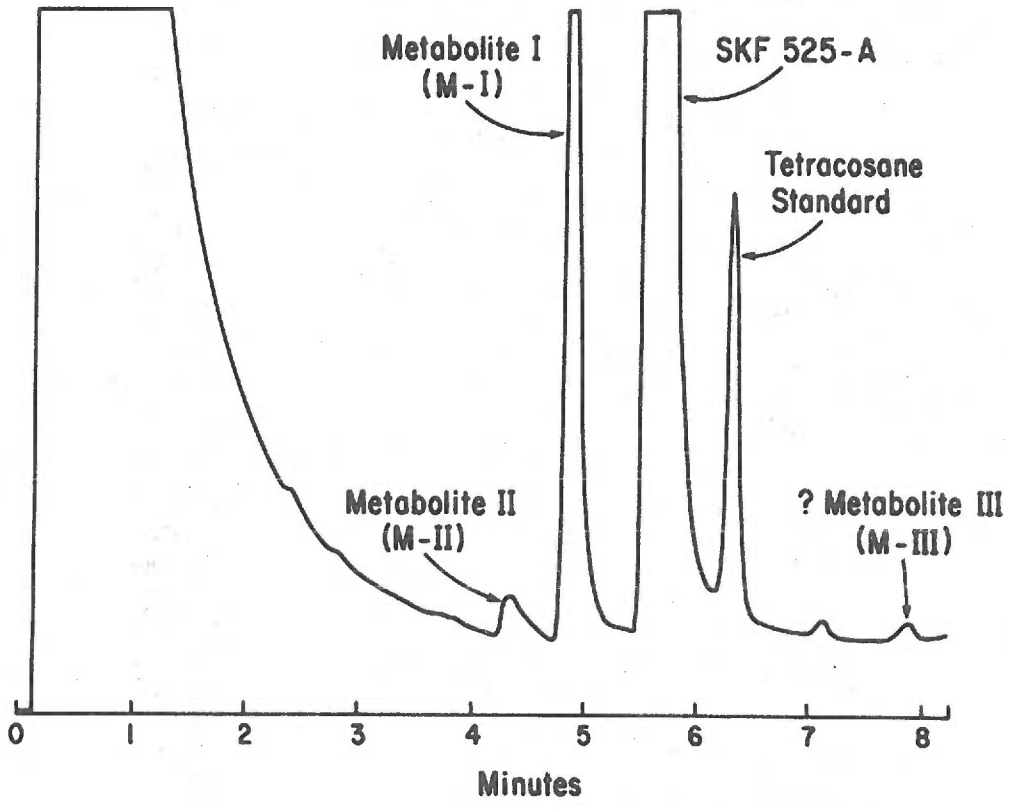
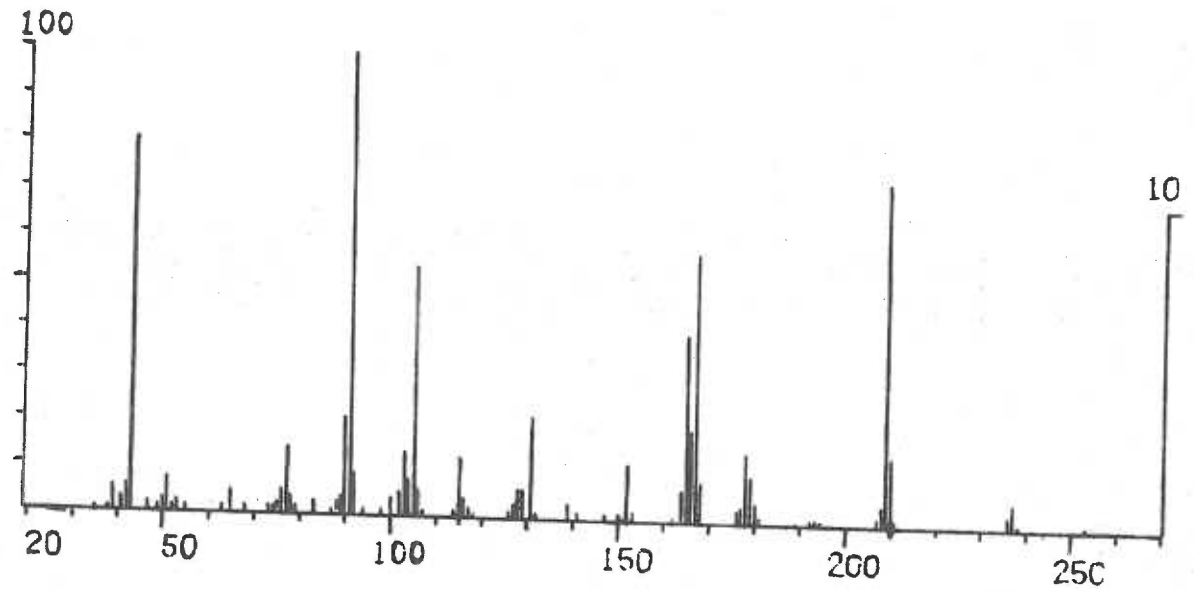

Metabolite I
(M-I)
SKF 525-A
Tetracosane
Standard
Metabolite II
(M-II)
? Metabolite III
(M-III)
0
1
2
3
4
5
6
7
8
Minutes

Figure 28: Mass spectra of two SKF 525-A metabolites: ethyl-aminoethyl diphenylvalerate (metabolite-I; SKF 8742-A) and aminoethyl diphenylvalerate (metabolite-II). Molecular ions for metabolite-I (lower spectrum) and metabolite-II (upper spectrum) were not visible. The fragmentation patterns of the two spectra are nearly identical to each other and to the pattern for the SKF 525-A spectrum at mass/charge ratios between 100 and 220. Ions in this range (100 to 220) represent fragmentation of the SKF 525-A molecule after removal of the amine group. Fragments below the 100 mass/charge ratio include ions derived from the amine group. Therefore, differences between the primary, secondary and tertiary amines become apparent at the lower mass/charge values.


