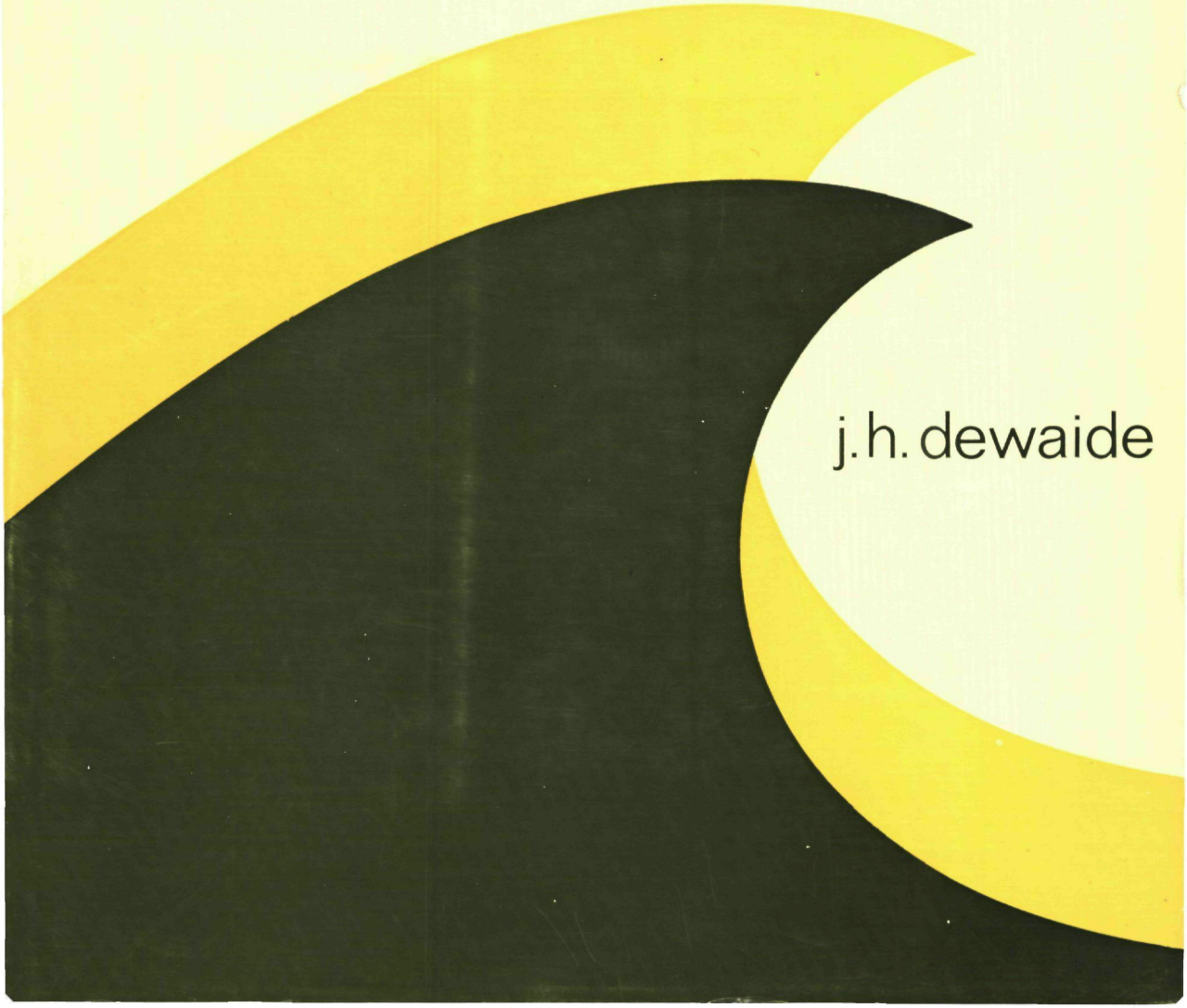
# METABOLISM OF XENOBIOTICS

## comparative and kinetic studies as a basis for environmental pharmacology



j.h. dewaide

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# METABOLISM OF XENOBIOTICS

## COMPARATIVE AND KINETIC STUDIES AS A BASIS FOR ENVIRONMENTAL PHARMACOLOGY

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE
KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG
VAN DE RECTOR MAGNIFICUS MR. W. C. L. VAN DER GRINTEN,
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VOLGENS BESLUIT VAN DE SENAAT IN HET OPENBAAR
TE VERDEDIGEN OP DONDERDAG 22 APRIL 1971,
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JOHANNES HENDRIKUS DEWAIDE

GEBOREN TE GELEEN

DRUKKERIJ LEIJN, NIJMEGEN, 1971

# PREFACE

During the last decades man has become more and more conscious that the use of his power to alter the appearance of his planet and to control life processes on it, has conjured up a number of unforeseen reactions which have become a substantial threat to various forms of life and, finally, to himself also.

As successful consequences of mankind's management in developed and thriving countries the increasing human population with a longer expectation of life, the expanded production of food by improved agriculture and cattle-breeding, and the effective preservation and distribution of nutritives by the food-processing industry and transport facilities can be mentioned.

However, the advances in science and technology do not produce merely beneficial effects. For instance, beside social and psychological problems with which man is confronted as a consequence of overpopulation and urbanization, detrimental effects of several factors on the various parts of the biosphere (air, soil, water, and biological objects) become manifest.

These factors may be divided in 4 categories:

*mechanical factors,* for instance, changes in nature by projects with reference to traffic, water-engineering, agriculture, and recreation, by dust formation, and accumulation of waste products such as rubbish, plastics, wrecks of cars, and refrigerators;

*physical factors,* for instance, noise, radioactive radiation, and thermal pollution of surface water;

*chemical factors,* for instance, stench, improper sewage disposal, combustion products of industrial furnaces, domestic incinerators, and internal-combustion engines, and extensive application of detergents and biocides;

*biological factors,* for instance, the introduction of foreign plant and animal species into certain areas, and the creation of monocultures.

These factors may be a source of hazard for public health. It will be obvious, therefore, that man ought to be concerned about the safety of the biosphere, the environment of living organisms. The awakening to a collective responsibility in order to maintain a habitable milieu has to result in actual practice of environmental hygiene. The intervention in natural processes in the future should be based on an orderly, well-considered development and application of the requisite knowledge. In this connection, the present study has to be considered as an approach from the viewpoint of pharmacology to one of the categories of the environmental problem outlined above: the pollution of the biosphere with chemical substances.

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This investigation was carried out in the Institute of Pharmacology, University of Nijmegen, The Netherlands.

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| AAP | 4-aminoantipyrine (4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) |
| DDT | 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane |
| DMAP | 4-dimethylaminoantipyrine, aminopyrine (4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediamine tetraacetate |
| G-6-P | glucose-6-phosphate |
| G-6-PD | glucose-6-phosphate dehydrogenase |
| ICD | isocitrate dehydrogenase |
| MMAP | 4-monomethylaminoantipyrine (4-monomethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) |
| NADH | nicotinamide-adenine dinucleotide in reduced form |
| NADP | nicotinamide-adenine dinucleotide phosphate in oxidized form |
| NADPH | nicotinamide-adenine dinucleotide phosphate in reduced form |
| SKF 525A | $\beta$-diethylaminoethyl diphenylpropylacetate |
| Tris | tris(hydroxymethyl)aminomethane |

# INTRODUCTION

## I, 1. ENVIRONMENTAL PHARMACOLOGY

The biosphere into which man introduces increasingly — both in quantity and variety — chemical substances, has a finite ability to dilute out the concentrations of the compounds to negligible levels. This may be elucidated by figure 1, in which the biosphere is divided schematically in 4 compart-

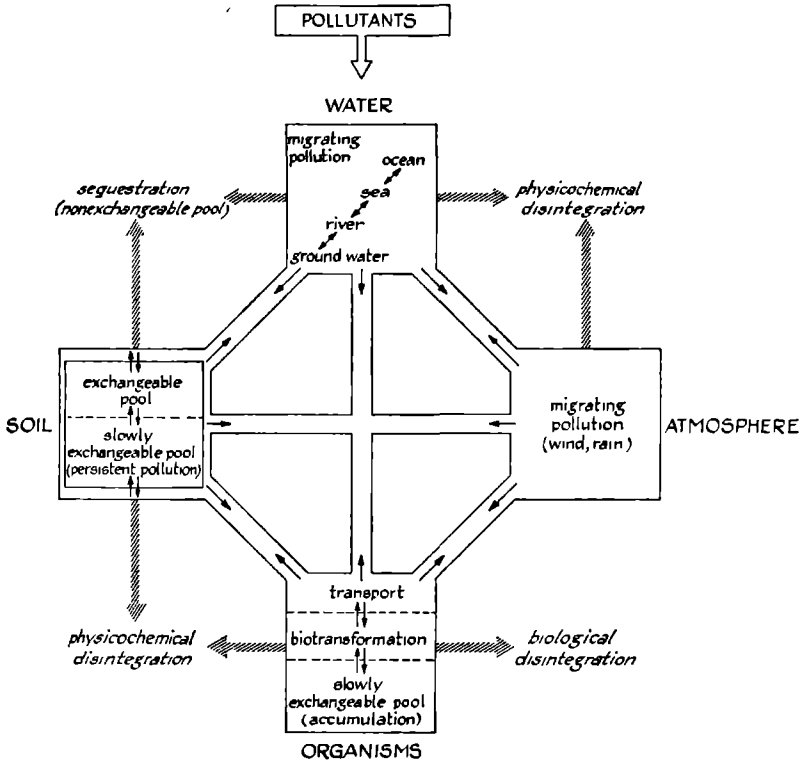



FIGURE 1. Schematic representation of some aspects of the fate of chemical pollutants in the biosphere.

ments, viz. air, soil, water, and biological objects, forming together a closed system. After being introduced into one of the compartments a chemical compound can move to other parts of the biosphere. The actual fate of a compound is determined by its physicochemical properties — especially in relation to its distribution over the various compartments — and by the processes occurring in the various compartments, especially the processes leading to elimination of the substance. Important routes for elimination of chemicals are:

*physicochemical disintegration,* for instance, the decay of radioactive compounds, and the photochemical decomposition of certain compounds;

*sequestration,* processes in which compounds precipitate in the biosphere by being bound to other substances;

*biotransformation,* processes in which compounds are converted by enzymatic reactions.

In view of the fact that some of the chemical pollutants are eliminated at very low rates, the biosphere is becoming loaded with persistent and potentially harmful materials. Due to their stability and relatively high lipid-solubility, certain compounds will accumulate in the various forms of life, the *biomasses*. The environmental fate of DDT-like compounds exemplifies this (figure 2). It is well-known that a long-term exposure, even to low concentrations of these compounds, via long-term accumulation, can lead to dramatic effects on a certain link of a food chain (cf. Dustman and Stickel, 1969).

Relevant information on the interaction between biological systems and alien chemical compounds and the effects of this interaction, can be obtained from pharmacology. Modern pharmacology is a multidisciplinary science which is neither restricted to drugs with a therapeutic or toxic action, nor to man as a biological object. It includes all living organisms and the multitude of chemical compounds in their environment. The chemical substances dealt with in pharmacology are commonly indicated by the terms *drug, pharmacon,* or *xenobiotic.* At the present time the word *drug* is mainly used to indicate the substances which are employed as therapeutics, whereas nowadays in the public conception the significance of the term has gradually shifted to the group of psychoactive compounds. The word *pharmacon* is used in a broader sense, indicating any bioactive nonnutrient agent, independent of whether it has a therapeutic (medicaments) or toxic (pesticides) action. According to this definition, substances which can be considered as normal food components do not belong to the class of pharmaca. The word *xenobiotic* is the most general term: it refers to the substances grouped together with the only criterion that they are alien to

living organisms (*foreign to the body*). Bioactive properties are of secondary importance. The indication *alien to living organisms* does not strictly mean foreign to all organisms, since various substances which are synthesized in certain plant species can be considered to be xenobiotics for animals. More-

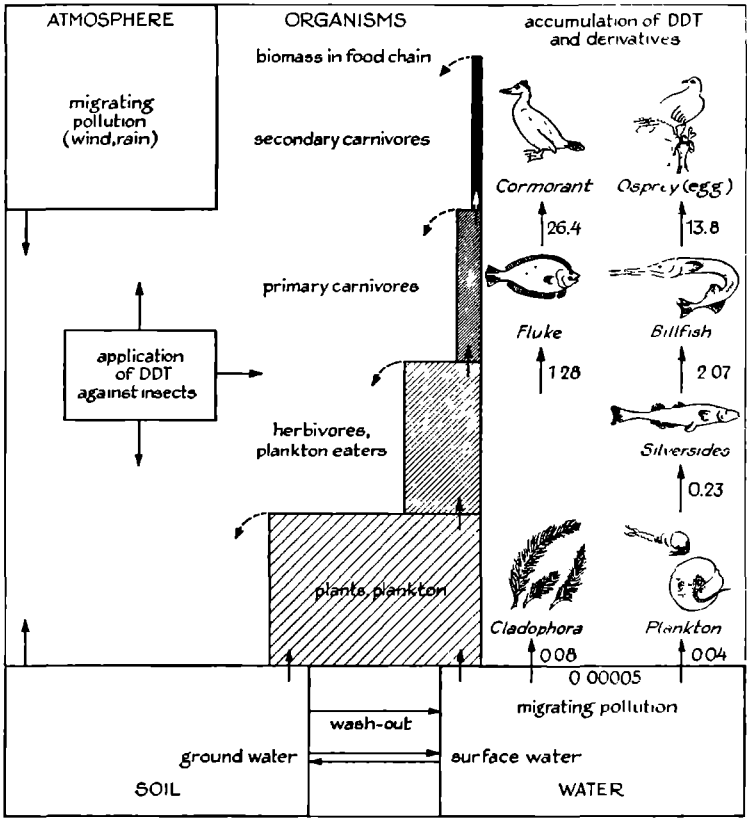



FIGURE 2. Schematic representation of the accumulation of DDT and its degradation product DDE (ppm) along food chains. The surface of the squares represents the size of the biomasses involved; the degree of shadowing represents the concentration of DDT and its product (Modified after Woodwell, 1967).

over, *foreign to the body* is not identical with *unfit for consumption*, since various xenobiotics — for instance certain detergents — can be used as food by various microorganisms. In view of the fact that in most of the literature no distinction is made between the terms drug, pharmacon, and xenobiotic, in this report, too, no distinction will be made between them. When the terms

drug or pharmacon are used they are meant to indicate xenobiotic substances as defined above.

It seems adequate to speak of *environmental pharmacology* with regard to that branch of pharmacology which deals with the exposure of biological objects to chemicals that are contaminants of their environment viz. food, air, soil, and water. In this field of study several aspects can be recognized, as for instance the problems relating to the flows of xenobiotics in nature, to and through the various compartments including the different forms of life, the biomasses. The study of these problems, dealing with the time-relationship in the distribution of the substances over the various compartments of the biosphere, can be described as *environmental pharmacokinetics* (Ariëns, 1969a and 1969b). Further, factors which control the concentrations of foreign compounds in nature, for instance, the degradation of the substances can be mentioned. In a number of cases the degradation of xenobiotics is performed by the biomasses themselves. This phenomenon is indicated with the terms *biological self-clearance* or *biological self-purification.*

Studies in the field of environmental pharmacology do not only deal with individual organisms but also with populations in relation to their environment, and consequently depend upon ecological knowledge. On the other hand, a rational approach to environmental pharmacology implies a study of the mode of action on, and the fate of foreign chemicals in, particular organisms and species, as for instance differences in the capability of metabolizing xenobiotics in different species. Pharmacological studies borrow freely from the knowledge of several of the basic sciences, such as biology, chemistry, and physics, and in a more specialized sense from biochemistry, physiology, and cytology. It will be clear that environmental pharmacology, too, is based upon these sciences and upon the specialized fields of general ecology, hydrobiology, geochemistry, meteorology, and climatology.

In conclusion, the fact that all forms of life are exposed to chemicals that are foreign to them, the fact that the number and variety of chemicals continuously increase, the fact that such chemicals can, potentially, produce harmful effects on individual organisms and consequently on whole populations, and the requirement of the contribution by a great variety of sciences in order to rationally approach the problem outlined, indicate the vast scope of study of environmental pharmacology.

In the study described here an effort has been made to investigate some particular aspects of environmental pharmacology. Firstly, attention is directed to factors playing a role in the disposal of xenobiotics from several vertebrates, especially aquatic species. Emphasis is put upon the differences in the capacity of different species to metabolize xenobiotics.

Secondly, attention is paid to some general phenomena which can be observed in the environmental pharmacokinetics of chemical pollutants. Emphasis is put upon the factors which determine the level of accumulation of pollutants in ecosystems.

## I, 2. BIOTRANSFORMATION OF XENOBIOTICS IN HIGHER ORGANISMS

In studies dealing with the action of drugs in organisms knowledge of the factors which control the concentrations of these compounds in the compartment in which the sites of action (*receptors*) are located — which can be defined as the concentrations in the biophase — is indispensable (figure 3). The concentration of a drug in the biophase is determined mainly by the

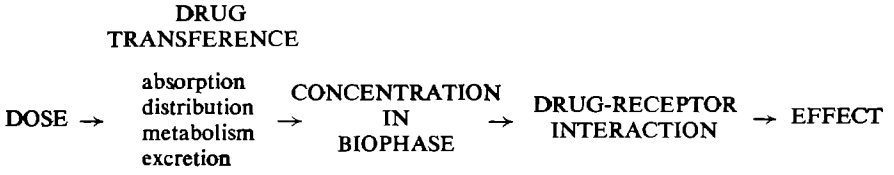



Figure 3. Schematic representation of the sequence of events occurring between the exposure to a drug and its effect. The biophase is the compartment in which the sites of action (receptors) are located.

relative rates of absorption, distribution — including factors such as transport across membranes and protein binding — and the rate of elimination. The rate of elimination of most drugs from the organism is controlled by the speed at which they are metabolized and excreted. It is known that the usual result of the metabolism of drugs *in vivo* is the formation of derivatives which are less active pharmacologically than the parent molecules. In general it may be stated that, due to the lipoidal character of most cellular and subcellular barriers across which drugs must pass in order to gain access to their sites of action, penetration is dependent upon the lipid-solubility of the drugs. Therefore, the observed reduction of drug activity is, at least in part, due to a decrease in the concentration of the relatively lipophilic drugs by enzymatic conversion to more hydrophilic metabolites which are less capable of crossing biological membranes to reach their sites of action.

A general trend of the processes involved in the metabolism of xenobiotics in vertebrate animals is the conversion to more polar, more water-soluble, substances. As a result, the distribution of the hydrophilic products in the body is more restricted to the extracellular fluid as compared with the lipophilic parent compounds. In this way the substances are put to a higher concentration at disposal of the excretory mechanisms. Moreover, the less the

degree of lipophility of a compound, the more rapidly it can be excreted by the kidney.

It should be emphasized that drug metabolism is not strictly identical with detoxication, since some chemical compounds are transformed in the organism to pharmacologically more active — in certain cases toxic — substances (Axelrod, 1965). Xenobiotics can be transformed in the animal body by a wide variety of metabolic changes. According to whether the products of the metabolic conversions are less active or more active than the parent compounds, the terms *bioinactivation* or *bioactivation* are applied. The formation of active or inactive metabolites will usually depend on the chemical structure of the parent compound as well as on the type of chemical reaction.

The biotransformation of xenobiotics can be broadly classified into oxidations, reductions, hydrolyses and syntheses. The literature in this field is extensive (Brodie *et al.*, 1958; Williams, 1959; Shideman and Mannering, 1963; Ariëns and Simonis, 1964; Remmer, 1965; Uehleke, 1965; Gillette, 1966; Dutton, 1966). Interest, however, has been so far nearly exclusively devoted to certain aspects of drug metabolism in mammals. Examples are: the mechanisms of various biotransformation reactions and the enzymes involved, and the influence on drug-metabolizing enzymatic activity of a number of factors which include genetic properties, age, hormonal changes in the body, and of environmental factors such as temperature, stress, diet and the administration of certain xenobiotics. In these studies it is common practice to investigate the various aspects of drug metabolism in small experimental animals, such as rat, mouse, rabbit, and guinea pig. Studies of drug metabolism in other vertebrates such as birds and reptiles are much less frequent, and for aquatic species and invertebrates other than insects, little or no information is available (cf. Smith, 1964; Symposium Comparative Patterns of Drug Metabolism, 1967). If one considers the connection and interdependency of the various forms of life, and the important role of drug metabolism with regard to the biological activity of xenobiotics and their fate in biological objects — especially in relation to the level of accumulation — it will be clear that knowledge of the quantitative and qualitative differences in the drug-metabolizing capacities of many more animal and plant species is essential for understanding the selective influence of drugs and poisons on organisms and therefore on populations. From a more practical point of view it will be obvious that a rational development and application of selective therapeutics and pesticides and prevention of persistent environmental pollution will only be possible with knowledge of the differences in the patterns of drug metabolism in various organisms.

Up to a short time ago it was a general idea that aquatic vertebrates (fishes, tadpoles of frogs and toads, and certain frogs), in contrast to terrestrial animals, were not provided with special mechanisms for detoxication of lipid-soluble substances, such as oxidative biotransformation or conjugation with glucuronic acid for instance (Brodie *et al.*, 1958; Brodie and Maickel, 1962). Lipid-soluble compounds would present no problem for water-dwelling animals since, because of the lipoidal character of gills and/or skin, they would readily diffuse from the body into a large volume of water: a continuous exchange (counter-current system) between gill and the infinite volume of environmental water. However, it has been totally overlooked in this theory, that not only mammals but fishes, too, will profit by the metabolic conversion of xenobiotics: the products are preferentially kept in the extracellular phase, which favours excretion.

On the other hand, lipophilic substances will easily penetrate, and possibly accumulate, in aquatic animals. This, at least in part, may explain the extreme sensitivity of amphibia and fishes to contamination of the surrounding water with lipophilic pesticides and other poisons. It is a well-known fact that lipid-soluble compounds from food or water strongly accumulate in aquatic animals (Allison *et al.*, 1964; Levy and Miller, 1965; Cope, 1966; Ferguson *et al.*, 1966; Holden, 1966; Mount and Putnichi, 1966; Koeman *et al.*, 1969a). This may result in the appearance of tumors and other diseases (Ashley *et al.*, 1964; Stanton, 1965; Sinhuber *et al.*, 1968), and often more dramatically in mortality on a large scale (Thiodan contamination of the Rhine, june 1969). Aquatic life finds itself in a vulnerable position because it is becoming increasingly exposed to industrial wastes, chemicals and pesticides in streams, rivers, lakes, and oceans, which communicate together over the earth and through which a distribution of especially the highly persistent chemicals can take place in an almost unlimited way. Not only are chlorinated hydrocarbon pesticide residues found in fish living in streams remote from the place of application, but they have also been found in penguins and seals from the Ross Sea area of Antarctica (Sladen *et al.*, 1966).

The aquatic populations consist of innumerable species of organisms. Fish alone comprise more than half the vertebrate animal species on earth. They frequently represent links in various food chains and are also of great concern to man as important sources of protein for food. From studies on the disposition of xenobiotic compounds in various aquatic species more basic knowledge of comparative physiology and pharmacology can be

obtained. These studies may also shed further light on the evolution of enzymes that metabolize drugs, on drug-metabolizing pathways and drug excretion in lower species.
If one considers the preceding remarks it will be obvious that many reasons indicate the necessity of a study of the disposition of xenobiotics from aquatic animals.

## I, 3. ENVIRONMENTAL PHARMACOKINETICS

Up to the present, the study of pharmacokinetics has been restricted to individuals, man or animals, as objects of study. It has been attempted in these investigations to understand aspects related to the distribution of drugs over the various compartments of the body and to describe these events mathematically. Attention is directed to plasma and tissue concentrations, levels of accumulation, and the half-life time of drugs. In relation to these aspects the rates of uptake, degradation and excretion of the compounds play a decisive role. With respect to the fate of drugs in individuals and that of chemical pollutants in the biosphere, which can also be considered as a multicompartment system, to a certain degree a parallelism can be observed. In this connection it is worthwhile to investigate whether the knowledge obtained in the study of individual pharmacokinetics can be used in order to understand the processes which determine the fate of chemicals in the biosphere.

## I, 4. THE AIM OF THIS INVESTIGATION

In the foregoing discussion the motives starting research from the viewpoint of pharmacology with reference to the contamination of the environment with xenobiotics, have been outlined. In the present study the problem was attacked from two different approaches.
In the first part of this study the metabolic conversion of xenobiotics in some freshwater fishes took the central position. Comparatively, the study has also been extended to certain homeothermic animal species. As a general parameter for the drug-metabolizing capacity of the animal species studied it was decided to take the enzymatic drug-metabolizing capacity of the liver, since this organ appeared to play the principal role in drug metabolism (see chapters II, 3 and VI, 2). The measurements were performed *in vitro*. Further the question was put whether the enzymatic drug-metabolizing capacity of fish is influenced by the same factors which affect hepatic drug metabolism in mammals, as for instance age, sex, and the administration of other xenobiotics. Enhancement of drug metabolism under the charge

of xenobiotics will be important for fishes in giving them a certain degree of protection against water pollution: enhancement of processes involved in drug elimination counteracts the accumulation of xenobiotics.
The purpose of the second part of the study was to demonstrate the utility of mathematical models which describe aspects of distribution of xenobiotics over the various compartments of ecosystems. Emphasis was put upon the shifts in concentration, the rates of elimination, and the levels of accumulation. The mathematical models were tested in the laboratory with an experimental system consisting of a relatively simple ecosystem: activated sludge from a sewage purification station.

The structure of this thesis can be outlined as follows:

Chapter II, an introduction to certain aspects of the biotransformation of xenobiotics in vertebrates, such as the enzymatic reactions involved and their specificity; a discussion of the factors which can affect the metabolism of xenobiotics, and of the comparative studies on drug metabolism in different animal species.

Chapter III, a description of the materials, animals, and methods used in this study on comparative drug metabolism.

Chapter IV, a description of the experiments performed in order to develop methods for the *in vitro* assay of the enzymes that catalyze the oxidative *N*-demethylation, the aromatic hydroxylation, and the transfer of glucuronic acid, the final step in glucuronidation. Emphasis is put on the differences in the nature of enzymes of different species, which necessitate adapted conditions for correct *in vitro* assays.

Chapter V, a start to a characterization of the mechanism of oxidative *N*-demethylation of aminopyrine *in vitro*.

Chapter VI, a comparison of the drug-metabolizing enzymatic activities of different species, in particular in species of fish.

Chapter VII, a report of the study of the influence of external factors on the level of hepatic drug metabolism in fish. Attention is directed to the influence of seasonal changes, of different ambient temperature, of alterations of the aquatic environment, and of treatment with xenobiotic agents.

Chapter VIII, a discussion of the fate of chemical pollutants in the biosphere. Some mathematical models are presented for a description of the kinetics of elimination and of accumulation of pollutants in ecosystems. An effort is made to fit some experimental data by the mathematical models described.

# SOME ASPECTS OF THE ENZYMATIC CONVERSION OF XENOBIOTIC CHEMICALS IN VERTEBRATES REVIEW OF THE LITERATURE

## II, 1. INTRODUCTION

The pharmacological effect produced by a drug depends on a number of events and factors of which the most important ones are schematically represented in figure 3 (chapter I, 2). In this scheme the concentration of the drug in the biophase is indicated as a factor influencing the intensity of drug action. The concentration in the biophase depends on the dose administered, the route of administration, and on several other processes indicated with the term *drug transference* (Ariëns, 1964). In this connection the absorption, distribution, metabolism, and elimination of the drug can be mentioned.

In this chapter attention will be focused on one important factor, the metabolic conversion of xenobiotics. Of the processes involved attention will be particularly focused on some catabolic reactions leading to the elimination of the drug and these will be reviewed. Anabolic processes in which assimilation — incorporation of xenobiotics into body constituents — takes place, will not be discussed extensively. Such an assimilation only occurs when the enzymes involved in intermediary anabolism cannot distinguish the xenobiotic compounds from the endogenous substrates, because of a close resemblance in physicochemical properties. In that case the drug may substitute for the natural metabolite both in a morphological as well as in a functional sense, or lethal synthesis may occur. Such a drug is indicated with the term *parametabolite*. When the drug substitutes for the natural metabolite only in a morphological sense with, as a result, a blockage of the normal biological process, it is called an *antimetabolite*. With respect to the fate of anti- and parametabolites in the body, reference may be made to the review of Ariëns and Simonis (1964). The following discussion will be restricted to the enzymatic conversion of xenobiotics with physicochemical properties rather different from the natural body constituents.

Most of the xenobiotic substances, when taken up into the body, undergo chemical transformation before they are eliminated into air, bile or urine. Due to the biochemical changes the products may differ not only chemically but, dependent on the events and factors outlined above, also pharmacolo-

gically from the administered compounds. The products may be less active than the parent compound or inactive; they can be equally active or more active. The bioactivity of the products usually does not primarily depend on the type of metabolic conversion involved but on their chemical structure and properties. From the viewpoint of detoxication generally, the metabolism of drugs in the body still will be of advantage for the organism since the products are less lipid-soluble in character than the parent drugs. The profits of a decrease in the lipid-solubility of xenobiotics for the organism can be explained by a teleological consideration of phenomena involved in distribution and excretion. The decrease in the lipophility of xenobiotic compounds as a result of the drug-metabolizing reactions causes a change in the distribution of the products over the various compartments of the body: hydrophilic substances are more restricted to the extracellular phase and can be offered so in a higher concentration to excretory mechanisms. The hydrophilization may also cause a decrease in the extent and firmness with which the substances are reversibly bound to proteins or other body constituents, or stored in adipose tissue. Thus again a substantial reduction of the volume of distribution occurs, whereas a decrease in the binding of drugs to blood proteins is directly in favour of excretory mechanisms based on ultrafiltration. Another important factor is that the kidney is poorly equiped to excrete lipid-soluble substances. Due to the lipoidal properties of the boundary between the renal tubuli and the blood capillaries — which consists of highly packed epithelial cells — lipid-soluble compounds would diffuse back into the plasma from the tubular compartment. Therefore, the reduction in the lipophilic character of the xenobiotics by the metabolic reactions results in a reduction of the back-resorption from the renal tubuli and therefore in a better fixation of the ultrafiltrated substances in the urine.

The phenomena described here can be considered as a serious objection against the hypothesis that aquatic animals would have no need for drug-metabolizing processes (Brodie *et al.*, 1958; Brodie and Maickel, 1962). Many xenobiotics would persist for too long a time in the body with as a result of prolonged exposure, accumulation, if the organisms did not have the ability to convert them into more polar derivatives. Since the products are kept in the extracellular phase, they can be excreted more rapidly by the kidney and/or gills.

## II, 2. THE GENERAL PATTERN OF THE METABOLISM OF XENOBIOTICS

The general pattern of the metabolic conversion of xenobiotics can be seen in the scheme in figure 4.

From the viewpoint of toxicity, the oxidative, reductive, and hydrolytic reactions can lead to less active (detoxication) or more active (toxication) products, which generally have a more hydrophilic character and can possibly be eliminated by the kidney or gill. Furthermore, the oxidative, reductive, and hydrolytic reactions lead to the introduction of groups into the xenobiotic, which are suitable for conjugation of the compound with physiologically occurring substances. The products of the conjugative reactions are always nontoxic and can be easily excreted by kidney or bile.

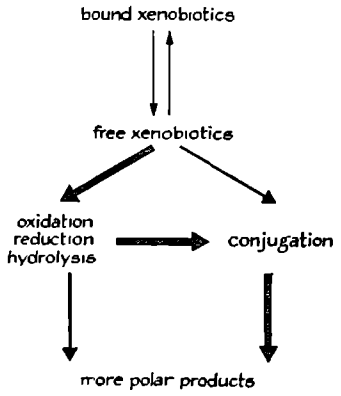



FIGURE 4. Schematic representation of the metabolism of xenobiotics. Lipophilic xenobiotics are transformed by enzymatic reactions into more water-soluble products.

The predominant pathways in the conversion of xenobiotics are the oxidative reactions. They include aromatic hydroxylation, *O*-, *N*-, and *S*-dealkylation, oxidation of alkyl chains, *S*-oxidation, oxidative deamination, aromatization, and epoxidation. Oxidation of xenobiotics will take the central position in this discussion.

For some xenobiotics the organism makes use of reductive reactions, such as the reduction of keto and nitro groups, and the reductive splitting of the azo-configuration, e.g. the conversion of prontosil (4'-sulfamyl-2, 4-diamino-azobenzene) into sulfanilamide (*p*-aminobenzenesulfonamide).

Enzymatic hydrolysis takes place in the case of some esters, and amides.

The conjugation of xenobiotics includes the synthesis of glucuronides, ethereal sulfates, mercapturic acids, amino acid conjugates, and acetylated aromatic amines. The methylation of nitrogen in heterocyclic rings, for instance in

nicotinamide, and of phenolic OH-groups in catecholamines also belong to synthetic reactions.
A detailed survey of the reactions involved in the metabolism and detoxication of xenobiotics has been given by Williams (1959).

## II, 3. LOCALIZATION OF THE ENZYME SYSTEMS INVOLVED IN THE CONVERSION OF XENOBIOTICS

In the metabolism of xenobiotics the liver plays the principal role. However, minor amounts of the drug-metabolizing enzymes have also been found in other tissues, such as kidney (Gelboin and Blackburn, 1964; Kato, 1966; Uehleke and Greim, 1968), lung, (Gelboin and Blackburn, 1964; Kato, 1966; Uehleke, 1968), gastrointestinal tract (Uehleke, 1968; Wattenberg *et al.*, 1962), placenta (Dixon and Willson, 1968; van Petten *et al.*, 1968), adrenal (Kupfer *et al.*, 1969), heart (Kato, 1966), spleen (Heinze and Kiese, 1968), pancreas (Heinze and Kiese, 1968), brain (Kato, 1966; Uehleke, 1968), testis and thyroid (Wattenberg and Leong, 1962) and muscle (Kato, 1966). Biotransformation of xenobiotics in nonhepatic tissues cannot be neglected, since compounds that did not yet reach, or that escaped the metabolic conversion by the liver can be altered near a possible site of action. For instance, the drug-metabolizing enzymes present in the gastrointestinal tract, lung, or skin may be of particular importance with regard to the direct contact of animals with foreign chemicals in the environment. Generally, the formation of toxic or nontoxic metabolites within the cells of nonhepatic tissue may be of importance with respect to, for instance, general and local toxicity, allergy, and chemical carcinogenesis.
As far as the intracellular localization of the drug-metabolizing enzyme systems is concerned extensive studies have been carried out with liver tissue. Almost all drug-metabolizing enzymes appear to be bound to the membranes of the endoplasmic reticulum. When the liver is homogenized, the disintegrated membranes of the endoplasmic reticulum form small vesicles. They can be isolated by centrifugation of the 9000 *g* supernatant of the homogenate at 105000 *g*. The sediment thus obtained is generally described as the microsomal fraction. This fraction also contains ribosomes which are either attached to a portion of membrane or exist freely. From a morphological point of view the subcellular fraction, which was indicated for the first time with the term *microsomes* by Claude (1943), must be regarded as an artefact. The composition of the microsomal fraction is closely related to the isolation procedure and varies according to the tissue of origin (Dallner and Ernster, 1968). Under the electron microscope that part of the membranes which carries

ribosomes, the so-called rough endoplasmic reticulum, can be distinguished from the membranes without ribosomes, the smooth endoplasmic reticulum. Microsomes therefore can be divided in two subfractions. The enzymes which oxidize xenobiotics are present in both subfractions. There are indications that they are more abundantly present in the subfraction containing the smooth membranes than in that containing the rough membranes (Gillette, 1966; Remmer, 1965; Holtzman *et al.*, 1968). Studies of Dallman *et al.* (1969) also suggest a nonrandom distribution of the enzymes in the membranes of the endoplasmic reticulum. NADPH-cytochrome c reductase and cytochrome P-450, involved in most of the drug-metabolizing reactions, are localized in a microsomal subfraction different from that containing NADH-ferricyanide reductase, NADH-cytochrome c reductase, and cytochrome $b_5$, which mainly function in intermediary reactions. In order to study the properties of the drug-metabolizing enzymes, in the past many attempts have been made to solubilize the enzymes. Separation of the protein fraction from the lipid part of the microsomal membranes has been performed, for instance, by treatment with phospholipase (Lumper *et al.*, 1969) and with deoxycholate (Gillette *et al.*, 1957; Ernster *et al.*, 1962). After solubilization, differences in the properties of the enzymes have been observed. Mostly, reduction or complete disappearance of enzymatic activity occurred for the structurally bound enzymes, e.g. the oxidative drug-metabolizing enzymes. Other microsomal enzymes which can be more easily solubilized, such as the enzymes involved in hydrolytic and conjugative reactions show after solubilization a similar or increased activity (Krisch, 1963; Henderson, 1970). From the alterations in the properties of drug-metabolizing enzymes when forced out of their natural relation with lipids, it appears to be essential for them to exist and to operate in special structures.

Since the mechanism of the drug-metabolizing reactions is rather complex, a spatial organization of the various components involved in the microsomal reactions is quite conceivable. Any change in the microenvironment of the enzyme proteins consequently may lead to a change in their activity.

## II, 4. SPECIFICITY OF THE DRUG-METABOLIZING ENZYMES

It has already been mentioned that foreign organic compounds may be metabolized in animal species if the substances are sufficiently like the natural metabolites to fit into the normal metabolic system. The metabolism of many foreign compounds probably occurs in this manner.

In the case of the microsomal metabolism of substances which the body has never "seen" before, questions arise about the specificity of the enzyme

systems. The enzymes of intermediary metabolism have a high specificity towards natural substrates so that they can achieve an effective organization of the cellular biochemical processes. In contrast, an attack on the xenobiotics, which may enter the organism in a great variety, requires a rather low discrimination by the enzymes in their acceptance of substrates. Therefore, the metabolic attack on xenobiotics by means of the already mentioned kind of reactions depends largely on the recognition of the presence of a vulnerable site in the molecule. The nature of the rest of the compound will have consequences almost solely for the processes of distribution, and to some degree, for the affinity between enzyme and substrate.

Gaudette and Brodie (1959) and McMahon (1961) presented experimental evidence that an important requirement for the substrate is that it be lipid-soluble. This proposal was particularly attractive in view of the lipoidal character of the membranes of the endoplasmic reticulum. It provided an explanation of why the oxidative enzymes have a predilection for foreign lipophilic compounds, while naturally occurring hydrophilic substances (e.g. sugars, amino acids) are not attacked. Dealkylation of normally occurring substrates such as sarcosine and dimethylglycine, and hydroxylation of L-tryptophan, L-phenylalanine, kynurenin, and phenylacetic acid, all poorly lipid-soluble substances, are not catalyzed by the microsomal enzymes, but by quite different selective enzyme systems in other parts of the cell, e.g. in mitochondria (Mackenzie *et al.*, 1953). Although there is little doubt that lipid-solubility is an important factor in controlling the rate of drug-metabolizing reactions, other physicochemical factors, such as the sterical structure and the charge distribution over the molecule, most certainly are also involved.

It can be asked whether the metabolism of xenobiotics is a special function rather than an involvement of the enzymes of physiological lipophilic substrates. It has been shown that many of the normal lipophilic body constituents are metabolized by NADPH-dependent enzymes in liver microsomes. For instance, it is probable that the enzyme systems which metabolize steroid hormones, e.g. estrogens, androgens, and glucocorticoids, convert barbiturates and other xenobiotics as well (Kuntzman *et al.*, 1964 and 1966; Conney *et al.*, 1965). Further the possible participation of the microsomal oxidative enzymes in ω-oxidation of fatty acids (Wada *et al.*, 1968a), in the formation of unsaturated fatty acids (Marsh and James, 1962) and bile acids (Wada *et al.*, 1968b) can also be mentioned. The enzyme system, involved in the formation of glucuronides seems to be also involved in the normal formation of bilirubine glucuronide (Schmid *et al.*, 1957).

Brodie and Maickel (1962) suggest that the properties of the drug-metabolizing

enzyme systems in microsomes fit those postulated for systems evolved to dispose of naturally occurring lipid-soluble, unwanted substances in the food, e.g. alkaloids, steroids, and terpenes. The data showing similarities between the microsomal metabolism of xenobiotics and of normal body substrates suggest that both enzyme systems if not identical, may have evolved from the same origin. It is not excluded that organisms, during their contact with foreign substances, have used certain enzyme systems of intermediary metabolism as the basis for the development of a more extended capacity to metabolize xenobiotics.

## II, 5. MECHANISM OF THE ENZYMATIC OXIDATION OF XENOBIOTICS

The microsomal enzyme systems for the oxidative biotransformation of xenobiotics were first extensively described by Brodie and collaborators (1958) and by Axelrod (1960). It has been shown by Mason (1957) that this type of enzymatic reaction requires both NADPH and free oxygen. He introduced the term *mixed function oxidase* for the enzyme system involved.
As far as the mechanism of the reaction is concerned, at the present time most of the oxidative drug-metabolizing NADPH-dependent reactions are considered to belong to the general category of hydroxylations (table 1).

TABLE 1 - Hydroxylation mechanisms in drug metabolism

| | |
|---|---|
| aliphatic hydroxylation | $R\text{-}CH_3 \rightarrow R\text{-}CH_2OH$ |
| aromatic hydroxylation | $R\text{-}C_6H_5 \rightarrow R\text{-}C_6H_4OH$ |
| oxidative dealkylation | $R\text{-}X\text{-}CH_2\text{-}R' \rightarrow [R\text{-}X\text{-}C(OH)(H)\text{-}R'] \rightarrow R\text{-}XH + O{=}\overset{H}{\overset{\vert}{C}}\text{-}R'$ (X = O, S, or NH) |
| oxidative deamination | $R\text{-}CH(NH_2)\text{-}R' \rightarrow [R\text{-}C(OH)(NH_2)\text{-}R'] \rightarrow R\text{-}CO\text{-}R' + NH_3$ |
| *S*-oxidation | $R\text{-}S\text{-}R' \rightarrow [R\text{-}SOH\text{-}R']^+ \rightarrow R\text{-}SO\text{-}R' + H^+$ |
| *N*-oxidation | $(CH_3)_3N \rightarrow [(CH_3)_3NOH]^+ \rightarrow (CH_3)_3NO + H^+$ |

Since the introduction of the concept by Fish *et al.* (1956) that *N*-dealkylation proceeds via intermediate *N*-oxides, several studies have been performed that argue for (Pettit and Ziegler, 1963; Ziegler and Pettit, 1964; Smuckler *et al.*, 1967; Dehner *et al.*, 1968) or against it (McMahon and Sullivan, 1964). The arguments have been reviewed by McMahon (1966) and Gillette (1966), and recently by Bickel (1969). A final answer concerning the role of *N*-oxides as intermediary metabolites in *N*-dealkylation has, however, as yet not been given.

