Methods for Assessing the Effects of Mixtures of Chemicals
Edited by V. B. Vouk, G. C. Butler, A. C. Upton, D. V. Parke and S. C. Asher

Toxicokinetics, Toxification, Detoxification: Methods to Assess the Effects of Exposure to Multiple Chemicals

James W. Bridges and Peter H. Bach

ABSTRACT

The methods used to identify toxicological interactions between xenobiotics (chemicals) include the assessment of the rates of absorption, distribution, metabolism, and excretion. The most likely forms of interactions between two or more xenobiotics in the toxicokinetic phase are:

(1) Direct competition for active transport systems, plasma protein or tissue binding sites, or for biotransformation reactions.
(2) Effects of one xenobiotic on the synthesis and/or degradation of specific proteins or enzymes involved in xenobiotic transport or metabolism.
(3) Xenobiotic-induced changes in the composition of biological fluids, or in membrane permeability, which alter the distribution kinetics of other xenobiotics.

A scheme is proposed for establishing the priorities for studying the interaction potential of a xenobiotic using non-toxic model compounds, the properties of which are well documented. This approach will facilitate the *in vivo* monitoring for adverse chemical interactions in experimental animals and man.

1 INTRODUCTION

The toxic effects associated with a chemical (xenobiotic) may arise at the site of exposure and/or at any other tissue if the xenobiotic reaches the systemic system. In order to assess the hazard associated with chemical exposure and to understand how toxicity arises, it is helpful to consider the toxic process as comprising a preabsorption phase, a toxicokinetic phase, and a toxicodynamic phase.

The preabsorption (exposure or chemical) phase describes the *actual* physicochemical properties of the relevant substance(s) and includes factors such as purity, polymorphism, chemical stability, general reactivity, particle size, water

or lipid solubility, absorption characteristics, etc., all of which may affect the bioavailability of a xenobiotic and/or lead to the formation of other potentially toxic xenobiotics.

The toxicokinetic (distributive, pharmacokinetic, or drug metabolism) phase describes the relationship between the total dose of the xenobiotic(s) and the resulting concentration of the xenobiotic(s) and/or its metabolites (including those produced by biotransformation and their chemical breakdown products) which are achieved in the vascular compartment, the body tissue and non-vascular tissue fluids. Thus the study of this phase involves an assessment of absorption, biotransformation, binding to tissues, distribution in tissue fluid compartments, and excretion.

The toxicodynamic (interactive, pharmacodynamic, or response) phase describes the interaction of the xenobiotic(s) and/or its metabolites with those cellular or extracellular components (receptors, macromolecules, enzymes and/or other sites of action) whose modified function(s) are involved with the immediate or subsequent expression of toxicity.

2 THE ROLE OF TOXICOKINETICS IN THE ASSESSMENT OF TOXIC HAZARD TO MAN

Characterization of the toxicokinetic phase is necessary in order to understand dose–response relationships and to provide a reliable basis for extrapolating toxicological data from animals to man. Commonly, the differences in toxic effects between structurally related xenobiotics and variation in toxic response between animal species can be ascribed to differences in toxicokinetics (see Table 1).

It is nearly always valuable to establish the relationship between the extent of

Table 1 Factors which suggest that xenobiotics should be subject to toxicokinetic evaluation

— Levels of the xenobiotic to be used in the test system are very high compared with those to which man is likely to be exposed.
— The exposure dose is not reproducibly related to the toxic effects.
— Large species differences in toxic response occur.
— The relationship between dose and toxicity is very steep, non-linear, or changes significantly between single and multiple dosing at levels close to those at which human exposure may occur.
— Structural considerations of toxicological properties indicate that the xenobiotic may exert its effects via an active metabolite(s).
— The structure of the xenobiotic indicates that it may be metabolized by a biochemical reaction for which marked genetic variations or polymorphisms are known to occur (e.g. *N*-acetylation).
— The xenobiotic is highly lipophilic.
— The route of exposure is dermal or by inhalation (large species variations may be expected when chemicals are administered by these routes).

absorption and the dose levels that have been chosen using toxicokinetic procedures. It should also be common practice to establish whether the toxicokinetics of the xenobiotic are altered during the multiple dosing regimens that are employed in most toxicity testing.

3 PREDICTABILITY OF TOXICOKINETICS FROM PHYSICAL AND CHEMICAL PROPERTIES ALONE

At present, the prediction of toxicokinetic behaviour is at the qualitative rather than the quantitative level. Lipophilicity appears to be an important determinant which influences membrane permeability (Hansch, 1976; Houston and Wood, 1980; Sargent *et al.*, 1980), substrate interaction with cytochrome P-450, and the binding to major sites of serum albumin (Al-Gailany *et al.*, 1978; Bridges and Wilson, 1976; Sargent *et al.*, 1981a,b). There is an approximate inverse relationship between lipophilicity and excretion of xenobiotics in urine and bile. Hydrogen bonding and steric factors affect intestinal absorption and, in certain circumstances, the binding to tissue proteins and xenobiotic metabolizing enzymes (Houston *et al.*, 1975). The presence of ionized groups tends to reduce the likelihood of effective absorption (except for very lipophilic xenobiotics); moreover, if ionizable groups are formed by metabolism this encourages urinary and/or biliary excretion. One of the most critical determinants which governs the route via which an anionic molecule and/or its anionic metabolite(s) is cleared to the urine or bile is its molecular weight. Compounds with high molecular weight (> 500 daltons) tend to be excreted by the biliary route, but there are marked species differences in the molecular weight cut-off for biliary excretion of anions; for example, in the rat the relevant threshold molecular weight is ~300 daltons whereas in man it is ~500 daltons (Smith, 1973).

It is usually possible to predict which metabolites will be formed from a particular xenobiotic (although present knowledge of the metabolic fate of heterocyclic ring compounds is still poor), but it is not yet possible to estimate the relative amounts of each metabolite which will be produced. There is a paucity of information on the influence of physicochemical properties on the tissue binding of xenobiotics, although it is often possible to predict the effects of structural changes on albumin binding. Albumin is exceptional because the interactions between xenobiotics and this protein have been the focus of many studies (see review by Bridges and Wilson, 1976), and recently the three-dimensional structure and primary binding sites on albumin for anions have been established (Behrens *et al.*, 1975; Brown, 1975).

4 DETERMINATION OF TOXICOKINETIC PROPERTIES *IN VIVO*

The techniques used for assessing interactions between xenobiotics are, in general, the same as those employed to define the toxicokinetic properties of

individual xenobiotics. Typically there is a direct relationship between the concentration of the xenobiotic(s) and/or its metabolites (particularly the proximate or ultimate toxin) in a target tissue and the extent of the initial toxic interaction(s). (NB: Hypersensitivity reactions are generally atypical in this respect.) Ideally a toxicokinetic study should include a time course analysis of the concentrations of xenobiotics and their metabolites *at* the target tissue sites. In practice, this is often frustrated by insensitive and non-specific analytical methods. Even if a suitable analytical method is available there are a number of problems which can compromise the design and completion of the toxicokinetic assessment of new chemical entities, namely:

(1) It is rarely possible to identify positively the nature of the cellular constituents which make up the critical molecular targets for toxicity.
(2) For ethical and practical reasons it is frequently not possible to sample the target tissue(s) during the *in vivo* development of a toxic lesion, and monitoring of tissue fluids must therefore be used instead.
(3) In many instances the chemically unstable metabolites which are responsible for toxicity have a very short lifetime, or they rapidly undergo further metabolism. This may frustrate the direct identification of their structure and their role in initiating the pathophysiological events which lead to toxicity.

