4.1

Biotransformation Processes

W. KLEIN and I. SCHEUNERT

Gesellschaft für Strahlen – und Umweltforschung mbH München
Institut für Ökologische Chemie
Ingolstädter Landstr. 1
D-8042 Neuherberg
Federal Republic of Germany

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4.1.1 INTRODUCTION

The transformation of chemicals by animals, plants or microorganisms is based on two major principles (Klein and Scheunert, 1978; Alexander, 1981). In the first case, the chemical is converted to inorganic products, for example carbon dioxide, chloride ion, etc., or to organic low molecular weight fragments joining the natural carbon pool. This conversion (mineralization) is accompanied by an increase in biomass of the respective organisms, indicating that they obtain carbon and energy for biosyntheses from this reaction. However, this kind of biological conversion is limited to a few chemicals and a small number of organisms, mostly microorganisms present in soil, water or sludge. Most chemicals are transformed by microorganisms, animals or higher plants by means of a second mechanism which is called cometabolism; the organisms effect alterations in the xenobiotic molecules but obtain neither carbon nor energy from the reaction which, consequently, does not sustain growth. Since total biodegradation by microorganisms is discussed in Section 4.2, this Section will be concerned mainly with biotransformations by cometabolism; mineralization is included only in so far as necessary for mass balance discussions.
The biochemical transformation of xenobiotics is the result of complex biochemical processes. Reviews on types and pathways of transformation reactions are given in literature (Klein and Scheunert, 1978; Alexander, 1981). The elucidation of transformation pathways, including chemical isolation, identification and quantitative determination of metabolites, is a major objective of environmental research. Methods must be specifically selected for individual chemicals. These procedures cannot be extended to the examination of all chemicals; therefore, comprehensive standardized tests for a variety of chemical classes have been difficult to develop.

Biotic transformation of chemicals is important to structuring of the environment within the ecosystem as well as to the individual toxic response of organisms. The environmental effects of chemicals are mediated by those organisms which occur in large numbers and are active in biochemical transformation processes, for example soil and water microorganisms and higher plants. The biotransformation of chemicals by these organisms may lead to environmental detoxification, the formation of new toxicants, or the biosynthesis of persistent products. In order to assess the potential increase in toxic xenobiotic in the environment, screening methods based on mass balance studies should be used to evaluate total biotransformation.

The toxicological aspects of biotransformation are particularly relevant to man in view of similarities in metabolism amongst mammals and in regard to the role other organisms may play in human nutrition. In these cases, research interest is focused on types and levels of biological conversion products present in the respective organism, without regard to the total mass balance of the xenobiotic in the environment. Such studies include classical in vitro or in vivo studies of animal or plant metabolism (Klein and Schuehert, 1978).

However, in both approaches it is difficult to recognize and quantify biological transformation products, as such, without the chemical identification and quantification of all individual metabolites, which, despite long-term sophisticated research efforts, remain incompletely described. To obtain screening estimates of the total amounts of biotransformation products, indirect methods often provide the greatest economy and utility. The difference between the amount of the parent compound applied and that recovered represents the sum of all metabolites. Other potential sources for loss can be considered as required by the specific investigation. Within the objectives of many investigations, the limiting of quantitative analysis of individual metabolites to those compounds which are either highly toxic or extensively produced may be a satisfactory goal. Such a selection can be based on the results of previous more exhaustive metabolite studies.

This appraisal of biotransformation tests for organic chemicals is not intended to be an extensive review of the subject, therefore references presented are only examples from the numerous publications discussing various aspects of the subject, which include not only methods for screening
chemicals but advanced procedures defining metabolic pathways as well as identifying and quantifying metabolites.

Before experimental test methods for total biotransformation and for residual conversion products in organisms are discussed in detail, a short summary of procedures for the estimation of biochemical reactions from chemical structure characteristics is provided.

### 4.1.2 PREDICTABILITY OF BIOTRANSFORMATION FROM STRUCTURAL CHARACTERISTICS

Long-term experience derived from extensive research work during approximately three decades has resulted in an empirical, mostly qualitative basis for the predictability of biotransformation of chemicals which possess certain structural characteristics. Table 4.1.1 presents some examples for the soil-plant system.