The oxidative reactions require the mediation of an electron-transporting chain which consists of NADPH-cytochrome c reductase (an FAD-containing flavoprotein), a non-heme iron-protein and cytochrome P-450 (Omura *et al.*, 1965; Orrenius, 1965). The sequence of the different steps is schematically represented in figure 5. Gillette and Gram (1969) propose the following scheme for the mechanism of the mixed function oxidase reaction. The substrate first forms a complex with the oxidized form of a microsomal

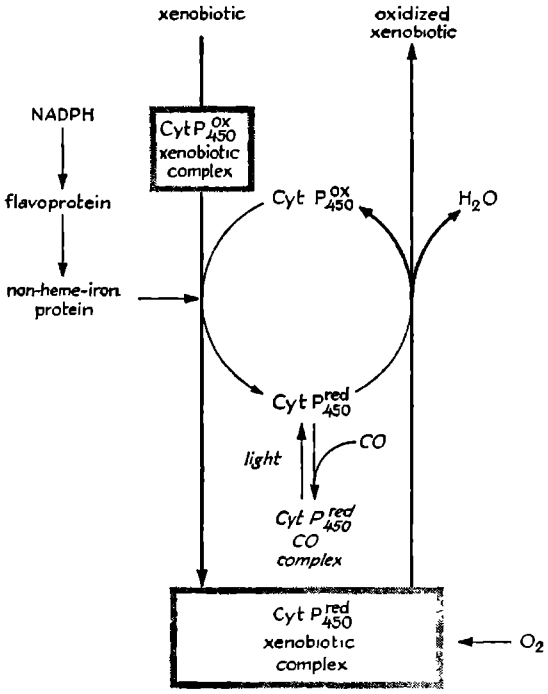



FIGURE 5. Sequence of steps in the oxidation of xenobiotics.

hemoprotein, the cytochrome P-450. This complex then is reduced through other microsomal components by NADPH. The reduced cytochrome P-450-substrate complex rapidly combines with molecular oxygen to form an $O_2$-cytochrome P-450-substrate complex, which quickly decomposes to form the oxidized substrate and the oxidized form of the cytochrome. The reduction of the oxidized cytochrome P-450-substrate complex is suggested as the rate-limiting step.

At present, the full identity of the non-heme iron-protein is not known.

Cytochrome P-450 is a specific cytochrome which is also called reticulochrome or CO-cytochrome. After its first description in 1958 (Garfinkel, 1958; Klingenberg, 1958), it was not until 1963 that the function of cytochrome P-450 in the oxygen activation and the associate reactions of microsomal electron transport became known (Estabrook *et al.*, 1963; Cooper *et al.*, 1965). Thereupon the cytochrome P-450 has been extensively investigated, especially its spectral properties (Omura and Sato, 1964a and 1964b; Omura *et al.*, 1965; Gillette, 1966; Remmer *et al.*, 1968a). The cytochrome P-450 can be distinguished from another microsomal pigment, the cytochrome $b_5$, since the spectral properties of the two cytochromes react in a different way to the presence of carbon monoxide. The presence of the cytochrome P-450 may be detected only in an atmosphere of carbon monoxide. In that case the reduced form of the pigment combines readily with CO to form a complex having an absorption maximum at 450 nm. As a result of the binding to carbon monoxide an inhibition of many drug-metabolizing reactions of various animal species have been observed (Gillette, 1966). Light, especially monochromatic light having a wavelength of 450 nm, can split the CO-cytochrome P-450-complex resulting in an abolishment of the inhibition.

More recent studies have been directed to the interaction between xenobiotics and the microsomal cytochrome P-450. It has been shown that the spectral properties of the microsomal suspensions can be altered by the addition of various drugs (Narasimhulu, 1963; Narasimhulu *et al.*, 1965; Remmer *et al.*, 1966; Imai and Sato, 1966). Two distinct types of spectral changes have been observed. A *type I* spectral change with an absorption minimum at about 420 nm and a maximum at about 390 nm caused by, for instance, barbiturates, cyclohexane, isooctane, aminopyrine, DDT, chlorpromazine, and SKF 525A, and a *type II* spectral change with a maximum at about 430 nm and a minimum at about 390 nm caused by, for instance, aniline, acetanilide, and metyrapone (Imai and Sato, 1967; Schenkman *et al.*, 1967b; Schenkman and Sato, 1968; Remmer *et al.*, 1968b; Leibman *et al.*, 1969). It is suggested that the difference in the two types of absorption spectra is caused by a difference in the nature of the complexes formed. A definite answer to the questions about the enzyme-substrate interaction requires further characterization of the structure of the hemoprotein involved. In the centre of interest is the question whether a single species of cytochrome P-450 exists in two or more functionally different, but interconvertible, forms capable of interacting with two different types of substrates (Hildebrandt *et al.*, 1968 and 1969; Leibman *et al.*, 1969; Schenkman and Sato, 1968; Schenkman *et al.*, 1969), or whether two or more hemoprotein species of cytochrome P-450 are present in microsomes (Alvares *et al.*, 1968a; Conney *et al.*, 1969;

Mannering *et al.*, 1969; Kuntzman *et al.*, 1968 and 1969). Up to now, however, this question is still a matter of debate.

## II, 6. REDUCTION, HYDROLYSIS, AND CONJUGATION REACTIONS IN THE METABOLISM OF XENOBIOTICS

A wide variety of xenobiotic substances used as food-additives, medicaments, and pesticides contain groups which can be biochemically reduced in the organism. As such can be mentioned the azo group in prontosil, azobenzene, and dimethylazobenzene which can be cleaved (thus forming primary aromatic amines), and the nitro group in chloramphenicol, *p*-nitrobenzoic acid, and nitrobenzene. The enzyme systems involved in reduction appear to occur mainly in the liver. Minor amounts are also observed in a variety of other tissues including kidney and lung (Fouts *et al.*, 1957; Fouts and Brodie, 1957). Intracellularly, the enzyme systems are mainly located in the endoplasmic reticulum, but are also found in the soluble fraction of the cell (Fouts and Brodie, 1957; Kato and Oshima, 1968; Umar and Mitchard, 1968). The studies of Kato *et al.* (1969) revealed different properties of the differently located reductase systems. For instance, the microsomal reductases are NADPH-dependent, whereas for the reductases found in the supernatant NADPH can be replaced by NADH. Reduction proceeds at a maximal rate under anaerobic conditions. The properties of the microsomal reduction mechanism resemble in some aspects those of the oxidative reactions since for the concomitant electron transport both types of reaction appear to make use of the same system (Gillette and Gram, 1969). NADPH acts as hydrogen donor. The reaction requires the mediation of NADPH-cytochrome c reductase (Hernandez *et al.*, 1965), and of cytochrome P-450. For details about the role of cytochrome P-450 in the reduction of *p*-nitrobenzoate by liver microsomes may be referred to the papers of Gillette *et al.* (1968) and Sasame and Gillette (1969).

In contrast to the oxidative and reductive drug-metabolizing enzymes, which only are located intracellularly, a considerable number of hydrolytic enzymes has been shown to be present both intracellularly as well as in blood plasma. Lee and Livett (1967) showed that various local anaesthetics containing an ester link are hydrolysed by esterases occurring in the liver. As examples of plasma esterases, aromatic esterases hydrolysing phenyl and *p*-nitrophenyl esters, aliphatic esterases hydrolysing aliphatic esters (e.g. tributyrin) and choline esterases (i.e. acetylcholine esterase and pseudocholine esterase) hydrolysing preferentially choline esters can be mentioned.

In conjugation, within the liver cells products suitable for excretion in urine

or bile are synthesized by the coupling of xenobiotics or waste products from intermediary metabolism with normal body substances indicated by the term *conjugating agents*. In order to be conjugated the unwanted molecule must possess one or more polar groups. Not only xenobiotics, which often have first to be modified by the previously discussed oxidative, reductive and/or hydrolytic reactions, but also products from normal carbohydrate, protein, and lipid metabolism are removed from the body in this way. Glucuronide formation may occur with compounds bearing OH-, COOH-, $NH_2$- and SH-groups. Conjugation with amino acids can take place in the case of aromatic and aliphatic carboxy-acids. Acetylation occurs with drugs containing hydroxyl or sulfhydryl groups, but more frequently with xenobiotics bearing amino groups. Phenolic hydroxyl groups may be conjugated with sulfuric acid. Methylation may occur with OH-, SH-, $NH_2$- and NH-groups. In view of the above mentioned possibilities, it will be clear that a drug, dependent on its structure, can be attacked at different points and by different types of reaction. In any case of conjugation it is a requirement that the drug or the conjugating agent is in an activated state. An example of the conjugation reactions in which the drug forms part of the active intermediate is the conjugation of acids with amino acids. For instance, benzoic acid is converted to an active form, benzoyl-coenzyme A, which is transferred by the enzyme glycine *N*-acylase to the conjugating agent glycine. Activation of the conjugating agent is more common. As such can be mentioned UDP-glucuronate in glucuronidation, acetyl coenzyme A in acetylation, adenosine-3'-phosphate-5'-phosphosulfate in sulfate conjugation, and *S*-adenosylmethionine in methylation. The final coupling between drug and conjugating agent is catalyzed by transferring enzymes. In this field extensive studies have been performed on the enzyme involved in glucuronide synthesis, the UDP-glucuronyltransferase. Kinetic evidence has been presented that this transferase exists in more than one molecular form (Dutton, 1966; Halac and Reff, 1967). Some of the products of the conjugation reactions may be removed from the body with the urine, but most of them are eliminated by biliary excretion (see Parke and Williams, 1969).

## II, 7. FACTORS INFLUENCING THE METABOLISM OF XENOBIOTICS

The extent to which the organism makes use of biotransformation processes in order to eliminate xenobiotics depends on various factors. Besides the chemical properties of the drug, its dose, route of administration, and effects of distribution and excretion, the qualitative and quantitative differences in the properties of the drug-metabolizing enzyme systems are especially

important. The capacity of animals to convert xenobiotics varies with several endogenous factors, such as genetic factors, hormonal changes, sex, and age, and with exogenous factors, among which certain environmental conditions, the nutritional status and the ingestion of other alien chemicals.

In this discussion attention will be mainly directed to the stimulation of drug biotransformation by the administration of alien chemicals *in vivo*. The enhanced drug metabolism evoked by pretreatment with drugs may lead to a tolerance or an increased sensitivity of the organism to the action of xenobiotics. The phenomenon of enhanced drug-metabolizing capacities was first described in 1954 by Brown and coworkers. They administered polycyclic hydrocarbons to rats and mice. Subsequently, a wide variety of drugs, insecticides, and carcinogens have been detected as stimulators of drug metabolism.

The increase in drug-metabolizing capacity appears to be mainly due to an increase in the concentration of enzyme protein; the stimulation observed is referred to as *enzyme induction*. The extensive studies in this field have been reviewed by Remmer (1962 and 1969), Burns *et al.* (1963), Conney (1967) and Street (1969). Recent studies have shown that enzyme induction in drug metabolism cannot only be observed in animals exposed to certain xenobiotics, but also *in vitro* in isolated rat liver cells (Henderson, 1971), and in hamster fetus cell cultures (Nebert and Gelboin, 1968).

A peculiar aspect of the situation is that the modification of the enzymatic activity is not restricted to the enzyme system converting the drug studied, but has a much more general character. Also compounds practically not metabolized themselves, such as the insecticide chlordane, are potent stimulators for a variety of drug-metabolizing enzyme systems (Hart and Fouts, 1965; Conney *et al.*, 1966). The inducers can be categorized into two groups, viz. a group of inducers mainly consisting of drugs and insecticides, that enhance the metabolism of a wide variety of drugs, and a group mainly comprising polycyclic hydrocarbons that stimulate the metabolism of only a few drugs. Phenobarbital is the prototype of the first group of inducers; 3-methylcholanthrene and 3,4-benzpyrene are the most frequently employed agents in the latter group. The different action of the two groups of inducers does not only reveal itself in relation to quite different types of reactions such as oxidation, reduction, hydrolysis, and conjugation (Conney 1967), but also when only one type of reaction is concerned. Here again the question arises whether in one type of reaction one or more enzymes are involved. The extensive studies of Rubin *et al.* (1964) supported evidence that a single enzyme system would be responsible for the metabolism of many drugs. They showed that many substrates are competitive inhibitors of each other's

enzymatic oxidation. These authors, however, already pointed out that these results were contradictory to the different inductive effects of the polycyclic hydrocarbons and phenobarbital. For instance, phenobarbital administered to male rats stimulated the *N*-demethylation of both ethylmorphine and 3-methyl-4-methylaminoazobenzene (3-MMAB), while 3-methylcholanthrene and 3,4-benzpyrene stimulated the *N*-demethylation of 3-MMAB only (Sladek and Mannering, 1969a and 1969b). These data suggest a diversity in the oxidative enzyme system, in which the two groups of inducers may produce an effect via different induction mechanisms. It was suggested that the administration of polycyclic hydrocarbons caused the synthesis of a microsomal hemoprotein differing from the original one in that it is capable of participating in the *N*-demethylation of 3-MMAB but not of ethylmorphine. These findings support the data which indicate the existence of more than one molecular species of cytochrome P-450 (Kuntzman *et al.*, 1968; Alvares *et al.*, 1968a; Levin and Kuntzman, 1969a and 1969b). However, at present data of the various investigators working in this field seem contradictory.

It must be remembered that the enzyme proteins involved have a complex structure and that they are "embedded" in microsomal lipids. Therefore, the differences in the metabolism of different substrates and the diversity in the induction processes may also be related to changes in the structure of the microsomal lipids and so to changes in the functional state of the enzyme systems.

The increase in the drug-metabolizing enzymatic capacities under the influence of inducing agents might be caused by several mechanisms. As such can be mentioned: a) enhancement of enzyme synthesis; b) synthesis of a more active enzyme; c) direct or indirect activation of existing enzyme, or d) stabilization of enzyme against degradation.

An effect of the agents on the possible level of activators or inhibitors has not been observed as yet. Removal of endocrine glands does not prevent the induction of drug metabolism. These data (cf. Conney, 1967) argue against an enhancement on the basis of a direct or indirect activation. At present there is much evidence that the increased enzymatic activity is mainly due to a *de novo* enzyme synthesis (Kato *et al.*, 1966; Piper and Bousquet, 1968; Baron and Tephly, 1969; Levin and Kuntzman, 1969a and 1969b; Bresnick and Madix, 1969). Inhibitors of protein or nucleic acid synthesis such as cycloheximide, ethionine, actinomycin D, and puromycin prevent enzyme induction (see Conney, 1967). Comparative data on the properties of the newly formed and the existing enzyme are somewhat contradictory. In the past it was generally accepted that the affinity of the enzyme for the substrates was not changed after induction (Leibman and McAllister, 1967; see

also Conney, 1967). The studies of Guarino *et al.* (1969) and Alvares *et al.*, 1968b), however, showed changes in the apparent Michaelis constants (apparent $K_m$) in drug-metabolizing reactions after induction. Concerning apparent $K_m$-values in drug-metabolizing reactions it has to be remarked that these values are determined by measuring only the formation of an end product, the disappearance of the initial substrate, or the disappearance of the initial electron donor. As has been discussed before in this chapter, the drug-metabolizing reaction includes several steps between the electron donor and the substrate. In view of this, and of the use of a rather impure isolated enzyme system such as the microsomal preparation, the kinetic data obtained in drug-metabolizing reactions are of limited value.

Some studies have shown that as well as *de novo* synthesis of microsomal components, a decrease in catabolism, by means of a stabilization of the components or by a repressing effect on catabolizing enzymes, is probably also involved in the induction phenomenon (Holtzman and Gillette, 1968; Greim and Remmer, 1969).

Treatment of animals with inducing agents not only enhances the drug-metabolizing capacity but also causes a broad spectrum of changes in the biochemical and morphological composition of the liver. The papers of Orrenius *et al.* (1965 and 1969), Kunz *et al.* (1966a and 1966b), and Conney (1967) are relevant in this respect.

## II, 8. SPECIES DIFFERENCES IN THE METABOLISM OF XENOBIOTICS

In pharmacology it is a normal observation that a certain dose of a drug has a profound effect in one animal species, but has little or no effect in another species. In many cases these differences are caused by differences in the pathways or in the rates of drug metabolism (Quin *et al.*, 1958; Kato and Gillette, 1965). Here again a parallel must be drawn with normal intermediary metabolism. In view of the possibility that the enzymes involved in the biotransformation of xenobiotics may also operate in the metabolism of physiological substances, the species variations in drug metabolism may well be a reflection of the variations in the metabolism of the normal intermediary compounds. Further, it is likely that in evolution the development of the qualitative and quantitative differences in the metabolism of xenobiotics is influenced by the environmental conditions of the organism. For example, an adaptation of the drug-metabolizing capacity may well be caused by the components in the food for which the animal is specialized, and by the exposure to various xenobiotics over long periods of time. It is likely that herbivorous animals in their normal diet encounter a larger amount and

diversity of foreign compounds, for instance alkaloids, terpenes, dyes, aromatic compounds, than carnivorous animals do.

It appears from the rather few surveys of the literature in this field of study that the basic pattern of drug metabolism is common to most animal species (Smith, 1964; Symposium Comparative Patterns of Drug Metabolism, 1967). A study of Strittmatter and Umberger (1969) shows similarities between the microsomal mixed function oxidase system of avian and mammalian liver. A comparison of some characteristics of several drug-metabolizing reactions in different animal species, especially fishes, will be presented in the next chapters.

In spite of the extensive studies on drug metabolism, in the last decade, comparative investigations with a wide range of species have scarcely been performed. Certain comparative studies (Dring *et al.*, 1966; Ellison *et al.*, 1966; Bridges *et al.*, 1969a and 1969b; see also review of Williams, 1967), which are only based on *in vivo* experiments with measurements of the metabolites appearing in urine and faeces are difficult to interpret. The data thus obtained can hardly be taken as representative of the respective importance of the various drug-metabolizing reactions. The rate of elimination of substances by the kidney depends on the lipid-solubility and on the degree of ionization (dependent on the $pK_a$-value of the compound and on the pH of the urine), and on the involvement of active transport systems. These factors may all result in variable patterns of the excreted metabolites. In studies where xenobiotics are orally administered to animals or man, the metabolism by gastrointestinal microorganisms and further enterohepatic circulation of the drug and metabolites is not always taken into consideration. This means that differences found in the metabolic fate may be a reflection of, for instance, differences in intestinal flora (Scheline, 1968a, 1968b, and 1968c). For DDT, for example, it has been shown that the intestinal microflora plays a major role in its detoxication in the rainbow trout (Wedemeyer, 1968).

*In vitro* studies have shown that species differences in drug-metabolizing reactions can be the result of variations in the absolute amount and the kinetic properties of the enzymes. Several investigators have shown this for the *N*-demethylation of ethylmorphine in mouse, rat, rabbit, and guinea pig (Castro and Gillette, 1967; Davies *et al.*, 1968; Davies *et al.*, 1969; Gillette and Gram, 1969). The data obtained in these studies cannot explain the interspecies differences in the reaction mentioned, on the basis of differences in the microsomal cytochrome P-450 content. That the amount of cytochrome P-450 in microsomes of various species does not differ very much has also been reported by Remmer *et al.* (1968a).

It has to be remarked that as well as differences in the amount of the enzymes

other factors may also be responsible for interspecies differences. For instance, the amount and properties of natural activators or inhibitors of the drug-metabolizing enzymes, the enzymes reversing the reactions, and the extent of competing reactions using the same substrate, may play a role.

The above discussion, which shows that rather little information is available in the area of comparative drug metabolism, indicates the urgency of the need of further study.

# MATERIALS AND METHODS EMPLOYED IN THE STUDY OF THE ENZYMATIC CONVERSION OF XENOBIOTICS

## III, 1. CHEMICALS

Biochemicals used were obtained commercially: glucose-6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), NADP (disodium salt), NADPH (tetrasodium salt), and UDPglucuronate (disodium salt) from C. F. Boehringer and Soehne GmbH, Mannheim, Germany; bovine serum albumin, DL-isocitrate (type I, trisodium salt), and isocitrate dehydrogenase (E.C. 1.1.1.42, Sigma type IV) from Sigma Chemical Company, St. Louis, U.S.A.; calf thymus DNA from the British Drug Houses Ltd., Poole, London, U.K.; saccharo-1,4-lactone from Calbiochem, Los Angeles, U.S.A.

Pyrazolone derivatives were obtained from Merck A.G., Darmstadt, Germany, except 4-monomethylaminoantipyrine which was donated by Farbwerke Hoechst A.G., Frankfurt, Germany.

Dieldrin (containing 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene) was obtained from Fluka A.G., Buchs, Switzerland; DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, technical grade) and atrazine (2-chloro-4-ethylamino-6-isopropylamine-s-triazin) were donated by N.V. Orgachemia, Boxtel, the Netherlands.

In thin-layer chromatography TLC-plates silicagel $F_{254}$ and TLC-plates silicagel without fluorescence indicator (layer thickness 0.25 mm or 2 mm) obtained from Merck A.G., Darmstadt, Germany, were used.

All other chemicals were obtained commercially in the highest purity available and were not further purified, except aniline which was distilled before use and *p*-aminophenol which was recrystallized from ethanol.

## III, 2. ANIMALS

Mammals: golden hamster (*Mesocricetus auratus*, L.), mouse (*Mus musculus*, L., Swiss mouse), rat (*Rattus norvegicus*, L., strain Wistar).

Birds: zebra finch (*Taeniopygia guttata*, L.), hen (*Gallus gallus gallus*, L.) pigeon (*Columba livia*, L.).

Reptiles: lizard (*Lacerta viridis*, L.).
Amphibians: frog (*Rana esculenta*, L.).
Fishes: hatchery-reared fishes, viz. golden orfe (*Leuciscus idus*, L. var. *Idus orfus*, L.) and rainbow trout (*Salmo irideus*, Gibbons) were furnished by the Koninklijke Nederlandse Heide-Maatschappij, Arnhem, the Netherlands; wild fishes were obtained from the river Waal (Rhine) near Nijmegen, the Netherlands, viz. bream (*Abramis brama*, L.), carp (*Cyprinus carpio*, L.), tench (*Tinca tinca*, L.), white bream (*Blicca bjoerkna*, L.), dace (*Leuciscus leuciscus*, L.), ide (*Leuciscus idus*, L.), roach (*Leuciscus rutilus*, L.), rudd (*Scardinius erythrophthalmus*, L.), twait (*Alosa finta*, L.), loach (*Nemachilis barbatula*, L.), houting (*Coregonus oxyrhynchus*, L.), salmon (*Salmo salar*, L.), eel (*Anguilla anguilla*, L.), perch (*Perca fluviatilis*, L.), pike (*Esox lucius*, L.). Besides the above mentioned animals two other water-dwelling animals have also been investigated, viz. the sea lamprey (*Petromyzon marinus*, L.) and an invertebrate species, the wool-handed crab (*Eriocheir sinensis*, De Haan), both obtained from the river Waal.
In the comparative study the wild animals investigated have been assayed for drug-metabolizing enzymatic activity on the day of capture. In certain experiments fishes were maintained alive in the laboratory, in tanks with continuously running tap water (600 l/h) until used experimentally.

### III. 3. PREPARATION OF TISSUE

Animals were killed by decapitation and exsanguinated. Tissues were immediately excised and placed in beakers immersed in ice. All subsequent operations were carried out at 0 - 4 °C. Twenty-five per cent (w/v) homogenates in 0.25 M sucrose containing $10^{-3}$ M sodium ethylenediamine tetraacetate (EDTA-Na) and 5 x $10^{-2}$ M Tris-HCl buffer (pH 7.4) were prepared with a Potter homogenizer having a Teflon pestle. The homogenization was performed during 1 min with 3 up and down strokes at 2500 revolutions per minute. The homogenates were centrifuged at 9000 *g* for 20 min. Either the "9000 *g* supernatant" fractions, or microsomes isolated from these fractions, were used in the enzymatic assays as the enzyme source. The microsomal fractions were isolated by centrifugation of the 9000 *g* supernatants at 105000 *g* during 60 min. After removal of the supernatant the sediments were suspended in the above mentioned homogenization medium, recentrifuged, and then resuspended in the homogenization medium to a final volume equal to the original 9000 *g* supernatant volume.
In the study of the intracellular localization of the enzymes in liver tissue besides the above mentioned fractions, the cell fractions sedimenting between

0 and 1000 *g*, 1000 and 9000 *g*, 9000 and 18000 *g* as well as the 105000 *g* supernatants were tested for enzymatic activity.
For the measurement of the *N*-demethylating and *p*-hydroxylating enzymatic activities the tissue preparations were used as described above. The UDP-glucuronyltransferase activity was assayed after ultrasonication of the tissue preparations according to Henderson (1970) in a MSE 100 Watt Ultrasonic Disintegrator for 60 seconds (6 x 10 seconds with intervals of 1 minute). To avoid heating, the tubes containing the homogenates were cooled with ice or a freezing mixture during the ultrasonic treatment.

## III, 4. ENZYME ASSAYS

Before a standardization of the enzyme assays applied in this study was accomplished, the influence of various reaction conditions on the enzymatic activity *in vitro* was evaluated. The experiments performed with respect to the influence of different reaction conditions are extensively described in chapter IV. The standard procedures and conditions of the enzymatic reactions finally employed during the whole investigation will be listed here. All enzyme assays were carried out in duplicate and referred to similarly treated blanks to detect non-enzymatic or endogenous formation of products and other interfering substances. Product formation was calculated by reference to calibration curves, obtained with solutions with decreasing concentrations of the substances being estimated, in mixtures which contained all reaction components including heat-denatured enzyme extract, and which were equally and simultaneously treated.

### III. 4.1. *Standard incubation procedure for oxidative N-demethylation and aromatic hydroxylation*

For the measurement of enzymatic activity in the *N*-demethylation of aminopyrine and *p*-hydroxylation of aniline *in vitro* the following incubation mixture was used: $5 \times 10^{-2}$ M tris-HCl (pH 8.0), $8 \times 10^{-4}$ M $MgCl_2$, $8 \times 10^{-6}$ M $MnCl_2$, $5 \times 10^{-3}$ M sodium isocitrate, 50 $\mu$g isocitrate dehydrogenase (capable of reducing 0.3 $\mu$moles NADP per min), $1.3 \times 10^{-4}$ M NADP, and the substrate, aminopyrine or aniline, of which the concentrations employed are presented in chapter IV, 3.4, table 9. The 9000 *g* supernatant or other tissue fraction was added in a suitable concentration, generally in amounts equivalent to 60 - 100 mg fresh tissue. The total volume was 3 ml. During the incubation the test media were saturated with air by shaking. The incubation

periods for the enzymatic reactions studied were short so that substrate conversion remained linear with time (chapter IV, 3. 1).
Enzyme reactions were started by adding the enzyme extracts after a preincubation of the assay media for 10 min to ensure temperature constancy and complete reduction of NADP to NADPH. The employment of the glucose-6-phosphate dehydrogenase reaction as an NADPH-generating system instead of the isocitrate dehydrogenase reaction — 5 x $10^{-3}$ M glucose-6-phosphate and 5 $\mu$g glucose-6-phosphate dehydrogenase (capable of reducing 0.7 $\mu$moles NADP per min) — proved as good.

### III. 4.2. *N-Demethylation of aminopyrine*

In the *N*-demethylation reaction with aminopyrine used as the substrate, enzymatic activity was defined as the amount of formaldehyde produced per hour per gram fresh tissue. In order to trap the formaldehyde formed, a neutralized solution of semicarbazide hydrochloride was added to the reaction mixture to a final concentration of 5 x $10^{-3}$ M.
After the incubation the reaction was stopped by the addition of 0.5 ml 25% $ZnSO_4.7\ H_2O$ and 0.5 ml saturated $Ba(OH)_2$. After cooling, the precipitated protein was removed by centrifugation at 18000 *g* for 10 min. The amount of formaldehyde in the supernatant was determined according to the method of Nash (1953) as modified by Cochin and Axelrod (1959): 2 ml of supernatant were mixed with 1 ml of double-strength Nash reagent (a solution of 4 M ammonium acetate, 0.1 M acetic acid, and 0.04 M acetylacetone in water); the mixture was heated for 15 min at 60 °C and cooled, and its extinction was determined at 412 nm.
The *N*-demethylated pyrazolone derivatives were quantified, in reaction mixtures in which semicarbazide was omitted, by the colorimetric methods of Brodie and Axelrod (1950) and of Brun (1951). In the first assay the protein in the reaction mixture was precipitated by the addition of 1 ml of a solution of 20% trichloric acid (La Du et al., 1955). After cooling, the precipitated protein was removed by centrifugation at 18000 *g* for 10 min. To 3 ml supernatant 0.3 ml 0.2% sodium nitrite solution was added, cooled for 10 min, and then thoroughly mixed with 0.3 ml 1% ammonium sulphamate solution. After 3 min 0.1 ml of a 3% solution of resublimed $\alpha$-naphthol in absolute alcohol was added, followed by 0.6 ml of 4N NaOH. After 10 min 0.3 ml of concentrated HCl was added, after which the dye was extracted in 1 ml isoamyl alcohol. The optical density of the solution was determined at 540 nm. In the assay according to Brun the protein in the reaction mixture was precipitated by the addition of 1 ml of a solution of 9% trichloroacetic

acid. After cooling, the precipitated protein was removed by centrifugation at 18000 *g* for 10 min. Subsequently 2 ml of the supernatant were mixed with 2 ml freshly prepared Brun reagent (0.07 M *p*-dimethylaminobenzaldehyde in 60% alcohol). After 15 min the extinction was determined at 430 nm. An evaluation of the various colorimetric assays applied in the studies on the *N*-demethylation of aminopyrine *in vitro* is given in chapter V. In the assays of *N*-demethylating enzymatic activity, incubated blanks containing all components of the incubation mixture except substrate, which was added after protein precipitation, were used to correct for formaldehyde produced during incubation by reactions not involving aminopyrine.

### III, 4.3. *p-Hydroxylation of aniline*

In the hydroxylation reaction in which aniline was used as the substrate, enzymatic activity was expressed as the amount of *p*-aminophenol produced per hour per gram fresh tissue. The amount of *p*-aminophenol was determined with a method based on the procedures of Brodie and Axelrod (1948), Dixon *et al.* (1964) and Jaccarini and Jepson (1968). After incubation the reaction mixture was extracted with ethyl acetate, once with 2 ml and subsequently twice with 1 ml. To the combined extracts 2 ml 0.01 N HCl were added. After shaking for 10 min, the ethyl acetate layer was removed and 2 ml of 5% phenol in 1 M $Na_2CO_3$ were well mixed with the aqueous layer. After 20 min the blue indophenol colour was determined at 630 nm. In the blanks no endogenous formation of *p*-aminophenol or interference by other substances could be detected.

### III, 4.4. *Glucuronidation of p-nitrophenol*

The activity of UDPglucuronyltransferase (UDPglucuronate glucuronyltransferase, E.C. 2.4.1.17) was determined using *p*-nitrophenol as acceptor substrate. The incubation mixture contained 5 x $10^{-2}$ M tris-HCl (pH 7.4), 3.3 x $10^{-3}$ M $MgCl_2$, 6.0 x $10^{-3}$ M uridine-5-diphosphoglucuronate (UDP-glucuronate), $10^{-3}$ M saccharo-1,4-lactone, 14 x $10^{-4}$ M *p*-nitrophenol, and tissue homogenate in concentrations equivalent to 18 - 75 mg fresh tissue in a total volume of 1.5 ml. Blanks contained all reagents except UDPglucuronate. The reaction was stopped by adding 1 ml ethanol. The glucuronide formation was measured indirectly by determination of the disappearance of *p*-nitrophenol. After centrifugation, aliquots of the deproteinized medium were diluted with 0.1 N NaOH and spectrophotometrically measured at 398 nm.

## III, 5. IDENTIFICATION OF PRODUCTS FORMED IN THE *IN VITRO* *N*-DEMETHYLATION OF AMINOPYRINE

In order to know whether in the *N*-demethylation of aminopyrine *in vitro* one or two methyl groups are split from the dimethylamino group in the molecule, reaction products were extracted from the reaction mixture, separated by thin-layer chromatography (TLC) and further analysed. In all experiments, appropriate blanks and standards were carried through the entire procedure.

### III, 5.1. *Extraction of reaction products*

Immediately after incubation the reaction mixtures, standard solutions with known concentrations of reference substances, and blanks, were brought to pH 9 with NaOH and extracted with 1,2-dichloroethane. The organic phases were evaporated to dryness in a stream of air and residues were taken up in ethanol.

### III, 5.2. *Thin-layer chromatography*

In preparative chromatography of extracts of large volumes — 0.5 to 1.0 ml, originating from reaction mixtures with a total volume of 150 to 300 ml — samples were applied in bands on plates coated with silicagel with a layer thickness of 2 mm. For the analysis of small samples 10 to 50 $\mu$l were applied in spots on plates with a silicagel layer, thickness of 0.25 mm.
The following solvent systems were used:
system A: chloroform — diethylether — methanol (70-30-10, vol.), and
system B: *n*-butanol — acetic acid — water (80-20-10, vol.).
Chromatography was carried out in the dark at room-temperature. Development time lasted until about 16 cm length of run.

### III, 5.3. *Identification of pyrazolone derivatives*

#### III, 5.3.1. Absorption measurements

After TLC, bands or spots were visible under ultraviolet light. $R_f$-values were calculated.
UV-absorbing areas were scraped from the plates, eluted with ethanol, and UV-spectra of the eluates were measured. The spectra were corrected for the absorption of an eluate from a blank area in the chromatogram.
In some experiments a quantitative estimation of the spots on the chromatograms was achieved. Reaction mixtures (3 ml) were extracted twice with

1.5 ml 1,2-dichloroethane as described above. Samples were subjected to TLC on plates without fluorescence indicator with solvent system A. After development of the chromatograms the absorption of ultraviolet light by the spots was directly measured with a Zeiss-chromatogram-spectrophotometer (PMQ II) at 275 nm, and recorded. Calculation was performed by referring the areas of the peaks found for the reaction products to those found for mixtures with known concentrations of the pyrazolone derivatives involved.

III, 5.3.2. Staining procedures

The spots were stained by spraying the chromatograms with the following reagents (cf. Stahl, 1967), which were freshly prepared before use:

a. 0.5% aqueous solution of Fast blue salt B, followed by 0.1 N NaOH.
b. 1% iron (III) chloride solution, followed by heating to 80 °C.
c. 1% 2,6-dichloroquinone chlorimide in ethanol, followed by exposure to ammonia vapour.
d. a mixture (1 : 1) of a 0.5% aqueous solution of phenol and a 5% ammonia solution, followed by 2% aqueous solution of potassium ferricyanide (Emerson and Beegle, 1943).

Colours formed by reaction of the reagents and the reference substances aminopyrine (DMAP), 4-monoethylaminoantipyrine (MMAP), and 4-aminoantipyrine (AAP) are given in table 2.

TABLE 2 - Staining of aminopyrine (DMAP), 4-monomethylaminoantipyrine (MMAP), and 4-aminoantipyrine (AAP) by various reagents

| | reference substances | | |
|---|---|---|---|
| reagent* | DMAP | MMAP | AAP |
| a | red-brown | yellow | red-brown afterwards brown |
| b | violet-blue | pink | red-violet |
| c | yellow | yellow afterwards grey-green | yellow |
| d | violet-blue | pink afterwards pink-violet | yellow |

* reagents a, b, c, d are described in the text; pyrazolone derivatives were stained after TLC.

### III, 5.3.3. Mass-spectrometry

Eluates were evaporated to dryness and the residues were subjected to mass-spectrometry in a LKB 9000, direct inlet, electron energy 70 eV.

## III, 6. ASSAY OF PROTEIN

The protein of liver homogenates was determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as a reference standard in the calculation of the protein content.

## III, 7. ASSAY OF DNA

DNA was determined by the diphenylamine test according to the method of Burton (1956) after a preextraction according to Wanka (1962). Calf thymus DNA was employed as a reference standard in the calculation of the DNA content.

# SOME CHARACTERISTICS OF THE ENZYMATIC *N*-DEMETHYLATION OF AMINOPYRINE, *p*-HYDROXYLATION OF ANILINE, AND GLUCURONIDATION OF *p*-NITROPHENOL *IN VITRO*

## IV, 1. INTRODUCTION

In this study an effort was made to obtain more knowledge of the quantitative differences in the xenobiotic-metabolizing capacities in different animal species. The parameter used was the biotransformation capacity of tissue preparations, especially of liver preparations. The terms *biotransformation capacity* and *enzymatic drug-metabolizing capacity* are used in this report to denote the maximally measurable rates of conversion *in vitro*. It remains disputable, however, whether the capacities measured *in vitro* can be considered representative for the drug-metabolizing capacity *in vivo*, especially as far as the quantitative aspects are concerned.

A comparison of the *in vitro* assays of drug-metabolizing reactions reported in the literature reveals a striking lack of uniformity in the methods used. For this reason the data obtained for different animal species in different laboratories are not readily comparable. Some standardized procedures for the assay of the drug-metabolizing enzymatic capacities of various animal species used in the present investigation, have been listed already in chapter III. This will be extended here by giving some elucidative examples of experiments performed for the determination of adapted reaction conditions.

This investigation concerned the following enzymatic reactions: the *N*-demethylation of aminopyrine, the *p*-hydroxylation of aniline, and the glucuronyl conjugation of *p*-nitrophenol. As outlined in chapter II, oxidative *N*-dealkylation, aromatic hydroxylation and glucuronidation represent major pathways in the hepatic processes involved in the biotransformation of xenobiotics. The compounds employed in this study are commonly used as model substrates for these types of reaction. The factors considered in the choice of the enzyme preparation and the reaction conditions employed will be discussed in detail now. In this connection the intracellular localization of

hepatic drug-metabolizing enzymes, and the influence of various treatments of the enzymatic preparations will be discussed. Further, various reactions conditions will be discussed such as temperature, incubation time, pH, nature of NADPH-generating system, concentrations of various additives, concentrations of substrates and cosubstrates, and enzyme concentration.

## IV, 2. THE ENZYME PREPARATION

As described in chapter II, most drug-metabolizing enzymes are localized within the liver cell, bound to the membranes of the endoplasmic reticulum. Therefore, the activity of these enzymes is generally measured with the supernatant obtained by centrifugation of a liver homogenate at 9000 *g*, indicated by the term *9000 g supernatant*, or with the sediment obtained by centrifugation of the 9000 *g* supernatant at 105000 *g*, indicated by the terms *105000 g pellet* or *microsomal fraction*. In order to choose the enzyme preparation considered in the present investigation as being representative for measurements of drug-metabolizing enzymatic capacities, homogenates and various fractions were tested for enzymatic activity.

### IV, 2.1. *Intracellular localization of hepatic drug-metabolizing enzymes*

Pilot experiments showed that the patterns of distribution of the enzymes over the various fractions studied were equal with respect to different animal species. Therefore, as an example, only the drug-metabolizing enzymatic activities observed in homogenate and fractions of the liver of one fish species, the rainbow trout, will be discussed here. The results are presented in figure 6.

It appears that, if the enzymatic activities are expressed as the amount of drug metabolized per g fresh liver, the highest values for the oxidative reactions are found in the 9000 *g* supernatant (fraction 4), and for the glucuronidation reaction in the homogenate. However, if the reaction rates are expressed per mg protein in the respective fractions, for the 3 reactions studied the highest activities are observed in the fraction sedimented by centrifugation of the 18000 *g* supernatant at 105000 *g* (fraction 6). This indicates a microsomal localization of the enzymes involved. From the activities expressed per g fresh liver, however, it can be seen that from the microsomal fraction (fraction 6) a considerable amount of the enzymatic activity has disappeared. It will be unlikely that the lower enzymatic activity is caused by a change in the enzyme-substrate interaction, since experiments in which the relationship between the enzyme preparations and different substrate concentrations was studied, revealed for the *N*-demethylation of

aminopyrine and for the *p*-hydroxylation of aniline, too, for 9000 *g* supernatant and 105000 *g* pellet, equal apparent Michaelis constants.

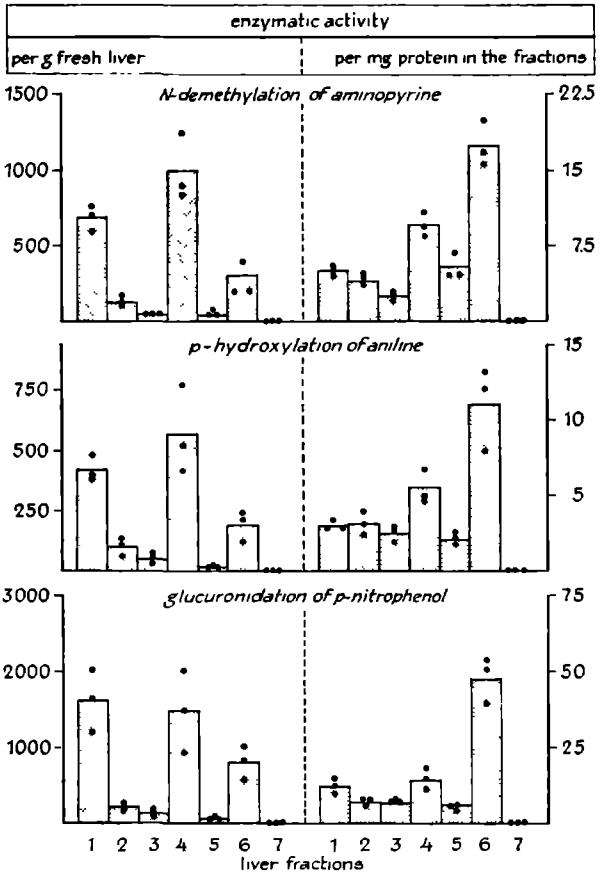



FIGURE 6. Intracellular localization of drug-metabolizing enzymes in trout liver. Blocks represent the means of 3 separate experiments. Enzymatic activity is defined as substrate turnover: in the *N*-demethylation of aminopyrine as nmoles formaldehyde produced per hour; in the *p*-hydroxylation of aniline as nmoles *p*-aminophenol produced per hour; in the glucuronidation as nmoles *p*-nitrophenol conjugated per hour. Fractions: 1, total homogenate; 2, 0 - 1000 *g* pellet; 3, 1000 - 9000 *g* pellet; 4, 9000 *g* supernatant; 5, 9000 - 18000 *g* pellet; 6, 18000 - 105000 *g* pellet; 7, 105000 *g* supernatant. Glucuronidation was measured after ultrasonic treatment of the fractions (for details see chapters III, 3 and IV, 2. 2).
Note: if substrate turnover is expressed per g fresh liver, the highest capacities for *N*-demethylation and *p*-hydroxylation are found with the 9000 *g* supernatant (fraction 4), and for glucuronidation with the total homogenate. If substrate turnover is expressed per mg protein of the fractions involved, the highest capacities for the 3 reactions are found with the microsomal fraction (fraction 6).

TABLE 3 - Microsomal *N*-demethylation in relation to the liver preparation

| enzyme preparation | mg tissue equivalent in reaction mixture | enzymatic activity* rat (n=9) | trout (n=6) |
|---|---|---|---|
| I 9000 *g* supernatant | 100 | 100 | 100 |
| II 9000 *g* supernatant** | 100 | 96 ± 2 | 69 ± 5 |
| III 9000-105000 *g* pellet § | 100 | | |
| + | + | 85 ± 2 | 66 ± 6 |
| 105000 *g* supernatant | 100 | | |
| IV 9000-105000 *g* pellet § | 100 | | |
| + | + | 70 ± 2 | 58 ± 6 |
| 105000 *g* supernatant | 10 | | |
| V 9000-105000 *g* pellet § | 100 | 79 ± 3 | 47 ± 4 |

* expressed as a percentage of the enzymatic activity in the 9000 *g* supernatant (100%); means ± S.E.M.

n the number of separate sets of experiments; in each set of experiments the *N*-demethylation activities of enzyme preparations I-V were assayed.

