

## GENERAL BACKGROUND

### TOXICITY OF ORGANIC SOLVENTS

As mentioned in the introduction, many solvents share several harmful effects on the living organism, some of them due to their common physico-chemical properties. Thus, their high fat solubility facilitates their absorption from the respiratory tract and in some cases through the skin. High fat solubility also enables them to pass into the central nervous system (CNS), where they exert an acute depressant effect. Skin contact with organic solvents may also defat the skin, often followed by infections, to cause one of the most frequent occupational diseases - dermatitis. The low surface tension of many organic solvents makes the aspiration of liquid solvents highly dangerous. They spread easily over large lung surfaces and cause a chemical pneumonitis. High concentrations of organic solvent vapors have also been shown to cause sudden deaths in ventricular fibrillation, presumably due to sensitization of the myocardium to the effects of endogenous catecholamines. This effect is especially pronounced at exposure to halogenated hydrocarbons (76, 166, 170, 180, 299) but has also been reported to occur at experimental acute benzene (289) and toluene (440) poisoning. In the case of halogenated hydrocarbons, their cardiac sensitizing potentials

have also been linked to their physico-chemical properties (76).

In addition to the above mentioned effects, generally considered to be caused by the solvents themselves, organic solvents also have the capacity to give specific organ injuries associated with products formed during their biotransformation.

#### *Nervous system effects of organic solvents*

The organic solvents belong to the general CNS depressants, i.e. they exert a depressant effect on all neurons in the CNS, expressed as initial sedation followed by hypnosis, general anesthesia, and if the exposure is intensive enough, coma and death. However, an excitatory effect on some functions, expressed as increased liveliness and motor activity, may be observed during exposure to low solvent vapor concentrations or during the initial stages of exposure to high concentrations.

Anesthesia is accomplished by a stabilization of neuronal membranes. The mechanism of membrane stabilization by anesthetics, including the organic solvents, is still not completely elucidated. Since studies of neuronal membranes are difficult to conduct, model systems using erythrocytes have been developed to investigate the membrane stabilization caused by anesthetics. Local and volatile general anesthetics have thus been shown to protect erythrocytes against hypotonic hemolysis at concentrations almost the same as those necessary to anesthetize nerve fibers (353, 379, 380). Also organic solvents have been found to be effective protectors against hypotonic hemolysis (184). There is substantial evidence that

the erythrocyte membrane stabilization is caused by an expansion of the membrane (382, 383, 384). The explanation of how anesthetics produce a membrane expansion rests basically on the early Meyer-Overton rule of anesthesia (272, 305), proposing a direct parallelism between the affinity of an anesthetic for lipid and its depressant action. However, the proposal explains only how anesthetics may gain access to cells of the CNS, inasmuch as nerve cells and their membranes contain lipids, and not how anesthesia is produced. The Meyer-Overton rule has been modified several times e.g. by Ferguson (127), stating that equal degrees of anesthesia occur at equal thermodynamic activities, and by Mullins (287), stating that equal degrees of anesthesia occur with equal volume fractions of anesthetic agents within the membrane. Anesthetic agents would thus simply occupy bulk space in the membrane, thereby increasing membranal pressure and membranal surface area but not membranal thickness. Quite a good correlation exists between lipid solubility and antihemolytic effect (184, 381), but the bulk volume of the anesthetic molecules dissolved in the erythrocyte membrane cannot completely account for the entire expansion of the membrane (237, 385). However, anesthetic gases have also been shown to interact with proteins (125, 126), which might contribute to the membrane expansion by changing the conformation of membranal proteins. The increase in membranal pressure during membrane expansion has been suggested to lead to a decrease of the radius of membrane pores, thereby reducing the passive sodium ion influx (380) resulting in a reduction of the excitability of the nerve membrane and consequent anesthesia (441). This suggestion would provide a link between the

Meyer-Overton lipid theory and accompanying physico-chemical theories of anesthesia and the cell permeability theory, proposing an interference of anesthetics with ionic movements necessary for nervous membrane depolarization (80). Theories of anesthesia have also been advanced on the basis of various biochemical phenomena occurring during anesthesia (80). However, it is still unclear whether these phenomena only accompany anesthesia rather than cause it.