It should be emphasized that, in many cases, biotransformations predicted from an individual substituent will not occur in more complex molecules, due to the interrelations between different elements of chemical structure. Chlorine substitution usually results in a lower biochemical reactivity of the respective carbon atoms. For aldrin, which has both a chlorinated and a non-chlorinated double bond, the non-chlorinated double bond is subjected

<table>
<thead>
<tr>
<th>Structure characteristic</th>
<th>Predicted biotransformation products</th>
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<tr>
<td>$-\text{C}=-\text{C}-$</td>
<td>$-\text{C}=-\text{C};-\text{C}=-\text{C};-\text{COOH}/\text{COOH};$ unextractable residues</td>
</tr>
<tr>
<td>$-\text{C}=\text{O}$</td>
<td>$-\text{COOH};$ unextractable residues</td>
</tr>
<tr>
<td>Aromatic rings</td>
<td>Phenols; phenylmethylene</td>
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<tr>
<td>$-\text{OH}$ (aromatic)</td>
<td>$\text{OCH}_2$; unextractable residues</td>
</tr>
<tr>
<td>$-\text{NH}_2$ (aromatic)</td>
<td>$\text{NHCHO;}\text{NHCOCH}_3;-\text{NO};-\text{NO}_2;-\text{N}=-\text{N}-;$ unextractable residues</td>
</tr>
<tr>
<td>$-\text{N}=-\text{C}=-\text{N}$ (aromatic)</td>
<td>$-\text{NH}_2$ (as intermediate) and resulting secondary products; unextractable residues</td>
</tr>
<tr>
<td>$-\text{Cl}$ (general)</td>
<td>Decreasing biotransformation with increasing substitution</td>
</tr>
<tr>
<td>$-\text{Cl}$ (aromatic)</td>
<td>Reductive dechlorination products; decreasing biotransformation with increasing substitution</td>
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to epoxidation, hydration, and oxidative cleavage in the soil–plant system, whereas the chlorinated double bond is not (Scheunert et al., 1977). Therefore, biotransformation is dependent on the position of chlorine atoms within the molecule; if the positions susceptible to enzymatic attack are substituted with chlorine atoms, the molecule will become less susceptible to biotransformation than its isomers bearing chlorine at non-critical positions. Furthermore, chlorine atoms may also inhibit reactions at other sites of the molecule for stereochemical reasons. Thus, the oxidation of anilines to azobenzenes in soil, which has been shown to occur with lower chlorinated anilines (Kearney et al., 1969; Kearney and Plimmer, 1972; Freitag, 1977; Viswanathan et al., 1978), has not been demonstrated for pentachloroaniline resulting from the application of the fungicide pentachloronitrobenzene to soil (Buser and Bosshardt, 1975). Both effects result in a general decrease of biotransformation with increasing chlorination of the molecule (for PCBs see Scheunert and Klein, 1979).

Establishing quantitative relationships between characteristics of molecular structure and individual biochemical reactions or even total biotransformation has been very difficult since the information available is often obtained under differing experimental conditions, and the data are insufficient to establish descriptive equations. By contrast, for abiotic reactions, e.g. hydrolysis, structure–activity relations may be quantitatively expressed in the form of linear free-energy relationships (Mabey and Mill, 1978; Mill, 1981). These equations may be applied indirectly to biotic processes if a correlation can be found between the rates of abiotic and biotic processes. This was achieved for OH-promoted hydrolysis and enzyme-mediated hydrolysis for diverse chemical structures (Wolfe et al., 1978; Mill, 1981).

In view of our weak understanding of structure–biotransformation relationships, the assessment of biochemical transformation has to be based on experimental procedures. In the following sections, the types of testing procedures presently used are presented and examples are selected for more detailed discussions. It should be mentioned that no test for biotransformation has yet been recognized as a standardized method by any official authority.

### 4.1.3 TESTS OF TOTAL BIOTRANSFORMATION

Tests assessing total biotransformation may be necessary as part of environmental hazard classifications of new chemicals. The amount of new xenobiotics formed by biochemical transformations of the test compound can be estimated by comparing the amount of the chemical applied with the sum of the parent compound recovered and the mineralization products (carbon dioxide) formed. The methods which provide this estimate generally
represent a combination of biomineralization tests—see Section 4.2—and analysis of the non-mineralized compound mixture by chromatographic separation, with the identification and quantitative determination of the parent compound. The technique of $^{14}$C-labelling of the xenobiotic is indispensable to the quantification of the carbon dioxide formed from mineralization of the xenobiotic in the presence of that produced by normal respiration. For microbial systems in soil and water, for higher plants and for plant–soil systems, apparatus have been developed to allow the separate determination of $^{14}$CO$_2$ formed by mineralization and of volatile organic substances. Table 4.1.2 presents some examples for such methods (Kearney and Kontson, 1976; Süß and Eben, 1978; Marinucci and Bartha, 1979; Kloskowski, 1981; Kloskowski et al., 1981; Vockel, 1981). Although most of these methods are aimed primarily at the determination of metabolically released carbon dioxide, the residual radioactivity in the medium may be used for the quantitative determination of the unchanged parent compound. In cases where the volatile organic fraction is significant, the parent compound and conversion products must be separated by chromatographic methods. The total of unchanged parent compound is then calculated as the sum of parent compound in the volatile fraction and in the media.