4.1 Sampling Consideration

Normally tissues that are the sites of toxic action cannot be sampled directly. The fluid most often sampled for pharmacokinetic purposes is blood plasma, but careful consideration must be given to the most relevant site for sampling if valid predictions are to be made about the levels of xenobiotics in a particular tissue. Ideally, samples should be taken from the arterial blood entering and the venous blood leaving the target tissue, and the clearance kinetics of the xenobiotic from the body should be established using urine, bile, and faeces, as well as blood. Most other tissue fluids, such as cerebrospinal fluid, testicular fluid, foetal tissue fluids, and breast milk, are separated from blood by permeability barriers. The first three of these fluids are much more difficult to sample than blood (for methodology see Evans *et al.*, 1980; Waites, 1977; Waynforth, 1980). A number of xenobiotics are either volatile or are converted to volatile metabolites.

4.2 Relationship between Tissue Levels of Xenobiotics and Levels in Body Fluids

4.2.1 Blood

The distribution of xenobiotics between blood and tissue depends on lipophilicity, plasma protein binding, the permeability of the membrane barriers, and the binding characteristic of the tissue itself. Xenobiotics with relatively high

octanol–water partition coefficients can readily cross lipid membrane barriers by passive diffusion, as long as their molecular size is not too large. Xenobiotics vary in their tendency to bind to plasma proteins (strongly bound compounds are restricted to the vascular compartment), and there are substantial species differences in plasma binding (Bridges and Wilson, 1976; Sturman and Smith, 1967). There are also differences in the permeability of the membrane barriers between blood and different tissues. For example, the liver is freely permeable to most lipid-soluble xenobiotics and it is also readily permeable to lipoprotein fragments of chylomicrons which may carry xenobiotics. The blood–brain barriers are also readily permeated by lipid-soluble xenobiotics, but not by large or ionized molecules. Finally, the uptake of xenobiotics may be influenced by binding within the tissue; for example, quinacrine is strongly bound in the liver (Evans *et al.*, 1980).

In certain instances, it is possible to provide a reasonable forecast of the tissue levels of xenobiotics and metabolites which might be attained at equilibrium, based on the levels found in blood (e.g. dieldrin (Moriarty, 1975) and polychlorinated biphenyl isomers (Lutz *et al.*, 1971)).

4.2.2 Bile and Urine

The liver plays a dominant role in converting xenobiotics to excretable conjugates; the monitoring of bile or urine may thus provide a means of studying these metabolic processes (Smith, 1973) although it does not give information on localized concentrations, or indicate the concentrations attained in other organs. For certain xenobiotics such as the dieldrin metabolite HCE, it is possible to obtain reasonable estimates of the rates of hepatic metabolism by using data from microsomal fractions, and knowing the concentration of substrate in the hepatic endoplasmic reticulum under *in vivo* conditions (Chipman *et al.*, 1979a,b). A technique for chronic bladder cannulation (which facilitates quantitative, timed recoveries) where renal blood can also be sampled (Gellai and Valtin, 1979) provides a very valuable measure of renal clearance and possibly also some information on renal metabolism.

4.2.3 Faeces

A number of substances undergo significant enterohepatic circulation (Smith, 1973). The monitoring of bile alone cannot give a reliable quantitative indication of this process and so it is desirable to monitor faeces. Also, gut microflora may metabolize chemicals to novel toxic compounds.

4.2.4 Exhaled Air

A variety of substances (including carbon dioxide) are in equilibrium with alveolar and exhaled air. There are a number of well-described examples where

monitoring the rate of respiratory clearance of $^{14}CO_2$ provides an accurate estimate of microsomal demethylation (of *N,N*-dimethyl-[^{14}C]aminopyrine) and plasma clearance (Lauterberg and Bircher, 1976).

5 CONDITIONS UNDER WHICH TOXICOKINETIC PROCESSES MAY BE SATURATED

The metabolism, excretion, and elimination of most xenobiotics from the body follow first-order kinetics, and parameters such as half-life ($t_{1/2}$) and clearance are independent of the size or frequency of dosing; thus xenobiotic availability to the tissues increases linearly with increasing dose. Some xenobiotics saturate one or more toxicokinetic processes, e.g. transport, binding, biotransformation, and excretion. As a consequence, a disproportionate change in the availability of the xenobiotic may occur, and cause a change in the quantitative and/or qualitative toxic effects (Watanabe *et al.*, 1980). To predict with any confidence the potential toxicity of a xenobiotic for another species, it is important to establish whether the saturation of rate-limiting toxicokinetic processes occurs over the dose range of interest. Both the degree and the nature of toxicity may be affected by the route of administration. Thus one route of administration (e.g. injection or gavage) may lead to sufficiently high concentrations of a substrate reaching the saturable site. The administration of the xenobiotic by an alternative route or form (e.g. diet, inhalation, topically), however, may give lower substrate concentrations which will not saturate the system (Wolf, 1980). Highly toxic compounds cause lethality before the saturation of the pharmacokinetic process is reached, but xenobiotics of relatively low intrinsic toxicity may depend on saturation of one or more of these processes for the expression of chronic toxicity (e.g. angiosarcoma of the liver with vinyl chloride monomer). The saturation of particular xenobiotic metabolizing enzymes may result in a change in metabolite profiles and low-dose levels in man may be difficult to relate to data obtained from high-dose levels in animals (Anderson, 1981). Several criteria (Table 2) can be used to indicate that saturation of crucial toxicokinetic processes for the elimination of a xenobiotic are occurring (Anderson, 1981; Gehring *et al.*, 1977; Levy, 1968).

5.1 Determination of Toxicokinetic Properties *In Vitro*

There are serious limitations in the extent to which toxicokinetic processes can be assessed in live animals. For example, those compounds (e.g. certain carcinogens) which form active metabolites which either bind rapidly to macromolecules or other cellular constituents, or are destroyed effectively by detoxification enzymes, will not appear in blood or excreta, and differences in metabolism are unlikely to be found by *in vivo* techniques alone. *In vitro* studies permit the investigation of metabolic processes under controlled conditions allowing the

Table 2 Criteria that can be used to indicate that saturation of toxicokinetic properties is likely

— The non-exponential decline of the blood or plasma levels of the xenobiotic following cessation of dosing.
— The rate constant of elimination decreases with increasing dose.
— The availability of the xenobiotic (as measured by the area under the curve) increases disproportionately with increasing dose.
— A change in the relative amounts of the excretory products occurs with increasing dose.
— The pharmacological, toxicological, or biochemical effects of the xenobiotic increase disproportionately with increasing dose.
— Other xenobiotics at relatively low concentrations, which are either metabolized or transported by the same saturable processes, competitively inhibit the elimination and/or metabolism of the xenobiotic under study.

identification and characterization both of the enzymes involved and of the formation of stable and unstable metabolites.

If toxicity in man is due to the parent compound (or a stable metabolite), an *in vitro* animal model system should be able to detect such toxicity provided that the half-life of the compound is similar both in man and in the model system(s) and assuming that there are no qualitative biochemical differences in man which are not mimicked by the system. If, as is often the case, toxicity is due to a reactive metabolite(s), then the nature of the drug metabolizing system involved in both its formation and detoxification also needs to be similar in the *in vivo* and the *in vitro* situation.