The different degrees of anesthesia caused by acute exposure to solvent vapors is generally reversible leaving no functional or pathological changes in the exposed subject. Today, such exposures are rare and long-term, low concentration exposures dominate in occupational settings. It is becoming increasingly evident that chronic solvent exposure may also cause nervous system impairment. Certain solvents, e.g. carbon disulfide and various hexacarbons, have established chronic neurotoxic effects, which are now beginning to be investigated and recognized also at exposure to other solvents (366). The observed signs and symptoms are indicative of both central and peripheral nervous system involvement. Chronic occupational or intentional exposure to organic solvents has been reported to produce signs and symptoms of CNS involvement such as neurasthenia and psychasthenia (25, 26, 28, 46, 70, 90, 92, 159, 168, 170, 216, 226, 228, 296, 299, 365, 396, 402, 430, 436, 471) but also peripheral neuropathy (1, 17, 41, 84, 90, 152, 157, 226, 227, 232, 252, 396).

Very little is known about the mechanisms and pathology of chronic nervous system damage due to solvent exposure.

The outbreak of peripheral neuropathy in 1973 in workers exposed to various hexacarbons (41) led to experimental investigations in animals of their neurotoxic properties (202, 414). Prolonged exposure of cats and rats to hexacarbons such as n-hexane and methyl-n-butylketone produced a wide-spread degeneration of both peripheral and central nerve fibres (414). There was a slowly progressing damage in vulnerable axons sited both in the peripheral and central nervous system, commencing distally in large peripheral myelinated axons and central ascending and descending tracts. Degenerative changes were also found in smaller myelinated and non-myelinated axons. Morphologically, the axonal changes were characterized by multifocal axonal swellings, accumulations of neurofilaments within axons and thinning of the myelin sheaths (370, 414). Similar changes have also been observed in nerve biopsies from human subjects with clinically manifest neuropathy after exposure to glues containing hexacarbons (17, 157, 232). The pathological mechanisms are still unclear but the degeneration of nerve fibers has been suggested to be connected with an impairment of nervous tissue metabolism (370). An interesting observation is that changes take place simultaneously in the peripheral and the central nervous system, suggesting that peripheral neuropathies and various signs of CNS damage cannot be regarded as two separate phenomena in organic solvent neurotoxicity.

In search of methods for the early detection of nervous system effects of solvent exposure various behavioral and psychological performance tests have been successfully

applied both in occupationally long-term exposed workers (167, 169, 171, 226) and in animal (191, 202, 367, 368) and human (137, 138, 359, 426) experimentation.

#### *Specific organ injuries of organic solvents*

Organic solvents taken up by the body may be eliminated via several routes. Many solvents are more or less completely eliminated in the expiratory air. Several solvents have also been shown to undergo biotransformation in the body to more water soluble products, which may be excreted via bile or urine (51, 146). The NADPH dependent cytochrome P450 mixed function oxidase system, localized in the microsomal fraction of several organs such as liver, lung, kidney, skin and small intestine, has been shown to be active in the biotransformation of many organic solvents. The products formed are not always innocuous but have been linked to various specific organ injuries caused by organic solvents such as liver and kidney lesions.

Results from relatively recent studies of chemical carcinogenesis have shown that most carcinogens bind covalently to structural and informational tissue macromolecules (275). The majority of the chemical carcinogens must also first be enzymatically biotransformed to reactive electrophilic intermediates or metabolites, which may then become covalently bound to nucleophilic sites in various macromolecules such as DNA, RNA and proteins. The phenomenon of covalent binding has also been implied to precede the development of other toxic effects such as tissue necrosis, bone marrow aplasia, hypersensitivity

reactions and mutagenic and teratogenic events (149, 150, 278). As will be discussed later, among the solvents studied in this monograph, the formation of reactive intermediates during biotransformation has been proposed or established for benzene, styrene, chloroform, carbon tetrachloride and trichloroethylene, which consequently has been recognized as a possible event in the development of specific organ injuries. There is, as yet, no satisfactory explanation concerning the mechanisms by which covalently bound toxic materials may cause any of the above mentioned effects. However, the strong correlation between covalent binding and carcinogenesis and other organ injuries justifies studies of covalent binding of foreign materials introduced into the living organism, including the organic solvents.