** 9000 *g* supernatant centrifuged for 1 hour at 105000 *g*, then gently remixed with a Potter homogenizer.

§ 9000-105000 *g* pellet gently resuspended in 0.25 M sucrose containing $10^{-3}$ M EDTA-Na and 5 x $10^{-2}$ M Tris-HCl.

significantly different, rat: I - II ($p < 0.05$); II - III ($p < 0.01$); III - IV ($p < 0.01$); IV - V ($p < 0.02$)
trout: I - II ($p < 0.01$); I - III ($p < 0.01$); III - V ($p < 0.01$)

The decrease in enzymatic activity observed for the microsomal fraction may be ascribed to mechanical damage to the enzyme system during the longer preparative procedure or to the absence of certain factors in the soluble fraction (fraction 7). That these factors may play a role is suggested by the data presented in table 3. If the 9000 *g* supernatant of liver homogenate was centrifuged at 105000 *g* for 1 hour, and remixed, both for the rat and the trout a significant decrease in the enzymatic activity was observed (enzyme preparation II). This may imply an injurious effect of the experimental procedure on the enzyme system studied. If the 105000 *g* pellet was separated from the supernatant, resuspended in the homogenization medium and assayed for *N*-demethylating enzymatic activity, both for the rat and the trout still lower enzymatic activities were observed (enzyme preparation V). The effect of the omission of the soluble fraction on the

enzymatic activity, which was more pronounced for the microsomes of the trout liver, indicates that the microsomal enzyme systems requires the soluble fraction for full activity. This may be confirmed by the data obtained in experiments in which the microsomal fraction in the reaction mixture was supplemented by the soluble fraction (enzyme preparation III), which suggest a restoration of enzymatic activity. A remarkable effect was observed in the rat, when only a small amount of the soluble fraction was added (enzyme preparation IV). In this case a significantly lower enzymatic activity appeared. An increase in microsomal drug oxidation by addition of soluble fraction has been described earlier by Chan *et al.* (1967). These investigators observed an enhancement of aldrin epoxidation by rat liver microsomes supplemented with either rat or trout liver soluble fraction. Later on, Terriere and Chan (1969) partially purified and characterized a heat-stable factor from the soluble fraction which increased aldrin epoxidation and ethylmorphine *N*-demethylation by rat liver microsomes.

### IV, 2.2. *Influence of various treatments on drug-metabolizing enzymatic activity*

The sensitivity of the drug-metabolizing enzyme preparations of rat liver to various experimental conditions has been investigated earlier (McLuen and Fouts, 1961; Leadbeater and Davies, 1964; Janson, 1968; Chan and Terriere, 1969). It appeared that various treatments resulted in decreased enzymatic activities in the oxidative reactions. In contrast, for the glucuronidation of *p*-nitrophenol an enhancement of the enzymatic activity has been observed by ageing and by treatment of enzyme preparations of rat liver with detergents (Winsnes, 1969; Mulder, 1970) or with ultrasound (Henderson, 1970). Table 4 shows the influence of various treatments on the enzymatic activities of the liver preparations in the present study. The data presented confirm the findings of the studies mentioned above. Positive or negative effects of the treatments appeared both in rat and roach liver preparations.

The effects observed may be explained by reference to the conception, that a disturbance of the structural properties of the lipoproteinic microsomal membranes on the one side leads to a decrease in the oxidative drug-metabolizing enzymatic activities by change of an essential lipophilic microenvironment of the enzyme proteins (see chapter II), on the other hand it may cause an increase in glucuronidating enzymatic activity by removal of barriers between protein and substrate and/or cosubstrate (Winsnes, 1969; Hänninen *et al.*, 1970; Henderson, 1970; Mulder, 1970).

TABLE 4 - Influence of various treatments on drug-metabolizing enzymatic activity

| treatment | per cent stimulation (+) or inhibition (—) | | | | | |
|---|---|---|---|---|---|---|
| | *N*-demethylation* of aminopyrine | | *p*-hydroxylation* of aniline | | glucuronidation** of *p*-nitrophenol | |
| | rat | roach | rat | roach | rat | roach |
| storage (5 h at room temperature) | —14 | —42 | —5 | —15 | +16 | +62 |
| storage (15 h at -15 °C) | —16 | —13 | —8 | —10 | +41 | +30 |
| storage (at -15 °C) with repeated freezing and thawing (24, 48, and 72 h after preparation) | —50 | —48 | —30 | —25 | +150 | +75 |
| bile fluid (enzyme preparation + bile = 4 + 1, vol.) | —23 | —86 | —9 | —81 | +65 | +40 |
| deoxycholate (added to enzyme preparation until 0.05% deoxycholate) | —80 | —92 | —68 | —75 | +1250 | +450 |
| ultrasound*** | —26 | —32 | —39 | —27 | +400 | +140 |

Each value is the mean of the results of 2 separate experiments which never differed more than 30%.

* assayed in 9000 *g* supernatant

** assayed in total homogenate

*** MSE 100 Watt Ultrasonic Disintegrator, 60 seconds at maximum output

### IV, 2.3. *Choice of enzyme preparation for the present study*

The data of IV, 2.1. indicate the occurrence of a decrease in enzymatic capacity during the preparation of the microsomal fraction and a requirement for the soluble fraction. The studies of Dallner and Ernster (1968) showed that the composition of the microsomal fraction is related to the origin of the tissue and to its sensitivity to the isolation procedure. On the basis of these data it seemed preferable, in the present comparative study about the differences in drug-metabolizing capacities of different animal species, to measure the capacities for oxidative reactions with the 9000 *g* supernatants of the liver homogenates, and for glucuronidation with the total homogenates. The preparative procedure had the advantage that there was only a short interval between the excision of the liver from the animal and

the final enzyme assay. The preparation of tissue was performed for each animal species in a standardized way (chapter III, 3). Reproducibility was proven for various liver samples of similar origin. Nevertheless, for this investigation it was necessary to presume that a possible difference in the sensitivity of the livers of different animal species to the preparative procedure had not led to essential differences in the composition of the homogenates and 9000 *g* supernatants employed.
According to the observations reported in this chapter (IV, 2.2.), for the oxidative reactions freshly prepared 9000 *g* supernatants were always used, and for the glucuronidation reaction freshly prepared homogenates. Before the assay of glucuronidation the homogenates were activated by ultrasonication (Henderson, 1970).

## IV, 3. MEASUREMENT OF THE ENZYMATIC ACTIVITY

In preliminary experiments, the influence of changes in temperature, incubation time, pH, and concentration of the various reaction components on the substrate turnover were studied. Each time only one of the reaction conditions was changed whereas the others were kept constant. The information obtained about the optimal range of the various conditions was used to perform the experiments described here. One of the reaction conditions was changed again, while all other factors were kept constant (chapter III).

### IV, 3.1. *Influence of temperature, incubation time, and pH on enzymatic activity*

The xenobiotic-metabolizing activities of 9000 *g* supernatants of liver homogenates from various animal species were determined at different temperatures, whereas the other reaction conditions (incubation time, pH, ionic strength) were kept constant (for details see chapter III). Figure 7 shows that differences can be observed in the temperatures at which the liver preparations studied exhibited their maximal *N*-demethylating activities (incubation time 10 min). The temperature for measurement of maximal enzymatic activity

FIGURE 7. *N*-Demethylation of aminopyrine by 9000 *g* supernatants prepared from liver homogenates of various animal species. *N*-demethylating activity was assayed in various separate sets of experiments. In each set activity was measured for 10 min at different temperatures. The values of each series were expressed in relation to the maximal value (numerically 100).
Note the differences in the temperature at which the enzyme preparations from different species exhibit their maximal activities.

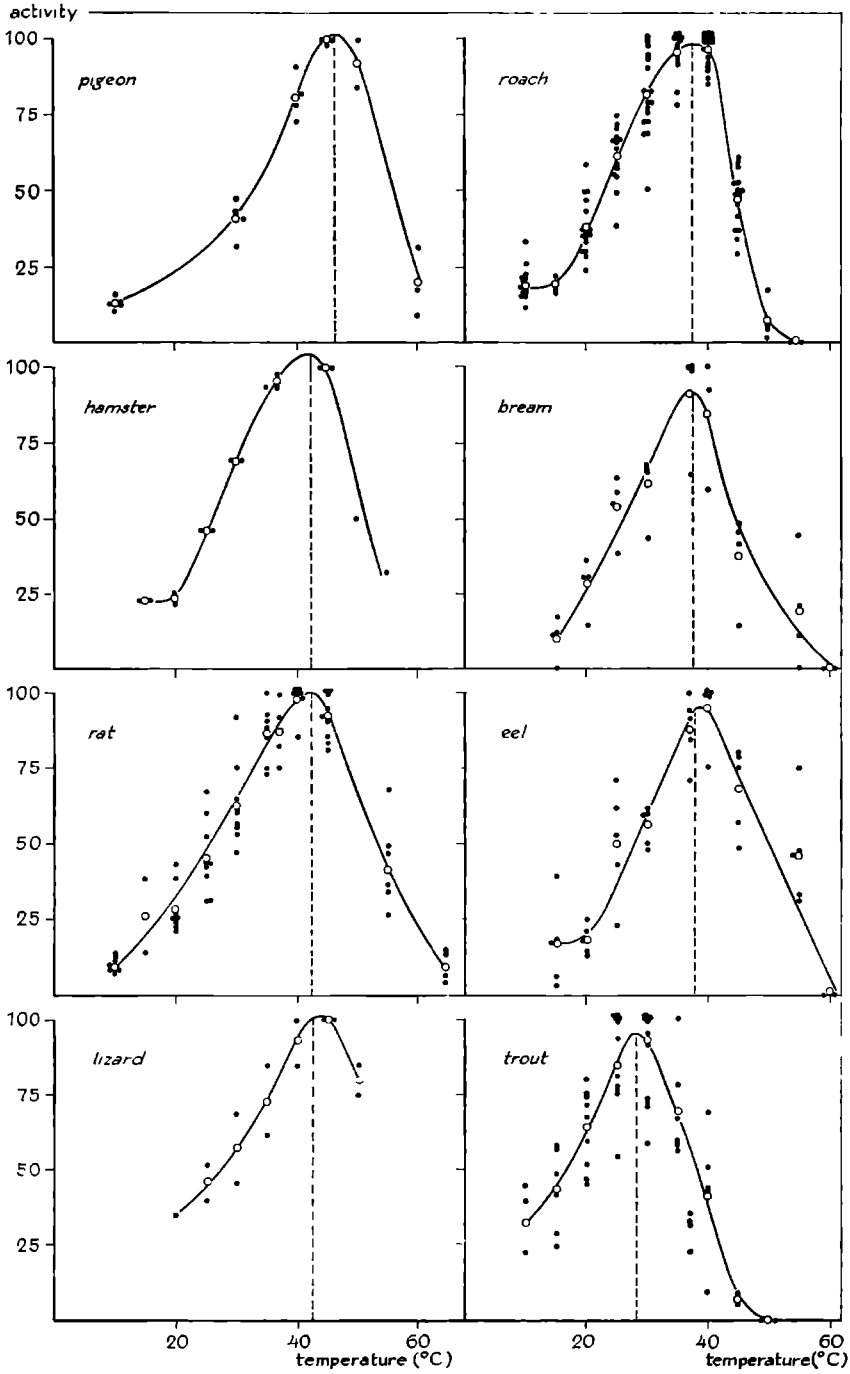

activity
100
75
50
25
pigeon
roach
hamster
bream
rat
eel
lizard
trout
20
40
60
temperature (°C)
temperature(°C)

TABLE 5 - *N*-demethylation and *p*-hydroxylation *in vitro* in relation to the incubation temperature

| animal species | incubation temperature (°C) range optimum* | operative temperature | per cent activity§ *N*-demethylation of aminopyrine | *p*-hydroxylation of aniline |
|---|---|---|---|---|
| pigeon | 45 — 50 | 42 | 83 ± 5 | 85 ± 3 |
| hen | 45 — 50 | 42 | 85 | 85 |
| hamster | 40 — 45 | 37 | 93 | 84 ± 4 |
| rat | 40 — 45 | 37 | 89 ± 3 | 72 ± 4 |
| lizard | 40 — 45 | 25 | 46 | |
| frog | 35 — 40 | 25 | 64 ± 8 | 86 ± 4 |
| bream | 35 — 40 | 25 | 53 ± 8 | 83 |
| white bream | 35 — 40 | 25 | 46 ± 7 | 87 ± 7 |
| orfe | 35 — 40 | 25 | 46 ± 3 | 67 ± 13 |
| roach | 35 — 40 | 25 | 61 ± 3 | 72 ± 3 |
| tench | 30 — 40 | 25 | 48 ± 6 | 68 ± 6 |
| eel | 35 — 40 | 25 | 50 ± 8 | 63 |
| perch | 35 — 40 | 25 | 54 | 75 |
| pike | 30 — 40 | 25 | 47 ± 4 | 77 ± 7 |
| salmon | 30 — 35 | 25 | 65 | 87 |
| trout | 25 — 30 | 25 | 85 ± 3 | 93 ± 4 |
| sea lamprey | 30 — 35 | 25 | 80 | 85 |

§ enzymatic activity measured at the operative temperature as a percentage of the maximal activity; means of at least 4 experiments ± S.E.M.

* temperature range of the two reactions for measurement of maximal activity; incubation time: *N*-demethylation, 10 min; *p*-hydroxylation, 30 min.

for the *p*-hydroxylation of aniline by liver preparations from various animal species (incubation time 30 min) were found within the same range as those for the *N*-demethylation of aminopyrine. In the present study, as the operative temperature for study of the enzyme reactions *in vitro* — unless otherwise stated — were chosen for the avian species 42 °C, for the mammalian species 37 °C, and for the poikilothermic species 25 °C. The main reasons for this choice were: the temperatures chosen approximate the physiological temperatures of the animals; they allow the employment of incubation periods during which the enzymes studied remain stable (product formation linear with time), whereas product formation can continue until a well-measurable amount. However it has to be stressed that the values obtained at the operative temperatures were not the maximally obtainable *in vitro* activities. The maximal enzymatic activities (obtained at fixed incubation times) can be derived from table 5.

Since the temperature at which maximal enzymatic activity can be measured

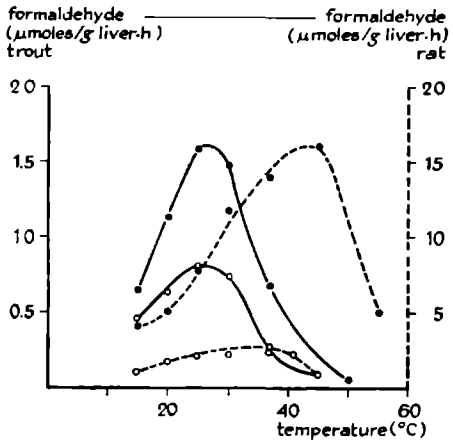



FIGURE 8. *N*-Demethylation of aminopyrine measured at different temperatures with 9000 *g* supernatants prepared from liver homogenates of rat and trout. The enzymatic activities are expressed as μmoles formaldehyde produced per g fresh liver per hour. The activities were measured during different incubation periods • —— • trout, 10 min incubation; o —— o trout, 60 min incubation; • - - - - • rat, 10 min incubation; o - - - - o rat, 60 min incubation.

is the resultant of two opposed factors — the increase in the substrate turnover and the enhanced time dependent denaturation of the enzyme at higher temperature — the length of the period during which the enzymatic activity is determined, is an important factor. Figure 8 illustrates this for the *N*-demethylation of aminopyrine by the 9000 *g* supernatant from liver homogenates of trout and rat. After an incubation time of 10 min a clear difference in the temperature at which the maximal enzymatic activities were found, became manifest. After much longer incubation periods — for instance 60 min — this distinction was less striking in view of the fact that the denaturation of the enzymes was further progressed.

The decrease in the enzymatic activities at different temperatures as a function of time is shown in figure 9. The *N*-demethylating activity of the trout liver enzyme at 25 °C remained constant for 20 - 30 min and then declined. At 37 °C the drop in activity started much earlier and was more pronounced. The *N*-demethylating enzyme of the rat, too, appeared to be more stable at 25 °C, but the maximal activity at this temperature was only a fraction of the maximal activity at 37 °C. At 37 °C the *N*-demethylating activity of the rat liver enzyme remained constant only for 5 - 10 min, and then rapidly declined.

Product formation in *N*-demethylation and *p*-hydroxylation, and the disappearance of free *p*-nitrophenol in glucuronidation in the course of the

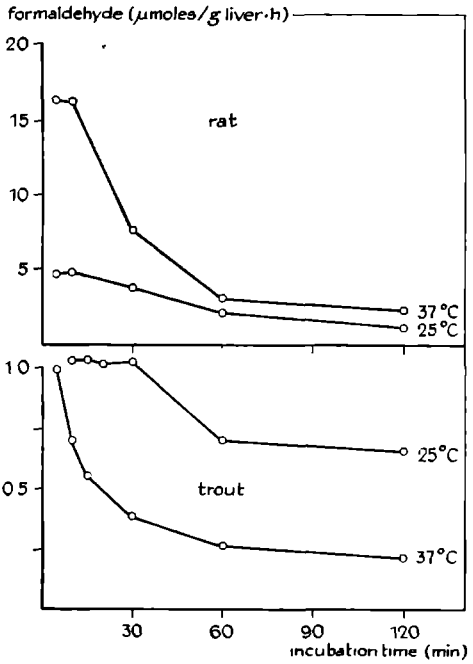



FIGURE 9. The decrease in the *N*-demethylating enzymatic activities in the 9000 *g* supernatants prepared from liver homogenates of rat and trout at 37 and 25 °C as a function of time. Enzymatic activities are expressed as μmoles formaldehyde produced per g fresh liver per hour.
Note: the initial reaction velocity remains constant only for a short period.

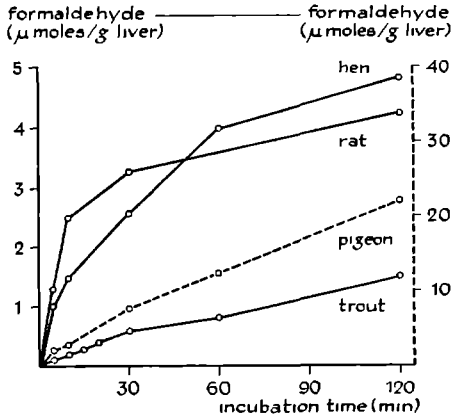



FIGURE 10. The production of formaldehyde in the *N*-demethylation of aminopyrine by 9000 *g* supernatants prepared from liver homogenates of various animal species as a function of time. The amount of formaldehyde produced is expressed as μmoles formed per g fresh liver. Incubation temperatures: 42 °C (hen, pigeon); 37 °C (rat); 25 °C (trout).

incubation period is shown for various species in figures 10, 11 and 12 respectively. In comparison with the *N*-demethylation of aminopyrine the two other reactions proceeded in a linear fashion over longer periods, which is in accordance with earlier observations on time course studies of these reactions in rat and rabbit (Leadbeater and Davies, 1964; Gram and Fouts, 1966a; Schenkman *et al.*, 1967a). In the figures 10, 11 and 12 it can be seen that during the incubation period the enzymatic activities for the

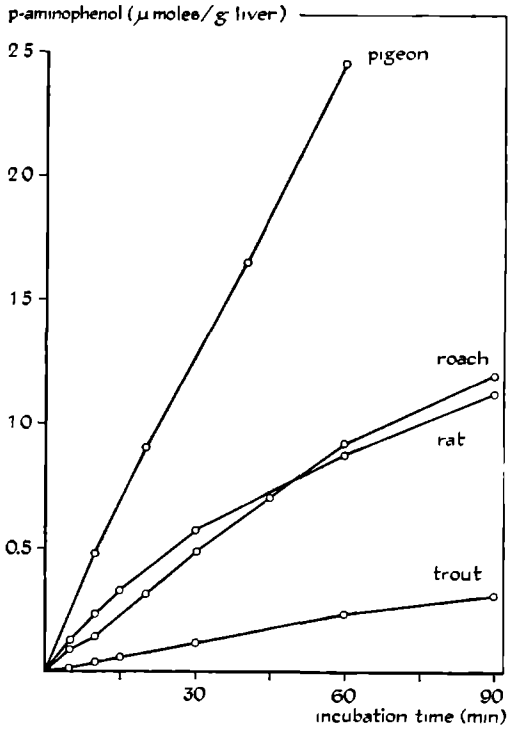



FIGURE 11. The production of *p*-aminophenol in the *p*-hydroxylation of aniline by 9000 *g* supernatants prepared from liver homogenates of various animal species as a function of time. The amount of *p*-aminophenol produced is expressed as μmoles formed per g fresh liver. Incubation temperatures: 42 °C (pigeon); 37 °C (rat); 25 °C (roach, trout).

various species may decrease at different rates. This confirms the work of Gram and Fouts (1966a), who reported the relative lability of several rat-liver microsomal drug-metabolizing enzymes and the relative stability of those in the rabbit.

The decrease in reaction velocity during the incubation period described

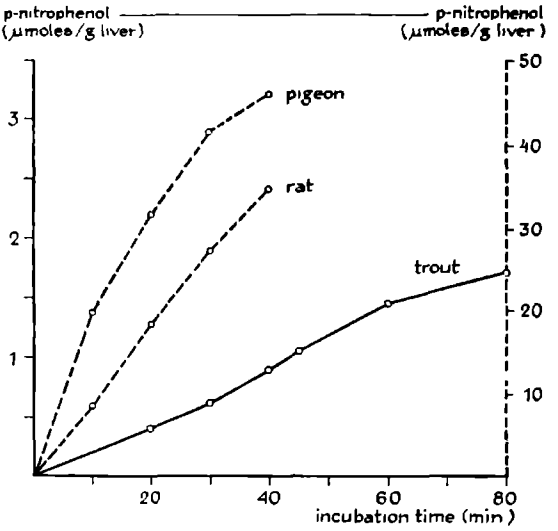



FIGURE 12. The disappearance of free *p*-nitrophenol in the glucuronidation reaction by total liver homogenates of pigeon, rat, and trout as a function of time. The amount of *p*-nitrophenol coupled is expressed as μmoles conjugated per g fresh liver. Incubation temperatures: 42 °C (pigeon); 37 °C (rat); 25 °C (trout).

above was not caused by exhaustion of the substrate or cofactor system, since these were added in saturating concentrations (chapter IV, 3.4), but rather by inactivation of the enzyme. This could be confirmed by experiments in which further addition of substrate and cosubstrate during the incubation period could not prevent the loss of activity, and by experiments in which preincubation of the enzyme preparation prior to the start of the *in vitro* reaction resulted in a substantial loss of activity. Gram and Fouts (1966a, 1966b) reported that preincubation of microsomal preparations, especially in the presence of NADPH, resulted in a deterioration of enzymatic activity similar to the inactivation that occurred in the reaction system. Schenkman *et al.* (1967a) confirmed this and noticed that during the enzyme inactivation clumps were appearing in the previously homogeneous suspension. This agglutination of particles was considered to contribute to the observed decline in enzymatic activity. Furthermore, it was suggested that a damage to enzymes and other proteins caused by lipid peroxidation (Roubal and Tappel, 1966) plays a role in microsomal deterioration. However, Gram and Fouts (1966b) showed that inhibition of the lipid peroxidation by the antioxidant α-tocopherol was without effect on the early decline in drug-oxidation activity. Moreover, in agreement with the work of Orrenius *et al.* (1964), Gram and Fouts (1966b) found that aniline and aminopyrine, the sub-

strates of drug-metabolizing reactions, inhibited lipid peroxidation. On the other hand, the inhibition of lipid peroxidation in microsomes has been found to stimulate aldrin epoxidation (Lewis *et al.*, 1967). It can be concluded that, at the present, the factors underlying the loss of microsomal enzymatic activity during incubation, which apparently depends upon the reaction and the animal species studied, are only partially understood.

In view of the decrease in enzymatic activity during incubation, for the assays of the initial velocities of the enzymes of the various species relatively short incubation periods were chosen in the present study (product formation remained linear with time), viz. for *N*-demethylation always shorter than 15 min, and for *p*-hydroxylation and glucuronidation always shorter than 30 min.

To study the influence of the pH, the drug-metabolizing enzymatic activities were compared in test systems prepared with Tris-HCl buffer solutions of different pH. In this way, information was obtained about the range of pH within which the hepatic enzyme preparations of various animal species exhibited their maximal capacities of substrate turnover (table 6). According to these observations pH 8.0 was chosen for the *in vitro* assay of the enzymatic activity of the oxidative reactions and pH 7.4 for the glucuronidation reaction. However, it can be questioned whether these pH-values are also optimal for the other tissue preparations studied.

TABLE 6 - pH-range for measurement of maximal activity*

| | *N*-demethylation of aminopyrine | *p*-hydroxylation of aniline | glucuronidation of *p*-nitrophenol |
|---|---|---|---|
| pigeon | 7.8 — 8.2 | — | 7.0 — 7.5 |
| rat | 7.9 — 8.1+ | 7.9 — 8.3 | 7.1 — 7.5 |
| roach | 7.0 — 8.0 | 7.1 — 8.1 | — |
| trout | 7.9 — 8.4+ | 7.7 — 8.2 | 6.5 — 7.6 |

* pH was determined in the reaction mixtures during the incubation period
\+ (Dewaide and Henderson, 1968).

IV, 3.2. *Effect of various additives on enzymatic activity*

Many investigators have reported that semicarbazide and nicotinamide are indispensable in measuring oxidative *N*-demethylating enzymatic activities (La Du *et al.*, 1955; Smith *et al.*, 1963; Orrenius, 1965; Anders and Mannering, 1966; Soyka, 1969; Ackermann, 1970). Semicarbazide is used to trap the formaldehyde, produced in the enzymatic reaction, as the semicarbazone. Nicotinamide, also employed in many other oxidative drug-metabolizing

TABLE 7 - Influence of nicotinamide and semicarbazide on the *N*-demethylation of aminopyrine

| nicotinamide concentration ($10^{-3}$ M) | semicarbazide concentration ($10^{-3}$ M) | per cent determined formaldehyde* rat liver (37°, 10 min incubation) | trout liver (25°, 20 min incubation) |
|---|---|---|---|
| 0 | 5 | 100 | 100 |
| 5 | 5 | 94 | 65 |
| 20 | 5 | 82 | 51 |
| 50 | 5 | 69 | 42 |
| 0 | 0 | 82 | 76 |
| 0 | 5 | 100 | 100 |
| 0 | 8 | 97 | 94 |
| 0 | 20 | 62 | 63 |
| 0 | 50 | 7 | 9 |

* The amounts of produced formaldehyde are expressed as percentages of the maximal values; means of the results of 3 experiments. Maximal and minimal value never deviated more than 5% of the mean given.

reactions, inhibits the breakdown of NADP by liver NADPnucleosidase (E.C. 3.2.2.6). However, an inhibition by nicotinamide of the aminopyrine *N*-demethylation and aniline *p*-hydroxylation has been reported by Schenkman *et al.* (1967a).

In the present study the decrease in the *N*-demethylating activity by the addition of nicotinamide to the reaction mixture in concentrations of 5 to 50 x $10^{-3}$ M was confirmed. Table 7 shows that the inhibiting action of nicotinamide was more pronounced in the *N*-demethylation by trout liver enzyme than by rat liver enzyme. In view of the inhibition of nicotinamide on the initial reaction velocity, nicotinamide was omitted in the assays described in this report.

Semicarbazide used in concentrations higher than 5 x $10^{-3}$ M also caused an inhibition (table 7). The decrease in the amounts of formaldehyde measured with the method of Nash (1953) was found to be due to an interference of semicarbazide with the colorimetric determination. This could be demonstrated by quantitative assays of standard formaldehyde concentrations in the presence of increasing amounts of semicarbazide, which below 5 x $10^{-3}$ M had no influence, but in concentrations of 20 x $10^{-3}$ M and 50 x $10^{-3}$ M depressed colour development to about 80 per cent and about 10 per cent respectively. In view of the data of table 7 it was decided to use semicarbazide in the reaction mixture in a concentration of 5 x $10^{-3}$ M.

As far as the application of divalent cations in the reaction mixtures is

concerned, it has been shown that $Mg^{++}$ and $Mn^{++}$ both stimulate the NADPH-generating enzyme systems employed (Kornberg, 1950; Cooper and Brodie, 1955), and many of the microsomal drug-metabolizing reactions (Trivus and Spirtes, 1964). Recently the influence of magnesium and some other divalent cations on hepatic oxidative microsomal drug metabolism *in vitro* has been extensively investigated by Peters and Fouts (1970a and 1970b), whereas the enhancement of glucuronidation reactions *in vitro* by $Mg^{++}$ has been reported by Mulder (1970).

In the glucuronidation reaction a possible enzymatic hydrolysis of the glucuronide by endogenous $\beta$-glucuronidase was prevented by the addition of saccharo-1,4-lactone ($10^{-3}$ M), which is a known inhibitor of the hydrolytic enzyme (Levvy and Conchie, 1966).

The inhibitory action of carbon monoxide and $\beta$-diethylaminoethyl diphenylpropylacetate (SKF 525A) on the oxidative drug-metabolizing enzyme systems of mammals is well-known (chapter II). In this study it appeared that the *N*-demethylating and *p*-hydroxylating activities of enzyme preparations from various fish species were also strongly inhibited (50% decrease) in the presence of these substances (30 sec bubbling of CO during incubation or addition of $10^{-4}$ M SKF 525A). This indicates similarities in the properties of drug-metabolizing enzymes of mammals and fish.

### IV, 3.3. *Effect of the nature of the NADPH-generating system*

The cofactor NADPH was supplied to the reaction mixture as NADP in combination with a reducing system, such as glucose-6-phosphate and glu-

TABLE 8 - Effect of the nature of the NADPH-generating system on the *N*-demethylation of aminopyrine

| cofactor system* | *N*-demethylation of aminopyrine+ ($\mu$moles/g liver . h) § | |
|---|---|---|
| | rat | trout |
| NADPH | 9.88 ± 0.74** | 1.40 ± 0.19** |
| G-6-PD | 14.81 ± 0.20 | 1.12 ± 0.03 |
| ICD | 14.35 ± 0.26 | 1.15 ± 0.07 |

+ concentration: $16.7 \times 10^{-3}$ M.

§ values are the means of 10 experiments ± S.E.M.

* NADPH ($13.3 \times 10^{-5}$ M) was added without reducing enzyme system; NADP ($13.3 \times 10^{-5}$ M) was added in combination with glucose-6-phosphate and glucose-6-phosphate dehydrogenase (G-6-PD) or with isocitrate and isocitrate dehydrogenase (ICD). Concentrations are given in chapter III, 4. 1.

** Significantly different from the values obtained with the reducing enzyme systems at $P < 0.01$ (Wilcoxon two-sided two-sample test).

cose-6-phosphate dehydrogenase, or isocitrate and isocitrate dehydrogenase. Table 8 presents the *N*-demethylating activities of rat and trout liver in the presence of either NADPH as such or NADP with the reducing systems mentioned. The results are in accordance with the findings of Gram and Fouts (1967) and of Janson (1968), who reported, for rabbit and rat respectively, lower activities with NADPH as such for the *N*-demethylation of aminopyrine and some other drug-metabolizing reactions. For the trout, however, in the case of NADPH addition, significantly higher activities have been observed. This increase in enzymatic activity appeared to be 10 - 25% in a number of fish species, e.g. roach, bream, and trout. For practical reasons — the rather low stability of the cosubstrate in the reduced form and for reasons of economy — it was decided, however, for the measurement of oxidative drug-metabolizing reactions for fish also to make use of one of the NADPH-generating systems described above.

### IV, 3.4. *Substrate and cosubstrate concentrations*

In enzymatic reactions for the enzymes of different origin differences may be observed in the interaction between the enzyme proteins and the substrate and cosubstrates, and in the rate of substrate conversion. According to the Michaelis-Menten kinetics for enzyme reactions mathematically two parameters can be derived: the maximum velocity of the reaction ($V_{max}$) and the affinity between the enzyme protein and the substrate. The latter is expressed as the dissociation constant ($K_s$), which is often assumed to equal the Michaelis constant ($K_m$), which represents the substrate concentration at $\frac{1}{2}V_{max}$. Both kinetic parameters can be derived from enzyme-substrate saturation curves: the transformation rates of the substrate at different concentrations by purified enzyme. It has to be noted, however, that from the viewpoint of enzymology the application of the above mentioned kinetic parameters in the study of oxidative drug-metabolizing enzyme reactions is disputable. The microsomal enzyme proteins are embedded in structural proteins and phospholipids, and it is the whole structure which participates in the metabolic process. In view of this complex structure, it appeared hitherto impossible to perform a reaction with the purified enzyme system. Moreover, regardless of the complexicity of the reaction system, which requires multiple steps between the initial electron donor and the substrate, the measurements are only based either on the formation of an end product, the disappearance of the initial substrate, or the disappearance of the electron donor. In this study, however, enzyme-substrate saturation curves were still made in order to determine the sub-

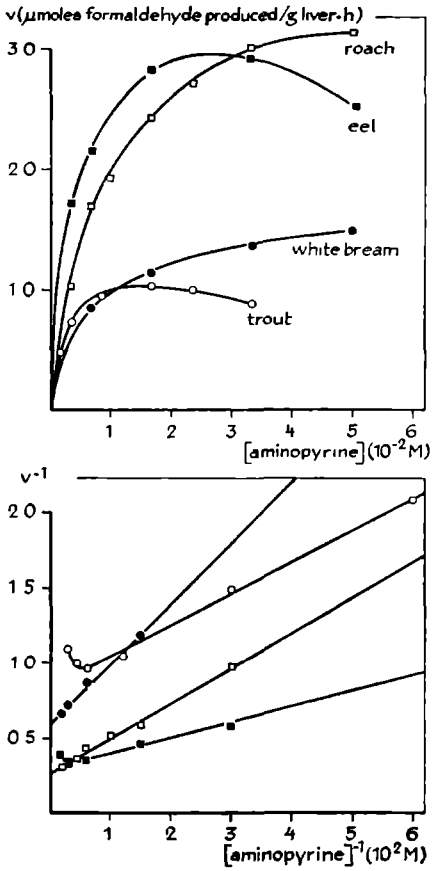



FIGURE 13. Initial reaction rates of the *N*-demethylation of aminopyrine by 9000 *g* supernatants prepared from liver homogenates of various fish species in relation to different concentrations of the substrate. The other reaction conditions (e.g. saturating concentrations of cosubstrate) are described in chapter III. In the lower part of the figure the kinetic data are plotted according to Lineweaver-Burk.

strate concentration at which the enzyme preparations exhibited their maximal transformation rates. This can be illustrated for the *N*-demethylation of aminopyrine (figure 13), the *p*-hydroxylation of aniline (figure 14), and the glucuronidation of *p*-nitrophenol (figure 15) for the enzyme preparations derived from various species. From the lower parts of these figures in which the values are plotted according to Lineweaver-Burk, it can be derived that the crude enzyme systems behave regarding the substrates as described in Michaelis-Menten kinetics. However, the question remains, as to how far the kinetic parameters derived from those graphs — indicated

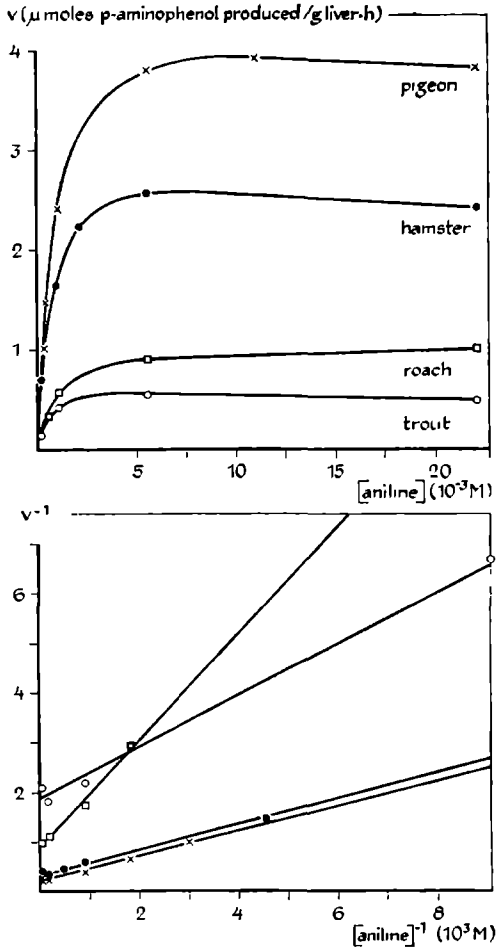



FIGURE 14. Initial reaction rates of the *p*-hydroxylation of aniline by 9000 *g* supernatants prepared from liver homogenates of various animal species in relation to different concentrations of the substrate. The other reaction conditions (e.g. saturating concentrations of cosubstrate) are described in chapter III. In the lower part of the figure the kinetic data are plotted according to Lineweaver-Burk.

further as "apparent $K_m$" and "apparent $V_{max}$" — represent the true kinetic properties of the enzymes. A comparative survey and a discussion of the results obtained are given in chapter VI. Here it will only be stressed that the concentration level at which the substrate and cosubstrate saturate the enzyme system applied can be different for the various animal species. Therefore, in the enzymatic reactions *in vitro* in order to measure maximal reaction velocities, the substrate and cosubstrate concentrations were kept at or above satur-

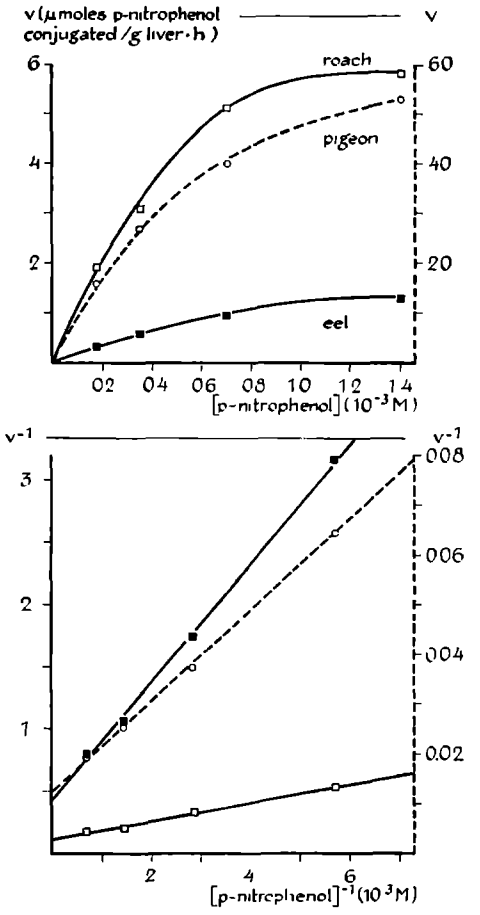



FIGURE 15. Initial reaction rates of the glucuronyltransferase in ultrasonicated total liver homogenates of various species in relation to different concentrations of the substrate. The other reaction conditions (e.g. saturating concentrations of cosubstrate) are described in chapter III. In the lower part of the figure the kinetic data are plotted according to Lineweaver-Burk.

ating levels. Table 9 presents the concentrations of substrates and cosubstrates used. These concentrations were standardized for the various groups of animals. In the experiments described under this heading it was verified that with these concentrations, for each animal species, maximal initial rates of substrate conversion were measured. In figure 13 it can be seen that, at high aminopyrine concentration, for the trout and the eel, an inhibition of the enzyme activity occurred.

TABLE 9 - Substrate and cosubstrate concentrations for measurement of maximal enzymatic activity in liver preparations of different animal species §

| enzyme reaction | *N*-demethylation | *p*-hydroxylation | glucuronidation |
|---|---|---|---|
| substrate | aminopyrine | aniline | *p*-nitrophenol |
| concentration | $33.4 \times 10^{-3}$ M fishes (except trout)<br>$16.7 \times 10^{-3}$ M all other animals (including trout) | $22 \times 10^{-3}$ M all animals (except trout)<br>$5.5 \times 10^{-3}$ M trout | $1.4 \times 10^{-3}$ M all animals |
| cosubstrate | NADP* | NADP* | UDPGA |
| concentration | $13.3 \times 10^{-5}$ M all animals | $13.3 \times 10^{-5}$ M all animals | $6.0 \times 10^{-3}$ M all animals |

§ animal species investigated are given in chapter III.

* NADPH was supplied via enzymatic reduction of NADP.

The saturation level of the cosubstrate NADPH in the oxidative reactions was determined by the addition of increasing amounts of NADP in combination with one of the reducing systems mentioned above (chapter III, 4.1. and chapter IV, 3.3.).

### IV, 3.5. *Enzyme concentration*

As a final test whether the assays described here could be used for measurement of drug-metabolizing enzymatic capacities *in vitro*, experiments were performed with decreasing amounts of the enzyme preparation in the reaction mixture. In the figures 16, 17 and 18, representing the *N*-demethylation, *p*-hydroxylation, and glucuronidation respectively, the amounts of enzyme prep-

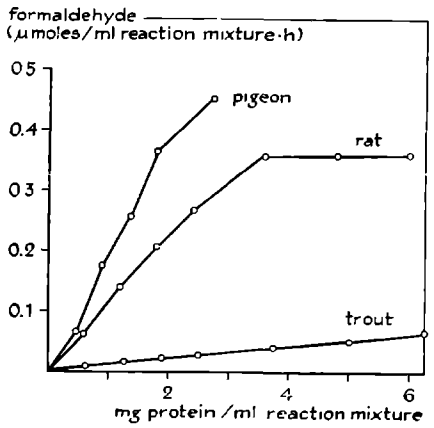



FIGURE 16. The formation of formaldehyde, expressed as μmoles produced per ml reaction mixture per hour, in the *N*-demethylation of aminopyrine by 9000 *g* supernatants prepared from liver homogenates of pigeon, rat, and trout in relation to the amount of enzyme preparation present. The amount of 9000 *g* supernatant employed is expressed as the amount of protein added to 1 ml reaction mixture.

arations are given in mg tissue protein per ml reaction mixture. Taking into account these observations, the standard assays of the capacity of the oxidative enzymes for the various animal species were carried out with enzyme preparations with amounts of liver protein within the range of 1.5 - 2.5 mg per ml reaction mixture (amounts of 9000 *g* supernatant equivalent to 20 - 33 mg fresh liver; see chapter III, 4.1.). The glucuronyl conjugation capacity of the fish species was measured with enzyme preparations with

an amount of hepatic protein within the range of 4 - 8 mg per ml reaction mixture (amounts of homogenate equivalent to 25 - 50 mg fresh liver). The glucuronyl conjugation capacity of the mammalian and avian species was measured with enzyme preparations with an amount of liver protein

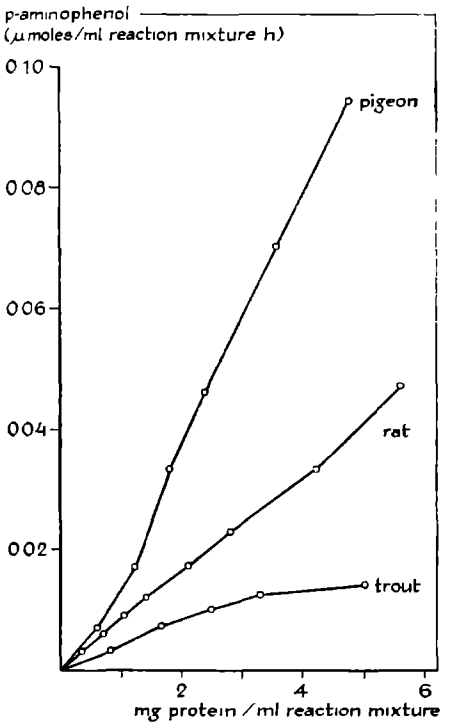



FIGURE 17. The formation of *p*-aminophenol, expressed as μmoles produced per ml reaction mixture per hour, in the *p*-hydroxylation of aniline by 9000 *g* supernatants prepared from liver homogenates of pigeon, rat, and trout in relation to the amount of enzyme preparation present. The amount of 9000 *g* supernatant employed is expressed as the amount of protein added to 1 ml reaction mixture.

within the range of 2 - 4 mg per ml reaction mixture (amounts of homogenate equivalent to 12 - 24 mg fresh liver) (see chapter III, 4.4.).
The proportionality between the rate of conversion and the amount of liver preparation employed has been verified for all species studied. This may indicate that the values obtained with the methods used in this study are useful parameters for the enzymatic capacities of the liver preparations investigated.