5.1.1 Choice of In Vitro Preparation

A variety of *in vitro* drug metabolizing systems are available which differ mainly in their levels of cellular and tissue integrity (Table 3). The majority of *in vitro* systems will (with varying degrees of success) mimic, at least qualitatively, the *in vivo* formation of metabolites. The disruption of the cellular integrity of any test system (e.g. by the use of microsomes) often enables the optimum generation of reactive metabolites to be expressed. In both the *in vivo* situation and intact cells there are several defence mechanisms that protect against the effects of reactive metabolites and which act as rapidly as the metabolites are formed. Thus, model systems which retain cellular or tissue integrity (isolated cells, or perfused organs) generally reflect the *in vivo* situation more closely than those where cellular integrity and detoxification systems have been disrupted (homogenates or subcellular fractions).

It must be emphasized that in general cell lines are deficient in a number of drug metabolizing enzyme activities, particularly those dependent on (phenobarbitone-type) cytochrome P-450, and this may restrict their value as *in vitro*

Table 3 Selected systems for the *in vitro* assessment of toxicokinetic parameters in the rat

Tissue	Reference
Liver	
Perfusion	Ross (1972)
Hepatocyte suspension/primary maintenance cultures	Berry and Friend (1969)
Microsomes	Fry *et al.* (1976)
Kidney	
Perfusion *in vitro* and *in situ*	Bach and Lock (1982)
Kidney cortex and medulla slices	Bach and Lock (1982)
Micropuncture and microinfusion	Diezi and Roch-Ramel (1982)
Tubular cells	Ormstad (1982)
Glomeruli	Ormstad (1982)
Cortex and medullary microsomes	Hewitt and Hook (1983)
Skin	
Skin strips	Maloney *et al.* (1982a)
Microsomes	Maloney *et al.* (1982b)
Gut	
Perfusion	Ross (1972)
Everted gut sacs	Wilson and Wiseman (1954)
Isolated epithelial cells	Dawson and Bridges (1981a)
Microsomes	Dawson and Bridges (1981b)
Lung	
Perfusion	Ross (1972)
Cells	Trump *et al.* (1980)
Subcellular fractions	Tredger and Chhabra (1976)
Purified enzymes (P-450)	Philpot and Wolf (1981)

models for toxicity testing (Bridges and Fry, 1979). This problem has been overcome to some extent by the addition of an *in vitro* drug metabolizing system (e.g. $9000 \times g$ liver supernatant microsomes, intact hepatocytes) to the cell line (Bridges and Hubbard, 1981). However, this approach is not always valid because the two systems may be incompatible, and/or metabolites may not reach the target sites in sufficient concentrations to cause toxicity. A particularly difficult problem with any *in vitro* system is to reflect realistically the *in vivo* situation with regard to the tissue–tissue interactions. For example, the lack of an excretory system often leads to dynamics *in vitro* which are never seen *in vivo* (see also below).

A number of extrahepatic organs may also metabolize xenobiotics (Connelly and Bridges, 1980), although often these are less effective in overall biotransformation. Studies on the *in vitro* extrahepatic biotransformation of xenobiotics have been much less extensive than have those on liver. Methods for

the preparation of subcellular fractions of tissues and for isolating those cells with particularly high concentrations of xenobiotic metabolizing enzymes are far from ideal for most organs. Much of the published quantitative data on xenobiotic biotransformation activity in extrahepatic organs is probably suspect because inappropriate methodology was employed in making the tissue preparation.

5.1.2 Use of Human Tissues

Metabolic studies using human tissues can provide a valuable complementary approach to the very limited *in vivo* studies that can be carried out in man. Studies on the metabolic fate and effects of chemicals in different human tissues should aid our understanding both of the mechanisms of chemical toxicity and also of the predisposition of certain target organs to toxicity.

Whilst there are many complications in the use of human tissues, these should not deter appropriate scientific investigations of the full metabolic potential of such tissues. Recent advances in organ culture techniques now permit the maintenance of normal morphology of various human tissues such as lung, bronchus, colon, breast, prostate, pancreas, oesophagus, urinary bladder, endometrium, and several types of renal cells. Many of these tissues have also been shown to be capable of metabolizing drugs and, in many cases, active carcinogens to reactive metabolites which bind to DNA (Autrup, 1982). The development of human tissue banks (Von Bahr *et al.*, 1980) which contain subcellular fractions of some of the major drug metabolizing organs stored at low temperatures is a promising development for the extension of toxicokinetic studies on human tissues.

6 METHODS FOR STUDYING ABSORPTION

6.1 Intestinal Absorption

Methods which may be used to determine the rates and extent of gastrointestinal absorption can be divided into three classes as listed below.

6.1.1 Methods Involving Unanaesthetized Animals

Determination of the extent and rate of recovery of xenobiotic in blood and/or urine provides useful information on absorption. However, unless both the oral and intravenous routes of administration are compared, only gross interpretations are possible. It is desirable to carry out cross-over studies where each animal acts as its own control. This may require large animal species unless a very sensitive assay is available, in which case small mammals such as rats can be used

for chronic repeated arterial or venous blood sampling via cannulae (Migdalof, 1976).

6.1.2 Methods Involving Anaesthetized Animals

Anaesthetization allows a much wider range of investigation to be employed than is otherwise possible *in vivo;* however, the use of anaesthetics may modify blood flow to the gastrointestinal tract and alter peristalsis and active transport. Absorption may be calculated either from the disappearance of the xenobiotic from the gastrointestinal lumen or from its appearance in the hepatic portal vein blood. The two most widely used disappearance methods are:

(1) The closed loop method (Verzar and McDougal, 1936) where the animal's small intestine is exposed and ligated. A solution containing the xenobiotic is introduced into the lumen, and the lumen is then replaced in the abdominal cavity and the incision closed. The animal is sacrificed at a predetermined period, a blood sample taken, and the intestinal loop is rapidly excised in order to quantitate the unabsorbed xenobiotic (Levine and Pelikan, 1961).
(2) The perfused loop method (Sols and Ponz, 1947) which requires cannulation of the duodenal and ileal ends of the small intestine (the stomach and caecum are closed off by ligatures) which is replaced in the abdominal cavity. Perfusion can then be undertaken via a single pass or a recycling system, while the time course changes in the blood and perfusate concentrations of the xenobiotic can be measured (Hayton and Levy, 1972).

6.1.3 In Vitro Methods Using Gastrointestinal Tissue

A large range of *in vitro* techniques have been used, the most popular of which is the everted gut sac method (Wilson and Wiseman, 1954). This method has the advantages of simplicity and sensitivity (the small volume within the sac makes it easy to measure 'absorbed' molecules) and the model is relatively reproducible. However, the physiological relevance of data from such a preparation is questionable because the integrity of the intestinal membranes is prone to rapid deterioration.

6.2 Absorption from the Skin

The percutaneous absorption of a xenobiotic is difficult to measure *in vivo* unless the compound is lipid soluble, in which case substantial quantities may reach the systemic system. Treherne (1956) showed a proportional relationship between the ether–water partition coefficient and the dermal penetration of model compounds. Dermal absorption can be measured by assessing the total quantity of parent compound and individual metabolites that have been excreted, but very sensitive assays are essential if the quantities of xenobiotic applied to the skin are

to be related to likely dermal exposure. The very low rates of absorption of most compounds necessitate the use of *in vitro* techniques where skin slices serve as the barrier between two compartments, one of which (epidermal side) contains the xenobiotic and the other is used for sampling and measuring the rates of transfer.