It is evident that mass balance experiments of this kind are not possible under field conditions. Not only is it generally impossible to determine all the volatile conversion products lost to the atmosphere, but also it is difficult to estimate total conversion rates in soil and plants because of unmeasurable losses due to chemical leaching into deep soil layers or into the ground water.

**Table 4.1.2** Laboratory tests to determine biodegradability and biotransformation of $^{14}$C-labelled chemicals

<table>
<thead>
<tr>
<th>Medium</th>
<th>Principle</th>
<th>References</th>
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<tr>
<td>Soil</td>
<td>Continuous trapping of volatile organic substances and CO$_2$</td>
<td>Kearney and Kontson (1976)</td>
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<tr>
<td>Soil</td>
<td>Discontinuous trapping of volatile organic substances and CO$_2$</td>
<td>Marinucci and Bartha (1979)</td>
</tr>
<tr>
<td>Soil–water suspensions</td>
<td>Discontinuous trapping of volatile organic substances and CO$_2$</td>
<td>Süß and Eben (1978)</td>
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<td></td>
<td></td>
<td>Kloskowski <em>et al.</em> (1981)</td>
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Therefore, field experiments have been restricted to the analysis of residual conversion products in those parts of the ecosystem which are of specific interest, such as edible plants and animals or fertilizer materials, as described in the second part of this chapter.

As an example of a technique for the determination of conversion rate within mass balance studies, Figure 4.1.1 diagrams a type of apparatus which enables within one week the determination of bioconversion or mineralization of chemicals in the plant-soil system (Kloskowski et al., 1981). As compared with other more complex apparatus for the same use, this set-up is relatively small and simple and, due to the short growing time of test plants (barley), procedures are not time-consuming. A desiccator is used as a plant growth chamber. It is connected to an appropriate chemical trapping system. Ten grains of summer barley are sown into 1 kg of soil treated with the $^{14}$C-labelled chemicals at a concentration of approximately 2 p.p.m. (dissolved in a minimum amount of organic solvent). Air is drawn through the system (10 ml/min). The volatile organic substances are absorbed in two special tubes each containing 15 ml of ethyleneglycol monomethyl ether. Carbon dioxide is absorbed in two tubes with 15 ml of a scintillation liquid containing phenethylamine. The air flow is interrupted automatically every 12 hours for 15 minutes in order to dissolve any crystals formed at the insets of the carbon dioxide absorption tubes. After one week, the radioactive residues in plants and soil are analysed, and levels of the parent compound determined. The volatile organic fraction may be analysed also.

### 4.1.4 TESTS OF RESIDUAL CONVERSION PRODUCTS IN ORGANISMS

The biochemical conversion rates of compounds can be assessed in vitro and in vivo, in terms of organ or tissue residues, in individual organisms or in media such as soil or water, containing various living organisms under both laboratory or field conditions. If these studies utilize radiolabelled substances, conversion rates may be determined as the per cent of parent compound to the total radioactivity recovered in the organism; if tissue residue levels are too low for quantitative analysis, similar determinations of labelled compounds in the excreta are often helpful in understanding the biochemical processes in the organism. When non-radiolabelled chemicals are used for such studies, the biochemical conversion rates cannot be established although the recovery of the parent compound, without any identification of the sources of loss, or metabolites produced, can be used to estimate relative transformation. The extrapolation of results from in vitro or in vivo laboratory studies to natural environmental conditions has to be done with considerable caution. As already mentioned, the overall biological conversion rate of the chemical cannot be determined from such experiments as the gas phase processes are not analysed.
4.1.4.1 In vitro studies

In vitro studies may be carried out with tissue homogenates or enzyme fractions of animals (Lay et al., 1974; Reddy and Khan, 1974; Chadwick et al., 1975; Greb et al., 1975) or plants (Lichtenstein and Corbett, 1969; Yu et al., 1971; McKinney and Mehendale, 1973; v.d. Trenck and Sandermann, 1980). Cell cultures or specific tissues or organs of animals and plants may also be used (Feung et al., 1971, 1972, 1973, 1975; Kadunce et al., 1974; Scheel and Sandermann, 1977; Sandermann et al., 1977; Scheel and Sandermann, 1981) in case of human cell culture experiments (e.g. Fox et al., 1975), results are especially relevant to the relationship of chemical toxicity and human health. Plant parts used for metabolic studies consist of epicotyls (Andreae and Good, 1957), coleoptile sections (Klámbr, 1961), or excised leaves (Haque et al., 1974). In animals, since the liver is regarded as the main site of chemical metabolism, experiments are designed to analyse $^{14}$C radioactivity of metabolites in the bile leaving the Ductus hepaticus after intravenous injection of $^{14}$C-labelled compounds (Mörsdorf et al., 1963). The chemical transformation in the liver may also be assessed in this isolated organ with liver-perfusion experiments (Altmeier et al., 1969).