#### UPTAKE, DISTRIBUTION, BIOTRANSFORMATION AND ELIMINATION OF ORGANIC SOLVENTS

The general principles governing the uptake, distribution and elimination of organic solvent vapors have been described in several papers both on basis of what is known from human experimentation (22, 162, 281, 282, 294), conclusions drawn from the construction of mathematical models in connection with human and animal experimentation (295, 363, 364, 474) and computer derived results (107, 128, 131, 263). The outlined theories correspond well with what is known about the uptake, distribution and elimination of clinical anesthetic agents (476). The effect of biotransformation as a modifying factor in the transfer of organic solvents in the body has only recently been included (107, 128, 131, 364).

Disregarding the influence of biotransformation, the uptake, distribution and elimination of organic solvent vapors follow relatively simple physico-chemical laws as described by Åstrand (22) and Gubéran (162) and shown to fit the computerized models (131, 263). The magnitude and rate of respiratory uptake of solvent vapors is thus dependent on the pulmonary ventilation, the diffusion of the vapor across the alveolar membrane, the partial pressure of solvent vapor in the inspired air, the solubility of the solvent in blood and the blood flow through the lungs (cardiac output). The pulmonary ventilation and the cardiac output are both determined by the degree of physical activity in a healthy person. An increase in physical workload has thus been shown to increase the amount of solvent taken up by human subjects (22, 23, 24, 64, 107). Ordinarily, the alveolar membrane allows free diffusion of the solvent in both directions. The solubility of a solvent in blood can be described by its blood/gas partition coefficient, i.e. the quotient between the solvent concentrations in blood and air at +37°C at equilibrium, which may vary considerably between different solvents (Appendix 2). Generally, the more soluble the solvent in blood the more is taken up and the slower is the approach of equilibrium between the tension of the solvent in the arterial blood and in the inspiratory air. This is due to the ability of the blood to act as a reservoir for large amounts of a solvent with a high blood solubility. The loss of solvent from the arterial blood to the tissues is determined by the partial pressure of the solvent in arterial blood and in the tissues, the solubility of the solvent in the tissues and the rate at which the solvent is delivered to the tissues, i.e.

the blood flow to the various parts of the body. Again, the solubility of a solvent in different tissues may be described by a tissue/blood partition coefficient. Tissue/blood partition coefficients for solvents have only rarely been determined; noteworthy in this respect is the work by Sato *et al.* concerning benzene, toluene (363) and trichloroethylene (364). As the tissues take up the solvent the partial pressure of the solvent in the tissue increases towards the arterial. The partial pressure increases most rapidly in tissues with high blood perfusion rates and lags in tissues where blood flow is slower. Blood flow to adipose tissue is very slow, but in spite of this adipose tissues can take up large amounts of solvents due to the high fat solubility of these agents. After exposure the solvent is eliminated from the tissues and the blood by various routes. Many organic solvents are mainly eliminated unchanged in the expiratory air. In this case the same physico-chemical laws govern the magnitude and rate of loss of solvent from the tissues and from the blood as govern the uptake. Uptake and elimination may be described by relatively simple exponential functions whose rate constants are determined by the above mentioned physico-chemical factors.

For the construction of mathematical and computerized models for the uptake, distribution, biotransformation and elimination of organic solvents pharmacokinetic theories have been applied. Accordingly, the complex living body is reduced to a number of compartments representing tissue groups with similar blood perfusion rates and similar solvent tissue/blood partition coefficients (131, 263, 363). This simplification makes experimental data relatively easy to handle. Usually,

three distinct pharmacokinetic compartments are recognizable: 1/ the tissues of the vessel-rich group such as liver, kidney and brain, sometimes also including blood, 2/ the low perfused tissue group such as muscle and skin, 3/ the poorly perfused fatty tissues with large solvent partition coefficients. This model has been applied to respiratory elimination curves of several solvents, which have mathematically been resolved into equations expressed by the sum of three exponential components assumed to correspond to the three compartments in the pharmacokinetic model (129, 295, 363, 364). One- and two-compartment models have also been shown to fit animal (185, 474) and human (105, 118, 136, 295) experimental data for some solvents.

# CHAPTER I

## GENERAL METHODOLOGY

### ADMINISTRATION OF ORGANIC SOLVENTS TO MICE

In animal experimental work with organic solvents several routes of administration have been used, including intraperitoneal or subcutaneous injection, gastric intubation and inhalation. Since the inhalational route represents the most realistic mode of contact with organic solvents, the solvents investigated in this monograph were administered to mice in a small inhalation apparatus (Fig. I:1), slightly modified after Cohen and Hood (81). Before the inhalation, carbogen (93.5% O<sub>2</sub>, 6.5% CO<sub>2</sub>) was circulated through the apparatus. For autoradiography, <sup>14</sup>C-labeled organic solvents were diluted with unlabeled material to final solutions of 1-25  $\mu$ Ci/ $\mu$ l. Five or 10  $\mu$ l of the solutions were added to the inhalation apparatus and evaporated by gentle heating. During the inhalation, which lasted for 10 minutes, the apparatus was completely sealed to prevent loss of radioactive material.