6.3 Absorption from the Lung

The very large surface area of the pulmonary epithelium represents a most important site for the absorption of molecules which have been inhaled, particularly lipid-soluble gases. High molecular weight chemicals (e.g. inulin, 5000 daltons) and several water-soluble molecules are also absorbed.

The pulmonary absorption of xenobiotics can be assessed by measuring the total quantity of parent compound and metabolites that are excreted, but this approach fails to give data on the rate of xenobiotic uptake or the amount of xenobiotic remaining in the lung at predetermined intervals following the instillation of metered quantities of liquid into the rat lung (Enna and Schanker, 1969).

7 METHOD FOR STUDYING PROTEIN AND TISSUE BINDING

7.1 *In Vitro* Methods

Several *in vitro* methods have been employed for assessing reversible and irreversible (covalent) binding to macromolecules.

7.1.1 Assessment of Reversible Binding

The most extensively used methods to determine reversible binding of xenobiotics to proteins remain equilibrium dialysis and ultrafiltration. In principle both methods are similar and the limitations and advantages of each have been reviewed by Bridges and Wilson (1976).

7.1.2 Assessment of Covalent Binding

Covalent binding may be assessed in tissues following *in vivo* treatment of animals with radiolabelled xenobiotic, or incubation of the xenobiotic with a drug metabolizing enzyme preparation. In order to obtain a reliable estimate of the amount of covalent binding, it is essential to carry out exhaustive extraction of the precipitated macromolecular material with several organic solvents (Mitchell *et al.*, 1973). The covalent binding of xenobiotics to nucleic acids has acquired a major significance in explaining the molecular processes that are involved in genotoxicity and carcinogenesis. It is possible to measure the formation of adducts by administering a suitably labelled xenobiotic *in vivo*,

followed by isolation, precipitation and exhaustive extraction and purification of nucleic acid (Swenson *et al.*, 1977), or by incubating pure nucleic acid with the xenobiotic in the presence of a metabolic system which activates the compound (Kadlubar *et al.*, 1982).

7.2 *In Vivo* Methods

In vivo methods have only rarely been used to assess serum protein binding despite their potential relevance to validate *in vitro* findings. McQueen (1968) has devised a simple method which involves the determination of xenobiotic concentration in a dialysis sac previously implanted in the peritoneal cavity. The assumption is made that the xenobiotic partitions freely between tissue compartments and hence free xenobiotic levels in the peritoneal cavity are a direct reflection of those in plasma. The method is unsuitable for xenobiotics which bind avidly to tissues.

Whole body autoradiography is widely used to assess the tissue distribution of radiolabelled xenobiotics in small animals *in vivo*, and offers more comprehensive information than techniques based on the quantitative analysis of dissected tissues. A great deal of information can also be derived on the distribution of radiolabelled material within specific tissues or organs and, if electron-microscopic techniques are used, within individual cells. However, the autoradiography does not allow the nature of the binding to be ascertained, the chemical structure of the label to be determined, or the amount of bound radiolabel to be quantitated (Rogers, 1979; Ullberg and Larsson, 1981).

8 METHODS FOR STUDYING BIOTRANSFORMATION

8.1 *In Vivo* Methods

Metabolites may be assayed in various biological fluids or in expired air (see sections 4.1 and 4.2). It may be necessary to assess the time course changes in the metabolite profile in several biological fluids in order to provide a reliable basis for comparing species differences and for identifying the effects of inducing agents or inhibitors. If reactive metabolites play a major role in the toxic process *in vivo*, it is normally necessary to resort to *in vitro* methods.

8.2 *In Vitro* Methods (see Table 3)

8.2.1 Preparation Methods for Cells and Subcellular Fractions

Methods for producing intact cells and cell fractions are generally well developed for mammalian liver, but have not yet been optimized for preparing intact cells and cell fractions from most extrahepatic organs, or from the liver of non-

mammalian species. Extrahepatic organs may be more resistant to homogenization than the liver, or not amenable to the enzyme-based cell dispersion and separation methods commonly used for hepatocytes; also organs such as the kidney and lung contain more biochemically different cell types. The temptation to continue to disrupt tissue until a visually homogeneous preparation is obtained must be resisted, because this approach will inevitably result in significant amounts of enzyme damage to cellular membranes and problems in cell fractionation. The presence of mucoid material in mucus-secreting tissues may cause the artifactual sedimentation of cell components at low centrifugation speeds (Shirkey *et al.*, 1979); also the commonly used marker enzymes and the characterization of the fractions are frequently inappropriate for extrahepatic organs. Thus all methods for preparing cells or subcellular fractions must be validated for each new tissue/species which is the subject of study if relevant data are to be obtained.

8.2.2 Incubation Systems for Biotransformation Studies

Conventionally *in vitro* investigations of xenobiotic biotransformation using cell fractions are conducted using saturating levels of substrate (xenobiotic) and cofactor to attain the maximum formation of metabolites. Short incubation times (< 15 min) are usually employed to retain a linear rate of metabolism. Optimal conditions must be ascertained for each new preparation, but for a number of purposes (e.g. extrapolation of *in vitro* results to the *in vivo* situation) such optimization may be inappropriate. For example, such an approach may mask the role of a high-affinity, low-capacity enzyme which at the low levels of xenobiotic encountered *in vivo* may be the predominant biotransformation enzyme; it may also be difficult to assess the contribution of enzymes which reverse the reaction. Although 37°C is generally used for incubations with mammalian tissues, it is not appropriate for systems from many fish, reptiles, etc. Typically, freshly isolated intact cell preparations provide more physiologically relevant results than those obtained using cell fractions, provided the cofactor levels are comparable with those *in vivo*.

9 METHODS FOR STUDYING EXCRETION

There are three major routes via which xenobiotics can be excreted: urine, bile, and exhaled air.

9.1 Urinary, Biliary and Pulmonary Excretion

The important factors which govern the renal excretion of a xenobiotic and its metabolites are water solubility, plasma binding, urinary pH, the functional state of the kidney (e.g. the glomerular filtration rate), and the integrity of the active

organic cation or anion transport systems. The renal clearance rate is best undertaken using conscious animals that have chronic cannulae in the bladder and in both the renal artery and vein (Gellai and Valtin, 1979).

The monitoring of a xenobiotic and its metabolites in faeces represents only one facet of biliary excretion, which may be difficult to interpret because of the enterohepatic circulation and the contribution made by gut microflora. For this reason it is essential to monitor biliary excretion directly: bile collected from anaesthetized animals is subject to flow artefacts and it is often desirable to prepare animals chronically, either with a 'T' piece cannula (Vig and Wostmann, 1979) to facilitate the measurement of the rate of excretion, or by implanting a glass vessel into the peritoneal cavity to collect bile continuously (Johnson and Rising, 1978).

The methods for measuring pulmonary excretion depend on suitable trapping of the constituents of exhaled air either by a physical method or by chemical trapping.

9.2 Other Routes

Several aspects of salivary gland excretion have been studied (Stephen *et al.*, 1980), but there are few kinetic data on the excretion of xenobiotics in sweat (or other sebaceous excretions) and tears. A large number of compounds are known to be excreted via the mammary glands (Welch and Findlay, 1981; Wilson *et al.*, 1980).