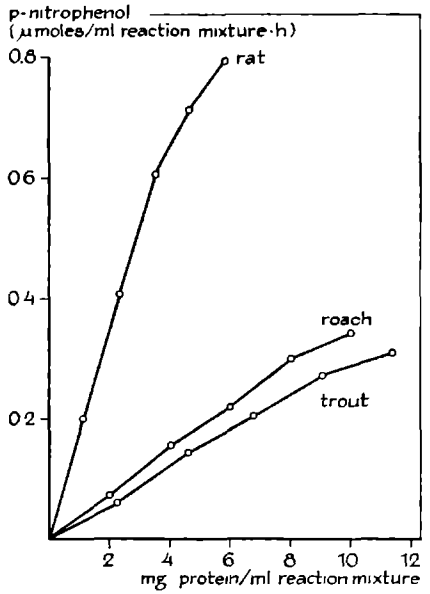



FIGURE 18. The glucuronidation of *p*-nitrophenol, expressed as μmoles conjugated per ml reaction mixture per hour by ultrasonicated total liver homogenates of rat, roach, and trout in relation to the amount of enzyme preparation present. The amount of homogenate employed is expressed as the amount of protein added to 1 ml reaction mixture.

## IV, 4. CONCLUSION

From the results presented in this chapter as a main conclusion it can be stated that xenobiotic-metabolizing activities can be observed in liver preparations from aquatic animal species. This is inconsistent with previous data from other studies in which these *in vitro* activities have not been detected. As far as general phenomena are concerned, such as the intracellular localization, the sensitivity of the enzyme preparations to various treatments, the requirements of various cofactors, and the inhibiting action of carbon monoxide and SKF 525A on the oxidative enzymes, the properties of the drug-metabolizing enzymes of the various animal species appear to have much in common. On the other hand, in a more detailed view, the nature of the enzymes of various species shows differences, for instance in stability, and optimal substrate and cosubstrate concentrations. The data from the literature about the lack of an enzymatic xenobiotic-metabolizing capacity in fish may be explained in view of the fact that the species differences discussed in this chapter were left out of account in the enzyme assays in previous studies.

# PRODUCT FORMATION IN THE *N*-DEMETHYLATION OF AMINOPYRINE *IN VITRO*

## V, 1. INTRODUCTION

Studies on the biotransformation of aminopyrine in various mammalian species revealed the presence of a great variety of metabolic products in blood and urine (Halberkann and Fretwurst, 1950; Brodie and Axelrod, 1950; Pechtold, 1964; Preuss and Voigt, 1965; Schüppel and Soehring, 1965; Vecerková *et al.*, 1967; Klug, 1970). From these studies it appeared that *N*-demethylation is of major importance in the metabolism of aminopyrine: in all of the metabolic products one or both methyl groups have been found to be split from the original dimethylamino group of the parent compound.
It can be expected that the *N*-demethylation of aminopyrine (DMAP)

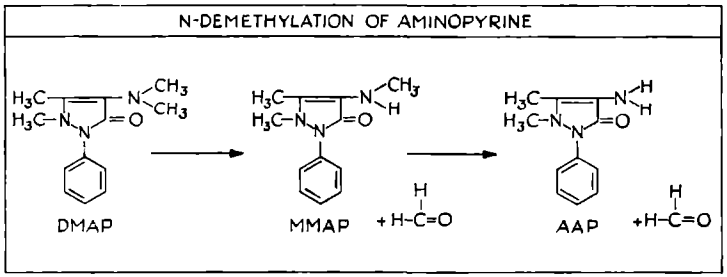



FIGURE 19. Two-step mechanism in *N*-demethylation of aminopyrine.

proceeds through two steps (figure 19). The first *in vitro* measurements of this reaction were performed by La Du *et al.* (1955). The assays were based both on the formation of formaldehyde and of 4-aminoantipyrine (AAP). La Du and co-workers found that the ratio of formaldehyde to AAP produced was 2 : 1, which indicated that the substrate DMAP was *N*-demethylated to AAP only. If the intermediate product 4-monomethylaminoantipyrine (MMAP) were also formed, then the ratio formaldehyde/AAP would have been greater than 2. A ratio for formaldehyde/AAP greater than 2 has

been reported by Gram *et al.* (1968), who studied the *N*-demethylation of DMAP both for rabbit and rat liver microsomes. Using thin-layer chromatography (TLC), they also showed that incubation of DMAP in reaction mixtures with 9000 *g* supernatants led to the formation of relatively large amounts of MMAP but only small quantities of AAP. The identification of the substances was only based upon comparison of $R_f$-values.

A more thorough identification of the *N*-demethylation products was performed in the present study. Experiments with regard to the interesting question whether the two steps of the *N*-demethylation of DMAP are catalyzed by the same enzyme or whether different enzymes are involved could not be performed, since the amount of MMAP available was too small for use as a substrate in the reaction. Therefore, the experiments described here were restricted to the oxidative *in vitro* *N*-demethylation of DMAP. Since the studies reported in the literature were all based upon colorimetric measurements of the formaldehyde and/or AAP produced, it was of interest to measure to what extent the *N*-demethylation of DMAP follows the two steps assumed (figure 19). Emphasis was put upon the methods for quantitative measurement of the reaction products MMAP, AAP, and formaldehyde.

## V, 2. IDENTIFICATION OF THE REACTION PRODUCTS

The extraction of the reaction products and the analytical methods employed in order to elucidate their nature are described in chapter III, 5. TLC of the extracts yielded spots of which the $R_f$-values are given in table 10. A comparison of these values already gives some indication about the identity of the products of the reaction. In the chromatograms 3 spots were mostly observed, with $R_f$-values corresponding to those of the reference pyrazolone derivatives: spot number 1, a very intense and large spot with an $R_f$-value corresponding to that of the substrate DMAP; spot number 2, a clear small spot with an $R_f$-value corresponding to that of reference MMAP; spot number 3, having an $R_f$-value corresponding to that of reference AAP. The latter spot had a very weak intensity, and was sometimes not detected. The spot was absent in the chromatograms developed for reaction mixtures in which high substrate concentrations (16.7 - 33.4 mM) and short incubation time (10 min) were employed. Therefore, in the methods used it can be imagined that the large quantity of DMAP present hampered the detection of a possibly small amount of AAP produced. Also the possibility exists that the second step in the *N*-demethylation reaction (from MMAP to AAP) is inhibited by high DMAP concentrations.

TABLE 10 - Thin-layer chromatography of pyrazolone derivatives

| samples subjected to TLC | | | | solvent system A | solvent system B | |
|---|---|---|---|---|---|---|
| sample number | origin of the sample | spot number | spot intensity | $R_f$-value | $R_f$-value | $R_f$-value correlates with |
| I | DMAP (reference) | 1 | | 0.69 | 0.29 | |
| II | MMAP (reference) | 2 | | 0.54 | 0.35 | |
| III | AAP (reference) | 3 | | 0.38 | 0.53 | |
| IV | blank reaction mixture, DMAP 16.7 mM*, 10 min incubation | 1 | ++ | 0.70 | 0.29 | DMAP |
| V | reaction mixture, rat liver preparation, DMAP 16.7 mM, 10 min incubation | 1 | ++ | 0.68 | 0.28 | DMAP |
| | | 2 | + | 0.51 | 0.33 | MMAP |
| | | 3 | — | 0.35 | 0.51 | AAP |
| VI | reaction mixture, rat liver preparation, DMAP 0.33 mM, 30 min incubation | 1 | ++ | 0.67 | 0.29 | DMAP |
| | | 2 | + | 0.53 | 0.32 | MMAP |
| | | 3 | — | 0.36 | 0.53 | AAP |
| VII | reaction mixture, roach liver preparation, DMAP 33.4 mM, 10 min incubation | 1 | ++ | 0.69 | 0.27 | DMAP |
| | | 2 | + | 0.53 | 0.34 | MMAP |
| VIII | reaction mixture, roach liver preparation, DMAP 0.33 mM, 30 min incubation | 1 | ++ | 0.69 | 0.29 | DMAP |
| | | 2 | + | 0.54 | 0.35 | MMAP |
| | | 3 | — | 0.37 | 0.54 | AAP |
| IX | reaction mixture, trout liver preparation, DMAP 16.7 mM, 15 min incubation | 1 | ++ | 0.70 | 0.28 | DMAP |
| | | 2 | — | 0.52 | 0.33 | MMAP |

solvent system A: chloroform - diethylether - methanol (70-30-10, vol.);
solvent system B: *n*-butanol - acetic acid - water (80-20-10, vol.);
spot intensity: —, weak; +, high; ++, very high;
* blank reaction mixture: DMAP was added after incubation;
Liver preparations used were 9000 *g* supernatant fractions.
The methods used are described in chapter III, 5.

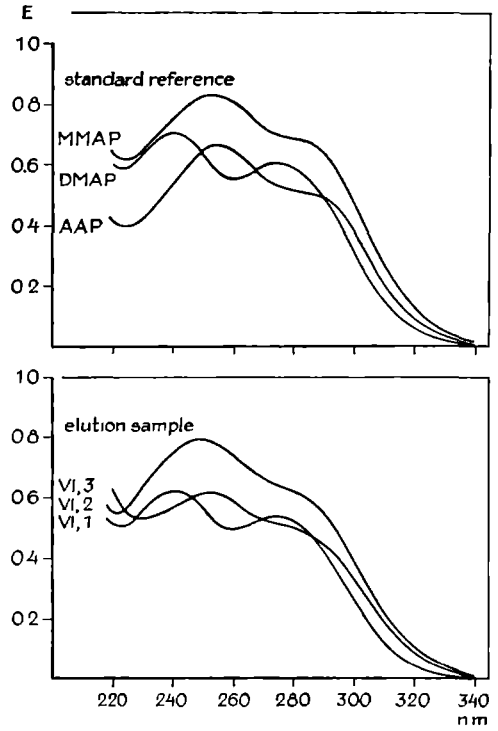



FIGURE 20. Qualitative absorption spectra of pyrazolone derivatives in ethanol. The upper part of the figure shows the spectra of reference substances, the lower part those of eluates from bands on thin-layer chromatograms. The code of the elution samples VI, 1.2.3. refers to the samples mentioned in table 10.

In the chromatogram spots were also observed the nature of which was unknown. The $R_f$-values for these spots in chromatograms developed with solvent system A were 0.80 and 0.30. Since these spots were also present in the control chromatograms, they must represent additional material extracted from the incubation mixtures as such and not be due to substrate, or metabolites thereof.

After staining of the chromatograms with various reagents, the spots in the colour patterns showed an exact resemblance to the corresponding colours obtained with the reference pyrazolone derivatives (table 2, chapter III, 5. 3. 2).

In order to obtain the reaction products in larger amounts, preparative TLC (solvent system A) was performed with extracts from reaction mixtures with a total volume of 240 ml (chapter III, 5.2). Bands were eluted with

ethanol and concentrated by evaporation to a concentration suitable for measurement in a spectrophotometer. However, a definitive proof about the identity of the reaction products could not be obtained on the basis of measurement of the absorption spectra of the eluates because of the close resemblance between the qualitative spectra of MMAP and AAP (figure 20). From this figure it may only be concluded that the substances in the elution samples originate from a pyrazolone molecule.
More relevant information could be obtained by again subjecting the differ-

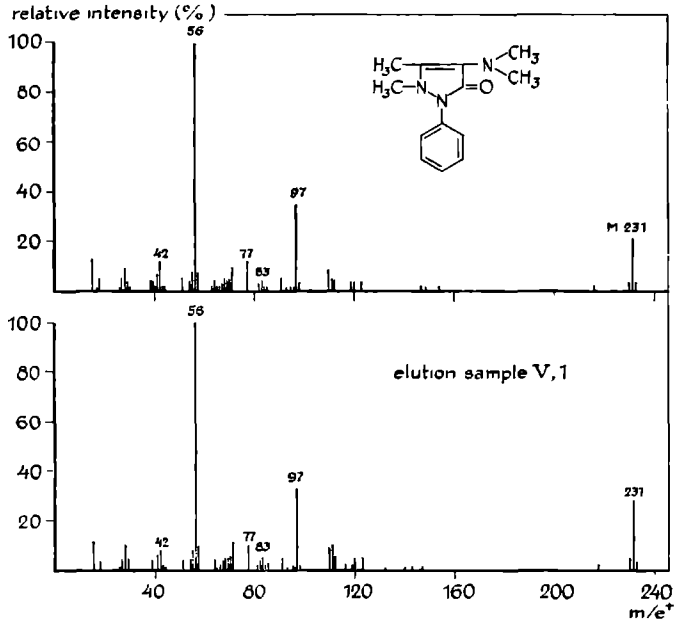



Figure 21. Mass spectra of DMAP and of the substance in the eluate of a band on thin-layer chromatograms. The code of the elution sample V, 1 refers to table 10. Note the peak for the molecular ion at m/e+ 231.

ent eluates to TLC, both separately and in combination with reference DMAP, reference MMAP or reference AAP (mixed-chromatograms). It was carefully ensured that equal quantities of the reaction products and references were applied on the plates. In TLC with both solvent systems the chromatograms for mixtures expected to contain MMAP showed a clear-cut resolution of the substance from the reference substances DMAP and AAP, but not from reference MMAP. The chromatograms for the mixtures expected to contain AAP showed a clear resolution of the substance from

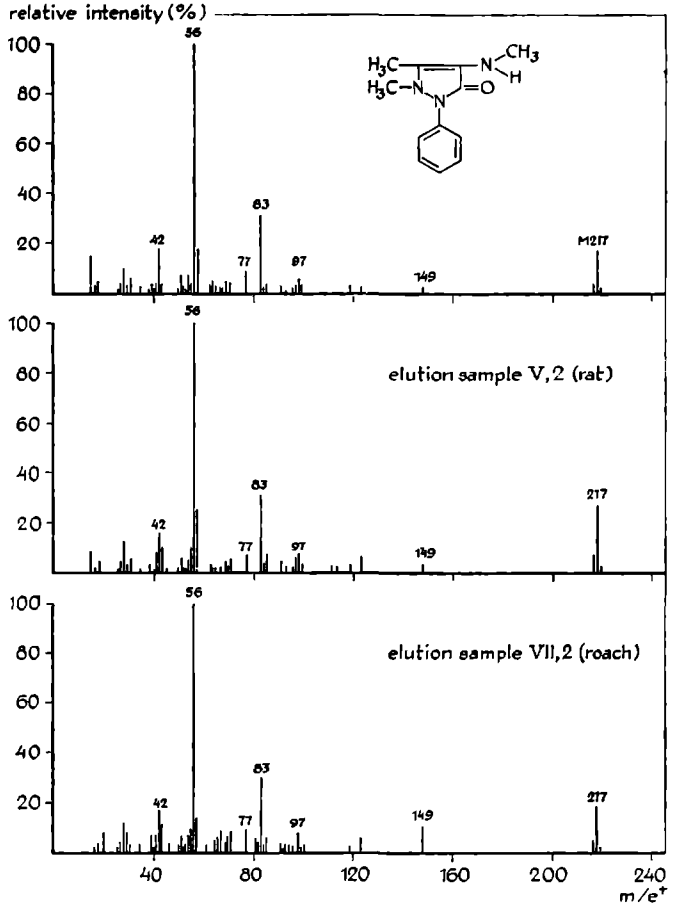



FIGURE 22. Mass spectra of MMAP and of the substances in eluates of bands on thin-layer chromatograms. The code of the elution samples V, 2 and VII, 2 refers to table 10.
Note the peak for the molecular ion at m/e+ 217.

the references DMAP and MMAP, but not from reference AAP. The shape and intensity of the unresolved mixed-spots, both under UV and after staining were identical to the spots obtained with the double concentration of the reference substances.

As a decisive test in the present study, after preparative TLC of extracts from reaction mixtures with a total volume of 300 ml, ethanol eluates of the bands on the chromatograms were evaporated to dryness and the residues subjected to mass spectrometry. The mass spectra of the reference pyrazolone derivatives and the samples are presented in figures 21, 22, and 23. In view

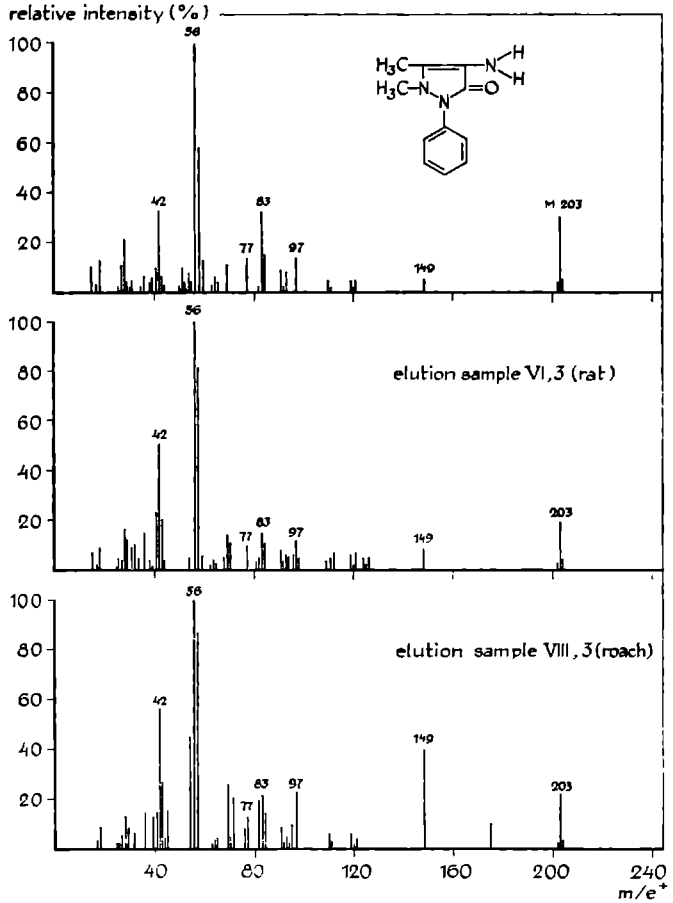



FIGURE 23. Mass spectra of AAP and of substances in eluates of bands on thin-layer chromatograms. The code of the elution samples VI, 3 and VIII, 3 refers to table 10.
Note the peak for the molecular ion at m/e+ 203.

of the close resemblance of the spectra an identification can only be done on basis of the distinction observed in the peaks at m/e· 231 (molecular ion, M) for DMAP, at m/e+ 217 (molecular ion, M) for MMAP, and at m/e+ 203 (molecular ion, M) for AAP. In the lower parts of the figures 21, 22, and 23 it can be seen that the substances from the elution samples which were expected to be DMAP, MMAP, and AAP respectively, show mass spectra with characteristic molecular peaks corresponding with those of the reference substances. It has to be noted that in the mass spectra of the samples derived from TLC eluates with low concentrations of AAP, in

some cases the base peak was shifted from m/e$^+$ 56 to m/e$^+$ 57, whereas an obvious peak at m/e$^+$ 149 appeared. The reason for this discrepancy is not clear as yet. The molecular ion, however, was still present at m/e$^+$ 203. The mass spectra of the residues of eluates from the unknown spots in the chromatograms mentioned above were quite different from those for the pyrazolone derivatives. It was not tried to identify these substances further.

### V, 3. *N*-DEMETHYLATION OF AMINOPYRINE *IN VITRO* AT DIFFERENT SUBSTRATE CONCENTRATIONS

In the TLC experiments performed in order to identify the *N*-demethylation products of aminopyrine (chapter V, 2) a difference was observed in the product formation at different substrate concentrations. At high substrate concentrations (16.7 - 33.4 mM) sometimes only one product (MMAP) was detected, whereas at low concentration (0.33 mM) the presence also of a second product (AAP) was observed. One of the explanations given was that the removal of the methyl group from the MMAP molecule might be inhibited by high DMAP concentrations. This possibility has already been suggested earlier by Gram *et al.* (1968). If such a reaction mechanism indeed is involved it will have consequences for measurements of enzymatic activity based upon the formation of formaldehyde. The formaldehyde, measured in mixtures with high (saturating) DMAP concentration, originates predominantly from the first step of the *N*-demethylation reaction. In reaction mixtures with low DMAP concentrations, however, the formaldehyde comes from a two-step *N*-demethylation mechanism in which the amount of MMAP produced is partly further *N*-demethylated to AAP. This complex reaction mechanism may explain, at least in part, that in certain cases a nonlinear relationship in the Lineweaver-Burk plots for the *N*-demethylating activities at increasing substrate concentrations are found (figure 24a, formaldehyde, solid line). The nonlinearity in the curves of Lineweaver-Burk plots was only observed with reaction mixtures containing liver preparations with relatively high enzymatic activity — as for instance from mammals and birds — if the aminopyrine was employed in a wide range of concentration (0.33 - 33.4 mM). For enzyme preparations with a relatively low enzymatic activity — as for instance for the various fish species investigated — the *N*-demethylation of aminopyrine stayed below the detectable level if low substrate concentrations (below 1.5 mM) were employed. At the relatively high substrate concentrations used the curves of the Lineweaver-Burk plots for the latter enzyme preparations were found to be linear (chapter IV, 3. 4, figure 13). The discrepancy in the Lineweaver-Burk

plots for liver preparations with different *N*-demethylating activity might be explained if the above assumption proves to be right, that at low substrate concentration the *N*-demethylation of aminopyrine by highly active enzyme preparations proceeds further until AAP. It may be expected therefore, that beside measurements of the amounts of formaldehyde formed, parallel assays of the amounts of MMAP and AAP will shed further light on the two steps involved in the *N*-demethylation of aminopyrine at different substrate concentration.

### V, 3. 1. *Quantitative determination of reaction products formed by N-demethylation in vitro*

The AAP produced in the *N*-demethylation of aminopyrine is generally measured by diazotization and coupling with $\alpha$-naphtol (Brodie and Axelrod, 1950). A colorimetric method not so extensively used is based on the yellow colour which compounds having an amino group give with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) in acid solution (Brun, 1951). The advantage of the latter method is that it is rather simple and rapid. In the present study, however, it appeared that neither colorimetric method is specific since not only AAP, but also MMAP develops a considerable colour in the presence of the reagents.

In order to control the possible mutual interference of the *N*-demethylated products in the colorimetric assays mentioned, incubation mixtures were prepared which corresponded to those involved in the assay of enzymatic activity (chapter III, 4.1). After protein precipitation various amounts of reference formaldehyde, MMAP, and AAP were added. After centrifugation the supernatants were subjected to the colorimetric assays of Nash (1953), Brun (1951), and Brodie and Axelrod (1950) (cf. chapter III, 4.2). The series of samples prepared for the assay of Nash contained $5 \times 10^{-3}$ M semicarbazide; in the series of samples for the assays of Brun, and of Brodie and Axelrod semicarbazide was omitted. Table 11 presents the extinctions found in the colorimetric assays on incubation mixtures containing 100 nmoles of the reference substances. That the colour formed with MMAP is really due to the pyrazolone derivative and not to impurities in the reference material used is indicated by the persistent positive reaction after recrystallization and TLC of the substance. Moreover, the MMAP isolated from the reaction mixtures (chapter V, 2) also showed a positive reaction with the assays of Brun, and of Brodie and Axelrod. Nevertheless, in the reports of all previous studies on the *N*-demethylation of aminopyrine in which these two colorimetric assays have been used the colours obtained are ascribed

TABLE 11 - Colour formation by formaldehyde, MMAP, and AAP in different colorimetric assays

| colorimetric assay according to | extinction (△ E) | | |
|---|---|---|---|
| | formaldehyde | MMAP | AAP |
| Nash | 0.115 | nil | nil |
| Brun | nil | 0.090 | 0.470 |
| Brodie and Axelrod | nil | 0.185 | 1.62 |

△ E: extinction of the sample minus the extinction of the blank (containing all reagents except the reference substance); the values given represent test solutions with 100 nmoles test substance in 3 ml incubation mixture.
Composition of incubation mixture and colorimetric assays are described in chapter III, 4.1 and 2.

solely to AAP. If the results of the present study (chapter V, 2) are taken into consideration, it may be clear that the colour obtained in colorimetric assays will be due to the presence of MMAP.
Quantification of the various products of the *N*-demethylation of aminopyrine at different substrate concentrations can still be achieved by the colorimetric assays mentioned if a suitable combination of simultaneous measurements of the amounts of formaldehyde and pyrazolone derivatives is applied. In this study this was performed by measurement of the total amount of formaldehyde produced in one series of reaction mixtures containing decreasing amounts of substrate with the method of Nash (1953), and by measurement of the total amount of AAP and MMAP in a corresponding series of reaction mixtures with the method of Brun (1951).
From the data presented in table 11 it will be clear that a sample containing $x$ nmoles AAP, $y$ nmoles MMAP and $2x + y$ nmoles formaldehyde gives an extinction with the assay of Nash:

$$\Delta E_N = (2x + y) \cdot 0.115 \cdot 10^{-2}$$

and with the assay of Brun:

$$\Delta E_B = x \cdot 0.470 \cdot 10^{-2} + y \cdot 0.090 \cdot 10^{-2}$$

From these equations the amounts of AAP, MMAP, and formaldehyde formed in the two *N*-demethylating steps can be derived. The total amount of DMAP which is passed through the first *N*-demethylating step is the sum of the amounts of MMAP and AAP produced.
As demonstrated in figure 24, the Lineweaver-Burk plot for the relationship between enzymatic activity and substrate concentration becomes more

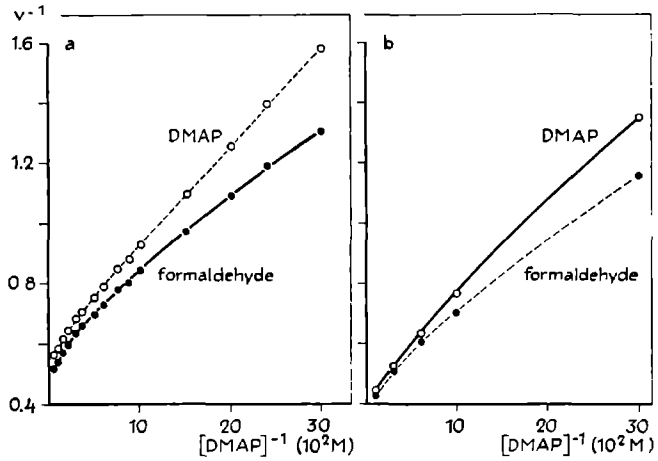



FIGURE 24. Lineweaver-Burk reciprocal plots of *N*-demethylating enzymatic activity (v) versus substrate concentration. Enzymatic activity is defined as the amount of product formed per g fresh liver per hour.

a. curve formaldehyde (solid line) is based on the total amount of formaldehyde produced. Formaldehyde is measured by the colorimetric method of Nash (1953). Curve DMAP (dotted line) represents a plot according to Lineweaver-Burk based on the amount of DMAP which has passed the first *N*-demethylating step (the sum of the amounts of MMAP and AAP produced). The amounts of MMAP and AAP are calculated by combination of the data obtained with simultaneous colorimetric assays according to Nash (1953) and Brun (1951). The method of calculation is given in the text.

b. Curve DMAP (solid line) represents the curve according to Lineweaver-Burk based on the amount of DMAP which has passed the first *N*-demethylating step. The amounts of MMAP and AAP are directly quantitated after extraction and TLC (chapter III, 5.3.1). Curve formaldehyde (dotted line) is based upon the total amount of formaldehyde produced. This amount is calculated from the amounts of MMAP and AAP measured directly.

linear if the above calculation is applied and enzymatic activity is based upon *N*-demethylation of only one methyl group (figure 24a, DMAP, dotted line).

The results of the rather cumbersome approach to the mechanism of the *N*-demethylation of aminopyrine discussed above could be confirmed by direct measurements of the amounts of MMAP and AAP after extraction of these substances from the reaction mixtures, TLC, and direct measurement of the absorption of ultraviolet light by the spots (cf. chapter III, 5. 3. 1). Figure 24b shows that the Lineweaver-Burk plot for the relationship between enzymatic activity and substrate concentration, when only the first *N*-demethylating step is considered, is almost linear (figure 24b, DMAP, solid

line). From the values for MMAP and AAP at direct measurements of TLC spots, the amounts of formaldehyde formed can be calculated. The Lineweaver-Burk plot with enzymatic activities based on these amounts shows an obviously nonlinear curve (figure 24b, formaldehyde, dotted line).
As far as the amounts of the products formed at different substrate concentrations are concerned, it was observed with both the combined-colorimetric assays and the direct quantitations via TLC that at increasing substrate concentrations the amount of AAP produced was nearly constant (3-4 nmoles per ml reaction mixture), whereas the amount of MMAP increased. The ratio between the two products at various substrate concentration is presented in table 12. From these observations it will be clear that at low substrate concentrations the second step in the *N*-demethylation reaction is of greater importance than at high, saturating, aminopyrine concentrations.

TABLE 12 - Product ratio in the *N*-demethylation of aminopyrine *in vitro* at different substrate concentration

| aminopyrine concentration (mM) | ratio MMAP/AAP |
|---|---|
| 0.33 | 8.2 |
| 1.00 | 12.5 |
| 1.67 | 17.4 |
| 3.34 | 18.9 |
| 10.0 | 30.0 |

The amounts of MMAP and AAP were determined by direct measurement of spots on thin-layer chromatograms after extraction of the substances from reaction mixtures containing increasing amounts of the substrate DMAP.

## V, 4. DISCUSSION

The results of the present study on the *N*-demethylation of aminopyrine *in vitro* indicate that one or both methyl groups can be split from the dimethylamino group in the substrate molecule. Formaldehyde, MMAP, and AAP were measured as products of the reaction. Quantitative measurements of these substances indicate that at increasing substrate concentrations the first step of the reaction occurs predominantly. Since at saturating substrate concentration the second step, the *N*-demethylation of MMAP to AAP, is negligible compared with the first one, the production of formaldehyde can be used for approximate determination of the enzymatic capacity of converting DMAP to MMAP.
It was shown that the demethylated pyrazolone derivatives formed in the

*in vitro* reaction — AAP as well as MMAP — give a colour formation in the colorimetric assays according to Brodie and Axelrod (1950) and Brun (1951). Since this was not recognized as yet, the results of previous studies in which the aim was to measure the formation of AAP only (whereas MMAP was the main reaction product), have to be interpreted with serious reservation.

Since the *N*-demethylation of aminopyrine involves the removal of one or two methyl groups, it is conceivable that in the reciprocal plot of enzymatic activity versus substrate concentration nonlinearity occurs, if enzymatic activity is based upon the total amount of formaldehyde produced. However, figure 24 shows that in this case the apparent $K_m$-value for the first step in the *N*-demethylation can be approximately estimated from the nonlinear plots when only that part of the curve is considered, which is obtained at relatively high substrate concentrations (3 - 20 mM).

On the basis of the nonlinearity in the Lineweaver-Burk plots in the *N*-demethylation of aminopyrine, Pederson and Aust (1970) concluded that the reaction is catalyzed by more than one enzyme. However, the importance of the two-step mechanism in this reaction was not fully evaluated. The question whether different enzymes catalyze the two steps of the *N*-demethylation of aminopyrine awaits further investigation.

# COMPARISON OF *N*-DEMETHYLATION, *p*-HYDROXYLATION AND GLUCURONIDATION IN VARIOUS ANIMAL SPECIES, PARTICULARLY IN SPECIES OF FISH

## VI, 1. INTRODUCTION

In chapter IV it has been shown that liver preparations from fishes, like those from other animal species, are capable of metabolizing compounds foreign to the body. The data presented confirm the findings of other studies on the occurrence of drug metabolism in fish. Enzymatic activities have been reported for the activation of thiophosphate insecticides (Potter and O'Brien, 1964; Murphy, 1966), the aromatic hydroxylation of 2-acetylaminofluorene (Lotlikar *et al.*, 1967), of biphenyl (Creaven *et al.*, 1965), and of aniline (Buhler and Rasmusson, 1968a), the dealkylation of alkoxybiphenyls (Creaven *et al.*, 1967), the epoxidation of aldrin (Chan *et al.*, 1967), the nitroreduction of various insecticides (Hitchcock and Murphy, 1967) and of *p*-nitrobenzoic acid (Adamson *et al.*, 1965; Buhler and Rasmusson, 1968b), and for the glucuronidation of *o*-aminophenol (Dutton and Montgomery, 1958). In general, the reaction mechanisms involved in the biotransformation of xenobiotics by tissue preparations from fish and other animal species have many properties in common. However, besides these resemblances the present study shows, too, that differences in the characteristics of the individual enzymes of the various species exist. For instance, the duration of incubation, pH, and concentrations of substrates and cofactors at which the enzymes exhibit their maximal activities, appear to be species dependent (chapter IV). It is of interest, therefore, to compare enzymatic activities assayed with the employment of adapted *in vitro* conditions.

In the present chapter attention will be mainly directed to the quantitative differences in the drug-metabolizing capacities of various animal species. In this study, the enzymatic activities which have been assayed in liver preparations at adapted reaction conditions are used as a measure for the drug-metabolizing capacity. Thus it is assumed that the enzymatic capacity measured *in vitro* represents to a certain degree the capacity *in vivo*. The imperfection of this extrapolation has to be realized well. The *in vitro* assays of the *N*-demethylating, *p*-hydroxylating, and glucuronidating enzymatic ac-

tivities were carried out under the conditions described in the previous chapters. In mammals the drug-metabolizing enzymes are mainly found in the liver (chapter II). It has been investigated in this study whether the drug-metabolizing enzymes of fishes are also situated mainly in the liver. Further it has been studied whether their level of hepatic drug metabolism is dependent upon the sex and the weight (age) of the animals.

## VI, 2. ORGAN DISTRIBUTION OF DRUG-METABOLIZING ENZYMES

Incubation of reactions mixtures with fractions from various tissues demonstrated that *p*-hydroxylation, *N*-demethylation, and glucuronidation may proceed not only in the liver but also in other organs. Biotransformation activity could not be observed when the tissue preparations were preincubated for 10 min at 70 °C. It has been assumed that the reaction conditions

TABLE 13 - *p*-Hydroxylation capacity of various organs in different species

| organ | pigeon | | rat | | trout | | roach | | crab | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $V_{max}$* | $K_m$** | $V_{max}$* | $K_m$** | $V_{max}$* | $K_m$** | $V_{max}$* | $K_m$** | $V_{max}$* | $K_m$** |
| liver | 4.15 | 1.31 | 1.10 | 0.05 | 0.49 | 0.48 | 0.88 | 2.4 | 0.14† | 50† |
| kidney | 2.20 | 7.5 | 0.15 | >50 | 0.30 | 0.16 | 0.15 | 13 | | |
| heart | 0.70 | 35 | 0.25 | 45 | 0.58 | 35 | 0.87 | >50 | 0.03 | |
| lung | 2.40 | 10 | 0.10 | 30 | | | | | | |
| gill | | | | | 0.18 | 20 | 0.20 | 20 | 0.42 | 0.29 |
| gut | | | 0.27 | 10 | 0.12 | 5 | | | | |
| muscle§ | 0.65 | >50 | 0.17 | >50 | nil | | nil | | | |
| blood | 0.50 | >50 | nil | | 0.10 | >50 | 0.35 | >50 | | |
| spleen | 1.80 | 15 | 0.10 | >50 | 0.23 | 40 | 0.50 | >50 | | |

*p*-Hydroxylation of aniline; each value represents the mean of at least 3 separate experiments; maximal and minimal values never deviated more than 25% from the mean.

* $V_{max}$: apparent maximal rate of conversion as measured in 9000 *g* supernatant fractions, expressed as $\mu$moles *p*-aminophenol produced per gram fresh tissue per hour.

** $K_m$: apparent $K_m$, substrate concentration at $\frac{1}{2}V_{max}$ in mM; apparent $V_{max}$ and $K_m$ were calculated from Lineweaver-Burk plots

† assay on hepatoprancreas

§ pigeon: breast muscle (*M. pectoralis major*), rat: leg muscle (*M. rectus femoris*), trout and roach: tail muscles (*M. obliquus abdominis*)

TABLE 14 - *N*-Demethylating and glucuronidating capacity of various organs in the trout

| organ | *N*-demethylation of aminopyrine* | glucuronidation of *p*-nitrophenol** |
|---|---|---|
| liver | 1.20 | 1.7 |
| kidney | 0.15 | 0.2 |
| heart | 0.15 | 0.3 |
| gill | 0.10 | nil |
| gut | 0.14 | |
| muscle | 0.06 | nil |
| blood | 0.02 | nil |
| spleen | 0.05 | 0.2 |

* $\mu$moles formaldehyde produced per gram fresh tissue per hour, assayed in 9000 *g* supernatant fractions.

** $\mu$moles *p*-nitrophenol conjugated per gram fresh tissue per hour, assayed in ultasonicated homogenates.

Each value represents the mean of at least 3 experiments; the maximal and minimal values never deviated more than 25% from the mean.

(temperature, incubation period, pH) for the measurement of maximal enzymatic activity would not greatly differ for the various organs of a certain species. Therefore, assay conditions were applied as described for the liver preparations (chapter IV). Tables 13 and 14 show that for the various species examined the greatest activity was found in liver preparations. Among the other organs investigated, the hydroxylating capacities of kidney and lung of the pigeon, and of the kidney of the trout were relatively high. For the *p*-hydroxylation of aniline in fish high activities were found in heart tissue. However, in view of the high apparent $K_m$-values found for extrahepatic aromatic hydroxylation the importance of the reaction for the situation *in vivo* may be considered low as compared with the biotransformation in the liver. The relatively high *p*-hydroxylating activity with a low apparent $K_m$-value for the substrate aniline found for the gill of the woolhanded crab may be considered a remarkable phenomenon. It would be an interesting point of study, therefore, whether this organ forms the main site of drug metabolism in crustacea.

### VI, 3. DRUG METABOLISM IN FISH IN RELATION TO SEX

A sex difference in drug metabolism has been reported for rats and mice. For instance, liver microsomal enzymes from adult male rats metabolize

TABLE 15 - Drug-metabolism in fish in relation to sex

| animal species | sex | n | *N*-demethylation of aminopyrine activity* per g fresh liver | mg liver protein | mg liver DNA | 100 g body weight | *p*-hydroxylation of aniline activity** per g fresh liver | mg liver protein | mg liver DNA | 100 g body weight |
|---|---|---|---|---|---|---|---|---|---|---|
| assay in march-april 1970 | | | | | | | | | | |
| trout | ♂ | 10 | 1.13 ± 0.32 | 0.0084 ± 0.0024 | 0.70 ± 0.16 | 1.65 ± 0.44 | | | | |
| | ♀ | 10 | 1.15 ± 0.48 | 0.0083 ± 0.0034 | 0.72 ± 0.14 | 1.69 ± 0.41 | | | | |
| bream | ♂ | 5 | 1.27 ± 0.53 | 0.0108 ± 0.0037 | 0.69 ± 0.33 | 2.47 ± 1.24 | 0.40 ± 0.18 | 0.0042 ± 0.0011 | 0.28 ± 0.12 | 1.27 ± 0.61 |
| | ♀ | 19 | 0.95 ± 0.54 | 0.0090 ± 0.0039 | 0.49 ± 0.20 | 2.02 ± 0.91 | 0.51 ± 0.16 | 0.0052 ± 0.0015 | 0.30 ± 0.09 | 1.28 ± 0.44 |
| pike | ♂ | 5 | 1.50 ± 0.73 | 0.0242 ± 0.0150 | 1.82 ± 1.03 | 4.13 ± 1.49 | 0.15 ± 0.06 | 0.0022 ± 0.0009 | 0.14 ± 0.06 | 0.29 ± 0.08 |
| | ♀ | 18 | 1.05 ± 0.45 | 0.0122 ± 0.0055 | 1.07 ± 0.53 | 2.76 ± 1.35 | 0.12 ± 0.04 | 0.0012 ± 0.0004 | 0.11 ± 0.04 | 0.29 ± 0.07 |
| roach | ♂ | 15 | 4.44 ± 1.15 | | 1.50 ± 0.39 | 7.97 ± 3.43 | 1.10 ± 0.23 | | 0.39 ± 0.08 | 1.99 ± 0.76 |
| | ♀ | 15 | 4.22 ± 0.97 | | 1.40 ± 0.32 | 7.22 ± 3.03 | 1.09 ± 0.25 | | 0.37 ± 0.06 | 1.90 ± 0.72 |
| assay in october 1970 | | | | | | | | | | |
| roach | ♂ | 10 | 6.10 ± 1.62 | 0.049 ± 0.013 | 2.75 ± 0.92 | 12.81 ± 3.10 | 1.65 ± 0.42 | 0.0116 ± 0.0052 | 0.63 ± 0.32 | 3.65 ± 0.98 |
| | ♀ | 10 | 5.85 ± 1.45 | 0.046 ± 0.015 | 2.84 ± 0.81 | 13.40 ± 3.30 | 1.80 ± 0.48 | 0.0129 ± 0.0043 | 0.75 ± 0.36 | 4.05 ± 1.08 |
| trout | ♂ | 10 | 1.20 ± 0.28 | 0.0090 ± 0.0024 | 0.66 ± 0.19 | 1.70 ± 0.40 | 0.31 ± 0.09 | 0.0022 ± 0.0009 | 0.17 ± 0.04 | 0.41 ± 0.10 |
| | ♀ | 10 | 1.16 ± 0.36 | 0.0088 ± 0.0029 | 0.69 ± 0.16 | 1.68 ± 0.45 | 0.29 ± 0.08 | 0.0023 ± 0.0008 | 0.13 ± 0.04 | 0.44 ± 0.11 |

n number of animals studied; values are means ± S.D.

* $\mu$moles formaldehyde produced per hour

** $\mu$moles *p*-aminophenol produced per hour

Note the great individual variations. With Wilcoxon's two-sided two-sample test no significant sex differences could be shown.