### THREE-STEP WHOLE-BODY AUTORADIOGRAPHY

Conventional whole-body autoradiographic techniques cannot be used with substances exerting significant vapor pressures below

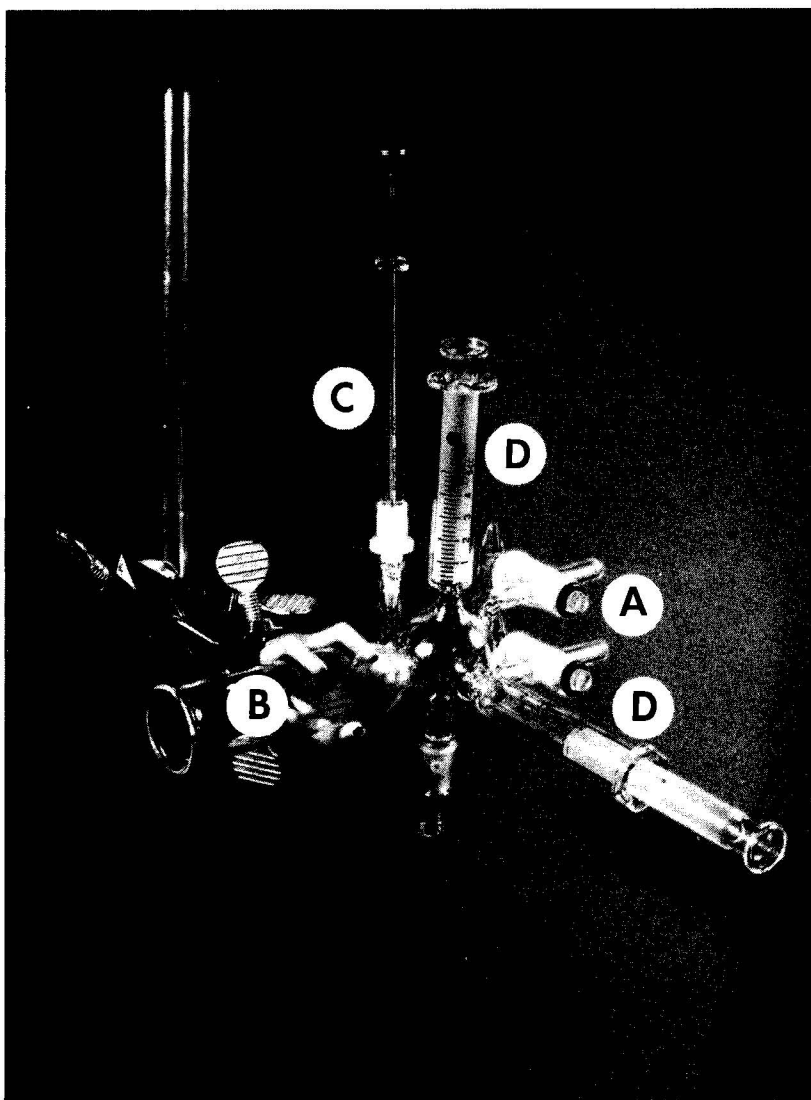


*Fig. 1:1.* Inhalation apparatus for the administration of radiolabeled organic solvents to mice. Before the inhalation, carbogen is circulated through the apparatus via the glass cocks (A). The mouse is placed in the tube (B) and the solvent added from the microliter syringe (C). During the inhalation, the radioactive vapors can be evenly distributed in the apparatus with the two glass syringes (D).