Reasons for choosing in vitro methodologies for testing biotransformation may be summarized as follows:

1. These methodologies are relatively simple as compared to in vivo experiments, and by rapid screening preliminary results can be obtained, which allow the setting of priorities for further testing.
2. It is possible to limit the investigation to specific enzymatic processes of the organism in question and to exclude the influences of other organisms, which often is difficult with whole organism studies. This reasoning applies to higher plants where biotic processes of microorganisms in soil or water can influence plant metabolism, or to mammals where intestinal bacteria may contribute significantly to the overall conversion of ingested compounds.
3. Specific information on mechanisms and sites of the biochemical reactions can be obtained.

However, the interpretation of results from in vitro experiments is complicated by the fact that only a part of an organism is studied, and only a small portion of the biochemical processes which normally occur in the intact organism are assessed. In the organism, transport of the chemical to the site of metabolism may represent a factor limiting the formation of metabolites. For example, the biotransformation of lipophilic chemicals in animals is dependent upon the rate at which they are available to the liver, and if this rate is much slower than the maximal velocity of enzymatic degradation, transport limits the rate of transformation (Walker et al., 1979). Additionally, competitive reactions may reduce or even prevent the reaction observed in
vitro, or the primary product formed by a cell fraction may be altered immediately by secondary reactions. The following examples help to demonstrate some of the problems limiting extrapolation of in vitro experiments to living animals.

For 2,2'-dichlorobiphenyl, all four theoretically possible monohydroxy derivatives and four dihydroxy derivatives have been found in rat cell fractions in vitro (Greb et al., 1975). In vivo experiments, however, only three monohydroxy isomers have been detected, one of which occurred only in very small amounts, and only three dihydroxy isomers isolated (Kamal et al., 1976). The only metabolite formed in vitro from the cyclodiene insecticide (trans)-chlordane was dehydrochlordane (Spitzauer, unpublished), while, in vivo, the epoxide and hydroxylated metabolites were the major conversion products (Schwemmer et al., 1970; Poonawalla and Korte, 1971; Barnett and Dorough, 1974). In human fat, the epoxide has been detected (Biros and Enos, 1973). In screening studies, only the relative rate of metabolism is determined without chemical characterization of metabolites, and the total rate of metabolism for such in vitro studies cannot be extrapolated to the intact organisms.

These examples show that in vitro experiments are suitable for rapid preliminary testing, but that extrapolation of such results to whole organisms should be done with caution, and that the evaluation of their significance should be made only in conjunction with in vivo experiments.

4.1.4.2 In vivo studies

Many of the difficulties encountered in the interpretation of in vitro studies are overcome by employing experimental procedures with intact organisms. These studies may be performed under laboratory or field conditions, with isolated organisms or some portion of the natural community. Ecosystem type studies include all kinds of field studies, but also laboratory studies with soils, higher plants grown in soil, or laboratory microcosms. The conversion rates obtained from organisms living within ecosystems account for the potential influences of the other biota as well as abiotic factors.

Usually, smaller organisms (microorganisms, invertebrates or smaller higher plants) are analysed whole such that the total conversion rate within the organism is obtained. The organism must be completely homogenized for determination of the conversion rate (Freitag et al., 1982). It is self-evident that for large plants (e.g. trees) the total conversion rate can be estimated only roughly, even if many samples of plant parts are analysed, since parent compounds as well as metabolites are heterogeneously distributed within the plant. Studies with higher animals (e.g. see review for PCB by Scheunert and Klein, 1979) are primarily focused on the determination of conversion rates in excreta or individual organs.
4.1.5 PREDICTABILITY OF ENVIRONMENTAL BEHAVIOUR FROM TESTS

It is assumed that results obtained under real field conditions are predictive of chemical behaviour in that environment. For studies under ‘restricted’ open-air conditions (e.g., plants grown outdoors in containers) and for laboratory studies, the extrapolation of results to predict behaviour under natural environmental conditions should be confirmed, if possible, by other experimental procedures, or should be made with caution and a thorough knowledge of the behaviour of similar compounds under natural conditions. Evidence available today seems to indicate that most biotransformation tests are qualitatively predictive of behaviour in the environment, with the exception of some in vivo procedures.