For the roach the values obtained in october were significantly different from those in the period march-april ($P < 0.01$, Wilcoxon, two-sided two-sample test).

various drugs, such as hexobarbital, pentobarbital, aminopyrine and morphine (Quinn *et al.* 1958; Henderson, 1971), and sex steroids (Kuntzmann *et al.*, 1964) more rapidly than those from female rats. This type of sex difference has been observed only in rats. The reversed situation has been found for several strains of mice (Rümke and Noordhoek, 1969). The factors underlying these differences in the metabolism of drugs are not fully understood up to now.

In view of the possibility that in fish a sex difference in drug metabolism might depend on annual hormonal cycles, the drug-metabolizing enzymatic capacities have been assayed in the spring (spawning period march-april) and in autumn. In order to check whether the apparent differences were generated by differences in the amounts of the main liver constituents between the sexes the enzymatic capacities were expressed in relation to various parameters. The results are presented in table 15. From the standard deviations of the various mean values it can be concluded that the individual variations in the drug-metabolizing capacities of the fishes studied are rather great. With these intraspecies fluctuations significant differences in the *N*-demethylating and *p*-hydroxylating capacities of males and females were not found. Further, table 15 shows that the drug-metabolizing capacity of the roach in autumn is different from that in the spring. This phenomenon will be discussed in detail in chapter VII.

## VI, 4. DRUG METABOLISM IN FISH IN RELATION TO BODY-WEIGHT

The drug-metabolizing capacity of rat liver is dependent upon the age of the animal. For example, Henderson (1971) showed that the level of the enzymatic activities for the *N*-demethylation of aminopyrine in adult rats is higher than in newborn animals. Female and male rats reach their adult levels within 30 and 60 days respectively after birth. As far as the activities in old rats (about 600 days old) are concerned, Kato and Takanaka (1968) found lower levels compared with animals of 100 days old.

In the present study the body-weight of the animals was taken as a rough parameter for the age of wild fishes. In order to check the influence of the body-weight on the drug-metabolizing capacity, wild roach were divided in three different weight classes in one set of experiments. The levels of the *N*-demethylating and *p*-hydroxylating capacities in these classes are presented in table 16. In order to exclude an apparent difference caused by possible variations in the overall composition of the liver, the enzymatic capacities are expressed in relation to different parameters. No significant

TABLE 16 - Hepatic drug metabolism of the roach in relation to body-weight

| *N*-demethylation of aminopyrine $\mu$moles formaldehyde produced per hour per: | body-weight 10 - 25 g (n=12) | 35 - 50 g (n=12) | 90 - 135 g (n=12) |
|---|---|---|---|
| g fresh liver | 8.44 ± 1.93 | 8.06 ± 1.65 | 6.16 ± 1.73* |
| mg liver protein | 0.0636 ± 0.0169 | 0.0583 ± 0.0120 | 0.0451 ± 0.0120* |
| mg liver DNA | 2.39 ± 0.53 | 2.54 ± 0.52 | 1.74 ± 0.36* |
| 100 g body-weight | 19.3 ± 7.0 | 21.9 ± 5.3 | 16.4 ± 5.5 |
| *p*-hydroxylation of aniline $\mu$moles *p*-aminophenol produced per hour per: | | | |
| g fresh liver | 2.26 ± 0.31 | 2.17 ± 0.44 | 1.90 ± 0.34** |
| mg liver protein | 0.0166 ± 0.0024 | 0.0142 ± 0.0032 | 0.0139 ± 0.0028** |
| mg liver DNA | 0.65 ± 0.10 | 0.63 ± 0.13 | 0.55 ± 0.10 |
| 100 g body-weight | 5.09 ± 1.34 | 5.35 ± 1.15 | 5.39 ± 1.48 |

Values are means ± S.D.
enzyme assay in july 1970
n represents the number of animals studied
* significantly different from the weight class 10-25 g at $P < 0.02$
** significantly different from the weight class 10-25 g at $P < 0.05$
(Wilcoxon two-sided two-sample test)

difference was found for the capacities in relation to the body-weight. However, if expressed per g fresh liver, per mg liver protein or per mg hepatic DNA, the *N*-demethylation of aminopyrine was significantly lower in the group of animals weighing 90-135 g than that observed in the group weighing 10-25 g. This was also the case for the *p*-hydroxylation of aniline, if expressed per g fresh liver or per mg hepatic protein. It has to be noted, however, that the difference in drug-metabolizing capacity between the groups of animals studied is relatively small. Nevertheless, in the comparative measurements described in chapter VII, the risk of a variation in enzymatic activity due to differences in the animal weight was reduced by the use of roach within a limited weight class (50-150 g). Within this class no individual correlation between the body-weight and the level of drug metabolism could be observed.

| -demethylation of aminopyrine activity* per | | | *p*-hydroxylation of aniline activity** per | | | | glucuronidation of *p*-nitrophenol activity*** per | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| mg liver protein | mg liver DNA | 100 g body-weight | g fresh liver | mg liver protein | mg liver DNA | 100 g body-weight | g fresh liver | mg liver protein | mg liver DNA | 100 g body-weight |
| 4 ± 0.02 | 12 ± 3 | 97 ± 16 | 2.8 ± 0.5 | 0.016 ± 0.003 | 1.4 ± 0.3 | 11 ± 2 | | | | |
| 1 ± 0.01 | 9.0 ± 1.4 | 90 ± 10 | 5.8 ± 0.6 | 0.034 ± 0.003 | 2.7 ± 0.4 | 28 ± 3 | 21 ± 3 | 0.12 ± 0.02 | 10 ± 2 | 99 ± 15 |
| ± 0.01 | 7.4 ± 1.5 | 59 ± 9 | 1.3 ± 0.2 | 0.008 ± 0.001 | 0.65 ± 0.10 | 5.0 ± 0.8 | 46 ± 13 | 0.26 ± 0.07 | 22 ± 6 | 184 ± 45 |
| 6 ± 0.004 | 1.03 ± 0.19 | 8.1 ± 1.6 | 1.82 ± 0.27 | 0.0134 ± 0.0018 | 0.54 ± 0.08 | 4.2 ± 0.9 | | | | |
| 6 | 4.58 | | 11.0 | 0.0602 | 5.0 | | | | | |
| 8 ± 0.033 | 9.57 ± 1.85 | 46 ± 15 | 3.74 ± 1.95 | 0.0275 ± 0.0063 | 1.65 ± 0.36 | 8.2 ± 2.4 | 85 ± 22 | 0.61 ± 0.17 | 37 ± 9 | 185 ± 50 |
| 3 ± 0.005 | 2.29 ± 0.74 | 14.3 ± 3.2 | 1.88 ± 0.23 | 0.0149 ± 0.0018 | 1.04 ± 0.36 | 6.5 ± 1.6 | 8.9 ± 2.3 | 0.071 ± 0.018 | 4.9 ± 1.8 | 31 ± 8 |
| ± 0.003 | 0.37 ± 0.11 | 2.1 ± 0.6 | 1.09 ± 0.35 | 0.0083 ± 0.0029 | 0.25 ± 0.08 | 1.42 ± 0.50 | 1.26 ± 0.47 | 0.010 ± 0.004 | 0.30 ± 0.08 | 1.60 ± 0.60 |
| ± 0.004 | 0.45 ± 0.24 | 2.1 ± 1.1 | 0.47 ± 0.15 | 0.0045 ± 0.0016 | 0.26 ± 0.12 | 1.19 ± 0.44 | 2.07 ± 0.98 | 0.019 ± 0.008 | 1.05 ± 0.44 | 2.51 ± 1.18 |
| ± 0.002 | 0.45 ± 0.18 | 2.0 ± 0.2 | 0.33 ± 0.24 | 0.0037 ± 0.0025 | 0.22 ± 0.12 | 0.56 ± 0.24 | 2.68 ± 0.65 | 0.028 ± 0.009 | 1.37 ± 0.39 | 6.64 ± 1.68 |
| ± 0.004 | 0.33 ± 0.17 | 1.2 ± 0.8 | 0.23 ± 0.09 | 0.0020 ± 0.0008 | 0.14 ± 0.09 | 0.40 ± 0.21 | 1.90 ± 0.53 | 0.018 ± 0.006 | 1.83 ± 0.82 | 3.60 ± 1.55 |
| ± 0.008 | 0.70 ± 0.39 | 3.7 ± 1.3 | 0.45 ± 0.29 | 0.0034 ± 0.0022 | 0.15 ± 0.09 | 0.73 ± 0.64 | 4.32 ± 1.20 | 0.033 ± 0.010 | 1.33 ± 0.50 | 3.34 ± 1.55 |
| ± 0.004 | 0.72 ± 0.22 | 3.8 ± 1.4 | 0.60 ± 0.23 | 0.0041 ± 0.0017 | 0.19 ± 0.09 | 0.94 ± 0.30 | 2.96 ± 1.06 | 0.020 ± 0.007 | 0.90 ± 0.41 | 5.06 ± 2.00 |
| | 0.62 | 2.3 | 0.33 | 0.0027 | 0.17 | 0.61 | 3.30 | 0.026 | 1.66 | 5.10 |
| ± 0.002 | 0.22 ± 0.07 | 0.7 ± 0.3 | 0.18 ± 0.03 | 0.0015 ± 0.0003 | 0.06 ± 0.02 | 0.17 ± 0.06 | 1.90 ± 0.33 | 0.015 ± 0.003 | 0.60 ± 0.10 | 1.73 ± 0.38 |
| ± 0.013 | 2.17 ± 0.75 | 13.2 ± 5.8 | 1.23 ± 0.60 | 0.0103 ± 0.0050 | 0.52 ± 0.22 | 3.40 ± 1.55 | 5.79 ± 1.15 | 0.048 ± 0.015 | 2.90 ± 0.79 | 13.3 ± 3.6 |
| ± 0.020 | 1.87 ± 0.89 | 10.8 ± 6.1 | 0.87 ± 0.45 | 0.0093 ± 0.0046 | 0.47 ± 0.25 | 2.76 ± 1.67 | 4.84 ± 0.36 | 0.043 ± 0.013 | 2.26 ± 0.63 | 12.0 ± 0.7 |
| | | | 0.30 | 0.0020 | 0.07 | | | | | |
| | | 1.2 | 0.32 | 0.0021 | | | | | | |
| | 0.55 | | | | | | | | | |
| ± 0.002 | 0.61 ± 0.16 | 1.7 ± 0.5 | 0.35 ± 0.10 | 0.0028 ± 0.0008 | 0.19 ± 0.05 | 0.53 ± 0.14 | 1.72 ± 0.25 | 0.014 ± 0.003 | 0.95 ± 0.25 | 2.60 ± 0.65 |
| ± 0.0009 | 0.05 ± 0.02 | 0.4 ± 0.2 | 0.07 ± 0.02 | 0.0005 ± 0.0002 | 0.02 ± 0.01 | 0.14 ± 0.04 | 2.10 ± 1.13 | 0.015 ± 0.010 | 0.72 ± 0.40 | 3.94 ± 1.24 |
| ± 0.007 | 1.22 ± 0.27 | 4.0 ± 1.6 | 0.39 ± 0.16 | 0.0036 ± 0.0016 | 0.30 ± 0.12 | 0.82 ± 0.46 | 1.32 ± 0.27 | 0.011 ± 0.003 | 0.73 ± 0.19 | 2.16 ± 0.85 |
| 0.002 | 0.29 ± 0.14 | 0.73 ± 0.23 | 0.13 ± 0.09 | 0.0011 ± 0.0008 | 0.09 ± 0.07 | 0.17 ± 0.13 | 2.06 ± 0.90 | 0.011 ± 0.005 | 1.30 ± 0.55 | 3.55 ± 1.46 |
| 0.009 | 1.23 ± 0.72 | 3.02 ± 1.4 | 0.12 ± 0.05 | 0.0014 ± 0.0007 | 0.12 ± 0.04 | 0.29 ± 0.08 | 1.57 ± 0.92 | 0.015 ± 0.012 | 1.43 ± 0.91 | 3.84 ± 2.71 |
| 0.008 | 0.38 ± 0.20 | 3.4 ± 2.3 | 0.05 ± 0.03 | 0.0004 ± 0.0002 | 0.015 ± 0.007 | 0.12 ± 0.05 | nil | | | |
| 0.0004 | 0.12 ± 0.07 | | 0.42 ± 0.18 | 0.0052 ± 0.0011 | 0.80 ± 0.18 | | | | | |

## VI, 5. SPECIES DIFFERENCES IN HEPATIC DRUG-METABOLIZING CAPACITIES

The capacities of the enzymes involved in the oxidative *N*-dealkylation and aromatic hydroxylation, and in the glucuronidation in the liver preparations from various animal species are presented in table 17. In this survey the enzymatic activities are expressed in relation to various parameters. The reason for this is that differences in the relative amounts of the various components may occur in the livers of different animal species. For instance, in relation to other fish species the livers of the pike and carp showed, in correspondence with a fatty appearance, relatively low amounts of protein and DNA per gram fresh liver. In order to reduce the risk of a picture with species differences as artefacts evoked by expression of the drug-metabolizing enzymatic activities only in relation to 1 g liver fresh weight, the activities are also expressed per mg hepatic protein and DNA, and in relation to the total livers normalized to animals of 100 g body-weight. For the present study the latter parameter may be considered to give the most rational impression of the differences in the drug-metabolizing capacities of the species investigated. In table 17 it can be seen that in the liver of all species tested enzymatic activity for the transformation of xenobiotics is present. Generally, the level of the drug-metabolizing capacity of the poikilothermic animals is lower than that of the homeothermic species. Aquatic animal species with relatively high hepatic enzymatic activities are: roach, rudd, eel, and bream, and those with relatively low activities are: orfe, perch, salmon, tench, and trout. The liver preparations from the sea lampreys investigated exhibited no glucuronidating activity. Possible explanations for the species differences in drug-metabolizing capacity will be given in the discussion at the end of this chapter.

## VI, 6. SPECIES DIFFERENCES IN APPARENT $K_m$-VALUES FOR SUBSTRATES AND COSUBSTRATES OF DRUG-METABOLIZING REACTIONS

The apparent Michaelis-Menten constants for the substrates employed in the enzymatic reactions with liver preparations from various species are presented in table 18. Generally, the apparent constants derived for the oxidative reactions appear to be higher in the poikilothermic animals than in the homeothermic ones. This phenomenon was also observed for the apparent $K_m$-values of the cosubstrate required in the *N*-demethylation of aminopyrine (table 19). For glucuronidation no striking differences in the

TABLE 18 - Apparent $K_m$-values of substrates for hepatic drug-metabolizing enzymes of different animal species

| | *N*-demethylation of aminopyrine* | *p*-hydroxylation of aniline** | glucuronidation of *p*-nitrophenol § |
|---|---|---|---|
| hamster | 0.8 | 0.8 | |
| mouse | 1.3 ± 0.5 | 0.29 ± 0.04 | 0.32 |
| rat | 0.65 ± 0.20 | 0.05 ± 0.02 | 0.72 ± 0.10 |
| hen | 0.6 | | |
| pigeon | 0.42 ± 0.07 | 1.31 ± 0.34 | 0.64 ± 0.14 |
| lizard | 3.23 | 1.67 | 0.33 |
| frog | 3.0 ± 0.7 | 2.4 ± 0.3 | |
| bream | 6.1 | 4.5 | 0.47 |
| carp | 1.5 | 4.2 | 0.83 |
| tench | | 5.3 ± 1.3 | 0.53 |
| white bream | 7.6 ± 2.7 | 10 | |
| roach | 9.3 ± 0.6 | 2.4 ± 0.4 | 0.97 |
| rudd | 6.5 | 2.6 | |
| rainbow trout | 2.8 ± 0.5 | 0.48 ± 0.12 | 0.48 |
| eel | 3.5 ± 0.9 | 1.7 ± 0.3 | 1.3 ± 0.3 |
| pike | 10 | 4.0 | 0.67 |
| sea lamprey | 8.0 ± 3.5 | 5.5 ± 2.0 | |
| wool-handed crab † | | 0.3 | |

$K_m$-values in mM; means of 2 or more determinations; for animals of which the apparent $K_m$-values are determined by 4-10 determinations the standard error of the mean is given;

* range of aminopyrine concentration used: 1.5 - 35 mM (see chapter V)

** range of aniline concentration used: 0.1 - 22 mM

§ range of *p*-nitrophenol concentration used: 0.14 - 1.4 mM

† assayed with 9000 *g* supernatant of gill homogenate

apparent $K_m$-values for substrate and cosubstrate between the homeothermic and poikilothermic animals investigated were observed (table 19).

As has been shown in chapter IV, species differences exist in the temperature at which drug-metabolizing enzymes exhibit their maximal activities (at fixed incubation period). The influence of different incubation temperatures on the *N*-demethylation of aminopyrine by the hepatic enzyme preparations of rat, roach, and trout were also studied in relation to the apparent $K_m$-values. The

TABLE 19 - Apparent $K_m$-values of cosubstrates in hepatic *N*-demethylation and glucuronidation in different species

| | *N*-demethylation of aminopyrine; cosubstrate NADPH* (x $10^{-6}$ M) | glucuronidation of *p*-nitrophenol; cosubstrate UDPglucuronate (x $10^{-3}$ M) |
|---|---|---|
| rat | 4 | 2.3 |
| hen | 0.6 | |
| pigeon | 0.5 | |
| lizard | 8 | 1.0 |
| frog | 7 | |
| roach | 8 | 1.0 |
| rainbow trout | 22 | 0.6 |

means of two determinations; calculation was done from Lineweaver-Burk plots.
* the cosubstrate NADPH was supplied by the addition of NADP and a reducing system (cf. chapters III, 4.1; IV, 3.3; and IV, 3.4)

results are plotted in figure 25. In order to eliminate the intraspecies variations, the biotransformation rates at the various temperatures are expressed as percentages of the maximum values in the respective temperature series (figure 25a). The temperature for the measurement of maximal enzymatic activity of the *N*-demethylation reaction *in vitro* (incubation for 10 min) for rat, roach, and trout is about 42, 37, and 28 °C respectively. It should be emphasized that these results were obtained using concentrations of aminopyrine and NADPH high enough to saturate the enzyme preparations at all incubation temperatures used. This is important since the apparent Michaelis-constant of the substrate aminopyrine for the enzymes in 9000 *g* supernatants appears to be temperature dependent (figure 25b). For the hepatic enzyme preparation of the roach the apparent $K_m$-value shows a minimum over a wide temperature range. In contrast, for the trout and the rat a distinct minimum can be observed at 25 and 40 °C respectively.

### VI, 7. *N*-DEMETHYLATING ACTIVITY OF COMBINED HEPATIC ENZYME PREPARATIONS FROM RAT AND TROUT

In order to test whether the low enzymatic activities observed in fishes are caused by inhibitory factors present in the liver, experiments were performed in which the *N*-demethylating enzymatic activity was measured by using a combination of 9000 *g* supernatant from the liver of an animal species with a relatively high activity, viz. the rat, and from the liver of a species with a relatively low activity, viz. the rainbow trout. The results are given

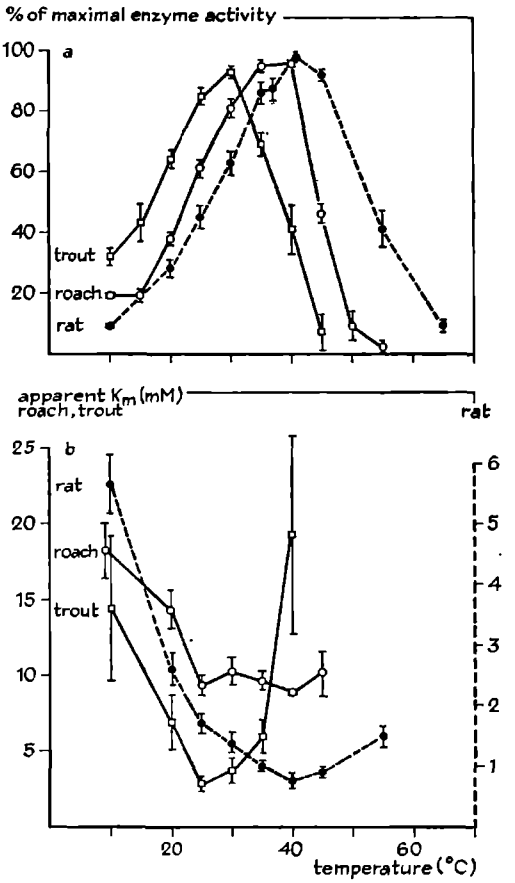



FIGURE 25. Oxidative *N*-demethylation of aminopyrine. Enzyme assays were carried out (incubation 10 min) with 9000 *g* supernatants from liver homogenates of trout, roach, and rat. Enzymatic activity was assayed with different substrate concentrations at various temperatures and expressed as the amount of formaldehyde produced per h per g fresh liver. a, Enzymatic activities are plotted as percentages of the maximum values of the respective temperature series. Mean values ± S.E.M. for at least 10 animals. b, Apparent $K_m$-values represent the substrate concentrations at ½ $V_{max}$ in mM. Calculation was done from Lineweaver-Burk plots. Means of at least 6 determinations ± S.E.M. (Dewaide, 1970).

TABLE 20 - *N*-demethylating activity of combined 9000 *g* supernatants from rat and trout livers

| | nmoles formaldehyde produced in 3 ml reaction mixture | | | |
|---|---|---|---|---|
| incubation temperature | 9000 *g* supernatant from rat liver (equivalent to 100 mg fresh tissue) (A) | 9000 *g* supernatant from trout liver (equivalent to 100 mg fresh tissue) (B) | combined 9000 *g* supernatants from rat and trout liver (equivalent to 50 mg fresh rat liver + 50 mg fresh trout liver (C) | (C) / ½(A) + ½(B) |
| 25 °C | 134 ± 12 | 24 ± 4 | 84 ± 3 | 1.06 ± 0.10 |
| 37 °C | 255 ± 25 | 17 ± 6 | 166 ± 8 | 1.22 ± 0.09* |

Means of 7 experiments ± S.E.M. Incubations were carried out at 25° and 37 °C under adapted assay conditions as described in chapter III and IV.
* In each experiment the value for C/(½A + ½B) was found to be greater than 1

TABLE 21 - Microsomal *N*-demethylating activity of rat and trout liver with interchange of the soluble fractions

| | nmoles formaldehyde produced in 3 ml reaction mixture | | | |
|---|---|---|---|---|
| incubation temperature | 9000 - 105000 *g* pellet R + 105000 *g* supernatant R | 9000 - 105000 *g* pellet R + 105000 *g* supernatant T | 9000 - 105000 *g* pellet T + 105000 *g* supernatant T | 9000 - 105000 *g* pellet T + 105000 *g* supernatant R |
| 25 °C | 112 ± 5 | 115 ± 6 | 15 ± 2 | 19 ± 4 |
| 37 °C | 207 ± 14 | 209 ± 15 | 12 ± 3 | 10 ± 4 |

Means of 7 experiments ± S.E.M.
R: fraction derived from rat liver (equivalent to 100 mg fresh tissue)
T: fraction derived from trout liver (equivalent to 100 mg fresh tissue)
Incubations were carried out for 10 min at 25 and 37 °C under adapted conditions as described in chapter III and IV.
Note that the activities of the microsomal fractions (9000 - 105000 *g* pellets) are not influenced by the interchange of the soluble fractions (105000 *g* supernatants).

in table 20. It appears that no inhibition occurs in the mixed enzyme preparation. On the contrary, a significant increase was found in the assay at 37°C: the favourable temperature for the rat enzyme. In chapter IV, 2. 1. with respect to the low *N*-demethylating activity in rat liver microsomes (9000 - 105000 *g* pellet) observed in the presence of small amounts of soluble fraction (105000 *g* supernatant), the possibility of inhibitory factors present in the rat liver was considered. It is of interest, therefore, to study whether the stimulation of the activity of rat liver in the presence of trout liver preparation, as described above, is the result of a suppression of possible inhibitors in the rat liver by factors from the fish. In order to check this, rat and trout liver microsomes were assayed for *N*-demethylating activity with interchange of the soluble fractions. The results presented in table 21 show no significant differences for fish and rat liver microsomes in the presence of the different soluble fractions. Thus, the results presented in chapter IV, 2. 1. and those described above indicate that the activity of the microsomal fraction can be increased by the addition of the soluble fraction, but, both for rat and trout it does not matter which soluble fraction is employed.

## VI, 8. DISCUSSION

This study shows that in all vertebrates investigated the liver has to be considered as a very important site for the biotransformation of xenobiotics (tables 13 and 14). For the reactions studied extracts of this organ exhibit higher rates of conversion and lower apparent $K_m$-values for the substrates as compared with other organs. In the wool-handed crab, the only invertebrate species investigated, the greatest *in vitro* capacity for aromatic hydroxylation was not found in the hepatopancreas — the organ in crustacea which is considered to resemble in some of its functions the liver of vertebrates (Vonk, 1960) — but in the gill (table 13). This may suggest that these water-dwelling arthropods make use of drug transformation in the gill in order to get rid of xenobiotics. This phemonenon contrasts with the rather poor drug-metabolizing capacity *in vitro* of the gills of fishes (tables 13 and 14).
In order to study the relationship between the age of the fishes and their drug-metabolizing capacity, efforts were made to correlate the level of drug transformation to the body-weight of the animals. The experiments performed with wild roach are suggestive of a slight decrease in the capacity of the drug-metabolizing processes in the animals of the heaviest weight class (table 16).
As far as the influence of the sex of the fishes is concerned, for 4 species

no significant differences in the drug-metabolizing enzymatic activities of males and females were observed (table 15). In relation to the possible presence of differences in drug metabolism under the influence of age and sex it has to be noted that in the present study differences may remain hidden by the great intraspecies differences observed. These intraspecies differences are determined by a lot of internal and external factors (chapter II, 7). In this connection it is well imaginable that small distinctions due to age or sex are overshadowed by more influential factors such as, for instance, exposure to certain xenobiotics. Therefore, a definite conclusion concerning the influence of age and sex on drug metabolism in fish cannot be made until a study has been performed with a sufficient number of animals with comparable genetic properties under controlled environmental circumstances.

As far as the interspecies differences in drug metabolism are concerned, with a view on the possible fluctuations in the relative amounts of the various components in the livers of different species, a comparison of the enzymatic activities is most reasonable if the activities are standardized in respect of a comparable parameter. Therefore, in this study enzymatic activities were expressed as a ratio to an amount of liver corresponding to a total body-weight of 100 g. With the present approach, these values are assumed to give a useful impression of the drug-metabolizing capacity of the various animals studied.

Generally, the enzymatic activities studied are found to be lower in the extracts of the livers from the poikilothermic animals than those observed for the homeothermic ones (table 17). That these lower activities represent lower concentrations of the drug-metabolizing enzymes rather than an inhibition of the enzymatic activity by unknown factors is suggested by the uninhibited, or even stimulated, activities of combined rat and trout enzyme preparations (tables 20 and 21). A similar phenomenon of an enhancement of enzyme activity with mixed rat and trout liver enzyme preparations has been observed earlier by Chan *et al.* (1967) for the microsomal aldrin epoxidation, whereas Buhler and Rasmusson (1968a) reported an unexpected increase in the enzymatic activity in the aromatic hydroxylation of aniline. At the present time, the mechanism through which this enhancement in the combined system of rat and trout liver is achieved, awaits further characterization.

As far as the apparent $K_m$-values of the substrates and cosubstrates for the oxidative reactions studied are concerned, the lower constants found for the homeothermic species indicate, too, more favourable conditions for the systems involved in the metabolism of xenobiotics in these animals as compared with the cold-blooded animals (tables 18 and 19). In this connec-

tion striking differences which were observed in the *N*-demethylation of aminopyrine in relation to the incubation temperature must be stressed. The temperatures for measurement of maximal reaction velocities for rat, roach, and trout liver preparations are about 42, 37 and 28 °C. As far as the variation of the apparent $K_m$-values of aminopyrine with the assay temperature is concerned species differences also appeared (figure 25). For the *N*-demethylating enzyme of the liver of the roach the minimum apparent $K_m$-value is rather temperature independent and ranges from 25 to 45 °C. In contrast, both for the rat and the trout, a more distinct minimum is found at 40 and 25 °C respectively. Correlations between the properties of other enzymes and the body or environmental temperature have been described earlier both for homeothermic and poikilothermic animals (Kaplan, 1965; Cowey, 1967; Hochachka and Somero, 1968). Possibly, the species differences in drug metabolism as described here may be a reflection of the different physiological conditions of the organisms, adapted to their natural habitat. The trout, which lives preferentially at low environmental temperatures, will be served best by enzymes showing maximal activities at low temperatures. The wild roach will be adapted to the naturally occurring thermal fluctuations of its aquatic environment. The properties of enzymes in the homeothermic rat will correspond with the higher internal body temperature.

It is difficult and hazardous to draw conclusions from the *in vitro* measurements about the rate of biotransformation of xenobiotics *in vivo*. Nevertheless, it is tempting to give an explanation for the differences in the xenobiotic-metabolizing capacities of the enzyme preparations from the various animal species. The speculative character of the following discussion, therefore, has to be stressed. One of the conclusions may be that the capacity of the poikilothermic animals to metabolize xenobiotics is lower than that of the homeothermic mammals and birds. The question remains, whether — besides the possibility that in water-dwelling animals other mechanisms for the disposition of xenobiotics are present, for example diffusion through the gills — the apparently low drug-metabolizing capacity in fish will be, nevertheless, sufficient to dispose of xenobiotics. Here the experimental data and conclusions of Brodie and coworkers (Brodie *et al.* 1958; Brodie and Maickel, 1962) must be recalled. Their conclusion that fishes do not possess drug-metabolizing capacity are clearly disproved by the experimental data of the present study. Their theory that fish would have no need for such a capacity can be invalidated by stating the importance of the drug-metabolizing reactions which decrease the lipophilic character of xenobiotics. The products are more restricted in their distribution

in the body to the extracellular phase. This favours, for fishes too, the disposal of xenobiotics from the body. Relating the different levels of drug-metabolizing capacity in totally different species, like homeotherms and poikilotherms, to a basic parameter such as standard metabolism — which in its turn is related to the food intake and therefore to the degree of exposure to xenobiotic compounds — might help to explain the difference in detoxificating capacities reported.

Further, it is of importance that the level of drug-metabolizing capacity of the wild fishes investigated may be dependent upon certain xenobiotic chemicals present in their polluted environment. Perhaps the observation that the enzymatic activities in the livers of the roach and the rudd are several times higher than those in the other species belonging to the family of the *Cyprinidae* has to be explained on this basis. It is imaginable that as compared with the white bream, the bream, the carp, and the tench — which are all relatively slow-swimming fishes, and which find their food consisting of plants, grubs, and worms near the bottom of the water — the roach and the rudd — which have a higher mobility in collecting a relatively great amount of food consisting of plankton, plants, small aquatic animals, and insects near the surface of the water — encounter in their environment a greater amount and variety of xenobiotics. The difference in drug-metabolizing capacity of roach belonging to different classes of weight (table 17) might also be explained on this basis: small roach, which are rapidly growing, consume a relatively greater amount of food and are thus possibly confronted with a greater amount of xenobiotics.

Among the family of *Cyprinidae* the hatchery-reared golden orfe shows a much lower drug-metabolizing capacity than the wild species. In this connection it may be asked to what degree the fact that the orfes have lived in non-polluted hatchery water, has played a role.

That external factors may play a role with regard to the level of the drug-metabolizing capacity will be supported by the experimental results presented in the next chapter.

Speculation about the dietary aspects of the evolution of enzymes dealing with the metabolism of xenobiotics, for instance between carnivorous and herbivorous fishes, cannot be done on basis of the data obtained in this study.

Finally, in relation to the chemical pollution of the water of the river Rhine, it may be asked whether the low detoxifying capacity of salmon and trout observed in this study and the high capacity especially of roach and rudd have played a role in the extinction of the first-mentioned species, and the rather fair maintenance of the last-mentioned species.

# THE INFLUENCE OF EXTERNAL FACTORS ON THE LEVEL OF HEPATIC DRUG METABOLISM IN FISH

## VII, 1. INTRODUCTION

Changes in the drug-metabolizing capacity of animals can be evoked by a wide variety of endogenous and exogenous factors, among which are age, sex, diet, environmental temperature, and exposure to xenobiotics (chapter II, 7). The observations of these phenomena mainly concern mammals.

As far as various fish species are concerned, studies in the past on the metabolism of xenobiotics failed to show alterations in their drug-metabolizing capacity under the influence of a change of diet, and of xenobiotics such as DDT and phenylbutazone (Buhler and Rasmusson, 1968a and 1968b; see also Adamson, 1967).

In the previous chapters evidence has been presented which may disprove the idea that water-dwelling animals do not have the ability to metabolize substances alien to the body. It was concluded that for the recognition of such a capacity *in vitro* the employment of adapted assay conditions is of primary importance (chapter IV).

In this study, besides the influence of internal factors such as sex and age of the animals (chapter VI, 3 and 4), the effect of certain external factors was also investigated.

## VII, 2. SEASONAL VARIATION IN HEPATIC DRUG METABOLISM IN THE ROACH

From March 1968 onwards, at set times, roach were caught in the river Waal (the main branch of the river Rhine delta flow) near Nijmegen, immediately transported to the laboratory, killed, and tested for hepatic drug-metabolizing enzymatic activities within 1 hour after arrival. From figure 26 it appears that the enzymatic activities involved in the *N*-demethylation of aminopyrine and in the *p*-hydroxylation of aniline at different times of the year were not constant. This was confirmed statistically ($P << 10^{-5}$) by the test of Kruskal and Wallis (1952). The highest drug-metabolizing capacities were found during the summer months. For both *N*-demethyl-

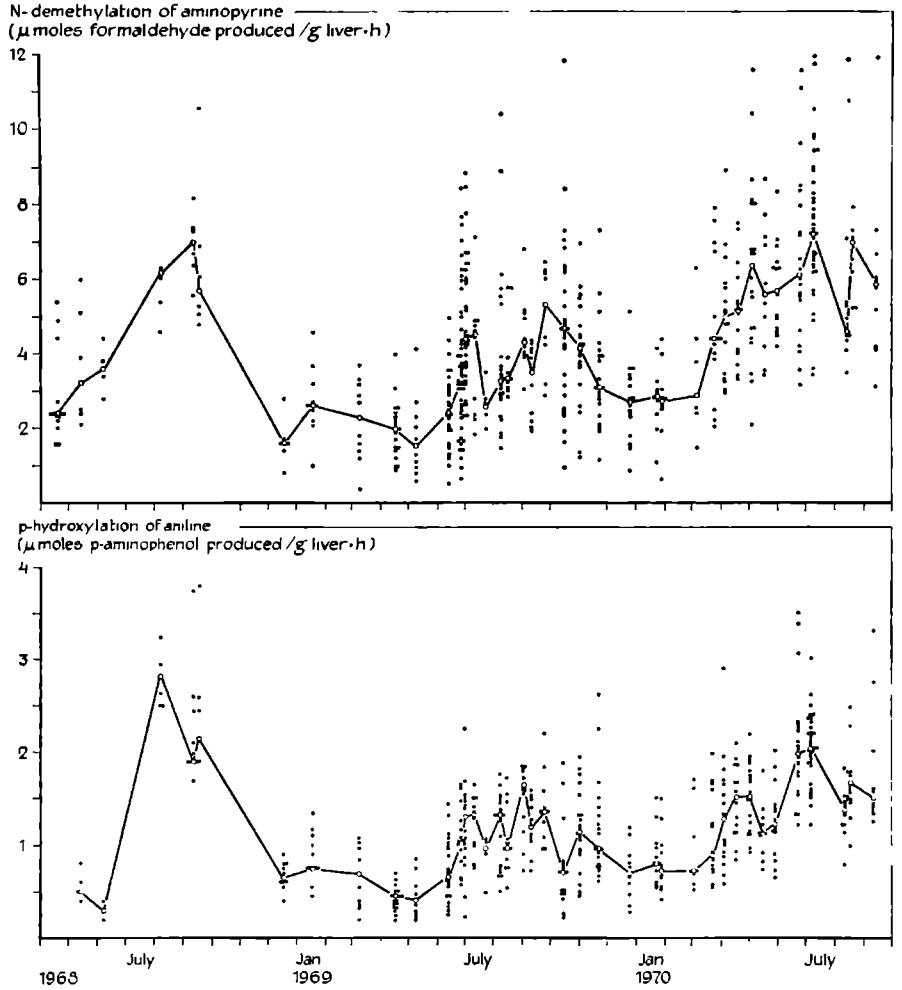



FIGURE 26. Seasonal variation in hepatic drug-metabolizing capacity in the wild roach. Measurements were performed with 9000 *g* supernatants derived from liver homogenates. Curves are drawn through the median values of the samples.
Note that the drug-metabolizing capacities in summer are higher than in winter.

ation and *p*-hydroxylation the capacities measured in July, August, and September were always significantly different from the capacities measured in December and January (Wilcoxon two-sided two-sample test, $P < 10^{-3}$).
The differences in the biotransformation capacities — defined in figure 26 as μmoles of the products formed per hour per gram liver — are not generated as artefacts under the influence of changes in the overall composi-

TABLE 22 - Liver weight, and hepatic protein and DNA contents of wild roach at different times of the year

| assay date | no. of animals | liver fresh weight (g/100 g body-weight) | protein (mg/g fresh liver) | DNA (mg/g fresh liver) |
|---|---|---|---|---|
| April 19, 1968 | 15 | 2.1 ± 0.4 | 141 ± 17 | 2.4 ± 0.4 |
| May 14, 1968 | 6 | 2.4 ± 0.5 | 156 ± 24 | 2.6 ± 0.4 |
| September 10, 1968 | 17 | | 140 ± 12 | 2.6 ± 0.3 |
| September 17, 1968 | 5 | | 150 ± 5 | 2.5 ± 0.3 |
| December 17, 1968 | 6 | | 138 ± 21 | 2.5 ± 0.2 |
| January 17, 1969 | 12 | | 150 ± 27 | 2.2 ± 0.3 |
| March 8, 1969 | 8 | | | 2.3 ± 0.4 |
| April 16, 1969 | 9 | | 167 ± 13 | |
| June 12, 1969 | 10 | | 162 ± 14 | |
| June 25, 1969 | 5 | | 150 ± 21 | |
| July 23, 1969 | 4 | | 152 ± 22 | 2.6 ± 0.5 |
| August 7, 1969 | 10 | | 161 ± 21 | 2.1 ± 0.3 |
| September 1, 1969 | 10 | | 138 ± 20 | 2.3 ± 0.2 |
| September 9, 1969 | 10 | | 145 ± 8 | 2.7 ± 0.4 |
| September 23, 1969 | 10 | | 163 ± 9 | 2.4 ± 0.4 |
| October 14, 1969 | 10 | | 120 ± 13 | 2.1 ± 0.4 |
| October 31, 1969 | 10 | | 155 ± 17 | 2.3 ± 0.4 |
| November 21, 1969 | 20 | | 152 ± 14 | 2.4 ± 0.4 |
| December 24, 1969 | 16 | | 126 ± 13 | 1.9 ± 0.3 |
| January 23, 1970 | 10 | 2.4 ± 0.4 | 115 ± 10 | 2.5 ± 0.2 |
| January 27, 1970 | 10 | 2.3 ± 0.4 | 124 ± 17 | 2.3 ± 0.2 |
| March 2, 1970 | 8 | 2.3 ± 0.5 | 130 ± 14 | 1.8 ± 0.3 |
| March 23, 1970 | 10 | 2.9 ± 0.6 | 120 ± 9 | 1.9 ± 0.4 |
| April 3, 1970 | 17 | 2.6 ± 0.4 | 135 ± 16 | 2.7 ± 0.4 |
| April 16, 1970 | 16 | 2.8 ± 0.6 | 125 ± 12 | 2.0 ± 0.6 |
| May 1, 1970 | 18 | 2.2 ± 0.6 | 138 ± 12 | 2.3 ± 0.5 |
| May 15, 1970 | 10 | 2.2 ± 0.6 | 163 ± 24 | 2.6 ± 0.5 |
| May 28, 1970 | 15 | 3.2 ± 0.8 | 148 ± 25 | 2.0 ± 0.5 |
| June 22, 1970 | 20 | 3.0 ± 0.7 | 147 ± 16 | 2.4 ± 0.5 |
| July 6, 1970 | 36 | 2.5 ± 0.5 | 138 ± 8 | 2.8 ± 0.4 |
| August 11, 1970 | 10 | 2.2 ± 0.5 | 151 ± 10 | 2.4 ± 0.4 |
| August 17, 1970 | 20 | 2.1 ± 0.3 | 146 ± 13 | 2.3 ± 0.6 |
| September 11, 1970 | 10 | 2.2 ± 0.5 | 167 ± 6 | 2.3 ± 0.3 |

Mean values with standard deviation

Note: the limits within which the liver weight, and the hepatic protein and DNA contents vary indicate that the seasonal variation in the drug-metabolizing capacities presented in figure 26 is not generated as an artefact by differences in the amounts of certain liver constituents.

tion of the liver. This can be derived from the relative constancy of the hepatic protein and DNA contents (table 22). Moreover, the fluctuations in the capacities plotted in figure 26 are not generated by a possible change in the water content of the fish liver because of the clear constancy observed for the liver dry weight (storage at 110 °C until weight remained constant): 24 5 ± 1.9 per cent of the liver fresh weight. The values for the liver fresh weight as a percentage of the body-weight (table 22, period 1970) indicate that, if the hepatic drug-metabolizing capacities were expressed per unit of body-weight, a picture similar to that of figure 26 would have been obtained.

The results presented above indicate that in wild roach the capability of metabolizing xenobiotics is high during the summer months. During this period the *N*-demethylation capacity of roach approximates that observed for rats, whereas the *p*-hydroxylation capacity exceeds even that found in rat liver (cf. table 17).

In another fish species, viz. the rudd, different levels of drug metabolism have also been observed at different times of the year (table 23).

TABLE 23 - Hepatic drug oxidation in the rudd at different times of the year

| | date of assay | |
|---|---|---|
| *N*-demethylation of aminopyrine activity* per | March 12, 1970 (n=25) | June 29, 1970 (n=11) |
| g fresh liver | 1.37 ± 0.52 | 3.60 ± 1.83 |
| mg liver protein | 0.0119 ± 0.0042 | 0.0379 ± 0.0197 |
| mg liver DNA | 0.63 ± 0.28 | 1.87 ± 0.89 |
| 100 g body-weight | 2.40 ± 0.86 | 10.8 ± 6.3 |
| *p-hydroxylation of* aniline activity** per | | |
| g fresh liver | 0.41 ± 0.17 | 0.87 ± 0.45 |
| mg liver protein | 0.0035 ± 0.0015 | 0.0093 ± 0.0046 |
| mg liver DNA | 0.19 ± 0.09 | 0.47 ± 0.25 |
| 100 g body-weight | 0.72 ± 0.28 | 2.76 ± 1.67 |

Means with standard deviation

n: number of animals investigated

* μmoles formaldehyde produced per hour

** μmoles *p*-aminophenol produced per hour

The corresponding values of the two dates are significantly different at $P < 0.01$ (Wilcoxon two-sided two-sample test)

Note: the drug-metabolizing capacities of the rudd in March and June are significantly different independent of the way of expression.