100
10
20
50
100
150
200
250

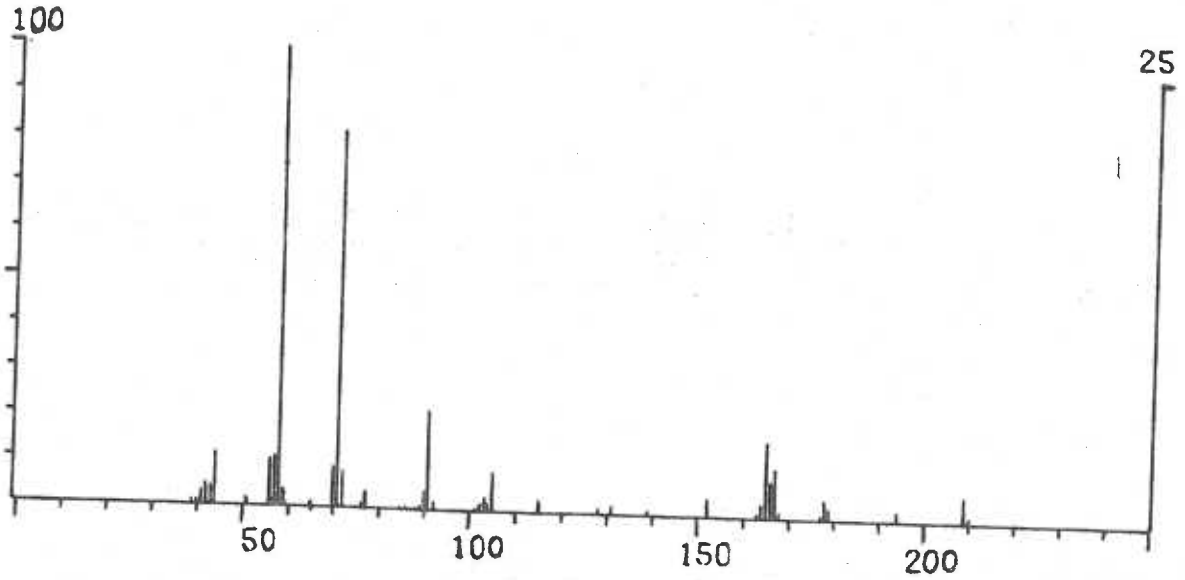

100
25
50
100
150
200

Figure 29: Mass spectrum of hydroxy SKF 525-A. Note the molecular ion at 369.