10 METHODS FOR STUDYING THE INTERACTIONS BETWEEN SEVERAL XENOBIOTICS IN THE TOXICOKINETIC PHASE

A wide range of interactions may occur between several xenobiotics in the toxicokinetic phase (see Table 4), and thereby affect absorption, distribution, biotransformation and/or excretion. In addition to considering the overall process involved, there are also at least four types of interaction mechanisms which must be considered:

(1) direct competition between two or more xenobiotics at sites of active transport systems, tissue binding, or biotransformation;
(2) xenobiotic-initiated changes in the synthesis and/or degradation of active transport components, tissue binding macromolecules, and/or biotransformation enzymes and their associated cofactors;
(3) xenobiotic-related changes in physiological factors such as blood flow (i.e. transport of other xenobiotics) or the pH of biological fluids; and
(4) xenobiotic-initiated pathophysiological changes affecting membrane permeability, fluid flow in ducts, tubules, etc.

It is obviously impractical to investigate each of the possible interactions which may occur between xenobiotics for each of the new chemical entities that will be

Table 4 Forms of xenobiotic interactions in the chemical and toxicokinetic phases

Nature of interaction
Preabsorption phase
Adsorption onto food
Chemical reaction
Physical association with other xenobiotics, etc.
Absorption
Active/facilitated transport
General changes in membrane permeability
Activity of gut microflora
Peristalsis
Blood flow
Intestinal pH
Metabolism
Competition for enzyme sites
Cofactor levels
Amount of active enzyme (apoprotein and/or prosthetic group)
Distribution
Competition for tissue binding sites
Amount of binding molecules
Blood flow
Excretion
Active/facilitated transport
Blood flow
Renal tubular pH

added to the environment, let alone the enormous number to which man and other species are already exposed. Thus it is essential that a scheme is introduced to establish the priorities for studying potential interactions, and identifying those that are likely to have major toxicological consequences. This approach is possible, provided we establish a systematic scheme to consider the potential for any one xenobiotic to interact with other chemicals, and provided we examine one facet at a time. Initially we need to address ourselves to two questions:

(1) Is the xenobiotic likely to influence the absorption, distribution, biotransformation and/or excretion of other chemicals in such a way as to significantly enhance the toxicity of these chemicals; and
(2) Is the toxicokinetic profile of the xenobiotic likely to be modified sufficiently by other chemicals such that its own toxicity will be significantly increased.

10.1 Establishing Priorities for Experimentally Studying the Interaction Potential of Particular Xenobiotics

In order to establish priorities for a particular xenobiotic we propose that a series of questions are answered which are outlined in Table 5. Predominantly positive or 'don't know' answers highlight a xenobiotic which is a high-priority candidate for a detailed investigation of its interaction properties.

The principle that has been employed is to establish the extent and amount of likely or actual human exposure to a particular xenobiotic, its physicochemical properties (particularly lipophilicity), its persistence in the body, the extent to which its toxicokinetic profile alters between single and multiple dosing, and its structural resemblance to known potent modifiers of distribution, absorption, biotransformation, and/or excretion.

A similar systematic evaluation (Table 6) allows us to assess the likely enhancement of toxicity. Some of these questions are obviously the same as those posed above, but more emphasis is given to the toxicological properties of the xenobiotic (i.e. the steepness of the toxicity dose–response relationship) at the

Table 5 Procedure for identifying xenobiotic interactions by establishing their potential to modify the toxicokinetics of other chemicals

	Score[a]
A. Exposure considerations	
— Number and range of population exposed (especially high-risk groups)	_____
— Level and duration of exposure	_____
— Environmental persistence	_____
— Bioavailability of exposure form	_____
B. Physicochemical properties	
— Lipophilicity	_____
— Structural relationship to other potent modifiers of toxicokinetic profiles	_____
C. Toxicokinetic profile	
— Persistence	_____
— Extent of alteration of toxicokinetic profile between single and multiple dosing	_____
— Degree of serum/tissue protein binding (or volume of distribution)	_____
D. Other biological properties	
— Ability to reduce blood flow	_____
— Effect on gastrointestinal mobility, or renal excretion function	_____
TOTAL	_____

[a] Scoring: 1 = low; 2 = medium; 3 = high; if not known assume scoring of 3. Score above 25 equates with a high-priority compound.

Table 6 Procedure for identifying xenobiotic interactions by establishing the susceptibility of other chemicals to modifying the toxicokinetics of a xenobiotic

	Score[a]
A. Exposure consideration	
— Number and range of population exposed (especially high-risk groups)	
— Level and duration of exposure	
— Bioavailability of exposure form	
B. Toxicological properties	
— Steepness of dose–response relationship at the possible exposure levels	
— Extent of conversion to toxic metabolites	
— Half-life	
— Extent of reversible tissue/serum protein binding	
C. Structural considerations	
— Structural resemblance to those xenobiotics which produce greatly enhanced toxicity interactions with other chemicals	
TOTAL	

[a] Scoring: 1 = low; 2 = medium; 3 = high; if not known assume scoring of 3. Score above 17 equates with a high-priority compound.

anticipated exposure levels, the extent of its chemical or biological conversion to toxic metabolites, and its structural resemblance to other xenobiotics which have already been shown to greatly enhance toxicity when exposed to other chemicals simultaneously.

10.2 Model Test Systems for Assessing Interaction Potency

At least two types of models are needed, one for assessing biotransformation effects, and another for assessing other aspects of toxicokinetics. Initially the most practical approach is to assess the effect of the xenobiotic on 'model' compounds *in vivo*.

The criteria for an ideal model compound that can be used for assessing effects of xenobiotics on biotransformation are:

(1) clearance should be limited by biotransformation;
(2) biotransformation should involve a range of important xenobiotic metabolizing enzymes (e.g. several P-450 species);
(3) metabolite formation should involve both hepatic and extrahepatic tissues;
(4) the analytical method for monitoring metabolites should be sensitive, simple and reproducible;

(5) the model compound should have minimal toxicity in various animal species and especially in man; and
(6) it should not, in itself, perturb the toxicokinetic system (i.e. it should not be an inducer, strongly plasma bound, etc.).

There are no model compounds which fulfil all of these criteria. The most suitable contenders include compounds such as aminopyrine, antipyrine, and phenacetin (see Table 7).

The criteria for an ideal model compound for assessing effects on other aspects of toxicokinetics are:

(1) clearance should be limited by excretion and depend on active transport through both bile and urine for elimination;
(2) the model compound (or one of its major metabolites) should be extensively bound by serum or tissue protein;
(3) gastrointestinal absorption rate should be moderate rather than rapid, but absorption should be complete;
(4) the analytical method for measuring metabolites should be sensitive, simple and reproducible; and
(5) it should have minimal toxicity in various animal species and in man.

Once again no model compound meets all of these requirements but the most suitable include acetaminophen (paracetamol), salicylate, probenecid and warfarin (see Table 8).

The model compounds would be administered simultaneously with the xenobiotic and also following several daily doses of the xenobiotic.

Model compounds with well-established interactive potential are needed to assess the extent to which the toxicokinetics of the xenobiotic are prone to variation. The model compounds might be administered as 'cocktails' to reduce the number of experiments required. Possible models are:

(1) inducers (Aroclor or naphthoflavone phenobarbital);
(2) inhibitors (1-substituted imidazole or piperonylbutoxide);
(3) protein binding agents (phenylbutazone, warfarin or salicylate and imipramine);
(4) kidney secretion competitors (probenecid, aminohippurate); and
(5) biliary secretion competitors (bromosulphthalein).