It has been reported that exposure of mammals to low environmental temperatures may result in an alteration in drug metabolism (Inscoe and Axelrod, 1960; Gillette, 1965; Inscoe *et al.*, 1965; Fuller and Bousquet, 1967; Stitzel and Furner, 1967; Furner and Stitzel, 1968). In view of the fact that especially poikilothermic animals, such as fishes, may be affected by changes in the environmental temperature, questions arise about their adaptability with respect to the biotransformation of xenobiotics. These questions are the more urgent as our technological society is modifying the aquatic environment in certain areas not only chemically but also thermally.

Also, in connection with the high level of hepatic drug metabolism in roach during the summer months which has been described above, it may be asked whether the difference in environmental temperature between summer and winter is the cause of the seasonal difference in their ability to metabolize xenobiotics. The drug-metabolizing abilities of hatchery-reared rainbow trout and wild roach were measured after keeping the animals in water of different temperatures. The drug-metabolizing capacities were also comparatively studied in two homeothermic species, Wistar rat and golden hamster, after exposure to different temperatures.

Before exposure to different ambient temperatures the fish species were kept for 1 week in tanks with running tap water (600 l/h) at 9 °C. Subsequently the fishes were kept in tanks with standing tap water at 5 or 18 °C for 2 weeks. Twice a week the water was refreshed. The mammals studied were exposed to an environmental temperature of 5 or 23 °C for 2 weeks. During the cold exposure the hamsters were not hibernating. During the whole period the roach and one group of trout were starved; another group of trout, the rats and the hamsters were fed *ad libitum*. Trout at 18 °C exhibited more aggressive feeding behaviour than those kept at 5 °C. In contrast, mammals at 5 °C tended to ingest greater amounts of food than those living at 23 °C.

After the period with different environmental temperatures the body-weight, the liver weight (wet and dry weight), the hepatic protein and DNA content, and the enzymatic capacities for *N*-demethylation and *p*-hydroxylation were measured. For the fish the whole procedure of temperature exposure and succeeding measurements was repeated 4 times with different groups of animals separately. The differences between the groups of animals exposed to different temperatures were statistically tested with a method based on a combination of independent two-sample tests of Wilcoxon (van Elteren, 1960).

TABLE 24 - Influence of change in environmental temperature on body-weight and liver weight

| | temperature °C | body-weight g | liver wet weight | |
|---|---|---|---|---|
| | | | g | g per 100 g body weight |
| hamster (fed) | 5 | 89 ± 12 (18) | 4.2 ± 0.6 (18) | 4.7 ± 0.4 (18) |
| | 23 | 98 ± 9 (17) | 4.0 ± 0.5 (16) | 4.1 ± 0.5 (16) |
| | | N.S. | N.S. | $P < 0.01$ |
| rat (fed) | 5 | 244 ± 19 (8) | 10.4 ± 1.1 (8) | 4.3 ± 0.1 (8) |
| | 23 | 271 ± 12 (8) | 10.7 ± 1.3 (8) | 4.0 ± 0.3 (8) |
| | | $P < 0.01$ | N.S. | N.S. |
| roach (starved) | 5 | 62 ± 18 (37) | 1.5 ± 0.6 (39) | 2.3 ± 0.7 (37) |
| | 18 | 62 ± 22 (32) | 1.1 ± 0.5 (55) | 1.6 ± 0.4 (29) |
| | | N.S. | $P < 0.01$ | $P < 0.01$ |
| trout (fed) | 5 | 145 ± 20 (26) | 2.4 ± 0.5 (43) | 1.6 ± 0.4 (26) |
| | 18 | 157 ± 19 (26) | 2.0 ± 0.5 (51) | 1.3 ± 0.3 (26) |
| | | N.S. | $P < 0.01$ | $P < 0.01$ |
| trout (starved) | 5 | 143 ± 15 (23) | 2.0 ± 0.6 (23) | 1.5 ± 0.4 (23) |
| | 18 | 139 ± 13 (14) | 1.6 ± 0.4 (14) | 1.1 ± 0.3 (14) |
| | | N.S. | $P < 0.01$ | $0.05 > P > 0.01$ |

Results are expressed as means with standard deviation; the number of animals is given in parentheses.
N.S. Not significantly different (Wilcoxon two-sided two-sample test)

The results are presented in tables 24-27, and figure 27. It appears that an alteration in the environmental temperature causes an obvious change in some components and enzymatic capacities of the liver. The effects are essentially equal both for the homeothermic mammals and for the poikilothermic fishes. For the trout they also appear independent of the nutritional status.

The influence of the different environmental temperature on the overall composition of the liver can be most clearly seen from the significantly lower weight of the total liver in the warmth-treated animals compared with the cold-treated ones, especially if the liver weight is related to the body-weight (table 24). The difference is not due to a variation (per g liver) in the water content of the liver tissue (figure 27). These findings are in accordance with earlier reports on the influence of low environmental temperature on the golden hamster (Smit-Vis and Smit, 1969), on the rat (Kalser and Kunig, 1969), and on the goldfish (Das, 1967).

Figure 27 shows that cold-exposed fish have a higher amount of total liver

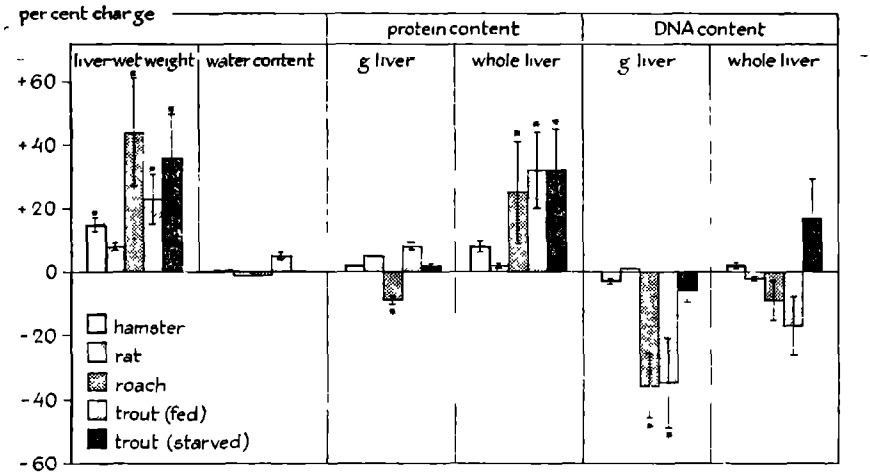



FIGURE 27. Per cent changes in the livers of cold-exposed animals compared with warmth-exposed animals. Calculation: (cold/warmth - 1) x 100%. Liver wet weight is related to 100 g body-weight. Water content has been derived from the dry/wet weight ratio. Blocks represent average changes ± S.D. Number of animals investigated is given in table 24. * indicates a significant difference at $P < 0.05$ (Wilcoxon two-sided two-sample test).

protein than the fish kept at elevated temperature. In earlier studies a clear-cut increase in the level of total hepatic protein and in the incorporation of $^{14}$C-leucine in total hepatic protein of fishes in cold water as compared with fishes in warm water has been observed for goldfish (Das, 1967; Das and Prosser, 1967) and for rainbow trout (Dean and Berlin, 1969). On the other hand no temperature-induced differences in the amount of hepatic protein of goldfish have been found by Caldwell (1969). Probably the reason for the inconsistent observations for the hepatic protein in the above mentioned studies is that a change in the amount of protein, if expressed per g fresh liver, is obscured due to a shift in the relative amounts of other liver components. Here also, species differences, the nutritional status of the animals, and the length of the period of exposure may play a role.

That an inaccurate picture of the overall change in the liver can be obtained if the components are expressed as concentrations (mg/g wet tissue) is also demonstrated in figure 27. As far as the concentrations of protein and DNA are concerned, it might be erroneously concluded that under the influence of exposure to different temperatures the amount of hepatic protein of the trout did not change and the amount of hepatic protein of the warmth-exposed roach increased, whereas in the liver of the warmth-exposed fishes a pronounced increase in DNA occurred. However,

TABLE 25 - Influence of change in environmental temperature on drug metabolism*

| | temperature °C | *N*-demethylation of aminopyrine | | | |
|---|---|---|---|---|---|
| | | activity per g fresh liver | activity per mg liver protein | activity per mg liver DNA | activity per 100 g body-weight |
| hamster (fed) | 5 | 28.0 ± 3.9 (18) | 0.162 ± 0.017 (18) | 15.2 ± 2.7 (18) | 132.7 ± 20.0 (18) |
| | 23 | 23.0 ± 3.7 (17) | 0.142 ± 0.020 (14) | 12.5 ± 2.4 (15) | 96.8 ± 16.0 (16) |
| | | $P < 0.01$ | $0.05 > P > 0.01$ | $P < 0.01$ | $P < 0.01$ |
| rat (fed) | 5 | 23.5 ± 2.8 (8) | 0.128 ± 0.010 (8) | 11.6 ± 1.0 (8) | 99.8 ± 9.3 (8) |
| | 23 | 20.4 ± 1.6 (8) | 0.116 ± 0.012 (8) | 10.2 ± 1.3 (8) | 80.7 ± 8.0 (8) |
| | | $P < 0.01$ | N.S. | $0.05 > P > 0.01$ | $P < 0.01$ |
| roach (starved) | 5 | 3.49 ± 1.17 (44) | 0.0278 ± 0.0096 (42) | 1.62 ± 0.61 (45) | 8.16 ± 2.06 (32) |
| | 18 | 2.45 ± 0.86 (39) | 0.0174 ± 0.0067 (39) | 0.74 ± 0.34 (38) | 4.01 ± 1.76 (28) |
| | | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ |
| trout (fed) | 5 | 1.51 ± 0.41 (38) | 0.0125 ± 0.0038 (38) | 1.05 ± 0.46 (38) | 2.57 ± 0.95 (26) |
| | 18 | 0.90 ± 0.30 (37) | 0.0083 ± 0.0036 (37) | 0.39 ± 0.23 (36) | 1.31 ± 0.39 (25) |
| | | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ |
| trout (starved) | 5 | 1.35 ± 0.50 (16) | 0.0114 ± 0.0051 (16) | 0.83 ± 0.51 (16) | 1.73 ± 0.59 (16) |
| | 18 | 0.71 ± 0.16 (9) | 0.0061 ± 0.0015 (9) | 0.35 ± 0.09 (9) | 0.77 ± 0.19 (9) |
| | | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ |

* Activity is defined as $\mu$moles formaldehyde produced per hour; assayed with 9000 *g* supernatants derived from liver homogenates at 37 °C for hamster and rat and at 25 °C for roach and trout; values are means ± S.D.; the number of animals is given in parentheses.

N.S. Not significantly different (Wilcoxon two-sided two-sample test).

TABLE 26 - Influence of change in environmental temperature on drug metabolism*

| | temperature °C | *p*-hydroxylation of aniline | | | |
|---|---|---|---|---|---|
| | | activity per g fresh liver | activity per mg liver protein | activity per mg liver DNA | activity per 100 mg body-weight |
| hamster (fed) | 5 | 3.99 ± 0.29 (12) | 0.024 ± 0.002 (12) | 2.15 ± 0.34 (12) | 18.87 ± 2.10 (12) |
| | 23 | 2.78 ± 0.46 (13) | 0.016 ± 0.003 (11) | 1.38 ± 0.29 (12) | 10.95 ± 2.10 (13) |
| | | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ |
| rat (fed) | 5 | 1.97 ± 0.45 (8) | 0.011 ± 0.003 (8) | 0.99 ± 0.28 (8) | 8.37 ± 2.00 (8) |
| | 23 | 1.41 ± 0.30 (8) | 0.008 ± 0.002 (8) | 0.71 ± 0.15 (8) | 5.60 ± 1.40 (8) |
| | | $P < 0.01$ | $0.05 > P > 0.01$ | $0.05 > P > 0.01$ | $P < 0.01$ |
| roach (starved) | 5 | 1.09 ± 0.46 (40) | 0.0082 ± 0.0038 (37) | 0.48 ± 0.23 (40) | 2.67 ± 0.93 (32) |
| | 18 | 0.62 ± 0.18 (36) | 0.0044 ± 0.0015 (36) | 0.19 ± 0.10 (36) | 1.12 ± 0.60 (28) |
| | | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ |
| trout (fed) | 5 | 0.39 ± 0.08 (38) | 0.0032 ± 0.0008 (38) | 0.27 ± 0.09 (38) | 0.63 ± 0.20 (26) |
| | 18 | 0.31 ± 0.11 (37) | 0.0027 ± 0.0010 (37) | 0.12 ± 0.04 (36) | 0.37 ± 0.15 (25) |
| | | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ |
| trout (starved) | 5 | 0.37 ± 0.07 (16) | 0.0030 ± 0.0008 (16) | 0.22 ± 0.12 (16) | 0.46 ± 0.10 (16) |
| | 18 | 0.17 ± 0.03 (9) | 0.0015 ± 0.0002 (9) | 0.09 ± 0.03 (9) | 0.18 ± 0.05 (9) |
| | | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ |

* Activity defined as $\mu$moles *p*-aminophenol produced per hour; assayed with 9000 *g* supernatants derived from liver homogenates at 37 °C for hamster and rat and at 25 °C for roach and trout; values are means ± S.D.; the number of animals is given in parentheses. Statistical comparisons were made by Wilcoxon's two-sided two-sample test.

TABLE 27 - Influence of change in environmental temperature on liver composition and hepatic drug metabolism of the roach in summer

| temperature (° C) | liver wet weight g per 100 g body-weight | liver protein mg per 100 g body-weight | liver DNA mg per 100 g body-weight | *N*-demethylation of aminopyrine activity* per 100 g body-weight | *p*-hydroxylation of aniline activity** per 100 g body-weight |
|---|---|---|---|---|---|
| 5 | 2.6 ± 0.4 | 345 ± 60 | 5.25 ± 0.60 | 13.0 ± 4.0 | 4.7 ± 1.5 |
| 18 | 1.8 ± 0.3 | 270 ± 35 | 5.10 ± 0.75 | 5.4 ± 2.9 | 2.7 ± 0.8 |
| | $P < 2 \times 10^{-3}$ | $P < 2 \times 10^{-2}$ | N.S. | $P < 2 \times 10^{-3}$ | $P < 10^{-2}$ |

Values are means ± S.D.; 2 groups of 8 roaches were exposed for 1 week (first week of September) to different environmental temperatures

* Activity defined as $\mu$moles formaldehyde produced per hour; enzyme assay at 25 °C

** Activity defined as $\mu$moles *p*-aminophenol produced per hour; enzyme assay at 25 °C

Statistical comparisons by Wilcoxon's two-sided two-sample test

Note the significantly higher values found for the animals kept at 5 °C

the relative constancy of the DNA content of the total liver indicates that the higher amounts of DNA observed per g liver in roach and trout (figure 27) are not caused by an increase in the absolute number of cells or absolute amount of DNA, but represent an increase of cells per g liver under the influence of changes in the amounts of other liver components.

In the present study the amounts of glycogen and lipids were not determined. It has to be noted, however, that during the preparation of the liver tissue of the cold-exposed fishes after centrifugation of the homogenates a white fat layer was always observed on the 9000 *g* supernatant. This layer was absent on the 9000 *g* supernatants of liver homogenates from the warmth-exposed animals.

In view of the changes observed for the hepatic constituents it seems reasonable to express the drug-metabolizing capacities in relation to various parameters (tables 25 and 26). The livers of cold-exposed animals show significantly higher capacities than those of the warmth-exposed ones. This change in drug-metabolizing capacities becomes manifest independent of whether they are expressed per unit of liver weight, per amount of protein, per amount of DNA, or per unit of body-weight.

The experiments described above were performed in winter. Repetition of the experiments with roach in summer showed a similar effect of the exposure to different temperatures on liver composition and hepatic drug-metabolism (table 27).

When the hepatic enzymatic activities of animals (hamster, rat, roach, and trout) exposed to different ambient temperatures were assayed at different incubation temperatures the enzymatic activities of the warmth-exposed animals were consistently lower over the entire temperature range. The apparent $K_m$-values for aminopyrine in the *N*-demethylation reaction, assayed at 4 temperatures (20, 25, 35, and 40 °C) were found to be unchanged after the exposure of the animals to different ambient temperatures.

### VII, 4. THE INFLUENCE OF AN ALTERATION OF THE AQUATIC ENVIRONMENT ON HEPATIC DRUG OXIDATION IN THE ROACH

Roach were caught and kept alive under different conditions. One group of fishes was kept in a fishing-boat in a reservoir freely accessible to the surrounding river water. Another group was transported to the laboratory and maintained for several weeks in tanks in running tap water (600 l/h, 9 °C). The roach in captivity refused the food offered.

At different times a sample of the various groups was taken and drug-metabolizing capacities were determined *in vitro*. The results of an experi-

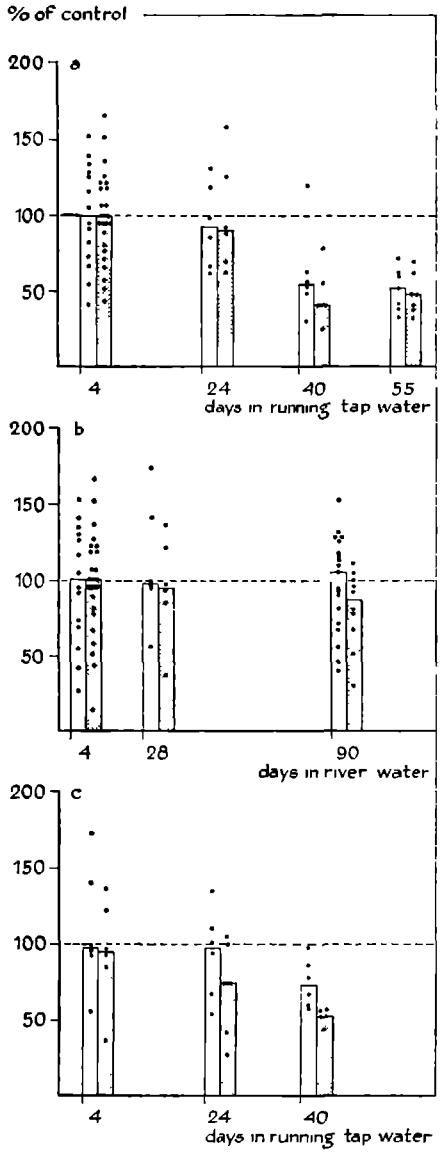



FIGURE 28. Drug-metabolizing capacities of roach in captivity under different conditions. Blocks represent the median value of each sample. All values are expressed as percentages of a control value, which was the median of freshly caught and immediately investigated fishes (at the start of the experiment). □, *p*-Hydroxylation of aniline; □ *N*-demethylation of aminopyrine. a, Roach living in captivity in running tap water (9 °C). b, Roach living in captivity in river water (5 °C). c, Roach living in captivity in running tap water after a preceding captivity period of 4 weeks in river water (Dewaide and Henderson, 1970).
Note: the drug-metabolizing capacities of roach living in running tap water decrease, those of roach living in river water do not decrease.

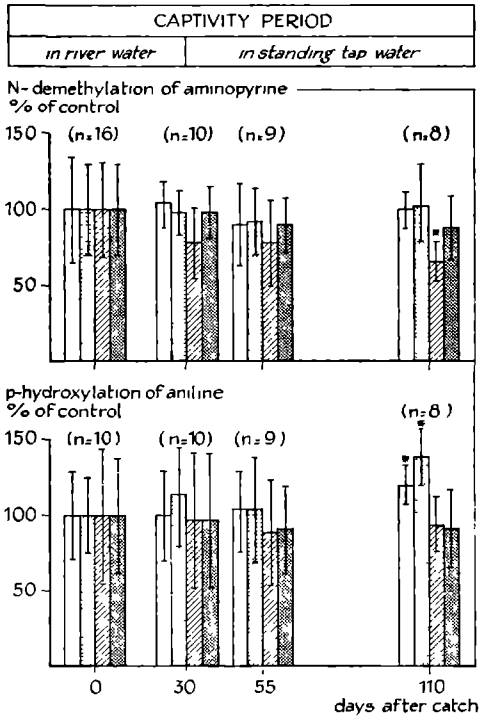



FIGURE 29. Drug-metabolizing capacities of the wild roach in captivity in river water and in standing tap water at 5 °C. Blocks represent mean values ± S.D.; the hepatic drug-oxidizing capacities are expressed as a percentage of a control value, which was the mean of a sample of freshly caught and immediately investigated fishes (at the start of the experiment); other fishes were kept for 30 days in river water and subsequently in the laboratory in standing tap water, which was refreshed twice a week; number of animals is given in parentheses. □ Enzymatic capacity expressed per g fresh liver; ⊡ enzymatic capacity expressed per mg hepatic protein; ▨ enzymatic capacity expressed per mg DNA; ▩ enzymatic capacity expressed per 100 g bodyweight. * significantly different from control at $P < 0.05$ (Wilcoxon two-sided two-sample test).

ment lasting from December 1968 to March 1969 are presented in figure 28. In figure 28a it can be seen that after transfer of the fishes to running tap water their drug-metabolizing capacities decreased in 40 days by 50 per cent. In the animals kept in captivity in the river water (± 5 °C) no clear decrease in the drug-metabolizing capacities could be observed even after 90 days (figure 28b). However, if a group of animals, after a captivity period of 4 weeks in river water, was transported to the laboratory and kept for 40 days in running tap water, then again a decrease in the enzymatic capacities became clear (figure 28c).

TABLE 28 - Decrease in hepatic drug-metabolizing capacities of roach living in running tap water (9 °C) in summer

| | per cent remaining capacity | |
|---|---|---|
| | stay in running tap water | |
| | 48 days (n=10) | 63 days (n=8) |
| *N*-demethylation of aminopyrine | | |
| a | 49 ± 15 | 41 ± 15 |
| b | 43 ± 11 | 40 ± 13 |
| c | 23 ± 11 | 20 ± 4 |
| d | 24 ± 6 | 23 ± 10 |
| *p*-hydroxylation of aniline | | |
| a | 43 ± 13 | 28 ± 7 |
| b | 34 ± 13 | 28 ± 6 |
| c | 18 ± 5 | 15 ± 5 |
| d | 22 ± 6 | 16 ± 7 |

Drug-metabolizing capacity expressed as percentage of the capacity at the time of catch (June 1970); mean values with standard deviation; n is number of animals;
a. capacity related to 1 g fresh liver; b. capacity related to 1 mg hepatic protein; c. capacity related to 1 mg DNA; d. capacity related to 100 g body-weight

On the other hand, if a group of roach was kept in the laboratory in standing tap water (5 °C), which was refreshed only twice a week no decrease in their drug-oxidizing capacities could be observed during a relatively long period. Figure 29 shows that the capacities were not reduced by a stay of 30 days in the fishing-boat in river water and a subsequent stay of 25 days in standing tap water at 5 °C. In this experiment the drug-oxidizing capacities were also related to various parameters. The changes in the capacities 110 days after catch show no uniform picture (figure 29). It seems to depend chiefly upon the parameter in terms of which the capacity is expressed. The situation of long-lasting starvation presumably causes changes in the amounts of the various liver components.

The phenomenon of the decrease of the drug-metabolizing capacity of roach due to a stay in running tap water was not only observed in winter but also in

summer (table 28). The observation that the decrease is most strongly pronounced if the enzymatic capacities are expressed in relation to the amount of DNA or to the amount of liver normalized to 100 g body-weight, also suggest changes in the overall composition of the liver.
The phenomena described above cannot be explained by reference to the difference in the aquatic temperature. The decrease in the drug-oxidizing capacity due to the transfer to running tap water becomes manifest both in summer, when the fishes are taken from an environmental temperature of about 18 °C to a temperature of about 9 °C, and in winter at a transfer from about 5 to 9 °C. Under these conditions a transfer to standing tap water causes no decrease. Also the nutritional status is of less importance with regard to the decrease observed: the animals were starved both in running and in standing tap water. The decrease in drug-oxidizing capacity observed presumably depends merely upon the continuous refreshment of the water.

## VII, 5. THE INFLUENCE OF XENOBIOTIC AGENTS ON THE DRUG-METABOLIZING CAPACITY OF FISH

As far as fishes are concerned no indications are available yet of an influence of the exposure to xenobiotic agents on the hepatic processes involved in drug metabolism. Buhler and Rasmusson (1968a and 1968b) reported that, in contradistinction to mammals, the enzymatic activities in oxidative and reductive drug-metabolizing reactions in rainbow trout were not affected by starvation, by feeding of diets high in protein or carbohydrate, nor by feeding or injecting DDT, chlordane, phenobarbital or phenylbutazone. In the present study, too, no changes could be observed in the drug-metabolizing capacity of rainbow trout and roach (living in standing tap water at 5 °C; cf. figure 29) during starvation. However, the other data already presented in this chapter (seasonal variation, effects of temperature change and water refreshment) argue against such a general view, i.e. that the drug-metabolizing capacity of fish would not depend upon factors in their environment.
As far as the administration of potentially enzyme-inducing compounds is concerned, it was observed in this study that most fish species are more sensitive to manipulation than the commonly used mammalian laboratory animals. It appeared that treatment with drugs has to be preferentially performed by the addition of the substances to the food or to the surrounding water, rather than by intramuscular or intraperitoneal injections. As far as oral treatment is concerned experiments could only be done with hatchery-reared animals in the present study since wild fishes refused the food offered. In an experiment with rainbow trout one group of animals was fed during

TABLE 29 - Effect of oral administration of dieldrin and DDT on the drug-metabolizing capacity of trout liver

| | *N*-demethylation of aminopyrine (μmoles formaldehyde produced per hour per g fresh liver) | *p*-hydroxylation of aniline (μmoles *p*-aminophenol produced per hour per g fresh liver) |
|---|---|---|
| control animals | 1.0 ± 0.3 (13) | 0.27 ± 0.09 (21) |
| animals treated with dieldrin* | 1.6 ± 0.5 (10)<br>$p < 0.05$ | 0.34 ± 0.05 (18)<br>$p < 0.01$ |
| animals treated with DDT* | 1.5 ± 0.4 (12)<br>$p < 0.05$ | 0.29 ± 0.08 (10)<br>N.S. |

* food with and without insecticide was supplied on alternating days; in a period of 36 days on 18 days 0.25 mg insecticide per 100 g animal per day was given. Statistical comparisons were made by using the two-sided two-sample test of Wilcoxon.
Note: enzymatic capacities of trout livers are significantly increased by a supply of insecticides in the food.

36 days with normal food (Clark's trout pellets obtained from the breeder); other groups were fed on alternating days with equal amounts of normal food and with this food containing sublethal doses of DDT or dieldrin. The control group and the insecticide-treated groups were supplied with the same amount of food and all the food offered was seen to be consumed. The diet was arranged in such a way that the dose of insecticide consumed at each feeding was approximately 0.25 mg insecticide per 100 g body-weight. After 36 days (18 treatments) the animals were killed and assayed for drug-metabolizing enzymatic activity. In this experiment the liver weight, and the hepatic protein and DNA contents of the animals were not determined. The results of the enzyme assays are given in table 29. The data show that the capacity for enzymatic *N*-demethylation in trout liver is significantly increased in animals supplied with a diet containing dieldrin or DDT. The capacity for *p*-hydroxylation is only significantly increased in dieldrin-treated trout.

In order to investigate whether the high drug-metabolizing capacities in wild roach and the changes therein during the various seasons of the year and during the stay in running tap water may be related to the presence of enzyme-inducing chemicals in the environment, experiments were performed in which roach were treated with dieldrin, DDT, and atrazin during the stay in running tap water. The treatment was performed by stopping the water flow and by adding the pesticide to the water in a dose of 0.1 mg per liter of water. After 1 hour the water flow was started up again. In a tank with

TABLE 30 - The influence of dieldrin on hepatic drug oxidation in the roach

| | enzymatic capacity | | |
|---|---|---|---|
| *N*-demethylation of aminopyrine<br>$\mu$moles formaldehyde produced<br>per hour<br>per | after catch<br>untreated animals<br>(n=10) | after a stay of 18 days in running tap water<br>untreated animals<br>(n=12) | <br>animals exposed to dieldrin*<br>(n=14) |
| g fresh liver | 5.4 ± 1.3 | 3.8 ± 0.4 | 4.8 ± 0.9 |
| mg liver protein | 0.036 ± 0.009 | 0.026 ± 0.007 | 0.034 ± 0.007 |
| mg liver DNA | 2.4 ± 0.6 | 1.8 ± 0.6 | 2.2 ± 0.6 |
| 100 g body-weight | 11.0 ± 2.5 | 8.6 ± 2.3 | 10.5 ± 1.9 |
| *p*-hydroxylation of aniline<br>$\mu$moles *p*-aminophenol produced<br>per hour<br>per | | | |
| g fresh liver | 1.65 ± 0.48 | 1.09 ± 0.36 | 1.54 ± 0.41 |
| mg liver protein | 0.0107 ± 0.0030 | 0.0078 ± 0.0025 | 0.0109 ± 0.0030 |
| mg liver DNA | 0.73 ± 0.25 | 0.55 ± 0.27 | 0.73 ± 0.27 |
| 100 g body-weight | 3.30 ± 0.58 | 2.50 ± 0.96 | 3.42 ± 0.95 |

Means with standard deviation; n is the number of animals; weight of the animals used was 65 - 90 g.

* water flow was stopped on 10 days for 1 hour and dieldrin was added to the water in a dose of 0.1 mg of insecticide per liter of water.

Enzymatic capacities of animals exposed to dieldrin are significantly different from those of untreated animals at $P < 0.05$ (Wilcoxon two-sided two-sample test).

control animals the tap water flow was stopped for 1 hour, but no pesticide was added. After various administration periods animals were killed and assayed for drug-metabolizing enzymatic activity. In control animals, and in DDT- and atrazin-treated animals, the decrease in drug-metabolizing enzymatic activities was similar to that normally observed in roach staying in running tap water. In the dieldrin-treated animals, however, the decrease in the drug-metabolizing enzymatic activities was less pronounced. In order to check this observation an experiment was performed in which roach were treated with dieldrin during their stay in the laboratory.

Freshly caught (September) roach were transported to the laboratory and kept in running tap water. After 4 days of acclimatization of the fishes to the laboratory conditions the exposure of the fishes to dieldrin was begun. On various days the water flow was stopped for 1 hour and dieldrin was added in a dose of 0.1 mg per liter of water. After arrival of the animals in the laboratory the scheme of treatment was as follows: 4 days of acclimatization, 5 days of dieldrin treatment, 2 days of rest, 5 days of dieldrin treatment, 2 days of rest. Subsequently the fishes were killed and assayed for drug-metabolizing enzymatic capacities. The results are presented in table 30 and show that a significant decrease in the drug-metabolizing capacity only occurred in the control animals, but not in the animals exposed to the insecticide.

## VII, 6. DISCUSSION

### *Changes in drug-metabolizing capacities of fishes*

The results presented in this chapter indicate that the drug-metabolizing capacity of fishes depends upon external factors. In the wild roach the hepatic drug-oxidizing capacity, which appears to be relatively high as compared with that in other fish species studied, varies with the season of the year. Highest enzymatic capacities were found during the summer months. The decrease in enzymatic capacities in the liver of wild roach kept in captivity in running tap water, and the differences caused by the exposure of fish to different environmental temperatures and to certain xenobiotics, are further evidence of the variability of the level of xenobiotic oxidation in fish.

For a clear view of the changes in drug-metabolizing capacity, it has been stressed that differences occur in the ratios of the various liver constituents in fishes living under different conditions. The changes in the relative amounts of the various liver components of fishes exposed to different aquatic temperatures may be taken as an example (figure 27). In connection with

the observed changes in liver weight and composition it should be emphasized that they are only rough indications of alterations in function. The changes can have a multifactorial cause since the liver participates in many metabolic processes and functions simultaneously as a store of carbohydrates, and in the case of fishes, of lipids also (Mead and Kayama, 1967). The differences in liver composition under different conditions of life may well be caused by a change in the synthesis or the mobilization of glycogen and lipids, which are used as fuel sources. For instance, an alteration in the level of hepatic energy reserves has been shown to occur in fish exposed to different temperatures (Dean and Goodnight, 1964).
In view of the overall changes in the liver of animals under different conditions, in the present study the parameters in relation to which the drug-metabolizing capacities are expressed were considered to be important. It will be obvious, that in the case of changes in the amounts of other hepatic components, differences in enzyme concentrations (enzymatic activity expressed per g tissue) may be evoked or obscured as artefacts. Therefore, in the present study the biotransformation rates are not only related to 1 g fresh liver, but also expressed per mg hepatic protein, per mg liver DNA, and to a parameter in which the overall situation is taken into account, viz. the amount of liver corresponding to 100 g body-weight.

*Seasonal variation in hepatic drug metabolism in the roach*

For the seasonal fluctuations in the drug-metabolizing capacity of wild roach several factors have to be considered to underlie this phenomenon.
Seasonal cycles in fishes with changes in metabolic and endocrine activity normally occur and seem to depend on external factors such as temperature and daylight (Hoar, 1957; Pavlovic, 1968).
Dietary factors may play a role. Hence, it is possible with respect to the drug-metabolizing capacity that the observed seasonal fluctuations are related to differences in food intake and therewith to the degree of exposure to foreign compounds. The higher biological activity of the fishes during the summer months implies more close contact of the animals with the environment, involving high mobility, an increased consumption of food and a higher aquatic respiration through the gills. If this is taken into account together with the possible presence of higher concentrations of enzyme-inducing chemical agents, among which are insecticides, in the river water due to a lower water level of the Waal, which is one of the chemically more polluted rivers in the Netherlands, an increase in the enzymatic capacities in the summer months is understandable. This hypothetical view is partially supported by the data plotted in figure 30, which represent some characteris-

tics of the river water at different times of the year. Periods with low water flow (low water level) appear to occur in both summer and winter. In general, figure 30 shows that during such periods peaks can be observed for the concentration of chemical substances (phenols, $Cl^-$) in the river water. Additionally, a high B.O.D.$_5$ (Biological Oxygen Demand in 5 days at 20 °C) can be observed in summer. The latter parameter is generally used as a

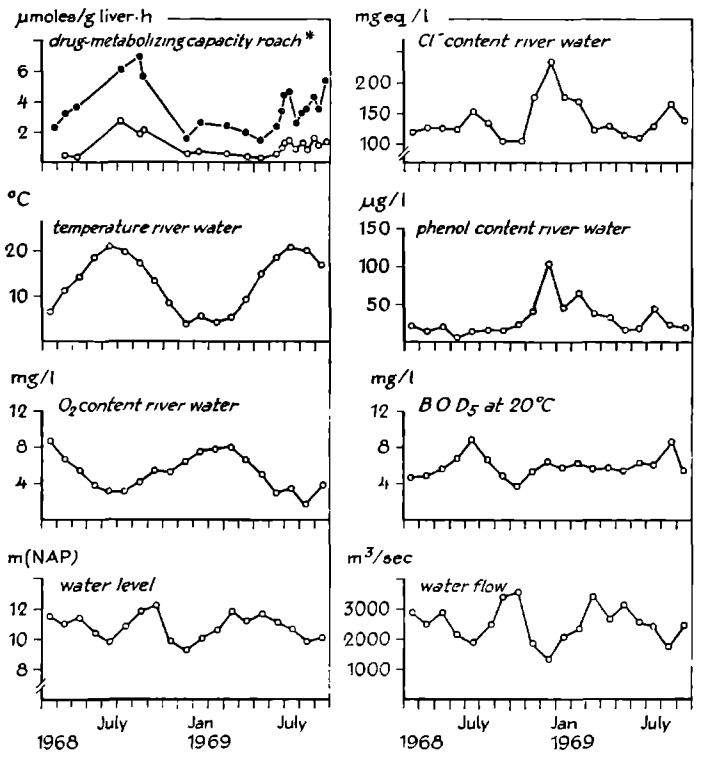



FIGURE 30. Drug-metabolizing capacity of the roach and characteristics of the river Waal, the main branch of the river Rhine delta flow.

* The values for the drug-metabolizing capacity of the roach correspond with those of figure 26: • —— •, *N*-demethylation of aminopyrine; o —— o, *p*-hydroxylation of aniline.

Parameters with respect to the river water were kindly furnished by RIZA (Rijksinstituut voor Zuivering van Afvalwater), Voorburg, the Netherlands. The data concern the water of the Rhine near Lobith.

Note the peaks for the B.O.D.$_5$ (Biological Oxygen Demand in 5 days at 20 °C), and for the concentrations of $Cl^-$ and phenol in the periods of low water flow (low water level). One may assume higher concentrations of chemical substances in the river water in these periods.

measure of the total amount of oxidizable organic matter. However, data about the concentrations of substances which are potential enzyme inducers, for instance insecticides and herbicides, are not yet available. The possibility exists that such substances occur in higher amounts in spring and summer. Moreover it seems reasonable to assume that a low water level also has a concentrative effect on these pesticides. The absence of an inducing effect of the substances during the period of low water level in winter might be due to the low biological activity of the roach at low aquatic temperatures (Fry, 1957). The suggestion of a higher entrance of xenobiotics into fishes during the summer months is supported by the findings of Robinson *et al.* (1967). They showed, in marine fish species from the Eastern Coast of Britain (young cods and sand eels), 2 to 3 times higher concentrations of organochlorine insecticides (*pp*'-DDE and dieldrin) in summer than in winter. In this connection the experiments of Koeman *et al.* (1969b), who found a higher degree of accumulation of the herbicide diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) in yearling carp during summer than in winter, are also significant.

*Changes in drug-metabolizing capacity due to a different environmental temperature*

Temperature differences between summer and winter are not the direct cause of the seasonal fluctuations in drug-metabolizing capacity. On the contrary, experiments in which various animals were exposed to a different ambient temperature showed that, just as happens in the livers of mammals (rat and hamster), in the livers of wild roach and hatchery-reared trout living at 5 °C, higher drug-metabolizing capacities are present than in animals living at 18 °C.

As far as metabolism in relation to temperature is concerned, studies are generally focused on the metabolic pathways involved with energy supply. Both in homeotherms and in poikilotherms it has been observed that exposure to low environmental temperatures is followed by an increased energy metabolism (Kanungo and Prosser, 1959; Freed, 1965; Hochachka, 1967; Liu *et al.* 1969). The increase is seen as a compensatory adjustment of metabolism in relation to the ambient temperature. Coincidental to the adjustment of energy metabolism quantitative and qualitative changes in the components participating in mitochondrial and microsomal electron transport systems have been observed (Caldwell, 1969; Aithal *et al.*, 1968). In view of these changes a concomitant change in the metabolic pathways involved in microsomal drug metabolism, which also requires electron transport, is conceivable.

From the exposures of animals to different environmental temperatures in this study it can be concluded that, in spite of the observed species differences in the hepatic metabolism of xenobiotics (chapter VI), in both mammals and fishes oxidative biotransformation rates are lowered by increase in the environmental temperature. Consequently, thermal pollution of the aquatic environment by increase in temperature may injure the drug-oxidizing capacity of water-dwelling animals.

As far as the influence of a higher aquatic temperature during the summer months is concerned, it can be supposed that the enzyme-inducing action of chemicals in the polluted environment predominates and masks the decreasing effect of the higher environmental temperature. This may be the case, since it has been found for the rat that chemical treatment and temperature stress produce their changes in drug-metabolizing enzymatic activities through different mechanisms (Stitzel and Furner, 1967; Furner and Stitzel, 1968).

*Influence of an alteration in the aquatic environment*

An indirect indication that not temperature differences but other factors are responsible for the seasonal fluctuations in drug-metabolizing capacity of the wild roach has been obtained from the experiments in which animals were kept in the laboratory in running tap water. It has been observed that during this stay both in summer, when the animals were transferred from an environmental temperature of about 18 °C to the laboratory water of 9 °C, and in winter from about 5 to 9 °C, the drug-metabolizing capacities decreased by 50 per cent. The influence of the deprivation of food during the captivity period is likely of minor importance for this decrease, since starvation was also involved in the experiments in which the animals were kept in river water or in standing tap water. In spite of starvation, even after a relatively long captivity period (3 to 4 months), no significant decrease in the drug-metabolizing capacity was observed.

An explanation for the decrease in the drug-metabolizing capacities to a low level in roach living in running tap water may be a termination of the exposure to inducing agents, presumably present in the chemically polluted river water. After removal of the animals from the river to the laboratory the foreign compounds may be removed from the body during the stay in clean, continuously running, tap water.

It has to be noted further that, in the liver of the rainbow trout, no changes in the drug-metabolizing capacity were found under the influence of starvation, season and removal from the presumably non-polluted hatchery water to the laboratory water.

*Influence of exposure to xenobiotics*

As far as the influence of xenobiotic agents on the drug-metabolizing capacity in fish is concerned, in the present study the exposure of trout and roach to enzyme-inducing compounds was not performed with a sufficiently wide variety of substances and dosage schedules to allow general conclusions to be reached. The experiments performed with rainbow trout and roach show that a slight increase in the hepatic *N*-dealkylating and aromatic hydroxylating capacities can be produced by exposure of the animals to the insecticides dieldrin and DDT, which are well-known inducers of drug metabolism in mammals. From this observation the expectation seems to be justified that further studies may show that the influence of xenobiotic compounds on the drug-metabolizing capacity of fishes and mammals will be much the same.

The experiments which show that the hepatic drug-metabolizing capacity, which decreases in the wild roach living in running tap water, remains on a high level when the animals are chronically exposed to an insecticide, agree with the idea that the high level of the drug-metabolizing capacities of wild fishes, at least in part, may depend on the presence of certain — as yet unknown — enzyme-inducing agents in their polluted environment.

CHAPTER VIII

# AN APPROACH TO ENVIRONMENTAL PHARMACOKINETICS

## VIII, 1. KINETICS OF CHEMICAL POLLUTANTS IN THE BIOSPHERE

The study of pharmacokinetics includes in a broad sense all the investigations directed to all aspects of the distribution of xenobiotic substances in systems consisting of interrelated compartments. Emphasis is placed upon the concentration-time relationships. As has been discussed in chapter I, the studies on the distribution of xenobiotic substances in human and animal bodies are indicated by the term *individual pharmacokinetics,* whereas studies dealing with the concentration-time relationships of chemical pollutants in the various parts of the biosphere are described by the term *environmental pharmacokinetics.* Both the biological objects and the biosphere can be considered as multicompartment systems, in which a multiplicity of factors and processes is present in a state of dynamic equilibrium. For a visualization of the kinetics of xenobiotic substances in the biological systems studied in individual pharmacokinetics, mathematical models are constructed which more easily permit an interpretation of the time-dependent nature of the various phenomena involved. In these formulations the events in the compartments are defined as mathematical entities. Thus, the word compartment is not only applied in order to indicate anatomically and physiologically well-defined parts of the body such as the vascular system, and the various organs and tissues, but refers also to constituents which are present in a more diffuse distribution in the biological object. Here can be mentioned the various body fluids, and the components (blood cells, proteins, and lipids), which are able to bind certain drug molecules. In all of these cases, the quantitative aspects of the interaction of a drug with a body constituent can be represented by a mathematical expression. Analogous to the procedures employed in individual pharmacokinetics, one may attempt in environmental pharmacokinetics to divide the biosphere into compartments, viz. atmosphere, soil, water, and biomass (figure 1), which in turn can be subdivided.