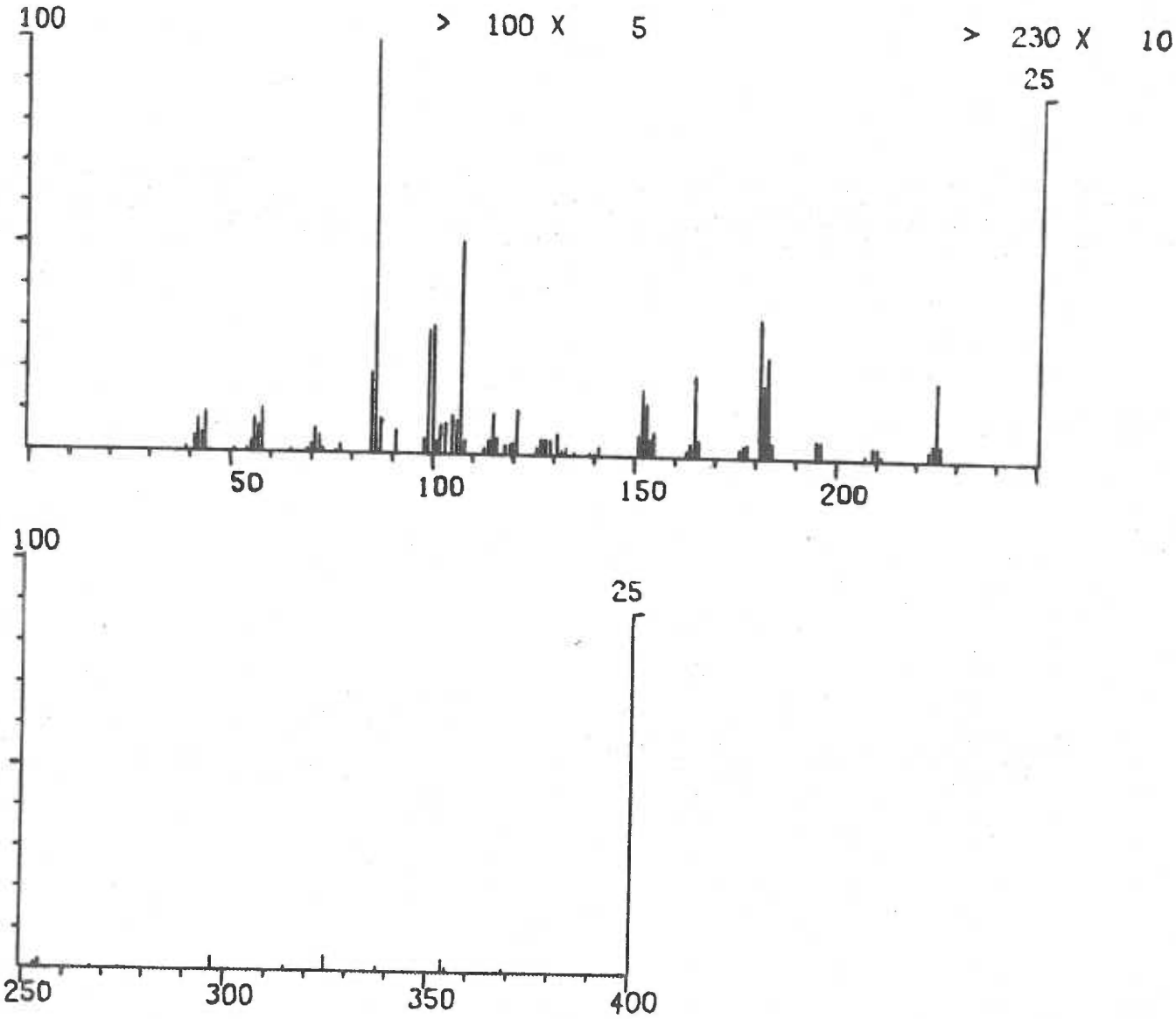

100
> 100 X 5
> 230 X 10
25
50
100
150
200
100
25
250
300
350
400

Figure 30: Mass spectrum of SKF 525-A. Note the molecular ion at 353.

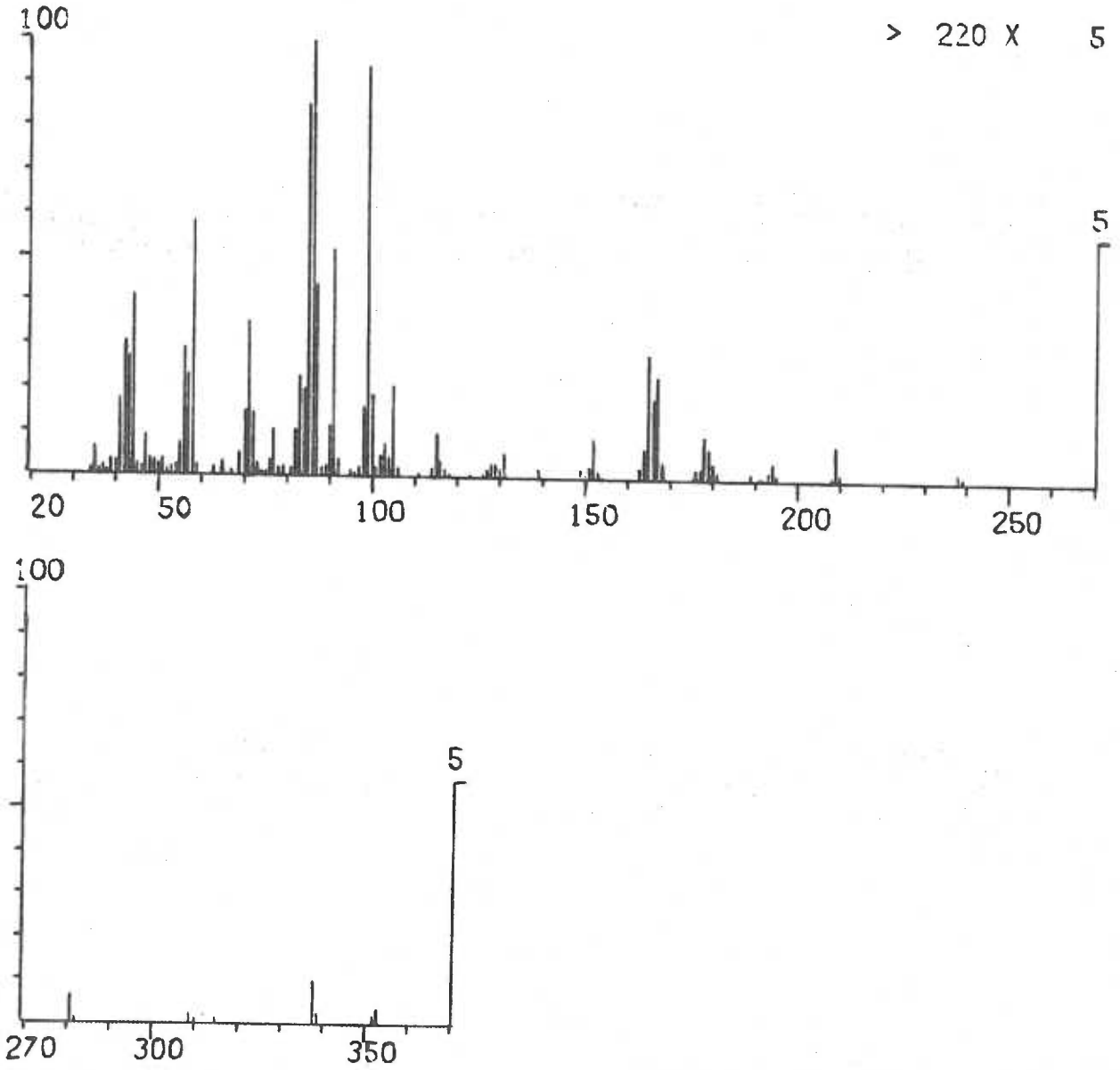

100
> 220 X 5
5
20
50
100
150
200
250
100
5
270
300
350

Figure 31: Biotransformation of SKF 525-A.