Model inducers would be administered daily for several days prior to exposing animals to the xenobiotic. Models for inhibition, protein binding displacement, and kidney and biliary transport competition should be administered simultaneously with the xenobiotic. In each case the toxicokinetic profile of the xenobiotic would need to be determined in blood, urine, and in many cases also breath and bile.

Table 7 Physicochemical, metabolic, kinetic, and toxicity characteristics of model compounds which could be used for assessing perturbed toxicokinetics at the level of biotransformation

	Aminopyrine (amidopyrine)	Antipyrine (phenazone)	Phenacetin
Nature of compound	Base	Base	Substituted amine
pK_a	5.0	1.4	–
Oral absorption	Moderate to good	Rapid and complete	Moderate to good
Metabolites	4-Aminoantipyrine 4-Acetylaminoantipyrine Monomethyl-4-amino-antipyrine Rubazonic acid and methylrubazonic formyl-4-aminoantipyrine plus minor metabolites	Glucuronic acid conjugate 4-Hydroxyantipyrine[a] 3-Hydroxyantipyrine[a] Norantipyrine[a] Up to 50% via ring opening where the metabolites have not been adequately characterized	Acetaminophen and its metabolites *p*-Phenetidine *p*-Ethoxyaniline 2-Hydroxyphenacetin [a, b] *S*-(1-acetamido-4-hydroxyl) cysteine
Clearance limited by biotransformation	< 3% excreted unchanged	Yes	Largely
Biotransformation via range of enzymes	Yes	Yes	Yes. Reactive metabolites formed
Metabolism in liver and extrahepatically	Defined in liver—not adequately in other regions	Defined in liver—not adequately in other regions	Defined in liver—not adequately in other regions

Table 7 (*Cont'd*)

	Aminopyrine (amidopyrine)	Antipyrine (phenazone)	Phenacetin
Sensitive reproducible assay	GC, HPLC	GC, HPLC	GC, HPLC
Percentage of parent compound excreted unchanged	3%	0–5% (6% norphenazone)	<1% (2–3% unconjugated acetaminophen)
Via urine	Mostly	Up to 50%	Largely
Via bile	?	?	Small percentage
Half-life	2–7 hours	6–24 hours	1 hour
	Poorly plasma bound (15–20%)	Poorly plasma bound (0–8%)	Poorly plasma bound (30%)
Likely effects on toxicokinetics of other compounds	Monomethyl-4-amino-antipyrine may inhibit *N*-demethylation	Weak inducer of mixed function oxidase enzymes	
Toxicity	Dosage should be limited to few 100 mg or less	Dose should be limited	Dose should be limited

[a] Glucuronic acid conjugate.

[b] Sulphate conjugate.

Data from Clark (1969), Pharmaceutical Codex (1979), La Du *et al.* (1971), Smith (1973), and Smith and Rawlins (1973).

Table 8 Physicochemical, metabolic, kinetic, and toxicity characteristics of model compounds which could be used for assessing altered absorption, distribution and excretion of chemicals

	Acetaminophen (paracetamol, *N*-acetyl-*p*-aminophenol)	Probenecid	Salicylate (aspirin, acetylsalicylic acid)	Warfarin
Nature of compound	Alcohol	Acid	Acid	Acid
pK_a		3.4	3.0 (salicylic acid 3.0)	
Gastrointestinal absorption	Rapid and extensive. Has been used for assessing gastric emptying and intestinal transit from peak plasma level and time to reach this	Rapid and complete	Rapidly absorbed intact, then hydrolysed to salicylic acid. Salicylic acid very rapidly absorbed. Likely to be affected by gastric pH	Relatively rapid and complete
Metabolism	Ethereal sulphate Glucuronic acid conjugate *p*-Aminophenol	Glucuronic acid conjugate and side-chain oxidation	Hydrolysed to salicylic acid. Metabolites include glucuronic acid conjugates (ether and ester), salicyluric acid, gentistic acid, 2,3-dihydroxybenzoic acid, 2,3,5-trihydroxybenzoic acid	Glucuronic acid conjugate Ring and side-chain hydroxylation
Methods for measuring metabolites	GC, HPLC	GC	GC, HPLC	GC, HPLC
Percentage of parent compound excreted unchanged	1–3%	3–35% (with increased urinary pH and urine flow)	< 1% aspirin 1.5% salicylic acid	0%
Excretion via urine	More than 85%	75–90%	More than 95%	60–90%

Table 8 (*Cont'd*)

	Acetaminophen (paracetamol, *N*-acetyl-*p*-aminophenol)	Probenecid	Salicylate (aspirin, acetylsalicylic acid)	Warfarin
Excretion via bile	Small percentage	± 3% (may be significantly increased in presence of extensive renal compromise)	Very little	
Half-life	1–4 hours	4–17 hours (depends on dose)	0.25 hour (salicylic acid 6–8 hours, depends on dose)	15–58 hours (differs for *R*- and *S*-enantiomer)
Clearance excretion limited—active transport to bile/urine		Rate of excretion affected by pH and competing molecules. Active renal transport process	Rate of excretion affected by urinary pH	
Significant plasma protein binding	Low (8–40%)	Yes (75–94%)	Minimal (75%)	Yes (97%)
Toxicity	Minimal in 'therapeutic' level, especially if limited to single dose	Minimal up to 1 g per day	Minimal in 'therapeutic' level, especially if limited to single dose	Decreased coagulation time unlikely with few doses but could offer an additional criterion for assessing interactive effects. Easily controlled
Effect on other toxicokinetic systems	No major effect known at low dose levels	Inhibits active renal and hepatic secretion of acidic molecules. May stimulate its own metabolism in animal	No major effect known at low-dose levels	Could displace some plasma bound molecules

Data from Clark (1969), Pharmaceutical Codex (1979), La Du *et al.* (1971), Smith (1973), and Smith and Rawlins (1973).

10.3 Subsequent Studies on Interactions Involving the Xenobiotic

Using the approach outlined above it should be possible to identify those xenobiotics which are likely to be involved in significant interactions at the toxicokinetic phase using model compounds, and then to define the most likely type of interaction. If major interactions are identified the most appropriate technique(s) should be selected (see sections 6–9) in an attempt to fully characterize their nature and dose–response relationship.

11 CONCLUSION

Because of the vast number of potential interactions which are possible between the plethora of the xenobiotics to which man is exposed, it is obviously impossible to assess directly the effects of every xenobiotic. The methodology is well established for studying the toxicokinetic phase interactions between xenobiotics and non-human mammalian liver, but the techniques for investigating similar interactions in extrahepatic tissues and non-mammalian organs are less well developed and frequently misapplied. Priority should be given to improving these *in vitro* preparations, particularly the preparation and storage of human cells and cell fractions (especially from liver) and cellular materials from the lung, kidney, and skin of other species.

Until such time that it is possible to predict toxicologically significant interactions from physicochemical considerations alone, we believe that the only logical approach to tackling the problem of chemical interactions leading to enhanced toxicity is through the type of scheme described above. The first part of this approach involves identifying those xenobiotics which have the highest priority for the investigation of interaction potential. The second part makes use of several model compounds to assess the magnitude of the interaction potential. Several suitable model compounds have been suggested, but detailed systematic studies are urgently needed to ensure that these compounds are, in fact, the most appropriate for the purpose.