According to the classification of compartment systems of Rescigno and Segre (1966) one can say that the biosphere belongs to the class of *strongly connected compartment systems,* since a chemical substance can in principle reach the various compartments from the initial compartment into which it is

introduced, and subsequently it remains possible to reach every other compartment from any compartment. For substances which are eliminated by processes of physicochemical and biological transformation the compartment system can be called *open,* for substances which cannot leave the biosphere the compartment system can be defined as a *closed* system. In a study on environmental pharmacokinetics it will hardly be possible to reckon with all the phenomena involved: every compartment or process which is taken into consideration may be subdivided on closer inspection into many more compartments or processes. Therefore, it has to be stressed that the mathematical expressions used in this study represent rather simplified descriptions of the real situations. However, it is an empirical finding of other fields of study in which mathematical models are applied (hydrodynamics, electronics, aeronautics, individual pharmacokinetics), that simplified models can simulate surprisingly well the major attributes of the real system and offer insight into the dominant forces operating in it. Since it is evident that certain processes are dominant whereas others may contribute to a much smaller degree, it seems reasonable to attempt to compile the main processes in simple mathematical formulations. Prior to initiating a mathematical consideration of the fate of chemical pollutants in the biosphere it will be informative to go briefly into some of the more important aspects of chemical contamination.

## VIII, 2. SOME GENERAL ASPECTS OF CHEMICAL POLLUTION

The chemical substances involved in environmental pollution can be divided into various categories. Table 31 represents a division on the basis of the susceptibility of the pollutants to biodegradation. The categories I and II include the pollutants that can be utilized as nutrients by organisms, especially microorganisms. These categories are predominantly made up of substances that are already naturally occurring: the compounds and minerals which are ordinary constituents of organic matter. For example, large amounts of organic material are introduced into the environment as domestic sewage, and as wastes from agriculture and food-processing industry. The presence of organisms in nature which are capable of breaking down the organic materials can, in principle, limit this kind of pollution. Thus, the introduction of organic matter is no real problem so long as ecosystems are not overloaded. If overloading occurs with respect to organic compounds and minerals utilized by only a few species of organisms, the *artificial eutrophication* will create several problems: e.g. an excessive increase in the number of adaptive organisms may eventually inhibit — or completely

TABLE 31 - Categories of chemical pollutants

| Susceptibility to biodegradation | | I highly susceptible | II moderately susceptible | III poorly susceptible | IV not susceptible |
|---|---|---|---|---|---|
| NON-TOXIC | elimination rate | high | moderate | low | nil |
| | environmental pollution** | low | moderate | high | unlimited |
| | harmful side-effect | eutrophication† | | | |
| TOXIC* | elimination rate | moderate | delayed | strongly delayed | nil |
| | environmental pollution** | moderate | progressive | strongly progressive | unlimited |
| | harmful side-effect | enhanced accumulation of other pollutants | progressive accumulation of other pollutants | strongly progessive accumulation of other pollutants | unlimited accumulation of other pollutants |

* potential damage to the biodegradation system

** accumulation at continual disposal

† excessive increase in the number of organisms utilizing the pollutants as nutrients

abolish — other forms of life (especially aerobic organisms). In the approach to environmental pharmacokinetics which will be developed in this chapter, the problems related to artificial eutrophication will not be considered.
The categories III and IV are largely made up of the poisons and chemicals that are not normally present in nature, but are synthesized and introduced by man. The diversity in this class of substances is great. Substances that can be easily broken down and utilized by organisms belong in principle to categories I and II. Generally speaking, however, most of the synthetic compounds, for instance many pesticides, detergents, lubricants, food-additives, and plastics, are rather resistent to biodegradation. Therefore, the risk of accumulation of these kinds of pollutants, which are being continuously introduced into nature, is great. This risk is still greater for substances that can damage the biodegradation processes: such substances can progressively accumulate and enhance the accumulation of other pollutants. In view of this, the substances which can damage biodegradation have to be considered as the most dangerous ones.

## VIII, 3. THE FATE OF CHEMICAL POLLUTANTS IN ECOSYSTEMS

For an understanding of the kinetics of chemical pollutants in the biosphere, the fate of the substances in *ecosystems* can be taken as a model. Ecosystems represent basic functional units in nature, including balanced populations of organisms and the nonliving environment, each influencing the properties of the other and both necessary for maintenance of life. An ecosystem taken as a multicompartment system, differs only from the biosphere in that an ecosystem is always open: a pollutant can move in principle from one ecosystem to another.
In the following discussion no emphasis is put upon differences in pharmacokinetics between the various types of ecosystems on the earth due to structural and functional variations in these systems. Attention is only directed towards factors which play a determining role with regard to the concentration-time relationship of the chemical pollutants in ecosystems in general.
After introduction of a dose ($Q_{x(o)}$) of a pollutant into an ecosystem, the fate of the compound can be represented by the very simplified scheme shown in figure 31.
From a kinetic point of view, when a given substance is introduced into an ecosystem that substance will enter one of the main compartments of the system, viz. air, soil, water, or organisms, and then be distributed to the other compartments and to their various parts. Later the substance may be

eliminated The elimination may proceed by migration of the pollutant to another ecosystem, by sequestration occurring when the substance is irreversibly bound to constituents of the ecosystem, by physicochemical disintegration, and by biodegradation. Migration of the chemical compounds to various ecosystems is important for the actual concentrations reached in the respective ecosystems, but not strictly for the total amount of pollutants present in the biosphere. Practically, however, the exchange between ecosystems will have an effective influence on the total amount of a substance

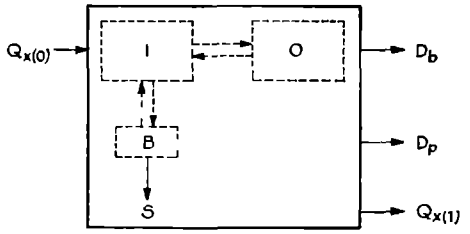



FIGURE 31. Schematic representation of some processes which determine the concentration-time relationships in environmental pharmacokinetics $Q_{x(0)}$ represents the dose of a chemical pollutant introduced into an ecosystem Arrows indicate possible flows of the compound in the ecosystem The introduction site compartment or initial compartment (I) can be the atmosphere, water, or soil The pollutant can exchange between these areas It can also be taken up by compartment O, which represents the biomass (all organisms) in the ecosystem The pollutant can reach the environmental compartments from compartment O when the substance is excreted chemically unchanged from the organisms, or when organisms decompose after death. In various compartments the pollutant can be reversibly bound to components of the ecosystem compartment B Compartment B functions as a depot. The pollutant can be eliminated from the ecosystem 1) by migration into another ecosystem (dose $Q_{x(1)}$, 2) by physicochemical disintegration ($D_p$), which can occur in all compartments, 3) by biological degradation ($D_b$), which can be performed by the various organisms living in the ecosystem, and 4) by sequestration (S), which implies irreversible binding of the pollutant to components of the ecosystem.

in the biosphere in the cases where the pollutant migrates into systems with different capacities of sequestration or biological degradation. The processes which effectively decrease the concentration of pollutants in the biosphere, viz. sequestration, physicochemical disintegration, and biodegradation, will be summarized as *purification.* Biodegradation plays a major role among the processes of purification. The breakdown of chemical pollutants in soil and water is mainly performed by microorganisms. This phenomenon is generally indicated with the term *biological self-purification.*

## VIII, 3.1 *Some aspects of biological self-purification*

In a brief discussion of some aspects involved in biological self-purification it seems valid to take as examples ecosystems with a high biodegradation capacity. As such the ecosystems artificially developed for the purification of sewage water may be used. The use of such a concrete model system may have the advantage that the present approach can be more accurately evaluated. Further it opens the possibility to control experimentally the mathematical expressions derived, since the biological systems operating in sewage purification stations lend themselves to studies on a small scale in the laboratory.

Most of the modern sewage purification stations make use of the *activated sludge procedure.* This procedure is based upon aerobic degradation of the substances in the sewage water which is performed by various populations of bacteria. The contact between microorganisms, dissolved oxygen and substrates is intensified by dispersing the bacteria through the sewage water into which air is being continuously dissolved. The sewage water flows into a tank — fitted with means for agitation and aeration — at such a rate that its retention time is long enough to achieve the desired strate of oxidation.

Many of the chemical pollutants which are degraded by bacteria have a nutritive function for the organisms. Therefore, a growth of the bacterial population will result.

The disappearance of chemical pollutants from soil and water is dependent upon the number of bacteria capable of degrading the substances and will be therefore directly related to the growth of the bacterial population. For the kinetics of growth of microbial populations the review of Painter and Marr (1968) may be referred to. In the discussion presented here only phases generally observed in the growth of bacterial populations will be taken into consideration. Generally the rate of bacterial population growth may be roughly divided into the phases described by Monod (1949):

1) Lag phase (initial stationary phase)
2) Exponential growth phase (logarithmic growth phase)
3) Stationary phase (maximum stationary phase)
4) Exponential death phase (logarithmic death phase).

Figure 32 gives a graphical representation of the various phases mentioned. With regard to the capacity of biological self-purification processes it seems adequate to differentiate between a *constant biological self-purification capacity,* when the number of organisms involved remains constant, an *expanding biological self-purification capacity,* when the population of

organisms is growing, and a *decreasing biological self-purification capacity,* when the number of organisms involved is decreasing.
In considering the events in sewage purification plants it seems reasonable to assume a steady state situation. The average composition of the sewage water will be nearly constant if it is considered over the time periods of weeks or months. Within these periods fluctuations in the sewage composition

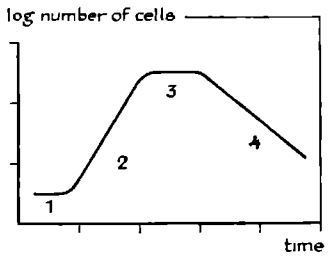



FIGURE 32. Phases of growth of microbial populations (generalized curve). Only 4 phases are considered here: 1, Lag phase; 2, Experimental growth phase; 3, Stationary phase; 4, Exponential death phase. The transition between two subsequent phases is not abrupt. These transitions will not be discussed.

can occur under the influence of daily different disposal of domestic and/or industrial materials and by environmental factors such as temperature and light. The steady state in the purification stations can be disturbed by accidental disposal of substances which do not normally occur in the sewage water. Changes in the size of the bacterial population are controlled by measurement of the amount of the activated sludge. On the basis of these measurements the number of bacteria which increases in the normal situation, is kept roughly constant by proper dilution of the activated sludge at regular times.

## VIII, 4. MATHEMATICAL MODELS IN ENVIRONMENTAL PHARMACOKINETICS

The mathematical models presented here are based on the factors which have been indicated above as playing a primary role in the kinetics of chemical pollutants in ecosystems. First of all attention will be directed to the concentration-time course of a pollutant introduced into an ecosystem in a single dose. This can be easily imagined to occur in practice in the case of accidental pollution. Later on the kinetics of pollutants will be considered at a constant rate of introduction. This may represent the case in which pollutants are continuously disposed of as industrial or domestic sewage.

TABLE 32 - Symbols employed in environmental pharmacokinetics

| symbols | description | example of units (dimensions) |
|---|---|---|
| $C$ | concentration | mg.l$^{-1}$; $\mu$g.ml$^{-1}$; M |
| $C_{x(o)}$; $C_{x(t)}$ | concentration of $X$ at time $o$; time $t$ | |
| $k_0$ | rate constant in zero-order kinetics | mg.h$^{-1}$; moles.h$^{-1}$; $\mu$g.min$^{-1}$ |
| $k_1$ | purification rate constant in first-order kinetics | l.h$^{-1}$; ml.min$^{-1}$ |
| $k_x$ | concentration of $X$ at $\frac{1}{2}$ $v_{max}$ | mg.l$^{-1}$; $\mu$g.ml$^{-1}$; M |
| $L$ | concentration at which the purification capacity is 50% destroyed ($LD_{50}$) | mg.l$^{-1}$; $\mu$g.ml$^{-1}$; M |
| $Q$ | quantity | mg; $\mu$g; moles |
| $Q_{x(o)}$; $Q_{x(t)}$ | quantity of $X$ at time $o$; time $t$ | |
| $r_{el0}$ | elimination rate constant in zero-order kinetics | mg.l$^{-1}$.h$^{-1}$; moles.l$^{-1}$.h$^{-1}$; $\mu$g.ml$^{-1}$.min$^{-1}$ |
| $r_{el0(o)}$; $r_{el0(t)}$ | $r_{el0}$ at time $o$; time $t$ (changing elimination capacity) | |
| $r_{el1}$ | elimination rate constant in first-order kinetics | h$^{-1}$; min$^{-1}$ |
| $r_{el1(o)}$; $r_{el1(t)}$ | $r_{el1}$ at time $o$; time $t$ (changing elimination capacity) | |
| $r_g$; $r_d$ | growth and death rate constants (exponentially expanding and decreasing purification capacity) | h$^{-1}$; min$^{-1}$ |
| $t$ | time | |
| $t\frac{1}{2}$ | half-life time (first-order elimination) | h; min |
| $\tau$ | time constant for first-order elimination | |
| $V$; $V_f$ | volume; fictive volume | l; ml |
| $v_{max}$ | maximal velocity of pollutant degradation | mg.h$^{-1}$; moles.h$^{-1}$; $\mu$g.min$^{-1}$ |
| $v_{max(o)}$ | $v_{max}$ initially present (changing degradation capacity) | |

It has already been pointed out that the concentration-time course of a pollutant in an ecosystem depends upon the rates of introduction and elimination. The kinetics of the processes leading to elimination of the pollutants will receive primary attention.
As far as the distribution of pollutants over the receiving systems is concerned, the binding of the substances to components of the system and the consequences thereof with regard to the volume of distribution will be concisely considered.
Finally the factors mentioned above will be discussed in relation to the phenomenon of accumulation.
For reasons of simplicity the mathematical models constructed will be mainly based upon the one-compartment model for which it is postulated that the amount of pollutant is completely and rapidly distributed through the total equilibrated volumes of distribution. This may be the case, for instance, for most of the substances in sewage purification plants. The symbols used to construct the mathematical formulations in this approach to environmental pharmacokinetics are listed in table 32.

VIII, 4.1. *Kinetics of elimination*

In view of the importance of biodegradation, the influence of this process on the kinetics of chemical pollutants in ecosystems will receive primary attention.
The kinetics of biodegradation can be considered by analogy with the kinetics for enzymatic reactions. Substitution of biodegradation kinetics into the Michaelis-Menten equation (according to which the velocity of enzymatic transformation of the substrate depends on the concentration of the substrate) gives

$$dQ_x/dt = -v_{max}\, C_x/(k_x + C_x) \qquad (1)$$

where $dQ_x/dt$ is the amount of pollutant $X$ disappearing per unit time (initial velocity), $C_x$ the concentration of pollutant $X$, $v_{max}$ the maximal velocity of degradation and $k_x$ corresponds to the Michaelis constant and is numerically equal to the concentration of pollutant $X$ at $\frac{1}{2}v_{max}$. For $k_x >> C_x$, that is when the concentration of pollutant $X$ is far below of saturating the degrading system, the expression $C_x/(k_x + C_x)$ can be approximated by $C_x/k_x$ and consequently, the degradation process can be regarded as being first order.
Once the degrading system is saturated by the pollutant ($C_x >> k_x$) the degradation will proceed at a constant rate. The process can be approximated by $-dQ_x/dt = v_{max}$ and consequently will become zero order.

In the discussion above a degrading system with constant capacity is assumed. When the capacity is changing, such as for example, when the pollutant induces or inhibits the growth of the bacterial population involved in the breakdown, the kinetics of elimination will become more complex.
Various possibilities in the kinetics of elimination will be considered in more detail during the following discussion.

### VIII, 4.1.1. First-order kinetics of elimination

It is assumed that a pollutant enters an ecosystem in a single, low dose ($Q_{x(o)}$). Further it is assumed that the concentration of the pollutant is far from saturating the degrading system and that the capacity of the system involved stays constant. This situation may be true if the breakdown is a metabolic process performed by higher organisms in the ecosystem. The same situation can be imagined when microorganisms are involved, provided that their number is not changing, for example in the stationary phases 1 and 3 presented in figure 32, and also in the exponential phase 2 if a proper continuous dilution of the bacterial population takes place (cf. sewage purification plants). Far below saturation the degradation process can be called *supply-limited* (cf. Riggs, 1963), which means that the rate of breakdown is limited by the dose $Q_{x(o)}$ and not by the capacity of the process itself. In this situation, a *fixed percentage* of the amount of pollutant $X$ is destroyed per unit time regardless of the concentration of $X$.
In the situation assumed above the number of molecules eliminated per unit time is directly proportional to the concentration at each moment. The elimination process corresponds to first-order kinetics which can be mathematically described as

$$dQ_x/dt = -k_1 C_x \tag{2}$$

where $Q_x$ is the quantity of pollutant $X$ present in the ecosystem (mg or mole), $k_1$ the purification rate constant for first-order elimination ($l.h.^{-1}$, $ml.min^{-1}$), corresponding to that part of the system completely purified in one unit time, and $C_x$ the concentration of the compound ($mg.l^{-1}$, $\mu g.ml^{-1}$, M).
$C_x$ is equal to the amount of pollutant ($Q_x$) divided by the apparent volume ($V$ in l; its significance will be discussed in section VIII, 4. 3) of the compartment over which it is distributed.
Accordingly

$$dC_x/C_x = -(k_1/V)dt \tag{3}$$

For $k_1/V$ the elimination rate constant $r_{el}$ ($h^{-1}$, $min^{-1}$) will be used. $r_{el}$ represents the rate at which the concentration of a pollutant $X$ in a compartment decreases; it represents the percentual decrease of the concentration per unit time.

Integration of equation (3) leads to the exponential expression

$$C_{x(t)} = C_{x(o)}e^{-r_{el}t} \tag{4}$$

where: $C_{x(t)}$ is the concentration of pollutant $X$ at time $t$, $C_{x(o)}$ the concentration of pollutant $X$ initially present, and $r_{el}$ the elimination rate constant. In individual pharmacokinetics $r_{el}$ is substituted by $\tau^{-1}$. $\tau$ is the time constant for the elimination process.

Accordingly equation (4) can also be written as

$$C_{x(t)} = C_{x(o)}e^{-t/\tau} \tag{4a}$$

A theoretical concentration-time curve for a pollutant $X$, which is degraded in the ecosystem (E) of a sewage purification plant with the assumptions made above, is graphically represented in figure 33a. According to equation (4), a straight line is obtained when the logarithms of the concentrations of the pollutant in the sewage water of the plant are plotted against time (figure 33b).

The time constant ($\tau$) determines the rate of the elimination process.

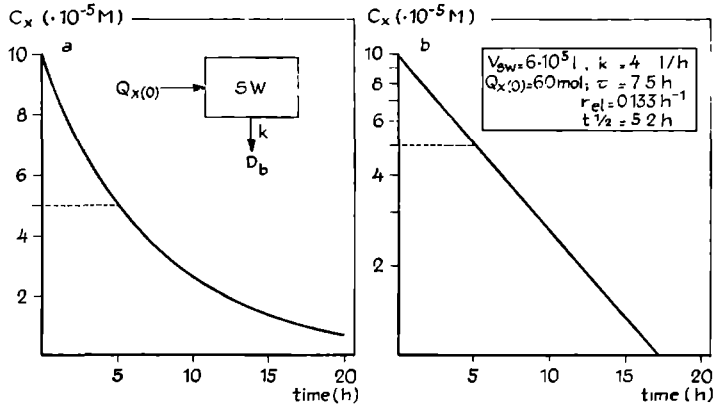



FIGURE 33. Theoretical concentration-time curves representing first-order kinetics of elimination ($D_h$). a, Exponential decline in the concentration of a chemical pollutant in the sewage water (SW). The decline is represented in a linear diagram according to the scheme of a one-compartment open system. b, The logarithm of the concentration of the pollutant in the sewage water declines linearly with time.

Usually the rate of a first-order reaction is represented by the half-life time of the compound involved ($t^1/_2$). The constant $t^1/_2$ corresponds to the length of time needed for the concentration to become 50% of its original value. $\tau$ and $r_{el}$ are related with $t^1/_2$ according to

$$t^1/_2 = 0.693 \times \tau = 0.693/r_{el} \text{ or } \tau = 1/r_{el} = 1.44 \times t^1/_2 \qquad (5)$$

It is obvious that the half-life time of a pollutant is the important parameter in elimination. In situations where the concentration of a pollutant can be measured (for example, in the water of sewage purification plants or lakes), and curves are found corresponding to those of figure 33, $t^1/_2$, and therewith, $\tau$ and $r_{el}$ can easily be calculated. $C_{x(o)}$ can be found as the intercept of the curve with the ordinate of the log concentration-time plot.

The discussion presented above concerns the kinetics of pollutants subjected to a supply-limited process of biodegradation with constant purification capacity. However, the parameters $t^1/_2$, $\tau$ and $r_{el}$ as such, do not give information about the mechanism of disappearance. It has to be realized that a similar picture can be observed when only physicochemical disintegration of the pollutants is involved, which also implies, first-order kinetics (cf. the decay of radioactive isotopes).

A picture of first-order elimination can also be observed if the pollutants are not broken down. This may be the case in a sewage purification station when a pollutant is introduced into, transported through, and removed from the system with the effluent without being chemically changed. It will be clear that in that case the time constant represents the retention time of the sewage water (containing the pollutant) in the purification station. A sewage purification station can be considered, therefore, to operate in an effective way only if the retention time of the pollutants in the station is greater than the time constant for the purification process.

When the pollutants in a sewage purification plant are partly subjected to first-order biodegradation, partly to physicochemical disintegration, and partly removed unchanged from the plant, the elimination rate constant found will be the sum of the elimination rate constants of the respective processes involved.

### VIII, 4. 1. 2. Zero-order kinetics of elimination

When a pollutant comes into a biodegradation system with a constant purification capacity in a concentration high enough to saturate this system, the process involved will become *capacity-limited* (cf. Riggs, 1963). The velocity of degradation will be maximal, which implies that a *constant*

*quantity* of pollutant $X$ is destroyed per unit time. The process of elimination then corresponds to zero-order kinetics. It can be described by

$$dQ_x/dt = -k_o \text{ or } dC_x/dt = -k_o/V = -r_{el0} \tag{6}$$

where $Q_x$ is the quantity of pollutant $X$ present in the ecosystem (mg, $\mu$g, or mole), $V$ the apparent volume of the ecosystem (1) (cf. section VIII, 4.3), $C_x$ the concentration of the compound ($mg.l^{-1}$, $\mu g.ml^{-1}$, M) $k_o$ the purification rate constant for zero-order elimination ($mg.h^{-1}$, $moles.h^{-1}$, $\mu g.min^{-1}$), and $r_{el0}$ the elimination rate constant for zero-order kinetics ($mg.l^{-1}.h^{-1}$, $moles.l^{-1}.h^{-1}$, $\mu g.ml^{-1}.min^{-1}$). One must realize well that the dimensions of the purification and elimination rate constants are different for zero-order and first-order kinetics.

Integration of equation (6) and solution for $C_{x(t)}$ gives

$$C_{x(t)} = C_{x(o)} [1 - (r_{el0}/C_{x(o)})t] \tag{7}$$

According to equation (7), a straight line will be obtained when the concentrations are plotted against time.

When the concentration of the pollutant decreases so strongly that a level far below saturation of the degrading system is reached, then the capacity-limited situation transforms into a supply-limited situation, in which an exponential decline (equation (4)) will be observed.

### VIII, 4.1.3. Kinetics of elimination in systems with changing capacity of elimination

In this section kinetics of elimination will be considered for ecosystems of which the capacity to eliminate pollutants is changing during the elimination process (expanding or decreasing purification capacity). Processes of elimination in which microorganisms are involved may examplify this. It has already been discussed that in bacterial populations (cf. figure 32, phases 2 and 4) exponential growth and death phases can be observed. As long as net change *(i.e.,* the difference between births and deaths) per unit of population and per unit of time is constant, the population will change exponentially.

It is assumed that the net rate of growth per unit of population is constant:

$$dN/dt = r_g N_{(t)} \tag{8}$$

where $N$ is the number of bacteria in the population, and $r_g$ the growth rate constant.

In the analogous situation when death exceeds birth

$$dN/dt = - r_d N_{(t)} \tag{8a}$$

where $r_d$ is the death rate constant.
Integration of the equations (8) and (8a) and solution for $N_{(t)}$ gives

$$N_{(t)} = N_{(o)} e^{r_g t} \tag{9}$$

and

$$N_{(t)} = N_{(o)} e^{-r_d t} \tag{9a}$$

If it is assumed that the elimination of pollutants from ecosystems depends on biodegradation by bacteria, then the rate of elimination ($r_{el}$) will depend on the number of bacteria present. If the number of bacteria is changing according to equations (9) and (9a), then $r_{el}$ will correspondingly change. In the case of growth (an expanding purification capacity):

$$r_{el(t)} = r_{el(o)} e^{r_g t} \tag{10}$$

In the case of death (a decreasing purification capacity):

$$r_{el(t)} = r_{el(o)} e^{-r_d t} \tag{10a}$$

If one considers an elimination process which is supply-limited, then, in the case of exponentially expanding purification capacity, equations (3) (with for $k_1/V = r_{el1}$) and (10) have to be combined:

$$dC_x/dt = - r_{el1(o)} e^{r_g t} C_x \tag{11}$$

Integration of equation (11) and solution for $C_{x(t)}$ gives

$$C_{x(t)} = C_{x(o)} e^{-(r_{el1(o)}/r_g)\,(e^{r_g t} - 1)} \tag{12}$$

In the analogous situation of exponentially decreasing purification capacity combination of equation (3) and (10a) gives as a mathematical expression

$$C_{x(t)} = C_{x(o)} e^{(r_{el1(o)}/r_d)\,(e^{-r_d t} - 1)} \tag{12a}$$

If one considers an elimination process which is capacity-limited, then, in the case of exponentially expanding purification capacity, equations (6) and (10) have to be combined:

$$dC_x/dt = - r_{el0(o)} e^{r_g t} \tag{13}$$

Integration of equation (13) and solution for $C_{x(t)}$ gives

$$C_{x(t)} = C_{x(o)} - (r_{el0(o)}/r_g)\,(e^{r_g t} - 1) \tag{14}$$

In the analogous situation of exponentially decreasing purification capacity equations (6) and (10a) have to be combined. The situation can be represented by the mathematical expression

$$C_{x(t)} = C_{x(o)} + (r_{clo(o)}/r_d)(e^{-rdt} - 1) \qquad (14a)$$

It will be evident that capacity-limited biodegradation processes (mathematical formulations (14) and (14a)) will become supply-limited when the concentration of the pollutant decreases far below the saturation level of the degrading system. Then, equations (12) or (12a) respectively, can be used for a mathematical description of the elimination process. It has to be noted, however, that the purification capacity of an ecosystem will preferentially expand at conditions of a good supply of nutrients. It can be expected, therefore, that in the case of an exponential growth of the degradation system caused by the pollutant (nutrient) itself, the kinetics of elimination will more probably occur according to equation (14) (a high saturating concentration of nutrient) rather than according to equation (12) (a low unsaturating concentration of nutrient).
Theoretical concentration-time curves calculated on the basis of equations (12) and (14) are graphically represented in figure 38.

VIII, 4.2. *Riversible and irreversible binding*

The amount of pollutant reversibly bound to components of an ecosystem can be considered to function as a depot. The substance remains available to processes of elimination. The amount of pollutant which is irreversibly bound to other substances present in an ecosystem and sequestered thus in a harmless form, can be considered as having been eliminated from the system.
The kinetics of the binding processes involving adsorption to the material of the receiving systems can be described according to the adsorption isotherms of Freundlich or Langmuir. The kinetics of binding will not be discussed here.
It has to be noted that binding of pollutants has a significance with regard to the volume of distribution and therefore ultimately to the kinetics of elimination.

VIII, 4.3. *Distribution volume and fictive distribution volume*

If the dose ($Q_{x(o)}$) administered to a compartment system is known, after determination of the initial concentration ($C_{x(o)}$) the distribution volume ($V$) can be calculated:

$$V = Q_{x(o)}/C_{x(o)} \qquad (15)$$

In a sewage purification plant the distribution volume may be the volume of water, or the volume of water plus one or more of the other constituents of the purification system. As such can be mentioned pollutant-adsorbing solid particles or activated sludge. When the substance is uniformly distributed over all of the components of the system (a similar partition coefficient), the value found for $V$ from equation (15), where $C_{x(o)}$ is the concentration in the water, resembles the virtual volume of the sewage purification system. This will not be the case, however, if the pollutant is unequally distributed over the various components. Solid particles or biological material may have different affinities for the pollutant chemicals and variations in exchange rate may govern the distribution process. If excessive binding of pollutants to constituents of the sewage plant occurs, the concentration found in the sewage water will be relative low so that on the basis of equation (15) a relatively extensive distribution volume will be found. Therefore, it seems to be more adequate to introduce the term *fictive* or *apparent distribution volume*. It will be clear that, in the case of excessive binding of the pollutant to the microorganisms present, changes in the number of organisms will be reflected in the value found for the fictive distribution volume.

### VIII, 4.4. *Kinetics of accumulation*

Accumulation of chemical pollutants in ecosystems will occur when the rate of introduction is higher than the rate of elimination. Considering an ecosystem as an open one-compartment system into which a pollutant is introduced in a continuous flow, whereas the elimination of the pollutant from the system proceeds according to first-order kinetics, one can suppose that the amount of pollutant in that system will increase until it reaches a plateau. Such a plateau occurs at the time that the rate of introduction equals the rate of output.

In the following mathematical descriptions of the kinetics of accumulation it is assumed that the main route of elimination of pollutants from an ecosystem is by means of biodegradation. The possibility of an expanding purification capacity will not be taken into consideration. This seems reasonable in a first approach of the accumulation phenomenon, since a certain limitation in the purification capacity is required in order to produce the phenomenon.

When a pollutant is introduced with a constant introduction rate ($k_o$) into a compartment with a fictive volume ($V_f$), whereas the concentration ($C_x$) of the pollutant in the system is decreased by biodegradation, the change in the concentration of pollutant per unit time ($dC_x/dt$) can be described by

$$dC_x/dt = [k_o - v_{max}C_x/(k_x + C_x)]\ (1/V_f) \qquad (16)$$

where $(v_{max}C_x)/(k_x + C_x)$ is equation (1); $v_{max}$ is the maximal velocity of degradation, and $k_x$ the concentration at $\frac{1}{2}v_{max}$.
When the plateau in the accumulation phenomenon is reached, $dC_x/dt = 0$.

The plateau concentration $C_{xpl}$ can be represented by

$$C_{xpl} = k_o k_x/(v_{max} - k_o)$$

It has already been discussed in this chapter (section VIII, 4.1) that, dependent on the concentration ($C_x$), the kinetics of elimination will be of first- or zero-order. For $C_x << k_x$, equation (16) can be approximated to

$$dC_x/dt = (k_o - v_{max}\ C_x/k_x)(1/V_f) \qquad (16a)$$

For $C_x >> k_x$, equation (16) can be approximated to

$$dC_x/dt = (k_o - v_{max})(1/V_f) \qquad (16b)$$

It can be expected that during the accumulation process at increase of $C_x$ the kinetics of elimination will pass from first-order to zero-order. When the elimination process proceeds according to first-order kinetics, that is when $k_o$ is so low ($k_o << v_{max}$) that the concentration remains far from saturating the degrading system, a plateau will be reached at

$$C_{xpl} = k_o k_x/v_{max}$$

When the process is transformed to zero-order kinetics a plateau will only be reached when $v_{max} = k_o$. When $k_o > v_{max}$ a plateau will never be reached and the increase in $C_x$ will continue.
It will be obvious that pollutants which can damage the degrading system — substances that decrease $v_{max}$ — may prevent the occurrence of a plateau.
The situation where a degrading system is damaged can be simplified by assuming the degrading system composed of a number of units ($N$). It is assumed that the velocity of degradation is proportional to the fraction of ($N$) involved. So it can be assumed that when all units ($N_{max}$) are involved in the degradation process, the breakdown proceeds at maximal velocity ($v_{max}$). The situation where a pollutant damages $N_{max}$ can be described as

$$\triangle N = N_{max(o)}C_x/(L + C_x) \qquad (17)$$

where $N_{max(o)}$ is the initial amount of $N_{max}$; and $L$ the concentration of $X$ where 50% of $N_{max(o)}$ is destroyed.
At any concentration the remaining amount of $N$ is $(N_{max(o)} - \triangle N)$.
Consequently

$$N_{max(o)} - \triangle N = N_{max(o)} - [N_{max(o)}C_x/(L + C_x)] = N_{max(o)}L/(L + C_x) \qquad (18)$$

Thus, assuming $N_{max(o)} = v_{max(o)}$ (where $v_{max(o)}$ is the initial undamaged $v_{max}$), in the cases where a pollutant damages $v_{max}$, the $v_{max}$ in equation (16) can be written as $(v_{max(o)}L)/(L + C_x)$. Therefore equation (16) can be written as

$$dC_x/dt = [k_o - v_{max(o)}C_xL/(k_x + C_x)(L + C_x)]\,(1/V_f) \qquad (19)$$

The differential equation (19) can be solved. However, the equation for the solution for $C_{x(t)}$ is implicit, and gives no further insight in the kinetics of the process.

Information about the course of the function $C_x = f(k_o, v_{max}, k_x, L)$ can be obtained from the situation where $dC_x/dt = 0$. For $dC_x/dt = 0$ equation (19) gives the quadratic equation

$$C_{xpl} = -\tfrac{1}{2}(k_x + L - v_{max(o)}L/k_o) \pm \tfrac{1}{2}\sqrt{(k_x + L - v_{max(o)}L/k_o)^2 - 4\,k_xL} \qquad (19a)$$

Real solutions (real values for which a plateau can be obtained) can be found for

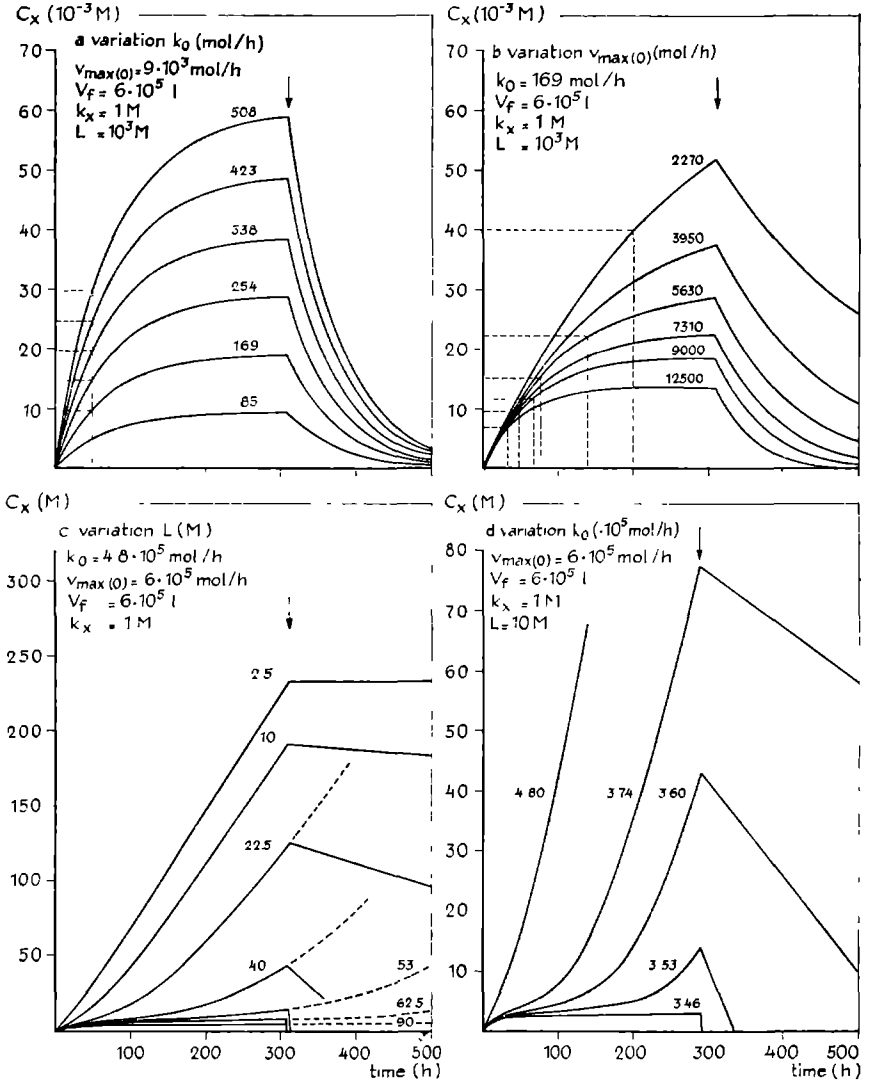
$$(k_x + L - v_{max(o)}L/k_o)^2 > 4\,k_xL \qquad (19b)$$

FIGURE 34. Kinetics of accumulation of pollutants in a one-compartment open system according to equation (19) at condition (19c). The pollutants ($X$) are introduced at constant rates. Elimination is performed by biodegradation. Arrows indicate the end of the introduction of the pollutants. Figures a and b represent accumulation curves for pollutants which are relatively nontoxic for the degrading system (relatively high value for $L$). Dotted horizontal lines indicate where the concentration is 50 per cent of the plateau concentration, whereas the dotted vertical lines indicate the time needed for reaching this value. Figures c and d represent accumulation curves for pollutants which are toxic for the degrading system (relatively low value for $L$). Family of curves a: variation of the introduction rate constant ($k_o$), whereas the other factors are kept constant. Note: increase in $k_o$ increases the plateau; the time required for reaching the plateau remains equal; after stopping the introduction of the pollutant, the concentration rapidly declines exponentially with time (equal $t\frac{1}{2}$). Family of curves b: variation of the maximal rate of degradation ($v_{max(o)}$), whereas the other factors are kept constant. Note: the accumulation curve with low $v_{max(o)}$ will reach a high plateau; the time required for reaching the plateau is relatively long; after stopping the introduction of the pollutant, the concentration declines exponentially with time (different $t\frac{1}{2}$). Family of curves c: variation of the toxicity constant ($L$). Note: the accumulation curve for a relatively nontoxic pollutant ($L = 90$) reaches a plateau; the accumulation curves for the more toxic pollutants surpass the plateau and the concentration increases progressively; after stopping the introduction of the pollutant, the highly toxic compounds (a highly damaged biodegradation capacity) show *persistent pollution*. Family of curves d: variation of the introduction rate constant ($k_o$) for toxic pollutants, whereas the other factors are kept constant. Note: at a critical value for $k_o$, the plateau is surpassed and the pollution transits from a ***biologically controlled*** steady state into ***biologically uncontrolled progressive pollution.***

Real positive solutions can be obtained for

$$k_o < v_{max(o)}L/(k_x + L + 2\sqrt{k_xL}) \qquad (19c)$$

For condition (19c) concentration-time courses were calculated according to equation (19) with variations for $k_o$, $v_{max(o)}$, and $L$. The calculations were performed with a computer of the General Electric Time-Sharing Service. Figures 34a and 34b represent the kinetics of accumulation for substances with low toxicity (a relatively high value for $L$). Figure 34a shows the effect



of variation of the rate of introduction ($k_o$), whereas the other factors were kept constant. The family of curves shows that the plateau concentration is increased with the increase of $k_o$. The time needed for reaching the plateau, however, remains equal. The plateau concentrations of figure 34a are far from saturation of the degrading system ($C_x << k_x$). When the introduction of pollutant is stopped a rapid first-order decline in the concentration becomes apparent. The half-life times for the declining curves are equal.

Figure 34b represents the kinetics of accumulation for pollutants with different rates of degradation (variation of $v_{max(o)}$), whereas the other factors are kept constant. The curve for the lowest $v_{max(o)}$ reaches the highest plateau. A second important phenomenon is that the time required for reaching the plateau is longer in the case of a lower $v_{max(o)}$. When the introduction of pollutant is stopped a first-order decline occurs. Since $v_{max(o)}$ differs for the various curves, the declining curves show different half-life times.

It seems adequate to speak of *biologically controlled pollution* with regard to the situations depicted by the figures 34a and 34b: the accumulation curves rapidly reach a plateau. With regard to a prevention of environmental pollution with chemical substances it can be concluded that lowering of their rate of introduction will result in a decrease in the plateau concentration. An increase in their susceptibility to biodegradation has a more drastic effect: the plateau concentration will be decreased, and the plateau will be reached more rapidly.

Figures 34c and 34d represent the kinetics of accumulation for pollutants which can damage the degradation capacity. In figure 34c pollutants are assumed with different toxicity (different values for $L$). The other factors are kept constant. It can be seen that, if $L$ is sufficiently high, the accumulation curve reaches a plateau. However, with increasing toxicity (decrease in $L$) the plateau is surpassed at a critical value for $L$ (determined by condition (19c)). With the increase in the concentration the damage to the degradation capacity will be enhanced. If it is assumed that no restoration of the degradation capacity occurs after stopping the introduction of pollutant, the remaining capacity for the various curves will require a long time for purifying the system. Since $C_x >> k_x$, the declining curves show zero-order kinetics. When the degradation capacity is completely destroyed, *persistent pollution* will occur.

Figure 34d represents the kinetics of accumulation of a toxic pollutant at different rates of introduction. If $k_o$ is sufficiently low, a plateau in the accumulation curve will be reached. If the introduction of pollutant is stopped, the concentration will rapidly level down. At a critical rate of introduction (determined by condition (19c)), however, the plateau will be sur-

passed. Then, the half-life time of the pollutant will be greatly prolonged because of further damage to the degrading system; the degree of accumulation then rises progressively. When the introduction of pollutant is stopped at a certain time the length of time required for purification of the system will depend on the remaining capacity for degradation. When the purification capacity is completely destroyed persistent pollution will result.

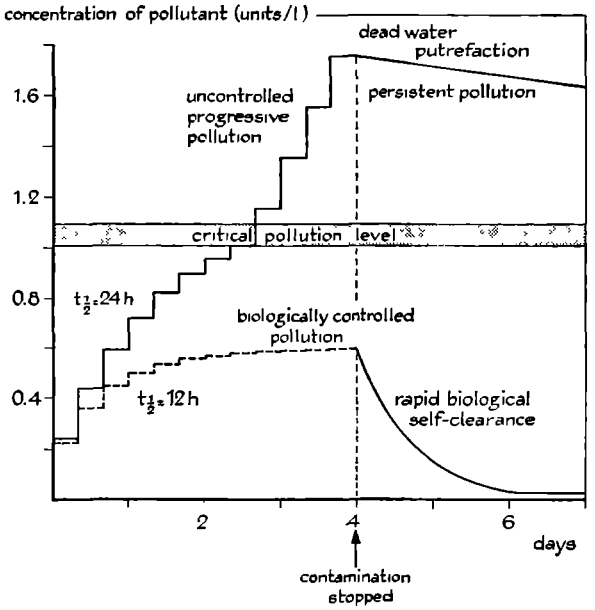



FIGURE 35. Critical pollution level in biological self-purification. Pollutants with relatively short half-life times can, in principle, be controlled by the biological self-purification process. The longer the half-life time of a pollutant, the greater the risk that its concentration reaches a critical level at which the biological self-purification capacity is affected. The resulting prolongation of the half-life time causes an uncontrolled progressive pollution. Strong reduction of the purification capacity results in persistent pollution (Ariëns, 1971).