-20°C. Registration of volatile substances can only be accomplished by working at low temperatures. The vapor pressures at the chosen exposure temperature, -80°C, of the solvents studied in this monograph may be found in Appendix 2. Low-temperature autoradiography has been successfully applied to distribution studies of volatile anesthetic agents (79, 81, 82, 83) and ethanol (8). The low-temperature technique used for the studies in this monograph was based on the method previously developed in our laboratory by the visiting scientists Cohen and Hood (81), modified by Åkesson (8). At different times after the inhalation the animals were sacrificed by CO<sub>2</sub> anoxia; the CO<sub>2</sub> was produced by adding a small amount of water to pieces of dry ice in a closed jar, and immediately immersed in isopentane cooled by liquid nitrogen. After at least 5 minutes they were rapidly mounted in a gel of carboxymethylcellulose reinforced with cotton wool and frozen in liquid nitrogen for about 30 minutes. The frozen blocks were divided mid-sagittally with a rotary saw through the animal body. X-ray film (Industrex®, Kodak), a blotter cushion, a lead paper and a metal plate were placed on the flat surface. Close contact between the surface and the film was achieved with two angle clamps. The hemisections were then packed in black plastic bags, labeled and transferred to a cold freezer (-80°C) for exposure. Dependent on the amount of radioactivity remaining in the animal body, the exposure time varied considerably between 3 weeks and up to 2 years. After exposure, the autoradiograms were developed by standard techniques at room temperature. The pictures obtained in the first low-temperature autoradiographic step were considered to represent the

distribution of total radioactivity, including both the volatile, non-metabolized part of the solvent and its non-volatile metabolites.

In the second step of the autoradiographic procedure, autoradiography was performed according to Ullberg (455). Accordingly, series of mice were sacrificed at different time intervals after inhalation of radioactive solvent vapors, embedded in carboxymethylcellulose gel, frozen in hexane cooled by dry ice and sectioned on tape (tape no. 800, Minnesota Mining and Manufacturing Company) at  $-20^{\circ}\text{C}$  in a microtome. Occasionally, the hemisections used for low-temperature autoradiography were sectioned in the second step of the autoradiographic procedure. The sections, usually 20 or 60  $\mu\text{m}$  thick, were dried in a freezer room at  $-20^{\circ}\text{C}$  for at least 2 days and then heated to  $+50^{\circ}\text{C}$  for 24 hours. This technique allows the evaporation of volatile material. The sections were pressed against X-ray film (Industrex<sup>®</sup>, Kodirex<sup>®</sup>, Kodak) and exposure took place at  $-20^{\circ}\text{C}$ . The autoradiograms obtained from dried and evaporated whole-body sections were considered to represent the distribution of non-volatile metabolites of the organic solvents.

The recent concern raised by the possibility of severe organ toxicities caused by covalently bound reactive intermediates or metabolites of foreign materials to tissue macromolecules led to a third step in the autoradiographic procedure. The determination of covalently bound material is usually accomplished using radiolabeled substances and extracting tissues repeatedly until no further radioactivity can be

removed (150, 278, 443). Eight 20  $\mu\text{m}$  thick adjacent sections were taken from solvent exposed animals at different sagittal levels on tape (tape no. 688, Minnesota Mining and Manufacturing Company) and dried and evaporated as above. Every other section was then stepwise extracted with water for 5 minutes, 10% trichloroacetic acid for 2 minutes and for 1-2 minutes with 50% methanol, butanol and heptane, respectively. Water soluble radioactivity and radioactivity bound to amino acids not yet incorporated into proteins would be extracted by the first steps of this procedure. The last of the extraction media would also extract tissue lipids where radioactivity could be covalently bound. The sections were pressed against Industrex<sup>®</sup> and exposure was performed at  $-20^{\circ}\text{C}$ . The autoradiograms were considered to represent the distribution of radioactivity firmly bound to tissue macromolecules such as proteins and nucleic acids. An extracted section was always developed together with its non-extracted adjacent section to enable a proper estimate of the amount of firmly bound radioactivity in relation to the total non-volatile radioactivity.

#### ELIMINATION STUDIES OF ORGANIC SOLVENTS

For the elimination studies of organic solvents  $^{14}\text{C}$ -labeled solvents were administered to mice in the inhalation apparatus. The same amount of solvent was added to the apparatus as for autoradiography, but the total radioactive content was less; approximately 1.3  $\mu\text{Ci}$ . The 10 minute inhalation time, used for autoradiography, was used also for the elimination studies. After the inhalation, the apparatus was quickly sealed and cooled in a freezer to  $-20^{\circ}\text{C}$ . The administered dose was then

estimated by rinsing the apparatus repeatedly with 10 ml portions of scintillation fluid (4.9 g PPO, 0.1 g dimethyl-POPOP/l toluene) until no further radioactivity could be removed. The rinsing fluid portions were counted in a liquid scintillation counter (Tri-carb 2425, Packard) and their total radioactivity subtracted from the radioactivity added to the apparatus.