ACKNOWLEDGEMENTS

We acknowledge the very helpful contributions of Drs L. F. Chasseaud, G. C. Cohen and C. Walker, in the preparation of this article, and Heather Scott and Janet Williams for typing the manuscript.

12 REFERENCES

Al-Gailany, K. A. S., Houston, J. B., and Bridges, J. W. (1978). The role of substrate lipophilicity in determining its Type I microsomal P-450 binding characteristics. *Biochem. Pharmacol.*, **27**, 783–788.

Anderson, M. E. (1981). Saturable metabolism and its relationship to toxicity. *Crit. Rev. Toxicol.*, **9**, 105–150.

Autrup, H. (1982). Carcinogen metabolism in human tissues and cells. *Drug Metab. Rev.*, **13,** 603–646.

Bach, P. H., and Lock, E. A. (1982). The use of renal tissue slices, perfusion and infusion techniques to assess renal function and malfunction. In Bach, P. H., Booner, F. W., Bridges, J. W., and Lock, E. A. (Eds.) *Monographs in Applied Toxicology, Vol. 1, Nephrotoxicity: Assessment and Pathogenesis*, pp. 128–143. John Wiley & Sons, Chichester.

Behrens, P. Q., Spiekerman, A. M., and Brown, J. R. (1975). Structure of human serum albumin. *Fed. Proc.*, **34,** 591.

Berry, M. N., and Friend, D. S. (1969). High yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.*, **43,** 506–520.

Bridges, J. W., and Fry, J. R. (1979). The use of cultured hepatocytes in biochemical toxicology. In Hodgson, E., and Philpot, R. (Eds.) *Reviews in Biochemical Toxicology*, Vol. 1, pp. 193–247. Elsevier/North-Holland, Amsterdam.

Bridges, J. W., and Hubbard, S. A. (1981). Problems of providing an appropriate drug metabolizing system for short-term screening tests for carcinogens. In Williams, G. M., Kroes, R., Waaijers, H. W., and van de Poll, K. W. (Eds.) *The Predictive Value of Short-term Screening Tests in Carcinogenicity Evaluation,* pp. 69–87. Elsevier/North-Holland, Amsterdam.

Bridges, J. W., and Wilson, A. G. E. (1976). Drug serum protein interactions and their biological significance. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 1, pp. 193–247. John Wiley & Sons, Chichester.

Brown, J. R. (1975). Structure of bovine serum albumin. *Fed. Proc.*, **34,** 591.

Chipman, J. K., Kurukgy, M., and Walker, C. H. (1979a). Comparative metabolism of a dieldrin analogue: hepatic microsomal systems in models for metabolism in the whole animals. *Biochem. Pharmacol.*, **28,** 859–865.

Chipman, J. K., Kurukgy, M., and Walker, C. H. (1979b). The metabolism of dieldrin and two of its analogues: the relationship between rates of microsomal metabolism and rates of excretion of metabolites in the male rat. *Biochem. Pharmacol.*, **28,** 1337–1345.

Clark, E. G. C. (1969). *Isolation and Identification of Drugs*. The Pharmaceutical Press, London.

Connelly, J., and Bridges, J.W. (1980). The distribution and role of cytochrome P-450 in extrahepatic organs. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 5, pp. 1–112. John Wiley & Sons, Chichester.

Dawson, J. R., and Bridges, J. W. (1981a). Guinea-pig intestinal sulphotransferases: an investigation using the cytosol fraction. *Biochem. Pharmacol.*, **30,** 2409–2413.

Dawson, J. R., and Bridges, J. W. (1981b). Intestinal microsomal drug metabolism. A comparison of rat and guinea-pig enzymes, and of rat crypt and villous tip cell enzymes. *Biochem. Pharmacol.*, **30,** 2415–2420.

Diezi, J., and Roch-Ramel, F. (1982). The use of single nephron, micropuncture and microcannulation techniques in assessing renal function and malfunction. In Bach, P. H., Bonner, F. W., Bridges, J. W., and Lock, E. A. (Eds.) *Monographs in Applied Toxicology, Vol. 1, Nephrotoxicity: Assessment and Pathogenesis*, pp. 144–160. John Wiley & Sons, Chichester.

Enna, S. J., and Schanker, L. S. (1969). Drug absorption from the lung. *Fed. Proc.*, **28,** 359.

Evans, W. E., Schentag, J. J., and Jusko, W. J. (Eds.) (1980). *Principles of Therapeutic Drug Monitoring*. Applied Therapeutics, San Francisco.

Fry, J. R., Jones, C. A., Wiebkin, P., Belbmann, H. P., and Bridges, J. W. (1976). The enzyme isolation of adult rat hepatocytes in a functional and viable state. *Anal. Biochem.*, **71,** 341–350.

Gehring, P. J., Watanabe, P. G., and Young, J. D. (1977). The relevance of dose-dependent pharmacokinetics in the assessment of carcinogenic hazards of chemicals. In Hiatt, H. H., Watson, J. D., and Winsten, J. A. (Eds.) *Origins of Human Cancer*, Book A, pp. 187–203. Cold Spring Harbor Laboratory, New York.

Gellai, M., and Valtin, H. (1979). Chronic vascular constrictions and measurements of renal function in conscious rats. *Kidney Int.*, **15,** 419–426.

Hansch, C. (1976). On the structure of medicinal chemistry. *J. Med. Chem.*, **19,** 1–6.

Hayton, W. L., and Levy, G. (1972). Effect of complex formation on drug absorption. *J. Pharm. Sci.*, **61,** 367–371.

Hewitt, W. R., and Hook, J. B. (1983). The renal excretion of drugs. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 7, pp. 11–76. John Wiley & Sons, Chichester.

Houston, J. B., and Wood, S. G. (1980). Gastrointestinal absorption of drugs. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 4, pp. 57–130. John Wiley & Sons, Chichester.

Houston, J. B., Upshall, D. G., and Bridges, J. W. (1975). Further studies on the role of lipophilicity in the gastrointestinal absorption of nutrients. *J. Pharmacol. Exp. Ther.*, **195,** 67–72.

Johnson, P., and Rising, P. A. (1978). Techniques for assessment of biliary excretion and enterohepatic circulation in the rat. *Xenobiotics*, **8,** 27–36.

Kadlubar, F. F., Fredrick, C. B., Weis, C. C., and Zenser, T. V. (1982). Prostaglandin endoperoxide synthetase-mediated metabolism of carcinogenic aromatic amines and their binding to DNA and protein. *Biochem. Biophys. Res. Commun.*, **108,** 253–258.

La Du, B. N., Mandel, H. G., and Way, E. L. (Eds.) (1971). *Fundamentals of Drug Metabolism and Drug Disposition*. Williams and Wilkins, Baltimore.

Lauterburg, B. H., and Bircher, J. (1976). Expiratory measurement of maximal aminopyrine demethylation *in vivo*. *J. Pharmacol. Exp. Ther.*, **196,** 501–509.

Levine, R. R., and Pelikan, E. W. (1961). The influence of experimental procedures and dose on the intestinal absorption of onium compounds, benzomethamine. *J. Pharmacol. Exp. Ther.*, **131,** 319–327.