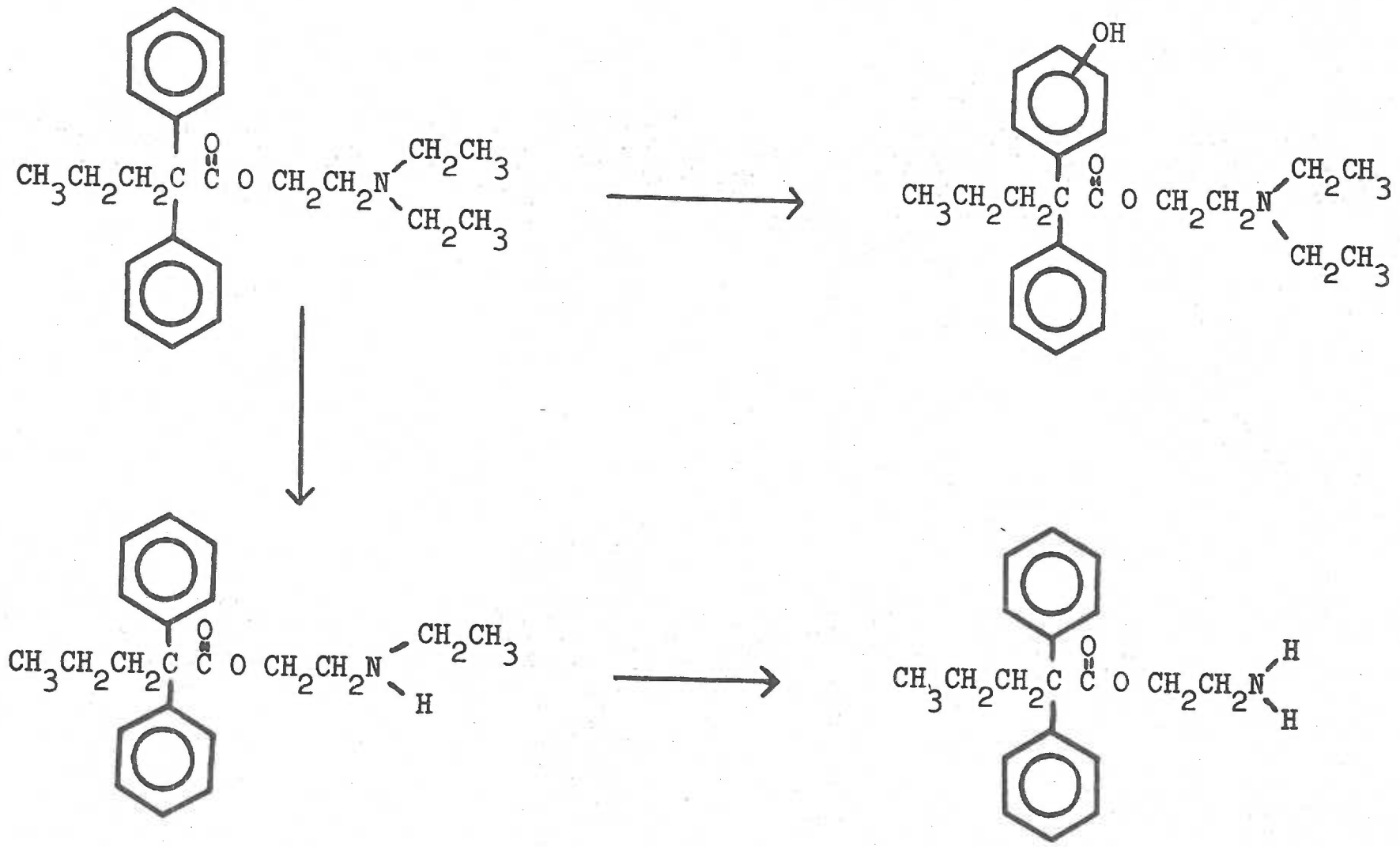


Figure 32: Gas-liquid chromatogram of SKF 525-A and its primary metabolite (metabolite-I; SKF 8742-A) extracted from a testis incubation.