It seems adequate to speak of *biologically uncontrolled progressive pollution* with regard to the situations pictured in figures 34c and 34d. The main aspects of the kinetics of accumulation discussed above are summarized in figure 35.

It can be concluded that the rate of introduction is a very critical parameter in environmental pollution with chemical substances that can damage the

biodegradation processes. If the rate of introduction is so high, that the plateau in the accumulation curve is surpassed, the pollutants will accumulate without limit and accelerate the accumulation of other substances.
In conclusion, it may be stated that a proper policy in the disposal of chemical wastes, or in the application of, for instance, pesticides, has to be based on knowledge of the susceptibility of the pollutants to biodegradation, and on knowledge of the potential toxicity of the pollutants for the degrading systems involved.
Factors which affect the processes involved in the degradation of pollutants will have an essential influence on the rate and the plateau of the accumulation. In this respect the influence of the environmental temperature on the growth of bacterial populations, and ultimately on the biodegradation of pollutants is important.
Long-lasting intervals in the introduction of pollutants into ecosystems can be important in that they will allow the system time for lowering an accumulation level. This may have a practical significance with regard to certain environmental chemical pollution caused by factories: ecosystems exposed to industrial waste products will have the possibility to "discharge" during the periods in which the rate of contamination is reduced (weekend, holidays).

## VIII, 5. FITTING OF EXPERIMENTAL DATA BY THEORETICAL EQUATIONS

The final and most important step in any approach to environmental pharmacokinetics is the fitting of the general mathematical models to the actual fate of pollutants in ecosystems.
In the foregoing discussion the fate of pollutants in sewage purification stations has repeatedly been considered as to relate the theoretical situation to a real-life situation. In principle it will be possible to follow experimentally the fate of pollutants in certain ecosystems, such as the aquatic ecosystems of a lake or a sewage purification station. The experimental data obtained can be compared with the mathematical models constructed.
The situation in a sewage purification plant or in the water of a lake or a river can be imitated on a small scale in the laboratory. In the past the biodegradability of chemical substances has been frequently tested in this way; for example the biodegradability tests on detergents (Huyser, 1960; Borstlap and Kooijman, 1963; Fischer, 1963; see also the review of Engelbrecht and Banerji, 1966).
In the present study some measurements with activated sludge from a sewage purification plant have been performed.

**Experimental procedure** — Activated sludge was obtained from the Sewage Works in Malden (near Nijmegen). Since the samples of sludge taken at different times from the sewage plant might have different biodegradation activity, for every separate set of experiments an aliquot of sludge taken from the same sample was used. In order to ensure constant bioactivity the activated sludge suspension (freshly taken from the plant) was centrifuged in the laboratory and stored at 0 °C. For each test 5 g of this stock were suspended in 250 ml of a salt solution containing 3.7 x $10^{-6}$ M $FeCl_3$. $6H_2O$, 2.5 x $10^{-3}$ M $CaCl_2$, $10^{-3}$ M $MgSO_4$. $7H_2O$, 0.15 x $10^{-3}$ M $(NH_4)_2SO_4$, 0.25 M $KH_2PO_4$ (pH 7.2) (Borstlap and Kooijman, 1963).
Bottles containing 250 ml sludge suspension were preincubated in a waterbath at 30 °C and aerated for 15 h. From some pilot experiments phenylethylamine appeared to be a useful substance for a study of elimination kinetics. In the preliminary experiments hardly any degradation of phenylethylamine was observed in the initial stage (5-10 h) of its incubation with the sludge suspension. This period may be interpreted as being necessary for the proper bacteria to develop a population consuming measurable amounts of the substrate offered. In the experiments presently described the initial stage of low degradation could be reduced by "activation" of the sludge suspension by adding some phenylethylamine at the beginning of the preincubation period. After 15 h preincubation with phenylethylamine in a concentration of 100 $\mu$g/ml, the substrate appeared to be fully eliminated from the mixture, whereupon the sludge suspension exhibited a greater bioactivity against a new supply of phenylethylamine
For measuring the disappearance of phenylethylamine samples of 3 ml homogeneous sludge suspension were taken at various times during incubation. After centrifugation 2 samples of 1 ml each were taken from the supernatant and extracted with 5 ml diethyl ether. Prior to the extraction each sample was alkalinized by adding 0.1 ml 2 N NaOH, and then 1 ml of an aqueous solution of *N*-benzylmethylamine-HCl of known concentration (ranging from 0.05 - 0.5 mg/ml) was added. The *N*-benzylmethylamine was carried through the whole procedure as an internal standard. The ether extracts were removed and concentrated by evaporation. The concentration of phenylethylamine in the extracts was assayed by gas chromatography on a column packed with 20% Apiezon, and 5% KOH on Diatoport S 60-89 mesh. The carrier gas was nitrogen, and the temperature of the column 150 °C. The concentration of phenylethylamine was calculated from the chromatograms by referring the area of the peak found for phenylethylamine to that of the internal standard *N*-benzylmethylamine. A blank was carried through the entire procedure: 250 ml solution of phenylethylamine in sterilized salt solution was incubated and analysed in the same way as the mixtures containing the activated sludge suspension. It appeared that the concentration of phenylethylamine in this blank remained constant during the whole incubation period.

Figure 36 represents the time course of the concentration of phenylethylamine in sludge suspensions incubated at different temperatures (0, 15, 30 °C). In the mixtures at 0 °C no decrease in the concentration could be observed over a period of 24 h. In the mixtures incubated at 30 °C the substrate had completely disappeared after 8 h. The shape of the disappearance curve at this temperature cannot be explained on the basis of simple zero-order or first-order kinetics of elimination. The curve represents an elimination process which is accelerating during the incubation; this indicates that a degrading system with an expanding purification capacity is operating. This phenomenon is explicable if the number of microorganisms, using phenylethyl-

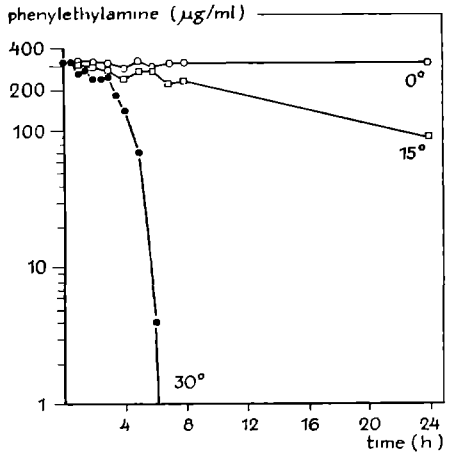



FIGURE 36. Time course of the phenylethylamine concentration in an activated sludge suspension at different temperatures.
Note the acceleration of the elimination process at 30 °C.

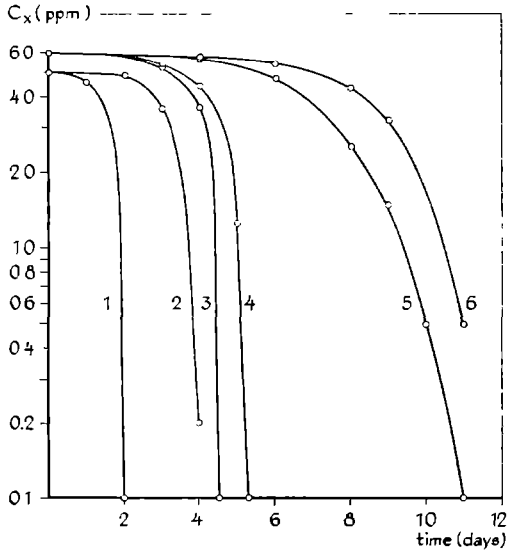



FIGURE 37. Time course of the concentration of surface-active compounds susceptible to biochemical oxidation. The substances were incubated in river water at 20 °C. 1, sodium *n*-decylsulphate; 2, sodium *p*-*n*-decylbenzene sulphonate; 3, sodium di(benzyl)sulphosuccinate; 4, sodium di(*n*-octyl)sulphosuccinate; 5, sodium di(2-ethylhexyl)sulphosuccinate; 6, sodium di(3,5,5-trimethylhexyl)sulphosuccinate. Curves are drawn according to data of Hammerton (1956).
Note the acceleration of the elimination process.

amine as a nutrient is increasing. This growth of the microbial population results in an ever-increasing consumption of substrate. The acceleration in the degradation of pollutants will presumably be a normally occurring phenomenon for the substances that are nutrients for the microorganisms involved. In this connection the data of Hammerton (1956) may be referred to (cf. figure 37).

For a mathematical description of the kinetics of elimination of a substance under the influence of microbial growth equations (12) and (14) may be

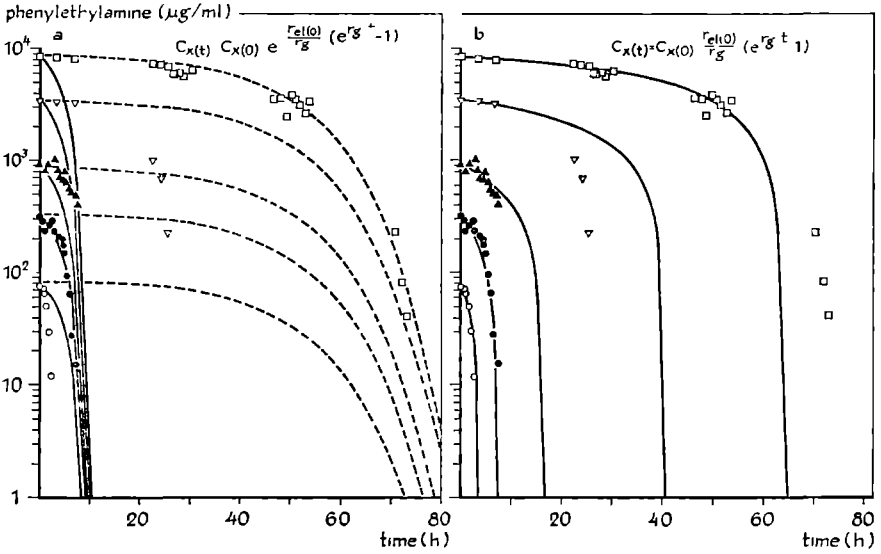



FIGURE 38 Time course of the phenylethylamine concentration in an activated sludge suspension at 30 °C with variation of the initial concentration ($C_{x(o)}$). Symbols represent the experimental values Curves represent the time courses according to mathematical equations based on the assumption that a purification process with exponentially expanding capacity is operating. a, Curves represent time courses of the concentration with the assumption that the purification process remains supply-limited at all concentrations employed Solid lines represent the curves according to equation (12) with $r_{el_1(o)} = 1.0 \times 10^{-3}$ min$^{-1}$, and $r_g = 6\,7 \times 10^{-3}$ min$^{-1}$. In this family of curves, only the curve starting at $3\,4 \times 10^2$ µg/ml gives a good fit with the experimental data Dotted lines represent the curves according to equation (12) with $r_{el_1(o)} = 3\,3 \times 10^{-5}$ min$^{-1}$, and $r_g = 1\,2 \times 10^{-3}$ min$^{-1}$ In this family of curves, only the curve starting at $8\,5 \times 10^3$ µg/ml gives a good fit with the experimental data
b, Curves represent time courses of the concentration with the assumption that the purification process remains capacity-limited at all concentrations employed. With equation (14) a family of curves can be obtained that fits the pattern of experimental data. The values $r_{el0(o)}$ (= 0 71 µg. ml$^{-1}$. min$^{-1}$), and for $r_g$ (= $5.0 \times 10^{-4}$ min$^{-1}$) were chosen on the basis of the experimental values in such a way that on the average a satisfactory fit was obtained.

used. It will be interesting, therefore, to investigate whether the experimental data found for the disappearance of phenylethylamine can be fitted by one of these equations.

Figure 38 represents the results of an experiment in which equal amounts of suspensions of activated sludge were incubated with different amounts of phenylethylamine. The temperature during incubation was kept at 30 °C. At various times the concentration of phenylethylamine was determined. The values found are represented by the symbols in figure 38a and 38b. The curves drawn in the figure represent the time course of the concentration according to the mathematical equations (12) and (14) respectively, for certain values of $r_{el1(o)}$, $r_{el0(o)}$, and $r_g$ at the initial substrate concentrations ($C_{x(o)}$) applied.

Kinetics of elimination according to equation (12) implies a supply-limited process of elimination, which means that the substrate offered is far from saturating the degrading system. It can be expected from equation (12), which assumes a proportionality between $C_{x(o)}$ and $C_{x(t)}$, that an increase in the initial substrate concentration will result in a shift of the curve upwards along the ordinates of the concentration-time course graph. The time required to reduce the concentration to zero will not markedly increase with increasing initial concentration, since it is assumed that the process remains supply-limited.

On the contrary, when a capacity-limited process of elimination is involved, (equation 14), a longer time will certainly be required for complete elimination of the substrate added at higher initial concentration. An increase in the initial concentration will shift the concentration-time course graph both upwards along the ordinates and along the abscissas from left to right.

The family of curves calculated according to equation (14) gives a better fit to the experimental data (figure 38b) than equation (12) does (figure 38a). The relatively good fit of equation (14) to the experimental data can be confirmed by plotting the difference between the various initial concentrations and the respective concentrations measured during the incubation period against time.

According to equation (14)

$$C_{x(o)} - C_{x(t)} = (r_{el0(o)}/r_g)\,(e^{r_g t} - 1)$$

The time course of this function with $r_{el0(o)} = 0.71$ $\mu$g.ml$^{-1}$.min$^{-1}$ and $r_g = 5.0 \times 10^{-4}$ min$^{-1}$ is represented by the curve in figure 39. This curve fits the experimental data well.

The correspondence between the experimental data and the mathematical description represented by equation (14) may allow the following conclusion

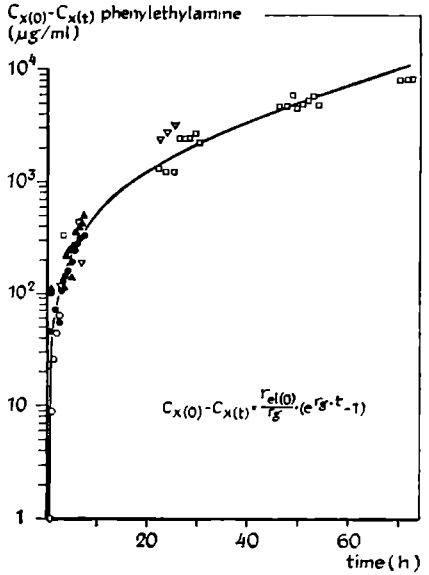



FIGURE 39. Time course for the decrease in the phenylethylamine concentration ($C_{x(o)}$ - $C_{x(t)}$) measured at different initial concentrations ($C_{x(o)}$). Symbols representing the experimental data correspond with the values plotted in figure 38. The curve drawn represents the theoretical time course according to equation (14) with $r_{el0(o)}$ = 0.71 $\mu$g. ml$^{-1}$. min$^{-1}$, and $r_g$ = 5.0 x 10$^{-4}$ min$^{-1}$.

about the elimination of phenylethylamine in the activated sludge suspensions: the kinetics of elimination presumably depends upon a capacity-limited exponentially expanding process of purification. In view of the parallel shift of the curves along both the ordinates and the abscissas of figure 38b it may be concluded that the degrading system remains capacity-limited at decrease of the initial concentration of the substrate from 8.5 x 10$^3$ to 85 $\mu$g/ml. The proportionality between the initial concentrations and the extent of the parallel shift of the corresponding curves indicates that, within the range of concentrations employed, no inhibition of the growth of the bacterial population involved occurs.

## VIII, 6. CONCLUSION

In this study, an approach to some problems related to environmental pharmacokinetics has been attempted.

Insight into the fate of chemical pollutants in ecosystems will be indispensable for control of environmental chemical pollution. The degree of admissibility of chemical pollutants to ecosystems has to be based upon objective criteria.

Such criteria can be obtained by quantitative tracing of pollutants. Pilot experiments with certain model systems can give useful information. The use of mathematical compartment models can contribute to a clarification of the insight into the phenomena observed.

Solid knowledge of the kinetics of accumulation is a primary condition for deliberate introduction of pollutants into nature. The present study emphasizes the importance of the rate of elimination. The rates of introduction and of elimination determine the level of the accumulation plateau. The time period within which an accumulation plateau is reached depends on the rate of elimination. Since biodegradation of pollutants is an important route for elimination, the factors which influence the biomass involved play a role in the accumulation phenomenon. In this study emphasis was put on the kinetics accumulation of pollutants that are toxic for the biological self-purification process. For such substances the accumulation curve may easily surpass the plateau. Thus, a steady state of biologically controlled pollution may transit into biologically uncontrolled progressive pollution. Finally, persistent pollution will result.

Far-reaching conclusions concerning the equations presented in this chapter cannot be given until adequate experimental data are available. One of the more complex models (including the kinetics of an exponentially expanding purification process) appears to describe well the kinetics of elimination of a substance in a simple ecosystem under laboratory conditions. This may be considered as encouraging for further study.

# SUMMARY

This thesis describes biological-pharmacological studies concerning some aspects of chemical pollution of the biosphere.
The first part of this thesis (chapters II — VII) concerns studies on the capacity of different animal species to metabolize compounds alien to them (xenobiotics). Biotransformation of xenobiotics is generally necessary for elimination of the compounds from the body.
In the second part of this report (chapter VIII) an attempt is made to describe the kinetics of the fate of chemical compounds in the biosphere. Emphasis is placed upon the factors which determine the occurrence of various forms of environmental pollution, viz. *biologically controlled, biologically uncontrolled progressive,* and *persistent* environmental pollution.

The above mentioned approaches are elucidated in chapter I.
Chapter II is a survey of data from the literature concerning some general aspects of the biochemical conversion of xenobiotics, particularly in mammals. The various types of drug-metabolizing reactions and their significance for the animal are discussed in this review. Further, the localization and specificity of the enzyme systems catalyzing the reactions, and the mechanism of the oxidative reactions are discussed in detail. The endogenous and exogenous factors which can influence the capacity for metabolizing xenobiotics are also reviewed. An important point in the latter discussion is the enhanced synthesis of enzymes in animals exposed to certain xenobiotics, among which are DDT, phenobarbital, and 3-methylcholanthrene. Finally, the possible species differences in drug-metabolizing capacities are considered.
Until recently, it was generally assumed that fishes and other aquatic animals were not provided with a drug-metabolizing ability. These animal species would have no need for this, since lipophilic xenobiotics would readily diffuse through the lipoidal membranes of gills and skin into the surrounding water. This argument can be invalidated, however, by referring to the consequences of the biotransformation of lipophilic xenobiotics into hydrophilic products. Such a conversion will also be in favour of aquatic animals, since the more water-soluble products are more restricted to the extracellular phase. In this way, the substances are offered in higher concentrations for excretion through gill, kidney and/or skin. The data in the present investigation show that tissue preparations from fish do possess a drug-metabolizing capacity. The enzymatic capacity was measured *in vitro* for reactions considered to be representa-

tive for biotransformation of xenobiotics: oxidative *N*-dealkylation, aromatic hydroxylation and glucuronidation. The substrates used in these reactions were aminopyrine, aniline, and *p*-nitrophenol respectively. The animals, materials, and methods employed in this study are listed in chapter III.

Among the various organs of fish, the greatest capacity for converting xenobiotics has been found in extracts from the liver. This agrees with the data in the literature for the drug-metabolizing capacities of birds and mammals, which are also mainly localized in the liver. The assays in this study were mainly performed, therefore, with this organ. Intracellularly, the hepatic drug-metabolizing enzymes of fish are bound to the (membranes of the) endoplasmic reticulum. This also agrees with the data from the literature about the intracellular localization of the enzyme systems in the mammalian and avian liver. Besides the similarities in localization, further similarities were found with respect to the enzyme systems of the varous species: the requirements for various cofactors and the sensitivity of the enzyme preparations to freezing and thawing, ultrasonic treatment, and treatment with bile fluid, deoxycholate, carbon monoxide, and SKF 525A. However, clear-cut species differences were also found, for instance, in the stability of the enzymes, and the optimal substrate and co-substrate concentrations. The data from the literature about the lack of a drug-metabolizing capacity in fish can be explained in view of the fact that the species differences mentioned above were left out of account in the enzyme assays in previous studies. In order to obtain correct comparable data for the enzymatic capacities of different animal species, the assays in this study were performed at adapted conditions as a standard procedure (chapter IV).

Chapter V is an analysis of the oxidative *N*-demethylation of 4-dimethylaminoantipyrine. It was shown that only one methyl group was split from the substrate predominantly. The identification of the reaction products (4-monomethylaminoantipyrine and 4-aminoantipyrine) was performed by thin-layer chromatography and mass spectrometry. The enzymatic conversion of 4-dimethylaminoantipyrine is generally measured *in vitro* with colorimetric assays on the products formaldehyde and 4-aminoantipyrine. However, the colorimetric assays on 4-aminoantipyrine appeared not to be specific. Since 4-monomethylaminoantipyrine, the main product in the *N*-demethylation of 4-dimethylaminoantipyrine, also shows a positive reaction in these assays, the results of previous studies in which the aim was to measure the formation of 4-aminoantipyrine only, have to be interpreted with serious reservations.

Chapter VI presents the results of the comparative study on the drug-metabolizing capacity of different animal species. The differences observed for the enzymatic activity in the *N*-demethylation and for the $K_m$-values of aminopyri-

ne for trout, roach, and rat liver preparations in relation to the incubation temperature are most probably a reflection of the different physiological conditions of the animals, adapted to their habitat. The trout, which lives preferentially at low environmental temperatures, will accordingly be cold-adapted. The wild roach will be adapted to the naturally occurring thermal fluctuations of its aquatic environment. The properties of enzymes in the homeothermic rat will correspond to the higher internal body temperature. As far as drug-metabolizing capacity is concerned, lower values were found for the poikilothermic animals studied than for the homeothermic ones. An explanation of this difference can presumably be found in the difference in standard metabolism, which in its turn is related to the food intake and therefore to the degree of exposure to xenobiotic compounds.

The drug-metabolizing capacity of wild fish is probably under the influence of enzyme-inducing xenobiotics in the chemically polluted environment. The difference in the drug-metabolizing capacity of the various species of *Cyprinidae* may be explained on this basis. The *Leuciscus* species are highly mobile fishes that attempt to collect a great amount of food consisting of plankton, plants, small aquatic animals and insects near the surface of the water. Thus they are presumably exposed to a great amount and variety of xenobiotics. The liver preparations from these fishes have a greater drug-metabolizing capacity than those from the white bream, bream, carp, and tench, which all are slow-swimming fishes, that collect their food consisting of plants, grubs, and worms near the bottom of the water.

It may be asked with respect to the increased chemical water pollution, to what degree the species differences in drug-metabolizing capacity have played a role in the shifts in the fish populations. *Salmonidae,* which have a very low detoxication capacity, have almost completely disappeared from the river Waal (Rhine). In contrast, *Cyprinidae*, which have a rather high drug-metabolizing capacity, remain in fairly good numbers.

That external factors have an influence on the drug-metabolizing capacity of fish is shown in chapter VII. The effect of exposure to insecticides and to differences in temperature appears generally to be equal for fish and mammals. Trout and roach were kept in water of different temperatures (5 or 18 °C in standing tap water for 14 days). The drug-metabolizing capacities of the fish kept at high temperature appeared to be lower (about 50%) than those of the fish kept at low temperature. This may indicate one of the negative effects of thermal pollution of the surface water, viz. damage to the systems necessary to the animals for the elimination of chemical pollutants.

The drug-metabolizing capacity of fish can be enhanced by exposure of the animals to certain enzyme-inducing xenobiotics (dieldrin, DDT). Roach from the

Waal staying in running tap water for 1 to 3 months, show a decrease in drug-metabolizing capacity of about 50%. This decrease does not occur in animals living in standing tap water or in river water. Additionally, the decrease can be retarded by treatment of the animals with an enzyme-inducing compound (dieldrin). This suggests that the drug-metabolizing capacity of wild fish is determined by certain chemical pollutants in the environment. These data argue for the hypothesis developed in this report that the seasonal varation in drug-metabolizing capacity observed for roach and rudd (high capacity in summer and low in winter), is largely due to a difference in the exposure of the animals to enzyme-inducing compounds in the chemically polluted environment. A difference in the contact of the fish with its environment (a difference in mobility, in food consumption), and a difference in the concentration of enzyme-inducing chemicals (e.g. insecticides and herbicides) in the water as a consequence of a different water level and of a seasonally dependent input of enzyme-inducing substances into nature, may play a role here.

The pharmacokinetic approach to the chemical pollution of the environment is worked out in chapter VIII. The procedure employed is similar to that utilized in individual pharmacokinetics, where the attempt is made to describe the fate of xenobiotics (e.g. medicaments, poisons) in man or animal.
Various aspects of environmental pollution are described with mathematical models. The rate of elimination is considered to be the most important factor. The main route for elimination of pollutants from ecosystems is the natural capacity of degradation, the biological self-purification capacity. Mathematical models are constructed for an expanding and a decreasing biodegradation capacity.
An *expanding biodegradation capacity* may occur in the case of contaminants that are nutrients for the degrading system. Two theoretical models for an expanding biodegradation capacity are compared using the degradation of phenylethylamine in an activated sludge suspension from a sewage purification station.
A *decreasing biodegradation capacity* may occur in the case of contaminants that are toxic for the degrading system. This case is theoretically worked out for a continual disposal of pollutants into ecosystems. Accumulation curves show that a plateau is rapidly reached for nontoxic chemicals with a short half-life time. The level of the plateau is dependent on the rates of introduction and of elimination. The time required for reaching the plateau is solely dependent on the rate of elimination. When the input is stopped the concentration will rapidly decrease to zero. It seems reasonable, therefore, to speak of *biologi-*

*cally controlled environmental pollution* with respect to the category of non-toxic substances mentioned above.

The most dangerous category of pollutants is that of substances that can damage the degrading system. It is shown in accumulation curves, that an ever-increasing damage to the decreasing system occurs with the increase of the pollutant concentration. This results in a progressive prolongation of the half-life time of the pollutant. Toxic pollutants accumulate, therefore, progressively since the biodegradation (biological control) diminishes further and further with the increase in the pollutant concentration. It seems reasonable to speak of *biologically uncontrolled progressive environmental pollution* with respect to the category of toxic pollutants.

When the degrading system is completely and irretrievably damaged, after stopping the input, the concentration will not decrease. In this situation the term *persistent pollution* may be used.

# SAMENVATTING

In dit proefschrift is biologisch-farmacologisch onderzoek beschreven betreffende enkele aspecten van de chemische verontreiniging van de biosfeer.
Het eerste gedeelte van het proefschrift (hoofdstukken II — VII) omvat onderzoek naar het vermogen van verschillende diersoorten tot biotransformatie van lichaamsvreemde verbindingen (xenobiotica). Biotransformatie van xenobiotica is als regel noodzakelijk om deze verbindingen uit het lichaam te elimineren.
Het tweede gedeelte van het proefschrift (hoofdstuk VIII) geeft een farmacokinetische beschrijving van het lot van chemische verbindingen in de biosfeer. Daarbij wordt de nadruk gelegd op factoren die bepalend zijn voor het optreden van verschillende vormen van chemische milieuverontreiniging, met name *biologisch gecontroleerde, biologisch ongecontroleerde progressieve,* en *persisterende* milieuverontreiniging.

De hierboven genoemde benaderingen worden in hoofdstuk I toegelicht.
Hoofdstuk II geeft een overzicht van literatuurgegevens betreffende algemene aspecten van de biochemische omzetting van xenobiotica, in het bijzonder bij zoogdieren. In dit overzicht worden onder meer de aard van de betreffende reacties en hun betekenis voor het organisme besproken. Verder wordt ingegaan op de localisatie en de specificiteit van de enzymsystemen betrokken bij de biotransformatie van xenobiotica, op het mechanisme van de oxydatieve reacties en op de endogene en exogene factoren die het vermogen van dieren om xenobiotica te metaboliseren kunnen beïnvloeden. Een belangrijk punt van bespreking is de verhoogde synthese van enzymen in dieren die blootgesteld zijn aan bepaalde xenobiotica, waaronder DDT, fenobarbital en 3-methylcholanthreen. Tenslotte wordt gewezen op mogelijke verschillen in het vermogen van verschillende diersoorten tot het metaboliseren van xenobiotica.
Tot voor kort werd algemeen verondersteld dat vissen en andere in het water levende dieren niet over een vermogen om xenobiotica te metaboliseren beschikken. Hieraan zou bij deze diersoorten geen behoefte bestaan omdat lipofiele xenobiotica gemakkelijk via de lipoidale membranen in kieuwen en huid naar buiten zouden kunnen diffunderen. Dit argument kan echter worden ontkracht door verwijzing naar de gevolgen van de biotransformatie van lipofiele xenobiotica tot hydrofiele producten. Een dergelijke omzetting is ook voor in het water levende dieren nuttig, alleen al omdat de in water beter oplosbare producten meer tot de extracellulaire fase worden beperkt en zo in

hogere concentraties voor verwijdering via kieuw, nier en/of huid beschikbaar komen. De gegevens van het hier beschreven onderzoek aan weefselextracten wijzen duidelijk uit dat vissen wel over een xenobiotica-metaboliserend vermogen beschikken. *In vitro* werd de enzymatische capaciteit gemeten voor reacties die representatief worden geacht voor de biotransformatie van lichaamsvreemde verbindingen: oxydatieve *N*-dealkylering, aromatische hydroxylering en glucuronidering. Bij deze reacties werden als substraten gebruikt respectievelijk aminopyrine, aniline en *p*-nitrophenol. De in het onderzoek gebruikte dieren, materialen en methodieken zijn beschreven in hoofdstuk III.

In aansluiting aan de gegevens uit de literatuur dat bij zoogdieren en vogels de enzymen die bij bovengenoemde reacties betrokken zijn, zich voornamelijk in de lever bevinden, werd bij vissen eveneens de hoogste enzymatische capaciteit in extracten uit de lever waargenomen. Derhalve werden de metingen voornamelijk aan dit orgaan verricht. Overeenkomstig de gegevens over de zoogdieren en vogellever bleek dat de onderzochte enzymsystemen ook in de visselever gebonden zijn aan (de membranen van) het endoplasmatische reticulum. Naast deze overeenkomsten voor vogels, zoogdieren en vissen werden verdere overeenkomsten waargenomen in de voor de reacties benodigde cofactoren en in de gevoeligheid van de enzympreparaten ten opzichte van invriezing en ontdooiing, ultrasone trillingen en behandeling met galvloeistof, deoxycholaat, koolmonoxyde en SKF 525A. Daarnaast werden echter toch ook duidelijke verschillen tussen diverse dierspecies voor de onderzochte enzymatische reacties waargenomen, bijvoorbeeld wat betreft de stabiliteit van de enzymen, en de optimale concentraties van substraten en cosubstraten. De literatuurgegevens over het ontbreken van een xenobiotica-metaboliserend vermogen bij vissen zijn verklaarbaar op grond van het feit dat men bij de enzymtesten in vroegere onderzoekingen te weinig rekening heeft gehouden met bovengenoemde verschillen tussen de species. Teneinde in het huidige onderzoek een verantwoorde vergelijking van de enzymatische capaciteiten van de verschillende dierspecies te realiseren werden gestandaardiseerde metingen verricht onder aangepaste condities (hoofdstuk IV).

Hoofdstuk V geeft een analyse van de oxydatieve *N*-demethylering van 4-dimethylaminoantipyrine (aminopyrine). Er werd aangetoond dat in deze reactie voornamelijk slechts één methylgroep van het substraat wordt afgesplitst. Identificatie van de reactieproducten (4-monomethylaminoantipyrine en 4-aminoantipyrine) vond plaats door middel van dunnelaag-chromatografie en massaspectrometrie. In het algemeen wordt bij de enzymatische omzetting van 4-dimethylaminoantipyrine *in vitro* de vorming van formaldehyde en 4-aminoantipyrine gemeten door middel van colorimetrische bepalingen. De colorimetrische testen op 4-aminoantipyrine bleken echter niet specifiek te zijn.

Omdat ook 4-monomethylaminoantipyrine, het belangrijkste product van de *N*-demethylering van 4-dimethylaminoantipyrine, reageert in deze testen, moet ten opzichte van de resultaten van vroegere studies, waarbij men dacht 4-aminoantipyrine te meten, een grote reserve in acht genomen worden.
Hoofdstuk VI omvat de resultaten van het vergelijkende onderzoek naar het xenobiotica-metaboliserend vermogen van verschillende diersoorten. Hoogstwaarschijnlijk zijn de waargenomen verschillen voor de enzymatische activiteit in de *N*-demethylering en de $K_m$-waarden van aminopyrine voor de extracten uit de levers van forel, blankvoorn en rat met betrekking tot de incubatietemperatuur een weerspiegeling van de fysiologische aanpassing van de dieren aan hun habitat. De eigenschappen van de enzymen van de forel, welke bij voorkeur leeft in water van lage temperatuur, getuigen van een aanpassing aan deze lage temperatuur, die van de in het wild levende blankvoorn aan de nogal wisselende temperatuur van de omgeving, terwijl die van de rat corresponderen met de hogere lichaamstemperatuur. Wat de capaciteiten tot het metaboliseren van xenobiotica betreft werden bij de onderzochte poikilotherme dieren lagere waarden waargenomen dan bij de homoiotherme dieren. Een verklaring voor dit verschil zal vermoedelijk gezocht moeten worden in een verschil in standaardmetabolisme, dat op zijn beurt gecorreleerd is met de voedselopname en daarmede met de mate van blootstelling aan xenobiotica.
De capaciteit tot het metaboliseren van xenobiotica bij in het wild levende vissen wordt mogelijk beïnvoed door enzym-inducerende verbindingen in het chemisch verontreinigde milieu. Op deze basis zouden de waargenomen verschillen binnen de diverse soorten der *Cyprinidae* verklaard kunnen worden. De verschillende soorten voorn, erg beweeglijke vissen welke een grote hoeveelheid voedsel bestaande uit plankton, plantendelen, kleine waterdieren en insecten aan het wateroppervlak bemachtigen — en derhalve vermoedelijk blootgesteld worden aan een relatief grote hoeveelheid en variatie van xenobiotica — vertonen een groter vermogen tot omzetting van xenobiotica dan de bliek, brasem, karper en zeelt, vrij langzaam zwemmende vissoorten, welke hun voedsel, onder meer bestaande uit plantendelen en wormen, dieper in het water verzamelen.
Men kan zich afvragen in hoeverre de toegenomen chemische waterverontreiniging heeft bijgedragen tot speciesverschillen wat betreft het vermogen om xenobiotica te metaboliseren en zodoende tot verschuivingen in de vispopulaties. *Salmonidae,* welke over een zeer geringe detoxificatiecapaciteit beschikken, komen vrijwel niet meer voor in de Waal, terwijl *Cyprinidae,* welke een vrij hoge capaciteit hebben, zich betrekkelijk goed weten te handhaven. Dat externe factoren een invloed hebben op de capaciteit van vissen om lichaamsvreemde verbindingen te metaboliseren, is aangetoond in hoofdstuk

VII. Het effect van blootstelling aan insecticiden en aan temperatuursverschillen blijkt bij vissen in grote lijnen gelijk te zijn aan dat bij zoogdieren.
Na een verblijf van forellen en blankvoorns in water van verschillende temperatuur (14 dagen bij 5 of 18 °C in stilstaand kraanwater) bleken de metabole capaciteiten van de vissen gehouden bij hoge temperatuur veel lager (ongeveer 50%) te zijn dan die van de vissen gehouden bij lage temperatuur. Dit wijst op een van de gevaren die een thermische verontreiniging van het oppervlaktewater kan inhouden, namelijk beschadiging van juist die systemen die vissen gebruiken bij de eliminatie van lichaamsvreemde verbindingen.
Het vermogen van vissen om xenobiotica te metaboliseren kan worden verhoogd door blootstelling van de dieren aan bepaalde enzym-inducerende stoffen (dieldrin, DDT). Blankvoorns uit de Waal vertonen na een verblijf van 1 tot 3 maanden in stromend kraanwater een daling van omzettingscapaciteit tot ongeveer 50%. Deze daling treedt niet op wanneer de dieren worden gehouden in stilstaand kraanwater of rivierwater. Zij kan bovendien worden geantagoneerd door de dieren in stromend kraanwater te belasten met een enzym-inducerende verbinding (dieldrin). Dit wijst erop dat het xenobiotica-metaboliserende vermogen van in het wild levende vissen beïnvloed kan worden door bepaalde chemische contaminanten in het milieu. Deze gegevens pleiten sterk voor de in dit proefschrift uitgewerkte hypothese dat de waargenomen seizoensvariatie in het vermogen om xenobiotica te metaboliseren bij blank- en ruisvoorn (hoge waarden gedurende de zomer, lage gedurende de winter) in belangrijke mate veroorzaakt wordt door een verschil in blootstelling van de dieren aan enzym-inducerende verbindingen in het chemisch verontreinigde milieu. Factoren, die vermoedelijk hierbij een rol spelen zijn: een verschillend contact van het dier met zijn milieu (verschil in beweegijkheid, in voedselopname), en een verschillende concentratie van enzym-inducerende verbindingen (bijvoorbeeld insecticiden en herbiciden) in het water als gevolg van verschillende waterstand en een seizoensafhankelijke introductie van inducerende verbindingen in de natuur.

De farmacokinetische benadering van de chemische milieuverontreiniging is uitgewerkt in hoofdstuk VIII. De gevolgde werkwijze vertoont een grote overeenkomst met die toegepast in de individuele farmacokinetiek, waarmede men het lot van xenobiotica (bijvoorbeeld geneesmiddelen, vergiften) in de mens of in het proefdier tracht te beschrijven.
Een aantal aspecten van de milieuverontreiniging is beschreven met behulp van mathematische modellen. In deze modellen wordt de eliminatiesnelheid als de belangrijkste factor beschouwd. De snelheid van eliminatie van chemische verbindingen in ecosystemen wordt voornamelijk bepaald door de natuurlijke

capaciteit tot degradatie, het biologisch zelfreinigend vermogen. Mathematische modellen zijn geconstrueerd voor een toenemende, respectievelijk afnemende biodegradatiecapaciteit.
Een *toenemende biodegradatiecapaciteit* is denkbaar in het geval dat de contaminanten voedingsstoffen voor het afbrekende systeem zijn. Een tweetal modellen die deze situatie beschrijven, is experimenteel getoetst aan de hand van de afbraak van phenylethylamine in een suspensie van actief slib uit een rioolwaterzuiveringsinstallatie.
Een *afnemende biodegradatiecapaciteit* is denkbaar in het geval dat de contaminanten voor het afbrekende systeem toxisch zijn. Dit geval is theoretisch uitgewerkt voor situaties waarbij continue lozing van chemische verbindingen in ecosystemen plaatsvindt. In cumulatie-curven is te zien dat vrij snel een plateau bereikt wordt in geval van continue lozing van verbindingen met geringe toxiciteit en korte halfwaarde-tijd. De hoogte van het plateau is afhankelijk van de snelheden van introductie en eliminatie. De tijd waarin het plateau wordt bereikt is alleen afhankelijk van de snelheid van eliminatie. Als de aanvoer van de stof wordt gestopt, zal de concentratie weer snel tot de nulwaarde dalen. Het lijkt daarom adequaat ten opzichte van de hier bedoelde categorie van stoffen te spreken van *biologisch gecontroleerde milieuverontreiniging.*
Toxische stoffen die het degradatiesysteem kunnen beschadigen, vormen de gevaarlijkste categorie van verbindingen in de milieuvervuiling. In cumulatiecurven wordt weergegeven dat bij toenemende concentratie een steeds sterkere beschadiging van het biodegradatiesysteem optreedt. Als gevolg hiervan wordt de halfwaarde-tijd van de stof progressief verlengd. Toxische verbindingen cumuleren derhalve progressief doordat de biodegradatie (biologische controle) met het toenemen van de concentratie steeds geringer wordt. Het lijkt daarom adequaat met betrekking tot de hier bedoelde categorie van toxische verbindingen te spreken van *biologisch ongecontroleerde progressieve milieuverontreiniging.*
Wanneer het biodegradatiesysteem volledig en onherstelbaar wordt beschadigd, zal bij beëindiging van de aanvoer van stof de concentratie niet afnemen. Er kan dan worden gesproken van *persisterende milieuverontreiniging.*

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# STELLINGEN

I

De bewering van Brodie en Maickel dat vissen niet over het vermogen tot biotransformatie van xenobiotica beschikken houdt geen stand in het licht van recent onderzoek.

BRODIE, B. B. en MAICKEL, R. P., *Proc. 1st Intern. Pharmac. Meet. 6,* 299 (1962)

Dit proefschrift

II

De conclusies van Buhler en Rasmusson betreffende de *N*-demethylering van aminopyrine zijn niet verantwoord gezien de door deze onderzoekers gevolgde analytische methoden.

BUHLER, D. R. en RASMUSSON, M. E., *Comp. Biochem. Physiol. 25,* 223 (1968)

Dit proefschrift

III

Een kinetische benadering van de chemische verontreiniging van het milieu, analoog aan de farmacokinetische benadering toegepast bij de farmacotherapie, biedt goede perspectieven bij pogingen tot het verkrijgen van inzicht in, en tot het onder controle brengen van de milieuvervuiling.

IV

Het getuigt van meer zin voor realiteit ernaar te streven de chemische verontreiniging van het milieu onder controle te brengen dan haar in elke vorm te verbieden.

V

Een van de dringend om voorziening vragende vormen van milieuverontreiniging wordt geleverd door de verbrandingsprodukten van tabak, in het bijzonder daar waar organismen aan inhalatie daarvan zijn blootgesteld.

VI

De conclusies van Corona en Facino betreffende de metabole omzetting van het antidepressivum amitriptyline zijn aan ernstige bedenkingen onderhevig.

CORONA, G. L. en FACINO, R. M., *Biochem. Pharmac. 17,* 2045 (1968)

## VII

Een analyse van de dosis-werkingscurven van $\beta$-adrenerge stoffen levert tegen de achtergrond van de nieuwere opvattingen betreffende het zogenoemde functionele antagonisme de mogelijkheid tot een eenduidige differentiatie tussen de affiniteit en de intrinsieke activiteit van de betrokken farmaca.

## VIII

De anti-aritmische stof iproveratril wordt in de literatuur bij herhaling ten onrechte geclassificeerd als een $\beta$-adrenerge blokkeerder.

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## IX

Bij het gebruik van plastics voor verpakking van, in het bijzonder vethoudende, voedingsmiddelen en voor bepaalde geneeskundige doeleinden, bijvoorbeeld bij bloedtransfusies, verdient het aanbeveling alleen plastics toe te laten met weekmakers die in het organisme kunnen worden omgezet in niettoxische, goed wateroplosbare produkten, zodat cumulatie in het organisme en schadelijke nevenwerkingen voorkomen worden.

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Nijmegen, 22 april 1971

J. H. DEWAIDE