Levy, G. (1968). Dose dependent effects in pharmacokinetics. In Tedeschi, D. H., and Tedeschi, R. E. (Eds.) *Importance of Fundamental Principles in Drug Evaluation*, pp. 141–172. Raven Press, New York.

Lutz, R. J., Dednick, R. L., Matthews, H. B., Eling, T. E., and Anderson, M. W. (1971). A preliminary pharmacokinetic model for several polychlorinated biphenyls in the rat. *Drug Metab. Dispos.*, **5,** 386–396.

Maloney, S.J., Fromson, J. M., and Bridges, J. W. (1982a). The metabolism of 7-ethoxycoumarin and 7-hydroxycoumarin by rat and hairless mouse skin strips. *Biochem. Pharmacol.*, **31,** 4005–4009.

Maloney, S. J., Fromson, J. M., and Bridges, J. W. (1982b). Cytochrome P450 dependent deethylase activity in rat and hairless mouse skin microsomes. *Biochem. Pharmacol.*, **31,** 4011–4018.

McQueen, E. G. (1968). Comparison of tissue fluid and plasma concentrations of a protein bound drug sulphormethoxine by *in vivo* dialysis in rats. *Br. J. Pharmacol.*, **33,** 312–317.

Migdalof, B. H. (1976). Methods for obtaining drug time course data from individual small laboratory animals. Serial microblood sampling and assay. *Drug Metab. Rev.*, **5,** 295–310.

Mitchell, B. R., Jollow, D. J., Potter, W. Z., Davis, D. C., Gillette, J. R., and Brodie, B. B. (1973). Acetaminophen induced hepatic necrosis. *J. Pharmacol. Exp. Ther.*, **187,** 185–194.

Moriarty, F. (1975). *Exposure and Residue in Organochlorine Pesticides: Organic Pollutants*, p. 29. Academic Press, New York.

Ormstad, K. (1982). The use of isolated kidney cells, glomeruli and subcellular fractions for assessing renal function. In Bach, P. H., Bonner, F. W., Bridges, J. W., and Lock, E. A. (Eds.) *Monographs in Applied Toxicology, Vol. 1, Nephrotoxicity: Assessment and Pathogenesis*, pp. 161–168. John Wiley & Sons, Chichester.

Pharmaceutical Codex (1979). The Pharmaceutical Press, London.

Philpot, R. M., and Wolf, C. R. (1981). The properties and distribution of the enzymes of pulmonary cytochrome P-450 dependent mono-oxygenase systems. In Hodgson, E., Bend, J. R., and Philpot, R. M. (Eds.) *Reviews in Biochemical Toxicology*, Vol. 3, pp. 57–76. Elsevier, Amsterdam.

Rogers, A. W. (1979). *Techniques in Auto-radiography*, 3rd edition. Elsevier/North-Holland, Amsterdam.

Ross, B. D. (1972). *Perfusion Techniques in Biochemistry*. Clarendon Press, Oxford.

Sargent, N. S. E., Upshall, D. J., and Bridges, J. W. (1980). The relationship between chemical structure and the *in vivo* metabolism of an homologous series of *n*-alkyl carbamates. *J. Pharm. Pharmacol.*, **34,** 367–372.

Sargent, N., Upshall, D., and Bridges, J. W. (1981a). Absorption of drugs from the urinary bladder. *Biochem. Soc. Trans.*, **7,** 129–131.

Sargent, N. S. E., Upshall, D. J., and Bridges, J. W. (1981b). The relationship between binding to cytochrome P-450 and metabolism of *n*-alkyl carbamates by isolated rat hepatocytes. *Biochem. Pharmacol.*, **31,** 1309–1313.

Shirkey, R. J., Kao, J., Fry, J. R., and Bridges, J. W. (1979). A comparison of xenobiotic metabolism in cells isolated from rat liver and small intestinal mucosa. *Biochem. Pharmacol.*, **28,** 1461–1466.

Smith, R. L. (1973). *The Excretory Function of Bile*. Chapman and Hall, London.

Smith, S. E., and Rawlins, M. D. (1973). *Variability in Human Drug Response*. Butterworths, London.

Sols, A., and Ponz, F. (1947). A method for the study of intestinal absorption. *Rev. Espan. Fisiol.*, **3,** 207–211.

Stephen, K. W., McCrossan, J., MacKenzie, D., MacFarlane, W., and Spiers, C. F. (1980). Factors determining the passage of drugs from blood into saliva. *Br. J. Clin. Pharmacol.*, **9,** 51–55.

Sturman, A., and Smith, M. A. H. (1967). The binding of salicylate to plasma proteins in different species. *J. Pharm. Pharmacol.*, **19,** 621–623.

Swenson, D. H., Lin, J. K., Miller, E. C., and Miller, J. A. (1977). Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxin B_1 and B_2 to rat liver DNA and ribosomal RNA *in vivo*. *Cancer Res.*, **37,** 172–181.

Tredger, J. M., and Chhabra, R. S. (1976). Preservation of various microsomal drug metabolizing components in tissue preparations from liver, lung and small intestines of rodents. *Drug Metab. Dispos.*, **4,** 451–459.

Treherne, J. E. (1956). The permeability of skin to some non-electrolytes. *J. Physiol.*, **133,** 171–180.

Trump, B. F., Resau, J., and Barrett, L. A. (1980). Methods of organ-culture for human bronchus. *Methods Cell Biol.*, **21,** 1–14.

Ullberg, S., and Larsson, B. (1981). Whole body autoradiography. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 6, 249–289. John Wiley & Sons, Chichester.

Verzar, F., and McDougal, E. J. (1936). *Absorption from the Intestine*. Longmans, London.

Vig, M. M., and Wostmann, B. S. (1979). Exteriorization of the bile duct in germfree rats. *Lab. Anim. Sci.*, **29,** 759–764.

Von Bahr, C., Groth, C. G., Jansson, H., Lundgren, C., Lind, M., and Glaumann, H. (1980). Drug metabolism in human liver *in vitro*: establishment of a human liver bank. *Clin. Pharmacol. Ther.*, **27,** 711–725.

Waites, G. M. H. (1977). Fluid excretion in the testis. In Johnson, A. D., and Gomes, W. R. (Eds.) *Advances in Physiology, Biochemistry and Function, Fluid Excretion*, pp. 91–123. Academic Press, New York.

Watanabe, P. G., Ramsey, J. C., and Gehring, P. J. (1980). Pharmacokinetics and metabolism of industrial chemicals. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 5, pp. 311–343. John Wiley & Sons, Chichester.

Waynforth, H. B. (1980). *Experimental and Surgical Techniques in the Rat*. Academic Press, New York.

Welch, R. M., and Findlay, J. W. A. (1981). Excretion of drugs in human breast milk. *Drug Metab. Rev.*, **12,** 261–277.

Wilson, J. T., Brown, R. D., Cherek, D. R., Dailey, J. W., Hilman, B., Jobe, P. C., Manno, B. R., Manno, J. E., Redetzki, H. M., and Stewart, J. J. (1980). Drug excretion in human breast milk: principles, pharmacokinetics and projected consequences. *Clin. Pharmacokinet.*, **5,** 1–66.

Wilson, T. H., and Wiseman, G. (1954). The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. *J. Physiol.*, **123,** 116–125.

Wolf, P. J. (1980). Effect of overloading pathways in toxicology. *J. Environ. Pathol. Toxicol.*, **3,** 113–134.