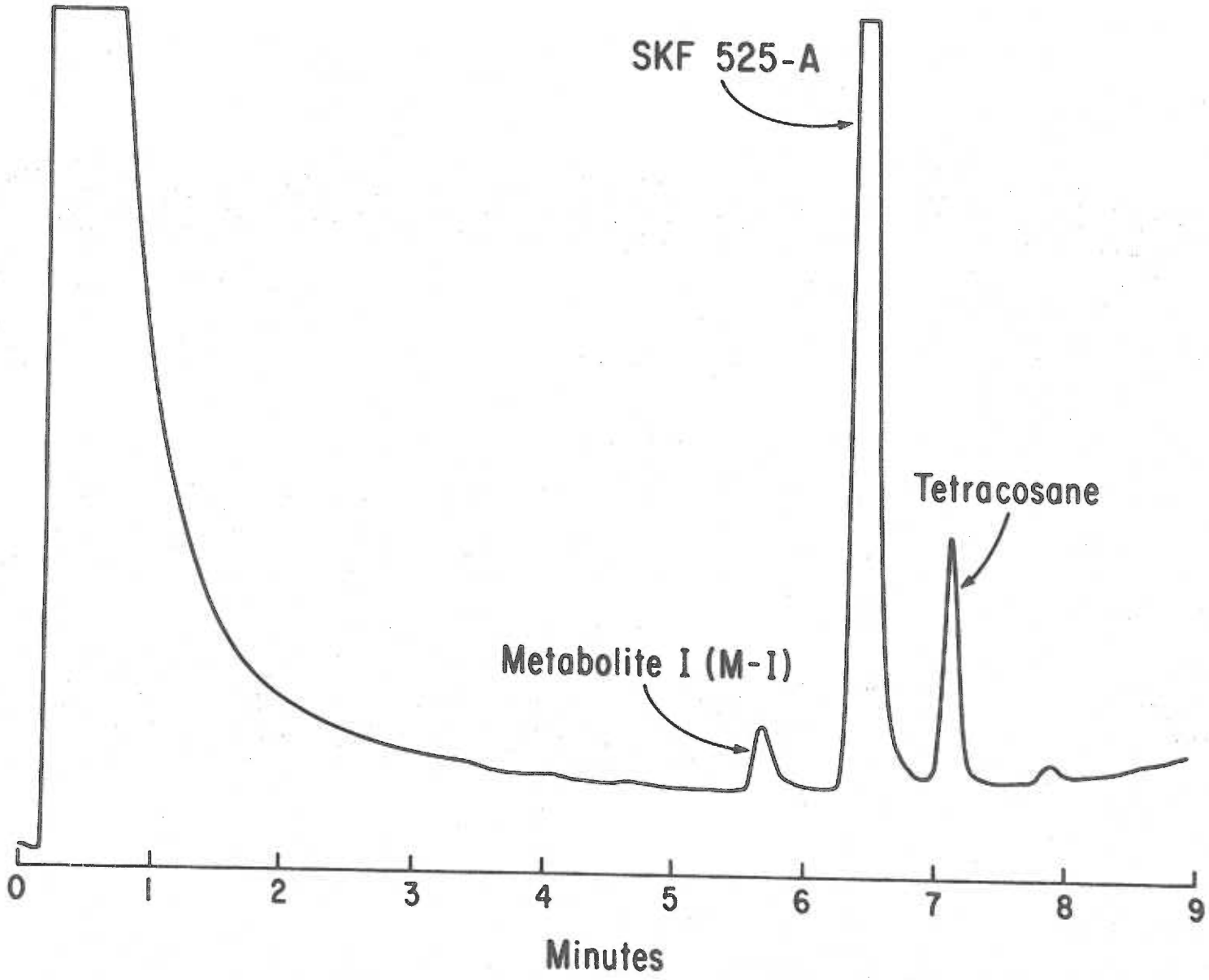

SKF 525-A
Tetracosane
Metabolite I (M-I)
0
1
2
3
4
5
6
7
8
9
Minutes

Figure 33: Disappearance of floctafenin and appearance of floctafenic acid in testis and liver incubations. The weight of tissue per volume of incubation mix was the same for both tissues. Points and bars represent means and standard deviations, respectively, for four determinations.