Immediately after the inhalation the mouse was placed in an all-glass metabolic cage permitting the collection of urine and feces and supplied with an inlet and outlet for air. Air was drawn through the cage with a small vacuum pump. The flow rate was maintained at 250 ml/min with a gas flow meter placed before the metabolic cage. The outcoming air was bubbled through a series of absorption fluids contained in scintillation vials, essentially according to Kasprzak and Sunderman (214), except that a trapping device for exhaled, volatile material was added. The incoming and outgoing air was also passed through Drierite® to remove moisture. The first part of the assembly consisted of four vials each containing 5 ml of toluene immersed in a hexane-dry ice bath. The radioactivity trapped in these vials was taken to represent exhaled volatile material. Expired  $^{14}\text{CO}_2$  was then collected in three vials, each containing 2 ml ethanolamine. For the collection of  $^{14}\text{CO}$  the air was then passed through a rinsing vial containing concentrated  $\text{H}_2\text{SO}_4$  and over hot red mercuric oxide. The mercuric oxide was placed in a glass pipe wired with a high resistance Kanthal thread heated electrically to  $+200^\circ\text{C}$  with a variable transformer. The  $^{14}\text{CO}$ , oxidized to  $^{14}\text{CO}_2$  by the mercuric oxide, was collected in two ethanolamine vials. All vials were re-

placed after 15 minutes, 30 minutes, 1 hour and then every hour up to 8 hours after the start of the experiment. For measurement of radioactivity, 10 ml scintillation fluid (4.9 g PPO, 0.1 g dimethyl-POPOP/1 toluene) was added to each of the toluene vials. Four ml methanol and 10 ml scintillation fluid (15 g PPO, 1 g bis-MSB/1 toluene) was added to each of the ethanolamine vials.

After 8 hours the mouse was sacrificed and organ samples taken for analysis of the remaining radioactivity. The samples were frozen on dry ice to prevent evaporation of volatile material and transferred to previously tarred scintillation vials containing 1 ml Soluene®-350. The vials were weighed and allowed to stand for 24 hours at room temperature for dissolution of the organ samples. Ten ml of scintillation fluid (4.9 g PPO, 0.1 g dimethyl-POPOP/1 toluene) was added. The wet weights of the remaining parts of the organs were noted to permit calculation of the total organ radioactivities. Blood samples, 0.25 ml, were taken from the heart, dissolved in 1.5 ml Soluene®-100: isopropanol (1:1 v/v), bleached with 0.5 ml 30% H<sub>2</sub>O<sub>2</sub> and counted in 15 ml Instagel®: 0.5 M HCl (9:1 v/v). Urine was collected after 8 hours by gently pressing the abdomen of the animal and counted in Instagel® together with the urine collected in the metabolic cage. Quench corrections were made using an external standard.

#### PHARMACOKINETIC CALCULATIONS

The liquid scintillation measurement data for the amount of radioactivity collected as expired volatile unchanged solvent

were subject to mathematical and computerized compartmental analysis as follows. The total amount of radioactivity registered at each time of measurement was calculated as percentage of dose and the mean excretion rate (% of dose exhaled/min) of generally four experiments for each time interval was plotted against time on a semilogarithmic paper. Visual inspection of the elimination curves gave at hand that the time course of elimination of unchanged solvent in the expired air might be assumed to follow the equation:

$$Y = \sum_{i=1}^n K_i \cdot e^{-k_i t}$$

where:  $Y$  = mean excretion rate (% of dose/min)

$K_i$  = constant (% of dose/min)

$k_i$  = rate constant ( $\text{min}^{-1}$ )

$n$  = the number of exponential terms

$t$  = time (min)

Initial estimates of the exponential functions were made using an electronic desk calculator (Hewlett-Packard 97). Non-linear regression compartmental analysis was performed with the NONLIN digital computer program (271) with the number of exponential terms,  $n$ , indicating the minimal number of compartments necessary to fit the chosen compartment model. Before computer analysis, the data were weighted according to their observed reciprocal variances, using the weighting factor  $\frac{1}{SD^2}$ . Since the data differed by several orders of magnitude, weighting was necessary to prevent a biased fit due to the relatively greater importance of high  $y$ -values as compared to low  $y$ -values in calculating the exponential curve equation.