The conversion rates obtained under ‘restricted’ field conditions or in the laboratory may be also quantitatively predictive of transformation processes in the environment under the following conditions:

1. The organisms must be kept under conditions approximating their normal environment and, consequently, must be healthy. The effects of the chemicals on the organisms must correspond to those in the environment, i.e. the concentration of chemicals applied should not exceed that observed under environmental conditions.

2. The experimental time should be sufficiently long to approximate environmental exposures considered. Extrapolation from short-term experiments to long-term behaviour in the environment will give correct results only if the time-dependent kinetics of the conversion reactions are known. If this premise is not assured, short-term laboratory tests can be used only to predict long-term environmental behaviour on the basis of relative ranking of substances.

3. If conditions 1 and 2 have been followed, the conversion rate obtained for a species in the laboratory is predictive of the conversion rate for the same species in its natural environment. However, to assess the environmental relevance of this conversion rate, the species must be representative of the environment, i.e. its frequency of occurrence in the environment or its importance must be great so that the biotransformation within this species is not overwhelmed by competitive reactions within other organisms present in larger numbers or which are significantly more active. Experiments in the laboratory with isolated microbial strains which are relatively uncommon in natural soil or water are cases which violate the above restriction.

In the following paragraphs, an example of methods for assessing the conversion of chemicals in a plant-soil systems presented to evaluate the
predictability of specific test results for quantifying behaviour in the environment (Kloskowski et al., 1981). Quantitative results of laboratory experiments carried out with the plant–soil system as shown in Figure 4.1.1 were compared with those obtained under outdoor conditions (Kohli et al., 1973; Sandrock, 1974; Moza et al., 1976, 1979a, b; Freitag, 1977; Scheunert et al., 1977; Scheunert, unpublished; Fragiadakis et al., 1979). These were shown to be of the same order of magnitude as results from natural field experiments (Scheunert et al., 1977). The experimental times for these methods differed: one week for the laboratory experiments and one vegetation period (about four months) for the outdoor experiments. Therefore, their comparability is limited to a relative ranking of test substances.

Figure 4.1.2 presents the comparison of the conversion of 11 chemicals in terms of percentage of unchanged parent compounds based on the total radioactivity recovered in soil, from the laboratory test system and from outdoor plots. In spite of large differences in experimental time, the conversion rates were within the same order of magnitude and the ranking of substances was similar for laboratory and outdoor experiments.

Figure 4.1.3 shows an analogous comparison of the conversion of the same compounds to bound residues in soil. The longer experimental time in the

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**Figure 4.1.1** Apparatus to determine biodegradability and biotransformation of $^{14}$C-labelled chemicals in the soil–plant system (Kloskowski et al., 1981). 1. Activated charcoal. 2. Ethyleneglycol monomethylether. 3. Empty tube. 4. Phenethylamine-containing scintillation cocktail. 5. Valve. 6. Timer
outdoor tests resulted in a higher percentage of bound residues. However, the rating of substances was fairly comparable between laboratory and field studies.

Figure 4.1.4 presents results of chemical conversion in plants. Between these laboratory and field experiments there was also comparability of relative ranking except in the case of aldrin and hexachlorobenzene.
4.1.6 CONCLUSION

Laboratory and field tests are available for determination of the biological conversion rates of chemicals. No method has been recognized, so far, as a standard test by any official authority. The tests available apply to the overall conversion including volatile products as well as to the residues in those organisms of special interest. Available evidence seems to indicate that conversion products found in in vivo experiments will also occur in nature. Thus, it seems that, at least, metabolic products can be qualitatively predicted in nature. The quantitative prediction of metabolites in the environment must
be otherwise ascertained for all experiments which are not carried out under natural environmental conditions.

In most tests, residual products not identified as either the parent compound or as carbon dioxide are regarded as bioconversion products. However, for non-persistent compounds these products are not exclusively xenobiotic conversion products but may include also ‘bound’ mineralization products, for example carbonates or assimilation products formed from the carbon dioxide resulting from total degradation of the xenobiotics. These ‘natural’ substances cannot be separated from the xenobiotic residues by simple standard tests. More complex procedures are needed to perform this differentiation. Thus, the determination of total biotic transformation of
chemicals remains a difficult problem. We must generally rely upon the results from available laboratory and simplified field methods, but must also keep in mind the potential gaps and deficiencies in extrapolating such test results to predict transformation behaviour in natural ecosystems.

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