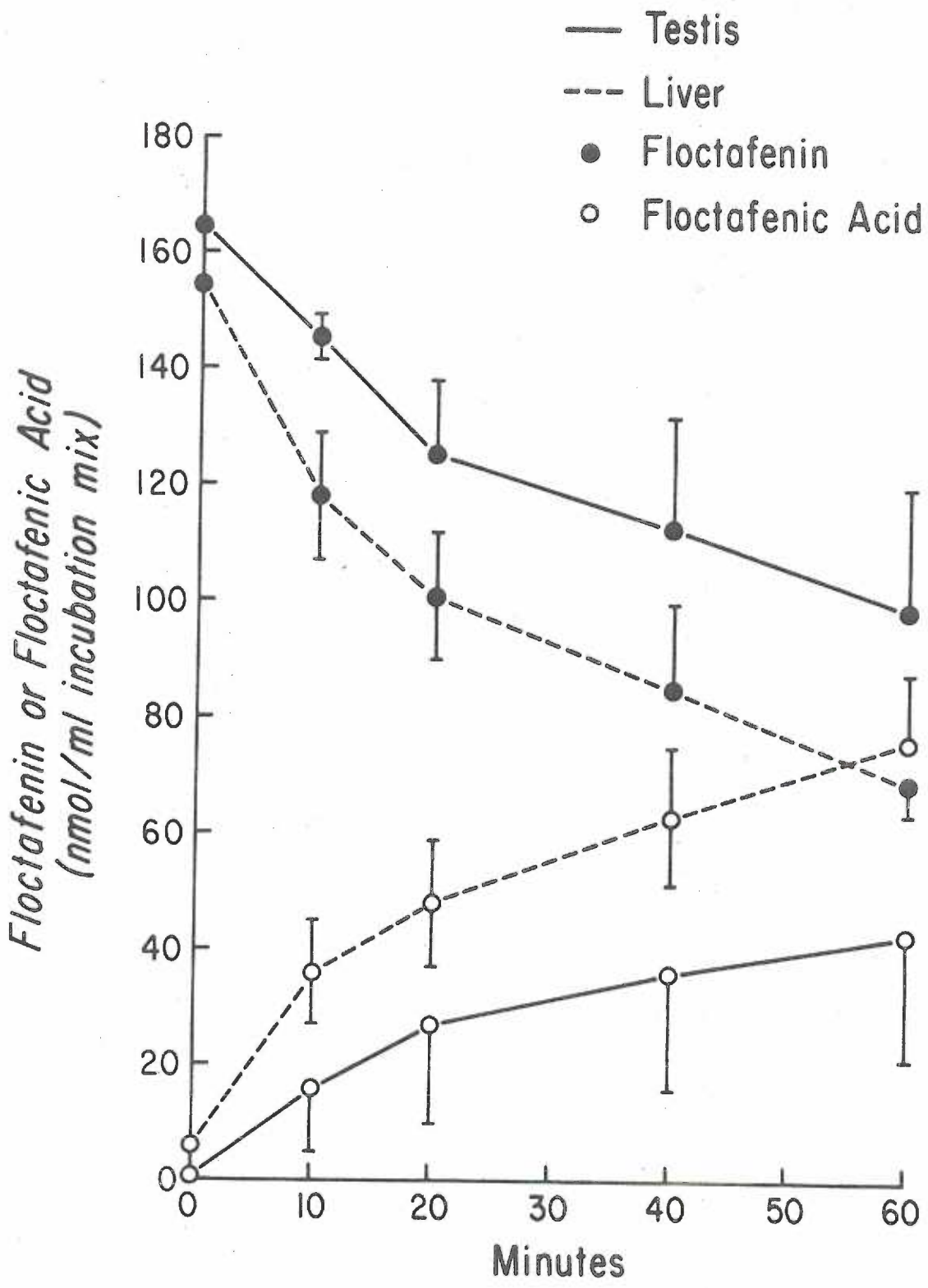

Testis
Liver
Floctafenin
Floctafenic Acid
Floctafenin or Floctafenic Acid
(nmol/ml incubation mix)
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Minutes

Figure 34: Disappearance of tricaine and appearance of its metabolite m-aminobenzoic acid in testis incubations. Points and bars represent means and standard deviations, respectively, for four determinations.

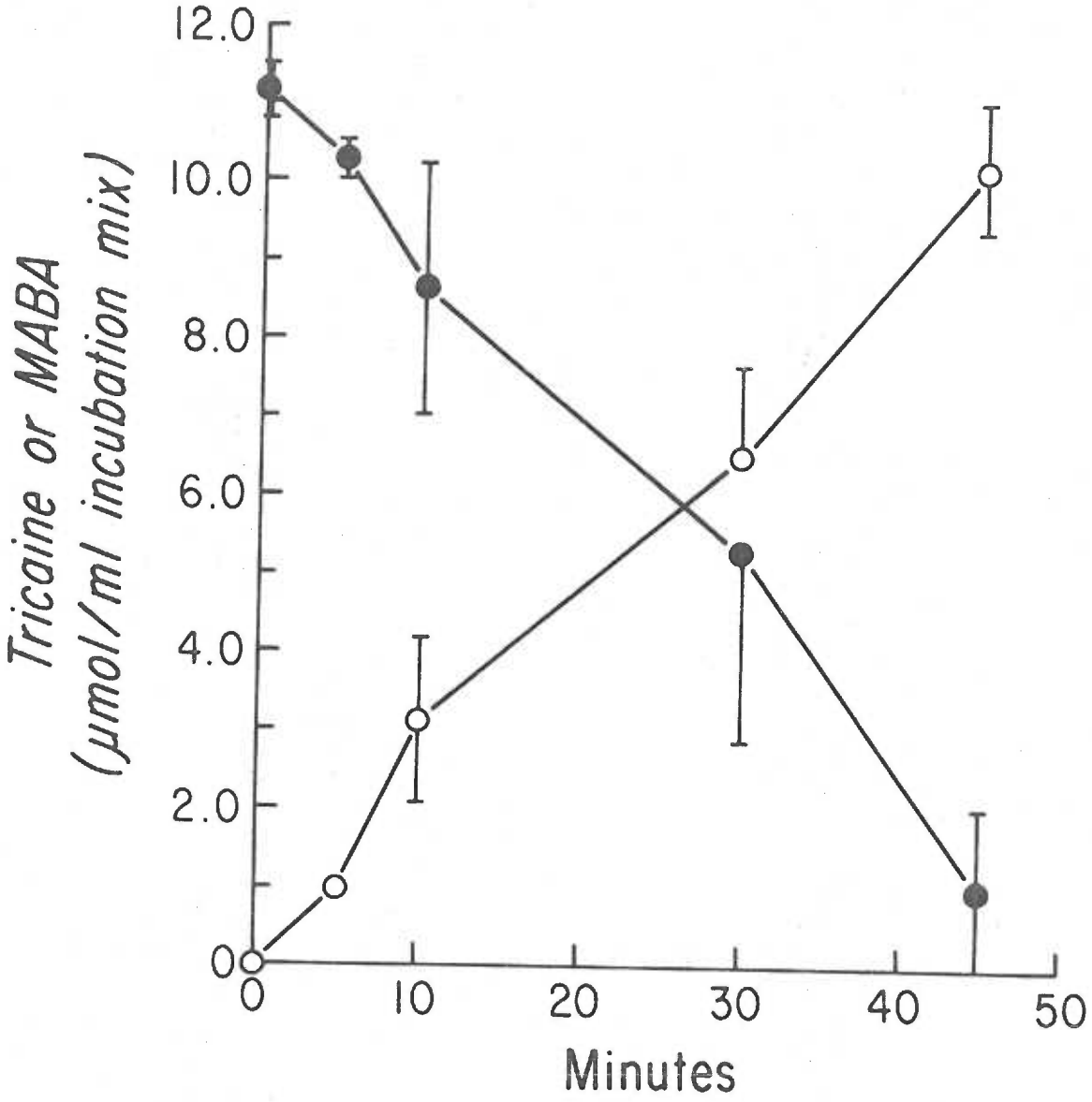
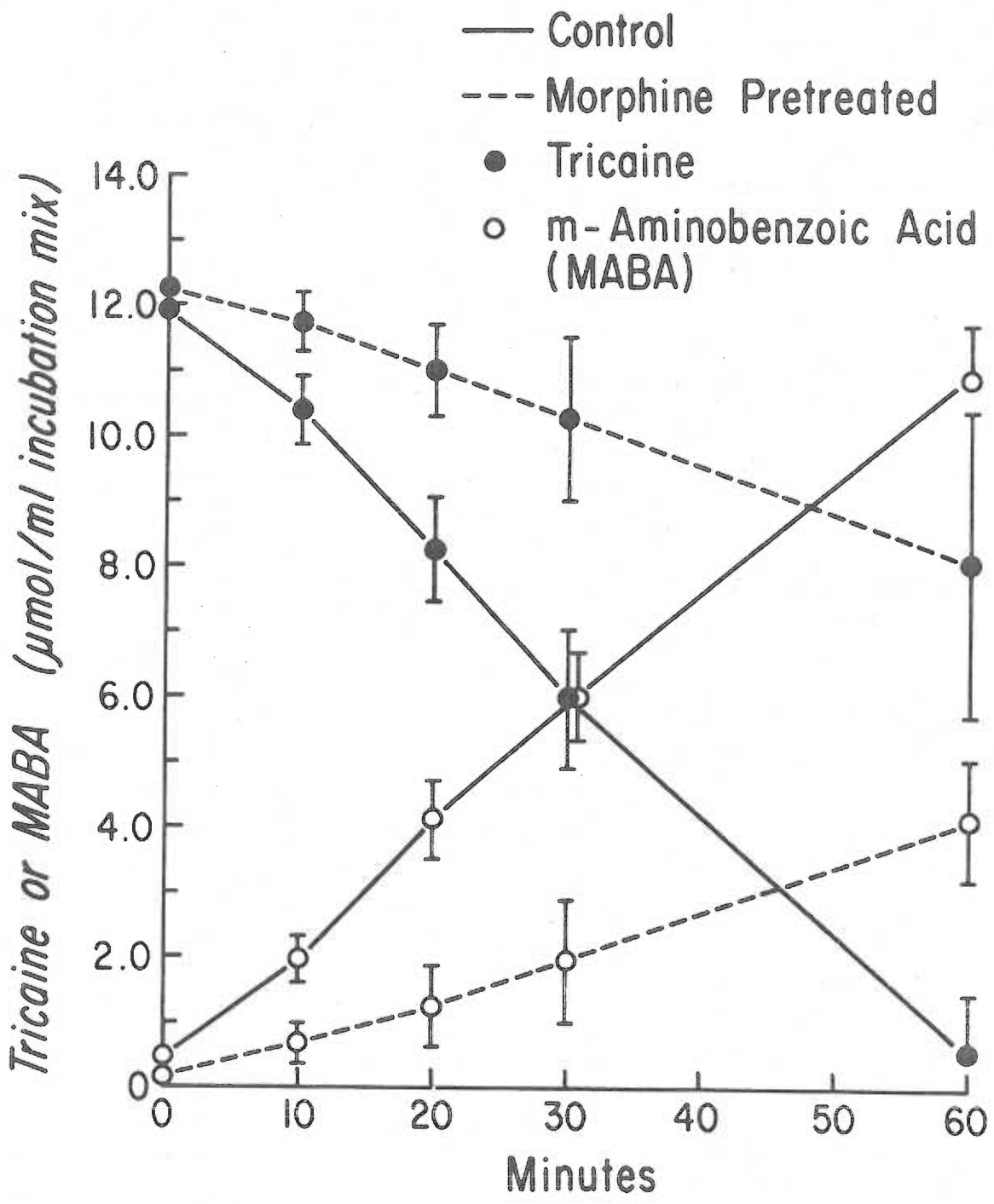

Tricaine or MABA
(μmol/ml incubation mix)
12.0
10.0
8.0
6.0
4.0
2.0
0
0
10
20
30
40
50
Minutes

Figure 35: Disappearance of tricaine and appearance of m-aminobenzoic acid in testis incubations: effect of morphine administration. Points and bars represent means and standard deviations, respectively, for four determinations.


Control
Morphine Pretreated
Tricaine
m-Aminobenzoic Acid (MABA)
Tricaine or MABA (μmol/ml incubation mix)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
0
10
20
30
40
50
60
Minutes

APPENDIX A

Chemicals

All chemicals were of Analytical Reagent grade (AR) or better unless otherwise indicated. Aqueous solutions were prepared using deionized-glass distilled water.

Sources of some of the chemicals were as follows:

Chemical	Source
Solvents:	
1-chlorobutane	Burdick & Jackson, Muskegon, Mich.
chloroform (AR)	J. T. Baker, Phillipsburg, N.J.
chloroform (nanograde)	Mallinckrodt, St. Louis, Mo.
ethanol (USP quality)	IMC Chemical Group, Agnew, Cal.
p-dioxane (reagent grade)	J. T. Baker, Phillipsburg, N. J.
Aquasol (universal scintillation cocktail)	New England Nuclear, Boston, Mass.
Drugs and Drug Metabolites:	
d-, l-, and racemic methadone HCl	Eli Lilly, Indianapolis, Ind.
^{14}C-methadone (racemic)	California Bionuclear, Sun Valley,Ca.
EDDP (methadone metabolite) HI	Applied Science Labs, State College, Pa.
Phenytoin (99+%)	Aldrich Chemical, Milwaukee, Wisc.
Phenytoin sodium (ready mix)	Parke-Davis, Detroit, Mich.
^{14}C-phenytoin	New England Nuclear, Boston, Mass.
^{14}C-hexobarbital	Milton Busch, PhD, Vanderbilt Univ, Nashville, Tenn.
Floctafenin	Hoechst-Russel, Somerville, N.J.
Floctafenic acid	R. K. Lynn, PhD, Univ Oregon Health Sciences Center, Portland, Or.
Tricaine methanesulfonate	Ayerst Labs, New York, N.Y.
m-Aminobenzoic acid	Eastman Chemical, Rochester, NY
Dipropylacetate sodium	Abbott Labs, North Chicago, Ill.
Dipropylacetic acid (capsules)	Abbott Labs, North Chicago, Ill.
SKF 525-A	Smith Kline & French, Philadelphia,Pa
SKF 8742-A	Smith Kline & French, Philadelphia,Pa

(continued)

Appendix A (continuation)

Testosterone propionate	Invenex, San Francisco, Cal.
Testosterone	Sigma Chemical Co., St. Louis, Mo.

Enzyme Cofactors

Nicotinamide	Sigma Chemical Co., St. Louis, Mo.
Glucose-6-phosphate	Calbiochem, San Diego, Cal.
Nicotinamide adenine dinucleotide phosphate	Sigma Chemical Co., St. Louis, Mo.
Glucose-6-phosphate dehydrogenase (Type XII lyophilized)	Sigma Chemical Co., St. Louis, Mo.

APPENDIX B

Commercial Sources of Animals

Species	Breed	Source
Rabbit	New Zealand White	David Robb, Sheridan, Or.
Rat	Sprague-Dawley	Simonsen Labs, Gilroy, Cal.
Mouse	Swiss-Webster	Simonsen Labs, Gilroy, Cal.

APPENDIX C

A New Method for Chronic Administration of Narcotics

During the course of the thesis work a new method was developed for the chronic administration of opiates to experimental animals. This method could prove to be useful for studies on drug tolerance, induction or inhibition of enzymes with opiates, and chronic effects of narcotics on tissues. Silicone tubing (.132 in. ID x .183 in. OD, Silastic, medical grade, Dow Corning Corp., Midland, Mich.) was chopped into 2.4 cm segments. One end of each segment was plugged with silicone adhesive (Silastic, medical grade, silicone type A). The adhesive was allowed to cure overnight, and l-methadone (about 40 mg) was packed into each capsule. The capsules were completed by plugging the remaining ends with the adhesive. After an overnight curing period the capsules (final chamber size = 2 cm) were autoclaved for 20 minutes. Each capsule was then inserted under the interscapular skin of rats, 280 to 320 g. The rats experienced typical opiate effects (glary stare, hunched back, occasional trembling and diminished motility) for two days. Thereafter, they returned to normal behavior. Large quantities of methadone and methadone metabolites were present in urine from the animals for the duration of the study (five days). It is obvious from this work that the silicone capsules permitted systemic absorption of the narcotic, but at a much slower rate than normally observed after subcutaneous administration. Single, subcutaneous injections of l-methadone in doses as low as 30 mg/kg can kill rats, and the usual half-life of methadone after parenteral injection into the rat is only about four hours. In contrast, silicone capsules containing 40 mg of l-methadone base were not lethal and continued to deliver the drug for more than five days.

The advantage of silicone implants of narcotics compared with most other means of drug administration is that moderate levels of a drug can be maintained in the animal for long periods of time. In rodent species narcotics are rapidly absorbed and rapidly metabolized. Thus, intermittent delivery of these drugs results in large fluctuations in drug

concentrations in tissues. Tolerance to narcotics is likely to occur more rapidly if tissues are exposed to low doses of narcotics throughout the day rather than to high doses once or twice a day.

APPENDIX D

Method for the Determination of Protein

The concentration of protein in tissue homogenates and incubations was measured according to the method of Lowry, et al. (J. Biol. Chem. 193: 265, 1951).

Reagents:

A. 2% sodium carbonate in 0.1 N sodium hydroxide. Dissolve 20 g sodium carbonate in 900 ml distilled water, add 10 ml 10 N sodium hydroxide and dilute to 1000 ml total volume.

B. 1% copper sulfate (pentahydrate)

C. 2% potassium-sodium tartrate

D. Mix 0.5 ml solution B with 0.5 ml solution C, then add 50 ml solution A to this mixture.

E. Phenol reagent: Make a 1:1 dilution of 2 N Folin's reagent with water.

F. Bovine serum albumin standard, 500 µg/ml BSA

Procedure: To each 1.0 ml sample (standard, water blank or unknown) add 5 ml reagent D. Mix well and let stand at room temperature for 10 minutes.

Then add 0.5 ml reagent E, mix thoroughly and let stand for 30 min at room temperature.

Determine the optical density of each sample at 660 nm in a spectrophotometer.

Standards should cover the range, 10 to 200 µg protein.

Particulate suspensions of tissue should be pre-digested with base. Thus, 0.2 ml homogenate is mixed with 0.4 ml 1 N sodium hydroxide, incubated at 70° C for 5 min, and then diluted to 100 ml total volume with water.